

Assessment of genetic diversity of seagrass populations using DNA fingerprinting: Implications for population stability and management

(hypervariable minisatellite DNA/eelgrass/population genetics/gene flow/marine macrophyte)

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ABSTRACT Populations of the temperate seagrass, *Zostera marina* L. (eelgrass), often exist as discontinuous beds in estuaries, harbors, and bays where they can reproduce sexually or vegetatively through clonal propagation. We examined the genetic structure of three geographically and morphologically distinct populations from central California (Elkhorn Slough, Tomales Bay, and Del Monte Beach), using multilocus restriction fragment length polymorphisms (DNA fingerprints). Within-population genetic similarity (S_w) values for the three eelgrass populations ranged from 0.44 to 0.68. The Tomales Bay population located in an undisturbed, littoral site possessed a within-population genetic similarity ($S_w = 0.44$) that was significantly lower than those of the other two populations. Cluster analysis identified genetic substructure in only the undisturbed subtidal population (Del Monte Beach). Between-population similarity values (S_b) for all pairwise comparisons ranged from 0.47 to 0.51. The three eelgrass populations show significantly less between locale genetic similarity than found within populations, indicating that gene flow is restricted between locales even though two of the populations are separated by only 30 km. The study demonstrates that (i) natural populations of *Z. marina* from both disturbed and undisturbed habitats possess high genetic diversity and are not primarily clonal, (ii) gene flow is restricted even between populations in close proximity, (iii) an intertidal population from a highly disturbed habitat shows much lower genetic diversity than an intertidal population from an undisturbed site, and (iv) DNA fingerprinting techniques can be exploited to understand gene flow and population genetic structure in *Z. marina*, a widespread and ecologically important species, and as such are relevant to the management of this coastal resource.

Seagrasses form extensive meadows along the shores of all but the polar seas (1). Where they are found, these submerged marine angiosperms structure nearshore food webs and are highly productive (2, 3). Seagrass systems worldwide serve as important and often critical habitats for a broad diversity of invertebrate and fish species, many of which are economically important (3, 4). In addition, seagrasses protect coastlines by minimizing erosion; increasing sedimentation, leading to enhanced recycling of nutrients; and improving water clarity (5).

Zostera marina L., or eelgrass, is the dominant seagrass species in temperate waters and can achieve production rates exceeding 4 g of carbon $m^{-2} \cdot day^{-1}$ (2). Eelgrass reproduces both sexually and vegetatively and can colonize to depths of 30 m in clear waters but typically is restricted to shallow or

intertidal depths in many estuaries (1–3, 6, 7). Although eelgrass is ecologically successful in very low-light environments ($<100 \mu mol$ of quanta $m^{-2} \cdot s^{-1}$; refs. 6 and 7), the reduction in light penetration found in most industrialized coastal regions has severely restricted the depth distribution and abundance of eelgrass and other seagrass species (8–11).

Losses worldwide of seagrass beds have accelerated at alarming rates in the last two decades because of physical disturbance (e.g., dredging, coastline development, fishing practices) and water quality deterioration most often realized as enhanced light attenuation by the water column because of particle loading, eutrophication, and nuisance algal blooms (8–11). Though some of the proximal causes for seagrass loss are increasingly evident (8–12), the importance of genetic diversity and gene flow for resource stability is unknown. The poor knowledge of the minimal habitat requirements for seagrass growth, colonization and establishment mechanisms, genetic diversity, and reproductive modes requisite for the maintenance of ecologically successful populations hinders the development of sound management criteria (see ref. 13).

Previous investigations examining isozyme polymorphisms revealed essentially no genetic diversity within populations and a low level of genetic distinction between geographically disjunct populations of eelgrass (14, 15). These findings, in conjunction with the known vigorous rhizomatous growth of this and other seagrass species, have led to the notion (3, 15) that the wide distribution and general ecological success of seagrasses are based upon a vegetative growth strategy. Consequently, a high degree of genetic similarity within populations would be expected.

