Estuaries and Coasts
July 2010, Volume 33, Number 4 : Pages 878-889
http://dx.doi.org/10.1007/s12237-009-9238-9

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*

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 & 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 & 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 & 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 & 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 & 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 & 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 pre-disturbance 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 *Posidonia 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 & Piazzi 2001), but lower mortality rates have been reported in *Posidonia 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 *Posidonia 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 and nutrients to coastal areas fuel sediment microbial activity, increasing the production of sulfide (e.g. Holmer et al 2008; Mascaró 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 organic matter 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 (Díaz-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 gDW organic matter m^{-2} day^{-1}, 50 mg P m^{-2} day^{-1} or 40 mg N m^{-2} day^{-1} (Díaz-Almela et al 2008).

Here, we test the prediction that genetic diversity plays a role in the resistance of *Posidonia oceanica* to environmental pressures, particularly inputs of organic matter and nutrients (nitrogen and phosphorous) 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 organic matter and particulate nitrogen and phosphorous).

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 1600-m^2 (80 x 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 & 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 & 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 & Werth 1993) to estimate the allelic frequencies at nuclear loci in each population.

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

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 i^{th} locus (f and g are identical for homozygotes; $F_{is(i)}$ is the F_{is} estimated for the i^{th} locus using the round-robin method; and z_i=1 for the i^{th} locus if it is homozygous and -1 for the i^{th} 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 & Werth 1993):

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

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

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

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

Genotypic diversity estimates

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

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

The Pareto distribution, which describes the skewed distribution of genotypes among lineages in clonal organisms (Arnaud-Haond et al. 2007a, Arnaud-Haond et al. 2007b), was estimated for each meadow. The parameters of that distribution, beta (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 & 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 & 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 & Belkhir 2007, Arnaud-Haond et al. 2007a).

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

Demography

We assessed the demography of the seagrass (*Posidonia 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 (Díaz-Almela et al. 2008). In fourteen meadows, the level of disturbance, as represented by inputs of organic matter 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 organic matter 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.

211 Censuses were separated by about one year (from 307 to 386 days). During the first census, all
212 shoots within each plot were counted and marked by placing a plastic cable tie around the rhizomes.
213 During the second census, the number of surviving shoots (identified as those marked in the previous
214 census) and the number of recruited ones (identified as young unmarked shoots) were counted. We
215 calibrated the counting error by having two plots counted by independent observers, yielding an
216 estimated error of ± 0.2% and ± 3.5% of the total shoot population for recruits and lost shoots,
217 respectively.
218
219 The repeated censuses allowed direct estimates of specific rates (yr^{-1}) of shoot mortality, recruitment
220 and net population growth rate (Marbà et al. 2005).
221 The specific shoot mortality rate (*RMR*, in year^{-1}, yr^{-1}) was estimated, assuming an exponential
222 population growth model, as
223

224 $$\mathbf{RMR} = -\frac{(\ln N_{S1} / N_{t0}) \times 365}{t_1 - t_0} \qquad (4)$$

225
226 where N_{t0} is the total number of shoots (vertical and horizontal apices) counted in the initial census
227 (t_0, days) at each plot and N_{S1} is the total number of surviving shoots at the second census (t_1, days).
228 The specific shoot recruitment rate (*RRR*, in yr^{-1}) was estimated, assuming an exponential population
229 growth model, as
230

231 $$\mathbf{RRR} = \frac{\ln\left((N_{r1} + N_{s1}) / N_{s1}\right) \times 365}{t_1 - t_0} \qquad (5)$$

232
233 where N_{r1} is the total number of recruited shoots observed at t_1 and N_{s1} is the number of survivors at
234 t_1.
235 Specific net population growth rates (NPG, in yr^{-1}) were estimated as
236

$$NPG = \mathbf{RRR} - R\mathbf{MR} = \frac{\ln(N_{t1} / N_{t0})}{t_1 - t_0} \times 365 \quad (6)$$

Where N_{t1} 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 re-suspension. The contents of 1-3 tubes were combined and collected on a combusted, pre-weighed Whatman GF/F filter. Dry weight of total sediment deposition was obtained after drying the filters at 60ºC to constant weight. Dry weight of organic matter (OM) deposition was measured through combustion of some of the filters. Total P (TP) was obtained after boiling combusted materials in 1 M HCl for 15 min followed by spectrophotometric determination of phosphate (Koroleff 1983). We analyzed the un-combusted samples for total N content with an elemental analyzer (Iso-Analytical Ltd., United Kingdom). Further information on these analyses and spatial patterns of fish-farm inputs are shown in Holmer et al. (2008). We estimated total matter, organic matter, N and P sedimentation rates from these measurements according to Blomqvist and Håkanson (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 beta and maximum MLL size) or genetic diversity (allelic richness, unbiased heterozygosity) and seagrass demography (specific mortality, specific recruitment, specific net population growth) 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 net population growth (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 M 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, 2004; Storey et al., 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 et al., 2002).

