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ITS DNA Barcoding Reveals That *Halophila stipulacea* Still Remains the Only Non-Indigenous Seagrass of the Mediterranean Sea

Catalina A. García-Escudero [1,2,*], Costas S. Tsigenopoulos [3], Vasilis Gerakaris [4], Alexandros Tsakogiannis [3] and Eugenia T. Apostolaki [1]

[1] Institute of Oceanography, Hellenic Centre for Marine Research, P.O. Box 2214, GR-71003 Heraklion, Crete, Greece; eapost@hcmr.gr
[2] Biology Department, Voutes University Campus, University of Crete, P.O. Box 2208, GR-70013 Heraklion, Crete, Greece
[3] Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, P.O. Box 2214, GR-71003 Heraklion, Crete, Greece; tsigeno@hcmr.gr (C.S.T.); tsakalex@hcmr.gr (A.T.)
[4] Institute of Oceanography, Hellenic Centre for Marine Research, P.O. Box 712, GR-19013 Anavyssos, Attiki, Greece; vgerakaris@hcmr.gr
* Correspondence: cagarcia3@uc.cl; Tel.: +30-698-587-4976

Abstract: Non-indigenous species (NIS) are one of the major threats to the native marine ecosystems of the Mediterranean Sea. *Halophila stipulacea* was the only exotic seagrass of the Mediterranean until 2018, when small patches of a species morphologically identified as *Halophila decipiens* were reported in Salamina Island, Greece. Given the absence of reproductive structures during the identification and the taxonomic ambiguities known to lead to misidentifications on this genus, we reassessed the identity of this new exotic record using DNA barcoding (rbcL, matK and ITS) and the recently published taxonomic key. Despite their morphologic similarity to *H. decipiens* based on the new taxonomic key, the specimens showed no nucleotide differences with *H. stipulacea* specimens (Crete) for the three barcodes and clustered together on the ITS phylogenetic tree. Considering the high species resolution of the ITS region and the common morphological variability within the genus, the unequivocal genetic result suggests that the *Halophila* population found in Salamina Island most likely corresponds to a morphologically variant *H. stipulacea*. Our results highlight the importance of applying an integrated taxonomic approach (morphological and molecular) to taxonomically complex genera such as *Halophila*, in order to avoid overlooking or misreporting species range shifts, which is essential for monitoring NIS introductions.

Keywords: biological invasions; species range shifts; species monitoring; integrative taxonomy; seagrass barcoding; *Halophila decipiens*; morphologic variability; phenotypic plasticity; species misidentification

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1. Introduction

The natural ranges of species are inherently dynamic, but in recent decades globalization and climate change have accelerated the pace of change by facilitating the introduction of species outside their natural ranges [1–3]. Non-indigenous species (NIS) that become established and spread can pose a major threat to native biodiversity and community structure, affecting the integrity and function of natural ecosystems [4,5]. This is particularly evident in the Mediterranean Sea, which is currently considered the most invaded marine basin globally [6,7], with nearly 700 multicellular established NIS documented up to March 2021 [8,9]. The vast majority occur in the eastern subregion and probably entered the basin through the Suez Canal, which since 1869 connects the Mediterranean Sea with the Indo-Pacific region [10–12]. This artificial passage, combined with the high volume of shipping routes, aquaculture, aquarium trade [12,13], and recent warming of Mediterranean waters due to climate change, makes the basin vulnerable to the introduction of NIS [14,15]. This

is especially true for the eastern subregion, which is warming faster than the rest of the basin [16], leading to an assemblage restructuring shaped by the native species in peril and a replacement by tropical ones [17–19].

In the Mediterranean Sea, seagrass communities dominate the sublittoral environment and provide several important ecosystem services [20,21]. Of the seagrass species that occur in this basin, *Halophila stipulacea* (Forsskål) Ascherson, 1867 is the smallest species known to be among the first Lessepsian migrants [22–24]. Originally native to the western part of the Indian Ocean, including the Red Sea, the Arabian Sea, and the Persian Gulf [25], this species was first reported in Rhodes, Greece, in 1894 [26]. Since then, it has progressively spread throughout the Mediterranean, colonizing the eastern and central subregions and, in recent decades, the western subregion [27], including populations off the coasts of Italy [9,28,29], Tunisia [30,31], and, more recently, Cannes on the French Riviera [32]. So far, the Mediterranean invasion can be described as slow and punctuated in space [27]. It generally colonizes habitats devoid of native macrophytes or occasionally forms mixed meadows with the native *Cymodocea nodosa* (Ucria) Ascherson, 1870, which is opposite to its invasion of the eastern Caribbean islands, where it spreads rapidly and displaces several native macrophytes [27,33]. However, its invasion dynamics are expected to change as the basin becomes saltier and warmer, favoring the establishment of tropical and subtropical species [14,34], and, as the endemic meadows of *Posidonia oceanica* (L.) Delile, 1813 continue to decline [35,36] leaving new suitable habitats available for fast-growing opportunistic macrophytes to recolonize. Invaders tend to be stronger colonizers than native species, so the recolonization of declining meadows could likely be dominated by invasive macrophytes such as *H. stipulacea*, *Caulerpa taxifolia*, and *Caulerpa racemosa* over the natives *C. nodosa* and *Caulerpa prolifera* [37,38]. Changes in the seagrass biogeography, including the replacement of native *P. oceanica* by species with a lower habitat complexity and the shift from seagrass meadows to algae, could inevitably lead to dramatic changes in the dynamics and function of coastal ecosystems [31,34].

