Genetic distances between three Ushaar (*Calotropis procera (Ait) f.*) genotypes as measured by RAPD and ISSR techniques

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ABSTRACT

Three Ushaar genotypes from three different locations (El Maghara, Shalateen and Sant Kathrine) were screened using two different molecular Techniques; RAPD-PCR and ISSR. Both of these techniques were used to detect some molecular markers associated with the genotype identification. RAPD-PCR results, from using seven arbitrary primers, revealed 50 amplified fragments, 24 of them were polymorphic (48.00 %). The results revealed that the presence of 18 positive and 6 negative molecular markers for the genotypic identification. ISSR-PCR results with five specific primers revealed 41 amplified fragments, 12 of them were polymorphic (29.27 %). The results revealed that the presence of 4 positive and 8 negative molecular markers for the genotypic identification. Similarity indices and dendrogram for the genetic distances were estimated for each technique. The Similarity indices and dendrogram for the genetic distances of the combination between the two techniques revealed that the highest similarity was 44.40 % between the Ushaar genotypes from El Maghara and Shalateen regions. Meanwhile, the lowest similarity index was between the Ushaar genotypes from El Maghara and Sant Kathrine regions (22.20 %). The dendrogram resulting from the combination of the two techniques separated the three Ushaar genotypes into one cluster (including El Maghara and Shalateen genotypes) with the Sant Kathrine genotype alone.

INTRODUCTION

Medicinal plants have acquired increasing significance over the last few years, not only because they healing ability, but also due their minor side effects comparing of that the chemical medicine. Worldwide, 35000 plant species are used for medicinal purposes but only 90 species are considered in the most important industrial medicines. In developing countries traditional medicine is

spread because natural remedies are cheaper than chemical medicine and are often the only medicine available in the remote rural area. Besides serving medicinal functions, medicinal plants in developing countries have an important economic role because they have 90% of the earth genetic diversity exists in the developing countries.

Family Asclepiadaceae composed of about 2,000 species of flowering herbaceous plants or shrubby climbers in more than 280 genera. Most family members have milky juice, podlike fruits, and tufted silky-haired seeds that drift on wind currents to new locations for sprouting such as *Calotropis procera* Ait. f. commonly known, as 'Arka' is a popular medicinal plant found throughout the tropics of Asia and Africa and is used in many traditional systems of medicine **(Sharma and Sharma 1999)** as a purgative, anthelmintic, anticoagulant, anticancer as well as. tntipyretic, analgesic arid antimicrobial **(Jain *et al* 1996)** and is used also as an antiseptic for skin infection (**Fleurentin and Pelt 1982).**

Random amplified polymorphic DNA (RAPD) markers and inter - simple sequence repeats (**ISSR**) markers are two molecular typing approaches that have been used to detect variation among plants. Each method has been used extensively to identify and determine relationships between the species and cultivar levels **(Rajaseger** *et al*.**, 1997; Ratnaparkhe,** *et al*.**, 1998; Raina** *et al*.**, 2001** and **Martins** *et al*.**, 2003)**. These methods are widely applicable because they are rapid, inexpensive, simple to perform, do not require prior knowledge of DNA sequence and require very little starting DNA template **(Esselman *et al*., 1999).**

The present work aimed to identify some genotypes of Egyptian Ushaar (*Calotropis procera* Ait.f.) using molecular genetic fingerprints (RAPD and ISSR) techniques. Also the result of the combination between the RAPD and ISSR techniques analysis revealing the genetic distance and the dendrogram of the henbane genotypes under study.

MATERIALS AND METHODS:

A. Materials:

Three Ushaar genotypes (*Calotropis procera Ait f.*), (Table 1), were collected from three different locations El Maghara, Shalateen and Sant Kathrine, Egypt.

B. Methods:

1. DNA isolation:

Total DNA was isolated from young leaves of the Ushaar genotypes (*Calotropis procera* Ait (Ait).) according to **Dellaporta *et al.*, (1983).**

2. RAPD-PCR analysis:

Reaction conditions for amplification were optimized according to **Williams *et al.*, (1990)** using seven arbitrary primers synthesized by Operon Technology, Inc. (USA). The amplification was performed for 42 cycles, using a Coy thermocycler programmed, as follows; initial denaturation at 94°C for 3 min, one cycle, denaturating at 94°C for 45 sec., annealing at 37°C for 30 sec., extension at 72°C for 2 min., (40 cycles) and final extension at 72°C for 12 min., (one cycle).

