JJBS

Volume 7, Number 1, March. 2014
ISSN 1995-6673
Pages 13 - 18

Jordan Journal of Biological Sciences

Genetic Variability Evaluation Among Iraqi Rice (*Oryza sativa* L) Varieties using RAPD Markers and Protein Profiling

Nawroz Abdul-razzak Tahir*

University of Sulaimanyah, Faculty of Agricultural Sciences, Bakrajo, Sulaimanyah, Iraq

Received: August 30, 2013 Revised: October 25, 2013 Accepted: November 1, 2013

Abstract

Genetic variability and relationships among ten rice varieties were studied by using 20 decamer random primers and SDS-PAGE methods. Out of 22, 20 Random Amplified Polymorphic DNA (RAPD) primers revealed polymorphism, while the remaining 2 primers showed no reaction. The primers produced a total of 109 bands, of which 62 (56.88%) were polymorphic. The number of polymorphic fragments for each primer varied from 1 to 9 with an average of 3.1 polymorphic fragments. The primer OPC-07 produced the maximum number of polymorphic bands. The RAPD data were analyzed to determine the genetic distance coefficients which ranged from 0.30 to 0.76. Cluster analysis was performed using the Jaccard's coefficient. The dendrogram resolved the selected rice varieties into four major clusters. On the other hand, similar varieties of rice were analyzed for endosperm storage proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to estimate their genetic diversity. Seed storage profiling led to the detection of a total of 12 polypeptide bands. The molecular weights of peptides ranged from 284 to 11 kDa. Polymorphism was not evident in all seed proteins of diverse molecular weights among all rice varieties, but two polymorphic bands (63 and 120 kDa) were found in a high molecular weight region. The results of the genetic diversity, obtained during this study, will be useful for the selection of the parents for developing rice breeding varieties in the future.

Keywords: *Oryza sativa*, Seed, Genetic Variation, RAPD, Polypeptide.

1. Introduction

Rice (*Oryza sativa* L.), belonging to the family Graminae, is the staple food for one third of the world's population (Chakravarthi and Naraveni, 2006). Approximately, 90% of the world's rice is grown in the Asian continent and constitutes a staple food for 2.7 billion people worldwide (Salim *et al.*, 2003; Paranthaman *et al.*, 2009). The world's rice production has doubled during the last 25 years, largely due to the use of improved technology such as high yielding varieties and better crop management practices (Byerlee, 1996). Further scope of crop improvement depends on the conserved use of genetic variability and diversity in plant breeding programmes and use of new biotechnological tools. Molecular characterization can reveal the maximum genetic variation or genetic relatedness found in *Vigna angularis* population (Xu *et al.*, 2000). Chakravarthi and Naravaneni (2006) reported that the usefulness of preservation and conservation of genetic resources since genetic diversity provides information to monitor germplasm and prediction of potential genetic gains. DNA based molecular markers have been proven to be powerful tools in the assessment of genetic variation and in the elucidation of genetic relationships within and among the species of rice (Shivapriya and Hittalmani, 2006). Several molecular markers have been used for studying the genetic variation and relatedness among different populations, species and genes such as RFLP and RAPD (Tingey and Deltufo, 1993; Wu *et al*., 2006; Rahman *et al.*, 2007; Abdul-razzak, 2008; Rawashdeh, 2011; Rajani *et al.*, 2013), SSRs (Prabakaran *et al.*, 2010), AFLP and SNPs (Joshi *et al*., 2000; Liu *et al*., 2011).

Nowadays, the PCR-based RAPD is technically simple and cheaper compared to the others methods of molecular markers. RAPD requires small quantity of DNA, and ability to regenerate polymorphisms. In many instances, the small number of primers is necessary for polymorphism identification within species.

On the other hand, genotyping of different species is also necessary for characterization of different accession of crop germplasm, testing varietal purity and registration of newly developed cultivars (Choudhury *et al.*, 2001). Among numerous techniques available for assessing the genetic variability and relatedness among crop germplasm, seed storage protein analysis represents a valid alternative and/or improved approach to varietal identification

* Corresponding author. e-mail: nawroz.tahir@univsul.net or nawrozbiology@gmail.com.

