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Feed-backs between genetic structure and perturbation-driven decline in seagrass (*Posidonia oceanica*) meadows

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Abstract We explored the relationships between perturbation-driven population decline and genetic/genotypic structure in the clonal seagrass *Posidonia oceanica*, subject to intensive meadow regression around four Mediterranean fish-farms, using seven specific microsatellites. Two meadows were randomly sampled (40 shoots) within 1,600 m^2 at each site: the ''impacted'' station, 5–200 m from fish cages, and the ''control'' station, around 1,000 m downstream further away (considered a proxy of the pre-impact genetic structure at the site). Clonal richness (R), Simpson genotypic diversity (D^*) and clonal sub-range (CR) were highly variable among sites. Nevertheless, the maximum distance at which clonal dispersal was detected, indicated by CR, was higher at impacted stations than at the respective control station (paired t-test: $P < 0.05$, $N = 4$). The mean number of alleles ($\hat{A}$) and the presence of rare alleles ($\hat{A}_r$) decreased at impacted stations (paired t-test: $P < 0.05$, and $P < 0.02$, respectively, $N = 4$). At a given perturbation level (quantified by the organic and nutrient loads), shoot mortality at the impacted stations significantly decreased with CR at control stations ($R^2 = 0.86$, $P < 0.05$). Seagrass mortality also increased with $\hat{A}$ ($R^2 = 0.81$, $P < 0.10$), R ($R^2 = 0.96$, $P < 0.05$) and D^* ($R^2 = 0.99$, $P < 0.01$) at the control stations, probably because of the negative correlation between those parameters and CR. Therefore, the effects of clonal size structure on meadow resistance could play an important role on meadow survival. Large genotypes of *P. oceanica* meadows thus seem to resist better to fish farm-derived impacts than little ones. Clonal integration, foraging advantage or other size-related fitness traits could account for this effect.

Keywords Clonal sub-range · Genetic diversity · Population decline · Genotypic diversity · Fish-farm impacts

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Introduction

The interactions between perturbation-driven population decline and genetic diversity are currently the focus of an intense research activity, both for its fundamental interest and for its implications to conservation biology. But the dissection of their influence on each other is a complex task, because a circular feedback is expected between both factors: population decline may affect population genetic resources, and the genetic diversity present in the population prior to perturbation may influence its response.

Strong reductions in population size are expected to erode genetic variability, first through direct loss of genotypes and alleles, and thereafter through increased random genetic drift and elevated inbreeding within the remnant population offspring (Wright 1931; Nei

Journal : 10592 | Dispatch : 29-1-2007 | Pages : 15
Article No. : 9288 | LE | TYPESET
MS Code : COGE-06-496 | CP | DISK

1975; Young et al. 1996). Although most experiments and field observations support positive interactions between population size and genetic diversity (Leimu et al. 2006), the effects of population decline in the genetic diversity of the adult remnant populations are highly variable (e.g. Young et al. 1996; Lee et al. 2002; Edwards et al. 2005; Lowe et al. 2005; Reusch 2006). This variability can be accounted for by the role of life-history traits, such as the generation time or the breeding regime in the speed of genetic diversity erosion (Young et al. 1996; Collevatti 2001; Lee et al. 2002; Lowe et al. 2005; Leimu et al. 2006). Moreover, intermediate perturbation levels may enhance genetic diversity in populations, producing space available for new genotypes to install, as has been described among several clonal plants, in which developed and stable populations show dominance by a few clones (McNeilly and Roose 1984; Watkinson and Powel 1993).

Among seagrasses (clonal plants), there is evidence that perturbation-induced regression may reduce meadow genetic polymorphism (Alberte et al. 1994; Micheli et al. 2005). Therefore, the empirical evidence suggests the existence of species-specific thresholds of population reduction and isolation under which population genetic diversity would not be significantly affected (Leberg 1992; Young et al. 1996; Lowe et al. 2005).

At a given perturbation level, populations bearing high genetic diversity are expected to be more resistant (i.e. to be less affected by a given perturbation), and to exhibit faster recovery than homogeneous ones because the probability of occurrence of resistant variants is expected to be higher and/or through processes of functional complementarity (Loreau and Hector 2001; Reusch and Hughes 2006). Overall, a majority of empirical studies indicate positive interactions between population genetic diversity and fitness (Leimu et al. 2006). But more studies are needed to confirm this tendency (Leimu et al. 2006), specially for the population fitness components of resistance to and recovery from perturbations. In the seagrass *Zostera marina*, higher genetic diversity (in terms of allelic richness and/or heterozygosity) increased survival, growth and flowering rates of transplants (Williams 2001; Hämmerli and Reusch 2003).

Among clonal plants, another component of population genetic diversity is genotypic diversity (clonal diversity), the number and evenness of genetic individuals (genets) represented among the ramets. Recent experiments indicate that genotypic diversity can increase resistance (Reusch et al. 2005) and speed of recovery (Hughes and Stachowicz 2004) of the clonal seagrass *Zostera marina* facing perturbations (Reusch and Hughes 2006).

The seagrass *Posidonia oceanica*, is a slow-growing (Marbà and Duarte 1998) and extremely long-lived clonal plant (Mateo et al. 1997). Its primary reproductive mode is vegetative, with sparse sexual reproduction (Gambi and Guidetti 1998; Balestri and Cinelli 2003; Díaz-Almela et al. 2006). *P. oceanica* is endemic to the Mediterranean coasts (den Hartog 1970), where its meadows are the dominant ecosystems between 0.3 and 45 m depth (Bethoux and Copin-Monteagut 1986; Pasqualini et al. 1998). These meadows provide important ecosystem functions, both in terms of production and biodiversity (Hemminga and Duarte 2000), which are being jeopardised by their tendency towards a substantial decline (e.g. Marbà et al. 2005).

One of the major threats to *P. oceanica* meadows is the growing marine aquaculture activity (Holmer et al. 2003). Fish farm effluents produce rapid reductions in meadow shoot density, which are particularly fast in the areas next to fish cages (Delgado et al. 1997, 1999; Ruiz et al. 2001). If there is an effect of this perturbation on the genetic diversity and clonal structure of *P. oceanica* meadows, it should be best detected in these areas.

In the present work, we use seven microsatellite markers (Alberto et al. 2003; Arnaud-Haond et al. 2005) to investigate the variability in genetic diversity and genotypic structure of *P. oceanica* meadows situated around four fish farms across the Mediterranean, for which demographic trajectories have been evaluated (Diaz-Almela et al. submitted). Our objectives are (1) to elucidate the effects of shoot density regression on meadow clonal structure and genetic diversity and (2) to derive insights into the possible importance of the clonal structure and genetic diversity of the meadow previous to perturbation on its resistance to fish-farm impacts.