The product was resolved on agarose gel (1.2 %) in TAE buffer and stained with ethidium bromide. Two different DNA markers were used; a 100 bp ladder mix and a 1kb DNA ladder mix. The seven arbitrary primers used for RAPD-PCR amplification, their names and sequences are found in Table (2). The run was performed for about 1 h at 80 V in a mini gel agars electrophoresis apparatus (Biorad). The products were visualized by UV light. PCR products were photographed by gel electrophoresis system (Gel Doc. BIORAD 2000) under transilluminator.

3. Inter simple sequence repeats (ISSRs)-PCR analysis:

The ISSR method has been reported to produce more complex marker patterns than the RAPD approach (**Parsons *et al.*, 1997**). In addition, ISSR markers are more reproducible than RAPD markers, because ISSR primers, designed to anneal to a micro-satellite sequence, are longer than RAPD primers,

allowing higher annealing temperatures to be used (**Goulao and Oliveira, 2001**).

The five used primers in PCR amplification of Inter simple sequence repeat regions (Table 3) were synthesized on an ABI 392 DNA/RNA synthesizer (Applied Biosystems) at AGERI, ARC.

The amplification was performed for 42 cycles, using a Coy thermocycler programmed, as follows; initial denaturation at 94°C for 3 min, one cycle, denaturating at 94°C for 45 sec., annealing at 55°C for 30 sec., extension at 72°C for 2 min., (40 cycles) and final extension at 72°C for 12 min., (one cycle).

The product was resolved on agarose gel (1.2 %) in TAE buffer and stained with ethidium bromide. Two different DNA markers were used; a 100 bp ladder mix and a 1kb DNA ladder mix. The run was performed for about 1 h at 80 V in a mini gel agars electrophoresis apparatus (Biorad). The products were visualized by UV light. PCR products were photographed by gel electrophoresis system (Gel Doc. BIORAD 2000) under transilluminator.

4. Genetic distance relationships:

The banding patterns of the seven RAPD and five ISSR primers were scored and data were feed to the PC computer as 1 and 0 for the presence and absence of bands, respectively. Pair-wise comparisons of genotypes, based on the presence or absence of unique and shared polymorphic products, were used to regenerate similarity coefficients, according to **Jaccard (1908)**. The similarity coefficients were then used to construct dendrograms, using the unweighted pair group method with arithmetic averages (UPGAMA) employing the SHAN (Sequantial, Agglomerative, Hierachical, and Nested clustering) from the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), version 2.1 Program (**Rohlf, 2000**).

RESULTS AND DISCUSSION

In the present investigation, many of random primers were used to differentiate between *Calotropis procera Ait f.* genotypes which collected from three different locations (El Maghara, Shalateen and Sant Kathrine). However,

only seven arbitrary primers (OP A19, OP B12, OP DO7, OP I15, OP L20, OP M17 and OP M20) gave reproducible results.

1. Randomly amplified polymorphic DNA (RAPD) analysis:

The banding patterns of RAPD-PCR fragments using the seven arbitrary primers with the three Ushaar genotypes (Fig. 1) showed 50 amplified fragments; 24 of them were polymorphic (48.00 %). The total number of amplified and polymorphic fragments obtained with each primer is found in Table (4). According to the resulted data, it was revealed that presence of 18 positive RAPD molecular markers in addition to 6 negative molecular markers. The results indicated that it was possible to discriminate between Ushaar genotypes which collected from three different locations, since each plant banding pattern was not similar to the others. Based on RAPD analysis, the same results was obtained by **Kochieva** *et al.,* **(2001)** who used RAPD analysis to study 53 species and cultivars of the genus *Lycopersicon* (Tourn.) Mill. revealed their high genetic polymorphism (Tourn.) Mill. based on which their phylogenetic relationships. The results showed that in intra-specific polymorphism was maximum (79.00 %) in *L. peruvianum* and minimum (9.00 %) in *L. parviflorum***.** Moreover, **Fu** *et al.,* **(2003)** studied the genetic variation, genetic erosion, and genetic relationship of 54 North American flax (*Linum usitatissimum* L.) cultivars using random amplified polymorphic DNA (RAPD) markers. The variations observed at the 84 polymorphic RAPD loci were relatively moderate with respect to primer, polymorphism, and cultivar. The same results was obtained by **Khan, *et al.,* (2007)** used RAPD analysis to construct phylogenetic trees of fifty six *Nicotiana* species using Phylogenetic Inference Package (PHYLIP). The results showed that RAPD analysis is suitable for studying phylogenetic relationships between related species.

