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Numerous mitigation transplants of the eelgrass *Zostera marina* in southern California shuffle genetic diversity and may promote hybridization with *Zostera pacifica*

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ABSTRACT

Intensive human pressures along the southern California coast have led to >50 mitigation transplants of eelgrass over the past 30 years. We analyzed diversity and population structure of *Zostera marina* and *Zostera pacifica* at 36 locations to identify potential management units and further develop transplant guidelines. Normalized allelic diversity of *Z. marina* was uniformly moderate to high (4.78; 3.48–6.44) and nearly two-fold higher than mainland *Z. pacifica* (2.70; 1.74–4.89). More than half of the *Z. marina* populations exhibited strongly significant inbreeding coefficients coupled with strong linkage disequilibrium attributable to transplant effects; neither attribute was found in *Z. pacifica*. Both species were characterized by high genotypic diversity and an absence of large clones. A Bayesian analysis of population structure suggested 6 potential management units for *Z. marina* and 3 for *Z. pacifica;* some units included disjunct locations associated with transplants. Hybridization between *Z. marina* and *Z. pacifica* was documented at Newport Bay Entrance Channel and south San Diego Bay. The presence of two species requires management plans for each, as well as avoidance of potential transplant-induced hybridization. Although transplant admixtures elevate diversity, shuffling among locations may potentially reduce the genetic potential necessary to ensure rapid adaptation, even though overall transplant success has been successful. Given that transplants will continue (from both plants and seeds), we recommend that the current requirement for "two additional distinct donor sites" be restricted to within a management unit for small, routine mitigations and expanded to among-management units for wholesale *de novo* restorations.

1. Introduction

Zostera marina (narrow-leaved eelgrass) is the most widely distributed seagrass in temperate, northern hemisphere regions of both the Pacific and Atlantic. Along the eastern Pacific coast, it extends from Arctic Alaska to southern Baja California Mexico where it forms meadows in fjords, bays, lagoons and portions of the open coast characterized by soft sediments (Green and Short, 2003). *Zostera pacifica* (wide-leaved eelgrass) is restricted to the California Channel Islands and the adjacent mainland north to at least Monterey Bay and south to San Diego Bay (Watson, 1891; Engle and Miller, 2003; Coyer et al., 2008). Recent reviews of the biology, morphology and conservation of *Zostera* species can be found in Larkum et al. (2006), Waycott et al. (2006, 2009), Procaccini et al. (2007) and Short et al. (2011).

The maximum extent of eelgrass in southern California is less than 5000 acres (~2000 hectares) based upon available information from large-scale surveys. San Diego Bay and Mission Bay collectively comprise approximately 90% of the known mapped extent of eelgrass. However, a number of coastal embayments have experienced limited eelgrass monitoring and open coast populations have not been comprehensively assessed; thus, significant potential for greater eelgrass habitat probably exists. Furthermore, distinction between the two eelgrass species has not been a focus of regional eelgrass monitoring (Bernstein et al., 2011).

Many bays and lagoons along the Southern California Bight have undergone multiple eelgrass transplants as compensatory mitigation following filling, dredging and placement of structures. Eelgrass restoration has also occurred as a component of various large-scale wetland and lagoon restoration efforts with >50

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mitigation transplants documented over the past 30 years (NMFS, 2011). Even though these activities have resulted in an expansion of eelgrass habitat beyond the direct losses authorized by permitted actions (Bernstein et al., 2011), genetic effects on pre-/post-population reestablishment and fitness remain unknown.

The importance of genetic biodiversity for eelgrass health and ecosystem function is now well established. Experiments with *Z. marina* showed that increased genotypic diversity led to: (a) increased growth rates and competitive superiority of some clones and seed production (Williams, 2001; Hammerli and Reusch, 2002); (b) greater biomass production and recovery following grazing (disturbance) by geese (Hughes and Stachowicz, 2004); (c) enhanced shoot density (reflecting habitat quality) and biomass of epiphytic algae (a measure of food resource availability) (Hughes and Stachowicz, 2009a,b); and (d) a "high-disturbance" response and better resilience (Hughes and Stachowicz, 2011). At the community level, seagrass genotypic diversity was strongly correlated with an increase in the biodiversity of the associated community, thus adding complexity and greater insurance effects for resistance and resilience (Reusch et al., 2005; Eklöf et al., 2012). In a word of caution, however, Massa et al. (2013) experimentally showed that effects attributed to genotypic diversity alone need to be dissected and reconsidered to include the embedded allelic diversity, as one may not be a simple proxy for the other. In any case, however, the amount of genetic variation in a population affects its evolutionary potential and capacity to rapidly adapt to new circumstances, a process characterized by the occurrence of local ecotypes. For example, experimental studies have documented differences in gene expression and photosynthetic performance between intertidal and subtidal temperature- and light-ecotypes of *Z. marina* (Oetjen and Reusch, 2007; Bergmann et al., 2010; Oetjen et al., 2010; Franssen et al., 2011, 2014; Winters et al., 2011). In short, whereas ecological factors affecting eelgrass meadows have been well studied (reviewed in Larkum et al., 2006), consideration of evolutionary factors (reviewed in Waycott et al., 2006; Procaccini et al., 2007) is gaining importance in both primary research and improved conservation management because it is increasingly recognized that "evol-eco" processes occur in real time (Spielmann et al., 2004; Allendorf and Luikart, 2007). Finally, the evolutionary dimension of genetic-level diversity is an explicit goal of the International Convention on Biological Diversity (Laikre et al., 2010).

In the present study, we focus on mainland *Z. marina* and *Z. pacifica* populations along the Southern California Bight, from Point Conception to San Diego Bay (including additional sampling from north of Point Conception and south along the Pacific coast of Baja California). The aims were to: (1) establish the current baseline distribution of allelic diversity in *Z. marina* and *Z. pacifica* as an indicator of evolutionary potential for adaptation; (2) assess genotypic diversity (clonal diversity) as a reflection of local meadow persistence, stability and sexual reproduction; (3) compare genetic population structure and gene flow within and among bays and harbors that have experienced one or more mitigation transplants over the past 30 years; (4) determine whether interspecific hybridization has occurred between the two species; and (5) utilize the above "status" information to help define management units and modify mitigation guidelines that will minimize the risk of inadvertently reducing long-term meadow fitness.

2. Materials and methods

2.1. Sample collection

Samples ($n = 48$) of both *Zostera* species were collected from 36 sites (meadows) from Morro Bay, California to Magdalena Bay, Baja California Sur, Mexico: 25 with *Z. marina* and 11 with *Z. pacifica* (Fig. 1, Table 1, Fig. A1). Samples were collected by divers using scuba at all sites in California; samples from Mexico were collected at low tide. In all cases, shoots were collected at intervals of approximately 1.5 m along transects, a standard interval used in genetic baseline studies which facilitates comparisons among studies. Transects were perpendicular to shore where extensive beds were present. However, many areas exhibited fringing eelgrass beds along narrow margins of bays and channels and in these areas, transects ran horizontal to the shore. Each sample was isolated in a separate bag and placed in a cooler until further processing later in the day. Leaves (2–3) from each shoot were blotted dry and cut into 5–10 mm lengths before placement into 1.7-mL plastic tubes filled with silica gel crystals for rapid dehydration and subsequent storage.