Materials and methods

Samples of the seagrass *Posidonia oceanica* were collected in meadows located around four fish farms along the Mediterranean (Fig. 1; Table 1), at water depths ranging between 16 and 28 m among sites. The farms in Cyprus, Italy and Spain were located in open coasts about 1 km from shores, whereas the farm in Greece was located in a strait about 300 m from shore and was the shallowest (16 m). All studied meadows near (i.e. 5–15 m) the cages exhibited high rates of shoot decline, as reflected by the annual balance between shoot recruitment and mortality rates assessed by shoot census in permanent plots (Table 1). Conversely, shoot populations were in steady state or declining at slow

Journal : 10592 Dispatch : 29-1-2007 Pages : 15
Article No. : 9288 LE TYPESET
MS Code : COGE-06-496 CP DISK

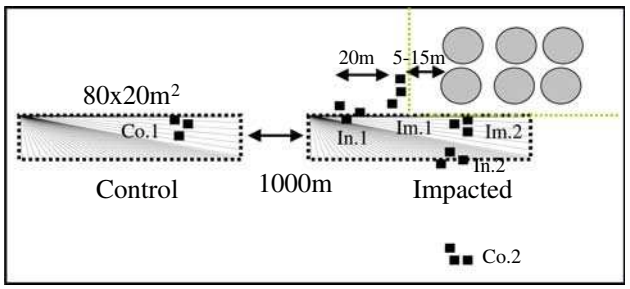

Fig. 1 Above: locations of the fish farm sites analysed in this study. Circle: El Campello (Spain), square: Porto Palo (Sicily), diamond: Sounion (Greece), triangle: Amathous (Cyprus). Below: sampling scheme of the genetic sampling stations (Impacted, Control). The genetic sampling areas encompass a variable number of demographic census plots, belonging to impacted (Im) and intermediate (In) demographic stations, in the case of the genetic impacted station, or to a control (Co) demographic station, in the case of the genetic control station

rates, similar to those observed in other *P. oceanica* meadows elsewhere (Marbà et al. 2005), when growing at 800–1,200 m away from the cages (Table 1).

The sampling for genetic structure was performed in each site, within two stations (i.e. hereafter called ''impacted'' and ''control'' stations), encompassing an area of 80×20 m^2 each. These stations contained the permanent plots where annual shoot demographic parameters were estimated (Table 1). Mean shoot densities within the ''impacted'' stations, located at the edge of the meadow nearest to fish cages, ranged from 20 (El Campello, Spain) to 165 (Sounion, Greece) shoots m^{-2} and the meadow showed very rapid net population decline. The ''control'' station, situated 1,000–1,200 m away from cages, in the direction of the main current, had mean shoot densities of 68 (El Campello, Spain) to 395 (Porto Palo, Sicily) shoots m^{-2}.

A total of 38–40 ramets (i.e. leaf shoots) were collected within each genetic sampling station, at randomly drawn coordinates, within a rectangular area of 80×20 m^2. The base of each leaf bundle, including the shoot apical meristem, was preserved in silica crystals until DNA extraction. Distributions of distances between pairs of collected samples (normal, slightly skewed towards low distances) were not significantly different among sampling sites and stations.

Genomic DNA was extracted following a standard CTAB extraction procedure (Doyle and Doyle 1988). The sample polymorphism was analysed with the most efficient combination (Arnaud-Haond et al. 2005) of seven nuclear microsatellites reported by Alberto et al. (2003) to allow the resolution of clonal membership, using the conditions described by Arnaud-Haond et al. (2005). The number of alleles and size range (bp, Table 2) of some of the microsatellite loci was enlarged in this study as compared with the initially described by Alberto et al. (2003).

Table 1 Location, water depth, distance to fish cages and year of initiation of fish farm activities of each sampling site and station

Site	Coordinates	Depth (m)	Distance to cages (m)	Fish farm initiated in:	Demography station	Shoots m^{-2}	Relative mortality rate (yr^{-1})*	Relative recruitment rate (yr^{-1})*
Amathous (Cyprus)								
IMPACTED	34°41′96N 33°12′00E	20.5	300	1992	Im. 1, 2	454 ± 42	0.186 ± 0.050	0.141 ± 0.041
CONTROL	34°41′99N 33°12′36E	19.5	1,200		Co. 1, 2	491 ± 51	0.185 ± 0.067	0.139 ± 0.047
Sounion (Greece)								
IMPACTED	37°39.586′N 23°57.291′E	15.5	10–30	1996	Im.-In. 1, 2	165 ± 25	1.606 ± 0.479	0.095 ± 0.034
CONTROL	37°39.550′N 23°58.240′E	16.2	1,200		Co 1	365 ± 34	0.070 ± 0.020	0.056 ± 0.013
Porto Palo (Sicily)								
IMPACTED	36°42.710′N 15°8.438′E	22.5	5–50	1993–1994	Im.-In. 1	156 ± 17	1.241 ± 0.491	0.004 ± 0.003
CONTROL	36°43.307′N 15°8.474′E	20	1,000		Co. 1, 2	395 ± 35	0.577 ± 0.275	0.027 ± 0.009
El Campello (Spain)								
IMPACTED	38°25.300′ N 0°20.829′W	28	10–30	1995	Im.-In. 1, 2	20 ± 6	0.617 ± 0.128	0.091 ± 0.027
CONTROL	38°24.875′N 0°21.139′W	28	1,000		Co. 1	68 ± 4	0.056 ± 0.029	0.106 ± 0.019

The demographic stations encompassed by the genetic sampling stations at each site are also provided, as well as the mean shoot densities and mean mortality, and recruitment rates at the genetic sampling stations (Mean ± SE)

Journal : 10592 | Dispatch : 29-1-2007 | Pages : 15
Article No. : 9288 | LE | TYPESET
MS Code : COGE-06-496 | CP | DISK

Table 2 Total alleles per locus across the four Mediterranean meadows and microsatellites size ranges found in this study

Locus name	PO 15	PO 5	PO5-40	PO5-49	PO5-10	PO4-3	PO5-39
Base pairs range	141–167	154–198	194–288	208–252	159–171	168–178	176–182
Number of alleles	15	10	36	15	6	5	4

Clone discrimination

We used the round-robin method (Parks and Werth 1993) to estimate the allelic frequencies in each population sample. This sub-sampling approach avoids the overestimation of the rare alleles, by estimating the allelic frequencies for each locus on the basis of a sample pool composed of all the genotypes distinguished among all the loci, except the one for which allelic frequencies are estimated. This procedure is repeated for all loci, taking into account Wright's inbreeding coefficient estimated for each loci after the exclusion of identical multi locus genotypes (Young et al. 2002), and the probability that the same multi-locus genotype is produced by different sexual events (P_{gen} (f)) is then estimated as:

$$P_{\text{gen}}(f) = \prod_{i=1}^{l} [(f_i g_i) * (1 + (z_i \times (F_{is(i)})))]2^h \quad (1)$$

where l is the number of loci, h is the number of heterozygous loci, f_i and g_i the allelic frequencies of the alleles f and g at the ith locus (with f and g identical for homozygotes), the F_{is} estimated for the ith locus with the round-robin method, and $z_i = 1$ the ith locus that is homozygous and $z_i = -1$ for the ith locus that is heterozygous.

