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Genetic variation within and among the wild populations of *Alhagi graecorum* using ISSR markers

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***Abstract*:**

***Alhagi graecorum* Boiss. (Camel thorn) (Family Fabaceae) is a wild plant which grows in salty soils and dry environment. It is native to Mediterranean and central Asia. Several studies have revealed the use of *Alhagi* plants in treating a wide spectrum of diseases. In this study, eight ISSR primers were used to assess genetic diversity of five populations of *A. graecorum* (El-Dakhla Oasis; Botanical garden of botany department, Ain Shams University; Wadi El-Rayan; Qarun Lake and Siwa Oasis) using individual - and bulked samples-based approaches. Twenty five individuals were sampled, amplified separately and amplified as bulked DNA to identify polymorphism among the studied populations. In total, 101 and 97 bands were scored with 83.3% and 78.4% polymorphism, respectively. Average polymorphism information content (PIC) for amplified DNA of individual samples was 0.875 and bulked-samples was recorded 0.69. Analysis of molecular variance (AMOVA) showed that 63% of the total variation was observed among populations while variation within populations accounted for the remaining 37%. The highest genetic similarity index was (0.99) among the studied *Alhagi* genotypes was recorded between the two genotypes 6 and 7 both individuals were belonging to Ain Shams University population, while among inter populations was (0.853%) between Qarun Lake and Siwa Oasis populations. The UPGMA cluster analysis divided the 25 individuals and bulked samples into two main groups. Each group was further subdivided into many sub-clusters, PCoA was also carried out and in agreement with the UPGMA. The obtained results demonstrate that the ISSR markers were efficient for evaluation of the genetic relationships between intra- and inter-population due to their efficiency in revealing polymorphism.**

Key words: Medicinal plant, *Alhagi graecorum*, Genetic diversity, Molecular markers, ISSR.

INTRODUCTION

The *Alhagi* genus (commonly known as Camel thorn, Caspian manna or Persian manna) belongs to family Fabaceae and has been placed under sub-family Papilionaceae **(Ali, 1977)**, commonly occur in dry lands associated with low rainfall, high salinity and alkalinity in waste places **(Kawase and Kanno, 1983)**. *Alhagi* genus comprises different species such as *A. pseudalhagi* M. Mieb. Desv., *A. graecorum* Boiss., *A. sparsifolia* Shap., *A. maurorum* Medik. **(Badshah and Hussain, 2011)**. *Alhagi graecorum* Boiss is a wild plant which grows in Nile and canal banks, roadside and lake borders. It is distributed all over the world as in North Africa, South East Europe, East Mediterranean and Western Asia **(Boulos, 1966)**. **Boulos (1999)** mentioned that, *A. graecorum* has long been treated as *A. maurorum* Medik. in Egypt **(Täckholm, 1974)**. On the other hand, **Meikle (1977)** distinguishes 2 species in Cyprus: *A. graecorum* and *A. maurorum*, the latter species being represented by two varieties.

The rate of seed germination is very low (**Hassanein and Mazen, 2001**), although various species of *Alhagi* propagates

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conventionally through seeds. Therefore, an efficient large scale propagation of this rare plant species is an essential prerequisite for meeting the upcoming pharmaceutical requirements, as well as for its sustainable utilization **(Agarwal *et al*., 2015).**

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**). It is generally recognized that plant genetic diversity changes in time and space. The extent and distribution of genetic diversity in a plant species depends on its evolution and breeding system, ecological and geographical factors, past bottlenecks, and often by many human factors **(Rao and Hodgkin, 2002).** One of the most important decisions to be used is DNA fingerprinting technique. Molecular markers have proven to be valuable tools in the evaluation of genetic variation both within and between species **(Powell *et al*., 1996)**. Molecular biology has contributed to the development of DNA- based markers that can be used for genotype identification, fingerprinting, genetic mapping and diversity assessment (**Khazaei *et al*., 2016**). On the other hand, ISSRs are DNA-based markers permit detection of polymorphisms in inter-microsatellite loci **(Karaca and Izbirak, 2008)**. These markers cover a large portion of the genome; ISSR may reveal a high number of polymorphic fragments per primer **(Meyer *et al*., 1993)**. Moreover, ISSRs have high reproducibility possibly due to the using of single longer primers (16–25 mers) that can be di-, tri-, tetra- or penta-nucleotide as compared to RAPD primers (10- mers) which permits the subsequent use of high annealing temperature (45–60 ^{o}C) leading to higher stringency **(Tikunov *et al*., 2003; Rakoczy- Trojanowska and Bolibok, 2004)**. ISSRs segregate mostly as dominant markers following simple Mendelian inheritance. However, they have also been shown to segregate as co-dominant markers in some cases thus enabling distinction between homozygotes and heterozygotes **(Akagi *et al*., 1996; Ratnaparkhe *et al*., 1998; Wang *et al*., 1998; Sankar and Moore, 2001)**. ISSR technique possesses a number of limitations. For instance, there is the possibility that fragments with the same mobility originate from non-homologous regions, which can contribute to some distortion in the estimates of genetic similarities. The molecular nature of the polymorphism can be known only if the fragments extracted from the gel are sequenced **(Reddy *et al*., 2002)**.

ISSR molecular markers are widely used for population genetic analysis of different plants generating more reliable and reproducible bands than RAPD (**Nagaoka and Ogihara, 1997; Zhang and Dai, 2010)**. It is used efficiently for studying genetic diversity of plants **(Ma *et al*., 2008; Etminan *et al*., 2016)**. Many investigators studied the genetic diversity and polymorphism using ISSR molecular primers on different plant species as *Salvadora persica* **(Monfared *et al*., 2018)**, *Lotus* species **(Ducar *et al*., 2018)**, *Phaseolus vulgaris* **(Cabral *et al*., 2018)**, *Teucrium polium* **(Tapeh *et al*., 2018)**, *Pongamia* plants **(Aldoori *et al*., 2019)**, *Capsicum* species **(Olatunji and Afolayan, 2019)** and *Stylosanthes scabra* **(Costa *et al*., 2019).** Currently, there is no report on genomic DNA isolation and the use of ISSR markers for molecular identification and genetic diversity characterization of collected *Alhagi graecorum* from Egypt. Therefore, isolation, amplification, and characterization of genomic DNA of 25 *A. graecorum* genotypes from different habitats in Egypt using eight ISSR molecular markers were carried out.

MATERIALS AND METHODS

Plant material: The material comprises 25 genotypes of *Alhagi graecorum* were collected from different five regions (five individuals from each region) were estimated. **(1-5);** El-Dakhla Oasis, **(6-10);**

Botanical garden of botany department, Ain Shams University, **(11-15);** Wadi El-Rayan, **(16-20);** Qarun Lake and **(21-25);** Siwa Oasis.

