Evolution of Population Structure and Life History Types in Eelgrass (*Zostera marina*)

Bryan Antonio Briones Ortiz

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Reading Committee:
Kerry Naish, Chair
Jennifer Ruesink
Lorenz Hauser

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Abstract

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Chair of the Supervisory Committee:
Kerry Naish
School of Aquatic and Fishery Sciences

Foundation species structure the ecosystems they inhabit, and their resilience is key to maintaining the stability of the environments that they support. The seagrass *Zostera marina* (eelgrass) is one such species with high ecological relevance, distributed across geographically complex estuarine systems throughout the northern temperate hemisphere. Rapid meadow declines have drawn increasing conservation attention, yet the success of recovery strategies may be hindered by a lack of data on population genetic diversity, connectivity, and the adaptive variation underlying ecologically important traits. In eelgrass, the annual strategy, which reproduces predominantly through seeds, may aid recovery following disturbance, while perennial clonal growth supports persistence under stable conditions. This dissertation employs genomic approaches to characterize the genetic structure, local adaptation, and evolution of key life history variation in eelgrass. (Chapter 1) I first characterized population genetic structure and connectivity across the Puget Sound and outer Pacific Coast, where eelgrass is distributed across geographically complex habitats and has experienced population declines. Using RAD sequencing across 16 Washington locations and outer-coast sites in Oregon and California, I found that clonality was more prevalent within the Puget Sound than along the outer coast, and that pairwise differentiation varied markedly (F_{st} = 0.02–0.42), with significant isolation by

distance. Population assignment identified four major groups corresponding to the outer coast, north and south Puget Sound, and the Hood Canal. All populations exhibited small effective sizes and little contemporary gene flow, and outlier analyses revealed signatures of local adaptation that primarily reflected geography. Altogether, these findings provide a basis for informing management across the State, such as delineating management units. (Chapter 2) I next asked whether annual and perennial life histories in eelgrass that occur in close proximity represent locally adapted genetic variation or plastic phenotypes. Combining a common-garden reciprocal transplant experiment with population genetic analyses in a single estuary (Willapa Bay), I found no differential survival to maturity, but a greater likelihood of flowering in annual-sourced plants and branching in perennial-sourced shoots. Reproductive trait performance was greater for local individuals, indicating adaptive differentiation, and fine-scale structure separated life history types, distinguished mainly by whether seedlings flowered, regardless of geographic source or outplant location. These results provide the first evidence of genetic control of life history in the species, offer insight into the adaptive capacity of this variation, and establish a basis for considering it in restoration practices. (Chapter 3) Last, genetic differentiation between isolated regions exceeds that between life histories within a site, raising the possibility of independent evolutionary transitions between annual and perennial strategies. Thus, I investigated whether life history variation represents replicated evolution across the species range, and whether there was a shared genomic basis across locations. I used whole-genome sequencing to reconstruct evolutionary relationships and test for parallel divergence for annual and perennial plants across ten sites spanning the eastern Pacific and both Atlantic coasts. Phylogenetic inference supported multiple transitions between strategies, with the grouping of samples driven primarily by geography (a major division between Pacific and Atlantic samples). Parallel genomic divergence was identified across Pacific but not Atlantic life history types, and repeated allele frequency change at a subset of candidate loci (some implicated in life history divergence in other plant systems) indicated a partially shared genomic

basis. From these results, I concluded that annual and perennial strategies have evolved repeatedly across the range, with shared standing variation underlying parallel divergence in the Pacific and more independent evolution of life histories in the Atlantic. Together, these chapters show that eelgrass population structure is governed by geography and oceanography, that annual and perennial strategies can be adaptively differentiated when co-located in close proximity, and that these life histories have evolved repeatedly across ocean basins, with parallel divergence within the Pacific likely originating from shared standing variation and a transition toward more independent genomic changes in the Atlantic. These findings contribute to our understanding of population structure, adaptation, and replicated evolution in a foundation species, informing the trait-based management of a declining foundation species and advancing a broader understanding of replicated evolution in the wild.

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ii) DEDICATION

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CHAPTER 1: POPULATION GENETIC STRUCTURE OF *ZOSTERA MARINA* (EELGRASS) IN PUGET SOUND AND OUTER PACIFIC COAST

1.1. ABSTRACT

Eelgrass (*Zostera marina*) is a foundation species distributed across geographically complex estuarine systems along the Pacific coast of North America, including the Puget Sound in Washington State, USA. Rapid meadow declines within this inland sea have drawn increased conservation attention; however, the success of conservation and recovery strategies may be hindered by a lack of data on population genetic diversity and connectivity. We used RAD sequencing to characterize population genetic structure across 16 Washington locations and two outer coast populations in Oregon and California. Clonality was more prevalent within the Puget Sound than along the outer coast; notably, two sites in the southern Sound were dominated by only a few highly clonal genotypes. Pairwise differentiation varied markedly (*Fst* = 0.02-0.42), with significant isolation by nautical distance that was greater for comparisons within Puget Sound. Population assignment within Washington identified four major groups that largely corresponded with the outer coast, north and south Puget Sound, and the Hood Canal. Along the Pacific coast, Washington and Oregon populations were more related to one another than to California meadows. All populations exhibited small effective sizes and little to no contemporary gene flow among locations. Outlier analyses identified signatures of local adaptation, with allele frequencies primarily reflecting geographic patterns of variation. Our findings indicate that eelgrass population structure in Washington is shaped by regional oceanographic patterns, heterogeneous geography, and broad environmental regimes. The genetic groupings identified here provide a basis for delineating management units and guiding restoration strategies that explicitly account for population structure across the State.

1.2. INTRODUCTION

Describing the population genetic structure of ecologically relevant species is a key step towards developing effective management and conservation strategies (Pazzaglia et al., 2021). Characterizing spatial patterns of genetic variation is essential for assessing genetic connectivity and evaluating the evolutionary potential of populations under environmental change (Procaccini et al., 2001). For species of conservation concern, information on genetic composition across the landscape can guide efforts to prioritize populations for protection,

design plans to maintain or enhance connectivity, and implement other interventions aimed at ensuring their long-term survival in the face of anthropogenic and natural pressures (Allendorf et al., 2022). When these species play important roles in the environment, their conservation may have cascading effects that support the enhancement of biodiversity and the provision of ecosystem services (Cardinale et al., 2012; Orth et al., 2020).

The seagrass *Zostera marina*, commonly known as eelgrass, is a marine angiosperm that plays a critical role in maintaining the stability of nearshore environments (Gutiérrez et al., 2011; Duarte et al., 2013). Eelgrass is the most widely distributed seagrass species, found in shallow coastal waters across the northern hemisphere (Green and Short, 2003). As both a foundation species and an ecosystem engineer, this species provides essential ecosystem services such as complex habitat structuring, carbon sequestration, shoreline protection, and nursery grounds for marine life (Hemminga and Duarte, 2000; Bos et al., 2007; Sato et al., 2016). Describing patterns of population genetic structure in *Z. marina* is therefore a crucial step towards enhancing conservation efforts and informing management strategies in eelgrass-dominated areas of conservation concern (Pazzaglia et al., 2021).

Population genetic structure in eelgrass is strongly influenced by diversity in the species' reproductive and dispersal strategies (Talbot et al., 2016; Kim et al., 2017; Ries et al., 2023; Yu et al., 2023). Notably, eelgrass is a partially asexual species that may reproduce sexually through seeds and asexually via lateral rhizome expansion (Phillips, Stewart Grant and Peter McRoy, 1983; Yang et al., 2013). Most eelgrass beds are perennial, primarily propagating clonally and with less than 10% meadow-wide flowering rates (Olesen, 1999). In contrast, rare annual populations can exhibit nearly 100% flowering within a year, producing large quantities of seeds that may facilitate habitat recolonization (Meling-López and Ibarra-Obando, 1999; van Katwijk and van Tussenbroek, 2023). The extent of clonality or sexual reproduction within seagrass meadows is closely linked to population performance, with higher genotypic and genetic diversity often associated with improved resilience, recovery, and productivity (Hughes and Stachowicz, 2004, 2009; Reusch et al., 2005). Further, while longer-distance gene flow (up to 300 Km) could be facilitated by rafting plant material (Harwell and Orth, 2002; Källström et al., 2008) or biotically through herbivore consumption (Sumoski and Orth, 2012), dispersal through pollen and seed is typically restricted to a few tens of meters from the parent bed (Ruckelshaus, 1996; Hosokawa et al., 2015). This diversity of reproductive and dispersal strategies, combined with the fragmented nature of marine habitats, can contribute to the formation of strong population genetic structuring in eelgrass even across small spatial scales (<1 Km) (Oetjen and Reusch, 2007; Hays et al., 2021; Briones Ortiz et al., 2025).

The population structure of eelgrass along the Pacific Coast of North America is characterized by pronounced spatial genetic differentiation shaped by a combination of historical events, patterns of gene flow, and local adaptation (Talbot et al., 2016; Yu et al., 2023; Briones Ortiz et al., 2025). Across this region, eelgrass meadows are found from the Baja California peninsula in Mexico to the Bering Sea in Alaska (Muñiz-Salazar et al., 2005; Talbot et al., 2016). Historically, the eastern Pacific populations arose following two trans-Pacific colonization events from Japan, an earlier dispersal into Southern California and a later one into Alaska (Yu et al., 2023). Eelgrass populations along the outer coast of Washington through northern California are considered a likely admixture zone between these two lineages (Yu et al., 2023). Moreover, regional genetic stratification largely driven by ocean currents and land barriers has been described in detail mainly at the northern (Talbot et al., 2016) and southern (Muñiz-Salazar et al., 2005; Olsen, Coyer and Chesney, 2014) regions of the Eastern Pacific distribution. A strong inverse relationship between latitude and genetic diversity and heterozygosity at the northern part of this range indicates the influence of postglacial northward expansion on shaping regional diversity (Talbot et al., 2016; Yu et al., 2023). At finer-scales, within bays or between adjacent ones, genetic differentiation may arise due to spatially limited gene flow (Reynolds et al., 2017), which could be enhanced by adaptive processes such as life expectancy variation (Briones Ortiz et al., 2025) or temperature gradients (DuBois et al., 2022; Schiebelhut et al., 2023). These multiscale patterns of population genetic structure highlight the complex interactions of broad-scale and localized events in shaping genetic composition of eelgrass populations along this extensive coastline.

Washington State in the United States occupies a central position within the Pacific Coast distribution of *Z. marina* in North America, an area where the population structure of this seagrass remains comparatively less well resolved. In Washington, eelgrass meadows occur along the outer coast in semi-enclosed estuaries, and across a highly fragmented inland coastline shaped by fjord-like inlets and numerous islands throughout the Puget Sound. Population genetic stratification within and between nearby bays has largely been attributed to spatially-limited gene flow or potential microgeographic adaptation (Ruckelshaus, 1998; Briones Ortiz et al., 2025). However, the region's geographical and oceanographical complexity likely contributes to shaping population stratification across larger spatial scales. Prominent features such as the Olympic Peninsula and shallow sills in Admiralty inlet and the Tacoma Narrows have been recognized as local barriers to gene flow and drivers of population structure in other marine species with diverse dispersal capacities (Small et al., 2005; Gierke et al., 2023;

Wray et al., 2025). However, broader-scale descriptions of eelgrass genetic diversity and population structure throughout Washington remain largely lacking.

Protecting or conserving threatened *Z. marina* meadows is a priority in Washington State (Washington State Department of Natural Resources, 2015). Regional and site-specific declines of eelgrass meadows have prompted an increase in efforts to protect this species locally (Shelton et al., 2017; Graham et al., 2024). In fact, dramatic losses of eelgrass in the region resulted in the creation of legislature aiming to conserve the species through a variety of strategies that include transplanting adult shoots or seeds to accelerate habitat recolonization and expansion (Washington State Legislature, 2022). Despite the implementation of such efforts, recent assessments indicate that the conservation goals in Washington State have yet to be met (Christiaen et al., 2022). However, the success of such interventions may be hindered by a lack of data on the genetic diversity and population structure of the target populations. Pronounced genetic stratification across Washington eelgrass populations may have direct implications for restoration efforts, because mismatches between donor and transplant sites and the presence of local adaptation in population persistence can significantly affect transplantation success (Ort et al., 2014; Pazzaglia et al., 2021).

This study aimed to describe the population genetic structure of *Z. marina* meadows across a geographically complex area of the species' range, thus providing valuable insights for local management and conservation efforts. To achieve this goal, we conducted a population genomic survey of eelgrass beds across major oceanographic regions of Washington State [Fig. 1]. We then extended our population genetics analyses to a broader distribution of *Z. marina* – by incorporating more distant sites along the US Pacific Coast in Oregon and California – to evaluate the influence of long-distance connectivity on shaping local genetic composition [Fig. S1]. Our first objective was to evaluate clonal diversity at each site, in order to assess the relative effort in cloning compared to sexual reproduction across locations and oceanographic areas. Our second objective was to characterize the genetic diversity and population differentiation of eelgrass meadows within Washington and along the broader outer coast range to identify patterns of genetic variation at multiple spatial scales. We hypothesize that populations in more topographically and oceanographically complex regions, such as the Puget Sound, may exhibit stronger genetic population stratification compared to those located along more continuous habitats, such as the Pacific Coast, where gene flow may be less impeded. And last, given ongoing population declines in Washington, we evaluated which populations are most at risk. This final objective was accomplished by: 1) assessing demographic independence through estimates of migrant ancestries among sites to determine the extent to which populations are

reproductively isolated, and 2) estimating effective population size, which serves as a critical indicator of a population's long-term evolutionary potential under environmental change. Together, these analyses provide a perspective on population vulnerability to further inform local management and conservation.

1.3. METHODS

1.3.1. Sample Collection & Genotyping

1.3.1.1. Sampling, DNA Extraction & Library Construction

Eelgrass tissue samples were collected along the continental US West Coast to investigate population structure [Fig. 1A, S1] [Table S1]. We surveyed a total of 20 sites, including 16 locations in Washington State and four additional sites along the Pacific coastline.

At each site, we sampled 50 adult shoots near mean lower low water (except for the San Juan Islands where divers were involved) at regular intervals of 3-5 m between plants to reduce the likelihood of collecting clonemates. Although the density of eelgrass beds may vary considerably even across spatially-proximate meadows (Ruesink et al., 2022), significant kinship structure associated with clonality has been detected in spatial scales as small as two to four meters within beds (Hämmerli and Reusch, 2003a). Thus, sampling shoots over several meters apart likely minimizes clonal bias by reducing the probability of repeatedly sampling the same genet. Pale meristems and young green leaves were targeted to reduce the presence of polyphenol compound contaminants. The tissues were rinsed in distilled water and flash frozen in liquid nitrogen before being transferred to a -80°C freezer until DNA extraction.

High molecular weight DNA was extracted using the DNeasy Plant Pro Kit (Qiagen Inc., Valencia, CA, USA). A TissueLyser II (Qiagen Inc., Valencia, CA, USA) was used to homogenize frozen tissues before DNA extraction. Genomic libraries were constructed for restriction-site associated DNA sequencing (RAD-seq) (Ali et al., 2016) with the restriction enzyme Sbf1. The genomic libraries were sequenced using the Illumina NovaSeq 4000 (Genomics & Cell Characterization Core Facility, Eugene, Oregon), the NovaSeq 6000 SP (Northwest Genomics Center, Seattle, Washington), and the S4 (Genewiz, South Plainfield, New Jersey) platforms with 150 bp paired-end settings.

1.3.1.2. SNP Genotyping and Filtering

Raw sequence files were processed using STACKS (Catchen et al., 2013). Pooled-library reads were demultiplexed using the process_radtags program with –best-rad settings and trimmed to 137 bp based on Phred scores greater than 30 per base sequence quality in FASTQC (Babraham-Bioinformatics, 2015). The single-end reads were aligned to the published genome of *Zostera marina* (Ma et al., 2021), using BWA-MEM (Li, 2013), with minimum alignment and mapping Phred quality scores of 30.

The sequenced reads were divided into two datasets for analysis. One dataset included samples from all 16 eelgrass collections in Washington State to analyze fine-scale population structure [Fig. 1], and the second comprised individuals from six sites on the Pacific coastline, two each in Washington, Oregon, and California [Fig. S1]. Treating the two datasets as separate facilitated variant detection for each analysis, because loci that are fixed between more divergent populations (Washington and California) may be polymorphic at a finer scale (within Washington). Both datasets underwent similar bioinformatic processing and analyses unless otherwise stated [Table S2].

The genotyping of polymorphic loci was conducted separately for each dataset using the ref_map pipeline in STACKS (Rochette and Catchen, 2017). Unfiltered bi-allelic base pairs scored in all populations were initially retained. Subsequent filtering was performed using the R-package SNPFILTR (DeRaad, 2022) [Table S2]. Individual genotype calls with a sequence depth ≥ 10x and an allelic balance 0.2 < 0.8 in heterozygotes were retained. SNPs with more than 20% missing genotypes across all individuals were subsequently removed. At each RAD tag (137 bp), the SNP with the highest minor allele frequency (MAF) was retained and subsequently designated as a locus (other SNPs on the same RAD tag were removed to minimize the effects of potentially linked markers). Individuals with more than 20% missing genotypes across all loci were also removed. Finally, loci with minor allele frequencies less than 0.05 or with significant deviations from Hardy-Weinberg equilibrium (significant Fis values) in all the samples were removed (Pearman, Urban and Alexander, 2022).

1.3.2. Population Genetic Analyses

1.3.2.1. Clonality

Throughout, we followed naming conventions for clonal species (Tuomi and Vuorisalo, 1989); ramets for a single demographic individual produced by clonal propagation, and genets for a distinct genetic group of ramets that originate from a single seed (Harper, 1977). Clone mates were identified using measures of genetic similarity between pairs of ramets (shoots in

our collection). The genomes of clonal individuals are identical-by-descent (IBD), except for the occurrence of rare somatic mutations and genotyping errors. Therefore, we estimated the proportion of genome-wide IBD SNPs between pairs of individual plants using the function –genome in PLINK (Chang et al., 2015). Pairwise genetic similarity is expected to be considerably higher between clonal compared to unrelated plants, therefore thresholds for the detection of clone mates were determined after visually examining the relatedness distributions for each dataset [Fig. S2]. We predicted a bimodal pattern in the frequency distribution of pairwise genetic similarities that could be used to determine this threshold. Multiple shoots that were identified as IBD were designated as belonging to a multilocus lineage, MLL (Arnaud-Haond et al., 2007), and thus to the same genet. For subsequent genet-based population genetic analyses, only one representative genotype per MLL was retained.

We calculated genotype richness (R) for all locations, a measure of relative number of genotypes generated through sexual processes:

$$R = \frac{nMLLs-1}{nInd-1}$$

Where nMLLs and nInd represent the number of unique genotypes (MLLs) and all genotypes at each location, respectively. Both genet and ramet-based data were analyzed for the Washington and Pacific Coast regions.

1.3.2.2. Within-Population Genetic Diversity

We used the R-package DartR (Gruber et al., 2018) to evaluate site-specific within-meadow diversity richness (percent of polymorphic loci, % Pol) and evenness (observed heterozygosity, Ho; and expected heterozygosity, He). The number of private alleles (Pa) was used to identify rare variation found in only one population.

At each site, departure from random mating according to Hardy Weinberg equilibrium expectations was assessed site by estimating inbreeding coefficient (Fis) (Hoban et al., 2022). Confidence intervals (95%) were generated through bootstrapping over 10,000 permutations.

1.3.2.3. Population Differentiation

Population genetic structure was investigated using individual- and population-based analyses to describe spatial patterns of genetic divergence. Genetic differentiation between pairs of sample sites (pairwise *Fst*) (Weir and Cockerham, 1984) was quantified using the R-package HIER*FST*AT (Goudet, 2005; de Meeûs and Goudet, 2007), using 10,000 bootstrap permutations to estimate 95% confidence intervals (CI).

We tested whether there was a relationship between genetic and geographic distances among sites using isolation-by-distance analyses to assess the degree of population structuring in the geographically complex Puget Sound and along the continuous outer coast. Specifically, we conducted Mantel tests to assess the correlation between pairwise *Fst* and minimum distance over water using the mantel.test function in the R-package ncf (Bjornstad, 2022), with 100000 resamples for assessing statistical significance. Distinct relationships were observed between subgroups within the datasets; therefore, separate Mantel tests were conducted for the following comparisons: (1) between the Puget Sound and the Washington Pacific Coast, (2) within the Puget Sound, (4) across all sites in Washington State, and (5) across all sites on the outer Pacific Coast.

Hierarchical population structure was inferred using Bayesian individual assignment analysis implemented in STRUCTURE v2.1.1.5 (Pritchard, Stephens and Donnelly, 2000). Identification of distinct genetic clusters was based on an admixture model with correlated allele frequencies (burn-in period of 10,000 iterations and 100,000 MCMC repetitions), which assumes that populations share recent common ancestry and allele frequencies have diverged by drift from this shared ancestral state. The number of clusters of individuals (K) tested ranged from 1 to 17. The most likely K was evaluated using multiple approaches: ΔK statistic (Evanno, Regnaut and Goudet, 2005) and mean L(K) (Pritchard, Stephens and Donnelly, 2000), both of which rely on changes in the log likelihood between successive K values. We also used two conservative metrics from the Puechmaille method (Puechmaille, 2016), MedMedK and MedMeanK, which estimate the median of the median and mean, respectively, of assignment probabilities across clusters for each population. These estimates evaluate the consistency of individual assignments to the same cluster across replicates and account for uneven sampling among populations.

Genetic relatedness within and between populations were visualized by conducting a principal components analysis (PCA) using the R-package ADEGENET (Jombart and Ahmed, 2011).

1.3.2.4. Estimates of Recent Migration

To assess demographic independence within and among populations, we estimated recent migrant ancestries among sites (Wilson and Rannala, 2003). Such estimates can provide insight into the designation of management units and are especially relevant to understanding how dispersal from local eelgrass meadows supports regional population structure across the State, given the aforementioned complexity in oceanographic features. We calculated the

proportion of individuals within sites originating from the same location (mii) or from other (mij) sampled sites per generation using the function BA3SNP in the program BA3-SNPs (Mussmann et al., 2019), based on 100,000 total MCMC iterations, a burn-in of 10,000, and sampling every 1,000 iterations. Mixing parameters for migration rates (-m), allele frequencies (-a), and inbreeding coefficients (-f) were obtained using BA3-SNPS-autotune.py (Washington: -a 0.2125 -m 0.075 -f 0.1; Pacific Coast: -a 0.2125 -m 0.1 -f 0.1) (Mussmann et al., 2019).

1.3.2.5. Effective Population Size

Estimates of recent effective population size (Ne) based on linkage disequilibrium (LD) were generated to evaluate long-term population viability in the context of genetic variation driven by drift (Waples, 1991). Here, the magnitude of LD is inversely related to Ne under the assumption that non-random associations between alleles at different loci in closed, randomly mating populations primarily arise from genetic drift, rather than from selection or migration (Waples and Do, 2008; Waples, Larson and Waples, 2016). Ne estimates and their 95% CI were obtained for both datasets using the function ldNe from the R-package StrataG (Waples, Larson and Waples, 2016; Archer, Adams and Schneiders, 2017).

1.3.2.6. Detection of loci under selection

Considering the highly variable physical and environmental conditions of eelgrass habitats across Washington State, and the reduced neighborhood sizes of eelgrass meadows (Ruckelshaus, 1996), it is possible that local selective forces have contributed to fine-scale population divergence and adaptation (Briones Ortiz et al., 2025). To investigate whether selection has played a role in shaping genetic variation in Washington, we applied three population-level outlier detection approaches to identify SNPs putatively under selection. We excluded the highly clonal populations of Dyes Inlet and Rocky Bay to avoid bias in outlier detection, as their reduced genotypic diversity can inflate signals of differentiation and potentially lead to the detection of false positives. First, PCAdapt was used to detect outlier loci that are highly correlated with the principal components of overall population genetic variation, an approach suitable for detecting selection when population structure is continuous or complex (Luu, Bazin and Blum, 2017). BayeScan uses a Bayesian framework to model the effect of selection on allele frequencies (Foll and Gaggiotti, 2008). This method identifies SNPs with unusually low and high genetic differentiation (*Fst*), indicative of balancing and divergent selection, respectively, by comparing models with and without selection at each locus. And last, we used OutFLANK, a more conservative approach that controls for false positives especially in datasets with unequal sample sizes or hierarchical population structuring (Whitlock and

Lotterhos, 2015). This *Fst*-based frequentist method identifies outlier loci by fitting an empirical distribution of neutral *Fst* values and detecting SNPs with higher differentiation.

SNPs were identified as outliers if they were labeled as such in at least two of the three approaches applied. Outliers for each method were identified using a false discovery rate (FDR)–corrected p-value threshold of 0.05 (q-value). The frequencies of the most common allele for each of the identified outliers were examined to investigate the spatial pattern associated with the signals of putative selection. A BLAST (Altschul et al., 1990) search for the gene sequences of the loci putatively under selection was conducted against the reference genome of *Zostera marina* "Zmarina v3.1" (Ma et al., 2021) in the Phytozome hub (Goodstein et al., 2012). BLAST hits were retained if they met the following criteria: alignment length ≥ 140 bp, e-value ≤ 1 × 10−25, and sequence similarity ≥ 99%. The possible functional roles of the closest matches within 10000 base pairs of the annotated regions identified through BLAST were analyzed for their biological relevance.

1.4. RESULTS

1.4.1. SNP genotyping & filtering

A total of 688 eelgrass plants across 16 sites in Washington were successfully genotyped at 563 SNPs before removal of clones [Full details provided in Tables S1, S2], while 252 plants in the Pacific Coast dataset were genotyped at 555 biallelic SNPs [Table S1, S2]. Individuals from Willapa Bay and Grays Harbor were included in both datasets.

1.4.2. Population Genetic Analyses

1.4.2.1. Clonality

Analyses of the incidence of clonality highlight the varying role of asexual reproduction across populations in shaping genetic variation across the State. A 90% similarity threshold in pairwise IBD was used to detect clonemates within the Washington dataset [Fig S2], resulting in a total of 428 unique genotypes (61.8 % of all collected shoots) identified for genet-based analyses [Table S2, S3]. The proportion of unique genets per population varied widely across the State (0-100%) [Fig. 2]. Padilla Bay (genotypic richness, R = 1), Grays Harbor (R = 0.98), and Cherry Point (R = 0.95), comprised almost entirely unique individuals. In contrast, some sites were dominated by a few clones, such as Dyes Inlet (R = 0.02) and Rocky Bay (R = 0.04), where only two and three MLLs were detected, respectively, with a single genet within each site accounting for more than 70% of the sampled ramets. The remaining locations exhibited

intermediate levels of genotypic richness (R = 0.35 - 0.84). Across all sites, there was an average of 5.1 ramets per genet; removal of Dyes Inlet and Rocky Bay reduced this value to 2.9 ramets per genet per site [Fig. 2].

The results also suggest a comparatively lower prevalence of clonality along the Pacific Coast relative to the inland Puget Sound. A total of 218 unique genotypes were identified in the Pacific Coast dataset following clone filtering using a 91% similarity threshold [Table S2, S3]. Between 4% and 65 % of shoots within locations were assigned to a clonal genet, with the lowest proportion observed at Valino Island, Oregon, and the highest at Newport Beach, California [Fig. 2]. Most sites had high genotypic richness (R > 0.80), except for Newport Beach (R = 0.52), indicating that most individuals represented unique genotypes. Further, there was an average of 2.48 ramets per genet across all sites.

1.4.2.2. Within-Population Genetic Diversity

After removing clones, within-population genetic diversity differed considerably between and within regions across Washington. In general, polymorphism and heterozygosity were comparatively lower in geographically secluded sites, such as those in the Hood Canal as well as in inlets and embayment in the North and South Sound [Table S3]. Notably, the low polymorphism and low genotypic richness in Dyes Inlet and Rocky Bay suggest that these meadows are dominated by a few highly homozygous genotypes Nearly half of the scored biallelic loci (% Pol = 46.8 – 61.2%) were polymorphic across locations, except for Nisqually, Dyes Inlet and Rocky Bay (% Pol ~ 30%) [Table S3]. Observed and unbiased expected heterozygosity exhibited narrow ranges across all sites (Ho = 0.114 – 0.189; uHe = 0.114 – 0.186) but were marginally higher in the central part of the Sound [Table S3]. All sites except Padilla Bay contained unique alleles (Pa = 2 – 18) not shared with other meadows, with a higher frequency of rare alleles in locations along the main channel of the Sound [Table S3]. Eleven sites, spanning all regions, showed significant but low departures from HWE expectations based on Fis estimates (Fis = -0.028 – 0.056) [Table S3], with mostly positive values indicating slight heterozygote deficits consistent with some inbreeding or substructuring.

Within-population genetic diversity patterns along the outer coast were not explained by latitudinal gradient. Within-population genetic diversity was greater diversity in central populations, while southern sites exhibit lower diversity but a higher frequency of unique alleles. Measures of polymorphism and estimates of heterozygosity were highest in Oregon (%Pol = 69%; average uHe and Ho = 0.21), followed by Washington (%Pol = 59%; average uHe and Ho = 0.16), and lowest in California (%Pol = 32%; average uHe and Ho = 0.08) [Table S3]. Fewer

private alleles, a rare variation found in only one population, were identified in both Washington and Oregon (Pa = 1–8) compared to California (Pa = 21–32) [Table S3]. Only Valino Island in Oregon exhibited a significant but small deviation from HWE expectations (Fis = 0.078).

1.4.2.3. Population Differentiation

All measures of population structure across the State revealed extensive differentiation, particularly in the inland waters of Puget Sound [Fig. 1, S5], and highlighted the role of geographic isolation and oceanographic features in shaping broad and fine scale population genetic structure in eelgrass.

Measures of pairwise genetic differentiation (*Fst*) between sites within the State spanned a 17-fold range [Table S4] and were significantly correlated with geographic distance ($R = 0.68$; $p < 0.05$) [Fig. 3]. The highest *Fst* values were between Willapa Bay on the outer coast and Nisqually in the inner South Puget Sound (*Fst* = 0.421), and between sites in the southern range of the Hood Canal and Nisqually (up to *Fst* = 0.399). In addition, relatively lower levels of genetic divergence were observed in all comparisons involving exposed locations along the main channel of the central Sound basin (Port Gamble, Dumas Bay, and Maury Island), and for comparisons within the sheltered cluster of sites east of the San Juan archipelago (Padilla Bay, Fidalgo Bay, and Ship Harbor) [Table S4]. While the number of sites was smaller in the analyses comparing outer coast populations [Table S5], average pairwise genetic differentiation was four times lower between Washington and Oregon populations (WA-OR *Fst* = 0.176) than between these states and California (WA-WA *Fst* = 0.715; CA-OR *Fst* = 0.621).

Isolation by distance was steeper within Puget Sound (Slope = 0.00092 km-1; $R = 0.66$; $p < 0.001$) [Fig. 3] compared to an analysis across all populations in Washington (Slope = 0.00044 km-1; $R = 0.68$; $p < 0.001$), suggesting that the local geographic configuration of the inland sea results in greater population structure across smaller spatial scales.

Population assignment (STRUCTURE) within Washington State supported four groupings as the most likely (Evanno method, K = 4), largely explained by oceanographic distances and finer scale features within the Puget Sound [Fig. 1B, S6]. The clusters distinguished regions characterized by different oceanographic regimes: Outer Coast, South Puget Sound, North of Puget Sound, and the Hood Canal [Fig. 1B]. Evidence of admixture at some sites suggests some localized gene flow. First, Port Gamble is located at the mouth of Hood canal – this site appears to be influenced by gene flow from both Hood Canal and South Sound, as well as from North Sound to a lesser extent. [Fig. 1B]. Eastsound in the North has evidence of admixture between the North Sound and Hood Canal clusters only, which differentiates this site

from all other North Sound sites. Along the Pacific coast, both methods used to evaluate the outputs from STRUCTURE supported K = 3 as the most likely number of genetic clusters, with each corresponding primarily to a different State [Fig. S7, S8]. Low levels of admixture were observed between Oregon and Washington (K = 3), and Oregon and California (K = 2) [Fig S7].

A multivariate approach used to infer genetic relationships between individuals revealed hierarchical population structure within the State (PCA, Fig 1C). The first axis of the PCA separated the coastal and inland populations [Fig. 1C], explaining 14.07% of the variance. The second axis further resolved regional differentiation within inland Washington waters (explaining 9.05% of the variance), first discriminating Hood Canal from the mainstem Puget Sound, and then North from South Sound populations. As in the STRUCTURE analyses, Port Gamble was placed intermediate between the mainstem and Hood Canal populations. Analyses of the coastal populations only separated the sites in California from those in Washington and Oregon along the first axis, explaining the greatest variance [Fig S9; 74.6%], with Axis 2 further subdividing the latter two populations [Fig. S9; 19.1%].

1.4.2.4. Estimates of Recent Migration

Estimates of self-recruitment (mii) [Table S6] for all sites across Washington were significant. The highest estimates were in Willapa Bay and Potlatch (~ 0.91), suggesting the greatest isolation. Willapa Bay is the southernmost coastal population, whereas Potlatch is found in South Hood Canal. But in contrast, one of the lowest estimates was in Belfair (0.79), just south of Potlach. Both Dyes Inlet and Rocky Bay also had lower estimates (0.72) but were represented by very few genets (Fig 2).

Most pairwise estimates of recent immigrant ancestry (mij) were non-significant (Fig 4, Table S6). Five significant values suggested recruitment from neighboring populations: Willapa Bay north to Grays Harbor on the Outer Coast (0.12), Dumas Bay east to Maury Island in the South Sound (0.12), Potlach south to Belfair in the Hood Canal, Ship Harbor north to Padilla Bay (0.16) and Cherry Point (0.08) in Northern Puget Sound.

Sites on the Pacific Coast also exhibited significant rates of self-migration (mii >0.81), with the highest estimates in Willapa Bay in Washington (0.96) and in Seal Beach, California (0.95). The difference between the Willapa Bay estimates between the two datasets was likely explained by variation in the polymorphic loci that were scored. Such dataset differences may also explain the change in magnitude of significant migration observed from Willapa Bay north to Grays Harbor (0.25), in contrast to the State only analyses. Significant and higher recent

between population migration was observed in Oregon, with an estimated 25% of migrants in the more northerly Clam Island originating from Valino Island.

1.4.2.5. Effective Population Size

Estimates of effective population size based on genets ranged 100-fold across sample sites [Table S3]. The highest Ne estimates were observed in Potlatch (Ne = 103.8) and Grays Harbor (Ne = 120.5), and the lowest in Dyes Inlet and Port Gamble (Ne < 1), where there were very few unique genotypes. The remaining sites across the State had intermediate values (Ne = 32.5–74.2), suggesting small and relatively isolated populations across Washington.

Along the Pacific Coast, Ne estimates had a wider range [Table S3], from the highest at Clam Island in Oregon (Ne = 145.03), followed by Grays Harbor in Washington (Ne = 118.91) to the lowest at both sites in California (Ne = 13.6 – 43.05).

1.4.2.6. Detection of loci under selection

A total of seven outlier loci were detected across two methods in the Washington State dataset (Fig S11, Table S7). PCAdapt and BayeScan detected 9 and 40 outlier SNPs, respectively, while OUTFlank did not detect any. Two pairs of the shared outlier SNPs (four total loci) were found in adjacent RAD tags on chromosomes one and three – 40 and 189 bp of separation between each pair of neighboring SNPs, respectively [Fig. S11, S12] [Table S7].

Allele frequencies at five of the outlier loci reflected geographic structuring, suggesting the influence of correlated environmental conditions on adaptive diversity across Washington (Fig S12). However, the exact relationship with geography differed across outlier loci. For example, the most common allele for the outlier SNP 1_3125291 was prevalent in most Puget Sound populations and absent along the outer coast; however, the major allele for outlier SNP 6_20585521 had low presence in both the outer Coast and the South Sound compared to the North Sound and Hood Canal [Fig. S12]. These contrasting patterns suggest that factors influencing adaptation vary across the State and differ in their action on specific loci. Interestingly, the frequencies of adjacent outlier SNPs 3_5296412 & 3_5296601 (189 bp apart) on chromosome three were not correlated [Fig. S12]. Such a result may indicate elevated recombination within this genomic region, or structural differences between the genome in Washington State and the European genomic reference.

All but two of the sixteen genes located overlapping or within 10,000 bp of the BLAST queries were successfully annotated [Table S8]. Broad functional annotations suggest potential roles of abiotic stress response (Zosma05g33460–33500), as well as multi-layered control for

gene expression and cellular/membrane integrity in adaptive processes, including cellular transport (Zosma01g22590, Zosma06g19100) and ribosome structure (Zosma03g07180).

1.5. DISCUSSION

This study aimed to test the effects of complex bathymetry and geography on the population genetic structure of *Zostera marina* across a portion of its range along the West Coast of the United States, and particularly, throughout the Puget Sound, a fjord-like inland sea, and the adjacent Pacific Coast of Washington State. We surveyed genetic variation across 16 locations within the inner fjord system in Washington, and six meadows that represented the outer coastline that included two outgroup populations south of the focal study area. We found a higher prevalence of clonality in the Puget Sound compared to the outer coast; notably, two sites in the southern Sound were dominated by only a few highly clonal genotypes. Overall, genetic diversity was lower within Puget Sound. Pairwise measures of genetic differentiation (*Fst*) revealed significant structure between all populations, with values ranging from 0.02 to 0.42, and significant isolation by distance over water. The slope of the relationship was greater within Puget Sound than across all populations including the outer coast. Population assignment within Washington State identified four major groups that largely corresponded with the outer coast, north of Puget Sound, south Puget Sound, and the Hood Canal. An additional multivariate analysis identified hierarchical population structure, with most of the variance in the data set explained by differentiation between the outer coast and Puget Sound, followed by geographic structuring within the Sound itself. A focused analysis on the coastal populations alone revealed closer grouping between the Washington and Oregon populations compared to the sites in California. All populations had small effective population sizes and little contemporary gene flow among locations, although weak asymmetrical dispersal was estimated between a few adjacent sites. Outlier analyses identified signatures of local adaptation, with allele frequencies primarily reflecting geographic patterns of variation. Broad annotations of the loci putatively under selection pointed towards the role of cell structural integrity and transport, and control of gene expression. Together, these results highlight the role of geographic processes, coupled with extensive isolation, in shaping the population structure of eelgrass meadows along the US West Coast, with elevated differentiation within the inland sea.