When the same genotype is detected more than once (n) in a population sample composed of N ramets, the probability that the samples 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):

$$P_{\text{sex}} = \sum_{i=n}^{N} \frac{N!}{i!(N-i)!} [P_{\text{gen}}]^i [1 - P_{\text{gen}}]^{N-i} \quad (2)$$

where n is the number of sampled ramets with the same multi-locus genotype, N is the sample size, and P_{gen} is the probability of the common genotype. Estimates were performed using the software GENCLONE 1.0 (Arnaud-Haond and Belkhir in press)

Clonal diversity and structure

The clonal, or genotype diversity (R) at each station has been estimated as:

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

where G is the number of genotypes in the sample and N is the number of ramets analysed, as was recommended by Dorken and Eckert (2001) and Arnaud-Haond et al. (2005). Using this estimator, the minimum value for clonal diversity in a monoclonal stand is always 0, independently of sample size, and the maximum value is still 1, when all the different samples analysed correspond to distinct genotypes.

The complement of Simpson index (Pielou 1969) for genotypic diversity in each station, representing the probability of encountering distinct Multi-Locus Genotypes (MLG) when randomly taking two sample units was estimated as:

$$D* = 1 - \sum_{i=1}^{G} \left[\frac{n_i(n_i-1)}{N(N-1)}\right] \quad (4)$$

where N is the number of sample units (ramets sampled), G the number of multi-locus genotypes, and n_i is the number of sample units sharing the ith MLG.

The clonal sub-range (i.e., the maximum distance in meters between two identical genotypes belonging to the same clone) was estimated for each station (Harada et al. 1997; Alberto et al. 2005). All clonal diversity and structure parameters were calculated with GENCLONE 1.0 (Arnaud-Haond and Belkhir in press).

Genetic diversity and structure

Genetic diversity within populations was estimated with the mean number of alleles per locus, which was standardized ($\hat{A}$) to the lowest sample size collected in a station (33 samples in Greece, control station), using GENCLONE 1.0 (Arnaud-Haond and Belkhir in press). After identification of ramets belonging to the same genets, replicates were removed from the dataset to perform the following calculations using the Genetix 4.0 package (Belkhir et al. 1996–2001). Unbiased (H_{E}) and observed (H_{O}) gene diversities (Nei 1987) were calculated. A permutation procedure (1,000 permutations) was used to test whether a particular estimate of the overall inbreeding coeffi-

Journal : 10592 Dispatch : 29-1-2007 Pages : 15
Article No. : 9288 LE TYPESET
MS Code : COGE-06-496 CP DISK

Table 3 Number of distance pairs per distance class in each station, with and without genet replicates

Station	No. distance pairs per distance class	$b_F \pm$ SE	$Sp \pm$ SE
Cyprus impacted			
Ramets	130	$-0.009 \pm 0.006^{P = 0.08}$	0.009 ± 0.006
Genets	27 (18 higher class)	-0.011 ± 0.005^{ns}	0.010 ± 0.005
Cyprus control			
Ramets	130	-0.006 ± 0.004^{ns}	0.006 ± 0.004
Genets	54 (55 lower class)	0.003 ± 0.002^{ns}	0.003 ± 0.002
Greece impacted			
Ramets	111	**–0.030 ± 0.005*****	**0.031 ± 0.005**
Genets	95	***–0.030 ± 0.001*****	***0.030 ± 0.001***
Greece control			
Ramets	88	**–0.010 ± 0.002***	**0.010 ± 0.002**
Genets	84 (76 higher class)	***–0.009 ± 0.002****	***0.009 ± 0.002***
Italy impacted			
Ramets	130	**–0.022 ± 0.006****	**0.022 ± 0.006**
Genets	79 (70 higher class)	**–0.015 ± 0.002****	**0.015 ± 0.002**
Italy control			
Ramets	130	**–0.012 ± 0.005***	**0.012 ± 0.005**
Genets	69 (61 higher class)	**–0.014 ± 0.002***	**0.014 ± 0.002**
Spain impacted			
Ramets	123–124	**–0.020 ± 0.003***	**0.020 ± 0.003**
Genets	54 (55 lower class)	**–0.041 ± 0.009****	**0.042 ± 0.009**
Spain control			
Ramets	130	**–0.032 ± 0.006****	**0.033 ± 0.006**
Genets	42 (43 lower class)	**–0.044 ± 0.007****	**0.046 ± 0.007**

The observed regression coefficient b_F between mean $\hat{F}_{ij}$ and the Log_e of mean geographic distance within each distance class ± SE and the *Sp* statistic for each spatial autocorrelation analysis. The significant values are in bold. The b_F and *Sp* values underlined or marked in italics indicate significant differences between the stations signalled in this way

cient (F_{is}), was significantly different from 0. Heterozygosity was also calculated for each genotype, and relationships of genotype heterozygosity with genotype frequency and clonal sub-range were explored through regression analysis.

Spatial autocorrelation within stations was assessed using the kinship estimator coefficient of Ritland ($\hat{F}_{ij}$) as a genetic relatedness statistic (Ritland 1996), calculated using the GENCLONE 1.0 software (Arnaud-Haond and Belkhir in press). We performed regression analyses of mean $\hat{F}_{ij}$ against the Log_e of mean geographic distance, within each distance class. This allowed the test of the adequacy of two dimensional isolation-by-distance models in each station (Rousset 1997).

The autocorrelation analyses were performed twice for each station and site: (i) first including all samples, which mostly estimates the genetic neighbourhood of ramets of the same genet and (ii) using permutations (1,000) in order to include at each permutation only one ramet (and one of the possible corresponding coordinates, randomly chosen for each permutation step) from each genet. This approach removes the influence of the spatial pattern of clonal growth from estimates of the relationship between genetic and geographic distance, allowing us to test for limitations to gene dispersal through seeds and pollen. The spatial scale (80×20 m^2) and number of distance classes (6) were the same across stations. For each autocorrelation analysis the upper levels of distance classes were defined in order to include, as much as possible, an even number of distance pair comparisons among classes (Table 3). Among stations, the minimum geographic distance between pairs of samples was of 0.3–0.7 m (0.6–1.6 m when genotype replicates were excluded), and the maximum distance ranged between 63.4 and 76.9 m. We tested the significance of the regression slopes using 1,000 random permutations of the sample coordinates.

