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Understanding the ecological and evolutionary dynamics of tropical seagrasses and the factors driving habitat distribution

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Understanding the ecological and evolutionary dynamics of tropical seagrasses and the factors driving habitat distribution

A dissertation submitted in partial satisfaction of the requirements

for the degree of Doctor of Philosophy in Biology

by

Kelcie Lorena Chiquillo

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ABSTRACT OF DISSERTATION

Understanding the ecological and evolutionary dynamics of tropical seagrasses and the factors driving habitat distribution

by

Kelcie Lorena Chiquillo

Doctor of Philosophy in Biology

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Professor Paul Henry Barber, Chair

Seagrasses occur worldwide, and are essential primary producers that uptake carbon dioxide, fix nutrients, stabilize sediments, prevent reef degradation, filter bacteria, provide food and nursery habitats to marine organisms. When seagrass meadows disappear, carbon is released back into the water column, sediments get stirred, water clarity decreases, and reefs become infected, with negative impacts on marine biodiversity and maritime economy. My thesis utilizes multidisciplinary ecology and evolutionary biology approaches to better understand the biology of seagrasses, particularly an invasive seagrass, to help improve management strategies for seagrass conservation.

Seagrasses frequently display distinct depth distribution, although drivers of these patterns can be spatially and temporally variable. Chapter 1 examines the factors that influence the depth distribution of a circumtropical seagrass, *Halophila decipiens*. While *H. decipiens* can

grow in waters as shallow as 1 m, in Moorea, French Polynesia we only found it in waters deeper than 6.4 m. To understand why *H. decipiens* did not grow in shallower habitats, we transplanted it into 3 habitats: the existing seagrass bed (control), just outside the seagrass bed, and shallower habitat adjacent to a fringing coral reef. Results showed that growth was not significantly different between the seagrass bed and just outside of the seagrass bed; however, its growth was significantly reduced when adjacent to the reef. We then transplanted seagrass into a shallower reef site with and without herbivore exclusion cages, and the results showed that *H. decipiens* grew best when herbivores were excluded, but lost growth when herbivores were allowed access. These results indicate that *H. decipiens* can grow in shallow habitats adjacent to reefs, but herbivory pressure from the reef limits its depth distribution.

Seagrass meadows are in decline around the world. Biological invasions can magnify threats to seagrass ecosystems with detrimental consequences to seagrass biodiversity. In Chapter 2, I used mesocosm experiments to investigate the interactions between the invasive seagrass *Halophila stipulacea* and native seagrasses to determine whether species interactions can drive, prevent, or facilitate invasions in both the Mediterranean and Caribbean Sea. In the Caribbean, invasive *H. stipulacea* increased in growth when grown with the native *Syringodium filiforme*, and lost shoots when grown alone, while *S. filiforme* only increased in shoots when grown alone. This pattern was the same in the Mediterranean; when invasive *H. stipulacea* grew with the native *Cymodocea nodosa,* it gained more shoots than when grown alone, but *C. nodosa* only did better when grown alone. Results suggest that the invasive seagrass *H. stipulacea* can drive its own success by negatively affecting native seagrasses and benefiting from that negative interaction. This novel example of native species facilitating the success of an invasive provides one possible mechanism for the widespread success of this invasive species.

Mechanisms that influence invasion success can further be understood by understanding how it was introduced to a specific region. In Chapter 3, I used genomic tools to reconstruct the origins of the globally invasive seagrass *Halophila stipulacea* in the Mediterranean and Caribbean Seas. While *H. stipulacea* almost certainly invaded the Mediterranean from native populations in the Red Sea through the Suez Canal, it is unclear whether the Caribbean invasion represents stepping stone colonization from the Mediterranean, an independent introduction from the native range, or an admixture from multiple native/invasive populations. To test these hypotheses, we examined population genetic structure and genetic diversity from multiple locations spanning across the native, historic, and recent invasive ranges of *H. stipulacea*, including the Indian Ocean and Red Sea, Mediterranean Sea, and the Caribbean Sea, respectively. Data from 524 SNP loci and restrictive, 45 SNP loci at >10x coverage revealed significant genetic structure among all five regions. The analyses revealed that the widespread invasion of *H. stipulacea* into the Caribbean Sea came from multiple introductions originating from the Mediterranean. This work provides a baseline for the distribution of the invasive *H. stipulacea* in the Caribbean and may help predict how to minimize detrimental impacts of a non-indigenous seagrass across its invaded ranges.

Life history differences can provide a link in invasion potential and dispersal. In Chapter 4 I investigated the life history of seagrass *Halophila stipulacea* in the Caribbean. Reports of asexual and sexual reproduction are common in its native range, with sexual reproduction being less common in the Mediterranean Sea. Here we make the first report of *H. stipulacea* male flowers in the Caribbean and suggest that asexual fragmentation is the main strategy of expansion. These findings have important implications for the future dispersal, survival, and maintenance of the non-native populations in the Caribbean.

The dissertation of Kelcie Lorena Chiquillo is approved.

Peggy Marie Fong

Pamela J. Yeh

Richard F. Ambrose

Paul Henry Barber, Committee Chair

University of California, Los Angeles

2021

DEDICATION

To my ancestors, my grandparents, my parents, my brother,

my tios, my tias, my cousins, my friends,

thank you,

you've given me porpoise.

I dedicate this dissertation to my loved ones,

the living and the ones who have departed—Moses Avalos, Eduardo Chiquillo, Jaime Avalos,

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Que descansen en paz

#MoePower

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- Kelcie L. Chiquillo contributed to the conceptualization, methodology, formal analysis, data collection, data curation, visualization, and original writing and review editing of this work.
- Brianna M. Mims contributed to the conceptualization, methodology, formal analysis, data collection, and writing
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PUBLICATIONS

2021	Fong, C.R, **Chiquillo, K.L**., Gaynus, C.J., Grier, S.R., Ha, B.A., Ryznar, E.R., Smith, L.L., Sura, S.A., Anggoro, A.W., Moore, T.N., Fong, P. Flip it and reverse it: reasonable changes in designated controls can flip synergisms to antagonisms. *Science of The Total Environment*.
2020	Smulders, F.O.H., **Chiquillo, K.L**., Willette, D.A., Barber, P.H., Christianen, M.J.A. Inconclusive evidence of sexual reproduction of invasive *Halophila stipulacea*: a new field guide to encourage investigation of flower and fruit production throughout its invasive range. *Botanica Marina.*
2020	**Chiquillo, K.L.,** Mims, B.M., Chong, A.U., Johnson C., Habtes, S.Y., Fong, C.R., Barber, P.H., Fong P. Little giants: Herbivory as a limiting factor for seagrass proximity to fringing reefs in Moorea, French Polynesia. *Aquatic Botany.*
2020	Winters, G., Beer, S., Willette, D.A., Viana, I.G., **Chiquillo, K.L.,** Beca-Carretero, P., Villamayor, B., Azcárate-García, T., Shem-Tov, R., Mwabyu, B., Migliore,L., Rotini, A., Oscar, M.A., Belmaker, J., Gamliel, I., Alexandre, A., Ashwin, H., Procaccini, G., & Riloy, G. The tropical seagrass *Halophila stipulacea*: reviewing what we know from its native and invasive habitats, alongside identifying knowledge gaps. *Frontiers in Marine Science.*
2019	Willette, D.A., **Chiquillo, K.L.,** Cross, C., Fong, P., Kelley, T., Toline, C.A., Zweng, R., & Muthukrishnan R. Growth and recovery after disturbance of a rapidly-expanding invasive seagrass. *Journal of Experimental Marine Biology and Ecology.*
2019	Muthukrishnan, R., **Chiquillo, K.L.,** Cross, C., Fong, P., Kelley, T., Toline, C.A., Zweng R., & Willette D.A. Little giants: A rapidly invading seagrass alters ecosystem functioning relative to native foundation species. *Marine Ecology Progress Series*

2018 **Chiquillo, K.L.,** Barber, P.H & Willette, D.A. Fruits and flowers of the invasive seagrass *Halophila stipulacea* in the Caribbean Sea. *Botanica Marina.*

2014 **Chiquillo, K.L.,** Barber, P.H & Willette, D.A. Ebert, D.A., Slager, C.J., & Crow, K.D. The secret of the mermaid's purse: Phylogenetic affinities within the Rajidae and the evolution of a novel reproductive strategy in skates. *Molecular Phylogenetics and Evolution.*

TEACHING APPOINTMENTS

2021 EEB 109- Introduction to Marine Science (Virtual)
2019 EEB 123B – Field Marine Ecology
2019 EEB 109- Introduction to Marine Science
2018 EEB 111 – Vertebrate Zoology
2017 EEB 109 – Introduction to Marine Science
2017 2BRAD Genomic Sequencing Workshop
2017 The Diversity Project -
2017 EEB 101 – Marine Botany

CIVIC ENGAGEMENT

2021 Peer Mentor for E. Cota Robles Foundation
2020 Co-founder of A WOC Space, LLC. *https://awocspace.com*
2019 Notre Dame Academy Elementary School. Guest Lecture. "Seagrass is Grass."
2018 UCLA EEB Department. Faculty Graduate Student Liaison
2017 Westwood Charter Elementary School Science Slam. "Hunger Games"
2017 Explore your Universe. Volunteer
2016 SACNAS Chapter at UCLA. Graduate Student Representative.
2016 Educational Film. "Out of Thin Air..." *https://youtu.be/FZwq2yzsA20*

CERTIFICATIONS

2021 PADI® SCUBA Divemaster
2019 Yin Yoga Teacher 50 hr– RYT®
2019 UCLA Entering and Mentoring (EMT) Program
2018 Vinyasa Yoga Teacher 200 hr – RYT®
2016 RNAseq – Conservation and Gene Expression Training
2015 Eco Stats- Technological Advances between Ecology and Statistics Training
2015 Environmental DNA (eDNA) Training
2015 2b-RADseq Genomic Sequencing
2014 U.S. Boat Operations
2010 AAUS Scientific Diving– Moss Landing

CHAPTER 1

HERBIVORY AS A LIMITING FACTOR FOR SEAGRASS PROXIMITY TO FRINGING REEFS IN MOOREA, FRENCH POLYNESIA

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Short communication

Herbivory as a limiting factor for seagrass proximity to fringing reefs in Moorea, French Polynesia

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ABSTRACT

Seagrasses are important foundational species that frequently display distinct depth distributions, although the drivers of these patterns can be spatially and temporally variable. While the pantropical seagrass *Halophila decipiens* is known from waters as shallow as 1 m deep, in Moorea, French Polynesia we only found it > 6.4 m deep. To explore factors affecting depth distribution, we transplanted *H. decipiens* into 3 habitats: the existing seagrass bed (control), just outside the seagrass bed, and shallower habitat adjacent to a fringing coral reef. Results showed that growth was not significantly different between the control and just outside of the seagrass bed; however, number of shoots and rhizome length were significantly reduced adjacent to the reef. Transplanting seagrass into the shallow reef site with and without herbivore exclusion cages showed that *H. decipiens* grew in herbivore exclusion treatments, but lost both shoots and rhizomes in the control. These results indicate that *H. decipiens* can grow in shallow habitats adjacent to reefs on Moorea, but that herbivory pressure, presumably from the reef, limits its depth distribution.

1. Introduction

Seagrass beds are some of the most productive ecosystems worldwide, providing important ecological and economic benefits to coastal regions. They are the primary food resource for many organisms, provide shelter and nurseries, sequester carbon (Larkum et al., 2006), attenuate water flow, and reduce vertebrate pathogens (Lamb et al., 2017). While seagrasses play key roles in coastal ecosystems, knowledge of the factors that limit their depth distribution is constrained by high spatial variability in a complex mosaic of abiotic and biotic conditions regulating their distribution.

Some of the factors that can shape local or small-scale distribution of seagrasses include chemical characteristics of sediment (Krause-Jensen et al., 2011) and physical disturbances (El Allaoui et al., 2016). Seagrasses also display distinct patterns of depth zonation as a function of abiotic factors such as light attenuation (Duarte, 1991), desiccation (Kahn and Durako, 2009), and characteristics of the seagrass itself such as the ability to tolerate high irradiance (Björk et al., 1999), among others. Similarly, biotic interactions, including competition for resources (Greve and Binzer, 2004) and grazing (Hay, 1981), can control local patterns of distribution. Herbivory may play an important role in controlling local distributions as seagrasses provide a suite of resources for grazing organisms (Heck et al., 2008). Further, grazing pressure can vary as fish communities are impacted by fishing pressure and can be spatially variable as a result of fear of or release from predation (Madin et al., 2011). For example, in the Caribbean Sea, grazing by surgeonfishes, parrotfishes (Randall, 1965), and the urchin *Diadema* (Ogden et al., 1973) can completely eliminate seagrasses from habitats adjacent to coral reefs. While it is clear that herbivory can control local distribution patterns of seagrass (Nowicki et al., 2018), what remains unknown is whether top down control by grazing can shape depth distributions.

The seagrass *Halophila decipiens* is pantropical and can inhabit a broad depth range (0–85 m deep) (Den Hartog, 1970). However, on the

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island of Moorea, French Polynesia, *Halophila decipiens* is known from only three sites, all reported at > 6 m deep (Moorea Berkeley Biocode-biocode.berkeley.edu). Given that *H. decipiens* can occur in other parts of world as shallow as the intertidal, it is unclear what limits this species from occupying shallower waters of Moorea. Here we examine two factors that may limit the distribution of *H. decipiens* in Moorea. We characterized the shallowest depth limit of *H. decipiens* at two sites and conducted two *in situ* experiments to evaluate the abiotic and biotic processes that shape these distribution patterns. We hypothesize that *H. decipiens* cannot survive and grow outside the limits of the existing seagrass beds. Further, we hypothesize that proximity of *H. decipiens* to the reef is limited by herbivory.

2. Materials and methods

Our study occurred in July 2017 at two sites along the northern shore of Moorea, French Polynesia. Site 1 (17° 29′ 34′′ S, 149° 51′ 4′′ W) is at the mouth of Opunohu Bay and consists of a sandflat ~100 m wide bordered by a fringing reef. Site 2 (17° 28′ 51′′ S, 149° 49′ 27′′ W) is on the backreef near the mouth of Cook's Bay and is bordered by a patch reef system and a deep natural channel.

To determine the upper depth limits of *H. decipiens*, we quantified seagrass density along the shallow margin of the seagrass bed at both sites using visual transects and noted the shallowest occurrence (Duarte and Kirkman, 2001). We deployed a 30 m baseline transect parallel to the shore along a contour ~two meters inside the upper edge of the seagrass bed. We then placed perpendicular transects from five randomly-selected points along this baseline, extending each towards the shore until encountering the shallowest seagrass shoot. At every meter along each perpendicular transect we recorded depth, counted shoots per 0.25m^2 quadrat (pairs of leaves were recorded as one shoot), and extrapolated shoot density to m^2. Because tidal amplitude only varied ~ 0.15 m at our sites (Hench et al., 2008), we conducted surveys regardless of tidal height.

To determine if *H. decipiens* can survive and grow outside the limits of the existing seagrass beds, we conducted a single factor experiment at Site 1, where we transplanted seagrass into three habitats. First, to determine whether *H. decipiens* could survive in the shallower habitat near the fringing reef, we transplanted seagrass to the sand flat at 2–4 m depth and 1 m from the fringing reef (~18 m from the seagrass bed). Second, to determine whether *H. decipiens* could survive just outside the seagrass bed, we transplanted seagrass within 1–2 m of the margin at 6–8 m depth. Third, as a control for excavation and replanting effects, we excavated and replanted seagrass inside the donor bed at 8–9 m deep.

To begin the experiment, we carefully excavated 30 individual rhizomes with attached roots, shoots, and growing tip from inside the seagrass bed. We counted the initial number of shoots and trimmed rhizomes to similar lengths, making sure to leave the apical tip. Rhizomes averaged 16.5 cm (± 0.7 SE) in length with an average of 8.3 (± 0.3) SE shoots. We transplanted 10 rhizomes into each of the three habitats by excavating a shallow trench of the approximate depth where the rhizomes were extracted, laying roots and rhizomes into the trench, and covering them with the excavated sediment ensuring the shoots remained above the sediment. We then secured each transplant to the benthos with metal U-shaped stakes and ensured blades and shoots were clear of sediment. In this experiment, we lost two ramets transplanted outside the seagrass bed, and two ramets transplanted to the shallow reef.

After 20 days, we re-excavated each transplant, re-counted the number of shoots, and re-measured rhizome length. We calculated change from initial for each response variable (*i.e.* shoot number and rhizome length). After data met assumptions of normality and homogeneity of variance, we conducted one-way ANOVAs using 'stats' package for each response variable (R Core Team, 2019). Significant ANOVAs were followed by Tukey's honest significant difference test (Tukey HSD) to identify treatments that differed.

To explore whether herbivory limits the distribution of *H. decipiens* adjacent to the reef, we performed a paired transplant experiment manipulating herbivore access. We transplanted 20 rhizomes into the same shallow sand flat as in the first experiment using the same methods. Transplants initially averaged 17.9 cm (± 0.8 SE) in length and included 10.4 (± 0.4 SE) shoots. To test for herbivory effects, we deployed transplants in pairs, one rhizome protected by herbivore exclusion cages, while the other was placed in an open cage that allowed herbivory while controlling for cage effects. Closed and open cages were 10 × 40 × 10 cm (L x W x H) and constructed from hardware cloth with 1 cm openings (Carpenter, 1986). Open cages were identical to the closed cages, but with the top and one long side of the cage removed, allowing access for herbivorous fishes and invertebrates. No transplants were lost in this experiment.

After 16 days, we re-measured rhizome length and shoot number. The data conformed to assumptions of parametric statistics, and we performed a paired *t*-test to determine significant differences in growth due to herbivory (R Core Team, 2019).

3. Results

The shallowest recorded depth of *Halophila decipiens* in our survey occurred at 6.4 m at Site 1 (n = 82) and 8.6 m at Site 2 (n = 24). The density of seagrass shoots varied greatly, ranging from 0 to 706.3 shoots/m^2 at Site 1 and 0–433.3 shoots/m^2 at Site 2. However, average densities were similar with Site 1 at 165.8 shoots/m^2 (± 18.2 SE) and Site 2 at 158.9 shoots/m^2 (± 26.2 SE) (t = test, p = 0.829). Distribution was patchy, with no seagrass recorded in numerous quadrats.

Changes in both number of seagrass shoots and length of rhizomes differed across transplant habitats (Fig. 1; shoots: ANOVA, p = 0.0002, df = 27, $F_{2,27} = 11.6$; rhizomes: ANOVA, p = 0.0003, df = 27, $F_{2,27} = 10.84$). After 20 days, transplants inside and just outside the existing seagrass bed nearly tripled in shoot number while rhizome length doubled. However, there was no difference in growth between these two habitats for either variable (Tukey HSD; $p > 0.05$ for both comparisons). Increases in the number of seagrass shoots averaged 16.2 shoots 20 d^{-1} (± 3.5 SE) and 11.6 shoots 20 d^{-1} (± 4.9 SE) for inside and outside, respectively. Rhizome length increased by 23.6 cm (± 6.5 SE) and 13.9 cm (± 6.9 SE), for inside and outside respectively.

In contrast, transplants in the shallow habitat lost both shoots and rhizomes, making changes in these metrics significantly different than in the other two experimental treatments (Tukey HSD, $p < 0.05$ for both comparisons). Shallow transplants decreased in number of shoots by 77 % over the course of the experiment (decreased by 6.4 shoots 20 d^{-1} ± 1.1 SE), while rhizome length decreased by 72 % from initial values (decreased by 11.9 cm ± 1.6 SE in length). Rhizome growth was 1.2 cm d^{-1} (± 0.33 SE) within the seagrass bed, 0.7 cm d^{-1} (± 0.4 SE) just outside the seagrass bed, and -0.6 cm d^{-1} (±0.08 SE) at the shallow site. Similarly, shoots increased by 0.8 cm d^{-1} (± 0.2 SE) within the seagrass bed, 0.6 cm d^{-1} (± 0.2 SE) just outside the seagrass bed, and lost -0.3 cm d^{-1} (± 0.05 SE) at the shallow site.

After 16 days, shoots of seagrass transplanted to shallow depths and within herbivore exclusion cages increased by an average of 4.9 (± 2.4 SE), which was a 51.1 % (± 20.8 SE) increase (Fig. 2). Rhizome length also increased over initial values by 7.1 cm (± 2.5 SE) or 39.9 % (± 15.4 SE). However, in open cages accessible to grazers, seagrass transplants lost both shoots and rhizomes; shoots decreased 76.6 % (± 8.4 SE) (on average -8.2 shoots) and rhizomes decreased 7.6 % (± 7.1 SE) in length (on average -1.2 cm). These differences resulted in a significant difference between final number of shoots (paired *t*-test, p = 0.0001) and final rhizome length (paired *t*-test, p = 0.005).

Caged seagrass near the reef experienced average rhizome growth of 0.44 cm 16 d^{-1} (± 0.16 SE), compared to uncaged treatments, which were -0.07 cm 16 d^{-1} (± 0.08 SE). Similarly, growth of shoots increased by 0.31 cm 16 d^{-1} (± 0.12 SE) in caged treatments and decreased by -0.51 cm 16 d^{-1} (± 0.07 SE) in uncaged treatments.