(Mennella *et al.,* 1999; Galani *et al.,* 2011). Seed storage protein markers are polymorphic and environmental influence on their electrophoretic pattern is limited (Sadia *et al.,* 2009). It is a useful tool for studying genetic diversity via sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Abdul-razzak, 2009; Sadia *et al.,* 2009) in a short period of time (Netra and Prasad, 2007). The objective of this study is to determine the genetic relationships among several Iraqi rice varieties based on RAPD markers and protein profiling.

2. Materials and Methods

2.1. Plant Materials and Genomic DNA Isolation

The present paper was carried out at the University of Sulaimani in Iraq during 2012-2013. Ten Iraqi rice varieties (*Oryzae savtiva* L.) have been used for molecular and biochemical analysis. The genetically pure rice varieties, named [Delfino/M239 (Italian origin), Azmar56R (Iraqi origin), Bcdo/M245 (Italian origin), Loto/M269 (Italian origin), Azmar56W (Iraqi origin), Kalar-2 (Iraqi origin), Shawre (Iraqi origin), Zangiswr (Iraqi origin), Kalar-1(Iraqi origin), Pitwen (Iraqi origin)], were collected from the Agriculture Department of Ministry of Agriculture in Sulaimanyah, Iraq. Those varieties were widely cultivated at Sulaimanyah region. The analysis of these varieties leads to the collection of information about the genetic diversity at the genome level. In a 9 cm pot, the healthy seeds were covered with compost, and then the pots kept warm at 25ºC. The seedlings germinate moved to a 30 cm pot. Transplants should be placed slightly deeper in the compost than they were previously growing; as this will help them produce side shoots. Plants put in full sun and kept well watered. The pots incubated in greenhouse for six weeks under natural conditions (temperature 28-30ºC). The DNA extraction was carried out from the fresh leaves following cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987) with minor modifications. Fresh 500 milligrams of young leaves were ground to a very fine powder in liquid nitrogen and dispersed in 3 mL of pre-warmed (65°C) CTAB DNA extraction buffer [2% CTAB; 1.4 M NaCl; 100 mM Tris-HCI (pH 8.0); 20 mM EDTA (pH 8.0); 0.2% 2-Mercaptoethanol (added just before use)]. Tubes containing samples were incubated at 65 °C for 60 min in a water bath. The samples were swirled every 10 min. After incubation, the mixture was cooled down to room temperature and emulsified with an equal volume of chloroform: isoamyl alcohol (24:1), and centrifuged at 8000 rpm for 10 min. Following centrifugation, the aqueous phase was collected and nucleic acid was precipitated by mixing with equal volume of chilled isopropanol. The precipitated nucleic acid was centrifuged at 12000 rpm for 10 min and the pellet was washed with 70% ethanol. The DNA pellet obtained was dried and stored in 100 µL TE buffer. In order to remove RNA, DNA was treated with 40 µg RNAse-A at 37 °C for 1 h and stored at 4 °C until use.

2.2. Determination of DNA Concentration by Spectrophotometer

The DNA samples were diluted 100 times with TE buffer. The optical density (OD) of each sample was read at wave length of (260 and 280 nm) with a spectrophotometer. The DNA concentration was calculated by this equation:

DNA concentration (µg/µl) = (50 µg/ml x ODA_{260} x dilution factor)/1000

The OD of diluted dsDNA at 260nm was measured. The dilution factor was 100 (10 µl of extracted DNA in 990 µl of high pure water or ddH_2O). The D260/280 ratio for pure DNA was 1.78-1.85 (Sambrook *et al*., 1989). The final concentration of extracted DNA was 0.5-0.6 µg/µl. A solution of 20 ng/µl was prepared by adding of 20 µl of extracted DNA in 480 µl of ddH_2O. 2.5 µl (50 ng) of this dilution was used for PCR reaction.

