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Assessment of genetic diversity among moderately drought tolerant landraces of rice using RAPD markers

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ABSTRACT

Genetic diversity and relationships among six rice genotypes were investigated using five random amplified polymorphic DNA (RAPD) markers. A total of 69 alleles were amplified, of which 66 were polymorphic. The size of the amplified alleles was between 0.25 and 2.35 kbp. The number of polymorphic alleles detected with each primer ranged from 7 to 24 with an average of 13.2 per primer and the polymorphism information content (PIC) values varied from 0.8672 to 0.9471. Pair-wise similarity estimated the range of 0.308 to 0.718 among all the genotypes and the highest genetic similarity was found between Maloti and BRRI dhan53. Cluster analysis using UPGMA (unweighted pair group method with arithmetic averages) revealed three clusters at genetic similarity of 46%. A moderately drought tolerant landrace, Boalia, formed a single cluster and the remaining genotypes grouped into distinct clusters based on their relatedness. The results showed a high level of genetic diversity among studied genotypes and this information will assist in conservation as well as selection of parents during breeding programs for the development of drought tolerant rice varieties in near future.

Key words: drought, genetic diversity, polymorphism information content (PIC) value, RAPD marker, UPGMA dendrogram

Introduction

Rice is the world's most important food in terms of the number of people dependent on it as a direct source of calories (Long & Ort, 2010). It occupies almost one-fifth of the total land area covered under cereals (Chakravarthi & Naraveneni, 2006) and more than 90% of the world's rice is grown and consumed in Asia, where 60% of the earths' people live (Khush, 2005). Rice has most diversified crop species and is grown under diverse eco-geographical conditions in various tropical and subtropical countries including Bangladesh. In Bangladesh, rice accounts for 92% of production of total food grains (Rahman et al., 2007), covering 77% of the total cropped area and provides 75% of the calories and 55% of the proteins of the population's average daily diet (Bhuiyan et al., 2002).

Rice is sensitive to different abiotic stresses like drought, salinity and cold. Among these stresses drought is more complex phenomenon than others as it can occur at any point during production. It is becoming an increasingly severe problem in many regions of the world (Passioura, 2007) and the percentage of drought affected land areas more than doubled from the 1970s to the early 2000s in the world (Isendahl & Schmidt, 2006). About 45% of the world's rice is cultivated in rainfed ecosystems (Nazari & Pakniyat, 2008) and in these areas drought poses the major environmental

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constraints to rice productivity (Serraj et al., 2009). Thus developing drought tolerant variety will improve productivity to cope with increasing demand for food.

Landraces are geographically or ecologically distinctive population, which are endowed with tremendous genetic variability and represent unique source of genetically variable traits for rice genetic improvement (Ram et al., 2007). Landraces are excellent source of various genes for adaptability, resistance to drought, pests and fungal diseases (Prabakaran et al., 2010; Ganesh et al., 2004; Siddiq et al., 2005). Nearly 10,000 landraces are considered to exist in Bangladesh (Kaul et al., 1982). More than 4000 traditional Bangladesh rice accessions or landraces have been collected and registered at a rice gene bank in the Bangladesh Rice Research Institute (BRRI) for medium-term storage and an identical set is held in trust at IRRI for longer storage (Jackson, 1999). As exact genetic potential remain uncategorized (Ram et al., 2007) and estimated that not even 15% of potential diversity utilized (Hossain et al., 2007), there is an urgent need to conserve landraces as well as to reveal the diverse gene pool and unzip genes for agronomically important traits for crop improvement. Adequate knowledge about genetic diversity of landraces will assist in selection of parents during rice breeding programs.

