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Full Length Research Paper

Genetic analysis of some Egyptian rice genotypes using RAPD, SSR and AFLP

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Information of genetic similarities and diversity among superior Egyptian rice genotypes is necessary for future rice breeding programs and derivation of plant lines. Genetic variability and relationships among seven Egyptian rice genotypes namely Giza 178, Giza177, Giza 175, Giza171 Giza 172, Sakha 102, and Sakha 101 were established by using eight RAPD primers, six SSR primer pairs, eight AFLP primer combinations. The level of polymorphism as revealed by RAPD, SSR and AFLP was 72.2, 90, and 67.9%, respectively. The highest genetic relationship as revealed by combined RAPD, SSR, and AFLP was detected between Giza 175 and Giza177 (83.4%), while the lowest similarity was found between Giza 178 and Sakha 101 (61.5%). Dendrograms derived from different techniques include minor differences in clustering pattern but did not affect the main grouping of the different genotypes. Moreover RAPD, SSR and AFLP-based dendrograms clustered the two genotypes Giza171 and Giza 172 together and the two genotypes Giza 175 and Giza 177 in the same cluster. RAPD, SSR, and AFLP techniques characterized the seven rice varieties by a large number of unique markers, which revealed 17, 4, and 65 unique markers, respectively. It could be concluded that each type of the three molecular approaches of DNA analysis could identify the different rice genotypes, and some of the Egyptian rice genotypes under investigation have probably originated from closely related ancestors and possess high degree of genetic similarity.

Key words: *Oryza sativa*, DNA markers, genotype identification.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important crops that provide food for about half of the world population. In areas such as Asia, Africa and Latin America where the demand for rice is a top priority, the population is expected to increase 1.5-fold by 2025 (Sasaki, 1999 and 2002). Egypt is one of the few countries which produce high yielding rice varieties. From the commercial point of view, DNA fingerprinting is a useful tool for varietal protection to prove ownership or derivation of plant lines. Moreover, the analysis of genetic diversity and relatedness between or within different species, populations and individuals is a prerequisite towards effective utilization and protection of plant genetic resources (Weising et al., 1995). With DNA being the only basis of genetic differences between distinct organisms, DNA fingerprinting is presently the ultimate method of biological individualization. In principle, genetic uniqueness is brought about by two factors, inheritance and new mutations, and since all genetic differences between individuals are laid down in the primary sequence of their genomic DNA, the most straightforward method is identifying an individual sequence for genomes under comparison (Krawczak and Schmidtke, 1994).

Unlike the morphological and biochemical markers which may be affected by environmental factors and growth practices (Xiao et al., 1996, Ovesna et al., 2002,

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Higgins 1984). DNA markers portray genome sequence composition, thus, enabling to detect differences in the genetic information carried by the different individuals. A wide variety of DNA-based markers have been developed in the past few years. Restriction fragment length polymorphism (RFLP) was the first molecular marker (Botstein et al., 1980), generated for genome analysis and mapping. However, the development of the polymerase chain reaction (PCR) technology has introduced a considerable number of useful molecular markers, e.g., RAPDs (Welsh and McClelland 1990, Williams et al., 1990), AFLPs (Vos et al., 1995) and SSRs (Cregan 1992). Because each marker system has specific advantages and disadvantages, the choice of the marker system to be used is the most important decision. To compare the effectiveness, several comparisons among the different classes of PCR-based markers were carried out. For instance, Palombi and Damiano (2002) compared RAPD and SSR-markers to detect genetic variability in kiwifruits. Similarly, Ferdinandez and Coulman (2002), Corazza-Munes et al. (2002), Archak et al. (2003) and McGregor et al. (2000) either compared the efficiency of RAPD, SSR and AFLP or used the different classes of markers for genotype identification. Recently, the efficacies of different classes of PCR-based markers were also used to characterize barley and rice cultivars (Saker et al., 2005 and Virk et al., 2000).

The objectives of this study were to compare the effectiveness of different PCR-based molecular approaches to determine the genetic relationships among several Egyptian rice genotypes and the suitability of the different approaches for developing unique molecular markers characterizing the seven rice varieties and therefore, developing unique fingerprint for each variety.

