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Comparative Analysis of Stability—Genetic Diversity in Seagrass (*Posidonia oceanica*) Meadows Yields Unexpected Results

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Abstract The diversity–stability relationship is the subject of a long-standing debate in ecology, but the genetic component of diversity has seldom been explored. In this study, we analyzed the interplay between genetic diversity and demographic responses to environmental pressures. This analysis included 30 meadows formed by the Mediterranean endemic seagrass, *Posidonia oceanica*, showing a wide range of population dynamics ranging from a near equilibrium state to steep decline due to strong environmental pressures close to aquaculture installations. Our results show that sedimentation rates are much better predictors of mortality than clonal or genetic components. An unexpected positive trend was observed between genotypic diversity and mortality, along with a negative relationship between allelic richness and net population growth. Yet such trends disappeared when excluding the most extreme cases of disturbance and mortality, suggesting the occurrence of a threshold below which no relationship exists. These results contrast with the positive relationship between genotypic diversity and resistance or resilience observed in previous manipulative experiments on seagrass. We discuss the reasons for this discrepancy, including the difficulties in designing experiments reflecting the complexity of natural meadows.

Keywords Seagrass · Demography · Clonality · Genetic richness · Diversity–stability · *Posidonia oceanica*

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Introduction

The relationship between diversity and stability has been the subject of a long-standing debate in ecology. Many components of diversity have been tested for their effect on resistance or resilience to environmental perturbations at levels ranging from populations to ecosystems. Among other components of diversity, clonal diversity, taxa diversity (mainly from species to genera), and functional diversity have been investigated (Naeem and Li 1997; McCann 2000). Depending on the study and the proxies measured, various trends or lack of trends have been observed (Johnson et al. 1996; Loreau et al. 2001; Pfisterer and Schmid 2002; Worm et al. 2006).

The genetic component of biodiversity has so far been largely neglected in such observational and experimental studies. At best, some authors working on resistance and resilience of clonal organisms to environmental pressures have experimentally tested the effect of genotypic diversity (Hughes and Stachowicz 2004; Reusch et al. 2005), which reflects the number of clonal lineages (roughly the number of genetic individuals arising from distinct events of clonal reproduction) but does not necessarily correlate with genetic diversity (Hughes and Stachowicz 2004; Hughes et al. 2008). Nevertheless, the existence of effects of genetic diversity on the potential of populations and species to overcome environmental fluctuations is a strong underlying

assumption driving most interpretations and conclusions in molecular ecology in general and in conservation genetics in particular (Frankham 1995; Spielman et al. 2004; Frankham 2005).

It is widely assumed that genetic impoverishment affects the resistance and resilience of populations and species against future environmental changes, thereby potentially threatening their mid-term survival. This expectation results from two complementary effects. First, the loss of polymorphism would lower the "evolutionary potential" of populations (Frankel 1974), the capacity to adapt to a range of variable environmental conditions. Second, loss of polymorphism below a certain threshold would increase the likelihood of inbreeding with deleterious effects (Frankel and Soulé 1981) and of accumulation of deleterious mutations (Lande 1995; Lynch et al. 1995). Both the overall consequences of the loss of diversity and the effects of inbreeding alone have been demonstrated empirically for the demography of several species (Newman and Pilson 1997; Saccheri et al. 1998; Spielman et al. 2004). In contrast, there is still little evidence for the theoretically expected negative effect of the loss of "evolutionary potential" and the nature of the hypothetical relationship between genetic diversity and population vulnerability.

A well-established trend, both theoretically (Kimura 1983) and empirically (Spielman et al. 2004), is that large populations with positive demographic balance sustain higher genetic diversity than small declining populations. What remains to be empirically demonstrated is, apart from inbreeding, whether higher diversity in terms of allelic variants may in turn provide populations with a greater likelihood of adapting to a wide range of environmental conditions, thereby positively influencing their demography. A positive relationship between demography and genetic diversity may be expected for both reasons, preventing the assessment of whether demography drives genetic diversity or vice versa. The possible circular nature of the arguments challenges efforts to dissociate the respective influence of these two putative drivers, demographic and genetic status, on the basis of empirical relationships between demographic status and genetic diversity in natural populations. However, in long-living (longevity of centuries or millennia), slow-growing organisms, these relationships cannot be tested by assessing the demographic performance of experimental populations across a gradient of genetic diversity because the duration of the experiments would span across several human generations. Conversely, the effect of genetic diversity on demographic responses to environmental pressures could be examined across a range of populations for which genetic diversity prior to disturbance was known, but such predisturbance information is largely lacking.

Seagrass meadows are highly valuable marine habitats experiencing a worldwide decline at rates of about 1% to 3% per year due to the combination of a number of pressures leading to increasing anthropogenic pressure on coastal areas (Orth et al. 2006). Meadows formed by the Mediterranean species *Posidonia oceanica*, a long-lived, highly clonal species (Arnaud-Haond et al. 2007b), are declining at a rate of about 5% per year (Marbà et al. 2005). Testing for a possible role of genetic diversity in the resistance of *P. oceanica* meadows to environmental pressures is an important goal in order to infer which meadows may be more vulnerable to these pressures. Preliminary transplant experiments have provided some evidence indicating that genetic diversity enhances survival of transplants (*P. oceanica*; Procaccini and Piazzi 2001), but lower mortality rates have been reported in *P. oceanica* meadows subjected to aquaculture impacts where genotypic and genetic diversity were lower (Diaz-Almela et al. 2007). Hence, the role of genetic diversity in the response of *P. oceanica* meadows to disturbance remains unclear.