Results

Clonal diversity descriptors

The probabilities of obtaining the same multi-locus genotype (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 5 to 34 multi-locus lineages (MLLs) per meadow revealed in sample sizes of 31 to 40 sampling units, 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 sampling units (SUs) belonging to the same MLL] falling between 1 and 35, and Pareto β ranging from 0.03 to 2.35. The clonal subrange also varied widely, from 12.7 m to 78 m in the standardized sampling area of 20 x 80 m.

Genetic descriptors

Allelic richness in Spanish meadows, standardized to the maximum common sampling size observed (N=31) using a sub-sampling approach, varied from 2.6 to 5 alleles per locus. Allelic richness was somewhat higher (4 to 7 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

304 shoots per m^2 in the deep and heavily impacted station of El Campello to 1550 shoots per m^2 in the
305 shallower meadow of Fanals, along the Spanish mainland coast. Specific shoot mortality rates ranged
306 between 0.02 and 0.28 shoot per year in meadows unaffected by aquaculture operations, compared
307 to 0.19 to 1.5 shoot per year at those impacted by fish farm effluents. Low recruitment (0.00 to 0.18
308 shoot per year) was unable to balance the high mortality rate in most meadows (28 of the 30
309 meadows studied). This resulted in declining densities (i.e., declining population growth) at rates of
310 up to -0.25 shoot per year when unaffected by fish farm effluents (in a deep meadow, Sa Paret,
311 Cabrera Island, dominated by a very large clone) and up to -1.39 shoots per year when impacted by
312 fish farming activity (Greek impacted meadow).
313
314 *Sedimentation rates*
315 Ranges of levels of organic matter, N and P and total benthic sedimentation rates, estimated
316 in grams of dry weight.m^2.day^{-1}, were 0.44-3.80, 0.01-0.11, 0.01-0.08 and 5.3-8.94, respectively, at
317 stations located near aquaculture cages (Table 2). Except for total organic matter at Fanals (Table 2),
318 lower values were observed in control stations and in other meadows sampled along the Spanish
319 coasts, which had ranges of 1.59-11.54 for total sedimentation, 0.42-2.09 for organic matter and
320 0.01-0.06 for nitrogen and a noticeably lower range of values for phosphorus (0.00-0.01).
321
322 *Tests for correlations*
323 There was a significant, positive relationship (Table 3) between mortality rates and genotypic
324 evenness (Pareto beta: r^2=0.54, p <0.01, q-value=0.00) across all populations, along with negative
325 relationships between net Population growth and clonal richness and clonal evenness (Pareto beta:
326 r^2=0.51, p=0.00, q-value=0.00) and allelic richness (Â: r^2=0.20, p=0.04, q-value=0.47), although the
327 latter q-values (significance corrected for multiple tests) reflect a non-negligible probability of type I
328 error. In any case, the significance of any of these relationships was entirely dependent on the high
329 evenness and allelic richness in the four meadows highly impacted by fish cages, and no relationship
330 was evident when these populations were excluded. No other correlation was observed between any
331 of the other clonal or genetic *versus* demographic parameters.
332

Mortality rates were generally positively related to sedimentation rates as represented by inputs of organic matter and nitrogen when the heavily impacted meadows were excluded (Table 4). When all meadows were included, this positive relationship was significant for phosphorous. 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 vs. 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 *Posidonia oceanica* meadows, less clonal (more genotypically diverse) populations are associated with higher mortality and that populations with more alleles (more genetically diverse) have lower net population growth. This is shown by the positive relationship observed between mortality rate and genotypic diversity (i.e., clonal richness and evenness as estimated with R and Pareto beta) and the negative relationship between the net rate of population growth and genetic diversity (allelic richness) (Table 3, Figure 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 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- 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 *Posidonia 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, Marbà et al. 2005, Diaz-Almela et al. 2008). The relationship between shoot mortality and sedimentation inputs explains so much variance