MATERIALS AND METHODS

Plant material

The seeds of seven Egyptian rice cultivars, namely Sakha 101, Sakha 102, Giza 171, Giza172, Giza 175, Giza177 and Giza 178 were secured from the Rice Research Department, Agricultural Research Center, Ministry of Agriculture, Egypt. The cultivars were grown in the greenhouse of the Genetic Engineering Research Center, Faculty of Agriculture, Cairo University. All cultivars are diploid with chromosome no. 2n=24.

RAPD analysis

Total DNA was isolated from young leaves of 10 day-old seedlings, grown in the green house, according to Walbot (1988). RAPD analysis was performed according to Williams et al. (1990) with minor modifications. PCR reactions were carried out in 25 µl volume containing 25 ng of total genomic DNA, 10 pmol primer, 200 µM dNTPs, 2 mM MgCl2, 1X PCR buffer and 0.4 µl (2 units) AmpliTaq Polymerase. Ten 10-mer oligonucleotide random primers were selected for analysis. They were obtained from Carl Roth GmbH Co, Germany. Amplification was performed in a Perkin-Elmer 9600 thermal cycler with the following profile: 94^{0}C for 5 min (initial denaturation), 94°C for 1 min, 36°C for 1 min, 72°C for 90 sec for 40 cycles with a final extension at 72°C for 7 min.

SSR analysis

A set of SSR primers (Panaud et al., 1996) were used. The PCR reaction was carried out using golden Taq polymerase (Promega) in 20 µl reaction volume containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 1 µM of forward and reverse primers, 1 unit golden Taq polymerase and 25 ng genomic DNA. Profile was used as follows: an initial hot start and denaturing step at 94ºC for 10 min followed by 35 cycles at 94ºC for 1 min, appropriate annealing temperature for 1 min, and primer elongation at 72ºC for 1 min. A final extension step at 72ºC for 7 min was performed.

AFLP analysis

AFLP was performed as described by Vos et al. (1995) using the GIBCO BRL system I (Cat. No. 10544) according to the manufacturer's protocol.

Electrophoresis

The RAPD-PCR products were analyzed directly on 1.5% agarose gels in TAE buffer, visualised by staining with ethidium bromide and transillumination under short-wave UV light. Amplified DNA products using SSR primers were examined by horizontal electrophoresis in 3.5% Metaphor (FMC Bioproducts, Rockland, ME) agarose gels. AFLP amplification products were separated in a vertical denaturing 6% polyacrylamide gel in a Sequi-Gen Cell (BioRad Laboratories Inc.) as described by Bassam et al. (1991). The DNA fragments were visualized by autoradiography.

Data analysis

Pairwise comparison of genotypes, based on the presence (1) or absence (0) of unique and shared polymorphic products was used to generate similarity coefficients using statistical software package STATISTICA-SPSS (Stat Soft Inc.). The similarity coefficient was used to construct a dendrogram by the unweighted pair group method with arithmetic averages (UPGMA) according to Rohlf (1993).

RESULTS

RAPD analysis

Genetic relationship among the seven Egyptian rice genotypes has been carried out using RAPD. Eight primers generated reproducible, informative and easily scrabble RAPD profiles were preselected. These primers produced multiple band profiles (Figure 1) with a number of amplified DNA fragments varying from 8 to 14, with a mean number 10.9 markers per primer. The primer OPO-10 gave the highest number of fragments (14), while the minimum number of fragments (8) was amplified with primers OPA-10 and OPB-05. A total of seventy six