1.5.1. Factors Influencing Population Structure

The interaction between circulation patterns and physical features shape the population genetic structure of eelgrass in Washington State. The outer coast and the inland sea are

separated by the Strait of Juan de Fuca [Fig. 1], a long and deep channel that is characterized by a two-layer flow typical of estuaries: deep inflow of cold saltwater from the Pacific Ocean and shallow outflow of warmer freshwater originating from the Puget Sound and the Strait of Georgia (Cannon, 1978). This pattern of water circulation likely facilitates outward gene flow from the inland sea toward the Pacific through the dispersal of rafting plant material closer to the water surface (Kendrick et al., 2012). However, the pronounced genetic differentiation between these regions indicates that gene flow across the Strait is highly restricted, promoted by discontinuity of suitable eelgrass habitats on the outer coast near the Olympic Peninsula (Christiaen et al., 2022). Genotyping of plants from rare meadows along the Strait would help to characterize connectivity from the outer Washington Pacific coast to the Puget Sound, as has been done for other marine macrophytes with similar range distributions (Gierke et al., 2023; Bemmels et al., 2025). Broadly, these results reflect those observed in many other marine species (Drinan et al., 2018; Gierke et al., 2023; Bemmels et al., 2025; Wray et al., 2025), which suggests that the Olympic peninsula acts as a strong biogeographic barrier isolating populations on the outer coast and the inland sea for diverse taxa including eelgrass.

Eelgrass population structure within the Puget Sound reflected well-defined oceanographic subregions: north of the Puget Sound, southern Puget Sound, and the Hood Canal. In the inland sea, water flow is primarily driven by large tidal exchanges with the Pacific Ocean where current speeds can reach high velocities in some locations of the San Juan Islands and Admiralty Inlet at the entrance of Puget Sound proper (Rice et al., 2015). Such hydrodynamic conditions could facilitate the mixing of floating and suspended plant materials across the northern and southern subregions. However, rafting shoots may accumulate away from the shoreline in nearshore eddies and tidal fronts that form as the tides interact with the irregular bathymetry of the Sound (Cannon, 1978; Yang and Wang, 2013). A large number of eddies on the eastern side of the San Juan Islands (Cannon, 1978) may explain the significant population structure between sites in the island archipelago and those across the adjacent strait in the north Sound. South of Admiralty Inlet, a series of shallow sills further constrain circulation within Puget Sound proper and generate residual tidal eddies that limit water exchange among the central and south Sound subbasins, and the Hood Canal (Babson, Kawase and MacCready, 2006; Yang and Wang, 2013). The effect of these sills on water circulation differs among passages; for example, mixing is more widespread at the Tacoma narrows in the South than at the head of the Hood Canal (Yang and Wang, 2013). This differential pattern of hydrodynamic isolation might explain the greater differentiation between eelgrass populations in the Hood Canal and those in the central Sound, compared with those in the more connected

central and south Sound basins. The north-south differentiation in the main basin is likely due to both greater geographic isolation and longer water residence times in the south (Babson, Kawase and MacCready, 2006; Rice et al., 2015).

Long-distance connectivity among eelgrass populations along the outer Pacific Coast also appears to be influenced by circulation patterns and physical features. Realized dispersal of eelgrass along the outer coastline is likely bidirectional, facilitated by the transport of floating material southward by the California Current during the summertime and northward by the Davidson current during the winter months (Hickey, 1979; Yu et al., 2023). This pattern of bidirectional gene flow may explain the higher estimates of genetic diversity for meadows in Oregon. However, this region has also been identified as a putative admixture zone between two hypothesized trans-Pacific colonizations (Yu et al., 2023). While no consistent barriers to gene flow have been identified between our Washington and Oregon sampling sites, several features including Cape Blanco, Cape Mendocino, and Point Conception have shaped the genetic structure of marine taxa along the Oregon-California coastline (Kelly and Palumbi, 2010).

Historical events and changes in connectivity have also likely played important roles in shaping eelgrass genetic variation across Washington. It has been hypothesized that many marine species in the area colonized the Salish Sea from the Pacific Ocean following the last glacial maximum (Sotka, Hempelmann and Biermann, 2005; Petrou et al., 2013; Gierke et al., 2023; Wray et al., 2025). The comparatively lower genetic and genotypic diversities observed within the Puget Sound, such as Dyes Inlet, Rocky Bay, Nisqually, and Eastsound, suggest post-glaciation population expansion into the inland sea, possibly from refugia populations along the outer coast (Yu et al., 2023). As the ice sheets retreated from the Puget Sound area following the Vashon Glaciation nearly 16,000 years ago (Haugerud*, 2021), successive founder events at the leading edge of the expansion back into the Sound from the Pacific Ocean may have eroded genetic diversity through repeated founder events. In partially clonal species such as eelgrass, this process may result in the establishment of clonal lineages derived from sexual populations with low genetic diversity and reduced capacity for sexual reproduction (van der Kooi and Schwander, 2014; Pereyra et al., 2023; Ries et al., 2023). Such a landward colonization pattern may explain the higher clonality observed in Puget Sound meadows, where some locations were dominated by few clonal lineages.

Gene flow in *Z. marina* is also influenced by differences in the dispersal capacity of sexual propagules. Pollen tends to sink quickly and remain viable for only a few hours, which makes it an ineffective vector for long distance dispersal through rafting (Kendrick et al., 2012).

Seeds, although capable of remaining viable for longer periods, also exhibit limited dispersal potential due to their rapid sinking and burial in sediments (Harwell and Orth, 2002; Li et al., 2018; Sotka et al., 2024). The long-distance transport of seeds requires resuspension from the benthos during deep-water high-energy current events (Alvarez, 2019; Zhao et al., 2021). Such scenarios are rare in the Puget Sound because the strong water column stratification and the depth of local basins promote deep water retention that could restrict the transport of eelgrass seeds on the substrate. Rafting flowering (or vegetative) shoots could also contribute to dispersal over distances exceeding 150 km (Harwell and Orth, 2002; Källström et al., 2008), however, such drifting material may become trapped in hydrographic barriers such as eddies and tidal fronts closer to shallow sills (Yang and Wang, 2013). Thus, while high reproductive effort may enhance the potential for gene flow originating in genotypically diverse meadows, the realized connectivity in Washington eelgrass is largely modulated by the dispersal ecology of their propagules through local hydrodynamic conditions.

The composition of populations by life history types, and the frequency of flowering across meadows, are expected to influence the opportunities for dispersal by pollen and seed and hence population structure (van Katwijk and van Tussenbroek, 2023; Briones Ortiz et al., 2025). Eelgrass in Washington State exhibits annual and perennial life histories (Ruesink et al., 2022; Briones Ortiz et al., 2025), characterized by differences in lifespan and seed productivity, which is five times higher in annual populations compared to conspecific perennial ones (van Katwijk and van Tussenbroek, 2023). However, all the meadows included in this study, and most meadows in Washington, are perennial, and thus, exhibit limited sexual reproductive effort. Such reduced investment in reproduction may limit the availability of propagules mediating gene flow both at local scales via pollen and seeds, and at larger scales via positively buoyant flowering shoots. The prevalence of clonality in the Puget Sound, compared to the outer coast suggests reduced investment into sexual reproduction, which in turn could partly explain the higher genetic differentiation in the inland seas.

The low contemporary estimates of effective population size (Ne) suggest that genetic drift has played an important role in shaping population divergence across Washington. Such low Ne values (<121) indicate that the number of genetically effective breeders per generation is much smaller than the number of adult plants present (Christiaen et al., 2022; Sotka et al., 2024). Consequently, reproductive success is skewed to a subset of plants that contribute disproportionally to the gene pool in each generation. This disparity is more pronounced for locations with higher clonality and reduced flowering effort because fewer distinct genotypes are available for outbreeding (Hämmerli and Reusch, 2003b). As a result, stochastic changes in

allele frequencies may be pronounced, leading to random loss or fixation of alleles within each population. Drift-driven allele frequency fluctuations can further reinforce genetic structure in the absence of strong selection or substantial gene flow among meadows (Kawecki and Ebert, 2004).

We found some evidence for local adaptation, characterized by regional changes in allele frequencies at outlier loci. There are well-defined environmental regimes that differ across basins within the State (Rice et al., 2015; MacCready et al., 2021; Alin et al., 2024), which may have influenced these changes. Differential freshwater inputs, combined with oceanic upwelling and local circulation patterns, generate strong spatial variability in salinity and temperature across subregions within the Puget Sound (Banas et al., 2015). In addition, the deep bathymetry and longer water residence times of the inland sea facilitate the development of regional low-oxygen zones as plankton consume resources at depth (Ciesielski, 2015; MacCready et al., 2021). Thus, the variable environmental regimes between oceanographic regions in Washington could have driven selection on genes related to osmoregulation, cell wall integrity, and stress regulation in eelgrass, though experimental validation is still needed.

1.5.2. Implications for Conservation and Management

Evidence for hierarchical population structure across Washington State, shaped by well-defined water basins, provides a population genetic framework for advancing science-based decision-making. In the Puget Sound, conservation planning relies on site suitability assessments that evaluate success based on environmental conditions and their predicted effects on plant growth. However, the predictive accuracy of such approaches could be enhanced with the incorporation of complementary genetic information, such as diversity, population structure, and adaptive capacity (Jahnke et al., 2020). The genetic relationships among populations described in this study are key to identifying drivers of population divergence and informing the development of strategies aimed at maintaining population resilience. Such efforts may include delineating management units that reflect ecologically and evolutionarily relevant boundaries; identifying priority sites for protection based on uniqueness, genetic diversity, or adaptive potential; and selecting appropriate sites for transplant-based restoration to maximize success via similar genetic composition between source and target meadows (Pazzaglia et al., 2021; Orth and Heck, 2023; Jahnke et al., 2025). Our work therefore provides valuable information on spatial genetic variation to further refine conservation planning and restoration strategies in the region.

The data revealed broad genetic groupings that provide a basis for the delineation of management units in Washington State. Genetic similarity within genetic clusters suggests shared ancestry and comparable gene flow regimes, which implies that plants are more likely to be adapted to similar environmental conditions and thus may be interchangeable for restoration within independent units. Transplanting individuals between genetically distinct units risks disrupting long-term natural patterns of population structure and associated ecological networks, and introducing maladapted genotypes that may perform poorly under different environmental conditions (Pazzaglia et al., 2021). Both of these outcomes could negatively affect restoration success and long-term meadow persistence, therefore, the identification of distinct genetic clusters has direct practical implications to inform restoration efforts. For example, regional admixture provenancing is a strategy often recommended for seed-based eelgrass restoration, which involves sourcing seeds from multiple locations within the same region to increase genetic diversity while retaining locally adapted variation (Bucharova et al., 2019; Jahnke et al., 2025). Our work therefore provides genetically informed boundaries that may be incorporated into management planning that explicitly accounts for population structure and local adaptation.

The highly restricted connectivity between sites suggests that eelgrass meadows in Washington depend minimally on distant beds for recruitment. Thus, the growth and recovery of individual beds is expected to depend primarily on local processes, such as vegetative growth (lateral branching) and seed production, instead of successful establishment of propagules dispersing from neighboring meadows (Kendrick et al., 2012; Sotka et al., 2024). The natural rate of meadow colonization and expansion may be insufficient to offset population declines caused by rapid environmental change, and thus, active restoration practices may be needed to recover the loss of threatened beds and their associated ecosystem benefits (Orth et al., 2020; Christiaen et al., 2022). The isolation by distance relationship among meadows provides insight into the spatial scale over which transplant material can be sourced for restoration. Since genetic differentiation increases with geographic distance, geographically proximate donor and target meadows are more likely to share a higher degree of genetic background and may be predicted to perform comparably under local conditions following transplantation, which could in turn minimize establishment failure and support restoration success (Jahnke et al., 2020, 2025).

The highly clonal meadows in Dyes Inlet and Rocky Bay may represent unique life histories due to their highly reduced genetic and genotypic diversity (Stoeckel, Porro and Arnaud-Haond, 2021). Since higher genotypic diversity has been linked to increased population

resilience, the limited diversity in these sites may constrain the potential of eelgrass meadows for adaptation under changing environmental conditions (Waples and Naish, 2009). However, the remarkably large genet sizes at these locations may reflect higher fitness of dominant genotypes, as it has been proposed that there may be a greater potential for sexual reproduction compared to smaller genets of less successful genotypes (Hämmerli and Reusch, 2003b). Whether the prevalence of clonality in these sites reflects natural drift, historical disturbance regimes, or a fitness advantage remains to be explored.

1.5.3. Conclusion

Our findings provide a detailed characterization of the population genetic structure of *Zostera marina* meadows in the State of Washington, USA, a central portion of the species' range in the Northeastern Pacific. We identified four major genetic clusters of population divergence that broadly correspond to the State's main oceanographic basins: the outer Pacific Coast, the Hood Canal, north of Puget Sound, and the southern Puget Sound. This structure likely reflects the combined influence of historical connectivity between the Puget Sound and the outer Pacific coast, contemporary circulation patterns shaped by the complex physical configuration of the inland sea, differential rates of clonal propagation and investment in sexual reproduction, as well as potential adaptation to environmental variation across basins.

Our work provides baseline information on the population genetic structure of eelgrass meadows in Washington State that is critical to inform the local coastal management and conservation. By characterizing how genetic variation in eelgrass is distributed across the State, our work may guide decisions on defining management units, as well as inform recovery strategies to boost population resilience (Pazzaglia et al., 2021). For example, matching donor and recipient meadows based on genetic similarity could enhance restoration success by maintaining adaptive variation and reducing risks of outbreeding depression (Breed et al., 2018; Tan et al., 2020).

1.6. FIGURES

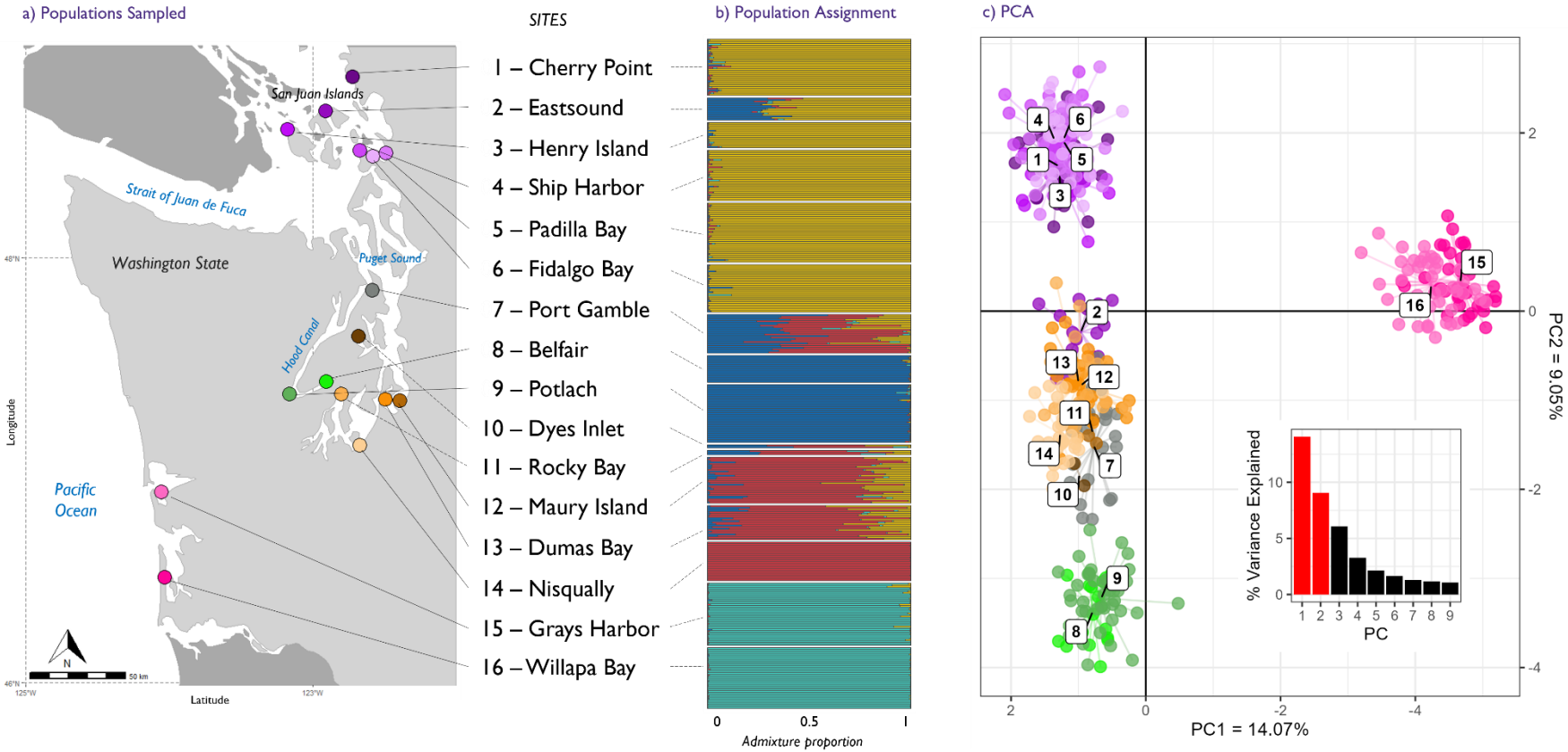

Figure 1.1. Geographic sampling and population genetic structure of *Zostera marina* in Washington State, USA. (A) Map showing 16 eelgrass meadow sampling locations for population genetics analyses. (B) Population structure of genets inferred using an admixture model in STRUCTURE. Each bar represents an individual plant, and the coloration displays the probabilities of assignment to K = 4 genetic clusters. (C) Principal Component Analysis (PCA) scatterplot illustrating genetic relationships among individual genets. Data points indicate individuals, which are colored according to their population of origin. The variance of the first and second PCs are displayed on the x and y axis, respectively. The barplot inset displays the percent variance explained by the first nine principal components.

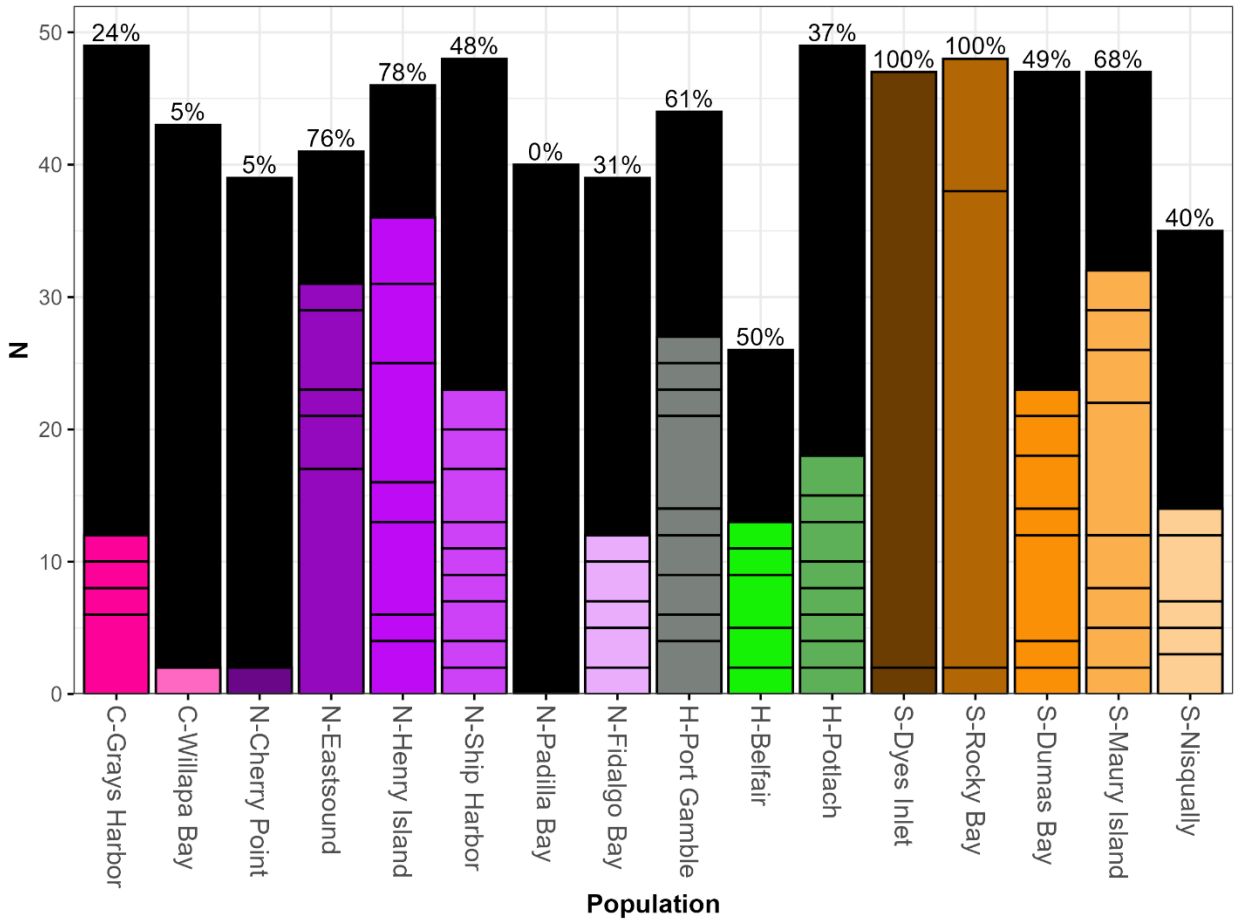

Figure 1.2. Distributions of identical multilocus genotypes (MLGs) across 16 eelgrass meadows in Washington State. Solid black bars display the total number of samples genotyped per site. Percentages on top of the bars indicate the proportion of sampled individuals within each site that were assigned to a clonal genet, each of which is shown as a contoured colored box containing multiple sampled ramets within locations. Bar colors correspond to individual populations as depicted in Figure 1A.

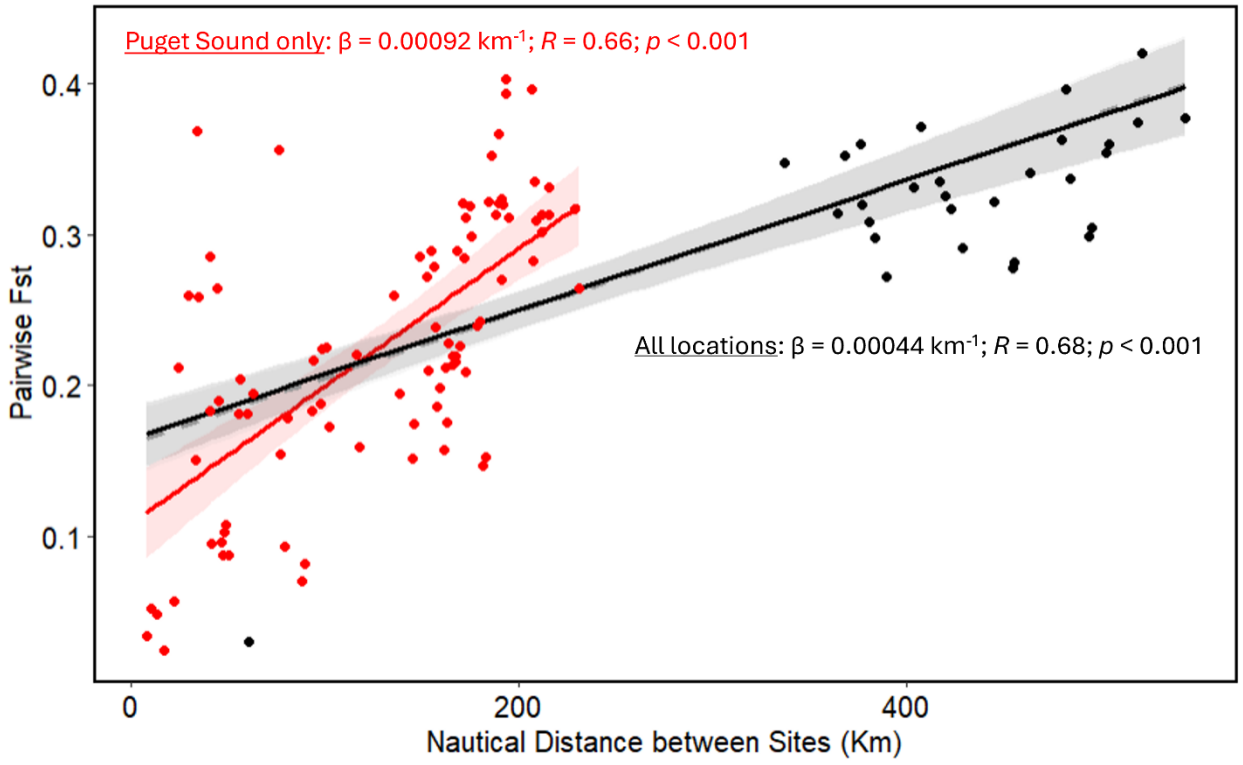

Figure 3. Isolation by distance analysis showing the relationship between genetic differentiation (pairwise Fst) and nautical distance (km) among 16 eelgrass (*Zostera marina*) meadows in Washington State based on genets. Colored points and regression lines represent analyses among all sites (black) and between those in the Puget Sound only (red). Mantel tests were used to assess correlations between geographic and genetic distances. The regression slope (β, change in Fst per Km), mantel R correlation coefficient, and significance (p-value) are provided for each test.

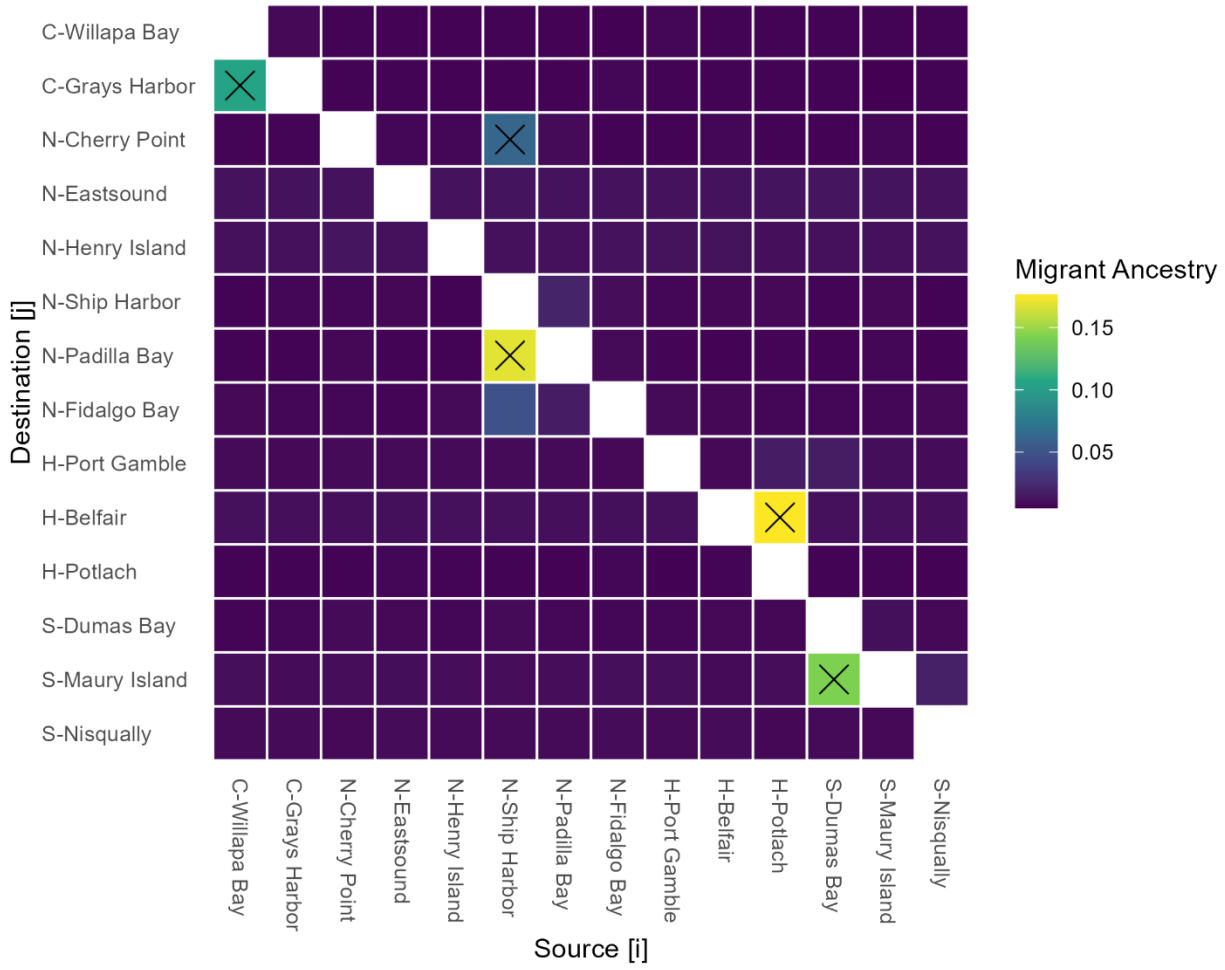

Figure 4. Heatmap of recent migration rates (mij) into each eelgrass meadow sampled across Washington State using genets. Rows and columns represent the source and recipient sites of migrants, respectively. Estimates for self recruitment (mii) are given in Table S6; all exceeded 0.72. Significant estimates are indicated with an X.

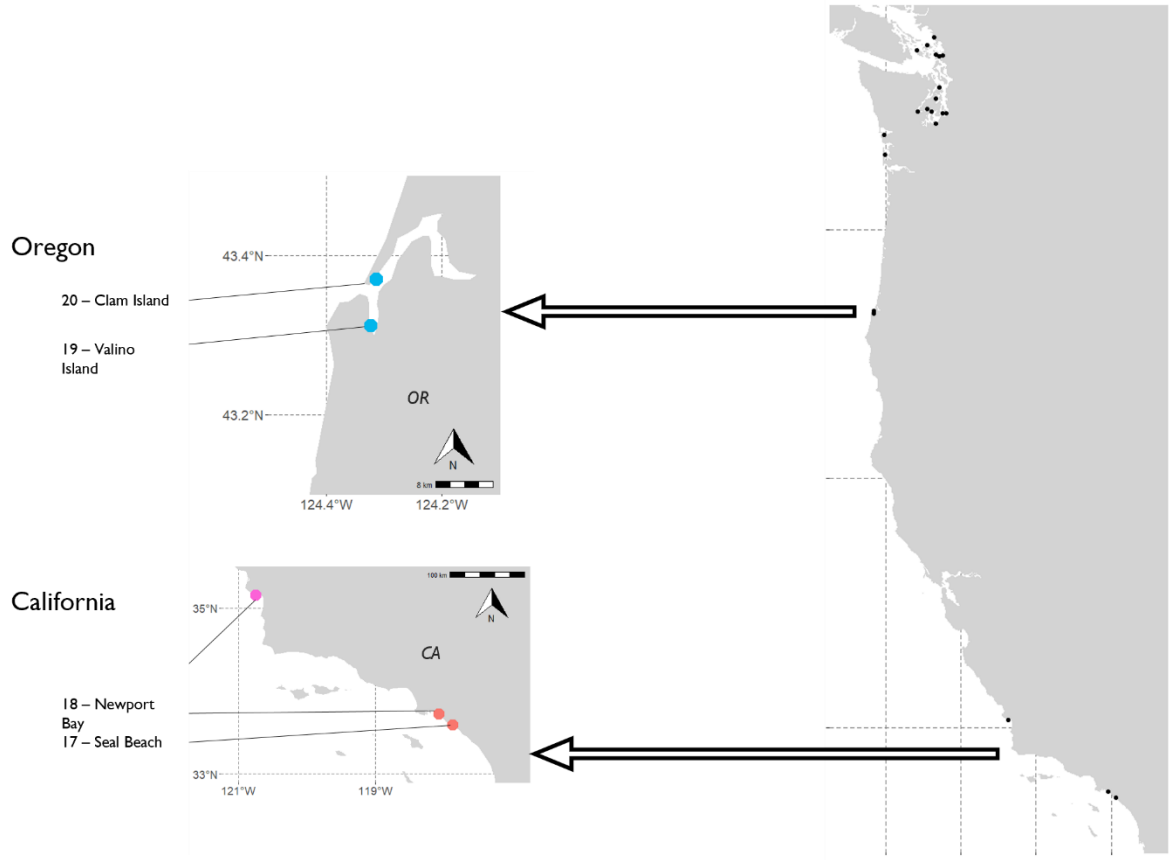

Figure S1.1. Geographic sampling of eelgrass meadows along the Pacific Coast of the US. (A) Map of sampled locations along the coasts of Washington (see Fig. 1), (B) Oregon, and (C) California for population genetic analyses.

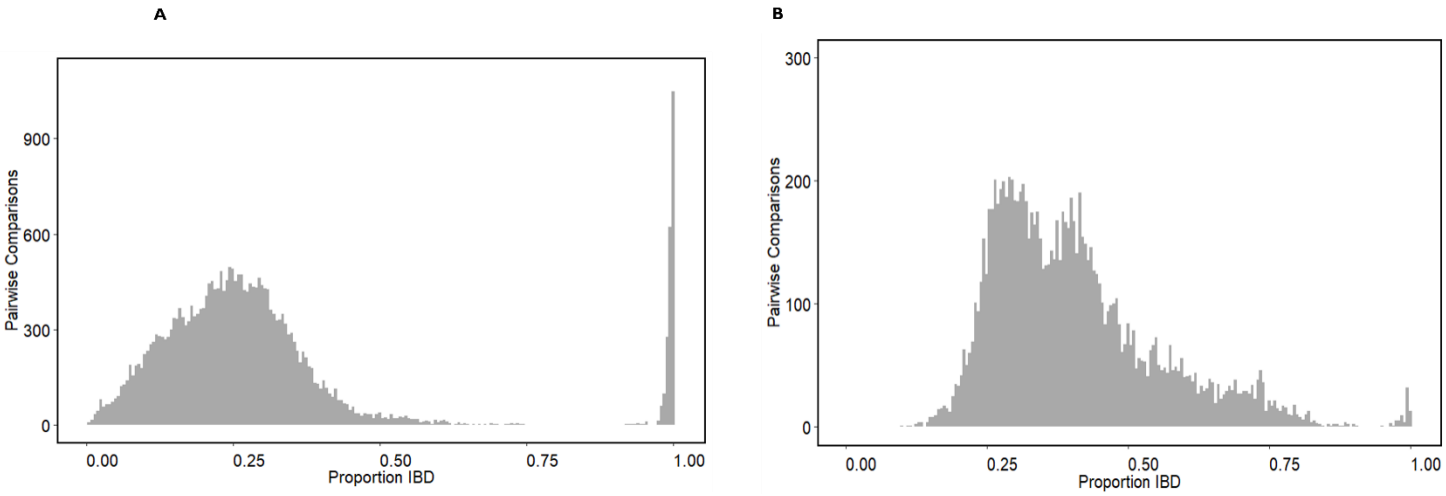

Figure S1.2. Distributions of pairwise identity-by-descent (IBD) estimates used to assess the occurrence of shared MLLs. The analyses were based on A) 689 individual plants genotyped at 560 SNPs across Washington State, and B) 252 plants genotyped at 555 SNPs from the Pacific Coast. Both analyses evaluated shared genetic variation between pairs of individuals to identify identical MLLs within and among populations.

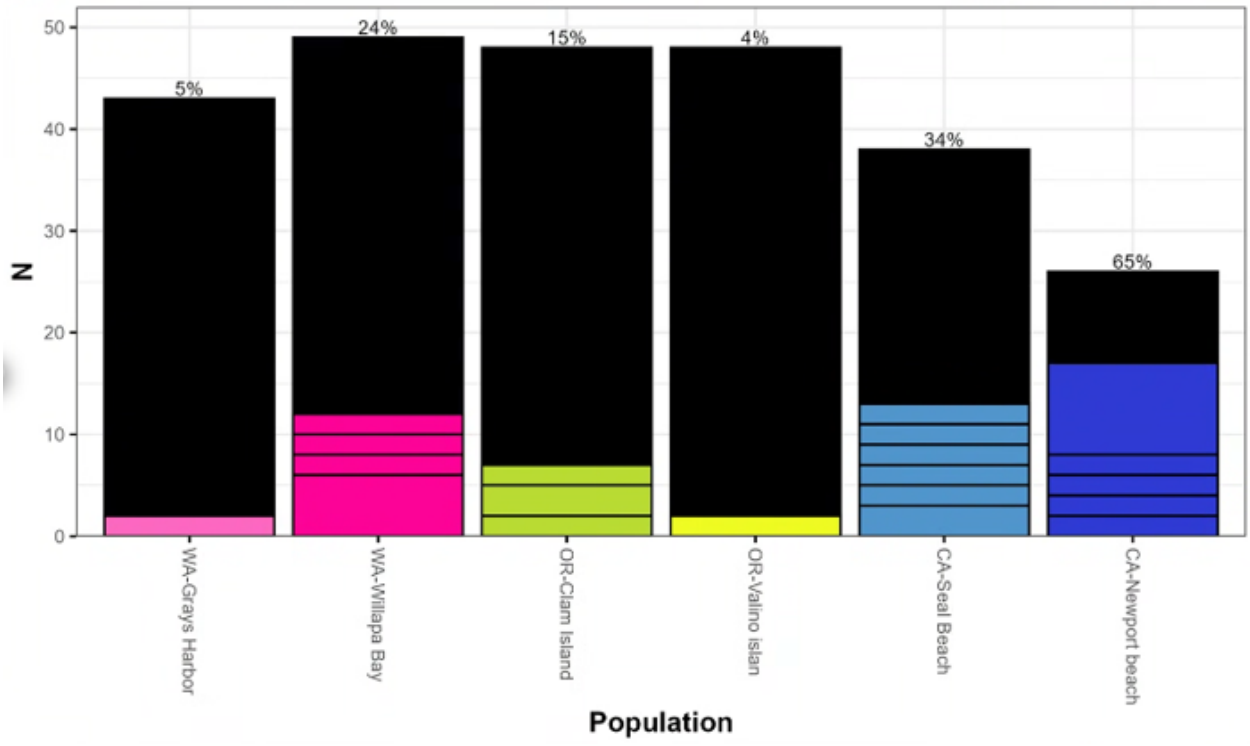

Figure S1.3. Distributions of identical multilocus lineages (MLLs) across six eelgrass meadows along the Pacific Coast of the US. Solid black bars display the total number of samples genotyped per site. Percentages on top of the bars indicate the proportion of sampled individuals within each site that were assigned to a clonal genet, each of which is shown as a contoured colored box containing multiple sampled ramets within locations.