Coastal eutrophication is the major cause of seagrass decline worldwide (Orth et al. 2006). Excessive inputs of organic matter (OM) and nutrients to coastal areas fuel sediment microbial activity, increasing the production of sulfide (e.g., Holmer et al. 2008; Mascaro et al. 2009) and ammonium (e.g., Frederiksen et al. 2008) and causing sediment anoxia. Eutrophication may also reduce light availability to seagrass meadows (due to the proliferation of phytoplankton and macroalgae) and increase grazing pressure. All of these processes are detrimental to seagrass survival and growth. Benthic sedimentary inputs of OM and particulate nutrients to *P. oceanica* meadows have been demonstrated to be a useful proxy for coastal eutrophication, particularly when deterioration of the water column is not evident, as is often the case in Mediterranean coastal areas (Diaz-Almela et al. 2008). Although seagrasses are often nutrient limited and their productivity could thus increase with coastal nutrient enrichment, it has been demonstrated that organic and nutrient loading to *P. oceanica* sediments triggers plant mortality and meadow decline when it exceeds 1.5 g dry weight OM m^{-2} day^{-1}, 50 mg P m^{-2} day^{-1}, or 40 mg N m^{-2} day^{-1} (Diaz-Almela et al. 2008).

Here, we test the prediction that genetic diversity plays a role in the resistance of *P. oceanica* to environmental pressures, particularly inputs of OM and nutrients (nitrogen [N] and phosphorus [P]) to the sediments. We first examine the genetic diversity of 30 *P. oceanica* meadows across the Mediterranean Sea in order to test for the existence of a possible relationship between the demographic dynamics and the genotypic (i.e., clonal) and genetic (allelic) composition of the meadows. We then assess, for 14 meadows receiving different amounts of organic and nutrient benthic inputs, whether genetic diversity affects the relationship between *P. oceanica* demography and environmental pressures (i.e., inputs of OM and particulate N and P).

Materials and Methods

Sampling

A total of 30 *P. oceanica* populations spanning the Mediterranean basin from Spain to Cyprus were sampled from summer 2001 to summer 2003. About 40 shoots were collected at randomly selected coordinates within a 1,600-m^2 (80×20 m) area at each site (Arnaud-Haond et al. 2007b; Diaz-Almela et al. 2007). The basal, meristematic section of the leaves was removed and preserved in silica crystals for later analysis. Twelve of these populations were analyzed in a previous study on biogeography (Arnaud-Haond et al. 2007b); eight were sampled in the context of a study on the effects of aquaculture installations on *P. oceanica* meadows (Diaz-Almela et al. 2007; Holmer et al. 2008); and ten others were newly added for this study (Table 1).

Genotyping

Genomic DNA was isolated using a CTAB extraction procedure (Doyle and Doyle 1987). All meadows were analyzed with the most efficient combination (Arnaud-Haond et al. 2005) of seven dinucleotide microsatellite markers (Alberto et al. 2003), as described in Arnaud-Haond et al. (2007b).

Details of the Methods Used for Clone (Genet) Discrimination

When the same genotype was detected more than once, the probability that the samples actually originated from distinct reproductive events (i.e., from separate genets) was estimated (Tibayrenc et al. 1990; Parks and Werth 1993), taking into account Wright's inbreeding coefficient estimated for each locus (Young et al. 2002). The procedure was based on the round-robin method (Parks and Werth 1993) to estimate the allelic frequencies at nuclear loci in each population:

$$p_{\mathrm{gen(fis)}} = \prod_{i=1}^{l} \left[(f_i g_i) \times \left(1 + \left(z_i \times \left(F_{\mathrm{is}(i)}\right)\right)\right)\right] 2^h \qquad (1)$$

where l is the number of loci, h is the number of heterozygous loci, and f and g are the allelic frequencies of the alleles f and g at the ith locus (f and g are identical for homozygotes; $F_{\mathrm{is}(i)}$ is the F_{is} estimated for the ith locus using the round-robin method; and z_i=1 for the ith locus if it is homozygous and −1 for the ith locus if it is heterozygous).

When the same genotype is detected more than once (n) in a population sample composed of N ramets, the probability that these sample units actually originate from distinct reproductive events (i.e., from separate genets) is described by the binomial expression (Tibayrenc et al. 1990; Parks and Werth 1993):

$$p_{\mathrm{sex(fis)}} = \sum_{i=n}^{N} \frac{N!}{i!(N-i)!} \left[p_{\mathrm{gen(fis)}}\right]^i \left[1 - p_{\mathrm{gen(fis)}}\right)\Big]^{N-i} \qquad (2)$$

where n is the number of sampled ramets with the same multilocus genotype (MLG), N is the sample size, and $p_{\mathrm{gen(fis)}}$ is the probability of the common genotype.

The possible occurrence of somatic mutations or scoring errors resulting in slightly distinct MLG actually derived from a single reproductive event and, therefore, belonging to a single clone, was tested for. When significant results were obtained, multilocus lineages (MLL) were then defined, including the slightly distinct MLGs (Arnaud-Haond et al. 2007a, b).

All calculations were performed using the software GenClone2.1 (Arnaud-Haond and Belkhir 2007).

Genotypic Diversity Estimates

Genotypic richness was estimated from the number of ramets sampled (N) and the number of MLG detected (G), as suggested by Dorken et al. (2002):

$$R = \frac{(G-1)}{(N-1)}. \qquad (3)$$

The Pareto distribution, which describes the skewed distribution of genotypes among lineages in clonal organisms (Arnaud-Haond et al. 2007a, b), was estimated for each meadow. The parameters of that distribution, β (derived from the slope) and the maximum MLL size (in terms of number of replicates), were used as indicators of the evenness and diversity of each meadow, as proposed in Arnaud-Haond et al. (2007a) and implemented in Genclone 2.1 (Arnaud-Haond and Belkhir 2007).

The clonal subrange (Harada et al. 1997), an estimate of the maximal extent of clones, was also estimated for each population using Genclone 2.1 (Arnaud-Haond and Belkhir 2007).

Genetic Diversity Estimates

Allelic richness was estimated after randomly subsampling each sample to standardize it to the maximum common sample size between populations after removing clonal replicates (Arnaud-Haond and Belkhir 2007; Arnaud-Haond et al. 2007a).

Expected heterozygosity was estimated on the set of MLL defined after removing ramets derived from the same zygote ancestor according to $p_{\mathrm{sex(fis)}}$ using Genetix (Belkhir et al. 1996–2001).