2.2. DNA extraction and microsatellite amplification

Template DNA for PCR reactions was obtained from 2 to 3, 5–10 mm pieces of silica-dried leaves. Six microsatellite loci were used for both *Z. pacifica* and *Z. marina*: Zosmar-CT3, CT12, CT19, GA2, GA3, and GA6 (Reusch et al., 1999; Reusch, 2000; Olsen et al., 2004). Locus CT20 is a diagnostic locus, as it does not amplify in *Z. pacifica* (Coyer et al., 2008). Consequently, it was not included when both *Z. marina* and *Z. pacifica* populations were considered simultaneously, but was included when only *Z. marina* populations were evaluated (see Table A1 for *Z. marina* diversity based on 7 loci). The hypervariable loci CT17H and CT35, which are commonly utilized for *Z. marina*, were not used in the present study because their genotypes revealed mosaic alleles in some, but not all populations, suggesting the presence of multiple cell lineages within the same ramet (=somatic mutation) (Reusch and Boström, 2011). DNA extraction was based on a method developed for the seaweed *Fucus* (Hoarau et al., 2007) with subsequent modification for *Zostera* by heating the CTAB mixture to 60 °C (Coyer et al., 2009). PCR amplification and genotyping are described elsewhere (Coyer et al., 2004; Olsen et al., 2004). Genotypes were visualized on an ABI 3730 gene analyzer (Applied Biosystems) and analyzed using GENOTYPER (Applied Biosystems) software.

2.3. Genets and ramets

A genetic individual (genet) consists of many shoots (ramets) that can extend for several meters along a rhizome. Sampled shoots can, therefore, have the same multilocus genotype (MLG) if derived from the same large clone. The relative number of genets and ramets sampled in a given area was distinguished with GENCLONE 2.0 (Arnaud-Haond and Belkhir, 2007). Probabilities of identity by chance (*P*sex (F_{IS})) were calculated for each sample to avoid false assignment of individual ramets sharing the same MLG by chance to the same genet (clone). *P*sex (F_{IS}) accounts for departure from Hardy–Weinberg equilibrium (HWE) and provides the most conservative estimates of clonal identity (Arnaud-Haond and Belkhir, 2007).

All ramets reported as identical were identical due to clonality, not chance ($P < 0.05$). All subsequent analyses utilized genets only, i.e., duplicate MLGs removed.

Clone size was estimated by the spatial resolution of the linear sampling method (i.e., 1.5 m), which provided a coarse minimum value only; shoots were not sampled in a quadrat or mapped. For example, if three consecutive samples had the same MLG, the clone was estimated as minimally 4.5 m in size.

2.4. Data analysis

Allelic richness ($\hat{A}$) is the mean number of alleles^{-locus}. Allelic richness was standardized to $N = 20$ genets (smallest number for

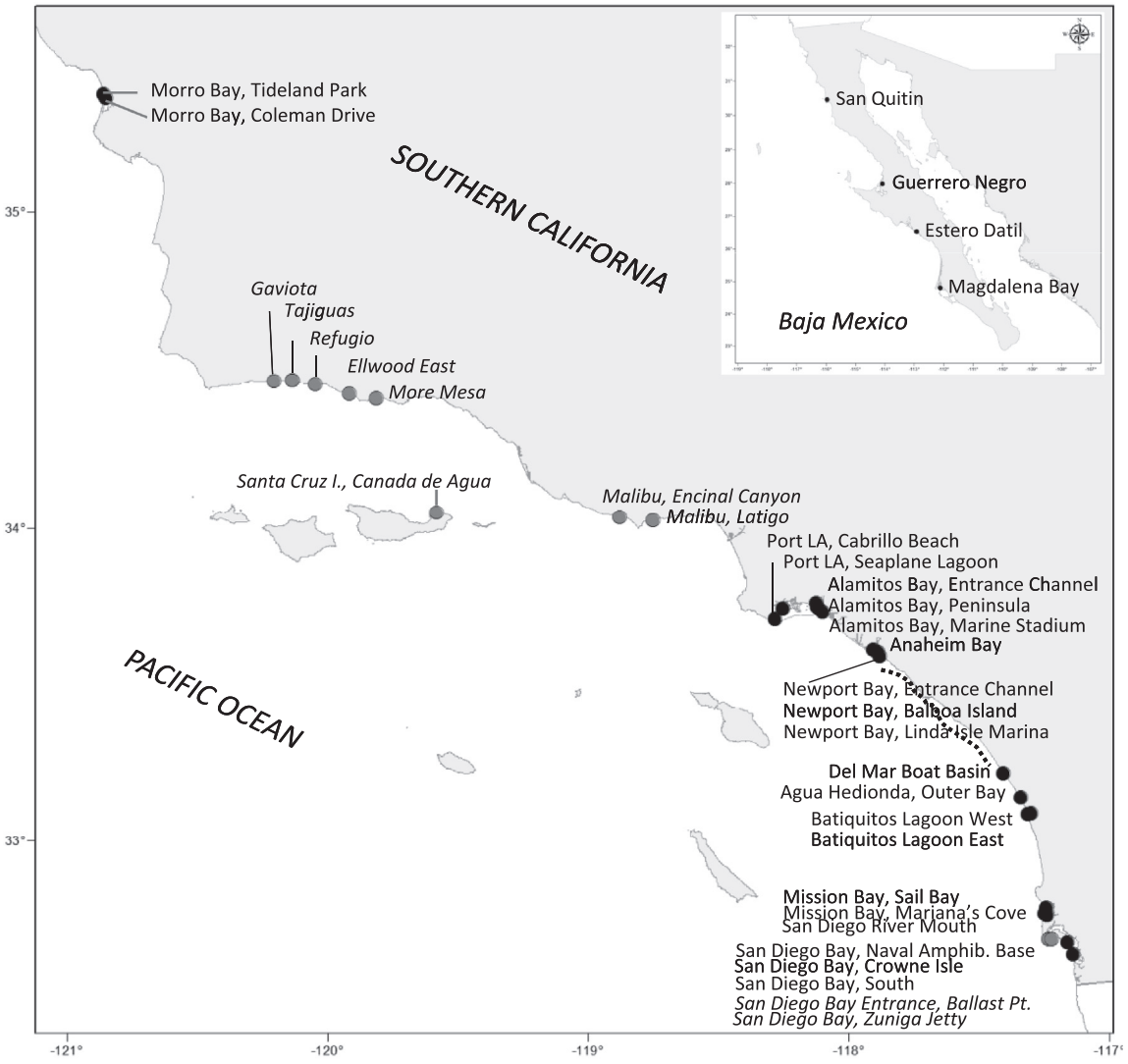

Fig. 1. Location of sampling sites for *Z. pacifica* (gray) and *Z. marina* (black). The dotted line indicates a putative disjunction in the distribution of *Z. marina*. For detailed maps per location see Fig. A1.

a sampled population) for both *Z. pacifica* and *Z. marina*. Genotypic diversity (*R*) is a measure of degree of clonality, which ranges from 0 where every shoot is genetically identical, to 1 where every shoot is a unique genotype and is defined as: $G-1/N-1$, where G is the number of genets and N is the number of shoots. The number and size of clones were estimated using GenClone 2.0 (Arnaud-Haond and Belkhir, 2007). Within- and among-population genetic diversity (number of alleles; Nei's gene diversity, H_{exp}) (Nei, 1978) Wrights fixation indices (F_{ST} as a measure of population differentiation, θ; and F_{IS} as a measure of departures from Hardy Weinberg Equilibrium and inbreeding, f) (Weir and Cockerham, 1984) were estimated using Genetix 4.02 (Belkhir et al., 2001). The significance of pairwise F_{ST} and F_{IS} estimates was tested using 1000 permutations. Sequential Bonferroni corrections (Rice, 1989) were not applied.

Linkage disequilibrium (LD) was assessed in Arlequin 3.5 (Excoffier and Lischer, 2010). Pairwise comparisons of all loci ($n = 6$) per population were compared ($n = 15$) using a likelihood ratio test (Slatkin and Excoffier, 1996) and tested for significance ($p = 0.05$) with 10,000 permutations.

