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Meadow-scale genetic structure in *Posidonia oceanica*

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ABSTRACT: *Posidonia oceanica* (L.) Delile meadows are long-lived systems that persist in the field for millennia. The age and size of single clones have not been clearly assessed, nor has the within-meadow dispersal of sexual propagules and neighbourhood size. The present study describes the genetic structure of an ancient and large *P. oceanica* meadow, extending from 3 to 33 m depth, that has been analyzed utilizing 13 variable microsatellite loci. A total of 180 single shoots (ramets) was sampled in 21 areas selected at nodes of a 160 × 400 m grid superimposed on the meadow. For each area, shoots were collected at a reciprocal distance of 1 to 5 m. The number of distinct genotypes was assessed for each sampling area and mapped on the grid using a kriging technique. Neighbourhood size and meadow-scale gene flow were assessed by means of autocorrelation analysis. Data indicate that the meadow is composed of a number of distinct clones, some of which might be hundreds of years old. Different sample groups were identified within the meadow by means of a Bayesian approach. The pattern of genetic diversity is not always related to shoot density, but it increases in the deepest stand, where density is lower. Spatial autocorrelation analysis showed a significant correlation up to 70 or 40 m, considering all samples or only distinct genotypes, respectively. The *P. oceanica* meadow analyzed seems to result from initial recruitment events and active clonal growth of originally established genotypes.

KEY WORDS: *Posidonia oceanica* · Microsatellites · Genetic diversity · Kriging · Clonal reproduction · Spatial autocorrelation · Bayesian analysis

INTRODUCTION

Spatial distribution of genetic variation has important implications for processes related to species persistence, distribution and evolution. This is particularly true in sessile animals and in plants (Epperson & Chung 2001, Cottrell et al. 2003, Escudero et al. 2003, Oostermejer et al. 2003). Knowledge of genetic structure also allows us to make inferences on demographic processes that have happened in the past. However, the real effects of different levels of spatial genetic structure (SGS) on species persistence and the scale at which processes have to be analyzed must be ascertained more effectively (Escudero et al. 2003).

Genetic structure within single populations is defined as the non-random spatial distribution of alleles and genotypes and results from effective gene dispersal and local selection in space and time (Wolf et al. 2000, Birch 2001, Epperson 2003, Vekemans & Hardy 2004). In sexual plants, vectors for gene dispersal are pollen and seeds (Dieckmann et al. 1999, Ouborg et al. 1999). Effective dispersal of these 2 vectors within meadows will have an effect on the size of neighbourhoods with genetically related individuals, possibility of inbreeding and overall levels of genetic variation. Limited dispersal over time will increase isolation by distance (Epperson 2003).

Processes are more complex in clonal plants, where replication of the same genotype through clonal growth adds a potential source of genetic structure (Harada & Iwasa 1996, Ivey & Richards 2001, Hangelbroek et al. 2002). In fact, genetic diversity and structure in clonal plants populations results from the interplay between sexual reproduction and clonal

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propagation (Widén et al. 1994, Waycott et al. in press). As for sexual reproduction, where different mating systems and seedling recruitment strategies have been identified (Eriksson 1989, 1993), clonal propagation also can follow different strategies. Clones are less differentiated and more intermingled in plants growing with a so-called guerrilla strategy, whereas they are bigger and discrete in plants adopting a so-called phalanx strategy (Lovett-Doust 1981).

Marine angiosperms (seagrasses) possess different mating systems, from hermaphroditic to dioecious, and clonal strategies. The spatial distance of genetically related genotypes depends on the dispersal of reproductive structures and on the drifting of vegetative fragments (Ruckelshaus 1996, Harwell & Orth 2002, Waycott et al. in press). However, the rhizome elongation rate and rhizome branching pattern determine intermingling between clones and the size and shape of clonal patches, which can also persist in the meadow for long periods of time (see Reusch et al. 1998, Reusch 2000, Procaccini et al. 2001, Kendrick et al. 2005, Sintes et al. 2005, Waycott et al. in press), depending on the fitness of their genotypes (Hämmerli & Reusch 2003a). In species with different mating systems one predicts a diverse distribution of genotypes. This result depends on whether fertilization between different genets in outcrossing plants or the existence of geitonogamy in selfing species is prevalent (Reusch 2001, Ruggiero et al. 2005a, Waycott et al. in press).

Spatial autocorrelation analysis, together with the assessment of clone distribution and size, provides insight into the evaluation of SGS in seagrasses, as well as in clonal plants in general (Epperson 2003). To date, meadow genetic structure and genetic similarity among pairs of ramets have been analysed as a function of their pairwise distance in only a few seagrass species. A positive spatial autocorrelation of kinship coefficients was found at a distance of 2 to 5 m and 2 m in the monoecious *Zostera marina* and *Z. noltii*, respectively (Hämmerli & Reusch 2003b, Coyer et al. 2004), and at a distance of 10 to 16 m in the dioecious *Cymodocea nodosa* (Ruggiero et al. 2005b).

Posidonia oceanica is a hermaphroditic seagrass species, endemic to the Mediterranean basin. *P. oceanica* meadows can persist *in situ* for millennia, building an organogenic structure (i.e. matte) that rises for meters above the sediment level (Mateo et al. 1997, Procaccini et al. 2003). *P. oceanica* is characterized by slow rhizome elongation rates, a high dispersal potential of reproductive structures and drifting vegetative fragments (Procaccini et al. 2003). In accordance with these characteristics, we would predict a high clonal structure and a large neighbourhood size for this species.