403 (96%) in *P. oceanica* mortality rates that any effect of genetic diversity must necessarily be small, as
404 the residual error is already close to the uncertainty of mortality estimates. Indeed, no significant
405 relationship is observed between the residuals of the relationship between mortality and
406 sedimentation rate and genotypic or genetic diversity descriptors of the studied meadows.
407
408 The comparative analyses presented here highlight the challenges of detecting relationships between
409 demography and genetic traits in the presence of other sources of variance, including differential
410 environmental pressures. In order to resolve the influence of genetic composition on the ability of
411 populations to respond to environmental stress, *in situ* observation requires the availability of pre-
412 disturbance genotypic and genetic parameters, which are seldom available because most studies on
413 declining populations are initiated in response to observed demographic decline. One alternative is to
414 estimate these parameters in non-impacted areas belonging to the same meadow, as reported
415 previously (Diaz-Almela et al. 2007); another is to design experimental manipulations controlling initial
416 genotypic and/or genetic parameters and environmental variability. Such experiments, focused on the
417 role of *Zostera marina* genotypic diversity in responses to disturbances, have been conducted in the
418 field by Hughes and Stachowicz (2004) and Reusch et al. (2005) and in laboratory conditions by
419 Ehlers et al. (2008).
420 These experimental studies have revealed the positive influence of genotypic (i.e., clonal) diversity on
421 the ability of experimental populations of *Zostera marina* to successfully overcome major
422 environmental stresses, such as massive grazing (Hughes & Stachowicz 2004) and an exceptional heat
423 wave (Reusch et al. 2005, Ehlers et al. 2008). Yet, *in situ* comparative analyses of genetic structure
424 under heavy mortality induced by fish farm effluents has revealed better performance of *Posidonia*
425 *oceanica* meadows bearing larger clones and lower allelic diversity before the impact (where the
426 genetic structure at the control stations was used as a proxy for the genetic structure near the cages
427 before the impact: Diaz-Almela et al. 2007). This finding implies that populations with low genotypic
428 and genetic diversity are more resistant to disturbance, a process likely to result from the fitness
429 advantages of individuals with large clonal sizes (Diaz-Almela et al. 2007). This discrepancy in
430 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 *Zostera marina* and *Posidonia 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). 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 & 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 i) their higher fitness, possibly selected for 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 ii) 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 & 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 amongst 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

489 studies (e.g., Frankham et al. 2005a). Genotypic diversity may enhance the ability of a particular
490 clonal population to cope with environmental changes that occur too suddenly to leave time for sexual
491 reproduction to play a role in the immediate response of the population by rearranging the 'genomic
492 diversity' into new clonal lineages. Yet, 'genomic diversity' recovering the existence of different allelic
493 forms of the same genes in a given population, which might perform differently under different
494 environmental conditions, is the component of genetic diversity with the greatest bearing on the
495 'evolutionary potential' of populations, operating at mid- to long- time scales to buffer them against
496 environmental changes. Indeed, 'genomic diversity' is the concept that most biologists not specifically
497 concerned with clonal organisms identify with the term 'genetic diversity', as 'genotypic diversity' is a
498 concept applicable only to clonal organisms. Therefore, it is important to emphasize that although
499 theory and some empirical observations support the hypothesis of an influence of genetic diversity (in
500 the classical sense of this term, reflecting 'evolutionary potential') on the ability of populations or
501 species to cope with significant environmental changes, this remains to be tested. Future experiments
502 to address this may also focus on allelic richness by designing controlled plots comparable to those
503 designed for clonal organisms for genotypic richness but also manipulating allelic richness and/or
504 heterozygosity, allowing the importance of 'genomic' diversity for the evolutionary potential of both
505 non-clonal and clonal species to be tested.

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

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Acknowledgments

This research was funded by projects M&MS (EVK3-CT-2000-00044) and MedVeg (Q5RS-2001-02456 of FP5), a project funded by the BBVA Foundation, the Portuguese Fundação para a Ciência e a Tecnologia (FCT) and FEDER through project DIVSTAB-(POCI 2010) and a FCT and FSE postdoctoral fellowship (S.A.-H.).

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Table 1: Sampling locations (country, location, GPS coordinates) and number of sampling units collected and analyzed (N_{SU}). For each location, the clonal descriptors include the number of clonal lineages (N_{MLL}), the genotypic richness (R), and evenness (Pareto beta), as well as maximum clonal size (quantified as number of SU's = 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 (Â), unbiased (H_{nb}) and observed heterozygosity (H_{obs}), departure from Hardy-Weinberg equilibrium (F_{IS}). Demographic data are detailed as Density, Relative Mortality Rate (RMR), Relative Recruitment Rate (RRR) and Net Population Growth (NPG).