Table 1 Sampling locations (country, location, and GPS coordinates) and number of SU collected and analyzed (N_{SU})

	Sampling data			Genotypic data						Genetic data				Demographic data			
	Locality	GPS coordinates		N_{SU}	N_{MLL}	R	Pareto β	max	CR	$\hat{A}$	H_{nb}	H_{obs}	F_{IS}	Density (shoots m^{-2})	RMR (shoot per year)	RRR (shoot per year)	NPG (shoot per year)
Spain (peninsula)	Roquetas	36°43.26′ N	2°37.09′ W	40	26	0.64	1.08	6	47.76	3.72	0.56	0.66	−0.18	425			
	Rodalquilar	36°51.21′ N	2°00.53′ W	40	21	0.51	0.65	10	44.92	4.43	0.58	0.65	−0.12	966			
	Campomanes	38°37.54′ N	0°0.57′ E	31	22	0.70	0.76	7	48.00	4.43	0.58	0.64	−0.09	427	0.28	0.06	−0.23
	Torre de la Sal	40°8.13′ N	0°10.72′ E	39	20	0.50	0.35	7	52.92	3.57	0.51	0.55	−0.08	350	0.21	0.05	−0.16
	El Arenal	38°38.37′ N	0°3.06′ E	39	32	0.82	1.25	4	31.98	4.43	0.54	0.56	−0.04	431	0.24	0.15	−0.09
	El Campelo impacted	38°25.30′ N	0°20.83′ E	39	26	0.66	0.93	6	70.90	2.86	0.40	0.51	−0.27	20	0.55	0.11	−0.05
	El Campelo control	38°24.88′ N	0°21.14′ E	40	23	0.56	0.94	6	68.70	4.00	0.57	0.71	−0.24	68	0.06	0.11	0.05
	La Fossa	38°33.59′ N	0°4.56′ E	40	31	0.77	0.97	5	68.73	4.43	0.54	0.49	0.09	1,551	0.24	0.03	−0.19
	Fanals	41°41.58′ N	2°50.56′ E	38	26	0.68	0.45	11	53.45	3.71	0.48	0.58	−0.21	121	0.14	0.02	−0.12
	Cala Giverola	41°44.15′ N	2°57.37′ E	38	17	0.43	0.23	19	61.09	3.00	0.39	0.43	−0.12	326	0.17	0.09	−0.08
	Cala Jonquet	42°18.19′ N	3°17.36′ E	39	20	0.50	0.56	9	38.33	4.14	0.53	0.51	0.05	207	0.28	0.07	−0.21
	Port Lligat	42°17.61′ N	3°17.58′ E	40	12	0.28	0.25	17	67.07	3.29	0.54	0.67	−0.25	192	0.23	0.18	−0.05
	Xilxes	39°45.13′ N	0°8.07′ E	32	12	0.36	0.49	9		3.14	0.51	0.67	−0.13		0.16	0.14	0.30
Spain (Balears)																	
Formentera	Es Caló des Oli	38°43.49′ N	1°24.16′ E	40	15	0.36	0.45	11	46.04	5.00	0.61	0.56	0.07	403			
	Cala Torreta	38°47.45′ N	1°25.18′ E	40	21	0.51	0.62	10	59.20	4.29	0.54	0.52	0.04	527	0.12	0.03	−0.10
	Ses Illetes	38°45.37′ N	1°25.83′ E	36	22	0.60	0.50	10	35.11	4.29	0.54	0.64	−0.18	667	0.02	0.03	0.01
	Es Pujols	38°43.74′ N	1°27.27′ E	40	27	0.67	0.88	11	72.53	4.29	0.51	0.47	0.08	746	0.04	0.02	−0.02
Cabrera	Es Castel (5 m)	39°9.16′ N	2°55.83′ E	40	5	0.10	0.05	33	73.25	2.71	0.53	0.61	−0.17	704	0.11	0.04	−0.06
	Sa Paret (18 m)	39°8.81′ N	2°55.86′ E	40	5	0.10	0.03	35	78.06	2.57	0.60	1.00	−0.83	259	0.28	0.05	−0.25
	Cala Sta. María (13 m)	39°9.07′ N	2°56.92′ E	35	20	0.56	0.85	7	52.96	3.14	0.51	0.53	−0.04	762	0.21	0.02	−0.19
	Cala Sta. María (7 m)	39°9.00′ N	2°56.96′ E	40	22	0.54	0.75	8	43.78	3.72	0.42	0.47	−0.11	1,000	0.18	0.03	−0.15
Mallorca	Magalluf	39°30.25′ N	2°32.59′ E	38	26	0.68	1.18	5	34.79	4.29	0.56	0.52	0.07	563	0.12	0.04	−0.08
	Porto Colom	39°25.05′ N	3°16.18′ E	35	16	0.44	0.40	12	56.68	3.72	0.57	0.78	−0.38	415	0.17	0.06	−0.11
Menorca	Cala Fornells	40°03.39′ N	4°08.26′ E	40	5	0.10	0.04	34	68.41	2.57	0.46	0.40	0.15	935			
	Addaia	40°00.97′ N	4°12.42′ E	37	25	0.67	1.06	5	50.59	3.42	0.56	0.61	−0.08	1,090			
Italy (Sicily)	Porto Palo impacted	36°42.71′ N	15°8.44′ E	40	31	0.77	1.48	4	60.50	5.42	0.63	0.59	0.06	156	1.18	0.00	−1.18
	Porto Palo control	36°43.31′ N	15°8.48′ E	40	29	0.72	0.84	5	41.68	5.71	0.61	0.64	−0.04	395	0.28	0.03	−0.25
Greece	Sounion impacted	37°39.59′ N	23°57.29′ E	37	34	0.92	2.35	3	29.90	6.00	0.51	0.52	−0.01	165	1.50	0.11	−1.39
	Sounion control	37°39.55′ N	23°58.24′ E	33	29	0.97	2.00	1	12.70	7.00	0.57	0.58	−0.02	372	0.07	0.06	−0.01
Cyprus	Amathous impacted	34°41.96′ N	33°12.00′ E	40	18	0.44	0.28	10	76.56	4.14	0.51	0.58	−0.14	454	0.19	0.16	−0.03
	Amathous control	34°42.02′ N	33°12.99′ E	40	25	0.62	0.65	9	65.10	4.57	0.47	0.46	0.01	491	0.19	0.16	−0.03