Although the ecological importance of *Posidonia oceanica* meadows to the structure and dynamics of the coastline has been widely recognized (Procaccini et al. 2003), the extent to which the environmental parameters that shape meadow architecture play a role has not been determined (e.g. Borg et al. 2005) and only a few studies have dealt with an analysis of the fine-scale genetic structure of this species. Information obtained with the use of 6 variable microsatellite loci and RAPD markers indicated low genetic variability and high clonality in *P. oceanica* meadows throughout the Mediterranean basin (Procaccini et al. 2001, 2002, Dalmazio et al. 2002, Ruggiero et al. 2002). The use of additional variable microsatellite loci suggested higher genetic diversity and allelic richness in distinct *P. oceanica* meadows (Arnaud-Haond et al. 2005). Based on this result, the relatively low resolution of the first set of 6 microsatellite markers could have affected estimates of genetic diversity at small spatial scales. Therefore, we reason that the combination of 6 'old' (Procaccini & Waycott 1998) and 7 'new' (Alberto et al. 2003) microsatellite loci might provide new and more reliable insights into the analysis of meadow-scale genetic structure in *P. oceanica*.

The aim of the present study is to assess spatial genetic structure in a large *Posidonia oceanica* meadow that extends along a wide depth gradient. Genetic analyses were performed using 13 microsatellite loci and a spatial interpolation method (kriging; Matheron 1971, Davis 1973) which shows the distribution of genetic diversity, in relation to shoot density and depth, over the extent of the entire meadow. Autocorrelation analysis was also performed in order to relate genetic and spatial distance among pairs of ramets.

MATERIALS AND METHODS

Study site and sampling. The *Posidonia oceanica* meadow off Lacco Ameno Bay in Ischia (Naples, Italy) was selected for this study. The meadow extends continuously from 1 to 33 m depth, covering a surface of about 3.08 km^2. In 1992, an area of 64 000 m^2 (400 m × 160 m) was chosen within the meadow and divided in an imaginary sampling grid, made up of 10 m-sided quadrats containing 640 nodes (Fig. 1; Zupo et al. in press). The position of each node was detected in the field using an integral topographical station consisting of an electronic distance meter and centesimal theodolite (Nikon NTD-2) positioned on the coast, and by 3 optical prisms, located on a boat. Shoot density and depth were measured directly for each node. In spring 2000, a total of 21 nodes (sampling points) was selected on the basis of the distributions of shoot density reported in 1992 (Zupo et al. in press). Shoot density and depth were recorded at each node by SCUBA divers who also sampled shoots for genetic analysis.

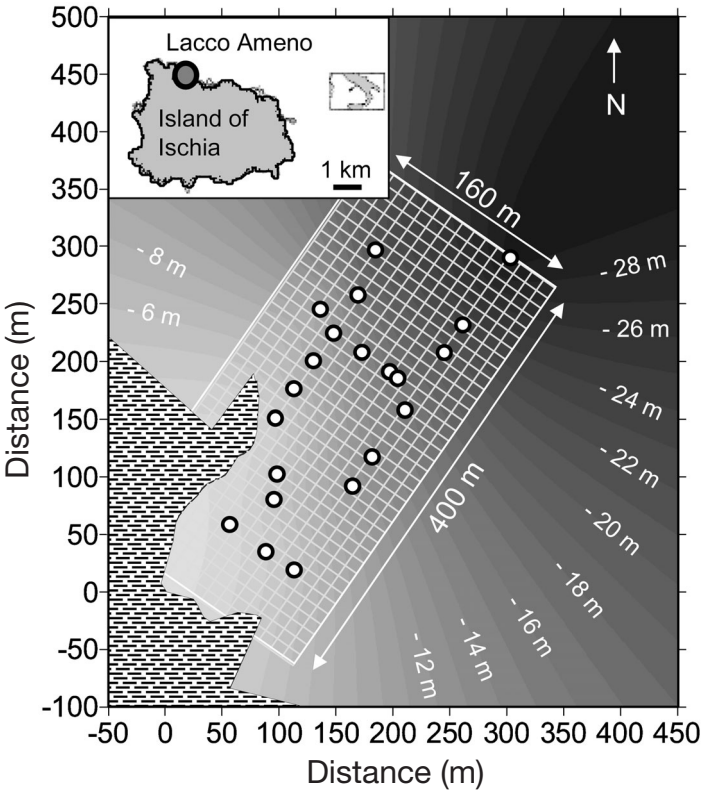

Fig. 1. Location of sampling points analyzed within the *Posidonia oceanica* meadow (Lacco Ameno Bay, Island of Ischia, Tyrrhenian Sea). Nine individual shoots were sampled for each sampling point. Depth profile, obtained through kriging, is also reported on the map

A total of 180 shoots (ramets) was collected at the 21 sampling points at depths ranging from 2.5 to 29.4 m. Nine shoots were collected at each point at a reciprocal distance of 1 to 5 m (Fig. 1).

DNA extraction and microsatellite genotyping. For each sampled shoot, 1 fresh leaf (approximately 100 mg wet weight) was removed and placed into a 1.5 ml tube. Samples were ground under liquid nitrogen, and genomic DNA was extracted using a modified hexadecyltrimethyl ammonium bromide (CTAB) method (Procaccini et al. 1996). Thirteen polymorphic microsatellite regions, 12 nuclear and 1 chloroplast (Procaccini & Waycott 1998, Alberto et al. 2003), were used to obtain individual multilocus genotypes. In order to give better amplifications, new PCR primers were selected for some of the loci in addition to the published ones (Table 1). Approximately 15 ng of DNA extract was amplified in 10 µl PCR reactions under the following conditions: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl^2$, 0.2 mM dNTPs, 0.5 U *Taq* polymerase (BioGeM), 5 pmol ul^{-1} of each primer. PCRs were programmed for 1 cycle of 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s annealing (58 to 52°C), and 40 s at 72°C. Final extension was carried out for 10 min at 72°C. Only locus *Po-5* was amplified using a touchdown strategy: 1 cycle of 3 min at 94°C, followed by 24 cycles of 30 s at 94°C, 30 s at 64°C (with a decrement of 0.5°C/cycle) and 30 s at 72°C, thereafter 10 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 30 s and a final elongation at 72°C for 10 min.