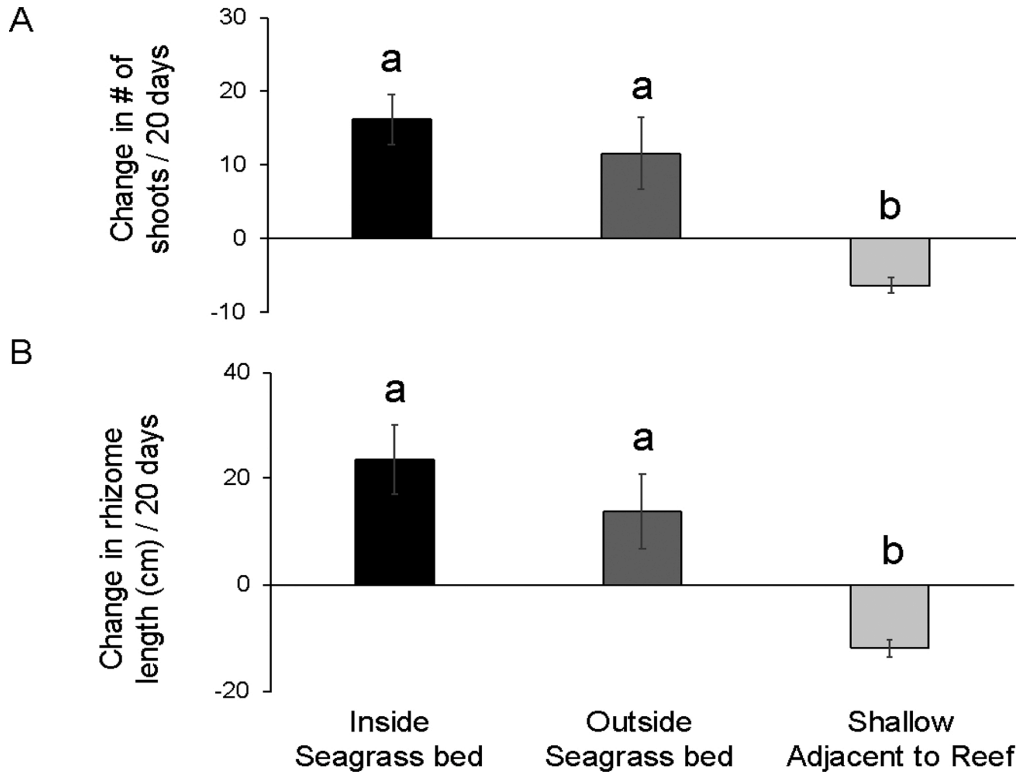

Fig. 1. Changes in (a) shoot number and (b) rhizome length of seagrass transplanted into three habitats: inside seagrass bed, 8 – 9 m deep, outside seagrass bed, 6 – 8 m deep, and shallow habitats, 2 – 4 m deep, 1 m adjacent to fringing reef. Bars are means (± SE). Bars that have different lowercase letters are significantly different.

4. Discussion

The distribution of the pantropical seagrass *Halophila decipiens* in Moorea, French Polynesia was quite limited, occurring no shallower than 6.4 m deep. In contrast, other studies found this seagrass is capable of inhabiting depths as shallow as 0.3 m deep (the lowest intertidal) in Bocas del Toro, Panama (Schubert and Demes, 2017) and 1−2 m deep in Hawaii (McDermid et al., 2002). Although the seagrass we transplanted into shallow (~ 2 m deep) habitat lost shoots and rhizomes, this was not a function of unsuitable habitat. Instead, loss was only observed in shallow water habitats when herbivores had access to the transplants. When transplants in shallow water were protected from herbivory by caging, *H. decipiens* survived and exhibited vigorous growth in both length and number of shoots. Combined, these results demonstrate that *H. decipiens* is likely excluded from shallow reefs habitats of Moorea by herbivory pressure, possibly arising from proximity to shallow fringing reefs inhabited by herbivorous fishes.

Our findings also suggest that shallow water habitats of Moorea may not be optimal for *H. decipiens* growth, even in the absence of herbivores, because the seagrass transplanted to the shallow habitat and caged had a slower daily growth rate compared to transplants to deeper water. Shallow transplants possibly experienced photoinhibition, a process noted in the same species (Durako et al., 2003) and other seagrasses (Björk et al., 1999), which would support the growth rate differences we observed. However, an important caveat to this result is that our two experiments from which we compare growth rates (transplants in deep and shallow water without caging, and caged/uncaged shallow water transplants) were conducted sequentially, not simultaneously. As such, differences in growth may be confounded by time.

Seagrasses can be limited by unstable sediment caused by dredging and sediment infilling (Erftemeijer and Lewis, 2006). All three of the habitats into which we transplanted seagrass were once dredged, and therefore differences in sediment instability due to dredging are not likely contributing to among-habitat differences in growth. The lower growth rates of shallow water *H. decipiens* protected from herbivory indicate that abiotic controls, in addition to herbivory, likely contribute to preventing *H. decipiens* in Moorea from successfully establishing in shallow water habitats. Our findings support the current paradigm that *H. decipiens* has a patchy and discontinuous distribution (Den Hartog, 1989; Fonseca et al., 2008). One possibility could be foraging by animals; for example, bioturbators, such as fishes in the family *Haemulidae, Mullidae* and *Lethrinidae*, feed on endofauna in the sediment and disturb vegetation patterns while foraging up to 25 m from reefs (Madin et al., 2019, Steiner and Willette, 2014). Patchiness of seagrass could be a function of changes in herbivory pressure due to proximity of apex predators, as fear of predators can alter foraging behavior, reducing herbivore movement and consumption (Catano et al., 2016, Rizzari et al., 2014).

Although our results demonstrate that *H. decipiens* is limited by herbivory pressure, we did not observe the taxa responsible for grazing. However, other studies that surveyed herbivorous fishes on the reef adjacent to Site 1 found members of the families *Acanthuridae* (surgeon fishes) and Labridae, subfamily *Scarinae* (parrotfishes) (Gaynus, 2019; Keeley et al., 2015) as well as Siganidae (rabbit fishes) in Site 2 (Poray and Carpenter, 2014). Common species of herbivorous fishes in Moorea include *Zebrasoma scopas, Acanthurus nigrofuscus, Chlorurus sordidus,*

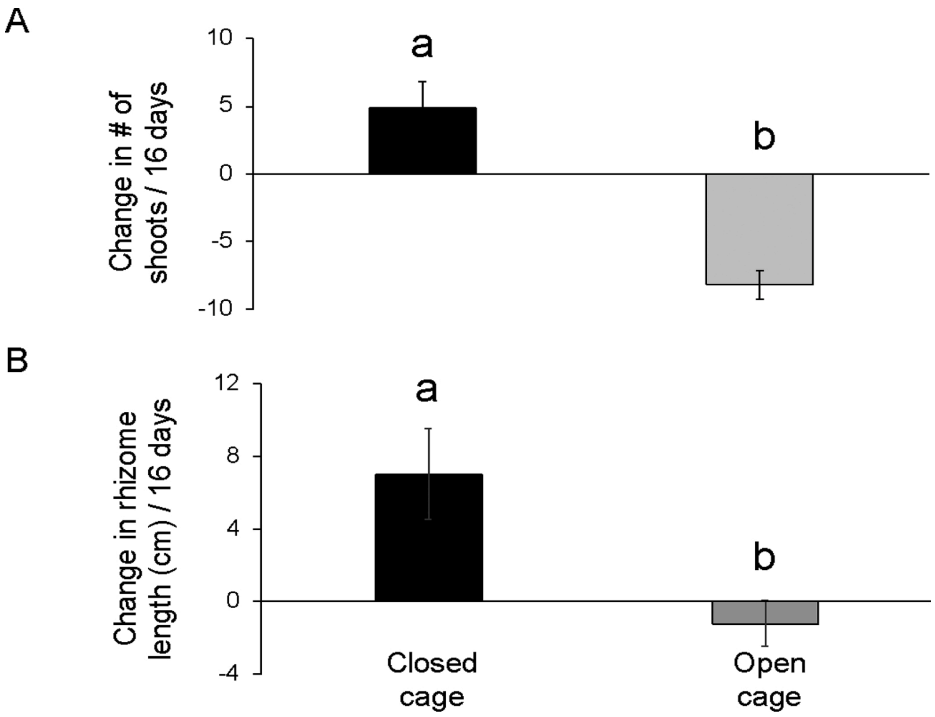

Fig. 2. Changes in (a) number of shoots and (b) rhizome length of seagrass transplanted into closed cages and open cages deployed in the shallow site 2 – 4 m deep, and 1 m adjacent to a fringing reef. Bars are means (± SE). Bars that have different lowercase letters are significantly different.

Ctenochaetus striatus, Naso unicornis, Naso lituratus (Fong et al., 2018). Globally, a wide variety of species graze on seagrass (Scott et al., 2018), and stomach content analyses of butterflyfishes (Chaetodontidae) in Moorea detected the presence of seagrass fragments, although the overall frequency was low (Harmelin-Vivien and Bouchon-Navaro, 1983). In the Caribbean, herbivory on seagrasses by fishes and urchins (Randall, 1965, Ogden et al., 1973) resulted in conspicuous zones of bare sand proximal to reefs. These studies show that herbivores in the western Caribbean can be important regulators of seagrasses near areas of refuge (Armitage and Fourqurean, 2006). The high herbivory next to the reef we found in our experiment suggests the same regulatory role for herbivory may occur near Pacific reefs.

Overall, the depth distributions of *H. decipiens* in Moorea appears to be a function of the interaction of herbivory pressure and bathymetry and that if there were shallow soft bottomed areas not in close proximity to reef habitat, *H. decipiens* could grow there. While further study is required to conclusively determine the source of seagrass herbivory proximal to reef habitat, our results provide clear evidence that *H. decipiens* in Moorea is subject to top-down control by herbivores, influencing its depth distribution.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquabot.2020.103294.

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CHAPTER 2

Interspecific interactions between invasive versus native seagrasses shows it is both a driver of its own success, and benefitting from the negative effects

2.1 Abstract

1. The nature and strength of interactions between native and invasive species can determine invasion success. Species interactions can drive, prevent, or facilitate invasion, making understanding the nature and outcome of these interactions critical.
2. We conducted mesocosm experiments testing the outcome of interactions between *H. stipulacea,* a seagrass that invaded the Mediterranean and Caribbean Seas, and native seagrasses (*Cymodocea nodosa* and *Syringodium filiforme,* respectively) to elucidate mechanisms explaining the successful invasions. Mesocosms were intact cores with species grown either mixed or alone. In the Mediterranean, an added treatment tested for density effects.
3. In both locations, the invasive grew faster with the native than when alone, while also negatively affecting the native, with similar patterns for shoot density, aboveground, and belowground biomass. Average internode length only differed between species, with the invasive having shorter horizontal internodes.
4. In the Caribbean, *H. stipulacea* increased by 5.6±1.0 SE shoots in 6 weeks when grown with the native, while when alone there was a net loss of -0.8±1.6 SE shoots. The opposite pattern occurred for *S. filiforme* where it only gained shoots when alone (+3.6±0.5 SE) and lost them when with the invasive (-3±3.0 SE) with final above and belowground biomass mirroring this response.

5. In the Mediterranean, the invasive grown with the native increased shoots (+3.7±1.3 SE) more than when it grew alone (+0.5 ± 0.9 SE). However, the native *C. nodosa* did better when grown alone (+1.7 ±0.4 SE shoots) than with the invasive (-1.0 ± 0.4 SE), and this pattern is similar for biomass measures. Further, growth was strongly density-dependent for both species, suggesting considerable intraspecific competition. In both seas, invasive plants had shorter internodes than native seagrasses, indicating a phalanx resource acquisition strategy
6. *Synthesis.* Our results suggest that a seagrass that invaded two seas drives its own success by both negatively affecting native seagrasses and benefiting from that negative interaction. This a novel example of native species facilitating the success of an invasive, providing one possible mechanism for the widespread success of this invasive species.

2.2. INTRODUCTION

Understanding whether species interactions play a key role in determining the success or failure of invasive species is critical for predicting future invasion success and subsequent spread (Schultheis & MacGuigan, 2018; Theoharides & Dukes, 2007). Species interactions that may promote invasion success can have negative effects on native species, such as competition (Dueñas et al., 2018), or positive effects on invasive species, such as facilitation (McIntire & Fajardo, 2014). Alternatively, invasion success may be propelled by natural or anthropogenic disturbance with species interactions playing a minor role (King & Tschinkel, 2008). Thus, to predict whether an invasive species can succeed in an invaded habitat it is critical to understand whether the invader is a driver of its own success, a passenger of community change (reviewed by (MacDougall & Turkington, 2005), or the result of other species interactions, such as facilitation (Bertness 1989, Bruno et al. 2003).

In one conceptual framework for successful invasion, invasive species can be “drivers” of their own success by readily displacing native species through direct interspecific interactions, such as competition and allelopathy, or by enacting changes in surrounding environmental conditions (HilleRisLambers et al., 2010; Lambers & Oliveira, 2019). The success of many invasive species has been linked empirically to superior competitive abilities (Strain & Johnson, 2009; Vilà et al., 2011) that result in direct density-dependent, negative impacts on native species (van de Voorde et al., 2012; Zhang & van Kleunen, 2019). Invasive species can be competitive dominants through superior capacities to acquire resources such as nutrients, light, or space (Gioria & Osborne, 2014; Goldberg et al., 1999). As an example, internode length may indicate different resource acquisition strategies, wherein plants with long internodes exhibit a "guerilla strategy" that promotes exploring new patches (Doust, 1981, 1987), while shorter internode

lengths represent a "phalanx strategy" that facilitates remaining in a resource-rich patch (Sutherland & Stillman, 1988). Other mechanisms where invasive species drive their own success include production of allelopathic chemicals that inhibit growth of co-occurring native species (Bauer et al., 2012; Callaway & Ridenour, 2004; Lambers & Oliveira, 2019) or release of substances that change soil or water chemistry (He et al., 2009; Raniello et al., 2007; Tuya et al., 2013; Weidenhamer & Callaway, 2010). Alternatively, invasive primary producers can generate positive environmental feedbacks, like enhanced nutrient cycling, that amplify their growth compared to natives (Chen et al., 2009). Thus, it is critical to explore species interactions to determine whether an invader can be a driver of its own success under certain environmental contexts.

Another conceptual framework for species invasions posits that invasive species can be “passengers”, with invasion success enhanced with disturbance or environmental change that removes native species and/or prevents their recovery (Didham et al., 2005; MacDougall & Turkington, 2005). Theory predicts that disturbance-mediated passengers are usually not competitive dominants, but readily colonize open space (Bulleri et al., 2010). Empirical studies have shown that early successional habitats are especially vulnerable to species invasions (Johnson et al., 2006), while the ability to invade is reduced in late successional stands (Cunard & Lee, 2009). For example, highly disturbed habitats have been linked to the invasion and spread of grass species such as *Microstegium vimineum* (Barden, 1987; Rauschert et al., 2010) as growth of the invasive plants was greater in more disturbed areas (Averill et al., 2011). Some invasive species are better at acquiring critical resources (e.g., light, nutrients) after a habitat is disturbed (D’Antonio et al., 1998). Thus, exploration of species interactions may also provide

insight into whether an invasive species can be a passenger of community change under a given environmental context.

Facilitation is a positive species interaction that may influence invasion success, though these interactions are often overlooked (Bruno et al. 2003). Facilitation of species invasions can be interspecific, with native species facilitating invasive species (McIntire & Fajardo 2014), or intraspecific with invasive species exhibiting positive feedbacks (Fajardo et al. 2011). Facilitation by native species can enhance invasion success with a resulting decline in native species and alteration of ecosystem function (Bronstein et al. 2009). Examples of facilitation by natives include reducing thermal stress by shading (Altieri et al. 2010), increasing access to nutrients through symbiosis (Callaway & Walker 1997), or removing/deterring predators (Stachowicz 2001). Further, native nitrogen-fixing plants can facilitate invasions by increasing available soil nitrogen, which is often a limiting resource (Kuebbing & Nunez 2015, 2016). Alternatively, invasive plants can facilitate their own invasion by reducing native fitness (Reinhard 2002; Jordan et al. 2008) or positively supporting their own growth (Vitousek et al. 1987; Ehnefeld et al. 2001; and Poulette and Arthur 2012; Ehrenfeld 2003). For example, some invasive plant species fix nitrogen or create conditions that promote fire, both of which can facilitate their own success (Stachowicz and Byrnes 2006). Therefore, it is important to examine whether facilitation plays an important role in enhancing the success of invasive species.

Native to the Indian Ocean and Red Sea, the seagrass *Halophila stipulacea* has invaded both the Mediterranean Sea where it coexists with native seagrasses (Sghaier, 2014; Winters et al. 2020), and the Caribbean Sea where it is displacing native seagrasses (Steiner et al. 2015; Smulders et al. 2017; Winters et al. 2020). Thus, this seagrass is the only known marine plant to have invaded both eastern and western hemispheres, including both temperate and tropical zones

(Winters et al., 2020), motivating reach into the mechanisms underlying these successful invasions. In this study, we examine ecological interactions of *H. stipulacea* and native seagrasses in its two invasive ranges. Specifically, we test whether *H. stipulacea* is a driver of its own success through superior competitive abilities, or whether other interspecific interaction, such as facilitation, may contribute to invasion success. Our overall approach in both seas was to quantify interactions among native and invasive seagrass species by growing them mixed and alone in flow through mesocosms and measuring growth responses.

2.3 MATERIALS AND METHODS

2.3.1 Study Species and Sites of Donor Seagrass Beds

The seagrass *Halophila stipulacea* is native to the Western Indian Ocean, Eastern Africa, Arabian Sea, Persian Gulf, and the Red Sea. *H. stipulacea* functions as a pioneer species in its native range, where it is a short-lived opportunist that colonizes and grows rapidly in disturbed areas (Coppejans et al., 1992). *H. stipulacea* has many disturbance-mediated traits, including high turnover (Azcárate-García et al., 2020), reduced longevity (Rindi et al., 1999), high tolerance to a wide range of irradiance (Lee et al., 2007), short leaf life span (Hemminga et al., 1999), high leaf production, and rapid rhizome elongation rate (Azcárate-García et al., 2020; Duarte, 1991; reviewed by Winters et al., 2020)

Following the opening of the Suez Canal, *H. stipulacea* invaded the Mediterranean Sea where it was recorded in Akrotiri Bay, Limassol, Cyprus in 1968 (Lipkin, 1975). Since then, *H. stipulacea* has flourished, expanding its range throughout the Mediterranean where it forms multi-species meadows with native seagrasses and algae (Sghaier et al., 2014; Winters et al., 2020) (Figure 2.2 a). A second invasion of *H. stipulacea* was recorded in 2002 on the Caribbean

Island of Grenada (Ruiz & Ballantine, 2004). Subsequently, it expanded its range over 700 kilometers by rapidly spreading to the north, reaching the Virgin Islands and, most recently, Puerto Rico, and west, reaching Curacao (Ruiz et al., 2017; Vera et al., 2014; Willette et al., 2014) (Figure 2.1 a). In contrast to the Mediterranean, *H. stipulacea* displaces native Caribbean seagrasses, forming monoculture meadows (Ruiz and Ballantine 2004; Willette et al. 2012; Willette et al. 2014).

There is limited evidence that *H. stipulacea* is a passenger invader as it can readily invade unvegetated habitat and proliferate under small scale disturbances (Steiner & Willette, 2015; Willette et al., 2020). Christianen et al. (2019) proposed that sea turtle grazing and catastrophic disruptions, such as hurricanes (Hernández-Delgado et al., 2020) that remove native species, favor the recruitment of the invader. However, one experiment showed *H. stipulacea* transplants could grow in an existing *S. filiforme* dominated seagrass bed, providing initial evidence it could be a driver (Willette & Ambrose, 2012). Finally, one study suggested *H. stipulacea* can be both a passenger and a driver depending on the environmental or community context (Muthukrishnan et al., 2020).

In both seas, we chose study sites comprised of natural, mixed species, relatively low-density seagrass beds. In the Caribbean, the donor seagrass bed was in Brewers Bay, St. Thomas, U.S. Virgin Islands, Eastern Caribbean Sea (18° 20’ 36.78’’ N, 64° 58’ 45.624’’ W) between 2 - 4 meters in depth (Figure 2.1 b). Shoot density of *H. stipulacea* in the donor bed averaged 786.3 shoots/m^2 ± 15.3 SE. This density is in the range of averages recorded across Brewers Bay of 742 - 3,850 shoots/m^2 (Olinger et al., 2017). However, it is denser than in Culebra, Puerto Rico, the site of the most recent expansion, where shoot density ranged from ~20 – 400 shoots/m^2 (Hernández-Delgado et al., 2020). In the Mediterranean, the donor seagrass bed was in Akrotiri

Bay at Dream Café site, Limassol, Cyprus (34°42′20″N, 33°07′24″E) between 3-5 meters in depth (Figure 2.2 b; same site as in Nguyen et al, 2018). Average shoot density was 231.9 shoots/m^2 ± 3.9 SE, which was lower than natural densities in other Mediterranean sites, including Akrotiri Bay, where averages ranged from ~ 800 - 2000 shoots/m^2 (Nguyen et al., 2020a) and Italy with an average of 10,500 shoots/m^2 (Gambi & Barbieri, 2009). However, density of the donor bed appeared typical of other sites around Limassol (K. Chiquillo, pers. obs).

2.3.2 Experimental design:

St. Thomas, USVI, Caribbean experiment

To examine the effects of species interactions on the growth of native and invasive seagrasses in the more recent range expansion into the Caribbean, we used mixed seagrass beds comprised of the invasive *H. stipulacea* and the native Caribbean *Syringodium filiforme.* (Figure 2.1 b). *Syringodium filiforme* is a dominant native seagrass in coastal regions of some Caribbean islands, including the Virgin Islands (Kendall et al., 2004; Willette & Ambrose, 2012). We explored the nature of species interactions with three experimental treatments: 1) native and invasive species mixed in ambient densities, 2) invasive species alone, and 3) native species alone (Figure 2.3 a). This mesocosm experiment was conducted for 6 weeks from February 1 to March 21, 2017 at the University of Virgin Islands (USVI), St. Thomas, Caribbean.

To create the three treatments, we constructed outdoor mesocosms consisting of intact cores (sediment with seagrass and epiphytes) from a natural, mixed community of *H. stipulacea* and *S. filiforme.* Individual experimental mesocosms were 24-L cylinders that were 52 cm in height and 24 cm in diameter and lined with clear 46 cm x 61 cm (Width x Height) flat

rectangular polyethylene bags (Figure 2.1 d-e). Intact cores were collected by SCUBA using clear polycarbonate cores 30 cm in height and 7.2 cm in diameter (Figure 2.1 c). Two intact cores were placed into each experimental mesocosm. To ensure roots were covered and there was sediment into which they could expand, we filled the spaces between and around the cores in each experimental mesocosm with sand from the collection bed until the sediment depth was the same as the cores (~15 cm in depth). We counted initial shoots in each experimental unit, but did not measure the initial aboveground and belowground biomass nor internode length within the experimental units of the treatments as these methods are disturbing or destructive. Instead, we randomly collected an additional five cores to estimate initial above- and belowground biomass and internode length. We cleaned sediment and debris from the seagrasses in these cores, then separated into aboveground (blades and shoots) and belowground (roots and rhizomes) portions, spun for one minute in a salad spinner to remove excess water and wet weighed (g). We measured internode length between each shoot with a ruler and took the average for each mesocosm (cm). We did not count rhizome apical meristems in these initial samples though including sufficient numbers can be a concern for *S. filiforme* (Schwarzschild & Zieman, 2008). However, another study showed that 10 cm diameter cores contained enough rhizome apical meristems of *H. stipulacea* to grow as transplants (Willette and Ambrose 2012).

