

The genetic diversity and relatedness of rice (*Oryza sativa* L.) cultivars as revealed by AFLP and SSRs markers

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Abstract: A set of SSR and AFLP markers was applied to assess the levels of genetic diversity in eight rice genotypes. A total of 12 SSR and 3 AFLP markers combinations revealed 100% and 77.56% polymorphism, respectively. A total of 46 and 245 polymorphic bands were detected in both SSR and AFLP markers, respectively. The number of SSRs polymorphic bands per locus varied from 2 for *RM133*, *RM 215* and *RM433* to six for *RM271*, with a mean of 3.83. Whereas, the number of AFLPs polymorphic bands ranged from 19 for E37/M41 to 176 for E36/M41 with an average of 81.67. PIC value for SSR and AFLP was 0.825 and 0.967, respectively. A significant correlation coefficient between gene diversity and the number of polymorphic bands for SSR and AFLP was high, $r = 0.096$ and $r = 0.742$ ($P < 0.01$), respectively. Pair-wise genetic similarity ranged from 0.101 to 0.682 and from 0.393 to 0.783 and for SSR and AFLP, respectively. Cluster analysis was achieved based on SSRs and AFLP data and the relationship between the studied rice cultivars were addressed.

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1. Introduction

Rice (*Oryza sativa* L.) is the second most important cereal crop in the world exceeded only by wheat (Poehlman & Sleper, 1995). It is grown in the temperate zone; (91%) in Asia, (5.8%) in North and South America, (2.8%) in Africa and (0.4%) in the southern part of Europe (Konokhova, 1985). The detection and characterization of genetic variation present in germplasm collections is important for plant breeding programs seeking to widen the genetic base of breeding populations.

Rice possesses the smallest genome size (400-500 Mbp) in monocotyledonous *Gramineae*, for this reason, it is considered the model system for studying *Gramineae* genome (Hart and Jones, 2001). Based on comparative mapping with other *Gramineae*, the rice genome has extensive synteny with other *Gramineae* such as maize, sorghum, wheat or barley (Moore *et al.*, 1995; Saghai-Marouf *et al.*, 1996; Gale and Devos 1998 and Smilde *et al.*, 2001). Dennis (2001) reported that genomics has led to a new paradigm for the study of biology and the research will focus on studying all the genes in an organism instead of studying individual genes. In plants, genomics has been very dependent on the use of two model organisms, *Arabidopsis* as a general model for dicots and rice as model for monocots.

Molecular markers provide novel tools to differentiate among different plant genetic resources. Different marker systems are currently available for monitoring genetic diversity. Model markers with contrasting characteristics include amplified fragment length polymorphism (AFLP) as dominant and biallelic markers (Vos *et al.*, 1995) and simple sequence repeat (SSR) as co-dominant markers that usually reveal a high number of alleles (Tautz, 1989). Also, AFLP technique provides many markers that randomly spread in the genome, whereas in a diversity study, the number of analyzed microsatellites is usually low. The comparative analysis between different markers is therefore intended to reveal the balance between different evolutionary forces contributing to genetic diversity.

Great efforts have been undertaken to obtain detailed genetic and physical maps of the rice genome (Ideta *et al.*, 1994 and Yano *et al.*, 1996). Several scientists have interest to study and analyze the rice genome in order to localize the economic characters to their positions on the rice chromosomes (Mackill *et al.*, 1996; Powell *et al.*, 1996 and Lin *et al.*, 2000). Causa *et al.* (1994) and Kurata *et al.* (1994) developed RFLP maps for rice in Cornell and Japan respectively. The results derived from these two maps clarified that polymorphism is very high between

Indica and *Japonica* subspecies and most mapping studies utilize *Indica* x *Japonica* crosses.

Gustafson and Yano (2000) generated DNA fingerprints for rice genome analysis utilizing several rice derived minisatellites containing different sequences and number of repeat units, followed by assessing their potential for use as genetic markers when mapped to rice recombinant inbred lines (RILs) population. Therefore, Sakata *et al.* (2000) established a new rice genome database in Japan called INE (integrated rice genome explorer) in order to integrate all the genomic information that has been accumulated so far and to correlate these data with the genome sequence.