Population structure and species identification of *Z. marina* and *Z. pacifica* were visualized with the Cavalli-Sforza and Edwards chord distance (Cavalli-Sforza and Edwards, 1967) applied to the microsatellite data. A neighbor-joining tree was constructed (bootstrap = 1000) using the Phylip software package (Felsenstein, 1994).

Hybridization between *Z. marina* and *Z. pacifica* was analyzed using Structure 2.3.3 (Pritchard et al., 2000). For this analysis, we assumed two parental populations or taxa (e.g., $K = 2$) corresponding to *Z. marina* and *Z. pacifica*. The degree of admixture of the two taxa within a single individual revealed the presence of F_1 hybrids (e.g., equal proportion of each taxa), as well as the degree/direction of introgression (unequal proportion of each taxa). The analysis was repeated five times (10^6 iterations; Burn-in = 100,000) to avoid dependence on starting values.

Isolation by distance (IBD) (Wright, 1943; Slatkin, 1993) was evaluated by correlating estimates of $F_{ST}/1-F_{ST}$ (Rousset, 1997) using the θ estimator (Weir and Cockerham, 1984) with geographical distances using matrix correlation methods based on the Mantel test (Manly, 1994) and 10,000 randomizations with IBD Web Service v.3.23 (Jensen et al., 2005). Linear distances were determined with the Path Analysis feature in Google Earth. Geographic distances were not log transformed in accordance with the linear coastline and a one-dimensional stepping stone model.

Population structure was further analyzed in a Bayesian framework implemented in the software Structure 2.3.3 (Pritchard et al., 2000). In this approach the *a priori* designation of "populations"

Table 1
Location of sampling, genetic diversity and clonality in *Z. pacifica* and *Z. marina* using six microsatellite loci. Latitude/Longitude; *N*, number of shoots; *G*, number of genets; *R*, genotypic diversity ($G-1/N-1$); $\hat{A}$, allelic richness standardized to 20 genets; $G > 1$, number of genets with >1 ramets; *nR*, mean number of ramets/genet (and range); H_{exp}, expected heterozygosity (Nei, 1978); F_{IS}, estimated as *f* (Weir and Cockerham, 1984), $^{*}p < 0.05$); LD, significant linkage disequilibrium, $p < 0.05$ expressed as percent e.g., 15/15 = 100% pairwise comparisons of the 6 loci for *Z. pacifica*; leaf width, *w* (wide), *m* (mixed), *n* (narrow). Mixed width leaves are not included in the mean values. All California collections were made by NOAA-SSCRP personnel; Baja California collections by L. Ladah, Centro de Investigación Cientifica Y de Educacóon Superior de Ensenada (CICESE).

	Coordinates	*N*	*G*	*R*	$\hat{A}$	$G > 1$	*nR*	H_{exp}	F_{IS}	LD	Width
California											
Z. pacifica											
Gaviota	N34.4675, W120.2041	33	33	1.000	–	1	–	0.3241	−0.1449	0.00	*w*
Santa Barbara, Tajiguas	N34.4690, W120.1338	48	46	0.957	1.91	2	2.5 (2,3)	0.3478	0.0000	0.00	*w*
Santa Barbara, Corral Canyon	N34.4583, W120.0478	48	43	0.894	1.74	3	3.7 (2–4)	0.3400	−0.0808	0.00	*w*
Santa Barbara, Refugio	N34.4690, W120.1338	48	43	0.894	4.89	4	2.2 (2,3)	0.5646	*0.4400	100.0	*w*
Ellwood East	N34.4272, W119.9171	34	12	0.333	–	4	6.5 (2–11)	0.3134	−0.1602	0.00	*w*
Santa Barbara, More Mesa	N34.4113, W119.8117	48	47	0.979	2.97	1	5.0	0.3452	−0.0764	0.00	*m*
Malibu, Encinal Canyon	N34.0349, W118.8796	48	34	0.723	–	3	7.3 (7,8)	0.3630	0.0909	0.00	*w*
Malibu, Latigo	N34.0252, W118.7501	48	16	0.319	–	4	9.0 (2–21)	0.3148	−0.0667	0.00	*w*
Canada de Agua, Santa Cruz Island	N34.0505, W119.5806	48	37	0.766	2.92	6	3 (2–5)	0.3767	0.0328	0.00	*w*
San Diego Bay Entrance, Ballast Pt	N32.6822, W117.2324	48	33	0.681	2.36	3	6 (2–14)	0.3424	−0.3539	0.00	*w*
San Diego Bay, Zuniga Jetty	N32.6814, W117.2191	48	43	0.894	2.42	5	2.4 (2–4)	0.3049	−0.1241	0.00	*w*
Mean				**0.767**	**2.70**	**3.5**	**5.3 (2–21)**	**0.5766**			
Z. marina											
Morro Bay, Tidelands Park	N35.3589, W120.8516	48	46	0.957	4.54	2	2.0 (2)	0.6146	*0.2178	0.44	*m*
Morro Bay, Coleman Dr.	N35.3719, W120.8611	46	42	0.911	3.96	4	2.5 (2,3)	0.5616	−0.0706	0.66	*m*
Port of Los Angeles, Cabrillo Beach	N33.7123, W118.2827	48	45	0.936	4.36	2	2.5 (2,3)	0.5211	*0.1916	0.77	*n*
Port of Los Angeles, Seaplane Lagoon	N33.7438, W118.2519	48	48	1.000	6.44	0	–	0.7341	*0.5344	0.11	*n*
Alamitos Bay, Entrance Channel	N33.7450, W118.1150	48	38	0.787	4.30	4	3.2 (2,5)	0.5604	0.0695	0.05	*n*
Alamitos Bay, Alamitos Peninsula	N33.7480, W118.1190	48	47	0.979	4.44	1	2	0.5465	*0.1488	0.39	*n*
Alamitos Bay, Marine Stadium	N33.7620, W118.1240	48	47	0.979	3.67	1	2	0.4743	0.1030	0.11	*n*
Anaheim Bay	N33.7342, W118.1000	46	31	0.667	4.42	8	2.9 (2–7)	0.5558	*0.1606	0.72	*n*
Newport Bay, Entrance Channel	N33.5921, W117.8803	48	48	1.000	5.30	0	–	0.6471	−0.0976	0.11	*m*
Newport Bay, Balboa Island	N33.6042, W117.8857	48	47	0.979	5.77	1	2.0 (2)	0.5131	0.0765	0.11	*n*
Newport Bay, Linda Isle Marina	N33.6133, W117.9033	48	26	0.532	4.46	6	4.5 (2–12)	0.5635	0.0452	0.11	*n*
Del Mar Boat Basin	N33.2148, W117.4043	43	36	0.833	4.52	3	2.7 (2,3)	0.5196	*0.1532	0.39	*n*
Agua Hedionda, Outer Bay	N33.1403, W117.3376	48	37	0.766	5.05	5	3.2 (2–4)	0.5561	*0.1185	0.44	*n*
Batiquitos Lagoon East	N33.0890, W117.2977	48	43	0.894	5.53	5	2.0 (2)	0.6285	−0.0116	0.22	*n*
Batiquitos Lagoon West	N33.0849, W117.3100	48	37	0.766	4.90	7	2.5 (2–3)	0.6538	−0.0900	0.22	*n*
Mission Bay, Sail Bay	N32.7813, W117.2398	48	46	0.957	6.32	2	2.0 (2)	0.6550	*0.3276	0.55	*n*
Mission Bay, Marina's Cove	N32.7646, W117.2473	48	27	0.553	4.18	9	3.3 (2–8)	0.5678	*0.1324	0.33	*n*
San Diego River Mouth	N32.7581, W117.2374	48	41	0.851	5.66	6	2.2 (2,3)	0.6965	0.0197	0.23	*n*
San Diego Bay, Naval Amphib. Base	N32.6724, W117.1576	48	48	1.000	4.95	0	–	0.5937	*0.3767	0.44	*n*
San Diego Bay, Crown Isle	N32.6342, W117.1364	48	44	0.915	4.15	3	2.0(2)	0.4861	*0.1965	0.33	*n*
San Diego Bay, South	N33.6133, W117.9033	47	41	0.870	3.48	5	2.8 (2–6)	0.4817	−0.0911	0.11	*n*
Mean				**0.847**	**4.78**			**0.5660**			
Baja, Mexico (*Z. marina*)											
San Quintin	N30.4524, W115.9548	48	45	0.936	4.31	3	2.0 (2)	0.4600	0.0748	0.11	*n*
Magdalena Bay	N24.7996, W112.1155	48	20	0.404	4.26	5	5.8 (2–23)	0.5940	*0.2046	0.44	*n*
Estero Datil	N26.5325, W112.9160	48	43	0.894	3.64	5	2.0 (2)	0.5759	0.0040	0.38	*n*
Guerrero Negro	N27.9811, W114.0759	48	35	0.723	7.55	7	1.9 (2–5)	0.7486	0.0090	0.15	*n*
Mean				**0.737**	**4.83**						

