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Genetic Variation of the Species *Atriplex halimus* L. (Chenopodiaceae) Using the ITS1-5.8s-ITS2 Region of the Ribosomal DNA

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Abstract: *Atriplex halimus* L. (Chenopodiaceae) is well adapted to dry saline habitats and widely distributed in the Mediterranean Basin. The ITS1-5.8S-ITS2 region of the ribosomal DNA was used to study the genetic variation of 13 *A. halimus* populations collected from five countries in the Mediterranean Basin (France, Spain, Tunisia, Morocco and Algeria). For the study of the Phylogenetic relationships of the 13 samples were obtained by analyzing and sequencing of the ITS1-5.8S-ITS2 region of the ribosomal DNA. The present work indicates that two genetics groups of *A. halimus* can be distinguished after analyzing the genetic variation of 13 populations from five countries in the Mediterranean Basin.

Key words: *Atriplex halimus* L. · Genetic variation · ITS1-5.8S-ITS2 region · rDNA

INTRODUCTION

Atriplex halimus L. (Chenopodiaceae) a monoecious, highly outbreeding, C_4 perennial shrub, is found in semi-arid and arid environments. This species is interesting because of its tolerance to environmental stresses, its use as a fodder shrub for livestock in low rainfall Mediterranean areas [1-4] and its value as a promising forage plant for large-scale plantings [5]. It has been divided into two subspecies: subsp. *halimus* (2n = 2x = 18) and subsp. *schweinfurthii* (2n = 4x =36) [6, 1]. The subspecies are based on differences in morphological characters. However the existence of intermediate morphotypes complicates the designation of plants as one or the other subspecies [7]. The two sub-species show relatively large levels of morphological variability. It seems that there is a large genetic variability in *A. halimus* L. populations of different Mediterranean origins [8, 9] and such heterogeneity could be exploited to develop adequate populations with a combination of good traits such as high adaptability to limiting factors in semi-arid Mediterranean environments. Haddioui and Baaziz [4] performed isozyme polymorphism in *A. halimus* and found high degree of genetic diversity within population. Ortiz-Dorda *et al.* [10] studied the RAPD-PCR marker polymorphism and sequenced the ribosomal DNA ITS1-5.8S-ITS2 region of *A. halimus* populations from ten countries of the Mediterranean Basin. They found that the populations separated in two genetic groups. Description and Conservation of *A. halimus* L. genetic resources seem particularly important for the rehabilitation of disturbed areas by salt and low rainfall. However, few genetic investigations have been carried out in this species. The aim of the present work was to study the genetic variation and phylogenetic relationships of *A. halimus* L. by sequencing the ITS1-5.8S-ITS2 region of the ribosomal DNA [11].

MATERIALS AND METHODS

Plant Material and Genomic DNA Isolation: A total of 13 populations of *A. halimus* L. from five countries in the Mediterranean Basin were collected and analyzed (Table 1). Four individuals for each population were analyzed. In order to assess the phylogeny of *A. halimus* L., the entire ITS1 and ITS2 regions, together with the 5.8S gene sequence between them, were sequenced by using the universal primers ITS4 and ITS5 [11].

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Table 1: List of the populations of *Atriplex halimus* L. used in the study with their origin

No	Sample	Origin	Country
1	M.1	Tensift	Morocco
2	M.2	Moulay Hafid	Morocco
3	A.1	Djelfa	Algeria
4	A.2	Hoggar	Algeria
5	T.1	Gabes	Tunisia
6	T.2	Sidi Bouzid	Tunisia
7	T3	Medenine	Tunisia
8	T4	Kairouan	Tunisia
9	E.1	Kairouan	Spain
10	E.2	Aranjuez	Spain
11	E.3	Madrid	Spain
12	E.4	Murcia	Spain
13	F.1	Marseille	France

