# THE PHYLOGEOGRAPHY AND CONSERVATION GENETICS OF THE ENDEMIC SEAGRASS *HALOPHILA HAWAIIANA* ON O‘AHU, HAWAI‘I

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This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in Marine Science at Hawai‘i Pacific University. We the undersigned have examined this document and found that it is complete and satisfactory in all respects, and all revisions required by the final examining committee have been made.

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# DEDICATION

This thesis is dedicated to Dr. Catherine Unabia, without her this research would not have been possible. May her memory live on in all of us as we strive to treat others with kindness, patience and compassion while asking difficult scientific questions.

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ABSTRACT

Seagrasses are globally important in coastal marine ecosystems primarily for a number of ecological services they provide: extensive habitat and food for fish, invertebrates, epiphytic algae and microbes; carbon sequestration; forage for rare and endangered species including manatees and sea turtles; improvement of water quality by absorbing nutrients; and the stabilization of sediments in meadows that protect coastal zones and reefs from wave damage and erosion. Seagrass populations are declining globally, but in order to work towards restoration there is a need for ongoing and enhanced assessments of the ecology and conservation status of seagrass populations, including those in Hawai‘i. The endemic species *Halophila hawaiiana* is patchily distributed on multiple islands in the Hawaiian high islands, but this species is understudied especially in regard to its genetic diversity. This study uses a noncoding nuclear region, the internal transcribed spacer regions (*ITS1* and *ITS2*) to create a phylogeny of *H. hawaiiana* relative to other *Halophila* species. Haplotype networks also clearly show the close genetic affinity between *H. hawaiiana* and the Indonesia/Australia lineage of *H. ovalis*. The haplotype network is also an unambiguous way to visualize this close relationship as well as the difference in patterns of haplotype diversity between a sexually reproducing seagrass in *H. ovalis*, and an asexually reproducing species, *H. hawaiiana*. In addition, seven microsatellite loci were amplified and scored to compare the genetic diversity among and between Windward and Leeward seagrass populations on O‘ahu. Our results indicate *H. hawaiiana* is most closely related to *H. ovalis* from Australia and Indonesia based on the *ITS* gene fragment. We see two subunits in the clade of *H. ovalis* where the phylogeographic pattern shown seems to correspond to and follow the biogeographic division depicted by Wallace’s Line. This is the first time that this theoretical phylogeographic break has been observed in a marine angiosperm. When assessing genetic diversity between the Windward and Leeward *H. hawaiiana* populations, we saw uniformity in the results at all seven microsatellite loci. The lack of genetic diversity in both the microsatellite alleles and *ITS* haplotypes may have arisen due to predominately asexual reproduction. These low genetic variability patterns raise questions of how the species would be impacted by future changes in environmental pressure.

CHAPTER I:

# LITERATURE REVIEW OF HAWAIIAN SEAGRASSES

## *Introduction*

Seagrasses are globally important in coastal marine ecosystems primarily for a number of ecological services they provide: extensive habitat and food for fish, invertebrates, epiphytic algae and microbes; carbon sequestration; forage for rare and endangered species including manatees and sea turtles; improvement of water quality; and the stabilization of sediments in meadows that protect coastal zones and reefs from wave damage and erosion. Despite the high proportion of the length of coastlines to geographic area in the Hawaiian Islands, the importance of seagrasses is elevated, and yet are rarely recognized.

Marine angiosperms can store up to 10% of the total ocean organic carbon in their sediment and generate ten liters of oxygen for every one square meter of seagrass meadow per day making them important ecosystems for offsetting global climate change (Fourqurean et al., 2012; Orth et al., 2006). Seagrass meadows are considered a major blue carbon sink, with inorganic carbon levels estimated to be 0.87 ± 0.19% and 1.3 ± 0.3 mg C $cm^{-3}$ in *Halophila* seagrass beds in Australia (York et al., 2018). Seagrasses have efficient photosynthetic systems allow seagrass beds to serve as nutrients for diverse members of the ecosystem including microbes, protists and invertebrates in the sediments, epibiota on the leaves, and herbivores in the water such as the threatened Hawaiian green sea turtle (Russell et al., 2003). Their extensive root systems stabilize sediments, creating mounds of dense seagrass that promote particle deposition and serve as rich habitats for infaunal species (Evans et al., 2018).

Globally, seagrass populations are declining, but in order to understand the factors that may be contributing to these declines, there is an urgent need for ongoing assessments of the

status of seagrass populations (Waycott et al., 2009). In Hawai‘i, there are two species of seagrass, both in the genus *Halophila. Halophila hawaiiana* was long considered the only species of seagrass in the Hawaiian Islands and is especially important for its status as an endemic Hawaiian marine species (Fig. 1C), as well as being the obligate host plant for one of only four endemic Hawaiian marine gastropods, *Smaragdia bryanae* (Fig. 1A,B) (Unabia, 2011).

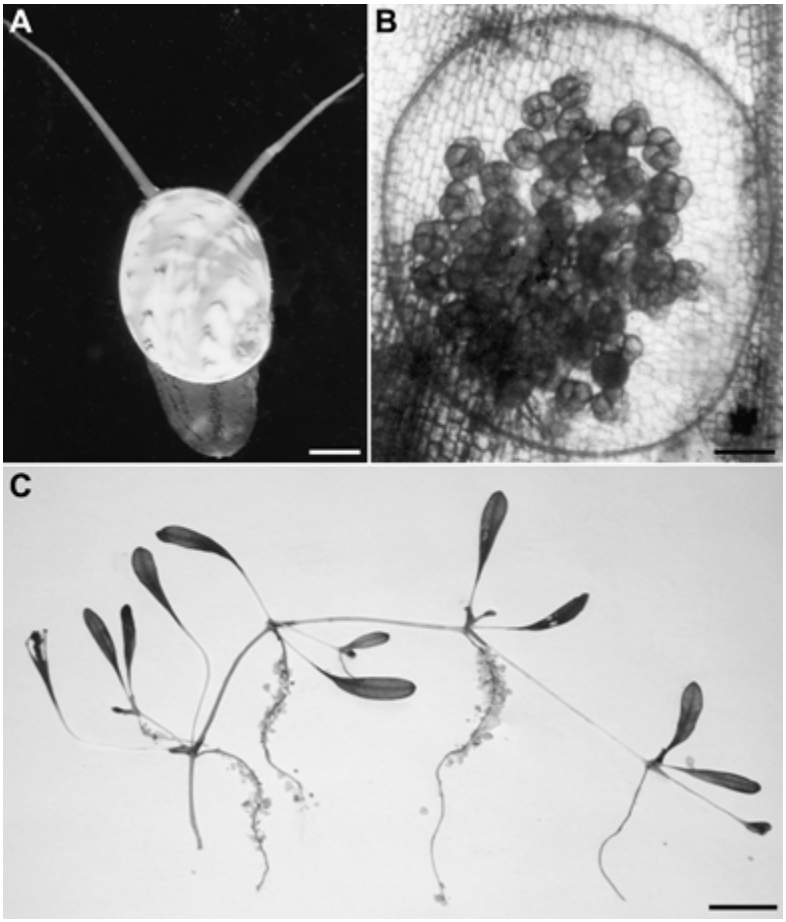


**Figure 1**. The endemic marine snail *Smaragdia bryanae* and the seagrass *Halophila hawaiiana*. A. Typical individual of *S. bryanae* (T. Burch, photo) (scale bar 0.8 mm). B. Transparent egg capsule of *S. bryanae* containing developing embryos, laid on a leaf basal section (scale bar=0.15 mm). C. The seagrass *H. hawaiiana*. Rhizome, roots, and most of the leaf petioles are normally buried under the surrounding sediment (scale bar 8 mm) (Unabia, 2011).

A second species, the cosmopolitan *Halophila decipiens,* was relatively recently documented in the Hawaiian Archipelago (Fig. 2; McDermid et al., 2002; Russell et al., 2003).

More study is needed regarding the current distribution and potential interactions between these seagrasses. Specifically, there is a need to understand whether there is evidence of cooperation, i.e. does one species favorably alter habitat for the other, versus whether there might be competition for resources and habitat. Other topics that merit additional study include differences in the communities they support, in levels of carbon fixation and sediment stabilization as well as other aspects of their roles and services provided to the coastal marine ecosystem.

**Figure 2.** Image of *Halophila decipiens* (Spielman, 2012).

This chapter will review what is presently known about *H. hawaiiana* and *H. decipiens* ecology, evolution and conservation status in the Hawaiian Archipelago, and will examine factors that contribute to ecosystem services provided to coastal marine communities in the islands, including their role in substrate stabilization, food, habitat, and filtration services. Here I will describe the life history and habitat of the two seagrasses, and will then examine differences in their life cycles, habitat preferences and reproductive mechanisms of each angiosperm in comparison to other species of *Halophila* as well as other seagrasses. Synthesizing and promoting awareness of basic information regarding the conservation status, habitat requirements and ecological roles played by both *H. hawaiiana* and *H. decipiens* has important

implications for the conservation of these species.

Halophila

*Halophila* is a genus in the family Hydrocharitaceae, and has a circumglobal distribution throughout tropical and subtropical coastal zones around the world (Hemminga & Duarte, 2000). Members of *Halophila* are identified by several morphological characteristics: petiolate leaves found in pairs, small shoots (1-20 cm), thin rhizomes at each node and placentas coming from the ovary with seeds being released when the outer layer of the ovary decays (Doty & Stone, 1966). There are ten described species of *Halophila*: *H. ovalis*, *H. madagascariensis, H. ovata, H. minor, H. australis, H. johnsonii, H. decipiens, H. capricorni, H. stipulacea,* and *H. hawaiiana* (Kuo and den Hartog, 2001). *Halophila ovalis* is a widespread species found in the Indo-West Pacific, east Asia and the tropics and its populations are genetically diverse (Ruggiero and Procaccini, 2004). Molecular sequence data from nuclear loci suggest that *H. hawaiiana* and *H. johnsonii* (an endemic species in Florida) both evolved from *H. ovalis* (Waycott et al., 2002), although a recent molecular systematic analysis concluded that *H. johnsonii* is actually a population of *H. ovalis* (Waycott et al., 2021). Additionally, previous molecular phylogenetic reconstructions suggest that *H. decipiens* is ancestral to *H. ovalis,* although not as closely related as *H. hawaiiana* is to *H. ovalis* (Short et al., 2007). *Halophila decipiens* is biogeographically similar to *H. ovalis* in that both species can be found widely distributed across the northern and southern hemisphere as compared to *H. hawaiiana*, which is just found in the Hawaiian Islands (Fig. 3 & 4).


**Figure 3.** Distribution of *Halophila decipiens* (McMahon & Waycott, 2009).

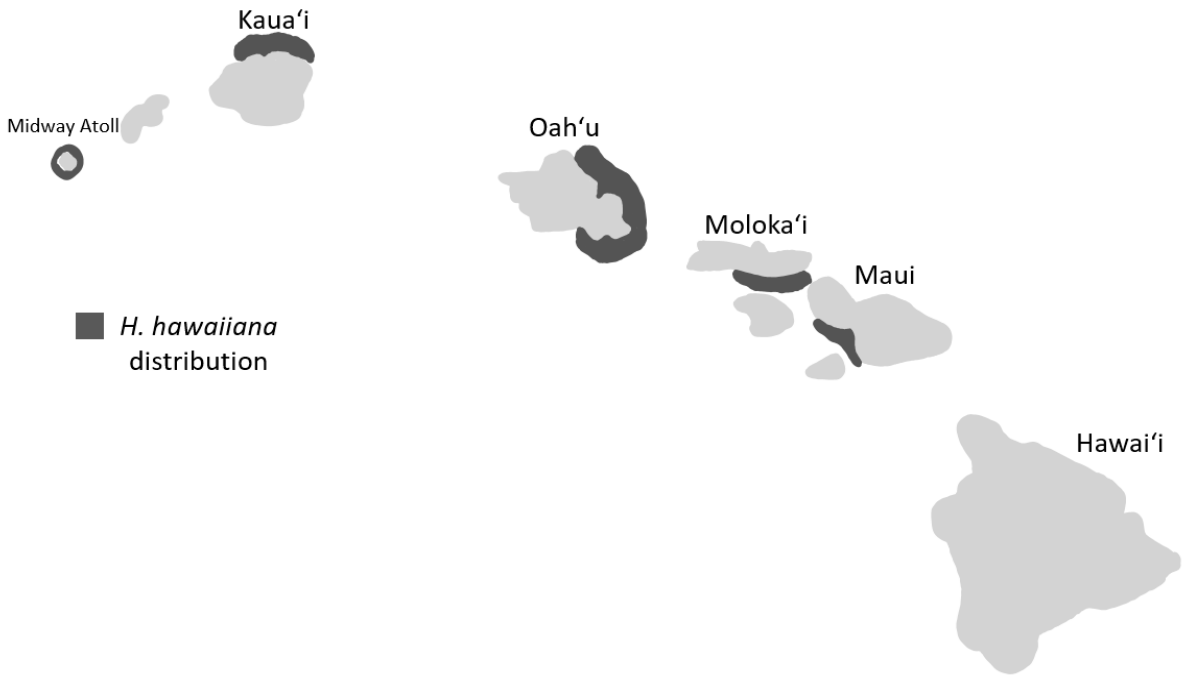


**Figure 4.** Distribution of *Halophila hawaiiana*.

High levels of morphological diversity tend to suggest relatively high genetic diversity in populations however, this may not be true in *H. hawaiiana.* McDermid et al. (2003) examined morphological characteristic and performed DNA sequence analysis on 64 samples of *H.*

*hawaiiana* collected from 18 different sites across the Hawaiian Archipelago and found that there were significant differences in leaf length, leaf width, leaf length to width ratio and internode length among the samples (ANOVA, $F_{0.001(1)17,200} = 2.56$, $p<0.001$). However, when DNA sequences from the chloroplast *trnL* intron were compared, only two closely related haplotypes were found (McDermid et al., 2003).

McDermid et al. (2003) demonstrated that there may be low genetic diversity amongst *H. hawaiiana* populations. The author acknowledged that it was possible that environmental conditions had contributed to the low levels of genetic diversity, but then failed to identify or suggest any specific contributing environmental conditions. It is also important to note that this study only amplified a single small DNA marker from the chloroplast, and it is possible that the low variability reflects a low mutation rate associated with this gene fragment, and that more rapidly evolving gene sequences might be more appropriate to further explore this issue. Based in part on the low variability found in phylogenies using the *trnL* locus, we decided to use *ITS* gene fragments, which has been used extensively in phylogenetic studies in many angiosperm families and has been particularly useful in improving our understanding of species relationships in these families (Waycott et al., 2002; Baldwin et al., 1995). Additionally, we used seven previously published microsatellite loci to examine phylogenetic status and population structure in *H. hawaiiana*, as these markers tend to provide appropriate levels of variability to address these questions (Xu et al., 2010). Microsatellite loci were used to evaluate gene flow in the seagrass *Posidionia oceanica* and could potentially be used to study gene flow patterns in *H. hawaiiana* (Migliaccio et al., 2005).

*Genetic Diversity in Seagrass*

Genetic diversity is defined as nucleotide diversity, haplotype diversity, allelic richness, and heterozygosity within and among populations (Oliva et al., 2014). Patterns in genetic diversity can provide valuable information on evolutionary processes and connectivity within and among populations (Ehrlich & Wilson, 1991). McDermid et al. (2003) compared differences in the morphological characteristics and DNA sequences of *H. hawaiiana* samples across the Hawaiian Archipelago. This study found significant morphological differences between populations in leaf length, leaf width, leaf length to width ratio and internode length. However, when the sequences from the cpDNA intron *trnL* were compared, low genetic variation among samples was revealed. Low genetic variation in *H. hawaiiana* suggests several possible mechanisms may be at play, including asexual reproduction, low mutation rates, strong selection, genetic drift, and a lack of gene flow among populations. Extremely low haplotype diversity was exhibited within collection sites (McDermid et al., 2003). It is important to note that McDermid et al. (2003) only sequenced a single cpDNA fragment and there are other sequences with higher substitution rates which could be used to further explore this issue (Shaw et al., 2005). Chloroplast DNA sequences are a common source of data for inferring plant phylogenies (Shaw et al., 2005). Specifically, noncoding regions of the chloroplast have been used to explore intraspecific relationships among populations because noncoding regions, both organellar and nuclear, tend to exhibit faster mutation rates than coding regions (Gielly & Taberlet, 1994). Of the plant studies investigating cpDNA, 84% of these studies utilize the following regions: *rpS16, rpL16, trnK-matK-trnK*, or *trnL-trnL-trnF*, showing a reliance on specific regions (Shaw et al., 2005). However, organellar DNA sequence fragments have shown no differences between populations of *H. hawaiiana* (McDermid et al., 2003). Noncoding nuclear regions such as the

internal transcribed spacer regions (*ITS1* and *ITS2*) have also proven informative in plant studies quantifying and partitioning genetic diversity for phylogenetic and phylogeographic studies, although some systems have shown multiple divergent copies can be present in a genome (Won & Renner, 2005). However due to concerted evolution, this locus has been shown to be reliably consistent in certain lineages, therefore many plant phylogenies focus on *ITS* sequences, and this marker is considered a barcoding region (Shaw et al., 2005).