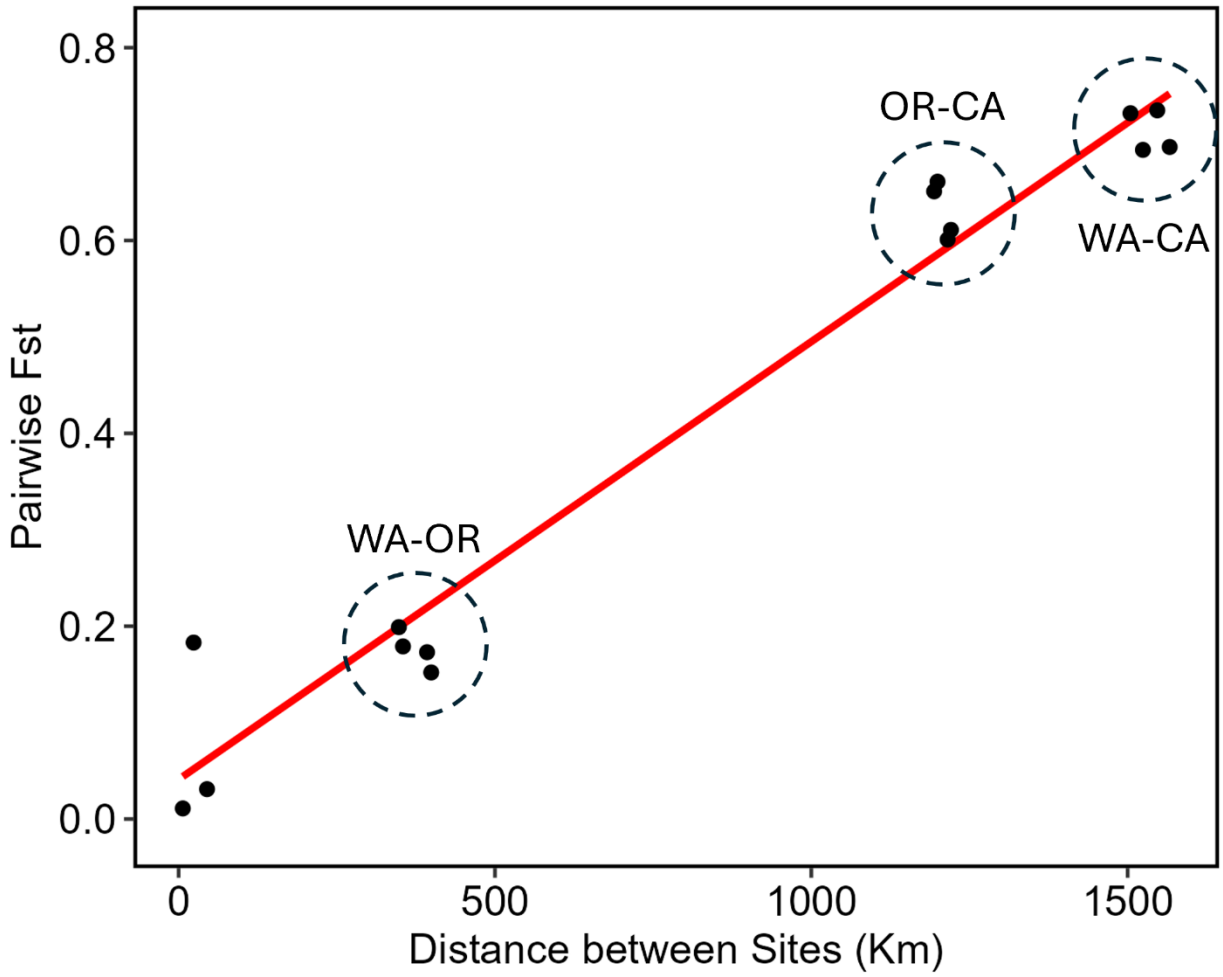

Figure S1.4. Isolation by distance analysis showing the relationship between genetic differentiation (pairwise Fst) and nautical distance (km) among six eelgrass (*Zostera marina*) meadows along the Pacific Coast based on genets. Mantel tests were used to assess correlations between geographic and genetic distances. Comparisons between states are indicated as dashed circles.

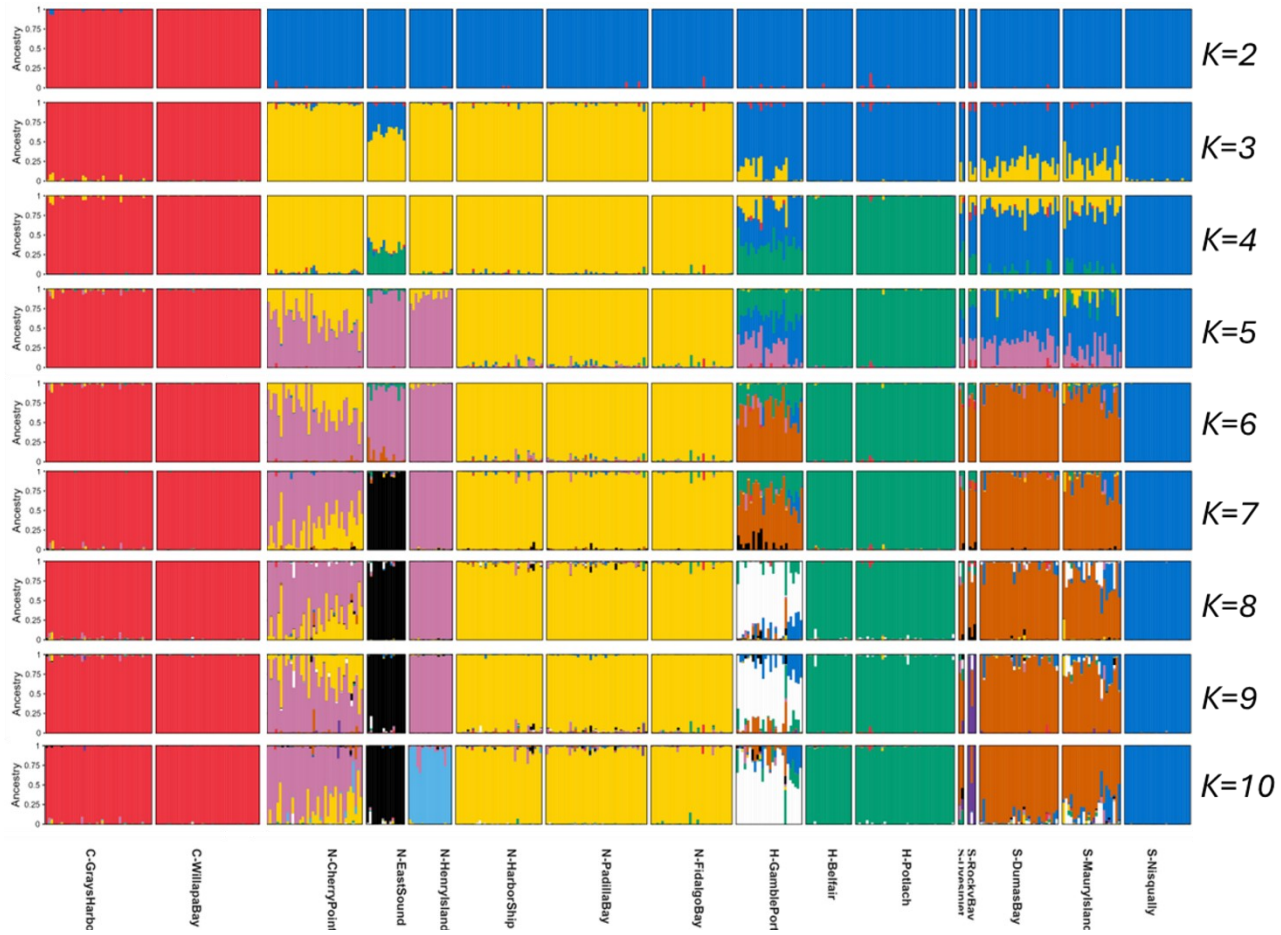

Figure S1.5. Hierarchical population stratification of eelgrass meadows in Washington inferred using Bayesian assignment of individuals. Each individual plant is represented by a stacked column indicating the probabilities of assignment in K = 2-8 constructed ancestral populations for the Washington State populations.

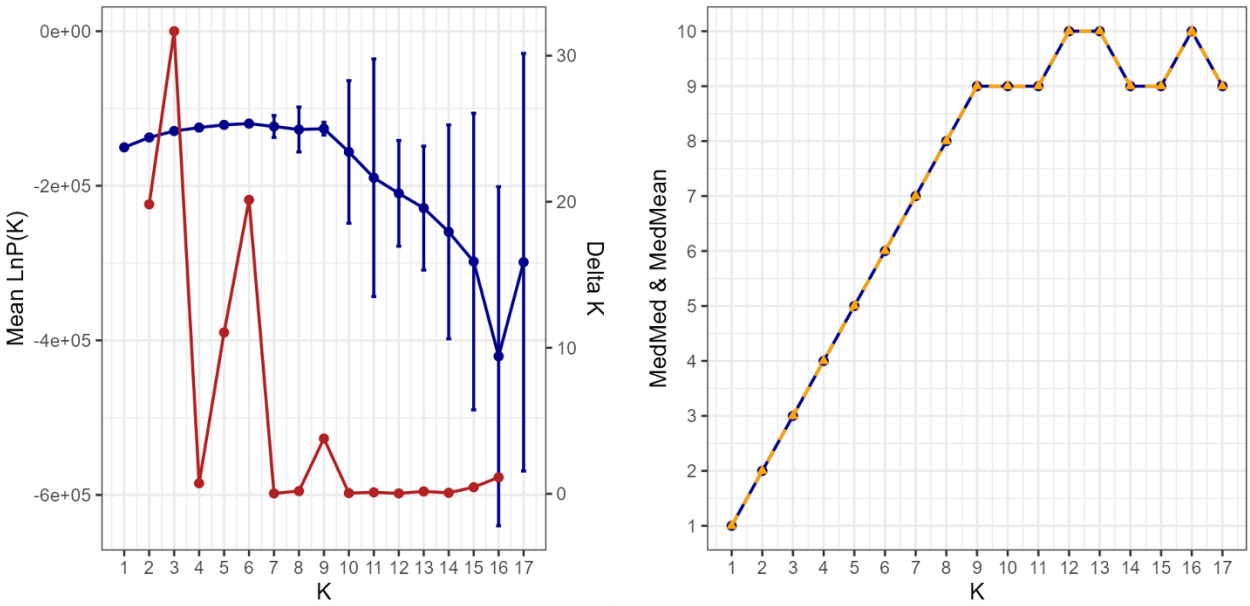

Figure S1.6. Evaluations of the STRUCTURE results for eelgrass sites across Washington State. Panels display diagnostics based the A) Evanno method: Delta K (red) and change in mean log likelihood with standard deviations (blue); and the B) Puechmaille approach: MedMed (solid blue) and MedMean (dashed yellow).

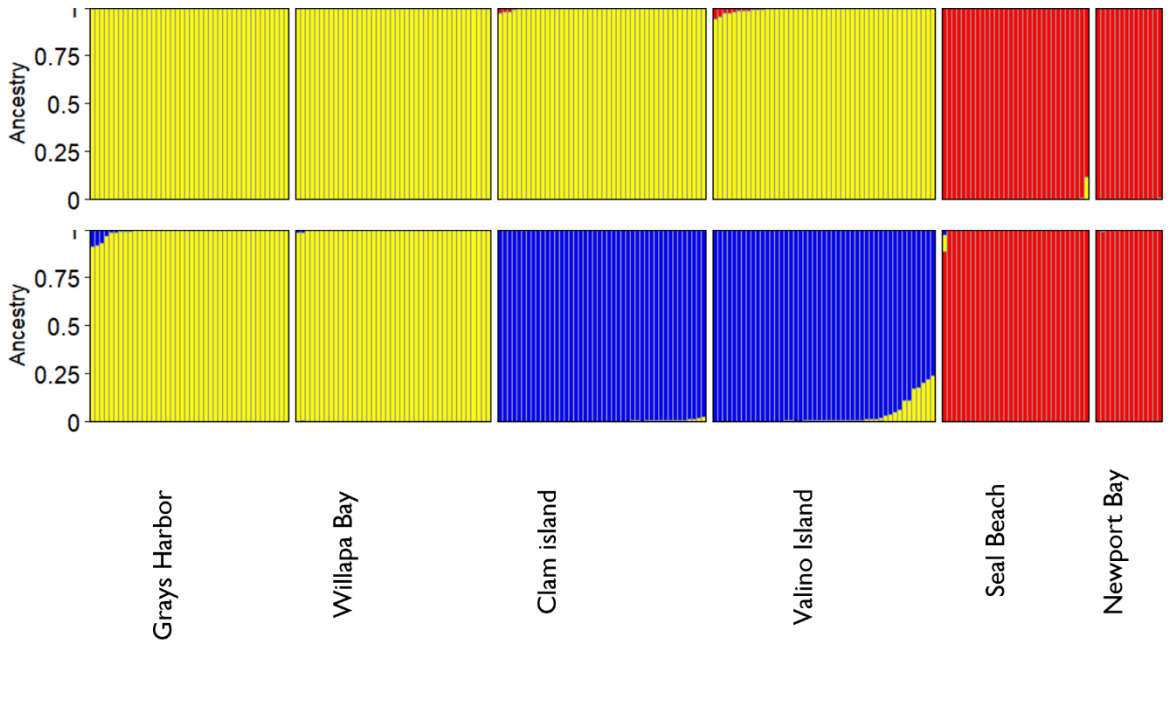

Figure S1.7. Hierarchical population stratification of eelgrass meadows along the outer Pacific Coast inferred using Bayesian assignment of individuals. Each individual plant is represented by a stacked column indicating the probabilities of assignment in K = 2-3 constructed ancestral populations for the Pacific Coast sites.

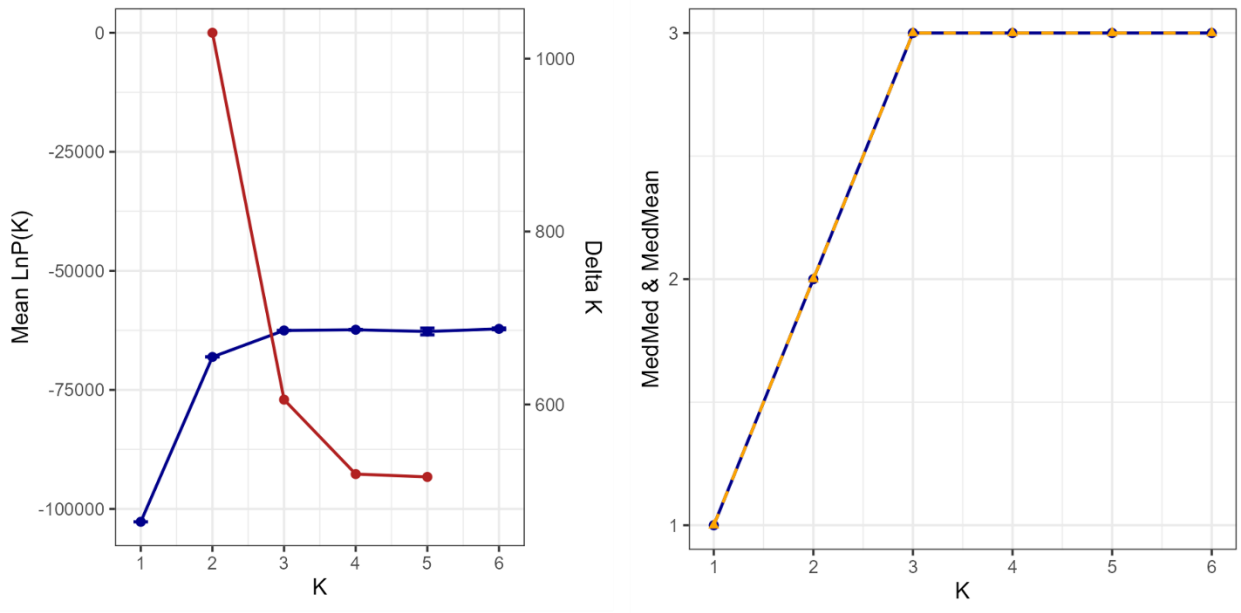

Figure S1.8. Evaluations of the STRUCTURE results for eelgrass sites along the outer Pacific Coast. Panels display diagnostics based the A) Evanno method: Delta K (red) and change in mean log likelihood with standard deviations (blue); and the B) Puechmaille approach: MedMed (solid blue) and MedMean (dashed yellow).

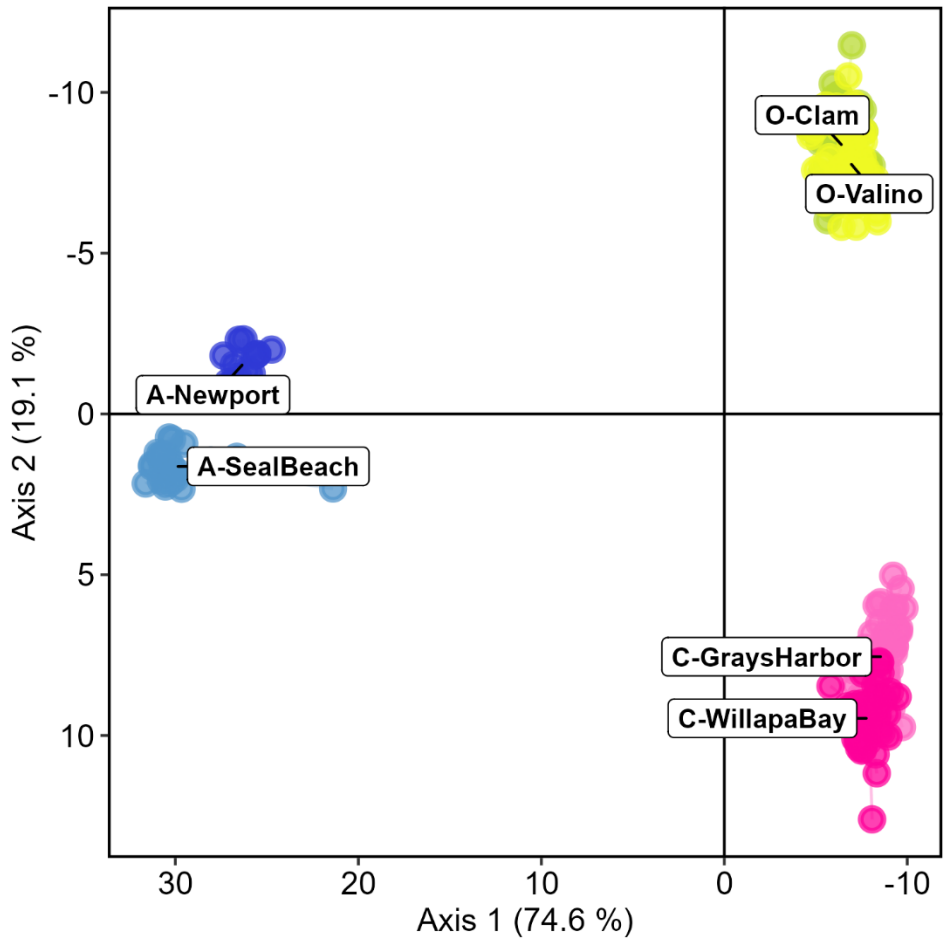

Figure S1.9. Discriminant Analysis of Principal Components (DAPC) scatterplot illustrating genetic relationships among individual genets for sites along the outer Pacific Coast. Data points indicate individuals, which are colored according to their population of origin. Variance explained by the first and second components is shown in parentheses.

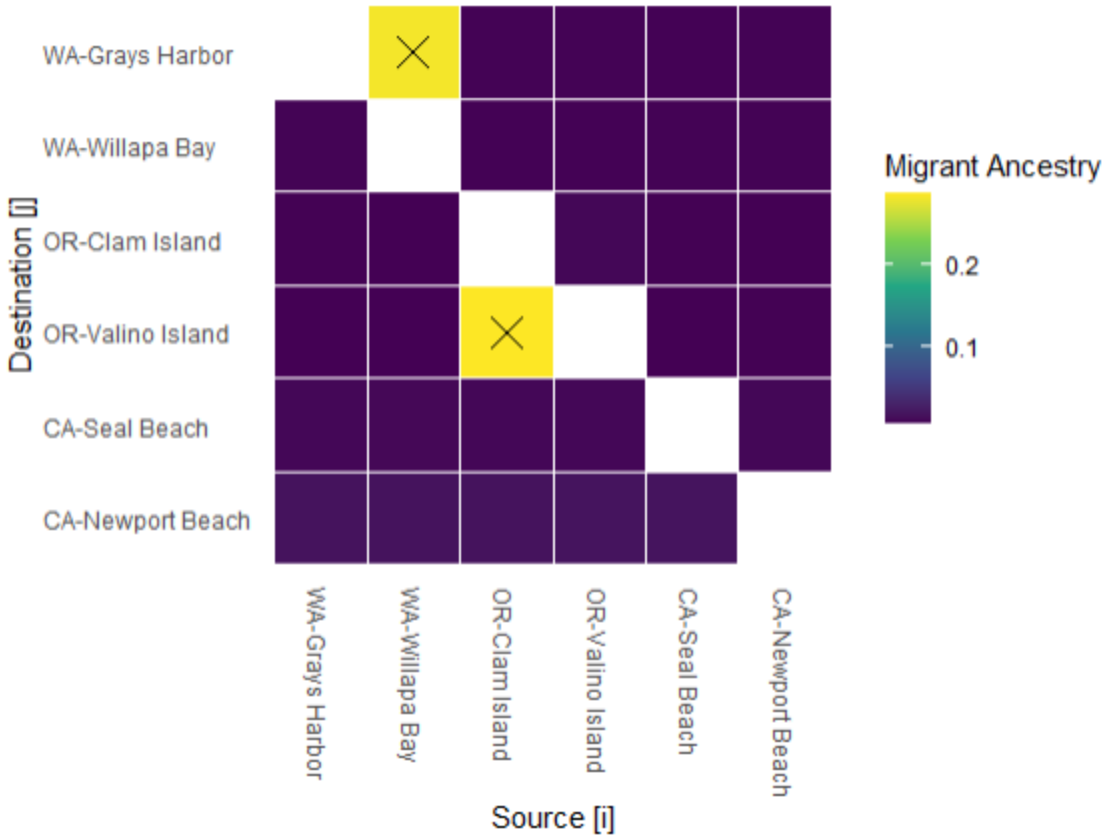

Figure S1.10. Heatmap of contemporary migrant ancestry estimates (mij) for eelgrass meadows along the outer coastline. Columns and rows represent the source and recipient sites of migrants, respectively. Migrant ancestries (mii) for the same sites, all above 0.7, have been removed to better visualize the lower estimates across different sites (mij).

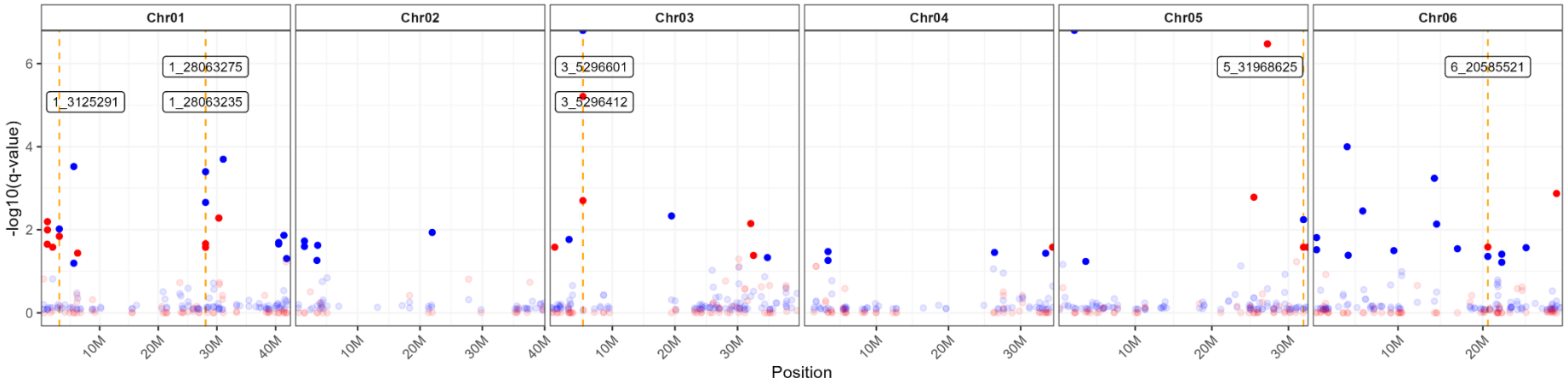

Figure S1.11. Manhattan plot of SNPs identified as putative outliers. Each dot represents a SNP variant plotted along the genome of eelgrass (x axis) against its FDR-corrected significance (-log10(q-value); y-axis. Colors correspond to outlier detections with BayeScan (blue) and PCAdapt (red), with significant SNPs shown in solid colors. Orange dashed lines mark loci identified as outliers by both methods, with labels corresponding to SNP *chromosome_position*.

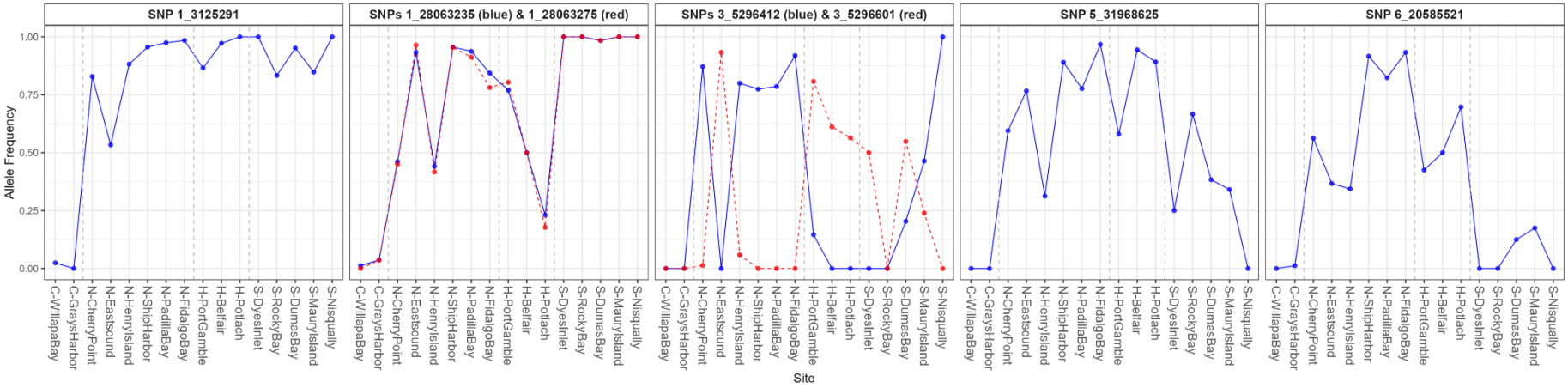

Figure S1.12. Allele frequencies for seven SNPs identified as putatively under selection. Lines and dots indicate the frequency of the major allele for each SNP across all sampled locations (x axis). Panels with dashed red and solid blue lines correspond to putative outliers located within 190 bp of each other.

1.7. TABLES

Table S1.1. Sampling site information for 20 eelgrass meadows included in this study. Details include site names and labels, coordinates, state, and oceanographic region.

n	Site	Label	Longitude	Latitude	State	Region
1	Cherry Point	CP	48.850	-122.720	Washington	North of Puget Sound
2	Eastsound	ES	48.692	-122.906		
3	Henry Island	HI	48.603	-123.174		
4	Ship Harbor	SH	48.505	-122.671		
5	Padilla Bay	PB	48.493	-122.488		
6	Fidalgo Bay	FB	48.477	-122.578		
7	Port Gamble	PG	47.847	-122.583		Hood Canal
8	Belfair	BE	47.417	-122.902		
9	Potlach	PO	47.360	-123.157		
10	Dyes Inlet	DI	47.630	-122.677		South Puget Sound
11	Rocky Bay	RB	47.359	-122.796		
12	Dumas Bay	DB	47.329	-122.390		
13	Maury Island	MI	47.335	-122.491		
14	Nisqually	NI	47.117	-122.668		
15	Grays Harbor	GH	46.899	-124.050		Outer Pacific Coast
16	Willapa Bay	WB	46.496	-124.026		
17	ClamIsland	CI	43.371	-124.314	Oregon	
18	Valino Island	VI	43.312	-124.322		
19	Seal Beach	SE	33.729	-118.080	California	
20	Newport Bay	NE	33.596	-117.880		

Table S1.2. Filtering steps applied to the sequenced reads in the bioinformatic pipeline. Rows describe individual filtering steps, and columns describe the number of individuals, polymorphic sites (SNPs), and missing data (proportion of missing genotypes across all loci and individuals) in the Washington and Pacific Coast datasets after each step.

	Dataset	*Washingon State*			*Pacific Coast*		
	Step / Filter	**Individuals**	**SNPs**	**Missing Data**	**Individuals**	**SNPs**	**Missing Data**
1	Unfiltered Single Nucleotide Variant calling with ref_map.pl in STACKS	691	46202	88.17%	265	20606	73.48
2	Genotype depth ≥ 10	691	46500	92.65%	265	20606	80.93
3	Genotype Allelic Balance 0.2 ≥ 0.8	691	46500	92.75%	265	20606	81.12
4	Loci with Minor Allele Count > 1 (remove fixed loci)	691	6591	54.65%	265	6369	45.97
5	Loci with < 20% Missing Genotypes across individuals	691	1617	3.92%	265	1968	5.63
6	One locus per rad-tag (locus with highest Minor Allele Frequency)	691	780	3.21%	265	810	5.03
7	Individuals with < 20% Missing Genotypes across all loci	688	780	3.15%	252	810	3.37
8	Loci with Minor Allele Frequencies ≥ 0.05 in at least one subpopulation	688	563	3.57%	252	555	3.77
9	Loci meeting Hardy Weinberg Equilibrium expectations in at least one subpopulation	688	563	3.57%	252	555	3.77
10	Identical Multi-Locus Lineages (MLLs)	427	562	3.77%	219	555	3.84

Table S1.3. Genetic diversity estimates for 16 eelgrass meadows across Washington and six sites along the Pacific Coast. Information includes the collection site, the number of shared multi-locus lineages (n MLLs) and individuals collected (n Ind), the proportion of polymorphic loci per population from the total SNPs for the corresponding dataset (% Pol), the number of private alleles per site (Pa), observed heterozygosity (Ho), unbiased expected heterozygosity (uHe), inbreeding coefficient with confidence intervals (Fis (95% CI)), effective population size with confidence intervals (Ne (95 % CI)), and genotypic richness (R). The estimates are provided for both genet (unique genotypes) and ramet-based (all genotypes) analyses.

		Ramets						Genets							
n	Site	n Ind	% Pol	Ho	uHe	Pa	Fis (95% CI)	n MLLs	% Pol	Ho	uHe	Pa	Fis (95% CI)	Ne (95% CI)	R
Washington State dataset															
1	Willapa Bay	49	58.5	0.162	0.169	7	**0.031 (0.022, 0.050)**	41	58.4	0.158	0.167	7	**0.043 (0.027, 0.088)**	73.93 (64.97, 85.28)	0.83
2	Grays Harbor	43	59.9	0.166	0.170	3	**0.026 (0.017, 0.041)**	42	60.0	0.165	0.170	3	**0.03 (0.005, 0.056)**	120.52 (96.36, 158.61)	0.98
3	Cherry Point	39	60.6	0.169	0.173	2	**0.028 (0.020, 0.052)**	38	60.7	0.170	0.174	2	0.03 (-0.002, 0.046)	99.53 (83.92, 121.32)	0.97
4	Eastsound	41	47.6	0.147	0.136	15	**-0.036 (-0.057, -0.005)**	15	46.8	0.144	0.150	16	0.024 (-0.004, 0.089)	17.38 (15.14, 20.14)	0.35
5	Henry Island	46	55.1	0.164	0.156	18	**-0.032 (-0.041, -0.016)**	17	55.0	0.159	0.163	18	0.018 (-0.01, 0.061)	45.04 (36.64, 57.59)	0.36
6	Ship Harbor	48	59.1	0.144	0.146	3	**0.014 (0.010, 0.027)**	34	58.9	0.145	0.148	3	0.021 (-0.011, 0.049)	91.20 (76.71, 111.52)	0.70
7	Padilla Bay	40	58.8	0.146	0.148	0	**0.019 (0.014, 0.035)**	40	58.9	0.146	0.148	0	0.019 (-0.014, 0.037)	117.81 (95.38, 152.20)	1.00
8	Fidalgo Bay	39	55.2	0.143	0.141	3	-0.006 (-0.016, 0.016)	32	55.0	0.140	0.141	3	0.004 (-0.021, 0.036)	101.63 (81.42, 133.44)	0.82
9	Port Gamble	44	60.4	0.197	0.182	2	**-0.055 (-0.074, -0.024)**	26	60.1	0.189	0.186	2	-0.016 (-0.051, 0.011)	18.85 (17.42, 20.43)	0.58
10	Belfair	26	50.0	0.161	0.154	3	**-0.027 (-0.038, -0.003)**	18	49.9	0.165	0.159	3	**-0.021 (-0.071, -0.001)**	32.53 (27.68, 38.98)	0.68
11	Potlach	49	51.9	0.156	0.153	1	-0.002 (-0.011, 0.018)	39	52.0	0.154	0.154	3	0.01 (-0.029, 0.029)	103.83 (83.34, 135.69)	0.79
12	Dyes Inlet	47	31.1	0.171	0.095	3	**-0.377 (-0.406, -0.318)**	2	29.1	0.156	0.163	2	-0.02 (-0.153, 0.126)	0.07 (0.07, 0.07)	0.02
13	Rocky Bay	48	35.3	0.191	0.136	11	**-0.298 (-0.345, -0.234)**	3	32.6	0.157	0.151	11	-0.078 (-0.124, 0.042)	0.24 (0.23, 0.25)	0.04
14	Dumas Bay	47	60.6	0.169	0.171	10	**0.027 (0.018, 0.044)**	31	60.5	0.170	0.176	11	**0.036 (0.003, 0.062)**	38.30 (34.76, 42.46)	0.65
15	Maury Island	47	61.1	0.160	0.170	6	**0.052 (0.038, 0.085)**	23	61.2	0.162	0.173	7	**0.056 (0.036, 0.1)**	40.48 (35.55, 46.68)	0.48
16	Nisqually	35	33.6	0.116	0.112	4	-0.022 (-0.049, 0.018)	26	32.9	0.114	0.114	4	0.003 (-0.034, 0.041)	36.88 (30.55, 45.71)	0.74
Pacific Coast dataset															
17	Grays Harbor, WA	43	0.59	0.163	0.169	1	0.032 (-0.117, 0.087)	42	0.589	0.162	0.169	1	0.035 (-0.069, 0.108)	118.91 (95.06, 156.49)	0.98
18	Willapa Bay, WA	49	0.58	0.163	0.170	8	**0.034 (0.003, 0.146)**	41	0.580	0.159	0.168	8	0.042 (-0.008, 0.146)	77.10 (67.39, 89.54)	0.83
19	ClamIsland, OR	48	0.68	0.200	0.211	1	**0.050 (0.002, 0.120)**	44	0.679	0.200	0.212	1	0.052 (-0.008, 0.102)	145.03 (122.16, 177.27	0.91
20	Valino Island, OR	48	0.69	0.198	0.217	2	**0.079 (0.036, 0.194)**	47	0.686	0.198	0.217	2	**0.078 (0.024, 0.194)**	99.87 (86.37, 117.60)	0.98
21	Seal Beach, CA	38	0.33	0.077	0.085	21	0.082 (-0.088, 0.163)	31	0.324	0.076	0.086	21	0.094 (-0.029, 0.321)	13.60 (10.73, 17.33)	0.81
22	Newport Bay, CA	26	0.34	0.099	0.095	32	-0.020 (-0.068, 0.031)	14	0.326	0.091	0.095	32	0.031 (-0.058, 0.417)	43.05 (27.59, 86.05)	0.52

Table S1.4. Pairwise genetic divergence among 16 eelgrass meadows in Washington State. Tables display Fst measurements (below diagonal) and the corresponding 95% confidence intervals (above diagonal). Fst estimates are provided for genet- (unique genotypes, top) and ramet-based (all genotypes; bottom) analyses. Significance tests were conducted using 10,000 permutations.