From the slopes of the regressions of genetic distance to geographic distance within each distance class, we calculated the *Sp* statistic (Vekemans and Hardy 2004), following the equation (5):

$$Sp = -\frac{\hat{b}_F}{(1 - \hat{F}_{(1)})} \quad (5)$$

where $\hat{b}_F$ is the slope of the linear regression and $\hat{F}_{(1)}$ represents the mean Kinship coefficient within neigh-

Journal : 10592 | Dispatch : 29-1-2007 | Pages : 15
Article No. : 9288 | LE | TYPESET
MS Code : COGE-06-496 | CP | DISK

bours (i.e. the lowest distance class). We tested for differences between regression slopes from impacted and control stations within each site performing *F*-tests of the slopes, for the spatial autocorrelation with genet replicates. In the case of the spatial autocorrelation without genet replicates, we simply compared the 95% confidence intervals of the permutations performed with one genet real coordinate each time.

Testing for the impact of perturbations on the genotypic and genetic variability in the meadows

In the absence of pre-disturbance samples, we have considered the genetic structure at control quadrats to provide a proxy for the genetic structure of the meadow next to the fish farm prior to disturbance. We based this assumption on the fact that the distance between stations (800–1,200 m) was relatively low for a species forming long-lived large clones (Sintes et al. 2006) in which, for a large proportion of meadows, the genetic neighbourhood has been shown to exceed the sampling area of stations sampled in this work (1,600 m^2; Arnaud-Haond et al. in press). Moreover, the sampling was parallel to the coast at uniform depths between stations.

We therefore compared genetic structures at control and impacted stations among sites. We considered the four sites across the Mediterranean as independent replicates to test for a consistent impact of fish farms on the genetic and clonal diversity of the seagrass meadows. Differences in Clonal sub-range (CR), Genotypic richness (*R*), Simpson Diversity Index (*D*), the mean number of alleles ($\hat{A}$) and expected (H_E) and observed (H_O) heterozygosities between impacted and control stations was analysed performing pairwise *t*-tests over data around the Mediterranean. When significant pairwise differences between stations were detected in a parameter, we searched for correlations between the magnitude of the differences and benthic sediment inputs (total, organic matter and nutrients), which provides a metric for the intensity of fish farm pressures on the farms (Holmer et al. in press) and shoot density between stations.

Testing for the influence of genetic diversity components on demographic responses to perturbation

Data on meadow shoot recruitment and mortality were obtained by direct census of tagged plants within three permanent plots installed in each demographic station (genetic sampling stations encompassed a variable number of demographic stations, see Table 1) and site, as described in Diaz-Almela et al. (submitted). In that work, shoot mortality and recruitment variability have been shown to change exponentially, or in some cases following a power-law with the total, organic and nutrient benthic input rates measured in situ. Therefore, the possible influences of genotypic and genetic diversity components on the demographic response at a given environmental forcing were assessed by comparing the residuals (averaged within each genetic station, Table 4) of mortality and recruitment versus

Table 4 Genotypic structure parameters at the stations investigated in terms of number of multilocus genotypes discriminated (*G*) in *N* genotyped samples, the unbiased genotypic richness (*R*), Simpson diversity (*D*) and the clonal sub-range (CR)

	Genotypic structure					Genetic structure			Mean residuals of mortality with inputs			
Sampling locations	*N*	*G*	*R*	*D*	CR	$\hat{A}$	F_{is}	F_1	Total	OM	N	P
Amathous												
IMPACTED	40	18	0.44	0.880	76.6	29	**–0.14**	–0.02	–0.85	–0.23	–0.07	–0.18
CONTROL	40	25	0.62	0.937	65.1	30	0.01	–0.03	–0.24	–0.68	–0.29	–0.30
Sounion												
IMPACTED	37	31	0.92	0.994	29.9	41	–0.01	0.01	0.98	1.26	0.68	0.24
CONTROL	33	29	0.97	0.998	12.7	48	–0.02	–0.01	–0.27	–1.01	–1.19	–1.06
Porto Palo												
IMPACTED	40	34	0.77	0.981	60.5	38	0.06	–0.01	0.19	–0.06	0.01	–0.17
CONTROL	38	32	0.72	0.971	41.7	40	–0.04	0.00	–0.48	–0.49	–0.18	0.23
El Campello												
IMPACTED	39	26	0.66	0.961	70.9	20	**–0.27**	0.02	–0.25	–0.36	–0.20	–0.18
CONTROL	40	23	0.56	0.953	68.7	28	**–0.24**	0.04	–0.66	–1.34	–1.23	

Genetic structure parameters: the mean number of alleles ($\hat{A}$), the mean inbreeding coefficient (F_{is}, marked in bold when it deviates significantly from Hardy–Weinberg equilibrium), and the mean Ritland kinship coefficient between neighbour samples ($\hat{F}_{(1)}$, without genet replicates). The residuals of regressions between mortality and total, Organic Matter, Nitrogen and Phosphorus sedimentation rates are also provided

Journal : 10592 | Dispatch : 29-1-2007 | Pages : 15
Article No. : 9288 | LE | TYPESET
MS Code : COGE-06-496 | CP | DISK

sediment inputs at impacted stations with the genetic and genotypic structure at control stations. Control stations were assumed to provide a proxy for the genetic and genotypic structure prior to the impact at each site.

Results

Genetic variability

Clonal structure and genetic diversity showed high variability among sites (Table 4). Genotypic richness (R) ranged between 0.44 (Amathous, ''Impacted'', Cyprus) and 0.92 (Sounion, Control, Greece). The number of genotypes differing in just one dinucleotide repetition at a unique locus varied among sites and stations (1 at Sounion Control station to 16 at El Campello impacted station). The frequency of such genotypes did not depend on the station, the mean number of samples per genotype or the clonal sub range, but it was negatively correlated to the allelic diversity, suggesting that those very similar genotypes did not derive from somatic mutations and arose naturally from the lower number of possible allelic combinations. The standardized mean number of alleles ($\hat{A}$) present in each station ranged between 20 (El Campello, ''Impacted'', Spain) and 48 (Sounion, ''Control''), and the allelic frequencies were more similar between stations that between localities (see annex tables). The chances of obtaining the same multi-locus genotype by sexual recombination were very small (all $P_{sex} < 0.01$). Therefore, all identical genotypes were considered members of the same clone.

As clonal richness, Simpson diversity was minimum at Amathous (''Impacted'', $D = 0.880$) and was highest at Sounion (''Control'', $D = 0.998$, Table 4). On the contrary, the clonal sub-range was minimum at the Sounion ''control'' station (CR = 12.7 m) and maximum at the Amathous ''impacted'' station (CR = 76.6 m, Table 4). Genotypic and allelic diversity decreased with increasing clonal sub-range, as the maximum clonal size was linked to the dominance of the sample by a few clones (CR and R: $R^2 = 0.80$, $P < 0.002$; CR and D*: $R^2 = 0.49$, $P < 0.04$; CR and $\hat{A}$: $R^2 = 0.79$, $P < 0.003$, $n = 8$).