Amplification products were separated by electrophoresis on a 1.5% Agarose 1× TAE gel.

Allele detection was conducted using an automated sequencer (CEQ 2000XL DNA Analysis system, Beckman Coulter) and electropherograms were analysed with the Beckman CEQ 2000 ver. 3.0 software. Details on PCR and fragment multiplex reactions are given in Table 1.

Statistical analysis. The genetic variation within the sampled population was calculated using the GIMLET software (Valière 2002). The following estimates were calculated: number of polymorphic loci (P), mean number of alleles per locus (ANA), allele frequencies for each locus, observed (H_o) and expected (H_e) heterozygosity. Deviation from Hardy-Weinberg proportions was evaluated at the genet level using a Markov-chain algorithm (Guo & Thompson 1992) carried out in GenePop 3.3 software (Raymond & Rousset 1995). Fix-

Table 1. Complete sequences for forward (F) and reverse (R) primers selected in this paper are indicated. The appropriate reference is given for those taken from the literature. Composition of multiplex reactions has been specified both for PCR and automatic sequencer (AS) reactions. Loci included in the same multiplex reactions are indicated by the same number (PCR) or letter (AS)

Locus name	Primer (5′–3′)	Multiplex reactions PCR	AS
Poc-5	Procaccini & Waycott (1998)	1	a
Poc-26	F-GTC ACC TTA AAT CAT CGG AG R-AAC GAG GAC ATA GCG AGT AC	2	b
Poc-35	Procaccini & Waycott (1998)	2	b
Poc-42	F-CTC CTT CCT GTA CAT TCC CCT C R-TGG TCT CTC CCC TTC TCT CAC T	1	a
Poc-45	F-AAA TTG CCA GAT TCT GGT GTC A R-AAC CAC GTG AGT AAG GGA GGG	2	b
Poc-trn	F-GGG CAA TCC TGA GCC AAA TCC R-TTG ATA TGT CAG TAT GTA TAC GTA CG	1	a
Po5-49	Alberto et al. (2003)	4	d
Po5-40	Alberto et al. (2003)	4	d
Po4-3	Alberto et al. (2003)	5	d
Po5-10	Alberto et al. (2003)	3	c
Po5-39	Alberto et al. (2003)	3	c
Po-5	Alberto et al. (2003)	6	e
Po15	Alberto et al. (2003)	7	e

ation index (f) was calculated based on Weir & Cockerham (1984), after exclusion of replicated genotypes. Significance of f was assessed by estimation of 95% confidence interval (CI) after 1000 bootstraps. This index was calculated with the software GENETIX 4.1 (Belkhir et al. 2001).

In order to establish the theoretical number of genotypes according to the given number of loci and alleles, the N_g value (Parks & Werth 1993, Ruggiero et al. 2005b) was calculated

$$N_g = \prod_{i=1}^{L} [a_i (a_i + 1)]/2$$

where L is the number of loci, and a_i is the number of alleles at locus i. The probability of identity (Pi) of each single genotype (Waits et al. 2001) was also estimated using the GIMLET software (Valière 2002). The Pi index allows one to distinguish genotypes that are identical by descent from genotypes identical by chance. Pi was also calculated for each locus in order to evaluate the power or ability of the microsatellite markers utilised to effectively resolve 2 distinct individuals.

For the evaluation of clonal structure the genetic diversity index (G/N) was used, where G is the number of genets and N is the total number of sampled ramets (Pleasants & Wendel 1989). G/N is equal to 1 when all individuals are genetically different. G/N was calculated both for the whole meadow and for each sampling station. Significance of differences in G/N values between the stations located above and below the summer thermocline was tested using the Mann-Whitney test (van der Waerden 1969). A clonal map of the meadow was developed manually by connecting those sampling points that shared the same genotype. A rough estimate of clone age was performed assuming a centrifugal growth pattern of clonal patches and a rhizome horizontal growth rate ranging from 1.1 to 7.4 cm yr^{-1} (Buia et al. 2000).

To test the spatial autocorrelation of genotypes the kinship coefficient f_{ij}, which is computed as a correlation coefficient between allelic states (Loiselle et al. 1995), was calculated using the software SPAGeDi (Hardy & Vekemans 2002). This is a statistical procedure which relates the probability of identity by descent to the distance between pairs of genotypes (Wright 1943, Sokal et al. 1997, Epperson 2003). Single sampling stations were treated as spatial groups and all individuals belonging to the same station were assigned the same spatial coordinates.

Eleven distance classes were fixed, taking into consideration the size of the grid together with the reciprocal distance between samples. Distance classes ranged from 5 to 337 m, and were selected in order to include where possible a similar number of pairs for each class. For a given distance, 95% CIs were defined. In order to identify differential effects of clonal reproduction and isolation by distance on population genetic structure, 2 different data sets were compared: one including all sampled individuals (ramets) and the second including only the distinct genotypes (genets; Reusch et al. 1999b). Significant differences between ramets and genets were assessed using 2-tailed t-tests. In order to compare the genetic structure of *Posidonia oceanica* to other species, we estimated Sp statistics (Vekemans & Hardy 2004) as in Ruggiero at al. (2005b). The rationale is based on the expectation that in the presence of isolation by distance correlation parameters decrease linearly with the logarithm of the distance at least in a spatial range depending on gene dispersal and effective population density.

