AFLP fingerprinting of some Egyptian date palm (*Phoenix dactylifera* L.) cultivars

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ABSTRACT

PCR-based DNA profiling of five Egyptian date palm (Phoenix dactylifera L.) cultivars was conducted using AFLP's. DNA samples from ten individual trees representing each of the five cultivars were pooled to form the sample representing the cultivar. A total of 433 amplification products were generated from the five cultivars using six primer pair combinations (EcoR1 and Mse1) with a mean of 72.17 amplicons per assay. The information about genetic variation determined from AFLP data was employed to estimate genetic similarity matrix value based on Jaccard's coefficient. The similarity values were further used to construct a phonetic dendrogram revealing the genetic relationships. The dendrogram generated by the UPGMA (un-weighted pair group method using arithmetic averages) formed two major clusters with Siwi and Hayany being the most genetically similar cultivars, and in the second cluster Amhat and Samany being next, while Zaghloul was the most distinct cultivar. ALFP analysis also permitted the distinction of unique markers among the five studied date palm cultivars. A total of 78 positive and 48 negative markers were identified by the six AFLP primer combinations. The total number of unique markers per genotype ranged from 13 to 51. The cultivar Zaghloul was characterized by the highest number of unique markers (51), while a total of 16, 23, 13 and 23 unique markers characterized the cultivars Siwi, Hayani Amhat, and Samany, respectively.

Key words: *Date palm, AFLP, DNA fingerprinting, genetic similarity.*

INTRODUCTION

Molecular genetic marker technologies are playing an increasingly important role in the assessment of genetic diversity, genetic relationships and fingerprinting in germplasm collections. Due to advances in the field of molecular genetics a variety of different techniques to analyze genetic variation has emerged during the last few decades. Several PCR-based genetic markers have been used to provide information on genetic variation in plant species. Initially, RAPD markers were employed for genetic analyses, but problems regarding reproducibility were reported (Jones *et al.*, 1997). AFLP technique was introduced as a reliable and reproducible marker system (Vos *et al.*, 1995).

AFLPs have proven to be extremely proficient in revealing diversity at the species level and provide an effective means of covering a wide area of the genome in a single assay (Ellis *et al.*, 1997; Zhu *et al.*, 1998). It was preferred over other DNA-based markers mainly because of its high multiplex ratio and non-requirement of prior sequence information (Breyne *et al.*, 1997; Pejic *et al.*, 1998; Garcia-Mas *et al.*, 2000; Vuylsteke *et al.*, 2000 and Yuan *et al.*, 2000).

The major disadvantage of RAPD and AFLP markers is that they are dominant and cannot detect heterozygosity. However, comparative analysis using several PCR-based markers showed that the multi-locus AFLP technology is one of the best methods available for evaluation of germplasm (Powell *et al.*, 1996; Russell *et al.*, 1997). Powell *et al.* (1996) and Russell *et al.* (1997) claimed that AFLPs are more efficient than SSR markers even though SSRs are co-dominant and detect the highest level of polymorphism per locus. Aggawal *et al.* (1999) reported that the results of various tests done to check robustness of the dendrogram/estimates of phylogeny, clearly establish that the polymorphism revealed by AFLP is not only abundant but also stable and statistically reliable.

Date palm (*Phoenix dactylifera* L.) is an arborescent, dioecious, highly heterozygous, monocotyledonous plant with a very slow growth rate and a late reproductive phase. It is an ancient crop and one of the five fruit trees among the seven species mentioned in the Bible and the Holy Koran. It is of great economic importance to oasis agriculture and creates favorable conditions for improving secondary crop culture thus; it is a strategic crop for the entire region of North Africa and the Middle East. The average number of date palm in the Arab countries is estimated to be about 62 million trees of which 7.5 million trees are cultivated in Egypt.

Little is currently known about the molecular characterization of date palm varieties, though a great effort is now on its way in this particular discipline. Thus, the objectives of this study were: (1) to optimize the AFLP conditions in date palm, (2) to estimate the level of polymorphism among the five cultivars used in this study, (3) to identify unique DNA markers and generate fingerprints for each cultivar, (4) to determine the genetic relationships among these genotypes using Jaccard's coefficient.

MATERIALS AND METHODS

Plant material

Five Egyptian date palm cultivars (Zaghloul, Samany, Hayany, Siwi and Amhat) collected from different locations in Egypt were used in this study.