Halophila stipulacea was considered the only non-indigenous seagrass species in the Mediterranean until October 2018, when several small patches (1 to 10 m^2) of a species identified as *H. decipiens* were found in Salamina Island in the Saronikos Gulf, Greece [39]. *H. decipiens* is a pantropical species with a wide geographic distribution, originally occurring in tropical, subtropical, and warm-temperate systems in both hemispheres [40,41]. Similar to *H. stipulacea*, it is a fast-growing species with a high phenotypic plasticity and the ability to live in a wide range of temperatures, salinities, light irradiances, and substrates [42–44]—all typical characteristics of invasive species. The introduction of another exotic seagrass would pose a new, unpredictable threat to native coastal ecosystems. Close monitoring would be required as it is difficult to predict at an early stage whether a new introduction would be an ephemeral event or whether it would become established and spread throughout the basin. Containment, eradication, and management plans become more difficult or even impossible when an NIS becomes abundant and widespread [45,46]. Therefore, the ability to rapidly and accurately identify and monitor NIS introductions plays an essential role in mitigating the threats posed by them [47].

Traditional morphology-based species identification works as a standard method for many taxa. However, early life stages of species, ambiguous or uninformative morphological characters, high phenotypic plasticity, morphologically cryptic species, and a lack of taxonomic expertise can compromise the accuracy of this method, leading to misidentifications or uncertainties that can obscure invasion histories and preclude appropriate management strategies [48]. In the face of these and other difficulties, traditional taxonomy has evolved into an integrative approach in which species are studied from multiple complementary perspectives, including morphological, molecular, behavioral, developmental, and ecological characterizations [49]. Most molecular studies have focused on animals, given the remarkable success of the mitochondrial cytochrome oxidase c subunit 1 (COI) gene as a universal single DNA barcode for metazoans [50,51]. In contrast, plants' much slower substitution rate of the COI and other mitochondrial genes does not generate a suffi-

cient intergenetic distance to discriminate between species in most plant groups [52]. This has led to an extensive and difficult search for an alternative region in the mitochondrial, plastid, and nuclear genome [52–54]. Multiple candidates have been proposed, however, no consensus has been reached on a single universal plant DNA barcode, limiting the application of this technique to these organisms [55]. Currently, although they are taxa-specific and achieve different degree of success, the plastid rbcL (ribulose-bisphosphate carboxylase) and matK (maturase K) and the nuclear trnH-psbA and ITS (internal transcribed spacer) regions, are widely used and considered effective markers for species identification and phylogenetic reconstruction for land plants and seagrasses [56–61].

The taxonomic classification of the genus *Halophila* is a major challenge, and changes in species delimitation and misidentifications occur frequently [62]. The difficulty lies in the high fragility and small size of the species, the simplicity and frequent absence of reproductive structures (e.g., petals, sepals, stamens, fruits, and seeds), and the limited number of vegetative characters (e.g., the plant's appearance, leaf length and width, leaf margin and tips, number of cross-veins, and branching), which occasionally show considerable variation and overlap with species living in similar environments [25,63]. Molecular analyses have already helped to clarify some species delimitations and resolve previous morphological misidentifications [57,64,65], supporting the idea that an integrative taxonomic approach is necessary for a taxonomically complex genus such as *Halophila*. In the case of the recent first record of *H. decipiens* in the Mediterranean, its taxonomic identification was based only on vegetative morphological characters, as the reproductive structures found were still at an early stage. Considering the importance of species-level accuracy for reporting and monitoring NIS introductions and the problematic taxonomy of the genus *Halophila*, the aim of this study was to reassess the species identification of the first record of *H. decipiens* in the Mediterranean Sea using DNA barcoding. By doing so, we support the idea that DNA barcoding can be employed as a rapid and accurate complementary tool for seagrass species identification and assist with monitoring range shifts in taxonomically complex and potentially invasive genera such as *Halophila.*

2. Materials and Methods

2.1. Morphological Identification

The specimens of *Halophila decipiens* found in Salamina Island [39] were identified following the descriptions of Phillips and Mehez (1988) [66] and the taxonomic key of Kuo and den Hartog (2001) [63]. Since then, a new taxonomic key for the *Halophila* genus was published by Kuo (2020) [62]. Here we used the compiled information of the latter key with the older keys to re-identify the collected specimens. Generative characters were not available (i.e., reproductive structures were not fully developed), so identification of *Halophila* species was based only on vegetative morphological characters. Reference images of *H. stipulacea* specimens were included for visual comparison.

2.2. Sample Collection

For the DNA barcode identification, samples of the specimens morphologically described as *H. decipiens*, named *Halophila* sp. for this study, were collected in November 2019 from the exact location where the first population was reported. A patchy meadow located on a shallow (3–4 m deep) sandy area on the south coast of Salamina Island, Saronikos Gulf, Aegean Sea, Greece (37°52′44.4″ N, 23°27′39.6″ E). For comparison, samples of *H. stipulacea* were collected in May 2019 from a 20 m depth meadow near Hersonissos, Crete, Greece (35°18′53.74″ N, 25°25′7.23″ E). The populations correspond to monospecific seagrass meadows. On both samplings, four individual plant modules (each module consisted of a section of rhizome, a node, and one mature leaf pair) were randomly collected by hand at 1–2 m from each other using scuba-diving. The entire plant modules were submerged in RNAlater^{TM} Stabilization Solution and stored at 20 °C for future molecular analysis.