Recently, Fain *et al.* (16) used comparative restriction analyses of nuclear DNA encoding RNA (rDNA) [restriction fragment length polymorphisms (RFLPs)] to demonstrate genetic distinctions between geographically disjunct eelgrass populations from California that were correlated with leaf morphology and habitat depth. Estimated sequence variations (17) between the four eelgrass populations examined ranged from 0.00 to 0.69, and three of the populations could be distinguished by unique RFLPs (16). These studies verified genetic diversity between seagrass populations (14, 15) but did not reveal significant within-population genetic diversity.

We used multilocus minisatellite DNA fingerprinting techniques, which have been shown to resolve genetic variation at the level of individuals (18) and populations (19), to assess the genetic structure and the extent of gene flow between three geographically disjunct eelgrass populations from the central California coast. We sought to determine whether (i)

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Abbreviation: RFLP, restriction fragment length polymorphism.

populations that were morphologically distinct and/or showed different depth distributions could be distinguished by DNA fingerprinting, (ii) eelgrass was characterized by a high degree of clonal reproduction, and (iii) physical disturbance influences genetic diversity. It is anticipated that molecular characterizations such as those described here can be used for mitigation, restoration, and management of this and other marine macrophyte resources.

MATERIALS AND METHODS

Individual *Z. marina* L. plants were collected at 5-m intervals along transects within three central California locations (Fig. 1). The Del Monte Beach population forms an extensive subtidal meadow from -3 to -13 m depth (meters below sea level at low tide); samples were collected from -7 to -9 m depth. At the Elkhorn Slough site, plants occur in a patchy, exclusively intertidal bed in a disturbed habitat (20), while in Tomales Bay plants extend from the intertidal region to depths of -5 m.

Plants were cleaned of epiphytes and stored frozen (-70°C) until DNA was extracted from leaf tissue (21) and purified on CsCl gradients. DNA (6 µg) was digested with 40 units of seven restriction enzymes (*Rsa* I, *Bam*HI, *Hae* III, *Dra* I, *Eco*RI, *Hin*fI, and *Alu* I) according to the manufacturer's protocols (New England Biolabs). The restriction enzyme *Hae* III consistently yielded the largest number of fragments over the range in length from 2 to 23 kb and, therefore, was used in all subsequent analyses. DNA restriction fragments were size-fractionated in 0.8% agarose gels at 40 V for 30 hr with 45 mM Tris borate/1 mM EDTA buffer, transferred to nylon membranes (HyBond-N, Amersham) by standard capillary methods, and cross-linked to membranes by baking at 80°C for 30 min (22). Membranes were prehybridized for 1 hr at 37°C in hybridization buffer [6× SSC (0.9 M NaCl)/0.09 M sodium citrate dihydrate, pH 7.0]/1× Denhardt's reagent (0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/1% SDS] and then transferred to hybridization buffer containing a digoxigenin-conjugated dUTP-labeled 781-bp *Cl*a I/*Bsm* I fragment from M13mp18 replicative form 1 (RF1) DNA (Sigma) (23) for 16 hr at 37°C according to Boehringer Mannheim protocols. Membranes were washed twice for 10 min at room temperature in 2× SSC/0.1% SDS, once in 2× SSC/0.1% SDS at 42°C for 10

min, once in 0.7× SSC/0.1% SDS at 42°C for 15 min, and twice more with 2× SSC at room temperature for 5 min. Chemiluminescent detection of hybridized probe was accomplished by incubating the membranes with LumiPhos according to manufacturer (Boehringer Mannheim Biochemicals) recommendations and exposing to Kodak XAR-5 film.

