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Linking Spatial Patterns of Adult and Seed Diversity Across the Depth Gradient in the Seagrass *Zostera marina* L.

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Abstract

Sexual reproduction is the primary source of genotypic diversity in seagrass meadows, with important implications for both the evolutionary potential and ecological function of these habitats. However, relatively little is known about seagrass pollination dynamics or how genetic diversity in life history stage may influence genetic diversity in another. We used microsatellite DNA markers to assess how genetic diversity of adult shoots and seeds covaried across the depth gradient in two *Zostera marina* L. (eelgrass) meadows in Massachusetts (USA). Despite strong spatial patterns in the density of reproductive shoots and genetic evidence for limited pollen dispersal, we found modest corresponding variation in outcrossing rates and paternal diversity. Across spatial scales from quadrats to sites, there was a positive association between the density of flowering shoots and clonal richness; however, other measures of diversity (e.g., allelic richness and heterozygosity) showed weak or opposing patterns with reproductive effort, and neither flowering effort nor local genotypic richness of adults were good proxies for seed diversity. Self-pollinated seeds were significantly smaller than their outcrossed half-siblings, and our data suggest that selection against selfed seeds varies spatially within and among meadows, possibly due to high competition with vegetative shoots in the dense shallow margin. Understanding the processes that shape genetic diversity in seagrass populations is key to their conservation and management; our study highlights the challenges of inferring the contribution of sexual reproduction to population structure using a single metric or life history stage.

Keywords *Zostera marina* · Eelgrass · Genetic diversity · Sexual reproduction · Seeds · Microsatellites

Introduction

Genetic diversity is a fundamental characteristic of populations, key to their evolution, ecology and conservation, and the raw material for adaptive evolution. As such, genetic diversity can constrain a population's response to a changing environment (Fisher 1930). Standing genetic diversity can also play a critical role in ecological function (Whitham et al. 2006; Hughes et al. 2008), especially in those systems where one species provides and physically structures the habitat. For example, in marine and terrestrial communities where a clonal plant functions as a foundation species, genetic diversity within that species can impact community stability and productivity in ways analogous to species diversity in other ecosystems (e.g., Wimp et al. 2004; Crutsinger et al. 2006; Crawford and Rudgers 2013). While multiple processes may act to maintain existing diversity within clonal plant populations (e.g., balancing or frequency-dependent selection; Browne and Karubian 2016), the generation of genotypic diversity over short-time scales depends primarily on sexual recruitment from seed. Thus, understanding the processes that govern seed production and diversity within and among populations is a necessary first step in understanding spatial variation in population genetic structure in clonal foundation species, and ultimately informing their management and conservation.

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Some of the most striking evidence for the ecological impacts of genetic diversity comes from the study of seagrasses (Reusch and Hughes 2006; Procaccini et al. 2007; Duffy et al. 2013), marine angiosperms that form highly productive meadows in nearshore areas and provide habitat for a wide variety of invertebrates, fishes, birds, and mammals during one or more stages of their life cycles (Orth et al. 1984; Heck et al. 1997). *Zostera marina*, or eelgrass, is the dominant species of seagrass in the northern hemisphere and increasingly a model organism for studying the importance of genetic variation (e.g., Hughes et al. 2010; Tomas et al. 2011; Abbott et al. 2017; Evans et al. 2017). Seagrasses reproduce asexually through rhizomatous growth and sexually through the seasonal production of flowering shoots and seeds. Thus, as in all clonal plants, genetic diversity can be assessed with different metrics, including genotypic diversity (i.e., the number and relative abundance of unique clonal lineages), allelic diversity (e.g., allelic richness at the population scale or heterozygosity at the scale of individuals), and the relatedness of individual genotypes (Arnaud-Haond et al. 2007). Genotypic and allelic diversity are correlated at the lowest levels, but at the intermediate to high levels of clonal richness increasingly documented in seagrass meadows (e.g., Reusch et al. 2000; Williams and Orth 1998; Olsen et al. 2004), different measures of diversity do not necessarily covary predictably and may have independent effects (Massa et al. 2013; Abbott et al. 2017). In *Z. marina*, experimental manipulations demonstrate that the number of clones in a plot influences both resistance and resilience to natural disturbances (Hughes and Stachowicz 2004; Reusch 2006), and patch-scale genotypic and genetic diversity (i.e., allelic diversity and relatedness) have both been linked to increased biomass and productivity in adult shoots (Hughes and Stachowicz 2009; Stachowicz et al. 2013), as well as for seeds/seedlings (Reynolds et al. 2012; Hughes et al. 2016).

Despite abundant evidence for the importance of *Z. marina* genetic diversity, we have limited understanding of the links among allocation to sexual reproduction, seed production, seedling recruitment, and genetic diversity in natural populations. Across the species' geographic range, *Z. marina* populations display considerable variation in allocation to sexual reproduction (e.g., including fully annual versus perennial life histories; Robertson and Mann 1984; Jarvis et al. 2012; Kim et al. 2014). Even in the same region, the frequency of flowering and seed production varies widely within and among sites for reasons still largely unknown (Xu et al. 2018; von Staats et al. 2020). While clonal growth has traditionally been assumed to be the primary method of reproduction in perennial populations, there is an increasing recognition of the contribution of seeds and seedlings to seagrass dynamics (Orth et al. 2000; Kendrick et al. 2012). In fact, seedling recruitment has been documented over multiple years in largely continuous perennial meadows (Reusch 2006; Becheler et al. 2014; Johnson et al. 2020), demonstrating that ongoing sexual reproduction can shape the genetic structure and diversity of established populations, as well as contribute to meadow expansion (Furman et al. 2015) and recovery from disturbance (Plus et al. 2003; Reusch 2006 ; Becheler et al. 2010).

Beyond flowering shoot density, little is known about basic characteristics of the sexual mating system in *Z. marina* (e.g., outcrossing rate, correlation in paternities, gametic, and zygotic dispersal), or how and why these may vary among populations. This knowledge gap is problematic, as such characteristics interactively determine how genetic diversity is structured within and among offspring (e.g., heterozygosity, allelic richness, and relatedness). Flowering shoots produce a variable number of inflorescences (spadices) that mature acropetally and contain both male and female flowers (Churchill and Riner 1978; De Cock 1980). Within each spathe, the female flowers mature hours to days before males; this temporal separation of function (dichogamy) may prevent self-fertilization within inflorescences but not necessarily between spathes on the same shoot, or between reproductive shoots produced by the same clone (i.e., geitonogamy; Ackerman 2006). Thus, the frequency of selfing in eelgrass will depend on multiple factors that vary spatially, including (1) the clonal structure of the meadow (i.e., proximity and diversity of potential pollen sources); (2) local fluid dynamics (e.g., effects of currents and/or eelgrass canopy characteristics on pollen supply); as well as (3) any traits of reproductive shoots that restrict or promote self-pollination, such as possible cryptic self-incompatibility mechanisms (Hammerli and Reusch 2003) or synchronization of flowering within shoots and among clonemates (Jahnke et al. 2015). These factors are likely (or known) to change within sites as well as among them, across the depth gradient spanned by eelgrass meadows. For example, Ruckelshaus (1995) found higher selfing rates in subtidal versus intertidal seeds, possibly linked to differences in flowering phenology with depth: maternal shoots displayed reduced dichogamy in the subtidal compared to the intertidal. To date, only a handful of studies thus far have used molecular genetic techniques to examine the link between seed and adult genetic diversity in any seagrass species (Reusch 2001; Zipperle et al. 2011; Sinclair et al. 2014; Furman et al. 2015; Lloyd et al. 2018), which limits our ability to generate or test hypotheses about the processes that mediate that relationship and how they may vary spatially.