The variability in genetic structure between stations was much lower than among sites. Moreover, common Multilocus genotypes (MLG) were found between impacted and control stations at Amathous (1 MLG), Porto Palo (2 MLG) and El Campello (2 MLG).

Genotype heterozygosity was not correlated to genotype frequency or clonal sub-range (data not shown). Significant heterozygote excesses were detected at the ''control'' station of El Campello (Spain, $P < 0.001$) and at the ''impacted'' station of Cyprus. The remaining stations did not differ significantly from Hardy–Weinberg equilibrium (Table 4). The mean Ritland kinship coefficient between neighbours was near 0 at all stations and sites (Table 4).

Significant ($P < 0.001$ to $P < 0.05$) spatial autocorrelation patterns were detected either with or without genotype replicates in all sites and stations with the exception of Cyprus (Table 3), revealing a significant relationship between genetic and geographic distance. The spatial autocorrelation patterns varied widely across sites: comparing control stations among sites, it was lowest in the shallowest site (Greece: $Sp = 0.010 \pm 0.002$, Table 3) and highest at the deepest site (Spain: $Sp = 0.032 \pm 0.006$, Table 3). The removal of the MLG replicates did not affect the strength and patterns of the spatial autocorrelation in any consistent way (Table 3).

Impact of perturbations on the genotypic and genetic variability in the meadows

The slope of the spatial correlation and the Sp statistic were not significantly different between stations, except in Greece, where Sp at the impacted station was three times higher than at control station ($P < 0.05$). Such difference persisted when the autocorrelation was performed without MLG replicates (Table 3).

The observed heterozygosity H_o was lower at impacted than at control stations in every site with the exception of Cyprus, in which no significant differences were found in shoot density and net population growth between the so called ''impacted'' and ''control'' stations. Nevertheless, the reduction was not significant, even excluding this site (Pairwise t-test, two tails, $P = 0.17$, $n = 3$).

In turn the clonal sub-range was systematically and significantly higher at ''impacted'' stations than at control ones (paired t-test, $P < 0.05$, $n = 4$, Fig. 2). Despite their negative relationship with clonal sub-range, no consistent variation was found in clonal richness R or Simpson diversity index between impacted and control stations across sites (Fig. 2). Nevertheless, the mean number of alleles (also inversely related to clonal sub-range) significantly decreased, as compared to their respective control stations (paired t-test, $P < 0.05$, $n = 4$, Fig. 2). The mean number of rare alleles (frequency < 5% at any station of a given site) was also significantly lower at impacted stations as

Journal : 10592 Dispatch : 29-1-2007 Pages : 15
Article No. : 9288 LE TYPESET
MS Code : COGE-06-496 CP DISK

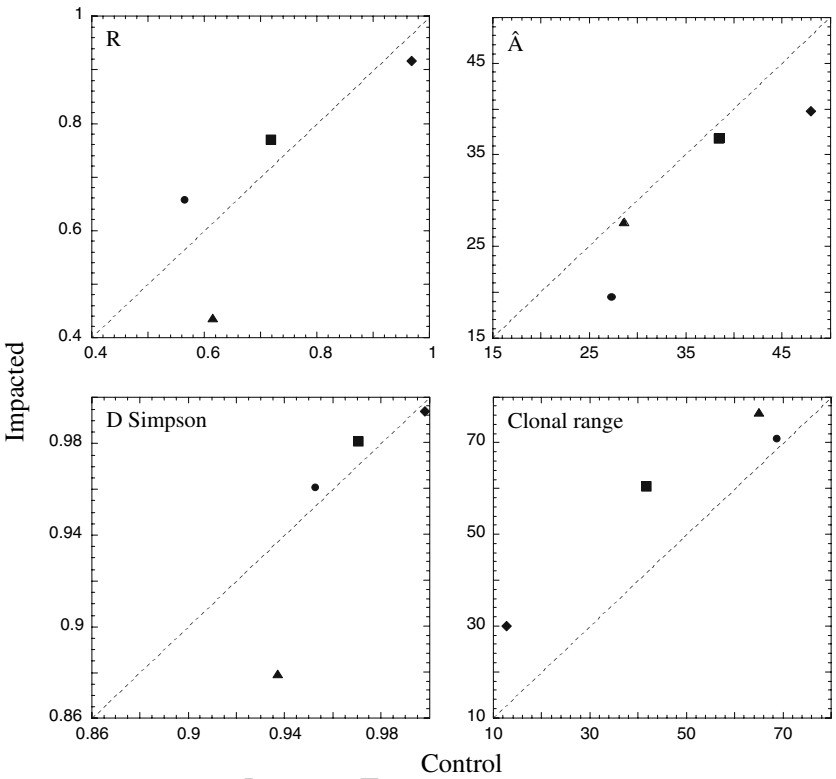

Fig. 2 Diagrams of clonal richness (R), mean number of alleles ($\hat{A}$), Simpson genotypic diversity (D) and clonal sub-range (CR) at impacted and control stations. The symbols correspond to the sites indicated in Fig. 1

Table 5 Coefficient of determination of linear regressions describing the relationship between differential shoot mortality at impacted stations (i.e. the residuals of shoot mortality with sedimentation rates) and clonal richness (R), Simpson clonal diversity (D), mean number of alleles ($\hat{A}$) and maximum clonal range (meters) at the respective control stations

Demographic residuals at impacted stations	Genetic structure at control stations ($n = 4$)			
	R	D	$\hat{A}$	Clonal range (m)
Mortality-Total inputs	$R^2 = 0.70$, ns	$R^2 = 0.99$**	$R^2 = 0.79$, ns	$R^2 = 0.79$, ns
Mortality-OM inputs	$R^2 = 0.94$*	$R^2 = 0.70$, ns	$R^2 = 0.78$, ns	$R^2 = 0.85$, ns
Mortality-N inputs	$R^2 = 0.96$*	$R^2 = 0.67$, ns	$R^2 = 0.81$, ns	$R^2 = 0.86$*
Mortality-P inputs	$R^2 = 0.83$, ns	$R^2 = 0.61$, ns	$R^2 = 0.62$, ns	$R^2 = 0.70$, ns

ns: $P > 0.05$; *: $P < 0.05$; **: $P < 0.01$

compared to their respective control stations ($P < 0.02$, $n = 4$).

The increase in clonal sub-range at impacted stations showed no significant correlation with differences in shoot mortality rates and shoot densities between impacted and control stations ($R^2 = 0.66$, $P = 0.121$, $n = 4$; $R^2 = 0.43$, $P = 0.211$, $n = 4$, respectively). The systematic reduction in the mean number of alleles at impacted stations also showed a non-significant relationship with differences in shoot mortality rates (expressed as $\ln(\text{year}^{-1})$, $R^2 = 0.73$, $P = 0.096$, $n = 4$) and with differences in sediment input rates (expressed as $\ln(g(\text{DW})\text{m}^{-2}\,\text{d}^{-1})$, $R^2 = 0.49$, $P = 0.189$, $n = 4$).

