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Investigation of Genetic Diversity and Relationships among a Set of Rice Varieties in Iraq Using Random Amplified Polymorphic DNA (RAPD) Analysis

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Abstract

This study is an attempt to determine the genetic diversity and relationships among seven local rice varieties (*Oryza sativa* L.) and one commercial genotype by using the Random Amplified Polymorphic DNA (RAPD) technique. Seven universal primers used in this study produced (110) bands across eight varieties. Of these bands, 96 bands or 87.3% were polymorphic. The size of the amplified bands ranged between 190-3620 bp. The genetic polymorphism value of each primer was determined and ranged between 83-93%. In terms of unique banding patterns, the most characteristic banding pattern was for the Amber variety with primer A13 and for the Al-abasia variety with primer P07. Genetic distances ranged from 0.177 to 0.992 among rice varieties. Cluster analyses were performed to construct a dendrogram among studied rice varieties. The cluster analysis places most of the aromatic varieties into a close relation (subcluster) showing a high level of genetic relatedness and were distinct from non-aromatic (the other subcluster) with a few of independent varieties. Interestingly, a number of varieties originating from the same sources did form well defined groups, indicating association between the RAPD patterns and the geographic origin of the varieties. Therefore, cluster analysis grouped the eight varieties into three main clusters depending on their geographic origin; their ancestor and their aroma characteristics. The information generated from this study can be used in the future for rice breeding programs.

Keywords: Rice, DNA, RAPD, genetic distance, *Oryza sativa*.

1. Introduction

Rice (*Oryza sativa* L.) is one of the world's most important food crops, providing food for more than one third of the world's population. It is no longer a luxury food but has become the cereal that constitutes a major source of calories for the urban and the rural populations (Sasaki and Burr, 2000). Rice is grown in wide range of environments worldwide, even on a steep hill or mountain (Chakravarty, 1976). Most of the world's rice is grown and consumed in Asia, which constitutes more than half of the global population (Chakravarthy and Naravaneni, 2006). Iraq is one of the Asian countries which had suitable agro-climatic conditions for rice growing. Rice is the staple food for the greater majority of the Iraqi population. In Iraq, a number of traditional and improved varieties have been released for cultivation in different regions, such as the "Amber" variety, the most important traditional Iraqi rice variety (Chakravarty, 1976), and also "Furat" and "Yasmin", as introduced varieties.

There are numerous techniques available to investigate different genotypes of crop species and determine the purity of the variety to help in plant breeding programs, through improving the rice crop for the long-term and reducing of vulnerability of the crop. Prior to the availability of DNA-based markers, most genetic diversity studies in various crops were carried out using morphological and biochemical markers. Morphological and biochemical markers can be affected by environmental factors, growth practices and they are taking a long time to access, unlike DNA-based markers, which are not affected by environmental factors. Therefore, a DNA-based marker became one of these techniques which are very effective and reliable tools for measuring genetic diversity and relatedness among crop germplasm that was a major goal in evolutionary biology. The DNA based markers chosen for this study are Random Amplified Polymorphic DNA (RAPD). These markers were preferred because of the relative ease with which PCR assays can be carried out compared to other molecular markers. Advantages include rapid analysis, highly informative results, low cost and simplicity (Williams *et al.*, 1990). Single primers as short as decamers with random sequences are used to prime on both strands, producing a diverse array of PCR products (Sobral and Honeycutt, 1993). Prior knowledge about the genome is also not a prerequisite (Rabbani *et al.*, 2008; Shaptadvipa and Sarma, 2009), which makes RAPD a

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common method for such studies in different crops (Nair *et al.*, 2002; Gorji *et al.*, 2010; Szilagyi *et al.*, 2011).

Although there are a number of traditional and improved varieties of rice available in Iraq, no complete characterization or systematic analysis has been carried out so far into their genetic base and diversity, with the exception of one study by Al-Judy (2004) that studied other varieties using other universal primers. The aims of the current study are (i) identification and differentiation of various Iraqi rice varieties by generating a DNA fingerprint for each variety, (ii) estimation of the genetic diversity and determination of the genetic relationship among studied varieties by using RAPD markers.