A video-image analysis system (BioImage, Millipore) was used to determine relative migration distances of all restriction fragments and to estimate the length of fragments by using standard curves generated from *Hind*III-digested phage λ DNA marker. The λ DNA size fragments (23–2 kb) were visualized with ethidium bromide, and a ruler was used to measure migration distances. Standard deviations for all sample bands on each gel were calculated from least-squares residual variation in migration distance of the phage λ DNA standards on each gel after correction for systematic variation in migration distance across lanes using polynomial regression. Coefficients of variation were then regressed against molecular size, and the resulting equations were used to calculate standard deviations in molecular size for each sample band.

Average genetic similarity was assessed by making all possible pairwise comparisons among all individuals and calculated as $S = 2m_{ij}/n_i + n_j$, where m_{ij} is the number of matching bands between a pair of individuals i and j , and n is the number of bands possessed by individuals i and j . Bands were scored as matching when the calculated molecular sizes fell within 3 standard deviations of each other. To determine if average genetic similarity, both within (S_w) and between (S_b) populations, was significantly different among the populations, band-sharing data were analyzed with SIM, an MS-DOS-based program developed by us that corrects the variances for nonindependent sampling as described by Lynch (24).

Cluster analyses of the within-population similarity (S_w) matrices were performed with the CSS (Complete Statistical System, version 2) statistical package using the amalgamation procedure of Ward (25) to examine substructure in the populations based on S_w . Statistical significance of the relationships between physical distance and genetic distance within populations ($D = 1 - S_w$) was determined by comparing the mean physical distances (in meters) between the most genetically similar pairs of individuals identified by the cluster analysis within each population to the mean physical distance (in meters) of 1000 randomly paired synthetic individuals by means of Student's t test. Our purpose was to determine if pairs of most genetically similar individuals were physically located closer to each other than might be expected from a random distribution.

RESULTS AND DISCUSSION

DNA fingerprints of the three geographically disjunct *Z. marina* populations from central California typically revealed ≈20 bands in the 2- to 23-kb range with a high degree of polymorphism evident above 4.4 kb (Fig. 2). Fingerprints generated from the same plant always revealed complete identity of the banding patterns, and calculated S values using SIM were not significantly different from 1 (data not shown; see below).

Average within-population genetic similarity (S_w) for the Del Monte Beach, Elkhorn Slough, and Tomales Bay seagrass beds ranged between 0.44 and 0.68 (Table 1). In addition to occurring over different depth ranges, the different eelgrass populations can be distinguished by shoot morphology (Table 1; see ref. 26). The Del Monte Beach plants are exclusively subtidal and have tall shoots (1 m) with wide blades (1.5 cm). Elkhorn Slough plants are only intertidal, and shoots are short (0.5 m) with narrow blades (0.5–1.0 cm). In Tomales Bay, plants are distributed over a wide depth

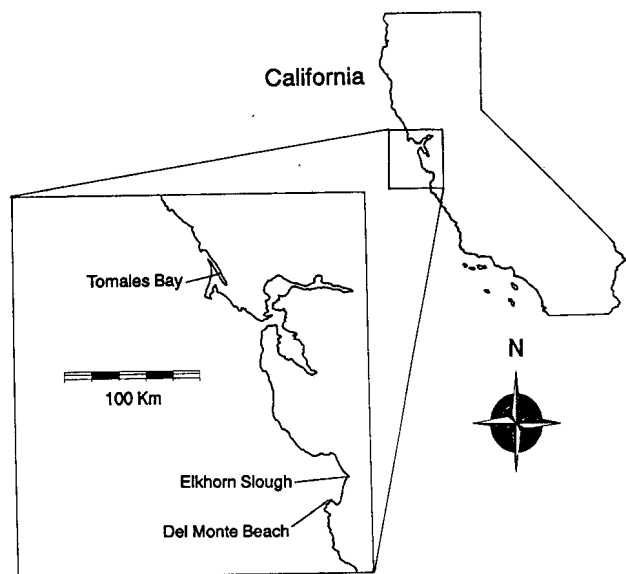


FIG. 1. Map of California indicating the locations of the three *Z. marina* populations (Tomales Bay, Elkhorn Slough, and Del Monte Beach) examined in this study.