Here, we explore how the genetic diversity of adult shoots and seeds covaries across a depth gradient in two *Z. marina* meadows in Massachusetts (USA). We used microsatellite DNA markers to assess the clonal richness, allelic richness, and genetic relatedness of adult vegetative shoots at hierarchical spatial scales, ranging from the local neighborhood scale (i.e., within quadrats, at the scale of interacting shoots) to the scales of depth (shallow, middle, and deep) and site. We

genotyped developing seeds collected from flowering shoots at each depth to quantify selfing rates, pollen diversity, and spatial genetic structure, and we examined the correlations between adult and seed traits as a first step in understanding how spatial variation in the mating system may influence population structure across the life history of *Z. marina*.

Methods

Study Sites and Sample Collection

We collected samples from two coastal eelgrass meadows in the Gulf of Maine, USA, separated by approximately 48 km: Curlew Beach in Nahant, MA (hereafter Curlew) and Niles Beach in Gloucester, MA (hereafter Niles). Both sites support perennial *Z. marina* meadows that extend approximately 300 m offshore. Eelgrass coverage at the two sites was estimated in 1995, 2001, 2006, and 2012 as part of a statewide mapping effort by Massachusetts Department of Environmental Protection (e.g., see Costello and Kenworthy 2011): the meadow at Niles is ~ 53 acres while the Curlew meadow wraps across several embayments to cover ~ 334 acres. Areal coverage fluctuated at both sites but showed no consistent trend over the four sampling periods (data from Massachusetts DEP). In late July and early August of 2014, we used SCUBA to sample *Z. marina* from three depths at each site: the center of the meadow (hereafter mid) and approximately 5 m from the inshore and offshore edges (hereafter shallow and deep, respectively). The depth range spanned by *Z. marina* was similar at both sites, such that the depth of our shallow, mid, and deep samples was approximately 1, 3, and 5 m MLLW, respectively. At each depth, we collected 8–10 flowering shoots separated by at least 2 m to minimize the likelihood of resampling the same clone; from each of these focal flowering shoots, we harvested a leaf tissue sample and ~ 8–15 developing seeds from within a single spathe on the highest and youngest rhipidia, or cluster of inflorescences (n = ~ 250 seeds per site). To characterize the genetic structure of neighboring plants, we haphazardly selected up to 10 vegetative shoots from within a 0.25-m^2 quadrat set around each focal flowering shoot (n = ~ 250 shoots per site). Leaf tissue was preserved in silica or frozen at − 20 °C until DNA extraction; seeds were frozen at − 80 °C.

To compare shoot density and morphology between sites and among depths, we harvested all aboveground biomass from 0.0625-m^2 quadrats set adjacent to the focal flowering shoots. In the lab, we recorded the number of vegetative and flowering shoots and measured the maximum length of 10 vegetative shoots per quadrat as an estimate of canopy height, which could influence pollen and seed dispersal dynamics. In both meadows, eelgrass cover was patchy along the deep transect, and we deliberately set quadrats to capture areas where eelgrass was present; thus, deep quadrats inflate any area-based estimate of total eelgrass density but describe the local density of interacting shoots. To estimate seed production, we recorded the total number of spathes per flowering shoot and the number of seeds per spadix on the focal shoot and up to two additional flowering shoots from the associated quadrat (n = 10–24 flowering shoots per depth). Most samples were spatially paired as described above, except at the mid zone at Gloucester, where flowering shoots were collected independently of the other samples.

Molecular Methods

We extracted DNA from leaf tissue by grinding each sample with a Retsch mixer mill MM400 and using the Omega Bio-Tek E.Z.N.A.® Plant DNA Kit. DNA from seeds was extracted with Chelex 100 (Bio-Rad). A subset of seeds (n = 279 of the total 498, representing 33 families) were weighed to the nearest 0.1 mg before DNA extraction. Individual seeds were ground by hand in microfuge tubes with micropestles after removing the seed coat; samples were then incubated at 55 °C for 8–24 h, gently mixed, heated to 98 °C for 10 min, vortexed, centrifuged at 14,000 rpm for 5 min, and the supernatant stored at − 20 °C until PCR.

We genotyped each leaf sample using 12 microsatellite loci developed for *Z. marina*, multiplexed in three 11 μl PCR reactions (ESM Table 1). Each reaction consisted of 1 μl DNA template, 5 μl 2X Type-It multiplex master mix (Qiagen), and 0.25 μl of each 10 μM primer. PCR cycling conditions included initial activation/denaturation at 95 °C for 5 min, followed by 28 cycles of 95 °C for 30 s, 60 °C for 90 s, and 72 °C for 30 s, and final extension at 60 °C for 30 min. PCR products were separated on a 3730xl Genetic Analyzer (Applied Biosystems) at the Yale University DNA Analysis Facility, and fragment analysis was performed using GeneMarker version 2.6 (SoftGenetics). Nine of the 12 markers used for adult shoots proved useful for analyzing seed diversity. ZMC-12075 was eliminated due to a pattern of stutter around peaks that made scoring paternal alleles unreliable; two other markers (GA-3, CT-12) showed low allelic diversity in the adult populations and thus low power to discriminate among fathers (ESM Table 2).

Analyses

Diversity and Relatedness of Adult Shoots

First, we used GENCLONE 2.0 (Arnaud-Haond and Belkhir 2006) to determine clonal identity by calculating p_{sex}, the probability that two sampled ramets with the same multilocus genotype (MLG) could have arisen independently, via separate sexual events. When $p_{sex} < 0.01$, ramets sharing the same MLG are considered as belonging to the same clonal lineage

(Arnaud-Haond et al. 2007). Subsequent molecular analyses were conducted with GENODIVE 6.4 (Meirmans and van Tienderen 2004), unless stated otherwise.