Genetic diversity can be evaluated on both morphological and molecular levels and development of different marker systems helps in this regard. Molecular markers are reliable tools in the evaluation of genetic variation and in the elucidation of genetic relationships within and among species (Tehrim et al., 2012) and serve as a valuable guide for effective collection and use of genetic resources (Rahman et al., 2007). Unlike the morphological and biochemical markers, molecular markers are not stressed by environmental factors and growth practices (Ovesna et al., 2002). Among several molecular markers, RAPD markers have been proved to be an efficient tool used in genetic research due to its simplicity, low cost and less performing time, ease of essay by PCR and no prior knowledge about genome is required (Williams et al., 1990; Rekha et al., 2011; Rabbani et al., 2008). RAPD markers have been successfully employed in rice for identification and classification of cultivars (Choudhury et al., 2001), identifications of hybrids (Hashemi et al., 2009), genetic diversity analysis (Saker et al., 2005; Kanawapee et al., 2011; Ogunbayo et al., 2005). RAPD markers were found to be more useful than restriction fragment length polymorphism (RFLP) markers (Williams et al., 1990; Fukuoka et al., 1992) and serve as excellent tools for plant breeders (Lima-Brito et al., 2006). There is very little information available on genetic diversity of drought tolerant landraces of rice in Bangladesh. Effort has been made to assess genetic diversity and relatedness at molecular level among six rice genotypes using RAPD markers because of their advantages over other markers. The present study demonstrated the utility of RAPD markers in revealing genetic diversity among moderately drought tolerant landraces of rice and will serve as a basis for selection, germplasm collection, and conservation of moderately drought tolerant landraces.

Materials and Methods

Plant materials

Six rice genotypes were used as plant materials in the present study (Table 1). Seeds were collected from Genetic Resource Centre (GRC) of Bangladesh Rice Research Institute (BRRI) and Sylhet district of Bangladesh. Ten seeds of each genotype were placed into petridish with filter paper soaked by distilled water for germination under aseptic condition and then germinated seeds were grown into labeled pots.

Genomic DNA extraction

Genomic DNA was extracted from the healthy portion of young leaves harvested from 21 days old plant. DNA isolation was carried out using a mini prepararion modified CTAB method, which did not require liquid nitrogen and only a very small amount of tissue samples needed (Zheng et al., 1995). Leaf tissues were cut into small pieces, homogenized and digested with extraction buffer (1 M Tris, 0.5 M Na_2EDTA, 5M NaCl and distilled H_2O, pH 8.0) and 20% SDS. Following incubation of leaf extracts for 10 min at 65°C in water bath, 100 µl of 5M NaCl was added and mixed well by gentle inversion. Then 100 µl 10X CTAB was added and again incubated for 10 min at 65°C in water bath. After that, 900 µl of a mixture of chloroform and isoamyl alcohol (24:1) was added and centrifuged for 8 min at 11,000 rpm in a microcentrifuge. Then 500 µl of upper aqueous layer was separated and 600 µl of ice cold isopropanol was added to it, mixed and centrifuged for 12 min at 13,200 rpm. A small pellet was visible and supernatant was decanted. The pellet was then washed with 200 µl cold 70% ethanol and

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centrifuged at 13,200 rpm for 12 min. After removing ethanol followed by air drying the DNA pellets were resuspended into 100 µl of 1X TE buffer and dissolved the pellet by warming in a 65ºC water bath for up to 1 hour (with frequent mixing or flicking the tube with finger). Then the pellet was stored at -20ºC in an ultra freezer. Quality of DNA was estimated by agarose gel (0.8%) electrophoresis and visualized with UV light.

RAPD primers selection and PCR amplification

A total of five decamer primers belonging to different groups including OPA, OPB, OPC, OPF and OPL of Operon Technologies Inc. (Alameda, California, USA) were selected (Table 2). Various groups of researchers previously used these primers to assess genetic diversity in different rice genotypes and found them highly polymorphic (Rabbani et al., 2008; Kanawapee et al., 2011; Hashemi et al., 2009; Raghunathachari et al., 2000). Initially, these primers were evaluated in two genotypes to detect their banding pattern. As banding pattern was good they were used for analysis in all six genotypes.

Amplifications were performed in a total volume of 25 µl containing 5 µl of 5X buffer, 0.6 µl of 10 mM of dNTPs, 1 µl of primer, 1.5 µl of 25 mM $MgCl_2$, 1 unit *Taq* polymerase, 1 µl template DNA and 15.7 µl of ddH_2O. PCR was carried out in a MultiGene gradient thermal cycler (Labnet International Inc., USA) and it was set at 1 cycle for 5 min at 94°C (pre-denaturation) followed by 40 cycles of 1 min at 94°C (denaturation), 1 min at 35°C (annealing) and 2 min at 72°C (extension) and a final extension in 1 cycle of 10 min at 72°C followed by a hold at 4°C. PCR products were stored at -20°C for further use. Amplified products were analyzed by electrophoresis in 1.8% agarose gel containing ethidium bromide using 0.5X TBE buffer. Electrophoresis was carried out at 75V for 50 minutes. Bands were imaged under UV transilluminator and photographed by a digital camera (Panasonic DMC-FS20).

