



Halophila ovalis in the Tropical Atlantic Ocean

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ABSTRACT

A species of seagrass in the genus *Halophila* was found growing in a shallow lagoon on the west shore of Antigua in the Caribbean West Indies. Genetic analysis showed the plants were *Halophila ovalis*. In addition, the samples had no genetic deviation (using nrDNA sequences) from *Halophila johnsonii*, considered to be an endemic and endangered species in Florida, USA. Morphological analysis demonstrated the Antiguan *Halophila* to be well within the range of plant characteristics previously described in the literature for *H. ovalis*, except for leaf width and number of seeds per fruit, and again, not different from *H. johnsonii* and very closely related to *H. ovalis* from the Indo-Pacific. Ours is the first report of *H. ovalis* in the Tropical Atlantic bioregion.

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1. Introduction

The *Halophila* genus is a group of paddle-bladed seagrasses in the Hydrocharitaceae (den Hartog, 1970) comprising diminutive, paired-leaved species. Worldwide naturalistic investigations, as well as insights from phylogenetic approaches, underline a need for species realignment (Waycott et al., 2006). Fifteen *Halophila* species have been reported, distributed among five of the world's six bioregions (Short et al., 2007); den Hartog (1970) suggested that several fall within the dioecious *Halophila ovalis* species complex. The *H. ovalis* species complex has little genetic variation but wide morphological plasticity; the complex is dominated by *H. ovalis* itself, and includes *Halophila australis* (may be *Halophila euphlebia*, Uchimura et al., 2006a), *Halophila hawaiiiana*, *Halophila johnsonii*, and *Halophila minor* (Waycott et al., 2002). With two newly described species of *Halophila* having adequate reproductive or genetic evidence for species designation, *Halophila sulawesii* (Kuo, 2007) and *Halophila nipponica* (Kuo et al., 2006; synonymous with *Halophila japonica*, Uchimura et al., 2006b), a total of 17 *Halophila* species is now recognized: *H. australis*, *Halophila baillonii*, *Halophila beccarii*, *Halophila capricorni*, *Halophila decipiens*, *Halophila engelmanni*, *H.*

euphlebia, *H. hawaiiiana*, *H. johnsonii*, *H. minor*, *H. nipponica*, *H. ovalis*, *H. ovata*, *Halophila spinulosa*, *Halophila stipulacea*, *H. sulawesii*, and *Halophila tricostata* (Livingston et al., in preparation).

Halophila species occur predominantly within the two tropical bioregions (Tropical Atlantic and Tropical Indo-Pacific), as well as in southern Australia (Short et al., 2007). *H. decipiens*, a monoecious species, is the most widely distributed of any seagrass species in the tropical oceans and, together with *H. capricorni* and *H. sulawesii*, forms a second complex of *Halophila* species, characterized by serrate leaf margins (*H. decipiens* complex). The species *H. ovalis* has been found only in the Tropical Indo-Pacific and the Southern Oceans bioregions (Short et al., 2007).

Halophila species in the Atlantic include the ubiquitous *H. decipiens* (Creed et al., 2003; Short et al., 2006); two species, *H. baillonii* and *H. engelmanni*, that are considered “vulnerable” on the IUCN Red List (Livingston et al., in preparation); a species endemic to Florida, *H. johnsonii* (Eiseman and McMillan, 1980), considered endangered by the U.S. Fish and Wildlife Service (NMFS, 2002); and, an exotic species, *H. stipulacea*, that was recently reported in the eastern Caribbean (Ruiz and Ballantine, 2004; Willette and Ambrose, 2009). *Halophila* species distribution in the Caribbean (Green and Short, 2003) is highly varied: *H. decipiens* and *H. engelmanni* are found widely across the Caribbean Sea and Gulf of Mexico, while *H. baillonii* has an infrequent, discontinuous distribution, and *H. johnsonii* and *H. stipulacea* have narrow geographic

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Table 1List of *Halophila* taxa included in our molecular analysis, in order of their position in Fig. 2, with geographic locations and GenBank Accession numbers.