Several studies concerning investigating the phenotypic and genetic variability linked to biotic and abiotic stresses tolerance of the Egyptian rice cultivars (Sehly and Bastawisi, 1993; El Keredy *et al.*, 1994 and Abdel Hafez *et al.*, 1994), as well as studying the SDS protein banding pattern and RAPD-based fingerprinting for discrimination between Egyptian rice cultivars and testing hybrid purity (Aly *et al.*, 2000 and Wang *et al.*, 2001). Mackill *et al.* (1996) compared the levels of polymorphism of 14 rice accessions as revealed by AFLP. They concluded that AFLPs are promising markers for mapping important genes. Powell *et al.* (1996) suggested that AFLPs provide high levels of delineation of complex genetic structures.

AFLPs fingerprinting technology was used in studying and mapping of rice economic traits such as submergence tolerance in rice and studying the level of polymorphism in rice. It was also used in studying the comparative mapping of rice chromosome 1 and barley chromosome 3 (Saghai-Marooif *et al.*, 1996; Smilde *et al.*, 2001) and drought tolerant (Price *et al.*, 2000). Ishimaru *et al.* (2001) constructed a rice function map by collating the results on quantitative trait loci (QTLs) for 23 important physiological and agronomic traits using EST (expressed sequence tags). Saini *et al.* (2004) assessed the genetic diversity within and among Basmati and non-Basmati rice varieties using AFLP, ISSR and SSR markers. They emphasized on the need for using a combination of different marker systems for a comprehensive genetic analysis of Basmati rice germplasm.

Saker *et al.* (2005) analyzed seven Egyptian rice genotypes using RAPD, SSR and AFLP markers. They 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. Kumagai *et al.* (2010) studied the genetic diversity and evolutionary

relationships in genus *Oryza* using highly variable regions of chloroplast DNA.

Characterization of genetic diversity and cultivars relationships based on genetic similarity is the major goal of the current research project. Both DNA fingerprinting using AFLP technology (Vos *et al.*, 1995) and microsatellite marker (Litt and Luty, 1989 and Hossain *et al.*, 2012) were used as genetic tools for fingerprinting rice cultivars under study. The objectives of current study were to (i) generate a comparative assessment the potential of AFLP and SSR markers; ii) estimate genetic diversity among Egyptian rice genotypes; (iii) evaluate the application of AFLP and microsatellite markers as a tool for detecting genetic diversity and (iv) analyze the genetic structure of the Egyptian rice genotypes.

2. Materials and methods

Plant materials

Eight rice genotypes were obtained from Rice Research Department, Field Crop Research Institute, Agriculture Research Center (ARC), Ministry of Agriculture, Giza, Egypt. Details on these genotypes are given in Table (1).

DNA isolation

Young leaves of 15 days-old seedling were harvested and immediately frozen in liquid nitrogen. DNA was extracted from the frozen leaves using a modified CTAB method as described by (Saghai-Marooif *et al.*, 1984).

Microsatellite assay

Twelve microsatellite markers were selected to represent the entire rice genome based on the published framework map (Akagi *et al.* 1996 and Temnykh *et al.*, 2000). The loci, chromosomal location, primer sequence, annealing temperature (°C) and fragment size are presented in Table (2). DNA amplification was carried out in 25 µl reaction mixture, each containing 5 µl rice genomic DNA (50 ng), 2.5 µl 10X PCR buffer, 0.65 µl of both forward and reverse primers (250 nM), 2 µl 0.2 nM dNTPs and 0.3 µl *Taq* DNA polymerase and then completed to 25 µl with sterilized ddH₂O. PCR amplification was carried out as described by Temnykh *et al.* (2000) as follows: 5 min at 94°C followed by 35 cycles (1 min at 94°C, 1 min at 55°C or 60°C and 2 min at 72°C), with a final extension of 5 min at 72°C. The amplification product was resolved on 10% polyacrylamide gel electrophoresis (Chen *et al.*, 1997 and Temnykh *et al.*, 2000).