2.3. DNA Isolation, PCR Amplification, and Sequencing

The plant material (leaf tissue) of each sample was homogenized using a mortar and pestle under a constant addition of liquid nitrogen. From the finely powdered leaf produced, 100–150 mg was used for the DNA isolation following a modified cetyltrimethylammonium bromide (CTAB) chloroform/isoamyl alcohol (24:1) isolation protocol including an RNase treatment (RiboShredder RNase Blend, Epicentre, Madison, WI, USA) of 1 h at 37 °C. The final DNA pellet was resuspended in 50 µL of Buffer AE (QIAGEN, Hilden, Germany). The DNA quality was checked on a 1% agarose gel stained with ethidium bromide. The concentration and purity were quantified using a NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, DE, USA). Based on the Consortium for the Barcoding of Life (CBOL) plant barcoding recommendations [58] and previous seagrass DNA barcoding studies [56,57,60,61], the ITS1-5.8S-ITS2 (ITS), rbcL and matK regions were selected for the study. The primers P609 (5′-GTAAAATCAAGTCCACCRCG-3′) and P610 (5′-ATGTCACCACAAACAGAGACTAAAGC-3′) were used to amplify sequences of ~600 bp corresponding to rbcL. Primers P646 (5′-TAATTTACGATCAATTCATTC-3′) and P647 (5′-GTTCTAGCACAAGAAAGTCG-3′) were used to amplify sequences of ~945 bp corresponding to matK. Last, primers P674 (5′-CCTTATCATTTAGAGGAAGGAG-3′) and P675 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify sequences of ~700 bp corresponding to the ITS region. The PCR amplifications were performed in a 15 µL final reaction volume consisting of 30 ng of template DNA, 0.45 µM of forward primer, 0.45 µM of reverse primer, and 7 µL of DreamTaq Hot Start PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). PCR conditions consisted of an initial denaturation step at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for ITS/rbcL and at 50 °C for matK for 35 s, and elongation at 72 °C for 1 min. The 30 cycles were followed by a final extension at 72 °C for 8 min. The PCRs were performed in a Bio-Rad T100 Thermal Cycler with a heated lid. All PCR reactions were repeated four times independently for the same individual to keep potential errors in the final consensus sequence to a minimum. A manual ethanol/sodium acetate precipitation protocol was used to purify the PCR products. DNA sequencing reactions were performed using the Applied Biosystems™ BigDye™ Terminator (Thermo Fisher Scientific) in a 10 µL total volume and run on an ABI 3730 automated DNA sequencer.

2.4. Data Analysis

Consensus sequences were assembled by combining the forward and reverse sequences previously end-trimmed based on average quality scores, using CodonCode Aligner v9.0.1.3 (CodonCode Co., Centerville, MA, USA). The sequences were aligned using the CLUSTAL W [67] algorithm in MEGA 7 [68], and the alignments were checked and adjusted by eye to exclude obvious alignment errors. For the phylogenetic analysis, known ITS sequences of *H. decipiens*, *H. stipulacea*, and other *Halophila* species were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/ (accessed on 13 December 2020)) and included in the alignment (Table 1). The sequences of *Halophila beccari* and *Halophila engelmannii* were used as the outgroups. The jModelTest version 2.1.6 [69] was used to find the model of nucleotide sequence evolution that best fit our data. The maximum likelihood (ML) phylogenetic tree reconstruction was performed in W-IQ-TREE [70–72] with the TIM2 + G model and visualized with the Interactive Tree Of Life (iTOL) v5 [73]. The neighbor-joining (NJ) phylogenetic tree reconstruction was performed in MEGA 7 [68] with the default Tamura-Nei + d model. Bootstrap values of the ML and NJ tree were estimated using 1000 replicates. The barcoding gap between *H. decipiens* and *H. stipulacea* was calculated based on the Automatic Barcode Gap Discovery (ABGD) method on the ABGD graphic web version using the default settings [74].

Table 1. GenBank accession numbers of the sequences included in the present ITS phylogenetic analysis.