Taxa	Geographic source	Herbarium voucher number or citation	GenBank accession number
<i>Halophila beccarii</i> Ascherson	Viet Nam	Waycott et al. (2002)	AF366441
<i>Halophila engelmanni</i> Ascherson	Florida, USA	Waycott et al. (2002)	AF366404
<i>Halophila tricostata</i> Greenway	Australia	Waycott et al. (2002)	AF366438
<i>Halophila spinulosa</i> (R. Brown) Ascherson	Malaysia	Waycott et al. (2002)	AF366440
<i>Halophila decipiens</i> Ostenfeld	Malaysia	Waycott et al. (2002)	AF366412
<i>Halophila decipiens</i> Ostenfeld	Hawaii, USA	Waycott et al. (2002)	AF395673
<i>Halophila decipiens</i> Ostenfeld	Australia	Waycott et al. (2002)	AF366411
<i>Halophila decipiens</i> Ostenfeld	Okinawa, Japan	Uchimura et al. (2006a)	AB243979
<i>Halophila decipiens</i> Ostenfeld	Costa Rica	Waycott et al. (2002)	AF366409
<i>Halophila decipiens</i> Ostenfeld	Panama	Waycott et al. (2002)	AF366408
<i>Halophila decipiens</i> Ostenfeld	Netherlands Antilles	Waycott et al. (2002)	AF366413
<i>Halophila stipulacea</i> (Forsskål) Anderson	Italy	Waycott et al. (2002)	AF366436
<i>Halophila ovalis</i> (R. Brown) Hooker f.	Philippines	Waycott et al. (2002)	AF366416
<i>Halophila euphlebica</i> Makino	Tokushima, Japan ^a	Uchimura et al. (2006a)	AB243958
<i>Halophila euphlebica</i> Makino	Tokushima, Japan ^a	Uchimura et al. (2006a)	AB243957
<i>Halophila australis</i> Doty et Stone	Australia	Waycott et al. (2002)	AF366414
<i>Halophila euphlebica</i> Makino	Okinawa, Japan	Uchimura et al. (2006a)	AB243979
<i>Halophila ovalis</i> (R. Brown) Hooker f.	S. Queensland, Australia	Waycott et al. (2002)	AF366415
<i>Halophila ovalis</i> (R. Brown) Hooker f.	Viet Nam	Waycott et al. (2002)	AF366437
<i>Halophila ovalis</i> (R. Brown) Hooker f.	Okinawa, Japan ^a	Waycott et al. (2002)	AF366421
<i>Halophila ovalis</i> (R. Brown) Hooker f.	Okinawa, Japan ^a	Uchimura et al. (2006a)	AB243974
<i>Halophila ovalis</i> (R. Brown) Hooker f.	Malaysia	Waycott et al. (2002)	AF366420
<i>Halophila ovalis</i> (R. Brown) Hooker f.	Thailand	Uchimura et al. (2006a)	AB436939
<i>Halophila hawaiiiana</i> Doty et Stone	Hawaii, USA	Waycott et al. (2002)	AF366426
<i>Halophila hawaiiiana</i> Doty et Stone	Maui, HI, USA ^b		P219
<i>Halophila ovalis</i> (R. Brown) Hooker f.	N. Queensland, Australia	Waycott et al. (2002)	AF366435
<i>Halophila ovalis</i> (R. Brown) Hooker f.	Flores, Indonesia	Uchimura et al. (2006a)	AB436930
<i>Halophila johnsonii</i> Eiseman	Florida, USA	Waycott et al. (2002)	AF366425
<i>Halophila ovalis</i> (R. Brown) Hooker f.	Antigua		P479
<i>Halophila ovalis</i> (R. Brown) Hooker f.	Antigua		P475
<i>Halophila ovalis</i> (R. Brown) Hooker f.	Zanzibar, Tanzania		P394
<i>Halophila nipponica</i> (japonica)	Shimane, Japan	Uchimura et al. (2006b)	AB436933
<i>Halophila nipponica</i> (japonica)	Okinawa, Japan	Uchimura et al. (2006b)	AB436937
<i>Halophila minor</i> (Zollinger) den Hartog	Philippines	Waycott et al. (2002)	AF366406
<i>Halophila minor</i> (Zollinger) den Hartog	Guam	Waycott et al. (2002)	AF366405
<i>Halophila nipponica</i> (japonica)	Guam	Uchimura et al. (2006b)	AB436924

^a See Fig. 2.^b Type locality.

ranges that are restricted to Florida and the Lesser Antilles, respectively.