DNA barcoding is a widely applied method that uses relatively short (usually <800 bp), orthologous DNA sequences to represent the far larger and more complex genomes of plants and animals, to identify species and facilitate evolutionary studies (Herbert et al., 2003). The barcoding sequences *ITS* and *rbcL* (ribulose-bisphosphate carboxylase) are fairly widely applied in plant studies, and have been used to provide phylogenetic information and clarify patterns of evolution within and among taxa in the genus *Halophila* (García-Escudero et al., 2022; Kim et al., 2017; Lucas et al., 2012; Waycott et al., 2006). In a study by Kim et al. (2017), a phylogenetic analysis of the fragment was used to reconstruct the patterns of genetic variability of *Halophila nipponica*, an eastern Asian seagrass found in coastal Korea and Japan. Through the use of DNA barcoding it was confirmed first, that *H. nipponica* was a member of the genus *Halophila,* and second that the populations found in Korea were genetically connected with the species found in Japan. Researchers used DNA barcoding to infer the evolutionary history of *H. nipponica,* suggesting that it had diverged from a tropical ancestral species and was transported via ocean circulation and then adapted to a more temperate environment (Kim et al., 2017). Kim et al. (2017) showed *H. nipponica* is endemic to Japan and likely derived from *H. ovalis*. Similarly, *H. hawaiiana* is considered a close relative of *H. ovalis* and the application of *ITS* DNA barcoding could provide a better understanding of the evolutionary history of this endemic

Hawaiian seagrass.

Numerous molecular studies (Danchin et al., 2011; Dias & Ressler, 2014; Gluckman et al., 2007) as well as neo-Darwinian synthesis theory have shown that levels of population genetic diversity are strongly influenced by reproductive strategy, ranging from random mating (panmixia) to the opposite extreme of full-sib or parent-offspring mating, also known as strong inbreeding. In addition, sexual reproduction is a powerful force in maintaining genetic diversity levels from generation to generation (Noble, 2015). However, although rare in the animal kingdom, asexual reproduction is relatively widespread in angiosperm biology (Hemminga & Duarte, 2000), and this can have important implications in terms of genetic homogenization (Arnaud-Haond et al., 2012). Sexual reproduction is well-known to maintain higher genotypic diversity, relative to that of asexual taxa, due to meiotic recombination at prophase I during gamete formation, the random process of fertilization, and pollen / seed dispersal (Arnaud-Haond et al., 2012). However, there is relatively little known about the reproductive strategies that many seagrasses use. This is one reason why studies based on molecular markers are useful in gaining a better understanding of how reproductive strategy shaped population structure, which in turn is helpful in determining the relative level of resilience of different species of seagrasses, particularly in the case of significant environmental disturbance (Phan et al., 2017).

Based on the different reproductive strategies of *H. hawaiiana* and *H. decipiens*, it will be interesting to compare levels of genetic diversity within and between populations of each species at different locations throughout their respective ranges on O‘ahu. Obtaining a better understanding of patterns of gene flow and genetic diversity in Hawaiian seagrasses will be an important step in developing successful management strategies for the conservation of these species.

*Geographic Distribution*

There are established populations of *H. hawaiiana* and *H. decipiens* throughout the Hawaiian Archipelago. However, *H. hawaiiana* has not been documented on the island of Hawai‘i, however, *H. decipiens* is found there (McDermid et al., 2002). The absence of *H. hawaiiana* on the island of Hawai‘i could be due to a lack of suitable habitat, or possibly due to the low vagility of *H. hawaiiana* and the relatively young geological age of Hawai’i (~0.53 myr), the most recently formed of the high islands (Doty & Stone, 1966; Neall & Trewick, 2008).

*Halophila decipiens* has been anecdotally reported by Navy divers as occurring in large meadows offshore of Reef Runway at depths in excess of 20 m (C. Unabia personal communication, 2020). Although the geographic source of *H. decipiens* is unknown, this seagrass has been documented on O'ahu since 2001, when McDermid et al. (2001) first recorded it. Russell et al. (2003) found evidence of *H. decipiens* in the stomachs of Hawaiian Green Turtles in 1998, which suggests that this seagrass has been around for at least two decades.

On O‘ahu, neither *H. hawaiiana* nor *H. decipiens* is known to occur on the North Shore, possibly due to high wave energy and strong coastal currents at this location (C. Unabia personal communication, 2020). A previous study in Florida showed that the seagrass *Zostera marina* experienced substantial damage and loss when surface current flow reached 4 m/s. On O‘ahu’s North Shore average current velocity has been measured at 6 m/s during relatively common high surf events (wave heights of 10 - 15 m) in the winter (Firing, 1996; Gaylord & Denny, 1997). This suggests that it may be difficult for seagrass to establish populations on the North Shore.

There are locations on O‘ahu where both *H. hawaiiana* and *H. decipiens* occur in close proximity, however there is no documentation of both species growing together in a single shared patch. Both species can be found in Kāne'ohe Bay, however, *H. decipiens* occurs in

deeper water, typically deeper than 1-2 m, while *H. hawaiiana* prefers shallower depths, 0-1 m (Chan, 2014). It is not yet clear what the relationship between these two species is, for example, whether *H. decipiens* and *H. hawaiiana* are engaged in substrate competition. Although competition between marine plants has been studied, these studies have generally been done in rocky intertidal areas where plants are competing for space (Williams, 1987). *Halophila decipiens* and *H. hawaiiana* have not been observed growing in the same patch and it appears that the two seagrasses prefer different habitats. The relationship between these seagrasses warrants further investigation because if there was competition for resources it could be detrimental to the endemic *H. hawaiiana* due to its presumed low dispersal ability. Competition would also be detrimental to species that rely on *H. hawaiiana* for food and shelter such as *Smaragdia bryanae,* which would likely starve if *H. decipiens* were the only available food resource (Unabia, 2011).

It is also possible that *H. hawaiiana* and *H. decipiens* coexist without competition, as competitive exclusion is known to be a powerful ecological force where two species have identical resource needs. One personal observation suggests that *H. decipiens* patches have remained in the same location for over ten years with no noticeable expansion (C. Unabia personal communication, 2020). *Halophila hawaiiana* and *H. decipiens* may have different microhabitat requirements, which prompts questions of what these requirements are, and this is another area that warrants additional field studies.

*Habitat Preferences*

All seagrass species have four fundamental habitat requirements: a marine environment, sufficient sunlight for photosynthesis, a suitable substrate that allows root establishment, and

adequate depth for complete immersion in seawater even during extreme low tide events (Hemminga & Duarte, 2000). With 18 species, *Halophila* is the largest genus of seagrasses in the world, and its species are globally distributed. Some *Halophila* species are restricted to a particular location such as *H. hawaiiana,* which is only known from a few locations in Hawai‘i (Figure 4). In Hawai‘i, *H. hawaiiana* is found growing subtidally at depths of 0.5 to 4 meters compared to *H. decipiens* which grows in depths of 0.5 to 35 meters (Dawes et al., 1989; Doty & Stone, 1966). There may be differences in the photosynthetically active radiation (PAR) profile of these angiosperms due to their depth preferences, where *H. decipiens* is found at deeper depths (35 m) than H. hawaiiana (5 m) (Spielman, 2012). Since *H. hawaiiana* prefers shallower depths, it tolerates higher levels of solar radiation and shorter wavelengths due to differences in UV-absorbing pigments compared to *H. decipiens* (Durako et al., 2003). Both *H. hawaiiana* and *H. decipiens* can be found in areas where they may experience occasional aerial exposure during extreme low tides, although *H. decipiens* tends to be found in deeper locations (Duarte, 1991).

There are differences in the sediment sizes where *H. hawaiiana* establishes populations compared to *H. decipiens*. Spielman (2012) collected sediment cores from seagrass beds of *H. hawaiiana* and *H. decipiens* at eight sites around the island of O‘ahu, Hawai‘i. Sediment particle size was analyzed at these sites, and phi values were generated based on the Udden-Wentworth scale, where larger values indicate smaller grain size. *Halophila hawaiiana* was found occupying habitats with significantly larger sediment particles, with a phi value of 1.77 compared to *H. decipiens,* which is found in muddy substrates with smaller sediment grains with an average phi value of 3.24 ($p<0.01$; Spielman, 2012). Additionally, by looking at the rhizome biomass information can be gained regarding the type of sediment grain sizes these seagrass species prefer. A study by Fonseca & Bell (1998) found that larger rhizome biomass was correlated with

larger sediment grain size, this is consistent with the results of Spielman (2012) where rhizome biomass of *H. hawaiiana* was 20% larger than that of *H. decipiens,* and *H. hawaiiana* was consistently found growing in patches with larger sediment grain size compared to *H. decipiens.*

Differences in organic content of sediments in *H. decipiens* versus *H. hawaiiana* were also found by Spielman (2012). On average, seagrass occurs in sediment with an organic carbon content below 5% (Barko & Smart, 1983; Koch, 2001) however, the organic carbon content in the sediment composition of *H. decipiens* was over 12% in several locations on O‘ahu (Spielman, 2012). High organic content is strongly influenced by the location of seagrass beds, as well as grain size and other aspects. But, under these conditions, sediments tend to become anoxic, and seagrass roots and organisms inhabiting the sediment can be negatively impacted, as they become anoxic reducing environments high in sulfide (Koch, 2001). Since we see populations of *H. decipiens* and associated infauna thriving in these conditions, it is possible that *H. decipiens* uses its roots to provide oxygen below the sediment surface, counteracting harmful impacts and creating suitable habitat (Hemminga & Duarte, 2000). The ability of *H. decipiens* to establish populations in such environments imparts a significant ecological service by absorbing nutrients.

*Reproduction and Growth*

Both seagrasses and terrestrial angiosperms reproduce either sexually or asexually, and some species use both strategies. Seagrasses are generally dioecious, with separate male and female plants, and have highly specialized mechanisms for dispersing pollen in the marine environment (Ewanchuck & Williams, 1996). Physical properties such as current flow and ocean temperature have a strong effect on seagrass pollen dispersal (Hemminga & Duarte, 2000).

*Halophila hawaiiana* is thought to be dioecious, and appears to form staminate and ovulate flowers in separate populations. Female flowers have been observed, but fruit with seeds have not been documented. The release of pollen by male flowers of *H. hawaiiana* was observed in aquaria where Herbert (1986) documented the anthesis and release pollen of a staminate flower over an eight hour period. Under natural conditions it is expected that currents might speed up this process. However, there are no published observations of staminate and ovulate flowers in the same seagrass patch in the field. It is challenging to estimate or predict pollen dispersal patterns. Doty & Stone (1986) found that *H. hawaiiana* pollen is negatively buoyant so this could also limit pollen dispersal distance. Additionally, it is possible that pollen release occurs at night, as in other species in Hydrocharitaceae, and is therefore very challenging to studies aimed at understanding this process (Cox & Tomlinson, 1988).

Although most seagrasses are dioecious there are four species in *Halophila* that are known to be monecious, meaning both male and female floral shoots are produced on a single rhizome (Fig. 5; Kuo et al., 1995). *H. decipiens* is monecious and produces male and female flowers at the base of each leaf pair along the rhizome. In locations outside of Hawai‘i, fruits are located directly below the sediment where seeds are deposited. McMillian (1991) found that these buried seeds could remain dormant for up to two years in the laboratory. However, there is no record of how long these seed banks remain dormant in the natural setting.

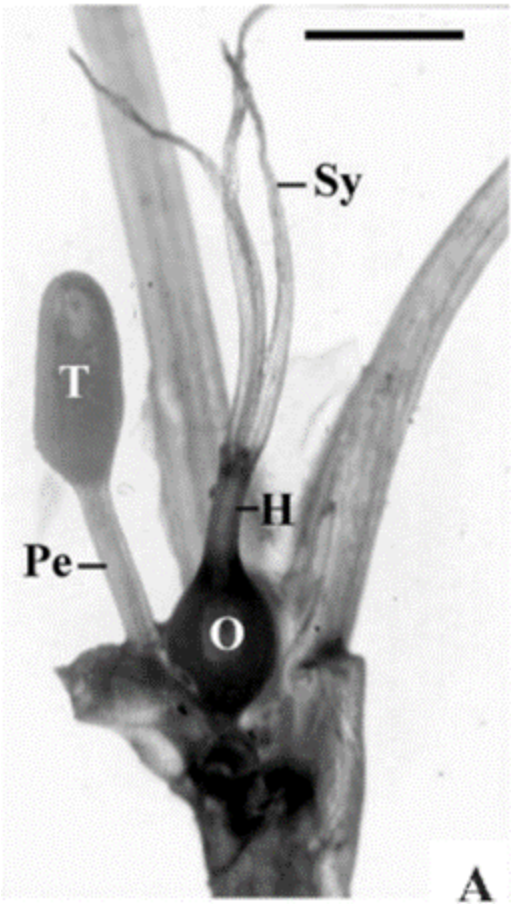


**Figure 5.** The reproductive anatomy of *Halophila decipiens* as seen on the same shoot, featuring a male pedicel (Pe) and tepals (T) and a female the hypanthium (H) and three styles (Sy) growing out of an ovary (O). (Scale = 4 mm; Kuo et. al., 1995).

The amount of time seed banks can lay dormant could have important implications for restoring populations after mass mortality events (Hammerstrom et al., 2006). Hammerstrom et al. (2006) collected 122 seed density cores (5 cm in diameter, 3 cm in depth) in five different *H. decipiens* beds in Southern Florida and found an average of 4.95 ± 5.7 seeds per core. More seeds were found in the nearshore cores (10 m from shore) compared to the offshore cores (20 m from shore). Seeds were found in the core samples year-round, which supports the idea of seagrasses storing a seed reserve for potential mortality events. Hammerstrom et al. (2006) estimated the seed bank to be about 134–3,414 seeds $m^{-2}$ which is lower than previous research

by McMillian & Soong (1989) which estimated the seed bank for *H. decipiens* in Panama to be between 600 and 13,500 seeds $m^{-2}$. This difference could have to do with time of year the seed density cores were collected, for example, seagrasses often flower in the summer, or might be due to physical differences in substrates that make it hard for seeds to persist and survive in the sediment.

Since *H. hawaiiana* seed production has not been observed, it has been assumed to grow primarily through rhizome clonal expansion, which according to Tomlinson & Vargo (1966) has greater importance for seagrass growth since it requires less energy than reproduction. *Halophila hawaiiana* growth via rhizome elongation is estimated at 89 cm per year, while *H. decipiens* growth rate was estimated at 215 cm per year (Marba & Duarte, 1998).

*Ecological Role and Importance of Seagrass Beds*

Sediment Stabilization

Seagrasses form a dense system of roots and rhizomes that hold and stabilize the underlying sediment and prevent particle resuspension, which in turn increases the nutrient content below the surface, and in turn increases substrate surface elevation and prevents coastal erosion (Potouroglou et al., 2017). The roots and rhizomes form a strong, secure mat that strengthens coastal sediments, a phenomenon that is particularly important during tropical depressions and hurricanes that threaten coastline integrity. The ability of seagrasses to prevent resuspension of particles is important in decreasing turbidity and thus creating clearer water that allows more light to penetrate into the water column, which is beneficial for seagrasses growth (Newell & Koch, 2004).