The total genomic DNA of *A. halimus* L. was isolated from fresh leaf material by using the modified CTAB (cetyl-trimetyl ammonium bromide) method according to Torres *et al.* [12]. Leaf material was ground to a fine powder in liquid nitrogen using a pestle and mortar. The DNA was extracted with 1 ml of extraction buffer [1 M Tris-HCl, 0.5 M EDTA (pH 8.0), 5 M NaCl, 2% CTAB, 1% PVP-40, 0.1% $NaHSO_3$, 0.2% β-mercaptoetanol] and transferred to a 1.5 ml Eppendorf tube. The samples were then mixed with 100 µl of chloroform-isoamyl alcohol (24:1) and incubated in water bath at 60°C for 20 to 30 min and then centrifuged at 10.000 g for 4 min at 5°C. The aqueous phase was transferred to another tube containing 1 ml of 95% cold-ethanol and was rest for 20-60 min at -20°C. The crude DNA was spooled out with a glass rod and suspended in 1 ml of 0.2 M sodium acetate in 76% ethanol and after it was transferred to another tube which contained 1 ml of 0.01 M ammonium acetate in 76% ethanol. The DNA was air dried, redissolved in 200 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and stored at 4°C.

PCR Reactions and Dna Sequencing: PCR-amplification was performed according to Williams *et al.* [13] with some modifications. The reaction volume was 25 µl containing 2.5 µl reaction buffer 10x (160 mM $(NH4)_2SO_4$, 670 mM Tris-HCl, pH 8.8 at 25°C and 0.1% Tween-20), 2.5 µl Cl_2Mg 2.5 mM, 2.0 µl dNTPS 0.2 mM, 0.3 µl *Taq* polymerase 5 / µl of Ecotaq Replitherm, (Perkin Elmer Geno Tech), 0.5 µl of each primer (5 µM) and 4.0 µl of DNA. The amplification was performed in a Gene Amp 9700 PCR System (Perkin-Elmer (Norwalk, CN).

The amplification program consisted of 2 min at 95° for initial DNA denaturation followed by 30 cycles of 35 sec at 94°C, 1 min at 56°C, 1 min at 72 °C and final cycle was followed by 5 min at 72°C.

The amplification product was analyzed by electrophoresis in 1.6% agarose gel in 1x TBE buffer (89 mM Tris-base pH 8.0, 89 mM boric acid and 5 mM EDTA) and run in the same buffer for 2 h at 160 V. The gel was stained with 0.5 µg/ml of ethidium bromide and photographed under UV light.

DNA was directly amplified by PCR using the ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5' GGAAGTAAAAGTCGTAACAAGG-3') primers according to White *et al.* [11]. The amplified fragments were directly sequenced in an ABI Prism 310 sequencer (Applied Biosystems, Foster City, CA, USA) at the University of Valencia (Burjassot, Valencia, Spain).

Data Analysis: Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 3.0 [14]. The genetic distance was calculated by the method of Kimura 2-parameter [15]. Bootstrap was calculated after 2000 permutations. The tree was represented by the method Neighbour Joining.

RESULTS AND DISCUSSION

After sequencing the ribosomal DNA fragments amplified by PCR (Fig. 1.) we obtained 13 sequences (Fig. 2.). The sequences were 615 nucleotides long and nucleotide 1 to 238 corresponded to the ITS1 region; nucleotide 239 to 400, corresponded to the 5.8S gene and nucleotide 401 to 615 corresponded to ITS2 region (determined by comparison with sequences in the GenBank). The points indicated that the four nucleotide letters (A, G, C, T) were identical to the nucleotides of the first row.

Fig. 1: Example of an agarose gel showing the ribosomal DNA fragments amplified by PCR using the ITS4 and ITS5 primers and four plants of three populations G, Sb and Me (reference to populations in Table 1), M: marker (100 bp)

#A.1-Algerie	CGGAAGGATC	ATTGTCGAAA	CCTGCCCAGC	AGAGCGACCA	GAGAACGTGT	TTATCATGAA	CGGGGTCGGG	GTGAAAGCCC
#A.2-Algerie	....	....	....	....	....	....	....	....
#T.1-Tunisie	.......T..	....	....	....	....	....	....	....
#T.2-Tunisie	.......T..	....	....	....	....	....	....	....
#T.3-Tunisie	....	....	....	....	....	....	....	....
#M.1-Maroc	....	....	....	....	....	....	....	....
#T.4-Tunisie	....	....	....	....	....	....	....	....
#M.2-Maroc	....	....	....	....	....	....	....	....
#E.1-Espagne	....	....	....	....	....	.......T..	....	....
#F.1-France	....	....	....	....	....	.......T..	....	....
#E.2-Espagne	....	....	....	....	....	....	....	....
#E.3-Espagne	....	....	....	....	....	....	....	....
#E.4-Espagne	....	....	....	....	....	....	....	....