While establishing SeagrassNet sites in Antigua and Barbuda in the Caribbean Sea (www.SeagrassNet.org), we surveyed seagrasses around both islands and discovered a *Halophila* species with single pairs of leaves lacking leaf hairs, and having smooth leaf margins and female flowers, growing with *Halodule wrightii* in a tidal salt lagoon on Antigua. The morphological characters of this *Halophila* species excluded its identification as *H. decipiens*, *H. baillonii*, *H. engelmanni*, or *H. stipulacea* but not as the geographically restricted *H. johnsonii*. To investigate the taxonomic status of the Antiguan *Halophila* material, we examined vegetative and reproductive morphology and analyzed molecular phylogenetic sequences obtained from nrDNA internal transcribed spacers (ITS).

2. Methods

2.1. Collection and morphometry

Specimens of seagrass were collected by hand on 26 April 2009 from the south shore of Salt Pond adjacent to Deep Bay in north-west Antigua (17°07.6'N, 61°53.0'W) between –0.2 and 1.7 m depth mean low water. Leaf morphometry was conducted on 20 leaves from different leaf pairs taken haphazardly across the bed; flowers and fruits were counted and measured. Specimens were identified using Kuo and den Hartog (2001). Fresh and dried specimens were photographed and vouchers were deposited in the Albion R. Hodgdon Herbarium at the University of New Hampshire and in the International Seagrass Herbarium collection at Smithsonian Natural History Museum, Washington, DC.

2.2. Phylogenetic analysis

DNA sequence data from the nuclear ribosomal DNA target region that included ITS1, 5.8S and ITS2 were used to compare the *Halophila* species collected from Antigua to other *Halophila*, including sequence data from previously published work (Table 1). Two replicates from the Antiguan population of *Halophila* were extracted, amplified and sequenced. DNA was isolated from 0.01 g of silica-preserved samples ground in liquid nitrogen using the DNeasy Plant minikit (Qiagen, Valencia, CA, USA) and following the manufacturer's protocol.

PCR reactions were prepared for 25.0 µL final volume containing 13.3 µL of sterile Nanopure water; 2.5 µL of 10× NH₄ polymerase reaction buffer (Bioline, London, UK); 2.0 µL of 25 µM dNTPs, 1.5 µL 50 µM MgCl₂; 0.5 µL of 10 mg mL⁻¹ bovine serum albumin (BSA; BioLabs, Beverly, MA, USA); 1.0 µL for each (forward and reverse) of the 10 µM primers for ITS4 and ITS5 (White et al., 1990); 0.7 µL Biolase Red DNA polymerase (Bioline); and, 2.5 µL of template genomic DNA. The PCR was performed on a Dyand Thermal Cycler (MJ Research, Boston, MA, USA) under the following conditions: one cycle each of 1 min at 94 °C, one cycle of 40 s at 92 °C, 40 s at 50 °C and 90 s at 75 °C followed by 30 cycles of 40 s at 92 °C, 40 s at 50 °C, and 90 s at 75 °C. The final extension step was at 75 °C for 5 min. PCR products were quantified by 1% (w/v) agarose gel electrophoresis with DNA Hyperladder IV (Bioline). Prior to DNA sequencing, enzymatic removal of primers and excess dNTPs from PCR products was done by adding 3.2 µL of 1 unit µL⁻¹ shrimp alkaline phosphatase and 1.6 µL of 10 units µL⁻¹ exonuclease (USB Corporation, Cleveland, OH, USA) to 8 µL of PCR product and incubating at 37 °C for 30 min followed by 80 °C for 15 min.



Fig. 1. *Halophila ovalis* from Antigua. Clockwise from upper left: intertidal population growing with *Halodule wrightii*; typical asymmetric leaf; fruit attached to rhizome; ovules released from fruit; ovary and styles of the female flower.

Both strands of the PCR products were sequenced using Applied Biosystems (ABI, Carlsbad, CA, USA) BigDye terminator chemistry run on an ABI 3730XL capillary-based DNA sequencer. Using Sequencher version 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA), electropherograms of the sequence fragments were visually inspected, edited and assembled.

2.3. Statistics

Sequences of *Halophila* were aligned using MULTIPLE Sequence Comparison by Log-Expectation MUSCLE (Edgar, 2004). Consistent with an earlier analysis (Waycott et al., 2002), *H. beccarii* and *H. engelmanni* were used as the outgroup. Phylogenetic relationships were inferred using a Bayesian analysis (Metropolis-coupled

Markov chain Monte Carlo method) performed in MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). A general time reversible substitution model with invariable sites and gamma distribution (GTR+I+G) was used because it was the best-fitting base substitution model determined by a maximum-likelihood ratio test using Modeltest 3.7 (Posada and Crandall, 1998). The two parallel runs with four chains (three heated and one cold) each were run for 1 million generations, sampling a tree every 100 generations. Only trees sampled after convergence were used to make inferences about the phylogeny and to compute a 50% majority-rule consensus tree. The program MEGA4.1 beta was used to draw the tree and estimate pair wise sequence divergence (Tamura et al., 2007).