Sites (Genets)	C-WillapaBay	C-GraysHarbor	N-CherryPoint	N-Eastsound	N-HenryIsland	N-ShipHarbor	N-PadillaBay	N-FidalgoBay	H-PortGamble	H-Belfair	H-Potlach	S-DyesInlet	S-RockyBay	S-DumasBay	S-MauryIsland	S-Nisqually
C-WillapaBay		0.021, 0.04	0.287, 0.346	0.341, 0.405	0.332, 0.397	0.295, 0.37	0.293, 0.359	0.294, 0.369	0.26, 0.321	0.334, 0.404	0.316, 0.386	0.296, 0.376	0.328, 0.419	0.279, 0.329	0.267, 0.328	0.38, 0.448
C-GraysHarbor	0.03		0.272, 0.326	0.32, 0.381	0.316, 0.374	0.28, 0.347	0.278, 0.336	0.281, 0.354	0.244, 0.298	0.324, 0.398	0.305, 0.372	0.279, 0.361	0.319, 0.402	0.258, 0.303	0.25, 0.302	0.361, 0.425
N-CherryPoint	0.317	0.298		0.176, 0.241	0.077, 0.117	0.076, 0.11	0.072, 0.099	0.091, 0.124	0.13, 0.183	0.246, 0.314	0.237, 0.296	0.17, 0.242	0.225, 0.3	0.129, 0.176	0.124, 0.171	0.276, 0.341
N-Eastsound	0.372	0.352	0.212		0.22, 0.298	0.223, 0.298	0.218, 0.3	0.238, 0.327	0.145, 0.2	0.268, 0.355	0.257, 0.327	0.189, 0.285	0.272, 0.363	0.187, 0.257	0.186, 0.253	0.356, 0.447
N-HenryIsland	0.36	0.348	0.096	0.259		0.151, 0.216	0.146, 0.207	0.166, 0.234	0.189, 0.256	0.288, 0.368	0.285, 0.36	0.234, 0.314	0.287, 0.385	0.181, 0.245	0.176, 0.244	0.35, 0.433
N-ShipHarbor	0.331	0.314	0.095	0.26	0.19		0.018, 0.031	0.038, 0.057	0.15, 0.197	0.281, 0.349	0.254, 0.309	0.213, 0.309	0.264, 0.366	0.15, 0.208	0.129, 0.171	0.279, 0.345
N-PadillaBay	0.326	0.308	0.087	0.265	0.181	0.024		0.039, 0.061	0.159, 0.211	0.274, 0.343	0.246, 0.308	0.223, 0.312	0.267, 0.367	0.151, 0.209	0.136, 0.177	0.293, 0.353
N-FidalgoBay	0.335	0.32	0.107	0.285	0.204	0.048	0.051		0.179, 0.243	0.277, 0.357	0.251, 0.313	0.229, 0.329	0.281, 0.388	0.167, 0.235	0.157, 0.213	0.32, 0.389
H-PortGamble	0.291	0.272	0.159	0.172	0.225	0.178	0.188	0.217		0.156, 0.206	0.13, 0.172	0.06, 0.12	0.16, 0.223	0.066, 0.097	0.055, 0.081	0.191, 0.25
H-Belfair	0.375	0.363	0.283	0.32	0.324	0.321	0.313	0.322	0.183		0.044, 0.069	0.175, 0.27	0.276, 0.362	0.206, 0.274	0.201, 0.269	0.357, 0.438
H-Potlach	0.355	0.341	0.27	0.299	0.319	0.289	0.284	0.289	0.154	0.057		0.17, 0.252	0.257, 0.346	0.192, 0.256	0.172, 0.237	0.323, 0.404
S-DyesInlet	0.338	0.321	0.209	0.238	0.279	0.259	0.272	0.285	0.093	0.226	0.21		0.163, 0.285	0.05, 0.117	0.064, 0.138	0.297, 0.408
S-RockyBay	0.377	0.36	0.264	0.313	0.331	0.312	0.313	0.336	0.194	0.317	0.301	0.224		0.153, 0.229	0.146, 0.224	0.319, 0.406
S-DumasBay	0.305	0.282	0.152	0.22	0.215	0.174	0.176	0.198	0.082	0.242	0.228	0.087	0.194		0.025, 0.041	0.156, 0.207
S-MauryIsland	0.299	0.278	0.146	0.22	0.214	0.152	0.157	0.186	0.07	0.24	0.211	0.102	0.181	0.033		0.134, 0.177
S-Nisqually	0.421	0.397	0.31	0.403	0.393	0.311	0.321	0.353	0.22	0.396	0.366	0.356	0.369	0.183	0.151	

Sites (Ramets)	Cherry Point	Eastsound	Henry Island	Ship Harbor	Padilla Bay	Fidalgo Bay	Port Gamble	Belfair	Potlach	Dyes Inlet	Rocky Bay	Dumas Bay	Maury Island	Nisqually	Grays Harbor	Willapa Bay
Cherry Point		0.208, 0.276	0.098, 0.14	0.084, 0.118	0.072, 0.102	0.092, 0.133	0.15, 0.2	0.256, 0.329	0.237, 0.305	0.361, 0.441	0.28, 0.354	0.137, 0.19	0.139, 0.19	0.287, 0.356	0.266, 0.328	0.285, 0.349
Eastsound	0.242		0.27, 0.342	0.254, 0.335	0.248, 0.332	0.271, 0.357	0.196, 0.26	0.319, 0.403	0.293, 0.37	0.434, 0.519	0.343, 0.427	0.232, 0.305	0.237, 0.305	0.38, 0.473	0.358, 0.436	0.376, 0.454
Henry Island	0.118	0.306		0.175, 0.238	0.161, 0.221	0.181, 0.249	0.235, 0.298	0.309, 0.389	0.294, 0.369	0.419, 0.503	0.355, 0.437	0.217, 0.28	0.226, 0.289	0.368, 0.444	0.331, 0.404	0.341, 0.417
Ship Harbor	0.101	0.295	0.206		0.023, 0.035	0.044, 0.064	0.166, 0.224	0.296, 0.371	0.255, 0.328	0.386, 0.469	0.311, 0.393	0.158, 0.215	0.145, 0.197	0.281, 0.357	0.281, 0.355	0.3, 0.374
Padilla Bay	0.087	0.291	0.19	0.028		0.045, 0.067	0.172, 0.228	0.286, 0.359	0.248, 0.315	0.399, 0.487	0.31, 0.395	0.152, 0.212	0.15, 0.199	0.292, 0.366	0.27, 0.342	0.291, 0.362
Fidalgo Bay	0.111	0.315	0.214	0.054	0.055		0.194, 0.263	0.291, 0.37	0.25, 0.326	0.404, 0.496	0.323, 0.409	0.178, 0.244	0.169, 0.236	0.32, 0.406	0.282, 0.358	0.296, 0.374
Port Gamble	0.175	0.227	0.266	0.195	0.2	0.229		0.166, 0.221	0.133, 0.181	0.293, 0.365	0.218, 0.285	0.09, 0.128	0.076, 0.107	0.184, 0.251	0.251, 0.312	0.272, 0.334
Belfair	0.293	0.361	0.349	0.334	0.323	0.331	0.194		0.055, 0.08	0.397, 0.493	0.329, 0.406	0.226, 0.295	0.227, 0.295	0.364, 0.449	0.331, 0.409	0.344, 0.424
Potlach	0.271	0.332	0.332	0.292	0.282	0.288	0.157	0.068		0.345, 0.432	0.299, 0.376	0.204, 0.269	0.191, 0.255	0.329, 0.414	0.303, 0.38	0.319, 0.396
Dyes Inlet	0.403	0.478	0.462	0.429	0.445	0.452	0.329	0.446	0.39		0.394, 0.484	0.279, 0.36	0.287, 0.367	0.453, 0.548	0.445, 0.528	0.455, 0.537
Rocky Bay	0.317	0.386	0.397	0.352	0.353	0.366	0.251	0.367	0.337	0.439		0.221, 0.281	0.205, 0.272	0.328, 0.414	0.371, 0.45	0.384, 0.465
Dumas Bay	0.164	0.269	0.248	0.186	0.182	0.21	0.109	0.261	0.237	0.32	0.25		0.056, 0.083	0.169, 0.226	0.257, 0.323	0.282, 0.35
Maury Island	0.164	0.271	0.258	0.171	0.174	0.202	0.091	0.261	0.223	0.328	0.238	0.069		0.142, 0.196	0.255, 0.323	0.274, 0.346
Nisqually	0.322	0.427	0.406	0.319	0.33	0.364	0.217	0.407	0.372	0.503	0.371	0.198	0.168		0.37, 0.445	0.391, 0.465
Grays Harbor	0.298	0.397	0.368	0.318	0.306	0.32	0.282	0.37	0.342	0.488	0.411	0.291	0.29	0.407		0.026, 0.044
Willapa Bay	0.317	0.415	0.38	0.337	0.326	0.335	0.304	0.384	0.358	0.498	0.425	0.316	0.31	0.428	0.035	

Table S1.5. Pairwise genetic divergence among six eelgrass meadows along the Pacific Coast of the US. Tables display *Fst* measurements (below diagonal) and the corresponding 95% confidence intervals (above diagonal). Fst estimates are provided for genet- (unique genotypes, top) and ramet-based (all genotypes; bottom) analyses. Significance tests were conducted using 10,000 permutations.

Sites (Genets)	**WA-Grays Harbor**	**WA-Willapa Bay**	**OR-Clam Island**	**OR-Valino Island**	**CA-Seal Beach**	**CA-Newport Bay**
WA-Grays Harbor		0.022, 0.041	0.148, 0.199	0.129, 0.177	0.713, 0.758	0.673, 0.722
WA-Willapa Bay	0.031		0.171, 0.228	0.153, 0.206	0.708, 0.755	0.669, 0.72
OR-Clam Island	0.173	0.199		0.007, 0.014	0.637, 0.685	0.584, 0.638
OR-Valino Island	0.152	0.179	0.011		0.627, 0.676	0.574, 0.628
CA-Seal Beach	0.735	0.732	0.661	0.651		0.141, 0.225
CA-Newport Bay	0.697	0.694	0.611	0.601	0.183	

Sites (Ramets)	**WA-Grays Harbor**	**WA-Willapa Bay**	**OR-Clam Island**	**OR-Valino Island**	**CA-Seal Beach**	**CA-Newport Bay**
WA-Grays Harbor		0.026, 0.046	0.15, 0.201	0.129, 0.177	0.72, 0.764	0.688, 0.736
WA-Willapa Bay	0.036		0.175, 0.23	0.156, 0.207	0.71, 0.755	0.678, 0.728
OR-Clam Island	0.174	0.202		0.009, 0.016	0.647, 0.692	0.608, 0.657
OR-Valino Island	0.152	0.181	0.013		0.64, 0.686	0.6, 0.649
CA-Seal Beach	0.742	0.733	0.67	0.663		0.173, 0.249
CA-Newport Bay	0.713	0.704	0.633	0.625	0.211	

Table S1.6. Inferred proportion of migrants within (mii, values on the diagonal) and between (mj) sites among eelgrass populations in Washington (top) and along the Pacific Coast of North America (bottom) based on genets. Rows and columns represent source and destination populations, respectively, with corresponding standard deviations in parenthesis. Significant estimates are displayed in bold.

Sites	N-CherryPoint	N-EastSound	N-HenryIsland	N-HarborShip	N-PadillaBay	N-FidalgoBay	H-GamblePort	H-Belfair	H-Potlach	S-DyesInlet	S-RockyBay	S-DumasBay	S-MauryIsland	S-Nisqually	C-GraysHarbor	C-WillapaBay
N-CherryPoint	**0.8878 (0.0218)**	0.0083 (0.0095)	0.0065 (0.0057)	0.0205 (0.0122)	0.0054 (0.0044)	0.0053 (0.0046)	0.0047 (0.0042)	0.0064 (0.0065)	0.0065 (0.0063)	0.0057 (0.0057)	0.0082 (0.0090)	0.0057 (0.0063)	0.0088 (0.0073)	0.0081 (0.0074)	0.0064 (0.0059)	0.0056 (0.0055)
N-EastSound	0.0096 (0.0074)	**0.8343 (0.0305)**	0.0086 (0.0083)	0.0106 (0.0114)	0.0113 (0.0095)	0.0103 (0.0114)	0.0148 (0.0187)	0.0100 (0.0090)	0.0102 (0.0125)	0.0118 (0.0132)	0.0101 (0.0081)	0.0151 (0.0168)	0.0099 (0.0090)	0.0123 (0.0126)	0.0115 (0.0124)	0.0096 (0.0092)
N-HenryIsland	0.0156 (0.0127)	0.0109 (0.0111)	**0.8393 (0.0283)**	0.0103 (0.0099)	0.0099 (0.0088)	0.0107 (0.0115)	0.0116 (0.0089)	0.0079 (0.0067)	0.0099 (0.0115)	0.0095 (0.0099)	0.0135 (0.0105)	0.0106 (0.0103)	0.0096 (0.0088)	0.0134 (0.0131)	0.0087 (0.0086)	0.0086 (0.0078)
N-HarborShip	0.0070 (0.0072)	0.0059 (0.0057)	0.0058 (0.0057)	**0.8958 (0.0215)**	**0.0127 (0.0095)**	0.0057 (0.0065)	0.0065 (0.0057)	0.0078 (0.0067)	0.0055 (0.0056)	0.0066 (0.0050)	0.0062 (0.0057)	0.0057 (0.0056)	0.0084 (0.0071)	0.0081 (0.0068)	0.0064 (0.0068)	0.0060 (0.0056)
N-PadillaBay	0.0063 (0.0059)	0.0067 (0.0059)	0.0063 (0.0073)	0.0702 (0.0271)	**0.8457 (0.0275)**	0.0070 (0.0079)	0.0065 (0.0057)	0.0061 (0.0047)	0.0054 (0.0051)	0.0049 (0.0039)	0.0047 (0.0048)	0.0046 (0.0049)	0.0076 (0.0064)	0.0060 (0.0053)	0.0070 (0.0057)	0.0051 (0.0048)
N-FidalgoBay	0.0073 (0.0072)	0.0064 (0.0064)	0.0088 (0.0089)	0.0210 (0.0157)	0.0145 (0.0083)	**0.8753 (0.0261)**	0.0070 (0.0068)	0.0078 (0.0085)	0.0058 (0.0052)	0.0069 (0.0057)	0.0055 (0.0060)	0.0060 (0.0055)	0.0062 (0.0071)	0.0067 (0.0059)	0.0069 (0.0052)	0.0078 (0.0080)
H-GamblePort	0.0072 (0.0070)	0.0083 (0.0082)	0.0072 (0.0066)	0.0084 (0.0075)	0.0071 (0.0054)	0.0083 (0.0074)	**0.8672 (0.0236)**	0.0080 (0.0077)	0.0170 (0.0124)	0.0097 (0.0080)	0.0076 (0.0069)	0.0099 (0.0090)	0.0106 (0.0078)	0.0081 (0.0101)	0.0079 (0.0061)	0.0078 (0.0081)
H-Belfair	0.0096 (0.0083)	0.0097 (0.0089)	0.0085 (0.0071)	0.0117 (0.0116)	0.0095 (0.0087)	0.0087 (0.0082)	0.0111 (0.0122)	**0.7931 (0.0422)**	0.0690 (0.0402)	0.0103 (0.0100)	0.0108 (0.0088)	0.0075 (0.0072)	0.0107 (0.0089)	0.0083 (0.0086)	0.0113 (0.0114)	0.0101 (0.0080)
H-Potlach	0.0040 (0.0036)	0.0061 (0.0053)	0.0068 (0.0061)	0.0066 (0.0071)	0.0063 (0.0061)	0.0056 (0.0046)	0.0072 (0.0065)	0.0052 (0.0051)	**0.9103 (0.0180)**	0.0056 (0.0052)	0.0064 (0.0059)	0.0056 (0.0049)	0.0060 (0.0062)	0.0066 (0.0061)	0.0061 (0.0057)	0.0057 (0.0056)
S-DyesInlet	0.0151 (0.0131)	0.0234 (0.0212)	0.0144 (0.0131)	0.0157 (0.0122)	0.0208 (0.0179)	0.0306 (0.0366)	0.0182 (0.0180)	0.0166 (0.0148)	0.0175 (0.0133)	**0.7180 (0.0276)**	0.0207 (0.0156)	0.0232 (0.0199)	0.0147 (0.0152)	0.0149 (0.0152)	0.0210 (0.0187)	0.0151 (0.0145)
S-RockyBay	0.0159 (0.0132)	0.0161 (0.0149)	0.0211 (0.0213)	0.0215 (0.0225)	0.0179 (0.0168)	0.0190 (0.0178)	0.0156 (0.0124)	0.0154 (0.0131)	0.0186 (0.0156)	0.0204 (0.0189)	**0.7169 (0.0281)**	0.0237 (0.0139)	0.0201 (0.0203)	0.0161 (0.0146)	0.0162 (0.0128)	0.0258 (0.0259)
S-DumasBay	0.0068 (0.0069)	0.0071 (0.0067)	0.0078 (0.0071)	0.0061 (0.0058)	0.0068 (0.0060)	0.0076 (0.0085)	0.0059 (0.0056)	0.0068 (0.0061)	0.0076 (0.0062)	0.0058 (0.0052)	0.0076 (0.0070)	**0.8930 (0.0207)**	**0.0096 (0.0077)**	0.0080 (0.0052)	0.0060 (0.0067)	0.0074 (0.0062)
S-MauryIsland	0.0087 (0.0094)	0.0072 (0.0073)	0.0084 (0.0096)	0.0074 (0.0061)	0.0064 (0.0065)	0.0098 (0.0084)	0.0081 (0.0080)	0.0100 (0.0100)	0.0073 (0.0082)	0.0091 (0.0079)	0.0088 (0.0086)	0.0493 (0.0228)	**0.8380 (0.0308)**	0.0075 (0.0067)	0.0082 (0.0066)	0.0060 (0.0055)
S-Nisqually	0.0071 (0.0066)	0.0093 (0.0090)	0.0080 (0.0077)	0.0059 (0.0074)	0.0077 (0.0071)	0.0067 (0.0061)	0.0087 (0.0077)	0.0077 (0.0067)	0.0073 (0.0066)	0.0116 (0.0106)	0.0081 (0.0071)	0.0071 (0.0059)	0.0061 (0.0059)	**0.8833 (0.0233)**	0.0071 (0.0063)	0.0081 (0.0068)
C-GraysHarbor	0.0074 (0.0072)	0.0063 (0.0074)	0.0051 (0.0052)	0.0058 (0.0054)	0.0047 (0.0047)	0.0067 (0.0063)	0.0065 (0.0054)	0.0044 (0.0047)	0.0060 (0.0055)	0.0058 (0.0047)	0.0062 (0.0064)	0.0056 (0.0060)	0.0066 (0.0064)	0.0054 (0.0039)	**0.8744 (0.0251)**	0.0431 (0.0188)
C-WillapaBay	0.0067 (0.0059)	0.0079 (0.0069)	0.0059 (0.0054)	0.0061 (0.0059)	0.0060 (0.0049)	0.0060 (0.0063)	0.0048 (0.0041)	0.0055 (0.0054)	0.0053 (0.0048)	0.0045 (0.0057)	0.0051 (0.0059)	0.0074 (0.0066)	0.0063 (0.0049)	0.0056 (0.0050)	**0.0069 (0.0073)**	**0.9098 (0.0192)**

Sites	WA-Grays Harbor	WA-Willapa Bay	OR-Clam Island	OR-Valino Island	CA-Seal Beach	CA-Newport Bay
WA-Grays Harbor	**0.8101 (0.0705)**	0.1622 (0.0686)	0.0066 (0.0062)	0.0075 (0.0063)	0.0070 (0.0066)	0.0066 (0.0073)
WA-Willapa Bay	**0.0113 (0.0091)**	**0.9627 (0.0135)**	0.0078 (0.0071)	0.0060 (0.0059)	0.0059 (0.0060)	0.0062 (0.0059)
OR-Clam Island	0.0082 (0.0076)	0.0075 (0.0065)	**0.9123 (0.0300)**	**0.0590 (0.0284)**	0.0065 (0.0070)	0.0064 (0.0072)
OR-Valino Island	0.0060 (0.0051)	0.0063 (0.0059)	**0.1471 (0.0720)**	**0.8293 (0.0715)**	0.0051 (0.0046)	0.0060 (0.0059)
CA-Seal Beach	0.0090 (0.0091)	0.0110 (0.0099)	0.0106 (0.0111)	0.0087 (0.0078)	**0.9504 (0.0226)**	0.0104 (0.0103)
CA-Newport Bay	0.0165 (0.0168)	0.0148 (0.0142)	0.0182 (0.0159)	0.0193 (0.0169)	0.0187 (0.0180)	**0.9126 (0.0277)**

Table S1.7. Alignments to the *Z. marina* genome for all outlier loci identified in two of three detection approaches for the Washington dataset based on genets. Alignment match descriptions include the locus ID, SNP base pair location on chromosome (Chrom), the nucleotide identities of the major and minor variant alleles, base pair ranges for sequence queries (BP from & BP to), the probability of the alignment occurring by chance (e- value), percent similarity (%), alignment length in base pairs, and name of gene(s) located on or within 10,000 bp from each BLAST hit.

N	Chrom	SNP BP position	BayeScan q-value	PCAdapt q-value	Major Allele	Minor Allele	Query BP from	Query BP to	Strand	e-value	Similarity (%)	Alignment Length (bp)	*Z. marina* gene
1	1	3125291	0.0190	0.0255	G	A			+				Zosma01g04820, ~500 bp downstream
2	1	28063235	0.0018	0.0003	T	C	28063107	28063248	-	7.66 E-63	98	141	Zosma01g22590, 200 bp upstream Zosma01g22610, 1500 bp downstream
3	1	28063275	0.0006	0.0011	T	G	28063245	28063385	+	4.27 E-66	99	142	
4	3	5296412	0.0000	0.0198	G	A	5296336	5296492	-	6.51E -84	100	172	Zosma03g07160, ~5000 bp upstream Zosma03g07170, on intron Zosma03g07180, ~2000 bp downstream
5	3	5296601	0.0001	0.0001	T	C	5296489	5296660	+	4.23 E-73	99	157	
6	5	31968625											
7	6	20585521	0.0586	0.0429	G	A	20585458	20585608	-	5.99 E-71	99	151	Zosma06g19100, on intron

Table S1.8. Putative functions of the *Z. marina* genes located within 10,000 bp of the SNPs identified as outliers in the Washington dataset based on genets. The information presented orginates from both the GO and InterPro annotation databases.

Outlier SNP	Gene Name	GO ID	GO Description	InterPro I	InterPro Description	Example related Literature
1_3125291	Zosma01g04810					
	Zosma01g04820	GO:000367	nucleic acid binding	IPR00408	K Homology domain	
		GO:000372	RNA binding	IPR00408	K Homology domain, type 1	
				IPR036612	K Homology domain, type 1 superfamily	
	Zosma01g04830					
	Zosma01g04840	GO:000551	protein binding	IPR00288	Pentatricopeptide repeat	Involved in nearly all aspects of psot-transcriptional processing in plant mitochondria and plastids (Wang & Tan 2025)
				IPR011990	Tetratricopeptide-like helical domain superfamily	
				IPR04684	E motif	
1_28063275 & 1_28063235	Zosma01g22580	GO:000819	UDP-glycosyltransferase activity	IPR00221	UDP-glucuronosyl/UDP-glucosyltransferase	
	Zosma01g22590	GO:001711	Golgi transport complex	IPR00725	Conserved oligomeric Golgi complex subunit 8	Important for glycosylation, mutant plants show defects in cell wall components (Blackbrun 2019). Linked to plant pathogen response (Lawaju 2020)
				IPR016159	Cullin repeat-like-containing domain superfamily	
				IPR016632	Conserved oligomeric Golgi complex subunit 8, Metazoal and Viridiplantae	
	Zosma01g22610			IPR011991	Winged helix-turn-helix DNA-binding domain	
3_5296601& 3_5296412	Zosma03g07160			IPR00686	Domain of unknown function DUF632	
				IPR00686	Domain of unknown function DUF630	
	Zosma03g07170			IPR01287	Protein of unknown function DUF1666	
	Zosma03g07180	GO:000373	structural constituent of ribosome	IPR00059	Large ribosomal subunit protein uL3	Transcriptional response is upregulated in eelgrass under hypo- and hyper-saline conditions (Zhao 2025) and under temperature gradients (Schiebehult
		GO:000584	ribosome	IPR00900	Translation protein, beta-barrel domain superfamily	
		GO:000641	translation	IPR04489	Ribosomal protein L3, domain 3, archaeal-type superfamily	
5_31968625	Zosma05g33460	GO:000551	protein binding	IPR00062	Ubiquitin-like domain	
		GO:005108	protein-folding chaperone binding	IPR00310	BAG domain	
				IPR02907	Ubiquitin-like domain superfamily	
				IPR03653	BAG domain superfamily	
	Zosma05g33470	GO:001529	antiporter activity	IPR00252	Multi antimicrobial extrusion protein	
		GO:001602	membrane			
		GO:004291	xenobiotic transmembrane transporter activity			
		GO:005508	transmembrane transport			
	Zosma05g33480	GO:000551	protein binding	IPR00288	Pentatricopeptide repeat	
		GO:000827	zinc ion binding	IPR011990	Tetratricopeptide-like helical domain superfamily	
				IPR03286	DYW domain	
				IPR04684	E motif	
	Zosma05g33490	GO:000367	DNA binding	IPR001471	AP2/ERF domain	Involved in controlling hormone and stress responses in Arabidopsis (Xie et al. 2019)
		GO:000370	DNA-binding transcription factor activity	IPR016177	DNA-binding domain superfamily	
		GO:000635	regulation of DNA-templated transcription	IPR03695	AP2/ERF domain superfamily	
	Zosma05g33500	GO:000367	DNA binding	IPR00608	XPG/Rad2 endonuclease	Arabidopsis homolog linked to repair in plant tissue from UV or oxygen damage (Liu et al 2001)
		GO:000382	catalytic activity	IPR00608	XPG, N-terminal	
		GO:000451	nuclease activity	IPR00608	XPG-I domain	
				IPR00891	Helix-hairpin-helix motif, class 2	
				IPR02906	PIN-like domain superfamily	
				IPR03627	5'-3' exonuclease, C-terminal domain superfamily	
6_20585521	Zosma06g19100	GO:000660	protein import into nucleus	IPR01292	Nucleoprotein TPR/MLP1	TPR containing proteins are essential for osmotic stress regulation in Arabidopsis (Rosado 2006)

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CHAPTER 2:ADAPTIVE GENETIC DIFFERENTIATION BETWEEN SPATIALLY PROXIMATE ANNUAL AND PERENNIAL LIFE HISTORY TYPES OF A MARINE FOUNDATION SPECIES

2.1. ABSTRACT

Diversity in life expectancy is common in flowering plants. In the seagrass *Zostera marina*, a vital foundation species in estuarine ecosystems, annual and perennial varieties occurring in close proximity raise the question of whether these lifespan strategies represent locally adapted genetic variation or plastic phenotypes influenced by the environment. Our study combined field transplant experiments and population genetic analyses to investigate the phenotypic (juvenile survival, flowering, and branching) and genetic differentiation between paired annual and perennial eelgrass meadows in a single estuary (Willapa Bay, northeast Pacific Ocean, USA), over one growing season. A common garden reciprocal transplant experiment based on seedlings demonstrated no differential survival to maturity but revealed a greater likelihood of flowering in annual-sourced plants and branching in perennial-sourced shoots. Further, reproductive trait performance was greater for local individuals compared to non-local ones, which indicates adaptive differentiation. Experimental transplants of annual-sourced seeds into both annual and perennial-dominated sites flowered within a few months regardless of overwintering conditions. Estimates of population structure based on 325 SNPs (RAD-seq) revealed fine-scale population structure between life history types. Population assignment tests identified two distinct groups, distinguished mainly by whether the seedling flowered or not, regardless of geographic source or outplant location. Tests for outlier loci between the two life histories provided further evidence of local adaptation. These insights shed light on the factors governing life cycle variation and resilience in *Z. marina*, offering implications for the evolution and trait-based management of eelgrass populations.

2.2. INTRODUCTION

Diversity in life expectancy strategies is common in flowering plants (Friedman, 2020). Annual and perennial life histories represent distinct endpoints along a continuum of lifespans linked to reproductive allocation (Salguero-Gómez et al., 2016; Hughes, 2017). Annual plants concentrate their reproductive effort into a single episode of flowering and seed set before their life cycle concludes, whereas perennials persist for many years and may reproduce over multiple

seasons (Friedman, 2020). While the evolution of both annual and perennial life histories has been documented across multiple plant families and genera (Datson, Murray and Steiner, 2008; Herron et al., 2021), it is relatively uncommon for individual species to exhibit conspecific life cycle variation (Friedman, 2020). From an evolutionary perspective, plant species with flexible life histories (e.g., both annual and perennial life cycles) may prove to be model systems that offer insights into the origin and maintenance of such strategies, as well as their evolutionary trajectories in response to environmental change.

Zostera marina is a critical ecosystem engineer with annual and perennial life history types that may occur in close proximity (Kendrick et al., 2017; van Katwijk and van Tussenbroek, 2023). Commonly known as eelgrass, this dominant seagrass species has a wide distribution across the Northern hemisphere, where it is a foundation species within intertidal and subtidal ecosystems, maintaining the stability of coastal and estuarine areas (Beck et al., 2001; Gutiérrez et al., 2011; Duarte et al., 2013). Eelgrass meadows generate complex habitat structure that supports community assemblages of economically and ecologically important species, facilitating ecosystem functioning through wave attenuation, carbon sequestration, and nutrient recycling (Hemminga and Duarte, 2000; Bos et al., 2007; Sato et al., 2016).

Notably, *Z. marina* populations may display high phenotypic variability in the allocation of sexual and asexual reproduction across their range (Keddy, 1987), with increasing investment in the former as a response to environmental disturbance and nutrient enrichment (Cabaço and Santos, 2012; Johnson, Moore and Orth, 2017). Annual eelgrass appears to have higher fitness in the presence of frequent environmental disturbances, such as ice-scour, heat stress, grazing, and light limitation (Robertson and Mann, 1984; van Lent and Verschuure, 1994; Santamaría-Gallegos, Sánchez-Lizaso and Félix-Pico, 2000; Kim et al., 2014). By allocating energy preferentially into sexual reproduction, annual eelgrass populations produce seeds that survive during unfavorable conditions or disperse to new habitats (Greve et al., 2005; Lee et al., 2007). In contrast, perennial populations display low frequency of flowering and instead achieve meadow maintenance and growth primarily through rhizome branching when conditions are benign (Olesen, 1999). While this species mainly occurs in a perennial form (characterized by low sexual reproductive effort, for example, less than <10% of a population flowering within a season (Olesen, 1999)), true annual populations where there is high reproductive effort and whole-plant senescence within a year have been reported around the world for decades (Keddy and Patriquin, 1978; van Katwijk and van Tussenbroek, 2023). However, annual and perennial eelgrass meadows mainly exhibit sexual or asexual reproduction, respectively, when coexisting in proximity (Jarvis, Moore and Kenworthy, 2012; Ruesink et al., 2022). The co-occurrence of

different life history strategies in close proximity to each other has been considered as a potential mechanism for population recovery following widespread losses caused by heavy disturbance (Jarvis and Moore, 2010). This resilience may be achieved by maintaining overall population biomass through a combination of seed production for meadow reestablishment and clonal growth for lateral expansion (Jarvis, Moore and Kenworthy, 2012; Thom et al., 2012; Yang et al., 2013).

It is still unclear whether annual and perennial strategies in *Zostera marina* are modulated by adaptive genetic variation or simply represent plastic phenotypes that respond to environmental influences (van Katwijk and van Tussenbroek, 2023). In general, the annual and perennial life histories of plants are considered to be genetically fixed across individual plant taxa (Van Kleunen, 2007; Friedman, 2020; Hjertaas et al., 2022). However, there is little evidence of genetic differentiation between eelgrass life history types found in close proximity with each other (< 20 km) (Gagnon et al., 1980; Keddy, 1987; Ort et al., 2012; Reynolds et al., 2017; Kim et al., 2019; Allcock et al., 2021). In contrast, genetic distinctions have appeared between annual and perennial eelgrass that occupy distinct habitats or have different geographic distributions. Comparisons between paired intertidal (mainly sexually reproducing and annual) and subtidal (mainly clonal and perennial) populations in Germany revealed support for genetic divergence associated with habitat-dependent selection (Oetjen et al., 2010). At greater distances, differentiation between disturbed (mainly annual) and undisturbed (mainly perennial) populations in the southern Korean peninsula were attributed to genetic drift associated with meadow size and clonal reproduction (Kim et al., 2019). And at an even larger scale, genetic differentiation was detected between annual eelgrass in the Gulf of California and perennial populations along the Pacific Coast (Muñiz-Salazar et al., 2005), which may be explained by physical barriers to gene flow and interregional differences in the timing of flowering development. However, it is unresolved whether genetic differentiation underlies phenotypic divergence in annual and perennial *Z. marina* that are growing in close proximity to each other, and whether this differentiation is adaptively significant (van Katwijk and van Tussenbroek, 2023).

Whether adaptive variation underlies phenotypic divergence is best assessed using a combination of common garden experiments and measures of genetic divergence between life history types (De Villemereuil et al., 2016). Common gardens test local adaptation by investigating fitness differences between plants from different sources when grown under common environmental conditions (Núñez-Farfán and Schlichting, 2001). More specifically, reciprocal transplant experiments can elucidate the degree to which environmental and genetic

variation, and their interaction, contribute to phenotypic diversity and performance (Kawecki and Ebert, 2004; Johansson et al., 2017; Evans et al., 2018). Local adaptation requires that individuals with a given attribute (e.g., annual life history) exhibit higher fitness in their "home" environment compared to others from a different environment with distinct attributes (e.g., perennial life history). Differences in fitness-related traits in reciprocally transplanted populations may provide insight into whether these changes are associated with the shared characteristics of the populations where individuals originated (Genotypes, G), the sites where they were translocated into (Environment, E), or a combination of these factors (Genotype-by-Environment, GxE) (Kawecki and Ebert, 2004; Johnson et al., 2022; Lortie and Hierro, 2022). In addition, molecular markers can be used to determine the amount of gene flow between life history types using measures of genetic divergence. If dense marker sets are used, comparative analyses of genetic diversity across the genome (genome scans) may also detect genetic variants correlated to elevated divergence between different life history types (Luikart et al., 2003). Such signals might be indicators of adaptive divergence and can be combined with the results of common garden experiments to infer local adaptation (De Villemereuil et al., 2016).

The aim of this study was to determine whether eelgrass plants with annual and perennial life histories in close proximity within a single estuary represented adaptive genetic divergence or phenotypic variation influenced by the environment. Using reciprocal transplants of seedlings between proximate meadows that differ in the incidence of annual and perennial plants, we investigated whether trait expression (juvenile fitness and reproductive phenotypes) over a growing season were modulated by their site of origin (G), the location where they were outplanted (E), or their interaction (GxE). We conducted additional seedling translocations into sites with similar environmental conditions to those in the reciprocal transplant experiment, to examine the consistency of patterns in phenotypic expression across different locations within an estuary. Seeds sourced from plants exhibiting an annual life history were also transplanted into beds dominated by either annual or perennial plants to investigate whether flowering occurs due to the environment experienced by the seed (vernalization) (Michaels and Amasino, 2000). We then combined the results of these field experiments with a population genetics study, where we described the genetic diversity and population structure among plants with different life history strategies, using reduced representation sequencing (Ali et al., 2016). The integration of these investigations allowed us to identify potential differences between annual and perennial eelgrass and investigate whether these ecotypes may play a role in shaping the diversity and structure of local populations. More broadly, these results may benefit the

conservation of eelgrass by informing the selection of phenotypic variants to propagate as part of restoration efforts.

2.3. MATERIALS & METHODS

2.3.1. Study System

We conducted our study in Willapa Bay, Washington, United States (46° N, 124° W), where annual and perennial eelgrass plants are known to occur within several hundreds of meters of each other (Ruesink et al., 2022) [FIG 1]. Eelgrass meadows can be found in low-intertidal and shallow-subtidal zones of the estuary and their total distribution has been estimated to range from 3000 to 7000 ha, corresponding to 13-29% of the bay area at mean tide level (Ruesink et al., 2006; Dumbauld and McCoy, 2015). Environmental conditions in eelgrass habitat are highly variable. In the growing season during the summer, salinity and water temperature range 20–29 ppt and 15–20 °C, respectively, and water level oscillates between −1m to +2m relative to mean lower low water [TABLE S1].

2.3.2. Field Transplants

2.3.2.1. Seedlings

To investigate the potential for local adaptation associated with life history variation in eelgrass, we translocated *Z. marina* seedlings (first growing season) among co-occurring meadows (found within 200 m to 12 km from each other) that are known to differ in their incidence of annual and perennial plants. We selected three meadows in the northern area near the estuary mouth (one dominated by annual plants, Site A_a, and two dominated by perennial shoots, Sites B_p and C_p), and two meadows approximately 12 km to the south (Sites D_a and E_p, dominated by annual and perennial plants respectively) [FIG 1]. The annual-dominated meadows were identified prior to the transplants in 2019 because nearly all shoots were flowering in September and were no longer present by December of the previous year. Due to logistic constraints, not all study sites served as donor sources or were designated as outplant locations [FIG 1]. Sites A_a and B_p sourced seedlings that were transplanted to all three outplant sites in the northern region, whereas D_a and E sourced seedlings that were only outplanted to site E_p in the south.

Sexually produced seedlings that measured less than 15 cm in total length and comprised two to three leaves were carefully removed from the sediment at each source site for transplantation on that same day right after collection. To avoid targeting vegetatively produced

shoots, small plants attached to a rhizome with multiple internodes were not collected. Therefore, no clonal shoots were included in the experiments and complementary molecular genetic studies. All plants were rinsed with seawater and tracked using colored zip ties over the duration of the experiment. Outplant locations were cleared of other seagrasses and a total of ten experimental plots measuring 0.25 m2 were established at each outplant site. A total of 25 randomly selected seedlings that shared life history and source site were arranged in 5 x 5 grids within each plot between 10 and 22 May 2019. Half of the plots at each outplant site contained transplants from an annual population and the other half from a perennial source. The study species *Z. marina* cooccurs with Z. japonica, which also appears seasonally; however, Z. japonica outplants (n = 6) were clearly identifiable after a few weeks and were omitted from further data collection.

We collected individual data on post-transplant juvenile presence as a proxy for early life stage fitness. Differences in survival between adult life histories are uninformative for investigating local adaptation because annual plants senesce after the occurrence of flowering. However, differential mortality during the juvenile stage (before the appearance of any reproductive traits) may reflect fitness costs associated with the sources or outplants of seedlings (Huang et al., 2010). Therefore, survival (two weeks after translocation) was recorded as the presence or absence of shoots before widespread flowering. Similarly, we tracked reproductive strategies – flowering and cloning – every two weeks from July to August to evaluate the performance of these traits across transplants within a single growing season. Flowering was designated as differentiation of the terminal shoot into a reproductive type (characterized by round rhizome extension above ground and the development of spathes). Cloning was defined as the formation of lateral branches.

We determined whether phenotypic expression in transplanted seedlings was influenced by source or outplant sites using generalized mixed effects models (GLMMs; glmer function in the R-package LME4) (Bates et al., 2015). First, we determined whether trait expression (phenotype) was influenced by genotypes (G), the environment (E), or their interaction (GxE), using the fully reciprocal transplants between predominantly annual site A_a and perennial dominated site B_p, separated by 200 m on a tidal flat. We investigated the following full model:

$$(Model\ 1)\ Phenotype \sim Source * Outplant + (1|Plot).$$

The response variables (phenotypes) were coded as binary traits: juvenile survival as present or absent after 14 days, and flowering and branching as whether these traits were

phenotypically expressed or not during the duration of the experiment for the shoots that survived to adulthood. The predictor variables Source and Outplant (both indicated as A_a and B_p, corresponding to annual and perennial dominated meadows, respectively) were treated as fixed effects to test if the responses varied according to the predominant life history of the sources (Genotypes) or the site characteristics of the outplants (Environment). We also included an interaction term to evaluate whether the effect of the predictor variables on the responses were conditioned by each other (Genotype by Environment). The unique identity of 20 total plots across the two outplant sites (plot ID; 1-20) was included as a random effect to control potential finer-scale spatial variation on the phenotypic responses.

Second, we assessed whether the phenotypic responses in plants sourced from sites A and B were consistent at a muddier site where perennial plants predominate – site C_p [Table S1]. Therefore, we evaluated the full model:

$$(Model\ 2)\ Phenotype \sim Source_{Life\ History} * Outplant + (1|Plot).$$

The response variables were treated similarly to those in Model 1 (Bernoulli distributions = Y/N). Source Life History was included as a predictor to test whether trait expression was different when shoots were sourced from predominantly annual or perennial sites (source A = annual dominated, source B = perennial dominated). Outplant (sites B_p and C_p) was also treated as a fixed effect to evaluate if environmental variation between outplant sites had different effects on the phenotypic responses. An interaction term was included to test whether the influence of source life history on phenotypic expression may be conditioned by specific outplant sites. The random effect Plot ID (11-30) was added to control for potential phenotypic variation attributed to plot-level differences.

In a third analysis, we determined whether the responses in phenotypes from annual (A_a and D_a) and perennial-sourced populations (B_p and E_p) were replicated across regions within the bay. We examined the phenotypic response in perennial dominated outplants only, sites B_p and E_p, corresponding to the North and South regions, respectively. The full model analyzed was:

$$(Model\ 3)\ Phenotype \sim Source_{Life\ History} * Outplant\ region + (1|Plot).$$

The trait responses were analyzed as in the previous models (Bernoulli distributions = Y/N). We included the variables $Source_{Life\ History}$ (annual; perennial) to evaluate differential trait expression between source sites with similar predominantly annual (A_a and D_a) and perennial (B_p and E_p) life history characteristics, and Outplant region (North and South) to

test if the responses varied by bay region. The interaction term was added to determine whether the influence of source life history on the response depends on the region where the sites are located. Plot ID (11-20, 31-40) was included as a random effect to control for finer-scale variation across plots.