2.3. RAPD Amplification Procedure

Twenty two different 10-mer oligonucleotide RAPD primers (Operon Technologies Inc., USA) (Table 1) were used. Each polymerase chain reaction (PCR) was carried out in a 25 µl volume containing 50 ng template DNA, 1.5 mM $MgCl_2$, 0.32 mM dNTPs, 1X *Taq* DNA polymerase buffer, 10 pmol decanucleotide primer and 2 units of *Taq* DNA polymerase. Amplification was performed in a thermal cycler (Corbett Research, Australia) using the following conditions: denaturation at 95ºC for 3 min; 36 cycles of 1 min denaturation at 94ºC, 1 min annealing at 34, 35, 36, 37, and 38ºC and 2 min extension at 72ºC; and a final extension at 72ºC for 5 min. The RAPD-PCR products were analyzed directly on 1.5% agarose gel in 1X TBE buffer. The DNA was stained with 0.5 mg mL^{-1} Ethidium bromide and visualized and photographed under a UV transilluminator.

Table 1. Twenty two RAPD primers, their sequences and annealing temperature.

	Name of primer	Sequence (5' to '3)	Annealing temperature
1	OPC-07	GTCCCGACGA	38
2	OPC-15	GACGGATCAG	38
3	OPD-08	GTGTGCCCCA	38
4	OPF-06	GGGAATTCGC	38
5	OPF-13	GGCTGCAGAA	38
6	OPF-14	TGCTGCAGGT	38
7	OPF-17	AACCCGGGAA	38
8	OPJ-13	CCACACTACC	38
9	OPK-11	AATGCCCCAG	38
10	OPR-2	GATTCCGCGG	36
11	OPJ-08	CATACCGTGG	35
12	OPD-05	TGAGCGGACA	34
13	OPD-06	ACCTGAACGG	34
14	OPD-07	TTGGCACGGG	34
15	OPD-02	GGACCCAACC	37
16	OPD-10	GGTCTACACC	37
17	OPD-18	GAGAGCCAAC	37
18	OPD-20	ACCCGGTCAC	37
19	OPF-03	CCTGATCACC	37
20	OPH-13	GACGCCACAC	37
21	OPG-14	GGATGAGACC	37
22	OPJ-10	AAGCCCGAGG	37

2.4. Protein Extraction

Variability of the total seed storage proteins was investigated by using SDS-PAGE.

Protein was extracted from dry seeds by grinding them into a fine powder. For total seed protein extraction from individual seed samples, 0.5 g of each variety was taken and then, 1 mL Tris urea buffer [containing 0.05 M Tris-HCl (pH 8.0); 0.4% SDS; 5 M Urea; 2.5% glycerol; 1.5% 2-Mercaptoethanol; 1 mM EDTA and 0.01% (w/v) Bromophenol blue)] was added. The sample was vortexed for 5 min followed by incubation for 3 hours at room temperature. The crude homogenate was centrifuged at room temperature at 15000 rpm for 10 min. The extracted protein samples were collected as supernatant and pellets were discarded and then stored at (-20°C).

2.5. SDS-PAGE (Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis) Gel

The soluble seed proteins were subjected to SDS-PAGE in gel slabs of 1.5 mm thickness (3.5 cm, 4.5% stacking and 15.5 cm, 15% resolving gels) as described by Laemmli (1970). Electrophoresis was carried out at 80 V for 5 h. The gels were then fixed in solution (10% acetic acid and 40% ethanol) for 15 min with constant shaking and then stained with 0.2% (w/v) Commassie brilliant blue R250 overnight on an electrical shaker. Distaining of gels was carried out for a couple of hours followed by gel preservation, scanning and photography.

2.6. Data Analysis

The data obtained with the technique RAPD were scored in a binary form as the presence or absence (1/0) of bands per each sample. Program of SPSS (version 19) was used to calculate Jaccard coefficient and construction a hierarchical cluster analysis.