To establish our treatments, we left 5 experimental mesocosms as mixed communities, removed *S. filiforme* from 5 experimental mesocosms, and removed *H. stipulacea* from the remaining 5 experimental mesocosms. To control for disturbance effects, we disturbed sediment by hand digging to the rhizome level in mixed treatments to mimick the disturbance caused from the removal of seagrass. We randomly assigned mesocosms to different positions in a large flow-through tank (2 m x 0.6 m x 1 m) that acted as a water bath to keep a constant temperature

among replicates. We filled each mesocosm to the top with unfiltered seawater and used an overflow system to replace water. Each mesocosm had an independent water source with a constant flow of seawater; there were approximately 16 turnovers/per day. Positions of the mesocosms within the large tank were re-randomized every 7 days to account for any spatial variation within the large tank. To minimize the impacts of other species interactions (e.g. herbivores) we observed mesocosms every other day and removed invertebrates as needed. After six weeks, we removed mesocosms from the tank, counted the live shoots in each mesocosm, and calculated the net change in shoot density for each species present. We then measured the final aboveground and belowground wet biomass and internode length as described above.

To contextualize our experimental conditions, we measured water temperature and light in the donor seagrass beds and mesocosms. Light and temperature in the field were measured with a pendant HOBO Temperature/Light 64K logger (Model UA-002-64; Microdaq, USA) placed on the benthos within the donor seagrass canopy at 2 - 4 m depth. In the field, readings were taken every 15 min for 24 hr the day before and three days after the experiment. During the experiment, a logger was placed on the sediment of one experimental unit and both temperature and light measurements were recorded every 15 minutes for 41 days (Feb 8 – March 21); mesocosms and loggers were cleaned of epiphytes at least every other day. The logger remained in the same experimental unit with weekly re-randomization in the location of this unit within the water table. Following Apostolaki et al. (2014), daytime light levels were calculated by averaging light intensity measures from one hour after sunrise until one hour before sunset in both the field and mesocosms.

Limassol, Cyprus, Mediterranean experiment

To examine the effects of species interactions on the growth of native and invasive seagrasses in the historic invaded range, we conducted a similar mesocosm experiment in Cyprus, in the Mediterranean as described above (including re-randomization every 7 days), with six key differences. First, we chose the native seagrass, *Cymodocea nodosa* (Figure 2.2 b), a common seagrass in the Mediterranean (Pérez & Romero, 1992). Second, we added another treatment to test for density effects (Figure 2.3 b), where native and invasive species were grown mixed, but we reduced densities by one-half. Third, due to differences in facilities, the mesocosm, tank and core sizes were different. Mesocosms (n=40) were smaller 17-L cylinders (compared to the 24-L used in the Caribbean) that were 52 cm in height and 20 cm in diameter lined with clear 31cm x 20cm x 61 cm (Width x Depth x Height) three-dimensional (rather than flat as in the Caribbean) polyethylene bags (Figure 2.2 d-e). We randomly assigned mesocosms to a flow-through circular tank (0.8 m x 3 m diameter). Cores tubes were slightly larger, at 30 cm in height and 9 cm in diameter, made from clear polycarbonate (Figure 2.2 c). Thus, we placed only one intact core (sediment with seagrass and epiphytes) in each mesocosm (unlike in the Caribbean, where we placed two cores in each mesocosm). Fourth, we increased the number of experimental replicates to 10. Fifth, initial shoot densities and biomasses were lower than in the Caribbean due to differences in ambient densities (see results). And sixth, temperature and light measurements in the mesocosms were recorded every 3 minutes for 10 days during the experiment using HOBO loggers, and data were processed as above. However, we were unable to record field measurements of these parameters.

This mesocosm experiment was maintained daily (as above) and ran for six weeks from October 15 to December 3, 2018 in Larnaka, Cyprus, at the Cyprus Marine Aquaculture Center

of the Department of Fisheries and Marine Research, Ministry of Agriculture, Rural Development and Environment in Meneou, Cyprus.

Response variables and statistical analysis

Change in shoot density was calculated as the difference in number of shoots over the 6-week experiment; shoots were counted both initially (I) and finally (F) and change calculated as F-I so increases were positive values. Because initial above and belowground biomass as well as internode length were taken from cores not used in the experiment, we did not calculate change per mesocosm. Thus, the response variables used for these metrics were final measures, with initial values indicated on each graph for visual comparison.

We used R stats package to test data for assumptions of normality using the Shapiro Wilk Test "shapiro.test" and for homoscedasticity using Bartlett's Test "bartlett.test" and the "leveneTest" in car package (Fox & Weisberg, 2018) . In the Caribbean, final belowground biomass met assumptions untransformed, while changes in shoot density were squared and final aboveground biomasses were square root transformed; however, even after transformations, internode length violated assumptions of normality for ANOVA models. In the Mediterranean, changes in shoots and final aboveground biomass data were square-root transformed and belowground biomasses were log transformed to increase conformance to normality. Similar to the Caribbean, internode length failed to meet assumptions of normality.

Change in shoots, final aboveground biomass, and final belowground biomass were analyzed using two-factor ANOVAs with two categorical predictor variables. In the Caribbean predictor variables were species (native vs invasive), species treatment (alone vs mix), and their interaction, using the "aov" stats package in R version 3.6.1 (R Core Team 2020). For the

Mediterranean, the analyses were the same, but the species treatment had three levels (alone, mixed, and mixed reduced density). We used a Linear Mixed-Effects model for final internode length using "lme" function from the "nlme" stats package (Pinheiro et al., 2019) in R v 3.1-152 as this analysis is robust to violation of assumptions of normality (Schielzeth et al., 2020). We used Tukey's multiple comparison test to determine which treatments were significantly different from each other using the package and function "lsmeans" in R (Lenth 2016). Tukey's *post hoc* values less than 0.05 were considered to be significantly different between treatments.

2.4. RESULTS:

St. Thomas, USVI, Caribbean experiment

Initial seagrass shoot densities per mesocosm averaged 28.8 ± 0.2 SE shoots for the native *S. filiforme* and 25.6 ± 0.5 SE shoots for the invasive *H. stipulacea.* Initial biomass and internode lengths are indicated with dashed horizontal lines in Figure 2.4. Initial aboveground biomass for the invasive averaged 1.2 ± 0.3 SE g and belowground was 2.1 ± 0.5 SE g. The initial aboveground biomass for the native was 2.3 ± 0.6 SE g and belowground biomass was 6.1 ± 1.8 SE g. Initial internode length of the native *S. filiforme* was 2.4 ± 0.2 SE cm and of the invasive *H. stipulacea* was 0.9 ± 0.1 SE cm.

The invasive seagrass increased shoot density over the 6-week experiment when grown in a mixed community but lost shoots when grown alone (Figure. 2.4 a). This pattern reversed for the native seagrass, with positive growth occurring only when it was alone, resulting in a significant statistical interaction (Table 1a; ANOVA $p < 0.01$). The average increase in number of *H. stipulacea* shoots was 5.6 ± 1.0 SE when grown with the native, an increase of 22.0% ± 4.3 SE. However, when grown alone there was overall loss in the number of shoots, with an average change of -0.8 ± 1.6 SE shoots (-3.9% ± 5.8 SE); these means were significantly different

(Tukey's *post hoc* p = 0.02). In contrast, when the native species, *S. filiforme*, was grown alone, it increased by 3.6 ± 0.5 SE from initial values (12.5% ± 1.7 SE). When mixed with *H. stipulacea*, *S. filiforme* lost shoots, with an average loss of 3.0 ± 3.0 SE (average number of lost shoots: -10.2% ± 10.3 SE); however, the Tukey's *post hoc* test did not detect a significant difference between alone and mixed treatments for *S. filiforme* (p=0.10), likely due to high variability in the mixed community (Figure 4.4 a).

There was a significant difference in final aboveground biomass between the two species of seagrass (Table 1b), with aboveground biomass of the native species (averaged across all treatments where it occurred) almost double that of the invasive (Figure 4.4 b; ANOVA p < 0.01). In contrast, there was no effect of being grown mixed or alone or an interaction. Patterns for aboveground biomass were similar to that for shoots, with the invasive seagrass *H. stipulacea* having higher final aboveground biomass (2.0 g ± 0.8 SE) when grown in a mixed community and lower when grown alone (1.3 g ± 0.3 SE), while the native *S. filiforme* performed best when grown alone (4.4 g ± 0.7 SE). However, these results did not produce in a significant interaction (Table 1b; ANOVA p = 0.13). However, the Tukey's *post hoc* test revealed that the significant species effect was driven by the large amount of aboveground biomass when *S. filiforme* was grown alone (Figure 4.4 b; Tukey's *post hoc* p = 0.02).

The invasive seagrass also had more final belowground biomass when it grew in a mixed community than when growing alone and this pattern was the opposite for the native (Figure 4.4 c), resulting in a significant interaction (Table 1c; ANOVA p = 0.02). When the invasive grew mixed with the native, the belowground biomass (2.6 g ± 0.7 SE) was nominally higher than when the invasive grew alone (1.5 g ± 0.3 SE); however, this difference was not significant (Tukey's *post hoc* p = 0.60). Similarly, when the native grew mixed with the invasive species, its

average belowground biomass was 5.6 g ± 0.5 SE, less than when it was growing alone (7.9 g ± 0.9 SE), but this difference was also not significant (Tukey's *post hoc* p = 0.08).

There was a significant difference in length of the rhizomal internodes (i.e. length of the rhizome between individual shoots) with average internode length of the native species being 3-fold longer than the invasive (Fig. 4d; Table 1d; LME $p < 0.01$). In contrast, there was no effect of being grown mixed or alone, nor was there an interaction between factors. Mean internode length across both treatments containing the invasive was 0.7 ± 0.1 SE and the native was 2.3 ± 0.1 SE.

Water temperature in the field ranged between 22-26 ºC; average light in the field from Jan to March was 428 ± 67 Lux with maximum intensity of 6200 Lux. Temperatures recorded in our mesocosm ranged between from 24-28 ºC with an average light intensity of 1177 ± 37 Lux (8.13 ± 1.27 μmoles/m^2/s), with the highest intensity of 8266 Lux (117.8 μmoles/m^2/s; see Thimijan & Heins, 1983 for conversion formula). Although light intensity was higher in our mesocosm than in the field, average light intensities of shallow tropical seagrass beds reported in another Caribbean study were also much higher, ranging from 1566 to 7577 Lux (29.75 to 143.96 μmoles/m^2/s) (Kantún-Manzano et al., 2018).

Limassol, Cyprus, Mediterranean experiment

Initial seagrass shoot density in full density treatments (mixed and alone) averaged 5.9 ± 0.1 SE shoots for the invasive *H. stipulacea* and 3.0 ± 0.2 SE shoots for the native *C. nodosa*. Initial shoot densities in reduced density treatment averaged 2.9 ± 0.1 for the invasive and 1.6 ± 0.2 for the native. Initial biomasses and internode lengths are indicated with dashed horizontal lines in Figure 2.5 b-d). The initial aboveground biomass for the invasive *H. stipulacea* averaged

0.47 g ± 0.1 and belowground was 1.2 g ± 0.2, while the native *C. nodosa* aboveground initial biomass was 0.43 g ± 0.1 SE and the belowground biomass was 2.5 g ± 0.6 SE. Reduced density treatments were assumed to be half of the aboveground and belowground biomass as full density treatments (indicated with dotted horizontal lines). Initial internode length of the invasive *H. stipulacea* was 1.2 cm ± 0.2 SE and the native *C. nodosa* was 3.6 cm ± 1.0 SE.

Similar to the response in the Caribbean, when grown in mixed communities the invasive *H. stipulacea* increased shoot density over the 6-week experiment but did not change shoot density substantially when grown alone (Figure 2.5 a). This pattern is reversed for the native seagrass, *C. nodosa,* with growth occurring only when it was alone, resulting in a significant interaction (Table 2 a; ANOVA $p < 0.01$). The average increase in number of *H. stipulacea* shoots was 3.7 ± 1.3 SE when grown with the native, an increase of 59.2% ± 23.8 SE growth. Reducing density in this mixed community enhanced growth of the invasive species by 4.3 ± 1.5 SE shoots, an increase of 150.0% ± 50.7 SE (initial shoot density in this half density treatment was 2.9 ± 0.1). When grown in isolation, the invasive seagrass *H. stipulacea* experienced marginal growth of 0.5 ± 0.9 SE shoots (+8.3% ± 16.5 SE) while the native species, *C. nodosa,* increased by 1.7 ± 0.4 SE shoots from initial values (or 56.7% ± 12.2 SE growth). When in ambient density and mixed with *H. stipulacea*, *C. nodosa* lost shoots, with an average loss of -1 ± 0.4 SE shoots (-29.2% ± 13.7 SE reduction). However, when density was reduced in the mixed community, the native increased slightly, with an average change of 0.2 ± 0.1 SE shoots (+15.0% ± 10.7 SE; initial shoot density in this half density treatment was 1.6 ± 0.2). Tukey post-hoc tests revealed that the loss of shoots in the native species in the treatment with full density and mixed with the invasive was driving the interaction found in the ANOVA (Table 2.2 a, Figure 2.5 a; Tukey's *post hoc* $p < 0.01$ Cn mixed vs Hs reduced; $p = 0.02$ Cn mixed vs Hs full).

When the invasive seagrass grew in mixed, ambient-density treatments there was a pattern of more final aboveground biomass than when it was grown alone or in reduced mixed communities (Figure 2.5 b); in contrast the native grew best when alone, resulting in a significant interaction between the two factors (Table 2.2 b; ANOVA $p < 0.01$). When the invasive *H. stipulacea* was grown in ambient density with the native species, final average aboveground biomass was 0.46 g (± 0.08 SE), which was very similar to initial biomass (0.47 g ± 0.06 SE). When the invasive grew in reduced mixed treatments, final aboveground biomass was 0.37 g ± 0.08 SE, similar to when it was grown alone (final 0.38 g ± 0.06), despite being reduced by half six weeks earlier (estimated initial 0.24 g ± 0.03 SE), suggesting considerable regrowth during the experiment (Figure 2.5 b; compare to horizontal dashed and dotted lines). None of the final biomass values for the invasive species differed from each other (Figure 2.5 b). In comparison, when the native seagrass (*C. nodosa*) grew mixed with the invasive species in ambient density its average aboveground biomass (0.18 g ± .03 SE) decreased compared to initial values (0.43 g ± 0.11 SE), and was significantly lower than when it grew alone (average of 0.55 g ± .06 SE) (Tukey's *post hoc* $p=0.01$). When the native seagrass grew in a reduced density mixed treatment, its aboveground biomass was not different than when grown in ambient density mixed treatment (Tukey's *post hoc* $p=0.99$), despite the experimental reduction that we initially imposed (final 0.22 g ± .04 SE). It was also significantly lower than when it was grown alone (Tukey's *post hoc* $p < 0.01$).

The invasive *H. stipulacea* had more final belowground biomass when grown in either ambient or reduced mixed communities than when grown alone, and this pattern was opposite for the native species (Figure 2.5 c), resulting in a significant interaction (Table 2.2 c; ANOVA $p < 0.01$). Comparing final belowground biomass in reduced and ambient-density mixed

treatments suggested considerable regrowth after initial reductions. When the invasive *H. stipulacea* was grown in ambient densities mixed with the native species, the invasive had a nominally higher average final belowground biomass of 2.20 g (± 0.39 SE) compared to when it grew in reduced density (final 1.79 g ± 0.26) and when it grew alone (final 1.32 g ± 0.23 SE), but this difference was not statistically significant. Average belowground biomass of *H. stipulacea* was higher than initial values in both mixed ambient and reduced density treatments, but not in the alone treatment (Figure 2.5 c; compare final bars to horizontal dashed and dotted lines). When the native seagrass *C. nodosa* grew with the invasive in mixed, ambient density communities, final belowground biomass was 1.92 g ± 0.35 SE. while it was 3.15 g ± 0.7 SE when it grew alone, though these differences were not statistically significant (Tukey's *post hoc* $p = 0.37$).

There was a significant difference in internode length between the two species of seagrass; internode length of the native species was more than 2-fold longer than the invasive (Table 2.2 d, Figure 2.5 d). There was no effect of being grown in a mixed treatment or alone, nor was there an interaction between factors, nor did post hoc tests detect differences within species across treatments (LME $p = 0.33$). Average internode length across all replicates of *H. stipulacea* was 1.0 cm ± 0.1 SE, n= 29) and of *C. nodosa* was 2.8 cm ± 0.2 SE, n=21).

In the experimental outdoor mesocosms, temperature ranged from 23-25 ºC and average light intensity was 417 ± 7 Lux (7.92 ± 0.13 µmoles/m^2/s) with maximum intensity of 2670 Lux (50.73 µmoles/m2/s). While we did not measure light and temperature in the field, Nguyen et al. (2020) measured temperature at the same site and during the same months (Oct-Dec) in Akrotiri Bay and found it ranged from 21-26 º C. In addition, these mesocosm values were similar to our field measurements in St. Thomas, Caribbean, although maximum temperature was lower.

Further, other studies found average light intensity of seagrass meadows in Balearic Islands, Mediterranean ranged between 5442 to 6711 Lux (103.4 to 127.51 μmoles/m2/s) at 5 -12 m (Hendriks et al., 2014), albeit these islands are more than 2700 km from Cyprus.

2.5. DISCUSSION

Controlled mesocosm experiments in both the Caribbean and Mediterranean demonstrate higher growth of *H. stipulacea* in the presence of native seagrasses and lower growth alone, suggesting that native species may facilitate the success of invasive *H. stipulacea* populations. While positive effects of native on invasive species have been documented in terrestrial and other marine systems (Cavieres, 2021; McIntire & Fajardo, 2014), ours is the first study to document positive effects for invasive seagrasses.

At the same time, native seagrasses performed better alone than in the presence of *H. stipulacea,* indicating strong negative effects of invasive *H. stipulacea* on native seagrass populations. That this pattern occurred with different native species and in two invaded seas at opposite ends of the world implies these coupled facilitative for the invasive and antagonistic for the native relationships may be an important aspect of the invasion ecology of *H. stipulacea,* making it a driver of its own invasion success. Willette & Ambrose (2012) demonstrated the ability of *H. stipulacea* to expand when transplanted into a native *S. filiforme* bed, but this work was only in the Caribbean and, as a field experiment, was unable to identify mechanisms of success. Knowing that *H. stipulacea* can be a driver of its invasion success is important because it indicates that it does not require disturbance and recently opened space to succeed, but instead can invade and thrive in intact native communities. These findings are critical for managers to understand and predict the future spread of this very successful invasive species worldwide, as human impacts increase invasions in the Anthropocene (Ricciardi, 2007).

2.5.1 Native species as facilitators of invasion success at their own cost

Results demonstrate that native seagrasses enhance growth of *Halophila stipulacea* when grown in mixed communities. Similar examples of native species facilitating invasive species are well-documented in terrestrial and other marine systems (e.g., Bronstein, 2009; Flory & Clay, 2010; Sueiro et al., 2013), but not for invasive seagrasses. Importantly, this facilitation occurred consistently across both the historic (the Mediterranean Sea) and the much more recent (the Caribbean Sea) invasive habitats. The implication of these results is that the positive effects of multiple native seagrass species can contribute to *H. stipulacea*'s invasion success (Cavieres, 2021; McIntire & Fajardo, 2014), potentially explaining the widespread success of this invasive species.

One possible mechanism underlying negative impacts on native species is that *H. stipulacea* carries microbial or fungal associations (Gribben et al., 2017) that release compounds that negatively affect the native species (Orr et al., 2005); this negative effect may release resources for use by the invasive. While it is well-established that seagrasses are generally devoid of mycorrhizae symbionts (Nielsen et al., 1999), arbuscular mycorrhizal fungal associates have recently been found in mature *Posidonia oceanica* in the Mediterranean (Borovec & Vohník, 2018; Vohník et al., 2019). It is also possible that *H. stipulacea* can form positive associations with novel epibionts that enhance nutrient or vitamin acquisition to the cost of the native, as seen in the invasive seaweed *Gracilaria vermiculophylla* (Saha et al., 2016). Alternatively, positive associations that favor invasive species may act by enhancing nutrient acquisition (Tharayil et al., 2009) or decreasing positive microbial associations with other plants (Mummey & Rillig, 2006). Further the release of allelopathic chemicals, known as 'novel

weapons' (Callaway & Ridenour, 2004), is an invasive strategy that results in negative effects on native species of terrestrial grasses (Greer et al., 2014). It is also possible that these negative effects release limited resources for natives. All of these possible mechanisms have yet to be explored for this invasive seagrass and deserve further attention.

Another possible mechanism underlying the positive effects of the native on the invasive seagrass is that *H. stipulacea* may be able to take advantage of recycled nutrients, deriving a nutritional benefit from the dying native seagrasses. Support for this explanation comes from Di Genio et al. (2021, which demonstrated *H. stipulacea* can grow on dead matte (a bioconstruction comprised of large quantities of buried organic debris) of the native seagrass *Posidonia oceanica*, also suggesting decomposition as a potential source of limiting nutrients (Gambi & Barbieri, 2009). Further evidence for the role of nutrients in facilitation comes from the short internodes of *H. stipulacea* compared to native species. Short internodes suggest a "phalanx strategy" (sensu Sutherland & Stillman, 1988), which can effectively enable the invasive to take advantage of a localized nutrient benefit produced by dying or dead native plants. Moreover, *Halophila*-dominated beds are nutrient limited, but native *Thalassia testudinum* dominated beds are not (Muthukrishnan et al., 2020), suggesting that *H. stipulacea* more efficiently sequesters nutrients.

Despite similar results in both invasive ranges, it is possible that the positive effect of the native on the invasive was an artifact of the disturbance due to the experimental removal of the native. While we tried to disturb all mesocosms equally to reduce the likelihood of this artifact, we cannot know this was efficacious. However, we argue that this explanation is unlikely as *H. stipulacea* grew vigorously in the reduced density treatment, which was also created by removing seagrass, suggesting that disturbance alone did not produce negative effects. Further,

H. stipulacea has many traits of opportunists that thrive on disturbance (Willette et al. 2020), suggesting that ecological interactions with native seagrass, not experimental artifacts facilitated growth of invasive *H. stipulacea*.