#A.1-Algerie	CTTCCTCAAG	CCGGGGAATC	GCTTCGCCTT	GGCGGGGCGT	CCTTCCCGGC	ACAATAACGA	ACCCCGGCGC	GGTCTGCGCC
#A.2-Algerie	....	....	....	....	....	....	....	....
#T.1-Tunisie	....	....	....	....	....	....	....	....
#T.2-Tunisie	....	....	....	....	....	....	....	....
#T.3-Tunisie	....	....	....	....	....	....	....	....
#M.1-Maroc	....	....	....	....	....	....	....	....
#T.4-Tunisie	....	....	....	....	....	....	....	....
#M.2-Maroc	....	....	....	....	....	....	....	....
#E.1-Espagne	....	....	....	....	....	....	....	....
#F.1-France	....	....	....	....	....	....	....	....
#E.2-Espagne	....	....	....	....	....	....	....	....
#E.3-Espagne	....	....	....	....	....	....	....	....
#E.4-Espagne	....	....	....	....	....	....	....	....

#A.1-Algerie	AAGGAACATG	AATACAAGCG	TGCCCTTCTC	CGACCGGTTC	GCCGGTCGTG	GACGTGGCAC	CAAGTCGTAT	ATAACATTAA
#A.2-Algerie	....	....	....	....	....	....	....	....
#T.1-Tunisie	....	....	....	....	....	....	....	....
#T.2-Tunisie	....	....	....	....	....	....	....	....
#T.3-Tunisie	....	....	....	....	....	....	....	....
#M.1-Maroc	....	....	....	....	....	....	....	....
#T.4-Tunisie	....	....	....	....	....	....	....	....
#M.2-Maroc	....	....	....	....	....	....	....	....
#E.1-Espagne	....	....	....	.........T	..T.......	....	....	....
#F.1-France	....	....	....	.........T	..T.......	....	....	....
#E.2-Espagne	....	....	....	.........T	....	....	....	....
#E.3-Espagne	....	....	....	.........T	....	....	....	....
#E.4-Espagne	....	....	....	.........T	....	....	....	....

#A.1-Algerie	ACGACTCTCG	GCAACGGATA	TCTCGGCTCT	CGCATCGATG	AAGAACGTAG	CGAAATGCGA	TACTTGGTGT	GAATTGCAGA
#A.2-Algerie	....	....	....	....	....	....	....	....
#T.1-Tunisie	....	....	....	....	....	....	....	....
#T.2-Tunisie	....	....	....	....	....	....	....	....
#T.3-Tunisie	....	....	....	....	....	....	....	....
#M.1-Maroc	....	....	....	....	....	....	....	....
#T.4-Tunisie	....	....	....	....	....	....	....	....
#M.2-Maroc	....	....	....	....	....	....	....	....
#E.1-Espagne	....	....	....	....	....	....	....	....
#F.1-France	....	....	....	....	....	....	....	....
#E.2-Espagne	....	....	....	....	....	....	....	....
#E.3-Espagne	....	....	....	....	....	....	....	....
#E.4-Espagne	....	....	....	....	....	....	....	....

#A.1-Algerie	ATCCCGTGAA	CCATCGAGTC	TTTGAACGCA	AGTTGCGCCC	GAAGCCTTTA	GGTTGAGGGC	ACGCCTGCCT	GGGCGTCACG
#A.2-Algerie	....	....	....	....	....	....	....	....
#T.1-Tunisie	....	....	....	....	....	....	....	....
#T.2-Tunisie	....	....	....	....	....	....	....	....
#T.3-Tunisie	....	....	....	....	....	....	....	....
#M.1-Maroc	....	....	....	....	....	....	....	....
#T.4-Tunisie	....	....	....	....	....	....	....	....
#M.2-Maroc	....	....	....	....	....	....	....	....
#E.1-Espagne	....	....	....	....	....	....	....	....
#F.1-France	....	....	....	....	....	....	....	....
#E.2-Espagne	....	....	....	....	....	...C......	....	....
#E.3-Espagne	....	....	....	....	....	...C......	....	....
#E.4-Espagne	....	....	....	....	....	...C......	....	....

