

## Article

# Phylogenomic Insights into the Phylogeography of *Halophila baillonii* Asch.

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**Abstract:** A molecular genetic approach was used to elucidate the phylogeographic relationships of the clover grass (*Halophila baillonii* Asch.) from three key regions within its current distributional range. *Halophila baillonii* is a small seagrass that has historically been only found in a few locations in the Caribbean and Atlantic coast of Brazil. In the past few decades *H. baillonii* has also been observed in the Eastern Tropical Pacific (ETP). Very little is known about the relationship between the ETP populations and the Caribbean and Atlantic ones. To study their relationship, we used a hybrid capture approach targeting chloroplast loci on samples from Belize, Brazil, and the Pacific coast of Costa Rica. Phylogenetic analyses resolved *H. baillonii* as monophyletic and placed the samples from Belize and Costa Rica in a clade sister to the Brazilian ones. The results clearly indicate that the ETP samples are very closely related to Belize and that the most likely explanation of its occurrence in the ETP is a recent introduction, possibly as a consequence of the opening of the Panama Canal.

**Keywords:** Costa Rica; Belize; Brazil; clover grass; Isthmus of Panama; dispersal



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## 1. Introduction

Seagrass meadows, including those dominated by smaller seagrass species such as *Halophila* are a key coastal habitat and provide many ecosystem services. For example, these small seagrasses serve as habitat for fish and invertebrates, provide sediment stabilization, water purification and serve as a food source for megaherbivores such as dugongs [1]. *Halophila* species are considered to be colonizing seagrasses [2,3], where despite low resistance to perturbations they have substantial capacity to grow back following loss. This recovery potential is linked to having fast shoot turnover rates, a short sexual maturity period, and production of a large number of seeds able to persist in a dormant state thereby forming a seed bank in the sediment [3]. Their capacity for recovery means that *Halophila* species play a key role in disturbed or highly dynamic seagrass meadows often acting as the ruderal occupants in these sites [3].

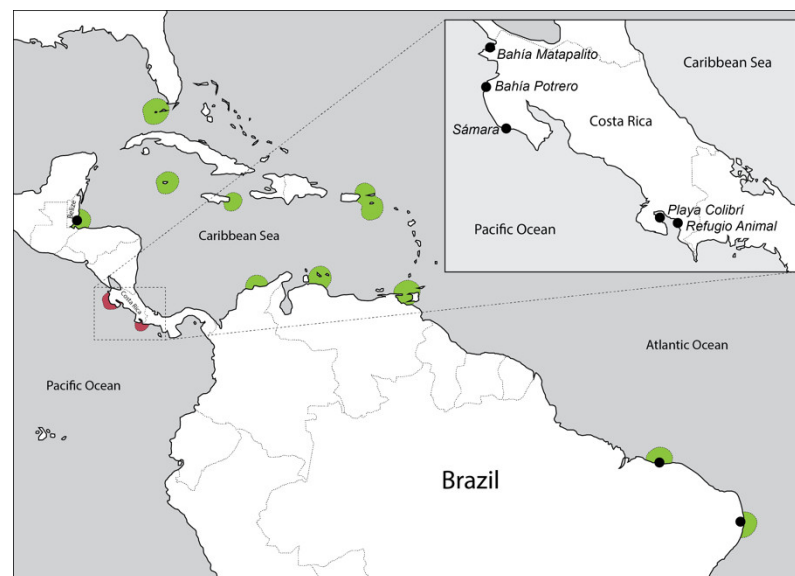
*Halophila* species are found in most seagrass bioregions around the world, being absent only in the Temperate North Pacific and Temperate North Atlantic regions [4]. Some *Halophila* species, such as *Halophila decipiens* Ostenf., have a very wide distribution, being found in all four bioregions where *Halophila* is present [4]. Other species are only found in the southern temperate waters of Australia, such as *Halophila australis* Doty & B.C.

Stone [5]. Species of *Halophila* are easy to recognize as the only seagrass genus with ovate and usually petiolate leaves. Leaf shape is one of the main morphological characteristics that is used for species identification. However, the leaves of some *Halophila* species look very similar to each other and other characters such as leaf venation, leaf margin, and leaf hairs have been used to discriminate species. The *Halophila ovalis* (R.Br.) Hook.f. complex is particularly controversial as key characters (such as leaf size and shape) overlap and do not match the phylogeny e.g., [6]. On the other hand, some *Halophila* species are easier to recognize as these have higher leaf numbers clustered at each node, such as *Halophila spinulosa* (R.Br.) Asch., *Halophila tricostrata* M.Greenway, *Halophila engelmannii* Asch., and *Halophila baillonii* Asch.