2.5.2 *H. stipulacea* as a driver of its own invasion success

Our results demonstrate that *H. stipulacea* can be a driver of its own success, at least in the relatively low-density seagrass beds in both the Mediterranean and Caribbean that we studied. Our results contrast previous observational studies of active invasion fronts in the Caribbean that indicate that *H. stipulacea* is a passenger, opportunistically establishing and expanding into open habitat or areas of recent disturbance. For example, field surveys in Bonaire found that *H. stipulacea* only expand into seagrass-dominated sites only after first settling in deeper areas devoid of seagrass (Debrot et al., 2012; Smulders et al., 2017), or where natural and anthropogenic disturbances created open space (Smulders et al., 2017). Similarly, data in Dominica and St. John found expansion of *H. stipulacea* in locations that were devoid of native seagrasses (Willette et al., 2014). Combined, these studies support the hypothesis that *H. stipulacea* expansion is mediated by the presence of open spaces or disturbances such as turtle grazing, storms, damage by propellers or anchors, eutrophication and bioturbators (Steiner & Willette, 2015; van Tussenbroek et al., 2016; Willette et al., 2020).

However, results from our studies clearly demonstrate that *H. stipulacea* can also be a driver of its own success in both invasive ranges. Previous studies (e.g. Winters et al. 2020) commonly report *H. stipulacea* existing in mixed species seagrass communities, consistent with our finding that *H. stipulacea* can be successful in intact native communities. Once present, it can negatively impact native seagrasses, such as in Tunisia where expansion of *H. stipulacea*

correlates with a dramatic decrease in shoot density of the native seagrass, *Cymodocea nodosa*, even in the absence disturbance (Sghaier et al. 2014), supporting the driver hypothesis. Willette and Ambrose (2012) also provide evidence that Caribbean *H. stipulacea* can expand into existing seagrass beds. These field studies combined with the results of our mesocosm experiments provide clear and strong support that *H. stipulacea* can be a driver of its own success in both regions it invaded.

Whether *H. stipulacea* acts as a driver of its own success, is a passenger of community change, or both, may depend on the ecological or environmental context (Muthukrishnan et al., 2020). For example, in dense and healthy native seagrass beds, *H. stipulacea* may play the role of a passenger, invading only after disturbances such as hurricanes (Hernández-Delgado et al., 2020) or intensive grazing by a megaherbivore (Christianen et al., 2019) reduces or removes the native species. However, shoot densities in the seagrass beds used in this study had densities on the lower end of reported ranges. Thus, our results imply that in relatively sparse native communities, *H. stipulacea* can be a driver, invading and replacing native species, even without disturbance.

While invasive species that are drivers are generally superior competitors (Valley & Newman 1998, Fleming & Dibble 2015), we did not find evidence of interspecific competition. Rather, our results in the Mediterranean showed strong density-dependent effects where reductions in density caused rapid regrowth across all metrics for both species. This result suggests strong intraspecific competition, in contrast to other studies showing strong negative effects of interspecific competition among seagrasses (Bando, 2006; Davis & Fourqurean, 2001).

The potential dual nature of *H. stipulacea,* as a driver that is also facilitated by native species, is important because recent models of the Mediterranean Sea predict that this invasive

species will keep expanding as climate change produces more tropical conditions (Beca-Carretero et al., 2019; Nguyen et al., 2020b). However, the results of our study suggest that predictions of future invasiveness may also need to consider the density of the native seagrass in the "receiving" habitat, which also may be affected by climate change. Based on our findings, we predict that *H. stipulacea* will spread more vigorously in habitats that may be marginal for other seagrasses, without the need for disturbance that removes native seagrass species.

2.5.3 Concluding statement

Our results demonstrate *H. stipulacea* may not require disturbance or community change to invade, but can be a driver of its own success in low density native seagrass communities. Worldwide, native seagrasses are declining (Orth et al. 2006) due to multiple stressors in the Anthropocene (i.e. climate change, coastal development, and degraded water quality) (Waycott et al. 2009). These declines, and associated low-density states, may make native seagrass habitats more invadable, providing one explanation of the broadening invasion success of *H, stipulacea*. As such, the sustainability of native seagrass beds and the ecological goods and services they provide may hinge on maintaining dense, healthy seagrass beds capable of fending off invasives.

2.6. ACKNOWLEDGEMENTS:

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2.7. FIGURES:

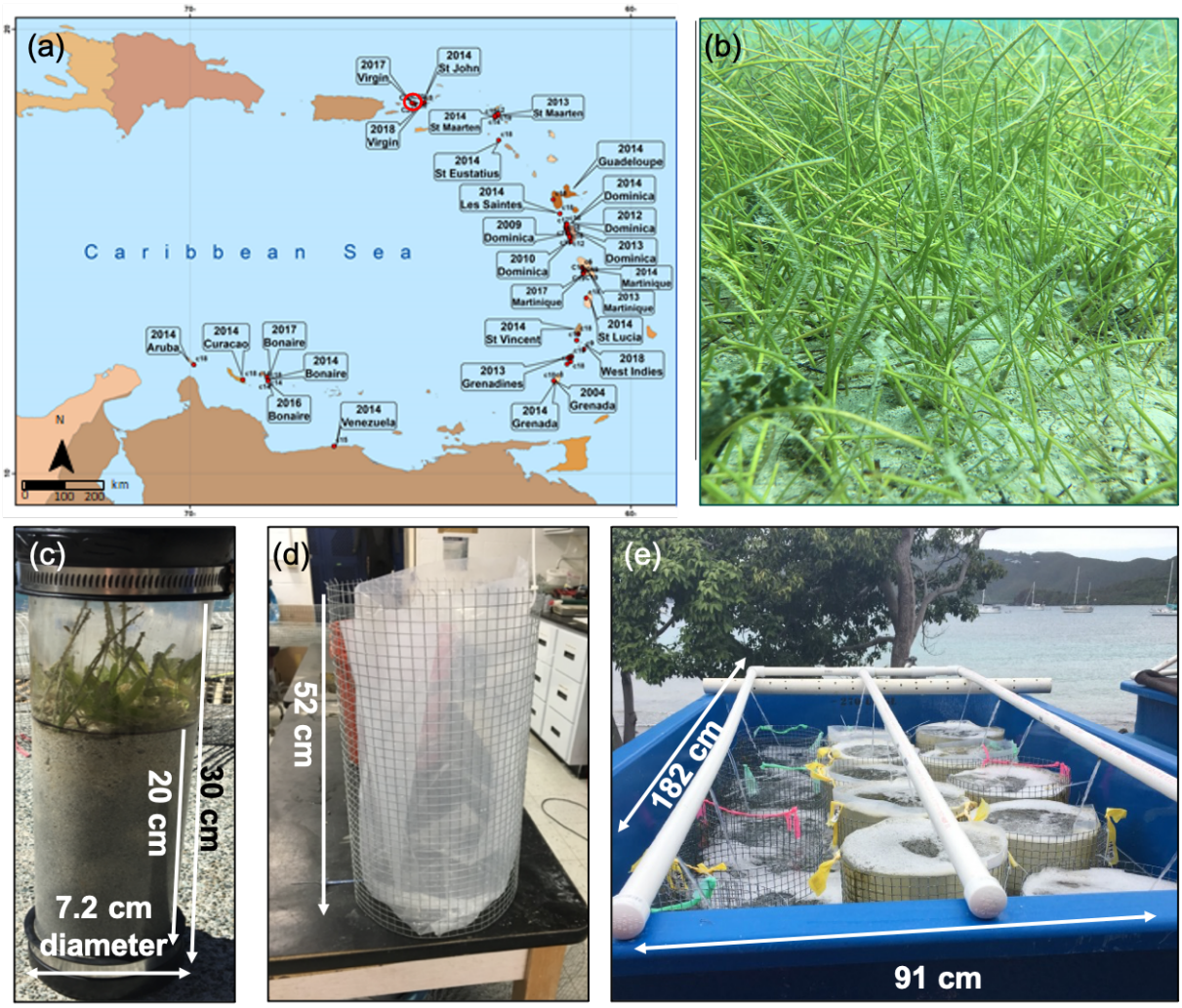


FIGURE 2.1 Experimental setup in Caribbean (a) Geographic distribution of published studies on *Halophila stipulacea* (red colored dots) in the Caribbean Sea labeled by year of the first report in each location (adapted from Winters et al. 2020). The red circle encloses our site in Brewers Bay, St. Thomas, U.S. Virgin Islands), (b) Native Caribbean seagrass *Syringodium filiforme* growing in a single species meadow, and (c) experimental core of mixed with the invasive *H. stipulacea*. (d) Cores from these mixed meadows were inserted into 24 L mesocosms for our experiment. (e) Mesocosms were set into flow-through water baths for the six weeks duration of the experiment.

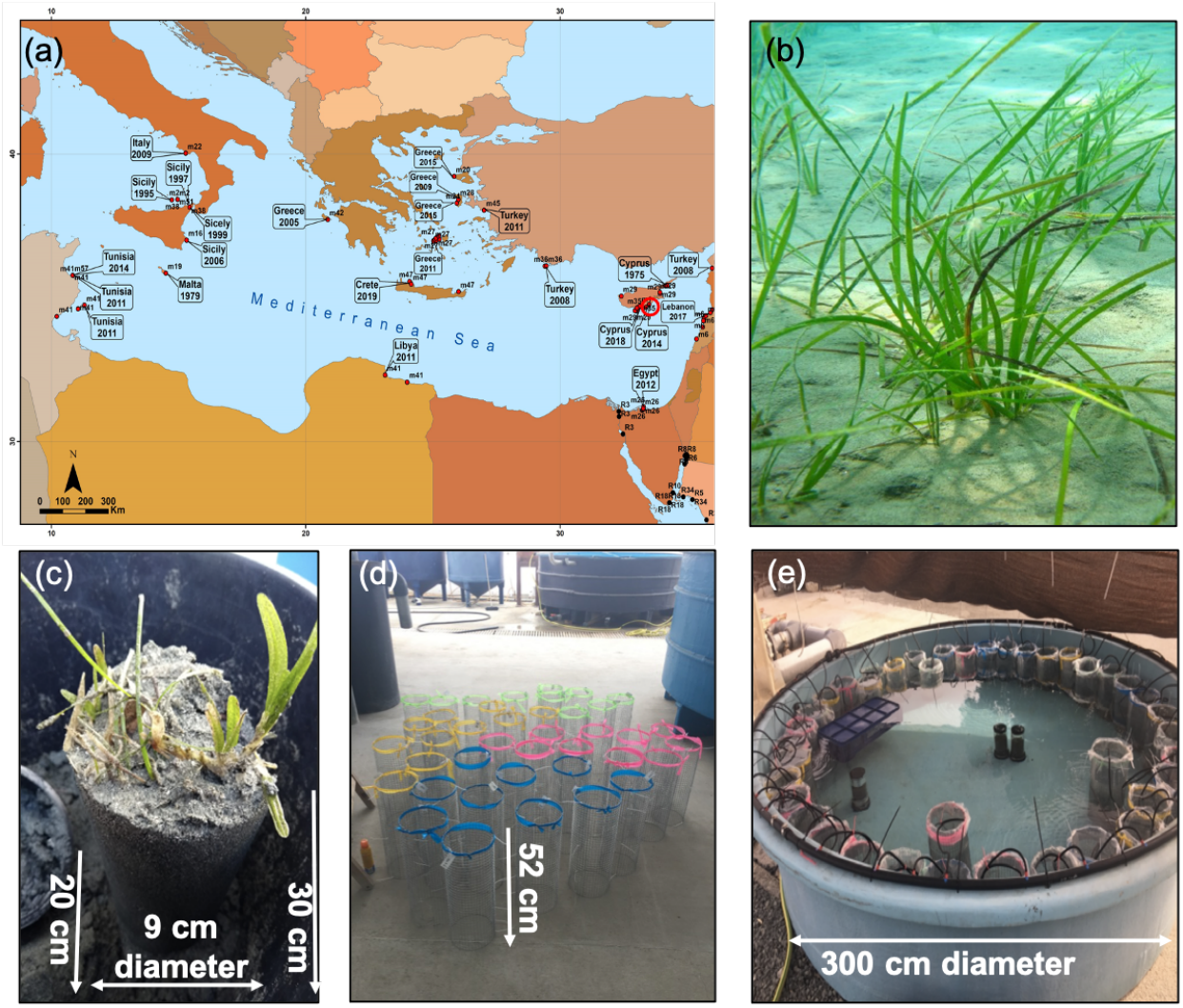
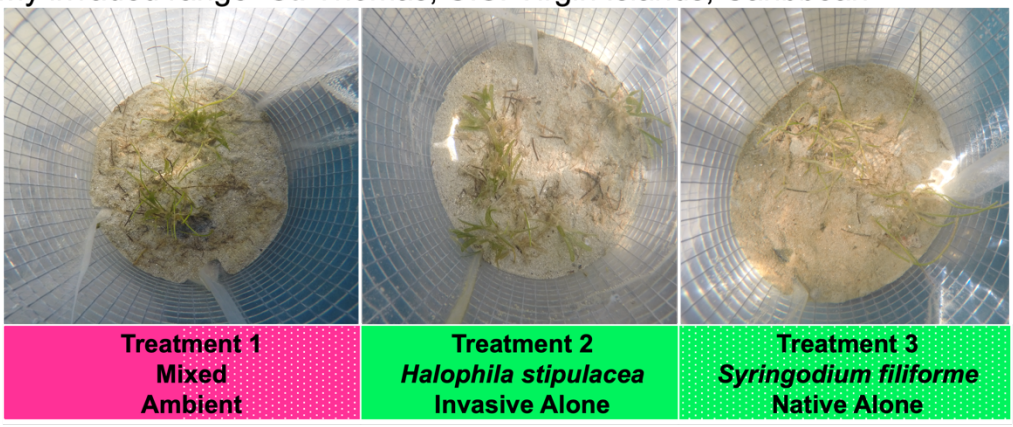


FIGURE 2.2 Experimental setup in Mediterranean (a) Geographic distribution of published studies on *Halophila stipulacea* (red colored dots) in the Mediterranean Sea labeled by year of the first report in each location (adapted from Winters et al. 2020). The red circle encloses the country of the site in Akrotiri Bay at Dream Café, Limassol, Cyprus. (b) Native Mediterranean seagrass *Cymodocea nodosa* growing in a single species meadow, (c) mixed with the invasive *H. stipulacea*. (d) Cores from these mixed meadows were 20 cm into the sediment deep and inserted into 17 L mesocosms. (e) Mesocosms were set into flow through water baths for the six weeks duration of the experiment.

(a) Newly invaded range- St. Thomas, U.S. Virgin Islands, Caribbean



(b) Historically invaded range- Limassol, Cyprus, Mediterranean

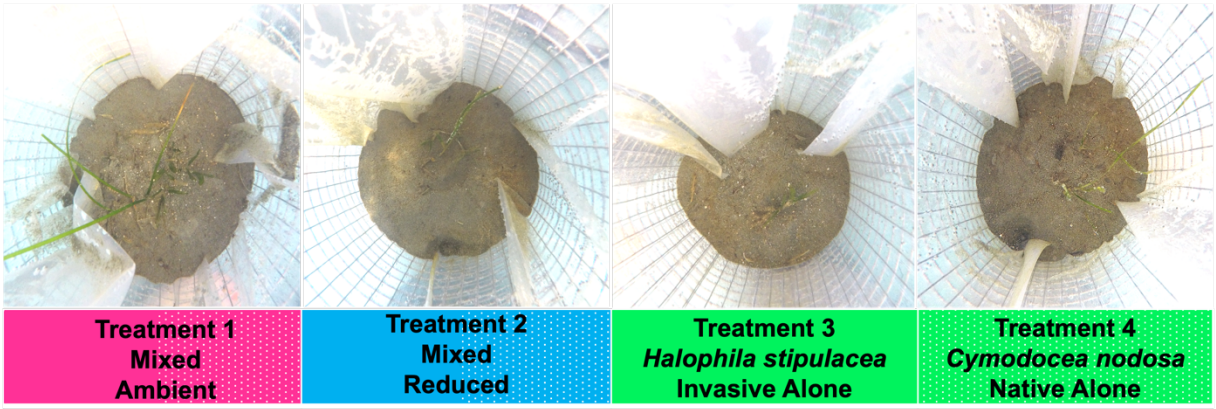


FIGURE 2.3 (a) Top view of experiment design in the newly invaded range- Caribbean. Treatment 1 Ambient represents mixed conditions where both species are present. Treatment 2, Hs, is the invasive *H. stipulacea* alone. Treatment 3, Sf, is native *S. filiforme* alone. Each of the three treatments has 5 replicates; N= 15. (b) Top view of experimental design in the Historical invaded Range- Mediterranean. Treatment 1, ambient, represents mixed conditions where both species are present. Treatment 2, half density, is where the mixed treatment was reduced by 50%. Treatment 3, Hs, is the invasive *H. stipulacea* alone. Treatment 4, Cn, is native *C. nodosa* alone; N=40.

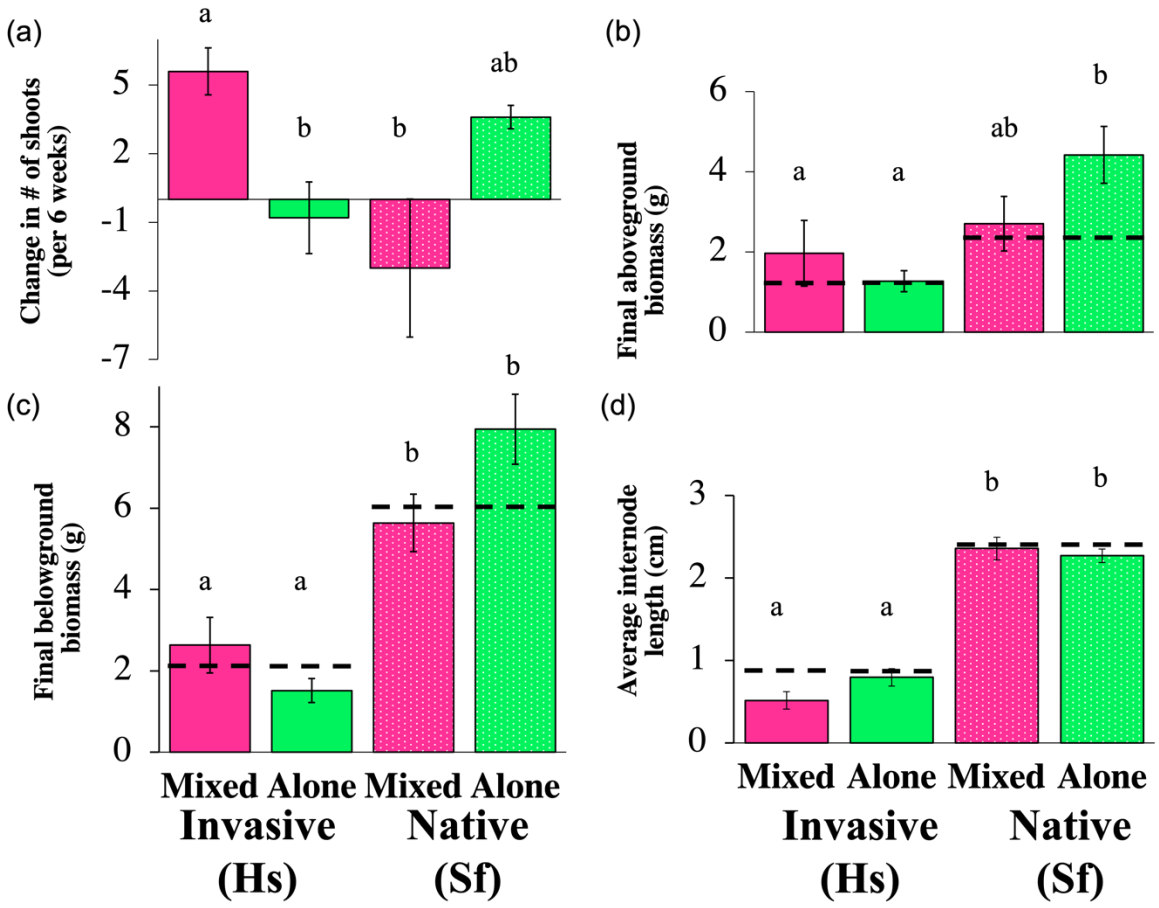


FIGURE 2.4 Results of the mesocosm experiment in the Caribbean for (a) the change in number of shoots after 6 weeks, (b) final aboveground biomass, and (c) final belowground biomass (d) average internode length. Pink represents the ambient, mixed treatment for both species. Green is Hs, the invasive *H. stipulacea* alone and stippled green is *Sf* for *S. filiforme* alone. Bars represent mean +/- Standard Error. Dashed lines represent estimates of initial biomass. Tukey's post hoc test reveal pairwise comparisons across treatments and different letters indicate significant differences ($P < 0.05$), while bars with the same letters are not significantly different from each other.