We measured genotypic diversity, allelic diversity, and genetic relatedness in adult shoots at hierarchical spatial scales from quadrats to depth to site. Genotypic richness was measured as $R = (G-1)/(N-1)$, where G is the number of unique genotypes and N is the number of ramets sampled. We determined the genotypic richness (R), average allelic richness (A), and observed heterozygosity (H_O) of the 10 ramets sampled within each 0.25-m^2 quadrat, and we used STORM (Frasier 2008) to calculate their mean pairwise relatedness (r; all with all, including clonemates where $r = 1$). Pooling across quadrats and restricting the analysis to unique MLGs, we calculated the same metrics (R, Ho, and r) for the unique genotypes collected at each depth and site. To compare allelic diversity across locations that differed in total clonal richness, we calculated rarified allelic richness (A_E) and adjusted to reflect the smallest number of unique MLGs sampled using HP-rare (Kalinowski 2005).

Restricting the dataset to unique clonal lineages, we calculated standard population genetic indices for each depth and site, including expected heterozygosity (H_e) and the inbreeding coefficient F_{IS}. We calculated pairwise F_{ST} (Weir and Cockerham 1984) as a measure of population structure and used analysis of molecular variance (AMOVA) to partition adult genetic variance among depths and sites (Excoffier et al. 1992).

Diversity and Relatedness of Seeds

We measured allelic richness (A_E) in each seed family (i.e., all seeds collected from a single flowering shoot), rarified to the smallest maternal family size (6 seeds). To explore patterns of paternity, we first determined the minimum outcrossing rate in each seed family by scoring seeds for the presence of any non-maternal alleles. For those seed families with both putatively selfed and outcrossed seeds, we used a mixed model (random effect of family, fixed effect of seed mating type) to test whether half-siblings differed in mass depending on pollen source.

We then used two programs, GERUD 2.0 (Jones 2005) and COLONY (Jones and Wang 2010), to determine the number of possible paternal genotypes contributing to each seed family. GERUD uses an exclusion approach to determine the minimum number of sires necessary to produce a given progeny array when the maternal shoot's genotype is known; given a small number of informative loci, it will reconstruct possible paternal genotypes and rank the probability of different father combinations based on allele frequencies in the larger population (Jones 2005). COLONY uses maximum likelihood methods to infer parentage and assign offspring to full and half-sib groups, with fewer computational restrictions on the number of loci (Jones and Wang 2010). We compared paternal diversity across spathes that differed in the number of seeds genotyped by calculating a standardized number of sires as $S = (\text{sires} - 1)/(\text{seeds-1})$ for each maternal family, analogous to R for clonal richness.

To explore pollen dispersal distances, we used the likelihood ratios calculated in COLONY to infer potential fathers from candidate individuals (i.e., all MLGs detected at each depth). We also used a complementary approach to estimate genetic structure in pollen (*PhiFT*) based on gametic AMOVA (i.e., variation in alleles among sampled seed families) using TWOGENER (Smouse et al. 2001), executed within GENALEX (Peakall and Smouse 2006, 2012).

Statistical Analyses

To assess how adult and seed traits varied across the spatial scales sampled, we used linear models to test the fixed effects of site, depth, and their interaction, and we visually inspected residuals for normality and homogeneity. Count data (flowering and vegetative shoot density) and proportions (outcrossing rate) were overdispersed, and thus were analyzed with generalized linear models fit to a quasi-poisson and quasi-binomial error distribution, respectively (Zuur et al. 2009; Crawley 2015).

We used a generalized linear mixed model selection approach to test two sets of a priori hypotheses about possible relationships between adult and seed traits at the smallest (quadrat) scale. First, we tested for a relationship between the density of flowering shoots and local adult diversity (clonal richness, relatedness, and mean heterozygosity). Second, we explored relationships between adult traits (total shoot density, local clonal richness, and percent flowering) and seed diversity, measured as the number of unique sires per maternal seed family (S). We used maximum likelihood to fit nested models that included all individual and additive effects of fixed factors and a random effect of sampling location (depth within site), as well as a null model with an intercept of 1 and only the random effect. Model selection was based on Akaike information criterion corrected for small sample sizes (AICc; Burnham and Anderson 2002). The identification of the best candidate model was based on Akaike weight (wi), which was calculated as the model likelihood normalized by the sum of all model likelihoods. Alternate models were considered to have substantial support if their ΔAICc score was less than 2.0, and weights close to 1 indicate greater support for the model in the data (Johnson and Omland 2004).

Statistical analyses were conducted with R v. 3.6.0 (R Core Team 2019), using the packages lme4 (Bates et al. 2015), lmerTest (Kuznetsova et al. 2017), and "car" (Fox and Weisberg 2011). The package "AER" (Kleiber and Zeileis 2008) was used to test for overdispersion, "bbmle" (Bolker 2020) was used for model selection, and "visreg" (Breheny and Burchett 2017) was used to visualize the effects of factors in fitted models.

Results

Shoot Density, Morphology, and Seed Production

The density of vegetative shoots was greater at Niles than Curlew ($\bar{x} \pm SD = 17.8 \pm 11.02$ versus 12.5 ± 7.98 shoots per quadrat, corresponding to average densities of 280.9 ± 184.6 versus 208.0 ± 129.3 shoots per square meter, respectively; $\chi^2(1) = 9.26$, $p = 0.002$). However, both sites displayed the same qualitative pattern with depth: densest in the shallow areas at both sites and decreasing with increasing distance offshore ($\chi^2(2) = 115.15$, $p < 0.0001$; Fig. 1a). The density of flowering shoots, on the other hand, was highly variable and showed different patterns across the depth gradient at the two sites (depth*site: $\chi^2(2) = 16.59$, $p < 0.0003$). We recorded the highest floral densities in the shallow and mid regions at Curlew Beach (80.0 ± 60.2 flowering shoots/m^2); in contrast, flowering shoots at Niles showed an overall mean of less than one per 0.0625-m^2 quadrat (13.04 ± 23.5 shoots/m^2), with fewest in the center of the meadow (Fig. 1b).

Two other measures of sexual reproduction, the mean number of spathes and the mean number of seeds per spathe, also depended interactively on both site and depth (site × depth; spathes: $F_{2,54} = 12.7$, $p < 0.0001$; seeds: $F_{2,54} = 11.78$, $p < 0.0001$). Within each site, flowering shoots in the deep zone at Curlew had the most spathes, while those at Niles had the fewest (Tukey, $p < 0.05$; Fig. 1c). Flowering shoots at Curlew showed equivalent seed numbers regardless of location ($F_{2,27} = 0.96$, $p = 0.39$). In contrast, the number of seeds per spathe varied with depth at Niles ($F_{2,28} = 18.74$, $p < 0.0001$), with the lowest seed production occurring in inflorescences at the deep edge of the meadow (Tukey, $p < 0.05$; Fig. 1d).