3. Results

3.1. RAPD Analysis

The genetic relationship among the ten rice varieties has been carried out using RAPD markers (Figure 1). The results of present study indicate a considerable level of genetic diversity among the studied varieties. Among the 22 primers, 20 primers showed high polymorphic bands (Table 2).

A total of 109 DNA fragments was generated by 20 primers, out of which 62 were polymorphic (56.88% polymorphism) (Table 2). These primers produced multiple band profiles with a number of amplified DNA fragment varying from 2 to 14.

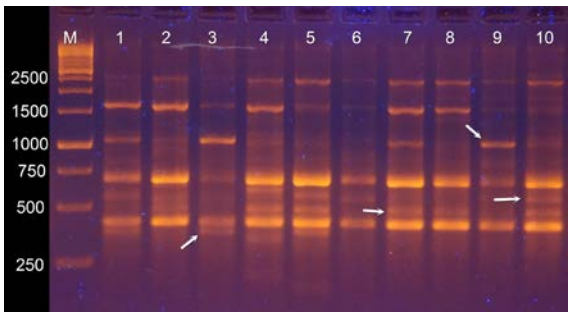

Figure 1. RAPD profiles of ten different rice varieties using primer OPC-15. Lane M: 1kb ladder, lane 1: Pitwen, lane 2: Kalar-1, lane 3: Zangiswr, lane 4: Shawre, lane 5: Kalar-2, lane 6: Azmar56W, lane 7: Loto/M269, lane 8: Bacdo/M245, lane 9: Azmar56R, lane 10: Delfino/M239. The white arrows show polymorphic bands.

Table 2. Total amplified fragments, number of polymorphic bands and percent of polymorphic bands of 20 random primers used under this study.

Primers	Total amplified fragments	Number of polymorphic bands	% of polymorphic bands
OPC-07	14	9	64.3
OPC-15	7	4	57.14
OPD-08	7	2	28.6
OPF-06	10	6	60
OPF-13	6	4	66.67
OPF-14	5	3	60
OPF-17	5	4	80
OPJ-13	5	3	60
OPK-11	9	6	66.67
OPR-2	4	4	100
OPJ-08	2	1	50
OPD-05	6	3	50
OPD-06	2	1	50
OPD-07	8	6	75
OPD-02	3	1	33.33
OPD-10	4	1	25
OPD-18	3	1	33.33
OPD-20	3	1	33.33
OPG-14	2	1	50
OPJ-10	4	1	25
Total	109	62	
Average per primer	5.45	3.1	

The primer OPJ-08 and OPJ-14 gave the minimum number of fragments (2), while the highest number of fragments (14) was obtained by primer OPC-07. Out of 20 primers, only 14 primers exhibited 50% or more polymorphism. The number of polymorphic fragments for each primer varied from 1 to 9 with an average of 3.1 polymorphic fragments. The primer OPR-2 yielded 4 bands that were all polymorphic (100%). The primer OPC-07 produced the maximum number of polymorphic bands (9). The genetic distance coefficients, among 10 rice varieties based on the RAPD fragments, were used to construct a dendrogram (Figure 2).

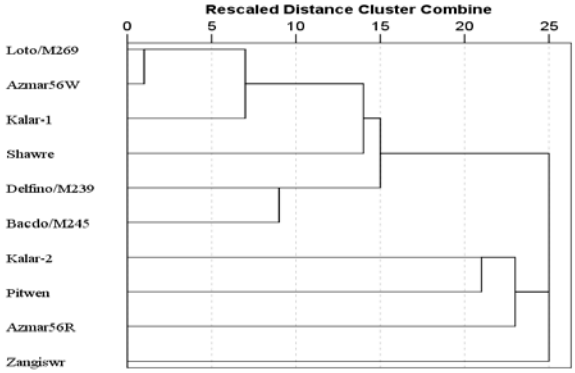

Figure 2. Adendrogram among rice varieties using polymorphic RAPD primers based on Jaccard coefficients.

