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Genetic Variation among Fragmented Populations of *Atriplex halimus* L. Using Start Codon Targeted (SCoT) and ITS1-5.8S-ITS2 Region Markers

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Abstract

Two forms of *A. halimus* shrubs: erect habit (*A. halimus*) and bushy habit shrub (*A. schweinfurthii*) are used naturally isolated by a considerable distance from each other and occupy the same area. To explore the effect of natural isolation on the genetic basis of the two forms, Start Codon Targeted (SCoT) and the phylogenetic relationships of *A. halimus* by sequencing ITS1-5.8S-ITS2 regions of the ribosomal DNA are used. Significant isolation-by-distance relationship was found (r = 0.62, *P* = 0.001). Soil factors did not influence molecular variations. The natural isolation of *A. halimus* habitats restricts gene flow among the populations and the observed high within-population genetic diversity (74.19%) in this species is best explained by its outcrossing behaviour, long-lived individuals and overlapping generations. The UPGMA analysis of the SCoT results showed that all the studied populations were divided into two discrete genetic groups with significant separation of the two forms in Burg El-Arab area (Populations 1 and 2) and insignificant separation between two forms in El-Hammam area (population 5 and 6). The sequencing of the ITS1-5.8S-ITS2 rDNA regions also showed the insignificant separation of the two *A. halimus* forms. We conclude that gene flow depending on habitat fragmentation was the main factor affecting the population genetic differentiation. We suggest that the two forms do not merit specific rank in presence of interference between the two forms and absence of a breeding barrier fail to separate the different populations when they become sympatric.

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Keywords

Atriplex halimus*, SCoT, ITS1-5.8S-ITS2 rDNA Regions, *Atriplex schweinfurthii

1. Introduction

Atriplex halimus L. (Chenopodiaceae), a C4 perennial shrub, highly outbreeding, is found in semi-arid and arid environments. This species, particularly well adapted to arid and salt-affected areas, is valued as livestock forage in low rainfall Mediterranean areas [1]-[4] and is considered as a promising forage plant for large-scale plantings [5]. *A. halimus* is able to accumulate heavy metals in plant tissue without displaying symptoms of toxicity during growth [6] [7]. In Egypt only one species viz., *halimus* with one variety viz; *schweinfurthii* was mentioned by Täckholm [8], while Boulos [9] mentioned only one species *A. halimus*. Recently the taxonomic revision of *Atriplex halimus* L. in Egypt revealed the presence of two subspecies namely: subsp. *halimus* L. and subsp. *schweinfurthii* Boiss [10]. This treatment was based on the pollen diversity. Both subspecies of *Atriplex halimus* L., are extremely heterogenous in terms of their morphology, ecology and productivity [11]. Amer & Abdo [10] noted a high degree of morphological variations in 52 populations of *A. halimus* (subsp. *halimus* and subsp. *shweinfurthii*), which was supported by the earlier works cited by Le Houérou [1]. Moreover, the two subspecies were confirmed by Walker *et al.* [12], who divided *A. halimus* into two groups diploid (2×), named *A. halimus* subsp. *halimus* and tetraploid (4×), named *A. halimus* L. subsp. *Schweinfurthii* Bioss. Hcini *et al.* [13] mentioned that *A. halimus* is dominant in the semi-arid and sub-humid areas, while *A. schweinfurthii* is more common in arid areas. However, the existence of intermediate morphotypes complicates the designation of plants as one or the other subspecies [14] [15]. Determination of DNA content showed that certain populations with morphologies intermediate between those considered typical of subsp. *halimus* and *schweinfurthii* were tetraploid [14].

In recent years, changing environmental conditions and the resulting threats to the survival of existing populations have resulted in increased interest to study how genetic variation is maintained in natural populations [16]. Among the environmental factors, the habitat variability and gene flow by seed or pollen dispersal can affect genetic diversity [17] [18]. Habitat variation often generates ecological barriers against gene flow and thus enhances genetic differentiation between local populations [16] [19]. In the past, *A. halimus* occupied a large area of distribution in the Mediterranean area of Egypt. Currently, its habitat has been severely fragmented; thus its genetic resources is required to be catalogued.

The first study of genetic variability of *A. halimus* was made by Haddioui & Baaziz [4] analysing the isoenzyme polymorphisms of nine populations from several locations in Morocco. Ortíz-Dorda *et al.* [20] extended the study to include ten countries in the Mediterranean basin using RAPD and ITS markers marker. Bouda *et al.* [21] applied RAPD analysis and AMOVA technique to determine the pattern and extent of genetic variations within and between natural populations of *A. halimus* from Morocco. Morphological, physiological and isozyme-based studies showed high genetic diversity in Moroccan populations of *A. halimus* [4] [22] [23].

Recently, new marker techniques have been developed depending on gene-targeted markers. A novel marker system called Start Codon Targeted (SCoT) Polymorphism [24] was developed based on the short conserved region flanking the ATG start codon in plant genes. SCoT markers are generally reproducible, and are similar to RAPD and ISSR because the same single primer is used as the forward and reverse primer [24] [25]. It is suggested that primer length and annealing temperature are not the factors determining reproducibility. These dominant markers could be used for genetic analysis, quantitative trait loci (QTL) mapping and segregation analysis [24]. These markers have been successfully used to study diversity in peanut, grape, potato and *Dendrobium nobile* [26]-[29].

The present study used two forms of *A. halimus* shrubs: erect habit (*A. halimus*) and bushy habit shrub (*A. schweinfurthii*). The two morphotypes are used naturally isolated by a considerable distance from each other and occupy the same area to explore the effect of natural isolation on the genetic basis of the two forms using Start Codon Targeted (SCoT) and the phylogenetic relationships of *A. halimus*by sequencing ITS1-5.8S-ITS2 regions of the ribosomal DNA. Besides, the previous results obtained from both isozymes and RAPDs markers collected from the Mediterranean basin populations were compared with the results of the present markers.