The focus of this study is *H. baillonii*, commonly known as “clover grass”. In some instances, the species is likely to have been reported as *Halophila baillonis*, previously considered a synonym for *H. baillonii* [7]. This species has a distribution that is largely confined to the Tropical Atlantic seagrass bioregion [4,7]. It is easily recognizable with vertical stems arising from the rhizome that terminate in two pairs of serrated oval shaped leaves. The species is dioecious and flowers form in the center of the leaf whorls [8].

*Halophila baillonii* has been assessed as having a decreasing population trend and is currently categorized as Vulnerable under IUCN Red List criteria [9]. While largely restricted to the Caribbean and the Atlantic coast of Brazil, it has unexpectedly been reported in several locations in the Eastern Tropical Pacific (ETP) [7,10] (Figure 1). Seagrasses are rarely reported to occur along the tropical Pacific coastline of central America south of Baja California, Mexico. The first sightings of *H. baillonii* in the ETP were in Panama in the 1970s [11]. Further sightings of this species in the ETP were reported in the mid 1990s on the Pacific coast of Costa Rica, at a meadow that vanished following a storm event shortly after its discovery [11]. In recent years, however, *H. baillonii* has been reliably found along the Pacific coast of Costa Rica [10,12–16] and has now also been reported in Nicaragua [17]. Given that the first report of this species in the Pacific was in the 1970s, a reasonable inference is that this is a recent introduction, most likely from the Caribbean to the ETP, but this idea has yet to be investigated.

It would be reasonable, however, to hypothesize that there is more than one alternative scenario to consider. The first is a remnant species distribution hypothesis, where the current restricted and fragmented distribution of *H. baillonii* are remnants of an historically more widespread range. Given this, we would expect the populations in different regions to differ genetically from each other due to isolation over time. Under this model, there would be an assumption that ETP populations established before the formation of the Isthmus of Panama around 2 to 3 Ma and it would be inferred that the species was widespread across areas that were connected to both coasts of what is now Central America [18]. The creation of a land barrier would have limited gene flow between both water bodies eventually due to the total closure of the Isthmus. It is expected that 2–3 M years of isolation would lead to significant divergence and result in a strong phylogenetic signal between the *H. baillonii* in the Caribbean and Atlantic compared to the ETP. We would also expect the Atlantic populations to have a higher affinity with the Caribbean ones, as gene flow between these two is historically likely to occur. It is also likely that extant populations have very low diversity remaining where dispersal–recruitment events or gradients have occurred leading to random genetic drift and further genetic divergence.



**Figure 1.** Locations where *Halophila baillonii* populations have been reported in the Caribbean and Atlantic (Green) [19] and recently in the Eastern Tropical Pacific (Maroon). Sites collected for this study are shown as black dots: Costa Rica (5 sites), Brazil (2 sites), and Belize (1 site). See Table 1 and Supplemental Table S1 for further details on samples from each site.

**Table 1.** Name and origin of *Halophila* samples used in phylogeny. Additional information can be found in Supplemental S1. \* Provided for *H. baillonii* only.

