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A preliminary molecular variability within *Haloxylon salicornium* accessions growing in Saudi Arabia

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Abstract

Haloxylon salicornicum is a desert plant with medical importance as it is used in anti-diabetic, antiseptic and anti-inflammatory purposes. Six hundred and eighty nine nucleotides spanning ITS1, 5.8S rDNA exon, ITS2 and their flanking regions were amplified and sequenced for three samples of *H. salicornium* collected from the western region of Saudi Arabia. These data were analyzed by maximum-parsimony and neighbor-joining methods in order to investigate the molecular variability within the species. Both analytical methods revealed a single rooted tree. The tree showed clustering of all haplotypes of *H. salicornium* together (bootstrap supports for both methods are 97 and 99%, respectively). The genetic distance within *H. salicornium* was smaller than that was recorded between species. The slight genetic difference within *H. salicornium* could be attributed to the biogeographic difference between its haplotypes. More investigations are necessary to assess the molecular variability among the different populations of this species along with its distribution range.

Keywords: *Haloxylon*, Medicinal plant, Plant genome, ITS1, 5.8S rDNA exon, diversity.

Introduction

Haloxylon salicornicum is one of more than 1300 species belonging to the family chenopodiaceae (Ghazanfar, 1994). It is distributed in salty desert and semi desert areas (Mosallam, 2007; Al-Khamis et al., 2012). *H. salicornicum* is among 25 species of the genus inhabiting the region from Western Mediterranean to Arabia and China (Al-khamis et al., 2012). In Saudi Arabia, *H. persicum* and *H. salicornicum* (Shaukat, 2000) are grown. *H. salicornicum* is used in anti-diabetic (Ajabnoor et al., 1984), antiseptic and anti-inflammatory (Alqasoumi et al., 2012) purposes. The plant is known locally as Rimth and is widely distributed throughout the country. The phytochemical analysis of the aerial parts of *H. salicornicum* revealed the presence of alkaloids, cardiac glycosides, anthraquinones, flavonoids, saponins, coumarins, sterols, tannins, volatile oils and volatile bases (Ajabnoor et al., 1984).
The molecular studies on *H. salicornicum* are very limited focusing on RAPD (random amplified polymorphic DNA) and ISSR technologies. Al-Qurainy (2007) studied the genetic variability within and between two Saudi populations and found a high genetic variability. A recent study was performed on 9 Kuwaiti populations using similar techniques (AL-Salameen et al., 2013). Meghwal et al. (2014) have investigated the variability among different genotypes of *H. salicornicum* and *H. recurvum* using RAPD markers.

The internal transcribed spacer (ITS) region including 18S–26S nuclear ribosomal DNA has proven to be useful for genetic relationships within closely related genera of many angiosperm families (Baldwin et al., 1995) and even within animals (Sayed et al., 3013). The present study aimed to use the region spanning 18S-26S gene (ITS1, ITS2 and 5.8S) of the nuclear DNA to assess the molecular variability of the Saudi Arabian *H. salicornium*.

Results

Morphology

Rimth is the traditional Arabic name of *H. salicornicum*. The plant is a dwarf desert shrub with leafless woody stems (Fig. 1). It grows vegetatively between spring and summer with flowering between September and October (Abd El-Wahab et al., 2014).

Features of the sequenced fragment

In this study, the amplified and sequenced ITS region included 40 bp of the 3` end of 18S gene, the complete ITS1, 5.8S rDNA, ITS2 and 104 bp from the 5` end of 26S gene (Fig. 2). The sequenced fragment (approximately 689 bp) was aligned with its counterparts from *Haloxylon* and *Salsola* in the Genbank database. The length of ITS1, 5.8S and ITS2 regions, frequency of different bases and adenine-thymine and cytosine-guanine ratio (AT/CG and CG/AT) for the studied taxa are illustrated in Table 1. The entire ITS region is 545 bp in Saudi Arabian *H. salicornium* and was 544 bp in the NCBI *H. salicornium*. In *H. persium* and *H. ammodendron*, this region was 546 bp. The number of nucleotides in the ITS1 is 197 bp in the three studied *Haloxylon* species while it was 198 bp in *H. persium*. The 5.8S region is 162 bp long in the three *Haloxylon* species while it was 161 bp in *H. salicornicum*. The ITS2 region is 186 bp in all studied *Haloxylon* species except *H. ammodendron* (it was 187 bp). The AT/CG ratio is 39.08/ 60.92 % in Saudi Arabian *H. salicornicum* and 39.14/ 60.86 % in NCBI *H. salicornicum*.

Both 5.8S gene and ITS2 region showed more homogenous in both haplotypes of *H. salicornicum*. The aligned nucleotide

Table 1. Base frequency and length of the sequenced region for the studied *Haloxylon*.