2. Material and Methods

2.1. Plant Populations

A total of 18 accessions of *A. halimus* were collected from six populations growing naturally in the Western Mediterranean Desert, Egypt (**Table 1**). Samples were collected from six populations. Three populations in the Mediterranean coastal land: Two distinctive forms (erect and bushy habit) from Burg Al-Arab (40 Km from Alexandria) and one population from El-Ghabaniat (45 Km from Alexandria). The other three populations were collected from El-Hammam (60 Km from Alexandria) (**Figure 1**). Soil physical and chemical characteristics were analysed by Richards [30] and Klimer & Alexander [31]. Soil characteristics supporting the six study populations are shown in **Table 2**.

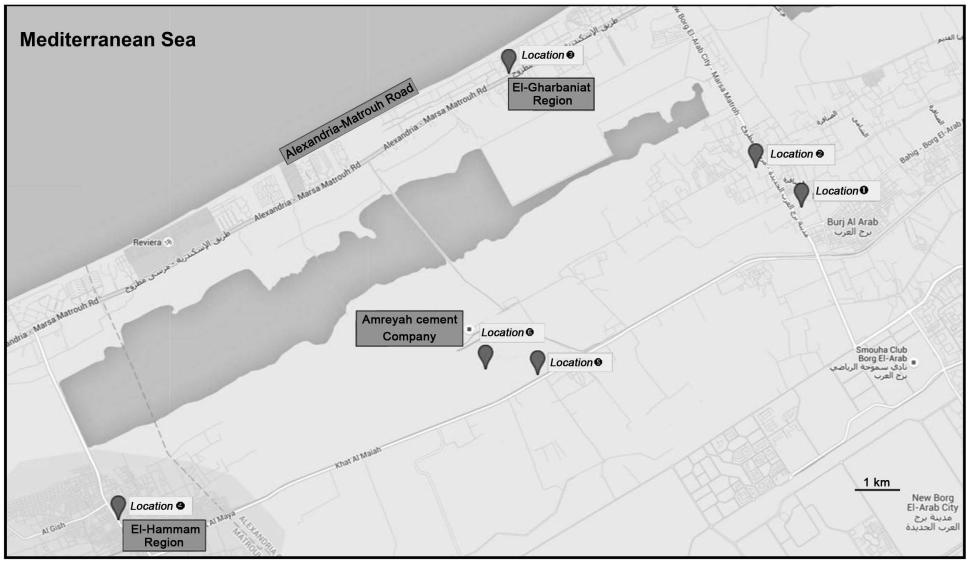

Figure 1. A map showing different locations from which the studied accessions of *A. halimus* were collected.

Table 1. Collection localities, habitats, dates and sample size of the studied populations of *A. halimus*.

Location	Population number	Site description	Growth form	Sample size	Sample code	Sampling site longitude and latitude
Burg El-Arab	1	Inland typical salt marches (Ideal habitat)	Bushy habit	4	1 - 4	30˚905' and 29˚536'
	2	Edges of run-road-transported sand to this area	Erect habit	3	5 - 7	30˚909' and 29˚533'
El-Gharbaniat	3	North slope, costal rocky ridge	Erect habit	3	8 - 10	30˚929' and 29˚474'
	4	Road run, Sandy plain	Erect habit	2	11 - 12	30˚847' and 29˚386'
El-Hammam	5	Polluted area (Cement factory)	Bushy habit	3	13 - 15	30˚862' and 29˚478'
	6	Polluted area (Cement factory)	Erect habit	3	16 - 18	30˚874' and 29˚475'

Table 2. The soil characteristics supporting the six studied populations of *A. halimus.*

Soil factor	Locations			
	Burg El-Arab Pop.1 & 2	El-Gharbaniat Pop.3	El-Hammam Pop.4	El-Hammam Pop.5 & 6
pH	8.6	8	8.3	8.5
Conductivity mmhos/cm	125	55.8	9.1	71.3
Sand%	13	60	48	31
Silt%	37	18	23	51
Clay%	50	22	29	18
$CaCO_3$ (mg/L)	45.6	55.8	37	16.5

2.2. DNA Extraction and SCoT-PCR Amplification

DNA was extracted from 2 g of young leaf tissue using DNA Plant Minipreps Kit (Bio Basic INC, Canada.), following the manufacturer instructions. All the PCR reactions were carried out in 25 µL volumes containing 50 ng of template DNA, 12.5 µl of PCR master mix buffer (2×) (Thermo, USA), and 20 pmol for each primer (**Table 3**). The reaction programs were set at 95˚C for 3 min, followed by 35 cycles of 30s at 95˚C, 30s at annealing 30˚C and 1 min at 72˚C, with a final extension at 72˚C for 10 min in a thermal cycler MyGene MG96+ (LongGene , USA). After completion of the amplification, 2.5 µL of 10× blue dye was added to the samples, and the amplified DNA was analyzed on 1.5% agarose gel in 1× TAE buffer at 65 - 70 V for 3 - 4 h. The amplified products were stained with ethidium bromide and photographed under UV illumination. Scoring for the presence or absence of DNA fragments was aided by the use of a 1 kb DNA ladder.

2.3. ITS1-5.8S-ITS2 rDNA Gene

For the phylogenetic the internal transcribed spacer ITS1 and ITS2 regions and the 5.8*S ribosomal DNA* (rDNA) regions were amplified by using universal primers ITS1 (5'-TCCGTA GGTGAACCTTGCGG-3') and ITS-4 (5'-TCCTCC GCTTATTGATATGC-3') [32]. Amplifications were performed in 25 µL volumes containing 50 ng of template DNA, 12.5 µl of PCR master mix buffer (2×) (Thermo, USA), and 20 pmol for each primer. PCR cycles were as follows: initial denaturation for 5 min at 95˚C followed by 34 cycles of 1 min at 95˚C, annealing at 58˚C for 1 min and extension at 72˚C for 1 min, and a final elongation step of 10 min at 72˚C. PCR products were then separated electrophoretically on agarose gel using 2% (w/v) agarose in 0.5× TBE buffer. The gel was stained with ethidium bromide. Then the PCR products were purified by EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic, Canada), following the manufacturer's instruction. Cycle sequencing was performed using T7SequencingTM kit (Pharmacia, Biotech, USA) and model 310 automated sequencer (Applied Biosystems, Foster City, CA, USA). This analysis was performed using a single plant from the six studied populations (**Table 1**). The DNA sequences were determined by Macrogene Company (Korea). The DNA sequences are deposited in GenBank http://www.ncbi.nlm.nih.gov under the accession numbers: KU555430 to KU555435.