In terms of increasing coastal marine substrate elevation, a study in Scotland showed that

seagrass beds had a statistically significant increase in sediment elevation of 9.01 mm per year. This study utilized a field method called surface elevation change pin (SECP) which places metal rods in vegetated and unvegetated areas. The metal rods were surveyed every month for a year to see how much sediment had accumulated or eroded around the metal rod. In unvegetated locations there was an increase of sediment in the winter and decrease in summer which resulted in no net increase or decrease (Potouroglou et al., 2017). Increase in sediment helps to protect shorelines because as sediment accumulates water depths decrease and the force of waves lessens, effectively preventing coastal erosion (Christianen et al., 2013). In the Potoruoglou et al. (2017) study, the increase in sediment is associated with high canopy and biomass of the seagrass, however canopy height may not be the determining factor. A study by Christianen et al. (2013) showed that low canopy height (< 5 cm) and low-biomass (10 g / m) can still provide shoreline protection. *Halophila decipiens* has what is considered relatively small leaf biomass and was found to increase the threshold velocity for the movement of sediment compared to unvegetated sand, in similar fashion to the effect of other seagrasses (Fonseca, 1989). The leaf size of *H. decipiens* is comparable to *H. hawaiiana* so it is expected that this seagrass would also increase the threshold velocity of sediment, so both species are likely highly beneficial in mitigating erosion

Seagrass Beds as Nursery Habitats and Food for Coastal Marine Communities

In coastal ecosystems, seagrass canopies provide shelter for developing vertebrates and invertebrates. In Hawai‘i, post-settlement juvenile fishes, such as moi (*Polydactyl sexfilis*), 'omilu (*Caranx melampygus*), ula aukea (*Caranx ignobilis*), and ‘Ama‘ama (*Mugil cephals*) all utilize seagrass beds as habitats. For these species juveniles utilize seagrass bed habitats which

allows them to mature in an environment sheltered from predators and rich in nutrients (Leber et al., 1996; Waycott et al., 2009).

There are also endangered and endemic Hawaiian marine species that rely on seagrass for food. 95% of the diet of the threatened green sea turtle (*Chelonia mydas*) is seagrass, (Stokes et al., 2019), so any decline in seagrass bed coverage would be extremely detrimental to sea turtle health. Green sea turtles prefer young seagrass leaf tissue because it tends to be higher in nitrogen relative to older leaves, but removal of young leaves causes stress in the plants and sometimes leads to a decline in leaf production, which is the likely explanation for the tendency of sea turtles to continually move to different seagrass plots after some time (Stapel & Hemminga, 1997; Zieman et al., 1984). Additionally, the endemic Hawaiian marine snail *Smaragdia bryanae* is completely reliant on the presence of healthy *H. hawaiiana* beds for its entire life cycle, including feeding, reproduction and use of seagrass as obligate habitat. In fact morphological evidence of the coevolution of *S. bryanae* and *H. hawaiiana* can be seen in the snail's highly specialized radular teeth (Unabia, 2011).

Endemism in Hawaiian marine taxa is far lower than that in island terrestrial plants and animals. For example, there are no major marine adaptive radiations as there are for terrestrial species (Palumbi & Kay, 1987), the small radiation of Hawaiian *Cellana*, the marine gastropods known as opihi, with three endemic species, is a rare example (Bird et al., 2007; Bird et al., 2011). The distinct contrast between marine and terrestrial endemism levels highlights the importance of this endemic seagrass / gastropod relationship, and provides further impetus for its careful and effective conservation. Amplifying the urgency of this need for effective management is the fact that *H. hawaiiana* likely has restricted gene flow among populations, and its reproductive strategy of self-pollination, known as the ultimate form of inbreeding, and

together these factors both have important implications in terms of genetic diversity in this important native seagrass (McDermid et al., 2003). The genetic diversity of this species, both within and between populations of *H. hawaiiana* will be discussed in detail in Chapter 2 of this thesis.

## Seagrass Beds as Natural Filtration Systems

Seagrass meadows are known for improving water quality by absorbing dissolved nutrients and reducing suspended particle loads in the water column (Short et al., 2007). The ability to trap suspended particles leads to water clarity and thus less light is attenuated in seagrass beds (Hemminga & Duarte, 2000). In coastal cities that have limited resources for retaining wastewater, seagrass holds a particular importance. Lamb et al. (2017) tested water samples for the presence and levels of *Enterococcus* spp. in coastal Indonesia and found that there were reduced levels of the pathogen when seagrass meadows were present. Specifically, they found that seagrass beds reduced pathogen levels by 50% and that the coral reefs located adjacent to seagrass beds had a twofold reduction in pathogen levels.

### *Summary and Unresolved Issues*

Although *H. hawaiiana* and *H. decipiens* are the only two seagrass species in Hawai‘i, very little is known about their ecology. This literature review has discussed the tendency of *H. decipiens* to prefer sediments with smaller grain size (Speilman, 2012) and its deeper water relative to *H. hawaiiana* (Chan, 2014). It is likely that if there is competition for space, *H. decipiens* would have an advantage because of its ability to reproduce quickly and its theoretically higher dispersal ability (Hemminga & Duarte, 2000). Conversely, little is known

about the reproductive ecology of *H. hawaiiana* in nature. No studies to date have compared genetic diversity within and among *H. hawaiiana* populations. Analysis of genetic diversity and phylogenetic relationships among Hawaiian seagrass species and populations contributes to our understanding of these critical coastal marine ecosystem architects, and could provide crucial data for resource management strategy aimed at conservation of both species and the coastal marine ecosystem as a whole.

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CHAPTER II:

# PHYLOGENETICS AND CONSERVATION GENETICS OF *HALOPHILA HAWAIIANA*

## *Introduction*

Seagrasses are important in coastal marine ecosystems for a number of reasons, including their productivity, improving water quality, providing food resources, the extensive habitats they provide for other organisms, and stabilization of sediments providing protection from erosion and wave stress for coastal zones and reefs. The efficient photosynthetic systems of seagrasses and their deep roots allow them to store up to 10% of the total ocean carbon in surrounding sediment and generate ten liters of oxygen for every one square meter of seagrass meadow per day (Fourqurean et al., 2012; Orth et al., 2006). These attributes make seagrasses major carbon sinks in coastal marine habitats, which can help mitigate global warming and ocean acidification. Seagrass populations are declining globally, but in order to work towards restoration there is a need for ongoing and enhanced assessments of the ecology and conservation status of seagrass populations (Orth et al., 2006), including those in Hawai‘i.

*Halophila* has the largest global distribution of any seagrass, with over twenty species in the genus (Kuo, 2007). Different species in the genus *Halophila* can be identified morphologically as having pairs of obovate leaves and short lateral shoots (Den Hartog & Kuo, 2007). Without taxonomic knowledge of this genus it is possible that distributions of *Halophila* species are inaccurate due to misidentification, which is why the use of DNA barcoding genes such as the internal transcribed spacers in ribosomal DNA (*ITS1* and *ITS2*) have been useful in providing species resolution within the genus.

In Hawai‘i, the seagrass *Halophila hawaiiana* is important as the only endemic Hawaiian seagrass species. *Halophila hawaiiana* was first noted in Hawai‘i on the islands of Kaua‘i, O‘ahu, Moloka‘i and Maui and was described as a new species in 1966. It is described as having leaves that are narrowly obovate with a slight curve and is morphologically different from other *Halophila* species by its leaf vein pattern, which are rarely forked (Doty & Stone, 1966). Doty & Stone (1966) noted that *H. hawaiiana* was likely a very close relative to the Indo-Pacific seagrass species *Halophila ovalis* and *Halophila major,* with the notable difference being in the number of ovules, *H. hawaiiana* having 12-15 where *H. ovalis* and *H. major* have over 20 seeds (Sachet & Fosberg, 1973). The similarity between the species *H. hawaiiana* and *H. ovalis* has been evaluated through morphological differences (Doty & Stone, 1966) and through newer technologies such as DNA sequencing of the internal transcribed spacer (*ITS*) region of the nuclear ribosomal DNA. However, the origin and evolutionary relationship among *H. hawaiiana* and *H. ovalis* remains uncertain.

Similarly to *H. ovalis, H. hawaiiana* is considered to be dioecious. However, *H. hawaiiana* male plants are considered rare, having only been observed in the field once (M. Weaver personal communication, 2023; Herbert, 1986). Fertilization of the styles in female plants requires the capture of drifting pollen, but it is not known how often pollen is being released and successfully arriving on female plants. No evidence of fertilized seeds has been observed or reported in *H. hawaiiana,* and this species is assumed to survive primarily through asexual expansion via growth of the rhizome. The lack of sexual reproduction occurring suggests an absence of gene flow among populations, although vegetative reproduction and dispersal, where floating clumps of this species might disperse and successfully recruit to a new location is a possibility (Herbert, 1986). Therefore, *H. hawaiiana* populations are expected to have low

levels of genetic diversity, rendering populations vulnerable to extirpation in the case of environmental change, novel pathogens, and competition (McMahon & Waycott, 2009).

This study examines the phylogenetic relationship of *H. hawaiiana* to the other members of the genus *Halophila*, and examines the genetic diversity between Windward and Leeward Oʻahu patches of *H. hawaiiana.* The objectives of this study include 1) creating a phylogenetic tree to show the position of *H. hawaiiana* in relationship to other *Halophila* species using the *ITS* region; 2) determining the phylogeographic position of *H. hawaiiana* in order to infer the colonization history of this species in Hawaiʻi; 3) examining the genetic diversity within and between Windward and Leeward Oʻahu patches of *H. hawaiiana* using seven microsatellite loci developed by Xu et al. (2010). Due to the uniqueness of *H. hawaiiana* as a marine endemic species and the importance of seagrasses in terms of ecological services and contributions (McDermid et al., 2003), a better understanding of the colonization history and genetic connectivity of *H. hawaiiana* has important implications for conservation.

*Methods*

Sampling Sites and Collection

Samples of the seagrass species *Halophila hawaiiana* were collected at eight distinct beds: Rabbit Island (1), Kāneʻohe Sandbar (2), Kāneʻohe Bay (3), Coconut Island Pier (4), Kuliouou Beach Park (5), Piako Drive (6), Kahala Hotel (7), and Sans Souci (8) around the island Oʻahu (Table 1; Fig. 1). The distances propagules would need to travel from sample sites ranged from 2 to 82 km. At each site 15 shoots were randomly collected for DNA extraction.

| Location number | Location name | Population name | Latitude (°) | Longitude (°) |
|---|---|---|---|---|
| 1 | Rabbit Island | Windward | 21.6696679 | -157.9233357 |
| 2 | Kāneʻohe Sandbar | Windward | 21.4525453 | -157.793539 |
| 3 | Kāneʻohe Bay | Windward | 21.4326727 | -157.7966637 |
| 4 | Coconut Island Pier | Windward | 21.4295868 | -157.7849372 |
| 5 | Kuliouou Beach Park | Leeward | 21.2829171 | -157.7211142 |
| 6 | Paiko Drive | Leeward | 21.2818679 | -157.7301742 |
| 7 | Kahala Hotel | Leeward | 21.2702422 | -157.7726961 |
| 8 | Sans Souci | Leeward | 21.2625591 | -157.8215115 |

**Table 1.** Sampling locations latitude and longitude.

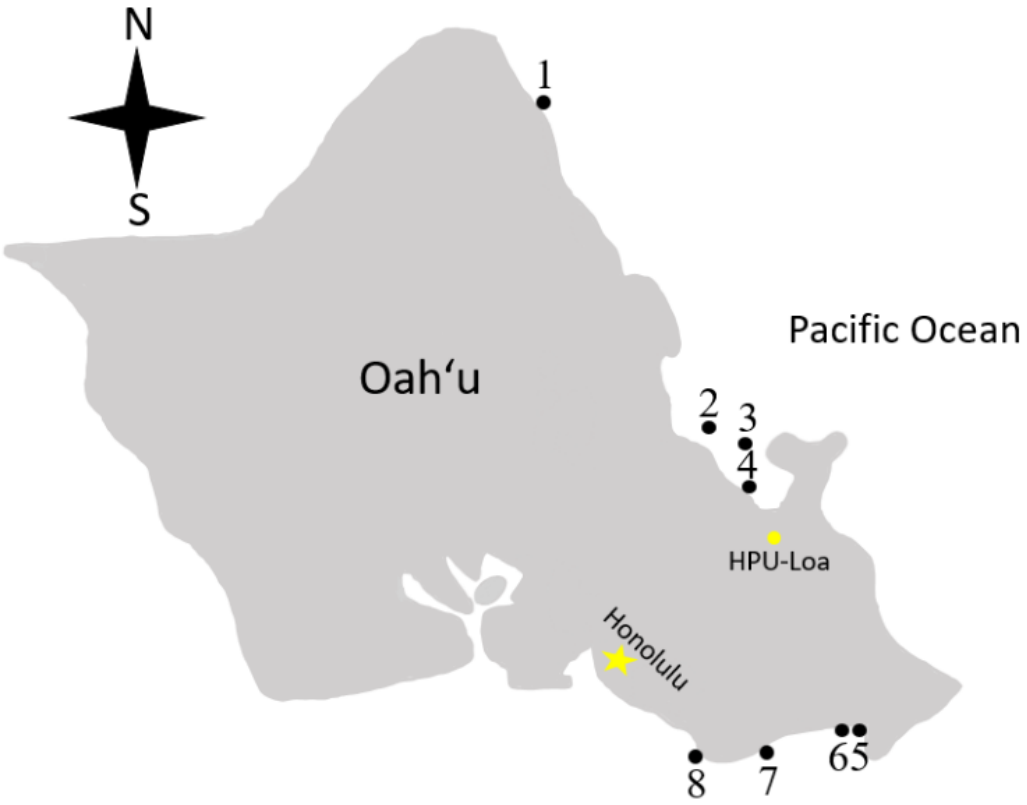



**Figure 1**. Map of Oʻahu and eight sampling sites. 1 = Rabbit Island, 2 = Kāneʻohe Sandbar, 3 = Kāneʻohe Bay, 4 = Coconut Island Pier, 5 = Kuliouou Beach Park, 6 = Piako Drive, 7 = Kahala Hotel, 8 = Sans Souci.

DNA Extraction

*Halophila hawaiiana* leaves were removed from the rhizome and dried in a 65°C oven overnight. Then 15 mg of dried leaves (about 10 leaves) were added to tissue disruption tubes that contained specially shaped beads for homogenization (Omega Bio-tek, Norcross, GA, USA). This translated to 30 samples collected on the Windward side and 30 samples collected on the leeward side of O‘ahu. The tissue distribution tubes were vortexed at 60 Hz on the Fisher Scientific Pulsing Vortex Mixer for two minutes to ensure homogenization. Genomic DNA was extracted from *H. hawaiiana* using the EZNA SP Plant DNA Kit following the manufacturer’s instructions (Omega Bio-tek, Norcross, GA, USA). The DNA quality was checked on 2% agarose gels stained with SYBR Safe (Invitrogen, Carlsbad, CA).

PCR Amplification Procedure and Sequencing

The region of the genome being analyzed in this study is in the nuclear ribosomal DNA internal transcribed spacer (*ITS*), which is the region between 18S rDNA and 26S rDNA and includes *ITS1,* 5.8S nrDNA and *ITS2*. Primers P674 (5’-CCTTATCATTTAGAGGAAGGAG-3’) and P675 (5’-TCCTCCGCTTATTGATATGC-3’) were be used to amplify fragments of ~700 bp corresponding to the *ITS* region (Fig. 2; García-Escudero et al., 2022).

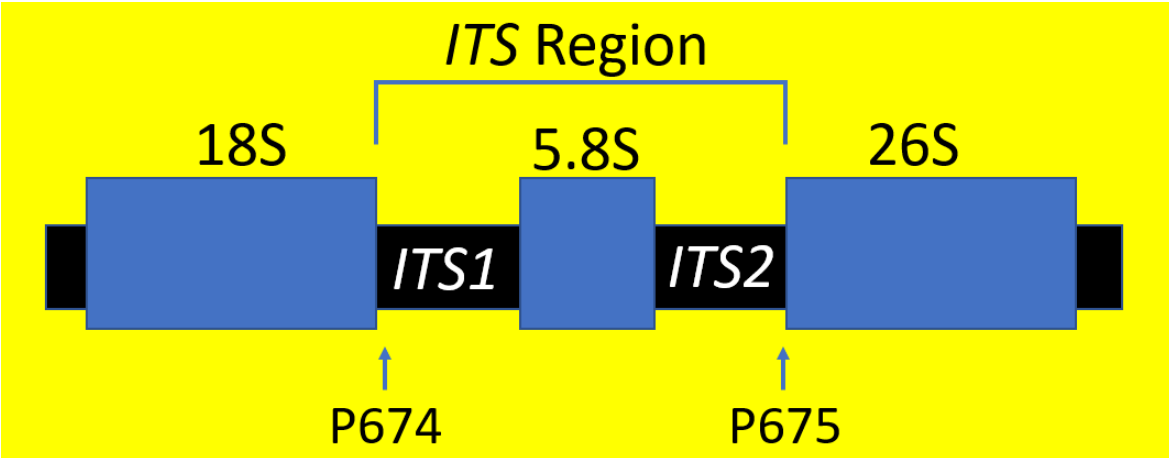



**Figure 2.** Nuclear ribosomal genes and their intron sequences, showing relative map positions of non-coding internal transcribed spacer regions (*ITS*) amplified and sequenced in this study. Approximate forward (P674) and reverse (P765) primer positions are indicated (Garcia et al., 2022).