Diversity and relatedness of adult shoots

All 12 loci used were polymorphic in both populations, displaying 3 to 21 alleles ($\bar{x} = 7.2 \pm 4.9$ alleles locus^{-1}; ESM Table 2). We identified 142 unique genotypes from the 541 samples collected from vegetative shoots (all MLGs had a $P_{sex} < 0.01$ with 12 loci). Most MLGs that were sampled more than once were collected from within a single 0.25-m^2 quadrat ($n = 57$ of 68 cases); none were sampled at more than one depth or site (ESM Table 3).

Each 0.25-m^2 quadrat sampled resulted in a range of one to six (Niles) or one to eight (Curlew) MLGs out of the ~10 ramets sampled. The genotypic richness (R) of interacting shoots showed contrasting patterns with depth at the two sites (site × depth, $F_{2,50} = 8.00$, $p = 0.001$; Table 1; Fig. 2a). At Niles, genotypic richness within quadrats was similar across the meadow ($F_{2,24} = 0.89$, $p = 0.4$), while at Curlew, local clonal richness varied with depth ($F_{2,26} = 9.89$, $p = 0.0006$) and was greater in the midzone than any other location sampled (Tukey, $p < 0.05$; Fig. 2a). Two measures of genetic diversity, allelic richness (A) and the pairwise relatedness of neighboring ramets (r), showed the same pattern as genotypic richness: the effect of depth depended on site (A: $F_{2,50} = 6.76$, $p = 0.003$; r: $F_{2,50} = 7.78$, $p = 0.001$), such that only Curlew

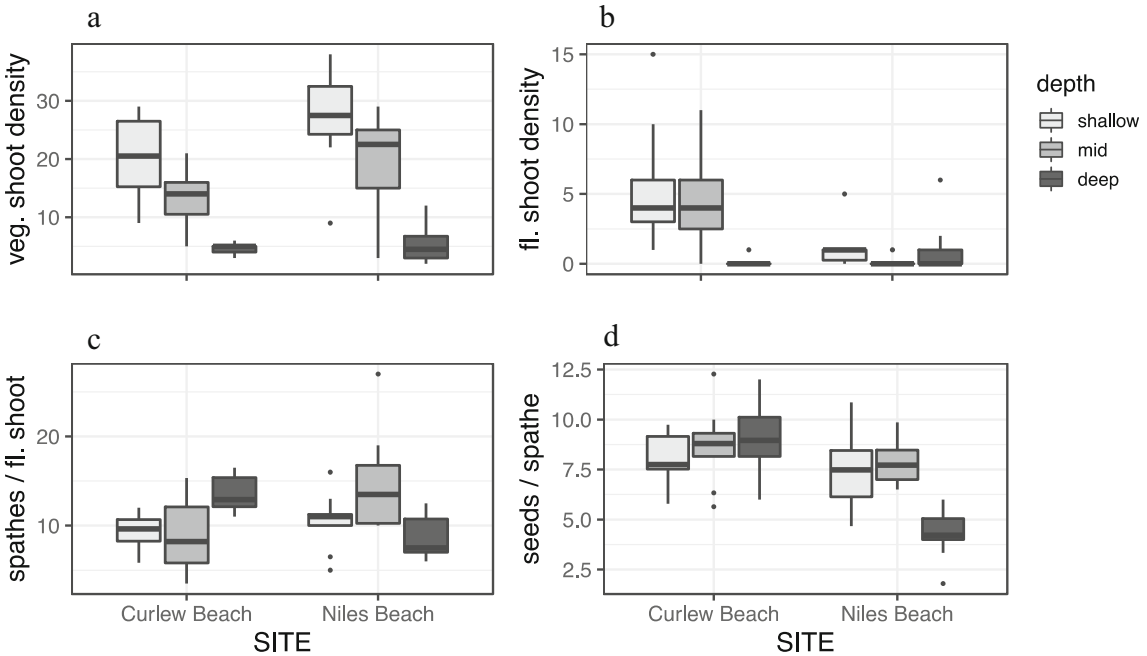

Fig. 1 Shoot morphology and reproductive effort. **a** Vegetative shoot density and **b** flowering shoot density (per 0.0625 m^2 quadrat; $n = 10$ quadrats per depth, 30 per site); **c** mean number of spathes per flowering shoot and **d** mean number of seeds per spadix ($n = 10$–24 flowering shoots per depth)

Table 1 Diversity of adult *Zostera marina* shoots at the quadrat scale (0.25 m^2). *N*, mean number of genotyped ramets within each quadrat; *G*, number of genets; *R*, genotypic richness, *H′*, Shannon index of genotypic diversity; *A*, mean number of alleles per loci; H_o, observed heterozygosity; *r*, mean pairwise relatedness, all with all (including clonemates). Values shown are the means calculated across $n = 8–10$ quadrats per depth, 27–29 per site

		N	*G*	*R*	*H′*	*A*	H_o	*r*
Curlew beach	Shallow	9.11	2.78	0.256	0.337	2.292	0.467	0.595
	Mid	10	5.56	0.511	0.649	2.975	0.488	0.207
	Deep	10	2.44	0.16	0.257	2.009	0.497	0.741
	Overall	9.72	3.72	0.314	0.42	2.44	0.484	0.506
Niles beach	Shallow	9.5	2.2	0.133	0.203	2.008	0.46	0.707
	Mid	9.88	1.75	0.085	0.139	1.844	0.616	0.882
	Deep	9.44	2.67	0.195	0.261	2.056	0.551	0.74
	Overall	9.59	2.22	0.14	0.203	1.975	0.536	0.77

showed an effect of depth (*A*: $F_{2,26} = 10.99$, $p = 0.0003$; *r*: $F_{2,26} = 10.90$, $p = 0.0004$), again with midzone quadrats displaying the greatest diversity and differentiation among neighbors (Tukey, $p < 0.05$; Table 1; Fig. 2b). Average heterozygosity, on the other hand, was marginally higher within quadrats at Niles than Curlew (site: $F_{1,50} = 3.68$, $p = 0.06$) and in the center of the meadow at both sites (depth: $F_{2,50} = 3.41$, $p = 0.04$; Table 1; Fig. 2c).