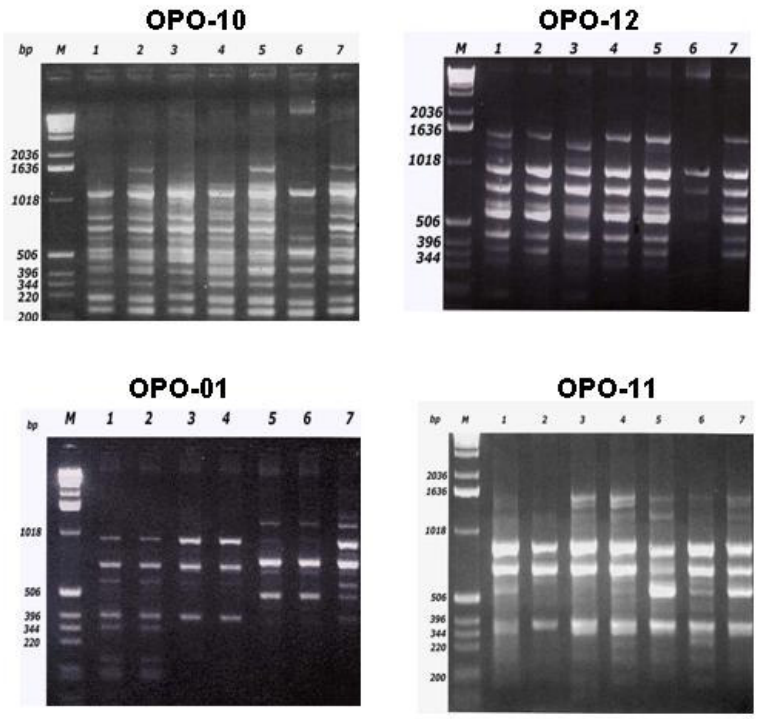

Figure 1. RAPD profiles of the seven rice genotypes.Lanes from: 1 to 7 represent Sakha 101, Sakha 102, Giza 171, Giza 172, Giza 175, Giza 177 and Giza 178.bM: DNA marker (1kb).

Table 1. Rice varieties characterized by unique positive and/or negative RAPD markers, marker size and the total number of markers identifying each variety.

Variety	Unique positive markers			Unique negative markers			Grand Total
	Size of the marker band (bp)	Primer	Total no. of markers/ variety	Size of the marker band (bp)	Primer	Total no. of markers/ variety	
Sakha 101	------	--------	0	250 450	OPA-05 OPB-04	1 1	2
Sakha 102	------	------	0	984,891,500 415,340 344 450 1565	OPO-12 OPO-11 OPO-10 OPB-10	5 1 1 1	8
Giza 172	2060	OPB-05	1	-------	------	0	1
Giza 178	2045 220 2016,495 210	OPO-01 OPO-12 OPA-05 OPB-05	1 1 2 1	985	OPO-10	1	6
Total			**6**			**11**	**17**

polymorphic bands were observed with the seven selected primers ranged from 6 to 10 per primer. The highest number of polymorphic bands (10) was obtained with primer OPO-11, with 83% polymorphism. The average number of polymorphic fragments per primer among the 7 rice varieties was 7.86.

The RAPD assay permitted the identification of only four rice varieties by unique positive and/or negative markers from the other varieties. Sakha 102 was characterized by eight negative unique RAPD markers, while only two unique negative markers were obtained with Sakha 101. Giza 172 was characterized by only one positive unique RAPD marker. On the other hand, Giza 178 was characterized by one negative unique RAPD marker and five positive unique RAPD markers. Among the tested primers three (OPA-05, OPB-05 and OPO-12) exhibited positive and negative markers, OPO-01 revealed only positive marker and OPO-10 and OPO-11 produced negative markers only (Figure 1). The RAPD primers which generate the different markers and the markers approximate size are listed in Table (1). Sakha 102 was characterized by the highest number of negative unique markers (8) but no positive markers, five of negative unique markers resulted from OPO-12. The size of the different unique markers ranged from 125 to 1565 bp. Certain primers, were more informative than the others e.g., OPB-05 which identified the highest number of varieties (two positive unique markers).