Haloxylon taxa	ITS1					5.8S					ITS2					Total base frequency				AT%	CG%	Tot. length
	A	T	C	G	Tot.	A	T	C	G	Tot	A	T	C	G	Tot.	A	T	C	G			
H. salicornicum (Saudi Arabia)	36	36	67	58	197	41	36	43	42	162	30	34	60	62	186	107	106	170	162	39.08	60.92	545
H. salicornicum (NCBI)	36	34	68	59	197	41	36	42	42	161	30	36	58	62	186	107	106	168	163	39.14	60.86	544
H. persicum	35	33	70	60	198	42	36	42	42	162	33	35	58	60	186	110	104	170	162	39.18	60.82	546
H. ammodendron	35	31	72	59	197	41	36	42	43	162	33	35	59	60	187	109	102	173	162	38.64	61.36	546

The NCBI accession numbers are EF453429 for *H. salicornicum*, EF453438 for *H. persicum* and EF453436 for *H. ammodendron*.

Fig 1. Woody stems of *H. salicornicum.*

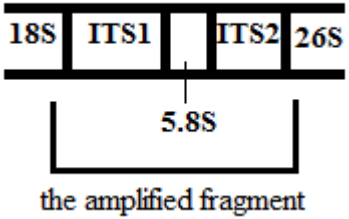

Fig 2. A schematic diagram of the nuclear DNA segment spanning the amplified fragment. It consists of 18S, 5.8S, ITS2 and 26S tracts. The ITSs are the internal transcribed spacers 1 and 2 as numbered from 5' end.

Table 2. Pairwise genetic distances among the different *Haloxylon* species as calculated from the sequenced fragment in this study.

	H. salicornicum (Saudi Arabia)	*H. salicornicum* (NCBI)	*H. persicum*
H. salicornicum (Saudi Arabia)	--		
H. salicornicum (NCBI)	0.025	--	
H. persicum	0.044	0.108	--
H. ammodendron	0.044	0.110	0.010

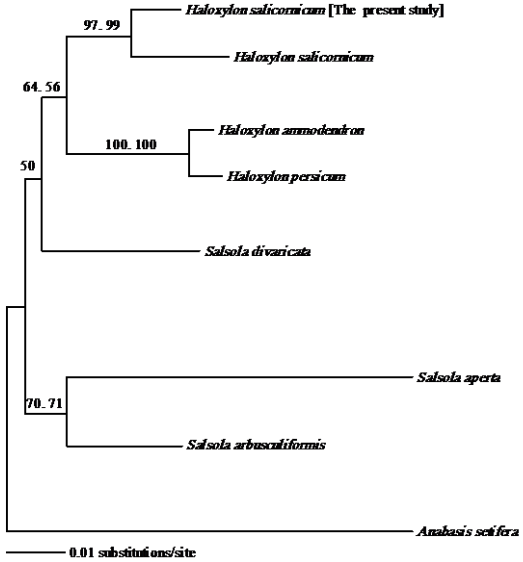

Fig 3. A neighbor-joining tree constructed from 608 bp sequenced fragment from ITS region of *Haloxylon* genome (one sample of this species is represented in the tree since the sequences from all individuals were identical). Values at nodes refer to the bootstrapping of maximum-parsimony and neighbor-joining methods which were shown when they were over 50%.

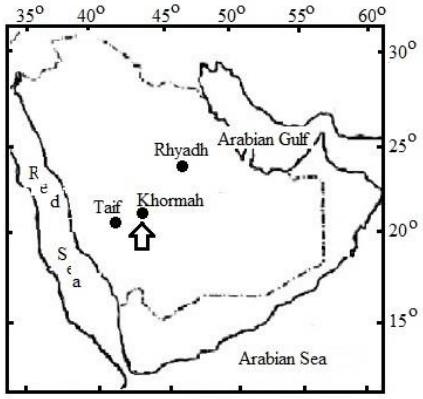

Fig 4. A map of Saudi Arabia. The arrow refers to the locality from which the samples were collected.

sequence of ITS1 region showed 9 base substitutions at positions 25, 90, 104, 107, 117, 127, 153, 155 and 196. Three of these substitutions were transversions and the other 6 were transitions. Two substitutions were found in 5.8S region, where A_{11} was substituted with G_{11} and G_{24} was substituted with A_{24} in the Saudi *H. salicornicum*. Three substitutions were found in ITS2 region. C_{15} was substituted with T_{15}, A_{69} was substituted with C_{69} and T_{76} was substituted with A_{76}. Only one insertion was found in the 5.8S where C_{20} was deleted from the non Saudi *H. salicornicum*.

The data showed base frequencies of A = 19.8%, C = 31.2%, G = 29.8% and T = 19.2%. After deletion of the gap-containing and the unaligned sites, 608 bp section was used for the analysis. Of the 608 nucleotides used for tree analyses, 488 were constant and 120 were variables. From the variable sites, 79 were parsimony uninformative and 41 were informative under parsimony criterion. The consensus parsimony tree constructed showed consistency index (CI = 0.860), homology index (HI = 0.301), retention index (RI = 0.639) and rescaled consistency index (RC = 0.550).

Species relationship

The sequenced fragment executed one parsimonious and one neighbor-joining tree and both trees showed similar topology. The neighbor-joining tree (Fig. 3) showed that the two haplotypes of *H. salicornicum* was grouped together (bootstrap support= 97 MP and 99 NJ) and the two *Haloxylon* species clustered with each other (100 % bootstrap support). All *Haloxylon* species formed one monophyletic clade.