#A.1-Algerie	CATCGCGTCT	CCCCCCACCA	CCCCGTGTGG	ATGGGGAGGA	GGA--TGATG	GCCTTCCATG	CCTCACCGGG	CGTGGATGGC
#A.2-Algerie	....	....	....	....	...--.....	....	....	....
#T.1-Tunisie	....	....	....	....	...--.....	....C.....	....	....
#T.2-Tunisie	....	....	....	....	...--.....	....C.....	....	....
#T.3-Tunisie	....	....	....	....	...--.....	....	....	....
#M.1-Maroc	....	....	....	....	...--.....	....C.....	....	....
#T.4-Tunisie	....	....	....	....	...--.....	....C.....	....	....
#M.2-Maroc	....	....	....	....	...--.....	....C.....	....	....
#E.1-Espagne	....	....	....	....	...--.....	....C.....	....	....
#F.1-France	....	....	....	....	...--.....	....C.....	....	....
#E.2-Espagne	....	....	...T......	....	...--.....	....	....	....
#E.3-Espagne	....	....	...T......	....	...--.....	....	....	....
#E.4-Espagne	....	....	...T......	.........G	A.G--.....	....C.....	....	....

Fig. 2: Continued

#A.1-Algerie	CTAAATATGG	AGCCCCCGGT	TACGAAGTGC	CGCGGCAATT	GGTGGAATAC	AAGGCCACGC	CTAGGATGA-	-AACGGTAGT
#A.2-Algerie	....	....	....	....	....	....	.........-	-.........
#T.1-Tunisie	....	....	....	....	....	....	.........-	-.........
#T.2-Tunisie	....	....	...C......	....	....	....	.........-	-.........
#T.3-Tunisie	....	....	......A...	....	....	........C.	...A.....-	-.........
#M.1-Maroc	....	....	......A...	....	....	........C.	...A.....-	-.........
#T.4-Tunisie	....	....	....	....	....	........C.	.........-	-.........
#M.2-Maroc	....	....	....	....	....	....	.........-	-.........
#E.1-Espagne	.......A..	.C........	....	....	....	.......T..	.........-	-.........
#F.1-France	.......A..	.C........	....	....	....	.......T..	.........-	-.........
#E.2-Espagne	.......A..	....	....	....	....	.......T..	.........-	-.........
#E.3-Espagne	.......A..	....	....	....	....	.......T..	.........-	-.........
#E.4-Espagne	.......A..	....	....	....	....	.......T..	.........-	-.........

#A.1-Algerie	CC-GCACATC	GTGGCTCTTG	AGGACTCGCA	AGACCCTTAC	TTGTTTGCCC	CTAGGGGCGG
#A.2-Algerie	..C.......	....	....	....	....	....
#T.1-Tunisie	.GC.......	....	....	G.........	....	....
#T.2-Tunisie	.GC.......	....	....	G.........	....	....
#T.3-Tunisie	..C.......	....	....	....	..TG......	....
#M.1-Maroc	.GC..C....	....	....	...A......	...G..TG..	...A......
#T.4-Tunisie	.GC.......	....	....	....	..TG......	....
#M.2-Maroc	..C.......	....	....	....	..TG..TG..	....
#E.1-Espagne	.GC.......	..A.......	....	G.........	...G......	....
#F.1-France	.GC..C....	..A..T....	....	G........T	...G......	....
#E.2-Espagne	.GC.......	..A..T....	....	G.........	...G......	....
#E.3-Espagne	..C.......	..AA..T...	....	....	.....G....	....
#E.4-Espagne	..C.......	..AA..T...	....	....	....	....