2.4. Statistical Analysis

SCoT bands were binary scored; presence (1) or absence (0) characters to assemble the matrix of the SCoT phenotypes. Then, the indices of genetic diversity were calculated using POPGENE 3.2 Software [33] on the basis of gene frequencies. Hierarchical analysis of molecular variance (AMOVA) within and among populations was done using allele frequencies with ARLEQUIN V. 3.11 [34]. The Mantel test was applied using XLSTATARTVIS Software to test the significance of the association between the genetic distance and geographic distance matrices. The Pearson correlation between the genetic diversity index within population and ecological factors was analyzed using the SPSS 17.0 Software.

Table 3. Data of SCoT primers used in the present study and the extent of polymorphism**.**

Primer name	Primer sequence (5'-3')	Total no. of bands	No. of polymorphic bands	No. of monomorphic bands	% of Polymorphism (P)
S4	CAACAATGGCTACCACCT	18	17	1	94.44
S6	CAACAATGGCTACCACGC	21	20	1	95.23
S7	CAACAATGGCTACCACGG	19	18	1	94.73
S9	CAACAATGGCTACCACGT	13	13	0	100
S10	CAACAATGGCTACCAGCC	19	19	0	100
S12	ACGACATGGCGACCAACG	15	13	2	86.66
S17	ACCATGGCTACCACCGAG	16	16	0	100
S32	CCATGGCTACCACCGCAC	19	19	0	100
S34	ACCATGGCTACCACCGCA	14	14	0	100
S36	CATGGCTACCACCCGCCC	23	23	0	100
	Total	177	172	5	
	Average	17.7	17.2	0.5	97.10

Genetic similarity was calculated on the basis of genetic distance coefficient using the NTSYS-pc program [35]. The similarity matrix was subjected to cluster analysis by Unweighted Pair Group Method with Arithmetic averages (UPGMA) [36].

Pairwise and multiple DNA sequence alignment were carried out using CLUSTAL W version 1.81 (http://seqtool.sdsc.edu/CGI/BW.cgi; Thompson *et al*. [37]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [38]. Neighbour-Joining (NJ) algorithm was employed to construct phylogenetic relationships. Using NJ, the evolutionary distances were computed using the Maximum Composite Likelihood model and reliability of the branches was assessed by bootstrapping the data with 1000 replicates. Phylogenetic comparisons included *A. canescens* (AM420672), *A. glauca* (AY873928) and *A. prostrate* (HM005857) as out-groups.

3. Results

3.1. SCoT-PCR

For analysis of variability of *A. halimus*, 10 primers were used for studying the SCoT banding patterns across the entire samples. A total of 177 amplification products were scored of which 172 were polymorphic, exhibiting 97.10% polymorphism. The amplification products using 10 primers ranged from 86.66% to 100% in producing polymorphic bands (**Table 3**). The primers S9, S10, S17, S32, S34 and S36 exhibited the highest level of polymorphism with the percentage of polymorphic bands to be 100% for all these primers (**Table 3** and **Figure 2**).