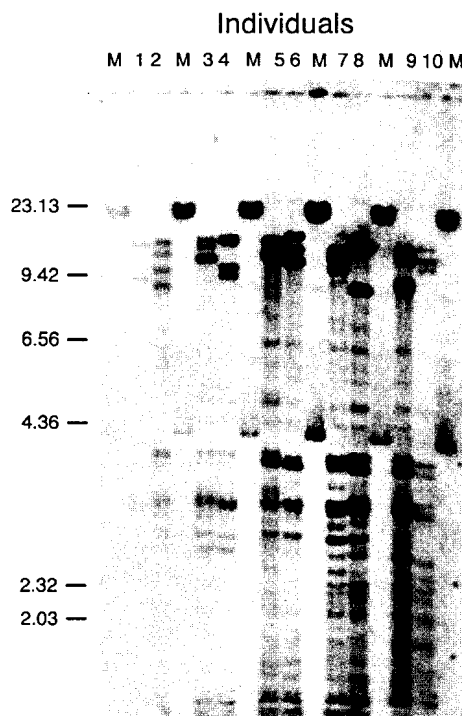


FIG. 2. Representative DNA fingerprints of individual plants of *Z. marina* from Tomales Bay, California. Each sample lane (1–10) contains 6 μ g of genomic DNA digested with *Hae* III and probed with M13. Molecular size markers (lanes M) are *Hind*III-digested phage λ DNA. Sizes are shown in kb.

range from the intertidal region to the subtidal region (0.5 to –5 m). These plants are characterized by narrow blades (0.5–1.0 cm) and show depth-dependent variation in canopy height (0.5–1.5 m).

While the Del Monte Beach and Tomales Bay habitats are relatively undisturbed, the Elkhorn Slough site is a highly disturbed intertidal environment that has lost 95% of its historical seagrass habitat through dredging activities and erosion (20). The Del Monte Beach population, which is subtidal and quite morphologically distinct from the other populations, possesses a relatively high level of within-population genetic similarity ($S_w = 0.68$; Table 1). The Elkhorn Slough population, which is intertidal, possesses a within-population genetic similarity of 0.62, which is significantly greater than that found in the other intertidal population in Tomales Bay ($S_w = 0.44$; Table 1). The greater degree of genetic diversity in Tomales Bay may result from a broader gradient in the physical environment (depth and exposure at low tides) relative to the Elkhorn Slough site, thereby accommodating discontinuous genotypic variation (27) that might lead to a high degree of ecotypic differentiation (26, 28). It is equally possible that the Elkhorn Slough population has a much reduced genetic diversity as a result

of the extensive physical disturbance at this site over the past 30 years.

Though not directly comparable because of differences in gel running conditions and data analysis, within-population genetic similarity values obtained from DNA fingerprints for two perennial species, box elder (*Acer negundo* L.; $S_w = 0.48$ – 0.68 ; ref. 29) and poplars (*Populus tremuloides*; $S_w = 0.35$ – 0.49 ; ref. 30) fall within the range reported here for perennial populations of *Z. marina*. Further, within-population similarity values obtained with the experimental techniques and data analyses described here on the giant kelp, *Macrocystis pyrifera*, which has only a sexual reproductive mode, are similar to those found for eelgrass ($S_w = 0.29$ – 0.81) (34).