PCR reactions were 25 µl including 12.5 µl of GoTaq® Green Master Mix (Promega, Wisconsin, United States), 2 µl (10µM) of each primer, 3 µl (10-20 ng) of DNA and 5.5 µl of nuclease-free water. PCR amplification was performed using the following profile: 2 min at 96°C; 30 cycles of 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C; with a final extension time of 10 min at 72°C. DNA sequencing was done at the University of Hawai‘i at Mānoa (http://www.hawaii.edu/microbiology/asgpb/). Of the 60 samples sequenced only 51 successfully amplified at the *ITS* region.

PCR Amplification and Analysis of Microsatellite Loci

Among the 50 microsatellite primer pairs developed in previous studies focusing on *Halophila* (Xu et al., 2010), we tested 10 of the most commonly used primer pairs (*Supplementary Materials; Table 1*) as recorded in previous *Halophila* research and seven amplified for *H. hawaiiana* (Table 2). Each forward primer was labeled with fluorescent dye (Table 2). The total reaction PCR is 25 µl including 12.5 µl of GoTaq® Green Master Mix (Promega, Wisconsin, United States), 2 µl (0.25 µM) of each primer, 3 µl (10-20 ng) of DNA and

5.5 µl of nuclease-free water. Microsatellite loci were amplified in the T100 Thermal Cycler (Bio-Rad, Hercules, CA) under the following conditions: initial denaturation for 3 min at 95 °C followed by 30 cycles of denaturation for 30 s at 94 °C, primer annealing for 30 s at 60 °C and extension for 30 s at 72 °C and termination with a final extension step of 72 °C for 2 minutes. Fragment analysis by capillary separation was performed using 3730xl DNA Analyzer (Applied Biosystems, Waltham, MA) at the University of Hawai‘i at Mānoa (http://www.hawaii.edu/microbiology/asgpb/).

Microsatellite alleles were scored with the microsatellite plugin in Geneious R7 version 7.1.7 (Fig. 3; Biomatters, Auckland, New Zealand). The potential for null alleles were evaluated for the seven microsatellite loci using Microchecker v.2.2.3 (Van Oosterhout et al., 2004). The number of alleles ($N_a$), observed heterozygosity ($H_o$) and expected heterozygosity ($H_e$) were calculated using GenAlex 6.5 (Peakall & Smouse, 2006). STRUCTURE 2.3 was used to assess genetic structure among the Windward and Leeward populations (Pritchard et al., 2000). Ten independent runs were completed using the default setting for K= 1-5. The most likely number of clusters was determined by Structure Harvester 0.6.94 (Earl & vonHoldt, 2012) using 1000 Markov chain-Monte Carolo repetitions and a burn-in period of 100,000 interactions in the admixture model (Jiang et al., 2014).

| Locus | | Primer Sequence (5'-3') | Motif | Product Size (bp) | Tm (°C) | Label | Reference |
|---|---|---|---|---|---|---|---|
| HO5 | F | GAATGGGAAGGTGAAAGAG | $(GA)_{13}$ | 187 | 60 | FAM | present study |
| | R | CACGGCACTGTTCATCTAC | | | | | |
| HO8 | F | ATAACCAAAGCCTCCCAAGC | $(AT)_9(GA)_{10}$ | 261 | 60 | FAM | present study |
| | R | AAATATCAAACGCCCCTCAC | | | | | |
| HO20 | F | AGAGGAAAAGAAAAGCGAG | $(AG)_{11}$ | 142 | 60 | NED | present study |
| | R | ATGTCACGTGGGACCATAT | | | | | |
| HO30 | F | CGCCGAAGGAAATGTGGAG | $(GA)_{10}$ | 127 | 60 | FAM | present study |
| | R | AACCCAACCGATCGACCCT | | | | | |
| HO31 | F | GGTTGTGCGTGAGGTGAAT | $(AG)_6$ | 223 | 60 | NED | present study |
| | R | ATACGCAGGTACGCACTCT | | | | | |
| HO48 | F | ATCGAACCCAATAGACACCAG | $(GA)_7GC(GA)_4$ | 235 | 60 | VIC | present study |
| | R | CAGGCAACTTAGCAAGAAACT | | | | | |
| HO51 | F | AGATAAGTTTCACTCCTGTG | $(GA)_{19}$ | 141 | 60 | VIC | present study |
| | R | ACCAGAACCAATCAAGAT | | | | | |

**Table 2**. Microsatellite PCR primers from Xu et al. (2010) that amplified *Halophila hawaiiana* individuals successfully for this study. Primer information includes repeat motif (with the repeated base pairs in parentheses and the average number of repeats outside of the parentheses), the product sizes of fragment lengths from PCR product sizes, annealing temperature for the seven microsatellite loci used, and the fluorescent dye labels attached to the forward primer in each pair.

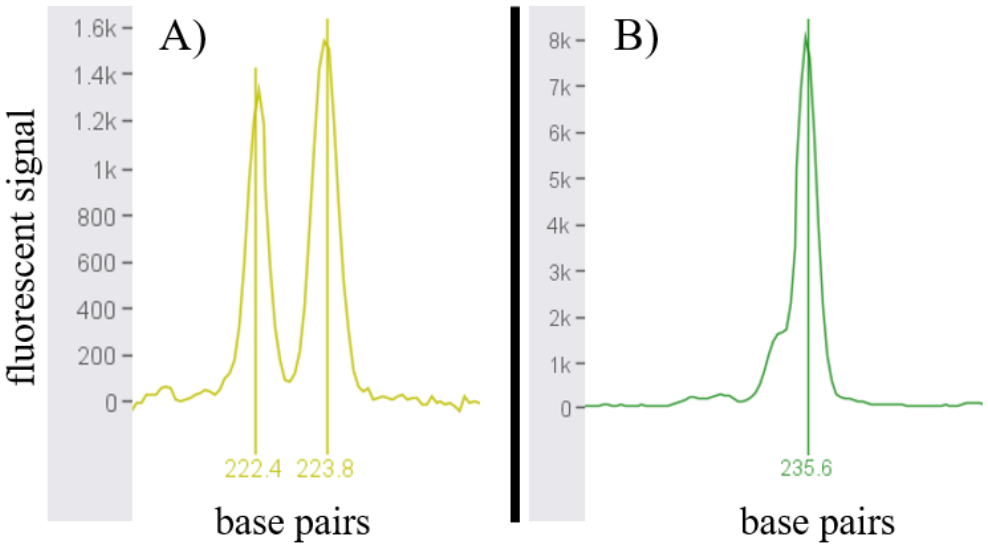



**Figure 3.** Example of scored microsatellite electropherograms for *Halophila hawaiiana* visualized using Geneious R7 version 7.1.7 (Biomatters, Auckland, New Zealand). The x-axis shows the fragment size in base pairs and the y-axis is an arbitrary unit, which is the fluorescent signal. A) shows the heterozygous genotype at locus HO31. B) shows a homozygous genotype at locus HO48.

Bioinformatic Analysis

A total of 359 *ITS* sequences of *H. ovalis, H. nipponica, H. major, H. decipiens, H. stipulacea, H. beccarii* and *Thalassia hemprichii* were downloaded from NCBI/GenBank database (*Supplementary Material; Table 2*; https://www.ncbi.nlm.nih.gov/) and were included in the alignment with 51 sequences from *H. hawaiiana,* for a total of 380 sequences, using Geneious R7 version 7.1.7 (Biomatters, Auckland, New Zealand). Identical sequences were excluded from the alignment in the final analysis for the reconstruction of phylogenetic trees, which resulted in a total of 70 unique *ITS* sequences and then we added 5 identical *H. hawaiiana* sequences from the Windward Side and 5 from the Leeward to highlight the lack of any variance between these sites. Phylogenetic analyses were implemented using the maximum likelihood (ML) model in the software MEGAX (Kumar et al., 2018) and RAxML (Stamatakis, 2014). Node support was assessed with 1000 bootstrap replicates using various substitution models. The tree was rooted with *Thalassia hemrichii,* a marine angiosperm in the family Hydrocharitaceae.

For intraspecific comparison, all *Halophila* sequences were initially included. The number of haplotypes (*N*), haplotype diversity (*h*), and nucleotide diversity ($\pi$) were computed within each species using DnaSP version 6 (Rozas et al., 2017). A haplotype network of the closely related species to *H. hawaiiana* were inferred by the TCS method (Clement et al., 2000) implemented in PopArt software (Leigh & Bryant, 2015) using statistical parsimony.

*Results*

Phylogeny Reconstruction

A final alignment of 665 bp was generated for 78 *ITS* sequence fragments for the seagrass genus *Halophila* sampled globally, together with a single sequence of the seagrass

*Thalassia* (downloaded from Genbank) to create maximum likelihood trees using MEGAX and RAxML (Stamatakis, 2014) (Fig. 4). In the tree presented, 10 sequences of *H. hawaiiana* were included and appear in a subunit in a monophyletic clade (Clade I subunit A), which also includes four *H. ovalis* sequences collected in Indonesia (1, 2) and Australia (2, 3) (Fig. 4). Sequence similarity of *H. hawaiiana* to *H. ovalis* was high, (98.6 - 99.5%) at this marker, and in fact the four Australia/Indonesian samples of *H. ovalis* represent a separate lineage from the remainder of this species. Clade I subunit B consists of *H. ovalis* samples that were collected from a number of Indian Ocean and Indo-Pacific regions, from southwest to northeast including Tanzania, Sri Lanka, India, Malaysia, Vietnam, Taiwan, Hong Kong and Korea

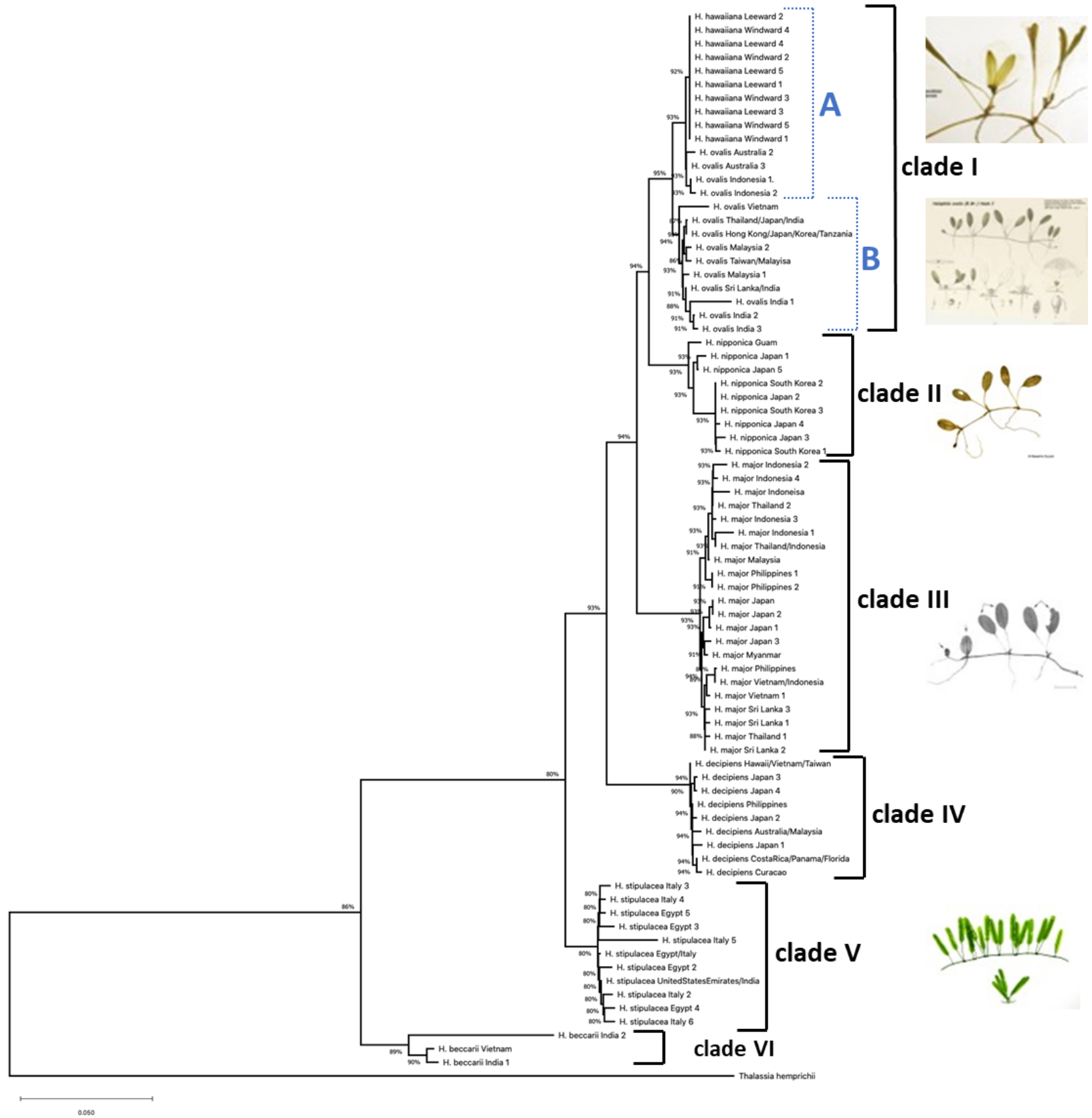

H. hawaiiana Leeward 2
H. hawaiiana Windward 4
H. hawaiiana Leeward 4
H. hawaiiana Windward 2
H. hawaiiana Leeward 5
H. hawaiiana Leeward 1
H. hawaiiana Windward 3
H. hawaiiana Leeward 3
H. hawaiiana Windward 5
H. hawaiiana Windward 1
H. ovalis Australia 2
H. ovalis Australia 3
H. ovalis Indonesia 1.
H. ovalis Indonesia 2
A
H. ovalis Vietnam
H. ovalis Thailand/Japan/India
H. ovalis Hong Kong/Japan/Korea/Tanzania
H. ovalis Malaysia 2
H. ovalis Taiwan/Malayisa
H. ovalis Malaysia 1
H. ovalis Sri Lanka/India
H. ovalis India 1
H. ovalis India 2
H. ovalis India 3
B
clade I
H. nipponica Guam
H. nipponica Japan 1
H. nipponica Japan 5
H. nipponica South Korea 2
H. nipponica Japan 2
H. nipponica South Korea 3
H. nipponica Japan 4
H. nipponica Japan 3
H. nipponica South Korea 1
clade II
H. major Indonesia 2
H. major Indonesia 4
H. major Indoneisa
H. major Thailand 2
H. major Indonesia 3
H. major Indonesia 1
H. major Thailand/Indonesia
H. major Malaysia
H. major Philippines 1
H. major Philippines 2
H. major Japan
H. major Japan 2
H. major Japan 1
H. major Japan 3
H. major Myanmar
H. major Philippines
H. major Vietnam/Indonesia
H. major Vietnam 1
H. major Sri Lanka 3
H. major Sri Lanka 1
H. major Thailand 1
H. major Sri Lanka 2
clade III
H. decipiens Hawaii/Vietnam/Taiwan
H. decipiens Japan 3
H. decipiens Japan 4
H. decipiens Philippines
H. decipiens Japan 2
H. decipiens Australia/Malaysia
H. decipiens Japan 1
H. decipiens CostaRica/Panama/Florida
H. decipiens Curacao
clade IV
H. stipulacea Italy 3
H. stipulacea Italy 4
H. stipulacea Egypt 5
H. stipulacea Egypt 3
H. stipulacea Italy 5
H. stipulacea Egypt/Italy
H. stipulacea Egypt 2
H. stipulacea UnitedStatesEmirates/India
H. stipulacea Italy 2
H. stipulacea Egypt 4
H. stipulacea Italy 6
clade V
H. beccarii India 2
H. beccarii Vietnam
H. beccarii India 1
clade VI
Thalassia hemprichii
0.050

**Figure 4.** The evolutionary history of seven species of the seagrass genus *Halophila* shown was inferred using the Maximum Likelihood method with the General Time Reversible (GTR) model with Gamma correction and Invariant sites (G+I). The tree with the optimal likelihood score (-3321.97) is shown. A discrete Gamma distribution was used to model the different rate differences among sites ( 5 site + G = 0.9361). The analysis is based on 79 *ITS* sequences and 665 aligned characters. The outgroup used is *Thalassia hemprichii.* The topology of this phylogram is characterized by six monophyletic clades with bootstrap values ranging from 80-95%. Each ingroup clade consists of 10 or more sequences, and each represents a single species with the exception of that labeled Clade I which consists of 10 *H. hawaiiana* sequences and four sequences from samples identified as *H. ovalis*, collected in Australia and Indonesia. Note that there is a second subunit in the clade labeled Clade I subunit B, which is comprised of the remainder of samples of *H. ovalis* sequences, sampled from various geographic localities from eastern Africa to Korea.