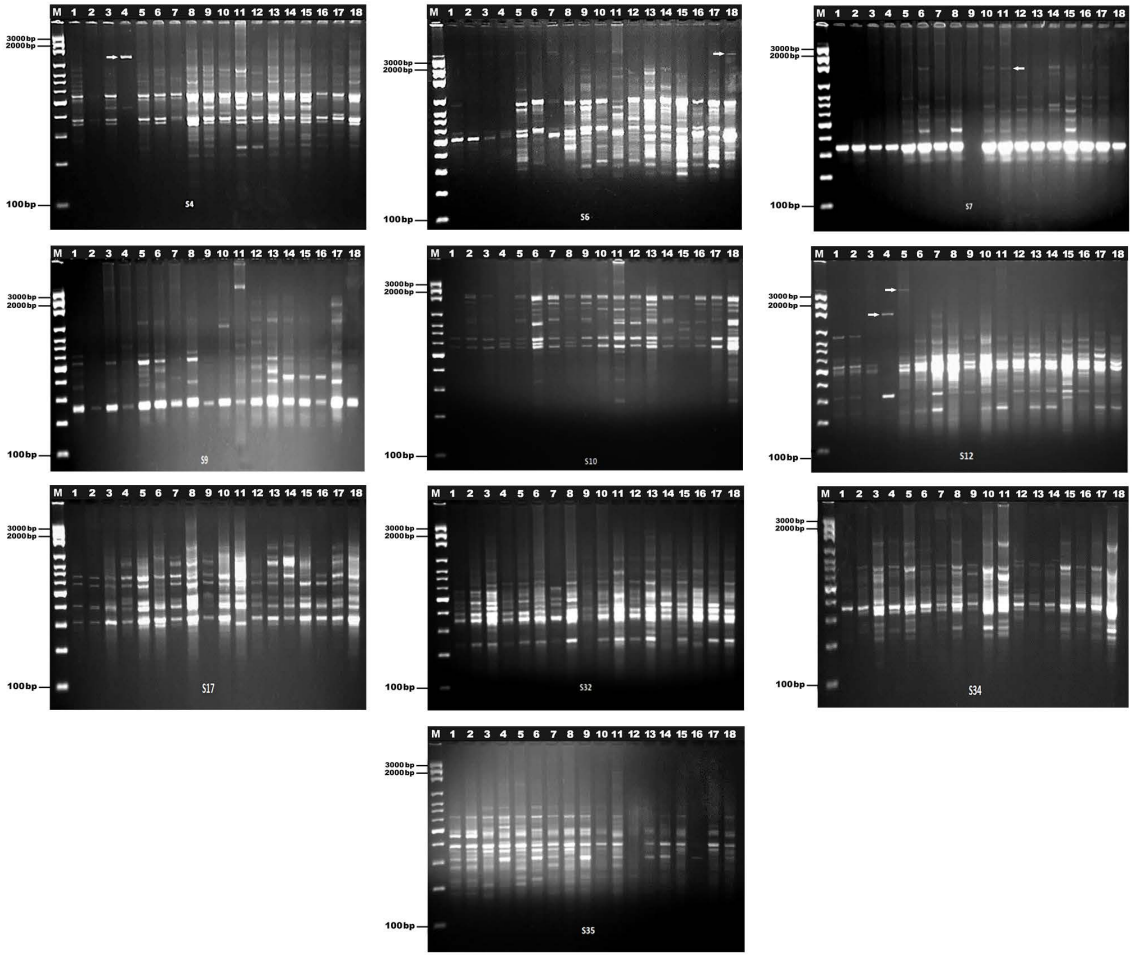

Figure 2. SCoT profiles generated from genomic DNA of *A. halimus* populations with primers; M: DNA marker, lanes 1-4: Burg El-Arab (1), lanes 4-7: Burg El-Arab(2), lanes 8-10: El-Gharbaniat, lanes 11-12: El-Hammam (1), lanes 13-15: El-Hammam (2) and lanes: 16-18 El-Hammam (3), arrows = monomorphic bands.

3.2. Genetic Diversity in Plant Populations

Ten primers (**Table 3**) generated a total of 177 SCoT bands (loci), 17.7 bands per primer on average. The number of amplification products per primer varied from 13 to 23, and these primers produced fragments ranging from 200 to 3000 bp in size (**Figure 2**). The observed number of alleles (N_a) and effective number of alleles (N_e) ranged between 1.355 - 1.553 and 1.284 - 1.412, respectively. Similarly, Nei's gene diversity (h) and Shannon's Information index (I) ranged between 0.226 - 0.327 with overall diversity of 0.28 and 0.13 - 0.24 with an average value of 0.43, respectively. The percentage of polymorphic loci (*PPL*) was estimated in the range of 35.51% to 55.37%. The gene flow value and the diversity among populations were found to be 0.362 and 0.881, respectively (**Table 4**). It was found that the genetic variation in population 6 growing in El-Hammam area reached the highest value (I = 0.327) and population 2 growing in road-run of Burg El-Arab attained the lowest value (I = 0.226).

3.3. Genetic Structure within and among Populations

The estimate of genetic structure of populations is significantly different from zero ($P < 0.0001$). Analysis of AMOVA showed that genetic variation (74.19%) was observed within the populations, whereas the variance among populations was 25.81% (**Table 5**), which was in accordance with the *Gst* (36.2%). The estimate of gene

Table 4. Genetic diversity and differentiation parameters for six natural populations of *A. halimus* in Northwest Egypt.

Populations	Sample size	Polymorphic loci (N_p)	Percentage population level (*PPL* %)	Observed number of alleles (N_a)	Number of effective alleles (N_e)	Shannon's Index of diversity (I)	Nei's gene diversity (h)	$h_S \pm$ SD	$h_t \pm$ SD	G_{ST}	*Nm*
Pop1	4	98	55.37	1.553	1.368	0.323	0.218				
Pop2	3	63	35.59	1.355	1.284	0.226	0.158				
Pop3	3	82	46.33	1.463	1.371	0.294	0.205				
Pop4	2	73	41.24	1.412	1.412	0.285	0.206				
Pop5	3	67	37.85	1.378	1.325	0.240	0.168				
Pop6	3	91	51.41	1.514	1.411	0.327	0.228				
Population level	3	79	44.63	1.445	1.361	0.282	0.197				
Species level	18	169	95.48	1.954	1.594	0.512	0.355	0.342 ± 0.022	0.218 ± 0.108	0.362	0.881

N_p = number of polymorphic loci; *PPL*% = percentage of polymorphic loci; Na = observed number of alleles; N_e = effective number of alleles; I = Shannon's information index; h = Nei's gene diversity; h_S = Gene diversity within population; h_t = total gene diversity; SD = standard deviation; G_{ST} = diversity among populations; *Nm* = gene flow 0.25 $(1 - G_{ST})/G_{ST}$.

Table 5. Hierarchical analysis of molecular variance (AMOVA) within and among natural populations of *A. halimus*in Northwest Egypt. The *P* values are the probabilities of having a greater variance component than the observed values by chance alone and are based on 1023 random permutations of the data matrix.

Source of variation	df	Sum of squares	Variance components	Percentage variation	*P*
Among populations	5	253.028	8.647 Va	25.81	***
Within populations	12	298.250	24.854 Vb	74.19	***
Total	17	551.278			
Fixation Index (*Fst*)		0.25813			***
Among regions	2	149.736	7.081	20.09	**
Among populations Within regions	3	103.292	3.310	9.39	**
Within populations	12	298.250	24.854	70.52	**

***$P < 0.0001$, **$P < 0.005$.

flow N_m based on *Gst* was 0.880 (**Table 4**). This result is equivalent to Fixation Indices (*Fst*) 0.258 $P < 0.0001$ calculated with the Arlequin program, which implies a low degree of differentiation among populations.

To identify the source of the highest genetic variation, AMOVA analysis was also performed among groups; Group (1) includes the erect form and Group (2) includes the Chameophyte form populations. Genetic variation among the two groups was found to be low, that reaching 1.9% of the total variation. The amount of genetic variation among regions and among populations was 20.09% and 9.39%, respectively, with the reminder (70.52%) occurring within populations, suggesting that there is no significant genetic differentiation of populations.

The Mantel test showed a significant correlation between genetic distance and geographic distance (r = 0.673, $P = 0.025$).

3.4. Cluster Analysis

The neighbour-joining dendrogram based on the genetic distance between populations revealed a similar pattern: the genetic distances among the populations showed a spatial pattern that corresponded to their geographic locations (**Figure 3**). Moreover, all six populations were clustered into two geographical groups. Clear geographical pattern of genetic diversity was identified between Burg El-Arab populations (1 and 2) and the rest of the studied populations.