N°	Species	GenBank Accession	Location	Source	Ref.
1	*Halophila decipiens*	AF395671	Hawaii	Waycott et al., 2002	[60]
2	*Halophila decipiens*	AF366411	Australia	Waycott et al., 2002	[60]
3	*Halophila decipiens*	AF366407	USA	Waycott et al., 2002	[60]
4	*Halophila decipiens*	AF366413	Curaçao	Waycott et al., 2002	[60]
5	*Halophila decipiens*	AF366409	Costa Rica	Waycott et al., 2002	[60]
6	*Halophila decipiens*	AF366408	Panama	Waycott et al., 2002	[60]
7	*Halophila decipiens*	AB243983	Japan	Uchimura et al., 2008	[57]
8	*Halophila decipiens*	KC175913	Vietnam	Nguyen et al., 2013	[65]
9	*Halophila decipiens*	MN200776	Malaysia	Rozaimi et al., 2020	[44]
10	*Halophila* sp.	OM162162	Greece	This study	-
11	*Halophila stipulacea*	OM162166	Greece	This study	-
12	*Halophila stipulacea*	AF366436	Italy	Waycott et al., 2002	[60]
13	*Halophila stipulacea*	AY352618	Italy	Ruggiero et al., 2004	[75]
14	*Halophila stipulacea*	AY352635	Greece	Ruggiero et al., 2004	[75]
15	*Halophila stipulacea*	KM609943	Egypt	Nguyen et al., 2015	[56]
16	*Halophila stipulacea*	KM609944	United Arab Emirates	Nguyen et al., 2015	[56]
17	*Halophila stipulacea*	KM609944	India	Nguyen et al., 2015	[56]
18	*Halophila ovalis*	KF620337	Hong Kong	Nguyen et al., 2014	[76]
19	*Halophila ovalis*	AF366430	Australia	Waycott et al., 2002	[60]
20	*Halophila ovalis*	AF366420	Malaysia	Waycott et al., 2002	[60]
21	*Halophila ovalis*	AB243975	Japan	Uchimura et al., 2008	[57]
22	*Halophila ovalis*	AB436939	Thailand	Uchimura et al., 2008	[57]
23	*Halophila ovalis*	AB436925	Hawaii	Uchimura et al., 2008	[57]
24	*Halophila ovalis*	KF620354	India	Nguyen et al., 2014	[76]
25	*Halophila ovalis*	KC175911	Vietnam	Nguyen et al., 2013	[65]
26	*Halophila hawaiiana*	AF366414	Hawaii	Waycott et al., 2002	[60]
27	*Halophila johnsonii*	AF366425	USA	Waycott et al., 2002	[60]
28	*Halophila major*	AB436929	Japan	Uchimura et al., 2008	[57]
29	*Halophila major*	AB436927	Thailand	Uchimura et al., 2008	[57]
30	*Halophila major*	KC175910	Vietnam	Nguyen et al., 2013	[65]
31	*Halophila major*	KF620340	Malaysia	Nguyen et al., 2014	[76]
32	*Halophila major*	KF620352	Myanmar	Nguyen et al., 2014	[76]
33	*Halophila major*	MT586874	Philippines	Kolátková et al., 2021	[77]
34	*Halophila major*	MT028353	Indonesia	Kolátková et al., 2021	[77]
35	*Halophila minor*	AF366406	Philippines	Waycott et al., 2002	[60]
36	*Halophila minor*	AF366405	Guam	Waycott et al., 2002	[60]
37	*Halophila nipponica*	AB36924	USA	Uchimura et al., 2008	[57]
38	*Halophila nipponica*	AB523410	Japan	Uchimura et al., 2008	[57]
39	*Halophila nipponica*	KX668188	Korea	Kim et al., 2017	[64]
40	*Halophila spinulosa*	AF366440	Malaysia	Waycott et al., 2002	[60]
41	*Halophila spinulosa*	AF366439	Australia	Waycott et al., 2002	[60]
42	*Halophila tricostata*	AF366438	Australia	Waycott et al., 2002	[60]
43	*Halophila engelmannii*	AF366404	USA	Waycott et al., 2002	[60]
44	*Halophila becarii*	KM609945	India	Nguyen et al., 2015	[56]

3. Results

3.1. Morphological Identification

Based on the compiled information of the current taxonomic key of the genus *Halophila* (Kuo, 2020) [62] and the two previous keys, Phillips & Mehez (1988) [66] and Kuo & Den Hartog (2001) [63], the vegetative morphological characters of the specimens found in Salamina Island (*Halophila* sp.) match better with *H. decipiens*'s diagnostic characters than to those of *H. stipulacea*, especially regarding the low number and type of cross-veins and the structure of the scales (Table 2 and Figure 1).

Table 2. Vegetative characters of *Halophila* sp. compared to *H. decipiens* and *H. stipulacea*.

	Phillips & Mehez (1988) [66]; Kuo & Den Hartog (2001 [63]); Kuo (2020) [62]		**This Study**
Character	***Halophila decipiens***	***Halophila stipulacea***	***Halophila* sp.**
Rhizome	Thin, fragile, fleshy, elongated, 1 mm diameter	0.5–2 mm wide	Thin, fleshy, smooth, elongated, <1 mm diameter
Leaf shape	Oblong to elliptic, apex obtuse or rounded, base cuneate	Linear to oblong, elliptic, cartilaginous to membranous, apex obtuse, base cuneate or gradually decurrent-petiolate	Oblong to elliptic, base cuneate, apex obtuse
Leaf dimensions	10–25 mm long, 2.5–6.5 mm wide	Up to 60 mm long, 10 mm wide	7–20 mm long, 2–4 mm wide
Cross-veins	5–9 pairs ascending, unbranched	10–40 pairs, branched, ascending at 45–60 degrees	6–9 pairs ascending, unbranched
Leaf margin	finely serrulate	Finely serrulate	Finely serrulate
Leaf surfaces	Membranous, hairy on both sides or only on the ventral side, sometimes glabrous	Glabrous, or with minute hairs; not papillous; occasionally bullate	Both surfaces covered in minute unicellular hairs
Petioles	Not sheathing, shorter than the blades, 3–15 mm long	Sheathing lopsidedly at the base, shorter than the blades, 5–15 mm long	Shorter than the blades, 1–26 mm long
Scales	Transparent, usually hairy outside	Large, elliptic, or obovate transparent scales; 12–17 mm long; 6–10 mm wide; folded at the rhizome nodes covering (sheathing) petioles	Short, obovate, transparent, not sheathing the petioles lopsidedly