DNA extraction: Genomic DNA was extracted and purified from young leaves of the samples using 2% CTAB extraction buffer according to (**Doyle and Doyle, 1990**). DNA concentrations of total genomic DNA in each sample were estimated using a spectrophotometer (TU 1880 Double Beam UV-VIS). All the DNA samples were stored at −20 °C.

ISSR-PCR analysis: ISSR-PCR reactions were conducted using anchored primers, which were synthesized by Eurofins, Germany. Their names and sequences are indicated in Table (1). Amplification was performed in a Gene Amp® PCR System 9700 thermal cycler (Applied Biosystems) programmed using the temperature conditions as shown in Table (2). Electrophoresis of DNA samples were performed on 1.7 % agarose gel and visualized with 0.5 mg/ml ethidium bromide. DNA bands were photographed under UV light. The size of each amplification product was automatically estimated using UV soft image analyzer system.

Bulking DNA

Aliquots (10 µl) of the same individuals DNA concentration were mixed into a bulk of DNA representing each region. Hence, the outcome was 5 bulked DNA samples representing the different populations of *A. graecorum* included in this study. Eight ISSR primers were used to amplify the bulked DNA samples with the same conditions.

Statistical analysis

The analysis of molecular variance (AMOVA): This analysis was performed to estimate the variance components and their significance levels of genetic variation within and among populations using GenALEx program.

Genetic relatedness and cluster analysis: The scored binary data generated by ISSR marker was compared to determine the genetic relatedness of the 25 *A. graecorum* genotypes. Similarity matrices and cluster analyses for ISSR markers were performed individually as well as collectively. The ISSR binary matrices were processed using the Bio-Rad diversity database software package and converted into similarity matrices according to Dice coefficient (**Dice, 1945; Sneath and Sokal, 1973**). The formula used by Dice to estimate the genetic similarity coefficient (GS) between two genotypes was as follows:

Dice formula: GSij = 2a/ (2a+b+c)

Where GSij is the measure of genetic similarity between individuals i and j, (a) is the number of bands shared by i and j, (b) is the number of bands present in i and absent in j, and (c) is the number of bands present in j and absent in i.

Determining true genetic dissimilarity between individuals is an important and decisive point for clustering and analyzing diversity within and among populations (**Kosman and Leonard, 2005**). The UPGMA (Unweighted Pair -Group Method with Arithmetic Mean) is the algorithmic

Table 1: The list of primers and their nucleotide sequences used for ISSR markers in amplification of *A. graecorum* DNA.

No.	Primer code	Primer nucleotide sequence (5'→3')
1	ISSR-1	5'-AGAGAGAGAGAGAGAGYC-3'
2	ISSR-4	5'-ACACACACACACACACYG-3'
3	ISSR-5	5'-GTGTGTGTGTGTGTGTYG-3'
4	ISSR-6	5'-CGCGATAGATAGATAGATA-3'
5	ISSR-7	5'-GACGATAGATAGATAGATA-3'
6	ISSR-9	5'-GATAGATAGATAGATAGC-3'
7	ISSR-10	5'-GACAGACAGACAGACAAT-3'
8	ISSR-18	5'-HVHCACACACACACACAT-3'

A: Adenine, T: Thymine, G: Guanine, C: Cytosine, H: (A or C or T) and V: (A or C or G).

Table 2: The ISSR-PCR reaction parameters.

Steps	Temperature	Time	Cycles
Initial denaturation	94 °C	7 min	1
Denaturation	94 °C	1min	
Annealing	40- 50 °C	1min	40
Extension	72 °C	2 min	
Final extension	72 °C	7 min	1

method used in this study, which uses a specific series of calculations to estimate a tree (**Sokal and Michener, 1958**).

Principal coordinates analysis (PCoA): Also known as classical multidimensional scaling which is a set of data analyses that show the structure of complex distance-like data represented in a high dimensional space into the lowest possible dimensional space. Prinicipal coordinates analysis (PCoA) resembles principal component analysis (PCA) but it takes dissimilarity as input (**Pavoine *et al*., 2004; Gower, 2015**). PCoA was performed based on the matrix calculated for each marker using PAST software (**Hammer *et al*., 2001**).

RESULTS AND DISCUSSION

Characterization of the capability of each ISSR primer to detect polymorphism

The percentage of polymorphism detected by the eight ISSR primers among *Alhagi* samples was shown in Table (3) and Figure (1). El-Dakhla Oasis exhibited the highest value of polymorphism 60% for ISSR-5, while 87.5% among Ain Shams University population with ISSR-9 as compared to the other primers**.** The selected primers, ISSR-5 and ISSR-9 produced the highest value of polymorphism 90% and 85.7%, respectively among Wadi El-Rayan population; also in Qarun Lake the same primers produced the highest value of polymorphism 100% and 77.78%**.** High level of genetic variation was observed using ISSR-5 and ISSR-10 primers among Siwa Oasis population with 71.43% and 69.23% polymorphism, respectively (Table 3). Inter simple sequence repeat markers involves PCR amplification of DNA using a single primer composed of microsatellite sequences, sometimes anchored at the 3` or 5` end by 1 to 4 nucleotides. These primers target the simple sequence repeats and amplify the intervening region between the two SSRs in opposite orientations (**González *et al*., 2002). Reddy *et al*. (2002)** reported that the extent of polymorphism varies with the nature (unanchored, 3`-anchored, or 5`-anchored) and sequence of the repeats (motif) in the primer employed. Generally when 5` anchored primers are used, the amplified products include the microsatellite sequences and their length variations across a genome and therefore give more number of bands and a higher degree of polymorphism. Usually di-nucleotide repeats, anchored either at 3` or 5` end reveal high polymorphism (**Blair *et al*., 1999**; **Joshi *et al*., 2000**; **Nagaoka and Ogihara, 1997**). The primers anchored at 3` end give clearer banding pattern as compared to those anchored at 5` end (**Tsumura *et al*., 1996**; **Blair *et al*.,1999).**

The present results showed that most of the primers revealed high polymorphism contained di- nucleotide repeats; GA, CA, AC and AG indicating abundance of these repeats in *A. graecorum* genome. These results are in accordance with results obtained by **Rawat *et al*. (2018)** who reported that di- nucleotide primers with repeats; GA, CT, CA, GT, AC, AG and TC

Table 3: The percentage of polymorphism recorded using eight ISSR primers in *A. graecorum* genotypes collected from five different locations.