| #  | Sample Name                        | Species               | Country of Origin | Coastline * | Sampling Site                     |
|----|------------------------------------|-----------------------|-------------------|-------------|-----------------------------------|
| 1  | <i>H. baillonii</i> Belize 1       | <i>H. baillonii</i>   | Belize            | Caribbean   | Placencia                         |
| 2  | <i>H. baillonii</i> Belize 2       | <i>H. baillonii</i>   | Belize            | Caribbean   | Placencia                         |
| 3  | <i>H. baillonii</i> Belize 3       | <i>H. baillonii</i>   | Belize            | Caribbean   | Placencia                         |
| 4  | <i>H. baillonii</i> Belize 4       | <i>H. baillonii</i>   | Belize            | Caribbean   | Placencia                         |
| 5  | <i>H. baillonii</i> Belize 5       | <i>H. baillonii</i>   | Belize            | Caribbean   | Placencia                         |
| 6  | <i>H. baillonii</i> Belize 6       | <i>H. baillonii</i>   | Belize            | Caribbean   | Placencia                         |
| 7  | <i>H. baillonii</i> Belize 7       | <i>H. baillonii</i>   | Belize            | Caribbean   | Placencia                         |
| 8  | <i>H. baillonii</i> Costa Rica 1.1 | <i>H. baillonii</i>   | Costa Rica        | Pacific     | Golfo Dulce, Refugio Animal       |
| 9  | <i>H. baillonii</i> Costa Rica 2.1 | <i>H. baillonii</i>   | Costa Rica        | Pacific     | Golfo Dulce, Playa Colibrí        |
| 10 | <i>H. baillonii</i> Costa Rica 2.2 | <i>H. baillonii</i>   | Costa Rica        | Pacific     | Golfo Dulce, Playa Colibrí        |
| 11 | <i>H. baillonii</i> Costa Rica 2.3 | <i>H. baillonii</i>   | Costa Rica        | Pacific     | Golfo Dulce, Playa Colibrí        |
| 12 | <i>H. baillonii</i> Costa Rica 2.4 | <i>H. baillonii</i>   | Costa Rica        | Pacific     | Golfo Dulce, Playa Colibrí        |
| 13 | <i>H. baillonii</i> Costa Rica 3.1 | <i>H. baillonii</i>   | Costa Rica        | Pacific     | Bahía Matapalito                  |
| 14 | <i>H. baillonii</i> Costa Rica 3.2 | <i>H. baillonii</i>   | Costa Rica        | Pacific     | Bahía Matapalito                  |
| 15 | <i>H. baillonii</i> Costa Rica 3.3 | <i>H. baillonii</i>   | Costa Rica        | Pacific     | Bahía Matapalito                  |
| 16 | <i>H. baillonii</i> Costa Rica 4.1 | <i>H. baillonii</i>   | Costa Rica        | Pacific     | Bahía Potrero                     |
| 17 | <i>H. baillonii</i> Costa Rica 4.2 | <i>H. baillonii</i>   | Costa Rica        | Pacific     | Bahía Potrero                     |
| 18 | <i>H. baillonii</i> Costa Rica 5.1 | <i>H. baillonii</i>   | Costa Rica        | Pacific     | Sámara                            |
| 19 | <i>H. baillonii</i> Costa Rica 5.2 | <i>H. baillonii</i>   | Costa Rica        | Pacific     | Sámara                            |
| 20 | <i>H. australis</i> Australia 1    | <i>H. australis</i>   | Australia         | -           | Port Noarlunga                    |
| 21 | <i>H. australis</i> Australia 2    | <i>H. australis</i>   | Australia         | -           | SA Pearsons Sanctuary Zone        |
| 22 | <i>H. australis</i> Australia 3    | <i>H. australis</i>   | Australia         | -           | VIC Flinders Island               |
| 23 | <i>H. beccari</i> Singapore        | <i>H. beccari</i>     | Singapore         | -           | Mandi                             |
| 24 | <i>H. decipiens</i> Australia      | <i>H. decipiens</i>   | Australia         | -           | QLD Lowe Isles                    |
| 25 | <i>H. decipiens</i> Spain          | <i>H. decipiens</i>   | Spain             | -           | Canary Islands El Hierro          |
| 26 | <i>H. engelmannii</i> Mexico       | <i>H. engelmannii</i> | Mexico            | -           | Puerto Progreso                   |
| 27 | <i>H. engelmannii</i> USA          | <i>H. engelmannii</i> | USA               | -           | Florida Black Water Sound         |
| 28 | <i>H. ovalis</i> Australia 1       | <i>H. ovalis</i>      | Australia         | -           | WA Rottneest Island<br>Parker Bay |

Table 1. Cont.