In Clade I, regardless of the substitution model or program used, *H. hawaiiana* and the four samples of *H. ovalis* from Indonesia and Australia were resolved in the Clade I subunit A. Although these two species are known to be morphologically distinct, we assume that the researchers who collected and identified these seagrass samples made the correct identification, but there is no way to confirm this as the DNA sequences were downloaded from Genbank. There was very little change within clade structure, and the position of Clade I subunit A relative to the overall tree topology remained stable, with Clade I subunit B sister to it, as well as the topology being stable in relation to the other five ingroup clades in the tree (Fig. 4). Relatively short within-clade branch lengths occurred throughout the tree, suggesting that within each species in this genus, genetic divergence was consistent, and low, with the exception of the four sequences of *H. ovalis* that grouped with *H. hawaiiana* in Clade A. Hawaiian sequences were included from the two geographic collection sites on the Windward and Leeward sides of the island of O‘ahu (Fig. 1). Five representative sequences from the two sides of the island again shared a single haplotype as seen in Clade I subunit A. Results of a pairwise genetic distance analysis (*Supplementary Materials; Table 3*) showed a distance of 0.004 between *H. ovalis* Australia and *H. hawaiiana* (Table 3). The percent identity (percent of bases that are identical) between *H. hawaiiana* and *H. ovalis* Australia was 98.6% similar, with a two base pair difference

among *H. hawaiiana* and *H. ovalis* Australia. Clade I subunit B is the second group of *H. ovalis*, which is more distantly related to *H. hawaiiana* with a genetic distance of 0.01 (Table 3).

| | (I-A) | (I-B) | (II) | (III) | (IV) | (V) | (VI) |
|---|---|---|---|---|---|---|---|
| *H. hawaiiana*<br>*H. ovalis*<br>(Indo/Aus)<br>(I-A) | - | - | - | - | - | - | - |
| *H. ovalis*<br>(I-B) | 0.01 | - | - | - | - | - | - |
| *H. nippoinica*<br>(II) | 0.03 | 0.03 | - | - | - | - | - |
| *H. major*<br>(III) | 0.04 | 0.04 | 0.05 | - | - | - | - |
| *H. decipiens*<br>(IV) | 0.5 | 0.06 | 0.06 | 0.06 | - | - | - |
| *H. stipulacea*<br>(V) | 0.06 | 0.07 | 0.07 | 0.07 | 0.06 | - | - |
| *H. beccarrii*<br>(VI) | 0.14 | 0.14 | 0.15 | 0.14 | 0.14 | 0.15 | - |

**Table 3.** Pairwise comparison of population differentiation among *Halophila* populations. Roman numerals correspond to the main clades in Figure 4. Clade I subgroup A contains 10 identical *H. hawaiiana* sequences from both Windward and Leeward O‘ahu, and the four *H. ovalis* sequences from Australia and Indonesia. Clade I subgroup B is made up of *H. ovalis* from multiple global sampling locations from above and west of Wallace’s Line, and this clade is sister to Clade I subgroup A.

Haplotype Diversity

We built haplotype networks with 329 unique genetic sequences used in the phylogenetic tree and added the 51 *H. hawaiiana* sequences generated for this study from eight sites on O‘ahu. Of the seven seagrass species used for the *ITS* phylogenetic tree, all species showed varying levels of haplotype diversity, with the exception of the endemic Hawaiian species *H. hawaiiana* (Table 3; *Supplementary Materials;Table 3*). The species *H. beccarii* shows three different

haplotypes between samples collected in India and Vietnam with a haplotype diversity of 0.84 (n=4). The seagrass species *H. stipulacea,* sampled from Italy, Egypt, the United Emirates and India, contained four distinct haplotypes with a haplotype diversity of 0.433 (n=65). The cosmopolitan species *H. decipiens* had the broadest geographic distribution withs samples from Japan, Hawai‘i, Vietnam, Taiwan, Philippines, Australia, Malaysia, Costa Rica, Panama, Florida and Curacao formed a monophyletic clade. In *H. decipiens* there were eight haplotypes and haplotype diversity was 0.54 (n=31). In *H. major,* we used 79 *ITS* sequences which comprised twelve haplotypes over the geographic regions of; Indonesia, Thailand, Vietnam, Sri Lanka, Malaysia, Myanmar, Japan and Philippines (n=79). *Halophila nipponica* sequences showed a haplotype diversity of 0.596 with 14 different haplotypes from Guam, Japan and South Korea (n=17). The species *H. ovalis* produced nine haplotypes from 163 *ITS* sequences across 12 different sampling locations; Vietnam, Tanzania, Taiwan Thailand, Sri Lanka, Egypt, India, Malaysia, Hong Kong, Australia, Indonesia and Japan. In Taiwan, *H. ovalis* showed two haplotypes and Malaysia had three haplotypes. In contrast, 51 *ITS* sequences generated for the present study) from *H. hawaiiana* showed a single haplotype from O‘ahu. Values of Tajima’s D were negative for all seagrass species except *H. hawaiiana* (Table 2)*,* which cannot be calculated since all 51 sequence fragments were identical, because there is no difference between observed (pi=$\pi$) and expected (theta=$\boldsymbol{\theta}$) nucleotide diversity. In general, negative values of Tajima’s test statistic signify an excess of observed low frequency, or rare substitutions relative to expectations, indicating population size expansion, where the negativity of the value is proportional to the magnitude of population expansion.

| Species | *N* | *h* | *Hd* | π | θ | *S* | Tajima's D | Significance |
|---|---|---|---|---|---|---|---|---|
| *H. ovalis* | 163 | 9 | 0.535 | 0.00439 | 5.44604 | 30 | -1.74001 | NS |
| *H. hawaiiana* | 51 | 1 | 0 | 0 | 0 | 0 | n/a | n/a |
| *H. major* | 79 | 19 | 0.711 | 0.00463 | 8.9063 | 44 | -2.3273 | p<0.01 |
| *H. decipiens* | 31 | 8 | 0.54 | 0.00172 | 2.25282 | 9 | -1.81265 | p<0.05 |
| *H. beccarri* | 4 | 3 | 0.833 | 0.00256 | 1.63636 | 3 | -0.75445 | NS |
| *H. stipulacea* | 65 | 14 | 0.433 | 0.00129 | 4.42675 | 21 | -2.57356 | p<0.001 |
| *H. nipponica* | 17 | 7 | 0.596 | 0.00475 | 4.73271 | 16 | -1.46757 | NS |

**Table 3.** Summarized *Halophila* sample size, number of haplotypes observed, and estimates of genetic diversity. *N*: Number of sequenced isolates, *h*: number of haplotypes, *Hd*: haplotype diversity, $\pi$: observed nucleotide diversity, $\boldsymbol{\theta}$: expected diversity, *S*: number of segregating sites, Tajima's D, statistical significance of Tajima's D.

The haplotype network produced based on the alignment of *ITS* sequences showed phylogeographic separation between *H. ovalis* collected in Australia and *H. hawaiiana* by two base pairs (Fig. 5). Of the 163 combined sequences of *H. ovalis,* 67% share the same haplotype which represents nine sampling localities. Again, *H. hawaiiana* showed a single haplotype for all 51 individuals (Fig. 5).

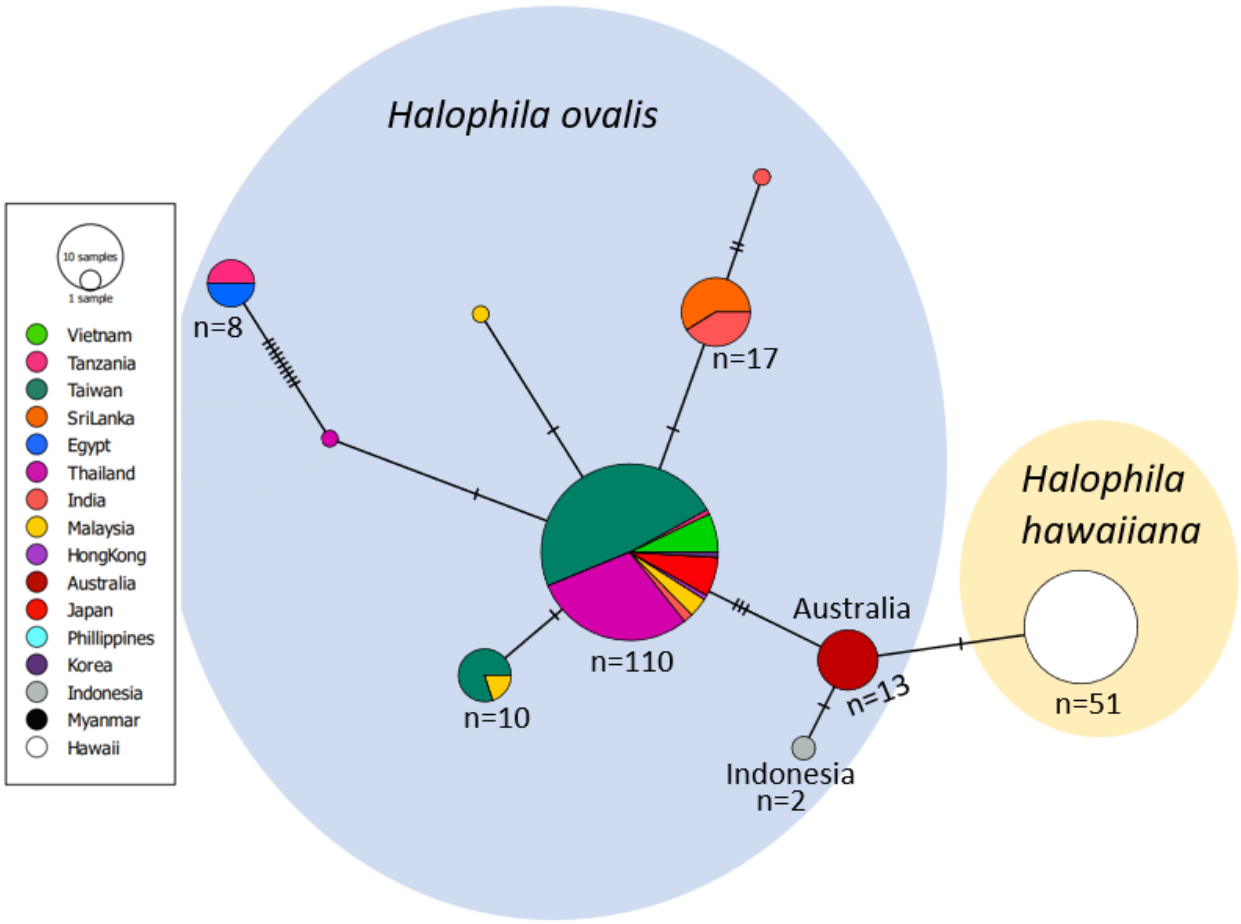



**Figure 5.** Haplotype network for *Halophila ovalis* and *H. hawaiiana* including global sampling of *H. ovalis,* where the maximum geographic sampling distance was over 11,000 km (Egypt to western Australia). The number of individuals that shared each haplotype is shown below each circle. As is standard for this type of figure, each line connecting haplotypes represents a mutational step. Missing intermediates are indicated by a perpendicular hatch mark on these lines. The figure was generated using the software PopArt (Leigh & Bryant, 2015), which uses statistical parsimony to generate unrooted network cladograms.

Genetic Diversity and Population Structure

No genetic diversity within or between the four Windward and four Leeward O‘ahu *H. hawaiiana* populations was revealed in this study. There were thirteen alleles in the seven microsatellite loci, with a maximum number of alleles per population of two at loci HO8, HO20, HO30, HO31 and HO51 (Table 4). The Windward and Leeward populations were dominated by

the same genotype at each of the seven microsatellite loci (Table 5). At locus HO5 Windward and Leeward populations were 100% homozygous. For locus HO51, 100% of samples were heterozygous. For locus HO48, homozygous individuals made up 93% of the samples. At loci HO8, HO20, HO30, HO31 and HO51 the most common genotype for both the Windward and Leeward side was heterozygous. For locus HO31, 79% of the samples were heterozygous. Additionally, at locus HO30 the homozygous genotype made up 7.4% of samples, but the dominant genotype for both Windward and Leeward populations at this locus was heterozygous. The observed heterozygosity was higher than the expected heterozygosity across five loci (Table 5). The software STRUCTURE clustered the Windward and Leeward populations into a single population (Fig. 6). Both Pritchard (2000) and Earl & vonHoldt's (2012) methods indicated that the optimal number of clusters was a single cluster, K=1 (Fig. 6).

| Pop/Locus | | HO5 | HO8 | HO20 | HO30 | HO31 | HO48 | HO51 |
|---|---|---|---|---|---|---|---|---|
| **Windward** | N | 15 | 12 | 26 | 27 | 25 | 27 | 30 |
| | Na | 1 | 2 | 2 | 2 | 2 | 2 | 2 |
| | Ar | 1 | 2 | 2 | 2 | 2 | 2 | 2 |
| | Ho | 0 | 1 | 1 | 0.852 | 0.76 | 0.074 | 1 |
| | He | 0 | 0.5 | 0.5 | 0.489 | 0.487 | 0.071 | 0.5 |
| overall heterozygosity | 0.435 | | | | | | | |
| **Leeward** | N | 14 | 10 | 28 | 27 | 24 | 28 | 30 |
| | Na | 1 | 2 | 2 | 2 | 2 | 2 | 2 |
| | Ar | 1 | 2 | 2 | 2 | 2 | 2 | 2 |
| | Ho | 0 | 1 | 1 | 1 | 0.833 | 0.71 | 1 |
| | He | 0 | 0.5 | 0.5 | 0.5 | 0.497 | 0.069 | 0.5 |
| overall heterozygosity | 0.367 | | | | | | | |

**Table 4.** Summary of genetic variation at seven microsatellite loci in the Windward and Leeward populations of *Halophila hawaiiana.* N - Sample size; Na - number of alleles; Ar - allelic richness; Ho - observed heterozygosity; He - expected heterozygosity.

| Locus/Pop | | Windward | | Leeward | |
|---|---|---|---|---|---|
| | | Allele Size (bp) | Frequency | Allele Size (bp) | Frequency |
| HO5 | Heterozygote | 187/187 | 15 | 187/187 | 14 |
| | | null | 20 | null | 21 |
| HO8 | Heterozygote | 259/261 | 12 | 259/261 | 10 |
| | | null | 18 | null | 20 |
| HO20 | Heterozygote | 140/142 | 26 | 140/142 | 28 |
| | | null | 4 | null | 2 |
| HO30 | Heterozygote | 125/127 | 23 | 125/127 | 27 |
| | Homozygote | 125/125 | 4 | | |
| | | null | 0 | null | 3 |
| HO31 | Heterozygote | 221/223 | 19 | 221/223 | 20 |
| | Homozygote | 221/221 | 1 | 221/221 | 1 |
| | Homozygote | 223/223 | 5 | 223/223 | 3 |
| | | null | 5 | null | 6 |
| HO48 | Heterozygote | 234/234 | 2 | 234/234 | 2 |
| | Homozygote | 235/235 | 25 | 235/235 | 26 |
| | | null | 3 | null | 2 |
| HO51 | Heterozygote | 135/141 | 30 | 135/141 | 30 |
| | | null | 0 | null | 0 |

**Table 5.** Microsatellite genotypes of *Halophila hawaiiana* at the Windward and Leeward populations in O‘ahu, Hawai‘i. Allele sizes are in base pairs.