Table 2

Comparison of vegetative and reproductive characters of *Halophila ovalis* from den Hartog (1970) with *Halophila johnsonii* (Eiseman and McMillan, 1980) and with *Halophila* from Antigua. Bold print indicates characters lacking overlap with *H. ovalis*; “–” indicates character not described.

Parameter	Character comparison		
	<i>Halophila ovalis</i> den Hartog (1970)	<i>Halophila johnsonii</i> Eiseman and McMillan (1980)	<i>Halophila</i> from Antigua Present study (n = 12)
<i>Vegetative morphology</i>			
Rhizome internode length (mm)	10–50	10–20	12–36
Scale length (mm)	3–8	3–4	2–6
Scale hairs	Absent	Absent	Absent
Petiole length (mm)	10–45 (–120)	10–20	8–26
Petiole length > leaf length	Yes	Sometimes	Sometimes
Leaf form	–	Asymmetrical	Asymmetrical
Leaf length (mm)	10–40 (–70)	5–25	(13–) 17–19
Leaf width (mm)	5–20	1–4	(2.5–) 3–4
Leaf surfaces	Glabrous	Glabrous	Glabrous
Leaf margins	Entire	Entire	Entire
Leaf margin to cross-veins distance (mm)	0.1–0.3	0.1–0.2	0.1–0.2
Number of cross-veins	10–25	5–10	8–10 (–12)
Cross-vein branching	Common	Occasional	Occasional
Cross-vein angle (°)	45–60	45	45
<i>Reproductive morphology</i>			
Flowering spathe hairs	Absent	–	Absent
<i>Male flower</i>			
	Known	Unknown	Unknown
<i>Female flower</i>			
Ovary length (mm)	1–1.5 (–2.5)	–	1.5–3
Style length (mm)	10–20	–	(12–) 16–22
Seeds (number per fruit)	20–30	10–15^a	4–10^a

^a Number of ovules, without evidence of fertilization; *H. johnsonii* from York et al. (2008).