was estimated using the admixture model, which uses the log probability $P(X|K)$ of each user-determined set of clusters/populations ($K = 2, 3, 4 \ldots 24$) by genetic assignment of individuals to the most likely clusters (i.e., independent from the geographic location of collection). The true number of clusters was estimated under two assumption sets using the web-based Structure Harvester (Earl and von Holdt, 2011). In the first analysis, posterior probabilities for a given *K*, $P(X|K)$ (Pritchard et al., 2000) were determined directly, whereas in the second analysis the *ad hoc* statistic ΔK (Evanno et al., 2005) was used; the latter is recommended when asymmetrical dispersal patterns exist among given locations. The ΔK method is based on the rate of change of $P(X|K)$ values between different *K* with the number of sampling locations used as priors and assigned to the most likely *K*. Each analysis was repeated five times (10^6 iterations; Burn-in = 100,000) to avoid dependence on starting values.

3. Results

3.1. Genetic diversity

Mean values of allelic richness ($\hat{A}$) and genotypic diversity (*R*) (Table 1) were relatively uniform in *Z. marina* with only two locations (Newport Bay, Linda Isle Marina and Mission Bay, Marina's Cove) exhibiting significant large-scale clonality. $\hat{A}$ ranged from 3.48 to 6.40 with a mean of 4.78. *Z. pacifica* also exhibited relatively uniform clone sizes with only two sites (Ellwood East and Malibu, Latigo) characterized by large clones; $\hat{A}$ ranged from 1.74 to 4.89 with a mean of 2.70. Overall, allelic richness was 2-fold higher in *Z. marina* than in *Z. pacifica* although this may be an artifact, as the microsatellite loci were originally developed for *Z. marina*. For *Z. marina*, diversity measures based on seven loci (including locus CT20 which was absent in *Z. pacifica*) were similar to those based on six (Table A1).

3.2. Departures from Hardy Weinberg Equilibrium and linkage disequilibrium

Mean H_e varied significantly in *Z. marina*, ranging from 0.481 at San Diego Bay-South to 0.734 at Port of Los Angeles–Seaplane Lagoon (Table 1); in *Z. pacifica,* mean H_e ranged from 0.304 at San Diego Bay–Zuniga Jetty to 0.564 at Santa Barbara–Refugio. Mean H_e was the same (0.576) for both species.

Departures from Hardy Weinberg Equilibrium (HWE) (11/21 locations) and extensive linkage disequilibrium (LD) were

observed in *Z. marina* (Table 1), whereas in *Z. pacifica*, only one location (Santa Barbara–Refugio) exhibited a strong inbreeding coefficient (0.440) and LD. Pairs of loci contributing to the LD within each population of *Z. marina* were not the same sets of loci contributing to LD among populations, and the number of loci involved was typically 1 or 2 except where inbreeding coefficients were significantly positive. In these cases, half or more of the pairwise comparisons showed significant LD. The most dramatic example of LD occurred in the Santa Barbara–Refugio population of *Z. pacifica* in which complete LD was observed (15/15 comparisons); all other populations exhibited no LD.

3.3. Population structure

Both *Z. marina* (bootstrap = 81%) and *Z. pacifica* (bootstrap = 85%) were resolved with a neighbor-joining tree (Fig. 2). Two populations, Newport Bay Entrance Channel (mixed leaf width) and San Diego Bay South (narrow leaf width), were intermediate between the *Z. marina* and *Z. pacifica* clusters, consistent with hybridization (see next subsection). Several strongly supported clusters were present within *Z. marina*: the two northernmost populations at Morro Bay, 300 km from the Port of Los Angeles (98% bootstrap); the 65-km region bounded by Agua Hedionda to the north and San Diego Bay to the south (95% bootstrap); the 90-km region bounded by Anaheim Bay and Del Mar Boat Basin (94% bootstrap); and the two innermost populations of Alamitos Bay, separated by 2 km (80% bootstrap). Although the Baja California populations clustered together, as did most of the other geographically-close groups, there was no bootstrap support.

Population structure was first analyzed in an analysis of variance framework in which populations were defined *a priori* by the locations sampled. For *Z. marina*, pairwise F_{ST} values among locations (1–500 km) were all significant but highly variable (ranging from 0.01 to 0.60); the same was true for *Z. pacifica* with pairwise F_{ST} values among locations (1–365 km) almost all significant and highly variable (ranging from 0.01 to 0.54) (Table A2). For *Z. marina*, within-location values were in the 0.01 range at Mission Bay, Batiquitos Bay and Morro Bay, consistent with minimal population substructure and high gene flow. In contrast, F_{ST} values were >10-fold higher (0.10–0.30) within the Port of Los Angeles, Alamitos Bay, Newport Bay and San Diego Bay populations, where significant F_{ST} was detected at as low as 1 km, indicating strong local population substructure. Almost all pairwise comparisons of F_{ST} for *Z. pacifica* were significant, ranging from 0.010 to 0.673 (Table A2). The main exception was between San Diego Bay–Ballast Point and San Diego Bay–Zuniga Jetty, separated by ~1 km.

Isolation by distance (IBD), though significant ($p = 0.05$), provided little explanatory power (Fig. 3) for either species, as strong differentiation could be found at both small and large distances. For *Z. marina* (excluding the Baja populations), the correlation was low ($r = 0.475$, $R^2 = 0.226$), as was also the case for *Z. pacifica* ($r = 0.2318$, $R^2 = 0.053$). There was no within-location sampling for *Z. pacifica*.