The genetic similarity among the seven genotypes ranged from 53 to 88.5%. The highest genetic similarity revealed by the RAPD analysis was between Giza 175 and Giza 177. This was followed by 85.2% between Giza 175 and Giza 178 and 82.6% between Giza 172 and Giza 171. On the other hand, the lowest percentage of similarity was obtained between Sakha 102 and Giza 178 (53%). The RAPD-based dendrogram (Figure 4) separates the Sakha102 variety from all the other rice varieties in the first cluster. While, the other rice varieties in the second cluster are divided into two subclusters. Each subcluster is subdivided into two groups, the first group in the first subcluster contains Sakha 101, and the second group includes Giza 171 and Giza 172. Also the other two groups in the second subcluster contain Giza 175, Giza 177 and Giza 178.

SSR analysis

Six primer pairs flanking dinucleotide simple sequence repeats (GA or AG) were used to investigate the level of polymorphism among the seven rice varieties. All primers produced fragments, even when using modified amplification conditions. The six SSR primer sets which are distributed through 6 different rice chromosomes revealed 25 alleles, eight alleles with RM-10 primer set, six with T92, and five alleles with RM13 primer set, four alleles to each RM-8 and two with RM-3 and RM-14 primer sets. All primers showed different levels of polymorphism except RM-14 (chromosome 1) which showed no polymorphism among the seven rice varieties (Figure 2). Most of the alleles were polymorphic, thus revealing 92% polymorphism. The number of alleles per locus ranged from 2 to 8 with an average of 5 alleles per locus. The size of the detected alleles produced from using the SSR primer sets ranged from 50- 500 bp which reflect a large difference in the number of repeats between the different alleles.

A total of 2 primers out of 6 revealed 4 unique SSR alleles (2 positive and 2 negative) for characterizing two rice varieties (Table 2). The primer RM-10 characterized Giza 175 by two positive unique SSR markers producing 300 and 450 bp alleles. On the other hand, the primer RM-13 characterized Giza 177 by two negative unique SSR markers giving 150 and 200 bp alleles.

The estimated similarities among the seven rice varieties ranged from 30.8 to 90% between Giza 178 and Sakha 102 and between Giza 171 and Sakha 102, respectively. These similarity levels were higher than those obtained by RAPD between Giza and Sakha varieties. The varieties of rice showed high genetic similarity percentages (84.2%) between Giza 177 and Giza 175, and between Giza171 and Giza 172 (82%). The SSR-based dendrogram (Figure 4) separated Giza 178 variety from all the other rice varieties in the first cluster. However, the second cluster divided into two subclusters; the first subcluster contained Giza 175 and Giza 177. The second subcluster contained two groups, Sakha 101 being in the first group. The second group is divided into two subgroups, Giza 172 in the first subgroup and Sakha 102 and Giza 171 in the last subgroup.

AFLP analysis

The AFLP analysis was performed using eight selective primer combinations and generated a total of 536 bands. Figure (3) illustrates the AFLP profile of the seven rice varieties as revealed by the eight primer combinations. The number of markers observed per primer combination ranged from 50 to 90 with an average of 67 markers per primer. The total accounting marker number was 536 amplified bands, representing 67.9% polymorphism and an average number of polymorphic bands of 45.5 per AFLP primer combination. The highest percentage of polymorphism was obtained with *E-CAC/M*-CAT (91.1%) then *E-AGT/M*-CTC (72.9%), and with *E-AGT/M*-CAG (65.8%). While *E-CAC/M*-CAT produced the highest polymorphic bands (82) and the lowest number was obtained with *E-AGT/M*-CAT (32). As shown in Figure 3, the size of AFLP fragments generated by the different primer combinations ranged from 25-350 bp and the polymorphic fragments were distributed across the entire size range. In addition, the number of fragments