DNA isolation

Genomic DNA was extracted from leaf samples according to Porebski *et al.* (1977). For each cultivar equal amounts of DNA from 10 individual plants were pooled together to form the sample representing the cultivar.

AFLP analysis

AFLP analysis was performed as described by Vos *et al.* (1995) using the Gibco BRL AFLP™ Analysis system I, and the AFLP starter primer kit (Cat. No. 10544-013 and 10483-014, respectively).

Preparation of template DNA for AFLP reaction

Genomic DNA (500 ng) was digested with two restriction enzymes (RE) simultaneously, *Eco*R1/*Mse*1 (1.2 units/µl each in 10 mM Tris-HCl (pH 7.5), 50 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA and 50 %

restricted DNA, 24 μl of the adaptor ligation solution (*Eco*R1/*Mse*1 adaptor, 0.4 mM ATP, 10 mM Tris HCl (pH 7.5), 10 mM Mg acetate) and 1 μl T_4 DNA ligase (1 unit/μl) were added.

Non-selective pre-amplification

Pre-amplification of DNA was performed in a total volume of 51 μl which consisted of 5 μl of the 10 fold diluted ligation mix, 40 μl preamp primer mix, 5 μl of 10 X PCR buffer plus Mg^{+2} and 1 μl *Taq* polymerase (RTS Gibco, BRL). PCR temperature profile was 20 cycles at 94°C for 30s, 56°C for 60s and a final extension cycle at 72°C for 60s then soaking at 4°C.

Selective amplification

For selective amplification of DNA, pre-amplified products were employed as templates using two AFLP primers, each containing 3 selective nucleotides. Two mixes were prepared: Mix 1 with a total volume of 50 μl (5 μl *Eco*R1 primer and 45μl *Mse*1 primer with dNTPs), Mix 2 with a total volume of 100 μl (97 μl dH_2O, 2 μl PCR buffer and 1 μl *Taq* DNA polymerase). The reaction was performed in a 20 μl total volume of 5 μl diluted preamp product, 5 μl Mix 1 and 10 μl Mix 2. The reaction was carried out using the following cycling parameters: One cycle of 30s at 94°C, 30s at 65°C, 1 min at 72°C followed by 12 cycles in which the annealing temperature decreases 0.7 °C per cycle, followed by 23 cycles of 1 min at 94 °C, 30s at 56 °C and 1 min at 72°C. All PCR reactions were performed using a Perkin Elmer 9600 thermocycler. Sequences of oligonucleotide adapters and sequences of primers used in pre-amplification and selective AFLP primer combinations are given in Table (1).

AFLP electrophoresis and staining

Products from the selective amplification were separated on a 6 % denaturing polyacrylamide sequencing gel. The DNA Silver Staining System (Promega Cat # DQ 7050) was used for band detection.

Data analysis

For AFLP analysis, only clear and unambiguous bands were visually scored as either present (1) or absent (0). Each band was interpreted as one allele. Bands with the same mobility were assumed to be homologous (Rouppe van der Voort *et al.*, 1977). Each marker was treated as an independent unit character.

The genetic similarities (GSs) and similarity matrix from AFLP data were calculated among cultivars using the Jaccard's coefficient.

$$Sim = 100\,x\,\frac{\sum_{i=1}^{B} Min(S_i, t_i)}{\sum_{i=1}^{B} Min(S_i + t_i) - \sum_{i=1}^{B} Min(S_i, t_i)}$$

Where:

$S_i = 1$ if the I'th band type is found in the lane.

$T_i = 0$ if it is not found

Dist. = 100 Sim

Cluster analysis was based on similarity matrix obtained with the un-weighted pair group method using arithmetic averages (UPGMA), (Rohlf, 1990) and relationships between cultivars were illustrated as a dendrogram. All data were scored in the form of a binary matrix. For each pair of cultivars, the Jaccard similarity index (GS) was calculated (Jaccard, 1908). The calculations were performed with the SAS software for data analysis (SAS Institute Inc.).

Table (1): Sequence of oligonucleotide adapters, primers used in pre-amplification, selective AFLP primer combinations and code number in parentheses.