The Bayesian analysis of population structure, as implemented in Structure, suggested $K = 5$ clusters for *Z. marina* when averaging over the replicates based on the posterior-probabilities and $K = 11$ clusters when using the ΔK *ad hoc* statistic (Fig. 4, Fig. A2). Both $K = 5$ and $K = 11$ revealed a major break in genotype distributions between Del Mar Boat Basin and Agua Hedionda, a distance of only 10 km. Under $K = 5$, four groups were recognized: Morro Bay (north of Point Conception) and the Baja, California, Mexico region from San Quintin southward. Within the Southern California Bight two

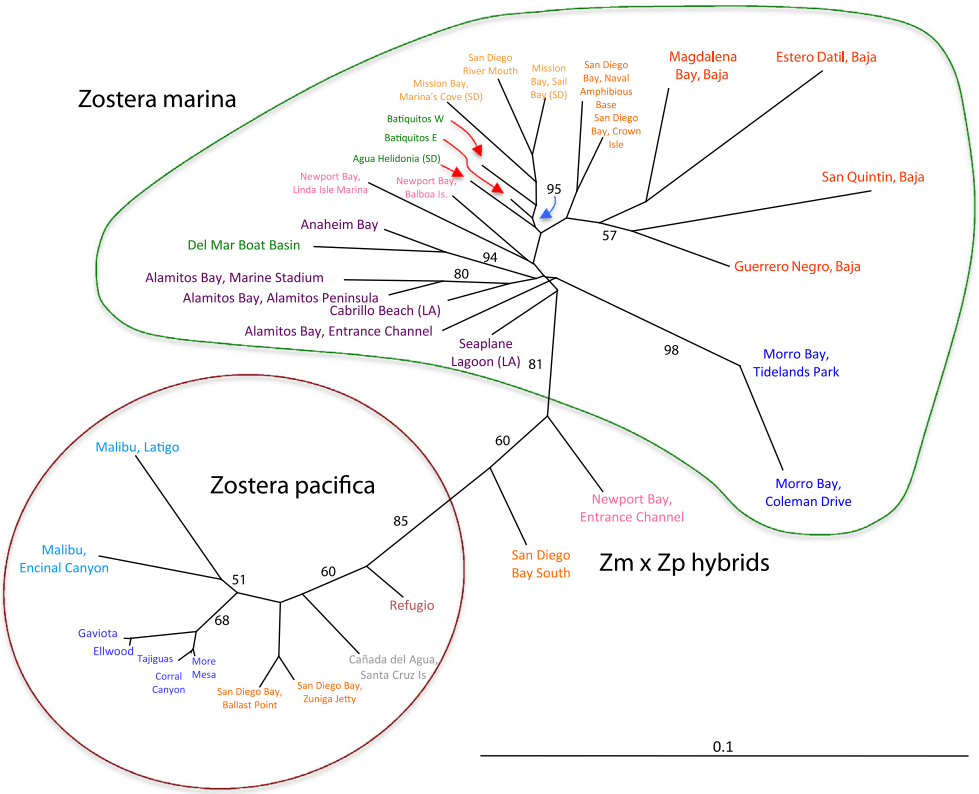

Fig. 2. Neighbor-joining tree of genetic distance relationships among populations of *Zostera pacifica* and *Z. marina* along the southern California Bight including Baja California, Mexico. The tree was based on pairwise Cavalli-Sforza and Edwards' chord distances derived from the six microsatellite loci (Cavalli-Sforza and Edwards, 1967) between genets only. Bootstrap values were derived from 1000 resamplings.

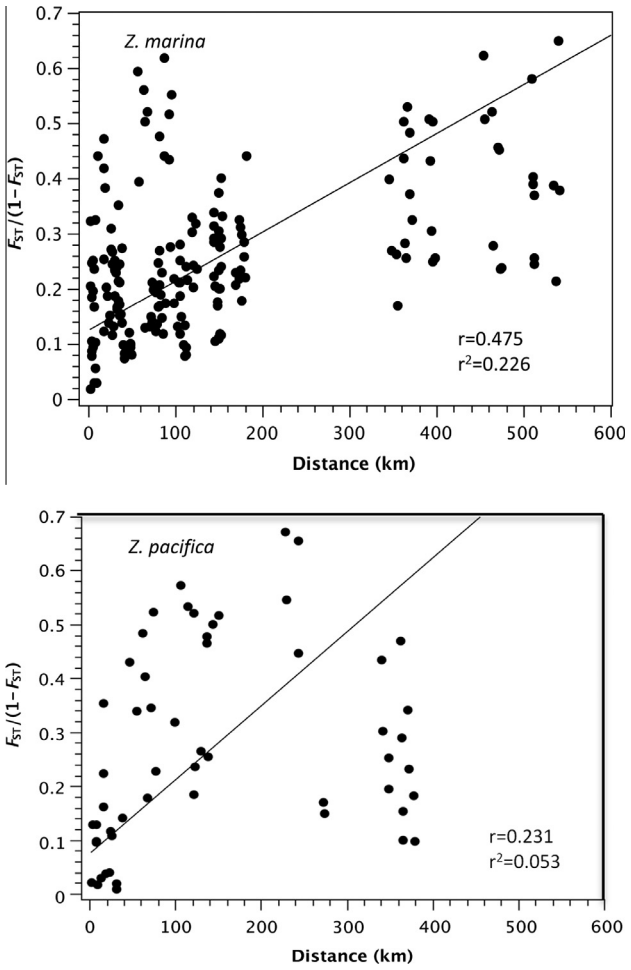

Fig. 3. Isolation by distance. Upper: *Zostera marina* (excluding Baja populations); lower: *Zostera pacifica*. The genetic and geographic distance matrix was compared with the Mantel Test and 10,000 randomizations; estimate of r^2 was calculated using reduced major axis (RMA) regression (Jensen et al., 2005). Geographic distance was not log transformed consistent with a one-dimensional stepping-stone model.

groups were recognized: the region from the Port of Los Angeles to Del Mar Boat Basin; and the region from Aqua Hedionda to San Diego Bay Crown Isle (Fig. 4 upper panel). Under $K = 11$, eleven groups were recognized (Fig. 4). These included: Morro Bay (north of Point Conception), a heterogeneous northern region from Los Angeles to Del Mar Boat Basin representing six groups (2a–2f), and a southern region including Aqua Hedionda and Batiquitos (3a), the Mission Bay complex (3b), the San Diego Bay complex (3c) and the Baja complex (4+). Groups 2a and 2b were themselves heterogeneous but distinct.

Group 2c included four non-contiguous locations as indicated by the arrows in Fig. 4. Group 2e included two non-contiguous locations. The three locations within Newport Bay (2d, 2e and 2f) were all distinct and highly differentiated with 2e having affinities to other locations.

The Bayesian analysis for *Z. pacifica* converged on $K = 3$ for both the ΔK *ad hoc* statistic and when averaging over the replicates based on the posterior-probabilities (Fig. 5 and Fig. A2). Samples from the Santa Barbara (Gaviota to More Mesa) and Malibu area (Encinal Canyon and Latigo) formed a contiguous group with the exception of Refugio. Samples from Santa Cruz Island (Channel Islands) were distinct as expected, but matched with those of Zuniga Jetty and Ballast Point in San Diego Bay.

3.4. Hybridization and introgression

The Newport Bay Entrance Channel and San Diego Bay South populations were intermediate to the clusters of *Z. pacifica* and *Z. marina* (Fig. 2) and revealed a signature of F_1 hybrids and introgression in the Structure analysis (Fig. 6). Both populations were centrally located within the coastal distribution of *Z. marina*, although 140 km and 20 km, respectively, from two adjacent populations of *Z. pacifica* at the entrance to San Diego Bay (Ballast Pt. and Zuniga Jetty). There was also some evidence for hybrids at Refugio even though no *Z. marina* was found at Refugio.

4. Discussion

4.1. Genetic variation, clonality and two species

An expected outcome of habitat loss and degradation is a reduction in population size and a loss of allelic diversity through genetic drift, which increases the likelihood of lower fitness and local extinction (Ruckelshaus, 1995; Williams, 2001; Procaccini et al., 2007). In seeking to restore meadows, the introduction of new genetic variation is expected to alleviate this process. Paradoxically however, the introduction of new individuals/genes can have variable effects. On the negative side, the new genes may potentially eliminate locally adapted gene complexes (ecotypes) through genetic recombination, dominance and/or outbreeding depression (Schaal and Leverich, 2005), especially in areas dominated by large clones. For example, in the southern California Channel Islands, where large clonal meadows and low allelic diversity are common, this possibility is of real concern (Coyer et al., 2008). Even if low clonal diversity is not an issue, low allelic diversity may be. For example, Campanella et al. (2010, 2013) compared allelic diversity between unrestored and restored meadows in Barnegat Bay, NJ and found that the genetic health of the restored populations was relatively better than the unrestored ones, but still found reduced levels of allelic diversity and connectivity after 10 years. On the positive side, however, new genetic variation may also stimulate new gene complexes and higher fitness. For example, Reynolds et al. (2012b) conducted a 9-year restoration experiment with *Z. marina* in Chesapeake Bay, VA. They showed that a small increase in allelic diversity enhanced ecosystem services (i.e., habitat complexity, primary production, nutrient retention) under natural, low-stress conditions. In their case, the donor material was characterized by high allelic and genotypic diversity, the situation found in most locations of the present study.