For each location, the clonal descriptors include the number of clonal lineages (N_{MLL}), the genotypic richness (R), and evenness (Pareto β), as well as maximum clonal size (quantified as number of SU=Pareto max=maximum number of clonal replicates or as clonal subrange (CR)=the maximum distance found between clonal replicates), and the genetic parameters are the allelic richness ($\hat{A}$), unbiased (H_{nb}) and observed heterozygosity (H_{obs}), and departure from Hardy–Weinberg equilibrium (F_{IS}). Demographic data are detailed as density, RMR, RRR, and NPG

Demography

We assessed the demography of the seagrass (*P. oceanica*) using repeated annual censuses of marked shoots in the same 30 meadows where genetic and genotypic diversity was assessed, from Cyprus to Spain. Seagrass demography at all Spanish meadows except El Campello was estimated from 2000 to 2002 (Marbà et al. 2005). Seagrass demography was quantified at Cyprus, Italy, and El Campello (Spain) from 2002 to 2003 and for Greece between 2003 and 2004 (Diaz-Almela et al. 2008). In 14 meadows, the level of disturbance, as represented by inputs of OM and particulate nutrients to the sediments, was measured. The meadows ranged in conservation status from protected, relatively pristine areas to highly disturbed sites located in the vicinity of fish farms that delivered important loads of OM and nutrients to the sediments (Diaz-Almela et al. 2007; Holmer et al. 2008). At each meadow, we installed three permanent plots at the bottom by scuba diving, using metal sticks, ropes, and buoys, as explained in detail in Marbà et al. (2005). The size of the triplicate quadrats was adjusted to encompass at least 100 shoots per quadrat. We performed two direct censuses of the shoots present within the permanent plots at each site. Censuses were separated by about 1 year (from 307 to 386 days). During the first census, all shoots within each plot were counted and marked by placing a plastic cable tie around the rhizomes. During the second census, the number of surviving shoots (identified as those marked in the previous census) and the number of recruited ones (identified as young unmarked shoots) were counted. We calibrated the counting error by having two plots counted by independent observers, yielding an estimated error of ±0.2% and ±3.5% of the total shoot population for recruits and lost shoots, respectively.

The repeated censuses allowed direct estimates of specific rates (per year) of shoot mortality, recruitment, and net population growth (NPG) rate (Marbà et al. 2005).

The specific shoot mortality rate (RMR, per year) was estimated, assuming an exponential population growth model, as:

$$\mathrm{RMR} = -\frac{(\ln N_{s_1}/N_{t_0}) \times 365}{t_1 - t_0} \tag{4}$$

where N_{t_0} is the total number of shoots (vertical and horizontal apices) counted in the initial census (t_0, days) at each plot and N_{s_1} is the total number of surviving shoots at the second census (t_1, days).

The specific shoot recruitment rate (RRR, per year) was estimated, assuming an exponential population growth model, as:

$$\mathbf{RRR} = \frac{\ln((N_{r_1} + N_{s_1})/N_{s_1}) \times 365}{t_1 - t_0} \tag{5}$$

where N_{r_1} is the total number of recruited shoots observed at t_1 and N_{s_1} is the number of survivors at t_1.

Specific NPG rates (per year) were estimated as:

$$\mathrm{NPG} = \mathrm{RRR} - \mathrm{RMR} = \frac{\ln(N_{t_1}/N_{t_0})}{t_1 - t_0} \times 365 \tag{6}$$

where N_{t_1} is the total number of shoots present at t_1.

Sedimentation Rates

We measured sedimentation rates at each station by deploying benthic sediment traps next to the plots for periods of about 48 h. The sediment traps were designed after Gacia et al. (1999) and consisted of two replicated arrays situated 20 cm above the bottom, each supporting five 20-ml cylindrical glass centrifugation tubes with an aspect ratio of 5 (16 mm diameter) in order to minimize internal resuspension. The contents of one to three tubes were combined and collected on a combusted, preweighed Whatman GF/F filter. Dry weight of total sediment deposition was obtained after drying the filters at 60°C to constant weight. Dry weight of OM deposition was measured through combustion of some of the filters. Total P was obtained after boiling combusted materials in 1 M HCl for 15 min followed by spectrophotometric determination of phosphate (Koroleff 1983). We analyzed the uncombusted samples for total N content with an elemental analyzer (Iso-Analytical Ltd., UK). Further information on these analyses and spatial patterns of fish farm inputs are shown in Holmer et al. (2008). We estimated total matter, OM, N, and P sedimentation rates from these measurements according to Blomqvist and Hakanson (1981) and Hargrave and Burns (1979), as described in detail in Gacia et al. (1999).

Using least squares linear regression analysis, we examined the overall relationship between genotypic diversity (R, Pareto β, and maximum MLL size) or genetic diversity (allelic richness and unbiased heterozygosity) and seagrass demography (specific mortality, specific recruitment, and specific NPG) as well as relationships between shoot mortality or net rate of population change and sedimentation rates. We also tested the relationships between the residuals of these regressions and genotypic and genetic diversity to examine whether high genotypic and genetic diversity lead to lower mortality and/or a higher net rate of population growth for a given degree of environmental pressure.

Because NPG is dependent on both the mortality (RMR) and recruitment (RRR; NPG=RRR−RMR) rates, the correlation obtained for NPG may not be independent from those for RMR and RRR. Depending on which of the two factors is the predominant force driving the demography of the meadow, the correlation observed for NPG may be negatively related to those obtained for RMR or positively related to those obtained for RRR.

Because multiple tests were performed to screen for the existence of a relationship between genetic and demographic data, we applied a q value correction for multiple tests using the QVALUE software (Storey et al. 2004; Storey and Tibshirani 2003) within the R 2.9.2 package (The R Development Core Team 2004). The q values indicate the probability of the null hypothesis being correct despite low p values. The bootstrap method was chosen as recommended by the authors for a limited number of p values (Storey 2002).