2. Materials and Methods

2.1. Plant material

Eight rice varieties *(Oryza sativa)* were used in this study. Among the rice varieties used, one is a local variety, six are local varieties introduced from different regions (these seven varieties represent the major rice varieties currently grow in central and south regions of rice cultivation areas of Iraq), while one commercial genotype (Indian genotype) from the market was included for comparison. A detailed description of the plant materials used in the present investigation is given in table (1).

2.2. Genomic DNA Isolation

Total genomic DNA of all the studied varieties was extracted from dry seed using a commercial kit, High Pure GMO Sample Preparation Kit (Roche – Germany), to produce a rapid extraction and high quality extracted DNA. Purity and concentration of DNA was measured by spectrophotometer (Sambrook *et al.*, 1989). Genomic DNA integrity was detected by running on 0.8% agarose gel electrophoresis followed by staining with ethidium bromide and visualized under UV light (Maniatis *et al.*, 1982). DNA samples were diluted to a working concentration of 50 ng/µl in order to be use in the RAPD-PCR experiments.

2.3. Primer selection and RAPD assay

To identify primers promising detectable polymorphisms among rice varieties, nine decamers of oligonucleotides (Alpha DNA-Canada) were tested. After an initial screening, the primers were classified into two groups according to results obtained. The first group gave no amplified products and this group included (R01 and R03). The second group gave results in term of amplification and polymorphism, including (A07, A13, C05, D20, P06, P07 and R02). Only primers that had been earlier found to be polymorphic among rice varieties were used in this study.

Amplification reactions were performed in a volume of 25µl containing 12.5µl of Go Taq®Green Master Mix (Promega-USA), with concentration (1X) containing (10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 200µM each deoxynucleotide triphosphate (dNTP) and 1 unit DNA polymerase), 10pmol of the primer, and 100 ng of template DNA. Amplification was carried out using a Mastercycler (Eppendorf-Germany), using the following program:- 1 cycle of 5 min at 94°C for initial strand separation, followed by 45 cycles of 1 min at 94°C for denaturation, 1 min at 36°C for annealing and 2 min at 72°C for primer extension. Finally, 1 cycle of 10 min at 72°C was used for the final extension, followed by a hold at 4°C (Rabbani *et al.*, 2008). Each PCR amplification reaction was repeated twice to ensure reproducibility.

Twenty microliter of PCR products were analyzed by electrophoresis in a 1.2% agarose gels at 5 Volt/cm for 2 hour in 0.5xTBE (10mM Tris-Borate, 1 mM EDTA) buffer, agarose gels were stained with ethidium bromide 0.5 µg/ml for 20-30 minutes. The 1 kb DNA ladder (250-10,000) bp (Promega-USA) was used as a molecular size marker. After electrophoresis, images of gels were captured using Gel Documentation System (Consort - Belgium).

2.4. Data analysis

A- Molecular Weight Estimation

Molecular weight was calculated by using the computer software M.W. detection program, Photo-Capture M.W. program from Consort, based on comparing the RAPD-PCR products with the known size of DNA fragments of a 1Kb DNA ladder (which consist of 14 bands from 250 to 10,000 bp).

B- Estimation of Genetic Distances

Only data generated from the detection of polymorphic fragments were analyzed. The amplification profile of all the used varieties for any given primer were compared with each other, the presence of band scored as "1" and the absence of the same band of the same size in other varieties scored as "0". The intensity of the bands was not taken into account. Only clear and reproducible amplified fragments were considered for genetic relationship analysis. Estimates of genetic distance (G.D) were calculated between all pairs of the varieties according to Nei and Li (1979) based on following formula:

$$G.D = 1-\{2Nab/(Na + Nb)\}$$

Where Na = the total number of fragments detected in individual 'a'; Nb = the total number of fragments shown by individual 'b' and Nab = the number of fragments shared by individuals 'a' and 'b'.