Possible influence of genetic structure components on demographic responses to perturbation

The residuals of shoot mortality with total, organic and nutrient inputs at the impacted stations were correlated with the clonal sub-range (CR) at the control stations (Table 5), assumed to be representative of meadow genetic structure in the area near the cages, before impact. The negative relationship was significant between CR and the residuals of shoot mortality with nitrogen input rates ($R^2 = 0.86$, $P < 0.05$, $n = 4$; Fig. 3, Table 5). The residuals of shoot mortality at the impacted stations were positively correlated with R, $\hat{A}$ and D* at control stations

Journal : 10592 Dispatch : 29-1-2007 Pages : 15
Article No. : 9288 LE TYPESET
MS Code : COGE-06-496 CP DISK

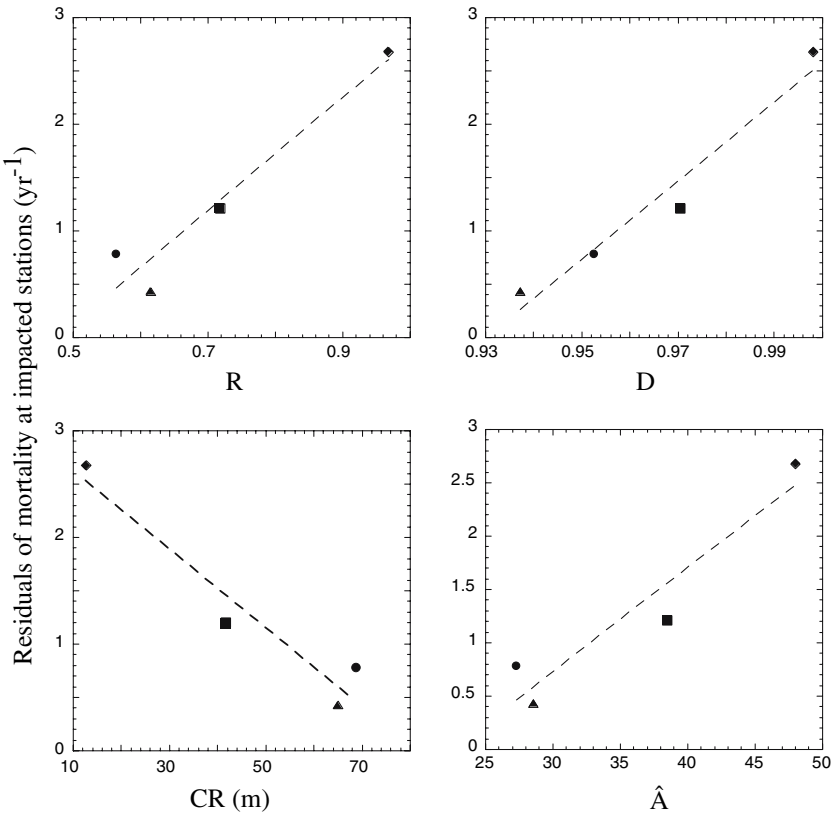

Fig. 3 Regressions of Clonal richness (R), Simpson clonal diversity (D), clonal sub-range (CR) and mean number of alleles ($\hat{A}$) at the control stations with the residuals of shoot mortality with N sedimentation rate

(Table 5). The strongest and most significant correlations occurred between residuals of mortality with nitrogen (N) inputs at impacted stations and R at control stations ($R^2 = 0.96$, $P = 0.014$, $n = 4$; Fig. 3, Table 5) as well as between residuals of mortality with total sediment inputs at impacted stations and D^* at control stations ($R^2 = 0.99$, $P = 0.003$, $n = 4$; Fig. 3, Table 5). Residuals of shoot recruitment vs. sediment inputs at impacted stations did not show any significant relationship with D^*, R, $\hat{A}$ or CR at control stations.

Discussion

The effect of disturbances on clonal structure and genetic diversity

In spite of the high mortality and rapid reductions on *P. oceanica* meadow density near fish cages, most variability in genetic parameters was still attributable to differences among sites rather than to differences between stations, indicating that the recent effects of population decline on genetic diversity have been lower than the longer term natural factors shaping the genetic structure across the species geographic range. Indeed, the similar genetic structure found at "impacted" and "control" stations within each site, as well as the existence of common genotypes between stations of the same site, support the assumption of similar patterns of clonal structure and genetic diversity between stations previous to impact.

Despite the low shoot densities at impacted stations (reaching 29% of shoot density at "control" station in El Campello) (which clearly compromise population viability in this slow growing species), effects on genetic diversity within the remaining meadows were limited to a reduction in the allelic richness, particularly affecting rare alleles. The lack of significant differences between stations for the observed heterozygosity or the inbreeding coefficient is consistent with predictions (Nei et al. 1975) and experiments (Leberg 1992), indicating that population bottlenecks have a stronger effect on allelic richness than on population heterozygosity (see also Widmer 2001). The latter would indeed require extreme bottleneck or founder effects through several generations to be clearly reduced (Leberg 1992). Such patterns of allelic richness

Journal : 10592 Dispatch : 29-1-2007 Pages : 15
Article No. : 9288 LE TYPESET
MS Code : COGE-06-496 CP DISK

reduction have also been observed in other long-lived species, like logged or fragmented populations of tropical trees (Hall et al. 1996, White et al. 1999). An extensive survey within this group of species indicates that genetic diversity loss through fragmentation or selective logging is better reflected in the resulting inbreeding in the progeny, or over longer time scales (Lee et al. 2002; Lowe et al. 2005). This suggests that genetic diversity may keep on being lost slowly in the subsequent generations (Lowe et al. 2005), still affecting the population a long time after the perturbation occurred.

Posidonia oceanica is an extremely long-lived species (Mateo et al. 1997) in which genets are expected to persist for centuries (Hemminga and Duarte 2000; Sintes et al. 2006), when they are allowed by the environmental conditions. The sparse sexual reproduction of the species (Gambi et al. 1984; Balestri and Cinelli 2003; Díaz-Almela et al. 2006) and its slow vegetative extension rate (Marbà and Duarte 1998) ensures that the genetic structure observed in a so short time scale (all fish farms initiated operation <10 years prior to this study) characterize basically the remains of the initial adult population, because any impact of the present shoot density reduction on the reproductive output would only affect the genetic structure of the meadow many decades after the onset of the impact. Indeed, no seedlings have been detected.