| #  | Sample Name                         | Species               | Country of Origin    | Coastline * | Sampling Site                         |
|----|-------------------------------------|-----------------------|----------------------|-------------|---------------------------------------|
| 29 | <i>H. ovalis</i> Japan              | <i>H. ovalis</i>      | Japan                | -           | Okinawa                               |
| 30 | <i>H. ovalis</i> Antigua            | <i>H. ovalis</i>      | Antigua and Barbuda  | -           | Antigua                               |
| 31 | <i>H. ovalis</i> Australia 2        | <i>H. ovalis</i>      | Australia            | -           | WA Two People Bay                     |
| 32 | <i>H. ovalis</i> Madagascar         | <i>H. ovalis</i>      | Madagascar           | -           | Andrevo Tulear                        |
| 33 | <i>H. ovalis</i> Mauritius          | <i>H. ovalis</i>      | Mauritius            | -           | Flic en Flac                          |
| 34 | <i>H. spinulosa</i> Singapore       | <i>H. spinulosa</i>   | Singapore            | -           | Check Java                            |
| 35 | <i>H. stipulacea</i> Zanzibar       | <i>H. stipulacea</i>  | Tanzania, Zanzibar   | -           | Chwaka Bay                            |
| 36 | <i>H. stipulacea</i> Virgin Islands | <i>H. stipulacea</i>  | USA, Virgin Islands  | -           | St Thomas                             |
| 37 | <i>H. stipulacea</i> UAE            | <i>H. stipulacea</i>  | United Arab Emirates | -           | Mubarras Island                       |
| 38 | <i>H. tricostrata</i> Australia     | <i>H. tricostrata</i> | Australia            | -           | QLD Lowe Isles                        |
| 39 | <i>H. baillonii</i> Brazil 1.1      | <i>H. baillonii</i>   | Brazil               | Atlantic    | Ponta do Socó,<br>Praia das Pedras    |
| 40 | <i>H. baillonii</i> Brazil 1.2      | <i>H. baillonii</i>   | Brazil               | Atlantic    | Ponta do Socó,<br>Praia das Pedras    |
| 41 | <i>H. baillonii</i> Brazil 1.3      | <i>H. baillonii</i>   | Brazil               | Atlantic    | Ponta do Socó,<br>Praia das Pedras    |
| 43 | <i>H. baillonii</i> Brazil 2.1      | <i>H. baillonii</i>   | Brazil               | Atlantic    | Barra do Mamanguape,<br>Banco d'areia |
| 44 | <i>H. baillonii</i> Brazil 2.2      | <i>H. baillonii</i>   | Brazil               | Atlantic    | Barra do Mamanguape,<br>Banco d'areia |
| 45 | <i>H. baillonii</i> Brazil 2.3      | <i>H. baillonii</i>   | Brazil               | Atlantic    | Barra do Mamanguape,<br>Banco d'areia |

The alternative hypothesis is that *H. baillonii* is a recent introduction into the ETP. There are multiple pathways and vectors through which this could have occurred. One possibility is the drifting/rafting of plant fragments from one ocean to the other via the Panama Canal, which was opened in 1914. However, the canal has locks and the inner sections are composed of freshwater so this seems unlikely. A second alternative is that the opening of the Panama Canal increased ship traffic, and that plant fragments or seeds were moved to the Pacific by large ships (e.g., on anchors, solid ballast, or ballast water). Seeds or plants could also be carried on small watercraft (e.g., on anchors or in storage lockers) hauled between seaboards. Recent biotic dispersal is also possible, and for example birds could have moved seeds from the Caribbean to the ETP, covering a distance of only ~80 km. This seems to be unlikely, however, as it could have occurred many times during the last 2–3 M years, and we would then expect that *H. baillonii* and other Caribbean seagrass species would be more common in the ETP. Under the premise that *H. baillonii* is a recent introduction to the ETP, we expect low genetic divergence between these populations and the source population(s) in the Caribbean and/or Atlantic. We would also expect the Caribbean and Atlantic populations to have higher genetic differentiation than the ETP.

This study aimed to assess whether *H. baillonii* populations in the ETP and those in the Caribbean and Atlantic are conspecific. Furthermore, we attempt to identify the origin of the disjunction in *H. baillonii* across the Isthmus of Panama. To further these aims, we use a phylogeographic genomic approach where multiple chloroplast gene regions were sequenced using hybridization capture.

## 2. Materials and Methods

### 2.1. Sampling, DNA Extractions and Library Preparation

Phylogeographic analyses were conducted on a total of 25 *H. baillonii* samples that were collected from Costa Rica in the ETP (12 samples), Brazil in the Atlantic (6 samples) and Belize in the Caribbean (7 samples, see Table 1 and Figure 1). Additionally, plant material from seven other *Halophila* species were added to the sample set to assess phylogenetic vs. phylogeographic distances between species and populations (Table 1). Samples of *H. baillonii* from Costa Rica and Brazil were specifically collected for this study. We

have made repeated attempts over the past 10 years to obtain samples of *H. baillonii* from other locations within the current distribution range of this species. However, our endeavors have proven extremely challenging given the limited abundance of this species in the field and of known current localities for active sampling. The remainder of the *Halophila* samples are part of a large dry sample collection that has been gathered over the past 25 years in the Waycott lab in Adelaide, Australia. Brazilian samples were collected under licence No. 80652-1 and genetic resources exported under SisGen/CGEN/MMA number R9C7FD8.