Cluster analysis was performed to construct genetic relationship tree diagrams among studied rice varieties using an Unweighted Pair-Group Method with Arithmetic Average (UPGMA). All computations were carried out using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc), Version 1.7 package (Rohlf, 1993). The percentage of polymorphic bands was defined as ratio of the number of polymorphic bands amplified by a single primer to that of the total number of bands produced by the same primer.

3. Results and Discussion

3.1. DNA amplification and variety identification

The genetic diversity and the relationships among rice varieties were evaluated using RAPD markers amplified from nine universal primers. Among the nine decamer primers, there were two primers (R01 and R03) that give no PCR products; hence, they were eliminated from the

Table 1. Local and improved rice varieties used in the study.

Varieties' name	Pedigree	Varietal group	Breeding Institute
1.*Amber	Local (Iraqi)	Aromatic	AMRRS & SBAR
2.*Furat	Introduced from (Vietnam) in 1996	Aromatic	AMRRS & SBAR
3.*Yasmin	Introduced from (Vietnam) in 1998	Aromatic	AMRRS & SBAR
4.*Mashkhab-	Introduced from IRRI (Philippines) in 1978	Non-Aromatic	AMRRS & SBAR
5.*Mashkhab-	Introduced from IRRI (Philippines) in 1987	Non-Aromatic	AMRRS & SBAR
6.*Brnamge -4-	Introduced from IRRI (Philippines) in 2001	Non-Aromatic	AMRRS & SBAR
7.AL-abasia**	Radiation grain of Mashkhab -1- by Gamma ray	Non-Aromatic	SBSTC
8.Daawat	Commercial	Aromatic	Market

* SBAR : State Board for Agricultural Research, and AMRRS: AL-Mashkhab Rice Research Station.

**SBSTC: State Board of Seeds Testing and Certificatio

analysis. Each of the remaining seven primers varied greatly in their ability to resolve variability among varieties. Some primers generated several bands, while others generated only a few bands.

A total of 110 useful bands were scored from the amplification products with the seven random primers of DNA from eight rice varieties (Table 2). The number of amplification products generated by each primer varied from 8 (R02) to 24 (C05) and ranged in size from 190 bp (C05) to 3620 bp (P07). In general, sufficient polymorphism existed to allow distinction between the varieties tested with, polymorphism ranged between (83-93%), primer P06 produced the highest percent of polymorphism compared with primer C05. Of these 110 PCR products generated, 12.7% (14 bands) were monomorphic across all varieties. Many bands appeared in most of the varieties and were absent in only a few varieties.

The remaining 96 bands (87.3% of the total products scored) were polymorphic among the studied varieties; this was a relatively high level of the percentage of polymorphic bands obtained by random primers compared to reports of other RAPD studies in rice which were 77.4% (Nadarajan *et al.*, 1999) and 78.46% (Kumar *et al.*, 2010), while this percentage was comparatively similar to other rice studies at 89% (Rabbani *et al.*, 2008).

A total of 96 (87.3%) polymorphic bands were observed, ranging from 7 (R02) to 20 (C05) bands with an average of (13.7) polymorphic bands per primer across all the eight rice varieties. This average was similar to that observed in other rice studies using RAPD markers with Indian scented basmati and Italian rice cultivars. These reports observed that the average number of polymorphic bands per primer were 13.7 and 14.0 (Raghunathachari *et al.*, 2000; Porreca *et al.*, 2001), respectively. The average number of polymorphic bands was relatively higher than earlier reports, with an average of 2.7, 4.4, 5.4 and 6.6 polymorphic bands per primer (Virk *et al.*, 1995; Parsons *et al.*, 1999; Choudhury *et al.*, 2001; Skaria *et al.*, 2011). This discrepancy may relate to varieties and the selection of RAPD primers with scorable bands or the use of more diverse varieties. The arbitrary primer A13 was useful for discriminating varieties of distinct characteristics (Figure 1).

Some varieties could be distinguished from all other varieties with a selection of these primers. For instance, Amber and Daawat gave specific banding patterns with primers A13, D20, P07 and R02, and Al-abasia gave unique banding patterns with primers P07 and R02; while Furat gave unique banding patterns with primers C05 and P06. In most cases, the varieties of Yasmin and Furat, Mashkhab-1- and Mashkhab-2-, and also Brnamge-4- and Al-abasia were genetically related, although clear differences between them could be seen.