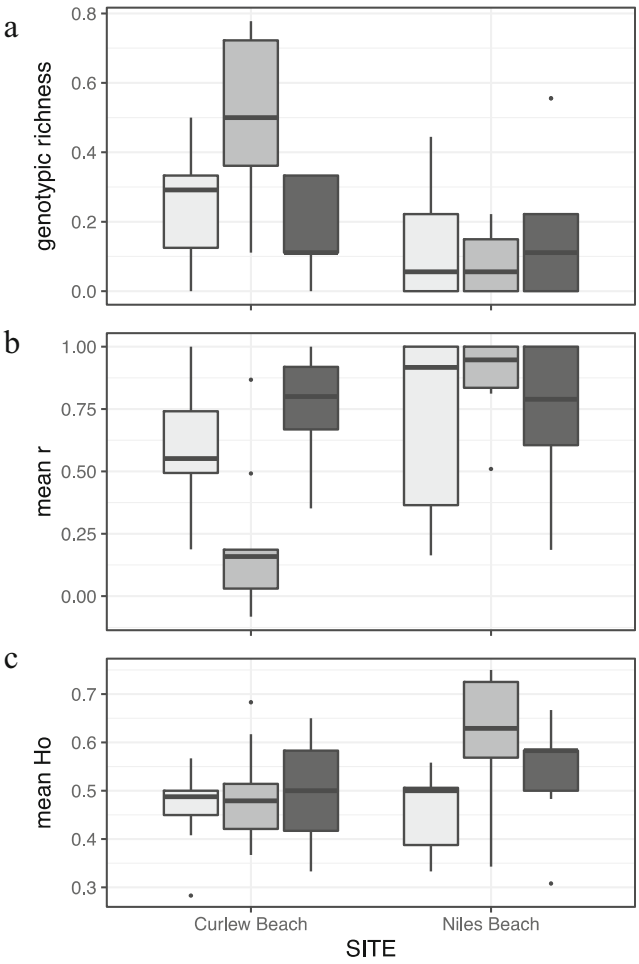

Fig. 2 Adult diversity at the quadrat scale. **a** *R*, mean genotypic richness; **b** *r*, mean pairwise relatedness, all with all (including clonemates); and **c** *Ho*, mean heterozygosity. $n = \sim 10$ ramets per quadrat, 8–10 quadrats per depth

Pooling across quadrats and using the reduced dataset (i.e., unique MLGs only), clonal diversity at Curlew followed the same spatial patterns seen at the smaller scale: total genotypic richness was highest in the center and lowest at the deep end of the meadow (resampling test implemented in GENODIVE: 1000 permutations, $p < 0.05$; Table 2). In contrast, at Niles, total genotypic richness was lowest in the mid zone (1000 permutations, $p < 0.001$), reflecting representation of some clones across multiple quadrats (ESM Table 3). The pattern of relatedness among unique genotypes also depended interactively on both depth and site ($F_{2,1405} = 32.69$, $p < 0.0001$). At Curlew, clones sampled within the shallow zone were most closely related to each other, while at Niles, the most closely related clones were found in the midzone (Tukey, $p < 0.05$, Table 2). The average pairwise relatedness of all unique genotypes at each site was weakly negative in both cases; this is consistent with limited gene flow, as negative values indicate lower relatedness than expected based on random mating.

Multilocus tests of HWE were nonsignificant at most sampling locations; the exceptions were the deep margin and overall at Niles, where we detected an excess of homozygotes (Table 2). Pairwise genetic distances (F_{ST}) indicated weak differentiation for all within-site comparisons except the mid and shallow at Curlew, which were not significantly differentiated (ESM Table 4). Hierarchical AMOVA also revealed modest structure across depth nested within site (3% of the total variation, ESM Table 5).

Diversity and Relatedness of Seeds

Allelic richness within each maternal seed family (rarified to six seeds, the smallest family size analyzed) ranged from 2.00

Table 2 Diversity of adult *Zostera marina* shoots at the scale of depth and site. *N*, number of genotyped ramets; *G*, number of genets; *R*, genotypic richness; *H′*, Shannon index of genotypic diversity; A_E, allelic richness rarefied to the minimum number of genets sampled within or between the sites; H_o, mean observed heterozygosity; H_e, expected population heterozygosity; F_{is}, inbreeding coefficient (italics indicate $p < 0.05$, significance tested by 10,000 permutations); *r*, the mean pairwise relatedness of unique MLGs

	Depth	*N*	*G*	*R*	*H′*	A_E	H_o	H_e	F_{is}	*r*
Curlew beach	Shallow	92	29	0.308	1.273	4.25	0.471	0.490	0.038	0.147
	Mid	100	42	0.414	1.421	4.69	0.551	0.542	− 0.017	− 0.071
	Deep	90	21	0.225	1.196	4.58	0.516	0.526	0.020	− 0.079
	Overall	282	92	0.324	1.964	5.60	0.513	0.519	0.007	− 0.031
Niles beach	Shallow	95	19	0.191	1.072	3.84	0.487	0.525	0.073	− 0.064
	Mid	79	9	0.103	0.675	3.17	0.537	0.498	− 0.078	0.115
	Deep	85	22	0.250	1.101	3.67	0.466	0.532	*0.124*	− 0.040
	Overall	259	50	0.214	1.699	5.50	0.497	0.519	*0.071*	− 0.037

to 3.67 alleles per locus. The pattern of diversity showed a marginal interaction between site and depth ($F_{2,45} = 3.05$, $p = 0.057$); when sites were analyzed separately, mean allelic richness was similar across the meadow at Niles ($F_{2,24} = 1.24$, $p = 0.31$) but differed with depth at Curlew ($F_{2,21} = 5.08$, $p = 0.016$), lowest for seed families produced in the shallow zone (Table 3).

Minimum outcrossing rates (scored unambiguously as the presence of a non-maternal allele at one or more loci) were high but variable across families ($\bar{x} = 0.79$; range = 0.33–1.0). Like allelic richness, outcrossing showed contrasting patterns with depth at the two sites (site*depth: $\chi^2(2) = 17.82$, $p = 0.0001$). Outcrossing rates were equivalent across the meadow at Niles Beach ($\chi^2(2) = 4.98$, $p = 0.08$) but varied with depth at Curlew ($\chi^2(2) = 19.18$, $p < 0.0001$), such that shallow seeds showed the lowest evidence of outcrossing (Tukey, $p < 0.001$; Table 3; Fig. 3a). Including all sampling locations and blocking by maternal family (to control for differences in seed development), seeds with non-maternal alleles were larger in mass than their half-sibs (10.28 ± 2.01 versus 9.130 ± 2.41 mg, $F_{1,172.9} = 17.35$, $p < 0.0001$; ESM Fig.1).