Data analysis

The molecular weight of each amplified products were calculated in base pair by comparing the migration of each fragment with that of a known size of fragments of Generuler™ 1 kb DNA ladder (Fermentas, USA) using Alpha-Ease Fc 4.0 software (Alpha Innotech, USA). Polymorphism Information Content (PIC) value for each primer was calculated according to the formula:

$$\mathbf{PIC = 1 - \sum Pij^2}$$

where Pij is the frequency of the $\mathbf{j}^{th}$ allele for primer **i** and the summation extends over $\mathbf{n}^{th}$ alleles (Anderson et al., 1993). All distinct bands were scored on the basis of their presence as (1) or absence as (0) separately for each genotype and each primer to create a data matrix.

Table 1. *List of rice genotypes used in diversity analysis.*

SI No.	Genotypes	BRRI accession No.	Special attributes
1	Panbira	4150	Moderately drought tolerant landrace
2	Dular	DA-22	Moderately drought tolerant landrace
3	Boalia	2068	Moderately drought tolerant landrace
4	Maloti	Collected from Sylhet	Landrace
5	BRRI dhan53	BRRI dhan53	Salt tolerant variety

Table 2. *Five RAPD primers with their sequences, total number and size range of alleles, PIC values found among six rice genotypes.*

Primer code	Sequence ($5^{/}$ - $3^{/}$)	Allele size (bp)	Total alleles	Polymorphic alleles	% Polymorphism	PIC
OPA-01	CAGGCCCTTC	600-2350	15	15	100	0.9159
OPB-07	GGTGACGCAG	250-2100	24	24	100	0.9471
OPC-05	GATGACCGCC	600-1350	10	10	100	0.8800
OPF-14	TGCTGCAGGT	250-1650	10	10	100	0.8878
OPL-03	CCAGCAGCTT	300-1650	10	7	70	0.8672
Total			69	66		
Average			13.8	13.2	94	0.8996

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Estimates of genetic similarity (F) were calculated between all pairs of the genotypes by the Dice algorithm. The Dice algorithm is identical to that of Nei & Li (1979) as follows:

$$\textbf{Similarity (F)} = 2N_{ab}/(N_a + N_b)$$

where N_a = the total number of fragments detected in individual 'a'; N_b = the total number of fragments shown by individual 'b' and N_{ab} = the number of fragments shared by individuals 'a' and 'b'. An unweighted pair group method with arithmetic averages (UPGMA) dendrogram based on these similarity coefficients was constructed by NTSYS-pc version 2.20 (Applied Biostatistics Inc., USA).

Results and Discussion

Assessment of genetic diversity is essential for germplasm characterization to identify potential parents, management and utilization of genetic resources. Molecular markers are valuable tools for the analysis of genetic relatedness and the identification and selection of desirable genotypes for crosses as well as for germplasm conservation in gene banks (Alvarez et al., 2007). In the present investigation, five RAPD markers were used to assess genetic diversity among six rice genotypes.

Overall genetic diversity revealed by RAPD markers

The genetic diversity among the rice cultivars was evaluated based on allele numbers and PIC values for each marker. A total of 69 alleles were amplified, of which 66 (95.65%) were polymorphic and 3 (4.34%) were monomorphic. The number of alleles amplified by each primer varied from 10 (OPC-05, OPF-14 and OPL-03) to 24 (OPB-07) with an average of 13.8 alleles per primer. The size of the amplified alleles ranged from 250 to 2350 bp. All five RAPD primers revealed polymorphism between genotypes, of which 4 primers were found to be 100% polymorphic and remaining primer (OPL-03) exhibited 70% polymorphism (Table 2). Figure 1 shows a gel image of amplified fragments produced by primer OPL-03.