To determine which explanatory variables best explained variation in the phenotypes in all analyses, we performed model selection by removing different fixed effects and selected the best fit using Akaike's Information Criterion adjusted for small sample sizes (AICc) (dredge function in R-package MUMIN) (Barton, 2023). Candidate models with ΔAICc values less than two were deemed to have comparable statistical performance (Burnham and Anderson, 2002). In these cases, only the model with the highest weighted Akaike Information Criterion value (AICw; (Symonds and Moussalli, 2011)) was described. This statistic is a commonly used measure of the probability of the model being correct given the data and suite of candidate models (Burnham and Anderson, 2004). In all cases, the random effects were retained to account for correlation between phenotypes across plots, and their significance was evaluated by examining their variance components in the best fit models. We transformed the original logistic model outputs to the probability scale for ease of model interpretation.

2.3.2.2. Seeds

We investigated whether the phenotypic expression of flowering in transplanted eelgrass may be cued during overwintering, before seed germination, by transplanting seeds sourced from annual plants at sites A_a and D_p in August 2019 to two different environments [FIG 1]. Approximately 30 maturing reproductive shoots with seeds were collected and placed in 1-mm mesh bags (20 cm diameter, 80 cm length) in situ. Plants in bags were submerged just under the surface of the water for continued seed maturation (hardened seed coats, darker color) and their posterior release from spathes. Each bag yielded over 500 seeds per site upon collection two weeks later.

Seeds were placed between two layers of paper towel within flat rectangular bags made of bridal veil mesh (10 x 15 cm) for transplantation on the same day of collection. In September 2019, seeds from each region (north, south) were back planted to their respective annual sources (North – A_a, South – D_a) and a nearby (100-200 m) perennial site (North – B_p, South – E_p). However, seeds were not moved between regions, north and south. At each outplant site, eight bags containing 10 seeds each were anchored with long stainless-steel wires and covered with 1 cm of sediment along a 2-m line. Seed bags were checked at two-month intervals through

the winter, when it was sometimes necessary to re-cover the bags with sediment, and at 2–4-week intervals in the summer.

The first seedlings appeared between May and June 2020 at all four outplant sites, at which point evaluation for the presence or absence of flowering began. Monitoring for this trait occurred every 2 weeks until August 2020, when all bags were excavated, and a final assessment of flowering was conducted for all plants. Because it was not possible to track individual seeds and seedlings, our analysis examined rates of flowering across germinated seedlings per transplant bag.

A Generalized Linear Model (GLM, glm function in R-package LME4) (Bates et al., 2015) was used to investigate the effects of region and outplant type on the likelihood of flowering in germinated annual-sourced transplanted seeds using the following model:

$$(Model\ 4)\ Phenotype \sim Outplant_{Life\ History} * Region$$

The response variable Phenotype was coded as whether the germinated seedlings had flowered or not by August and analyzed using a binomial distribution (Y/N). Regions (north vs south) and outplant type (annual dominated = A_a, D_a; perennial dominated = B_p, E_p) were included as fixed effects to test if flowering varied between sites that shared regions or life history types, respectively. Bags with no germinated seedlings at the time of collection were removed from the analysis. Model selection was conducted as described earlier.

2.3.3. Population Genetic Analyses

2.3.3.1. Tissue Collection & DNA Library Construction

Shoot samples for genotyping were collected from the seedling transplant study (sites A_a, B_p, D_a, and E_p; Fig. 1). Tissue from flowering plants was collected at the first sign of differentiation to flowering and from non-flowering plants at the end of the summer from any of the genets still present. Pale meristem and lower sections of the green leaves were targeted to reduce the presence of polyphenol compound contaminants. The samples were rinsed in distilled water and flash frozen in liquid nitrogen to preserve tissue integrity. The tissues were then transferred to a -80 C freezer and stored until DNA extraction.

High molecular weight DNA was extracted using the DNeasy Plant Pro Kit (Qiagen Inc., Valencia, CA, USA). A TissueLyser II (Qiagen Inc., Valencia, CA, USA) was used to homogenize frozen tissues immediately before DNA extraction. DNA quality was assessed using gel electrophoresis and the DNA concentrations for each sample were determined using a Qubit

4000 Fluorometer (ThermoFisher Scientific). Genomic libraries were constructed for restriction-site associated DNA sequencing (RAD-seq) (Ali et al., 2016) with the restriction enzyme Sbf1. The quality of libraries and fragment sizes were evaluated using gel electrophoresis and a Bioanalyzer (Agilent). The genomic libraries were sequenced using the Illumina NovaSeq 4000 (Northwest Genomics) and NovaSeq SP (Genewiz) platforms with 150 bp paired-end settings.

2.3.3.2. SNP Genotyping and Filtering

Raw sequence files were processed using STACKS (Catchen et al., 2013). Pooled-library reads were demultiplexed using the process_radtags program with –best-rad settings and trimmed to 138 bp based on Phred scores greater than 30 per base sequence quality in FASTQC (Babraham-Bioinformatics, 2015) The single-end reads were aligned to the published genome of *Zostera marina* (Ma et al., 2021), using BWA-MEM (Li, 2013), with minimum alignment and mapping Phred quality scores of 30.

Genotyping of polymorphic loci was conducted using the ref_map pipeline in STACKS (Rochette and Catchen, 2017). Bi-allelic base pairs scored in at least 80% of individuals in each population were initially retained, regardless of their proximity to each other within each sequenced RAD tag (138 bp sequences). Subsequent filtering was performed using the R-package SNPFILTR (DeRaad, 2022) [Table S7]. Only individual genotype calls with a sequence depth of at least 10x and an allelic balance (the proportion of reads that support an allele at a base pair position) between 0.2 and 0.8 were retained. Polymorphic base pair sites with more than 25% missing genotypes across all individuals were subsequently removed. At each RAD tag, the SNP with the highest minor allele frequency (MAF) was retained and subsequently designated as a locus (other SNPs on the same RAD tag were removed to minimize the effects of potentially linked markers). Individuals with more than 20% missing genotypes across all loci were then removed from the dataset. Finally, loci with minor allele frequencies less than 0.05 or with significant deviations from Hardy-Weinberg equilibrium (significant Fis values) in all the four populations were removed (based on best practice recommendations for HWE filtering using RAD-seq data) (Pearman, Urban and Alexander, 2022).

2.3.3.3. Population Genetic Tests

To describe genetic diversity within and between sites, we estimated standard population genetic parameters using the R-package PEGAS (Paradis, 2010). Observed (Ho) and expected (He) heterozygosity, and inbreeding coefficients (Fis) (95% confidence intervals generated

through bootstrapping using 10,000 permutations) were computed for each population and globally across all populations.

We examined whether meadows that were dominated by annual and perennial life history types were genetically differentiated from each other using pairwise population genetic differentiation (*Fst*) measures (Weir and Cockerham, 1984) using the package R-package HIER*FST*AT (Goudet, 2005; de Meeûs and Goudet, 2007). Confidence intervals (95%) were generated using bootstrapping with 10,000 permutations. Population structure was also inferred through population assignment of individuals performed in STRUCTURE v2.1.1.5 using an admixture model with correlated allele frequencies (Pritchard, Stephens and Donnelly, 2000), with a burn-in period of 10,000 iterations and 100,000 MCMC repetitions. The number of clusters (K) tested ranged from 1 to 5, and the most likely K was evaluated using the ΔK statistic (Evanno, Regnaut and Goudet, 2005) and mean L(K) (Pritchard, Stephens and Donnelly, 2000). Relationships between populations were visualized by conducting a discriminant analysis of principal components (DAPC) using the R-package ADEGENET (Jombart and Ahmed, 2011). Groups were defined a priori based on collection site. Because the DAPC analysis requires a complete data set, missing genotypes were replaced by the mean frequency of the most common allele in each population. Stratified cross-validation was performed to identify the optimal numbers of principal components to retain for the DAPC analysis (90% of observations used in the training dataset).

2.3.3.4. Outlier tests

To detect whether there were loci consistent with adaptive divergence, we conducted outlier tests using BAYESCAN with default parameters (Foll and Gaggiotti, 2008). The power of this program to detect outlier loci is known to be reduced with fewer than 6 populations, but we still used BAYESCAN because it has lower type I and II errors than other conventional methods (De Mita et al., 2013). Loci were determined to be outliers if their q-value was below 0.05 in each of five independent runs. A BLAST (Altschul et al., 1990) search for the gene coding sequences identified as outliers was conducted against the reference genome of *Zostera marina* "Zmarina v3.1" (Ma et al., 2021) in the Phytozome hub (Goodstein et al., 2012). We retained BLAST hits that met the following criteria: e-value ≤ 1x10-25, alignment length ≥ 140 bp, and sequence similarity ≥ 99%. The possible functional roles of the closest matches within or nearby annotated regions identified through BLAST were analyzed for their biological significance.

2.4. RESULTS

2.4.1. Field Transplants

2.4.1.1. Seedlings

We conducted field transplant experiments to test whether the early life-stage fitness and phenotypic expression of reproductive traits in eelgrass seedlings were influenced by their source and outplant locations. Table S2 describes overall survival and phenotypic expression across all four outplant sites A_a – E_p .

2.4.1.1.1. Test for effect of source and outplant sites on phenotypic expression in reciprocal transplant experiment

We first examined the reciprocal transplants between annual and perennial dominated meadows using GLMMs with three traits as response variables [Fig. 1]. For "juvenile survival", the best-fit model only included the Outplant variable as a significant predictor; however, two statistically equivalent models with similar weights (AICw) were also detected: one without fixed variables, and another including Source and Outplant [Fig. 2A; Tables 1 & S3]. The difference in probability of seedling survival for plants sourced from both locations was 12% higher at site A_a compared to site B_p, indicating that survival to adulthood differed only marginally between outplant sites. Given that there were two almost equally plausible models, we conclude that the effect of outplant site was small, given the experimental design. However, the candidate models also contained considerably high variances in the random effect Plot ID [Table S3], suggesting that the survival of juvenile eelgrass was also in part explained by heterogeneity at smaller spatial scales within sites.

The best fit model for the response variable "flowering" revealed that both the Source and Outplant sites (defined by dominant life history types) were significant predictors for this trait [Fig. 2B; Tables 1 & S3]. Additionally, two plausible models were identified: one including only the Source variable, and another including Source, Outplant, and their interaction [Table S3]. In the model with the highest weight (and in all top models), Source had a greater effect than Outplant site. The probability of flowering in plants sourced from site A_a was 70% greater than those from site B at both outplant sites [Fig. 2B]. Outplanting of both sources to Site B_p decreased the probability of flowering by 13% compared to site A_a [Fig. 2B]. Collectively, the models suggest the potential for both genotypic and environmental controls underlying the phenotypic expression of flowering in eelgrass. Specifically, the probability of flowering was substantially higher for plants originating from the annual-dominated meadow

compared to those sourced from the predominantly perennial site. But further, outplant conditions in site A_a favored greater rates of flowering regardless of site of origin.

The best-fit model for the trait "branching" also included both Source and Outplant, but not their interaction, as significant predictors for the response; an equally plausible model with lower AICw included the interaction [Fig. 2C; Tables 1 & S3]. Plants sourced from site B_p had a 43% higher probability of branching than those originating from site A_a at both outplant locations, indicating that shoots sourced from the perennial-dominated meadow were more likely to branch compared to those sourced from the predominantly annual site. Interestingly, branching was 27% more probable in site A_a compared to B_p for plants originating from both sources, implying that branching was generally favored by the environmental conditions of the annual-dominated location. Taken together, these results suggest that both genetic and environmental influences also play a role in the likelihood of branching in eelgrass.

2.4.1.1.2. Test for effect of source and outplant sites on phenotypic expression in perennial-dominated sites within a bay region

We then used GLMMs to evaluate whether the phenotypic expression of the three traits of transplanted seedlings in outplant B_p extended similarly to outplant C_p, another perennial-like site within the same bay region [Fig. 1].

The best-fitting model for the response "juvenile survival" included Outplant site as a significant predictor of the response [Fig. 2A; Tables 1 & S4]. Two other equally plausible models including Source, and Source and the interaction term, were also identified, but outplant site had the largest effect size in all three models [Table S4]. The probability of early life-stage survival was 24% higher for shoots from both source locations in outplant site B_p compared to C_p, implying that, for outplant sites with predominantly perennial plants within a bay region, survival to maturity was influenced by where the subjects were transplanted.

For the response variable "flowering", the best fit model included the Source variable as a significant predictor, whereas an equally plausible model with lower weight included both Source and Outplant [Fig. 2B; Tables 1 & S4]. The probability of flowering was 56% higher for plants originating from site A_a compared to those from B_p in both outplant locations. These findings indicate that the life histories characteristics of the source sites influenced the phenotypic expression of flowering in perennial-dominated outplant sites within the north region. There was a considerably higher probability of flowering for annual-sourced plants regardless of outplant location.

The best candidate model for the response “branching” included the variable Source as an explanatory predictor of the response, and another equally plausible model with lower AICw included both Source and Outplant sites [Fig. 2C; Tables 1 & S4]. Overall, plants sourced from site B_p exhibited 49% higher probability of branching compared to plants originating from site A_a in both outplants, which implies that branching in eelgrass across predominantly perennial outplants within the north region was largely driven by the life history characteristics of the source sites. Perennial-sourced shoots had a higher likelihood of branching, regardless of the outplant site, which did not considerably affect this trait's phenotypic expression.

2.4.1.1.3. Test for effect of source life history and bay region on phenotypic expression in perennial-dominated sites between bay regions

We investigated whether there was a regional effect on trait expression within the bay, by comparing the performance of seedlings were that sourced from annual (A_a and D_a) and perennial dominated (B_p and E_p) sites, and planted into a perennial dominated sites (B_p and E_p), in the north and south respectively [Fig. 1].

For “juvenile survival,” the best-fit model included Region and life history of the Source as significant predictors of the response – additional statistically equivalent models including only Region, and another including the interaction between Source life history and Region [Fig. 2A; Tables 1 & S5]. The overall probability of survival was 14% higher in the south region (outplant E_p) compared to the north region (site B_p) for plants originating from both annual and perennial sources. However, within both regions, perennial-sourced plants exhibited 11% higher probability of survival than annual-sourced ones. The results indicate that survival in juvenile eelgrass depends on the relationship between source life history and the regional outplant location. Broadly, survival differed between the north and south regions, but perennial-sourced shoots displayed marginally higher survival rates than annuals in both regions.

The best-fit model for “flowering” identified Source life history, Region, and their interaction as significant predictors of the response, with an equally plausible model including only the Source life history variable [Fig. 2B; Tables 1 & S5]. In all models, Source had a larger effect size. The probabilities of flowering were 62% and 82% greater for annual compared to perennial sourced plants in the north and south regions, respectively. This result suggests the presence of a genetic component underlying the difference in phenotypic expression of flowering that was consistent across the bay. However, the difference in flowering between regions was more pronounced for the annual-sourced plants (14% higher in the north) than for shoots originating from perennial beds (6% higher in the south). The inclusion of the interaction

term in the best-fit model may suggest the presence of interregional locally-adapted genetic differentiation that may impact flowering rates across the bay.

For the variable "branching", the best fit model included the Source life history type, outplant Region and their interaction as significant predictors, whereas a statistically equivalent model only included the Source life history and Region variables [Fig. 2C; Tables 1 & S5]. Branching was 40% and 48% more probable for perennial- compared to annual-sourced shoots in the north and south regions, respectively. Further, the outplant region also influenced the likelihood of branching, however, the difference in branching between regions for transplants sharing source life history was greater for perennial (20% greater in the north) compared to annual sourced-ones (12% greater in the north). Taken together, these results further suggest the existence of a genetic component modulating branching in eelgrass, with perennial-sourced plants exhibiting higher phenotypic expression of this trait.

2.4.1.2. Seeds

Annual-sourced seeds were transplanted before overwintering to evaluate differential phenotypic expression of flowering in transplanted eelgrass at an earlier life stage [Fig 1]. A total of 32 bags with seeds were outplanted to four locations in Willapa Bay, but only 18 bags had germinated seedlings. High rates of flowering occurred in each of the bags with germinated seedlings (50-100%) regardless of overwintering conditions experienced in the annual or perennial-dominated outplants [Table S6]. However, overall flowering rate appeared to be slightly higher when seeds were outplanted into the annual-dominated outplant sites A_a (93.3%) and D_a (100%), compared to the perennial-dominated sites B_p (86.1%) and E_p (85.2%) [Fig. 3].

The best-fitting model for "flowering" in transplanted eelgrass seeds revealed a null model without explanatory variables – a second statistically comparable model included the life history of the outplant site, and a third only the Region variable [Tables 1 & S6]. Here, we cannot reject the null hypothesis, and given the experimental design, we interpret these results as indicating that the dominant life history of the outplant and the bay region where the annual plants were sourced only marginally influenced the nearly fixed (~94%) flowering rate in overwintering eelgrass seeds originating from the annual-dominated sites A_a and D_a.

2.4.2. Population Genetic Analyses

2.4.2.1. Genotyping

To describe patterns of genetic diversity and population structure among meadows, we genotyped 199 individuals at 325 loci after performing filtering steps for genotype depth, allelic balance, missing data per variant site, minor allele frequency, and Hardy-Weinberg Equilibrium in at least one population [Table S8].

2.4.2.2. Genetic Diversity

Population-level observed and expected heterozygosity fell in the range of 0.24-0.26 and 0.25-0.28 across all loci, respectively [Table S8]. Population-specific inbreeding coefficients (Fis) were close to zero across populations (0.05 – 0.07), with the exception of site E_p (0.12), a perennial dominated site, but all were significantly different from zero [Table S9]. This finding suggests a small excess of homozygosity in seedlings produced in each of the populations compared to HWE expectations, particularly at site E_p, where Fis values are an order of magnitude higher compared to other sites. Per locus tests of Hardy-Weinberg Equilibrium identified less than 17% of loci (26-53 sites) significantly deviated from expected proportions in each population [Table S9]. These markers were retained for subsequent analyses because they met HWE expectations in at least one of the four populations tested and may therefore reflect true biological processes that contribute to genetic diversity across the bay.

2.4.2.3. Population Structure

Tests of pairwise population genetic differentiation (*Fst*) revealed values that ranged from 0.01 to 0.12, and all *Fst* measures were significantly different from zero. The greatest genetic distance was observed between the annual and perennial dominated meadows in the southern region (sites D_a and E_p), and the lowest between the annual dominated meadows (Sites A_a and D_a) in the North and South [Table 2].

The DAPC analysis revealed that 45% of the observed genetic variation among individuals was best explained by 20 Principal Components [Fig. 4]. The first discriminant function, which explained 63.6% of the retained variation, separated sites defined by dominant life histories. Interestingly, plants sourced from perennial-dominated sites B_p and E_p, that flowered by the end of the experiment clustered closer to samples sourced from annual dominated sites A_a and D_a. The second discriminant function explained 28.2% of the retained variation and separated sites from the north to south. These results indicate that the occurrence of flowering has a stronger influence on shaping eelgrass population structure than bay location [Fig. 4].

The most likely number of clusters identified in the STRUCTURE analyses was k=2 [Table S10]; these two clusters mostly corresponded to trait expression [Fig. 5]. Broadly, the majority of individuals in the annual-dominated sites A_a and D_a were defined as one genetic cluster, whereas most of the individuals in perennial-dominated sites B_p and E_p were identified as the second population. However, all individuals that flowered, regardless of population of origin, were characterized in the former group, suggesting that flowering and non-flowering plants, instead of population-wide life histories, represent genetically distinct populations. Similarly, branching plants fell largely in the second group, although there were more exceptions to this finding compared to the flowering trait. The presence of individuals with mixed assignment indicates gene flow between groups, which occurred at higher rates in plants sourced from annual-dominated sites.

2.4.2.4. Outlier Detection

A total of 28 loci were detected as outliers in five separate BAYESCAN assessments [$q < 0.05$; Table S11]. All loci were uniquely aligned to the genome of *Z. marina* – out of six chromosomes, 43% of outliers were localized on the sixth chromosome, while none were on the fourth chromosome. All but four of the outliers aligned with annotated coding regions within the *Z. marina* genome, and thus we report on these matches only, and not on additional flanking coding regions - we report the closest annotated regions within 1000 bp for each of the four exceptions: CLocus_156050, CLocus_221716, CLocus_221717, and CLocus_222314 [Table S11]. The 28 outliers aligned with 24 unique genes; two or more RAD loci aligned with four genes. We identified putative functions of the genes through literature searches [Table S12]. Many of the coding regions have been investigated in relation to reproduction in plants, but we note that their functions have not been explored specifically in eelgrass.

2.5.DISCUSSION

The aim of this study was to determine whether co-occurring *Z. marina* plants with annual and perennial life cycles, found in a single estuarine environment in the northeastern Pacific Ocean, were adaptively divergent or whether these life histories represented phenotypic variation influenced by the environment. Our findings provide evidence of fine-scale adaptive differentiation between lifespan strategies. Reciprocal transplants over less than 200 m demonstrated that the probability of flowering was significantly higher for seedlings sourced from a meadow dominated by annual plants compared to those sourced from a predominantly perennial site. Similarly, branching was higher for seedlings sourced from a predominantly

perennial site. We also found evidence for an environmental role in modulating the phenotypic expression of these traits; for example, both flowering and branching were higher in the annual dominated sites regardless of the source population, and branching rate differed between outplant sites dominated by perennial plants. Common garden experiments with seeds sourced from annual plants and grown overwinter in both annual- and perennial- dominated sites showed a fixed annual life history type. Population genetic tests revealed that flowering and non-flowering plants (most of which branched) largely represented two genetically distinct populations, regardless of distance between source populations. Detection of outlier loci further supports adaptive differentiation between life history types and functional annotation suggests a role of some of these loci in reproductive traits, although we note that their exact function in *Z. marina* has not been described. Overall, these results support the view that conspecific annual and perennial strategies in eelgrass may be differentiated and could reflect fine scale local adaptation.

The annual life history in *Z. marina* has been largely regarded as facultative and driven by the environment (van Katwijk and van Tussenbroek, 2023), but our investigation suggests that this view does not necessarily apply in the location we studied. First, the population differentiation observed here could not be explained solely by spatial proximity. Instead, the patterns of genetic (*Fst*) divergence were primarily explained by variation in life expectancy and the associated reproductive strategies. Further, there was evidence for a secondary role for spatial structuring, which conformed to bay regions within Willapa Bay. Spatial stratification is known to occur across a range of distances in *Z. marina* and has been linked to limited dispersal capacity (Kamel et al., 2012; Kim et al., 2019; Martínez-García, Hansson and Hollander, 2021), but we emphasize that life history played a greater role in explaining population structure in this study. Second, the reciprocal transplant study demonstrated that locally derived plants had greater reproductive trait performance compared to non-local plants, thus meeting the criterion for detecting local adaptation, and suggesting that such adaptation occurs on a fine spatial scale. Clear evidence for adaptive differentiation associated with life cycle variation traits has been demonstrated in other partially clonal plant species (Van Kleunen, 2007; Friedman et al., 2015), but not in *Z. marina* until this study (van Katwijk and van Tussenbroek, 2023). Third, the test for outlier loci provided additional support for local adaptation in shaping population structure. Broad annotation showed that some of the candidate genes [Table S11] have been linked to reproduction in plant model species. For example, in tobacco (*Thaumatococcus daniellii*) and rose (*Rosa chinensis*), thaumatin-like proteins have been associated with flowering differentiation under stress conditions (Richard et al., 1992; Su et al., 2021). In *Arabidopsis*

thaliana, the ribosomal protein L18 (AtPRPL18) is essential for seed development and has higher expression in inflorescences compared to roots (Chen et al., 2022). The sister chromatid cohesion complex (AtSCC2) is indispensable for fertility (meiosis) but not vegetative growth (mitosis) (Wang et al., 2020), microtubule-associated proteins (MAP65-1 and MAP65-2) are critical to promoting root cell growth and axial extension (Lucas and Shaw, 2012), and Type 2C Protein Phosphatases (POL) are involved in stem cell identification in shoot and flower meristems (Yu, Miller and Clark, 2003). Nevertheless, further experimental validation of the candidate outlier genes identified in this study is necessary to assess their functionality in the life cycle variation of *Z. marina*.

The outplant conditions had a comparatively lower effect size on the expression of reproductive traits than source population. Nevertheless, environment affected genotype performance; across annual and perennial sites, across perennial sites, and across regions. However, there were contrasting patterns explaining phenotypic change across the two traits in the reciprocal transplant experiment. Flowering exhibited a pattern of co-gradient variation, which occurs when both the genetic and environmental factors act in the same direction (Conover, Duffy and Hice, 2009). Here, the phenotypic difference between the two local populations A_a and B_p was pronounced across the environmental gradient [Fig 2B], which in turn can reinforce adaptive evolution (Conover, Duffy and Hice, 2009). In contrast, the trait "branching" exhibited a counter-gradient variation pattern [Fig. 2C], where environmental and genetic influences act in opposite directions; the probability of branching for annual plants at site A_a overlapped with that of perennial plants at site B p. Therefore, there was reduced phenotypic divergence across environmental clines (Conover, Duffy and Hice, 2009). Phenotypic similarity across ecological gradients represents a cryptic form of local adaptation, but may only be detectable through the use of common garden experiments (Conover and Schultz, 1995; Conover, Duffy and Hice, 2009). In this case, reproductive isolation between the two life histories may be partially achieved through differential reproductive phenology. Flowering in overwintering perennial shoots occurs while annual seedlings are still growing (Ruesink et al., 2022). However, local adaptation in reproductive traits detected in the seedling-based reciprocal transplant indicates the potential for outbreeding depression if interbreeding occurs between annual and perennial plants. This phenomenon encompasses the loss of fitness due to the inheritance of mismatched genetic combinations, which may manifest through the dilution of locally adapted genotypes or breakdown of co-adapted gene complexes (Lynch, 1991; Fenster and Galloway, 1997). The presence of fitness costs could potentially contribute to

intensifying the temporally driven differentiation between annual and perennial life histories, thereby maintaining the overall diversity of lifespan strategies in Willapa Bay.

Life cycle variation in eelgrass may also serve as an adaptive strategy in response to environmental factors and ecological pressures not captured in this study. In natural settings, light limitation from overshadowing by adult plants could potentially stunt seedling growth in established seagrass meadows (Ruesink et al., 2012; Statton et al., 2017), such as in perennial-dominated beds. This competition for resources likely limits the successful development of seeds, which are produced at higher rates by annual plants, and restricts their presence to open habitat spaces. These open areas may only remain viable for short periods due to natural and anthropogenic activities that can periodically wipe out entire meadows (Robertson and Mann, 1984; Kim et al., 2014). Such disturbances prevent the long-term persistence of perennial meadows and create opportunities for continuous seed recolonization. These processes likely enable the coexistence of annuals and perennials in unstable environments, with each life cycle strategy becoming adaptive based on the temporal and spatial availability of suitable habitats (Jarvis, Moore and Kenworthy, 2012).

The specific life stages examined in our study carry significant implications for the interpretation of our findings. Relying on sexually produced progeny for both seed- and seedling-based transplants, as well as the population genetic tests, allowed for consistency in both field experiments while also capturing the genetic diversity of the corresponding collection sites. Given that the dispersal of eelgrass pollen and seeds is typically restricted to less than 50 m from the source (Ruckelshaus, 1996), there is a high likelihood that the transplanted material in this study were produced by the annual and perennial dominated beds where the collections occurred, reflecting the local meadow genetic variation. However, there is a possibility that a small fraction of the transplanted seeds or seedlings originated elsewhere in the bay and were posteriorly displaced into one of our sampled sites. Our population genetic results revealed this pattern, notably the presence of annual plants with flowering phenotypes in perennial-dominated beds. These seeds may have dispersed from the predominantly annual sites, or they may be a legacy of annual plants that were previously more common but have been outcompeted by perennials over time, since seedling growth is stunted within established eelgrass meadows (Ruesink et al., 2012).

The evidence for fine-scale locally adapted genetic differentiation associated with reproductive strategies has practical implications for the trait-based management of eelgrass populations. Restoration strategies aimed to maintain or revive the ecosystem services provided

by seagrasses include the translocation and planting of donor plants or seeds (van Katwijk et al., 2016, 2021). However, careful considerations must be taken when selecting donor material, especially with seed-based approaches where a main challenge is finding donor sites with maximum and sustainable seed productivity to enhance the success of larger scale restoration (van Katwijk et al., 2016; Unsworth et al., 2023). Annual meadows, therefore, may appear as ideal donor candidates for seed sourcing given their increased seed productivity compared to their perennial counterparts (van Katwijk and van Tussenbroek, 2023). Nevertheless, seeds or shoots sourced from locally adapted annual or perennial meadows may experience lower fitness when transplanted into habitats different from their home site (Hufford and Mazer, 2003). This phenomenon may directly impact the phenotypic performance of the translocated material, such as survival or flowering, and consequently influence restoration outcomes.

Our transplant experiments suggest that annual-sourced seeds may serve as valuable tools for aiding in the restoration of perennial-dominated environments. In contrast to seedling survival rates that exhibited minimal variation across source or outplant conditions, annual-sourced seeds germinated at significantly higher rates in perennial compared to annual outplants [Table S6]. This variability indicates that selection may act at an early life stage in shoot development (Huang et al., 2010), but also that conditions replicating those in our experiments – such as overwinter seed maturation and ample spatial distribution – could enhance the successful germination of annual-sourced seeds in more stable environments. However, the absence of perennial-sourced material in the seed-based transplant precludes a comprehensive assessment of whether the rates of phenotypic expression observed in the perennial seedlings would persist under the seed-transplant conditions. Thus, conducting a fully reciprocal common garden setup including perennial-sourced seeds could offer additional valuable insights into the field conditions and demographic patterns that contribute to life history variation within natural *Z. marina* populations.

A caveat to this study's findings is the confined geographical extent of our investigation, which was conducted within a single estuarine environment. It remains unclear whether locally adapted genetic variation associated with life expectancy in *Z. marina* is also found at microgeographic scales elsewhere (van Katwijk and van Tussenbroek, 2023). Gene flow among regions harboring annual and perennial eelgrass in close proximity (van Katwijk and van Tussenbroek, 2023; Yu et al., 2023) is geographically restricted, which raises the possibility that the genetic variation underlying differences in lifespan diversity in *Z. marina* may differ across locations where distinct life cycle strategies coexist. Further investigation using combinations of common garden experiments with population genetics tests in other annual and perennial

meadows cooccurring in proximity may provide insight into whether these ecotype pairs also represent adaptively and genetically distinct varieties and potentially uncover shared genetic factors influencing life history variation of *Zostera marina* across its range.

2.5.1. Conclusion

This investigation underscores the pivotal role of life history trait variation in shaping the genetic diversity among cooccurring lifespan strategies within the seagrass *Zostera marina*. Previously, the annual life history in this species was largely regarded as an environmentally-driven facultative state (van Katwijk and van Tussenbroek, 2023). We offer compelling evidence of adaptive genetic differentiation between annual and perennial types on a microgeographic scale, evidenced in the greater reproductive trait performance of local individuals compared to immigrants (flowering advantage for transplanted annual eelgrass and branching for perennial plants), as well as by the genetic differentiation between types largely linked to reproductive strategies. This genetic structuring is likely maintained by adaptive life cycle strategies that respond to temporal and spatial habitat availability, combined with differences in flowering phenology between the life histories. Our findings not only contribute crucial insights into the potential origins and persistence of life cycle diversity in plant species but also pave the way for future research delving into the precise molecular mechanisms underpinning this life history variation across the distribution of *Z. marina* habitats. Moreover, our discoveries provide a science-based rationale for incorporating lifespan strategies in eelgrass management efforts aimed at preserving the resilience of critical eelgrass habitats in coastal and estuarine ecosystems.

2.6. FIGURES

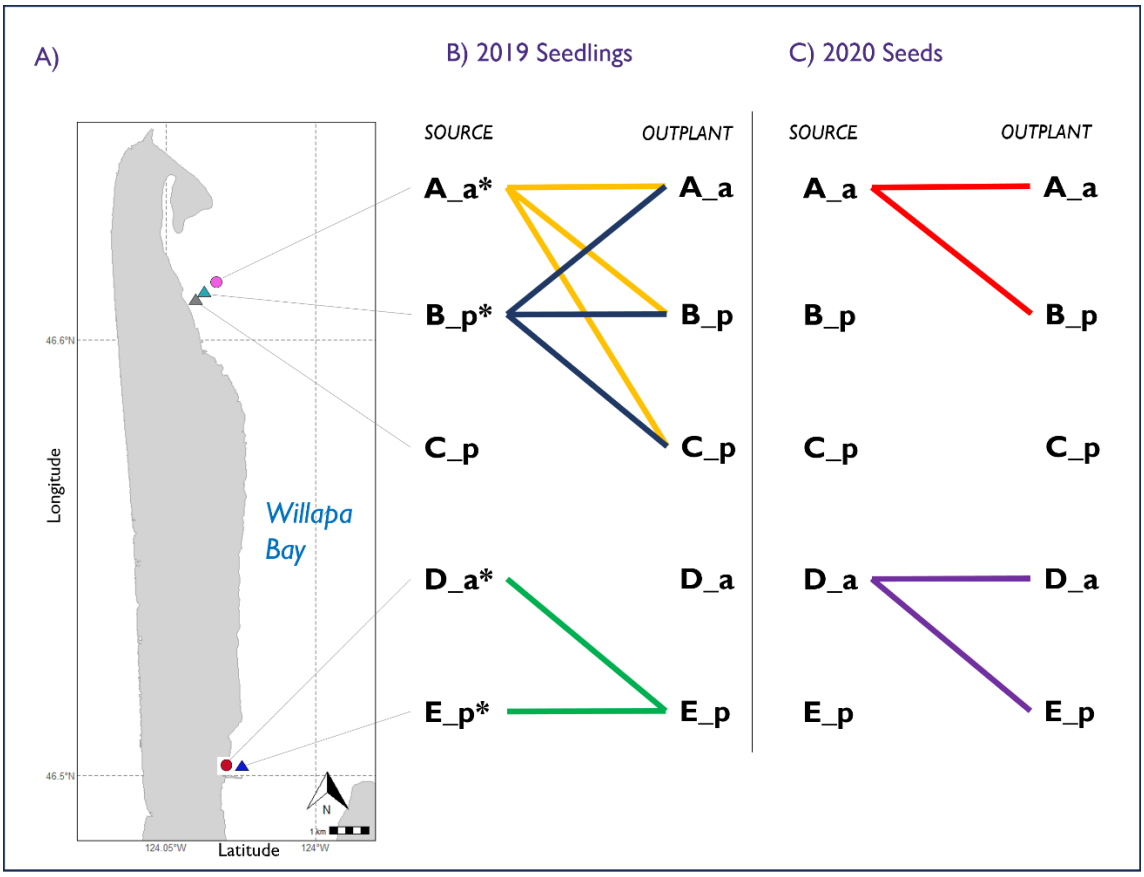

Figure 2.1. Description of experimental designs. A) Sites in Willapa Bay used in the transplant experiments with inset map of North America. Circles denote meadows dominated by annual plants, and triangles meadows dominated by perennial plants. Connecting lines indicate the directions of transplants from source to outplant sites in the B) seedlings & C) seeds experiments. Asterisks indicate locations where tissue was collected for population genetic analyses.

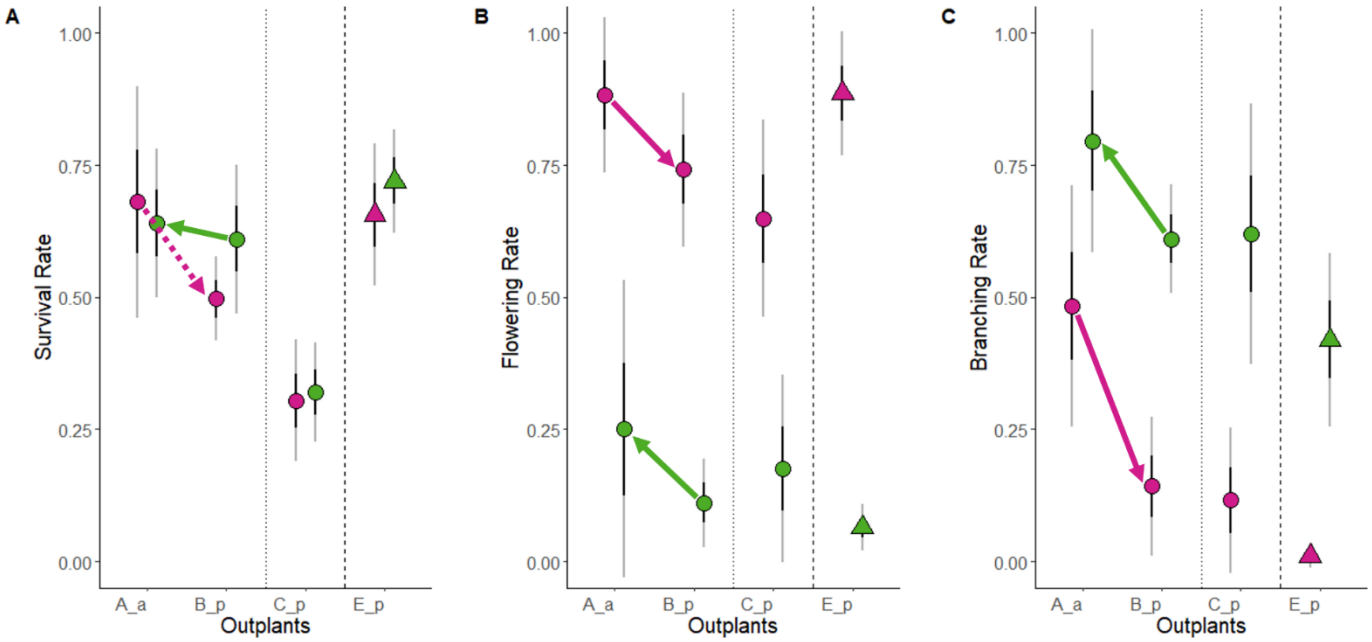

Figure 2.2. Early life-stage survival and phenotypic expression rates of transplanted annual and perennial eelgrass seedlings (2019 data). Average per-plot rates of A) juvenile survival, B) flowering and C) branching in translocated eelgrass seedlings within one season. Observations are grouped according to outplant sites (A_a, B_p, C_p, and E_p), colored based on the predominant life history in the source site (annuals A_a & D_a = pink; perennials B_p and E_p = green), and shaped by bay region (north = circle, south = triangle) – five plots per treatment. Black and grey error bars represent standard errors of the means and single standard deviations in the data, respectively. Arrows indicate the direction of phenotypic change from "home" to "away" transplant conditions.