The dissimilarity matrix among the 10 varieties ranged from 0.30 to 0.76 (Table 3).

Table 3. Dissimilarity matrix based on Jaccard coefficients among various rice varieties using RAPD data.

	Delfino/M239	Azmar56R	Bacdo/M245	Loto/M269	Azmar56W	Kalar-2	Shawre	Zangiswr	Kalar-1	Pitwen
Delfino/M239	0	0.692	0.421	0.561	0.55	0.61	0.524	0.683	0.591	0.619
Azmar56R		0	0.714	0.706	0.697	0.645	0.73	0.655	0.657	0.613
Bacdo/M245			0	0.486	0.375	0.658	0.526	0.737	0.486	0.632
Loto/M269				0	0.3	0.718	0.514	0.694	0.382	0.658
Azmar56W					0	0.711	0.5	0.757	0.412	0.649
Kalar-2						0	0.605	0.714	0.675	0.6
Shawre							0	0.684	0.474	0.579
Zangiswr								0	0.718	0.606
Kalar-1									0	0.579
Pitwen										0

The highest dissimilarity matrix revealed was between Azmar56W and Zangiswr (0.76), while the lowest dissimilarity matrix showed between Azmar56W and Loto/M269 (0.30). From the RAPD-based dendrogram, Zangiswr, which was the most genetically dissimilar, was separated from all other varieties. The varieties were separated into 4 groups at distance 22. Group I contains varieties including Loto/M269, Azmar56W, Kalar-1, Shawre, Delfino/M239 and Bacdo/M245. Group I could be further divided into three subgroups. Sub-group IA contained 3 varieties which include Loto/M269, Azmar56W and Kalar-1. Subgroup IB contained the Shawre variety only; subgroup 1C contained Delfino/M239 and Bacdo/M245. Kalar-2 and Pitwen comprising group II. The third group contained Azmar56R, and the last group comprised Zangiswr.

3.2. Protein Profiling

In this study, the SDS-PAGE of seed proteins of ten rice varieties was carried out to investigate the genetic diversity. Seed storage profiling showed distinct polymorphism in electrophoretic banding patterns and led to the detection of a total of 12 polypeptide bands (Figure 3).

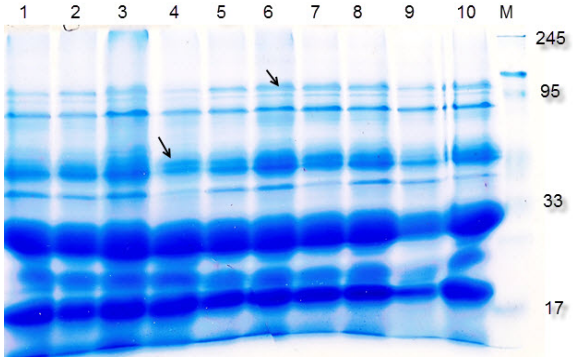

Figure 3. Proteins profiles of ten rice varieties. Lane M: Molecular weight marker size (Roti mark prestained (kDa)), lane 1: Delfino/M239, lane 2: Azmar56R, lane 3: Bacdo/M245, lane 4: Loto/M269, lane 5: Azmar56W, lane 6: Kalar-2, lane 7: Shawre, lane 8: Zangiswr, lane 9: Kalar-1, lane 10: Pitwen. The black arrows show polymorphic bands.

The number of SDS-PAGE bands per varieties varied from 11 to 12. The molecular weights of peptides ranged from 284 to 11 kDa. Polymorphism was not evident in all seed proteins of diverse molecular weights among all rice varieties but two polymorphic bands (63 and 120 kDa as shown in arrow) were found in high molecular weight region. Varieties Delfino/M239, Azmar56R and Kalar-1 possessed a band of 63 kDa. The rice varieties Kalar-2, Shawre, Zangiswr and Pitwen were showed subunit in of 120 kDa. These results suggested that by SDS-PAGE analysis, Iraqi rice varieties have a low diversity in storage protein profiles.