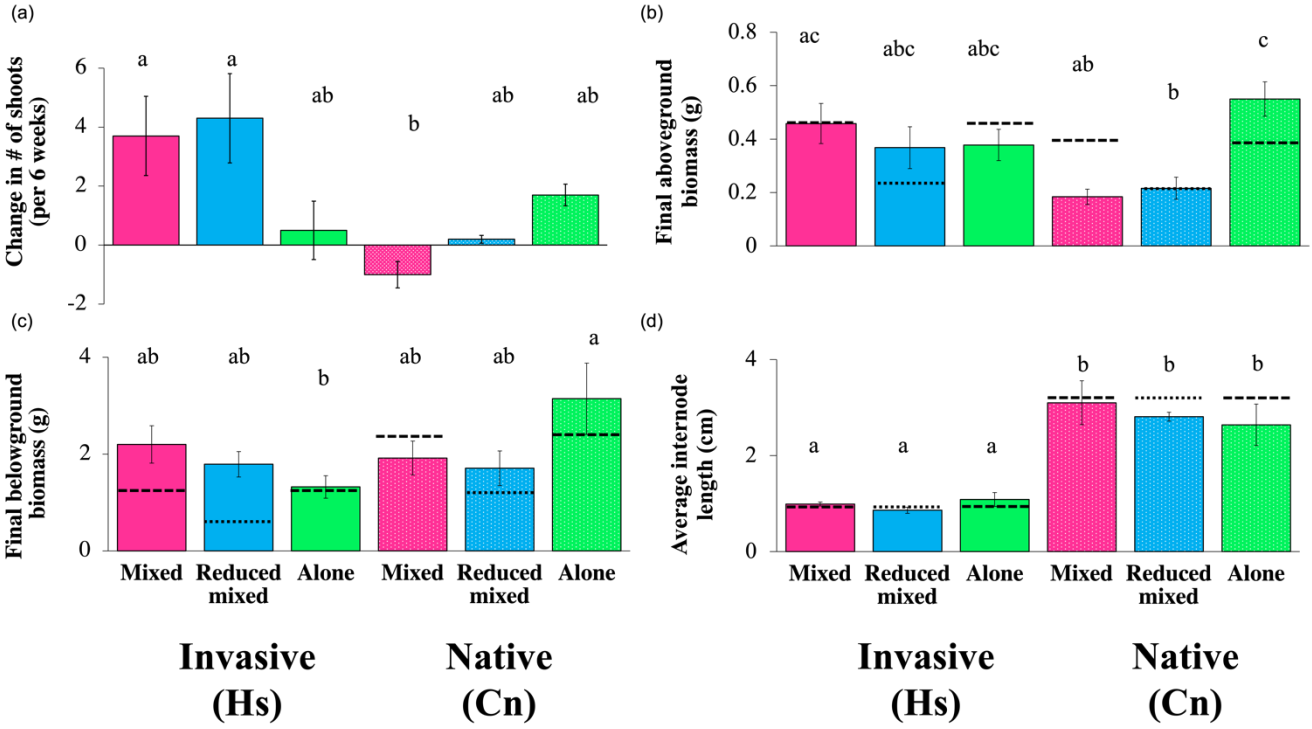


FIGURE 2.5 Results of the mesocosm experiment in the Mediterranean for (a) the percent change in number of shoots after 6 weeks, (b) final aboveground biomass, and (c) final belowground biomass in grams (d) average internode length. Pink represents the ambient, mixed treatment for both species. Blue represents reduced density for both species. Green is Hs for invasive *H. stipulacea* alone and stippled green is Cn for native *C. nodosa* alone. Bars represent mean +/- Standard Error. Dashed lines represent estimates of initial biomass and dotted lines are calculated values of the initial biomass assuming we removed exactly half.

2.8. TABLES:

Table 2.1 a-c. Statistical results of 2 factor ANOVAs for the Caribbean experiment. Table 2.1 d Statistical results from LME. P-values for significant interactions are in bold are significant.

(a) Response Variable: Difference in shoots after 6 weeks

Source of Variation	Df	SS	MS	F-value	P-value
Treatment	1	3302	3302	0.408	0.5320
Species	1	11472	11472	1.417	0.25118
Treatment x Species	1	137614	137614	17.004	**0.000796**
Residuals	16	129492	8093		

(b) Response Variable: Final aboveground biomass

Source of Variation	Df	SS	MS	F-value	P-value
Treatment	1	0.114	0.1145	0.529	0.47755
Species	1	2.059	2.0590	9.514	**0.00711**
Treatment x Species	1	0.537	0.5369	2.481	0.13481
Residuals	16	3.463	0.2164		

(c) Response Variable: Final belowground biomass

Source of Variation	Df	SS	MS	F-value	P-value
Treatment	1	1.76	1.70	0.897	0.3576
Species	1	111.16	111.1	56.716	**<0.0001**
Treatment x Species	1	14.64	14.6	7.469	**0.015**
Residuals	16	31.36	1.96		

(d) Response Variable: Final internode length

	Numerator df	Denominator df	F-value	P-value
Intercept	1	16	733.8279	<0.0001
Treatment	1	16	0.7616	0.3957
Species	1	16	229.333	**<0.0001**
Treatment x Species	1	16	2.8242	0.1123

Table 2.2 a-c. Statistical results of 2 factor ANOVAs for the Mediterranean experiment. Table 2.2 d. LME results. P-values in bold are significant

(a) Response: Difference in shoots after 6 weeks (Cn vs Hs Mediterranean)

Source of Variation	Df	SS	MS	F-value	P-value
Treatment	2	0.712	0.3560	0.879	0.4212
Species	1	2.768	2.7681	6.832	**0.0116**
Treatment x Species	2	4.916	2.4582	6.067	**0.0042**
Residuals	54	21.878	0.4051		

(b) Response: Final aboveground biomass (Cn vs Hs Mediterranean)

Source of Variation	Df	SS	MS	F-value	P-value
Treatment	2	0. 3174	0.15869	5.039	**0.00795**
Species	1	0.0757	0.075	2.525	0.11787
Treatment x Species	2	0.3194	0.25125	6.882	**0.00773**
Residuals	54	1.6187	0.02998		

(c) Response: Final belowground biomass

Source of Variation	Df	SS	MS	F-value	P-value
Treatment	2	0.062	0.0312	0.515	0.60049
Species	1	0.085	0.0850	1.403	0.24143
Treatment x Species	2	0.643	0.3214	5.305	**0.00788**
Residuals	54	3.271	0.0606		

(d) Response variable: Final internode length

	Numerator df	Denominator df	F-value	P-value
Intercept	1	43	97.94588	<.0001
Species	1	43	13.07934	**<.0001**
Treatment	2	43	1.67859	0.7697
Treatment x Species	2	43	1.15488	0.5478

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CHAPTER 3

Reconstructing the invasion history of the tropical seagrass *Halophila stipulacea* in the Caribbean Sea

3.1. ABSTRACT

Although seagrass declines are a major global concern, invasive seagrasses are a growing concern in certain marine ecosystems. Native to the Red Sea, Persian Gulf, and Indian Ocean, *Halophila stipulacea* first invaded the Mediterranean Sea in 1894, following the opening of the Suez Canal, where it coexists with native seagrass species. A second invasion into the Caribbean was first documented in 2002, and has quickly expanded across the Caribbean Sea displacing native Caribbean seagrass species, expanding into new habitats, and altering the Caribbean benthic landscape. Genetics may play key a role in determining vectors of transport, and whether an introduced species can become invasive. Here we examine multi-locus SNP data from *H. stipulacea* populations in both the native (Western Indian Ocean, Eastern Indian Ocean and Red Sea) and invasive (Mediterranean and Caribbean Seas) regions/habitats/locations/sites to test the hypotheses that the Caribbean introduction occurred via stepping stone colonization from the Mediterranean, an independent introduction from the native range, or an admixture from multiple native/invasive populations. Analysis of SNP datasets (524 loci or 45 loci) revealed significant genetic structure among all five regions, particularly among Eastern and Western Indian Ocean populations. Patterns of genetic structure confirm that invasive Mediterranean populations of *H. stipulacea* were most similar to native populations in the Red Sea, and invasive Caribbean Sea populations represent a secondary introduction from the Mediterranean. Despite introductions being, by definition, a subsample of source populations, both Mediterranean and Caribbean populations had high levels of genetic diversity, equal or greater to native populations,

suggesting introductions were either large, or a small number of genetically diverse individuals. Understanding the origins and the factors contributing to diversity of invasive populations provides insights into the success of this invasive species, and lays a foundation for future studies to advance our understanding of the success of invasive *H. stipulacea* in the Caribbean and Mediterranean.

3.2. INTRODUCTION

Invasive species can restructure natural habitats, change community composition, disrupt food webs, and degrade ecosystem services (Mollot et al., 2017). Globalization has accelerated the rate of species introductions, making the world more biologically homogenous (Ricciardi and Cohen, 2007). As a result, biological invasions have become one of the greatest threats to biodiversity, causing environmental change, agriculture, and human health (Park, 2004). Invasive species are also economically damaging, costing an estimated $120 billion dollars a year in the U.S. alone (Pimentel et al., 2005).

Species often invade by hitchhiking on human conveyances, stowing away in cargo, and taking advantage of human activities to get to distant new sites, however, not all introduced species become invasive. Some introduced species can be innocuous and integrate into the native environment, while other introduced species can become invasive and detrimental to the local environment (Simberloff, 2013). Non-native species can be invasive in one region, but not in others (Zenni and Nuñez, 2013; Gallagher et al., 2015). Factors such as number of introductions, number of source populations, and other factors that contribute to genetic diversity shaping invasion success (Stepien et al. 2005; Facon et al. 2008; Dlugosch et al. 2015). As such, an important aspect of understanding invasive species dynamics is reconstructing the potentially

complex history of their introduction into the invaded habitat (Sakai et al., 2001; Lee, 2002), often through the use of genetic data (Estoup and Guillemaud, 2010).

Previous studies reconstruct invasion history by comparing genetic variability and structure of invasive populations compared with those in the native range (Calderón et al., 2007; Pascual et al., 2007; Ciosi et al., 2008; Prentis et al., 2008; Lombaert et al., 2010). Genetic studies can also estimate the number of introductions from each source (Ficetola et al., 2008; Miller et al., 2005), and the dynamics of the geographical and demographic expansion (Kolar and Lodge, 2001)..Genetic diversity is of particular interest in the dynamics of an invasive species because genetic diversity influences the range of physiological and ecological tolerances of the invasive population, determines the genetic pool subjected to a new selection regime in the invasive habitat (Miura, 2007), and can help predict future spread (Hudson et al., 2021). For example, Tsusui and Case (2001) show that genetic diversity can play an important role in the survival, and adaptability of a population that is colonizing a new environment (Fisher, 1930; Frankham, 2008), as greater variation in genotypes maximizes the ability of individuals in this invasive population to adapt to environmental challenges in their invasive range (Sultan, 2007; Hoffmann and Willi, 2008).

A unique aspect of invasive species biology is that multiple introductions from genetically different source populations (Marrs et al., 2008; Prentis et al., 2008) can result in novel, admixed genotypes not seen naturally in the native range (Capo-chichi et al., 2008), potentially giving rise to unique physiological and ecological abilities that could aid in invasions. Conversely, invasions that result from a single origin or single introduction event with a few individuals often have limited genetic diversity, constraining the evolutionary potential of introduced populations (Frankham et al., 1999; Allendorf and Lundquist, 2003; Dlugosch and

Parker, 2008; Tabima et al., 2021). Limited genetic diversity can decrease the ability of introduced species to adapt to conditions in their new environments (Dlugosch et al., 2015), resulting in introduced species failing to become invasive (Wellband et al., 2017). However, if introduced individuals have a prominent genetic variant, such as an aggressive genotype still become invasive (Chambers et al., 1999; Zhao et al., 2006), genetic advantages may lead to genetic uniformity across an introduced range (Zhang et al., 2010). In addition, while a small number of individuals introduced from one source will limit genetic variation, a small number of individuals introduced from multiple source populations can minimize loss of genetic variability, providing the founder populations ample genetic diversity to respond to novel selection regimes (Novak et al., 2005; Roman and Darling, 2007).

Previous genetic studies of invasive species often employed single locus mtDNA sequence data (Lindholm et al., 2005; Arca et al., 2015; Valentin et al., 2017). However, modern genomic methods provide additional power for studying genetic patterns observed within and between populations (Keller and Taylor, 2010; Barker et al., 2017; Kotsakiozi et al., 2017). In particular, reduced representation genome sequencing, such as type 2 restriction site-associated DNA (2b-RAD; (Wang et al., 2012), simultaneously discover and genotype thousands of single nucleotide polymorphisms (SNP) markers across the genome, making this technique particularly useful for examining genetic diversity within and among populations (Varela and Amos, 2010). Although restriction associated DNA (RAD) sequencing technology has some disadvantages (O'Leary et al., 2018), proper SNP filtering can be advantageous for locus reconstruction and identifying shallowing divergences between populations (Hodel et al., 2017). Moreover, because these SNPs capture a larger number of loci, providing insights that single locus markers alone cannot (Hodel et al., 2017), SNPs can help to identify potential genotypes associated with the

source populations and infer ancestral or continental origin (Takahata, 1995; Reitzel et al., 2013; Andrews et al., 2016).

Globalization of shipping transport has accelerated the spread of invasive marine species (Kolar and Lodge, 2002) with pathways of dispersal closely linked to connected shipping ports and harbors (Molnar et al., 2008; Seebens et al., 2013; Souissi et al., 2017). Native to the Western Indian Ocean, Eastern Africa, Arabian Sea, Persian Gulf, and the Red Sea, the seagrass, *Halophila stipulacea* was first recorded in the Mediterranean Sea in 1894, 25 years after the opening of the Suez Canal (Fritsch, 1895; Lipkin, 1975). Since its introduction to the Mediterranean Sea *H. stipulacea* has expanded its range westwards and northwards, reaching Cyprus (Lipkin, 1975), Libya (Sghaier et al. 2011), Greece (Malea, 1994), Malta (den Hartog, 1972), Tunisia (Sghaier et al. 2011), Italy (Gambi et al. 2009), and France (Thibaut et al. 2022), where it forms low density seagrass meadows with other native seagrasses and algae (Sghaier et al., 2014; Nguyen et al., 2020). Although the *H. stipulacea* introduction has negative impacts on native Mediterranean species (Boudouresque and Verlaque, 2002; Sghaier et al., 2014) as a tropical native, *H. stipulacea* meadows shrink in the winter season, releasing pressure on local fauna (Winters et al., 2020). Recent studies also demonstrate potential positive effects on ecosystem processes of *H. stipulacea* in the Mediterranean, where it functions as an introduced carbon sink for the Eastern Mediterranean Sea (Apostolaki et al., 2019).

In 2002, the first Caribbean introduction of *H. stipulacea* was recorded on the island of Grenada (Ruiz and Ballantine, 2004). Unlike in the Mediterranean Sea, where it coexists with natives, in the Caribbean Sea *H. stipulacea* appears more aggressive, rapidly displacing local seagrass species (Willette and Ambrose, 2012; Smulders et al., 2017). Moreover, it is spreading quickly; within a decade, *H. stipulacea* expanded its Caribbean range over 700 kilometers,

reaching as far north as the Virgin Islands (Willette and Ambrose, 2012; Chiquillo et al., 2019) and Puerto Rico (Ruiz et al., 2017), as far west as Curacao (Willette et al., 2014) and south to Venezuela (Vera et al., 2014); it is predicted to reach the Gulf of Mexico in the upcoming years (Ruiz et al., 2017).

Genetic data indicates that invasive Mediterranean populations of *H. stipulacea* originated in the Red Sea (Ruggiero and Procaccini, 2004). However, this study only included a single population from the native range, and only used two genetic markers, limiting the strength of this inference. While Ruiz & Ballantine (2004) hypothesize that Caribbean populations originated from the Mediterranean Sea, this hypothesis has never been tested. As such, it remains unknown whether this introduction originated from the native range of *H. stipulacea* (Red Sea and Indian Ocean) or from the invasive Mediterranean range, and whether the introduction was single or multiple events, information that could shed light on the invasion success in the Caribbean.

In this study, we use multi-locus single nucleotide polymorphism (SNP) data to determine the geographic origins and the number of introductions of invasive *H. stipulacea* populations. Specifically, we test the hypotheses that the Caribbean population could have originated from a stepping stone colonization coming from the invasive Mediterranean populations, a single introduction, or a series of introductions from its native range: or an admixture introduction from multiple source populations, potentially contributing to the different dynamics observed in these two invasive ranges. Secondly, we test the Lessepsian migrant hypothesis, where populations from the Red Sea migrate to the Mediterranean Sea (and vice versa) via the Suez Canal, to understand its evolutionary dynamics from its native range.

3.3. MATERIALS AND METHODS

Sample collection

We collected individual *Halophila stipulacea* ramets (with shoots, attached roots, and rhizomes) from populations within its native and invasive ranges (Figure 3.1). Within the native range, we sampled populations from the Gulf of Aqaba (Israel) as well as the Western Indian Ocean (Mozambique, Mayotte) and Eastern Indian Ocean (Tamil Nadu, India). From the invasive range, we sampled populations from the Mediterranean (Tunisia, Italy, Greece, Cyprus) and the Caribbean (Curacao, St. Thomas, St John, Grenadines; Figure 3.1).

Following Migliaccio et al. (2005), we randomly sampled seagrass ramets at intervals of 5-10 meters at each of the collection sites to minimize the possibility of collecting clones. We prepared ramets by cleaning blades of epiphytes, blotting dry with paper towels and/or drying in the sun (i.e., solar dried) for ~ 1-2 days prior to preserving with silica gel in Ziplock bags. However, samples collected in the Red Sea, Curacao, and Grenadines were rinsed with sterile freshwater to remove sediment from the roots and shoots, blotted dry on a paper tissue, and immediately placed in RNA*later* (Sigma - Aldrich Inc. Missouri, USA*)*, stored at 4^{o}C for 24 hours, then transferred to -80^{o}C for long term storage. We extracted and retained high quality DNA from both silica air-dried samples and samples stored in RNAlater, and it did not affect the data.

gDNA extraction, RAD library preparation

We extracted genomic DNA from dried/frozen seagrass blades using standard protocols for seagrasses, following Jahnke et al. (2015), and used the Macherey-Nagel NucleoSpin® Plant II mini kit for DNA from plants (Macherey-Nagel GmbH & Co. Düren, Germany) to achieve 1

ug of total DNA. Next, we prepared 113 DNA samples (representing 15 populations) for 2b-RAD libraries, following the procedures described by Wang et al., (2012). Briefly, we digested genomic DNA from each sample to a uniform fragment length (i.e., 36 bp) using *Alf1* restriction enzyme. We constructed adaptors with 5'- NG and 3'-NG overhangs selected to capture $1/16^{th}$ of the *Alf*1 restriction sites, then ligated to restriction fragments. We amplified ligated products using unique 6-bp barcodes on a Biometra T-1 thermocycler (Serial # 2106184). PCR cycling conditions were: 98°C for 5 sec, 60°C for 20 sec, followed by an extension at 72°C for 10 sec. We performed the final amplification in reactions of 100 uL per library with 23 amplification cycles in each run and visualized the integrity of amplified PCR products by running them on a 2% agarose gel electrophoresis using 1x TBE (Tris/Borate/EDTA) buffer and ran at 150 V for 90 min. We excised target bands at ~170 bp and purified these using the QIAquick Gel Extraction Kit (Qiagen; Cat No./ID: 28704).

Because of changes in DNA sequencing technologies, we combined 57 samples (amplified PCR products that were extracted from the cut gel bands) into a single library in equimolar amounts and sequenced on Illumina HiSeq 3000 platform in a single lane of single-end 50 bp reads at the Oregon State University (OSU) Center for Genome Research and Biocomputing (CGRB; https://cqls.oregonstate.edu/core). We subsequently sequenced an additional 56 pooled equimolar using paired end 150-bp reads on an Illumina Hiseq 2000/2500 platform at the UCLA Technology Center for Genomics and Bioinformatics (https://www.uclahealth.org/pathology/tcgb).

2b-RADseq data processing: Amplification, Quality filtering and SNP calling

To process the data, we used custom scripts for analysis of 2bRAD sequence data developed by Eli Meyer (GitHub https://github.com/Eli-Meyer/2bRAD_utilities). We filtered all raw reads to eliminate low quality or uninformative reads, using a base call Phred quality parameter Q score value of > 20. Next, we employed end-trimming, reducing all sequences to the target 36bp recognition site, and then truncated reads with low quality (e.g., having > 5 bp positions with Phred quality scores Q > 20). We removed reads without the complete *Alf1* matching adaptor, or at least one 12-bp k-mer match, a process which resulted in a final subset of reads used to determine SNP genotypes.

To identify SNPs, we constructed a *de novo* reference genome library, by clustering sequence data from high quality reads (Build.Ref.pl program) to develop a catalog of reference loci. Since 2b-RAD sequences are double stranded (Wang et al., 2012), we used a pipeline to emulate the *Stacks* pipeline using "BuildRef.pl", a software pipeline for building loci from short-read sequences, such as those generated on the Illumina platform. Similar to *Stacks*, it counted unique tag sequences (minimum sequencing depth of 5x), and recorded numbers in reverse-complement orientation (http://eli-meyer.github.io/2bRAD_utilities/#reference).

We mapped reads per individual to the *de novo* reference genome using "gmapper" employed in SHRiMP (Rumble et al., 2009), and a minimum of three identical reads were aligned to create a stack. We then removed weak, partial, and ambiguous mappings by filtering by read depth (SamFilter.pl; -m) and excluded samples with matches lower than 30, strand bias (minimum 5) and mismatches (maximum 3 per read).To identify variable SNPs, we aligned the reference genome and used "SAMBaseCounts.pl" to genotype each locus, in each sample, to determine the significance level required to call a heterozygote or homozygote genotype. We

then applied allele filters (10x total depth, allele bias and strand bias), with an additional requirement that alleles appear in at least two individuals.

Individual sampling filtering steps:

To create a SNP dataset from the raw sequence data, we removed all individuals with low coverage and low-quality reads with <80% match to the reference genome. Of the total 113 samples sequenced only 96 individuals passed the initial QC filter. We kept the remaining samples and applied a locus filter allowing genotypes with a minimum of 10x coverage to be called with a maximum 0.01 major allele frequency (homozygous calls), and minimum 0.25 minor allele frequency (heterozygous calls).

We filtered low coverage samples, and low coverage loci in two ways. First, we filtered individuals with at least 1150 loci genotyped across all samples and genotyped in at least 75% of individuals. Because this filtering retained a relatively small number of loci, we created a second dataset based on genotyping at least 1000 loci across all samples and keeping loci genotyped in 60% of individuals. After removing low coverage and missing data, the remaining samples were used thinning the resulting SNPs to one per tag (the one with the highest minor allele frequency). The final data file was then arranged in a SNP matrix, and converted to other formats for downstream processing using "gt2structure.pl" from 2bRAD_utilities; adapted from PGDSpider v2.0.8.0 (Lischer and Excoffier, 2012).

Intrapopulation diversity and geographical variation

To test for reductions in genetic diversity associated with founder events, we compared common genetic diversity measures across all native and invasive populations. We conducted a Bartlett's test to test for homogeneity of variance in the "stats" package in R to test for

differences in expected and observed heterozygosity (Bartlett and Fowler, 1937; Jones et al., 1994). We used the "summary" function to calculate the number of alleles per populations and the "inbreeding" function to estimate the inbreeding coefficients with the "adegenet" package (https://cran.r-project.org/web/packages/adegenet/adegenet.pdf; (Jombart, 2008), using R V3.6.3 (R Core Team, 2020). To test for the proportion of loci out of Hardy-Weinberg Equilibrium we used the function "hw.test" in the "pegas" package (Paradis, 2010), using a chi-squared test to compare the expected genotype frequencies from allelic frequencies, assuming a false discovery rate of 0.05. Next, we tested the contemporary effective population size (N_e) using the program NeEstimator v2.1(Do et al., 2014), using a minimum allele frequency of 0.05 to calculate the upper and lower limits of N_e with linkage disequilibrium model assuming random mating in a GUI platform.