	Sampling data					Genotypic data				Genetic data				Demographic data			
	Locality	GPS coordinates		N_{SU}	N_{MLL}	R	Pareto beta	max	CR	Â	Hnb	Hobs	F_{IS}	Density (shoots .m^{-2})	RMR (shoot .yr^{-1})	RRR (shoot .yr^{-1})	NPG (shoot .yr-1)
SPAIN (peninsula)																	
	Roquetas	36º43.26'N	2º37.09'W	40	26	0.64	1.08	6	47.76	3.72	0.56	0.66	**-0.18**	425			
	Rodalquilar	36º51.21'N	2º00.53W	40	21	0.51	0.65	10	44.92	4.43	0.58	0.65	**-0.12**	966			
	Campomanes	38º 37.54' N	0º 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	40º 8.13' N	0º 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	38º 38.37' N	0º 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	38º 25.30' N	0º 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	38º 24.88' N	0º 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	38º 33.59'N	0º 4.56'E	40	31	0.77	0.97	5	68.73	4.43	0.54	0.49	**0.09**	1551	0.24	0.03	-0.19
	Fanals	41º 41,58'N	2º 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	41º 44.15'N	2º 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	42º 18,19'N	3º 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	42º 17,61'N	3º 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	39º 45,13'N	0º 8.07'E	32	12	0.36	0.49	9		3.14	0.51	0.67	-0.13		0.16	0.14	0.30

653 **Table 1** (continued):

	Sampling data			Genotypic data						Genetic data				Demographic data			
	Locality	GPS coordinates		N_{SU}	N_{MLL}	R	Pareto beta	max	CR	Â	H_{nb}	H_{obs}	F_{IS}	Density (shoots .m^{-2})	RMR (shoot .yr^{-1})	RRR (shoot. yr^{-1})	NPG (shoot. yr-1)
SPAIN (balears)																	
Formentera	Es Caló des Oli	38º43.49'N	1º24.16'E	40	15	0.36	0.45	11	46.04	5.00	0.61	0.56	0.07	403			
	Cala Torreta	38º47.45'N	1º25.18'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	38º45.37'N	1º25.83'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	38º43.74'N	1º27.27'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 5m	39º 9.16'N	2º55.83'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 (18m)	39º 8.81'N	2º55.86'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 13m	39º 9.07'N	2º56.92'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 7m	39 º9.00'N	2º56.96'E	40	22	0.54	0.75	8	43.78	3.72	0.42	0.47	**-0.11**	1000	0.18	0.03	-0.15
Mallorca	Magalluf	39 º30.25'N	2º32.59'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	39º 25.05'N	3º16.18'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	40º03.39'N	4º08.26'E	40	5	0.10	0.04	34	68.41	2.57	0.46	0.40	0.15	935			
	Addaia	40º00.97'N	4º12.42'E	37	25	0.67	1.06	5	50.59	3.42	0.56	0.61	-0.08	1090			
ITALY(Sicily)																	
	Porto Palo impacted	36º 42.71'N	15º8.44'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	36º 43.31'N	15º8.48'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	37º 39.59'N	23º 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	37º 39.55'N	23º 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

654

655 **Table 2:** Demographic and sedimentation data for 13 meadows sampled across the Mediterranean.
656 Demographic data are detailed as Density in shoots.m^{-2}, and as Relative Mortality Rate (RMR) , in
657 shoots.yr^{-1}. Total Sedimentation (Sed. Tot.) and the sedimentation of Organic Matter (Sed. OM), of
658 Nitrogen (Sed. N) and of Phosphorus (Sed. P) are indicated in (dry weight: g.m^{2}.day^{-1}).

	Demography		Sedimentation				Residuals
Sampling locations	Density	RMR	Sed. Tot.	Sed. OM	Sed. N	Sed P.	Mort. Vs 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 (18m)	259	0.28	9.00	2.09	0.06	0.00	0.07
Cala Sta. María 13m	762	0.21	2.97	0.57	0.01	-	