3.2. Genetic Distances

The ratio of genetic similarity among the eight varieties ranged from 0.007 to 0.822 (Table 3). The highest similarity (0.822) 82% was obtained between 'Furat' and 'Yasmin'. This was followed by (0.75) 75% similarity

between a pair of 'Mashkhab-1-' and 'Mashkhab-2-'. The lowest level of similarity (0.007) 0% was obtained between 'Amber' and 'Dawaat'.

In this study, the highest value of genetic similarity is relatively low when compared to the reports of other RAPD studies that obtained 0.50 to 0.96% genetic similarity among traditional and improved cultivars of Pakistani rice (Rabbani *et al.*, 2008), 49-89% genetic similarity among other Iraqi rice varieties (Al-Judy, 2004), and the Jaccard's similarity coefficient values ranged from 0.29 to 1.00% among the traditional medicinal rice cultivar of Kerala (Kumar *et al.*, 2010). However, similar values of similarity coefficients were obtained 0.83% among Indian elite rice varieties (Davierwala *et al.*, 2000) indicating a narrower genetic base in the improved varieties. Likewise, similarity coefficients ranging from 25 to 77.5% were observed among scented rice cultivars from India (Raghunathachari *et al.*, 2000).

3.3. Cluster analysis

Dendrogram was constructed based on Nei and Li's (1979) genetic distance using UPGMA cluster analysis and depicted genetic relationships among eight rice varieties, showing three major clusters I, II and III (Figure 2). As expected all introduced varieties: Yasmin, Furat, Mashkhab-1-, Mashkhab-2- and Brnamge-4-, were grouped into a cluster, including two subclusters, and other varieties Amber and Daawat into other two clusters. The first group, Yasmin and Furat formed one subcluster with the highest genetic similarity 82%. These were introduced from Vietnam and are aromatic varieties.

Table 2. Primers used for generating RAPD in traditional and improved varieties of rice from Iraq.

No.	Primer	Sequence (5'~3')	Total number of main bands	Number of polymorphic bands	Polymorphism %
1	A07	-GAAACGGGTG-	15	13	87
2	A13	-CAGCACCCAC-	15	13	87
3	C05	-GATGACCGCC-	24	20	83
4	D20	-ACCCGGTCAC-	20	18	90
5	P06	-TCGGCGGTTC	14	13	93
6	P07	-CTGCATCGTG-	14	12	86
7	R02	-GTCCTCGTGT-	8	7	88
8	R01	-CACACCGTGT-	---------	----------	----------
9	R03	-ACGGTTCCAC-	---------	----------	----------
	Total		**110**	**96**	

Table 3. Genetic distances among rice varieties based on the RAPD data.

Rice Varieties	Amber	Furat	Yasmin	Mashkhab-1-	Mashkhab-2-	Brnamge-4-	AL-abasia	Daawat
Amber	0.00000							
Furat	0.60391	0.00000						
Yasmin	0.47724	0.17784	0.00000					
Mashkhab-1-	0.52110	0.38819	0.27233	0.00000				
Mashkhab-2-	0.49471	0.42286	0.32547	0.24508	0.00000			
Brnamge-4-	0.56818	0.36115	0.33626	0.40510	0.31114	0.00000		
AL-abasia	0.71233	0.52131	0.50801	0.45009	0.52131	0.45383	0.00000	
Daawat	0.99257	0.64096	0.77022	0.75487	0.68018	0.68527	0.57503	0.00000