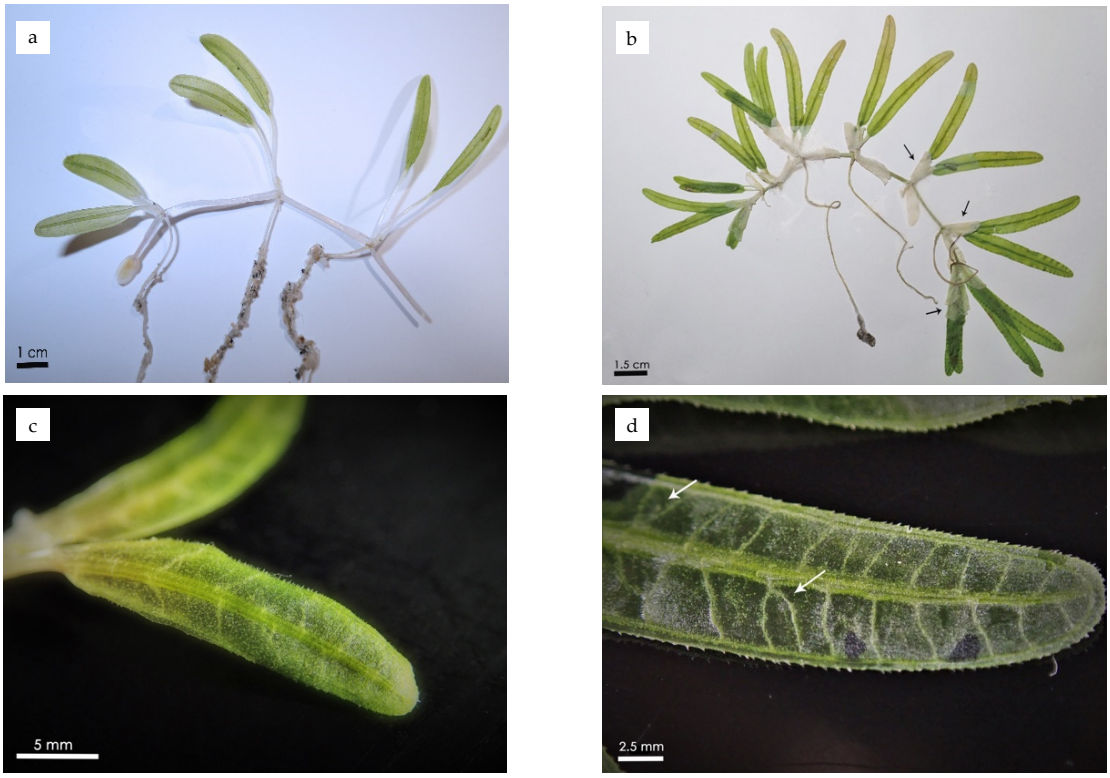

Figure 1. *Cont.*

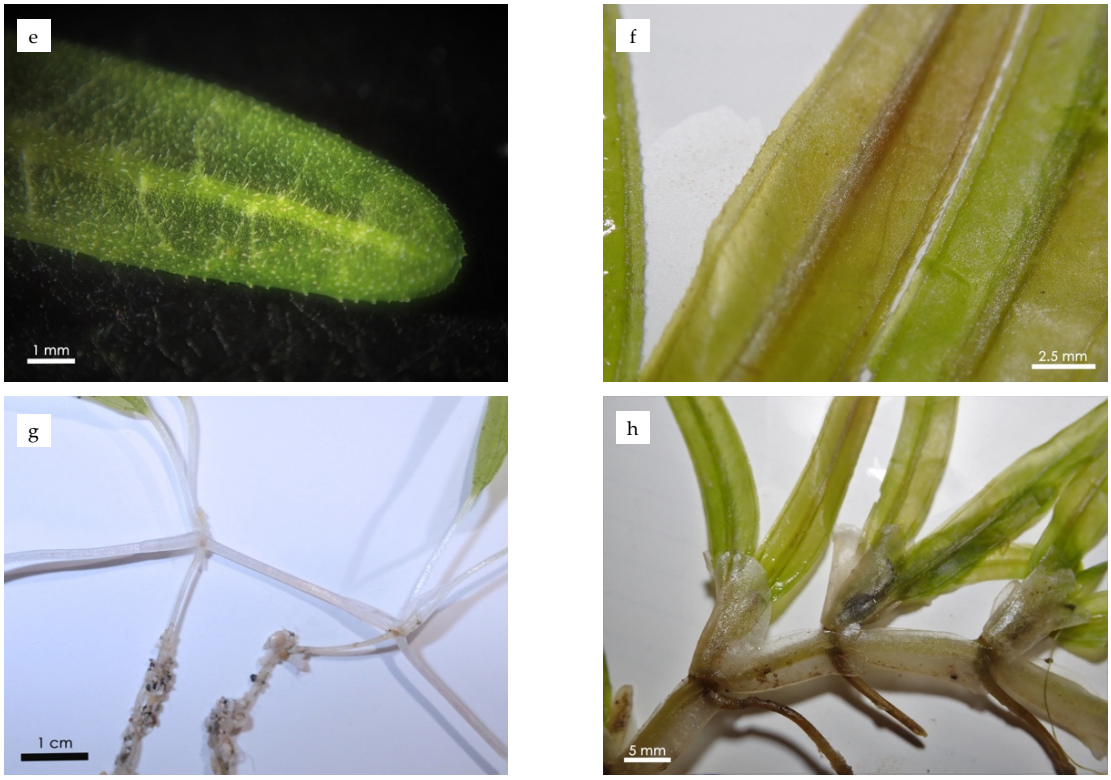

Figure 1. (**a**) A portion of *Halophila* sp. plant with leaf blades, apical meristem, rhizome, and roots. Scale bar = 1 cm. (**b**) A portion of *H. stipulacea* plant with linear to oblong leaf blades, scales (black arrows), rhizome, and roots. Scale bar = 1.5 cm. (**c**) *Halophila* sp. leaf blade with unbranched cross-veins, intra-marginal veins, and midrib. Scale bar = 5 mm. (**d**) *H. stipulacea* leaf blade showing serrated margin, numerous branched cross-veins (white arrows), intra-marginal vein, and midrib. Scale bar = 2.5 mm. (**e**) Close-up of *Halophila* sp. leaf blade showing serrate margin and a dense covering of minute unicellular hairs on the surface. Scale bar = 1 mm. (**f**) Close-up of *H. stipulacea* leaf blade with lack of minute hair and finely serrated margins. Scale bar = 2.5 mm. (**g**) *Halophila* sp. without persistent scales. Scale bar = 1 cm. (**h**) *H. stipulacea* showing large, persistent, transparent scales sheathing the petioles of leaf pairs. Scale bar = 5 mm. The photos from the left column are originally from Gerakaris et al. (2020) [39].