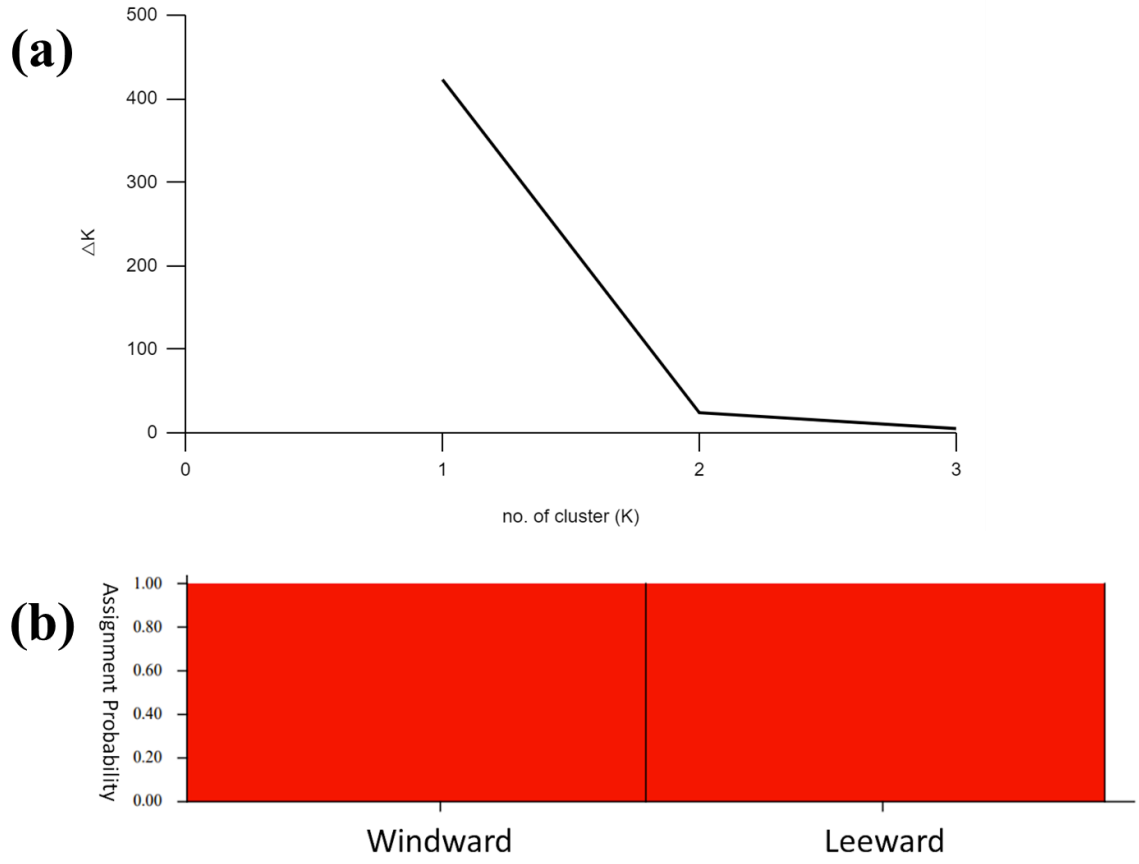



**Figure 6.** Cluster analysis graph of H. hawaiiana population using STRUCTURE (the same color represents the same group). a) the corresponding K statistic calculated using Structure harvester (Earl & vonHoldt, 2012). b) Histogram of the structure analysis for the model with K=1, the highest △ K.

*Discussion*

The focus of this investigation was several fold, and included gaining a better understanding of the evolutionary and possible biogeographic origin of the endemic Hawaiian species along with quantifying the genetic variation within O‘ahu populations. One of the most significant discoveries revealed by this study through analysis of both *ITS* sequence fragments and multiple microsatellite loci is the absence of genetic variation of both classes of nuclear loci, and the consequent conservation implications.

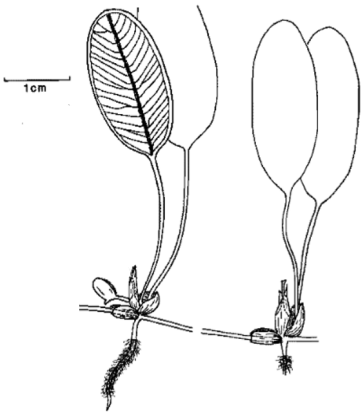
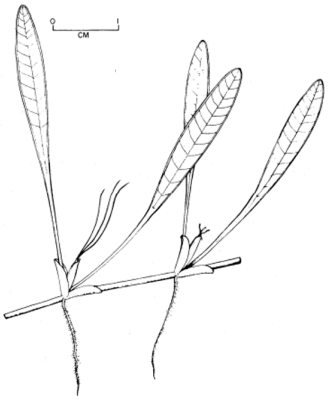
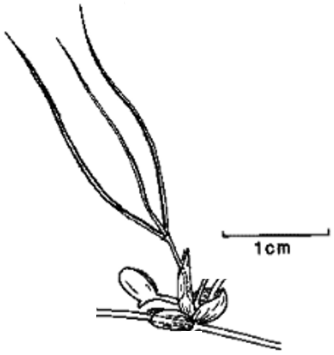
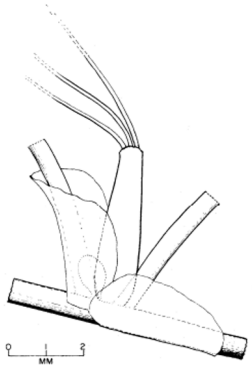
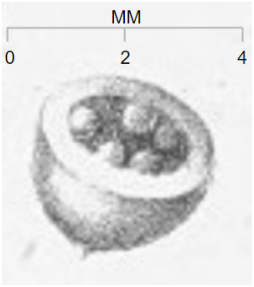
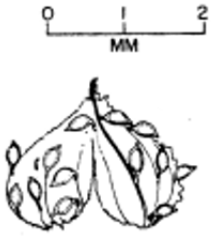
The phylogenetic reconstruction presented shows patterns in the evolutionary and biogeographic history of the seven species of *Halophila* sampled. Based on the position of *H. hawaiiana* in the phylogenetic reconstruction and morphological similarities, the endemic Hawaiian seagrass may have evolved from a most recent common ancestor of *H. ovalis.*

Previous studies based upon *ITS* sequencing, also determined a close genetic relationship between *H. hawaiiana* and *H. ovalis* (Waycott et al., 2002). However, Waycott et al. (2002) only included ten taxa total in their phylogenetic reconstruction with only one *H. hawaiiana* sequence, where our study included twice as many samples and 51 *H. hawaiiana* sequences. Additionally, Waycott et al., (2002) only included *H. ovalis* from five sampling sites and our study included *H. ovalis* from fifteen sampling sites. In the present study, based upon an expanded sampling of *H. ovalis*, new DNA sequences and microsatellite data we were able to better understand the evolutionary history of *H. hawaiiana* and its current population structure.

Taxonomic Status of *Halophila hawaiiana*

The endemic Hawaiian seagrass *H. hawaiiana* was first documented by Forbes (1911). Doty and Stone (1966) distinguished *H. hawaiiana* from *H. ovalis* due to morphological differences in the leaf blade structure. The leaf morphology of *H. hawaiiana* is elongated with narrowly obovate and broad spatulate leaves. Additionally, the basal lamina is narrowly cuneate at the point where the blade meets the petiole. The blades of *H. hawaiiana* are symmetric and have about 10-16 lateral veins that are rarely forked, where *H. ovalis* has less lateral veins and these veins are forked (Sachet & Fosberg, 1973). Another distinction can be seen in the width of the leaf and the length of the lamina as *H. hawaiiana* has broader leaves at 6 mm width and a shorter lamina when compared to *H. ovalis,* which has a leaf width of 4 mm (Fig. 7; Doty &

Stone, 1966; Sachet & Fosberg, 1973). The morphological differences are important for distinguishing the two species.

| | *Halophila ovalis* | *Halophila hawaiiana* |
|---|---|---|
| Shape of branch and female flowers, rhizome, roots and leaves |  (Womersley, 1984) |  (Doty & Stone, 1966) |
| Fertile leaf axil showing a rhizome supporting two stipular bracts, two petioles and a mature flower surrounded by a bract |  (Womersley, 1984) |  (Doty & Stone, 1966) |
| Fruit broken open to reveal seeds |  (Womersley, 1984) |  (Doty & Stone, 1966) |

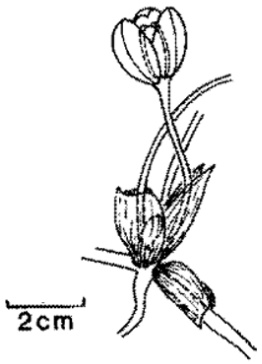
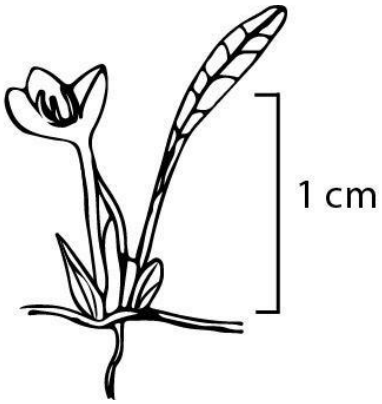
| | | |
|---|---|---|
| Portion of stolon showing male flower on elongated pedicel |  (Womersley, 1984) |  (Weaver, 2023) |

**Figure 7.** Morphologic differences between *H. ovalis* and *H. hawaiiana.*

Comparative Reproductive Ecology

Reproduction in *H. ovalis* and *H. hawaiiana* is hypothesized to occur sexually or asexually via clonal expansion and rhizome growth (Den Hartog, 1970; Herbert, 1986). Since both *H. ovalis* and *H. hawaiiana* are dioecious, meaning female and male plants are located on different rhizomes, sexual reproduction can be a challenge due to the unknown distance the pollen must travel to reach a female flower. Zipperle et al., (2011) recorded that pollen was traveling over 10 meters in the seagrass *Zostera noltii.* The number of female flowers differ between *H. hawaiiana* and *H. ovalis* in the: *H hawaiiana* has 12-15 seeds, while *H. ovalis* has 20 or more seeds (Table 6; Den Hartog, 1970; Doty & Stone, 1966; Sachet & Fosberg, 1973). Herbert (1984) only found female flowers of *H. hawaiiana* in the field and considered male flowers to be rare, which could mean sexual reproduction is either not occuring or rarely occurring. Our microsatellite data show a number of heterozygotes in the *H. hawaiiana* population, therefore if the seagrass was reproducing sexually we would expect to see different genotypes within populations. Previous research on different clonal seagrass species has observed similar levels of genetic uniformity as that observed in *H. hawaiiana* (McDermid et al.,

2003). The seagrass *Posidonia oceanica* in the family *Posidoniaeae* has been recorded having slow vegetative growth with inconsistent sexual reproduction and low genetic variability (Mari et al., 2020; Procaccini & Mazzella, 1998). The seagrass *P. oceanica* occurs across the Mediterranean Sea and 90% of the individuals sampled in the species are genetically identical throughout this geographic area (Procaccini & Mazellam, 1998). Another seagrass in the family Posidoniaeae, *P. australis* has been recorded as one of the largest clones at 16 m in diameter (Waycott, 1995). Additionally, a seagrass population of *Zostera marina* found in the Baltic Sea spanning 160 by 40 meters was dominated by a single genotype (Reusch et al., 1999). The infrequency of male flowers and lack of genetic diversity between Windward and Leeward *H. hawaiiana* supports the idea that this is a primarily a clonal species.

| **Reproductive Morphology** | ***Halophila ovalis* (Short et al., 2007)** | ***Halophila hawaiiana* (Short et al., 2007)** |
|---|---|---|
| Flowering spathe hairs | Absent | Absent |
| Male flower | Known | Known |
| Female flower | | |
| Ovary length (mm) | 1-2.5 | 1.2 |
| Style length (mm) | 10-20 | 4-6 |
| Seeds (number per fruit) | 20-30 | 12-15 |

**Table 6.** Comparison of reproductive morphology of *H. ovalis* and *H. hawaiiana.*

Phylogenetics and Biogeography

In this study maximum likelihood trees based on *ITS* showed two main relevant patterns, one was the identity of all of the *H. hawaiiana ITS* sequence fragments, and the second was paraphyly of *H. ovalis*. The phylogenetic break in the species appears to correspond to Wallace's

Line, a theoretical biogeographic construct based on the observations of the renowned British naturalist Alfred Russel Wallace, in 1859 (Fig. 7; Wallace, 1863).

Wallace's Line separates two biogeographical zones, originally based on Wallace's field observations of major groups, representing a floral and faunal transition between Asia and Australia. The line forms a diagonal division running from south west towards the north east, with lineages distributed above the line tend to derive from Asia, while those that occur below this line tend to be of Australian origin (Wallace, 1963; De Bruyn et al., 2011). The underlying mechanism driving the division along Wallace's Line is geological in nature, where the line roughly follows a trench that is substantially deeper than the ocean surrounding the Asiatic and Indo-Australian islands. This trench is a result of the collision of the Asian and Australasian tectonic plates, between c. 15 and 3 Ma, (Hall, 2002), and based on recurrent periods of low sea-level stands (of up to 120 m below present levels) associated with Pleistocene glaciations (2.4 Ma-10,000 years ago) (Voris, 2000). This vicariant biogeographic barrier has been present for at least several million years and is thought to have maintained separation of lineages including iconic mammals such as the Australian marsupials and monotremes (including platypus and kangaroos) from Asiatic placental mammals (including tigers, macaques and elephants) (Cowman & Bellwood, 2013). The biogeographic theory underlying the mechanism of action of Wallace's Line is thought to operate by limiting dispersal across this marine channel, despite fluctuations of sea level that periodically allowed terrestrial flora and fauna to be connected and separated again over shorter time intervals (during each glaciation) (Hall, 2002). Thus gene flow and connectivity among island species on either side of the line was intermittent, but across the line was generally restricted over this extended geological time, especially for those species with limited vagility, inducing certain birds, insects and plants (Hall, 2002; Voris,

2000). Based the phylogenetic results and sampling of *H. ovalis*, there is a distinct phylogeographic break that suggests a reproductive barrier in this lineage between Clade I subunit A and Clade I subunit B, and there is evidence of the possibility of colonization of Hawai‘i by the Australian/Indonesian lineage of *H. ovalis.* The evidence suggests that ancestors of the main *H. ovalis* lineage (Clade I subunit B) crossed Wallace's Line at some time in the past once or a limited number of times, then this lineage dispersed to Hawai‘i, followed by isolation and phyletic evolution yielding the endemic Hawaiian seagrass. The *ITS* phylogeny presented suggests paraphyly in this species and the potential for incipient speciation in the lineage, between *H. ovalis* from Clade I subunit A and subunit B (Ali et al., 2021).

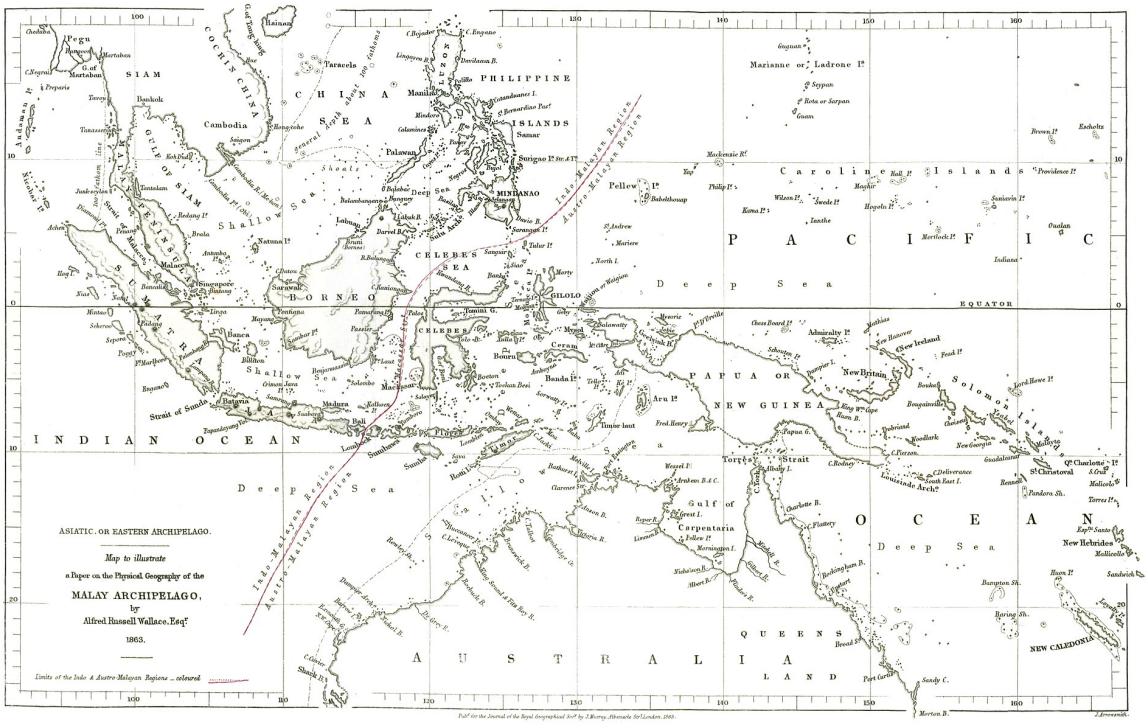



**Figure 7**. Reproduction of the original Indo-Pacific map showing Wallace's Line, hand-drawn by Alfred Russel Wallace in 1859, based on biogeographic distributions of Asiatic placental mammals, birds, insects and plants to the north west, and Australo-Asian monotremes, Marsupials, birds, Insects and plants to the south east.