Nevertheless it is realistic to expect that the genetic diversity of the remaining meadow will be reduced further in the following years due to the extreme seagrass decline rates registered at the impacted meadows, which may lead to complete plant depletion in the areas closest to fish cages in the short term (Diaz-Almela et al. submitted). The slow vegetative growth and the long generation time of the species would reduce the effects of genetic drift (Hamrick et al. 1979), but at the same time renders seagrass recovery in the affected areas unlikely. Demographic and genetic recoveries are expected to rely on recolonisation from the apparently genetically similar nearby meadow areas, which will probably require several centuries for the areas affected (Meinesz and Lefevre 1984; Marbà et al. 2002; Sintes et al. 2006).

The spatial autocorrelation patterns varied widely across sites, but within the range reported for other *P. oceanica* meadows (Arnaud-Haond et al. in press). Despite large density differences, the *Sp* statistic only increased at the Greek impacted station. These results only partially concur with those described by Hardy and Vekeman (2004), who report a negative relationship of *Sp* with plant density across four species. These authors interpret it as the combined action of stronger genetic drift and wider propagule dispersion in low-density populations. As explained before, the immediacy of the decline, combined with the long generation time of the species probably prevented the long-term cumulative action of gene flow, genetic drift and inbreeding to be expressed. However, the intense shoot declines in the meadows may have removed, if only through chance, many small genotypes from the meadow. The fact that the only site where we have detected an *Sp* increase with shoot density decline is that with the highest clonal richness and lowest clonal range suggests that the genetic drift derived from the intensive shoot decline was enough to alter the spatial autocorrelation patterns in the meadows composed of small clones, but not in the meadows dominated by larger clones. Nevertheless, as the number of shoots sampled is only a small fraction (in the order of 10^{-2} to 10^{-4}) of the shoots present in the area, the number of clones identified is a small sample of the actual number of clones present. Moreover, the sampling strategy implied that nearly 80% of distance pairs were greater than 10 m, while the loss of shoot density was observed at small spatial scales. Therefore, there could have possibly been changes in spatial autocorrelation patterns between impacted and control stations at other sites, which may have been undetected by our study.

The consistent and significant increase of the clonal sub-range observed in the impacted areas, suggests a higher mortality of small clones relative to large ones, even though we failed to detect significant effects on clonal richness. Such failure could have been caused for the same reasons advanced for the autocorrelation patterns. On the other hand, the lack of significant differences in clonal richness between impacted and control stations also suggests that allelic richness could have been reduced, at least in part, through non-random loss of genotypes containing rare alleles or with small clonal size.

Analysis of demographic answer to environmental forcing vs. genetic and genotypic diversity components

Unexpectedly, the mortality at impacted station for a given perturbation level increased with genotypic richness R and diversity D^*, and also with allelic richness $\hat{A}$ at control stations, assumed to approximate

Journal : 10592 | Dispatch : 29-1-2007 | Pages : 15
Article No. : 9288 | ☐ LE | ☐ TYPESET
MS Code : COGE-06-496 | ✔ CP | ✔ DISK

pre-impact conditions in the four sites. These observations were unexpected because of the evidence that genetic and genotypic diversity increase survival and growth after disturbance in the seagrass *Zostera marina* (Williams 2001; Reusch et al. 2005; Hughes and Stachowitz 2004). This contrast may derive from the dominant role of vastly different clonal sizes in our study, which appears to have greatly affected survival, whereas the experimental studies testing for the role of genotypic diversity did not test for the effects of clonal size (Williams 2001; Reusch et al. 2005; Hughes and Stachowitz 2004). The significant decrease in mortality with meadow clonal sub-range may explain the unexpected positive correlation of allelic and clonal richness with mortality, because those parameters decreased with clonal sub-range in the samples. Therefore genetic and genotypic richness may well have a positive effect on plant survival, once the parallel changes in clonal size are removed, as supported by experiments using uniform genet sizes (Hughes and Stachowitz 2005; Reusch et al. 2005).

Reusch et al. (1999), observing a meadow of *Z. marina* dominated by an ancient and large clone growing in the Baltic Sea, hypothesised that the relationship between meadow survival and genetic diversity could be not straightforward. Our results reinforce this idea, suggesting that the natural variability in genet size within seagrass meadows (e.g. Hämmerli and Reusch 2003; Alberto et al. 2005, present work) may also play a role in meadow survival. The observed significant reduction in shoot mortality at impacted stations with presumed larger initial clonal sub-range and number of shoots per genet suggests that mortality rates are slightly lower where clones are large and constituted of a high number of ramets.

While the observation of larger clones at impacted stations could be explained as a simple matter of probability (i.e. given an equal shoot probability to die, it is more likely for little clones to disappear completely than for large ones), the increased mortality observed within meadows initially composed of little clones would suggest that the shoot probability of dying decreases with the size of the clone it belongs to.

The results from this study have two main aspects: (1) the correlation of genetic structure at control stations (assumed to approximate that of impacted stations prior to the impact) with the demographic responses at impacted stations suggest that meadows dominated by larger clones would be less sensitive to fish-farm derived pressures, possibly through the greated resistance of large clones. (2) The comparison of genetic structures between impacted and control stations reinforces this suggestion, because the increased clonal range at the impacted stations with respect to their respective control stations implies a greater survival of larger clones following disturbance. A major uncertainty about these inferences is the lack of information on the meadow genetic structure previous to the impact, which does not allow us to validate that of the control areas as a proxy. Experimental studies are needed to test for our conclusions. Nevertheless the results are based on the observation of a consistent pattern across four sites in the Mediterranean, where a basic similarity in the genetic structure between impacted and control stations supports the likelihood of our assumption. A major role for chance in producing such patterns appears unlikely. Altogether, those observations strongly suggest that some size-related fitness traits may influence the seagrass resistance to perturbation.

Among clonal plants, clonal integration (share of resource and probability-to-die between ramets) has been shown to be a size-related adaptive trait (e.g. van Kleunen et al. 2000), which would provide a selective advantage in environments with a low proportion of suitable habitat (Oborny et al. 2000; Oborny and Kun 2002). It has been invoked to explain enhanced survival and accelerated growth of clone patches with clonal size in undisturbed conditions among several seagrass species (Olesen and Sand-Jensen 1994; Vidondo et al. 1997).

In *P. oceanica*, clonal integration has been experimentally proven to exist within at least 20–30 cm distance (Marbà et al. 2002). The ramets of a clone can remain connected during decades (as 40–50 years is the maximum life expectancy of *P. oceanica* shoots, Marbà and Duarte 1998) but given the slow horizontal growth rate of the species (1–6 cm year^{-1}, Marbà and Duarte 1998) we can hypothesize an upper limit for clonal integration in this species of 2.4–3 m, a range greater than the size estimated for most genotypes in this study, but much lower than the clonal sub-ranges registered at all the stations. This would suggest that other size-related fitness traits should account for the enhanced resistance to perturbation of large clones found in this work.