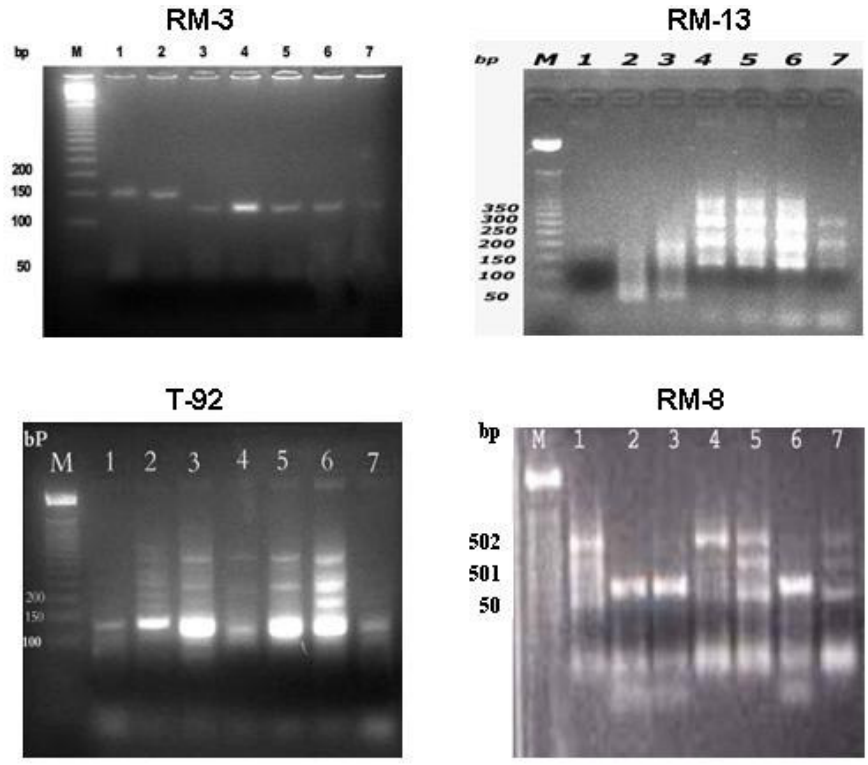

Figure 2. Multiple loci detected by SSR in between the seven rice genotypes. Lanes from: 1 to 7 reppresent Giza 178, Giza 177, Giza 172, Giza 171, Sakha 102 and Sakha 101. M: DNA ladder marker (50-bp).

Table 2. Rice varieties characterized by unique positive and/or negative SSR markers., marker size and total number of markers identifying each variety.

Variety	Unique positive markers			Unique negative markers			# of alleles
	Primer	Allele size (bp)	Total	Primer	Allele size (bp)	Total	
Giza 175	*RM-10*	450.300	2	------	------	------	2
Giza 177	------	------	------	RM-13	200,150	2	2
			2			2	4

produced by the different primer combinations ranged from 50 (*E-AGT/M*-CAT) to 90 (*E- CAC/M*-CAT).

The unique AFLP markers that characterized the seven rice varieties are listed in Table (3). In the present study, 7 primer combinations out of 8 the were able to characterize the seven rice varieties by a total of 65 unique AFLP markers (14 positive and 51 negative) with an average of 9.3 markers for each primer combination. Sakha 101 produced by the highest number of unique AFLP markers, giving 40 unique markers (3 positive and 37 negative), followed by Giza 171 which was characterized by 10 unique AFLP markers (3 positive and 7 negative). While Giza 172 was characterized by the lowest number producing only one unique positive marker. The primer combination *E-CAC/M*-CAT revealed the highest number of unique markers (2 positive and 33 negative markers). *E-CAC/M*-CAG produced 9 unique markers (7 positive and 2 negative markers). On the other hand, *E-AAC/M*-CTG and *E-AGT/M*-CAT are the same revealed the lowest number of unique markers (1 positive and 1 negative).

The similarity level among the seven varieties ranged from 61 to 81%. The highest similarity percentage was observed (81%) between Giza175 and Giza 172 followed by (79%) between Giza 177 and Giza 178, and between Sakha 102 and Giza 171. On the other hand, the lowest percentage of similarity was found between Giza 177 and Sakha 101 (61%). The AFLP based-dendrogram (Figure 4) separated Sakha 101 from the remaining 6 varieties in the first cluster. The second cluster contains two sub clusters. The first subcluster contains Giza 178 and Sakha 102 and the second was divided into two