3.2. Genetic Identification

DNA isolation and sequencing were successful for all three barcodes in all samples. After quality correction, a final sequence of 521 bp for rbcL, 814 bp for matK, and 646 bp for ITS was obtained. The sequences for all three barcodes were uploaded to GenBank under the accession numbers OM160754–OM160761 for rbcL, OM160762–OM160769 for matK, and OM162162–OM162169 for ITS. There were no nucleotide differences between replicates for each site; therefore, only one sequence per site was used for downstream analyses. No nucleotide differences were found between specimens morphologically identified as *H. decipiens* (*Halophila* sp.) from Salamina Island and *H. stipulacea* from Crete for any of the barcodes, suggesting that all samples belong to the same species. The ITS region was used for the phylogenetic analysis because it has the highest resolution [56,78] and the largest number of sequences available on GenBank NCBI, both in terms of the number of *Halophila* species and the number of samples within species. For the other two DNA barcodes, there are currently only a very small number of *Halophila* reference sequences available, not representative of the inter and intraspecific variability of the *Halophila* genus, required to build a well-founded phylogenetic tree and establish limits between species. Therefore, we based our genetic identification analysis mainly on the ITS region. A final alignment of 621 bp (including gaps) was made for the 44 *Halophila* ITS sequences, of

which 418 (67.31%) were conserved sites, 196 (31.56%) were variable sites, 145 (23.35%) were parsimony-informative sites, and 51 (8.21%) were singletons. Between *H. decipiens* (9 sequences) and *H. stipulacea* (7 sequences), 563 sites (90.66%) were conserved, 41 sites (6.6%) were variable, 36 sites (5.8%) were parsimony-informative, and 5 sites (0.81%) were singletons. There was no overlap between the greatest intraspecific distance (0.01) and the smallest interspecific distance (0.06), also known as the barcoding gap. As for the phylogenetic analysis, there were no meaningful topological differences between the ML and NJ inference trees. The tree obtained by the ML method is shown in Figure 2. The sequences of *H. decipiens* and *H. stipulacea* formed two clearly distinct monophyletic clades, regardless of the geographical origin of the samples. The specimens morphologically identified as *H. decipiens* (*Halophila* sp.) based on the recent taxonomic key clustered with *H. stipulacea* in a monophyletic clade, hence, the genetic result does not support the vegetative morphologic species identification and suggests that the *Halophila* population found in Salamina Island corresponds to a morphological variant of *H. stipulacea.*