Based on UPGMA clustering algorithm generated from the obtained SCoT dataset, the populations were grouped into two distinct groups (**Figure 3**). The four individuals (*A. halimus*) of population one of Burg El-Arab were included in one group and the other five populations were included in another group. Population 1 of Burg El-Arab is the most differentiated from the rest of the studied populations, even from population 2 which is the most geographically closely located population (4 Km apart). Calculated the cophenetic correlation coefficient (0.72) shows a relatively good fit of the data are obtained with the dendrogram. In the meantime, all populations of *A. halimus* could be discriminated from each other, except that of population 4, one individual (18) of population 5 from El-Hammam area and one individual (9) of population 3 from El-Gharbaniat area. The hierarchical AMOVA analysis indicating that the among-regions genetic variation was weak (25.81%) which agrees with the dendrogram which shows that most individuals from a given population tend to cluster together and are, therefore, more genetically similar than individuals from different populations.

3.5. Correlation between Genetic Diversity and Soil Factors

The correlation analysis indicated that the genetic diversity indices of different populations showed insignificant ($P > 0.05$) correlations between the genetic diversity indexes and the soil factors. This indicates that the soil factors had no effect on population's structure and there were no local adaptation of the studied populations.

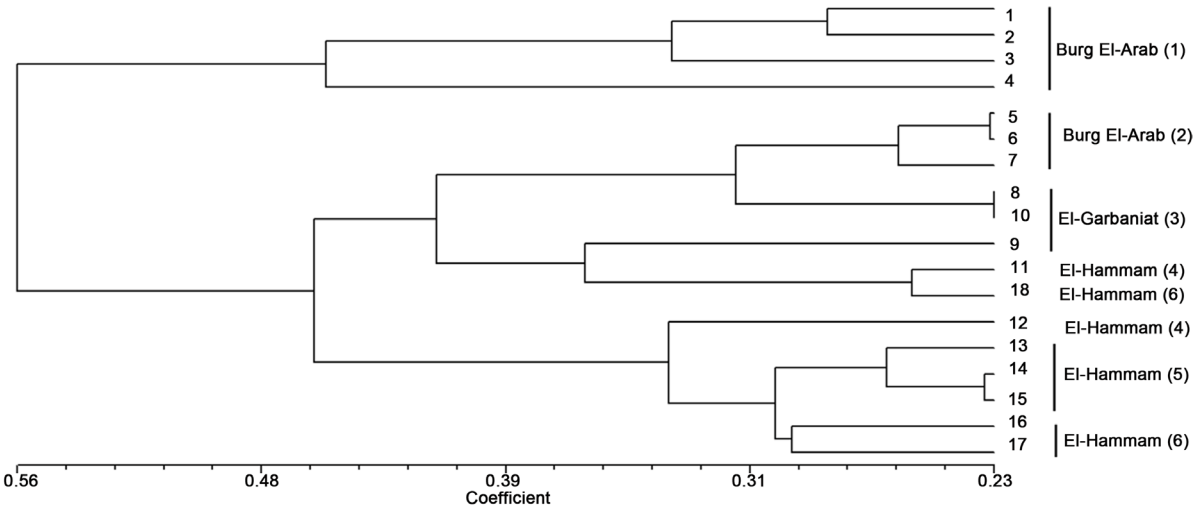

Figure 3. Dendrogram based on genetic distances and Jaccard coefficient computed from SCoT data using algorithm of Unweighted Pair Group Method with Arithmetic Averages (UPGMA) in the six *A. halimus* studied populations.

3.6. ITS1-5.8S-ITS2 rDNA Gene

The ITS1-5.8S-ITS2 rDNA gene was successfully amplified from the six individuals of the six populations (**Figure 4**). DNA sequencing results revealed an approximately 1200 bp fragment of the ITS1-5.8S-ITS2 rDNA. The edited nucleotide sequences of the six individuals ITS1-5.8S-ITS2 gene were subjected to sequence analysis using CLUSTAL W (1.81) multiple sequence alignment (**Appendix**). Comparative nucleotide sequence alignment revealed that the ITS1-5.8S-ITS2 rDNA gene was homologous between the studied individuals. The phylogenetic analysis based on ITS1-5.8S-ITS2 regions sequences presented in **Figure 5**. All population grouped together except population 4 from El-Hammam, which clearly separated in a node supported by a bootstrap value of 98. Using *A. canescens*, *A. glauca* and *A. prostrata* as outgroups in the phylogenetic analysis, *A. canescens*, *A. glauca* are the species closest to *A. halimus* from this group, while *A. prostrata* is the most distant.

4. Discussion

The study of genetic diversity of *Atriplex halimus* from diverse environment of the Mediterranean Basin showed a very high intra-populational diversity [4] [20] [21]. These authors found that two genetics groups of *A. halimus* can be distinguished and the genetic diversity of their collection was explained mainly by the within population component. The present work studied the two *A. halimus* forms (erect and bushy habit) occupying the same area and naturally isolated by a considerable distance from each other in Egypt with two types of genetic markers; SCoT and ITS.

Results of SCoT analysis showed differences in the genetic diversity among populations of *A. halimus* from different locations. The total gene diversity (hT) and the genetic parameters (*PPL*%, *I*, *h*, N_a, N_e) at population level were lower in Population 2 from Burg El-Arab and population 5 from El-Hammam than in the other

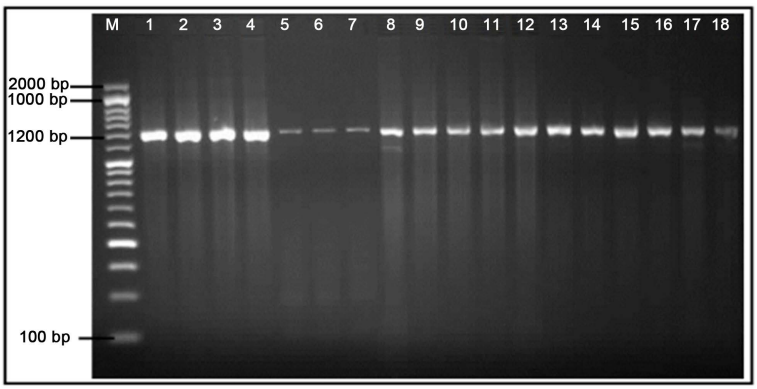

Figure 4. Amplified product of ribosomal DNA gene (approx. 1200 bp) of six *A. halimus* populations; M: DNA marker, lanes 1-4: Burg El-Arab (1), lanes 4-7: Burg El-Arab (2), lanes 8-10: El-Gharbaniat, lanes 11-12: El-Hammam (1), lanes 13-15: El-Hammam (2) and lanes 16-18: El-Hammam (3).