Estimating population divergence and gene flow:

To determine patterns of genetic exchange among populations within and among native and invasive ranges, we used the "StAMPP" (Statistical Analysis of Mixed Ploidy Populations; (Pembleton et al., 2013) package in R v1.6.2 to estimate gene flow, calculating pairwise F_{ST} values using the "stamppFst" function, testing for significance by bootstrapping across loci (1000 permutations). To detect population differentiation across sampling sites, we used a hierarchical analysis of molecular variance (AMOVA) using the "poppr.amova" and "randtest" function (1000 permutations) in the R package "poppr" v2.9.2 (https://cran.r-project.org/web/packages/poppr/poppr.pdf).

To further determine potential source populations, we examined population relationships in an evolutionary framework, constructing a neighbor joining (NJ) tree from Nei's genetic

distance (Nei, 1978), and calculating bootstrap support values (BSV) with 10,000 replicates using the "aboot" function of R package "poppr". For this analysis, we assumed that all loci/columns are independent, and missing values were replaced with the mean of the corresponding allele in the NJ tree construction.

Because invasive *H. stipulacea* populations could have multiple origins, we used a Discriminant Analysis of Principal Components (DAPC) using the "adegenet" v2.1.4 package (Jombart and Ahmed, 2011) in R. We used the "dapc" function and visualized using the "scatter" to determine population membership across pre-defined groups. We then used the "find.clusters" function to achieve the optimal number of clusters based on the Bayesian information criterion (BIC) values, and we used the function "compoplot" to estimate membership probability of individuals to the clusters in the "adegenet" package.

To examine genetic structure, we employed the Landscape and Ecological Associations (LEA) v1.81. package in R (Frichot and François, 2015), under the assumption that markers are not linked and populations are panmictic. We used LEA rather than STRUCTURE (Pritchard et al. 2000) because it can estimate population structure with more accuracy when there is inbreeding (Francois, 2016), which can be common in invasive populations (Schrieber and Lachmuth, 2017). To estimate the number of ancestral populations, K, we used cross-entropy criterions and least squares estimates. More specifically, we used the sparse non-negative matrix factorization "snmf" function to estimate the optimal number of clusters (k), or number of subpopulations, in 1,000 interactions over a range of K values. The number of ancestral populations, K, was based on the cross entropy curve, and we chose the K value with the lowest mean cross entropy for downstream analyses. We generated the ancestry matrix by estimating

individual admixture coefficients from the lowest cross entropy run and visualized using the "barchart" function using the LEA package.

3.4. RESULTS

Estimating de novo reference genome and polymorphic loci:

Sequencing runs yielded a total of 393,430,664 reads (TCGB: 292,813,197 and OSU 100,617,467 total reads), resulting in an average of 2,774,738 clean reads per sample. From these sequences, we constructed a *de novo* reference genome by combining a set of 10,967,421 high-quality, concatenated reads from 25 individuals (2-5 individuals from each sampled population). Quality control of the *de novo* reference genome identified 67,908 sequences with NAs, 5,561,303 low quality sequences, and 4,863,062 reads that perfectly matched to the restriction site were excluded and filtered out. Further parsing of the reference genome yielded a total number of 245,950 unique loci. We divided the total number of captured sites by the assumed site frequency rate and estimated the genome size to be approximately ~ 1.007 Gb.

We mapped a total of 273,745,564 reads to the reference genome, keeping reads that had a minimum similarity of 80% to the *de novo* reference genome. This process yielded an average of 2,422,527 (± 262,800 SE; range 1,041 - 13,167,537) mapped reads per individual. A total of 197,454,094 unique mapped reads (average 1,747,381 ± 193,681 SE; range 752- 9,462,557 reads per individual) remained after discarding mapped reads with short alignments and/or weak/ambiguous matches to the reference genome.

The 6,021,728 retained loci (60,054,980 genotypes all together) yielded a genotype matrix of 113 samples and contained 132,879 polymorphic loci, with individual genotypes

determined only at loci with >10x coverage, based on nucleotide frequency thresholds (Wang et al. 2010).

From the total SNP matrix, we created two datasets using different levels of stringency. In the most stringent, we limited the data set to individuals with at least 1150 variable SNPs allowing for up to 25% missing data, resulting in a final SNP matrix of 45 loci and 93 individuals. Because this stringency greatly reduced the number of loci, we created a second dataset that includes individuals genotyped at 1000 loci in a minimum of 60% of individuals, allowing for 40% of missing the data, yielding a final matrix comprised of 524 loci genotyped and 94 samples. We refer to these as the "smaller" and "larger" datasets, respectively.

Intrapopulation diversity and geographical variation

We detected significant difference between expected and observed heterozygosity, and populations did depart from Hardy-Weinberg Equilibrium (HWE) with the larger loci dataset (Bartlett's test; p=0.00002), however in the smaller data set we did not detect a departure from HWE (Bartlett's test: p=0.11). We report intrapopulation genetic diversity for the larger data set and include differences in smaller data set (Table 3.1). The global proportion of polymorphic loci for observed heterozygosity (H_o) was significantly lower (0.10 ± 0.03 SE) than H_e (0.35 ± 0.14 SE) (t-test P=0.02). The pattern was the opposite for smaller data set, where observed heterozygosity (H_o) was significantly higher (0.07 ± 0.01 SE) than the expected heterozygosity (H_e) or the mean genetic diversity within populations (0.06 ± 0.01 SE) (t-test P< 0.001). In larger the dataset, results showed varying levels of observed heterozygosity (H_o =0.002-0.185) and expected (H_e =0.065-0.986) across sampled populations. Similarly, in smaller dataset, results showed varying levels of observed heterozygosity (H_o=0.022-0.165) and expected (H_e= 0.011-

0.200) across the sampled populations. Heterozygosity levels in the larger dataset showed similar levels between WIO (0.695) and EIO (0.681) but were much higher than Red Sea (0.179), Mediterranean Sea (0.112) and Caribbean Sea (0.074). Conversely, heterozygosity levels in the smaller data set showed WIO (0.04) was higher than EIO (0.011), however, levels were similar to the Red Sea (0.047), but lower than the Mediterranean (0.102) and Caribbean (0.056).

In the native range populations in the Indian Ocean (WIO=205, EIO=227) had fewer private alleles than the Red Sea (n=907), but more in Mediterranean Seas (n= 771), and Caribbean (n=665). In the invasive ranges, the Mediterranean population in Cyprus had the highest number of private alleles (n=686), and the Grenadines in the Caribbean had the highest number of alleles (n=664) in the larger data set. Numbers of private alleles were higher in larger data set. In the smaller dataset, populations in the native range had a lower number of private alleles (WIO = 52, EIO = 46, Red Sea = 51) than the invasive populations, where the number of alleles were more similar (Mediterranean Sea = 68, Caribbean Sea =69). Similar to the larger data set, in the invasive range, the Mediterranean population from Tunisia had the highest number of private alleles (n=62), and in the Caribbean population from Grenadines had the highest number of private alleles (n=68).

Tests of Hardy-Weinberg equilibrium (HWE) in both data sets did not reveal significance difference. The patterns in the Eastern Indian Ocean (0.063; P=0.783), and Western Indian Ocean (0.021; P=0.856) had a lower proportion of loci out of HWE than Red Sea (0.246; P=0.578), Mediterranean (0.101; P=0.777) and Caribbean (0.129; P=0.842). HWE in the smaller dataset revealed populations from the Eastern Indian Ocean (0.044; P=0.978), Red Sea (0.044; P=0.927), and Caribbean (0.044; P=0.871) had a higher proportion of loci out of HWE compared to populations in the Mediterranean (0.022; P=0.823) and Western Indian Ocean (0.022;

P=0.894). In the larger dataset, the overall inbreeding coefficients (F_{IS}) were high among all populations with an average of 0.372 ± (0.04 SE), with the lowest values in the Western Indian Ocean (Mozambique, F_{IS} =0.119), and the highest in the Red Sea (Israel F_{IS} =0.527), similar to Greece in the Mediterranean Sea (n=0.524). This pattern was similar in the smaller dataset where the overall inbreeding coefficients (F_{IS}) were high among all populations (average of 0.35 ± 0.03 SE), however the lowest values were found in the Mediterranean (Tunisia, F_{IS} =0.161) and the highest in the Western Indian Ocean (Mayotte, F_{IS} = 0.574).

We were unable to estimate effective population for the larger data set as NeEstimator cannot handle large amounts of missing data, and we report for the smaller data set (Table 3.1). In the native range, we observed an infinite effective population size in the Red Sea; however, the effective population was lower in the Indian Ocean (WIO was 0.2 and EIO was 1.0). We found the effective population size was low in the invasive regions (0.2 for the Caribbean and 0.3 for Mediterranean). Additionally, we found extreme ranges in confidence intervals for N_e, making these estimates unreliable.

Estimating population divergence and gene flow:

Results showed high genetic differentiation between populations in all data sets. In the larger data set, 53 of 55 pairwise F_{ST} values were significant ($P<0.05$) in the larger data set, with values ranging from 0.02-0.975 (Table 3.2). Although the largest values were seen in comparisons of the native range and all other populations (Table 3.2) there was high genetic divergence among Indian Ocean populations (max F_{ST}=0.683, min F_{ST} =0.489 and among the Red Sea and Indian Ocean populations (max F_{ST} =0.892, min F_{ST}=0.878). There was also high genetic dissimilarity between populations in the Mediterranean Sea and Indian Ocean (min F_{ST}=

0.763, max F_{ST} = 0.959, average $F_{ST=}$ 0.876) and the Caribbean Sea and Indian Ocean (min F_{ST}= 0.830, max F_{ST} = 0.975, average $F_{ST=}$ 0.912).

Within the Mediterranean, genetic structure was lower, but significant (min F_{ST}= 0.100, max F_{ST} = 0.144, average F_{ST}=0.121), with the most similarity between Greece and Tunisia (F_{ST}= 0.100); similar F_{ST} values were found between the Red Sea and Mediterranean Sea (max=0.206, min=0.076, average=0.139), with the greatest similarity between Israel and Italy (F_{ST}= 0.076). The lowest levels of genetic structure occurred in the Caribbean (max=0.02, min=0.00, average=0.013), and Caribbean populations had lower F_{ST} values in comparison to the Mediterranean Sea (min F_{ST}= 0.105, max F_{ST} = 0.300, average $F_{ST=}$ 0.211) than in comparison to the Red Sea (min F_{ST}= 0.256, max F_{ST} = 0.293, average $F_{ST=}$ 0.277) or to the Indian Ocean (see above). Similarly, in the smaller data set, forty two out of the 45 pairwise F_{ST} comparisons were significant (P<0.05), ranging from 0.077 to 0.968 (Supplemental Table S3-1). Patterns of genetic structure obtained from the larger data set were very similar (Supplemental Table S3-1).

Results of hierarchical analysis of molecular variance (AMOVA) showed significant differences between populations, rather than within populations (Table 3.3). The global Φ_{ST} analysis of *H. stipulacea* indicated significant population structure (Φ_{ST} = 0.64, P=0.01) with 35.91% of the genetic variation within sites, while 62.59% of the genetic variation was among regions (Φ_{CT} = 0.63, P=0.01), and 1.49 % of the variation between sites within regions (Φ_{SC} = 0.04, P=0.01). Patterns for the smaller data set were very similar (Supplemental Table S3-2).

Assessing interpopulation genetic diversity and structure

A neighbor-joining tree constructed from both datasets were similar, grouping populations into five regions and differentiating individuals between the Indian Ocean, and

clustered together in the Red Sea, Mediterranean Sea, and Caribbean Sea (Figure 3.2, Supplemental Figure S3-1). The primary difference was the phylogenetic position of Tunisia.

Discriminant Analysis of Principal Components (DAPC) on the larger dataset yielded five discriminant functions with lowest Bayesian information criterion (BIC) and 85% of variance conserved from PCA, yielding five population clusters (Figure 3.3). Cluster 1 contains all Red Sea locations, cluster 2 includes all of the Caribbean and Mediterranean Sea populations, cluster 3 includes the eastern Indian Ocean population, and cluster 4 and cluster 5 consists of the western Indian Ocean populations (Figure 3.3). Results from DAPC using the smaller data set revealed equivalent patterns (Supplemental Figure S3-2). General patterns reveal individuals in the Caribbean Sea cluster together, and samples from the Mediterranean Sea cluster together. Samples originating from Israel show similarity with individuals from Cyprus, while the western Indian Ocean (WIO) and eastern Indian Ocean (EIO) cluster together.

Group assignment probability plots indicate five distinct genetic clusters (K=5). Regardless of dataset analyzed, the Eastern Indian Ocean, Western Indian Ocean and Red Sea form highly differentiated clusters with no evidence of admixture (Figure 3.4). However, the final two clusters, corresponding to the Mediterranean and Caribbean both show evidence of admixture.

Minimal cross-entropy values from Landscape Ecology Association (LEA) analyses also predicted five population clusters (ancestral populations), followed by a second minimum at 9 clusters (Figure 3.5). We were unable to perform cross-entropy analysis and an ancestry plot matrix for the larger data set as the LEA package cannot handle large amounts of missing data. LEA analyses distinguished ancestry between the Western Indian Ocean (dark blue), Eastern Indian Ocean (light purple) and the Red Sea (dark purple); Mediterranean and Caribbean

populations (green) were largely distinct, but with some shared clusters suggestive of admixture (Figure 3.6). For example, Mediterranean samples include genetic signatures associated with the Red Sea as well as the Western and Eastern Indian Ocean, and the Caribbean populations include genetic signatures from the Red Sea and Eastern Indian Ocean. Both Mediterranean and Caribbean populations contain a genetic cluster (light blue) that is not found in any sampled native population, despite both of these populations arising from native populations.

3.5. DISCUSSION

As human activities increase the rate of non-native species introductions and the number of invasive species (Britton-Simmons and Abbott, 2008), molecular tools provide a valuable framework for understanding the origins of these invasions (Muirhead et al., 2008; Tarin et al., 2013; Kinlock, 2021) and how their origins can affect invasion success (Wilson et al., 2009). Analysis of SNP variation in the invasive seagrass, *Halophila stipulacea,* supports a model of stepwise colonization, where *H. stipulacea* from the Red Sea first invaded the Mediterranean and subsequently populations from the Mediterranean (likely populations near Greece) invaded the Caribbean, confirming the hypothesis of Ruiz and Ballantine (2004).

However, both Mediterranean and Caribbean populations contain a genetic signal that is distinct from all native range samples and have higher genetic diversity than sampled native range populations, violating the assumptions of the genetic paradox hypothesis (Estoup et al. 2016). It is possible de novo mutations may contribute to genetic variation for adaptation after an invasion (Ossowski et al. 2010; Dlugosch et al. 2015), however ecological mutations are rare (Kotil et al. 2018). In a stepping stone colonization model, it is possible for beneficial alleles to become fixed in sub populations even with low migration rates, and can increase levels of gene

flow (Hartfield, 2012), however our F_{ST} values were high among regions and our data supports nucleotide diversity increasing with recent admixture. Combined, these results suggest that in addition to an original introduction from the Red Sea, there was likely a second introduction of individuals from an unsampled region of the native range, a result previously reported in Lessepsian marine invasive species (Negri et al. 2018; Stefani et al. 2012). Increased genetic diversity and admixture from distinct native source populations may play an important role in the success of this invasive seagrass in both invasive ranges through heterosis (Hahn et al. 2016)

Reconstructing Invasion History

Geographically and temporally, the origin of the Mediterranean invasion of *H. stipulacea* is consistent with the opening of the Suez Canal, with the source population assumed to be the Red Sea (Fritsch, 1895; Lipkin, 1975). Early genetic studies supported the hypothesis of a Red Sea origin of invasive Mediterranean populations (Ruggiero and Procaccini, 2004), but this result was based on a single genetic marker and did not examine other potential sources throughout the native Indian Ocean range. Our multi-locus SNP data, including both Red Sea and Indian Ocean populations reveals that invasive Mediterranean populations are genetically similar to the Red Sea, while Eastern and Western Indian Ocean populations are highly divergent, confirming that the Red Sea is one source of the Mediterranean *H. stipulacea* invasion.

Unlike the Mediterranean, there was no *a priori* assumptions regarding the origins of the Caribbean introduction of *H. stipulacea.* Our data supports the hypothesis that the invasion into the Caribbean Sea was the result of stepping-stone colonization via serial introduction (Corre et al., 1998; Estoup and Guillemaud, 2010), first from the Red Sea to the Mediterranean and subsequently from the Mediterranean to the Caribbean. Mediterranean and Caribbean

populations are the most similar (often overlapping) in the discriminant principles components analyses, and these two invasive populations form a highly supported clade in both of the neighbor joining analyses, with the Red Sea being the next most closely related group. Individual membership plot and structure analyses from LEA support the connectivity between Mediterranean Sea and Caribbean Sea, and these two invasive populations share ancestry (Figure 3.4 and Figure 3.6). In contrast, structure analyses, and pair-wise F_{ST} values indicate that extreme levels of isolation and genetic differentiation between the Caribbean and Eastern and Western Indian Ocean populations, excluding these regions as potential sources.

While the above data clearly show the Caribbean *H. stipulacea* originated from the Mediterranean, our sampling scheme and associated data do not allow us to pinpoint a specific founder population. The lowest F_{ST} values observed are between Greece and the Grenadines, an island group ~40 miles northeast of Grenada, the location where *H. stipulacea* was first observed in the Caribbean. However, while the F_{ST} value between these populations based on the smaller data set is small (0.007) and non-significant (Supplemental Table S3-1) as expected from a recent introduction, the larger data set has a sizable and significant value of 0.105. However, this value is still substantially less than any other sampled Mediterranean populations, indicating that *Halophila stipulacea* most likely arrived into the Caribbean Sea from the Eastern Mediterranean Sea, confirming the hypothesis of Ruiz and Ballantine (2004). Even though evidence from both datasets tend to conflict, the patterns remain consistent. The larger dataset provides a stronger support for a stepping stone colonization hypothesis, while smaller dataset was more conservative, containing fewer loci and less missing data.

Extensive sampling provides a strong support for a stepping-stone model of colonization, confirming the results of Ruggiero and Procaccini (2004) and the hypothesis of Ruiz &

Ballantine (2004), however, analysis of genetic structure and patterns of genetic diversity indicate a more complicated colonization history. Results from the LEA analyses indicate a distinct genetic cluster in both Mediterranean and Caribbean populations that is not present in any of the native range samples. Moreover, genetic diversity in both invasive populations was higher than native populations. Previous studies report high genetic diversity in invasive species despite stepping stone colonization (Tonione et al., 2011; Frisch et al., 2013; Mantovano et al., 2021), a pattern that could arise from multiple secondary introductions from already admixed populations (Kelager et al., 2013; Sherpa et al., 2019; Geburzi et al., 2020). Combined, these results suggest that there was likely two introduction events, with the second introduction coming from an unsampled source population, elevating levels of genetic diversity.

Given that both Mediterranean and Caribbean populations contain this unique genetic signal, it is not clear whether both the introduction of this second source population occurred independently, or whether there were multiple introductions into the Mediterranean, and it suggests that both of these genetic lineages were present in the individuals originally introduced into the Caribbean. However, what is clear is that this admixture increases genetic diversity, which increases adaptive potential (Crawford and Whitney, 2008; Kolbe et al. 2008) and that admixture could create novel genotypes that don't occur naturally in native populations (Verhoeven et al. 2011). Combined, these processes could aid the success of *H. stipulacea* following introduction, contributing to its success as an invasive species in both regions.

Gene flow within and among native and invasive ranges

Genetic differentiation was surprisingly high throughout the dataset, native and invasive, with the vast majority of pair-wise F_{ST} values being significant, indicating strong limits to gene

flow. Gene flow was almost completely absent between Eastern and Western Indian Ocean populations, and these populations were highly divergent from the Red Sea. More surprising was the strong genetic divergence between the Red Sea and Mediterranean populations, with F_{ST} values exceeding 10% in all comparisons, and nearly 50% in specific pair-wise comparisons.

Such strong genetic differentiation may seem unusual given that Mediterranean populations were established just over 100 years ago. However, similar results are reported in other Lessepsian bioinvasions, and have been alternately explained as resulting from founders effects (Golani et al., 2007) or population bottlenecks (Azzurro et al., 2006; Bernardi et al., 2016; Stagličić et al., 2020) that result in major changes in allele frequencies. In terrestrial taxa, colonization of new populations from a subsample of a larger source population can increase F_{ST} drastically (Yao et al., 2020; Hofmeister et al., 2021). Genetic drift, natural selection (Ramachandran et al., 2005; Praebel et al., 2013), and/or purging of deleterious alleles (Lombaert et al., 2010; Chen et al., 2021) could also drive strong changes in allele frequencies, leading to high F_{ST} values between Red Sea and Mediterranean population of *H. stipulacea.*

Interestingly, high F_{ST} values weren't just observed among regions, but were also seen among populations within the invasive Mediterranean Sea and Caribbean Sea populations (Table 3.2). As noted above, the serial introduction of *H. stipulacea* where new populations are colonized by a small subset of source population(s) could lead to substantial shifts in allele frequencies, inflating F_{ST} values (Handley et al. 2011, Lombaert et al. 2010). Primary introductions followed by subsequent dispersal/introductions and range expansions can contribute to differential invading potential (Golani and Bernardi, 2012), and higher invasiveness (Bertelsmeier and Keller 2018), potentially through the subsampling of allelic diversity associated with introduction and range expansion processes.

Conclusions

Although results show that the invasive populations of *Halophila stipulacea* in the Caribbean originated in the Mediterranean, the mechanism of this introduction is unknown. Dispersal of invasive populations is commonly linked to human commerce and trade networks (Bertelsmeir & Keller 2018), and maritime traffic the most likely source of introduction, possibly via anchor fouling or other forms of accidental transport. Entry of Indo-Pacific and Lessepsian migrants into the Eastern Mediterranean from the Suez Canal is one of the oldest maritime pathways of dispersal, transporting introduced species to a considerable number of marinas (Galil and Zenetos 2002). Further, popular maritime traffic highways between Northern European Seas and tropical ecosystems are strongly connected to the dispersal of invasive species (Seebens et al., 2013), and previous genetic studies demonstrate many Caribbean invasions originating from Europe (Gerlach, 1997; Besnier et al., 2014; Jeffery et al., 2017).

