Cah. Biol. Mar. (2011) 52 : 227-232

Molecular identification of the tropical seagrass *Halophila stipulacea* from Turkey

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Abstract: *Halophila stipulacea* (Forsskål) Ascherson, a tropical seagrass, is thought to be a Lessepsian immigrant that entered the Mediterranean Sea from the Red Sea after the opening of the Suez Canal (1869). Up to date, no genetic studies of *H. stipulacea* from Turkey are available. In order to verify the molecular identity of Turkish isolates of *H. stipulacea*, a part of the rDNA ITS region was sequenced. Comparisons of the genetic polymorphism of this region between isolates from the Turkish coasts of the Aegean Sea and individuals from putative native (Red Sea) and introduced (Mediterranean) populations deposited previously in GenBank were performed. No intra-individual variability was found in the region considered among the isolates from Turkey.

Résumé : *Identification moléculaire de la phanérogame marine tropicale* Halophila stipulacea *des côtes de Turquie. Halophila stipulacea* (Forsskål) Ascherson, une phanérogame marine tropicale, est probablement une espèce lessepsienne originaire de Mer Rouge entrée en Méditerranée depuis l'ouverture du canal de Suez (1968). Jusqu'à maintenant, aucune étude génétique d'*Halophila stipulacea* n'est disponible. Afin de vérifier l'identité moléculaire d'isolats turcs d'*H. stipulacea*, une partie de la région ITS de l'ADNr a été séquencée. Le polymorphisme génétique de cette région a été comparé entre isolats des côtes turques de la Mer Egée et des individus issus de populations de la zone d'origine probable (Mer Rouge) et d'autres populations introduites de Méditerranée et déposés à GenBank. Aucune variabilité intra-individuelle n'a été trouvée parmi les isolats turcs.

Keywords: Halophila stipulacea ● Seagrass ● rDNA ITS ● Lessepsian immigrant ● Red Sea

Reçu le 21 septembre 2010 ; accepté après révision le 17 janvier 2011.
Received 21 September 2010; accepted in revised form 17 January 2011.

Introduction

Halophila stipulacea (Forsskål) Ascherson is the most common seagrass in the northern Red Sea (Lipkin, 1975a) and it is widely distributed in the warm waters of the western Indian Ocean and Red Sea. This species was first recorded in the eastern Mediterranean Sea in 1895 (Fritsch, 1895), but only 30 years later it was recorded as developing well-established meadows at Rhodos, Greece (Forti, 1927; Issel, 1928). The species was initially considered a tropical relict in the eastern Mediterranean Sea (Pérès, 1967), but later studies hypothesized that *H. stipulacea* arrived from the Red Sea soon after the opening of the Suez Canal, about 130 years ago, in 1869, following the Lessepsian hypothesis (Lipkin, 1975b; Procaccini et al., 1999; Ruggiero & Procaccini, 2004). The species remained restricted to the eastern Mediterranean for several decades followed by a progressive expansion towards the western areas. It was first observed in 1995 at the Island of Vulcano, Sicily (Acunto et al., 1995) and recently on the coast of Salerno, Italy (Gambi et al., 2008). Genetic and morphological differentiations of western Mediterranean populations of *H. stipulacea* versus putative native populations from the Red sea have been compared using RAPD markers and the rDNA ITS region (Procaccini et al., 1999; Ruggiero & Procaccini, 2004). Although the existence of *H. stipulacea* from Turkish coasts was reported previously (Güner et al., 1985; Taskin et al., 2008), neither studies nor information on the genetic diversity and/or molecular identity of *H. stipulacea* from any coast of Turkey have been so far published.

The rDNA ITS region is a multi-copy, tripartite, and roughly 650-basepair (bp) segment that combines the advantages of resolution at various scales (ITS1: rapidly evolving, 5.8S: highly conserved, ITS2: moderately rapidly to rapidly evolving; Hillis & Dixon, 1991; Hershkovitz & Lewis, 1996) with the ease of amplification of a multi-copy region into a readily obtainable product. The ITS has been widely used in phylogenetic and phylogeographic studies in a variety of organisms (Baldwin et al., 1995), including the genus *Halophila*. Using the ITS, Waycott et al. (2002) inferred relationships among species and biogeographic patterns in the 13 species of the seagrass genus *Halophila*. In fact, out of the 137 sequences of *Halophila* published in GenBank up to date, the rDNA ITS represent the vast majority, with only 5 sequences belonging to other molecular markers.

In order to verify the molecular identity and possibly provide insights into the invasion paths of *H. stipulacea* in Turkey we compared genetic divergence between populations from Turkey versus populations from putative native (Red Sea) and introduced (The Mediterranean Sea) populations at a partial rDNA ITS region.