Long-distance dispersal and colonization of the Hawaiian islands by propagules of an ancestral lineage closely related to the Australian/Indonesian lineage (Clade I subunit A) (Fig. 4) is suggested by the results of this study. Although we do not know how this event may have occurred, nor do we know whether the species that arrived in Hawai‘i reproduced sexually or asexually. The floating seeds or vegetative pieces of rhizome may have dispersed via oceanic surface currents or by animal-mediated dispersal such as by sea turtles or birds, to the Hawaiian Archipelago (Wu et al., 2016; Cowie & Holland, 2006; Mari et al., 2019). This novel colonization would have created a small population, followed by bottleneck, and genetic drift, which tends to result in allele loss and fixation (Alotaibi et al., 2019), such as the pattern of microsatellite variation in *H. hawaiiana*.

The level of genetic homogeneity among all seven microsatellite loci in *H. hawaiiana* supports the possibility of a founder effect. However, the field observations of *H. hawaiiana* also are consistent with vegetative, clonal reproduction, so the two mechanisms cannot be ruled out as acting either individually or together. In other seagrass species, *Cymodocea nodosa,* an endangered seagrass in the Mediterranean sea, was recorded having lower genotype richness and allelic richness than *C. nodosa* outside of this area in the Mediterranean Sea. Alberto et al. (2001) concluded that low allelic richness of the peninsular population suggests a founder effect, where due to colonization by a single source population (Alberto et al., 2001). Similarity in the depauperate genotypic and allelic richness of the Iberian and Hawaiian seagrasses support the idea of a founder effect playing a role.

We do not know whether low allelic richness in *H. hawaiiana* is driven by population history or asexual reproductive strategy. Evolutionary events called selective sweeps can drive population genetic variation towards broad patterns of allele fixation via strong directional

selection, eliminating certain genotypes (Barrett et al., 1993). However, we have no way of determining whether this may have occurred in *H. hawaiiana*, other than to say that selective sweeps have sometimes been invoked in colonization events (Barrett & Schluter 2008; Lachmuth et al. 2010; Siol et al., 2010; Messer & Petrov 2013).

Sampling locations in Hawai‘i ranged in geographic distance from 2 to 85 km between sites. In a study by Liu and Hsu (2021), researchers used the same microsatellite markers as this study in *H. ovalis*, but results showed substantially higher genetic diversity from similarly closely spaced island sampling localities in Taiwan (for comparison, see Figure 6b and Figure 8). Future studies of *H. hawaiiana* should focus on including a larger number of samples.

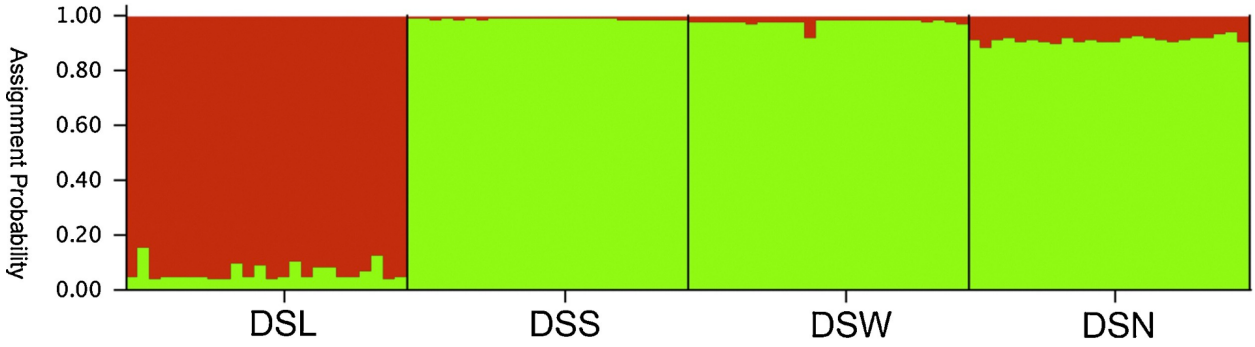



**Figure 8.** Multilocus structure plot showing assignment probabilities (K = 2) of 96 plants of *Halophila ovalis* from four sampling locations at Dongsha Island. DSL = Dongsha Island lagoon, DSN = Dongsha Island northern coast, DSW = Dongsha Island western coast, DSS = Dongsha Island southern coast (Liu & Hsu, 2021)


**Figure 9.** Sampling sites at Dongsha Island. DSL = Dongsha Island lagoon, DSN = Dongsha Island northern coast, DSW = Dongsha Island western coast, DSS = Dongsha Island southern coast (Liu & Hsu, 2021)

Conservation Genetics

Inbreeding depression and low genetic diversity lead to higher extinction risk in laboratory and wild populations of naturally outbreeding species (Frankham, 2005). Crossing individuals of unrelated populations, even if the populations have low genetic diversity, can mitigate the effects of inbreeding depression (Ralls et al., 2020). Outcrossing effectively removes inbreeding depression in laboratory and domestic animals and plants (Ralls et al., 2020; Adams et al., 2022; Spielman & Frankham, 1992; Falconer & Mackay, 1996), but only where populations show genetic differences in allele frequencies and diversity. Such recoveries have now been documented in multiple wild populations primarily of vertebrates such as deer mice (*Peromyscus maniculatus*; Schwartz & Mills, 2005), wolves (*Canis lupus*; Vila et al., 2003), greater prairie chicken (*Tymphanuchus cupido pinnatus*; Westemeier et al., 1998), snakes (*Vipera berus*; Madsen et al., 1999, 2004), fish (*Poeciliopsis monacha*; Vrijen-hoek, 1994), and some plants (*Ipomopsis aggregata*; Heschel & Paige, 1995; *Silene alba*; Richards, 2000).

The most widely recognized examples of the important link between evolutionary history, loss of genetic variation and conservation management include northern elephant seals (*Mirounga angustirostris*; Hoelzel et al., 2002), channel island foxes (*Urocyon littoralis*; Robinson et al., 2016), the Florida panther (*Puma concolor coryi*; Roelke et al., 1993) and the cheetah (*Axinonxyx jubatus;* O'Brien et al., 1985). In the case of the cheetah, nearly 12,000 years ago the species survived a population bottleneck, followed by a dramatic loss of about 75% of their habitat in the last century (Terrel et al., 2016). Cheetah numbers have decreased drastically due to habitat destruction, starvation and poaching. Population reduction, together with a historic natural bottleneck, has led to fragmented distributions and extremely low genetic diversity (Schmidt-Kuntzel et al., 2018).

There are many examples of efforts that focus on increasing genetic diversity as a management tool to enhance long-term population viability, and reduce vulnerability of endangered animals (Hedrick, 1995; Roemer & Donlan, 2004) and plant populations (Blythe et al., 2020). Enhancing genetic diversity is considered an integral part of rare plant and animal management (Williams et al., 2008; Holland & Hadfield 2002). The Hawaiian Islands are one of the 25 biodiversity hotspots in the world with more than 95% of native terrestrial species found only here, and are considered Hawaiian endemics (Myers et al., 2000). Hundreds of endemic Hawaiian species are Federally listed as endangered, with critical levels of genetic diversity (Juvik et al., 2008). Patterns of low genetic variability raise questions as to impacts of ongoing and future environmental stressors.

Genetic variation is a requirement for natural selection. In the absence of genetic variation, populations are unable to respond to changing environmental conditions and as a result, face increased risk of extinction (Jump et al., 2009). *Halophila hawaiiana* is endemic to

the Hawaiian Islands, and has very limited distribution, colony sizes, though both of these ecological metrics warrant immediate and thorough attention. Limited distribution may be a result of interspecific competition for space with the second species in Hawai‘i, *H. decipiens* but again relevant data are lacking.

Biogeographic theory suggests that all endemic species on remote volcanic islands arise via long-distance dispersal and colonization by an ancestral lineage, which by definition occurs via establishment of a small number of propagules, and thus strong population bottlenecks and resulting founder effects are built-in features of colonizing lineages (Whittaker et al., 2008). But importantly, there is also evidence that this species does not reproduce sexually (Herbert, 1986). Although the relative strength and roles of these potential mechanisms are difficult to predict, together they present a likely set of conditions that explain the lack of genetic diversity based on both *ITS* sequence analysis and microsatellite-based assays. The genetic data presented here along with the restricted geographic range, highly developed coastal habitat, and patchy distribution of *H. hawaiiana* are all cause for urgent conservation concern, and warrant consideration of *H. hawaiiana* for protected status. In order to achieve listing status, thorough, detailed quantitative field documentation of threats, population condition, distribution and stability of aerial coverage are all needed.

## Conclusions and Future Directions

In a recent study, Waycott et al. (2021) conducted a genome-wide analysis of the endemic seagrass *H. johnsonii,* endemic to the east coast of Florida and originally described as a sister species to *H. ovalis*. This study (Waycott et al., 2021) compared used (~18,000 bp) and low copy nuclear DNA (~6,500 bp) sequences and 900 single nucleotide polymorphisms (SNPs) and

found sufficient evidence to revise the taxonomic status of *H. johnsonii* as a synonym of *H. ovalis*. This genomic study reminds us that taxonomic conclusions based on phylogenies that rely on a barcoding approach, while expeditious and inexpensive, are tentative, and genomic-wide surveys are more likely to result in higher confidence in patterns of evolutionary relationships revealed (Rubinoff & Holland, 2005). As the biomass of seagrass has been shown to be in decline on a global scale, seagrass beds are now considered one of the most threatened coastal marine systems on earth (Orth et al., 2006; Waycott et al., 2009). Across both plant and animal lineages, the persistence of endemic island populations is dependent in part on adequate, healthy levels of genetic diversity. Populations with higher genetic variability have a better chance of being resilient in the face of new selective challenges and environmental disturbances (Connolly et al., 2018). Given a lack of genetic diversity, these populations have a substantially elevated vulnerability to extinction (Ellstrand & Elam, 1993).

The depauperate level of genetic diversity revealed in this study raises potentially alarming conservation concerns. These patterns and concerns warrant additional genetic sampling and analyses from populations throughout the range of the species in the Hawaiian Archipelago. In addition, field studies such as aerial coverage analyses and long-term monitoring of Hawaiian seagrass bed health should be instituted to better assess conservation status, and determine whether some level of protection is warranted, whether as a species of concern, a threatened species, or full protection under the endangered species designation.

In terms of a more thorough assessment of the taxonomy and genomic diversity in this species, it would be informative to survey additional genetic regions beyond a single barcoding gene (Rubinoff & Holland 2005). Especially in light of the recent taxonomic revision based on a genome-wide approach, where *H. johnsoniii* and *H. ovalis* were synonymized (Waycott et al.,

2021), *H. hawaiiana* and *H. ovalis* should also be comprehensively evaluated to confirm the systematic and thus conservation status of this endemic Hawaiian keystone species.

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*Supplementary Materials*

| Micostatelite Name | | Sequence (5’ to 3’) | Successfully amplified for *H. hawaiiana* |
|---|---|---|---|
| HO2 | F<br>R | GAGGTCTTCGTATCGTCTG<br>GCATCTTGTGGTGGTTCT | No |
| HO3 | F<br>R | CGAGGCCTATGTTCCAGAT<br>CAATGCCCAAGTAGGTGA | No |
| HO5 | F<br>R | GAATGGGAAGGTGAAAGAG<br>CACGGCACTGTTCATCTAC | Yes |
| HO8 | F<br>R | ATAACCAAAGCCTCCCAAGC<br>AAATATCAAACGCCCCCTCA | Yes |
| HO20 | F<br>R | AGAGGAAAAGAAAAGCGAG<br>ATGTCACGTGGGACCATAT | Yes |
| HO30 | F<br>R | CGCCGAAGGAAATGTGGAG<br>AACCAACCGATCGACCCT | Yes |
| HO31 | F<br>R | GGTTGTGCGTGAGGTCAAT<br>ATACGCAGGTACGCACTCT | Yes |
| HO36 | F<br>R | CAACTAACCAAACGAGAAAC<br>AACCTTGACACCTGCTAATA | No |
| HO48 | F<br>R | ATCGAACCCAATAGACACCAG<br>CAGGCAACTTAGCAAGAAACT | Yes |
| HO51 | F<br>R | AGATAAGTTTCACTCCTGTG<br>ACCAGAACCAATCAAGAT | Yes |

**Table 1.** Ten commonly used microsatellite primer pairs developed by Xu et al. (2010) and whether the primer amplified for *Halophila hawaiiana.*

| Species | Accession Number | Location | Species | Accession Number | Location |
|---|---|---|---|---|---|
| Halophila beccarii | KM609946 | India | Halophila ovalis | MZ191765 | Taiwan |
| Halophila beccarii | KM609945 | India | Halophila ovalis | MZ191764 | Taiwan |
| Halophila beccarii | KC175914 | Vietnam | Halophila ovalis | MZ191763 | Taiwan |
| Halophila beccarii | AF366441 | Vietnam | Halophila ovalis | MZ191762 | Taiwan |
| Halophila decipiens | AF366411 | Australia | Halophila ovalis | MZ191761 | Taiwan |
| Halophila decipiens | AF366410 | Australia | Halophila ovalis | MZ191760 | Taiwan |
| Halophila decipiens | AF366409 | Costa Rica | Halophila ovalis | MZ191759 | Taiwan |
| Halophila decipiens | AF366413 | Curacao | Halophila ovalis | MZ191758 | Taiwan |
| Halophila decipiens | AF366407 | Florida | Halophila ovalis | MZ191757 | Taiwan |
| Halophila decipiens | AF395673 | Hawaii | Halophila ovalis | MZ191756 | Taiwan |
| Halophila decipiens | AF395672 | Hawaii | Halophila ovalis | MZ191755 | Taiwan |
| Halophila decipiens | AF395671 | Hawaii | Halophila ovalis | MZ191754 | Taiwan |
| Halophila decipiens | AB243984 | Japan | Halophila ovalis | MZ191753 | Taiwan |
| Halophila decipiens | AB243983 | Japan | Halophila ovalis | MZ191752 | Taiwan |
| Halophila decipiens | AB243982 | Japan | Halophila ovalis | MZ191751 | Taiwan |
| Halophila decipiens | AB243981 | Japan | Halophila ovalis | MT347907 | Sri Lanka |
| Halophila decipiens | AB243980 | Japan | Halophila ovalis | MT347906 | Sri Lanka |
| Halophila decipiens | AB243979 | Japan | Halophila ovalis | MT347905 | Sri Lanka |
| Halophila decipiens | AB243978 | Japan | Halophila ovalis | MT347904 | Sri Lanka |
| Halophila decipiens | AB243977 | Japan | Halophila ovalis | MT347903 | Sri Lanka |
| Halophila decipiens | MN200776 | Malaysia | Halophila ovalis | MT347902 | Sri Lanka |
| Halophila decipiens | AF366412 | Malaysia | Halophila ovalis | MT347901 | Sri Lanka |
| Halophila decipiens | AF366408 | Panama | Halophila ovalis | MT347900 | Sri Lanka |
| Halophila decipiens | MT347851 | Taiwan | Halophila ovalis | MT347899 | Sri Lanka |
| Halophila decipiens | MT347850 | Taiwan | Halophila ovalis | MT347898 | Sri Lanka |
| Halophila decipiens | KC175913 | Vietnam | Halophila ovalis | MF371443 | Egypt |
| Halophila major | AB436926 | Indonesia | Halophila ovalis | MF371441 | Egypt |
| Halophila major | AB436928 | Indonesia | Halophila ovalis | MF371434 | Egypt |
| Halophila major | MT028353 | Indonesia | Halophila ovalis | MF371433 | Egypt |
| Halophila major | MT028354 | Indonesia | Halophila ovalis | KX668192 | Thailand |
| Halophila major | MT028355 | Indonesia | Halophila ovalis | KM609942 | India |
| Halophila major | MT028356 | Indonesia | Halophila ovalis | KM609941 | India |
| Halophila major | MW084345 | Indonesia | Halophila ovalis | KM609938 | India |
| Halophila major | MW084346 | Indonesia | Halophila ovalis | KM609937 | India |
| Halophila major | MW084347 | Indonesia | Halophila ovalis | KP408255 | Thailand |
| Halophila major | AB436929 | Japan | Halophila ovalis | KP408254 | Thailand |
| Halophila major | KF620340 | Malaysia | Halophila ovalis | KP408253 | Thailand |
| Halophila major | KF620341 | Malaysia | Halophila ovalis | KP408252 | Thailand |
| Halophila major | MT347852 | Myanmar | Halophila ovalis | KP408251 | Thailand |
| Halophila major | MT586874 | Philippines | Halophila ovalis | KP408250 | Thailand |
| Halophila major | AF366416 | Philippines | Halophila ovalis | KP408249 | Thailand |
| Halophila major | AF366417 | Philippines | Halophila ovalis | KP408248 | Thailand |