Primer code	Location	Total no. of bands	Monomorphic bands	Polymorphic bands	Percentage of polymorphism
ISSR-1	El-Dakhla oasis	3	3	0	00.00
	Ain shams university	11	9	2	18.18
	Wadi El-Rayan	7	3	4	57.14
	Qarun lake	10	3	7	70.00
	Siwa oasis	11	10	1	9.10
ISSR-4	El-Dakhla oasis	4	4	0	00.00
	Ain shams university	9	7	2	22.22
	Wadi El-Rayan	11	7	4	36.36
	Qarun lake	10	6	4	40.00
	Siwa oasis	8	6	2	25.00
ISSR-5	El-Dakhla oasis	5	2	3	60.00
	Ain shams university	10	10	0	00.00
	Wadi El-Rayan	10	1	9	90.00
	Qarun lake	12	0	12	100.00
	Siwa oasis	7	2	5	71.43
ISSR-6	El-Dakhla oasis	1	1	0	00.00
	Ain shams university	9	8	1	11.11
	Wadi El-Rayan	8	5	3	37.50
	Qarun lake	9	6	3	33.33
	Siwa oasis	11	7	4	36.36
ISSR-7	El-Dakhla oasis	7	5	2	28.57
	Ain shams university	7	5	2	28.57
	Wadi El-Rayan	4	4	0	00.00
	Qarun lake	7	6	1	14.29
	Siwa oasis	7	6	1	14.29
ISSR-9	El-Dakhla oasis	6	3	3	50.00
	Ain shams university	8	1	7	87.50
	Wadi El-Rayan	7	1	6	85.71
	Qarun lake	9	2	7	77.78
	Siwa oasis	9	4	5	55.56
ISSR-10	El-Dakhla oasis	10	7	3	30.00
	Ain shams university	11	10	1	9.10
	Wadi El-Rayan	11	6	5	45.45
	Qarun lake	13	5	8	61.54
	Siwa oasis	13	4	9	69.23
ISSR-18	El-Dakhla oasis	8	5	3	37.50
	Ain shams university	8	8	0	00.00
	Wadi El-Rayan	8	6	2	25.00
	Qarun lake	8	4	4	50.00
	Siwa oasis	8	4	4	50.00

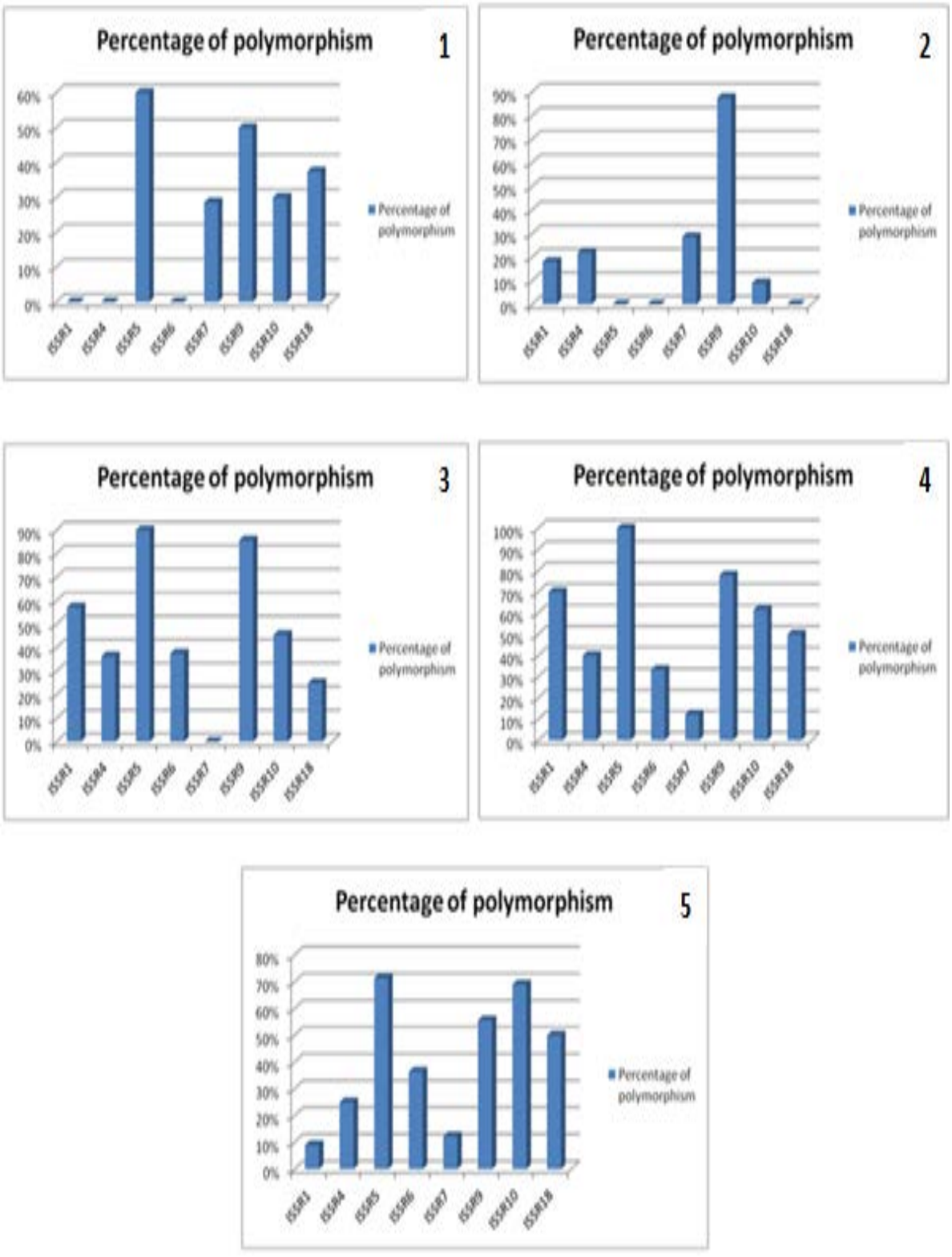

Figure 1: Polymorphism values detected using eight ISSR primers for *Alhagi* samples gathered from 1. El-Dakhla Oasis; 2. Ain Shams University; 3. Wadi El-Rayan; 4. Qarun Lake and 5. Siwa Oasis.

are abundant in the *Melia dubia* genome. This finding is reinforced by **Bhagyawant and Srivastava (2008)** who showed that in chick pea, dinucleotide repeats amplified more bands than tri-nucleotide and penta-nucleotide ones. **Rawat *et al*. (2018)** also reported that the high polymorphism is attributed to the abundance of dinucleotide repeats in the studied genotypes. Similarly, abundance of di-nucleotide repeats has been reported in other species like *Curculigo latifolia*, (**Ranjbarfard *et al*., 2014**). Also, abundance of tri-nucleotide repeats in *Azadirachta indica* (**Ranade and Farooqui, 2002**) and tri and tetra repeat in *Tectona grandis* (**Ansari *et al*., 2012**) has been reported. It was observed that AG and GA based primers were given high polymorphism **(Joshi *et al*., 2000; Reddy *et al*., 2002; Shilpa and Krishnan, 2016).** The differences between studies probably depend on the primer used and the band location **(Ismail *et al*., 2019)**.