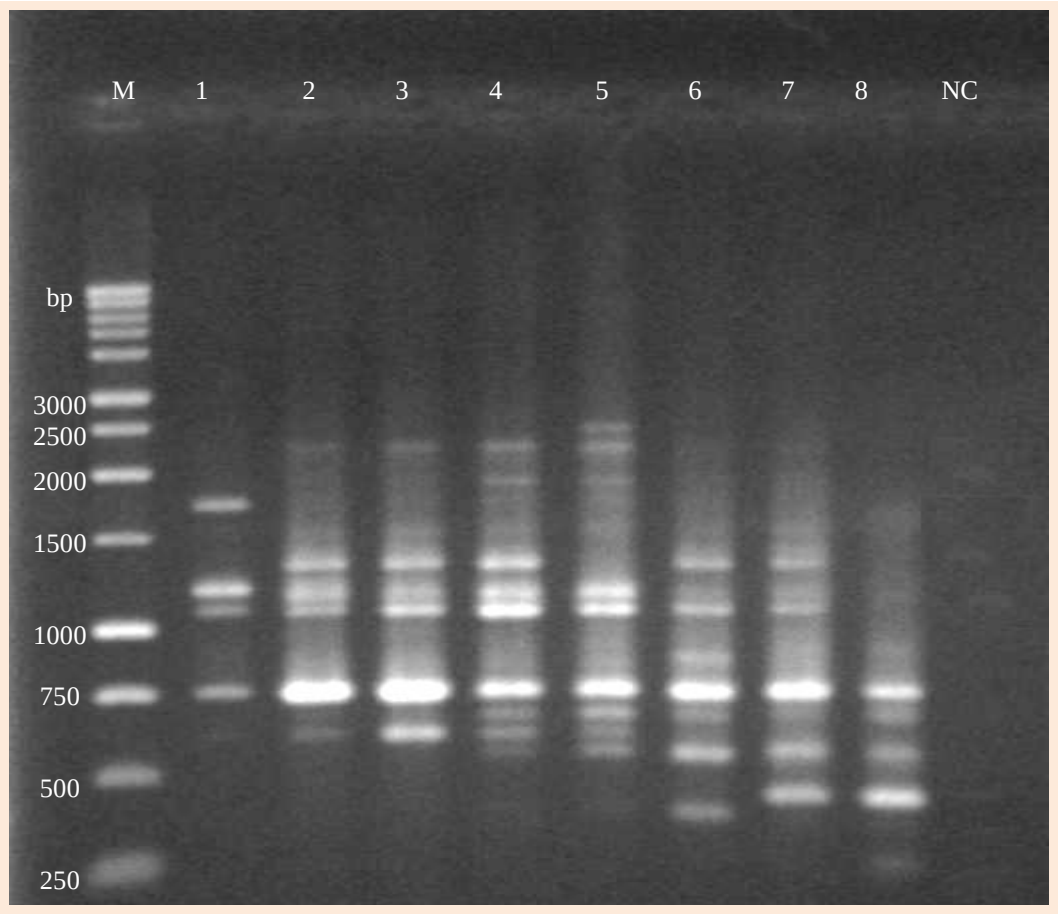

Figure 1. Agarose gel electrophoresis of a RAPD-PCR reaction for random primer A13 for DNA samples of the rice plants (under optimal conditions). Bands were fractionated by electrophoresis on a 1.2% agarose gel (2hr, 5V/cm, 0.5XTris-borate buffer) and visualized by ethidium bromide staining. M:1Kb ladder. Lanes: 1.Amber, 2.Furat, 3.Yasmin, 4.Mashkhab-1-, 5.Mashkhab-2-, 6.Brnamge-4-, 7.AL-abasia and 8.Daawat. NC: Negative control.