We calculated the expected exclusion probabilities for each marker with one parent (mother) known, and then selected the five most informative markers for paternity analysis in GERUD 2.0 (Jones 2005; ESM Table 6). Each maternal family required a minimum of two to four sires to generate each array of 6–12 seed genotypes in GERUD, while the maximum likelihood methods implemented in COLONY suggested four to ten sires per family. However, both programs showed the same qualitative patterns across space (Table 3). The effect of depth on paternal diversity differed between the two sites (e.g., data from COLONY, site × depth: $F_{2,45} = 3.59$, $p =$

Table 3 Diversity of *Zostera marina* seeds. *N*, number of maternal families (total seeds); A_E, mean number of alleles per loci rarefied to the smallest family size (6 seeds); *t*, minimum outcrossing rate; D_{Gerud}, mean number of sires per maternal seed family, exclusion-based approach; D_{Colony}, mean number of sires per maternal family, maximum likelihood-based approach; *PhiFT*, pollen structure, calculated via gametic AMOVA

		N	A_E	*t*	D_{Gerud}	D_{Colony}	*PhiFT*
Curlew beach	Shallow	9 (94)	2.28	0.65	2.44	6.44	0.215
	Mid	8 (77)	2.69	0.87	3.25	6.87	0.168
	Deep	7 (71)	2.59	0.88	3.43	8.14	0.061
	Overall	24 (242)	2.51	0.79	3.00	7.08	0.208
Niles beach	Shallow	10 (99)	2.62	0.83	3.00	6.90	0.146
	Mid	9 (88)	2.65	0.84	2.78	7.22	0.141
	Deep	8 (69)	2.39	0.74	2.25	5.25	0.325
	Overall	27 (256)	2.56	0.81	2.70	6.52	0.199

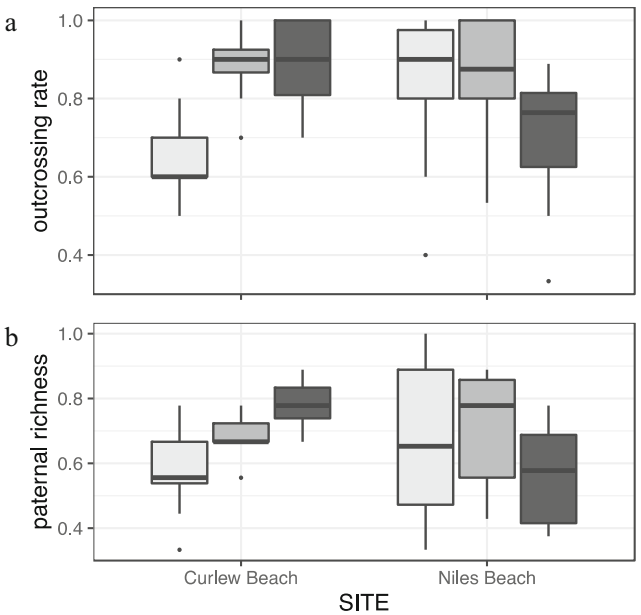

Fig. 3 Seed diversity. **a** *t*, minimum outcrossing rate; **b** paternal richness, measured as (1-number of unique sires)/(1-seeds genotyped) for each maternal seed family. $n = 242$ seeds from 24 families at Curlew Beach, 256 from 27 families at Niles Beach

0.036). Paternal diversity was equivalent across depth at Niles ($F_{2,24} = 1.36$, $p = 0.28$) but varied at Curlew ($F_{2,21} = 7.14$, $p = 0.0043$), such that the number of unique fathers contributing pollen to each seed family was lowest in the shallow zone (Tukey, $p < 0.05$; Fig. 3b).

COLONY identified candidate sires from our adult data set with likelihood ratios > 0.9 for 32% of seeds genotyped (81 of 242 seeds from Curlew, 75 out of 256 seeds from Niles). Of those sires, 79% were from the same depth zone as the maternal shoot at Curlew, and 87% of identified sires were from the same depth at Niles (Fig. 4). TWOGENER analyses provided additional evidence of limited pollen dispersal: *PhiFT* values were significantly greater than zero for all sampling locations (Table 3), indicating that individual flowering shoots were sampling partially non-overlapping pollen pools. Variation among mothers accounted for 16% (Curlew) and 18% (Niles) of the total variation in pollen genotypes at each site, more than that explained by depth in both cases (5% and 2%, respectively; ESM Table 7). When each sampling location was analyzed separately, the most spatial genetic structure in pollen was found in the shallow zone at Curlew (22% of the variation due to individual mothers), while at Niles, the greatest pollen structure was detected at the deep edge of the meadow (33%; ESM Table 8).

Linking Adult and Seed Characteristics Across Scales

Generalized linear mixed models support a relationship between neighbor genetic diversity and sexual reproductive effort: the best-selected model of flowering shoot density (lowest AICc) included significant additive effects of mean heterozygosity and clonal richness (Table 4), and two of the alternate models also provide support for mean pairwise relatedness (both with $\Delta AIC_c < 2.0$; ESM Table 9). The abundance of flowering shoots increased with clonal richness and decreased with the relatedness and mean heterozygosity of neighboring adult shoots (Fig. 5, ESM Table 10). However, AIC weights were modest (0.31), and the best-selected model explained only 26% of the total deviance.

Table 4 Analysis of deviance table for the best-selected mixed effect model of flowering shoot density (number counted per 0.0625-m^2 quadrat), fit to a negative binomial error distribution (theta = 0.85). Change in deviance and corresponding chi-square *p* values were obtained for each fixed predictor by sequentially dropping each one; model coefficients are reported in ESM Table 10

Effect	d.f	Deviance	Residual d.f	Residual dev.	*p*
Null			55	76.10	
Heterozygosity	1	10.47	54	65.63	0.0012
Genotypic richness	1	9.46	53	56.17	0.0021

We found no clear relationships between seed diversity (measured as sire richness) and adult traits measured at the quadrat scale: none of the individual or additive models of percent flowering shoots, total shoot density, or adult genotypic richness provided a better fit to the data than the null model (effect of sampling location only; ESM Table 11). The only candidate model with marginal explanatory power was total shoot density; this differed between sites, such that the number of unique sires pollinating each inflorescence decreased with increasing shoot density at Curlew ($F_{1,22} = 5.39$, $p = 0.029$) but not at Niles (ESM Fig. 2).

Discussion

Our study was motivated by the need to understand how fundamental characteristics of *Z. marina*'s sexual mating system contribute to variation in the diversity and kin structure of neighboring shoots, which has cascading effects at the community and ecosystem levels. We documented a wide range of relatedness and clonal richness in adult shoots at the quadrat

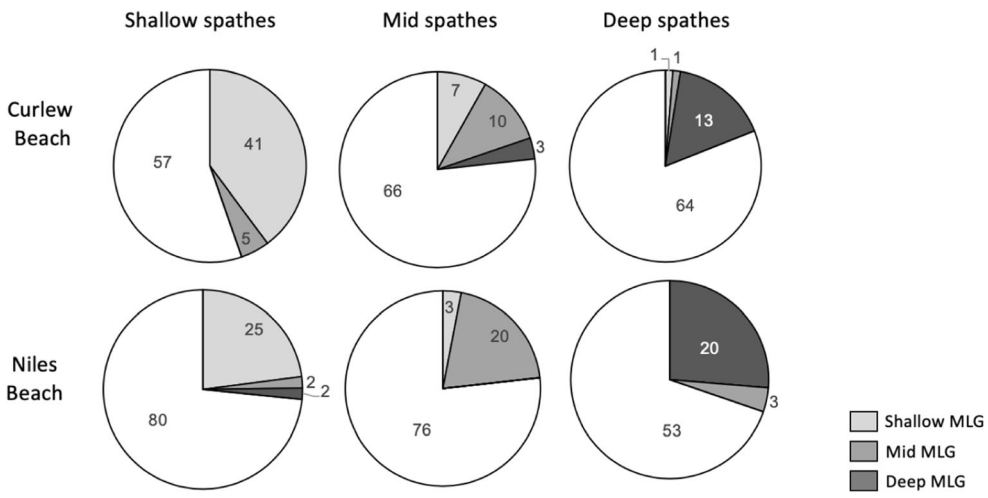

Fig. 4 Results of likelihood-based paternity assignments from the pool of candidate genotypes from each site using COLONY (Jones and Wang 2010). Color of the pie slice indicates location (depth) of identified father; white indicates seeds that could not be assigned with a likelihood ratio > 0.9