A further concern along the Southern California Bight is the presence of *Z. pacifica*, which increases the potential for inadvertent transplant-induced hybridization with *Z. marina*. Although hybridization can have a creative effect producing hybrids with higher fitness or even new species (Arnold and Hodges, 1995), it is more often deleterious leading to hybrid sterility, lower overall fitness, or "extinction by hybridization" (e.g., extensive introgression or hybrid swarms) (Mallet, 2005). At present, nothing is known about hybrid fitness or lack thereof in *Z. marina x Z. pacifica* hybrids. Hybridization/introgression of *Z. pacifica* and *Z. marina* has been confirmed genetically at Newport Bay Entrance Channel and San Diego Bay South (this study) and in Big Fisherman Cove on Santa Catalina Island and San Diego Zuniga Jetty (Coyer et al., 2008). The presence of hybrids at Refugio remains inconclusive in the absence of additional sampling. Hybrids in San Diego Bay-South may have resulted from inadvertent transplantation of *Z. pacifica* during one of the 26 transplant efforts between 1976 and 2009. The finding of hybridization in the San Diego Bay–Zuniga Jetty population by Coyer et al. (2008), but not in the present study,

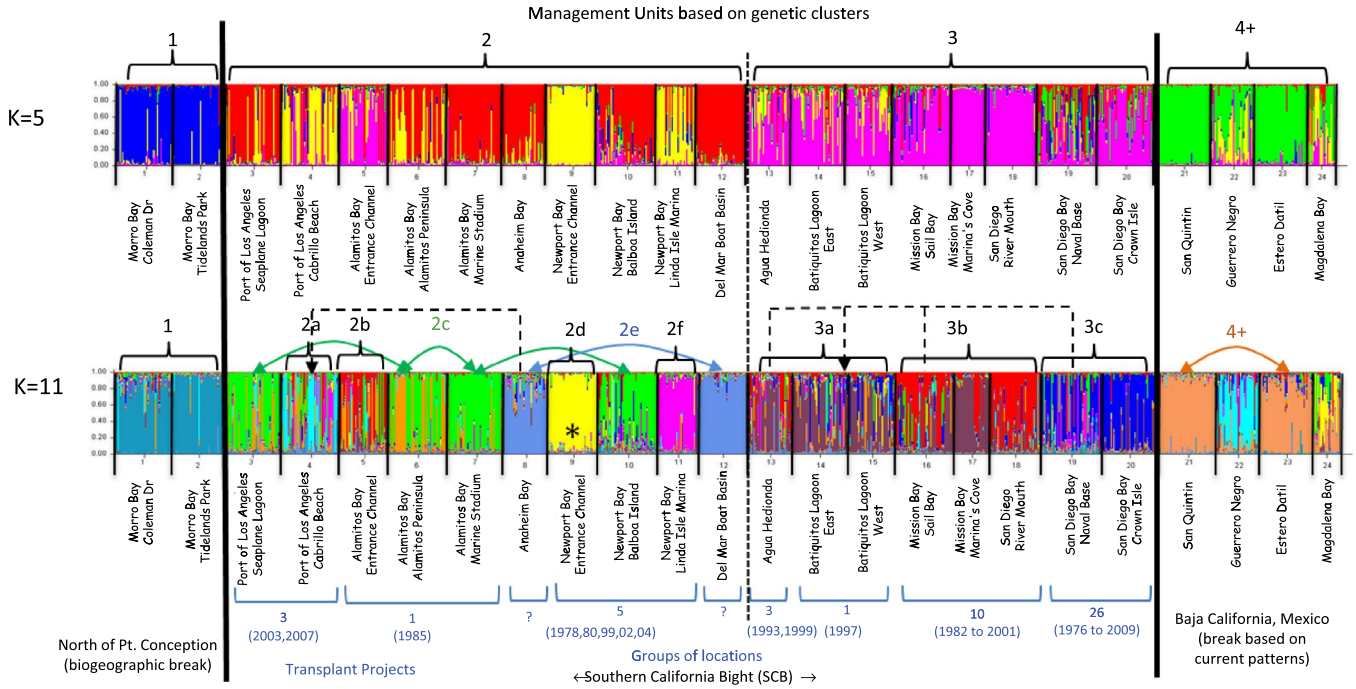

Fig. 4. Population structure of *Zostera marina* based on Bayesian inference as implemented in the software STRUCTURE (Pritchard et al., 2000). *Note:* Colors cannot be compared between the two panels; only within panels. Each cell within a panel represents a location. Within each cell, each individual sampled is represented by a vertical line partitioned into colored segments, the length of which is proportional to the individual's membership in each of *K* clusters designated. Top panel: $K = 5$ clusters, i.e., five colors as blue, red, yellow, magenta and green; Bottom panel: $K = 11$ clusters and 11 colors. The asterisk (*) indicates the presence of Zm–Zp hybrids. Possible management units are designated by horizontal braces (black) and arrowed-arcs (colored in the $K = 11$ analysis to match cells). Dotted arrow lines denote known transplants from source to target, i.e., from Anaheim Bay to Port of Los Angeles Cabrillo Beach and from three sources to Batiquitos Lagoon. For $K = 5$, two management units are suggested for the Southern California Bight; for $K = 11$, nine management units are suggested within the Bight. Recorded transplants (number and dates; are indicated along the lower panel. Localities alone are indicated by brackets (blue). The dotted vertical line marks a break in the distribution of *Z. marina* along a 65-km stretch of the coast as reflected in genotypic frequencies and clusters. The solid vertical black lines mark natural biogeographic boundaries related to ocean currents.

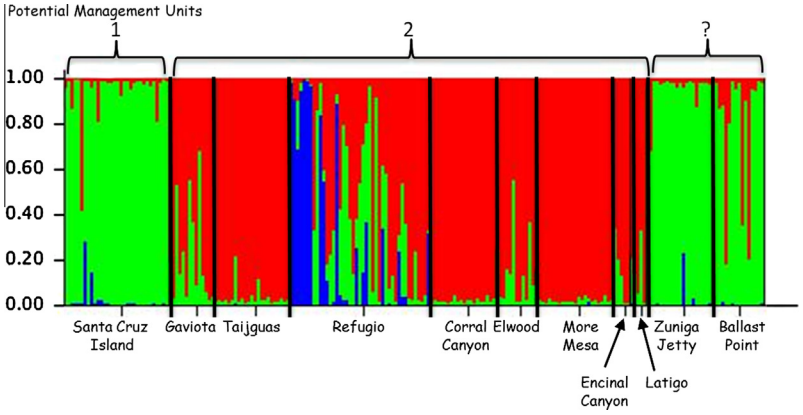

Fig. 5. Population structure in *Zostera pacifica* based on Bayesian inference as implemented in the software STRUCTURE (Pritchard et al., 2000). Each cell represents a location. Within each cell, each individual sampled is represented by a vertical line partitioned into colored segments, the length of which is proportional to the individual's membership in the $K = 3$ clusters designated by green, red and blue. Management units are indicated by braces. See Section 4 regarding the status of San Diego Bay–Zuniga Jetty and San Diego Bay–Ballast Point, as well as Refugio.

suggests that either this meadow is a mosaic (including areas with and without hybridization) or that selection against hybrids has occurred within the past several years. The most important point, however, is that the presence of two species of *Zostera* must be considered when evaluating and implementing eelgrass mitigation projects.