Genetic diversity revealed by individuals- and bulked-samples-based approaches using ISSR markers in *A. graecorum*

Genetic relatedness/diversity between the 25 *A. graecorum* genotypes was analyzed using ISSR markers. Polymorphism revealed by eight ISSR primers was shown in Table (4) and Figure 2. Generally, a total of 101 bands were amplified among the 25 individuals; including 16 monomorphic DNA fragments and 85 were polymorphic with percentage 83.3%, three of them are unique bands. Whereas the number of polymorphic amplicons varied from 7 to 15 for ISSR-18 and ISSR-1, respectively (Table 4).The polymorphic information content (PIC) varied from 0.778 to 0.948 values where primer ISSR-7 had the lowest PIC value while primer ISSR-1 had the highest value with average 0.875. On the other hand, The ISSR fingerprinting patterns revealed by the eight primers used among the five bulked DNA samples of *Alhagi* populations are showed in Figure (3). The genetic polymorphism obtained by the eight ISSR primers between the five *Alhagi* populations, which yielded total number of 97 bands; 76 of these were polymorphic (78.4%) throughout the five populations with 18 unique bands (Table 5**)**. The number of polymorphic bands varied from 3 to 16 with ISSR-18 and ISSR-10, respectively but the percentage of the polymorphism are varied between 42.8% and 100% with ISSR-18 and ISSR-9, respectively. Polymorphic information content (PIC) varied among different ISSR primers from 0.268 to 0.768 with average 0.69. Analysis of molecular variance (AMOVA), used to estimate the genetic variability among populations, revealed significant genetic variance between and within the studied populations (Table 6), being most of the genetic diversity found among the populations (63%). ISSR markers can be used in population genetic studies of plant species as they effectively detect very low levels of genetic variation **(Zietkiewicz *et al*., 1994)**. In the present study, ISSR markers were effective to characterize the relationship or genetic diversity between genotypes and among populations. They also may have potential for analysis biogeographic patterns among populations of a single plant species.

Geleta and Bryngelsson, (2009) studied the genetic variation on ten populations of Afroalpine giant lobelia, *Lobelia rhynchopetalum*, from results they found that, the percentage of polymorphic loci across all samples and within population was 78% and 27%, respectively. The present results in agreement with **Tanya *et al*. (2011)** who genetically characterized 30 accessions of *Jatropha curcas* using ISSR markers and the analysis of molecular variance showed a high level of polymorphism (63%) among populations and low polymorphism (37%) within populations. Also, **Mosula *et al*. (2014)** studied the genetic diversity among three populations of *Gentiana lutea* using nine ISSR primers and according to the results of AMOVA, the high level of genetic

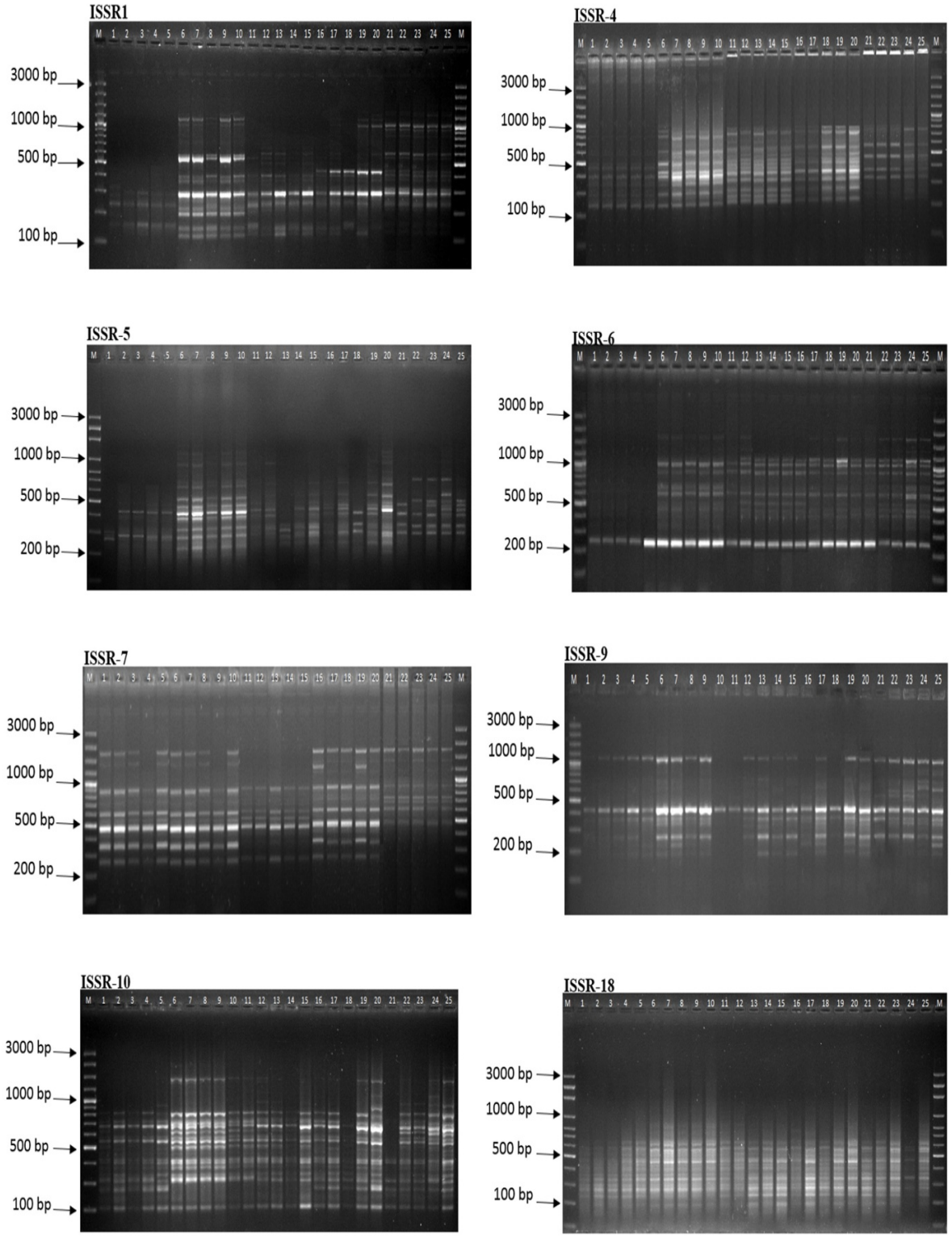

Figure 2: **Agarose gel electrophoresis result for PCR amplicons using eight ISSR primers to genetically characterize DNA of *A. graecorum* genotypes (numbered from 1-25). M refers to DNA marker 100 bp plus. (1-5); El-Dakhla Oasis, (6-10); Botanical garden of botany department, Ain Shams University, (11-15); Wadi El-Rayan, (16-20); Qarun Lake and (21-25); Siwa Oasis.**