Material and Methods

Description of material collected

Halophila stipulacea was collected from Gümüldür, Turkey on the 29th of November of 2006. The seagrass covered a surface of about 250 m^2 in a sandy area at the depth of 23 m. *H. stipulacea* formed a homogeneous population, in which no other large-sized macrophytes were observed. The meadow showed well-defined boundaries with *Posidonia oceanica* (Linnaeus) Delile, 1813 and the invasive green alga *Caulerpa racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman & Boudouresque, 2003. *H. stipulacea* consisted of thin creeping rhizomes, 0.25-0.30 cm wide, from which pairs of leaves were issued at regular intervals on the dorsal side. Pairs of leaves were attached to the rhizomes by petioles. The leaves were 3-5 cm long, 0.7-0.8 cm wide, 1 or 2 at each shoot node, linear-oblong, thin and hairy. The margin of the leaves was spinulose and their surface was crossed by 4 to 14 pairs of veins. The rhizomes were irregularly branched and were fixed to the sandy substratum by roots issued at each node of the rhizomes. Only non-reproductive plants were observed at the time of collection; no male or female flowers were observed. Some blades showed incisions, probably as a consequence of bites from grazers. Three *H. stipulacea* shoots connected by a rhizome were collected from Gümüldür, Turkey

Table 1. *Halophila stipulacea*. Details (collector, date, site, depth, coordinates and primers source) of samples used in this study.
Tableau 1. *Halophila stipulacea*. Détails (récoltant, date, site, profondeur et coordonnées géographiques et origine des primers) des échantillons utilisés dans cette étude.

Collector and date	Location, depth and GPS coordinates	Gene	Primers (Source: White et al., 1990)
Levent Cavas	Gümüldür, Izmir, Turkey, -23 m	Internal transcribed spacer 1 (ITS1) and	ITS 1_5′ TCC GTA GGT GAA CCT GCG G 3′
	38^{o}02’39.51’’ N	Partial sequence 5.8S	ITS 2_5′ GCT GCG TTC TTC ATC GAT GC 3′
	27^{o}00’54.07’’ E	Length: 325 bp	

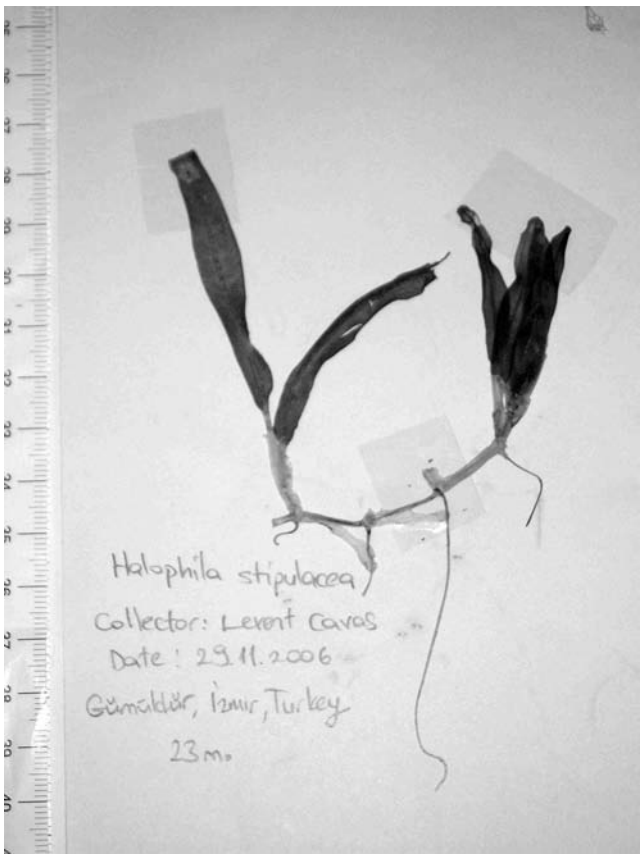

Figure 1. *Halophila stipulacea*. Herbarium specimens from Gümüldür, İzmir, Turkey.

Figure 1. *Halophila stipulacea*. Spécimens d'herbier de Gümüldür, İzmir, Turkey.

(geographical coordinates of the sampling site 38°02'39.51"N-27°00'54.07"E) by SCUBA diving and preserved in silica gel in a single bag for posterior DNA extraction and PCR amplification (Figs 1 & 2).

DNA extraction, PCR amplification and sequencing

DNA was extracted using the CTAB method of Doyle & Doyle (1987). The entire ITS1 and a partial region of the 5.8S rDNA gene were amplified by PCR using the universal primers ITS1 and ITS2 (White et al., 1990) (Table 1). PCR cycling conditions were hot start (95°C, 5 min), 40 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 1 min 30 sec, final extension of 5 min at 72°C. The PCR reactions

Figure 2. *Halophila stipulacea*. Map of sampling location at Gümüldür, İzmir, Turkey.