Eelgrass can reproduce sexually through seed production or vegetatively through rhizomatous growth or vegetative propagules. It has been inferred because of low rates of seedling recruitment, high seedling mortality, and minimal isozyme polymorphism among individuals (14, 15) that most eelgrass populations possess little genetic diversity and are predominantly clonal (3, 14, 15). Since S_w in the three populations examined is uniformly lower than would be expected for clonal or highly interbreeding populations (refs. 31 and 32; Table 1), two scenarios are possible. First, the immigration of new genotypes is infrequent, and existing populations were each founded by a few individuals. Although few in number, these founders would presumably be genetically distinct and would establish within-population S at some initial moderate value. As such, only sexual reproduction could maintain the levels of genetic variability presently observed in the populations. Second, the immigration of new genotypes is frequent, and existing populations (especially Del Monte Beach; see below) were founded by many genetically different individuals, providing very high initial genetic diversity. If eelgrass were clonal and clones persisted vegetatively for long time periods and if our analysis were comprised of individual samples from genetically distinct clones, then the second scenario would result in a vegetatively reproducing “population” of clones maintaining high genetic variability and thereby “appearing” to reproduce sexually. Since the localities we examined generally exhibited S_w values lower than would be expected for clonal populations and our sampling strategy precluded the possibility that we inadvertently sampled a number of distinct clones, it is most likely that the predominant mode of reproduction that leads to the current eelgrass populations was sexual.

To examine the spatial patterns of similarity along transects at each site, individuals were clustered according to their genetic distance by using S_w ($D = 1 - S_w$; Table 2 and Fig. 3). Only the Del Monte Beach plants showed a significant non-random spatial pattern of similarity among individuals distributed along a relatively constant depth transect through the bed (Fig. 3 and Table 2); random distributions of genotypes along the intertidal transects were observed at Elkhorn Slough and Tomales Bay. The high degree of genetic similarity among physically close individuals in the Del Monte population

Table 1. Within-population similarity indices, depth distributions, habitat types, and leaf and shoot morphologies for geographically disjunct populations of *Z. marina* from central California

Population	Depth distribution, m	Habitat	Plant morphology		Fingerprint analysis				
			Leaf width, cm	Shoot height, m	S_w	95% CI	n	N	n^*
Del Monte Beach	–3 to –13 (subtidal)	Undisturbed	1.5	1.0	0.68	0.60–0.76	20	21	190
Elkhorn Slough	0.3 to –3 (intertidal)	Disturbed	0.5–1.0	0.5	0.62	0.60–0.64	19	22	171
Tomales Bay	0.5 to –5 (intertidal)	Undisturbed	0.5–1.0	0.5–1.5	0.44	0.42–0.46	20	23	190

Within-population genetic similarity (S_w) was determined by making all possible pairwise comparisons within the sample set and correcting the variance for nonindependent sampling and variance in the migration distance of the marker (see *Materials and Methods*). 95% CI, 95% confidence interval derived by using critical values for two-tailed Student's t distributions; n , number of individuals; N , mean fragment number per individual; n^* , number of pairwise comparisons used to calculate mean similarity.

Table 2. Results of *t* tests comparing the mean physical distance among pairs of most genetically similar individuals within each population as identified by the cluster analysis to 1000 randomly generated synthetic pairs

Population	Pairs, no.	Mean distance, m	SD	df	<i>t</i>	<i>P</i>
Random pairs	1000	35	25	—	—	—
Del Monte Beach	8	15	12	1006	2.26	<0.05
Elkhorn Slough	8	28	21	1006	0.79	>0.40
Tomales Bay	9	33	15	1007	0.23	>0.50

The null hypothesis (H_0) is that the mean physical distance between pairs of randomly selected individuals is equal to the mean physical distance between genetically similar pairs identified by cluster analysis. Only the Del Monte Beach population shows a significantly nonrandom spatial distribution among pairs of genetically similar individuals.

suggests that seedlings were recruited successfully near parental genotypes. However, our casual observations in the past 5 years suggest that seedling recruitment is a rare event at the Del Monte site. Thus, the observed patterns might be a remnant of the original colonization event or may reflect seedling recruitment in the wake of periodic disturbances (such as heavy storms) that have not occurred in the past 5 years of unusual drought in California. In contrast, the Elkhorn Slough bed is extremely patchy, and shoot densities are low. High current speeds and chronic erosional disturbance in this locale probably help to randomize seed distributions and maintain ample space for seedling establishment, thereby randomizing the distribution of genotypes. The Tomales Bay meadow is distributed along a steep physical gradient from a high intertidal depth to a subtidal depth, yielding a diversity of potential establishment sites for seedlings that could account for a random distribution of genotypes.