Results

Clonal Diversity Descriptors

The probabilities of obtaining the same MLG through distinct sexual recombination events were very small (all $p_{sex}<0.01$). Identical MLGs were, therefore, considered as pertaining to the same clone. Genotypes differing by only one or two loci did not result in a $p_{sex}<0.01$ after removing the distinct loci. All MLGs were, therefore, considered to pertain to distinct MLLs. Despite standardized sampling area and size, highly variable levels of clonal richness were observed across the Mediterranean (Table 1) with five to 34 MLLs per meadow revealed in sample sizes of 31 to 40 sampling units (SU) and with R ranging from 0.1 to 0.97.

The Pareto descriptors of clonal diversity also revealed variable richness and evenness with the maximum clonal size [quantified as the number of SU belonging to the same MLL] falling between one and 35 and Pareto β ranging from 0.03 to 2.35. The clonal subrange also varied widely from 12.7 to 78 m in the standardized sampling area of 20×80 m.

Genetic Descriptors

Allelic richness in Spanish meadows, standardized to the maximum common sampling size observed (N=31) using a subsampling approach varied from 2.6 to 5 alleles per locus. Allelic richness was somewhat higher (four to seven alleles per locus) in the central (Sicily) and eastern (Greece and Cyprus) parts of the Mediterranean.

Expected and observed heterozygosity ranged from 0.4 to 0.6 and from 0.4 to 1, respectively. This discrepancy resulted in 13 of the 30 meadows significantly departing from Hardy–Weinberg equilibrium with negative F_{is} in 12 samples. Heterozygote excesses reached −1 in a meadow off of Cabrera Island (Sa Paret) dominated by a very large heterozygous clone, even though clonal replicates were not included in this estimation.

Shoot Demography

The density of meadows also varied immensely, by almost two orders of magnitude (Table 1), mostly due to very low density at impacted aquaculture stations and to depth differences, ranging from 20 shoots per square meter in the deep and heavily impacted station of El Campello to 1,550 shoots per square meter in the shallower meadow of Fanals along the Spanish mainland coast. Specific shoot mortality rates ranged between 0.02 and 0.28 shoot per year in meadows unaffected by aquaculture operations, compared to 0.19 to 1.5 shoot per year at those impacted by fish farm effluents. Low recruitment (0.00 to 0.18 shoot per year) was unable to balance the high mortality rate in most meadows (28 of the 30 meadows studied). This resulted in declining densities (i.e., declining population growth) at rates of up to −0.25 shoot per year when unaffected by fish farm effluents (in a deep meadow, Sa Paret, Cabrera Island, dominated by a very large clone) and up to −1.39 shoots per year when impacted by fish farming activity (Greek impacted meadow).

Sedimentation Rates

Ranges of levels of OM, N, P, and total benthic sedimentation rates, estimated in grams of dry weight per square meter per day, were 0.44–3.80, 0.01–0.11, 0.01–0.08, and 5.3–8.94, respectively, at stations located near aquaculture cages (Table 2). Except for total OM at Fanals (Table 2), lower values were observed in control stations and in other meadows sampled along the Spanish coasts, which had ranges of 1.59–11.54 for total sedimentation, 0.42–2.09 for OM, and 0.01–0.06 for N and a noticeably lower range of values for P (0.00–0.01).

Tests for Correlations

There was a significant, positive relationship (Table 3) between mortality rates and genotypic evenness (Pareto β: r^2=0.54, $p<0.01$, q value=0.00) across all populations, along with negative relationships between NPG and clonal richness and clonal evenness (Pareto β: r^2=0.51, p=0.00, q value=0.00) and allelic richness ($\hat{A}$: r^2=0.20, p=0.04, q value=0.47), although the latter q values (significance corrected for multiple tests) reflect a non-negligible probability of type I error. In any case, the significance of any of these relationships was entirely dependent on the high evenness and allelic richness in the four meadows highly impacted by fish cages, and no relationship was evident when these populations were excluded. No other correlation was observed between any of the other clonal or genetic versus demographic parameters.

Mortality rates were generally positively related to sedimentation rates, as represented by inputs of OM and

Table 2 Demographic and sedimentation data for 13 meadows sampled across the Mediterranean

Sampling locations	Demography		Sedimentation				Residuals
	Density	RMR	Sed. Tot.	Sed. OM	Sed. N	Sed P	Mort. versus sed.
Porto Palo impacted	156	1.18	8.94	3.80	0.11	0.08	0.03
Porto Palo control	395	0.28	7.00	2.35	0.04	0.01	0.26
Amathous impacted	454	0.19	6.98	1.12	0.01	0.01	−0.07
Amathous control	491	0.19	4.30	1.71	0.02	0.01	0.16
Sounion impacted	165	1.50	5.30	0.44	0.05	0.05	0.20
Sounion control	372	0.07	1.59	0.42	0.02	0.00	−0.27
El Campelo impacted	20	0.55	8.55	3.35	0.09	0.06	−0.23
El Campelo control	63	0.06	2.01	0.96	0.01	0.00	0.01
Fanals	121	0.14	11.54	1.96	0.04	0.01	−0.11
Magalluf	563	0.12	5.06	1.26	0.01	0.00	−0.03
Porto Colom	415	0.17	8.30	1.65	0.03	0.00	−0.03
Sa Paret (18 m)	259	0.28	9.00	2.09	0.06	0.00	0.07
Cala Sta. María (13 m)	762	0.21	2.97	0.57	0.01	–	

Demographic data are detailed as density in shoots per square meter and as RMR in shoots per year. Total sedimentation (Sed. Tot.) and the sedimentation of organic matter (Sed. OM), of nitrogen (Sed. N), and of phosphorus (Sed. P) are indicated in grams dry weight per square meter per day

N, when the heavily impacted meadows were excluded (Table 4). When all meadows were included, this positive relationship was significant for P. The q values ranged from 0.00 to 0.03 for all p values below 0.09, indicating that a low p value could be reliably interpreted as significant.

No significant relationship ($p>0.05$) was found between the residuals of these mortality versus pressure (i.e., sedimentation rate) regressions (which represent the extent of mortality for any given additional pressure) and clonal or genetic diversity parameters.