2. Inter simple sequence repeats (ISSR) analysis:

The banding patterns of ISSR-PCR fragments using the five specific primers with the three Ushaar genotypes (Fig. 2) revealed 41 amplified fragments; 12 of them were polymorphic (29.27 %). The total number of amplified and polymorphic fragments obtained with each primer is found in Table (4). ISSR-PCR data revealed 4 positive and 8 negative molecular markers for the three Ushaar genotypes.

3. Genetic relationships of the three Ushaar genotypes based on molecular markers:

The identification of the wild type germplasm is necessary for varietal improvement. Recently, RAPD-PCR and ISSR techniques are used to measure the degree of similarity / dissimilarity among genotypes and also to estimate the genetic distance between these germplasms. The obtained results of the banding patterns of RAPD-PCR and ISSR-PCR were used to study the genetic relationships of the three Ushaar genotypes at the levels of the markers and in combination between the two techniques.

RAPD-PCR amplification revealed different degrees of polymorphisms between the henbane genotypes. The obtained 50 bands were treated with Ntsyspc2 software to release the similarity matrices (Table 5) and the dendrogram of the genetic distances (Fig. 3). The genetic similarity matrices depended on all possible pairs of genotypes ranged from 20.80 % to 45.80 %. The highest genetic similarity indices were found between Ushaar genotypes from El Maghara and Shalateen (45.80 %). The lowest genetic similarity indices were found between Ushaar genotypes from El Maghara and Sant Kathrine (20.80 %). Meanwhile, the genetic similarity indices between Ushaar genotypes from Shalateen and Sant Kathrine were (33.30 %).

The ISSR data revealed that the genetic similarity indices ranged from 25.00 % to 41.70 % (Table 6 and Fig. 4). The nearest relationship was detected between the Ushaar genotypes from El Maghara and Shalateen (41.70 %). On the other hand, the highest relationship of similarity matrices was detected between the Ushaar genotypes from El Maghara and Sant Kathrine (25.00 %),

followed by Shalateen and Sant Kathrine (33.30 %). As indicated by the Fig. 4, the dendrogram based on the similarity matrices of ISSR-PCR banding patterns separated the three Ushaar genotypes into one cluster (including El Maghara and Shalateen genotypes) and Sant Kathrine alone.

Depending on the combination between the banding patterns of RAPD-PCR and ISSR-PCR data through using NTsyspc2 software, the similarity indices were 44.40 % between the Ushaar genotype from El Maghara and Shalateen regions, followed by 33.30 % between the Ushaar genotype from Shalateen and Sant Kathrine. The lowest similarity indices were detected between the Ushaar genotype from El Maghara and Sant Kathrine (22.20 %). The dendrogram resulted from the combination of the two techniques (Fig. 5) was found also in one cluster (including the Ushaar genotype from El Maghara and Shalateen regions) while the Ushaar genotype from Sant Kathrine found alone.

Table (1): Name and origin of the three Ushaar genotypes.

Number	Genotype name	Origin	Family
1	Ushaar	El Maghara	Asclepiadaceae
2	Ushaar	Shalateen	Asclepiadaceae
3	Ushaar	Sant Kathrine	Asclepiadaceae

Table (2): RAPD-PCR primers code and their sequences used for detection of banding patterns in three Ushaar genotypes.

Primer code	Sequence (5 → 3)	Primer Code	Sequence (5 → 3)
OP A19	CTGCTGGGAC	OP L20	TGGTGGACCA
OP B12	CCTTGACGCA	OP M17	CAAACAGCGT
OP D07	ACCGCGAAGC	OP M20	TCGCAGAACG
OP I15	AAGAGAGGGG		

Table (3): ISSR-PCR primers code and their sequences used for detection of banding patterns in three Ushaar genotypes.