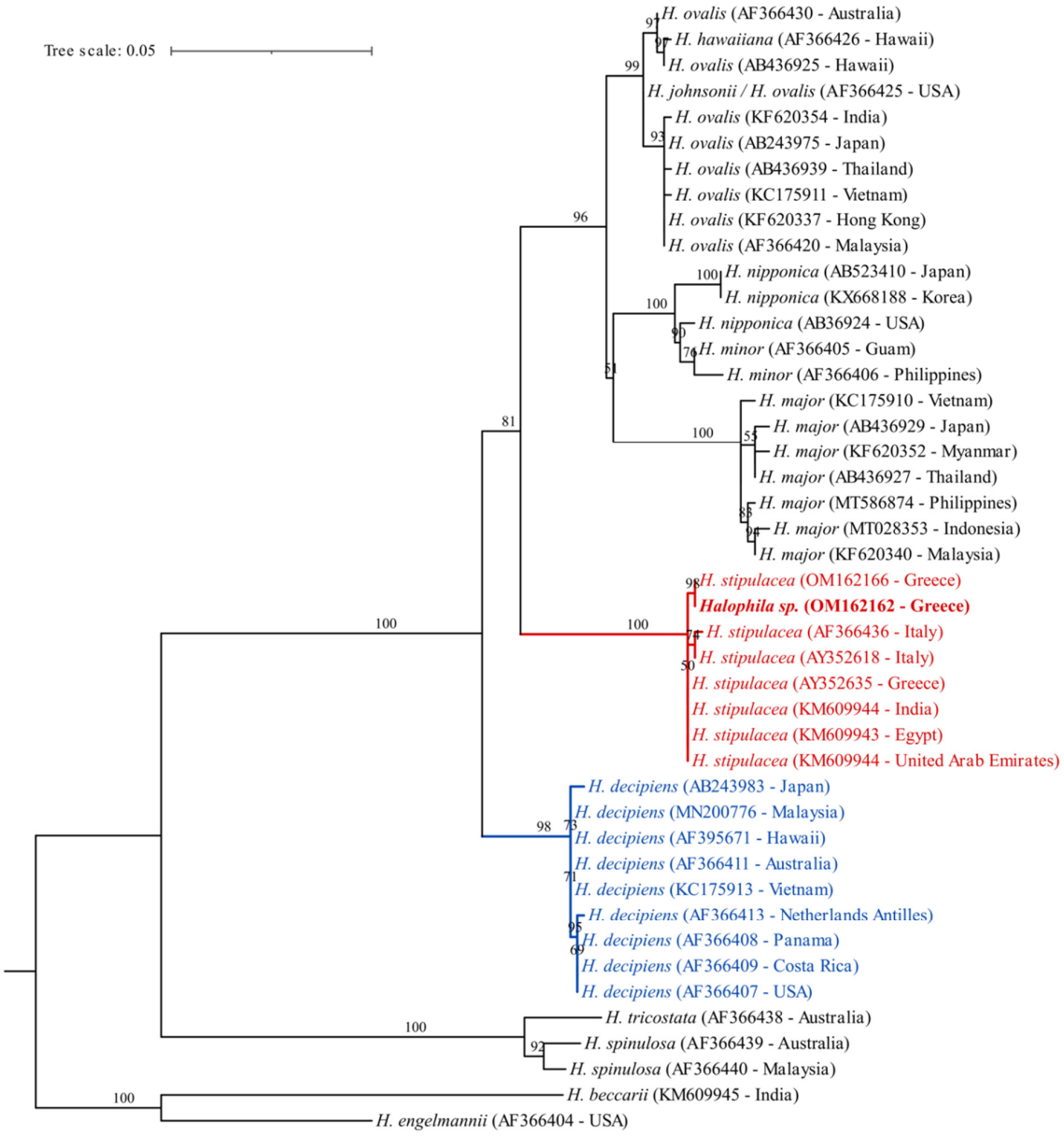

Figure 2. Phylogeny of the *Halophila* genus inferred from ML analysis based on 44 sequences of 621 bp (including gaps) of the ITS region. The bootstrap values of ML are shown in each node; values < 50 were

excluded. The species in bold corresponds to the specimen morphologically identified as *H. decipiens* found off Salamis, Greece. For sequence AF366425 we included the names *H. johnsonii* and *H. ovalis*, since recently a genomic-based phylogenetic and population analysis concluded that given the lack of genetic diversity, the ongoing recognition of *H. johnsonii* is unsupported and *H. johnsonii* and should be considered morphological variants of the same species [79]. Furthermore, the *H. hawaiiana* is currently considered an ecotype of *H. ovalis*; therefore, its taxonomic status should be taken with caution until a phylogenomic study takes place.

4. Discussion

The *Halophila* specimens from Salamina Island, morphologically described as *H. decipiens* (*Halophila* sp.) [39], did not show any nucleotide differences in the three DNA barcodes (ITS, rbcL, and matK) when compared to the *H. stipulacea* specimens from Crete, a population established in the Mediterranean Sea many years ago, suggesting that the specimens belong to the same species. This was further confirmed by the ITS maximum-likelihood and neighbor-joining phylogenetic trees, in which the *Halophila* sp. from Salamina Island formed a monophyletic clade together with *H. stipulacea*. The discriminatory power of the ITS region is not equal across the whole genus and is not able to resolve all morphologic and genetic conflicts, especially for the so-called *H. ovalis* complex (*H. ovalis*, *H. hawaiiana*, and *H. johnsonii*). However, in the case of *H. stipulacea* and *H. decipiens* the ITS region has a high species discriminatory power based on: (i) the two clearly distinct monophyletic clades containing a diverse representation of samples from widespread geographic origins; and (ii) the lack of overlap between the ITS greatest intraspecific distance (0.01) and the smallest interspecific distance (0.06), also called the barcoding gap, which is a condition necessary for the use of DNA barcoding in species identification. Apart from this study, ITS has also helped distinguish between *H. major* and *H. ovalis* in Japan [57] and Vietnam [65], confirmed that *H. nipponica* from Japan and Korea are the same species [64], identified the *H. ovalis subsp. bullosa* as conspecific with *H. ovalis* [80], confirmed the first record of *H. major* in Sri Lanka previously misidentified as *H. ovalis*, and helped find the first hybridization case of *Halophila* crossed between *H. ovalis* and *H. major* [78]. Above all, ITS has already been used in *H. decipiens* identification, by confirming the first report of this species in Kenya where it can easily be misidentified as *H. ovalis* [81]. The increase in successful studies based on the ITS region supports the idea that this marker can be an effective tool for species identification or confirmation of taxa where taxonomic ambiguity exists due to similar morphological characters and phenotypic plasticity [82], as is often the case in the widespread seagrass genus *Halophila*. However, the inclusion of the missing *Halophila* species, increasing the geographic cover, and resolving the *Halophila ovalis* complex and other unresolved species delimitations are still required to test the universality of this marker for the entire genus. If barcoding alone is insufficient, a comparative phylogenomic approach may be required to solve some of the current taxonomic ambiguities [79]. In addition, a revision of the current ITS barcoding database is needed to correct possible previous misidentifications and track changes in species delimitations, as these may lead to misinterpretations in future molecular analysis. As for the other two DNA barcodes included in the analysis, the rbcL and matK plastid genes have been widely used in plants and are currently recommended as the plant DNA barcode system by the CBOL [58]. However, the universality and effectiveness of these plastid regions varies among plant groups. The discriminatory power of a DNA barcode can be affected, among other things, by the inter and intraspecific divergence (barcoding gap) and the extent of the barcode library [83,84]. In the case of *Halophila*, these genes are highly under-sampled, so the current barcode library does not allow an appropriate characterization of the genetic variability. Moreover, based on the few rbcL and matK studies, these plastid markers have shown a low interspecific genetic variability among seagrasses, especially for the complex *Halophila* genus, limiting its resolution at the family and genus levels [56,61]. For these reasons, these regions were not considered for the species genetic identification here and the ITS region alone was used instead. Nevertheless, the sequences generated for the rbcL and matK

plastid barcodes and the lack of nucleotide differences among the specimens are important contributions to the seagrass DNA barcoding database for future phylogenetic studies.