Discussion

Global Patterns of Genetic Diversity Versus Demographic Status of Seagrass Meadows

This study reveals that, in *P. oceanica* meadows, less clonal (more genotypically diverse) populations are associated with higher mortality and that populations with more alleles (more genetically diverse) have lower NPG. This is shown by the positive relationship observed between mortality rate

Table 3 Overall regressions tested between genotypic (R, Pareto β, Pareto max, and CR) and genetic ($\hat{A}$, H_{obs}, H_{nb}, and F_{IS}) descriptors and demographic parameters (RMR, RRR, and NPG) as well as residuals of demographic versus sedimentation parameters

Demographic data		RMR (shoot per year)		RRR (shoot per year)		NPG (shoot per year)		Residuals multiple regression (mort. versus sed.)	
Genotypic and genetic data		All data	Without St.3	All data	Without St.3	All data	Without St.3	All data	Without St.3
Clonality	R	*0.13 p= 0.07*	–	–	–	*0.15 p= 0.05*	–	–	–
	Pareto max	–	–	–	–	–	–	–	–
	Pareto β	0.54[a] p=0.00	–	–	–	0.50[a] p=0.00	–	–	–
	CR	–	–	–	–	–	–	–	–
Genetics	$\hat{A}$	*0.13. p= 0.07*	–	–	–	0.20 p=0.02	–	–	–
	H_{nb}	–	–	–	–	–	–	–	–
	H_{obs}	–	–	–	–	–	–	–	–
	F_{IS}	–	–	–	–	–	–	–	–

Regression r values are detailed when analyzing all available data (all data) as well as when excluding the meadows specifically highly impacted by aquaculture installations (without impacted). When p values exceeded 0.1, no values are reported, else r and p values are detailed, with nonsignificant values at α=0.05 in italics

[a] Values still significant after correction for multiple tests (i.e., q values below 0.05)

Table 4 Multiple regressions of sedimentation (total, OM, N, and P) and demographic parameters (RMR and NPG) when analyzing all available data (all data) as well as when excluding the meadows highly impacted by aquaculture installations (without impacted)

Demographic data	RMR (shoot per year)		NPG (shoot per year)	
Sedimentation	All data (±SE)	Without impacted (±SE)	All data (±SE)	Without impacted (±SE)
Total sedimentation	*0.03 (±0.03) p= 0.32*	*−0.01(±0.00) p= 0.06*[a]	*−0.04 (±0.04) p= 0.55*	*0.04 (±0.10) p= 0.09*[a]
OM	−0.29 (±0.11) p=0.03[a]	0.12 (±0.03) p=0.02[a]	*0.36 (±0.16) p= 0.40*	*−0.07 (±0.05) p= 0.69*
N	*4.72 (±5.8) p= 0.44*	2.84 (±0.09) p=0.046	*−7.06 (±8.61) p= 0.06*[a]	*−4.52 (±1.68) p= 0.27*
P	15.36 (±15.7) p=0.01[a]	*5.75 (±5.80) p= 0.395*	*−12.76 (±6.95) p= 0.44*	*−6.73 (±11.29) p= 0.07*[a]
Overall r^2	0.88 p=0.00[a]	0.96 p=0.02[a]	0.75 p=0.02[a]	*0.91 p= 0.07*[a]

Contributions to the multiple regression are detailed for each of the four sedimentation parameters, and the overall regression coefficients, as well as corresponding p values, are detailed. Nonsignificant values at α=0.05 are in italics

[a] Values still significant after correction for multiple tests (i.e., q values below 0.05)

and genotypic diversity (i.e., clonal richness and evenness as estimated with R and Pareto β) and the negative relationship between the net rate of population growth and genetic diversity (allelic richness; Table 3, Fig. 1).

This is in contrast with theoretical expectations (Kimura 1983) and with some empirical observations (Spielman et al. 2004) of a positive relationship between population growth or effective size and genotypic or genetic diversity in natural populations. The unexpected relationship is, however, in line with the finding by Hämmerli and Reusch (2003) of a lower number of genets in a *Zostera marina* meadow under low disturbance in comparison with a highly disturbed one. It also agrees with the empirical results reported for the subset of meadows impacted by aquaculture effluents (Diaz-Almela et al. 2007), which are also included in the present, more extensive dataset. This previous study revealed lower specific mortality, reflecting higher resistance, in meadows impacted by aquaculture when the meadows initially harbored larger clones and consequently lower clonal diversity.

A negative relationship between current genetic or genotypic diversity and population dynamics is confirmed