Primer code	Sequence (5 → 3)	Primer Code	Sequence (5 → 3)
A 98	$(CA)_6$ GT	HB 11	$(GT)_6$ CC
HB 09	$(GT)_6$ GG	HB 13	$(GA)_6$ GC
HB 10	$(GA)_6$ CC		

Table (4): The total number of amplified and polymorphic fragments, percentage of polymorphism and specific markers in the three Ushaar genotypes using both of RAPD-PCR and ISSR-PCR data**.**

Primer Number	Primer code	TAF	PF	Polymophism %	SM
RAPD-PCR					
1	OP A19	8	3	37.50 %	+2 and -1
2	OP B12	7	2	28.57 %	+1 and -1
3	OP D07	10	8	80.00 %	+5 and -3
4	OP I15	5	0	00.00 %	0
5	OP L20	5	3	60.00 %	+3
6	OP M17	5	2	40.00 %	+2
7	OP M20	10	6	60.00 %	+5 and -1
		50	**24**	**48.00 %**	**+18 and -6**
ISSR-PCR					
1	A 98	9	2	22.22 %	-2
2	HB 09	8	1	12.50 %	+1
3	HB 10	7	3	42.29 %	+2 and -1
4	HB 11	10	5	50.00 %	+1 and -4
5	HB 13	7	1	14.29 %	-1
		41	**12**	**29.27 %**	**+4 and -8**

Table (5): Similarity index (Pair wise comparison) among the three Ushaar genotypes based on RAPD-PCR data.

Genotype	El Maghara	Shalateen	Sant Kathrine
El Maghara	1		
Shalateen	0.458	1	
Sant Kathrine	0.208	0.333	1

Table (6): Similarity index (Pair wise comparison) among the three Ushaar genotypes based on ISSR-PCR data.

Genotype	El Maghara	Shalateen	Sant Kathrine
El Maghara	1		
Shalateen	0.417	1	
Sant Kathrine	0.250	0.333	1

Table (7): Similarity index (Pair wise comparison) among the three Ushaar genotypes based on RAPD-PCR and ISSR-PCR data.

Genotype	El Maghara	Shalateen	Sant Kathrine
El Maghara	1		
Shalateen	0.444	1	
Sant Kathrine	0.222	0.333	1