A spatial model that depicted the distribution of structural and genetic parameters of the meadow was built using a kriging technique (Scardi & Fresi 1986, Wackernagel 1994). Kriging is a method of stochastic interpolation which predicts values from data observed at known locations (Matheron 1969, 1970). This method uses variograms to express spatial variation and calculates 2 different variograms: experimental and theoretical. The experimental variogram is a function of the spatial structure (Myers 1994a,b) and explains the covariance of a variable on a distance basis. In this case, the covariance of genetic diversity (G/N per sampling station) was analyzed through the use of a linear and a spherical model (Pannatier 1996, Kitanidis 1997). Values thus obtained were plotted as isolines on an x–y plane. The kriging map of G/N values was visually compared with a kriging map of shoot density obtained on the new values recorded in the 21 sampling stations. Computations were conducted using the Golden software program Surfer ver. 8.01 (Golden Software).

An unsupervised learning algorithm based on Bayesian statistics, Autoclass C ver. 3.3.4, was utilized in order to identify and locate similar groups within the meadow based on a comparison of multilocus allelic profiles. Autoclass C seeks a maximum posterior probability classification (Cheeseman & Stutz 1995) and finds the maximally probable set of clusters with respect to data and model. Values higher than 99% are considered significant. A matrix represented only by distinct multilocus genotypes was supplied to the program and a discrete model was applied. Only groups recovered with probability higher than 99.4% in all runs were mapped on the grid.

RESULTS

A total of 180 ramets was genotyped at 13 microsatellite loci, 12 of which were polymorphic (Table 2). A total of 136 genets was recognized (G/N = 0.75). The

Table 2. Size range of alleles, number of alleles, expected (H_e) and observed (H_o) heterozygosity, Hardy-Weinberg (HW) proportion and inbreeding coefficient (f) calculated for each microsatellite locus utilized. For the chloroplastic locus (*Poc-trn*) heterozygosity and f were not calculated. ** p value < 0.01

Locus name	Size range of alleles	No. of alleles	H_e	H_o	HW (p-value)	f
Poc-5	158–227	2	0.09	0.08	0.297	0.100
Poc-26	295–316	2	0.46	0.69	**	–0.413
Poc-35	194–206	4	0.14	0.13	0.435	0.650
Poc-42	201–216	2	0.42	0.47	0.038	–0.188
Poc-45	107–140	2	0.35	0.39	0.244	-0.108
Poc-trn	296–316	1	–	–	–	–
Po*5-49*	232–244	9	0.79	0.92	**	–0.095
Po*5-40*	186–226	10	0.81	0.88	**	–0.020
Po*4-3*	158–178	3	0.46	0.48	**	–0.038
Po*5-10*	151–165	6	0.61	0.66	0.067	–0.113
Po*5-39*	170–180	3	0.57	0.61	**	0.012
Po-*5*	164–188	3	0.66	0.79	0.027	–0.137
Po*15*	151–157	9	0.78	0.88	**	–0.082
Average		4.58	0.51	0.58	**	–0.098

theoretical number of genotypes possibly discriminated with the markers utilized (N_g) was very high (4.1×10^{11}).

The 12 polymorphic loci provided 56 alleles (with a maximum of 10 alleles observed at locus *Po5-40*). The mean number of alleles per locus was 4.58 (Table 2). Observed heterozygosity/locus ranged from 0.08 for *Poc-5* to 0.92 for *Po5-49*. Not all loci were at Hardy Weinberg equilibrium. The averages of observed and expected heterozygosity values for all loci were 0.58 and 0.51, respectively. The inbreeding coefficient (f) was negative for some loci (see Table 2) and averaged –0.098, which indicated overall an excess of heterozygosity.

The Pi for each multilocus genotype varied from 1.91×10^{-14} to 1.56×10^{-6}. Values were lower than the threshold of 0.001 recommended for the rejection of identity by chance matches of genotypes (Waits et al. 2001). By computing Pi values per locus and sequentially adding 1 locus at a time from the one with the higher rank, only the first 2 loci (*Po5-40* and *Po5-49*) were necessary to reach a Pi <0.001.

G/*N* values calculated per sampling station ranged from 0.22 to 1. Values lower than 0.5 occurred only at 4 sampling points above a 12 m depth (Fig. 2), where the average *G*/*N* value was 0.32. This value was significantly lower ($p < 0.001$) than the average (*G*/*N* = 0.89) of the remaining stations, where values were always higher than 0.66. We also compared the *G*/*N* value between stations located above and below the average position of the summer thermocline (15 m). The Mann-Whitney test shows significant differences among the 2 groups of stations ($U = 23.5$, $p(U) = 0.04258$).

Clonal structure

A clonal map shows the existence of few big clones, extending over different sampling points, and a series of small clones, mainly confined to a single sampling point, whose size is likely to be less than the distance to the nearest sampling station (Fig. 3). The approximate size of the clones was estimated by connecting the 2 most distant samples that shared the same genotype and assuming that clonal losses and the re-establishment of clonal fragments are negligible. The largest clones were present in the middle of the sampling grid, between a depth of 3 and 16 m. At a few sampling points all ramets represented different genets (Fig. 3). According to our estimates, the age of the largest clone, extending over an area of more than 100 m from a depth of 4.8 to 9.2 m (Fig. 3), may range between 4500 and 650 yr. Single and isolated clonal patches, with a diameter of about 4 m, should be between 30 and 200 yr old.