AFLP assay

AFLP analysis was performed as described by Vos *et al.* (1995). Briefly, 500 ng of rice genomic DNA was digested with *EcoRI* and *MseI* and double stranded adapters were ligated to the fragment ends. *EcoRI* adapters were ligated to the restricted fragments

and preamplified with two primers (A18: 5-GACGAT GAGTCTGAG; A19: CTCAGGACTCAT-5). *MseI* adapters were ligated to the restricted fragments and pre-amplified with two primers (M35: 5-bio-CTCG TAGACTGCGTACC; M36 CTGACGCATGGTTAA-5) containing one selective nucleotide. This was followed by pre-amplification step using non-selective primers. Selective amplification was performed on the pre-amplified fragment mixture using a total of five primer combinations. The reactions were performed in a Perkin-Elmer 9600 thermocycler. This was followed by pre-amplification step using non-selective primers. Selective amplification was performed on the pre-amplified fragment mixture using a total of three primer combinations namely, E36/M41, E37/M40, and E37/M41 were constructed by MWG-Biotech GmbH, Germany and used for PCR amplification. The sequence of these primers is as follows: (*EcoRI* primers: E36: 5-GAC TGC GTA CCA ATT C ACC-3, E37: 5-GAC TGC GTA CCA ATT C ACG-3, *MseI* primers: M40: 5-GAT GAG TCC TGA GTA A AGC-3, M41: 5-GAT GAG TCC TGA GTA A AGG-3). The Amplified DNA product was separated in a 4.5% denaturing polyacrylamide gel electrophoresis, visualized by autoradiography using P^{33} -radiolabelled *EcoRI* primers and scored for the presence or absence of bands.

Data analysis

Diversity values were calculated for each locus as polymorphic information content (PIC)

$$PIC = 1 - \sum_{i=1}^k P_i^2, \text{ where } k \text{ is the total number of}$$

alleles or polymorphic bands detected for a locus of each SSR and AFLP markers and P_i is the frequency of the i^{th} allele or polymorphic bands in the set of 8 rice genotypes (Botstein *et al.*, 1980). For genetic similarity estimates and cluster analysis, the allelic data were converted to a binary format, with the presence of a specific allele (band) scored as unity and its absence as zero. For a subdivided sample, the total diversity can be partitioned into its components resulting from differentiation among and within populations (Nei, 1973). Genetic similarities were estimated from the allele binary format dataset using the Dice method (Nei & Li, 1979). The binary data were used to compute pair-wise similarity coefficients. The similarity matrix was subjected to cluster analysis using the UPGMA (Unweighted Pair Group Method of Arithmetic Average) algorithm on NTSYS-pc version 2.1 software package (Rohlf, 2002).

3. Results

SSR analysis

Diversity of the twelve polymorphic SSR loci detected 46 alleles across the eight genotypes. The number of alleles per locus ranged from two for

RM133, *RM 215* and *RM433* to six for *RM271*, with a mean of 3.83 alleles per locus (Table 3). The percentage of polymorphic band (PPB) is 100% for each marker. Thus, this marker type seems to be the most informative for assessment of diversity and for discrimination among genotypes.

AFLP analysis

Three primer combinations were tested for their ability to generate AFLP banding patterns from rice genotypes. A total of 245 polymorphic bands were scored using the designated primer combinations (Table 3). The number of AFLP polymorphic bands varied from 19 for *E37/M41* to 176 for *E36/M41*, with a mean of 81.67. Thus, the primers tested are useful to reveal DNA polymorphisms in rice. Different levels of polymorphisms were detected, since the PPB ranged from 68.49% for *E37/M40* to 85.02% for *E36/M41* primer combinations (Table 3).

Comparative utility of marker systems

Polymorphic information content (PIC)

SSR and AFLP markers showed high levels of polymorphism in the examined rice germplasm as the PIC value for the SSR and AFLP markers was calculated with an average of 0.825 and 0.967, respectively, across rice genotypes assayed in Table (3). On the other hand, 12 SSRs and 3 AFLPs exhibited higher PIC values..

Gene diversity expressed as polymorphism information content (PIC) values for the 12 SSR loci varied from 0.444 for *RM 433* to 0.973 for *RM 215* with an average of 0.825. The correlation coefficient between gene diversity and the number of alleles was $r = 0.096$ ($P < 0.01$) and the linear relationship between them is shown in Figure (1). The PIC values of assayed AFLP were ranged from 0.935 for the *E37/M41* to 0.993 for *E36/M41* with an average of 0.967 as shown in Table (3). Also, there is an increase in genetic diversity as the number of polymorphic band increase. The correlation coefficient between gene diversity and the number of polymorphic bands was, $r = 0.742$ ($P < 0.01$) and the linear relationship between them is shown in Figure 2.