Primer combinations	Oligonucleotide adapters		Preamplification primers	Selective primers
*Eco*R1/*Mse*1 (Code No.)	*Eco*R1 forward adapter	*Mse*1 forward adapter	*Eco*R1 pre-selective primer (E+A)	*EcoR1* selective primers (E+3)
	*Eco*R1 reverse adapter	*Mse*1 reverse adapter	*Mse*1pre-selective primer (M+C)	*Mse1* selective primers (M+3)
E_{AAC} / M_{CAA} (1.1)	CTCGTAGACTGCGTACC	GACGATGAGTCCTGAG	GACTGCGTACCAATTCA	GACTGCGTACCAATTCAAC
	AATTGGTACGCAGTCTAC	TACTCAGGACTCAT	GATGAGTCCTGAGTAAC	GATGAGTCCTGAGTAACAA
E_{ACA} / M_{CAG} (3.3)	CTCGTAGACTGCGTACC	GACGATGAGTCCTGAG	GACTGCGTACCAATTCA	GACTGCGTACCAATTCACA
	AATTGGTACGCAGTCTAC	TACTCAGGACTCAT	GATGAGTCCTGAGTAAC	GATGAGTCCTGAGTAACAG
E_{ACC} / M_{CAT} (4.4)	CTCGTAGACTGCGTACC	GACGATGAGTCCTGAG	GACTGCGTACCAATTCA	GACTGCGTACCAATTCACC
	AATTGGTACGCAGTCTAC	TACTCAGGACTCAT	GATGAGTCCTGAGTAAC	GATGAGTCCTGAGTAACAT
E_{ACT} / M_{CTC} (6.6)	CTCGTAGACTGCGTACC	GACGATGAGTCCTGAG	GACTGCGTACCAATTCA	GACTGCGTACCAATTCACT
	AATTGGTACGCAGTCTAC	TACTCAGGACTCAT	GATGAGTCCTGAGTAAC	GATGAGTCCTGAGTAACTC
E_{AGC} / M_{CTG} (7.7)	CTCGTAGACTGCGTACC	GACGATGAGTCCTGAG	GACTGCGTACCAATTCA	GACTGCGTACCAATTCAGC
	AATTGGTACGCAGTCTAC	TACTCAGGACTCAT	GATGAGTCCTGAGTAAC	GATGAGTCCTGAGTAACTG
E_{AGG} / M_{CTT} (8.8)	CTCGTAGACTGCGTACC	GACGATGAGTCCTGAG	GACTGCGTACCAATTCA	GACTGCGTACCAATTCAGG
	AATTGGTACGCAGTCTAC	TACTCAGGACTCAT	GATGAGTCCTGAGTAAC	GATGAGTCCTGAGTAACTT

RESULTS AND DISCUSSION

In the present investigation AFLP analysis has been adapted to assay the level of polymorphism and to produce a fingerprint for the five studied date palm cultivars. Nevertheless, the technique required initial optimization to identify primer combinations that yield interpretable and reproducible fingerprinting profiles. Therefore, eight primer combinations were initially tested with the five bulked DNA samples representing the five date palm cultivars. Among the eight tested primer combinations, six *Eco*R1/*Mse*1 selective primer combinations yielded discernible reproducible profiles.

Polymorphism as detected by AFLP

AFLP analysis using the six selective primer combinations generated a total of 433 amplicons. Therefore, the mean number of amplicons per assay was 72.17. Meanwhile, the number of polymorphic amplicons was 233 representing a level of polymorphism of 53.81% and an average number of polymorphic bands of 38.83 per AFLP primer combination (Table2). The size of the AFLP amplified fragments ranged from 48 bp to 830

bp (Fig. 1). Figure (1) shows examples of two of the six combinations used in the present study. In this respect, similar findings were reported by Han *et al.*, (2000) studying tea species, they found that the number of amplified AFLP bands per assay ranged from 32 to 150 with a mean of 84.7 and an average of 10.5 polymorphic bands per primer combination. Le Febvre *et al.* (2001) analyzed 47 pepper inbred lines with 10 AFLP primer combinations and revealed 863 selectively amplified fragments of which 378 were polymorphic (34.8%). Matthes *et al.* (2001) used ten AFLP primer combinations with oil palm and reported that the average number of bands per primer combination was 82 which is in agreement with our results.

The number of distinguishable bands detected after selective amplification varied among the different primer combinations. The highest number of amplicons (106) was exhibited by the primer combination (4.4), whereas the lowest number was 33 as revealed by primer combination (6.6) (Table 2). In this concern, Goulao *et al.* (2001) reported a range of 34 to 66 fragments per primer pair in apple cultivars.