Figure 2. *Halophila stipulacea*. Carte de la zone d'échantillonnage à Gümüldür, İzmir, Turkey.

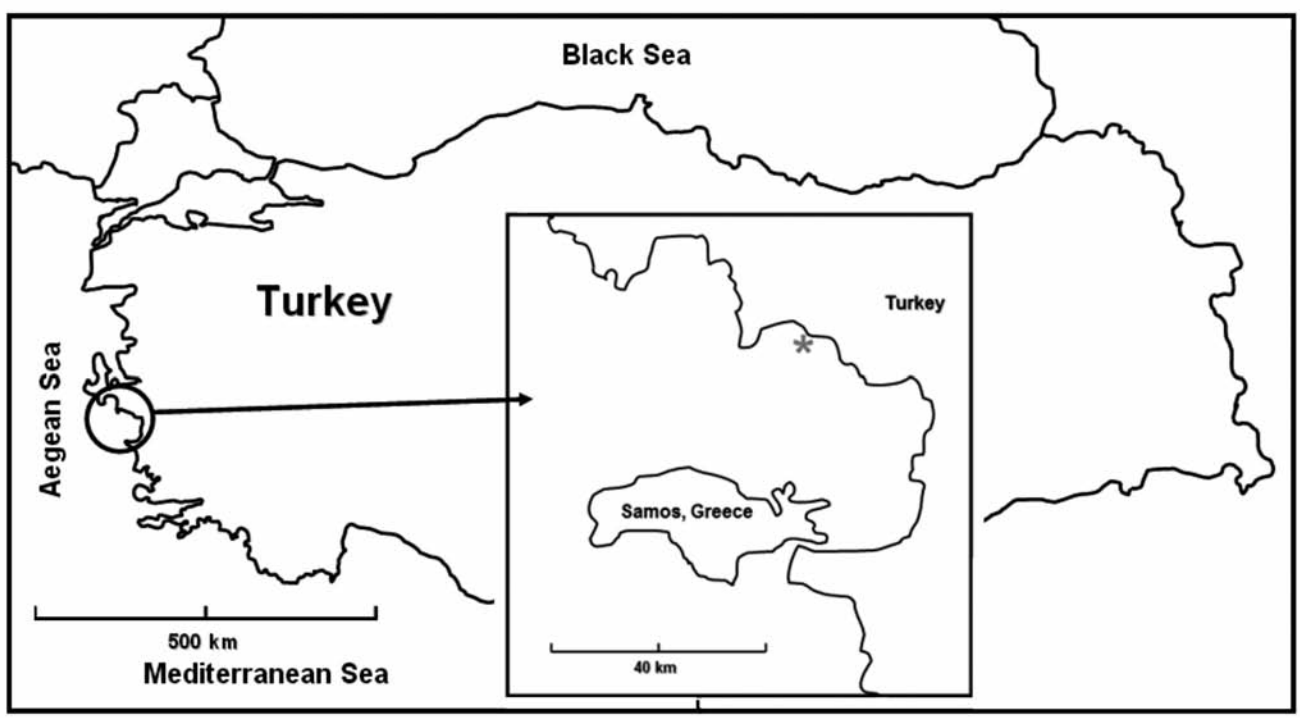

Table 2. *Halophila stipulacea.* rDNA ITS nucleotide divergence at intra-individual and intra-population level within complex, calculated comparing our data with sequences for all known sequences deposited in the NCBI data base up to date.

Tableau 2. *Halophila stipulacea.* Divergence nucléotidique de l'ITS de l'ADNr au niveau intra-individuel et intra-population du complexe, calculé en comparant nos données à toutes les séquences connues déposés dans la banque de données NCBI.

Reference	*H. stipulacea* localities	Number of individuals	Accession numbers of rDNA ITS *H. stipulacea* sequences used in this study	Averages intra-individual nucleotide diversity	Average nucleotide diversity per locality
Ruggiero & Procaccini, 2004	Vulcano Island, Italy	4	AY352637, AY352636, AY352634, AY352633, AY352632, AY352631, AY352630, AY352629, AY352628, AY352627, AY352626, AY352625, AY352624, AY352623, AY352622, AY352621, AY352620, AY352619, AY352618, AY352617	1.3-3.6%	2.0%
Waycott et al., 2002	Sicily, Italy	No info	AF366436	-	-
Ruggiero & Procaccini, 2004	Rhodes Island, Greece	1	AY352635	-	-
Ruggiero & Procaccini, 2004	Ras Mohammed, Egypt	2	AY352616, AY352615, AY352614, AY352613, AY352612, AY352611. AY352610, AY352609, AY352608, AY352607, AY352606	3.4-5	3.5%
Ruggiero & Procaccini, 2004	Small Creek Egypt,	2	AY352605, AY352603, AY352604, AY352602 AY352601, AY352600	1.5-4.4%	1.9%
Ficca et al., 2000	Unknown location	No info	AJ012307	0%	0%
This study	Izmir, Turkey	1	GU987099, GU987100, GU987101	0%	0%