Between-population similarity values (S_b) ranged from 0.47 to 0.51 (Table 3). The statistic of population subdivision, F' , which is a measure of genetic isolation among populations (see ref. 24), was 0.175 and was significantly different from zero [CI (confidence interval) = 0.107–0.253, $df = 58$, $P < 0.01$]. Thus, the populations are genetically distinct, and gene flow between them is restricted, even among populations (Del Monte Beach and Elkhorn Slough) separated by only 30 km in Monterey Bay. In a more extensive study of genetic structure of eelgrass populations ranging over 3000 km along the western coast of North America, DNA fingerprint analysis has revealed reduction in gene flow between geographically disjunct populations ($F' = 0.185$, $P < 0.01$; R.S.A., G.P., and R.C.Z., unpublished data). The mechanisms controlling genetic diversity of the three central California eelgrass populations and the dominant reproductive mode (sexual or clonal) currently are unknown, but it is clear that sexual reproduction has played an important role in the development of the populations studied here. DNA fingerprinting may allow us to investigate these questions directly by tracing the colonization and propagation of genotypes in areas cleared within eelgrass beds.

* The F' value (0.175) obtained for the three geographically disjunct eelgrass populations indicates a low but significant level of population subdivision occurring between seagrass beds as close as 30 km. Gene flow among these populations appears to be insufficient to maintain genetic homogeneity among them. It is possible that losses of seagrass populations and estuarine habitats or decimation of the coastal waterfowl populations throughout California during the last 100 years, or both, have severely restricted gene flow among these once luxuriant populations, and they have only recently begun to differentiate as a result of genetic isolation.

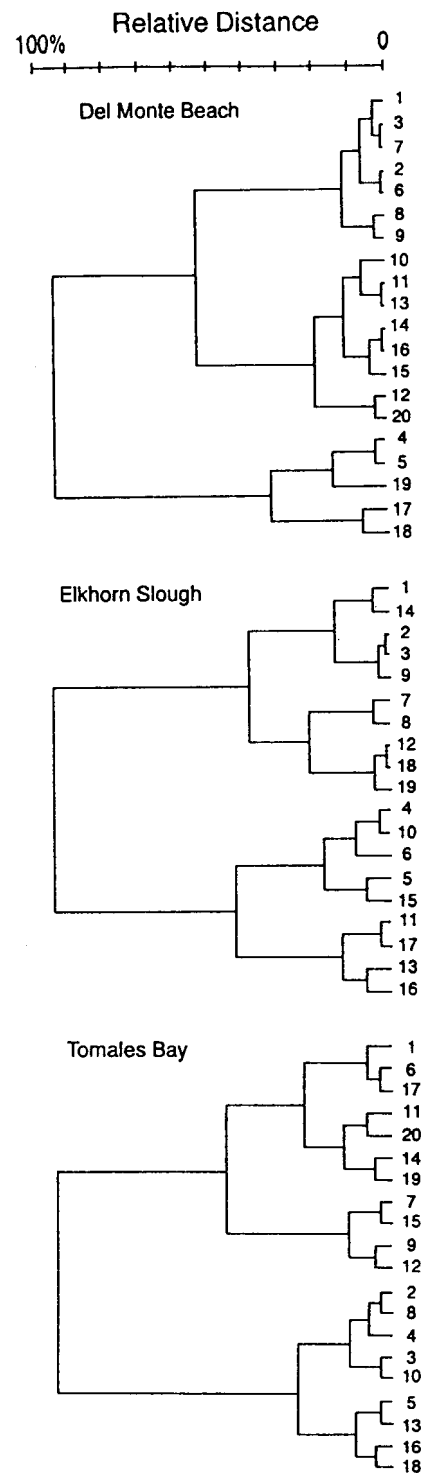


FIG. 3. Cluster analysis revealing the patterns of genetic distance in relation to physical distance (m) among individuals based on pairwise similarity values within the Elkhorn Slough, Tomales Bay, and Del Monte Beach populations of *Z. marina*. Individual numbers reflect physical positions along the transects. Physical distance between neighboring individuals was 5 m. Only the Del Monte Beach population displays a nonrandom pattern of distribution among genetically most similar pairs (see Table 2).