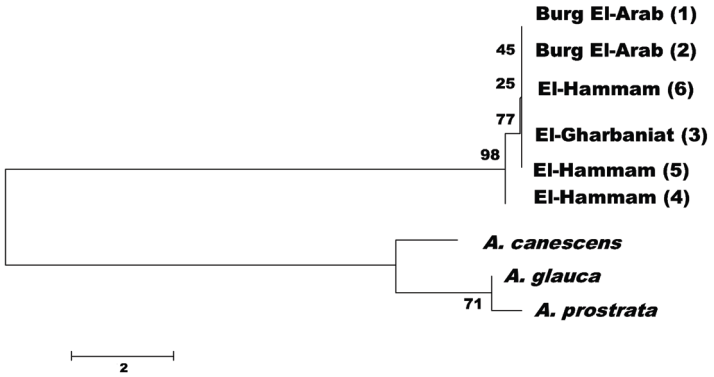

Figure 5. Phylogenetic tree calculated by Neighbor-Joining (NJ) method with 1000 bootstrap replications based on the nucleotide sequences of ribosomal DNA gene in the six *A. halimus* populations.

populations. In the present study, the mean value of Nei's gene diversity index (*h*) was 0.197, near to the minimum h value (0.174 - 0.328) of nine out-crossing plants summarized by Schoen and Brown [39]. The estimate of gene flow Nm based on *Gst* was 0.888, which indicates that gene flow among populations was low (**Table 5**). This result was equivalent to fixation indices (*Fst* = 0.258, *P* = 0.001) calculated with Arlequin program, which implies nearly moderate degree of differentiation among population according to Wright [40] (1965) (*Fst* > 0.15 and <0.25). This result agrees with Bouda *et al*. [41] who suggested that *A. halimus* populations from Morocco are very largely differentiated based on RAPD data (*Fst* = 0.334, *P* = 0.000). The estimate of *Gst* (0.362) at the species is consistent with the characteristics of outbreeding species, according to studies based on dominant markers (RAPD), which lead to the conclusion that population differentiation is lower in outbreeding than in inbreeding species (*Gst* < 0.23 and >0.5, respectively; Hamrick & Godt [42]; Nybom & Bartish [43]. The present study established that *A. halimus* in Egypt had a considerable amount of genetic variations at SCoT loci which are not correlated with soil factors. These results suggested that SCoT diversity is non-adaptive by natural selection and does not influence by soil factors and their interactions.

The analysis by AMOVA implied that 1/4 of genetic variation occurred among population and most of the variation (61.9%) settled within the populations. Our results are compatible with the pattern of species that are primarily outcrossing and long lived-wind pollinated shrub, which retain most of their genetic variability within populations [43]-[45]. The level of population differentiation 25.81% obtained in this study was lower than that found among 51 *A. halimus* populations (29.18%) from ten countries in the Mediterranean basin and 12 populations (33.43%) from Morocco, analysed by RAPDs [20]-[41]. However, Ortíz-Dorda *et al*. [20] studied six populations from Egypt (Matrouh area) and they concluded that these populations had the lowest value of genetic diversity among the populations studied and they all were clustered in one group.

Outcrossing species usually have a high within-population diversity and low population differentiation, whereas selfing species often have low within population diversity and high differentiation among populations [44] [46]-[49]. The genetic structure of plant populations is also influenced by the long-term evolutionary and ecological history of the species, which would include shifts in distribution, habitat fragmentation and population isolation [50]. Wu *et al*. [51] proposed that Tacca originated from the southern marginal area of the Palaearctic continent when Pangaea expanded to the Pacific Ocean for the first time. Later, this genus became differentiated in a succession of nearby environments.

The results (**Table 2**) indicate that 20.09% of the total variation is between regions (*P* < 0.001). The variation between regions is near to the variation among populations, which coincide with the isolation by distance (Mantel test r = 0.673, *P* = 0.025). Populations within regions accounts for 9% of the total variation and the variation within populations represent 74.19% of the total variations. This indicates that, there is a significant structuring and separation of populations.

The low estimates of gene flow (N_m = 0.881) among wind-pollinated *A. halimus* populations, correspond well with the geographic isolation of the populations, in which genetic differentiation among populations appears to be highly correlated with geographic distance between populations (r = 0.673, *P* = 0.025), although the detected geographical effect might be associated with differences between the type of isolation between populations (resort facilities, sand transport etc.). For example, Population 1 (bushy habit) and population 2 (erect habit) from Burg El-Arab are geographically close, but separated with a high genetic distance. The two locations are isolated by buildings of resorts, contrary to population 5 (bushy habit) and population 6 (erect habit) which are geographically and genetically close. The N_m would be low, with increased spatial isolation of small populations caused by habitat fragmentation [52]. Furthermore Limited gene flow due to habitat isolation could lead to a stronger genetic differentiation among populations compared with plants found in less-isolated habitats [49]. Our results from the cluster analysis based on the coefficients of genetic distances showed evident differentiation among the six *A. halimus* populations, where the genetic distance between two forms from Burg El-Arab is higher than the isolation by distance and a significant geographic sub-structure was confirmed by Mantel test. Genetic divergence was particularly low among populations of *A. halimus*, which may be explained by restricted gene flow. Similarly, significant correlation between genetic and geographic distances has been found in outcrossing species *Prunus mahaleb* [53] and long-lived perennial species *Quercus petrae* [54]. In contrast, no correspondence between geographic and genetic distances has been found in 12 populations from different locations in Moracco, long-lived, perennial species *Haloxylon ammodendron* [55] thus, isolation by distance has played an important role in establishing the genetic structure of this species. The significant correlation between the genetic and the geographic distances assumed that migration or gene flow rather than genetic drift or natural

selection was the main factors affecting the population genetic differentiation [56].