Genetic distances and relationships among genotypes

SSR analysis

Genetic distance matrix among genotypes ranged from 0.101 to 0.682 with a mean of 0.252. Genetic distance value (0.101) was observed between 'Sakha 101' [SK101] and 'Giza 181' [GZ181] genotypes (Table 4), which appear as the most similar accessions and closely related. The maximum distance value of 0.682 was obtained between 'Giza 172' [GZ172] and 'Giza 171' [GZ171] genotypes. The cluster analysis using UPGMA based on genetic distances from SSR markers analysis revealed that the eight genotypes were divided into two main clusters as shown in Figure (3). The first main cluster included

the *japonica* rice cultivars and was divided into two subclusters. The first subcluster included two sub-subclusters. The first sub-subcluster includes one rice variety Sakha 101. The other sub-subcluster includes four rice varieties; Giza 172, Giza 171, Giza 177 and Giza 176. The second subcluster includes one variety Sakha 102. The other main cluster includes two *Indica* and *Indica/Japonica* rice varieties Giza 181 and Giza 178.

AFLP analysis

To estimate the genetic relatedness among rice genotypes, pairwise distance matrix was obtained based on the formula of Nei and Li's (1979). Based on the 245 AFLP markers, estimates of the genetic distance exhibited values ranged from 0.393 to 0.783 with a mean of 0.554 signifying that the genotypes studied are characterized by large divergence at the

DNA level. Genetic distance value of 0.393 was scored between the two short-grain *Japonica* type cultivars 'Sakha 102' [SK102] and 'Giza 176' [GZ176] as shown in Table (5). However, 'Sakha 101' [SK101] and 'Sakha 102' [SK102] were most divergent, with the maximum genetic distance of 0.783. All others have displayed different middle levels of genetic similarity. The AFLP UPGMA dendrogram divided the eight tested genotypes into two main clusters as shown in Figure (4). The first main cluster included the two *japonica* rice cultivars Sakha 101 and Sakha 102. The second main cluster included two subclusters; the first includes two *japonica* rice cultivars namely; Giza171 and Giza176 and the other one includes four rice cultivars namely; Giza172, Giza 177, Giza 178 and Giza 181.

Table (1): List of rice genotypes used in the current study.

Genotype Name	Source	Subspecies Group	Grain type
Giza 171	ARC, Egypt	<i>Japonica</i>	Short grain
Giza 172	ARC, Egypt	<i>Japonica</i>	Short grain
Giza 176	ARC, Egypt	<i>Japonica</i>	Short grain
Giza 177	ARC, Egypt	<i>Japonica</i>	Short grain
Sakha 101	ARC, Egypt	<i>Japonica</i>	Short grain
Sakha 102	ARC, Egypt	<i>Japonica</i>	Short grain
Giza 178	ARC, Egypt	<i>Indica/ Japonica</i>	Short grain
Giza 181	ARC, Egypt	<i>Indica</i>	Long grain

Table (2): Description of 12 simple sequence repeats used in study.

Primers	Chromosome number	Relative distance from the centromere cM	Forward Primer sequence	Reverse Primer sequence	Annealing temperature (°C)	Fragment size
RM 5	1	94.9	TGCAACTTCTAGCTGCTCGA	GCATCCGATCTTGATGGG	55	113
RM 6	2	126	GTCCCTCCACCCAATTC	TCGTCTACTGTTGGCTGCAC	55	163
RM 11	7	47	TCTCCTCTTCCCCGATC	ATAGCGGGCGAGGCTTAG	55	140
RM 19	12	20.9	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA	55	226
RM 22	3	12.00	GGTTGGGAGCCCATAATCT	CTGGGCTTCTTCACTCGTC	55	194
RM 55	3	168	CCGTCGCCGTAGTAGAGAAG	TCCCGGTTATTTAAGGCG	55	226
RM133	6	0.00	TTGGATTGTTTTGCTGGCTCGC	GGAACACGGGTTCGGAAGCGAC	60	230
RM 215	9	99.4	CAAAATGGAGCAGCAAGAGC	TGAGCACCTCCTTCTCTGTAG	55	148
RM271	10	59.4	TCAGATCTACAATTCATCC	TCGGTGAGACCTAGAGAGCC	55	101
RM277	12	57.2	CGGTCAAATCATCACCTGAC	CAAGGCTTGCAAGGGAAG	55	124
RM307	4	0.00	GTACTACCGACCTACCGTTCAC	CTGCTATGCATGAACTGCTC	55	174
RM433	8	116	TGCGCTGAATAAACACAGC	AGACAAACCTGGCCATTAC	55	224