DNA extractions from Belize and Brazil were performed using the DNeasy® Plant Mini Kit (Qiagen) starting with ~10 mg of tissue. Grinding was done on a Bead Ruptor 24 (OMNI International Inc.) in 2 mL screw cap tubes. Initial lysis steps were done manually and after the ice precipitation step extractions were finalized on a QIAcube HT (Qiagen) following the manufacturer's instructions. Final DNA elutions were done in 100 µL EB buffer. Samples from Costa Rica were extracted locally using NucleoSpin Plant II (Macherey-Nagel), ground with liquid nitrogen starting with ~100 mg of dry tissue according to manufacturer's instructions. Total DNA was eluted two times with 25 µL PE buffer. The concentration and purity of DNA extracts were measured using a spectrophotometer (NanoDrop 2000c) and samples were sent to Adelaide, Australia for analysis.

For phylogenetic and phylogeographic data sets the myBaits® target capture kits (Daicel Arbor Biosciences, Ann Arbor, MI, USA) were used to isolate target genes. Illumina sequencing libraries and RNA bait captures were performed using the method described in Waycott, et al. [20]. For this study, a refined version of OzBaits chloroplast bait (myBaits design ID: D10125PtCOI) set was used to isolate informative loci [20], consisting of ~50 chloroplast loci instead of 19 loci (Design ID: D10122Plstd). Library preparation was done with the NEBNext® Ultra™ II FS DNA Library Prep kit with Fragmentase and Sample Purification Beads (New England Biolabs, Ipswich, MA, USA). Neat DNA extracts were used as the starting material and reactions were done in 1/3 volumes see details in [20]. To enable bioinformatics processing following myBaits capture, Y-adaptors (compatible to Illumina TruSeq primers) with synthetic "barcodes" were annealed to the ends of the DNA fragments. These barcodes allow for samples to be identified by a unique synthetic 8 nt DNA barcode situated on the 3' end of the Read 1 and 2 primer binding sites. Hybrid capture was performed following myBaits manufactures manual (chemistry v5.0), hybridization was done at 63 °C and incubated for 24 h. Post capture PCR was performed on the half build libraries to fuse the remaining sequencing adapters to the ends of the DNA fragments [20], as this library was sequenced with other libraries on the same Illumina lane 8nt unique P5 and P7 indexes were inserted. Libraries were pooled in equimolar concentrations and size selected to 350–600 bp with a Pippin Prep 2% agarose cassette (Sage Science, Beverly, MA, USA). Subsequently they were quantified on a 2100 Bioanalyzer (Agilent) using the high sensitivity DNA assay and molarity was calculated between 300 and 800 bp. Final libraries were sent for Illumina paired-end sequencing (2 × 150) on a NovaSeq 6000 S1 lane to the Australian Genome Research Facility (AGRF) in Melbourne.

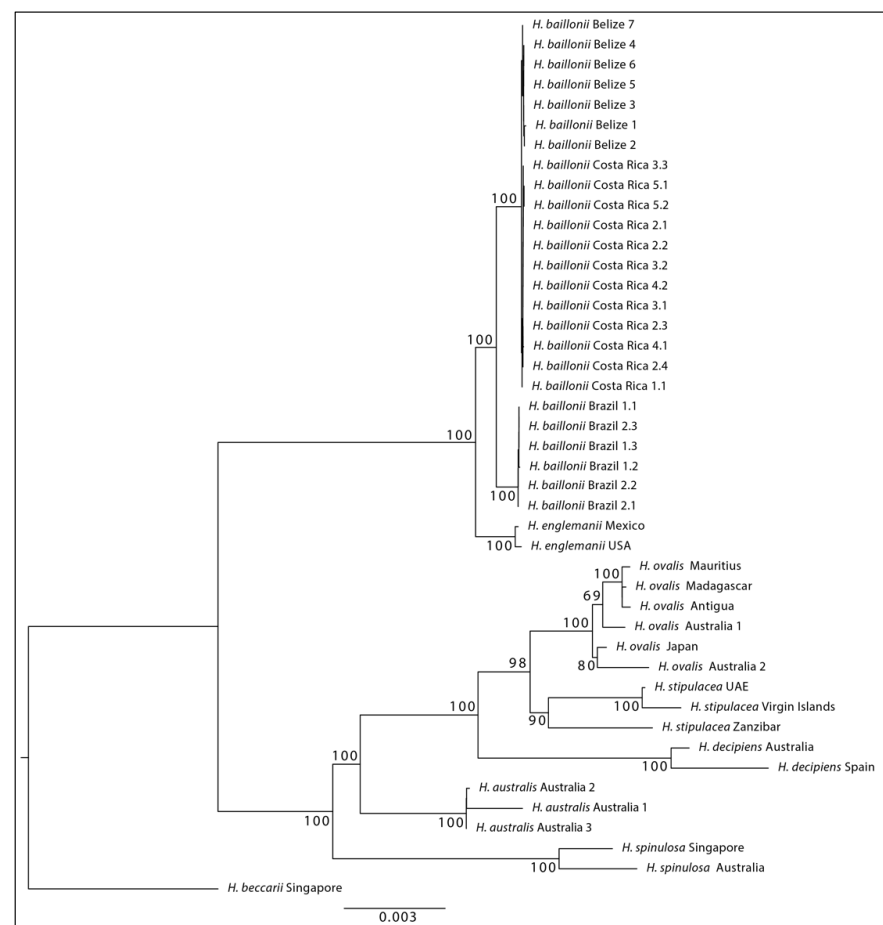
## 2.2. Post Sequencing Data Processing Targeted Genes