The level of polymorphism ranged from 42.42% to 59.02% in primer combinations 6.6 and 7.7, respectively. Similarly, Cervera *et al.*, (1998) applied the AFLP technique to characterize 67 different grapevine accessions. They obtained an average of 100 amplified fragments per primer combination, of which 49% were polymorphic. Moreover, Hussein *et al.*, (2002) reported that the level of polymorphism revealed by six AFLP primer combinations on cotton ranged from 38% to 65%.

Genetic relationships among the five date palm cultivars

To det ermine the genetic relationships among the five cultivars, the scoring data (1 for presence and 0 for absence) resulting from the six primer combinations were used to compute the similarity matrices according to Jaccard (Jaccard, 1908). These similarity matrices were used to generate a dendrogram using the UPGMA method. As shown in Table (3) the genetic similarity estimates ranged from 64.4% to 76.7%. This revealed moderate levels of genetic similarity among the studied cultivars. The highest genetic similarity (76.7%) was between Siwi and Hayany. This was followed by 75.2% between Samany and Amhat, while the lowest genetic similarity (64.4%) was detected between Zaghloul and Samany. These genetic relationships were reflected on the dendrogram which represents the graphical illustration of the genetic distances among the five date palm cultivars (Fig. 2). The dendrogram confirmed that the cultivars Siwi and Hayany were the most genetically similar among the studied cultivars, with Amhat and Samany next, while Zaghloul was the most distinct cultivar.

In this respect, Cervera et al., (1998) stated that genetic similarity measured on the basis of AFLP results is more reliable than other markers since they are based on the analysis of a large number of unbiased genetic markers. These markers represent a random sample of genetic loci distributed along the genome and thus reduce the variance of similarity estimate (Cervera et al., 1996 and Powell et al., 1996).

Table (2): AFLP primer combinations, total number of amplicons, number of polymorphic amplicons and level of polymorphism detected by the different primer combinations among the five date palm cultivars.

Primer combination	Total number of amplicons	Polymorphic amplicons	Polymorphism %
8.8	65	38	58.46
7.7	61	36	59.02
6.6	33	14	42.42
4.4	106	62	58.52
3.3	82	45	54.87
1.1	86	38	44.18
Total	**433**	**233**	**5381**

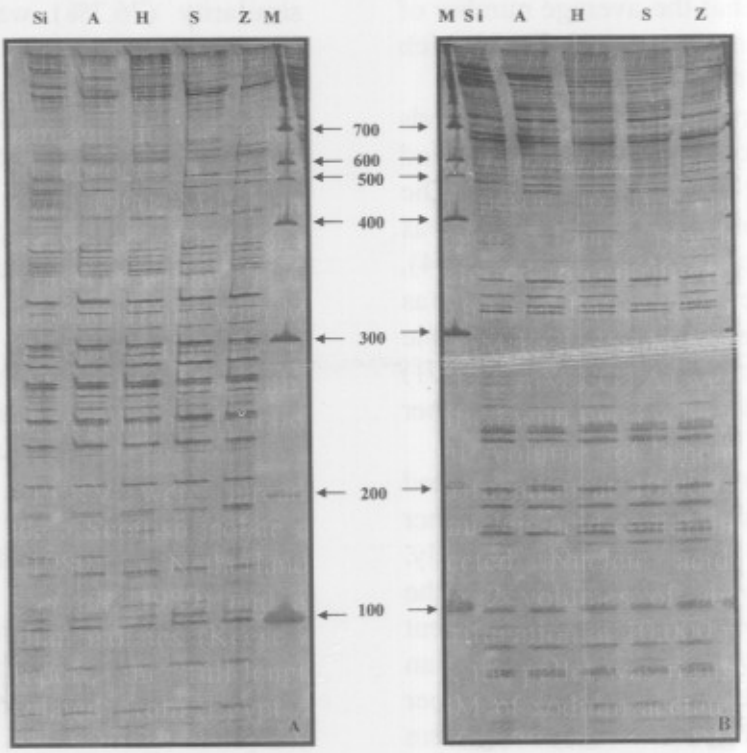

Fig. (1): AFLP profiles of the five date palm cultivars, Siwi (Si), Amhat (A), Hayany (H), Samany (S), & Zaghloul (Z). (A) Primer combination (3.3), (B) Primer combination (4.4). M DNA molecular weight standard (100 bp ladder).

Cultivar identification by unique markers

Unique markers are defined as bands that specifically identify an accession from the others by their presence or absence. The bands that are present in one accession but not found in the others are termed positive unique markers, in contrast to the negative unique

markers, which are absent in a specific genotype. These bands are useful for cultivar identification and fingerprinting.