were performed in a 20 µl volume containing buffer (10x), dNPTs (2 mM), $MgCl_2$ (50 mM), primers (10 mM), 0.3 U AmpliTaq Gold polymerase and approximately 5 to 10 ng of template DNA. 4 replicates were used. Amplified products were visualized on a 1% agarose gel and the excess of primers and dNTPs from the PCR product were removed using the Amersham PCR Purification Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Cloning was carried out using the pGEMT-Easy Vector system II (Promega, Madison, USA) as vector according to the manufacturer's instructions, with JM109 competent cells (high efficiency, > 108 cfumg1). Bacteria with cloned PCR products were incubated overnight at 37°C. Three clones were selected and sent for miniprep preparation and sequencing to MACROGEN (Seoul, Korea). Sequence alignments were conducted with alignment explorer in MEGA4 (Tamura et al., 2007).

Results and Discussion

The three sequences produced in this study were compared with 41 published rDNA ITS sequences available in GenBank using the Basic Local Alignment Search Tool (BLAST) to find regions of local similarity and then deposited at the NCBI database (accession numbers shown in Table 2). The amplifications gave a PCR product of 325 bp: from 1 to 271 bp corresponding to the ITS1, and from 272 to 325 bp corresponding to a partial region of the 5.8S gene. The three clones sequenced here produced identical sequences with the highest significant sequence alignment with *H. stipulacea* (AJ012307) (Evalue < e^{-15}).

The final dataset was composed by 43 sequences from 6 different localities. In all analyses, all

Table 3. *Halophila stipulacea*. Ranges of rDNA ITS nucleotide divergence and geographical distances of the Turkish isolated in relation to each locality studied by other authors.

Tableau 3. *Halophila stipulacea*. Gamme de divergence nucléotidique et distances géographiques des isolats turcs pour chaque site étudié par d'autres auteurs.

***Halophila stipulacea* localities**	**Distances in km (aprox.)**	**rDNA ITS nucleotide divergence ranges**
Gümüldür, Turkey vs. Sicily, Italy	1000	1.1%
Gümüldür, Turkey vs. Vulcano Island, Italy	1000	0.4-10.1%
Gümüldür, Turkey vs. Rothes Island, Greece	250	0.4%
Gümüldür, Turkey vs. Small Creek, Egypt	1000	0.7-4.3%
Gümüldür, Turkey vs. Ras Mohammed, Egypt	1000	0.4-11.2%
Gümüldür, Turkey vs. unknown location	-	0%

positions containing gaps and missing data were eliminated from the dataset (complete deletion option). We used for the analyses the common region for all the sequences. There were a total of 271 positions unambiguously aligned in the final dataset. ITS sequences were considerably divergent within the *H. stipulacea* complex (Table 2), both at intra-individual and intra-population level. At intra-individual level, isolates from Ras Mohammed and Small Creek in Egypt, and from Vulcano Island in Italy presented ranges of average sequence divergence within individuals from 1.3 to 5% (the highest at Ras Mohammed in Egypt). Conversely, no intra-individual nucleotide diversity was found in our isolate from Turkey. Table 3 shows ITS nucleotide divergence at inter population level between our isolate from Turkey and the other isolates from different locations. Divergences were quite high, with the highest pairwise uncorrected divergence observed between Turkey and Ras Mohammed Egypt (11.2%), and the lowest with a sample from an unknown location that was 100% identical to our sequences. The geographical distance in Km did not seem to have any relation with intra or interspecific diversity among isolates.

Using a partial nuclear ribosomal DNA region (rDNA ITS), we were able to confirm the molecular identity of *H. stipulacea* from İzmir, Turkey, but also to infer the lack of intraindividual diversity in this partial region of the ITS, in isolates from Turkey. This result is rather unexpected if compared with previous published results for other parts of the Mediterranean coasts (Ruggiero & Procaccini, 2004). The ITS sequences of *H. stipulacea* in İzmir, Turkey seem to be one single variant. It has still to be verified if this pattern is common to other *H. stipulacea* samples and/or populations of this region.

Acknowledgements

This work was financed through the project AXA Aliens, founded by the AXA Foundation to NM and the Portuguese Science Foundation (FCT), with co-funding by ESF and FEDER to EVA. We also thank Liam Cronin for reading and improving the English in the manuscript and Mar Huertas and Vincent Laizé for their help with the translation of the French abstract.

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