Table 4: The total number of bands, polymorphic bands, monomorphic bands, percentage of polymorphism, unique bands, fragment size range and polymorphic information content (PIC) as revealed by ISSR analysis of 25 *A. graecorum* genotypes.

S.	Primer name	Total no. of bands	Polymorphic bands	monomorphic bands	Percentage of Polymorphism (POL %)	Unique bands	Fragment size range (bp)	PIC
1	**ISSR-1**	16	15	1	93.8	-	1397-120	0.948
2	**ISSR-4**	15	11	4	73	2	870-177	0.868
3	**ISSR-5**	15	15	-	100	1	1188-180	0.9
4	**ISSR-6**	11	10	1	90.9	-	1819-225	0.849
5	**ISSR-7**	10	7	3	70	-	1919-253	0.778
6	**ISSR-9**	9	8	1	88.9	-	1240-172	0.92
7	**ISSR-10**	15	12	3	80	-	1643-195	0.92
8	**ISSR-18**	10	7	3	70	-	976-184	0.816
	Total	**101**	85	16	-	**3**	-	-
	Average	12.6	10.6	2	**83.3**		-	**0.875**

Table 5: Total number of bands, polymorphic bands, monomorphic bands, percentage of polymorphism, unique bands, fragment size range and polymorphic information content (PIC) as revealed by ISSR analysis of *A. graecorum* bulked DNA samples.

S.	Primer Name	Total no. of bands	Polymorphic bands	Monomorphic bands	Percentage of polymorphism (POL %)	Unique bands	Fragment size range (bp)	PIC
1	**ISSR-1**	13	8	5	61.5	4	1277-130	0.768
2	**ISSR-4**	16	12	4	75	3	1216-198	0.768
3	**ISSR-5**	9	5	4	55.6	-	1442-162	0.768
4	**ISSR-6**	13	12	1	92.3	2	1600-345	0.768
5	**ISSR-7**	9	7	2	77.8	1	1780-320	0.672
6	**ISSR-9**	13	13	-	100	3	894-153	0.768
7	**ISSR-10**	17	16	1	94	5	1779-185	0.768
8	**ISSR-18**	7	3	4	42.8	-	849-176	0.268
	Total	**97**	76	21		**18**		
	Average	12.1	9.5	2.6	**78.4**			**0.69**

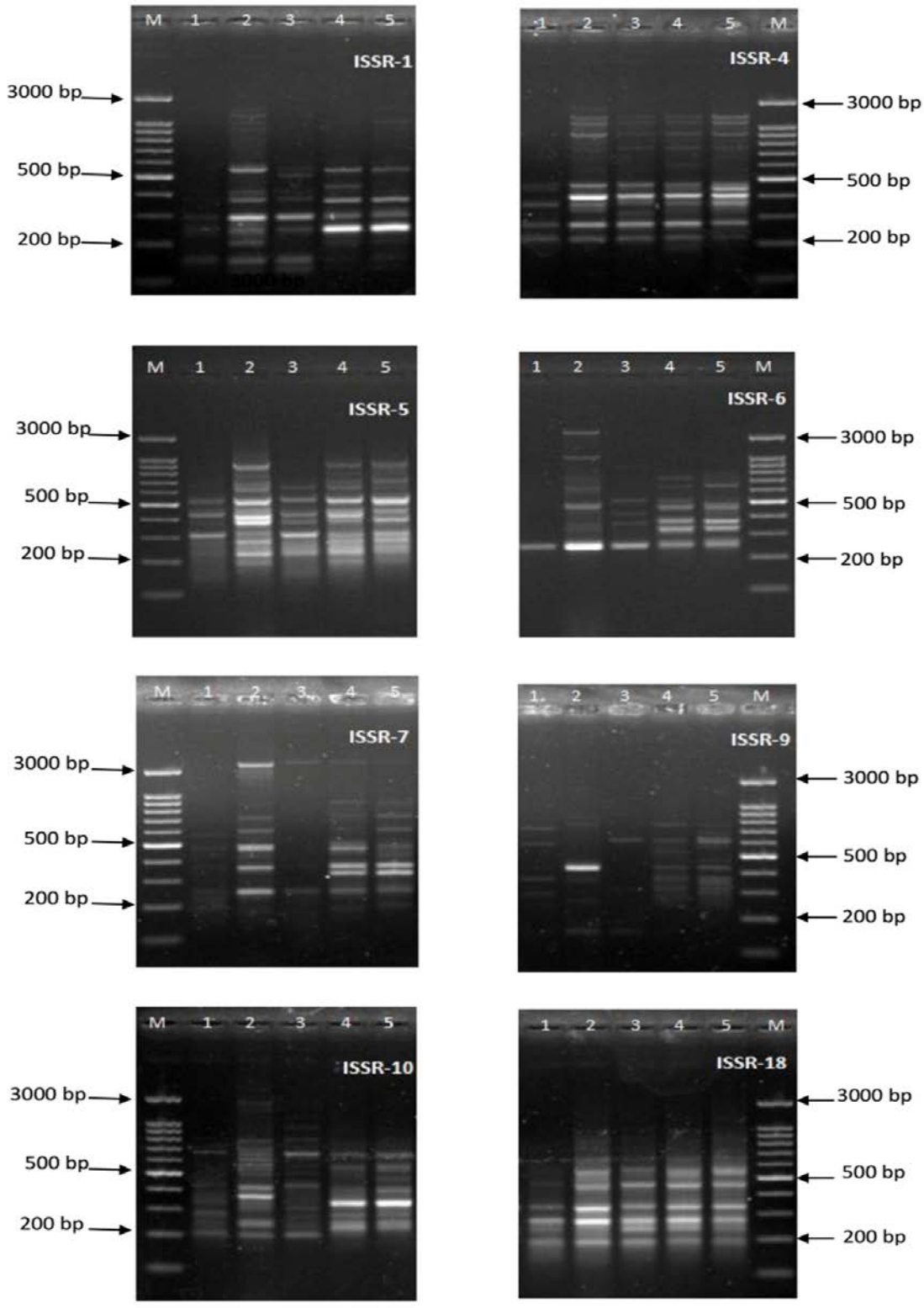

Figure 3: ISSR profiles (ISSRs 1, 4, 5, 6, 7, 9, 10 and 18) for *A. graecorum* bulked DNA samples. M refers to DNA ladder (100 bp) plus. *A. graecorum* populations are 1. El-Dakhla Oasis; 2. Ain Shams University; 3. Wadi El-Rayan; 4. Qarun Lake and 5. Siwa Oasis.