The level of polymorphism observed in the present study is in accordance with those reported by other researchers (Raghunathachari et al., 2000; Rabbani et al., 2008; Tehrim et al., 2012). On the other hand, Youssef et al. (2010) reported 73.02% polymorphism with five RAPD primers among 6 new drought tolerant lines and 4 sensitive cultivars and Rahman et al. (2007) reported only 53.85% polymorphism among six different rice cultivars and concluded low levels of genetic variation and high levels of genetic relatedness present in each of the rice cultivars. Lower level of polymorphism was also reported by Hashemi et al. (2009), Ashraf et al. (2007) and Choudhury et al. (2001) who found 65.8%, 66.7% and 67.5% polymorphism, respectively among tested rice varieties.

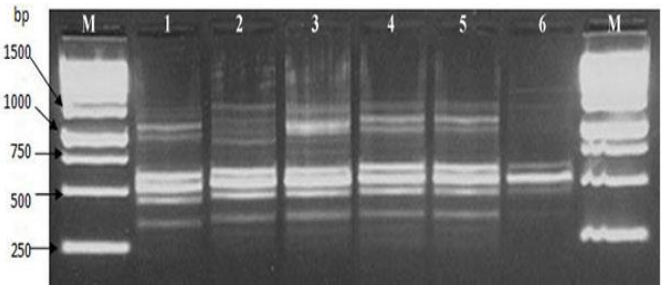

Figure 1. *DNA profiles of six rice genotypes amplified by primer OPL-03. Lane M: Molecular weight marker (Generuler™ 1 kb DNA ladder). Lanes 1, 2, 3, 4, 5 and 6 represent genotypes Panbira, Dular, Boalia, Maloti, BRRI dhan53 and Hashikalmi respectively. Images from different parts of the same gel have been grouped.*

Average number of polymorphic alleles per primer was 13.2 (Table 2). The average number of polymorphic bands obtained in our study is higher than those earlier reports using Indian scented rice and Italian rice cultivars (Verma et al., 1999; Porreca et al., 2001). These reports observed that the average number of polymorphic bands per primer were 13.7 and 14.0, respectively. Younan et al. (2011) reported on genetic diversity and relationships among a set of Iraqi rice varieties using 9 RAPD primers and detected 96 polymorphic bands with an average number of 13.7 polymorphic bands per primer, which is lower than our report. The average number of polymorphic bands per primer detected in the present study was also higher than earlier reports, who observed an average of 6.7, 7.4 and 7.8 polymorphic bands per primer (Ren et al., 2003; Rabbani et al., 2008; Saker et al., 2005). On the other hand, Rekha et al. (2011) reported an average of 32.3 polymorphic bands with 15 RAPD primers among ten rarely cultivated traditional indica rice varieties, which is higher than our report. Such big discrepancy using RAPD in the average number of polymorphic bands detected might be due to the diverse genotypes used and selection of RAPD primers with scorable bands.

The polymorphism information content (PIC) value is a reflection of allele diversity and frequency among varieties

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used in linkage analysis. The PIC value of each marker can be evaluated on the basis of its alleles and relative frequencies of these alleles. The polymorphism information content (PIC) values ranged from 0.8672 (OPL-03) to 0.9471 (OPB-07) with an average of 0.8996 (Table 2). The PIC values observed in present study were higher than previous estimate of RAPD marker analysis in rice by Kanawapee et al. (2011) (ranged from 0.08 to 0.73, average = 0.38). The PIC values indicate that all these primers were highly informative and capable of distinguishing between genotypes.

Similarity matrix

A similarity matrix based on the proportion of shared RAPD fragments was used to determine the level of relatedness among the genotypes. Pair-wise estimates of similarity ranged from 0.308 to 0.718 among all the genotypes. The highest genetic similarity (71.8%) was found between Maloti and BRRI dhan53. This pair was followed by BRRI dhan53 and Hashikalmi (57.9%), Panbira and Dular (50.0%), Dular and BRRI dhan53 (48.0%) and decreasing thereafter. The lowest genetic similarity (30.8%) was observed between Panbira and Hashikalmi (Table 3).

Our findings are lower than other estimates of pair-wise similarity observed in rice by Arif et al. 2005 (range from 0.42 to 0.85) and Ren et al. 2003 (range from 0.59 to 0.92). The similarity values are also lower than results of Rabbni et al. (2008) in which they reported genetic similarity ranged from 0.50 to 0.96 among 40 traditional and improved cultivars and Rekha et al. (2011) also observed similarity values ranged from 0.61 to 0.90 for Pakistani and traditional indica rice varieties using 15 RAPD primers. According to Tehrim et al. (2012) similarity values for 35 varieties and cultivars ranged from 0.53 to 0.94, which is markedly higher than the result obtained in our study. The lower range of similarity values for cultivars revealed by RAPD markers suggested that there is a high level of genetic diversity present among the studied genotypes.