Once introduced into the Caribbean, the primary vector of secondary spreading within this region is likely anchors of pleasure boats and/or commercial vessels moving between local ports and harbors (Murray et al., 2011; Zhan et al., 2015), as these vessels commonly anchor in the calm, protected, sandy bays utilized by *H. stipulacea*. Maritime traffic routes are popular throughout the Caribbean Sea for passengers and cargo, in particular the U.S Virgin Islands and Puerto Rico (https://invasions.si.edu/nbicdb/arrivals?state=VI&type=Passenger&begin=20040101&pg=2). However, hurricane activity may also contribute to the spread in the Caribbean through dislodging and transporting *H. stipulacea* (Hernández-Delgado et al., 2020).

Understanding the evolutionary origins of *H. stipulacea* in the Caribbean is an important step in predicting the vectors of transport, and its overall success. Seagrass ecosystems are already facing a global crisis due to both direct (reduced water quality, coastal development, and poor land use) and indirect (climate change, biological invasions) anthropogenic perturbations (Orth et al., 2006; Waycott et al., 2009; Short et al., 2011). Given the losses of native seagrass communities in the Caribbean (Smulders et al. 2017, Winters et al. 2020), it is essential to try to limit the spread of *H. stipulacea* into new habitats and address the underlying anthropogenic stressors driving native seagrass losses. Failure to do so will potentially alter the structure and function of Caribbean seagrass communities forever, compromising the valuable ecosystem services they provide.

3.6. ACKNOWLEDGMENTS

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3.7. FIGURES

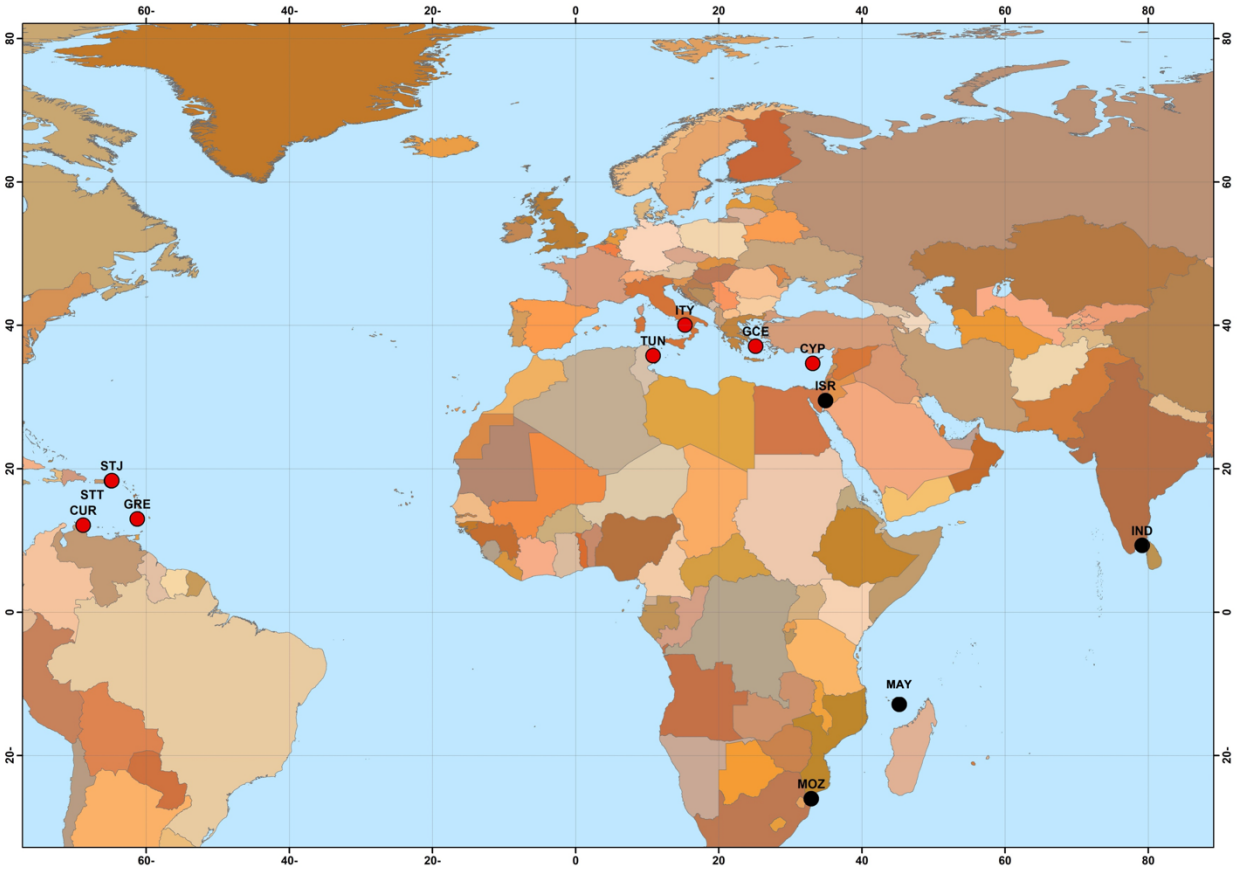


Figure 3.1. Sampling localities of *H. stipulacea* within the native range and invasive range of species. Circles represent localities from each population sampled. CUR, Curacao; STT, St. Thomas; STJ, St John; GRE, Grenadines; TUN, Tunisia; ITY, Italy; GCE, Greece; CYP, Cyprus, ISR, Israel; MOZ, Mozambique; MAY, Mayotte; IND, India.

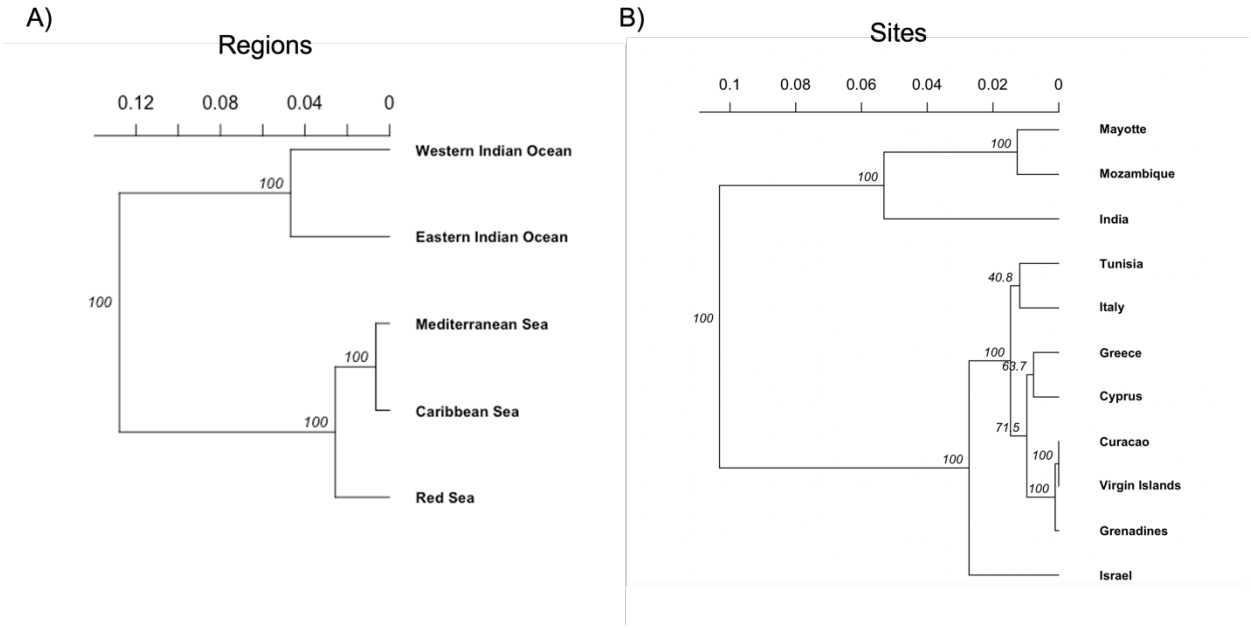


Figure 3.2. Neighbor joining dendrogram using "aboot" function with "nei.dist" based on Nei's genetic distance matrix of *H. stipulacea* based on n=94 and 524 loci. A) Neighbor joining tree is grouping across regions, and B) clustered by sites. The vertical axis represents closeness of populations, and the numbers on each branch represent the probability of encountering nth occurrence of a particular genotype. The x-axis above the dendrogram represents the distance or dissimilarity between clusters. Numbers on branches represent branch confidence levels. Prepared using the function "aboot" in "poppr" software.

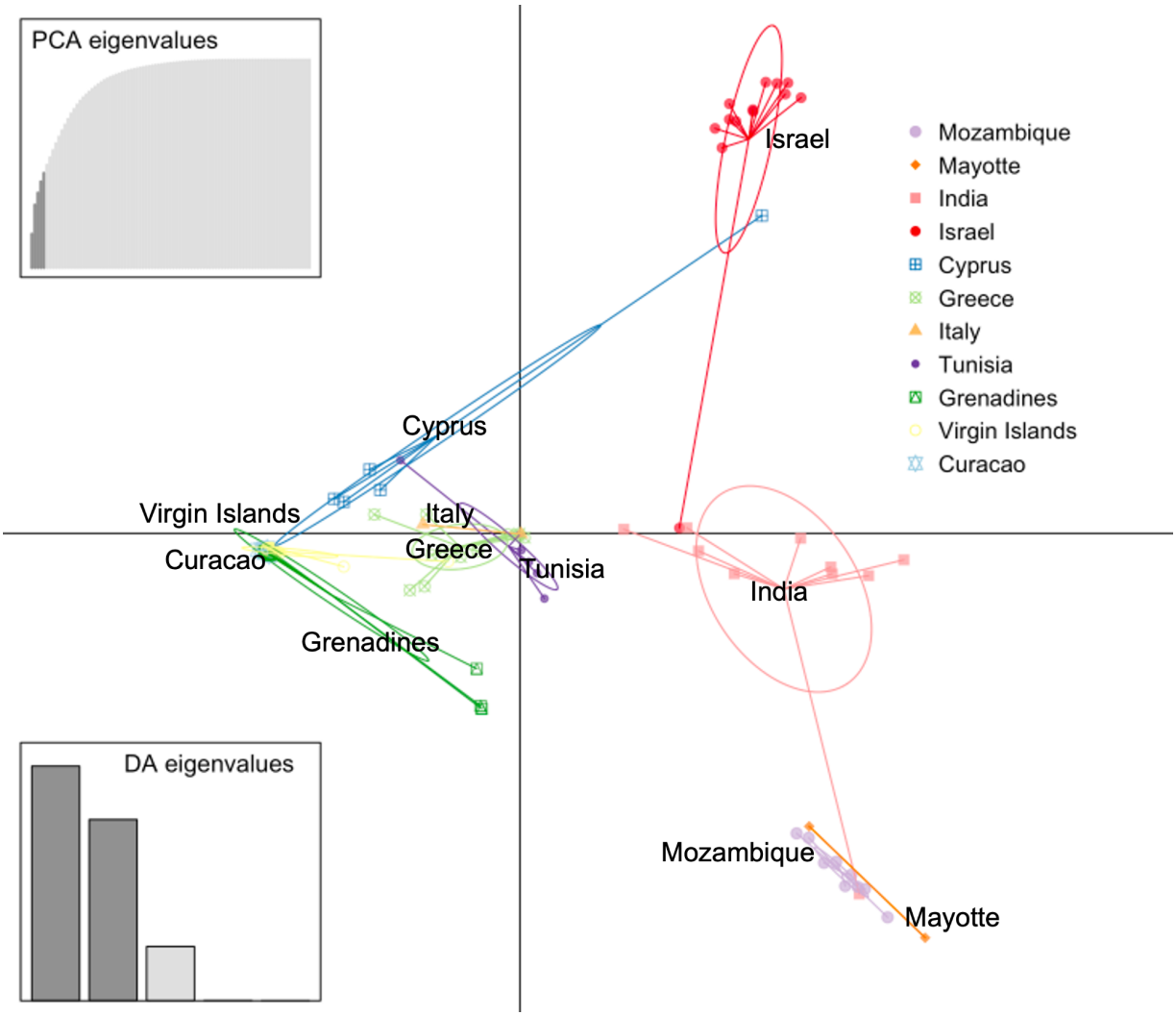


Figure 3.3: DAPC Discriminant Principles Components Analyses. 94 samples, 524 informative SNPS. The axes represent the first two Linear Discriminants (LD). Names on each circle represent a cluster and each symbol represents an individual.

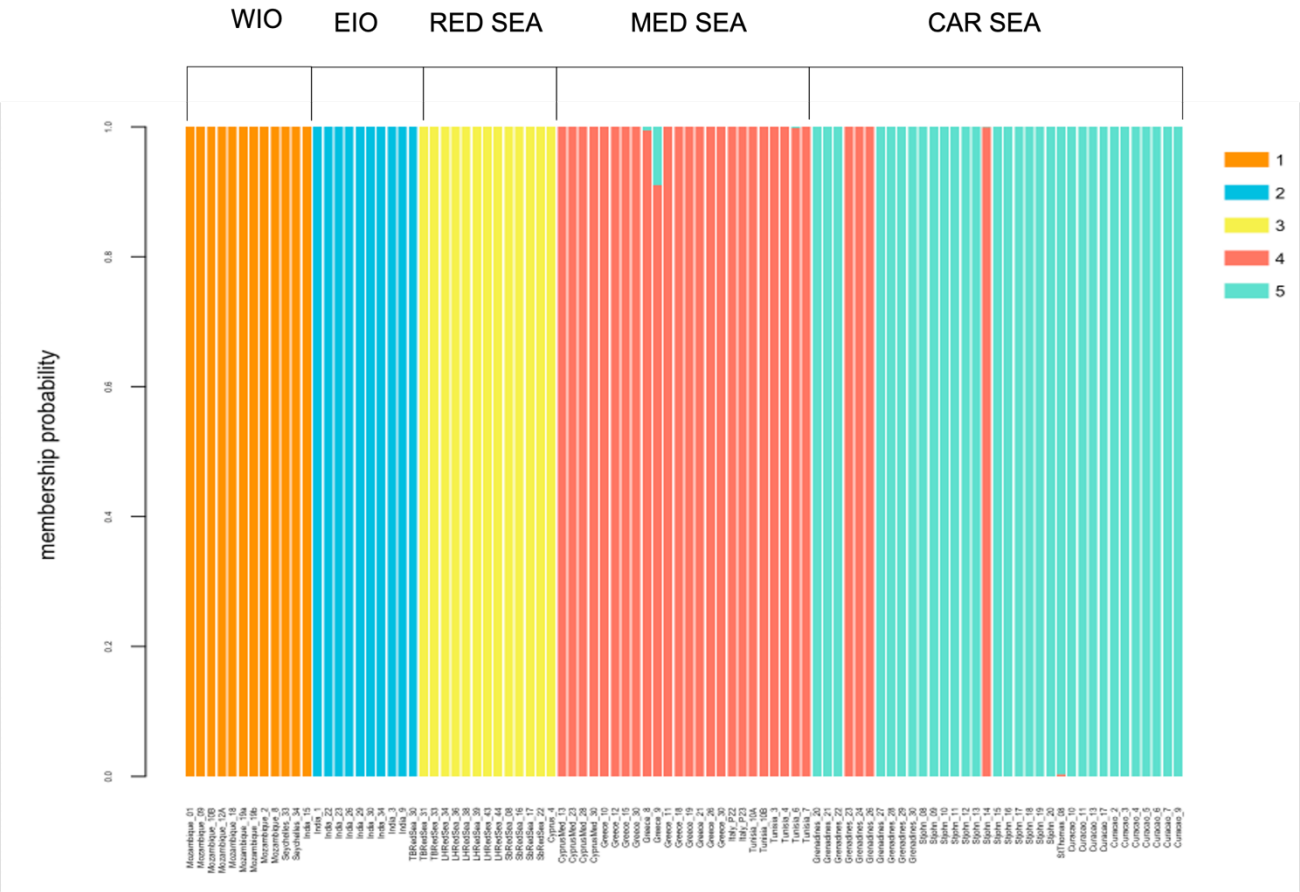


Figure 3.4. Individual assignment to group assignment probability by genotype composition. Curacao (n = 11), Cyprus (n=5), Greece (n=12), Grenadines (n=10), India (n=10), Light House Eilat, Israel (n=6), Mozambique (n=10), Mayotte (n=2), South Beach Eilat, Israel (n=4), St John (n=13), St Thomas (n=1), Taba Eilat, Israel (n=3), Tunisia (n=6) using adegenet package in R.

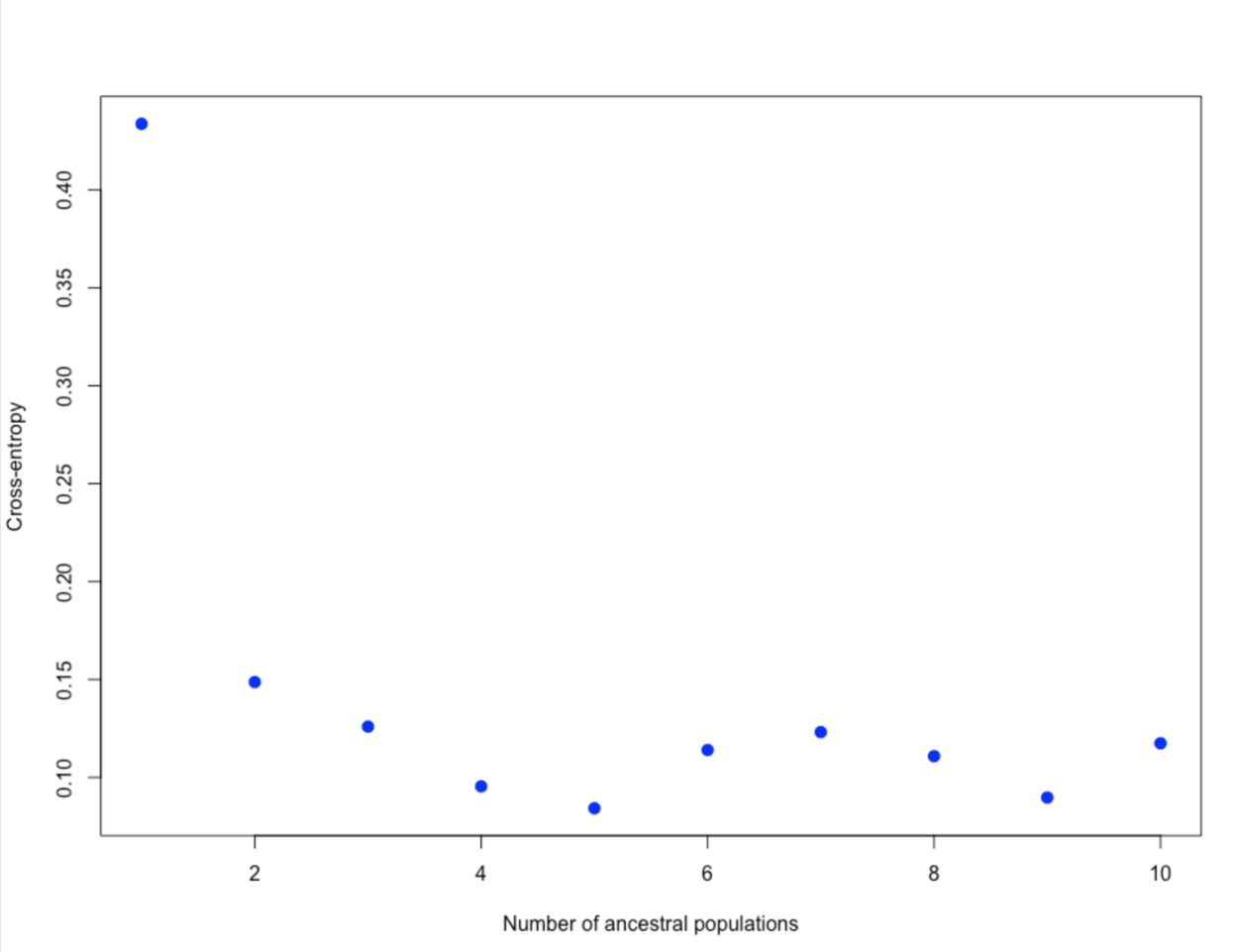


Figure 3.5. Cross- entropy values obtained for a given number of clusters (ancestral populations) for *H.stipulacea* using the LEA package.

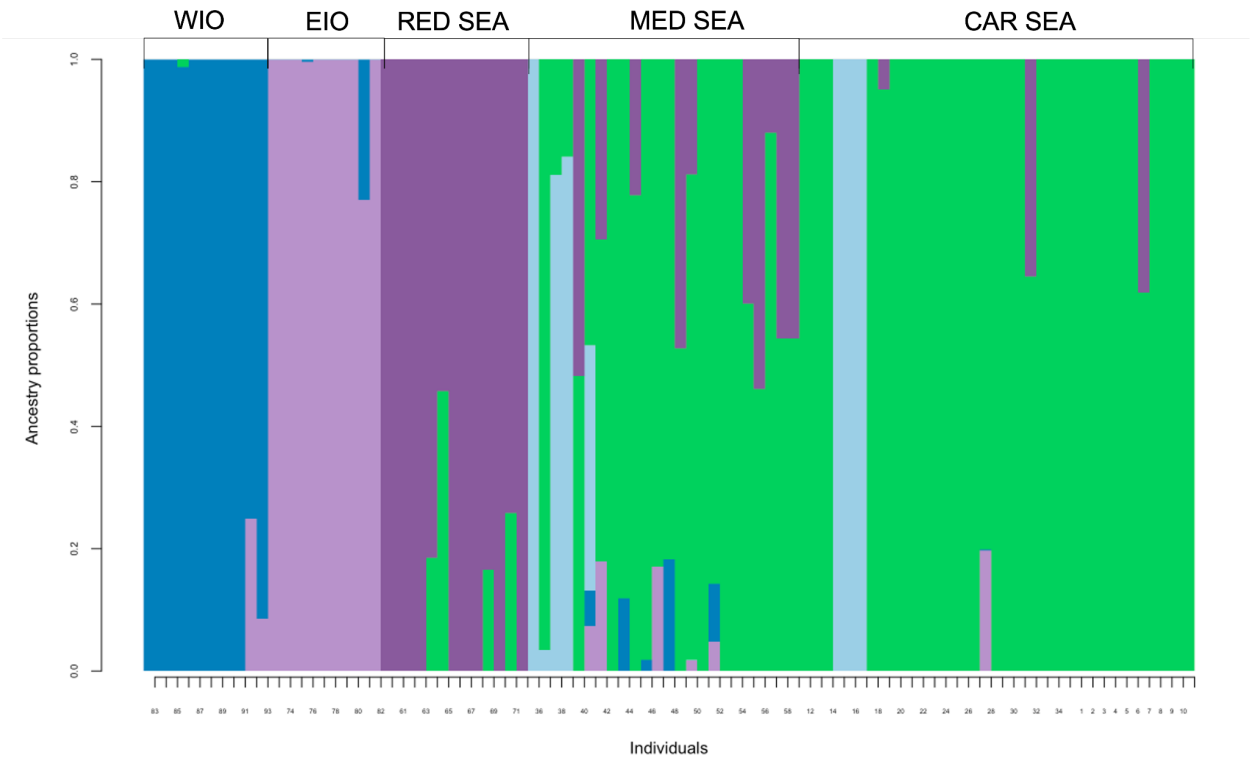


Figure 3.6. K=5. Ancestry matrix of 45 ancestry informative SNPs to distinguish among 35 Caribbean, 23 Mediterranean, 13 Red, and 22 Indian Ocean. Columns represent individuals that were placed in one of the five genetic clusters inferred by the program LEA.