variation 59% regard differences between populations and 41% within populations. Similarly, the molecular polymorphism was 67.18% with ten primers and within-population variation was 32.34% of *Pongamia pinnata* using ISSR markers **Sahoo *et al.* (2010)**. **Costa *et al.* (2019)** evaluated the genetic diversity of *Stylosanthes scabra* using ISSR molecular markers. They found from their results that ISSR primers revealed a total of 88 bands with 95% polymorphism at the species level. In addition, the AMOVA test revealed that 40% of the total genetic variation occurs within populations and 60% among populations.

In contrast, the molecular variance analysis (AMOVA) in four natural populations of *Croton tetradenius* using thirteen ISSR primers was found (87%) within populations with a high level of polymorphism 94.8% **(Almeida-Pereira *et al.*, 2017)**. Furthermore, many researches revealed that; the genetic variation within and among the populations of different plants by using ISSR markers and AMOVA analysis and concluded that this variation was higher within populations than among them as *Murraya koenigii* **(Verma and Rana, 2011)**, *Jatropha curcas* **(Arolu *et al.*, 2012)** and *Ziziphus spina-christi* **(Alansi *et al.*, 2016).** Finally, genetic variation is a necessary condition for a species to adapt to environmental changes (**Liu *et al.*, 2015**).

The using of ISSR primers is considered highly informative with the PIC value, this effectiveness of a marker depends on the information content and the number of markers produced individually **(Thimmappaiah *et al.*, 2009).** If a PIC value is >0.5, then polymorphism is at a high level, while if it is between 0.25 and 0.5, the polymorphism is normal (**Turhan-Serttas and Özcan, 2018**). In the present work, the mean averages of PIC in the genotypes and bulked DNA samples (0.87 and 0.69), respectively. These high values similar to **Gelotar *et al.* (2019),** who recorded the value of average PIC was 0.85 among 19 genotypes of *Amaranthus* using 11 ISSR markers. Also**, Monfared *et al.* (2018)** who found the mean polymorphism information content (PIC) value was 0.63 among 50 genotypes of *Salvadora persica* . Recently, the genetic relationships of the varieties of *Capsicum* species using 10 ISSR primers were performed. Furthermore, the average polymorphism information content (PIC) was 0.67 **(Olatunji and Afolayan, 2019)**. In other studies, the PIC values were mostly lower than our values; it is between 0.25 and 0.5, such as (**Moradkhani *et al.*, 2015; Araujo *et al.*, 2016 and Tapeh *et al.*, 2018).** The results of our study demonstrated that the ISSR method is sufficiently informative and powerful to estimate the genetic diversity in *Alhagi* populations.

Genetic similarities matrix of *A. graecorum* as revealed by ISSR markers

To investigate the genetic similarity among the 25 examined *A. graecorum* genotypes based on ISSR results, the scored data obtained from eight primers were analyzed using the Dice coefficient to compute the similarity matrix. This similarity matrix was used to generate a dendrogram using the UPGMA method. As shown in Table (7), the estimated similarities among the 25 *Alhagi* genotypes ranged from 0.49 to 0.99. The highest genetic similarity (0.99) was between genotypes 6 and 7 (both individuals from Ain Shams University population), while the lowest genetic similarity (0.49) was between genotypes 1 (individual from El-Dakhla Oasis population) and 9 (individuals from Ain Shams University population).

On the other hand, the data resulted from ISSR scoring for the 5 bulked DNA *A. graecorum* populations were shown in Table (8). The estimated similarities ranged from 0.519 to 0.853. The highest genetic similarity (0.853%) was between Qarun Lake and Siwa Oasis populations, while the lowest genetic similarity (0.519%) was between El-Dakhla Oasis and Ain Shams

Genetic variation within and among the wild populations

Table 6: Analysis of molecular variance (AMOVA) for eight ISSR markers among *A. graecorum* populations.

Source of variations	Degree of freedom	Sum of squares	Mean square	Variance components	% of total variance
Among populations	4	252.96	63.24	11.32	**63**
Within populations	20	134.00	6.7	6.700	**37**
Total	24	386.96		18.01	**100**