High-throughput 150 bp paired-end reads were processed using CLC Genomics Workbench v12 (<https://www.qiagenbioinformatics.com>, accessed on 27 May 2022). Reads were demultiplexed and named based on the 8 nt barcodes followed by trimming to remove residual adapter sequences and low-quality base calls (Phred-score threshold of 20). For the chloroplast genome recovery, a mapping reference was generated by mapping the reads of a single *H. baillonii* sample (GIBS 1973) to the chloroplast genome of *Halophila beccari* Asch. (NCBI accession number MN843749; CLC conditions 85% of length of each read had to map with 95% similarity). Consensus sequences were extracted using a coverage cut-off of 20, consensus length of ≥400 nt and using 'vote' to resolve conflicts (a total of 33 regions were retained, Supplemental S2). Reads from each sample were then mapped against

this reference (75% of length of each read had to map with 90% similarity) and consensus sequences extracted with a read threshold of 10 and calling ambiguous bases (Ns) below this cut-off. Consensus sequences for each sample were imported into Geneious Prime v2022 [21]. Alignments were generated for the 33 chloroplast regions using the MUSCLE [22] plugin for Geneious with default parameters and 24 regions were kept for further analyses. The concatenated alignment of the 24 chloroplast regions (Supplemental S3) was analyzed using the maximum likelihood phylogenetic software IQ-TREE 2 [23] with the best model estimated by ModelFinder [24] and branch support assessed with 1000 ultra-fast bootstrap (UFB) replicates [25]. A haplotype network was generated using a *H. baillonii* only sample set (see Table S1 and alignment; Supplemental S4) in PopArt (<http://popart.otago.ac.nz>, accessed on 4 June 2022) using the Median-joining algorithm [26] for network inference. For these analyses we first masked all ambiguous sites in the concatenated alignment to reduce the impacts of missing values.

### 3. Results

Sequencing outputs for the sample libraries varied between 200,000 to 4 M reads. Trimmed unprocessed reads can be found in the SRA database from NCBI (Accession numbers available in Table S1). Phylogenetic analyses of the chloroplast data resolved *H. baillonii* as monophyletic with strong statistical support (UFB  $\geq 95\%$ ; [27]) and placed the samples from Belize (Caribbean) and Costa Rica (Pacific) in a clade that is sister to the Brazilian (Atlantic) samples (Figure 2 and Supplemental S5 for *H. engelmannii* and *H. baillonii* only). Network analyses of the masked alignment indicates that of 25 segregating sites 21 separate the Brazilian from the Caribbean and Costa Rican samples (Supplemental S6).



**Figure 2.** Maximum-likelihood topology obtained from chloroplast hybridization capture of 24 chloroplast regions for 45 samples of *Halophila* using PhyML, *Halophila beccarii* was used to root the tree.