The pairwise genetic distances among the studied species are listed in Table 2. The distance showed the smallest values between the two haplotypes of *H. salicornicum* (0.025) and between the two *Haloxylon* species (0.010).

Discussion

H. salicornicum is an arid region shrub possessing multiple beneficial applications in food, fuel and medicine and it is highly endangered. Very recent study (Snigh et al., 2015) has surveyed its distribution, ecology, uses and diversity. However, the molecular studies on this respect are very rare or absent.

Most of the published studies, regarding the genetic variability within *H. salicornicum*, have focused on RAPD-PCR and/or ISSR (Al-Qurainy, 2007; AL-Salameen et al., 2013; Meghwal et al., 2014). One of the few studies was conducted by AL-Salameen et al. (2013) who found that there was genetic diversity within the populations of *H. salicornicum* in Kuwait. Within the Saudi populations, Al-Qurainy (2007) has found a high genetic variability within rather than between populations.

The ITS region consists of ITS1, ITS2 and the highly conserved 5.8S rDNA exon located between them (Fig 2) (Wheeler and Honeycutt, 1988). The total length of this region in *H. salicornicum* is in the range of other angiosperms (Baldwin et al., 1995). *Haloxylon* is one of the genera belonging to the subfamily Salsoleae. Pyankov et al. (2001) used ITS region to infer the geographic distributions of this subfamily. The authors suggested that Salsoleae originated in central Asia and dispersed to Africa, Europe and Mongolia. The present study agreed with the authors that reported clustering of *Haloxylon* and *Salsola* in one clade.

The present study; therefore, could be considered the first molecular investigation using sequence data for assessing the genetic variability within this medicinal plant. As the samples were collected from one locality (Western of Saudi Arabia), the molecular comparison was made between these samples (Saudi population) and that collected from the Genbank (non Saudi population). The compared data showed an obvious genetic variability between both populations on the level of fragment length, base substitutions, deletion and insertion.

Materials and Methods

Samples and DNA extraction

In this study, three samples of *H. salicornium* were collected from the region around Khormah 200 km east to Taif city, Western of Saudi Arabia (Fig. 4). The samples were labeled, sealed in sterilized polythene bags, transferred to the laboratory and stored at -20 °C till their use for DNA isolation. DNA was isolated and purified by DNeasy Plant Mini Kit following the manufacture protocol.

PCR experiments

The forward primer (ITS4) 5`- TCCTCCGCTTATTGATATGC-3` and the reverse primer (ITS5) 5`- GGAAGTAAAAGTCGT-AACAAGG -3` [14] were used for the amplification of the DNA fragment of this study. A total volume of 25 µL PCR mixture (1 µL genomic DNA, 12.5 µL PCR master mix, 0.5 µL of each primer and 10.5 µL distilled sterilized H_2O) was used. PCR amplification was carried out in a Techne thermocycler. The PCR condition was 94 ˚C for 5 min as an initial denaturation step followed by 35 cycles of 94 ˚C for 60 s denaturation, 56 ˚C for 60 s annealing and 72 ˚C for 60 s extension. The final extension was at 72 ˚C for 4 min. PCR products were run on 1% agarose gel containing ethidium bromide and visualized under UV. The PCR products were sent to Macrogen (www.macrogen.com) for sequencing using the same two primers of the PCR amplification.

Statistical analysis

Six hundred ninety one nucleotides spanning ITS1, 5.8S and 26S genes from the nuclear DNA for the collected samples were sequenced in this study. Comparisons with sequences in the GenBank database were conducted by BLASTN searches at the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov).

The sequenced data were aligned with their counterparts from the Genbank database for *H. salicornica* (EF453429), *H. ammodendron* (EF453436), *H. persicum* (EF453438), *Salsola arbusculiformis* (EF453468), *S. aperta* (EF453466), *S. divaricata* (EF453474) and *Anapasis setifera* (EF453389). The accession numbers of these sequences are enclosed within the brackets. The aligned data were used for phylogenetic analyses and the sequence for *Anapasis setifera* was used for tree rooting since rooting discriminatory power is shown to be stronger for more closely outgroups (Graham et al., 2002). The gap-containing sites and unambiguous nucleotides were deleted so that 608 bp were left for phylogenetic analysis (The aligned nucleotides can be obtained from the corresponding author upon request).

The phylogenetic analyses were conducted by maximum-parsimony and neighbor-joining methods with PAUP* 4.0b10 (Swofford, 2002) by heuristic searches with the TBR branch swapping and 10 random taxon additions. Bootstrapping replicates were set to 10,000 for both methods. The neighbor-joining method was adjusted by distance option of Tamura-Nei.

Conclusion

H. salicornicum is genetically homogenous along its distribution range and the slight genetic difference between its haplotypes is attributed, most probably, to the geographic factors. More molecular investigations are needed on the population level to assess the genetic framework of this medicinal plant.

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