CONCLUSIONS

The present investigation demonstrates that *Z. marina* populations along the central California coast possess a high degree of genetic diversity and are not clonal at spatial scales of 5 m. Though the circumstances of the founding and development of the currently disjunct eelgrass populations in

Table 3. Between-population comparisons of genetic similarity of three geographically disjunct populations of *Z. marina* from central California

Population comparisons	S_b	95% CI	n^*
Del Monte Beach vs. Elkhorn Slough	0.51	0.49–0.53	380
Del Monte Beach vs. Tomales Bay	0.49	0.46–0.52	400
Elkhorn Slough vs. Tomales Bay	0.47	0.43–0.51	380

Between-population similarity (S_b) was calculated as the mean of all possible pairwise comparisons between individuals from the different populations (see *Materials and Methods*). 95% CI, 95% confidence interval as derived in Table 1; n^* , total number of comparisons.

Tomales Bay, Elkhorn Slough, and Del Monte Beach are unknown, it appears from our analyses that sexual reproduction contributed to the expansion and maintenance of these populations. Often widespread or "weedy" species derive a significant part of their ecological success by invading disturbed or open habitats and reproducing clonally (28, 33). Though we cannot exclude this possibility for populations studied here, our data and a more extensive study of eelgrass over much larger geographic distances (R.S.A., G.P., and R.C.Z., unpublished data) do not substantiate clonal reproduction as the dominant means of expansion.

The significant but relatively low genetic distinction found between populations may be ascribed to a combination of loss of habitat and loss of a long-distance seed dispersal mechanism, such as the dramatic reductions in coastal waterfowl populations in California. Gene flow might be expected between populations in close proximity in the same body of water such as those in Elkhorn Slough and Del Monte Beach, unless mechanisms that control seed or vegetative propagule dispersal do not act at distances as great as 30 km. This investigation and a previous one that used RFLP analysis of rDNA (16) are the first to demonstrate significant genetic distinction among disjunct eelgrass populations (see also ref. 26) and may offer some insights into the widespread distribution and ecological success of this species in a diversity of temperate coastal habitats. The capacity for a high level of genetic variation in this cosmopolitan species probably also accounts for the diversity in leaf and shoot morphologies in different habitats (26) and argues that these features may not be as useful as taxonomic characters at the species level as previously thought (see refs. 1 and 26).

Losses in seagrass, and particularly *Z. marina*, in habitats degraded by anthropogenic events (i.e., eutrophication and particle loading) resulting in light limitation may lead to loss of genetic diversity because certain genotypes are less fit. The impact of such bottlenecks could be compounded by concomitant unsuccessful seedling establishment due to light-limitation at the base of the canopy (12). As such, only clonal growth could be supported in the near term, and genetic diversity could be reduced. In this regard, we show that an intertidal eelgrass population in a disturbed estuarine habitat (Elkhorn Slough) possesses much lower genetic diversity ($S_w = 0.62$) than a similar intertidal population growing in a pristine environment (Tomales Bay; $S_w = 0.44$, see Table 1). Studies that examine genetic structure of populations over time in disturbed and undisturbed habitats are needed so that the impacts of chronic habitat deterioration on genetic stability and resilience of this species can be ascertained. In this way it is anticipated that mitigation and restoration criteria could be established that would lead to preservation and ecologically sound resource management of seagrasses and other coastal macrophytes.

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