Table (3): Diversity analysis based on SSR and AFLP markers

	Total number of bands	Number of Polymorphic bands	Percentage of Polymorphic bands	PIC	Chromosomal Location	alleles Size (bp)	
						Min	Max
SSR							
RM 5	4	4	100	0.936	1	106	112
RM 6	3	3	100	0.862	2	144	162
RM 11	4	4	100	0.846	7	122	144
RM 19	5	5	100	0.964	12	213	248
RM 22	5	5	100	0.959	3	186	197
RM 55	5	5	100	0.773	3	210	237
RM133	2	2	100	0.764	6	232	234
RM 215	2	2	100	0.973	9	148	152
RM271	6	6	100	0.767	10	98	118
RM277	4	4	100	0.805	12	118	130
RM307	4	4	100	0.811	4	114	174
RM433	2	2	100	0.444	8	210	212
Total	46	46	100	-----	-----	-----	-----
Mean	3.83	3.83	100	0.825	-----	-----	-----
AFLP							
E36(GAC)/M41(ACC)	207	176	85.02	0.993	-----	-----	-----
E37(GAC)/M40(ACG)	73	50	68.49	0.974	-----	-----	-----
E37(GAC)/M41(AGC)	24	19	79.17	0.935	-----	-----	-----
Total	304	245	-----	-----	-----	-----	-----
Mean	101.33	81.67	77.56	0.967	-----	-----	-----

Table (4): Genetic similarity estimates based on SSR markers

	Sakha 101	Sakha 102	Giza 171	Giza 172	Giza 176	Giza 177	Giza 178	Giza 181
Sakha 101	1.000							
Sakha 102	0.133	1.000						
Giza 171	0.315	0.176	1.000					
Giza 172	0.200	0.167	0.682	1.000				
Giza 176	0.222	0.187	0.250	0.286	1.000			
Giza 177	0.353	0.267	0.421	0.350	0.333	1.000		
Giza 178	0.488	0.108	0.178	0.255	0.232	0.244	1.000	
Giza 181	0.101	0.114	0.232	0.133	0.243	0.103	0.391	1.000

Table (5): Genetic similarity estimates based on AFLP markers analysis

	Sakha 101	Sakha 102	Giza 171	Giza 172	Giza 176	Giza 177	Giza 178	Giza 181
Sakha 101	1.000							
Sakha 102	0.783	1.000						
Giza 171	0.516	0.457	1.000					
Giza 172	0.536	0.496	0.598	1.000				
Giza 176	0.432	0.393	0.604	0.534	1.000			
Giza 177	0.548	0.501	0.532	0.735	0.549	1.000		
Giza 178	0.535	0.480	0.559	0.607	0.574	0.736	1.000	
Giza 181	0.545	0.490	0.504	0.582	0.463	0.636	0.595	1.000