Table 7: Genetic similarity matrix among the 25 *A. graecorum* genotypes as computed according to Dice's coefficient as revealed by ISSR markers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	1																								
2	0.91	1																							
3	0.91	0.97	1																						
4	0.83	0.92	0.92	1																					
5	0.86	0.92	0.89	0.92	1																				
6	0.51	0.56	0.56	0.58	0.61	1																			
7	0.51	0.56	0.56	0.58	0.61	0.99	1																		
8	0.55	0.59	0.59	0.58	0.61	0.95	0.93	1																	
9	0.49	0.53	0.53	0.58	0.57	0.98	0.96	0.94	1																
10	0.51	0.56	0.54	0.53	0.58	0.94	0.93	0.95	0.93	1															
11	0.58	0.62	0.59	0.63	0.64	0.68	0.68	0.71	0.70	0.73	1														
12	0.56	0.57	0.57	0.60	0.61	0.73	0.73	0.75	0.74	0.72	0.87	1													
13	0.61	0.62	0.62	0.67	0.64	0.73	0.71	0.71	0.75	0.68	0.75	0.83	1												
14	0.66	0.64	0.67	0.70	0.64	0.67	0.65	0.67	0.68	0.64	0.75	0.80	0.92	1											
15	0.60	0.61	0.61	0.66	0.63	0.75	0.73	0.76	0.77	0.73	0.81	0.85	0.91	0.92	1										
16	0.66	0.62	0.62	0.63	0.63	0.63	0.63	0.64	0.61	0.64	0.71	0.72	0.69	0.73	0.69	1									
17	0.65	0.67	0.67	0.72	0.71	0.78	0.78	0.74	0.77	0.73	0.73	0.74	0.80	0.79	0.83	0.79	1								
18	0.68	0.69	0.69	0.67	0.66	0.62	0.62	0.64	0.61	0.64	0.71	0.68	0.73	0.75	0.71	0.80	0.81	1							
19	0.62	0.63	0.63	0.67	0.68	0.77	0.77	0.73	0.76	0.74	0.69	0.73	0.74	0.76	0.79	0.76	0.90	0.78	1						
20	0.60	0.59	0.57	0.58	0.63	0.72	0.72	0.69	0.72	0.70	0.65	0.72	0.74	0.69	0.75	0.69	0.79	0.73	0.83	1					
21	0.58	0.59	0.61	0.55	0.54	0.61	0.61	0.61	0.59	0.60	0.62	0.67	0.70	0.70	0.69	0.64	0.72	0.77	0.71	0.68	1				
22	0.58	0.59	0.61	0.60	0.61	0.69	0.69	0.66	0.69	0.63	0.65	0.74	0.76	0.71	0.71	0.67	0.76	0.75	0.74	0.74	0.87	1			
23	0.57	0.59	0.59	0.58	0.61	0.74	0.74	0.72	0.73	0.70	0.67	0.75	0.76	0.69	0.76	0.64	0.77	0.71	0.77	0.79	0.86	0.95	1		
24	0.54	0.53	0.53	0.50	0.53	0.71	0.70	0.69	0.70	0.66	0.61	0.71	0.70	0.65	0.70	0.63	0.70	0.63	0.73	0.75	0.78	0.85	0.90	1	
25	0.54	0.57	0.57	0.59	0.62	0.76	0.75	0.71	0.73	0.69	0.64	0.72	0.76	0.69	0.75	0.63	0.76	0.68	0.77	0.74	0.81	0.90	0.93	0.93	1

Table 8: Genetic similarity matrix of five *A. graecorum* populations (bulked DNA samples) as computed according to Dice's coefficient from ISSR data. *Alhagi* populations are numbered (1-5); 1. El-Dakhla Oasis, 2. Ain Shams University, 3. Wadi El-Rayan, 4. Qarun Lake and 5. Siwa Osis.

	1	2	3	4	5
1	1				
2	0.519	1			
3	0.595	0.689	1		
4	0.542	0.716	0.684	1	
5	0.547	0.778	0.645	0.853	1

University populations. The result of the similarity index values (0.99) as mostly similar to **Pérez de la Torre *et al* (2012)** who reported simple matching coefficient of similarity varied from 0.8875 to 0.6659 in *Calibrachoa caesia*, indicating high levels of genetic similarity among the genotypes studied. Also, this is in agreement with the findings of **Turhan-Serttaş and Özcan (2018)** they examined the similarity matrix with Jaccard's coefficient (0.933) in *Pistacia lentiscus* varieties. In addition, **Okon *et al*. (2013)**, who amplified chamomile germplasm using ISSR primers and calculate the mean genetic similarity at 0.653.

Cluster analysis as revealed by ISSR markers and principal coordinates analysis

Based on the polymorphic ISSR fragments generated by 8 primers for 25 individuals, a dendrogram was constructed by cluster analysis of ISSR-based genetic distance using UPGMA cluster analysis (Figure 4A). The obtained dendrogram was divided into two main clusters the first cluster comprises El-Dakhla population (1-5), while the second cluster is divided into two sub-clusters. The first includes Ain Shams University population (6-10) and the second sub-cluster could be further divided into two groups. Populations coming from Wadi El-Rayan (11-15) and Qarun Lake (16-20) were classified in group I and Siwa Oasis population (21-25) were clustered in group II. On the dendrogram, all the individuals collected from the same region were grouped together. The resulting ISSR dendrogram from bulked DNA *Alhagi* populations, are differentiated into two clusters, the first cluster represented by El-Dakhla Oasis population only. The second cluster differentiated into two sub-clusters, first sub-cluster contain Wadi El-Rayan population while other remained populations represented the second sub-cluster, which further differentiated into two groups; the first contain Ain Shams University population while the second include both Qarun Lake and Siwa Oasis populations together (Figure 4B).

The relationship observed in the principle coordinate analysis (PCoA) was in agreement with the UPGMA analysis of 25 individuals; where group A contains El-Dakhla Oasis population, group B comprised Ain Shams University population, group C having all Siwa Oasis individuals and group D comprised Wadi El-Rayan and Qarun Lake populations (Figure 5a). Whereas the PCoA resulted from ISSR marker classified the five *A. graecorum* populations into three groups (Figure 5b), group A comprises El-Dakhla Oasis population, and group B contains Wadi El-Rayan population and group C with the remaining three populations (Ain Shams University, Qarun Lake and Siwa Oasis populations). The populations in this study were clustered according to the degree of similarity of their habitats and had nothing to do with their geographical location, the populations with similar habitats firstly clustered together to form a specific clade as **Zou *et al.* (2011)** who constructed a dendrogram using the UPMGA method. Geographic difference caused great differentiation within populations. On the basis of genetic distances, we constructed a dendrogram of genetic similarities of the studied genotypes with the use of the method UPGMA (Figure 4). From the dendrogram, we see that, all the individuals collected from the same region were grouped together. The pattern of genetic subdivision can be clearly demonstrated in the UPGMA cluster analysis, in which similar to the populations of *Mallotus oblongifolius* which divided into two groups according to their habitat type (**Yan *et al*., 2019**). The results of PCoA analysis also support this habitat-specific genetic clustering model.

Also, **Sica *et al*. (2005)** studied a genetic diversity in Italian *Asparagus acutifolius* using twenty-three ISSR primers, the distance UPGMA tree grouped together the genotypes strictly according to their geographical origin, showing that each