4. Discussion

In this study, the inter-specific genomic polymorphisms in ten rice varieties were evaluated through RAPD-PCR and SDS-PAGE methods. The molecular technique RAPD-PCR analysis is currently used to discrimination of the closely related varieties in order to determine the genetic distance and genetic diversity. In general, RAPD fingerprinting has a number of potential applications including the determination of cultivar's purity, efficient use and management of genetic resources (Ahmed, 1999). The number of amplification RAPD bands (109) was more than that of SDS-PAGE (12). The total number of all amplification bands for each primer was between (2-14). The variation in the number of bands amplified by different primers is influenced by variable factors such as primer structure and less number of annealing sites in the genome. Moreover, the total number of polymorphic bands detected by RAPD primers was much higher than SDS-PAGE. However, the results in this study suggested that the RAPD markers were superior to SDS-PAGE markers in the capacity of revealing more information about the genetic diversity. It was observed, in this study, that the level of polymorphism with primers differed among the varieties. The total number of amplified and polymorphic bands was higher compared to those obtained by Mani *et al*. (2010). The proportion of polymorphism was higher compared to some previous RAPD analysis in rice e.g., 53.85% in six different rice cultivars (Rahman *et al*., 2007), and lower compared to 67.5% (Choudhury *et al*., 2001) 85.02% (Rajani *et al*., 2013) and 72.27% (Skaria *et al*., 2011) in some Indian rice varieties. One of the reasons for this high level of polymorphism can be due to intraspecific variation among the varieties. Information on intraspecific variation from the present study might be useful in making decision for

improvement of rice cultivars. The similarity level, up to 50% in cluster analysis, is indicative of plant derived from interspecific hybridization (Marsolais *et al.*, 1993). Also, Mackill (1995) stated that the use of RAPD markers in DNA fingerprinting of U.S. rice cultivars is feasible. The dendrogram, in this study, divided the rice varieties into 4 groups or clusters. The increasing of number of groups can be due to the presence of high variation among the rice varieties. The number of clusters was higher compared to a previous study, e.g., two clusters in some Indian rice varieties (Rajani *et al.*, 2013). Ren *et al.* (2003) reported that the dendrogram, constructed using UPGMA from a genetic-similarity matrix based on the RAPD data, supported the clustering of distinct five groups with a few exceptions. The success of RAPD analysis in *O. sativa* accessions were also reported earlier (Muhammad *et al.*, 2005; Malik *et al*., 2008; Rahman *et al*., 2007). The analysis of SDS-PAGE can be used to certify the genetic makeup of germplasm (Javid *et al.*, 2004; Iqbal *et al.*, 2005). Thus, profiling of total seed storage proteins through SDS-PAGE for differentiating rice genotypes is well established (Thanh and Hirata, 2002). It also estimates the extent of genetic variation and its geographical distribution in rice germplasm (Asghar, 2004). Tehrim *et al*. (2011) was studied the diversity in protein profiling in rice varieties. The authors found low genetic distance among the studied varieties. In this study, a wide range of protein peptides (low to high molecular weights) did not show the potential for discriminating rice varieties and creates additional variability to supplement existing germplasm. Seed storage proteins polymorphism can be used as a potential molecular marker for varietal identification and economic characterization of rice germplasm as reported earlier (Netra and Prasad, 2007).

5. Conclusion

In conclusion, it can be seen from the present study that there are real variations among the varieties studied. RAPD marker was found to be powerful tool to analyze the genetic structure of different varieties of rice. Using RAPD markers in this study give an early and correct result about the variations among the varieties before starting a breeding program. This study showed that the numerical analysis of seed protein profiles were not sufficient as a typing tool for the differentiation of rice varieties.

Acknowledgements

The author thanks the Department of Agriculture at Sulaimanyah for their providing us with seeds and also thanks extend to the staff of Biology Department at Faculty of Sciences for supporting this work.

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