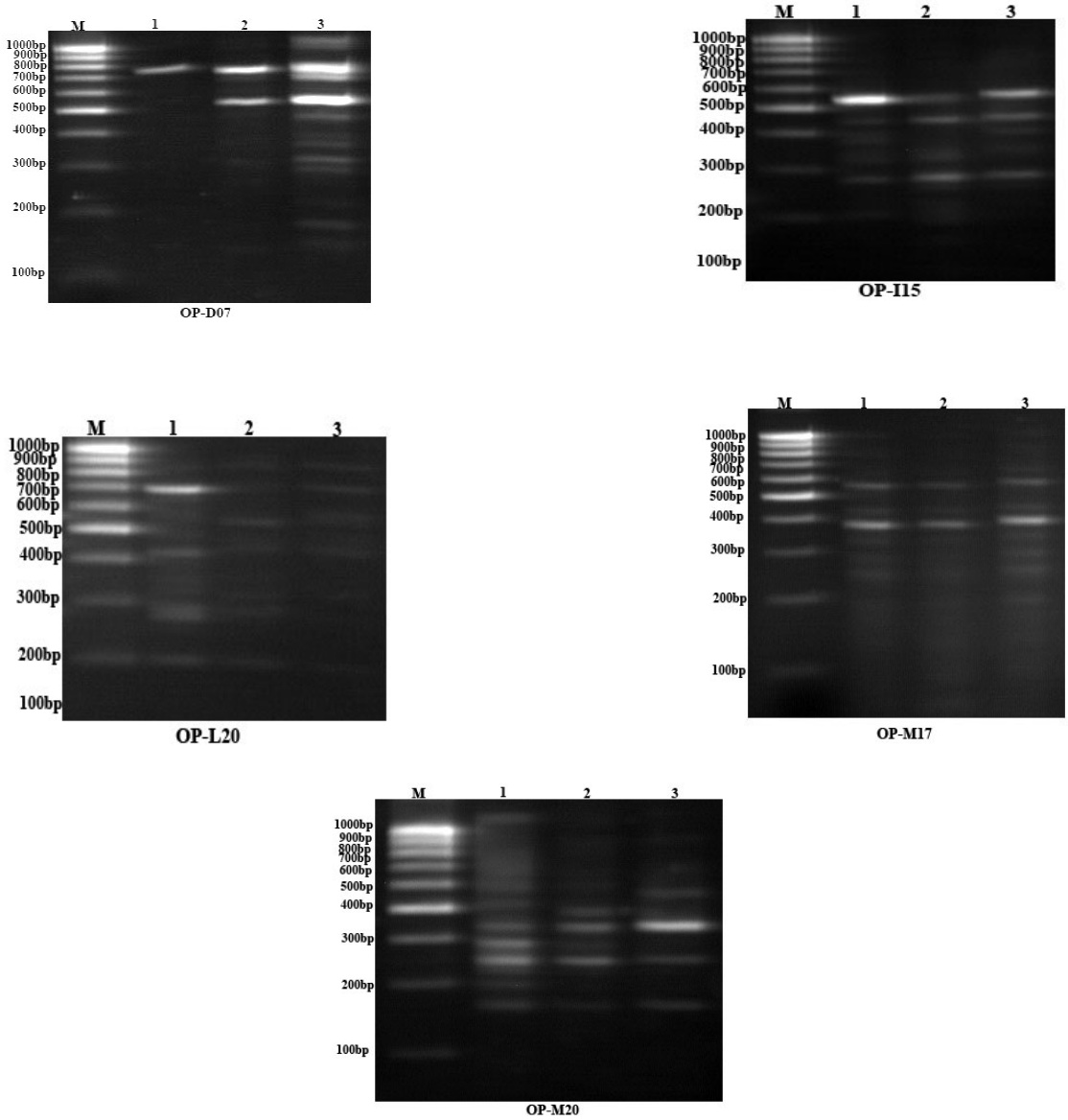


Fig. (1): RAPD banding patterns of the three Ushaar genotypes with seven arbitrary primers; M = 100-bp ladder