Autocorrelation

High genetic structure was observed, with kinship values (f_{ij}) for the first distance class ranging from 0.203 to 0.172 at ramet and genet levels, respectively (Fig. 4). Correlation was significant up to about 70 m when considering the contribution of clonal growth (Fig. 4a), while it decreased to about 40 m when considering dispersal of sexual products only (Fig. 4b). Differences between mean autocorrelation values of ramets and genets were not significant ($p = 0.936$). Sp values calculated for ramets and genets were 0.032 and 0.025, respectively. Neighbourhood size (Nb), calculated as number of individuals, was higher for genets (Nb = 39.4) than for ramets (Nb = 31.1).

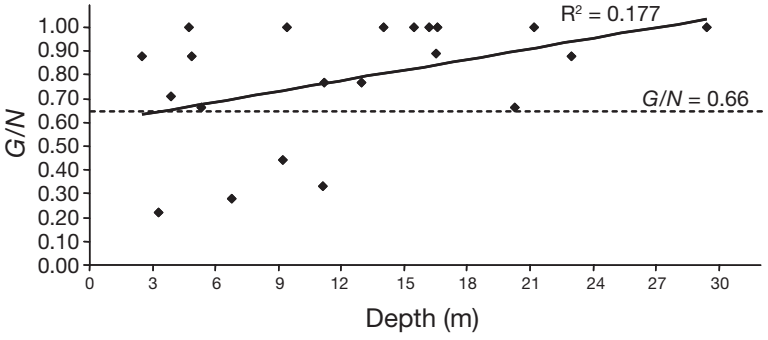

Fig. 2. *Posidonia oceanica*. Clonal diversity, expressed as number of genets (*G*)/total number of ramets (*N*), is reported for each sampling point as a function of depth. Only 4 points have *G*/*N* values lower than 0.66

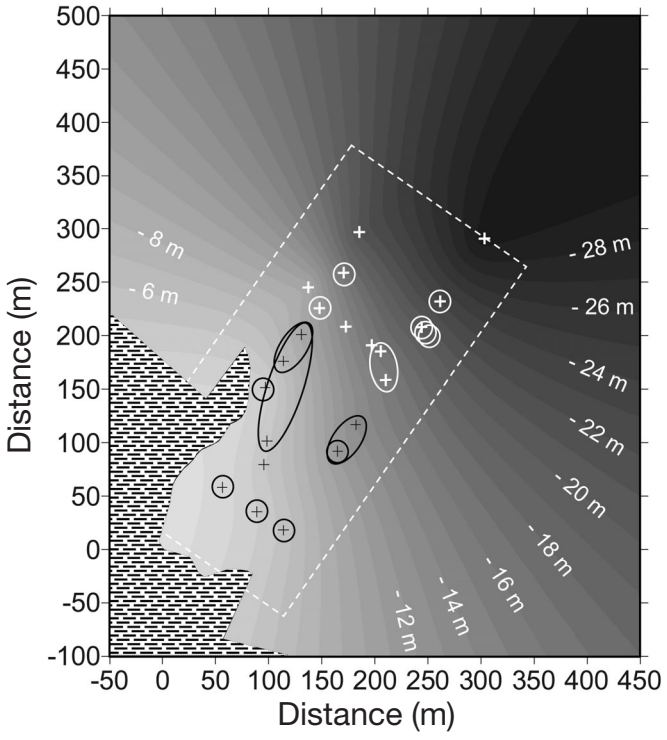

Fig. 3. *Posidonia oceanica.* Position of the 15 clones (genets represented by more than 1 ramet). Largest clones are between 5 and 16 m depth. Three distinct clones are present at a single station at 20 m depth. Single sampling stations are indicated by plus signs, circles indicate clones (in black and white for above and below the approximate position of the summer thermocline, respectively)

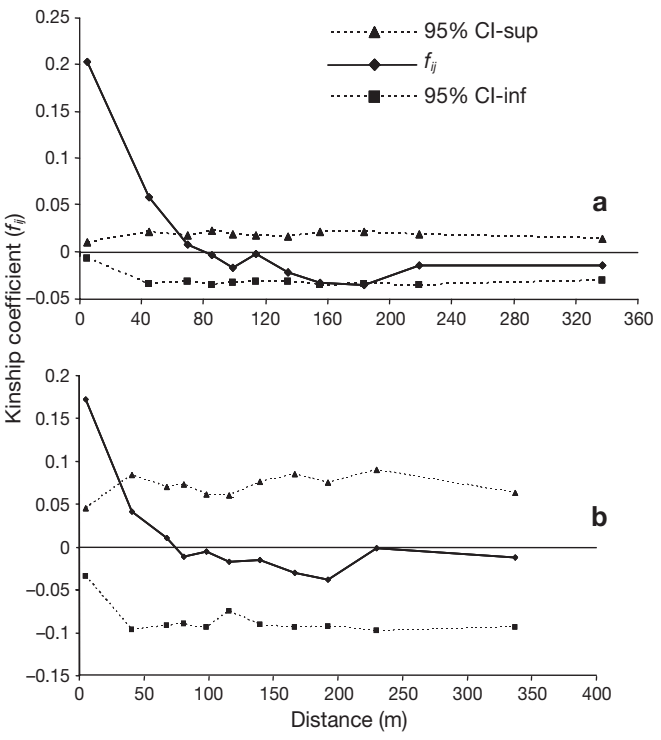

Fig. 4. *Posidonia oceanica.* Spatial autocorrelation of kinship (f_{ij}) over all loci, including (a) all sampled individuals and (b) a single individual for each clone. Representative individuals were chosen where possible from a central position within the clone. 95% confidence intervals (CI) are also shown. CI-sup: CI upper limit; CI-inf: CI lower limit