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|---|---|---|---|---|---|
| Halophila major | MT347853 | Sri Lanka | Halophila ovalis | KP408247 | Thailand |
| Halophila major | MT347854 | Sri Lanka | Halophila ovalis | KP408246 | Thailand |
| Halophila major | MT347855 | Sri Lanka | Halophila ovalis | KP408245 | Thailand |
| Halophila major | MT347856 | Sri Lanka | Halophila ovalis | KP408244 | Thailand |
| Halophila major | MT347857 | Sri Lanka | Halophila ovalis | KP408243 | Thailand |
| Halophila major | MT347858 | Sri Lanka | Halophila ovalis | KP408242 | Thailand |
| Halophila major | MT347859 | Sri Lanka | Halophila ovalis | KP408241 | Thailand |
| Halophila major | MT347860 | Sri Lanka | Halophila ovalis | KP408240 | Thailand |
| Halophila major | MT347861 | Sri Lanka | Halophila ovalis | KP408239 | Thailand |
| Halophila major | MT347862 | Sri Lanka | Halophila ovalis | KP408238 | Thailand |
| Halophila major | MT347863 | Sri Lanka | Halophila ovalis | KP408237 | Thailand |
| Halophila major | MT347864 | Sri Lanka | Halophila ovalis | KP408236 | Thailand |
| Halophila major | MT347865 | Sri Lanka | Halophila ovalis | KP408235 | Thailand |
| Halophila major | MT347866 | Sri Lanka | Halophila ovalis | KP408234 | Thailand |
| Halophila major | MT347867 | Sri Lanka | Halophila ovalis | KP408233 | Thailand |
| Halophila major | MT347868 | Sri Lanka | Halophila ovalis | KP408232 | Thailand |
| Halophila major | MT347869 | Sri Lanka | Halophila ovalis | KP408231 | Thailand |
| Halophila major | MT347870 | Sri Lanka | Halophila ovalis | KP408230 | Thailand |
| Halophila major | MT347871 | Sri Lanka | Halophila ovalis | KP408229 | Thailand |
| Halophila major | MT347872 | Sri Lanka | Halophila ovalis | KP408228 | India |
| Halophila major | MT347873 | Sri Lanka | Halophila ovalis | KF620355 | India |
| Halophila major | MT347874 | Sri Lanka | Halophila ovalis | KF620354 | India |
| Halophila major | MT347875 | Sri Lanka | Halophila ovalis | KF620353 | India |
| Halophila major | MT347876 | Sri Lanka | Halophila ovalis | KF620351 | India |
| Halophila major | MT347877 | Sri Lanka | Halophila ovalis | KF620350 | Thailand |
| Halophila major | MT347878 | Sri Lanka | Halophila ovalis | KF620349 | Thailand |
| Halophila major | MT347879 | Sri Lanka | Halophila ovalis | KF620347 | Thailand |
| Halophila major | MT347880 | Sri Lanka | Halophila ovalis | KF620346 | Malaysia |
| Halophila major | MT347881 | Sri Lanka | Halophila ovalis | KF620345 | Thailand |
| Halophila major | MT347882 | Sri Lanka | Halophila ovalis | KF620344 | Malaysia |
| Halophila major | MT347883 | Sri Lanka | Halophila ovalis | KF620343 | Malaysia |
| Halophila major | MT347884 | Sri Lanka | Halophila ovalis | KF620342 | Malaysia |
| Halophila major | MT347885 | Sri Lanka | Halophila ovalis | KF620339 | Malaysia |
| Halophila major | MT347886 | Sri Lanka | Halophila ovalis | KF620338 | Malaysia |
| Halophila major | MT347887 | Sri Lanka | Halophila ovalis | KF620337 | Hong Kong |
| Halophila major | MT347888 | Sri Lanka | Halophila ovalis | KC175912 | Vietnam |
| Halophila major | MT347889 | Sri Lanka | Halophila ovalis | KC175911 | Vietnam |
| Halophila major | MT347890 | Sri Lanka | Halophila ovalis | KC175909 | Vietnam |
| Halophila major | MT347891 | Sri Lanka | Halophila ovalis | KC175908 | Vietnam |
| Halophila major | MT347892 | Sri Lanka | Halophila ovalis | AF366437 | Vietnam |
| Halophila major | MT347893 | Sri Lanka | Halophila ovalis | AF366435 | Australia |
| Halophila major | MT347894 | Sri Lanka | Halophila ovalis | AF366434 | Australia |
| Halophila major | MT347895 | Sri Lanka | Halophila ovalis | AF366433 | Australia |
| Halophila major | MT347896 | Sri Lanka | Halophila ovalis | AF366432 | Australia |

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|---|---|---|
| Halophila major | MT347897 | Sri Lanka |
| Halophila major | AB436927 | Thailand |
| Halophila major | KF620348 | Thailand |
| Halophila major | KP408256 | Thailand |
| Halophila major | KP408257 | Thailand |
| Halophila major | KP408258 | Thailand |
| Halophila major | KP408259 | Thailand |
| Halophila major | KP408260 | Thailand |
| Halophila major | KP408261 | Thailand |
| Halophila major | KP408262 | Thailand |
| Halophila major | KP408263 | Thailand |
| Halophila major | KP408264 | Thailand |
| Halophila major | KP408265 | Thailand |
| Halophila major | KC175910 | Vietnam |
| Halophila major | MW850374 | Vietnam |
| Halophila major | MW850375 | Vietnam |
| Halophila major | MW850376 | Vietnam |
| Halophila nipponica | HQ687164 | S. Korea |
| Halophila nipponica | KX668190 | S. Korea |
| Halophila nipponica | KX668189 | S. Korea |
| Halophila nipponica | KX668188 | S. Korea |
| Halophila nipponica | KX668187 | S. Korea |
| Halophila nipponica | KX668186 | S. Korea |
| Halophila nipponica | KX668185 | S. Korea |
| Halophila nipponica | KX668184 | S. Korea |
| Halophila nipponica | AB523410 | Japan |
| Halophila nipponica | AB436937 | Japan |
| Halophila nipponica | AB436936 | Japan |
| Halophila nipponica | AB436935 | Japan |
| Halophila nipponica | AB436934 | Japan |
| Halophila nipponica | AB436933 | Japan |
| Halophila nipponica | AB436932 | Japan |
| Halophila nipponica | AB436931 | Japan |
| Halophila nipponica | AB436924 | Guam |
| Halophila ovalis | MW850379 | Vietnam |
| Halophila ovalis | MW850378 | Vietnam |
| Halophila ovalis | MW850377 | Vietnam |
| Halophila ovalis | ON684375 | Tanzania |
| Halophila ovalis | ON684374 | Tanzania |
| Halophila ovalis | ON684373 | Tanzania |
| Halophila ovalis | ON684372 | Tanzania |
| Halophila ovalis | OM478577 | Tanzania |
| Halophila ovalis | OM478576 | Taiwan |
| Halophila ovalis | OM478575 | Taiwan |
| Halophila ovalis | AF366431 | Australia |
| Halophila ovalis | AF366430 | Australia |
| Halophila ovalis | AF366429 | Australia |
| Halophila ovalis | AF366428 | Australia |
| Halophila ovalis | AF366427 | Australia |
| Halophila ovalis | AF366424 | Australia |
| Halophila ovalis | AF366423 | Australia |
| Halophila ovalis | AF366422 | Australia |
| Halophila ovalis | AF366421 | Japan |
| Halophila ovalis | AF366420 | Malaysia |
| Halophila ovalis | AF366419 | Philippines |
| Halophila ovalis | AF366418 | Australia |
| Halophila ovalis | AF366417 | Philippines |
| Halophila ovalis | AF366416 | Philippines |
| Halophila ovalis | AF366415 | Australia |
| Halophila ovalis | EU477609 | Korea |
| Halophila ovalis | KM609939 | India |
| Halophila ovalis | AB436940 | Indonesia |
| Halophila ovalis | AB436939 | Thailand |
| Halophila ovalis | AB436938 | Thailand |
| Halophila ovalis | AB436930 | Indonesia |
| Halophila ovalis | AB243976 | Japan |
| Halophila ovalis | AB243975 | Japan |
| Halophila ovalis | AB243974 | Japan |
| Halophila ovalis | AB243973 | Japan |
| Halophila ovalis | AB243972 | Japan |
| Halophila ovalis | AB243971 | Japan |
| Halophila ovalis | AB243970 | Japan |
| Halophila stipulacea | AF366436 | Italy |
| Halophila stipulacea | AY352601 | Egypt |
| Halophila stipulacea | AY352602 | Egypt |
| Halophila stipulacea | AY352603 | Egypt |
| Halophila stipulacea | AY352604 | Egypt |
| Halophila stipulacea | AY352606 | Egypt |
| Halophila stipulacea | AY352608 | Egypt |
| Halophila stipulacea | AY352609 | Egypt |
| Halophila stipulacea | AY352612 | Egypt |
| Halophila stipulacea | AY352613 | Egypt |
| Halophila stipulacea | AY352614 | Egypt |
| Halophila stipulacea | AY352615 | Egypt |
| Halophila stipulacea | AY352618 | Italy |
| Halophila stipulacea | AY352619 | Italy |
| Halophila stipulacea | AY352621 | Italy |
| Halophila stipulacea | AY352622 | Italy |

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|---|---|---|---|---|---|
| Halophila ovalis | OM478574 | Taiwan | Halophila stipulacea | AY352623 | Italy |
| Halophila ovalis | OM478573 | Taiwan | Halophila stipulacea | AY352624 | Italy |
| Halophila ovalis | OM478572 | Taiwan | Halophila stipulacea | AY352625 | Italy |
| Halophila ovalis | OM478571 | Taiwan | Halophila stipulacea | AY352626 | Italy |
| Halophila ovalis | OM478570 | Taiwan | Halophila stipulacea | AY352627 | Italy |
| Halophila ovalis | OM478569 | Taiwan | Halophila stipulacea | AY352628 | Italy |
| Halophila ovalis | OM478568 | Taiwan | Halophila stipulacea | AY352629 | Italy |
| Halophila ovalis | OM478567 | Taiwan | Halophila stipulacea | AY352631 | Italy |
| Halophila ovalis | OM478566 | Taiwan | Halophila stipulacea | AY352632 | Italy |
| Halophila ovalis | OM478565 | Taiwan | Halophila stipulacea | AY352634 | Italy |
| Halophila ovalis | OM478564 | Taiwan | Halophila stipulacea | AY352635 | Greece |
| Halophila ovalis | OM478563 | Taiwan | Halophila stipulacea | AY352636 | Italy |
| Halophila ovalis | OM478562 | Taiwan | Halophila stipulacea | AY352637 | Italy |
| Halophila ovalis | OM478561 | Taiwan | Halophila stipulacea | KM609943 | Egypt |
| Halophila ovalis | OM478560 | Taiwan | Halophila stipulacea | KM609944 | India |
| Halophila ovalis | OM478559 | Taiwan | Halophila stipulacea | MF371422 | Egypt |
| Halophila ovalis | OM478558 | Taiwan | Halophila stipulacea | MF371423 | Egypt |
| Halophila ovalis | OM478557 | Taiwan | Halophila stipulacea | MF371424 | Egypt |
| Halophila ovalis | OM478556 | Taiwan | Halophila stipulacea | MF371427 | Egypt |
| Halophila ovalis | OM478555 | Taiwan | Halophila stipulacea | MF371428 | Egypt |
| Halophila ovalis | OM478554 | Taiwan | Halophila stipulacea | MF371429 | Egypt |
| Halophila ovalis | OM478553 | Taiwan | Halophila stipulacea | MF371431 | Egypt |
| Halophila ovalis | OM478552 | Taiwan | Halophila stipulacea | MF371435 | Egypt |
| Halophila ovalis | OM478551 | Taiwan | Halophila stipulacea | MF371440 | Egypt |
| Halophila ovalis | OM478550 | Taiwan | Halophila stipulacea | MF371442 | Egypt |
| Halophila ovalis | OM478549 | Taiwan | Halophila stipulacea | MF371444 | Egypt |
| Halophila ovalis | OM478548 | Taiwan | Halophila stipulacea | MF371446 | Egypt |
| Halophila ovalis | OM478547 | Taiwan | Halophila stipulacea | MF371449 | Egypt |
| Halophila ovalis | OM478546 | Taiwan | Halophila stipulacea | MF371450 | Egypt |
| Halophila ovalis | OM478545 | Taiwan | Halophila stipulacea | MF371453 | Egypt |
| Halophila ovalis | OM478544 | Taiwan | Halophila stipulacea | MF371457 | Egypt |
| Halophila ovalis | OM478543 | Taiwan | Halophila stipulacea | MF371458 | Egypt |
| Halophila ovalis | OM478542 | Taiwan | Halophila stipulacea | MF371462 | Egypt |
| Halophila ovalis | OM478541 | Taiwan | Halophila stipulacea | MF371464 | Egypt |
| Halophila ovalis | OM478540 | Taiwan | Halophila stipulacea | MF371465 | Egypt |
| Halophila ovalis | OM478539 | Taiwan | Halophila stipulacea | MF371466 | Egypt |
| Halophila ovalis | OM478538 | Taiwan | Halophila stipulacea | MF371467 | United Arab Emirates |
| Halophila ovalis | OM478537 | Taiwan | Halophila stipulacea | OM162162 | Greece |
| Halophila ovalis | OM478536 | Taiwan | Halophila stipulacea | OM162163 | Greece |
| Halophila ovalis | OM478535 | Taiwan | Halophila stipulacea | OM162164 | Greece |
| Halophila ovalis | OM478534 | Taiwan | Halophila stipulacea | OM162165 | Greece |
| Halophila ovalis | OM478533 | Taiwan | Halophila stipulacea | OM162166 | Greece |
| Halophila ovalis | OM478532 | Taiwan | Halophila stipulacea | OM162167 | Greece |

| | | | | | |
|---|---|---|---|---|---|
| Halophila ovalis | MZ191767 | Taiwan | Halophila stipulacea | OM162168 | Greece |
| Halophila ovalis | MZ191766 | Taiwan | Halophila stipulacea | OM162169 | Greece |
| | | | Hydrilla verticilla | KU242358 | Connecticut |

**Table 2.** Species, collection location and GenBank accession numbers for the publicly available ITS sequence data used in the phylogenetic reconstruction.

| | *H. ovalis* I-A | *H. ovalis* I-B |
|---|---|---|
| *H. hawaiiana*<br>Clade I-A | 0.00371 | 0.016 |
| H. ovalis<br>Clade I-B | 0.0142 | |

**Table 3.** Distance matrix for well-supported, Clade I between species *H. hawaiiana* and *H. ovalis.* Clade I subunit A contains *H. hawiiana* species and *H. ovalis* species from Australia and Indonesia. Clade I subunit B is made up of *H. ovalis* and is more distantly related to *H. hawaiiana.*