1- Ushaar from El Maghara
2- Ushaar from Shalateen
3-Ushaar from Sant Kathrine

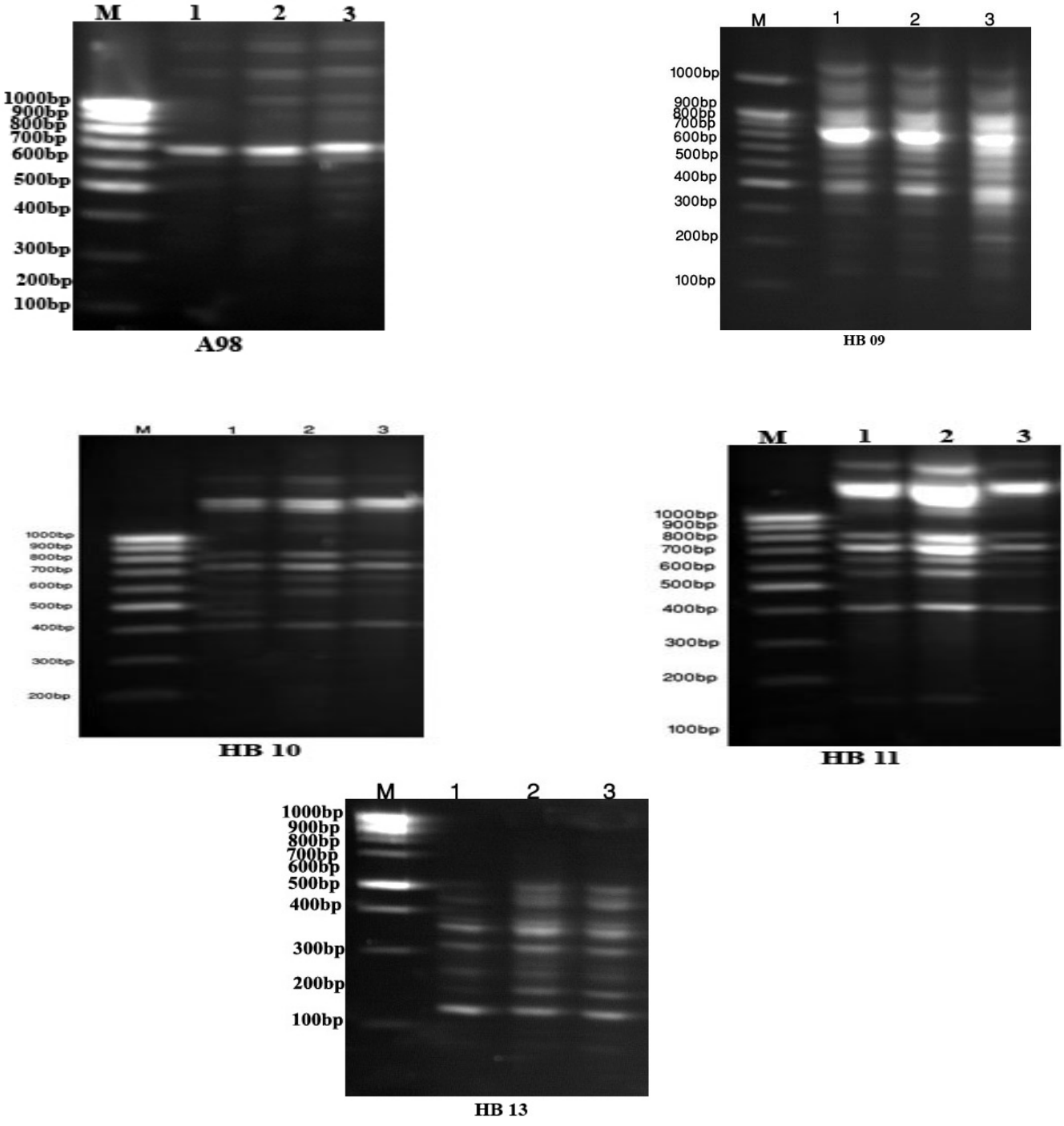


Fig. (2): ISSR banding patterns of the three Ushaar genotypes amplified with the five ISSR primers; M= 100 bp ladder.

1: Ushaar genotype from El Maghara**.**

2: Ushaar genotype from Shalateen

3: Ushaar genotype from Sant Catherin

Fig. 3: The genetic relationships among the three Ushaar genotypes based on RAPD-PCR data.

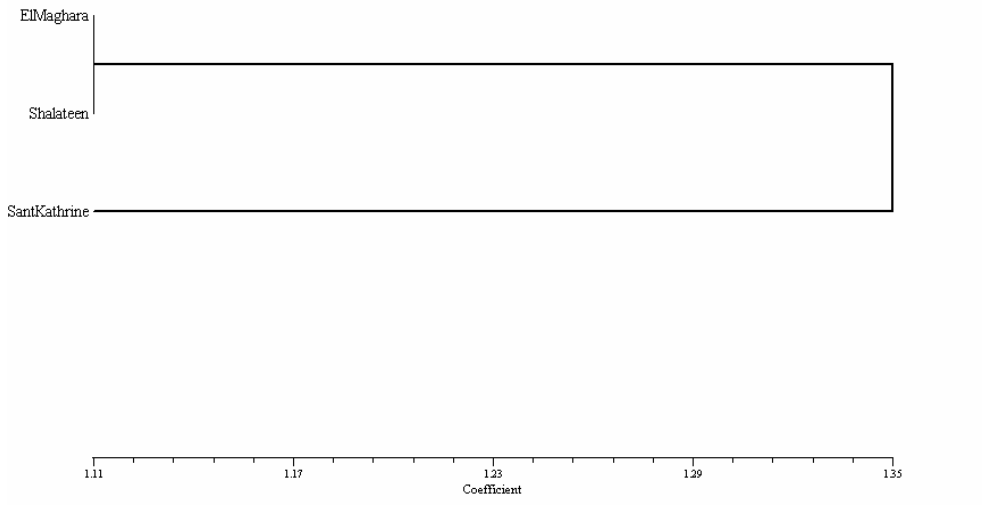


Fig. 4: The genetic relationships among the three Ushaar genotypes based on ISSR-PCR data.