Another factor may play an important role in the evolution of *A. halimus*; morphogenesis of reproductive structures is remarkably plastic in *A. halimus*, since flowers of both architectural patterns might be both male, female or hermaphroditic and their distribution along the reproductive axes as well as their relative occurrence were dependent on environmental conditions. *A. halimus* is mainly considered a monoecious species which is occasionally dioecious. However, some authors have found individuals that present unisexual and hermaphrodite flowers so this species could be polygamous or, more precisely, trimonoecious [57]. Amer & Abdo [10] established that there was a relation between polyploidy and pollen morphological variations in *Atriplex halimus* L., in case of erect habit (*A. halimus*) the pollen sterility increases with the increase of salinity and aridity. *A. schweinfurthii* showed more adaptive character to arid and saline soil, and the pollen fertility increased under these harsh environmental conditions.

The present study used two forms of *A. halimus* shrubs; erect habit (*A. halimus*) and bushy habit shrub (*A. schweinfurthii*). The two morphotypes are used naturally isolated by a considerable distance from each other and occupying the same area to explore the effect of natural isolation on the genetic basis of the two forms using Start Codon Targeted (SCoT) and the phylogenetic relationships of *A. halimus* by sequencing ITS1-5.8S-ITS2 regions of the ribosomal DNA. Besides, we compared the previous results obtained with isozymes markers and RAPDs from Mediterranean basin to the present marker. According to previous reports, *A. halimus* includes two quite different groups in terms of habitat and morphology; subspecies *halimus* and subspecies *schweinfurthii*. However, these subspecies described by Le Houérou are not accepted taxonomical units. Ortíz-Dorda [20] distinguished two genetic groups are rather coincident with the characters described by Le Houérou [1]-[11]. The connection between the two genetic groups described by Ortíz-Dorda [20] and the two subspecies described by Le Houérou [1] is uncertain particularly in our study which revealed the uncertain genetic separation between the two types of morphologies of *A. halimus* using Start Codon Targeted (SCoT) marker and the sequencing of ITS1-5.8S-ITS2 regions. According to Ortíz-Dorda, [20] all studied individuals from Egypt belong to subspecies *schweinfurthii* (Bushy habit) contrasting to our collection in which the majority characterized by erect habit (subspecies *halimus*). Moreover, it was found that *A. halimus* is dominant in the semi-arid, sub-humid areas (population 3) and also in arid areas of *A. schweinfurthii* habitat.

5. Conclusion

Habitat fragmentation through land use change can limit connectivity between populations and gene flow between *A. halimus* populations. There is interference between the two forms of *A. halimus* viz. *A. halimus* and *A. schweinfurthii*. We conclude that the two forms do not merit specific rank in presence of intermediate morphotypes between the two forms and absence of a breeding barrier.

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Appendix

The sequences of the fragment ribosomal DNA amplified by PCR of the six studied populations.

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El-Hammam     (6)     CCGGGGAATCGCTTCGCCTTGGCGGGGCGTCCTTCCCGGCACAATAACGAACCCCGGCGC
Burg El-Arab  (2)     CCGGGGAATCGCTTCGCCTTGGCGGGGCGTCCTTCCCGGCACAATAACGAACCCCGGCGC
El-Gharbaniat (3)     CCGGGGAATCGCTTCGCCTTGGCGGGGCGTCCTTCCCGGCACAATAACGAACCCCGGCGC
El-Hammam     (4)     CCGGGGAATCGCTTCGCCTTGGCGGGGCGTCCTTCCCGGCACAATAACGAACCCCGGCGC
El-Hammam     (5)     CCGGGGAATCGCTTCCCCTTGGCGGGGCGTCCTTCCCGGCATAATAACCAACCCCGGCGC
Burg El-Arab  (1)     CCGGGGAATCGCTTCGCCTTGGCGGGGCGTCCTTCCCGGCACAATAACGAACCCCGGCGC
                      ************** ************************ ****** **********

El-Hammam     (6)     GGTCTGCGCCAAGGAACATGAATACAAGCGTGCCCTTCTCCGACCGGTTCGCCGGTCGTG
Burg El-Arab  (2)     GGTCTGCGCCAAGGAACATGAATACAAGCGTGCCCTTCTCCGACCGGTTCGCCGGTCGTG
El-Gharbaniat (3)     GGTCTGCGCCAAGGAACATGAATACAAGCGTGCCCTTCTCCGACCGGTTCGCCGGTCGTG
El-Hammam     (4)     GGTCTGCGCCAAGGAACATGAATACAAGCGTGCCCTTCTCCGACCGGTTCGCCGGTCGTG
El-Hammam     (5)     GGTCTGCGCCAAGGAACATGAATACAAGCGTGCCCTTCTCCGACTGGTTCGCCGGTCGTG
Burg El-Arab  (1)     GGTCTGCGCCAAGGAACATGAATACAAGCGTGCCCTTCTCCGACCGGTTCGCCGGTCGTG
                      **************************************** ***************

El-Hammam     (6)     GACGTGGCACCAAGTCGTATATAACATTAAACGACTCTCGGCAACGGATATCTCGGCTCT
Burg El-Arab  (2)     GACGTGGCACCAAGTCGTATATAACATTAAACGACTCTCGGCAACGGATATCTCGGCTCT
El-Gharbaniat (3)     GACGTGGCACCAAGTCGTATATAACATTAAACGACTCTCGGCAACGGATATCTCGGCTCT
El-Hammam     (4)     GACGTGGCACCAAGTCGTATATAACATTAAACGACTCTCGGCAACGGATATCTCGGCTCT
El-Hammam     (5)     GACGTGGCACCAAGTCGTATATAACATTAAACGACTCTCGGCAACGGATATCTCGGCTCT
Burg El-Arab  (1)     GACGTGGCACCAAGTCGTATATAACATTAAACGACTCTCGGCAACGGATATCTCGGCTCT
                      ************************************************************