SGS by kriging

Spatial representation of genetic diversity using kriging showed the existence of 2 main centrifugal structures (with increasing diversity from the centre) in the middle of the grid, whose central points corresponded to the large clones described above. Diversity was highest at the shallowest and deepest stations and after a depth of 16 m showed a regular increase towards the deepest sites (Fig. 5; refer to Fig. 1 for depth profile). Comparison of kriging genetic diversity maps with kriging shoot density maps indicated a varying degree of correspondence in the spatial distribution of the 2

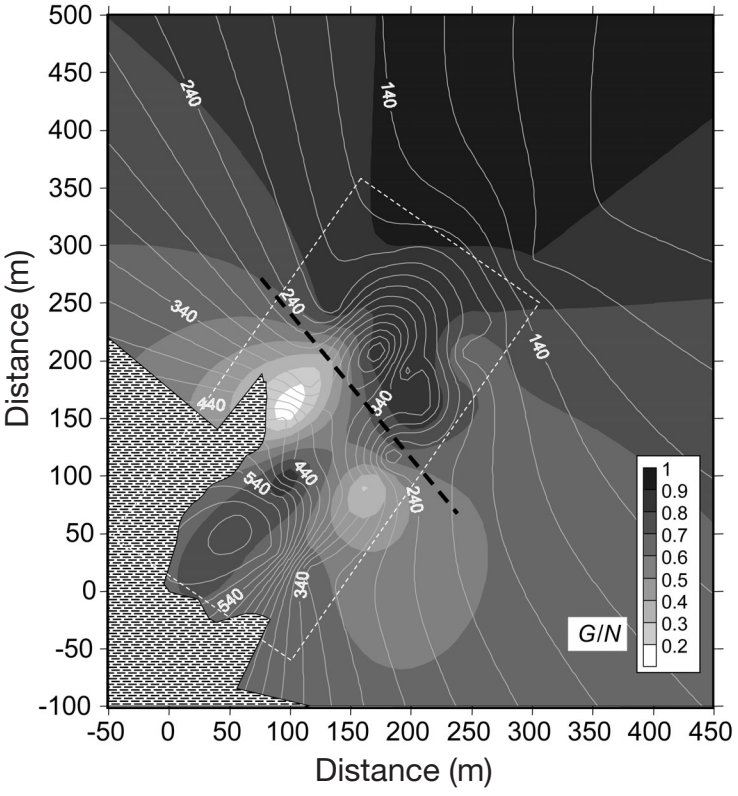

Fig. 5. *Posidonia oceanica.* Profile of clonal diversity (gray scales) and shoot density (white isolines), obtained through a kriging technique. The covariance of clonal diversity (G/N per sampling station) and shoot density (no. shoots m^{-2} per sampling station) were analyzed using a linear and a spherical model. For depth values refer to Fig. 1. Dashed black line represents the approximate position of the summer thermocline

values along the meadow (Fig. 5). In general, the 2 areas with higher shoot density (see white lines in Fig. 5) correspond to areas with high genetic diversity (dark areas). Hence, shoot density decreased towards the deepest stations while genetic diversity increased. The 2 areas with low genetic diversity located in the middle of the grid correspond to areas with average shoot densities.

SGS by Bayesian clustering

A clustering of the population genetic structure by an unsupervised learning approach showed the presence of 3 main groups. A major group that included all sampling points from 5 to 13 m depth was recovered in all cases with mean probability higher than 99.4% (Group 1 in Fig. 6). The maximum linear distance between points included in this group was about 200 m. A second group included the 4 deepest stations, all below a depth of 20 m (Group 3). The maximum linear distance between points included in this group was about 120 m. An intermediate group (Group 2) included 4 points between a depth of 13 and 20 m. Two other sampling points at a depth of about 16 m grouped with 1 point located above 4 m and these were about 140 m apart (Group 4).

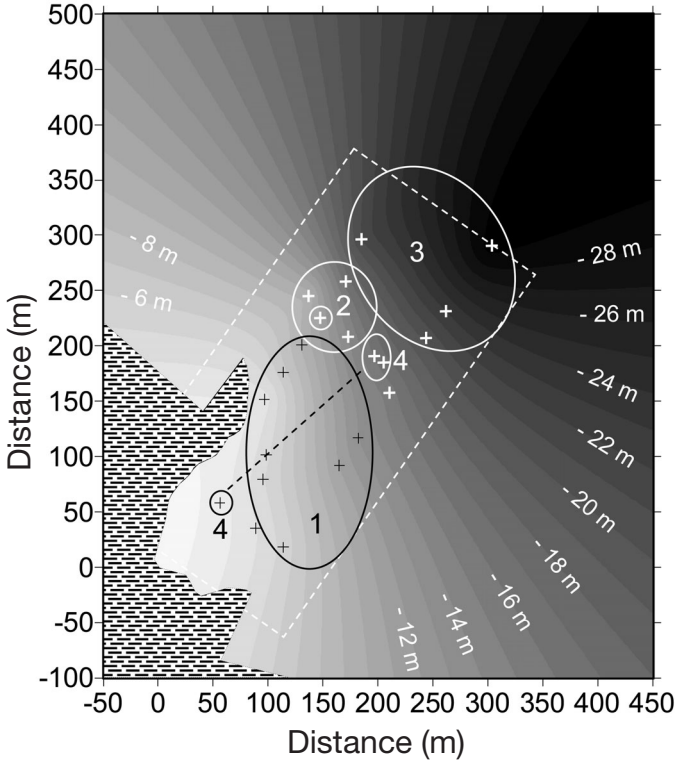

Fig. 6. *Posidonia oceanica*. Bayesian analysis of population clustering. A clear genetic subdivision is present within the meadow. In all the cases groups recovered with a probability higher than 99.4% were mapped on the grid. Single sampling stations are indicated by plus signs, groups are indicated by circles (in black and white for above and below the approximate position of the summer thermocline, respectively); the dashed black line connects sample stations belonging to the same group