#### 4. Discussion

This study investigated the phylogeographic relationships of *Halophila baillonii* from three key regions representing the extent of the current distributional range for this seagrass species. We aimed to establish if the occurrences of *H. baillonii* on the Pacific coast of Costa Rica in the Eastern Tropical Pacific (ETP) are: (1) remnant populations that established before the closing of the Panama Isthmus; or (2) they are the result of more recent dispersal and establishment from the Tropical Atlantic and/or Caribbean. The phylogeographic analysis demonstrated that *H. baillonii* from the Caribbean, Atlantic, and the ETP are genetically closely related and group separately from the other *Halophila* species analyzed (Figure 2 and Supplemental S6). If we accept a vicariance hypothesis, the divergence across the Panama Isthmus would be in the order of 3 M years (i.e., the timing of the seaway closure [18]). In light of recent molecular clock estimates for *Halophila* e.g., [28], this period of separation is similar to those estimated for the divergence among sister species and among major infra-specific lineages within the genus. If we are willing to accept these estimates, the limited genetic differentiation between the *H. baillonii* in the Caribbean, Atlantic, and in the ETP favors a hypothesis that populations from these regions separated substantially more recently than the formation of the Panama Isthmus.

The *H. baillonii* samples from the Atlantic are approximately 5500 and 7000 km distant from the Caribbean ones (Figure 1) and show a much higher level of genetic divergence relative to differences between the Caribbean and ETP ones (Figure 2 and Supplemental S6). While the origin of this divergence is unclear, we suggest that accepting the first hypothesis, above, would suggest an implausibly ancient separation of the Atlantic from the Caribbean lineages. One potential driver for the observed level of differentiation is the Amazon–Orinoco Plume, a major biogeographic barrier between the Caribbean and the Brazilian sites south of the Amazon sampled in the Atlantic, corresponding to the freshwater and sediment outflows of these major river systems e.g., [29]. This plume has been shown to be an important barrier to animal dispersal, particularly through the Pleistocene (c. 2.5–0.01 M years ago) e.g., [30], although its effect on seagrass dispersal is unclear. It remains possible that the distribution range of *H. baillonii* was more extensive in the past and that the Atlantic populations have become isolated over time.

Many *Halophila* species appear to be capable of very long-distance dispersal given their very widespread distribution. For example, *H. decipiens* is found globally and has very low genetic diversity among bioregions, implying regular and long-distance dispersal [6,31]. Other *Halophila* species have quickly and successfully colonized previous unoccupied coastal areas or seas. *Halophila ovalis* for example, which is not known to occur naturally in the Atlantic, has ‘recently’ been translocated to the Eastern coast of Florida. A comprehensive study by Waycott et al. [6] demonstrated that *Halophila johnsonii* Eiseman is conspecific with *H. ovalis* and is most likely an introduction from an east African population. This study also found that the Floridian population might be one huge clone. Another example is *Halophila stipulacea* (Forsskål and Niebuhr) Asch., a species that was originally only known to occur in the Indian Ocean, the Persian Gulf and the Red Sea [19]. However, *H. stipulacea* invaded the Mediterranean Sea about 150 years ago following the opening of the Suez Canal. In 2002, the species was reported for the first time in the Caribbean islands, quickly reaching the South American mainland [32–34]. It is therefore quite conceivable that *H. baillonii* has crossed to the Pacific Ocean and has established.

The above-mentioned ‘recent’ range expansions are most likely due to human mediated mechanisms as opposed to the natural dispersal pathways of these plants. These include the opening of shipping canals (e.g., Panama Canal and Suez Canal) connecting previously discrete waterbodies, and the movement of biological material by ships, increasing the dispersal potential beyond the species natural limits. We believe that the most likely origin for *H. baillonii* dispersal to Costa Rica (as a Phase II dispersal event [35]) is via ship anchor, solid ballast or ballast water through the Panama Canal, as this is the fastest and potentially less physiologically stressful mode of dispersal. We cannot immediately discount the possibility that fragments of the seagrass were dispersed by superficial currents through

the Panama Canal, but the internal waters of Gatun Lake are fresh, making survival far more challenging and thus unlikely. Our findings on the dispersal of *H. baillonii* to the ETP are the first confirmed report of a seagrass species crossing the American continental barrier through the Panama Canal. This highlights the potential for a similar dispersal pathway from the Caribbean into the ETP for other *Halophila* species that have been expanding their distributional range, such as *H. stipulacea* and *H. ovalis*.