3.8. TABLES

Table 3.1: Based on 524 loci, collection localities, samples sequenced at TCGB, samples sequenced at OSU, total number remaining after excluding low coverage loci, collector and depth. H_o is the observed heterozygosity. H_e is the expected heterozygosity. N_a is the number of alleles per population. HWE with the proportion of loci out using a chi-square test with all populations and conservative cut off, and the average F_{IS} per population. Average inbreeding coefficient F-statistic. N_e= effective population size median calculated using NeEstimator, 95% CI of Ne estimate using 45 loci.

Basin	Country/ Sites	TCGB	OSU	Total # samples after excluding low coverage loci	Depth	Ho	He	Na	Proportion of loci out of HWE	FIS	Inbreeding coefficient	Ne (95% CI)
Western Indian Ocean (12)						0.036	0.696	205	0.021	-0.894	0.145	0.2 (0.1-0.3)
	Inhaca Island, Mozambique	3	7	10		0.036	0.702	194	0	-0.912	0.119	
	Hajangoua, Mayotte	4	0	2		0.022	0.738	166	-	-0.834	0.162	
Eastern Indian Ocean (10)						0.023	0.681	227	0.063	-0.830	0.340	1.0 (0.3-24.0)
	Tamil Nadu, India	3	9	10		-	-	-	-	-	-	
Red Sea (13)	Eilat, Israel					0.178	0.179	907	0.246	-0.071	0.527	Infinite (133.9-Infinite)
	Taba, Eilat	0	3	3		-	-	-	-	-	-	
	Light House, Eilat	0	6	6	8 m	-	-	-	-	-	-	
	South Beach, Eilat	0	4	4	7-8 m	-	-	-	-	-	-	
Mediterranean (26)						0.143	0.112	771	0.101	-0.421	0.425	0.3 (0.2-0.4)
	Limassol, Cyprus	6	4	5		0.147	0.111	686	0.082	-0.175	0.524	
	Paros, Greece	5	7	12		0.114	0.081	648	0.059	-0.423	0.393	
	Palermo, Italy	0	3	3		0.002	0.986	9	0	-0.988	0.390	
	Malta, Tunisia	8	0	6		0.185	0.152	623	0.013	-0.632	0.220	
Caribbean (35)						0.139	0.074	665	0.130	-0.516	0.492	0.2 (0.2-0.3)
	Grenadines	15	0	10		0.162	0.093	664	0.128	-0.464	0.504	
	U.S. Virgin Islands, Great Lamshur Bay, St John	0	13	13		0.129	0.064	592	0.129	-0.457	0.468	
	U.S. Virgin Islands, Cay Bay, St Thomas	0	1	1	1-2 m	-	-	-	-	-		
	Spanish Waters, Curacao	11	0	11	2 m	0.130	0.065	593	0.130	-0.885	0.501	
Total	15	55	57	96								

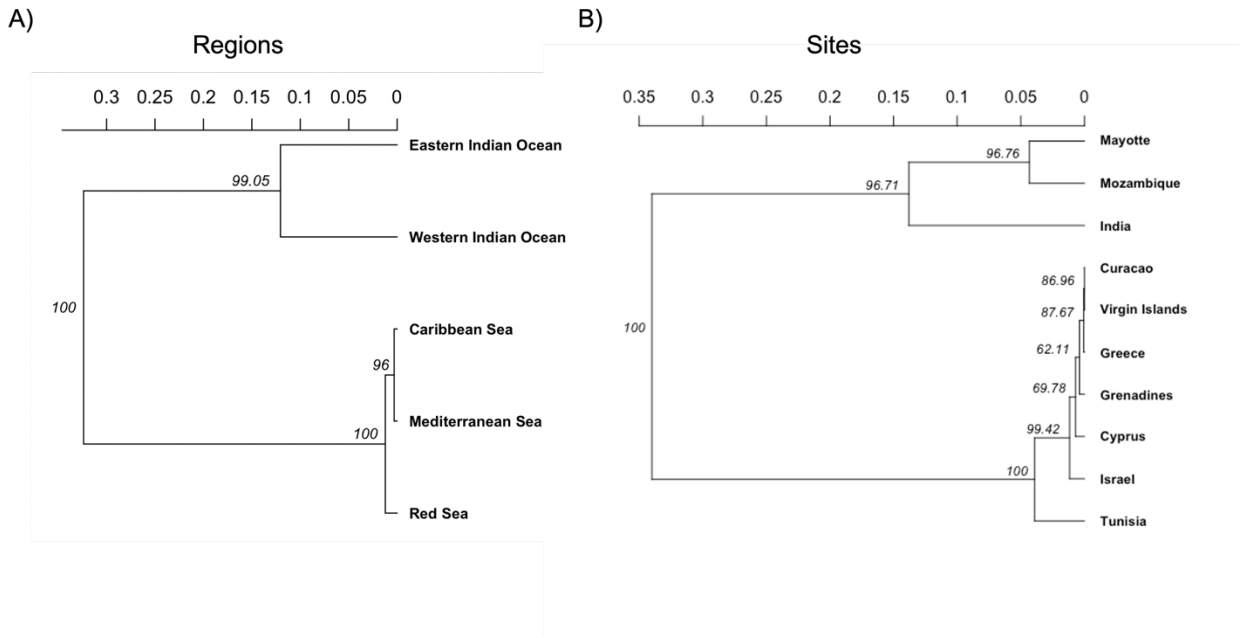
Table 3.2. Matrix of Pairwise F_{ST} values along (bottom-left, below the diagonal) and associated p-values (top-right, above the diagonal) for 11 sites (524 loci) of *H. stipulacea*. Bold are significant p-values.

	#	MOZ	MAY	IND	ISR	CYP	GCE	ITY	TUN	GRE	VI	CUR
Mozambique (MOZ)	9		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Mayotte (MAY)	2	0.498		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
India (IND)	10	0.666	0.683		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Israel (ISR)	13	0.881	0.892	0.878		**0**	**0**	**0**	**0**	**0**	**0**	**0**
Cyprus (CYP)	5	0.907	0.955	0.906	0.127		**0**	0.939	**0**	**0**	**0**	**0**
Greece (GCE)	12	0.896	0.959	0.882	0.206	0.103		**0**	**0**	**0**	**0**	**0**
Italy (ITY)	2	0.875	0.946	0.851	0.076	-0.032	0.144		**0**	**0**	**0**	**0**
Tunisia (TUN)	6	0.815	0.758	0.763	0.146	0.125	0.100	0.133		**0**	**0**	**0**
Grenadines (GRE)	10	0.830	0.830	0.839	0.256	0.148	0.105	0.236	0.173		**0**	**0**
U.S. Virgin Islands (VI)	14	0.940	0.975	0.941	0.293	0.193	0.154	0.300	0.295	0.020		0.391
Curacao (CUR)	11	0.938	0.974	0.939	0.283	0.188	0.151	0.298	0.291	0.0184	0.000	

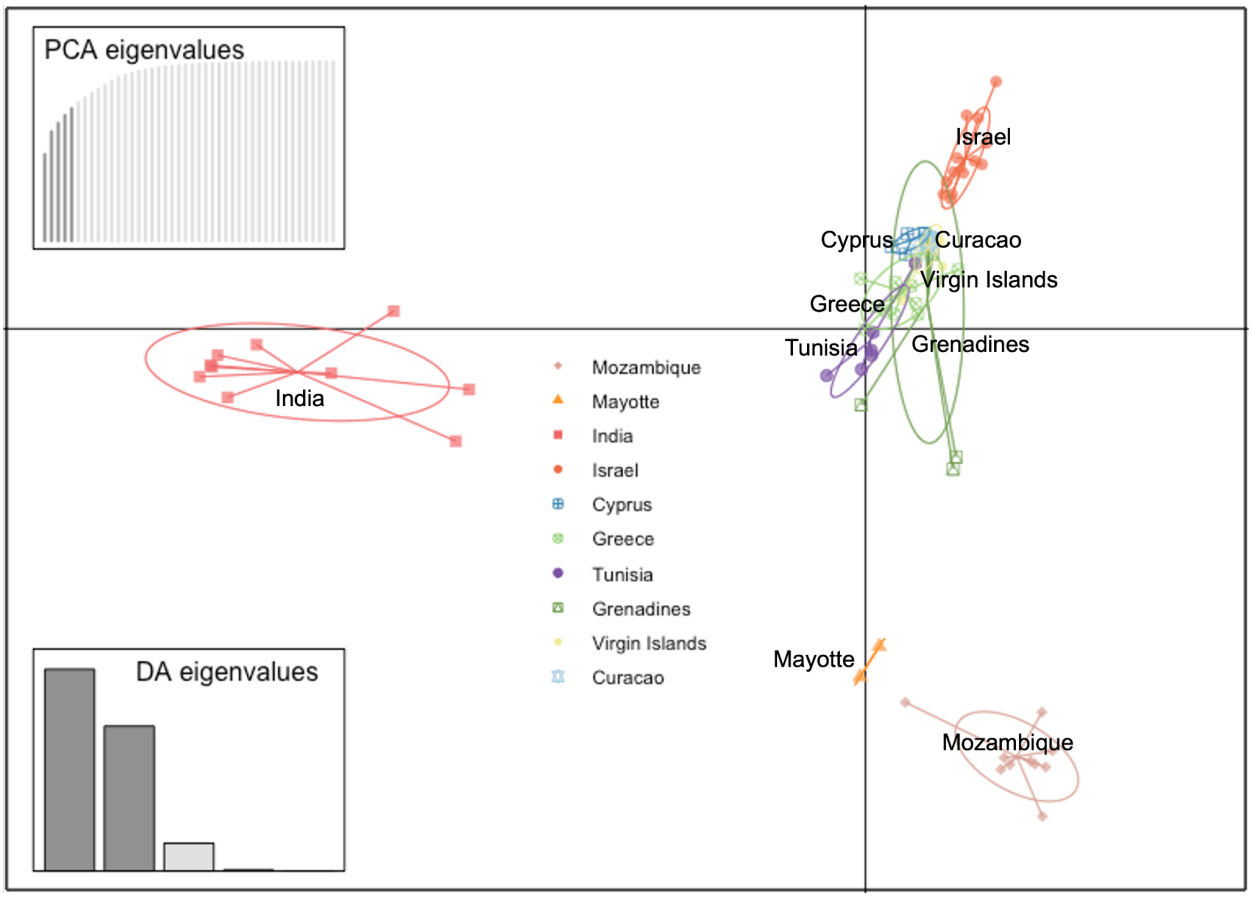
Table 3.3 Results of analysis of molecular variance (AMOVA) of *Halophila stipulacea* with 524 loci. Φ_{CT} is differences among regions, Φ_{SC} is the difference among sites within regions, Φ_{ST} is the differentiation among sites

Source	**Df**	**SSD**	**MSD**	**Variance Component**	**%**	**P value**	Φ_{CT}	Φ_{SC}	Φ_{ST}
Between regions	4	2092.86	523.22	28.50	62.59	0.01	0.63		
Between sites within regions	6	127.37	21.23	0.68	1.49	0.01		0.04	
Within sites	83	1357.19	16.35	16.35	35.91	0.01			0.64
Total	93	3577.42	38.47	45.53	100.00	-	-	-	-

3.9. SUPPLEMENTAL FIGURES AND TABLES



Supplementary Figure S3-1: Neighbor joining dendrogram using “aboot” function with “nei.dist” based on Nei’s genetic distance matrix of 10 sites, n=93, and 45 loci. A) Represents clustering by regions, and B) is clustering by sites. The vertical axis represents closeness of populations, and the numbers on each branch represent the probability of encountering nth occurrence of a particular genotype. The x-axis above the dendrogram represents the distance or dissimilarity between clusters. Prepared using the function “aboot” in “poppr” software.



Supplementary Figure S3-2: DAPC Discriminant Principles Components Analyses. 93 samples, 45 informative SNPS. The axes represent the first two Linear Discriminants (LD). Names on each circle represent a cluster and each symbol represents an individual.

Supplementary Table S3-1: Matrix of Pairwise F_{ST} values along (bottom-left, below the diagonal) and associated p-values (top-right, above the diagonal) for 10 populations (45 loci) of *H. stipulacea*. Bold are significant p-values.

	#	MOZ	MAY	IND	ISR	CYP	GCE	TUN	GRE	VI	CUR
Mozambique (MOZ)	10		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Mayotte (MAY)	2	0.631		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
India (IND)	10	0.894	0.956		**0**	**0**	**0**	**0**	**0**	**0**	**0**
Israel (ISR)	13	0.923	0.926	0.933		**0.006**	**0**	**0**	**0**	**0**	**0**
Cyprus (CYP)	5	0.939	0.968	0.968	0.408		**0.007**	**0**	**0**	**0.014**	**0.016**
Greece (GCE)	12	0.924	0.945	0.951	0.231	0.259		**0**	0.172	**0**	0.061
Tunisia (TUN)	6	0.798	0.674	0.813	0.489	0.351	0.297		**0.001**	**0**	**0**
Grenadines (GRE)	10	0.824	0.784	0.826	0.214	0.127	0.007	0.112		**0**	**0**
U.S. Virgin Islands (VI)	14	0.945	0.963	0.961	0.320	0.335	0.024	0.504	0.086		0.118
Curacao (CUR)	11	0.943	0.961	0.960	0.309	0.319	0.018	0.473	0.077	0.000	

Supplemental Table S3-2: Results of analysis of molecular variance (AMOVA) of *Halophila stipulacea* with 45 loci. Φ_{CT} is differences among regions, Φ_{SC} is the difference among sites within regions, Φ_{ST} is the differentiation among sites

Source	**Df**	**SSD**	**MSD**	**Variance Component**	**%**	**P value**	Φ_{CT}	Φ_{SC}	Φ_{ST}
Between regions	4	567.77	141.94	7.82	82.29	0.01	0.82		
Between sites within regions	5	23.64	4.73	0.43	4.52	0.01		0.25	
Within sites	83	104.00	1.25	1.25	13.19	0.01			0.87
Total	92	695.41	7.56	9.50	100.00	-	-	-	-

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CHAPTER 4

FRUITS AND FLOWERS OF THE INVASIVE SEAGRASS HALOPHILA STIPULACEA IN THE CARIBBEAN SEA

The following chapter is a reprint of Chiquillo, K.L., Barber, P.H & Willette, D.A. Fruits and flowers of the invasive seagrass *Halophila stipulacea* in the Caribbean Sea. *Botanica Marina.* https://doi/10.1515/bot-2018-0052/

Short communication

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Fruits and flowers of the invasive seagrass *Halophila stipulacea* in the Caribbean Sea

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Abstract: While the seagrass *Halophila stipulacea* reproduces both sexually and asexually in its native range, reproduction is largely asexual in its invasive range in the Mediterranean and the Caribbean Seas. Here we make the first report of fruit-bearing *H. stipulacea* in the Caribbean. Although the lack of reports of *H. stipulacea* fruit could be the consequence of past survey effort, multiple recent reports of both flowers and fruit across the invasive range strongly suggest that introductions of *H. stipulacea* in the tropical western Atlantic and Caribbean included both sexes of this dioecious seagrass. This finding may have important implications for the future dispersal, survival, and maintenance of the non-native population.

Keywords: Caribbean; *Halophila stipulacea*; introduced species; seagrass; sexual reproduction.

Halophila stipulacea (Forsk.) Ascherson is a small, tropical, dioecious, euryhaline, subtidal seagrass species. It is widely distributed in its native range along the western parts of the Indian Ocean, Eastern Africa, Arabian Sea, Persian Gulf, and Red Sea (Lipkin 1975a), where it forms monospecific and polyspecific meadows (Lipkin 1979, Malm 2006). Following the opening of the Suez Canal in 1867, the first fragments of *H. stipulacea* were observed in Rhodes, Greece in 1894 (Fritsch 1895, Lipkin 1975a). Since this first invasion, referred to as "the historical invasion", *H. stipulacea* has flourished, expanding its range broadly throughout the Mediterranean, forming meadows with native seagrass and algal species. Subsequently, in 2002, *H. stipulacea* invaded the Caribbean. It was first reported on the island of Grenada (Ruiz and Ballantine 2004), but rapidly spread west to Bonaire, north to Puerto Rico and south to Venezuela, spanning a distance of over 700 miles and forming monospecific and/or polyspecific seagrass meadows at a range of depths and substratum types (Vera et al. 2014, Willette et al. 2014, Ruiz et al. 2017).

Reproduction in *Halophila stipulacea* varies greatly in its native and introduced ranges. In its native range, the flowering season of *H. stipulacea* begins in May and ends in October, producing both staminate (male) and pistillate (female) flowers at depths of 2.5–5 m (Malm 2006), with a strong bias towards female flowers between July and October (Nguyen et al. 2018). Recent discoveries show that the flowering season begins later and/or lasts longer in the Mediterranean than it does in the Red Sea (Nguyen et al. 2018). Reports of sexual reproduction in the western Mediterranean show a stronger bias towards male flowers (Gambi et al. 2009) and that flowering can occur much deeper (5–25 m; Procaccini et al. 1999, Gambi et al. 2009). Only twice have fruits been reported in the Mediterranean (Lipkin 1975a, Gerakaris and Tsiamis 2015). Little is known about sexual reproduction of invasive *H. stipulacea* populations in the Caribbean Sea beyond a single report of male flowers only in one locality (Vera et al. 2014).

Given the strong differences in flowering and seed production in its native and invasive ranges, it is hypothesized that the spread and expansion rate of *Halophila stipulacea* into the Mediterranean and Caribbean Seas have occurred exclusively via asexual reproduction. If sexual reproduction is occurring, however, it would have a significant impact on our understanding of the potential for dispersal and the genetic potential of introduced populations.

In this study, we surveyed a population of *Halophila stipulacea* in the Caribbean to determine whether sexual reproduction occurs in this invasive population. Specifically, we examined a monospecific *H. stipulacea* meadow in Great Lameshur Bay, St. John, U.S. Virgin Islands (18° 19′ 6.6″ N, 64° 43′ 18.48″ W) in April 2017 to determine the presence of flowers and fruiting bodies.

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Surveys consisted of 10 predefined 1.02-m^2 removal plots at 5–6 m depth on sandy substrata with similar shoot density. Individual shoots were closely examined for reproductive structures (e.g. flowers and fruits). All flowers observed were collected, identified as female or male, individually photographed, and voucher specimens were frozen and stored at the University of California Los Angeles.

We found four male flowers and 27 fruits on *Halophila stipulacea* from three of the 10 experimental plots in Great Lameshur Bay, representing the first report of sexual reproduction in the Caribbean. As reported by Ackerman (2007), flowers were solitary, highly-reduced naked flowers on short pedicels close to the base of the plant. Lipkin (1975b) described *H. stipulacea* flowers as (1) oval in shape, and whitish to greenish towards the apex with small black dots; (2) pedicels 1.5 cm in length; (3) translucent tepals measuring 4.0–5.0 mm in length, 2.5 mm in width; (4) anthers bearing three stamens 3.0–5.0 mm in length; and (5) borne beneath the sediment. Flowers of Caribbean *H. stipulacea* largely conformed to this description (Figure 1, Table 1).

Although no pistillate flowers were observed, a total of 27 developed fruits were recovered, indicating that Caribbean populations of *Halophila stipulacea* produce both male and female flowers. The genus *Halophila* exhibits geocarpy – ripening below the sediment (Ackerman 2007), which was consistent with our observations. The density of fruits (27 fruits from three 1.02-m^2 plots) is higher than reports from the native Indian Ocean range (e.g. Gulf of Aqaba) where only one flower and several fruits were discovered along two 50-m transects (Hulings and Kirkman 1982). Size of fruits (Table 1) was comparable to those reported for *H. stipulacea* in the native range (den Hartog 1970) and Mediterranean Sea (Lipkin 1975c).

Most seagrasses produce flowers that yield fruits and seeds during reproductive periods (Kuo and den Hartog 2007). *Halophila stipulacea*, however, produces fewer flowers than many other seagrass species, including other species of *Halophila*, and reproduction is primarily vegetative in its native range (den Hartog 1970). Because previous studies of *H. stipulacea* found limited evidence of flowering or fruiting in the Mediterranean and Caribbean Seas, it had long been assumed that these invasive seagrass populations reproduced exclusively asexually; the results of this study, however, indicate that this is not the case. Sexual reproduction of *H. stipulacea* in the Caribbean may indicate that environmental conditions in the Caribbean are more similar to those in the native Indian Ocean range as light and temperature influence flower production in *H. stipulacea* (Procaccini et al. 1999). In contrast, fluctuations in sea temperatures in the Mediterranean Sea (Martrat et al. 2004, Shaltout and Omstedt 2014) may act to control the spread and growth of *H. stipulacea*, potentially limiting the availability of energy resources required for flowering and fruit formation.

The different reproductive ecologies of the two invasive *Halophila stipulacea* populations may impact their ability to spread, resulting in different population dynamics and underlying genetic architecture. For

Table 1: Plant part measures for *Halophila stipulacea* fruits and male flowers from 10 1.02-m^2 plots.

	Count	Average length (mm)	SE	Range
Fruits	27	4.38	0.009	3.90–5.39
Flowers				
Pedicel	4	15.2	0.073	13.20–16.70
Tepal	4	3.80	0.039	3.28–4.97
Anther	4	3.48	0.044	2.54–4.64

Measurements include total number of fruits and flowers, as well as average, standard error, and range of fruits and flower parts (pedicel, tepal, and anther).

Figure 1: Flower and fruit of Halophila stipulacea from St. John, United States Virgin Islands; (A) male flower showing pedicel (B) a developed fruit.
Scale bar: 2 cm.