DISCUSSION

The *Posidonia oceanica* meadow studied had a high clonal diversity with 75% of the sampled ramets representing distinct genotypes. This value is markedly higher than previous results obtained for the same population from 40 distinct samples that were not included in the present analysis (G/N = 0.33; Procaccini et al. 2001) and that were surveyed using only 6 of the microsatellite loci utilized here (*Poc* loci in Table 1). This discrepancy could originate from the higher number of samples analyzed, from the higher number and polymorphism of the loci utilized, or from recent changes which have occurred in the meadow over the past 4 yr. Among these 3 possibilities, we believe that the second provides the most reasonable explanation. The G/N value for the 180 samples analyzed in the present study using only the same 6 loci as the original study is 0.19, even lower than the one reported in Procaccini et al. (2001). Moreover, if we estimate the ANAs in the 180 samples considered here but only for the 6 loci used in the previous analysis (ANA = 2.2), this value is only slightly higher than that obtained for 40 samples (ANA = 1.83; Procaccini et al. 2001). Thus, we conclude that the higher polymorphism of the loci utilized here, more than the larger sampling effort, adequately explains the higher level of clonal diversity encountered with respect to the previous analysis (Procaccini et al. 2001). This result is also consistent with the findings of Arnoud-Haond et al. (2005), who described higher polymorphism in several *P. oceanica* populations when using the complete set of markers.

An excess of heterozygosity was encountered in the studied population. High heterozygosity could result from a selective heterozygote advantage due to local adaptation and hence could be correlated with individual fitness. Excess heterozygosity seems to be characteristic of seagrass meadows off the Island of Ischia, suggesting the presence of selective environmental conditions. We emphasize that although selectively neutral by definition, microsatellite loci can be linked to functional regions of the genome which are locally selected (Hämmerli & Reusch 2002, Li et al. 2004). Two other *Posidonia oceanica* meadows included in a previous study (Procaccini et al. 2001) also showed an excess of heterozygosity or very low homozygosity. Similarly, heterozygous excess was found in meadows

of *Cymodocea nodosa* and *Zostera noltii*, sampled along the coast of the Island (Ruggiero et al. 2005a,b).

Within a single meadow, Hämmerli & Reusch (2003a) found a significant correlation between clone size and heterozygosity levels in *Zostera marina*. However, in our analysis the largest clones did not show a higher number of heterozygous loci with respect to individual genotypes.

Genetic diversity was not evenly distributed throughout the meadow. Our sampling grid did not cover the whole extension of the meadow along the coastline but it did extend along the entire depth gradient. Our results showed consistently higher genetic diversity at the deep margins of the meadow and at the shallow stations located inside the Bay. Higher clonality was recorded at stations located in the intermediate positions, where most of the individuals belonged to the same clone. This result suggests that recruitment is more active at the meadow boundaries, while intra- and inter-clone competition do not allow new recruits to establish themselves in the middle of the meadow. Light extinction under the canopy can be very severe (Dalla Via et al. 1998) and can drop below survival values for young recruits that might settle at the bases and below the leaves of adult individuals. Instead, recruits are more capable of coping with the low light conditions typical of the deep margin, where competition for space is less of an issue. Once dropped from the surface, seedlings can be transported to the meadow by bottom currents and can remain trapped by the canopy at the meadow boundary. Moreover, limiting environmental conditions in the deep portion of the meadow do not allow single ramets to grow to a large size and this leads to a faster turnover of genotypes.

Strong hydrodynamic forces present at the shallow stations located off the cape that closes the Bay of Lacco Ameno could affect seedling establishment and account for the low genetic diversity recorded here.

A similar pattern of genetic distinction between the middle of the meadow and its boundaries was also found in the seagrass *Halophila stipulacea*, by means of RAPD markers (Procaccini et al. 1999), although without clear differences in levels of genetic diversity within each of the distinct areas. In that study samples collected at the meadow boundaries clustered separately from the samples collected within the meadow.

In the present work, different sample groups were identified within the meadow by means of a Bayesian approach. One big group included the largest clone and other nearby stations, while all the samples collected below a depth of 20 m clustered in the same group. Bayesian clusters that cover an area larger than the estimated neighbourhood size indicate a level of connection among different genotypes above the strict panmittic unit. Genetic structure is also confirmed by the unequal distribution of alleles within the meadow. Allele frequencies varied among the 3 groups of stations located above a depth of 10 m, between 11 and 15 m, and below 16 m. Four alleles were present exclusively in this last group of stations. The main reason for the genetic distinction between shallow and deep stations was the temporal shift in the ripening period for sexual structures at different depths (Buia & Mazzella 1991), as has already been suggested by Procaccini et al. (2001).