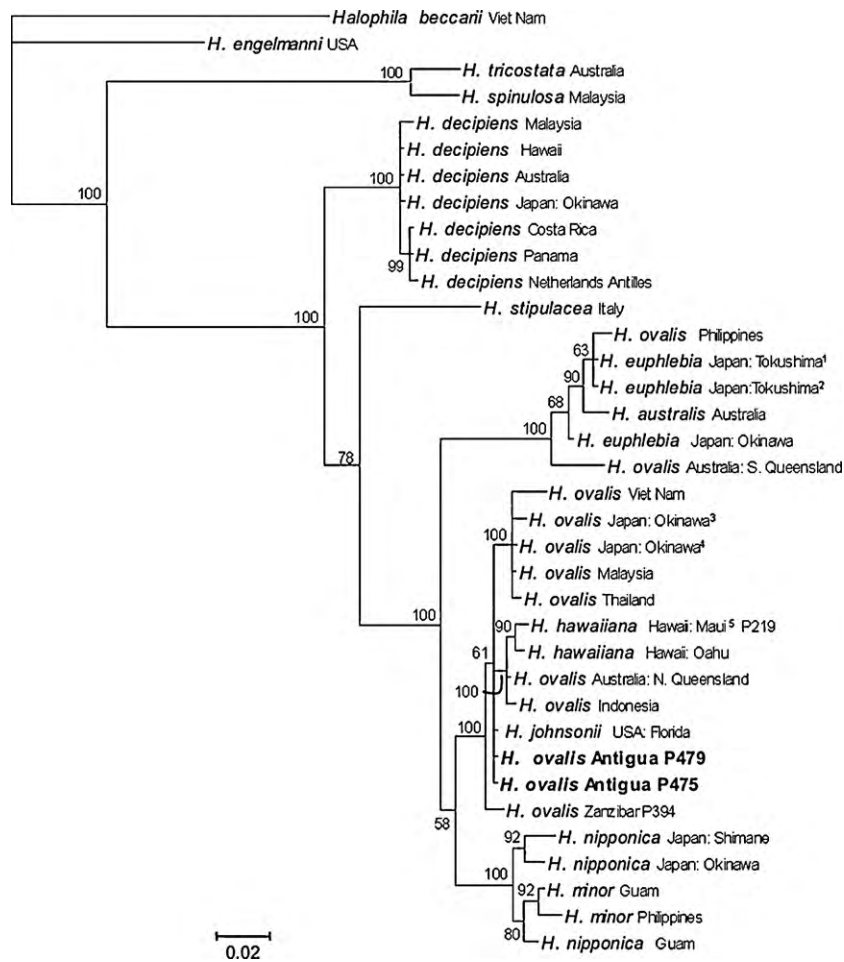


Fig. 2. Phylogeny of *Halophila* inferred from a Bayesian analysis and based on 787 base pairs of nrDNA sequences comprising ITS-1, 5.8S rDNA and ITS-2. The specimens collected from Antigua are in bold text. The branch lengths are proportional to the number of character changes. The posterior probabilities from a Bayesian analysis are included to the left of the nodes.

3. Results

Both morphological and genetic analyses of the *Halophila* species collected in Salt Pond, Antigua determined it to be *H. ovalis*. Our finding is the first report of the Indo-Pacific seagrass *H. ovalis* in the Atlantic Ocean. The smooth-surfaced, paddle-shaped, paired leaves, without serrated leaf margins, were typical of *H. ovalis* (den Hartog, 1970; Kuo and den Hartog, 2001), and the female flowers and fruits were also consistent with this species (Fig. 1). Morphologically, the *H. ovalis* collected in Antigua was similar to *H. johnsonii* described from Florida (Table 2). Moreover, both *H. ovalis* from Antigua and *H. johnsonii* fell well within the range of morphological characteristics previously described in the literature for *H. ovalis*, except for leaf width and number of seeds (or ovules in the case of *H. johnsonii* and possibly for the *H. ovalis* from Antigua) per fruit. Leaf cross-veins in the Antigua samples were both paired and alternate, often on a single leaf. The *H. ovalis* from Antigua and *H. johnsonii* both have somewhat asymmetrical leaves, a characteristic not originally reported for *H. ovalis* (Table 2).

A set of 36 ITS sequences and 787 characters (nucleotides and gaps) from the genus *Halophila* were included in our analysis. The two replicates of *H. ovalis* from Antigua were sequenced, with 0% genetic variation detected between replicates. Both replicate samples grouped with *H. ovalis/johnsonii/hawaiiiana* in a well-supported clade in the Bayesian analysis (Fig. 2). The percent sequence divergence of *H. ovalis* from Antigua with other taxa in this clade ranged

from 0% with *H. johnsonii* from Florida (USA) to 0.005% with *H. ovalis* from Indonesia and 0.016% with *H. ovalis* from Viet Nam.

4. Discussion

H. ovalis is present in the Tropical Atlantic bioregion, based on our morphological observations and a molecular systematic analysis using the ITS region of nrDNA. The phylogenetic analysis resolved that the *Halophila* collected from Antigua is *H. ovalis* (Fig. 2) and that there is a lack of sequence variation with *H. johnsonii* from Florida. Our results for the *H. ovalis* found in the Atlantic are consistent with an earlier analysis of the genus, which found low sequence variation within the *H. ovalis* species complex from the Indo-Pacific that also included *H. johnsonii* (Waycott et al., 2002).

Some small differences in plant morphology are found between the species descriptors of *H. ovalis* (den Hartog, 1970) and the samples collected in Antigua (Table 2). The differences occur in leaf width and the number of seeds per fruit, both of which are outside the range of what has been reported for *H. ovalis* but not different than that reported for *H. johnsonii*. The leaf width differences are not large (with leaves 5–20 mm wide for *H. ovalis*, 1–4 mm for *H. johnsonii*, and 3–4 mm for the Antigua samples). The leaf width range presented by den Hartog (1970) for *H. ovalis* does not include the width of the *H. ovalis* subspecies *linearis* (1.5 mm wide). Additionally, the leaf asymmetry of *H. johnsonii* and the Antigua samples (Fig. 1) is a feature not reported for *H. ovalis* (den Hartog, 1970;

Kuo, 2007) but leaf asymmetry has been seen in some populations of *H. ovalis* worldwide, including Egypt, Thailand, China, Guam, the Philippines and Fiji (Short, pers. obs.). *H. ovalis* (den Hartog, 1970) is reported to have twice the seed count per fruit of the Antigua sample, but we observed no male flowers (Table 2). Overall, the morphological characteristics of the Antigua samples are remarkably similar to *H. ovalis* especially when including the subspecies *H. ovalis* ssp. *linearis*, known only from Mozambique, east Africa.