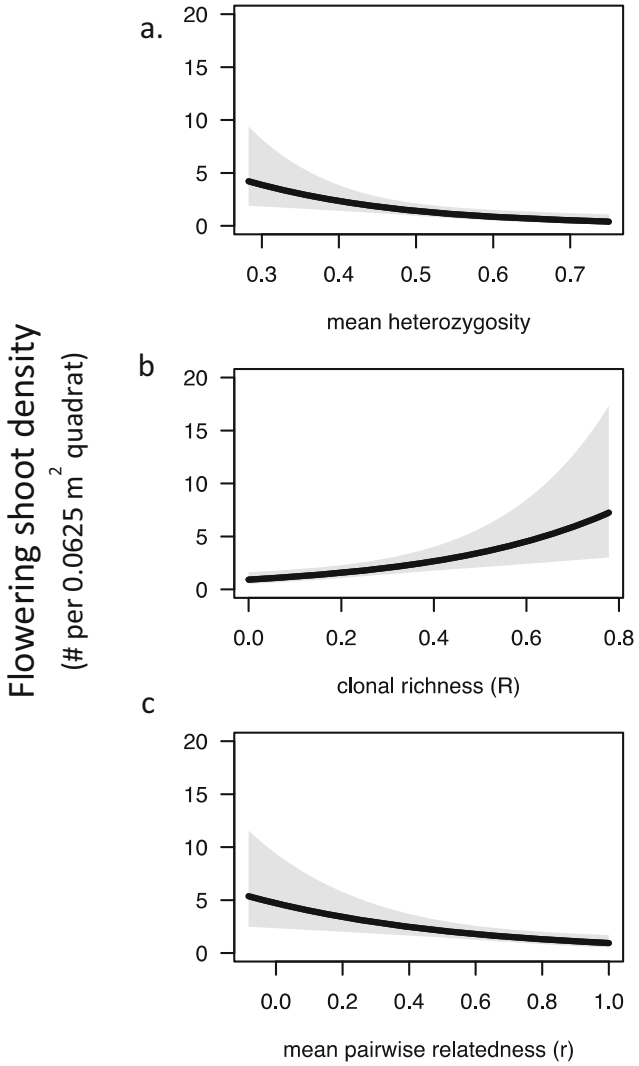

Fig. 5 Relationships between flowering shoot density and **a** mean heterozygosity, **b** clonal richness, and **c** mean pairwise relatedness of vegetative shoots at the local, quadrat scale (0.0625 m^2). Solid lines correspond to the predictions of the best-fit generalized linear models (ESM Table 5, ESM Table 9), shaded areas define the 95% confidence intervals around fitted values

scale, commensurate with that found in other studies (Hughes and Stachowicz 2009, Kamel et al. 2012, Reynolds et al. 2017). Yet, neighbor diversity showed strikingly different patterns across a similar range of depths (~ 1 to 4 m MLLW) spanned by *Z. marina* at the two sites (Fig. 2a): depth had a more consistent unimodal relationship with diversity at Curlew and was of varying importance at Niles. Kamel et al. (2012) also found that the effect of depth on quadrat-scale diversity varied across locations, documenting higher clonal richness and lower relatedness in the intertidal than in the shallow subtidal at some sites (Bodega Bay) but not others (Tomales Bay). Notably, differential patterns of local diversity across depths at these sites have remained stable for over a decade (Reynolds et al. 2017), suggesting that the underlying processes generating these differences are ongoing.

Total clonal richness (pooling across quadrats) also showed opposing patterns with depth at the two sites: the center of the meadow had the highest total genotypic richness at Curlew, but the lowest at Niles, where clones also appear to be larger (i.e., more cases where the same MLG was detected across multiple quadrats) with the highest mean heterozygosity (Table 2). Other studies that assessed *Z. marina* diversity across depth typically reported lower diversity in deeper water (e.g., Ruckelshaus 1998; Oetjen et al. 2010; Ort et al. 2012; Kamel et al. 2012; Reynolds et al. 2017; Kim et al. 2017), but they compared only two depths, and in most cases, depth represented different tidal regimes (i.e., intertidal vs. subtidal; Ruckelshaus 1998; Oetjen et al. 2010; Kamel et al. 2012; Reynolds et al. 2017). However, one of the Korean sites examined in Kim et al. (2017) was both fully subtidal and included a mid-depth comparison; in this seagrass meadow, clonal richness was highest at intermediate depths, similar to what we found at Curlew.

Across depths and sites, the density of flowering shoots correlated loosely with clonal richness, but no other measure of adult diversity (i.e., pairwise relatedness, allelic richness, or heterozygosity; ESM Fig. 3). We also found significant relationships between measures of adult diversity and flowering shoot density at the quadrat scale, where the underlying mechanism could be occurring in either direction. Increased flower density can lead to increased clonal richness if seed input limits recruitment (e.g., Johnson et al. 2020); however, the directionality may also be reversed, if the diversity and relatedness of interacting shoots influences how ramets allocate energy to sexual versus asexual reproduction. Jahnke et al. (2015) found that flowering intensity in *Posidonia oceanica* was strongly influenced by genetic factors, such that the number of flowers was positively related to individual heterozygosity and neighbor relatedness, and negatively related to clonal richness. We found the opposite pattern here: at the level of individual quadrats, flowering shoot density increased with clonal richness and decreased with the relatedness and mean heterozygosity of neighboring shoots (Fig. 5).