4.2. A closer look at diversity

Our initial prediction for the mainland populations of *Z. marina* was one of low allelic diversity ($\hat{A}$) corresponding to areas that have experienced strong anthropogenic pressures. This was not the case. Although only qualitative comparisons of mean $\hat{A}$ can be made due to differences in normalization and the number of loci used across studies, the mean of 4.78 (Table 1) for the southern California mainland populations is within the estimates of $\hat{A}$ for *Z. marina* populations along the US Pacific coast, i.e., San Juan Is, WA (2.74; Wyllie-Echeverria et al., 2010), Bodega Bay, CA (5.81; Olsen et al., 2004), San Francisco Bay (4.29; Ort et al., 2012), and Baja California, Mexico (4.74; Muniz-Salazar et al., 2005). In the global context, where >150 locations have been surveyed world-wide, the highest diversities found with these loci have been in

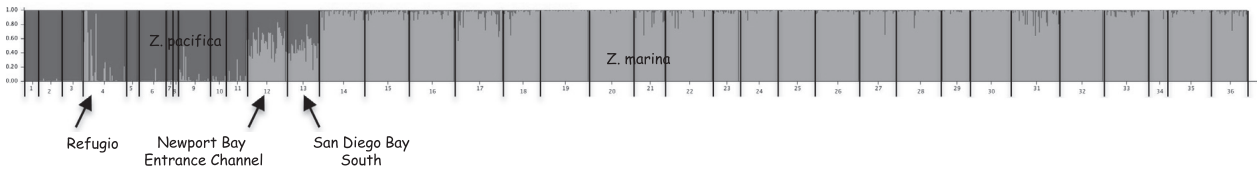

Fig. 6. Hybrids of *Zostera marina* (green) and *Z. pacifica* (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Japan (7.10, JLO unpubl.) and the lowest in the Baltic Sea (1.17) (Olsen et al., 2004). In *Z. pacifica*, higher mainland vs. island $\hat{A}$ may reflect sampling effects because Channel Island populations were typically characterized by larger clones than the mainland (Coyer et al., 2008). However, a real difference in $\hat{A}$ cannot be ruled out because little is known about the deep-water distribution of *Z. pacifica* along the mainland.

The positive correlation found between allelic and genotypic diversity is indicative of regular sexual reproduction and recruitment. However, high $\hat{A}$ also reflects admixture of local and non-local alleles as a consequence of transplant mitigations. This is supported by the presence of significant linkage disequilibrium (LD) for *Z. marina* (Table 1), whereas no LD (the Refugio population is discussed later) was detected for *Z. pacifica*, where no (intended) mitigative transplants have occurred. To assess whether the LD resulted from locus or population effects, we examined all pairwise comparisons per population (15) and pairwise comparisons across all populations ($25 \times 15 = 375$). Few matches were found (i.e., different locus pairs were linked in different populations), leading to the conclusion that the observed LD in *Z. marina* was a population effect associated with transplant activities over the past decades and not physical linkage.

In addition to LD, more than half of the locations exhibited positive inbreeding coefficients (F_{IS}). Positive values may be due to inbreeding and kinship (null alleles have seldom been encountered with these loci), which are characteristic of small populations or those dominated by large clones in which mating with relatives is unavoidable. A second and not mutually exclusive cause is a Wahlund effect (Hartl and Clark, 2007) which occurs when two overlapping populations are inadvertently sampled at the same time. Admixtures from local and non-local transplants of *Z. marina* are the most likely cause of the positive inbreeding coefficients and Wahlund effect although we cannot be absolutely certain. If the positive F_{IS} values are real, this could reflect low dispersal and kinship, and possibly a positive indication of niche differentiation and resource use (Stachowicz et al., 2013).

Although large clones are generally indicative of long-term stability, competitive superiority and enhanced niche differentiation (Stachowicz et al., 2013), the largest clones in the present study were still relatively small, less than 16 linear m (*Z. marina*) and 32 linear m (*Z. pacifica*) (Table 1). The higher frequency of smaller clones (4–6 linear m) is likely the consequence of most *Z. marina* meadows being located in sheltered bays and harbors characterized by more active dynamics, intra-specific competition, repeated seedling recruitment (Eriksson, 1993; Reusch, 2006) and, of course, human impact and mitigation. For *Z. pacifica*, large clonal meadows were initially predicted but found in only two of 11 meadows. At present we still know too little about the deep-water distribution and population structure of *Z. pacifica* to make firm conclusions about clonality.

The key unresolved question is whether the admixed allelic diversity from transplants observed at most sites will ultimately enhance fitness and ecosystem services, as this is the first baseline genetic survey in the region and little is known about the source locations used for most transplants. The presence of linkage disequilibrium, fixed loci and positive inbreeding coefficients most likely are transient artifacts, but they may also signal longer term fitness effects that will not be reflected functionally for many years.

4.3. Population differentiation, connectivity and management units

Poor correspondence between gene flow and geographic scale was evident in the isolation by distance analysis (IBD) (Fig. 3) in which IBD accounted for only 22% of the variation in *Z. marina* and 5% in *Z. pacifica*. This was also supported by the NJ tree (Fig. 2) in which within-site clusters generally conformed to geographic locations but were not well-supported by bootstrap values. Simulations of dispersal distances of *Z. marina* suggest that 50% of the floating rhiphidia remain within 500 m radius, with a highly skewed tail of long distance dispersal over a few km (Källström 2006). Assignment and other tests have detected rafting of seed-bearing shoots at 30–54 km (Reusch, 2002) and up to 150 km along the Wadden Sea coast in relation to local current patterns (Olsen et al., 2004; Ferber et al., 2008). At sub-kilometer scales, additional sampling strategies have revealed mosaic substructure at scales of 600 m^2 on the Brittany Peninsula of France (Becheler et al., 2010), in northern Norwegian fjords (Olsen et al., 2013) and parts of San Francisco Bay (Ort et al., 2012). Such patterns are typical of meadows that establish themselves but remain demographically disconnected, a feature of many of the sites in the present study (Fig. A1) and potentially exacerbated by inter-location transplants.

In both Bayesian analyses of population structure (i.e., $K = 5$ and $K = 11$; Fig. 4), Morro Bay (1) is clearly a different management unit and is likely connected to populations further north. Similarly, the Baja California region (4+) formed a separate regional unit. Both flanking areas are characterized by different current regimes that form significant dispersal barriers: for Morro Bay, the cold California Current to the north at Point Conception (a major biogeographic boundary); and for Baja-San Quintin, near shore countercurrents in an otherwise equatorial stream. All of the locations between the 'bookend' units have experienced multiple mitigation transplants (Fig. 4), often involving several sub-sites within a location (NMFS, 2011). The $K = 5$ analysis (Fig. 4 upper panel) separates a region from Port of Los Angeles to Del Mar Boat Basin (2) and the region from Agua Hedionda to San Diego Bay (3). These two regions are separated by 65 km of mostly exposed sandy beach and occasional rocky headland. Based upon available distribution information, this stretch of coast is apparently devoid of *Zostera* populations (Bernstein et al., 2011), although the area has not been thoroughly investigated. The $K = 11$ analysis (Fig. 4 lower panel) further divides the region between the Port of Los Angeles and San Diego Bay into potentially nine management units. Admixed populations are characteristic of 2a, 2b, and 2c, whereas 2d, 2e and 2f are internally more homogeneous. Notably, "like genetic populations" are not necessarily geographically contiguous as indicated by the arrows. For example, the 2c group involves subpopulations from three separate bays (Port of Los Angeles, Alamitos Bay and Newport Bay), and the 2e group involves two widely separated bays (Anaheim Bay and Del Mar Boat Basin). The disjunct distributions may reflect long-distance transplants although regional experts have no record of long distance material being used for

these systems (Chesney, pers. comm.). However, Anaheim Bay has been used as a source for Port of Los Angeles Cabrillo Beach. The southern region is also divided into potentially three groups (3a, 3b and 3c) thereby isolating Agua Hedionda and Batiquitos Bays from Mission Bay and San Diego Bay. However, it is known that Batiquitos Lagoon East and West received transplant material from Agua Hedionda, Mission Bay and Tidelands Park. This admixture is apparent in both $K = 5$ and $K = 11$. In contrast, San Diego Bay Naval Base and Crown Isle remain distinct (also in Fig. 2).