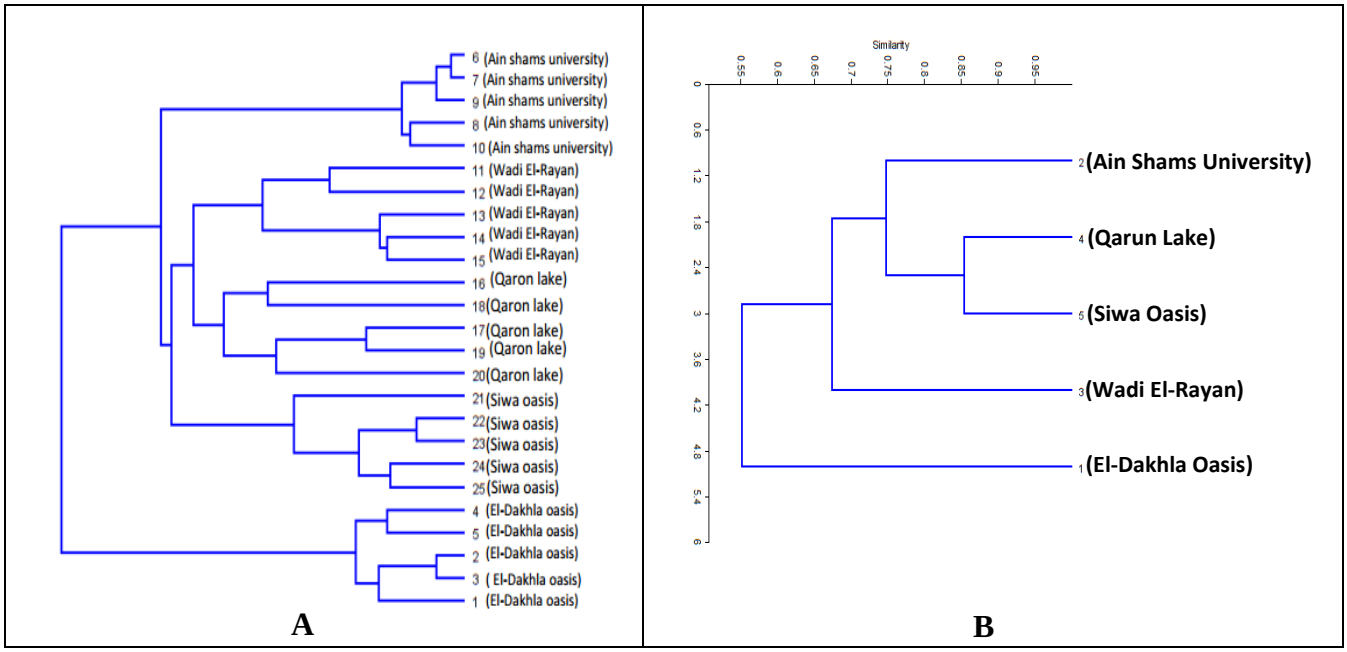

Figure 4: Dendrograms for A) 25 *A. graecorum* genotypes (1-25) B) five *Alhagi graecorum* populations (bulked DNA samples) constructed from the ISSR data using Unweighed Pair-group Arithmetic Average (UPGMA) and similarity matrix computed according to Dice's coefficient. Populations: 1. El-Dakhla Oasis; 2. Ain Shams University; 3. Wadi El-Rayan; 4. Qarun Lake; 5. Siwa Oasis.

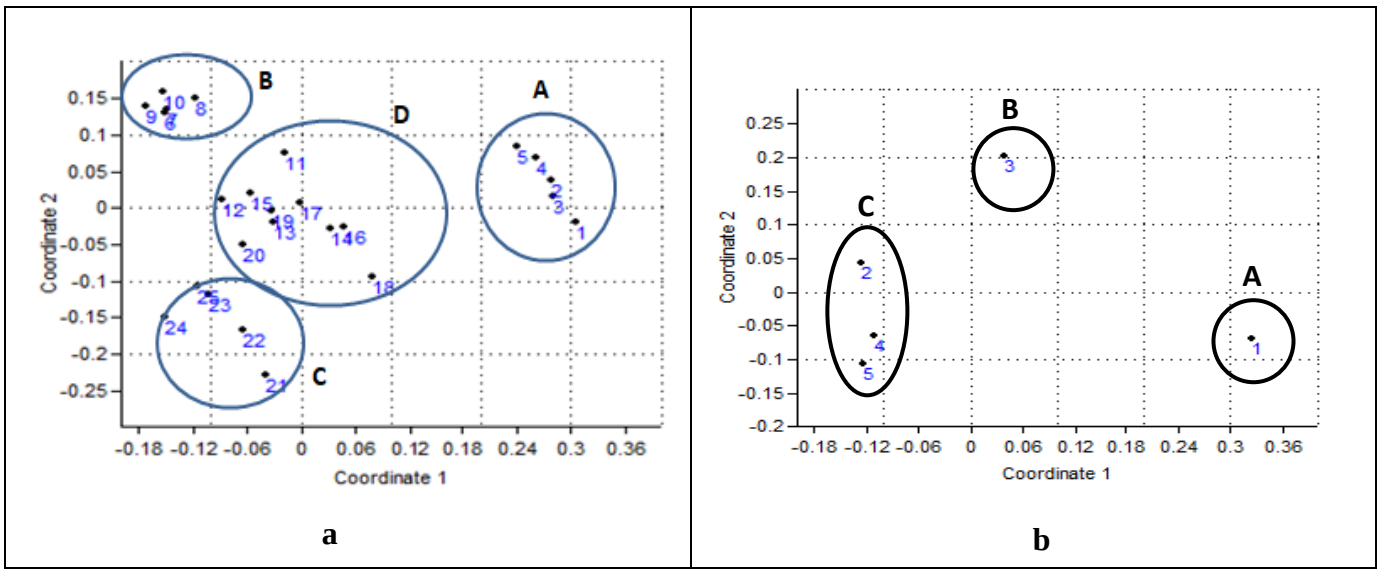

Figure 5: Scatter plot of principal coordinate analysis (PCoA) for a) 25 *Alhagi* individuals b) 5 *Alhagi* populations (bulked DNA samples) based on ISSR marker data: 1. El-Dakhla Oasis; 2. Ain Shams University; 3. Wadi El-Rayan; 4. Qarun Lake; 5. Siwa Oasis.

sample is genetically structured and can be considered as distinct population. There are many researchers determined the level of genetic diversity and relatedness in different plants as *Hagenia abyssinica* (**Feyissa *et al.*, 2007),** strawberry (**Debnath *et al*., 2008),** *Calibrachoa caesia* (**Pérez de la Torre *et al.*, 2012)** and *Pistacia lentiscus* (**Turhan-Serttaş and Özcan, 2018)** using UPGMA cluster and/or PCoA analysis.

CO*NCLUS*ION

To author knowledge, this is the first molecular marker reported based on genetic diversity study of *A. graecorum*. In the present study, the large number of polymorphic bands suggested that ISSR primers have been used successfully to study and analyse the genetic relationships within and among *Alhagi* populations. The molecular variations assessing of *A. graecorum* was 63% among populations while variation within populations accounted for the remaining 37%. The highest similarity was recorded between genotypes 6 and 7 (both individuals from Ain Shams University population) within the 25 *Alhagi* genotypes, while among the different five populations was between Qarun Lake and Siwa Oasis populations. The results obtained in this work indicated that ISSR are useful markers in genetic diversity assessment.

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