El-Hammam     (6)     CGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
Burg El-Arab  (2)     CGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
El-Gharbaniat (3)     CGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
El-Hammam     (4)     CGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
El-Hammam     (5)     CGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCACAATCCCGTGAA
Burg El-Arab  (1)     CGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
                      ********************************************** ***********

El-Hammam     (6)     CCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCTTTAGGTTGAGGGCACGCCTGCCT
Burg El-Arab  (2)     CCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCTTTAGGTTGAGGGCACGCCTGCCT
El-Gharbaniat (3)     CCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCTTTAGGTTGAGGGCACGCCTGCCT
El-Hammam     (4)     CCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCTTTAGGTTGAGGGCACGCCTGCCT
El-Hammam     (5)     CCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCTTTAGGTTGAGGGCACGCCTGCCT
Burg El-Arab  (1)     CCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCTTTAGGTTGAGGGCACGCCTGCCT
                      ************************************************************

El-Hammam     (6)     GGGCGTCACGCATCGCGTCTCCCCCCACCACCCCGTGTGGATGGGGAGGAGGATGATGGC
Burg El-Arab  (2)     GGGCGTCACGCATCGCGTCTCCCCCCACCACCCCGTGTGGATGGGGAGGAGGATGATGGC
El-Gharbaniat (3)     GGGCGTCACGCATCGCGTCTCCCCCCACCACCCCGTGTGGATGGGGAGGAGGATGATGGC
El-Hammam     (4)     GGGCGTCACGCATCGCGTCTCCCCCCACCACCCCGTGTGGATGGGGAGGAGGATGATGGC
El-Hammam     (5)     GGGCGTCACGCATCGCGTCTCCCCCCACCACCCCGTGTGGATGGGGAGGAGGATGATGGC
Burg El-Arab  (1)     GGGCGTCACGCATCGCGTCTCCCCCCACCACCCCGTGTGGATGGGGAGGAGGATGATGGC
                      ************************************************************

El-Hammam     (6)     CTCCCATGCCTCACCGGGCGTGGATGGCCTAAATATGGAGCCCCCGGTTACGAAGTGCCG
Burg El-Arab  (2)     CTCCCATGCCTCACCGGGCGTGGATGGCCTAAATATGGAGCCCCCGGTTACGAAGTGCCG
El-Gharbaniat (3)     CTCCCATGCCTCACCGGGCGTGGATGGCCTAAATATGGAGCCCCCGGTTACGAAGTGCCG
El-Hammam     (4)     CTCCCATGCCTCACCGGGCGTGGATGGCCTAAATATGGAGCCCCCGGTTACGAAGTGCCG
El-Hammam     (5)     CTCCCATGCCTCACCGGGCGTGGATGGCCTAAATATGGAGCCCCCGGTTACNAANTGCCG
Burg El-Arab  (1)     CTCCCATGCCTCACCGGGCGTGGATGGCCTAAATATGGAGCCCCCGGTTACGAAGTGCCG
                      ************************************************** ** *****

El-Hammam     (6)     CGGCAATTGGTGGAATACAAGGCCACGCCTAGGATGAAACGGTAGTCGCGCACATCGTGG
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Burg El-Arab  (2)     CGGCAATTGGTGGAATACAAGGCCACGCCTAGGATGAAACGGTAGTCGCGCACATCGTGG
El-Gharbaniat (3)     CGGCAATTGGTGGAATACAAGGCCACGCCTAGGATGAAACGGTAGTCGCGCACATCGTGG
El-Hammam     (4)     CGGCAATTGGTGGAATACAAGGCCACGCCTANGATGAAACGGTAGTCGCGCACATCGTGG
El-Hammam     (5)     CGGNNNTTGGTGGAATACAAGGNCACCCCTANGATGAAACGGTANTCGCGCACATCNTGG
Burg El-Arab  (1)     CGGCAATTGGTGGAATACAAGGCCACGCCTAGGATGAAACGGTAGTCGCGCACATCGTGG
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El-Hammam     (6)     CTCTTGAGGACTCGCAGGACCCTTACTTGTTTGCCCCTAGGGGCGGCAAAACCGTTGCGA
Burg El-Arab  (2)     CTCTTGAGGACTCGCAGGACCCTTACTTGTTTGCCCCTAGGGGCGGCAAAACCGTTGCGA
El-Gharbaniat (3)     CTCTTGAGGACTCGCAGGACCCTTACTTGTTTGCCCCTAGGGGCGGCAAAACCGTTGCGA
El-Hammam     (4)     CTCTTGAGGACTCGCAGGACCCTTACTTGTTTGCCCCTAGGGGCGGCAAAACCGTTGCGA
El-Hammam     (5)     CTCTTGANGACTNNNNNGACCCTTACTTGTTTGCCCCTANGGGCGGCAAAACCGTTGCGA
Burg El-Arab  (1)     CTCTTGAGGACTCGCAGGACCCTTACTTGTTTGCCCCTAGGGGCGGCAAAACCGTTGCGA
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El-Hammam     (6)     CCCC-AGGTCAGGCGGGGCTACCCGCTGAGTTTAAGCATAT
Burg El-Arab  (2)     CCCC-AGGTCAGGCGGGGCTACCCGCTGANTTTAAGCATAT
El-Gharbaniat (3)     CCCC-AGGTCAGGCGGGGCTACCCGCTGAGTTTAAGCATAT
El-Hammam     (4)     CCCC-AGGTCAGGCGGGGCTACCCGCTGAGTTTAANCATAT
El-Hammam     (5)     CCCCCAGGTCAGGCGGNGCTACCCGCTGAGTTTAAGCATAT
Burg El-Arab  (1)     CCCC-AGGTCAGGCGGGGCTACCCGCTGAGTTTAAGCATAT
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