The choice of $K = 5$ or $K = 11$ remains partly subjective. In general, the aim in a Structure analysis is to select the smallest value of K that captures the major structure of the data and although $K = 5$ is the smaller value, the uncertainty of K (Pritchard et al., 2000) and the different value of the ΔK *ad hoc* statistic makes a definitive choice difficult. To further guide our judgement, we compared the Bayesian clusters in Fig. 4 with the NJ tree in Fig. 2 and a Factorial Correspondence Analysis (not shown). Based on what we know about the coast and the transplant history, we conclude that the recognition of only two management units ($K = 5$) within the Bight is too broad, whereas nine management units ($K = 11$) is overly divisive. We suggest six potential management units (erring on the side of caution) with the northern group consisting of 2abc, 2d, 2e, 2f and the southern group of 3ab and 3c in Fig. 4. Our rationale for this choice is to minimize unnecessary long-distance admixtures that may compromise within-location adaptive potential. Additionally, because large clonal meadows of *Z. marina* are apparently uncommon along the mainland coast, concerns about the selection of inadequately diverse donor material is no longer warranted. However, this is not the case around the Channel Islands, where large clonal meadows generally prevail and we do not recommend island-mainland transplants for either species.

For *Z. pacifica*, the Bayesian analysis (Fig. 5) was based on $K = 3$ (Fig. A2) and identified two potential management units: Santa Cruz Island (1) and the mainland coastal group (2). Santa Cruz Island was distinct from the mainland populations in accordance with an earlier extensive survey of Santa Cruz Island (Coyer et al., 2008). The San Diego Bay–Zuniga Jetty and San Diego Bay–Ballast Point populations match those of the Channel Islands. Whether this match is the result of natural long distance dispersal or an inadvertent transplant remains unknown. However, the entrance to San Diego Bay represents a transition zone between open coast and bay environments so that, in principle, the admixture could be natural. The *Z. pacifica* populations from Santa Barbara to Malibu (2) represent an area of connectivity despite population differentiation (Fig. 5). Although still predominantly part of (2), Refugio exhibited extremely high allelic diversity, a high and significant inbreeding coefficient (0.440) and complete linkage disequilibrium (15/15 comparisons). Moreover, loci GA12 and GA23 were nearly fixed in surrounding populations and there was evidence of possible hybridization with *Z. marina* (Fig. 6). Accordingly, we speculate that the Refugio population of Z. *pacifica* has introgressed with *Z. marina* and/or with an unknown deep-water population of *Z. pacifica*.

4.4. Sources and type of donor material

Eelgrass has been recognized as a significant habitat in southern California by the National Marine Fisheries Service, the US Fish and Wildlife Service, and the California Department of Fish and Game, which collectively formulated a comprehensive mitigation policy for eelgrass transplantation (Anonymous, 1991). One requirement for transplant mitigation is that donor plants should be collected from the area of direct impact (whenever possible), as well as from "...two additional distinct sites to better ensure genetic diversity of the donor plants...".

We concur that the best approach is to choose neighborhood material and to select the individuals from similar microhabitat conditions, such as depth and exposure, both because it is prudent to assume that local ecotypic adaptation is present (Ort et al., 2012) and because natural replenishment of the restoration site via dispersal from more distant patches/meadows may be insufficient (Kendrick et al., 2012; Ort et al., 2012). However, the requirement of sampling from two additional "distinct sites" needs refinement, as it increases the unknowable risk of allelic admixtures and outbreeding depression (Mallet, 1995; Schaal and Leverich, 2005). We suggest that "distinct sites" be further defined as: (1) the utilization of donor material from different meadows/patches within a location undergoing routine mitigation (e.g., compensation for marina development); and (2) acquisition of donor plants from meadows in different bays/lagoons for total *de novo* restoration programs. The latter case would include areas where: (a) allelic diversity is demonstrated to be low and restoration of a pre-existing meadow is desired; or (b) establishing a meadow in an appropriate virgin habitat (e.g., as with Batiquitos Lagoon).

Current restoration practice still favors the use of adult plant material; a labor-intensive and expensive effort that severely restricts transplant diversity (both allelic and genotypic). A new transplant technique incorporating seeds has been shown to be very promising in Chesapeake Bay, VA (McGlathery et al., 2012; Orth et al., 2012; Orth and McGlathery, 2012; Reynolds et al., 2012a). In theoretical terms, the result is not surprisinout linkage disequilibrium. This work was supported g because the hundreds of thousands of planted seeds will contain many orders of magnitude more variation than a few hundred or thousand adult plants that may or may not survive and sexually reproduce. While the use of seeds is clearly superior from a genetic perspective, the same rules still apply (as outlined above) with respect to selection of donor meadows. Seeding in San Francisco Bay has so far produced variable responses (Boyer et al., 2007), possibly related to sediment quality, turbidity, and amphipod grazing. Seeding is being tested in southern California via a small-scale restoration project in Upper Newport Bay and results are pending (Chesney, pers. comm.).

5. Conclusions and recommendations

The baseline status of genetic diversity and structure for southern California populations of *Z. marina* and, to a lesser extent, *Z. pacifica*, are now established. Despite the shuffling of diversity through 30 years of transplant activity, allelic and genotypic diversity are relatively high, as is transplant success (as measured by survival and increase in areal cover). Nevertheless, long term genetic fitness components (e.g., shoot density, recruitment, resilience) associated with local adaptation and ecosystem services remains unknown, as the signature of linkage disequilibrium, positive inbreeding coefficients and strong local substructure likely reflect the combination of transplant activity in combination with natural fitness effects associated with limited dispersal.

Recommendations

- Modification of the "two additional distinct" donor sites requirement (in addition to the area of direct impact) to "within a management unit and as close as possible to the target site of mitigation" for routine, small mitigations in locations with multiple meadows; and to "among-management units to ensure the maximal starting diversity" for massive *de novo* restorations.
- All donor and recipient locations involved in future mitigations should be identified with precise GPS coordinates.
- The presence of two *Zostera* species must be considered when evaluating and implementing eelgrass mitigation projects. PCR diagnostics are available for definitive identification.

- As the relative abundance, distribution, and morphology of each species north of Morro Bay is currently unknown, transplantation programs in these areas should be accompanied by baseline genetic surveys to confirm species identity, diversity and population structure.
- No formal plans exist for restoration of *Z. pacifica*, an oversight that must be addressed if its distribution declines as a result of transplant programs and/or coastal restoration projects that favor *Z. marina*.
- Further efforts should be made to determine the distribution of *Z. pacifica*.

Author contributions

JLO and BC conceived the project; fieldwork was led by BC; JAC performed the lab work; JAC and JLO analyzed and interpreted the data; JLO led the writing with equal input from all.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocon.2014.05.001.

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