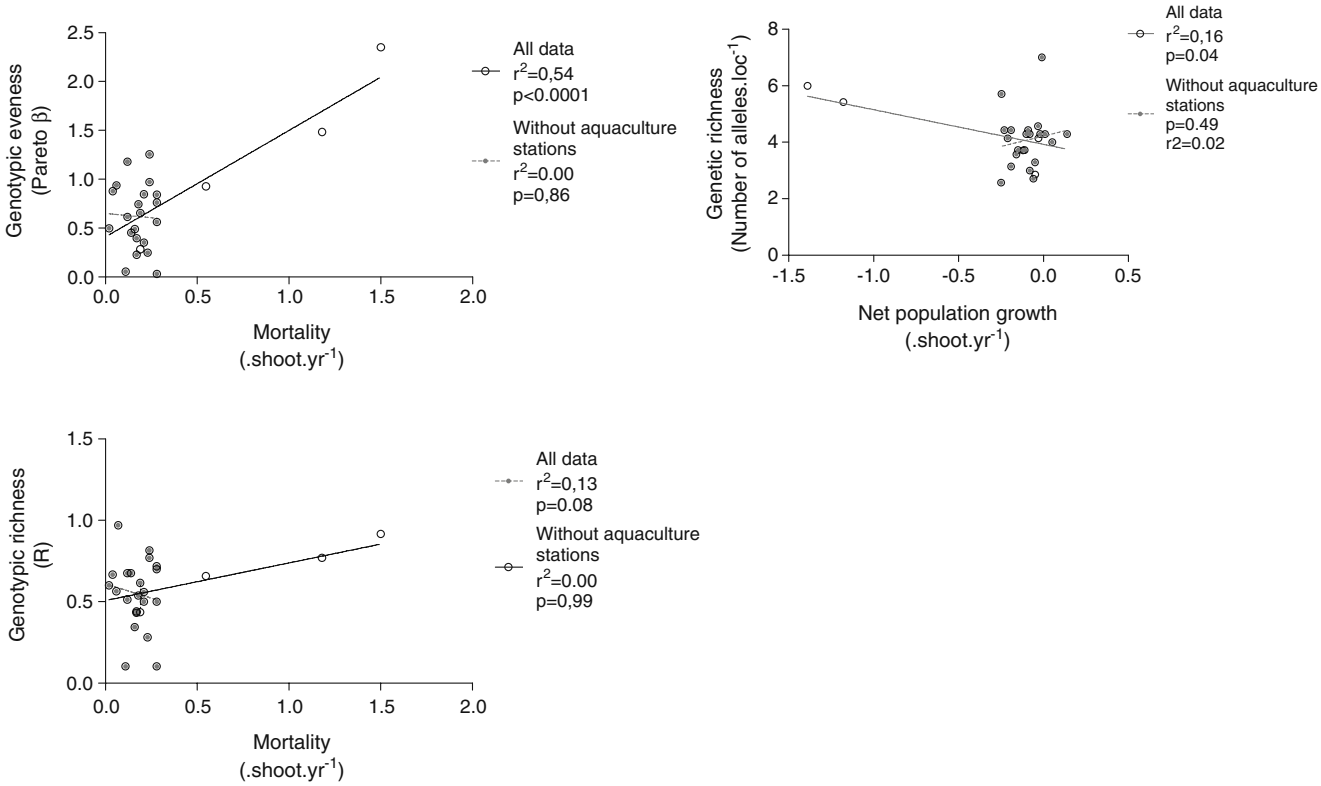

Fig. 1 Overall regressions between demography (RMR and NPG, shoot per year) and genotypic (richness [R] and evenness [Pareto β]) or genetic (allelic richness [$\hat{A}$]) descriptors

when including 17 additional meadows in the study. In contrast with previous studies, information on the genetic structure of the meadows *prior* to the impacts assessed here is not available. Accordingly, the relationship between the genetic structure and the demographic dynamics presented here does not allow causal inferences as to whether the genetic structure observed is derived from the demographic dynamics or vice versa. The general negative trends observed may be attributed to several causes. For instance, Diaz-Almela et al. (2007) observed that meadows with low genotypic diversity are more resistant to impacts and suggested that this may be linked to the presence of large, dominant clones that have been selected in the long term for phenotypic plasticity, thus causing lower genotypic diversity. Low genotypic diversity may also result from competitive exclusion of clonal lineages under mid-term or long-term demographic stability, as suggested for species coexistence models (Huston 1979). These models predict that stable environmental conditions may promote out-competition of clonal lineages and consequent reduction of genotypic diversity, whereas environmental fluctuations may promote clonal diversity by reducing the impact of competition. This is in line with the hypothesis of Hämmerli and Reusch (2003) to explain the lower diversity in more stable meadows by greater efficiency of competition resulting in relatively more outbred clones outcompeting more inbred ones under a lower disturbance regime.

Remarkably, no significant relationship between demography and genetic or genotypic diversity remains when meadows heavily impacted by fish farm effluents are excluded from the analysis, suggesting that such a global relationship emerges only beyond a critical mortality threshold. Although the genotypic and genetic variability observed in the meadows result from the total demographic history of the meadows, likely spanning across millennia, the estimated demographic rates derived here reflect short-term, annual estimates that may depend on current environmental pressures rather than on the history of the meadows. It seems that the global relationship observed here may be driven mostly by those meadows that have experienced extreme disturbance imposed by inputs of aquaculture effluents that have significantly altered the genotypic and genetic diversity of the meadows (Diaz-Almela et al. 2007). The lack of relationship observed when removing the outliers corresponding to highly impacted meadows is consistent with the results reported by Reusch (2006) who found no significant pattern in genotypic diversity across a gradient of moderate disturbance in natural *Z. marina* meadows.

Further support for the weakness of this relationship comes from the analysis of the sedimentation inputs, which shows a rather tight relationship with shoot mortality in *P. oceanica* across the Mediterranean, both when including highly impacted meadows and when excluding these (Table 3). This confirms the notion that *P. oceanica* meadows are strongly vulnerable to inputs of organic materials and nutrients to the sediments (Marbà et al. 1996, 2005; Diaz-Almela et al. 2008). The relationship between shoot mortality and sedimentation inputs explains so much variance (96%) in *P. oceanica* mortality rates that any effect of genetic diversity must necessarily be small, as the residual error is already close to the uncertainty of mortality estimates. Indeed, no significant relationship is observed between the residuals of the relationship between mortality and sedimentation rate and genotypic or genetic diversity descriptors of the studied meadows.

The comparative analyses presented here highlight the challenges of detecting relationships between demography and genetic traits in the presence of other sources of variance, including differential environmental pressures. In order to resolve the influence of genetic composition on the ability of populations to respond to environmental stress, *in situ* observation requires the availability of predisturbance genotypic and genetic parameters, which are seldom available because most studies on declining populations are initiated in response to observed demographic decline. One alternative is to estimate these parameters in non-impacted areas belonging to the same meadow, as reported previously (Diaz-Almela et al. 2007); another is to design experimental manipulations controlling initial genotypic and/or genetic parameters and environmental variability. Such experiments, focused on the role of *Z. marina* genotypic diversity in responses to disturbances, have been conducted in the field by Hughes and Stachowicz (2004) and Reusch et al. (2005) and in laboratory conditions by Ehlers et al. (2008).

These experimental studies have revealed the positive influence of genotypic (i.e., clonal) diversity on the ability of experimental populations of *Z. marina* to successfully overcome major environmental stresses, such as massive grazing (Hughes and Stachowicz 2004) and an exceptional heat wave (Reusch et al. 2005; Ehlers et al. 2008). Yet, *in situ* comparative analyses of genetic structure under heavy mortality induced by fish farm effluents has revealed better performance of *P. oceanica* meadows bearing larger clones and lower allelic diversity before the impact (where the genetic structure at the control stations was used as a proxy for the genetic structure near the cages before the impact; Diaz-Almela et al. 2007). This finding implies that populations with low genotypic and genetic diversity are more resistant to disturbance, a process likely to result from the fitness advantages of individuals with large clonal sizes (Diaz-Almela et al. 2007). This discrepancy in inferred roles of genotypic and genetic diversity in population stability in small-scale experiments versus larger-scale in situ observations raises the question of which spatial and temporal scales are captured in both kinds of studies.