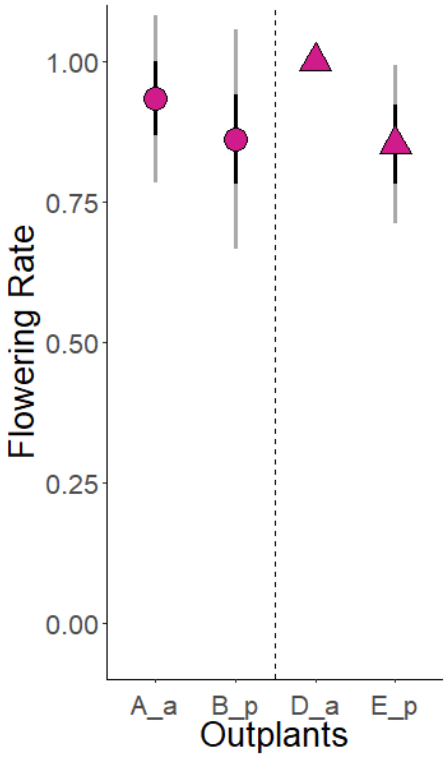

Figure 2.3. Flowering rates of transplanted overwintering eelgrass seeds in August following planting. Average per-bag flowering rates of annual-sourced seeds translocated to predominantly annual (A_a and D_a) and perennial (B_p and E_p) outplant sites; n bags = 1 – 6 per treatment (See Table S6). Black and gray error bars indicate standard errors of the means and one standard deviation in the data, respectively.

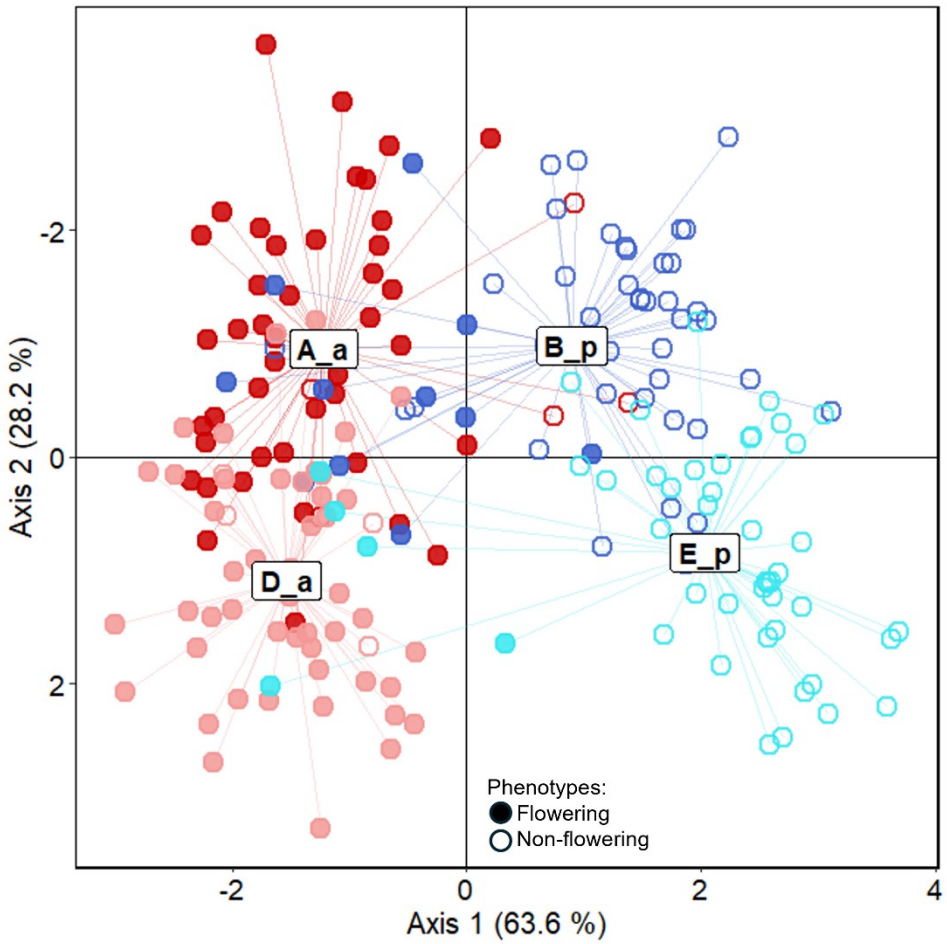

Figure 2.4. Scatterplot describing genetic relationships among individuals from the seedlings transplant experiment using the first two principal components of DAPC (variance explained in parentheses). Data points represent individual plants, colored by source site, with shape (filled or empty circles) denoting phenotype.

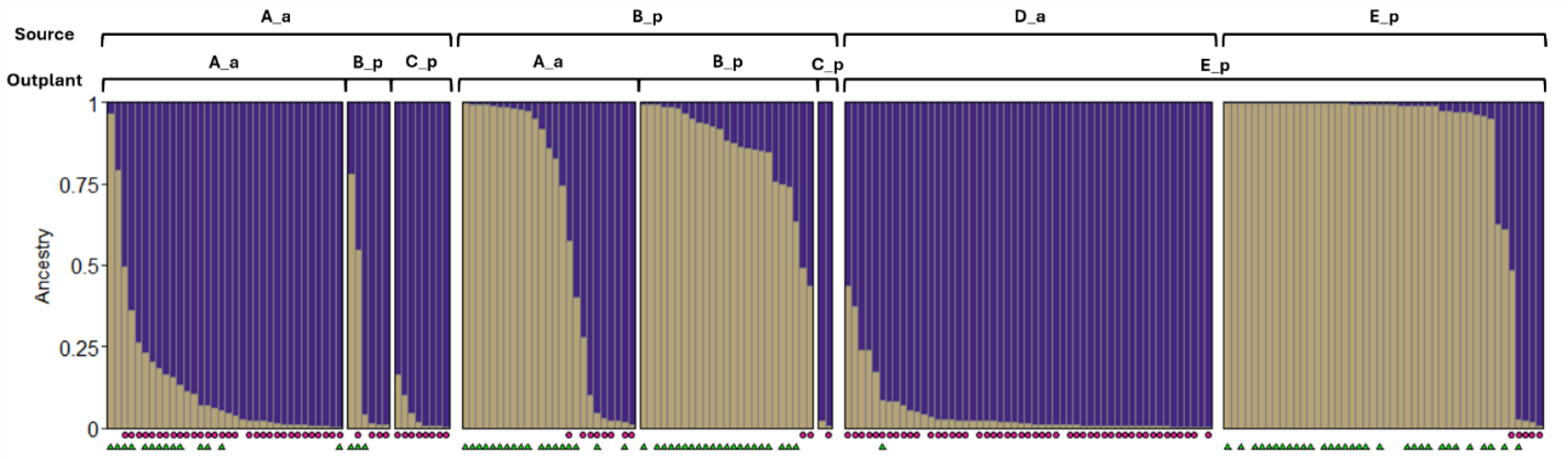

Figure 2.5. Population structure of the experimental seedlings inferred using ADMIXTURE analysis. Each individual plant is represented by a stacked column indicating the probabilities of assignment in $K = 2$ constructed ancestral populations. Pink circles and green triangles at the bottom of the plot indicate individuals that flowered and branched, respectively.

2.7.TABLES

Table 2.1. Best-fit candidate models describing the results of the seedling (GLMMs, models 1-3) and seeds (GLM, model 4) transplant experiments. Model estimates and 95% confidence intervals transformed to the probability scale (Log odds scores given in table S3-S6). The best-fit models were selected based on the lowest ΔAIC values (Tables S3-5, S7) and Akaike weight (AICw). The variance of the random effect (Var RE), Plot ID, is also reported.

Seedlings Models	Trait	Intercept (CI)	Source (CI)	Outplant (CI)	Interaction (CI)	AICw	Var RE
1: Reciprocal transplant between sites A_a and B_p	Survival	0.67 (0.57, 0.75)		0.55 (0.41, 0.69)		0.36	0.6
	Flowering	0.91 (0.79, 0.97)	0.21 (0.08, 0.46)	0.78 (0.54, 0.92)		0.50	0.72
	Branching	0.42 (0.27, 0.59)	0.85 (0.70, 0.93)	0.15 (0.07, 0.29)		0.62	0.62
2: Perennial sites within a region, B_p and C_p	Survival	0.55 (0.49, 0.62)		0.31 (0.19, 0.45)		0.40	0.51
	Flowering	0.70 (0.59, 0.79)	0.14 (0.03, 0.26)			0.61	0.51
	Branching	0.12 (0.07, 0.21)	0.61 (0.43, 0.77)			0.66	0.51
	Trait	**Intercept (CI)**	**Source (CI)**	**Region (CI)**	**Interaction (CI)**	**AICw**	**Var RE**
3: Perennial sites between regions, B_p and E_p	Survival	0.50 (0.42, 0.59)	0.60 (0.50, 0.69)	0.64 (0.54, 0.73)		0.48	0.51
	Flowering	0.74 (0.60, 0.84)	0.12 (0.05, 0.27)	0.88 (0.75, 0.95)	0.06 (0.02, 0.23)	0.51	0.51
	Branching	0.13 (0.07, 0.25)	0.61 (0.38, 0.80)	0.01 (0, 0.09)	0.41 (0.07, 0.87)	0.60	0.51
Seeds Model	**Trait**	**Intercept (CI)**	**Region (CI)**	**Outplant (CI)**	**Interaction (CI)**	**AICw**	
4: Annual and perennial sites between regions, A_a, B_p, D_a & E_p	Flowering	0.94 (0.66, 0.99)				0.5	

Table 2. Pairwise genetic divergence measured using *Fst* (below diagonal), with corresponding 95% confidence intervals (above diagonal) among four eelgrass meadows. Significance tests were conducted using 10,000 permutations.

Sites	A_a	B_p	D_a	E_p
A_a		0.041 - 0.066	0.011 - 0.022	0.081 - 0.128
B_p	0.053		0.053 - 0.090	0.014 - 0.025
D_a	0.016	0.071		0.092 - 0.140
E_p	0.104	0.019	0.116	

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CHAPTER 3:REPLICATED EVOLUTION OF ANNUAL AND PERENNIAL LIFE HISTORIES IN EELGRASS (*ZOSTERA MARINA*)

3.1. ABSTRACT

Variation in life expectancy has repeatedly evolved across distantly related plant lineages. Within the geographic range of a single species, the seagrass *Zostera marina*, annual beds are found sparsely across the northern temperate distribution of mainly perennial meadows. Both life histories can coexist in close proximity, where they may be adaptively differentiated. However, genetic differentiation between isolated regions is greater than between life histories within a bay, suggesting the possibility of independent evolutionary transitions between annual and perennial strategies. We investigated whether life history variation in eelgrass represents replicated evolution and investigated whether there was a genomic basis for parallel evolution. Plants were sampled across ten sites spanning the eastern Pacific and both Atlantic coasts. Five (Padilla Bay, Grays Harbor, Willapa Bay, Yaquina Bay, and San Francisco) of these sites included ecotypes co-located within 100s of m, another three (Machias Bay, Sylt, and Hooge) included ecotypes within the same bay, and two only had annuals plants (Oosterschelde and Middle Marsh). Whole-genome sequencing was carried out on four annual and four perennial (when available) individuals per site. Phylogenetic inference revealed support for multiple transitions between annual and perennial strategies in the species. Evolutionary relationships were driven primarily by geography, with a major division between Pacific and Atlantic samples, and additional subdivision within each ocean basin. Clear separation between life histories within some sites emerged only when gene tree discordance was minimized. Admixture analyses across sites revealed that genetic variation was structured hierarchically within subregions across ocean basins, with life history types separated at some geographic locations but not all, and shared covariation in allele frequencies between life histories across sites. Parallel genomic divergence was identified across Pacific but not Atlantic Ocean life history types. Repeated allele frequency change was observed across a subset of candidate loci in populations across both ocean basins, indicating a partially shared genomic basis across sites. Some candidate loci have been implicated in life history divergence in other plant systems. Importantly, several populations in both Oceans did not show significant divergence between annual and perennial plants, suggesting either environmental control for this life history or very recent divergence. Together, these results suggest that annual and perennial life histories have evolved repeatedly across ocean basins, with parallel genomic divergence within the Pacific that likely originated from shared standing variation, and a

transition toward more independent genomic changes between annual and perennial plants in the Atlantic.

3.2. INTRODUCTION

Transitions between annual and perennial life histories have repeatedly arisen across plant lineages (Friedman and Rubin, 2015; Hjertaas et al., 2022), with important consequences for individual fitness and population persistence (Vico et al., 2016). Annual and perennial strategies are commonly characterized as endpoints along a continuum of life expectancy that describes the distribution of reproductive investment among seasons (Friedman, 2020). Annuals concentrate reproductive effort into a single episode of flowering before senescence, whereas perennials may persist and reproduce over multiple seasons (Friedman and Rubin, 2015). The relative advantage of either strategy depends on the tradeoff between immediate reproduction and survival to future reproductive opportunities (Vico et al., 2016; Friedman, 2020), which is often associated with contrasting environments. Annual plant species are more common in unpredictable or highly disturbed habitats and perennial species often dominate where conditions are favorable for long-term persistence (Friedman, 2020; Poppenwimer, Mayrose and DeMalach, 2023). Shifts between strategies are often accompanied by coordinated changes in morphology, phenology, and reproductive mode, which are partly modulated by genetic variation linked to flowering time and vegetative growth (Remington et al., 2013; Friedman et al., 2015; Vico et al., 2016; Hjertaas et al., 2022). The repeated evolution of annual and perennial strategies across independent plant lineages suggests that selection repeatedly favors similar phenotypic solutions (Friedman, 2020; James et al., 2023). However, the outcomes of repeated evolution may vary even under similar selection pressures (Bolnick et al., 2018). Although life history variation is extensively documented among plant species, intraspecific annual and perennial populations have only been reported in few angiosperms (Evans et al., 2005; Duncan and Williams, 2020; van Katwijk and van Tussenbroek, 2023). Plant species with conspecific annual and perennial divergence provide an opportunity to examine the role of evolutionary processes on shaping the repeatability, and thus predictability, of life history evolution (Bolnick et al., 2018; James, Arenas-Castro, et al., 2021).

The repeated evolution of similar phenotypes may arise through substantially different genetic mechanisms across independent lineages (James et al., 2023). At the molecular level, the umbrella term “replicated evolution” is used to refer to both “convergent evolution”, when similar phenotypes repeatedly arise through different genetic changes, and “parallel evolution”,

when similar phenotypes arise from the same nucleotide variants (Bolnick et al., 2018; Cerca, 2023; James et al., 2023). There exists debate regarding the framework applied to distinguish modes of repeated evolution that result in similar phenotypic outcomes. The evolution of similar phenotypes from recent common ancestry has been typically classified as parallel. However, all lineages share recent ancestry within a single species, blurring the threshold used to classify evolutionary mode based on evolutionary relatedness alone. Replicated evolution may manifest at multiple levels of molecular organization: the same nucleotide, different mutations in the same gene, different genes involved in the same biological function, or different biological functions resulting in equivalent phenotypes (James et al., 2023). The likelihood of evolutionary replication across these levels is shaped by the genetic architecture of traits, time since divergence, amount of standing variation, and the degree of connectivity among populations (Bolnick et al., 2018). Closely related lineages that share more recent evolutionary divergence, and those connected by gene flow, are more likely to reuse the same polymorphisms when adapting to similar environments (Stern, 2013). The probability of genetic parallelism further increases when the trait is governed by few large-effect loci that may exhibit stronger responses to selection; for example, in *Mimulus guttatus* and *Arabidopsis lyrata*, where a small number of QTLs affecting both flowering time and vegetative growth contribute to annual-perennial divergence (Remington et al., 2013; Friedman et al., 2015). In contrast, lineages with deeper divergence may evolve convergent solutions through independent mutations, particularly when the genetic architecture of the trait is explained by many loci of small effect, or when the genetic architecture of the trait is redundant (Stern, 2013; Lee and Coop, 2017). Determining the level at which replication operates is therefore informative about both the constraints shaping adaptation and the evolutionary history of the lineages involved.

The seagrass *Zostera marina*, commonly known as eelgrass, is a well-suited system for investigating replicated evolution of life history across geographic and temporal scales within a species. The species likely originated in the Northwestern Pacific nearly 420,000 years ago (420 kya) (Yu et al., 2023) and expanded eastward to the western coast of North America through two dispersal events: first, a southern colonization near 30° N around 350 kya, and a second northern one near 45° N around 270 kya. An additional trans-Arctic dispersal around 243 kya led to the colonization of the Atlantic, where populations diversified across a much shorter timescale (<60 kya) (Yu et al., 2023). Contemporary eelgrass is predominantly perennial across this distribution, maintaining persistent biomass across seasons through clonal propagation via rhizome growth (Olesen, 1999; van Katwijk and van Tussenbroek, 2023). However, annual populations that complete their full life cycle within a single growing season have been

documented as occurring sparsely throughout the range (Keddy and Patriquin, 1978; Phillips and Backman, 1983; Robertson and Mann, 1984; van Katwijk and van Tussenbroek, 2023; Briones Ortiz et al., 2025; Nolan et al., 2026). Consistent with general patterns in angiosperms, annual eelgrass tends to occur in frequently disturbed environments, such as in the presence of ice scour, heat stress, or light limitation (Keddy and Patriquin, 1978; Phillips and Backman, 1983; Robertson and Mann, 1984; Kim et al., 2014). In such conditions, elevated seed production in annual plants may offer an avenue to escape unfavorable habitats for vegetative survival (Källström et al., 2008; Vercaemer et al., 2021). Although annual types in eelgrass and a few other seagrasses have been considered facultative states driven by the environment (van Katwijk and van Tussenbroek, 2023), recent evidence from one estuary (Willapa Bay, Northeastern Pacific) provided support for a genetic basis underlying lifespan variation for at least part of the range (Briones Ortiz et al., 2025). However, annual and perennial beds co-occurring in close spatial proximity (e.g. within a bay) show substantially lower genetic differentiation than meadows located in geographically isolated sites (e.g. the Pacific and Atlantic oceans) (van Katwijk and van Tussenbroek, 2023), which suggests that life history variation in eelgrass has repeatedly evolved across distant locations. Whether annual and perennial strategies represent truly replicated evolution of life history variation, and if so, whether the genomic basis for life history evolution is shared across sites, remains unexplored.

Our aim is to determine whether replicated evolution of annual and perennial life history strategies has occurred in *Zostera marina*, and whether such replication can be attributed to parallel molecular processes across populations. First, we characterize the evolutionary relationships among annual and perennial meadows across the Pacific and Atlantic ranges of the species using phylogenetic approaches based on nuclear and organellar genomes. We ask whether evolutionary relationships are driven by life history variation or geography to determine whether annual and perennial strategies have arisen once or multiple times. A single origin would be supported if life history types share a more recent common ancestor with each other than with the alternative type regardless of geography; multiple origins if annual and perennial individuals from the same location group together more closely than individuals of the same life history from different locations. Second, we describe hierarchical population structure to characterize genetic differentiation among annual and perennial samples. We examine the extent to which genome-wide variation is shared between life histories across and within locations using a multivariate analysis, Bayesian population assignment, and tests of shared drift among subsets of populations. Last, we examine whether there is a genomic basis for repeated parallel evolution between annual and perennial life histories across sites and evaluate

the organizational level where replication might occur. Repeated life history divergence may be driven in parallel by the same DNA loci across locations, or convergently through distinct genetic variants producing similar phenotypes independently at each location. Here, we identify candidate loci underlying annual and perennial differentiation using a Bayesian outlier detection approach, characterize the repeatability of outlier loci variation across life history pairs, and assess the genomic location and molecular context of candidate outliers. Together, these objectives address whether annual and perennial types in eelgrass have repeatedly evolved across a large part of the species' range, and whether such replication extends to the genomic level.

3.3. METHODS

3.3.1. Study System and Sample Collection

We sampled pairs of annual and perennial eelgrass meadows co-located within ~1 Km from each other in ten locations that spanned the Pacific and Atlantic ranges of *Zostera marina* [Table 1] [Fig. 1]. This sampling strategy aimed to capture the colonization history across the species distribution. Five sites were along the North American Pacific coastline, two on the North American Atlantic coast, and three in Europe [Fig.1]. Two locations (Middle Marsh in North Carolina, USA and Oosterschelde in the Netherlands) contained only annual meadows and were included to broaden the representation of annual diversity across the range.

A total of 72 eelgrass plants were sampled for genotyping. For each site, four individuals were included per available life history. Plants were identified based on morphology and advice from local biologists: annuals germinate from seed and flower within a season, and were distinguished by a scorpioid rhizome and the presence of flowering structures. Perennials were identified by the presence of thick underground rhizomes connecting to at least one other vegetative ramet, neither of which displayed signals of sexual reproduction through flowering at the time of sampling. We collected sheath meristems when possible because this tissue contains the least amount of photosynthetic compounds that may interfere with downstream molecular work. The whole shoot was sampled for annual plants that were too small for targeted tissue collection. Samples were subjected to a dark treatment of up to 72 hours at ~8°C to further minimize photosynthetic contaminants and subsequently stored in silica or at -80°C until further processing.

3.3.2. Laboratory Processing and Sequencing

DNA extraction was conducted using the Qiagen DNeasy Plant Pro kit following the manufacturer's recommendations. Genomic libraries were prepared for whole genome sequencing using the Illumina Nextera XT DNA Library Preparation Kit (Lou et al., 2021). Sequencing was carried out on a 10B NovaSeq X Plus lane (Genewiz, Azenta) with 150 bp paired-end settings. The quality of the raw demultiplexed reads was assessed using FastQC (Babraham-Bioinformatics, 2015). Adapter sequences and low-quality base calls with a Phred score below 30 (99.9% accuracy) in the leading and trailing ends of the reads were removed using Trimmomatic (Bolger, Lohse and Usadel, 2014).

3.3.3. Alignment and Genotyping

Trimmed reads were aligned to the *Zostera marina* v3.1 genomic reference derived from a plant from Finland (Ma et al., 2021), and to the chloroplast (RefSeq: NC_036014.1) (Xing and Guo, 2017) and mitochondria (RefSeq: NC_035345.1) (Petersen et al., 2017) organellar references using BWA-MEM with minimum alignment and mapping Phred quality scores of 30 (Li, 2013).

For phylogenetic inference (the first objective), reads were aligned to coding sequences (CDS; n = 19,810 loci across the six main chromosomes in the reference genome) because their conserved structure facilitates reliable alignment given extensive differentiation across the species range (Yu et al., 2023). Consensus sequences for each CDS were generated using iVar (Grubaugh et al., 2018) based on read depth and the frequency of alternate allele reads at each base pair position. A minimum read depth of 15 was required to call a base (those below this depth were set to ambiguity nucleotide "N"). A consensus base was called when a single nucleotide accounted for ≥70% of reads at that position; otherwise, the site was assigned an IUPAC ambiguity character reflecting the alternative nucleotides present. Samples with missing data at more than 60% of base pair positions within a given CDS were excluded from analysis of that locus. Zostera japonica CDS sequences (n = 615) were included as an outgroup to root the genomic phylogenetic inference of gene trees when the outgroup was scored at that locus (Yu et al., 2023).

For the second and third objectives (population structure and genomic evidence for parallelism), reads were aligned against the reference genome for *Z. marina* (Ma et al., 2021) to capture genome-wide variation suitable for population genetic approaches. Genotyping was conducted using GATK4 (Van Der Auwera and O'Connor, 2020). For each sample, read groups were assigned with AddOrReplaceReadGroups, PCR duplicates were marked with

MarkDuplicatesSpark, and genotypes were called with HaplotypeCaller in GVCF mode. Genotyping of SNPs across samples was performed using CombineGVCFs and GenotypeGVCFs (Van Der Auwera and O'Connor, 2020) for all samples and separately for Pacific and Atlantic site subsets because the deep divergence between clades reduced the power for detecting fine scale patterns of differentiation within the different ocean clades.

We retained high-confidence genotype calls by applying a series of SNP filters across all three datasets using SNPfiltr in R (DeRaad, 2022). Individual genotype calls were designated as missing if read depth was below 15 and above twice the mean read depth, or if allelic balance was outside the range of 0.2 to 0.8. Low read depth provides weak support for genotype calls, since few reads may fail to capture both alleles at a heterozygote sites, whereas high read depth could indicate the presence of paralogs or repeat regions mapping onto a single position. Deviations in allelic balance from the stipulated range could mean that heterozygote SNPs may have resulted from contamination, genotyping errors, or repeat mapping. Only those SNPs with zero percent missing data across all individuals were retained. The total number of SNPs were thinned to a minimum physical distance of 3,000 bp to reduce the influence of physical linkage among adjacent loci on downstream population genetic analyses, as non-independent variants can bias estimates of structure and selection through redundant signals among linked loci.

Phylogenetic inference of chloroplast and mitochondria organelles relied on genotypes that were called following the same approach as the nuclear genome, except that HaplotypeCaller in GATK4 was set to a ploidy of 1. Samples with less than 10% missing data across all SNPs were retained.

3.3.4. Phylogenetic Inference

3.3.4.1. Nuclear Phylogenies Based on Coalescence

Within species phylogenetic approaches were used to characterize evolutionary relationships among co-located annual and perennial meadows and to evaluate whether evolutionary relationships were structured by life history or geography. For each CDS, multiple sequence alignment was performed separately using MAFFT with the –localpair algorithm (Katoh and Standley, 2013). Alignments were trimmed to remove gap-rich columns using trimAl with the -automated1 setting (Capella-Gutiérrez, Silla-Martínez and Gabaldón, 2009). Given the recent origin of *Zostera marina* (~ 420 kya) (Yu et al., 2023), incomplete lineage sorting (ILS) was expected to generate phylogenetic discordance among gene trees (Degnan and Rosenberg, 2009). We addressed this potential issue by inferring a species tree under the multispecies coalescent instead of concatenating the sequences (Mirarab et al., 2014). Maximum likelihood

gene trees were constructed for each CDS using IQ-Tree with the automatic model finder selection via ModelFinder and 1000 ultrafast bootstrapping replicates (Minh et al., 2020). We retained gene trees from CDS alignments with a minimum of 30 individuals and three parsimony-informative sites, because loci with little variation may yield poorly resolved gene trees whose estimation may propagate error into the species tree (Zhang and Mirarab, 2022).

Coalescent species trees were estimated from all gene trees that were retained using two complementary quartet-based approaches in ASTER (Allman, Degnan and Rhodes, 2011; Zhang, Nielsen and Mirarab, 2025). Under the multispecies coalescent, the species tree is inferred as the topology that maximizes the number of concordant quartet subtrees across gene trees, which makes this approach robust to gene-tree conflict arising from ILS. ASTRAL-IV weighs all gene trees equally and estimates branch lengths in units of substitutions per site (Mirarab et al., 2014; Tabatabaee et al., 2023), displaying molecular divergence among lineages. In contrast, wASTRAL weighs gene trees by branch support and length to minimize the influence of poorly supported or long-branch gene trees (Zhang and Mirarab, 2022). Internal branch lengths are reported in coalescent units (1 CU = t/2Ne), which are inversely proportional to effective population size (Ne) and reflect the degree of gene tree discordance; t is time in generations. A comparative analysis of the two topologies may identify nodes whose resolution is sensitive to gene tree conflict, while comparisons of branch lengths provide insights into the relationship between accumulation of substitutions and gene-tree conflict.

3.3.4.2. Organellar Haplotype Networks

We constructed chloroplast and mitochondrial genome haplotype networks to visualize genealogical relationships independently of the nuclear genome. Because organellar genomes are uniparentally inherited and non-recombining, they preserve divergence history that may be obscured by recombination and gene flow within the nuclear genome. Thus, organellar DNA provides complementary evidence of evolutionary relationships among samples. Haplotype networks were constructed using the haploNet function in the R package pegas (Paradis, 2010), which uses uncorrected P or Hamming distances under a statistical parsimony model (Templeton, Crandall and Sing, 1992) with pairwise deletion of missing data. Haplotype frequencies for each organelle were separately summarized by site and life history.

3.3.5. Population Structure

3.3.5.1. Multivariate Analyses

We characterized genome-wide patterns of population genetic structure using principal component analysis. This approach was used to provide a model-free visualization of the major

axes of relationships among annual and perennial samples, assuming neither an evolutionary framework or a priori assignment of individuals to populations. Principal component analyses (PCA) of genome-wide variation were conducted using glPca in R (Jombart and Ahmed, 2011) for three datasets: all locations combined, Pacific sites only, and Atlantic sites only. Patterns of divergence were examined across the first six principal components (PCs) to evaluate the relative contributions of the axes in describing life history variation and geographic isolation to structure.

3.3.5.2. Bayesian Population Assignment of Individuals

Hierarchical population structure was examined using a Bayesian population assignment approach (Pritchard, Stephens and Donnelly, 2000) at three organizational levels: across all sampled sites to characterize broad scale structure among locations and life history types, within subsets of populations from the Pacific and Atlantic oceans, and within each geographical location to quantify population admixture between ecotypes. Analyses were conducted using STRUCTURE v2.3 (Pritchard, Stephens and Donnelly, 2000) based on an admixture model with correlated allele frequencies, with a burn-in of 100,000 iterations followed by 250,000 MCMC repetitions. We tested two to ten K genetic clusters for the analysis across all locations; for Pacific and Atlantic location subsets, we separately tested K from two to six. Ten independent runs were performed per K value, and the most likely number of clusters was identified using the Delta-K statistic (Evanno, Regnaut and Goudet, 2005). Within each site, only K = 2 was evaluated to assess the extent of admixture among samples and determine whether life history variation corresponded with distinct genetic groups.

3.3.5.3. Tests of Allele Frequency Covariation Among Subsets of Populations

We evaluated allele frequency covariance among groups of four populations to test finer-scale patterns of admixture identified in the STRUCTURE results (Patterson et al., 2012; Peter, 2016). We use f4 statistics in the form f4(A,B;C,D) to assess whether allele frequency differences between clades (A,B) and (C,D) are consistent with a tree formed by sister clades in the absence of admixture, or whether there is evidence of excess shared alleles between the two clades that is inconsistent with a strictly bifurcating tree model. For each locus, the f4 statistic is computed as the product of allele frequency differences between A and B and between C and D, f4 = (pA-pB)*(pC-pD), averaged across all loci. Under a bifurcating tree model, differences between A and B (A,B) are independent from differences between C and D (C,D), and the product is near zero. Positive estimates indicate that A and C share excess drift relative to the proposed tree (allele frequencies in A and C change in the same direction greater than expectations), and the

same applies to the relationship between B and D. Negative deviations would instead point to shared covariation between A and D, and between B and C. Confidence intervals for all f4 estimates were obtained using a block jackknife over blocks of 500 SNPs and statistical significance was assessed using a Benjamini-Hochberg (BH) false discovery rate below 0.05.

We first investigated whether annual and perennial plants within a site share excess alleles with either life history type at other sites (e.g. A and C, or A and D), because within-site admixture proportions were different between life histories at some locations. We used f4 statistics in the form f4(Outgroup, LifeHistory_1; Annual_2, Perennial_2): f4 = (pOutgroup – pLifeHistory_1)*(pAnnual_2 - pPerennial_2), where the outgroup is phylogenetically distant and is not expected to share recent admixture with the tested lineages. In this form, the outgroup provides a root of the tree, i.e. a reference for allele frequencies pre-dating the divergence among the tested lineages. The direction of the estimate reflects covariation in the allele frequency changes between a tested lineage from site 1 and either life history from site 2. Negative and positive deviations indicate shared excess drift between LifeHistory_1 at site 1 and the annual and perennial types at site 2, respectively, beyond background divergence captured by the outgroup. Estimates near zero instead indicate that LifeHistory_1 does not covary differently with either the annual or the perennial within site 2. We estimated f4 separately for plants in each ocean basin given their deep genetic divergence, and used Machias Bay perennials as the outgroup for Pacific analyses and Padilla Bay perennials for the Atlantic ones. Changing the outgroup to other lineages within the corresponding ocean basins did not result in different outcomes.

Second, because some annual and perennial pairs were genetically differentiated at some locations, we investigated whether co-located life histories pairs were evolving independently. We calculated f4 statistics in the form f4(Annual_1, Perennial_1; Annual_2, Perennial_2): f4 = (pAnnual_1-pPerennial_1)*(pAnnual_2- pPerennial_2), which measures covariance in allele frequency change between annual and perennial pairs across two locations. Under the null of independent divergence within each site, estimates were expected to be near zero. Deviations reject a tree in which annual and perennial lineages form sister clades within each location: positive estimates indicate shared excess drift between plants with the same life history types across sites (parallel genome-wide change), and negative values indicate covariation between opposite life histories (anti-parallel genome-wide change).

3.3.6. Genomic Signatures of Parallel Evolution

All analyses for the third objective were based on two subsets of populations. First, we analyzed all five populations where life-history pairs were divergent, identified in the population assignment tests. Second, we analyzed the four Pacific pairs alone, to determine whether there was evidence for parallel genomic divergence in this clade alone.

3.3.6.1. Principal Component Analysis

We used pcadapt (Luu, Bazin and Blum, 2017) to perform a PCA (K = 6) and determine whether any principal components separated annual and perennial types among sites. We used the "component wise" setting to compute per-PC loadings and test each SNP's loading against a Gaussian null distribution to identify SNPs with unusually large contributions to each axis relative to neutral expectation. Candidate loci on the axes that distinguished ecotypes (PC2 and PC3) were identified by converting pcadapt p-values to q-values and retaining those with FDR < 0.001.

3.3.6.2. Bayesian Outlier Detection

To identify genomic regions that may be indicative of selection, and specifically underlying life history divergence, we conducted *Fst*-based outlier detection analyses based on Bayesian approaches (Foll and Gaggiotti, 2008). Analyses were conducted at two hierarchical levels: first, all annual and perennial samples were pooled separately across groups regardless of location (life history combined analysis), and second, using life histories within each location as separate populations (location-specific analysis). These two separate analyses identified SNPs that are consistently differentiated by life history regardless of location from those that have diverged at specific locations, although we acknowledge reduced sample number and statistical power in the population-specific analyses. Outliers detected in both analyses were treated as the strongest candidates for a shared genetic basis of life history divergence across the sampled range (sensu James, Wilkinson, et al., 2021). Candidate outliers were identified with BayeScan using a false discovery rate below 0.01 (q < 0.01) (Foll and Gaggiotti, 2008).

3.3.6.3. Allele Frequency Change at Candidate Outlier Loci

We aimed to assess whether loci potentially explaining life history variation show repeated change across locations. A parallel genetic basis for life history variation would be reflected in shared allele frequency changes across co-located life history pairs at a given locus, while frequency changes specific to individual locations would suggest that similar phenotypic outcomes have arisen through convergent genomic changes. We calculated the difference in allele frequency change of the alternative allele to the one from the reference genome at each

candidate locus within each annual and perennial pair: Δp = (pA – pP), and evaluated the consistency of the sign across pairs.

3.3.6.4. Genomic Features and Functional Annotation of Candidate Outlier Loci

Candidate outlier loci were annotated using the *Z. marina* v3.1 genomic reference (Ma et al., 2021) to identify their position in introns, exons, or intergenic spaces, to evaluate the functional level at which replicated evolution might have occurred. The putative functional roles of genes harboring candidate outlier SNPs were assessed in Phytozome (Goodstein et al., 2012) to identify biological processes relevant to divergence between annual and perennial pairs.

3.4. RESULTS

3.4.1. Sequencing and Genotyping

A total of 71 individuals were successfully sequenced and genotyped for analyses. Alignment of the trimmed reads to the *Z. marina* CDS loci identified 18,572 loci; however, only 10,422 gene trees remained after filtering for phylogenetically informative loci based on parsimony-informative sites and number of taxa present.

Genotyping of genome-wide variation across all locations resulted in 7,552,441 genotyped SNPs, of which 21,559 remained following variant quality filtering [Table S1]. Mean read depth across all loci was 39.15x post filtering.

3.4.2. Phylogenetic Inference

3.4.2.1. Nuclear Phylogenies Based on Coalescence

Phylogenetic inference suggested multiple evolutionary transitions between annual and perennial strategies, rather than support for a single shared origin. Both nuclear phylogenies from ASTRAL-IV and wASTRAL revealed strong geographic structuring that separated samples into two well-supported clades corresponding to the Pacific and Atlantic basins regardless of life history [Fig. 2]. Phylogenies based on ASTRAL-IV (branch lengths indicate accumulation of DNA substitutions) and wASTRAL (branch lengths reflect the degree of gene tree discordance from ILS) [Fig. 2] revealed different relationships between individuals between ocean basins. Comparisons of branch lengths for samples from the Pacific Ocean between the two phylogenies show that long substitutions-per-site branches in the former phylogeny corresponded with short coalescent-unit branches in the latter, which indicates high sequence divergence accumulated among Pacific lineages alongside high gene-tree discordance. Within the Atlantic, shorter

substitution-per-site branches indicate lower sequence divergence compared to the Pacific; however, these were accompanied by longer internal and shorter shallower coalescent-unit branches. The San Francisco clade was a notable exception, where both metrics revealed long internal branches, which indicate low gene tree discordance together with high sequence divergence, and likely point to an isolated lineage and a well-differentiated ancestral group [Fig. 2]. Overall, comparison between branch across the trees revealed that molecular divergence tracked colonization history. Greater substitution accumulation occurred in older Pacific sites, while gene tree discordance was prevalent among more recent divergence events that likely reflect incomplete lineage sorting from large effective population sizes in the Pacific and additional limited evolutionary divergence time in the Atlantic (Yu et al., 2023).

We found evidence of repeated life history evolution in both phylogenies, but with different levels of support. When accounting for all gene trees in the ASTRAL-IV tree [Fig. 2A], geography had a clear role in shaping the observed phylogenetic relationships among samples, with only weak separation by life history. Samples grouped into clades corresponding to the Pacific and Atlantic oceans regardless of life history, which reflects colonization history and suggests separate evolution of annual and perennial strategies within each clade. Within the Pacific, plants from the outer Washington Coast (Willapa Bay and Grays Harbor) formed well-supported subgroups in which annual plants from both locations were more related to each other than to their respective co-located perennials. Padilla Bay annuals formed a sister clade to the Washington outer coast group, whereas the perennials from this location shared recent common ancestors with other locations or with the pacific clade as a whole. Similarly, Yaquina Bay annuals shared a common ancestor with the outer coast annuals and perennials, whereas the perennial plants from this location shared more basal divergence events. Overall, the phylogeny suggests that annual plants from Padilla and Yaquina Bays share a more recent common ancestor to annual and perennial plants on the outer Washington coast compared to their co-located perennials. Notably, all annual plants across these sites displayed longer substitutions-per-site branches than their perennial counterparts, which we interpret to mean that DNA sequences from annual plants were more divergent from the common ancestors shared with co-located perennials at each site. Plants from San Francisco Bay formed a distinct subgroup with no clear differentiation between life histories. Within the Atlantic ocean, separate European and North American clades were consistent with their previously described colonization history (Yu et al., 2023). North American clades had shorter substitutions-per-site branch lengths than those in Europe, indicating that western Atlantic lineages have accumulated

fewer substitutions and their DNA sequences are less divergent than those from Europe. There was no clear differentiation by site or life history within either Atlantic group.