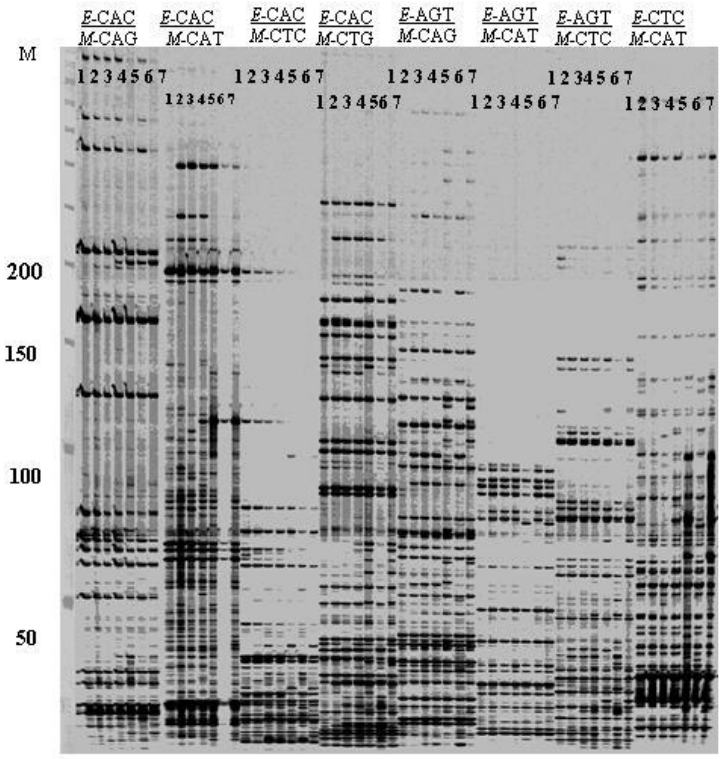

Figure 3. AFLP profile of the seven rice varieties as detected with eight primers combinations. Lanes: 1 to 7 represent Giza 178, Giza 177, Giza 175, Giza 172, Giza 171, Sakha 101, Sakha 102. M: 50 bp DNA ladder.

Table 3. Rice varieties characterized by unique positive and/or negative AFLP markers.

Variety	Unique positive primers			Unique negative Primers			Grand Total
	Primer combination	Molecular Weight (bp)	Total	Primer combination	Molecular Weight (bp)	Total	
Sakha 101	Ecac/ Mcag	175,90,55	3	Ecac/Mcat	250,210,205, 197,100,97, 95,89,84,83, 82,55,54,53, 51,48,49,45, 43,44,41,38, 36,35,34,30, 25,24,22,20, 19,15	33	40
				Eagt/Mctc	147,120,100 98	4	
Sakha 102				Ecac/Mcag Eagt/Mcag	53 145	1 1	2
Giza 171	Ecac /Mcag Ecac / Mctc Eagt / Mcag	96 60 174	1 1 1	Ecac / Mctc Ecac / Mctg Eagt / Mcag	40 220,196,194 250,180,50	1 3 3	10
Giza172	Ecac / Mcat	180	1				1
Giza 175	Eagt / Mctc	50	1	Ecac / Mctg	40	1	3
				Eagt / Mcat	49	1	

Table 3. contd.

Giza 177	Ecac /Mcag Ecac / Mcat	148 145	1 1	Eagt / Mcag	153	1	3
Giza 178	Ecac /Mcag Ecac / Mctg Eagt / Mcat	115,95 95 45	2 1 1	Ecac /Mcag Eagt / Mcag	375 185	1 1	6
Total			14			51	65