The findings of this study clarify the phylogenetic relationships between localities, but do not indicate if these dispersal event (s) happened once or multiple times, or if a single ramet (genetic individual) or multiple ramets were transported across the Panama Canal. The establishment and maintenance of a *H. baillonii* population at a new location can occur by seed dispersal and germination (sexual reproduction) or by the dispersal and elongation of vegetative fragments (asexual reproduction) [36]. *Halophila baillonii* is a dioecious plant, whereas for sexual reproduction to occur at least one flowering shoot of each sex is needed. Female and male flowers and fruits of *H. baillonii* have been found in Belize [8,37] and Brazil [38–40]. In contrast, no fruits or seeds have been reported to date in the Pacific coast of Costa Rica for this species [12,14–16] and only a few reports exist of female flowers for *H. baillonii* in the field or grown under experimental conditions (JSV pers. obs.). Similarly, *H. stipulacea* is also dioecious and successfully reproduces sexually in its native region; while in its non-native regions of the Mediterranean and the Caribbean it has shown limited fruit and seed production, along with dominance of male flowering [41]. Based on these observations it is likely that *H. baillonii* has colonized the ETP only once and that all populations are the results of fragmentation and dispersal of the same plant. This hypothesis is supported by our data as only one haplotype was detected in our samples for the ETP. Colonisation and expansion by fragmentation has been observed before in seagrasses e.g., [42,43], and one of the most recent studies proposes that all populations of *H. ovalis* (previously *H. johnsonii*) along the eastern Florida Coast are part of the same clone [6]. To establish if *H. baillonii* in the ETP is a single clone a population genetic study needs to be conducted using a high number of co-dominant markers. Co-dominant markers are essential as heterozygous loci in the ETP would result in offspring with mixed allele allocations, thus confirming sexual reproduction, if it exists. Co-dominant marker loci would also assist in identifying if reproduction is only clonal, and this would be manifested by the presence of only fixed heterozygotes among all samples, as stated that is very unlikely if alleles are exchanged. Therefore, the presence of *H. baillonii* in other locations of the ETP needs to be assessed actively to further understand the dispersal and maintenance mechanisms of this species.

The results of this study provide knowledge that will influence the management of this seagrass species. *Halophila baillonii* is currently classified as Vulnerable on the IUCN Red List, given its fragmented population of limited geographic range [9,12]. The ETP populations would have been seen as highly vulnerable if they had been found to be remnants of a broader distribution pre-dating the Panama Isthmus. Furthermore, the presence of *H. baillonii* on the Pacific coast of Costa Rica would not lead to a reclassification of this species to the category of Least Concern. *Halophila baillonii* disappeared from two sites on the Pacific coast of Costa Rica [11] and thus its current status is of critical importance. At the same time, the presence of this species in the southern Pacific coast of Costa Rica has been reported by the local community dating back to 1969 [15]. The presence of this species for over 50 years indicates that conditions in the ETP are favorable for growth of *H. baillonii*.

Regardless of its origin, *H. baillonii* provides ecosystem services in the ETP and is yet to show invasive tendency by displacing native species. *Halophila baillonii* has been found in the ETP coexisting with *Halodule beaudettei*, monospecifically in deeper portions of the meadow, and as the only seagrass species at multiple locations [12,14,16]. There is evidence of grazing on *H. baillonii* [10] and meadows containing this species store organic carbon in their sediment on the Pacific coast of Costa Rica [12,14–16]. The recent introduction of *H. stipulacea* to the Caribbean (20 years) has led to it being considered an invasive species, as it can form extensive meadows and can outcompete native Caribbean seagrasses [41]. At

other locations, *H. stipulacea* has quickly colonized previously unvegetated substrate and deeper areas, while it is also found growing in mixed meadows with native species [41]. Meanwhile, the introduction of *H. stipulacea* over 150 years ago to the Mediterranean only led to restricted and small populations [41]. Despite being an introduced species to the Mediterranean and Caribbean, where the seagrass *H. stipulacea* grows it changes ecosystem functioning and can provide or enhance some ecosystem services such as carbon sequestration and fish habitat [41,44,45]. At this point, we consider *H. baillonii* to be a recent introduction from the Caribbean to the ETP, where it is non-native and naturalized but not currently considered to be an invasive species.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d15010111/s1>, Supplemental Table S1: Metadata *Halophila baillonii* phylogenetic analysis samples; Supplemental S2: FASTA file with chloroplast reference sequences used for mapping; Supplemental S3: FASTA alignment of concatenated hybridization capture chloroplast loci; Supplemental S4: FASTA file with masked *H. baillonii* network alignment; Supplemental S5: Detailed chloroplast tree of *H. engelmannii* and *H. baillonii* only; Supplemental S6: *H. baillonii* haplotype network.

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