Experimental manipulations at small spatial and temporal scales may not capture the complexity of the genetic structure of natural seagrass meadows shaped across millennia. Both *Z. marina* and *P. oceanica* populations exhibit strong dominance by large clones with tens of thousands of shoots each, reflected in a Pareto distribution of clonal sizes typical of those observed in most clonal organisms tested to date (Arnaud-Haond et al. 2007a) that exhibit millenary life spans, as suggested in some locations for both *Z. marina* (Reusch et al. 1999) and *P. oceanica* (Arnaud-Haond et al., submitted for publication). The genetic structure of natural populations is, therefore, strikingly different from the even composition of experimental plots, which are typically designed with an equal number of ramets for each of the represented genotypes with rhizome connections broken to make small clusters of not more than three (Hughes and Stachowicz 2004) to six (Reusch et al. 2005) connected shoots. These are very small clones compared to those found in natural populations. At small spatial and temporal scales, genotypic richness in synthetic experimental plots may confer greater resistance to sudden imposed disturbances. At larger scales, the presence of large clones, which results in reduced genotypic diversity, may increase resistance to disturbances due to (1) their higher fitness, possibly selected through long periods of time by selective processes related to their ability to outcompete relatively less fit clonal lineages and to cope with environmental fluctuations occurring over large periods of time and (2) their ability to integrate resources and impacts in a possibly heterogeneous landscape.

In order to understand these discrepancies and test for possible effects of plasticity selected over centuries or clonal integration associated with large size, different kinds of experiments and *in situ* observations may be planned in the future. The effect of clonal integration may be tested for by designing plots bearing the same genotypes, but with series of interconnected shoots of different sizes. Better performance of plots with more interconnected shoots would reveal a positive effect of integration potential. As for the possible enhanced phenotypic plasticity of clones selected over decades or centuries in natural meadows, experiments have been designed to date with a selection of the largest clones available, as the aim has been to compare individual clonal fitness, and many replicates were, therefore, needed (Hughes and Stachowicz 2004; Reusch et al. 2005; Ehlers et al. 2008). In order to test for the putative increased fitness of existing large and old clones, experimental plots bearing comparable assemblages in terms of clonal richness may be designed with clones exhibiting a very restricted distribution in natural meadows versus clones known to extend across large areas in the field, likely representing the outcome of selective pressure and competition acting over large temporal scales. Such experiments would allow a better understanding of the evolution of genotypes and the importance of genotypic richness in natural populations, which are relevant questions for clonal organisms in general. As an example, most corals, which rank among the most threatened habitats in the world, are clonal with physical interconnection of the different clone mates for most species.

Finally, besides the importance of genotypic richness, a concept specific to clonal organisms that reflects the co-occurrence of distinct clonal lineages in a given population, experiments focused on genetic richness in a broader sense (i.e., genetic richness as estimated by allelic richness and diversity as estimated by, for example, heterozygosity) are needed. Reusch et al. (2005) proposed to decompose the genetic diversity of seagrass into a combination of "genomic diversity" (i.e., the level of genetic polymorphism) and "genotypic diversity" (i.e., clonal diversity, the number of genetic individuals or clonal lineages actually present in a set of samples that may include replicates of the same clonal lineages). Genotypic diversity estimates the relative abundance of distinct clonal lineages, reflecting the relative contribution of clonal versus sexual reproduction in a given population. For seagrass meadows where many shoots of a given clone may be distributed over tens of meters, genotypic diversity reflects only the relative abundance of genetically distinct individuals (originating from distinct events of sexual reproduction). Above a minimum number of clonal lineages, there is no support for the expectation of any relationship between the number of genotypes and the genetic richness or "diversity" (in the classical population genetics sense) in a given sample of clones (Hughes et al. 2008). Hughes and Stachowicz (2004) specifically state that "to avoid confounding the potential effects of genotypic diversity with those of multilocus heterozygosity on plant performance …, genotypes were assigned to treatments such that average multilocus heterozygosity did not vary with genotypic richness"; i.e., genomic diversity was set be uniform. However, the difference between genomic and genotypic seagrass diversity has not yet been clarified in the literature discussing such studies (e.g., Frankham 2005). Genotypic diversity may enhance the ability of a particular clonal population to cope with environmental changes that occur too suddenly to leave time for sexual reproduction to play a role in the immediate response of the population by rearranging the "genomic diversity" into new clonal lineages. Yet, "genomic diversity" recovering the existence of different allelic forms of the same genes in a given population, which might perform differently under different environmental conditions, is the component of genetic diversity with the greatest bearing on the "evolutionary potential" of populations, operating at mid-time to long-time scales to buffer them against environmental changes. Indeed, "genomic diversity" is the concept that most biologists

not specifically concerned with clonal organisms identify with the term "genetic diversity," as "genotypic diversity" is a concept applicable only to clonal organisms. Therefore, it is important to emphasize that, although theory and some empirical observations support the hypothesis of an influence of genetic diversity (in the classical sense of this term, reflecting "evolutionary potential") on the ability of populations or species to cope with significant environmental changes, this remains to be tested. Future experiments to address this may also focus on allelic richness by designing controlled plots comparable to those designed for clonal organisms for genotypic richness but also manipulating allelic richness and/or heterozygosity, allowing the importance of "genomic diversity" for the evolutionary potential of both nonclonal and clonal species to be tested.

In summary, the results reported here show that, in contrast to expectations, there is no evidence for a negative relationship between seagrass mortality and genetic diversity in the study area. Indeed, a positive relationship emerges when highly impacted meadows are included. A comparative analysis across Mediterranean *P. oceanica* meadows experiencing a broad range of disturbance provides no evidence that any component of genetic diversity significantly affects the level of mortality experienced for any given degree of environmental pressure. Although the importance of genetic diversity in seagrass conservation cannot be dismissed, the results available suggest that this influence emerges only against the variance introduced by other factors in highly simplified experimental situations or under extreme disturbance.

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