As shown in Table (4), the AFLP analysis permitted the distinction among the five studied date palm cultivars and the characterization of each cultivar by specific unique markers. A total of 78 positive and 48 negative markers were identified by the six AFLP primer combinations. The total number of unique markers per genotype ranged from 13 to 51. The cultivar Zaghloul was characterized by the highest number of unique positive markers (48), in addition to 3 unique negative markers. Siwi was identified by 9 positive and 7 negative markers. Hayany exhibited a total of 23 unique markers among which 10 were positive. Samani also exhibited

Table (3): Genetic similarity matrix computed according to Jaccard's coefficient based on AFLP data.

	Siwi	Hayani	Zaghloul	Samany	Amhat
1- Siwi	100.0				
2- Hayani	76.7	100.0			
3- Zaghloul	69.9	73.2	100.0		
4- Samany	69.4	73.2	64.4	100.0	
5- Amhat	69.2	73.1	64.9	75.2	100.0

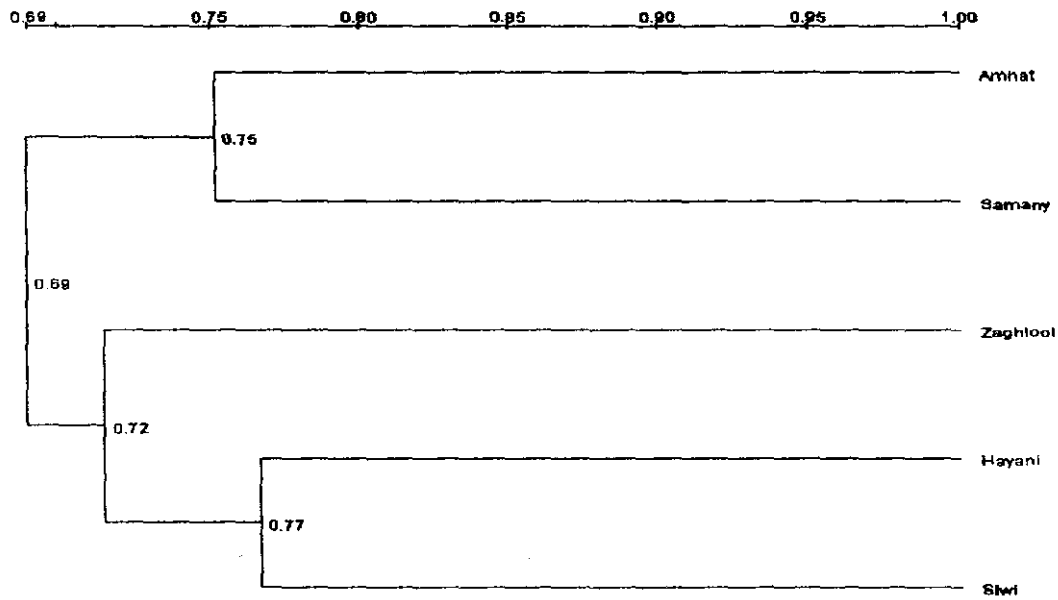

Fig. (2): Dendrogram constructed with UPGMA cluster analysis of AFLP data showing the genetic relationships among the five date palm cultivars.

Table (4): Unique positive and/or negative AFLP markers, markers size and total number of markers characterizing each of the five date palm cultivars.

Primer combination	Siwi		Hayany		Amhat		Samany		Zaghloul	
	UPM/bp	UNM/bp	UPM/bp	UNM/bp	UPM/bp	UNM/bp	UPM/bp	UNM/bp	UPM/bp	UNM/bp
1.1	183	486, 383		204, 135	239, 194	504, 94	269	318, 275	286, 197, 153, 95	
		212, 126						156, 102	92	
		114						93		
Total Unique bands for 1.1	1	5	0	2	2	2	1	5	5	0
3.3	305		244, 78	637, 446		625, 406	423, 205	161, 144	490,399,381,354	
				368, 332				51	308.273,262,257	
				311, 298					244.221,218,186	
				268					163, 48	
Total Unique bands for 3.3	1	0	2	7	0	2	2	3	14	0
4.4			734, 659	368,361	650	676, 242,	98	787,607	562.505,494,406	108
				260		201		293,144	398.386,370,353	
								111	350,302,279,275	
									237.229,215,178	
									168.164,86	
Total Uique bands for 4.4	0	0	2	3	1	3	1	5	19	1
6.6	105	231		137			295			177
Total Unique bands for 6.6	1	1	0	1	0	0	1	0	0	1
7.7		208	131				160, 142	240, 144	253,250,192,137	
									135,121,111,108	
Total Unique bands for 7.7	0	1	1	0	0	0	2	2	8	0
8.8	187,	---	189, 183,		84	123, 120		179	244, 148	142
	132,		160, 92							
Total Unique bands for 8.8	6	0	5	0	1	2	0	1	2	1
Total UPM&UNM	9	7	10	13	4	9	7	16	48	3
Grand Total	16		23		13		23		51	