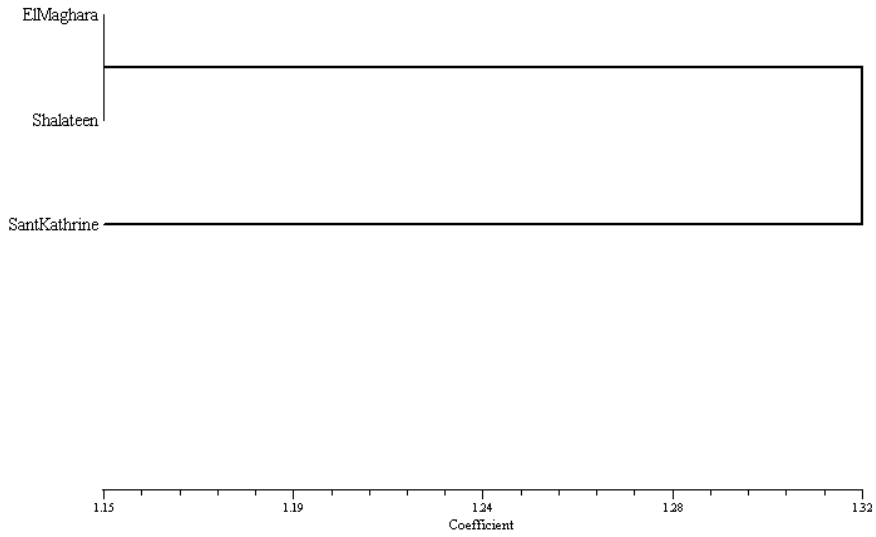
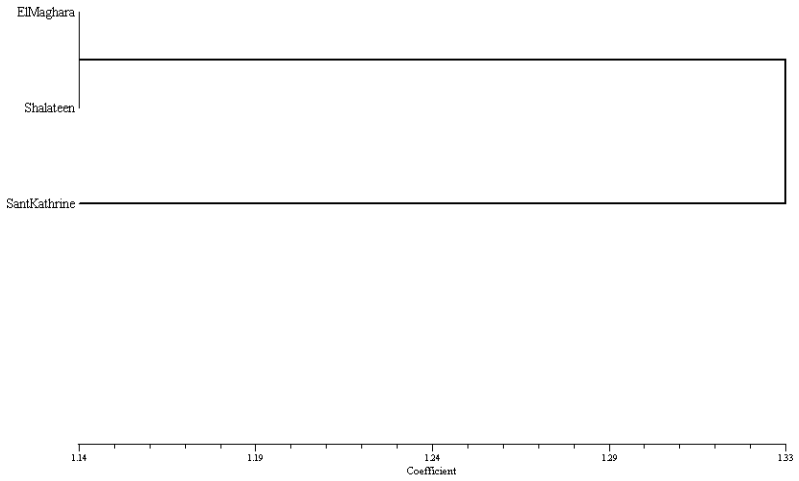


Fig. 5: The genetic relationships among the three Ushaar genotypes based on combination of RAPD and ISSR data.



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الملخص العربى

تقدير المسافات الوراثية بين ثلاثة تراكيب وراثية من نبات العشار (*Calotropis procera Ait f.*) باستخدام تقنيات ال RAPD-PCR و ال ISSR.

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إستخدامت **تقنيات ال RAPD-PCR و ال ISSR** لدراسة القرابة بين ثلاثة تراكيب وراثية من نبات العشارمن ثلاثة مواقع مختلفة (المغارة ، شلاتين و سانت كاترين). تم استخدام كل من الطريقتين للحصول على دلائل جزيئية مرتبطة بتعريف هذه التراكيب الوراثية. كانت نتائج طريقة ال RAPD-PCR من استخدام ٧ بادئات جزيئية عشوائية ٥٠ شظية مكبرة، ٢٤ شظية منها كانت متباينة بنسبة ٤٨,٠٠ % . أظهرت النتائج وجود ١٨ دليل جزيئى موجب و ٦ دلائل جزيئية سالبة بالنسبة لتعريف التراكيب الوراثية. وضحت نتائج الISSR بإستخدام ٥ بادئات متخصصة وجود ٤١ شظية مكبرة، ١٢ شظية منها كانت متباينة بنسبة ٢٩,٢٧ %. أوضحت النتائج وجود ٤ دلائل جزيئية موجبة و ٨ دلائل جزيئية سالبة بالنسبة لتعريف التراكيب الوراثية لنبات العشار.بينت نتائج القرابة الوراثية والشجرة الوراثية بالنسبة للمسافات الوراثية من التحليل المشترك لكل من الRAPD-PCR و الISSR أن أعلى نسبة تشابه كانت ٤٤,٤٠ % بين التراكيب الوراثية لنبات العشار من مناطق المغارة و شلاتين. بينما كانت أقل نسبة تشابه ٢٢,٢٠ % بين التراكيب الوراثية من مناطق المغارة و سانت كاترين. أوضحت نتائج الشجرة الوراثية الناتجة من التحليل المشترك بين الRAPD-PCR و الISSR حدوث انفصال الى عنقود واحد يضم كل من التراكيب الوراثية من مناطق المغارة و شلاتين و وجود التركيب الوراثى من منطقة سانت كاترين منفردا.