Among other benefits, foraging capacity is improved by clonal size (Oborny and Kun 2002), which means that a larger range of different micro habitats can be explored by the same genetic individual when its number of modular units increases, optimizing its capacity to reach micro-environments it is better

Journal : 10592 Dispatch : 29-1-2007 Pages : 15
Article No. : 9288 ☐ LE ☐ TYPESET
MS Code : COGE-06-496 ☑ CP ☑ DISK

adapted to. Also, large clones may have reached such large size because they may have surmounted various regimes of selection, being better adapted to a larger range of conditions. This could be an additional factor accounting for the greater survival of large clones relative to small ones when exposed to disturbance derived from fish farm operations. The lack of correlation between genotype heterozygosity and clonal sub-range with neutral markers is not enough to reject such hypothesis, because heterozygote advantage is not proven to occur in *P. oceanica*. Therefore, under disturbed conditions, such mechanisms (increased clonal integration, optimized foraging capacity, or dominance of the fittest genotypes) enhancing survival of larger clones could make a population constituted of a few large clones more resistant to perturbation than a diverse population consisting of many little clones, counterbalancing the potentially beneficial influence of genotypic and genetic diversity in population resistance to and recovery from perturbations (Reusch and Hughes 2006).

The experiments by Williams (2001), Hughes and Stachowitz (2004) and Reusch et al. (2005) suggest the existence of positive effects of genotypic diversity on survival and recovery of seagrasses for clones of similar size. As genotypic and allelic richness tend to be reduced with increased dominance of meadows by a few clones, the results of this study point to the existence of a trade-off between genetic or genotypic diversity and clone size in the potential of seagrass meadows to survive perturbations. This hypothesis deserves to be tested with experimental or field studies, which simultaneously test the effects of genotypic diversity with those of clonal size on plant survival and recovery. This study shows effects of fish farm-derived mortality on the clonal structure and genetic diversity of seagrass meadows. What are the consequences of those changes, on the scope of recovery after disturbance, is difficult to ascertain. Provided seagrass meadows are experiencing losses worldwide and will most likely continue to undergo in the near future (Duarte et al. 2005), to understand the feed-backs of genetic and clonal structure with disturbance may help to predict the trajectories of those meadows.

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Appendix

Table 6 Allelic frequencies of the seven loci at the four sites (C = Control station; I = Impacted station)

Locus 1	141	143	145	147	149	151	153	155	157	158	159	161	163	165	167	A	**Locus 7**	176	178	180	182	A					
Amathous C						0.60	0.22		0.16			0.02				4			0.40	0.60		2					
Amathous I	0.03					0.50	0.28		0.17			0.03				5			0.67	0.33		2					
Soumion C		0.02	0.19		0.02	0.52			0.19	0.05	0.02			0.02		8			0.52	0.45	0.03	3					
Soumion I			0.13			0.37			0.49		0.01					4		0.01	0.18	0.81		3					
Porto Palo C		0.02		0.02		0.50	0.22	0.09	0.05			0.07	0.03			8			0.55	0.45		2					
Porto Palo I				0.19	0.02	0.31	0.15	0.06				0.27				6			0.69	0.31		2					
Campello C			0.07	0.09		0.46			0.33		0.02		0.02		0.02	7			0.37	0.63		2					
Campello I						0.46			0.54							2			0.85	0.15		2					
Locus 2	154	156	164	166	172	174	182	184	188	198	A	**Locus 5**	159	161	163	165	A	**Locus 6**	168	170	172	174	178	A			
Amathous C			0.94	0.04	0.02						3				0.48	0.52			2								
Amathous I			0.89		0.11						2			0.03	0.33	0.06	0.58		4								
Soumion C		0.08	0.83		0.09						3			0.02	0.16	0.16	0.67		4								
Soumion I	0.04		0.88			0.07					3				0.34	0.03	0.63		3								
Porto Palo C			0.69				0.19	0.03		0.09	4				0.60	0.29	0.10		3								
Porto Palo I			0.50				0.47			0.03	3		0.16		0.44	0.31	0.06	0.03	5								
Campello C			0.20	0.09			0.61		0.11		4		0.17	0.07	0.43	0.33			4								
Campello I			0.02				0.77		0.21		3		0.15	0.1	0.46	0.29			4								
Locus 4	208	210	218	220	222	226	228	234	236	238	240	242	244	250	252	A			168	170	172	174	178	A			
Amathous C	0.02		0.84			0.08		0.06								4			0.74	0.26				2			
Amathous I			0.64			0.33										2			0.83	0.17				2			

Journal : 10592 Dispatch : 29-1-2007 Pages : 15
Article No. : 9288 LE TYPESET
MS Code : COGE-06-496 CP DISK

Table 6 continued

Sounion C		0.02	0.38			0.39		0.22								4		0.77	0.23				2
Sounion I		0.01	0.35	0.01		0.40	0.03	0.18				0.01				7		0.82	0.18				2
Porto Palo C			0.02			0.03	0.02	0.57	0.07	0.09		0.07	0.02	0.07	0.05	10		0.62	0.33	0.05			3
Porto Palo I					0.02	0.03		0.63	0.08	0.06	0.02	0.08			0.08	8		0.69	0.19	0.06		0.05	4
Campello C								0.91		0.09						2		0.20	0.39	0.41			3
Campello I								1								1		0.02	0.81	0.15	0.02		4
Locus 3	194	198	200	206	208	210	212	214	216	218	220	222	224	226	228	230	232	234	236	238			
Amathous C			0.02		0.06			0.22	0.10					0.16	0.02			0.02	0.08	0.12			
Amathous I					0.28	0.11			0.22			0.06								0.28			
Sounion C						0.02	0.05	0.02		0.09		0.06	0.11	0.06			0.02	0.03	0.02				
Sounion I						0.01	0.12	0.04	0.01		0.04	0.04	0.13		0.03	0.01	0.04	0.10	0.10				
Porto Palo C	0.05		0.03	0.02	0.19	0.24	0.16	0.03	0.07	0.19	0.02												
Porto Palo I	0.02	0.02			0.21	0.24	0.06	0.06	0.11	0.10	0.16			0.02									
Campello C						0.28	0.35		0.11	0.15		0.02	0.09										
Campello I						0.31	0.44	0.04	0.21														
Locus 3	240	242	244	246	248	250	252	254	256	260	262	264	266	268	282	288	A						
Amathous C	0.08	0.06	0.02			0.04											12						
Amathous I						0.06											5						
Sounion C	0.02	0.02	0.03		0.11	0.09	0.03	0.03	0.03	0.08	0.02	0.03		0.02	0.02	0.02	13						
Sounion I	0.03		0.07	0.09				0.03	0.04	0.01			0.01				14						
Porto Palo C																	10						
Porto Palo I																	10						
Campello C																	6						
Campello I																	4						

Journal : 10592
Article No. : 9288
MS Code : COGE-06-496
Dispatch : 29-1-2007
Pages : 15
LE
CP
TYPESET
DISK

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