Fig. 2: The sequences of the fragment ribosomal DNA amplified by PCR of 13 populations

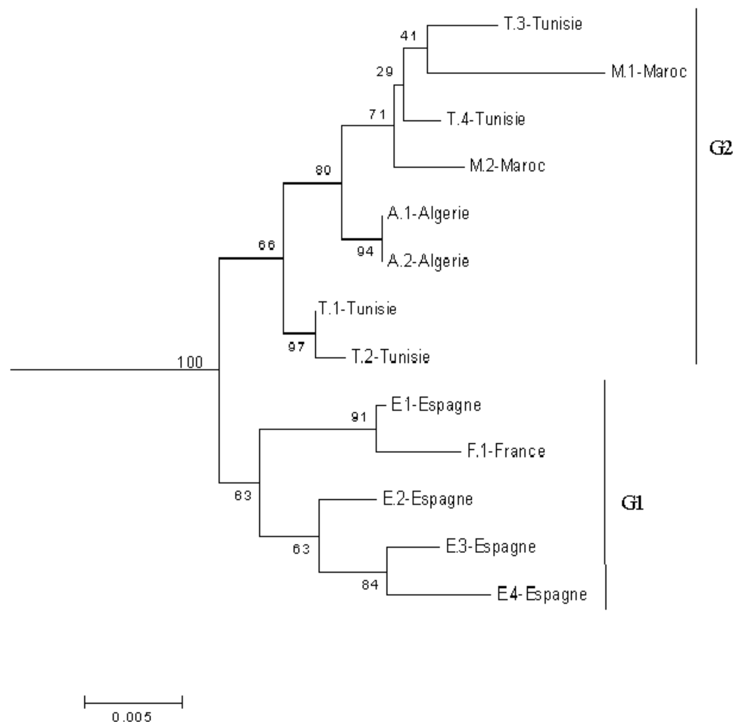

Fig. 3: Neighbour-joining dendrogram representing the genetic distance of eight plants of *A. halimus* group G2 and five plants of *A. halimus* group G1.

The phylogenetic analysis based on the ITS1-5.8S-ITS2 region sequences presented in Fig. (3). The populations from the Maghreb (Tunisia, Algeria and Morocco) (*A. halimus* subsp. *schweinfurthii*) were categorized in group G2 and clearly separated from the populations of group G1 (Spain and France) (*A. halimus* subsp. *halimus*). The two genetic groups of *A. halimus* were clearly separated in a node supported by a bootstrap value of 100.

Franclet and Le Houérou [6] and Le Houérou [1] divided *A. halimus* into two subspecies: *halimus* and *schweinfurthii.* There were few reports of genetic variation in *A. halimus*. Haddioui and Baaziz [4] analysed the isoenzyme polymorphisms of nine populations of *A. halimus* L. from several locations in Morocco and found very high intrapopulational diversity. Through the analysis of three enzyme systems (esterases, acid phosphatases and glutamate oxoloacetate transaminase), these authors found that the genetic diversity of their collection was explained mainly by the within population component. Only 8% of the whole diversity was explained by the between populations differentiation. Moreover, further work was done on chromosome counting and genome size by flow cytometry [7, 16], also confirmed such separation, where populations of group G2 are tetraploid (2n = 4x = 36) and contained twice as much DNA as populations of group G1, which are diploid (2n = 2x = 18).

The high levels of variability observed may be required to maintain plasticity in a highly fluctuating and diverse environment like the Mediterranean Basin. Another reason for the higher intrapopulational variation of subsp. *schweinfurthii* could be its polyploidal character. According to Soltis and Soltis [17] individuals and populations with polyploidy maintain higher levels of heterozygosity than do their diploid progenitors. Moreover, most polyploids may have a much better adaptability to diverse ecosystems, which may contribute to their success in nature. This illustrated in the case of *A. halimus* subsp. *schweinfurthii* by its much bigger distribution area than in *A. halimus* subsp. *halimus* and by its presence in very contrasting biotopes [7].

Little was known previously about the phylogenetic relationships within the genus *Atriplex*. The data presented here based on sequencing of the ITS1-5.8S-ITS2 region of ribosomal DNA indicated that the phylogenetic analysis of a group of five populations of *A. halimus* L. also sustained the separation between the two subspecies genetic groups.

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