Cluster analysis

Cluster analysis was performed to resolve the genetic relationships among genotypes based on the alleles detected by RAPD markers. Dendrogram based on Dice similarity coefficient using UPGMA grouped six genotypes into three clusters at the similarity coefficient of 46% (Figure 2). Panbira and Dular, which are moderately drought tolerant landraces, grouped in cluster I. Another genotype Boalia, which is also a moderately drought tolerant landrace grouped in a single cluster (Cluster II). Cluster III is the largest cluster and consisted of three genotypes namely Maloti, BRRI dhan53 and Hashikalmi. Maloti, a landrace and BRRI dhan53, a salt tolerant variety showed highest similarity and formed a subcluster within the cluster III. This dendrogram revealed that the genotypes derived from a genetically similar type clustered together more. Ogunbayo et al. (2005) reported differentiation among rice genotypes was higher for RAPD markers than for morphological classification.

High level of genetic diversity found among genotypes reveals the ability of RAPD markers as a suitable tool for diversity studies. As the use of molecular markers to investigate genetic diversity among drought tolerant landraces in Bangladesh is limited, information from present study will assist researchers in this regard. The use of RAPD markers provided sufficient information for discriminating rice genotypes. Therefore, it can be concluded that RAPD markers may be utilized for the assessment of genetic diversity in drought tolerant rice varieties. Our findings will be helpful in future breeding programs to identify suitable parents for the development of drought tolerant rice varieties.

Table 3. *Dice similarity coefficient matrix derived from RAPD analysis.*

Genotypes	Panbira	Dular	Boalia	Maloti	BRRI dhan53	Hashikalmi
Panbira	1.000					
Dular	0.500	1.000				
Boalia	0.393	0.373	1.000			
Maloti	0.340	0.392	0.417	1.000		
BRRI dhan53	0.346	0.480	0.426	0.718	1.000	
Hashikalmi	0.308	0.360	0.340	0.462	0.579	1.000

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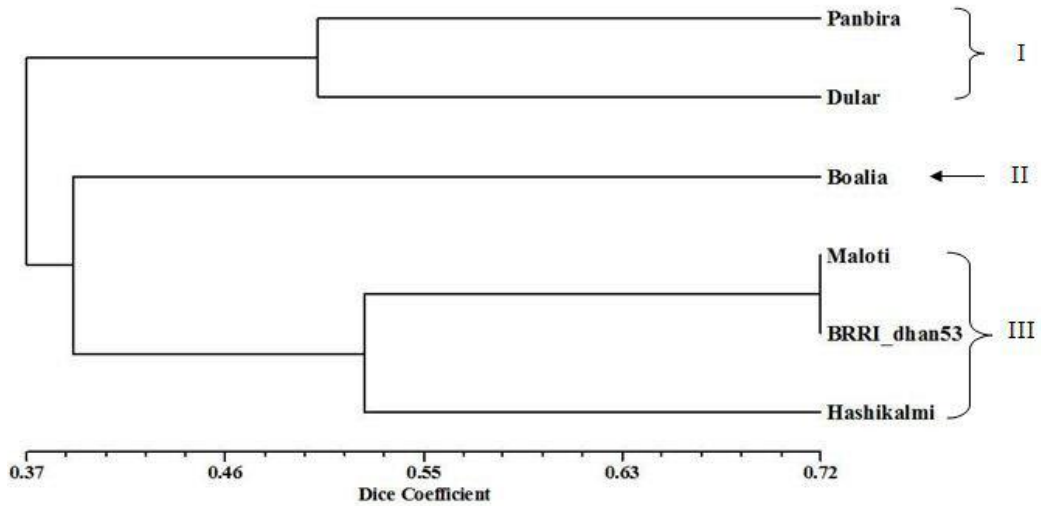

Figure 2. *Dendrogram showing genetic relationship among six rice genotypes based on Dice similarity coefficient.*

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