UPM/bp Unique Positive Markers UNM/bp Unique Negative Markers

a total of 23 unique markers, seven of which were positive while the other 16 were negative. While, Amhat revealed the lowest number of unique markers (13) with 4 positive and 9 negative markers.

Table (4) also illustrates that the different primer combinations identified different number of unique markers. Primer combination 4.4 exhibited the highest number of unique markers (35). While, primer combination 6.6 was the least efficient primer in revealing unique markers (5) among the studied cultivars. Moreover, each of the four primer combinations (8.8, 4.4, 3.3, and 1.1) had the potentiality to distinguish among the five cultivars.

The present results clearly demonstrate the efficiency of the AFLP marker system in date palm cultivar fingerprinting using a few number of primer combinations. This could be attributed to the high multiplex ratio of the AFLP technique. These results are in consistence with the findings of different authors on different plant species i.e. Zhu *et al.* (1998) in rice; Paran *et al.*, (1998) in pepper; Pillay and Myers (1999) in cotton; Negi *et al.*, (2000) in *Withania* species; Degani *et al.* (2001) in strawberry and Hussein *et al.* (2002) in cotton.

In a previous work the same authors (Adawy *et al.* 2002) employed ten RAPD and seven ISSR primers to investigate the genetic variability and the profiling of the cultivars used in the present study. They identified twelve unique RAPD markers and 24 unique ISSR markers. However, in the present investigation the AFLP analysis exhibited a total of 126 unique markers using only six primer combinations. These results highlight the distinctive nature of the AFLP assay in discriminating among the different genotypes. In this context Pillay and Myers (1999) reported that the AFLP is considered one of the most powerful high density marker system including (i) a 10 fold increase in the number of informative markers per analysis, (ii) its ability to give highly reproducible banding patterns and (iii) no prior sequence information of the DNA is necessary.

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الملخص العربي

تحديد البصمة الوراثية لبعض الاصناف المصرية من نخيل البلح بأستخدام تقنية الـ AFLP

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استخدمت في هذه الدراسه تقنيه الـ AFLP لتحديد البصمة الوراثية لخمسة اصناف مصرية من نخيل البلح. تم عزل الدنا من عشرة اشجار من كل صنف وخلطه لتكوين العينة الممثلة للصنف. تم اجراء تحليل الـ AFLP باستخدام ست توليفات من البادئات (*EcoR1* & *Mse1*) حيث اظهرت ٤٣٣ شظيه من الدنا على مستوى الاصناف الخمسة وبذلك يكون متوسط عدد الشظايا لكل زوج من البادئات هو ٧٢,١٧ شظية. وقد استخدمت المعلومات الناتجة من دراسة التباين الوراثي بهذه التقنية لتقدير درجة التشابه الوراثي باستخدام معامل جاكارد والذى استخدم بالتالي في رسم دندروجرام يوضح العلاقات الوراثية بين الاصناف تحت الدراسة. وقد أظهر الدندروجرام مجموعتين اساسيتين توضحان أن الصنفين سيوى وحيانى اكثر الاصناف تشابها، تلاهما الصنفين أمهات وسمانى أما الصنف زغلول فكان أكثر الاصناف تميزا. كما امكن تحديد واسمات فريدة مميزة لكل صنف من الاصناف تحت الدراسة وكان مجموعها ٧٨ واسماً موجباً و٤٨ واسماً سالباً وتراوح عدد الواسمات الفريدة للاصناف المختلفة مابين ٣١و ٥١ واسم حيث امكن تمييز الصنف زغلول بأكبر عدد من الواسمات الفريدة (٥١ واسم) بينما أظهرت الدراسة ١٦، ٢٣، ١٣، ٢٣ واسماً فريداً مميزاً للاصناف سيوى وحيانى وأمهات وسمانى على التوالى.