In terms of morphological identification, the new taxonomic key of Kuo (2020) [62] has only minor and non-decisive changes in the diagnostic characters of the species. Therefore, the morphological characters of the specimens of *Halophila* sp. collected from Salamina Island still match well with those of *H. decipiens*, especially the low number and type of cross-veins and the structure of the scales. Finding molecular and morphological discordances in species identification is not uncommon. Since the advent of molecular analyses, revision of taxonomic classifications based solely on morphology has led to numerous changes in species delimitations and correction of previously overlooked misidentifications. Discordances may be the result of hybridization, introgression, cryptic species, early speciation, or high phenotypic plasticity leading to morphological variability [85–90]. In the absence of previous records of *H. decipiens* in the Mediterranean Sea, crossing with *H. stipulacea* resulting in a hybrid is unlikely, and the high ITS region similarity also suggests this (Figure 2). On the other hand, environmentally induced phenotypic plasticity is a common response mechanism in seagrasses, including in growth, reproduction, and morphological variability [44,91,92]. High phenotypic plasticity is also a common feature of invasive species, which allows them to survive under changing environmental conditions [93,94]. Therefore, finding morphological differences among *H. stipulacea* populations throughout the basin is not surprising and may explain the current discordance between morphological and molecular analyses. Considering both taxonomic characterizations (morphological and molecular), the genetic result is strongly supported by the high discriminatory power of the ITS region. On the contrary, the known morphologic variability of the genus and the lack of reproductive features during identification makes the morphologic identification more susceptible to misidentifications. Therefore, based on the unequivocal genetic result, the specimens found in Salamina Island, despite their morphologic similarity to *H. decipiens*, correspond to a morphological variant of *H. stipulacea.*

The specific characteristics of the new morphological variant of *H. stipulacea* (i.e., its leaf length and width, number and type of cross-veins, size, type, and structure of scales) are of great taxonomic value, and their inclusion in future taxonomic keys is strongly recommended. However, the morphologic variability of *H. stipulacea*, which has led to its misidentification as *H. decipiens*, highlights the limitations of identifying species with overlapping and highly variable morphological characters using traditional morphological identification alone. This is even more true in the absence of reproductive structures, the main distinct sources for the species identification of flowering plants. In the case of these two species, *H. decipiens* is monoecious (male and female flowers on the same spathe) and *H. stipulacea* is dioecious (male and female flowers on different individual plants) [62]. Therefore, the use of an integrative taxonomy that includes morphological and DNA-based analyses is recommended to avoid future misidentifications and to help resolve current taxonomic discrepancies, which is needed to understand past and future range shifts in this highly complex, diverse, and widespread genus.

Further development and integration of DNA-based analyses into seagrass studies will not only aid species delimitation and reduce misidentification but will also allow the application of techniques such as metabarcoding and environmental DNA (eDNA) to monitoring shifts in native seagrasses ranges. Rapid and accurate identification of species is important for monitoring NIS, as it can impact efforts to mitigate the threats posed by them [46]. For seagrasses known to disperse by commercial vessels, molecular analysis of ballast water can be of great benefit to detecting potential sources of invasion [47], as can the inclusion of seagrasses in eDNA surveys of water and sediment near marinas or ports, which are common invasive habitats. A universal macrophyte minibarcode (18S DNA) has recently been developed; however, its current low species-level resolution [95] limits its use in monitoring species range shifts. Therefore, for monitoring *Halophila* and other seagrasses, ITS target species analysis remains a better option. Active monitoring is even more important now that *H. stipulacea* has reached the French Riviera 30 years earlier than

what habitat suitability models predicted, considering future changes in temperature and salinity under climate change [34]. This is an indication that we may be underestimating the ability of this species to invade new habitats and that its spread may be faster than originally thought [28,96]. A concerted effort is needed to expand the in-depth morphological, molecular, and ecological descriptions of *H. stipulacea* populations throughout the basin. This will help to establish a more representative taxonomic database for the identification of the species, as well as provide essential information on the plasticity and/or adaptability of the species, contributing to an understanding of the complex evolutionary and ecological mechanisms that govern its invasion dynamics. Furthermore, although it should be considered a positive outcome that *H. decipiens* has not yet entered the Mediterranean Sea, suitable environmental conditions already exist in the Levantine Sea and are expected to expand to other areas of the basin in the coming years [34], so its introduction can be expected in the future and active monitoring is required.

5. Conclusions

Considering the high species discriminatory power of the ITS DNA barcode, and the common morphological variabilities and taxonomic ambiguities within the genus, known to lead to misidentifications. We conclude that the unequivocal genetic result does not support the vegetative morphologic identification and suggests that the *Halophila* population found in Salamina Island can be considered a morphological variant of *H. stipulacea*. This means that *H. stipulacea* remains the only non-indigenous seagrass species in the Mediterranean Sea. Our results highlight the importance of integrating morphological and molecular analyses of taxonomically complex and widespread genera such as *Halophila,* to avoid overlooking or misreporting species range shifts, which are essential for monitoring and managing NIS introductions.

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Data Availability Statement: The sequences generated on this study are available on GenBank, NCBI (https://www.ncbi.nlm.nih.gov/) under the accession numbers OM160754–OM160761 for rbcL, OM160762–OM160769 for matK, and OM162162–OM162169 for ITS.

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