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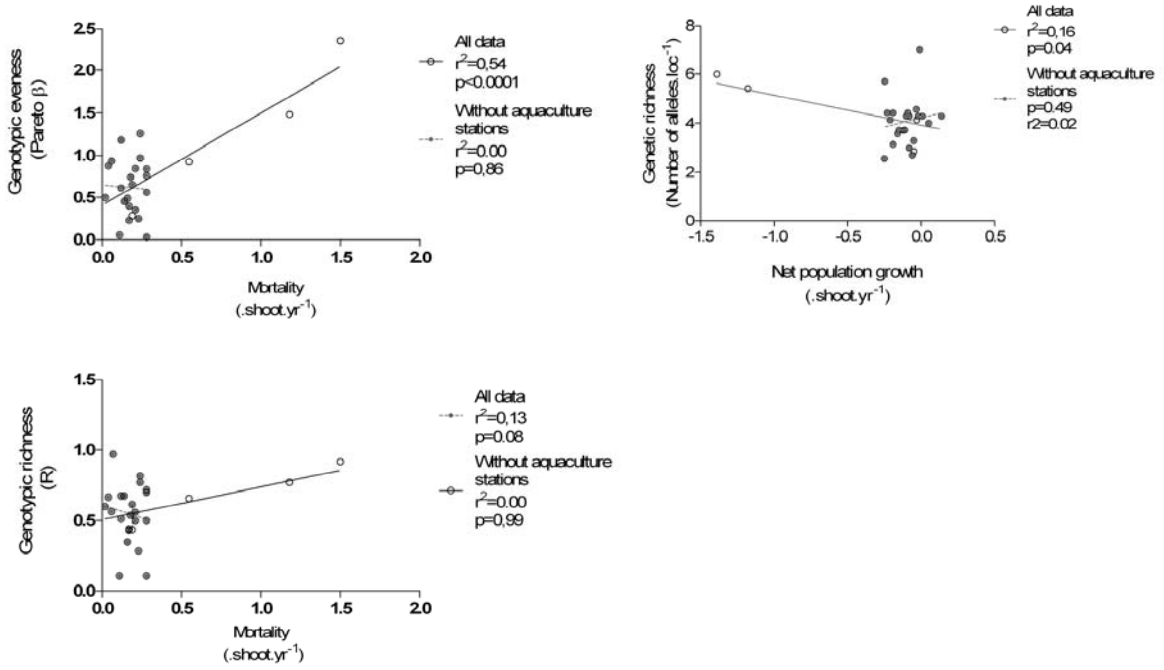
Table 3: Overall regressions tested for between genotypic (R, Pareto beta, Pareto max, CR) and genetic (Â, H_{obs}, H_{nb}, F_{IS}) descriptors and demographic parameters (relative mortality rate RMR, relative recruitment rate RRR, and Net Population Growth NPG) as well as residuals of demographic *versus* sedimentation parameters. 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 non significant values at α=0.05 indicated in grey. Values still significant after correction for multiple tests (i.e. q-values below 0.05) indicated by asterisks *.

	Demographic data	RMR (shoot.yr^{-1})		RRR (shoot.yr^{-1})		NPG (shoot.yr-1)		Residuals Multip. Reg. (Mort. Vs Sed.)	
Genotypic & 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 beta	0.54* p=0.00	-	-	-	0.50* p=0.00	-	-	-
	CR	-	-	-	-	-	-	-	-
Genetics	Â	*0.13. p=0.07*	-	-	-	0.20 p=0.02	-	-	-
	H_{nb}	-	-	-	-	-	-	-	-
	H_{obs}	-	-	-	-	-	-	-	-
	F_{IS}	-	-	-	-	-	-	-	-

667 **Table 4:** Multiple regressions of Sedimentation (Total Sedimentation: Total; Organic Matter: OM;
668 Nitrogen: N; Phosphorus: P) and Demographic parameters (Relative Mortality Rate RMR and Net
669 Population Growth NPG), when analyzing all available data (All data) as well as when excluding the
670 meadows highly impacted by aquaculture installations (Without impacted). Contributions to the
671 multiple regression are detailed for each of the four sedimentation parameters, and the overall
672 regression coefficients, as well as corresponding p-values, are detailed. Non significant values at
673 α=0.05 are indicated in grey. Values still significant after correction for multiple tests (ie q-values
674 below 0.05) are indicated by asterisks *.
675
676

Demographic data	RMR (shoot.yr^{-1})		NPG (shoot.yr-1)	
Sedimentation	All data (±SE)	Without impacted (±SE)	All data (±SE)	Without impacted (±SE)
Total	0.03 (± 0.03) p=0.32	-0.01(± 0.00) p=0.06*	-0.04 (± 0.04) p=0.55	0.04 (± 0.10) p=0.09*
OM	-0.29 (±0,11) p=0.03*	0.12 (± 0.03) p=0.02*	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*	-4.52 (± 1.68) p=0.27
P	15.36 (±15,7) p=0.01*	5.75 (± 5.80) p=0.395	-12.76 (± 6.95) p=0.44	-6.73 (± 11.29) p=0.07*
Overall r^2	0.88 p=0.00*	0.96 p=0.02*	0.75 p=0.02*	0.91 p=0.07*

677 **Figure 1:** Overall regressions between demography (Relative Mortality and Net Population Growth
678 .shoot.yr-1) and genotypic (richness R and evenness Pareto β) or genetic (allelic richness Â)
679 descriptors.
680



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