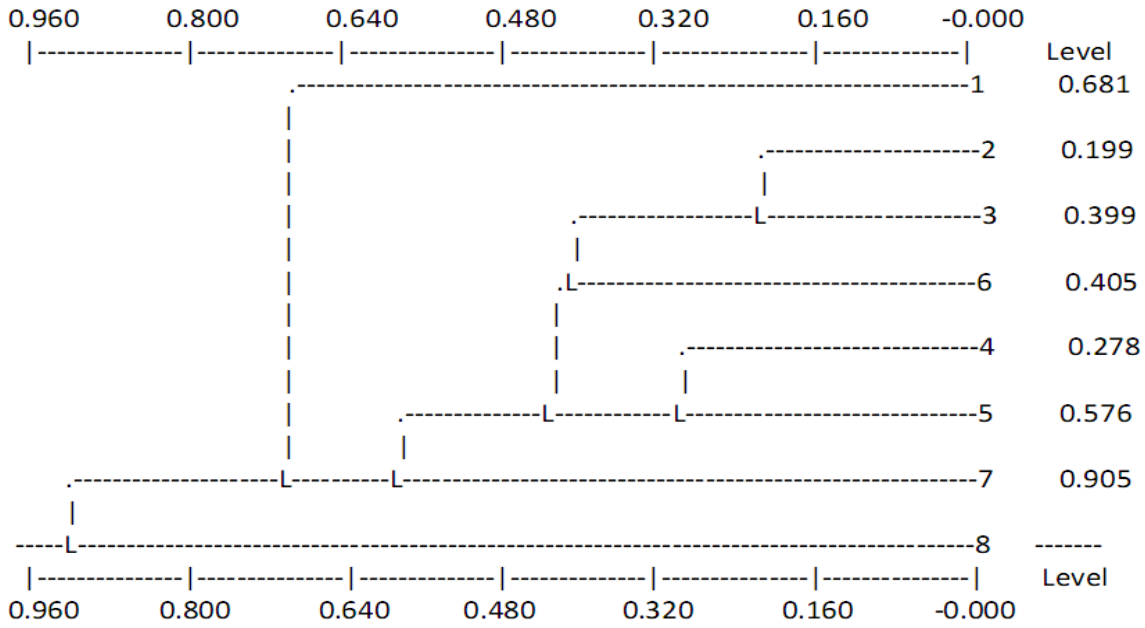

Figure 2. Dendrogram of rice varieties showing genetic distances based on RAPD data by using UPGMA cluster analysis. 1.Amber, 2.Furat, 3.Yasmin, 4.Mashkhab-1-, 5.Mashkhab-2-, 6.Brnamge-4-, 7.AL-abasia and 8.Daawat.

Mashkhab-1- and Mashkhab-2- formed another subcluster. These varieties were introduced from IRRI (Philippines) and are non-aromatic varieties. The Brnamge-4- variety was added to both subclusters. Though the variety Al-abasia was improved by radiating the grain of Mashkhab-1- with Gamma rays, it clustered with the IRRI varieties. In this analysis, the last Iraqi rice variety, Amber, appeared to be genetically distinct due to the lower level of the similarity with all other varieties. Therefore, it formed a separate group (II group). Likewise, the commercial variety 'Daawat' did not fall into any groups of Iraqi rice and it exhibited the lowest similarity with all varieties and also formed a separate group (III group).

Cluster analysis has placed most of the aromatic varieties together, showing a high level of genetic relatedness and these were distinct from those of non-aromatic varieties. Also the dendrogram indicates a clear pattern of division among the rice varieties based on geographic origin of the varieties. Therefore, cluster analysis grouped the eight varieties into three main clusters which correlated with their geographic origin; their ancestor and their characteristics. Similar results were reported in the study previously conducted using RAPD markers, where grouped rice varieties into different main groups depending on their geographic origin and their ancestor (Nadarajan *et al.*, 1999; Al-Judy, 2004; Skaria *et al.*, 2011) as also reported in other crops (Zhang *et al.*, 2005; Raju *et al.*, 2009; Al-Rawashdeh and Al-Rawashdeh, 2011; Kanbar and Kondo, 2011). The analysis clearly distinguished among studied rice varieties. Such studies can be used to study genetic differences of varieties for their identification. Therefore, it might be predicted that RAPD may be effective in analyzing polymorphism at the subspecies level in genus *Oryza*. In the present study RAPD markers provided sufficient resolution to distinguish closely related varieties.

The RAPD method may contribute to maximize the selection of diverse parent variety and to broaden the germplasm base in the future of rice breeding programs. The information generated from this study gives a clearer picture of their genetic relationship and might possibly be developed into a standard classification procedure in the future and will be used in identifying efficient strategies for the sustainable management of the genetic resources of rice crop.

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