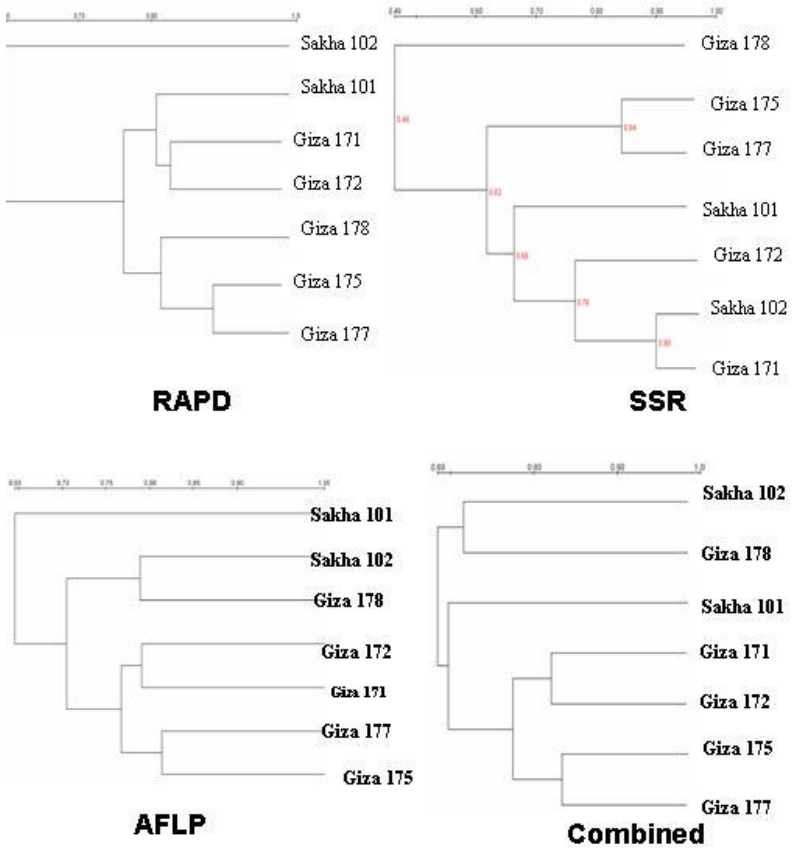

Figure 4. RAPD, SSR, AFLP and Combined (RAPD +SSR + AFLP)-based dendograms of the seven rice genotypes constructed using unweighted pair –group arithmetic average (UPGMA) and similiarity matrices computed according to Dice coefficients.

main groups and contains Giza171, Giza 172, Giza 175 and Giza 177.

Combined genetic relationships

The similarity matrices resulting from the combined DNA markers data were performed to generate correct relationships based on large and different genome regions. The highest percentage of similarity was detected between Giza 175 and Giza 177 (83.4%) followed by 82.1% between Giza 171 and Giza 172 and 81.3 % between Giza 172 and Giza 175. On the other hand the lowest similarity matrix (61.5%) was obtained between Giza 178 and Sakha 101. The dendrogram built on the basis of combined data from RAPD, SSR and AFLP analysis represents the genetic distances among the seven rice varieties (Figure 4). The dendrogram includes two clusters, the first containing Sakha 102 and Giza 178, while the second cluster contains two sub

clusters. The first subcluster contains Sakha 101 and the second is divided into two main groups which contain Giza171, Giza 172 in the first group and Giza 175 and Giza 177 in the last group.

DISCUSSION

The data of the present study indicate that SSR gave the highest level of polymorphism (90%) followed by RAPD (72.9%) and AFLP (67.9%). Yang et al. (1994) stated that the greater resolving power of the SSR assay can provide more informative data than other techniques. However, in the present study, SSR analysis was less informative in characterizing closely related Egyptian rice genotypes, compared with RAPD and AFLP. In this context, Choudhury et al. (2001) reported that RAPD analysis was able to distinguish between all of the rice genotypes. Although none of the primers individually was so informative as to differentiate all the genotype, highly polymorphic profiles were obtained with different primers. Also Mackill (1995) stated that the use of RAPD markers in DNA fingerprinting of U.S. rice cultivars is feasible. Moreover, 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. 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). Data of the present study confirmed the efficacy of RAPD as a molecular marker which could be used to distinguish different Egyptian (Japonica) rice genotypes Similarly, Mackill (1995) classified the Indica and Japonica cultivars into separate groups by cluster analysis. Clustering was less pronounced within the Japonica group. Ge et al. (1999) found that 61.8% of the genetic diversity is distributed between the populations of China and those of Brazil, whereas less than 40% of the genetic diversity resides between population within regions (14.9%) and between individuals within populations (23.3%).