We suggest that the morphology of *H. johnsonii* falls within the bounds of what has been reported for *H. ovalis*. The descriptors that Eiseman and McMillan (1980) used to identify *H. johnsonii* are completely encompassed by the descriptors of *H. ovalis*, particularly if one includes the descriptions of *H. ovalis* ssp. *linearis* from den Hartog (1970). The distinguishing morphological characteristics of *H. johnsonii* used in the species description (Eiseman and McMillan, 1981) are: (1) “exclusively linear-lanceolate blades” which are also seen in *H. ovalis* ssp. *linearis*; (2) “elongate-cuneate base” which also describes the narrowly triangular leaf base of *H. ovalis* ssp. *linearis*; and (3) “alternate cross venation,” a weak characteristic since venation varies widely in *H. ovalis* from paired to alternating, sometimes on the same leaf.

The discovery of a previously unreported seagrass species in a certain geographic area has been interpreted in various ways, including as a new record in a poorly cataloged marine flora (McDermid et al., 2002; Tsuda et al., 1977; Short et al., 2006; McMahon and Waycott, 2009), a range extension as a result of global climate change (Kim et al., 2009) or an exotic species (den Hartog, 1972; Lipkin, 1975; Lipkin et al., 2003; Ruiz and Ballantine, 2004; Willette and Ambrose, 2009). How we interpret seagrass distribution can have far-reaching implications in our perception of biogeography, biodiversity, and natural resource management. For example, certain species such as *H. johnsonii* are considered rare because of their limited distribution; hence the species is afforded protection (Notice for Critical Habitat of *H. johnsonii*, Federal Register 65(66): 17786). In contrast, a species with a history of invasion in one location (i.e., *H. stipulacea* in the Mediterranean, den Hartog, 1972; Lipkin, 1975) can receive a very different label; one that apparently constrains any explanation for its presence in a new biogeographic region to introduction via anthropogenic means, as recently suggested for *H. stipulacea* in the Caribbean (Ruiz and Ballantine, 2004; Willette and Ambrose, 2009). With the discovery of *H. ovalis* in the West Indies, we suggest two hypotheses to explain its presence in the Atlantic flora with the understanding that additional hypotheses may emerge.

Certain *Halophila* species, such as *H. stipulacea*, seem to be well-suited for dispersal via maritime traffic or other means (Ruiz and Ballantine, 2004; Willette and Ambrose, 2009). Like *H. stipulacea*, *H. ovalis* could also be characterized as an exotic species in the tropical Atlantic. The *H. ovalis* from Antigua was found beside a dredged channel, possibly introduced during dredging or another maritime activity. Although *H. stipulacea* appears to be dispersing in the Caribbean and was found around several islands, it was looked for, but not seen, in Antigua (Willette and Ambrose, 2009); the *H. ovalis* population we discovered in 2009 was apparently not found during their 2007–08 survey. The discovery of *H. ovalis* in Antigua suggests the species' capacity for dispersal, either natural or anthropogenic. Interestingly, both species are dioecious, meaning that both seeds or male and female plants would need to be transported to establish a viable, sexually reproducing population.

To provide an alternative explanation for the presence of *H. ovalis* in the Caribbean, we consider the case of *H. decipiens*, identified as the only pan-tropically distributed seagrass (den Hartog, 1970; Phillips and Meñez, 1988). To our knowledge, *H. decipiens* has not been regarded as an invasive species in either the Tropical Atlantic or Indo-Pacific bioregions. On the contrary, natural or “jump” dispersal of seagrasses via rafting/floating of vegetative

fragments has been asserted to explain *H. decipiens*' low genetic variability (0–0.7% sequence difference for ITS) between ocean basins (Waycott et al., 2002); fragment dispersal has also been used to explain the asexual recruitment of *H. johnsonii* in Indian River Lagoon, Florida (Hall et al., 2006). Like *H. decipiens*, *H. ovalis* has a wide geographic distribution which, taken together with its low percent sequence divergence, indicates that it too may have the capacity for long-distance natural dispersal, even between ocean basins.

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