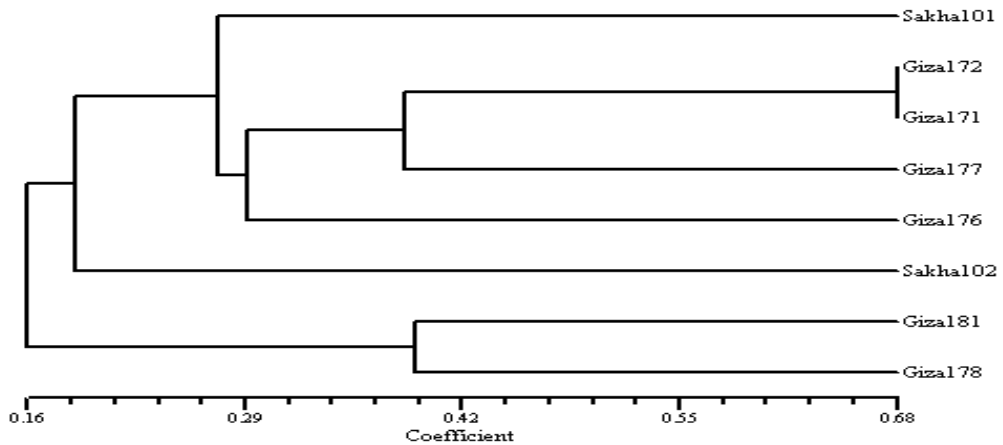
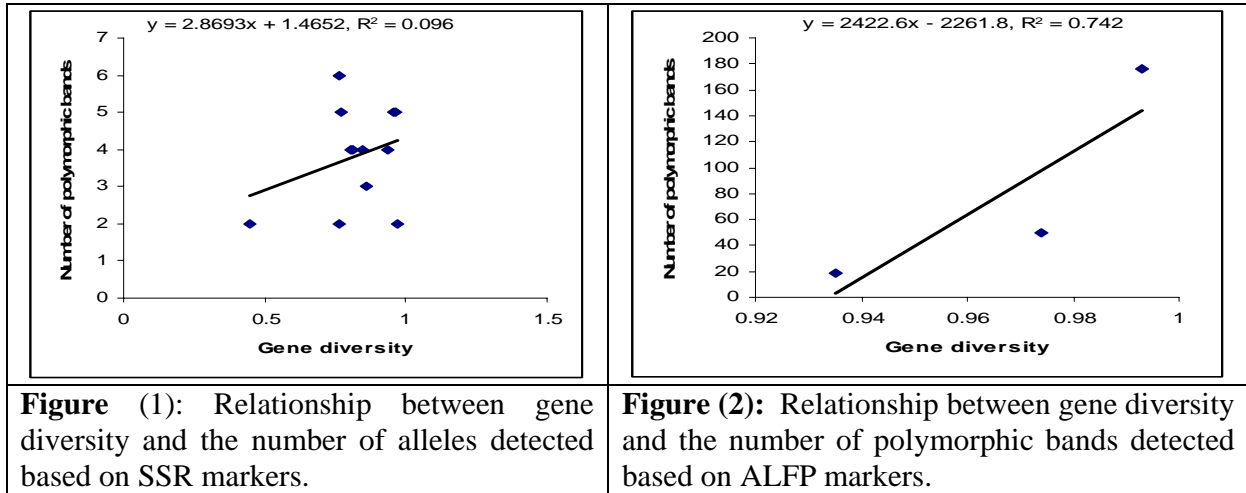


Figure (3): Dendrogram showing the genetic similarities among the studied rice cultivars based on twelve SSRs markers.

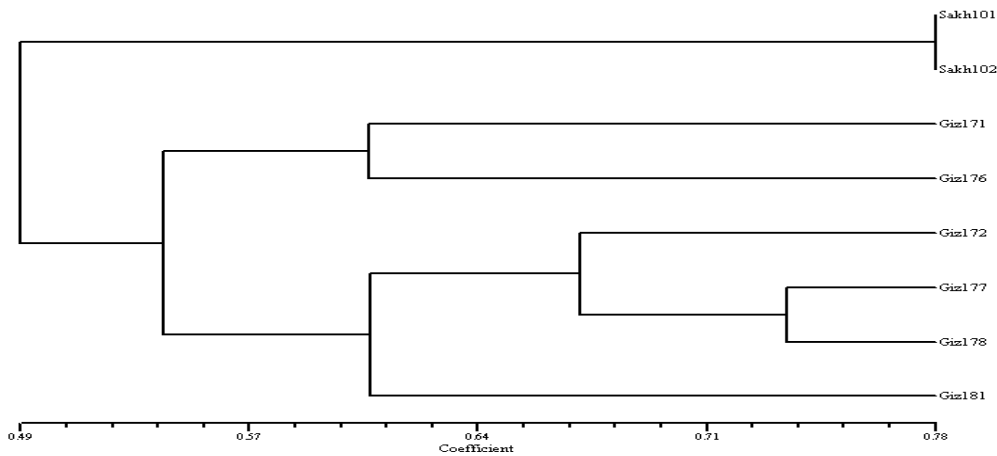


Figure (4): Dendrogram showing the genetic similarities among the studied rice cultivars based on AFLP markers