Outcomes of the correlation by distance analysis fit nicely with the range of estimates of clone size. Results obtained by considering only the distinct genotypes confirm that the within-meadow gene flow is behind the clonal spread of genotypes. Nb values for *Posidonia oceanica* (Nb = 39.4) are lower than in *Cymodocea nodosa* (Nb = 54 for the genet level; Ruggiero et al. 2005b). This result is not surprising considering that *Cymodocea* seeds develop in the sediment attached to the mother plant and their dispersal is potentially reduced. Coancestry values are among the largest observed previously for other clonal plant species. Our values for the 5 m interval are 0.2 and 0.17, including all samples and only distinct genets, respectively. Values for the monoecious seagrass *Zostera marina* are as low as 0.028 to 0.161 for all ramets and 0.009 to 0.036 for genets only (Hämmerli & Reusch 2003b). For the dioecious *C. nodosa*, values are 0.0456 and 0.0436 for all ramets and genets only, respectively, in a meadow located off the Island of Ischia (Ruggiero et al. 2005b). Values range instead from 0.133 to 0.094 at the ramet level and from 0.051 to 0.030 at genet levels, in 2 different *C. nodosa* meadows along the Spanish coast (Alberto et al. 2005). The large differences encountered among the 3 species when clonal spread is not considered are related to the different dispersal potentials of seeds and pollen (gene dispersal). Seed dispersal is assumed to be on the order of a few meters in *Z. marina* and *C. nodosa* (Ruckelshaus 1996, Alberto et al. 2005, Ruggiero et al. 2005b) whereas the buoyant seeds of *Posidonia oceanica* can be expected to cover much larger distances (Caye & Meinesz 1992). Hence, we can hypothesize that the high kinship values observed in *P. oceanica* derive from limited pollen dispersal or geitonogamy (mating within the same clone). Geitonogamy has already been suggested to occur in *P. oceanica* (Procaccini et al. 2001). Unfortunately, the low number of samples analysed with respect to the size of the entire sampling grid does not allow reliable estimates of pollen dispersal.

Differences existing between the 2 types of correlograms, although not significant, confirm the importance of clonal growth in shaping the genetic structure of *Posidonia oceanica* meadows, as already suggested by previous analyses (Procaccini et al. 2001, 2002).

Genetic structure assessed from the Sp value was also higher for the ramet (0.0322) than for the genet level (0.0253). A recent review on Sp values by Vekemans & Hardy (2004) allows a direct comparison to be made with terrestrial plants having different mating systems. The Sp values here encountered for the monoecious/hermaphroditic *P. oceanica* fall within the middle range of values reported for species having either a self-incompatible, outcrossing or mixed mating system. Previous studies on the congeneric *P. australis* (Waycott & Sampson 1997) indicated the existence of a mixed mating system, with possible selfing in some localities. Selfing was also hypothesized for *P. oceanica* by Sandmeier et al. (1999), although the experiments presented in that paper did not allow the exclusion of other reproductive mechanisms such as apomixis.

Our estimates of clone age, obtained using a simple approach based on centrifugal rhizome elongation rate, date the oldest clones (larger than 100 m for the longest axes) back to several hundreds or even several thousands of years. This great age is not surprising, considering that very large clones, which spread over whole meadows, have been found in different localities both in *Zostera marina* (Baltic Sea; Reusch et al. 1999a) and in *Posidonia oceanica* (North Adriatic Sea; Ruggiero et al. 2002). Nevertheless, patch expansion in seagrasses follows complex models and varies according to the age and size of single patches. Recent studies of *Cymodocea nodosa* report that patch growth accelerates through time according to an exponential increase in the number of new branches (Sintes et al. 2005). Within 6 yr, a patch can be up to 40 times bigger than it would be considering linear and constant rhizome elongation. If we presume that a similar process also applies to *P. oceanica*, clone age, although still notable, could have been greatly overestimated in our analysis. Moreover, the presence of additional replicates of the same genotypes within the meadow could also be related to mechanisms such as fragmentation, rift, and resettlement or to alternative clonal reproductive systems, such as pseudovivipary (Ballesteros et al. 2005).

The present study has shown the existence of a complex genetic structure in the *Posidonia oceanica* meadow studied. This structure could be due to the clonal spreading of genotypes and to the limited dispersal of pollen and/or seeds. High coancestry values at a distance of 5 m could suggest either continuous reproduction among spatially close individuals with nearby recruitment (Repeated Seedling Recruitment [RSR]; Eriksson 1989, 1993) or ancient colonization of the meadow by a few genetically related seeds (Initial Seedling Recruitment [ISR]; Eriksson 1989, 1993). In the first scenario, we would expect that during the long history of the meadow seeds are accidentally trapped in the canopy and grow close to the parental plants. However, a lack of records of seedlings growing *in situ* during the last 15 yr (Buia et al. 2000) seems to support the second scenario, i.e. the present meadow is the result of initial recruitment and continuous growth of a few established genotypes. Lack of recent recruitment could be related to high shoot density and inter-individual competition. The high genetic diversity characterizing areas with high shoot density could result from clonal growth of existing genotypes. More active recruitment should occur at the shallow and deep boundaries due to more relaxed competition.

A few large clones have been detected, together with many smaller but clearly distinct ones. This observation suggests that *Posidonia oceanica* grows according to a phalanx strategy, typical of long-living, slow-growing systems (Lovett Doust 1981). The high heterozygosity recorded in the meadow indicates adaptation and should facilitate persistence in the rapidly changing coastal environment. Our next challenge will be to assess the extent to which functional plasticity is present and to determine the mechanisms of adaptation to environmental features, such as light attenuation, within the same meadow and along the wide depth gradient.

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