In the case of SSR, the six SSR primer sets which are distributed through 6 different rice chromosomes revealed 25 alleles and the majority of the alleles were polymorphic, thus revealing 92% polymorphism and average number of allele per locus is 5. In this respect, Wu and Tanksley (1993) detected comparable number of alleles per locus, 2 to 5 at different loci within 6 cultivars of the Japonica subspecies and 2 to 6 alleles within 8 cultivars of Indica subspecies. While Yang et al. (1994) found that more alleles were resolved in Indica than in Japonica, in the total sample, ten (14%) more alleles were detected in Indica than in Japonica. They reported that there is a very good linear relationship between the number of alleles detected at a locus and the total number of SSRs within the target microsatellite DNA. Thanh et al. (1999) detected 41 alleles with an average of 2.9 alleles per locus using 14 primer pairs with 13 upland rice lines from Vietnam. In conclusion, SSR analysis of the present study clearly separated the seven Egyptian rice genotypes. Similarly, Maroof et al. (1997) reported that the cluster analysis revealed by SSR separated Jasmine 85 from the remaining seven lines. Thanh et al. (1999) found that the SSR-based dendrogram resolved the 31 Vietnamese upland rice accessions into two major groups.

AFLP amplified 50-90 fragments per primer combination in Egyptian rice cultivars and the percentage of polymorphism per primer assay is 45.5%. In this context, Mackill et al. (1995) used seven *Eco*RI and seven MseI primers in 18 combinations in AFLP analysis and generated 147 polymorphic bands among rice varieties (12 Japonica and 2 Indica varieties) with 24% polymorphism. Zhu et al. (1998) detected 179 polymorphic bands (44% polymorphism), when using four combinations of AFLP primers which generated a total of 410 AFLP bands. Mueller and Wolfenbarger (1999) stated that the AFLP markers were effective in the detection of polymorphism among closely related species; they have also been used to infer phylogenetic relationships based on measures of genetic distance. While, Bligh et al. (1999) reported that only 22 out of 142 amplification products were observed to be polymorphic among six long grain rice cultivars. On the other hand, Ovesna et al. (2002) reported that the AFLP technology is a powerful tool for the detection and evolution in germplasm collections and in the screening of biodiversity as well as for fingerprinting studies.

Analysis of the combined data obtained from the different DNA markers revealed that RAPD, SSR, and AFLP techniques characterized all investigated rice varieties by a large numbers of unique markers. It is clear that AFLP technique was able to differentiate rice varieties under study by a higher number of unique markers compared to RAPD and SSR techniques. This could be attributed to the different mechanisms of polymorphisms detection using different marker systems. AFLP assay reflects restriction size variation, depending on the number of markers used, genome coverage, degree of linkage unbalanced between individuals and on the type of DNA sequence variation detected with each marker system (Mueller and Wolfenbarger, 1999 and Ovesna et al., 2002). In this context, Luis et al. (1999) used data from isozyme, RAPD and AFLP analyses to compute matrices of genetic similarities. Although the genetic variability found among the different cultivars was low, both RAPD and AFLP markers proved to be efficient tools in assessing the genetic diversity of rice genotypes. Similarly, Mackill et al. (1996) found that the UPGMA analysis depending on the RAPD and AFLP data gave similar results; all cultivars were classified into the same subspecies (Indica vs. Japonica) and into the same group (temperate Japonica vs. tropical Japonica).

In conclusion, RAPD, SSR and AFLP-based dendrograms as well as combined RAPD, SSR, and AFLP dendrogram clustered the genotypes Giza 171 and Giza 172 in one cluster. These results are in agreement with the genetic background of Giza 175 and Giza 177. Both genotypes have a common ancestors, i.e. Giza 171 (Nahda x Calady40) and Giza 172 (Nahda x Kinmaze). Similarly Sakha 101 (Giza 176 x Milyang 79) and Sakha 102 (Giza 177 x Giza 4098-7-1) are clustered in a separate cluster as they have far genetic background from each other and from the rest of the investigated genotypes.

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