Downweighing of low-support and long-branch gene trees in the wASTRAL topology [Fig. 2B] resulted in phylogenetic relationships primarily shaped by geography between and within ocean basins, a result shared with the ASTRAL-IV tree [Fig. 2A]. However, wASTRAL revealed clearer separation by life history within several sites, which we attribute to its downweighing of gene trees with low phylogenetic support and long branches that may introduce phylogenetic conflict into the species tree estimation. Within the Pacific Ocean, annual and perennial plants formed separate monophyletic groups in Willapa Bay and Grays Harbor as in the previous topology. Both Yaquina and Padilla Bay individuals formed monophyletic clades by location and subsequently separated into annual and perennial groups within each. At Yaquina Bay, the internal branch lengths separating the ecotypes were short, indicating high incomplete lineage sorting between life histories. In contrast, longer internal coalescent-unit branches separating the co-located ecotypes in Padilla Bay indicate low gene tree discordance and greater divergence between life histories within this location. As in the first phylogeny, San Francisco samples also showed no separation between annual and perennial plants. Within the Atlantic, the separation between Europe and North America was more clearly resolved and supported by longer branch lengths in coalescent units, which is consistent with lower levels of gene-tree conflict at the deeper regional level. Shallower relationships displayed shorter branch lengths instead, and thus, reflect higher gene-tree conflict. Samples from the two sites in Germany did not separate between locations or life history, while the Netherlands annuals formed a monophyletic group that likely reflects geographic isolation. Along the North American coast, long coalescent-unit branch lengths separated samples from Maine and North Carolina, suggesting two well-defined and divergent lineages. Within Machias Bay, an additional separation occurred by life history, with longer coalescent-unit branch lengths in the perennials compared to the annuals, which instead suggests that annuals at this site are more recently derived from the isolated local perennial population.

3.4.2.2. Organellar Haplotype Networks

Evolutionary relationships were largely concordant between both organellar haplotype networks [Fig. 3], which also supported multiple origins of life history variation as well as historical events that shaped contemporary distribution (Yu et al., 2023).

The chloroplast network [Fig. 3A] revealed a total of 33 haplotypes that were distributed across three distinct lineages corresponding to the older San Francisco Bay clade with a subset

of Yaquina Bay plants, and the more recent north Pacific sites followed by all individuals from the Atlantic Ocean. The northern Pacific and San Francisco-dominant haplogroups were separated from the Atlantic lineage by 26 and 25 mutational steps, respectively, with both life histories occurring within each haplogroup. The placement of some Yaquina Bay annuals within the lineage containing the San Francisco plants suggests that Yaquina Bay is part of the hypothesized secondary contact zone between the two trans-Pacific colonization events (Yu et al., 2023).

The mitochondrial haplotype network [Fig. 3B] was based on fewer haplotypes (n = 17), where clades agreed with the three major lineages identified in the chloroplast network. However, the distance between the clades in the Atlantic to the one with San Francisco was only two mutational steps compared to 19 between the Atlantic and the other Pacific haplogroup. Such discordance in mutational steps between haplogroups compared to those in the chloroplast network may suggest differences in mutation rates or ILS acting independently on the different organellar genomes.

3.4.3. Population Structure

3.4.3.1. Multivariate Analyses

Genomic variation among samples predominantly reflected a geographic signal, as well as further support for multiple origins of life history diversity. In the PCA including all locations [Fig. 4A], the analyses recognized the three lineages described by the phylogenetic analyses. The first principal component (PC1; 69% of the variation explained) separated the Pacific and Atlantic ocean basins, with nearly complete overlap of sites and life histories within each of them. The second axis (PC2; 6.59%) primarily separated San Francisco Bay plants from the remaining Pacific sites, with a secondary separation between Washington and Oregon plants.

Finer scale analysis of populations within the Pacific Ocean [Fig. 4B] primarily separated sites along the coastline. The first axis (PC1; 26%), discriminated San Francisco samples from all other Pacific locations, with Yaquina Bay samples intermediate between this and all three northern sites. The second axis (PC2; 10%) separated Padilla Bay from the remaining populations in Washington State, despite being the northernmost population, and revealed a life history signal separating annuals and perennials within this site.

Within the Atlantic Ocean, the primary axis of genome-wide variation (PC1; 39%) largely separated North American from European locations, with some evidence of separation between

sites within Europe [Fig. 4C]. The second axis (PC2; 10%) reflected divergence among North American sites.

3.4.3.2. Bayesian Population Assignment of Individuals

Bayesian population assignment across all three datasets revealed strong geographic separation, with weak life history signals emerging at higher K values. In general, admixture proportions were structured hierarchically across and within ocean basins, which supports evidence for the colonization history of the species (Yu et al., 2023) as well as repeated evolution of life history strategies. The best-supported number of genetic populations (K) over the entire dataset was two [Fig. 5A], which corresponded to the two ocean basins with no evidence for admixture between Pacific and Atlantic samples.

Within the Pacific Ocean, K = 2 separated samples between San Francisco Bay and all remaining sites, with some evidence for gene flow from San Francisco to Yaquina Bay individuals [Fig. 5B]. The best supported number of populations was at K = 3, where San Francisco individuals were assigned to one population, Willapa Bay and Grays Harbor plants were assigned to a second population, and plants in Yaquina Bay shared admixture proportions with both the outer Washington coast and the San Francisco groups. Interestingly, perennial plants at Padilla Bay were assigned to a third group exclusively, while annual plants showed evidence of admixture between this third group and from the population representing the outer coast individuals, supporting results shown in the first phylogeny [Fig 2A]. At K = 4, the previous three assignments were retained, but Yaquina Bay individuals were largely assigned to a fourth cluster.

Within the Atlantic Ocean, the best supported number of populations at K=2 revealed two populations, where plants from North American versus European coasts assigned to different groups [Fig. 5C]. At K = 3, plants from Maine were additionally assigned to a third cluster, with some evidence for admixture in the North Carolina population, which comprised annuals exclusively. No additional geographic structure between samples was detected at higher K values.

STRUCTURE analyses by individual sites (n = 8) revealed variable degrees of admixture between annual and perennial plants across locations [Fig. 6A]. At five sites, annual and perennial plants were assigned to two separate groups: Grays Harbor, Willapa Bay, Yaquina Bay, Padilla Bay, and Machias Bay. In contrast, plants at San Francisco, Sylt, and Hallig Hooge had nearly equal admixture proportions across all individuals within each site, indicating no detectable differentiation between annual and perennial plants.

3.4.3.3. Tests of Allele Frequency Covariation Among Subsets of Populations

We tested whether a life history from one site (Site 1) shared different levels of drift with either life history types from another target site (Site 2) using f4-statistics in the form f4(Outgroup, LifeHistory_1; Annual_2, Perennial_2) [Fig. 5D]. In general, a pattern of shared excess alleles by life history emerged across all the tested groups from the Pacific (LifeHistory_1). Annual lineages showed more negative f4 estimates than their co-located perennials, indicating higher shared covariation among annual lineages across the Pacific. With Grays Harbor, Willapa Bay, and Yaquina Bay as target sites (Site 2), perennial lineages shared more drift with the perennial target, while annual lineages did so with the annual targets. With Padilla Bay as the target (Site 2), all but one of the tested Pacific lineages displayed significant negative estimates, indicating that all these populations share excess drift with annual plants in Padilla Bay relative to the co-located perennials, and suggesting that these perennials represent a highly divergent lineage within the Pacific. Although not all estimates were significant, we found that allele frequency covariation between sites tracks life history type rather than geography alone, particularly among annual lineages in the Pacific Ocean. Within the Atlantic, no significant f4 estimate was detected between Machias Bay as a target site and the remaining Atlantic groups as tested lineages.

We also tested whether genetically differentiated life history pairs across sites have evolved independently using an f4-statistics in the form (Annual_1, Perennial_1; Annual_2, Perennial_2) [Fig. 6B]. The results revealed high covariance of allele frequency differences between annual and perennial plants among a subset of sites, indicating similar genomic change between life histories across some locations. Positive and significant deviations from zero were detected for comparisons among Padilla Bay, Grays Harbor, Willapa Bay, and Yaquina Bay, all in the Pacific Ocean, which may be indicative of shared standing variation contributing to parallel divergence between life histories across the Pacific. The remaining comparisons, those including the Atlantic pair in Machias Bay, showed f4 values close to zero, indicating independent allele frequency change between life histories across ocean basins.

3.4.4. Evidence for a genetic basis underlying parallel evolution

3.4.4.1. Principal Component Analyses

Analyses of all five populations with differentiated life history pairs revealed no single axis that explained life history type [Figure S1]. In contrast, PCA analyses of the Pacific Ocean populations identified consistent loading of life history type onto some axes. While the first axis differentiated sites geographically along the outer coastline, separation by life history occurred

in Padilla Bay, with annuals positioned nearer the other sites compared to the perennials. The second axis (PC2; 26%) differentiated Yaquina Bay from the rest of the Pacific; in addition, annual individuals from Washington locations diverged in the same direction from each other, but in opposite direction to their co-located perennials, suggesting shared variation by life history across these three sites. The third axis (PC3; 15%) showed a clearer directional divergence between life histories, with perennials and annuals consistently positioned on opposite sides of the axis for all sites. The PCs 4 through 6 (<10%) displayed further differentiation between life histories within individual locations; however, the direction of divergence was not consistent across sites. Annuals and perennials shifted in opposite directions at different locations, indicating a degree of variation in the genomic basis of life history differentiation in the Pacific Ocean.

Life history-associated loci in the Pacific Ocean (PC2 and PC3) were distributed widely across all six chromosomes [Fig. 7B]. Chromosomes 3 and 5 had a higher number. A total of 1,214 and 479 SNPs loaded significantly onto the second and third axes of variation, of which 187 were shared between axes.

3.4.4.2. Candidate outlier loci detection

The detection of candidate outlier loci underlying annual and perennial variation varied substantially based on the subset of sites being compared. Analyses of all five differentiated life history pairs [Fig. S2] identified 41 and 196 outlier candidates in the combined and population-specific detection approaches, respectively. Only four candidate outlier SNPs overlapped across both detection approaches. The substantially higher number of outliers detected in the population-specific approach suggests that most of the differentiation detected is specific to individual locations rather than shared across pairs at different sites.

The separate analysis that excluded plants from Maine in the Atlantic Ocean identified a total of 19 candidate outlier SNPs [Fig. 8A]. The combined and population-specific detection approaches yielded 46 and 428 outlier candidate SNPs, respectively [Fig. S3]. These loci were concentrated on chromosomes three (n=8) and five (n=10), with one locus on chromosome four.

3.4.4.3. Allele Frequency Change at Candidate Outlier Loci

Directional change in outlier allele frequencies between annual and perennial plants [Fig. 8B] was both shared and unique across populations. These results suggest that there is evidence for parallel evolution at some loci, but not others. In the dataset containing all five differentiated sites, allele frequency change was shared across three (n = 1 locus) and four (n = 3

loci) sites in the Pacific Ocean. These shared directional changes were not observed at the Maine site in the Atlantic Ocean [Fig. S2]. However, a subset of loci in the population-specific detection approach had shared allele frequency change across some sites that included the pair from Maine, which suggests a potential shared genetic basis for life history differentiation between the Pacific and the Atlantic clades.

Within the dataset containing the four Pacific pairs, allele frequency changes at candidate outlier loci were more consistent across sites [Fig. 8B]. Twelve candidate outlier loci showed allele frequency change in the same direction across all four pairs in the Pacific, indicating repeated genomic divergence between life histories across the region. The remaining seven candidate outliers showed directional consistency across only three sites, which instead suggests additional partially parallel change at the genomic level.

3.4.4.4. Genomic Position and Functional Context of Candidate Outlier Loci

Differentiation between annual and perennial plants occurred in both coding and non-coding variation. Outlier SNPs found in the differentiated life history pairs in the Pacific Ocean were distributed across intergenic (n=9), intronic (n=4), and coding (n=6) regions [Fig. 8C] [Table 2]. Loci with parallel change in allele frequencies between life histories across all Pacific Ocean populations occurred across all three genomic feature classes, which indicates that both coding and non-coding variation differ repeatedly between life histories across sites.

Functional annotation broadly involved developmental timing and reproductive investment pathways relevant to life history divergence [Table 3]. A total of ten outlier loci mapped directly onto gene annotations, with some loci falling within the same gene. Among the candidate loci involved in life history divergence and showing parallel allele frequency change across all four Pacific pairs, Zosma03g23470 is associated with lipid transfer and seed storage; Zosma03g21210 encodes an Armadillo repeat protein involved in auxin signaling and protein-protein interactions; and Zosma05g10780 encodes a GRAS transcription factor of the SCARECROW-LIKE subfamily implicated in gibberellin signaling and meristem identity.

3.5.DISCUSSION

We investigated whether there was replicated evolution in annual and perennial life history strategies in eelgrass (*Zostera marina*) across the eastern Pacific Ocean and the Atlantic using phylogenetic and population genetic approaches. We also investigated whether there was evidence for repeated evolution at the genomic level. Phylogenetic inference revealed support

for multiple transitions between annual and perennial strategies in the species. Evolutionary relationships across the nuclear phylogenies were driven primarily by geography, with a major separation occurring between Pacific and Atlantic Ocean samples, and additional regional subdivisions within each ocean basin. Annual and Perennial life history strategies evolved independently within each clade. Clear separation between life histories was observed within three clades within the Pacific Ocean and one clade in the Atlantic Ocean sites, but only when gene tree discordance was minimized. Organellar haplotype networks were mutually concordant and showed geographic structuring across three major lineages, with evidence for secondary between two previously described transpacific colonization events at an eastern Pacific site that was intermediate between southern and northern locations. Population inference using admixture analyses supported these findings - individuals were assigned to two major groupings defined by ocean basins, with evidence of south to north structure in the eastern Pacific, and western to eastern separation in the Atlantic Ocean. Fine scale analyses on individual populations with both life histories identified five locations (four in the Pacific and one in the Atlantic) where life histories constituted distinct groups. Tests of shared drift based on allele frequency covariance within these five groups indicated disproportionate shared drift between life histories across the sites, and parallel genomic divergence between the genetically distinct life histories across Pacific sites, but not for the Atlantic population. Outlier analyses detected loci with parallel allele frequency change across multiple life history pairs in both Pacific and Atlantic basins, indicating some genomic evidence for parallel evolution underlying life history divergence at the nucleotide level. These candidate loci have been implicated in developmental timing and hormonal signaling pathways, such as gibberellin and auxin signaling, which are consistent with known genetic regulators of life history divergence in other plant systems (Friedman, 2020).

The distribution of life history variation in *Zostera marina* across the phylogeny and across populations does not support a single origin for annual and perennial strategies. We found high phylogenetic discordance among gene trees, which is expected under ILS and is likely explained by evolutionarily recent colonization events across the species range (Yu et al., 2023), and ongoing segregation of mutations within the species. Because branch lengths in coalescent units in the wASTRAL tree [Fig. 2B] scale with time (t) and inversely with effective population size (Ne) (1 CU = t/2Ne) (Zhang and Mirarab, 2022), short branches can arise either from large Ne or from recent divergence. The eastern Pacific Ocean was colonized twice, and there is some evidence of contact between lineages arising from these two events (Yu et al., 2023). The greater branch length between the southern San Francisco samples and the other

Pacific populations may reflect the greater time since divergence. On the other hand, the shorter coalescent branch lengths in the northern populations might reflect larger historical population sizes and recent times since divergence (Talbot et al., 2016; Yu et al., 2023), despite high divergence across these regions in population genetic studies.

In the Atlantic, bottlenecks during the more recent transarctic colonization event, combined with subsequent contractions through glacial cycles may have depressed Ne (Olsen et al., 2004; Campanella et al., 2010; Yu et al., 2023). These factors may explain the longer coalescent-unit branches and high quartet support recovered for earlier separations between American and European samples, and for those along the Eastern North American range. Within these coasts, shorter coalescent-unit branches in shallower relationships are likely attributed to recent divergence <60 kya, which instead reduces the time window for lineage sorting. Notably, the deeper divergence between ocean basins was concordant between both nuclear phylogenies, and robust to ILS compared to shallower divergent clades. Such consistent separation across all analytical approaches and marker types indicates strong divergence between eelgrass in the Pacific and the Atlantic, where annual and perennial strategies may have evolved independently within each basin.

There was significant discordance in the placement of annual and perennial plants in the phylogeny based on gene trees with low support and long branches (ASTRAL-IV) versus the phylogeny that downweighed these trees (wASTRAL) [Fig. 2]. For example, within the eastern Pacific Ocean, the differences in per-location placement of the two life histories relative to the common ancestor may indicate different processes that generate gene-tree conflict. In the first tree, Padilla Bay perennial plants were not sister taxa to the northern Grays/Willapa group compared to the wASTRAL tree and the life histories for the central coast Yaquina Bay were separated across distantly related clades. Across both topologies, there was considerable phylogenetic discordance among gene trees, particularly in the more recent nodes, although wASTRAL was better at resolving the deeper divergences. Possible explanations include incomplete lineage sorting following rapid radiation, or large effective population sizes in the common ancestor (Talbot et al., 2016). Within the Pacific Ocean, annual lineages at four sites also displayed longer branches in substitutions per site than their co-located perennials which may be explained by biological differences. First, generation time is estimated at close to three years for perennial eelgrass (Yu et al., 2023), compared to a single generation for annual plants. Over a similar timeframe, annual lineages therefore undergo more reproductive events, increasing the number of rounds of replication and meiosis in which mutations can arise (Smith and Donoghue, 2008). Second, because annual beds are patchy relative to continuous perennial

meadows (van Katwijk and van Tussenbroek, 2023), effective population sizes in annual lineages are likely smaller, where drift could accelerate the fixation of variants. In addition, the placement of some Yaquina Bay annuals across the two major Pacific organellar haplogroups provides evidence of historical connectivity between separate lineages that colonized the North American coast (Yu et al., 2023), which indicates historical gene flow among Pacific sites that may result in conflict between gene trees.

Evidence for parallel shifts in allele frequency at outlier loci among a subset of co-located annual and perennial plants along the Pacific coast points to some gene reuse rather than evolution of repeated phenotypes through independent variation. Thus, life history divergence in the Pacific likely reflects a partially shared genomic basis, which may follow collateral evolution (Stern, 2013) across sites drawing on a shared but incompletely distributed pool of standing variation. Because these sites are connected by gene flow and share recent common ancestors (see Chapter 1), the same variants may have been distributed among locations rather than originated independently within each. Interestingly, annual lineages consistently showed higher covariation in allele frequencies with one another than their co-located perennials did, which indicates that annual lineages share more similar levels of drift across locations while perennial lineages, particularly at Padilla Bay, have drifted more independently. This pattern may be explained by the reproductive biology of annual eelgrass, which has a higher investment in sexual reproduction compared to perennial plants; for example, annual plants may produce up to five times higher number of seeds than perennial shoots (van Katwijk and van Tussenbroek, 2023). Although eelgrass seeds typically disperse within neighboring meadows (Ruckelshaus, 1996), detached reproductive shoots can raft over much longer distances (Källström et al., 2008). While perennial shoots may also detach and drift with currents, they would only contribute to gene flow following successful reattachment and persistence to reproduction, or if flowering shoots were among the rafting individuals. Further, the lack of suitable habitats throughout the distribution of *Z. marina* (for example, along the outer Washington Coast (Christiaen et al., 2022)) may restrict the long-term establishment of perennial meadows while allowing the temporal persistence of patchy annual beds. Together, these processes could result in higher connectivity across annual meadows along the Pacific coastline and provide the variants necessary for repeated evolution of life history types through shared genetic variation rather than originating through independent mutations within different locations (Stern, 2013). Importantly, many SNPs were unique to ecotype divergence within individual locations, indicating that shared loci account for only part of the genomic basis of life

history differentiation. The detectability of shared loci was likely further limited by small sample sizes and SNP thinning during genotype filtering.

Repeated change across pairs at outlier SNP loci within coding sequences suggests that selection is acting in parallel on functional variation. Most notably, one candidate (Zosma05g10780) encodes a GRAS-family transcription factor of the SCARECROW-LIKE subfamily, a component of the gibberellin–DELLA signaling module that regulates germination in eelgrass seedlings (Pieraccini et al., 2025) and has been implicated in vegetative growth and the transition to flowering in other plants (Yamaguchi et al., 2014; Arro et al., 2019). An additional candidate, Zosma03g23470, encodes a bifunctional inhibitor/lipid transfer protein (Salminen, Blomqvist and Edqvist, 2016), which may be linked to the elevated seed production and reproductive investment characteristic of annual eelgrass. Notably, repeated changes across outlier loci also extended to non-coding DNA regions. Both intergenic and intronic spaces can modulate gene expression in plants (Rose, 2008; Friedman, 2020), and regulatory variation underlies annual-perennial divergence in other taxa (Kiefer et al., 2017). However, such non-coding changes may also represent linked variation hitchhiking with nearby coding loci under selection (Smith and Haigh, 1974). It is possible then that selection has repeatedly acted across multiple levels of molecular organization among distant co-located life history pairs.

Divergence between annual and perennial plants at Machias Bay in the western Atlantic Ocean did not match the parallel genomic signal of divergence recovered among the Pacific populations, indicating the potential for an independent genomic basis of life history divergence across ocean basins. Much of the shared ancestral polymorphisms within Pacific Ocean ancestors might have been absent or subsequently lost in the Atlantic Ocean, following the trans-Arctic colonization bottleneck (Olsen et al., 2004; Yu et al., 2023). Thus, the reduced genetic diversity of eelgrass in this basin relative to the Pacific Ocean populations might have limited the availability of standing variation for parallel genomic responses to selection, or, alternatively, that selection might have acted on different loci than in the Northeast Pacific. However, a small subset of outlier loci were shared between all populations with divergent life histories, suggesting shared ancestral polymorphisms.

The breadth of genomic variation shared across genetically different annual and perennial pairs in the Pacific Ocean suggest that many loci may be involved in life history divergence. At the same time, the high concentration of outliers on chromosome three at 29 Mb (~3 Mb in length) and chromosome five at 4 Mb (~5 Mb in length) might be explained by structural variants, such as chromosomal inversions (Wellenreuther and Bernatchez, 2018), or

by loci of large effect that have undergone a selective sweep (Smith and Haigh, 1974). In *Mimulus guttatus*, the transition between annual and perennial ecotypes is governed largely by a single chromosomal inversion that captures alleles for life history, local adaptation, and reproductive isolation, with restricted recombination (Lowry and Willis, 2010). It remains to be explored whether comparable structural rearrangements underlie annual and perennial life history divergence in eelgrass.

It is important to note that divergence between life history types was not supported at several sites across the Pacific and the Atlantic Oceans, despite observations of distinct annual and perennial phenotypes. At these sites, the annual phenotype may instead reflect environmentally induced variation. At San Francisco, this phenotype may be potentially driven in response to waterfowl grazing that prevent vegetative shoots from overwintering (Ganter, 2000; Kollars et al., 2017). Population genetic analyses did not place this annual population in a distinct lineage within San Francisco Bay (Ort et al., 2012). In the sites from Europe, eelgrass diversification has occurred only across <40 kya, possibly leaving insufficient time for genetic differentiation to accumulate between annual and perennial plants. This finding is in contrast with earlier microsatellite work reporting significant differentiation among the annual (subtidal) and perennial (intertidal) source sites used here (Oetjen and Reusch, 2007). The reduced accumulation of mutations between types plus the limited sample size per site may have restricted our power to detect differentiation at such scales, and thus, population-level approaches may further uncover finer-scale variation underlying life history divergence in these locations.

The occurrence of annual plants in disturbed and unpredictable environments (Poppenwimer, Mayrose and DeMalach, 2023) suggests that the environmental instability projected under climate change may favor an increased prevalence of the annual strategy in eelgrass. If standing variation for life history is already present in the species, as appears to be the case across much of the Pacific Ocean populations, shifts between strategies could occur rapidly through selection on existing alleles rather than requiring new mutations. Because eelgrass is a foundation species in nearshore environments, a greater prevalence of annual beds would alter the seasonality and persistence of large-scale perennial meadows. Such changes may have potential cascading effects on the many species that depend on seagrass habitats, as well as on the ecosystem services that these meadows provide (Bos et al., 2007; Serrano et al., 2021).

3.5.1. Conclusion

Together, our findings indicate that annual and perennial life history strategies in *Zostera marina* have evolved repeatedly across ocean basins, with parallel genomic divergence within the Pacific that likely originated from shared standing variation, and a transition toward more independent genomic changes between annual and perennial plants in the Atlantic. Annual and perennials also appeared to reflect environmentally-controlled phenotypes at some sites. Such a pattern corresponds with the role of colonization history in the species and connectivity potential between life histories and across sites in shaping the repeatability of genomic divergence in life history evolution.

3.6. FIGURES

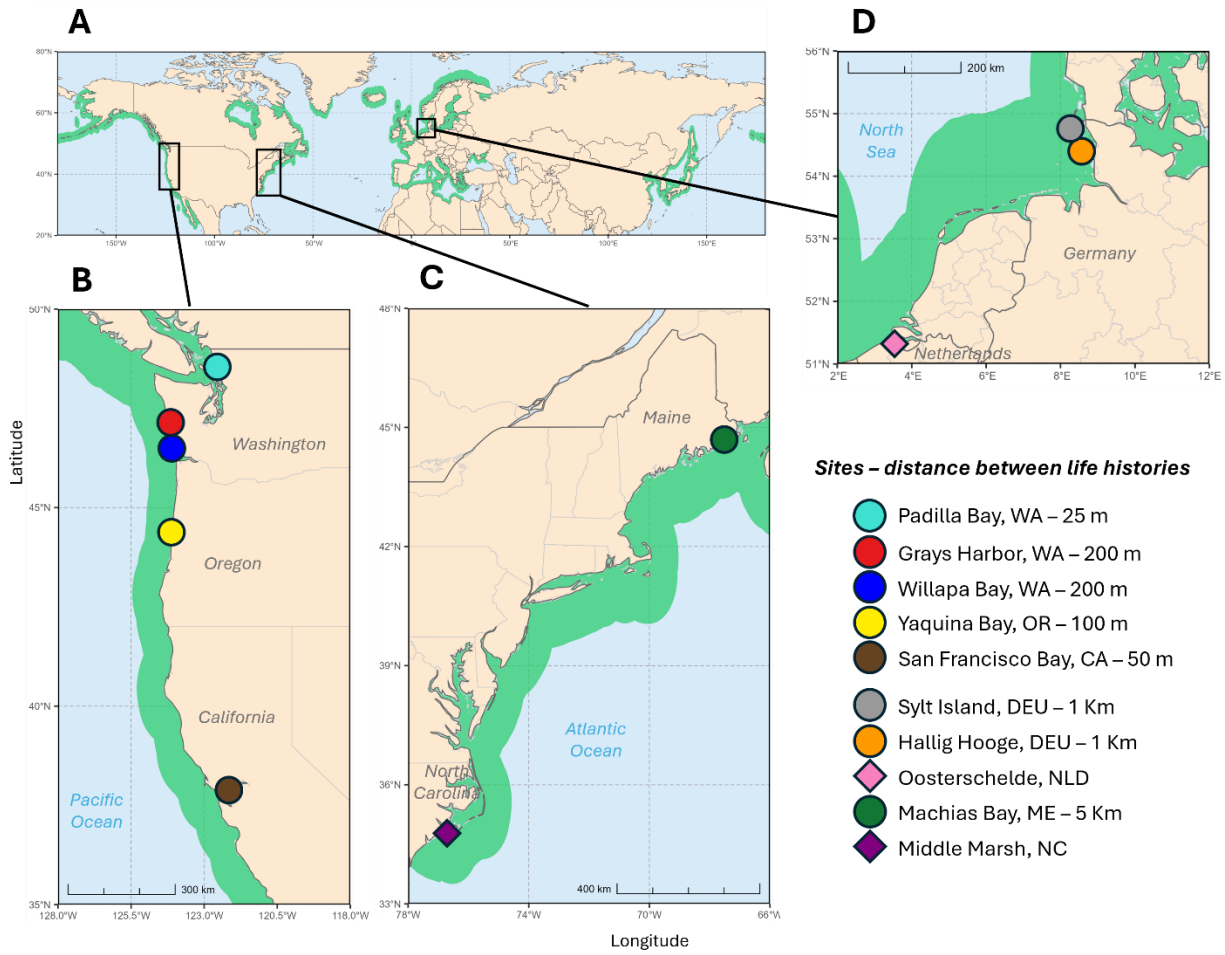

Figure 3.1. Sampling sites of annual and perennial eelgrass (*Zostera marina*) meadows across the species range (A). Plants were sampled along the (B) eastern

Pacific and (C) western Atlantic coasts of North America, and (D) in northern Europe. Marker color indicates sites of sampling. Sites where only annual plants were sampled are indicated by a diamond marker. Shading in green displays the extant range of eelgrass (IUCN data).

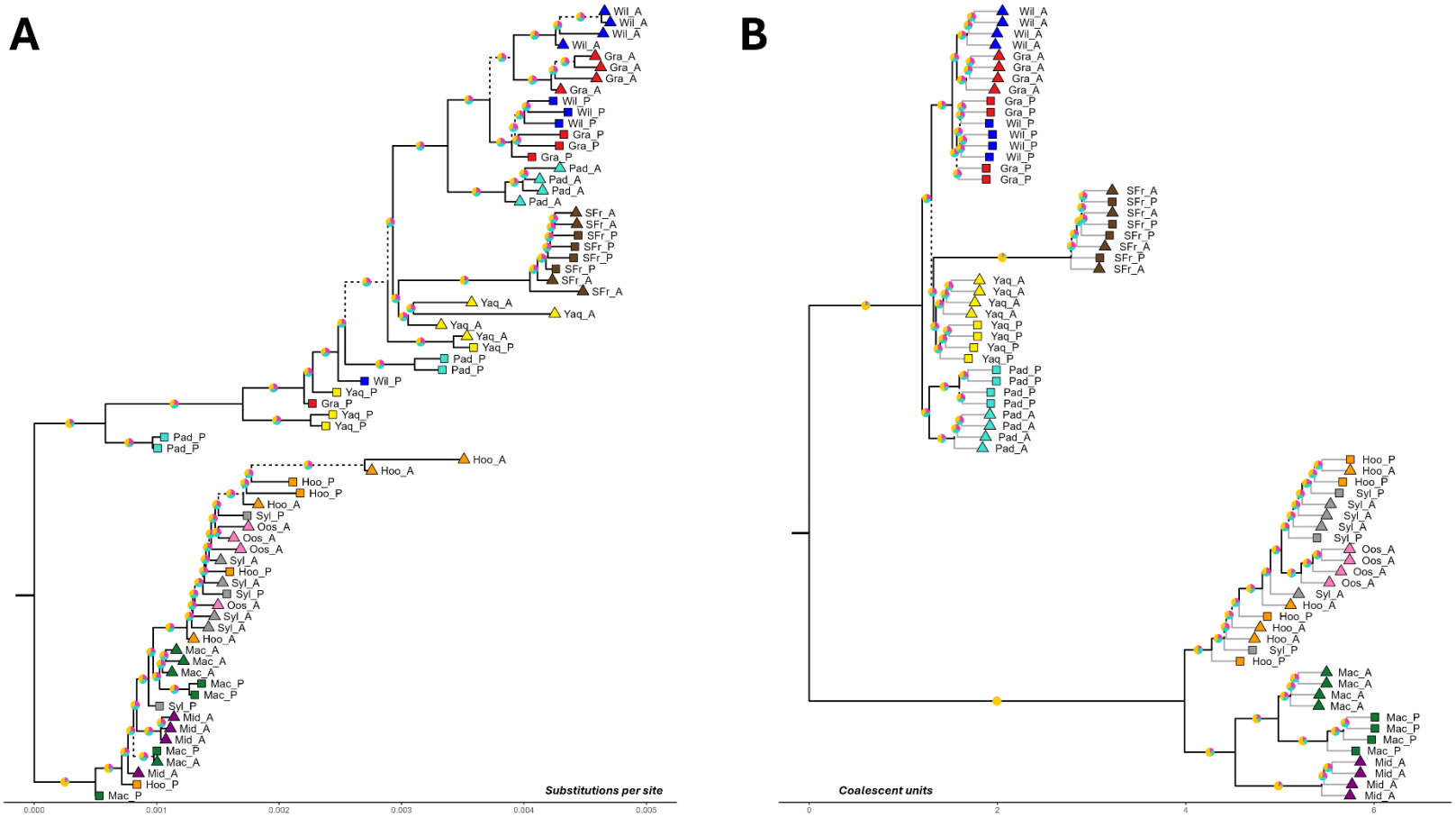

Figure 3.2. Nuclear Phylogenies Based on Coalescence of Annual and Perennial Eelgrass Plants Across the Sampled Range. Quartet-based phylogenetic inference was conducted using 10,870 CDS loci, including (A) all gene trees (ASTRAL-IV) and (B) those remaining after downweighing the low-support and long-branch gene trees (wASTRAL). Pie charts at internal nodes indicate the proportion of gene trees supporting the primary (yellow), first alternative (cyan), and second alternative (pink) quartet topologies. Solid branches indicate local posterior probability pp1 > 0.90 for the focal quartet topology; dotted branches indicate pp1 ≤ 0.90. Tip color indicates sample site (see Fig. 1), and marker shape indicates ecotype (triangles = annuals, squares = perennials). In (B), only internal branch lengths are inferred, and terminal branch lengths in gray are shown for visualization purposes only. Phylogenies were rooted using *Zostera japonica* CDS loci (n = 615), which was omitted from the plot to improve visual resolution within *Z. marina*.

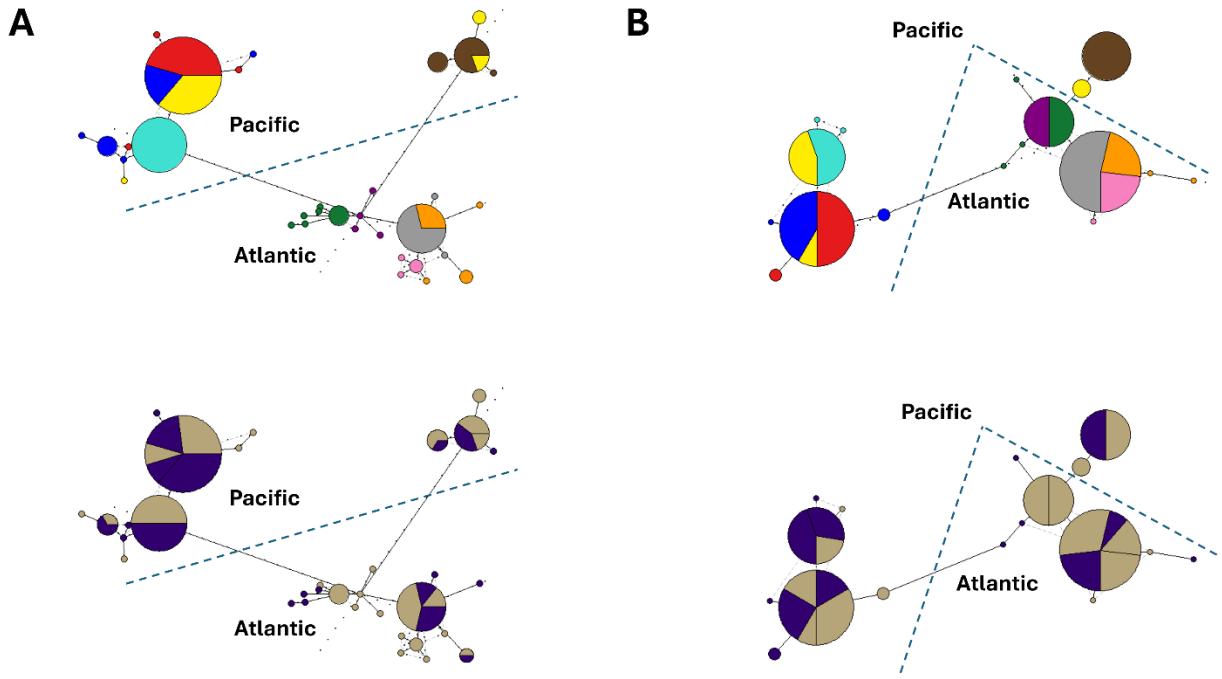

Figure 3.3. Organellar Haplotype Networks for Annual and Perennial Plants across the Pacific and Atlantic species range. (A) Chloroplast and (B) mitochondrial genome networks based on 121 and 323 SNP loci, respectively. Nodes are colored by population (top row, colors defined in Figure 1) or by ecotype, with perennials shown in purple and annuals in gold (bottom row). Node size is proportional to haplotype frequency. Dashed lines delineate Pacific and Atlantic clades.

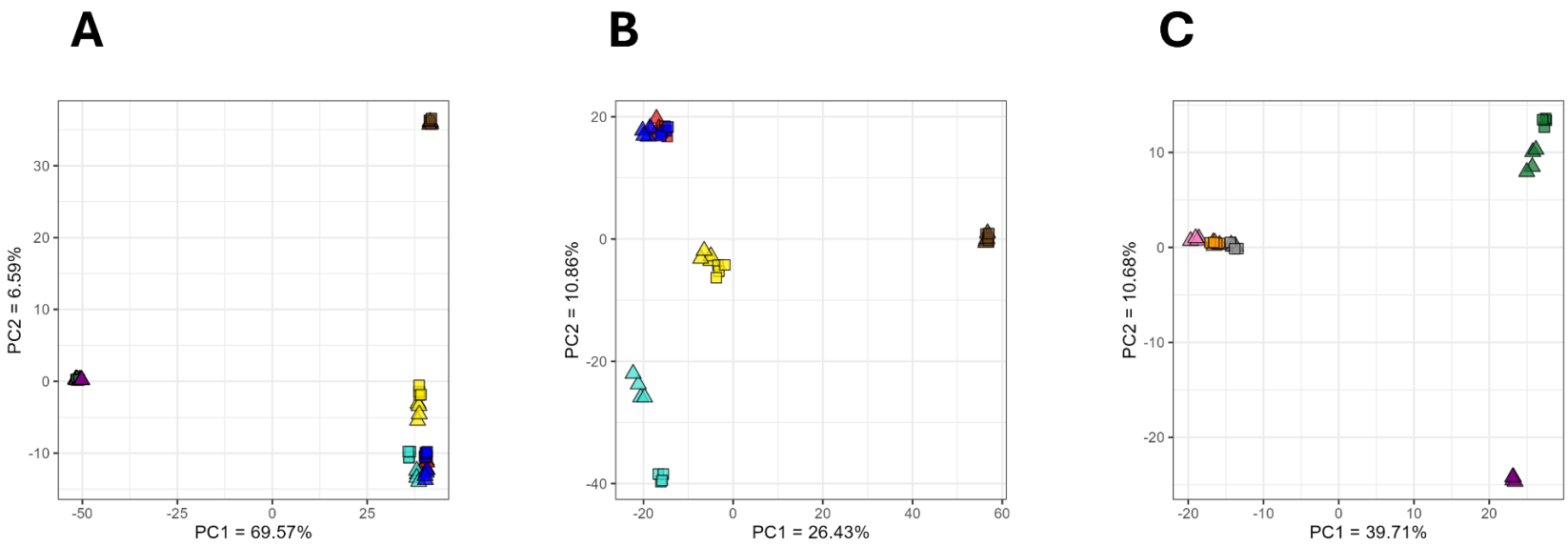

Figure 3.4. Principal Component Analyses of Genome-wide Genetic Variation Across Annual and Perennial Meadows. PCAs were conducted (A) across all sampled sites, and within the (B) Pacific and the (C) Atlantic subsets. Marker color indicates sampled site (see Fig. 1), and marker shape indicates observed phenotype (triangles = annuals, squares = perennials). The percentage of variance explained by each axis is shown in the axis label.