Two independent analytic approaches (likelihood-based paternity assignments and gametic AMOVA) confirmed restricted pollen flow at our sites. For those seeds for which a candidate sire could be identified (34%), the majority (83%) were matched to an adult genotype from the same depth as the maternal shoot, indicating local retention of pollen within depths. Prior estimates of pollen dispersal in *Z. marina* are low but variable, suggestive of site-specific hydrodynamic effects. Ruckelshaus (1996) measured dispersal directly (via capture of pollen grains on a slide array) and found a maximum distance of 15 m over unvegetated habitat. Reusch (2003) found even more restricted pollen dispersal, with pollen limitation occurring at densities of less than 5 flowering shoots per m^2 in a dense meadow. Furman et al. (2015)

recorded individual pollination events occurring from 0.57 to 73.91 m, with a median of 9.1 m, which is broadly consistent with our findings. Furthermore, the cross-shore distance from shallow to deep at both of our sites is approximately 300 m, well exceeding Furman et al.'s (2015) maximum, and differences in flowering phenology with depth may further restrict cross-gradient gene flow (von Staats et al. 2020). Nevertheless, individual flowering shoots within depths explained more of the total variance in pollen genotypes than depth per se (16–18% versus 2–5%), which suggests that the majority of pollen dispersal decays quickly with distance, irrespective of the depth gradient.

By genotyping multiple developing seeds from each spathe (6–15 seeds, mean = 9.7 per maternal family), we were able to explore relatedness among seeds produced by the same flowering shoot. *Z. marina* pollen is filiform and released from anthers in stringy clumps that tend to stick together (De Cock 1980); if pollen clouds remain cohesive in the water column, this could lead to correlation in pollination success and shape resulting seed diversity. Furman et al. (2015) tested single seeds from spathes and found evidence for spatial correlation (i.e., the same putative father pollinating adjacent flowering shoots), which they interpreted as evidence for pollen cohesion tens of meters from the point of release. Using both likelihood- and exclusion-based approaches to infer sibships in our dataset, we found that while all maternal families ($n = 51$) contained at least two seeds that were assigned to the same putative father, none had seeds that could have been sired by a single genotype. Thus, while pollination in *Z. marina* is not a "winner takes all" process, full sibling seeds are common, providing another mechanism (along with clonality and limited seed dispersal) that may lead to spatial clustering of kin groups in *Z. marina* and opportunity for ecological interactions among relatives across a range of relatedness values.

Surprisingly, the flowering shoots that produced the lowest diversity seeds were found at the shallow margin of the meadow at Curlew. This may reflect "pollen swamping" by nearby flowering shoots, via the combined effects of high shoot density and intermediate clonal richness. High shoot density reduces laminar flow in eelgrass meadows (Fonseca et al. 1983; Peterson et al. 2004); this could limit the area over which pollen could move, and lead to more paternities (i.e., larger family size) for more proximate sires. Generalized linear model selection found no relationship between seed diversity and adult clonal richness or percent flowering shoots, but modest support for an inverse relationship with shoot density, particularly at Curlew. Stubler et al. (2017) found seed production was negatively correlated with seagrass cover within a 2- or 4-m^2 buffer zone around their focal plants and interpreted this as a consequence of hydrodynamics. We saw no reduction in seed production in the shallow zone at Curlew, indicating that pollen was not limiting; however, the diversity of this pollen was apparently lower than elsewhere in the meadow, where either clonal richness was higher (the midzone) or shoot density was lower (deep). Niles complicates this interpretation: shallow and mid sites at Niles Beach had significantly higher vegetative shoot density and lower flowering shoot abundance, so we would predict more pollen limitation under this scenario than was observed. There was no clear effect of depth on paternal diversity in seeds produced at Niles, but variation among maternal families was much higher than at Curlew, suggesting that patchiness and unequal distribution of flowering shoots within the meadow (C. Hays, personal observation) and/or variation in the size of clones may be more important than depth per se.

The outcrossing rates documented here were variable across maternal families but high overall ($\bar{x} = 0.79$; range = 0.33–1.0), consistent with the relatively few other studies that have assessed mating system in *Z. marina* by genotyping seeds (e.g., Ruckelshaus 1995; Reusch 2000, 2001; Furman et al. 2015), as well as recent studies that found high outcrossing in other monoecious seagrass species (Sinclair et al. 2014; Zipperle et al. 2011). When seeds from the same maternal family were compared, those that were unambiguously outcrossed (i.e., possessing one or more non-maternal alleles) were significantly larger in mass than half-siblings that possessed only alleles shared with the maternal shoot. This difference could reflect differential maternal investment or possibly a difference in the timing of fertilization: in a greenhouse experiment, Hammerli and Reusch (2003) found that *Z. marina* stigmas exposed only to pollen from the same clonal lineage stayed receptive longer than those exposed to a mix of pollen genotypes, resulting in greater overlap between male and female function within spathes and ultimately a lower seed set. We genotyped embryos, not fully mature seeds; it is unknown whether observed differences in mass would be maintained until seed release. However, fitness costs of selfing (Ruckelshaus 1996; Reusch 2001) and biparental inbreeding (Billingham et al. 2007) have been documented in *Z. marina*, the magnitude of which are variable across sites and regions (e.g., see Rhode and Duffy 2004).

When we compare the selfing rates calculated from genotyped seeds to the genetic diversity of adults at our two sites, we find a disconnect between seed input and adult genetic structure. We detected a significantly positive F_{IS} in the deep zone and overall at Niles, indicating that clonal lines are more closely related than expected under a model of random mating, but the highest selfing rates documented in our study were at Curlew, in the shallow portion of the meadow. Seedling recruitment is likely to be negatively impacted by interactions with adults; for example, Johnson et al. (2020) found that experimental seed additions led to increased seedling recruitment, but seedlings survived only when they were greater than 15 cm from adults. Thus, competition with adults may generate stronger selection against selfed seeds in the high-density shallow and mid zones than

the deep zone, where patches and gaps in the canopy are more common and overall shoot density is lower (Fig. 1a). Olesen et al. (2017) observed natural seedling recruitment across a depth gradient in a temperate meadow, but no survival past the end of the summer, and found that experimentally outplanted seedlings survived only when planted in gaps at the deep edge. It is possible that the midzone functions as a buffer, supporting continued regrowth of shallow and deep margins of the meadow, but via different mechanisms: shallow regions may largely be regenerated from vegetative expansion and deep regions from seedling recruitment (Olesen et al. 2017).

By assessing patterns of genetic diversity at multiple scales and across a depth gradient in subtidal seagrass beds, we were able to identify key similarities across sites, as well as detect site-specific variation and patterns that would have been missed by comparing only the ends of the environmental gradient. Our results confirm the likelihood of spatially clustered kin groups and small-scale variation in diversity and relatedness in natural eelgrass populations that can impact population and community processes (e.g., Hughes and Stachowicz 2004, Stachowicz et al. 2013). However, we found a disconnect between seed and adult genetic structure across depths, suggesting that mating system variation within seagrass populations interacts with abiotic and biotic factors to determine genetic diversity across environmental gradients and life history stages. This disconnect complicates seagrass management efforts, as it suggests that a single, readily measured metric (e.g., proportion of flowering shoots) does not always predict patterns of genetic diversity or population structure. Future studies of spatial variation in mating system parameters are needed to understand when and how sexual reproduction acts to shape standing genetic variation in seagrass populations, particularly in light of global change.

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