4. Discussion

Given the proliferation of genetic markers, comparisons between techniques are inevitable. However, there is a need for such comparisons in order to decide on which technique is best suited to the issues being examined. In this study, two of the polymerase chain reaction (PCR) based systems (SSRs and AFLPs) have been compared. Each technique not only differs in principal, but also in the type and amount of polymorphism. The two techniques varied widely, ranging from a maximum 100% (SSRs) to only 77.56% (AFLPs). Indeed, whenever SSRs have been compared to other systems, they have always revealed the highest levels of polymorphism (Wu and Tanksley, 1993; Saghai-Marooof *et al.*, 1996; Powell *et al.*, 1996 and Morgante *et al.*, 2002). The average number of bands per lane or per PCR for AFLPs was 101.33, compared to 3.83 bands per lane or PCR for SSRs. Thus, when the overall diversity indices of the two techniques were compared, AFLP was the highest PIC value (0.967). Rice germplasm can be divided into four gene pools, *Japonica*, *Indica*, *Indica/Japonica* and *Javanica*, based on morphology distinctions and pedigree. Ni *et al.* (2002) using SSRs observed a clear separation between the *Indica* and *Japonica* types. In this study, similar results obtained with SSR markers. With SSRs there is a clear separation between the *Indica* and *Japonica* types. The most important phenomenon is the grouping of the two *indica* and *Indica/Japonica* genotypes with long grains (Giza 181 and Giza 178) together in one subgroup. This result is consistent with that based on proteins data analyses and that of Wang *et al.* (2001) and El Rabey (2003). In this study, genetic diversity and cultivars relationship of eight Egyptian rice cultivars were characterized on the basis of genetic affinity revealed by DNA fingerprinting using AFLP technique (Vos *et al.*, 1995). The AFLP data provided high degrees of polymorphism that supports the value of AFLP in fingerprinting. Rice cultivars under study revealed by 245 polymorphic bands that used efficiently in discriminating between various cultivars based on genetic diversity and addressed relationships among them.

The structural changes (addition or deletion events) in rice genome may contribute to DNA variation detected by southern analysis (McCouch *et al.*, 1988; Wang and Tanksley, 1989; Xu *et al.*, 1998 and Morgante *et al.*, 2002) or to point mutation (base substitution) (Ghareyazie *et al.*, 1995). Yano *et al.* (1996) reported that collecting all rice genome data such as physical map, RAPD, SSR, AFLP and RFLP linkage maps and genetic map of phenotypic trait genes, including *QTLs* and the sequenced markers will generate an informative chromosome map as a comprehensive genome map. Once a fully integrated

genome map is established, all the DNA clones such as plasmid, cosmid and YAC clones and all their data, will be very powerful tools not only for the analysis of rice genome structure and function, but also for practical rice breeding. The value of gene diversity increased with the number of alleles at a given locus. There was significant correlation between gene diversity and the number of alleles ($r = 0.309$, $P > 0.01$). Therefore, the number of alleles can be used to evaluate the genetic diversity. Similar results were obtained by Huang *et al.* (2002) and Salem *et al.* (2010). In the study herein, SSR was found highly polymorphic compared with AFLP markers. The high level of polymorphism linked with SSR is to be expected due to the unique mechanism responsible for generating SSR allelic diversity by replication slippage (Gupta and Varshney, 2000, Varshney *et al.*, 2005 and Hossian *et al.*, 2012), whereas the basis of AFLP polymorphism are single nucleotide mutations and insertions/deletions (Ridout and Donini, 1999 and Rafalski, 2002). Regarding the detection of polymorphism, SSR markers are better than that of AFLP as SSR are co-dominant and multiallelic markers in contrast to the nature of AFLP markers.

In the present study the use of AFLP for DNA fingerprinting of the rice cultivars addressed the genetic relationships based on the homology of the genomic DNA of each cultivar. El Rabey *et al.* (2002) compared common bands isolated from different *Hordeum* species, 80.05% of these bands were found homologous in sequence. However, the intercultivars relationships based on AFLP data is considered a relationship based on sequence data. So far, the genetic basis of several traits with economic and biological importance has been clarified by many rice geneticists and breeders. However, further analyses, at the molecular level, will be necessary for further improvement of rice varieties. Information generated from this study can be used to select parents for hybrid development to maximize the yield and development of segregating populations to map genes controlling yield and its component traits in rice.

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