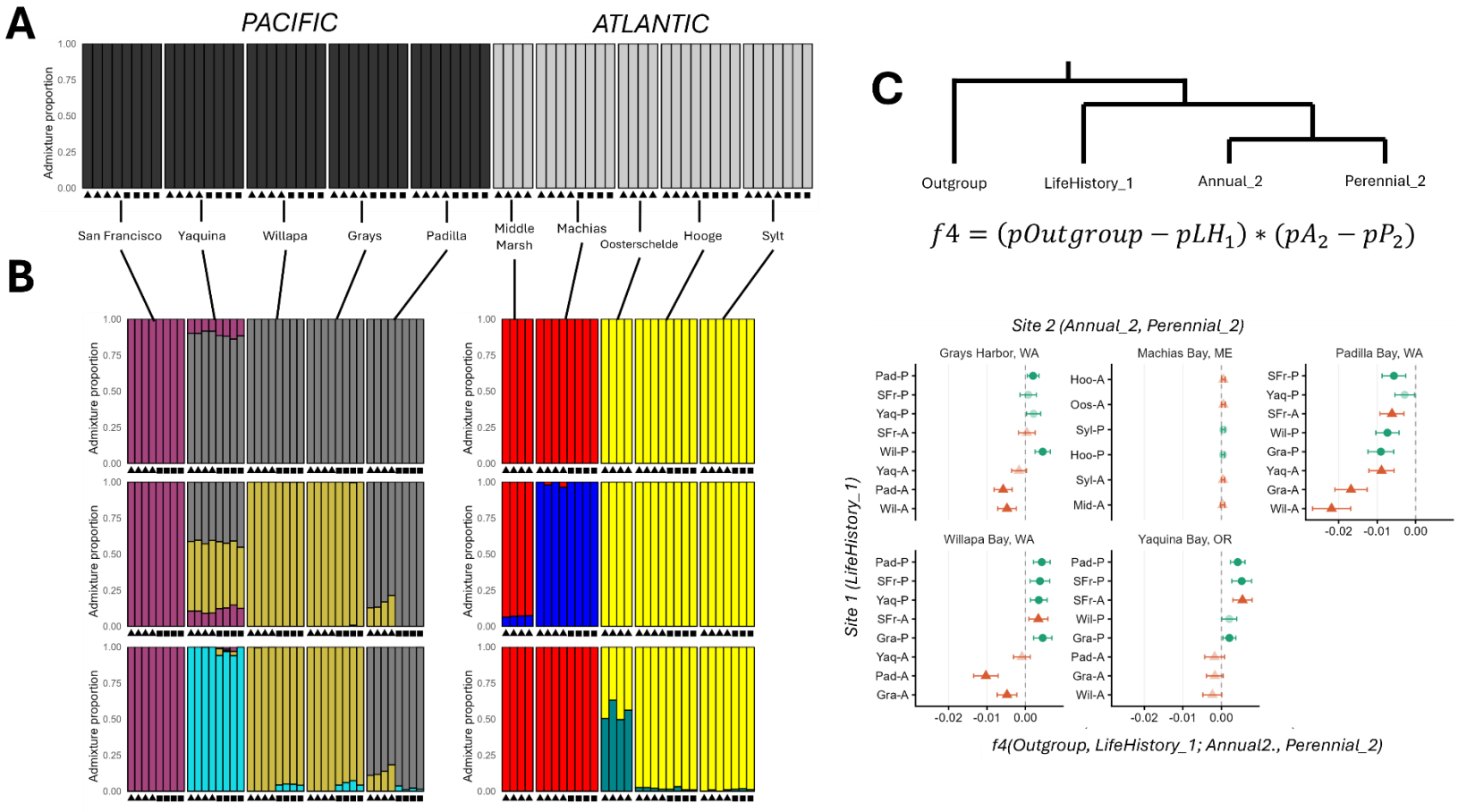

Figure 3.5. STRUCTURE analysis across locations and f4 statistics for sites with evidence of different admixture proportions between ecotypes. (A–C) Each bar represents the inferred ancestry of an individual partitioned into K genetic clusters: (A) all sites (K = 2), and (B) Pacific (left) and Atlantic (right) sites (K = 2–4, each). Markers below each individual indicate observed ecotype (triangles = annuals, squares = perennials). (C) f4 statistics of the form f4(Outgroup, LifeHistory_1; Annual_2, Perennial_2) estimated for sites showing within-site admixture differences in the STRUCTURE results. Machias perennial and Padilla perennial served as outgroups for the Pacific and Atlantic subsets, respectively. Error bars indicate one standard error estimated by block jackknife; significant values (bold color) were inferred using a Benjamini-Hochberg false discovery rate of 0.001. Labels on the y-axis that refer to LifeHistory_1 are abbreviated population names followed by "P" for perennials and "A" for annuals.

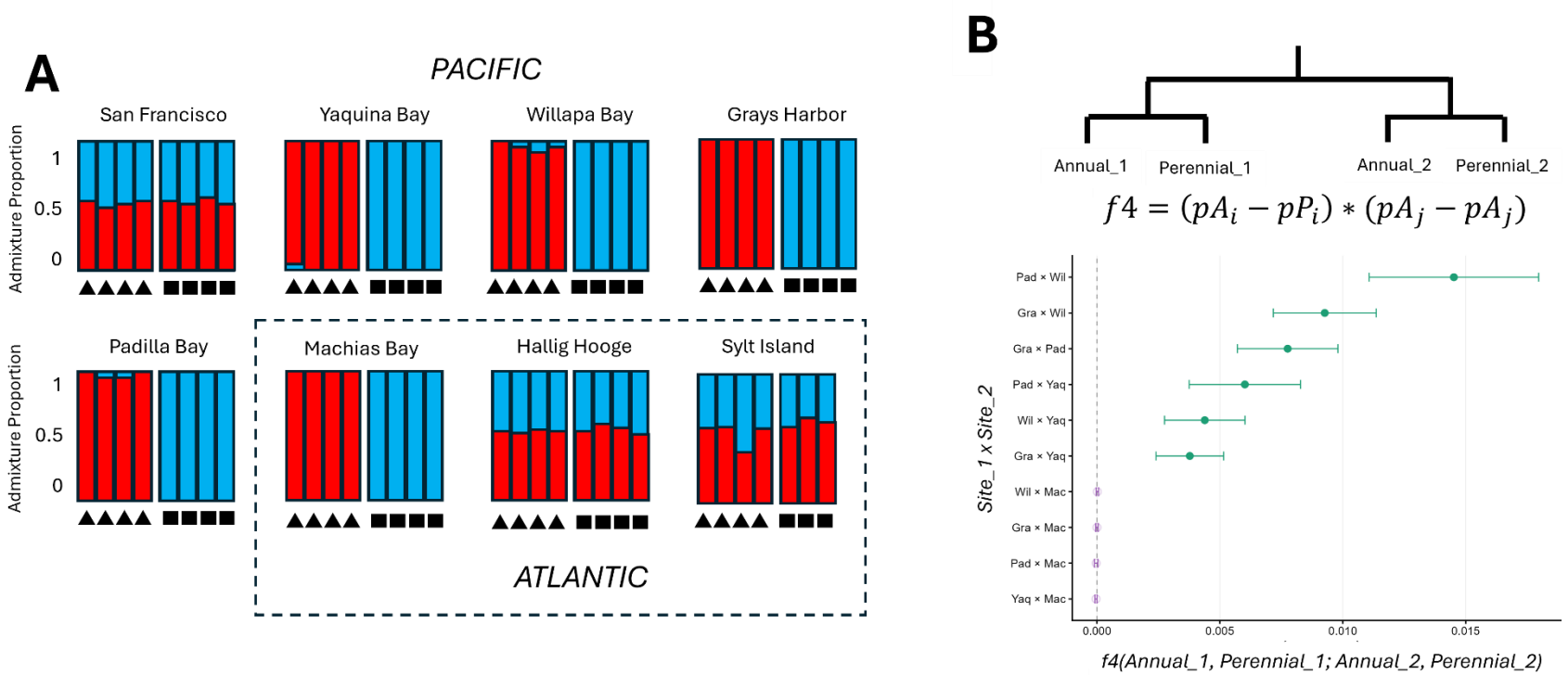

Figure 3.6. STRUCTURE analysis within locations and f4 statistics testing parallelism in allele frequency divergence across independently evolved annual and perennial pairs. (A) Inferred admixture proportions within each site at K = 2. Markers below each individual indicate observed ecotype (triangles = annuals, squares = perennials). (B) f4 statistics of the form f4(Annual_1, Perennial_1; Annual_2, Perennial_2) estimated for sites with genetically distinct annual and perennial groups. Estimate color indicates comparison type: green for comparisons within the Pacific and purple for comparisons between ocean basins. Error bars indicate one standard error estimated by block jackknife; significant values (bold color) were inferred using a BH correction.

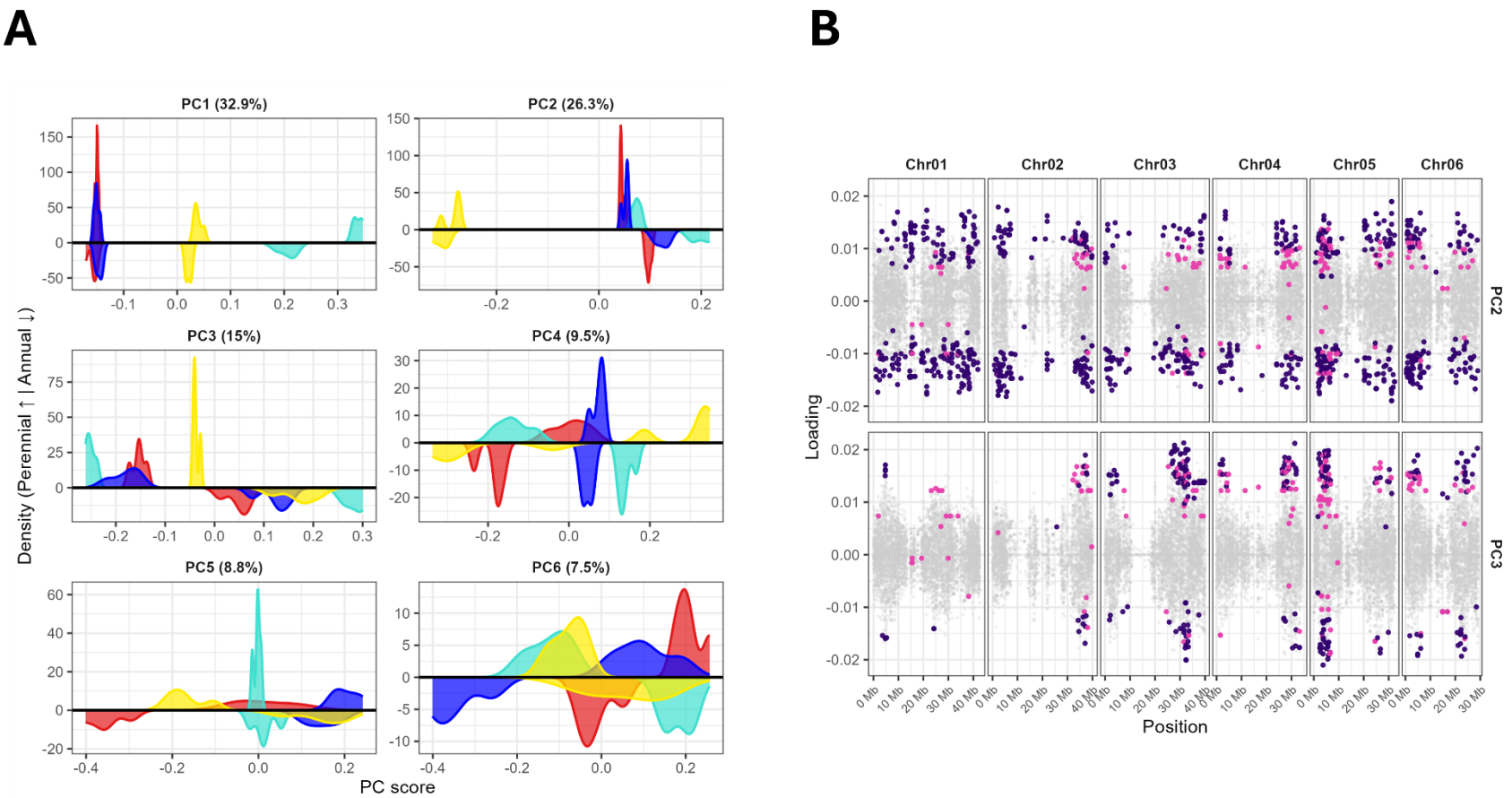

Figure 3.7. Principal Components of Genome-wide Variation Among Genetically Differentiated Annual and Perennial Plants Across Four Pacific Sites. (A) First six PCs, where each panel shows one axis; mirrored density plots give the distribution of individuals along that axis, with annuals below the midline and perennials above. Colors denote site. (B) SNP loadings onto PC2 (top) and PC3 (bottom) along the genome of eelgrass. Significant loadings (FDR < 0.001) are shown in color: purple indicates SNPs significant for that PC only, and pink indicates SNPs contributing significantly to both PC2 and PC3.

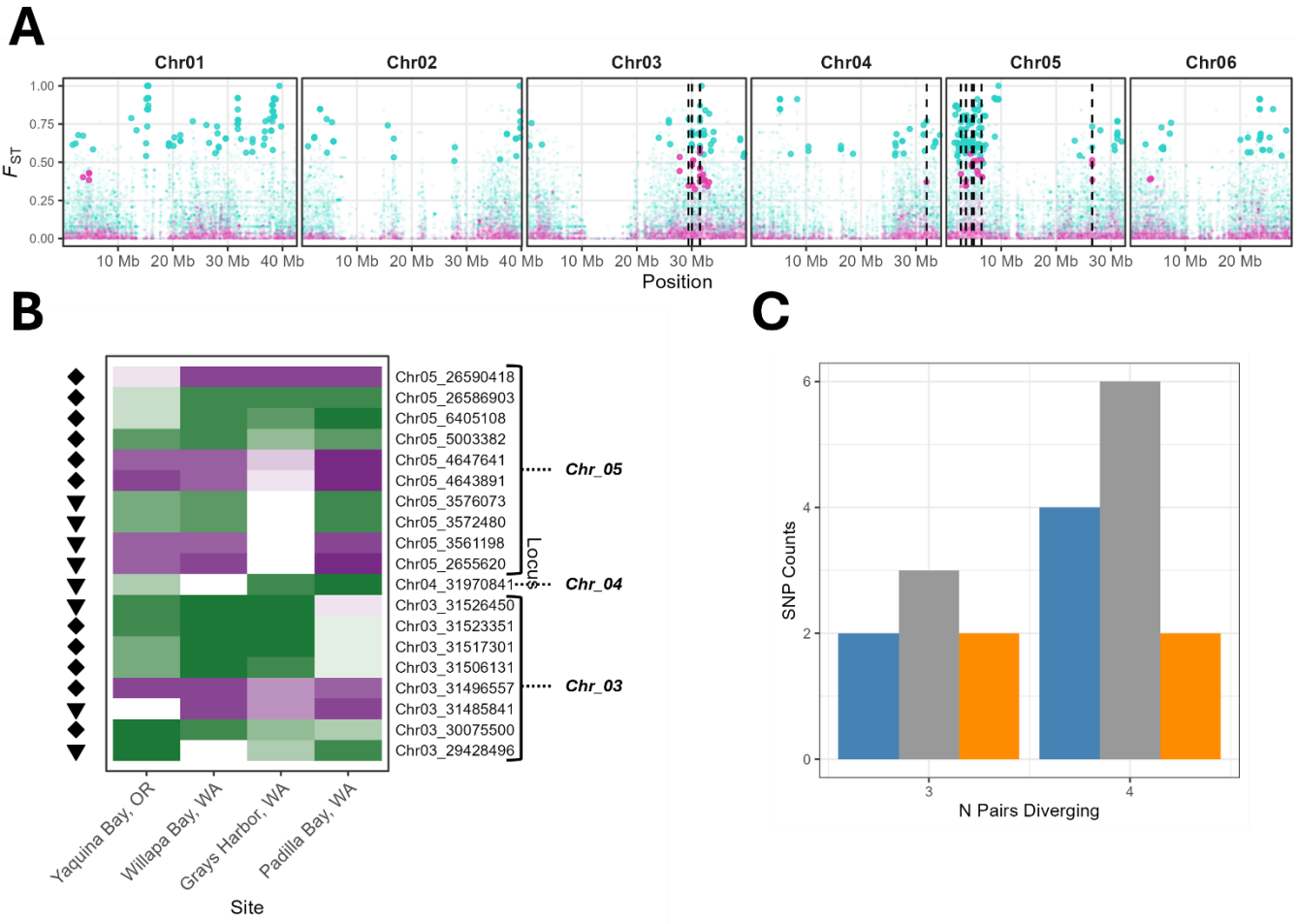

Figure 3.8. Candidate outlier analysis across the four genetically distinct co-located life history pairs in the Pacific. (A) FST distribution across the six major chromosomes. Marker color indicates outlier detection approach (combined = pink, population-specific = cyan). Significant outliers, identified at a 0.01 FDR threshold, are shown as bold markers; overlap between both approaches is indicated by dashed vertical lines. (B) Allele frequency difference (pA – pP) at candidate outlier loci across all tested pairs. Color shows direction of allele frequency change; purple and green indicate higher frequency of the alternative allele in annuals and perennials, respectively. Marker shape indicates the number of pairs showing consistent directional change (inverted triangle = three pairs, diamond = four pairs). (C) Bar chart of genomic location annotations for candidate outliers in CDS (blue), intergenic (gray), and introns (orange) and their repeated occurrence across pairs.

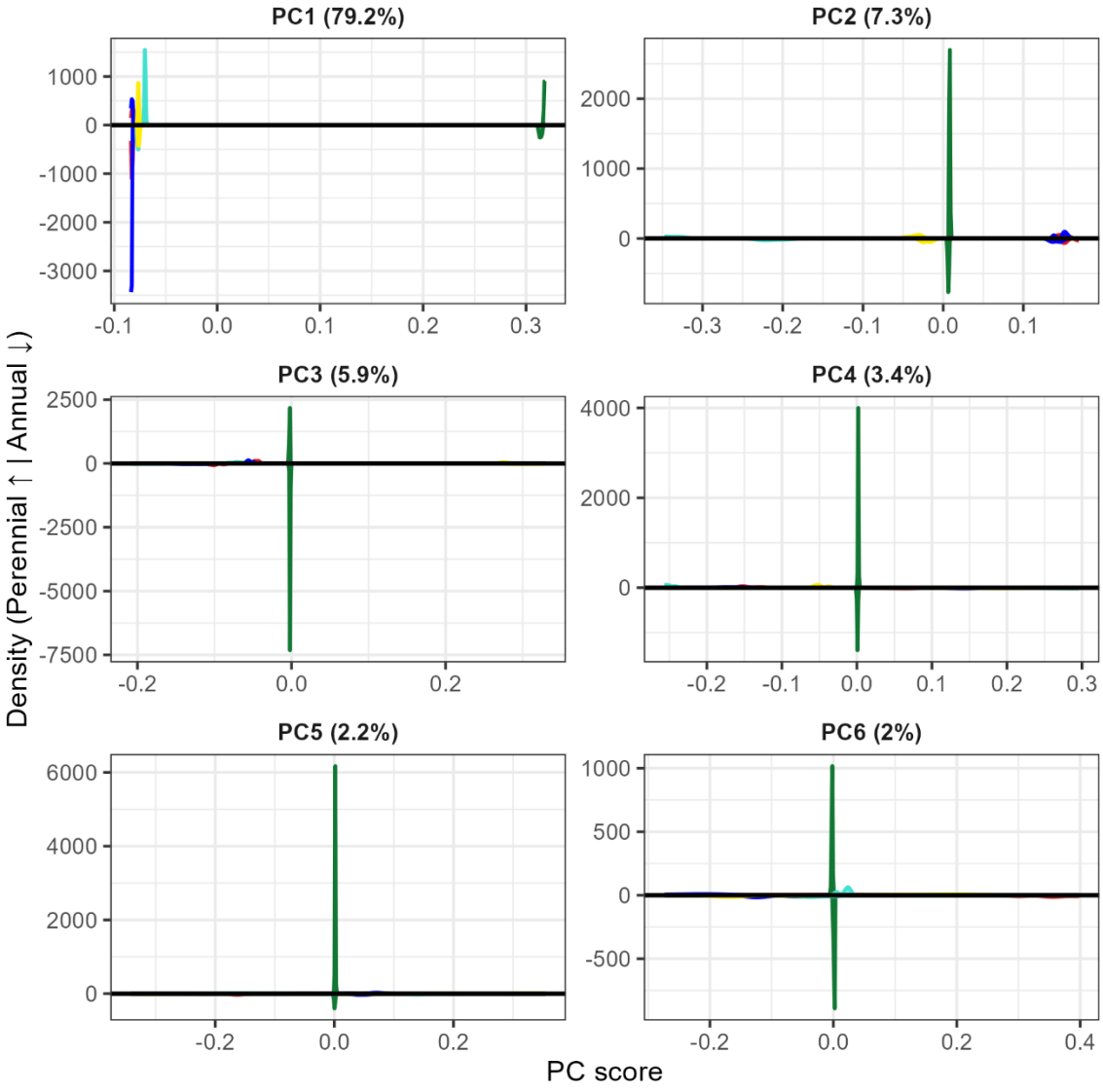

Figure S3.1. Principal Components of Genome-wide Variation Among Genetically Differentiated Annual and Perennial Plants Across Five Sites. Each of the first six PCs are displayed on one axis; mirrored density plots give the distribution of individuals along that axis, with annuals below the midline and perennials above. Colors denote site.

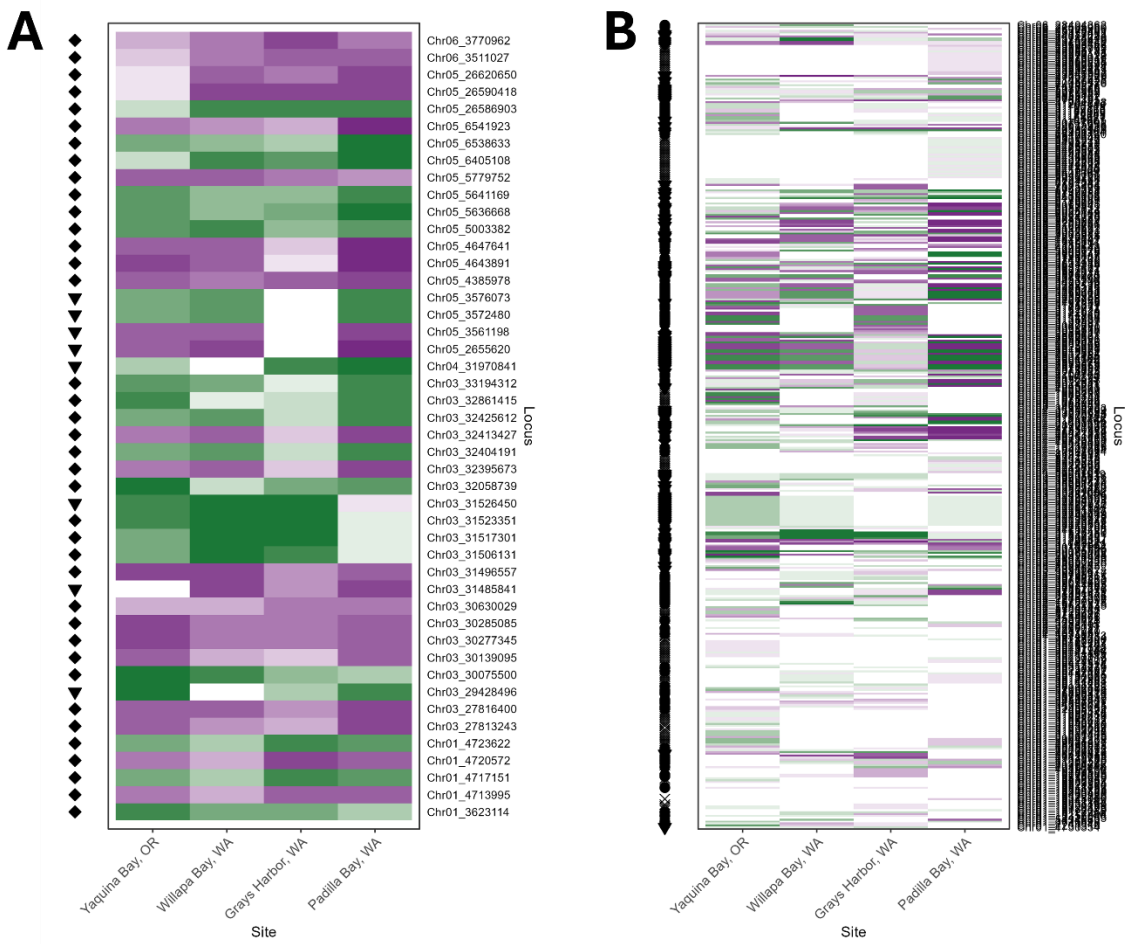

Fig S3.2. Allele frequency difference (pA – pP) at candidate outlier loci across all the four Pacific pairs for the (A) combined and (B) population-specific detection approaches. Color shows direction of allele frequency change; purple and green indicate higher frequency of the alternative allele in annuals and perennials, respectively. Marker shape indicates the number of pairs showing consistent directional change (empty = no shared change among pairs, cross = change in one pair, circle = two pairs, inverted triangle = three pairs, diamond = four pairs).

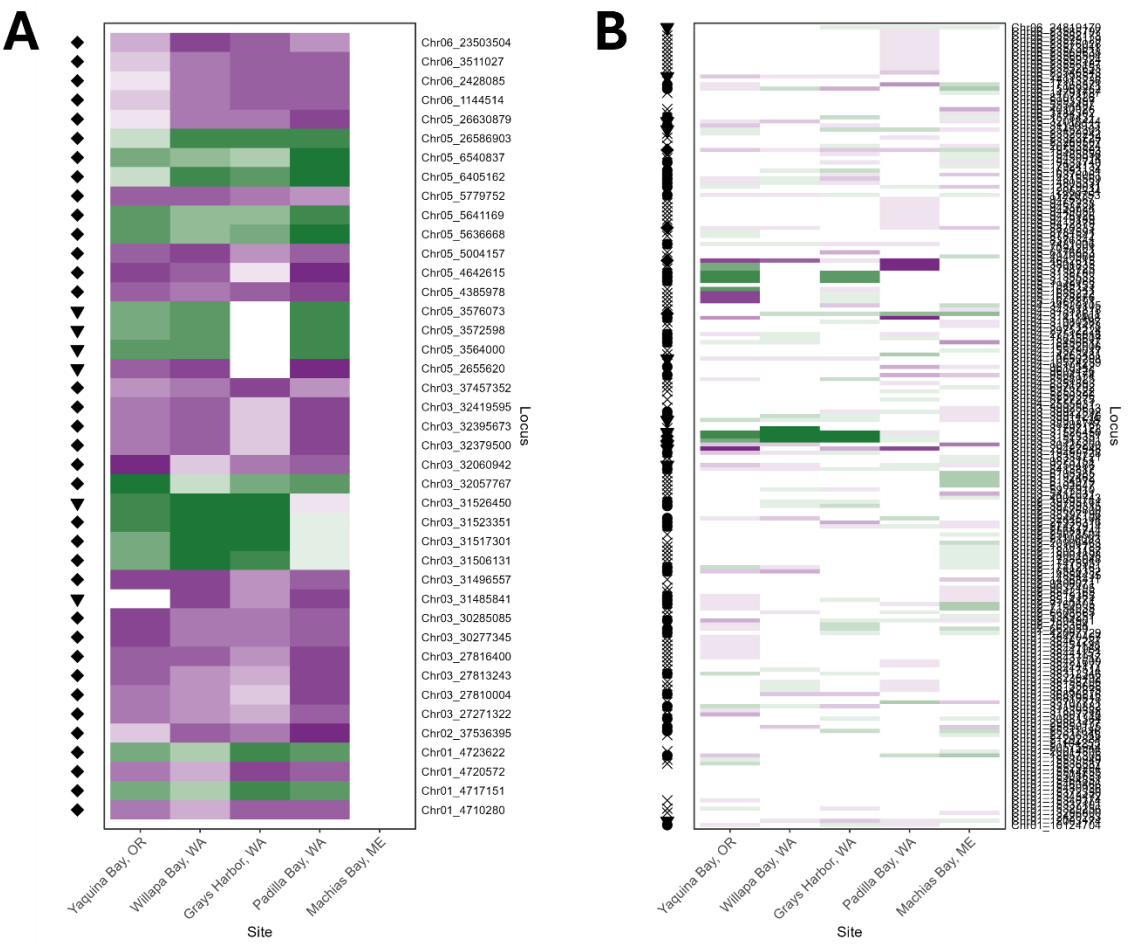

Fig S3.3. Allele frequency difference (pA – pP) at candidate outlier loci across all five genetically different life history pairs for the (A) combined and (B) population-specific detection approaches. Color shows direction of allele frequency change; purple and green indicate higher frequency of the alternative allele in annuals and perennials, respectively. Marker shape indicates the number of pairs showing consistent directional change (empty = no shared change among pairs, cross = change in one pair, circle = two pairs, inverted triangle = three pairs, diamond = four pairs).

3.7.TABLES

Table 3.1. Site information for annual and perennial plants sampled in this study.

Ocean Basin	Site	Region	Life History	Latitude	Longitude	Distance between pairs
Pacific	San Francisco Bay	CA, USA	Annual Perennial	37.690	-122.311	50 m
	Yaquina Bay	OR, USA	Annual Perennial	44.617	-124.025	100 m
	Willapa Bay		Annual Perennial	46.573	-124.012	200 m
	Grays Harbor	WA, USA	Annual Perennial	46.891	-124.084	200 m
	Padilla Bay		Annual Perennial	48.552	-122.534	25 m
Atlantic	Machias Bay	ME, USA	Annual Perennial	44.652	-67.345	5 Km
	Middle Marsh	NC, USA	Annual	34.693	-76.611	-
	Oosterschelde	Netherlands	Annual	51.592	3.930	-
	Hallig Hooge	Germany	Annual Perennial	54.577	8.569	1 Km
	Sylt Island		Annual Perennial	54.869	8.355	1 Km

Table 3.2. Outlier loci identified in the two Bayescan approaches across minimally admixed life history pairs. For each locus, information includes chromosome and base pair position, number of locations with parallel and anti-parallel allele frequency changes, genomic feature, and annotation gene based on the v3.1 *Zostera marina* genome reference.

n	Chromosome	BP position	Parallel	Anti-parallel	Genomic Context	Annotated Gene
1	3	29428496	3	0	intergenic	
2	3	30075500	4	0	CDS	Zosma03g21210
3	3	31485841	3	0	intergenic	
4	3	31496557	4	0	intergenic	
5	3	31506131	4	0	intergenic	
6	3	31517301	4	0	CDS	Zosma03g23470
7	3	31523351	4	0	CDS	Zosma03g23490
8	3	31526450	3	1	intron	Zosma03g23490
9	4	31970841	3	0	CDS	Zosma04g23650
10	5	2655620	3	0	intergenic	
11	5	26586903	4	0	intergenic	
12	5	26590418	4	0	intron	Zosma05g25730
13	5	3561198	3	0	intron	Zosma05g05510
14	5	3572480	3	0	intron	Zosma05g05520
15	5	3576073	3	0	CDS	Zosma05g05530
16	5	4643891	4	0	intergenic	
17	5	4647641	4	0	intergenic	
18	5	5003382	4	0	intergenic	
19	5	6405108	4	0	CDS	Zosma05g10780

Table 3.3. Putative functions of the *Z. marina* genes identified as outlier loci across minimally admixed annual and perennial pairs.

Gene Name	Panther ID	GO ID	Inter Pro ID	Panther Description	GO Description	InterPro Description
	PTHR461	GO:00	IPR00			
	68:SF9	05515	0225	ARMADILLO REPEAT ONLY 2	protein binding	Armadillo
	PTHR461	GO:00	IPR011			
	68:SF9	05515	989	ARMADILLO REPEAT ONLY 2	protein binding	Armadillo-like helical
Zosma0	PTHR461	GO:00	IPR01			
3g21210	68:SF9	05515	6024	ARMADILLO REPEAT ONLY 2	protein binding	Armadillo-type fold
			IPR01			Bifunctional inhibitor/plant lipid transfer
	PTHR31731:SF1		6140	OS06G0643500 PROTEIN		protein/seed storage helical domain
			IPR02			
	PTHR31731:SF1		7923	OS06G0643500 PROTEIN		Hydrophobic seed protein domain
Zosma0			IPR03			Bifunctional inhibitor/plant lipid transfer
3g23470	PTHR31731:SF1		6312	OS06G0643500 PROTEIN		protein/seed storage helical domain superfamily
Zosma0			IPR01			
3g23490	PTHR11477:SF37		2921	OS04G0644675 PROTEIN		Spen paralogue and orthologue SPOC, C-terminal
	PTHR125	GO:00	IPR01	EXOCYST COMPLEX		
	42:SF41	00145	6159	COMPONENT 7	exocyst	Cullin repeat-like-containing domain superfamily
	PTHR125	GO:00	IPR04	EXOCYST COMPLEX		
	42:SF41	00145	6364	COMPONENT 7	exocyst	Exocyst complex subunit Exo70, C-terminal
					phosphatidylinositol-	
	PTHR125	GO:00	IPR01	EXOCYST COMPLEX	4,5-bisphosphate	
	42:SF41	05546	6159	COMPONENT 7	binding	Cullin repeat-like-containing domain superfamily
					phosphatidylinositol-	
Zosma0	PTHR125	GO:00	IPR04	EXOCYST COMPLEX	4,5-bisphosphate	
4g23650	42:SF41	05546	6364	COMPONENT 7	binding	Exocyst complex subunit Exo70, C-terminal

	PTHR125	GO:00	IPR01	EXOCYST COMPLEX		
	42:SF41	06887	6159	COMPONENT 7	exocytosis	Cullin repeat-like-containing domain superfamily
	PTHR125	GO:00	IPR04	EXOCYST COMPLEX		
	42:SF41	06887	6364	COMPONENT 7	exocytosis	Exocyst complex subunit Exo70, C-terminal
	PTHR125	GO:00	IPR01	EXOCYST COMPLEX		
	42:SF41	00145	6159	COMPONENT 7	exocyst	Cullin repeat-like-containing domain superfamily
	PTHR125	GO:00	IPR04	EXOCYST COMPLEX		
	42:SF41	00145	6364	COMPONENT 7	exocyst	Exocyst complex subunit Exo70, C-terminal
					phosphatidylinositol-	
	PTHR125	GO:00	IPR01	EXOCYST COMPLEX	4,5-bisphosphate	
	42:SF41	05546	6159	COMPONENT 7	binding	Cullin repeat-like-containing domain superfamily
					phosphatidylinositol-	
	PTHR125	GO:00	IPR04	EXOCYST COMPLEX	4,5-bisphosphate	
	42:SF41	05546	6364	COMPONENT 7	binding	Exocyst complex subunit Exo70, C-terminal
	PTHR125	GO:00	IPR01	EXOCYST COMPLEX		
	42:SF41	06887	6159	COMPONENT 7	exocytosis	Cullin repeat-like-containing domain superfamily
	PTHR125	GO:00	IPR04	EXOCYST COMPLEX		
	42:SF41	06887	6364	COMPONENT 7	exocytosis	Exocyst complex subunit Exo70, C-terminal
	PTHR456	GO:00	IPR00	(WILD MALAYSIAN BANANA)	hydrolase activity, acting	
	48:SF177	16788	1087	HYPOTHETICAL PROTEIN	on ester bonds	GDSL lipase/esterase
Zosma0	PTHR456	GO:00	IPR03	(WILD MALAYSIAN BANANA)	hydrolase activity, acting	
5g05510	48:SF177	16788	6514	HYPOTHETICAL PROTEIN	on ester bonds	SGNH hydrolase superfamily
	PTHR456	GO:00	IPR00	(WILD MALAYSIAN BANANA)	hydrolase activity, acting	
	48:SF177	16788	1087	HYPOTHETICAL PROTEIN	on ester bonds	GDSL lipase/esterase
Zosma0	PTHR456	GO:00	IPR03	(WILD MALAYSIAN BANANA)	hydrolase activity, acting	
5g05520	48:SF177	16788	6514	HYPOTHETICAL PROTEIN	on ester bonds	SGNH hydrolase superfamily
Zosma0			IPR00			
5g05530	PTHR42874:SF1		2042	URICASE		Uricase
Zosma0			IPR00			
5g10780	PTHR31636:SF4		5202	SCARECROW-LIKE PROTEIN 1		Transcription factor GRAS

	PTHR31636:SF4	IPR005202	SCARECROW-LIKE PROTEIN 1	Transcription factor GRAS
Zosma05g25730	PTHR36048:SF1		RIBOSOME MATURATION FACTOR	

Table S3.1. Filtering steps applied to the sequenced reads for the genome-wide analyses. Datasets were genotyped and filtered separately for all samples, those in the Pacific and Atlantic ranges, the four genetically distinct life history pairs in the Pacific (Willapa Bay, Padilla Bay, Grays Harbor, and Yaquina Bay), and all five differentiated pairs including the Atlantic site from Maine (Machias Bay) in the Atlantic. Rows describe individual filtering steps, and columns describe the number of SNPs retained or percentage of genotypes removed after each step.

	Filtering Steps	Dataset				
		All	**Pacific**	**Atlantic**	**Four LH Pairs**	**Five LH Pairs**
1	Total initially genotyped SNPs	7,552,441	6,411,464	2,691,264	6,049,838	6,654,997
2	Retained only biallelic SNPs	6,612,090	5,689,690	2,438,528	5,389,357	5,909,179
3	Removed genotypes with depth < 15	31.35%	32.49%	38.73%	31.28%	29.59%
4	Removed genotypes with allelic balance outside 0.2 and 0.8	38.90%	38.27%	32.60%	36.31%	36.79%
5	Removed SNPs with minor allele count (mac) < 3	69.16%	76.29%	76.87%	80.89%	74.46%
6	Retained SNPs with no missing data across individuals	114,154	349,393	35,034	333,233	465,630
7	Retained SNPs after thinning to a minimum distance of 3,000 base pairs between loci	22,538	35,037	15,858	34,066	37,391
Total retained SNPs on the six main chromosomes		**21,559**	**32,578**	**12,861**	**33,384**	**36,732**
Total number of individuals		**71**	**40**	**31**	**32**	**40**

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