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## Genetic variability of *Posidonia oceanica* (L.) Delile in relation to local factors and biogeographic patterns

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### Abstract

To evaluate genetic differences of *Posidonia oceanica* (L.) Delile both at smaller (within a meadow) and larger scale (Mediterranean basin), plants of *P. oceanica* were analyzed by PCR technique and compared using random amplified polymorphic DNA (RAPD) markers. Results were associated to known differences in phenology. At the small-scale level, *P. oceanica* shoots collected in the bay of Monterosso al Mare (Liguria, NW Mediterranean Sea) showed genetic differences among sampling stations, with a decrease in genetic diversity along an anthropogenic disturbance gradient. At basin level, genetic differences were detected among 11 *P. oceanica* shoots coming from different regions of the Mediterranean, and transplanted to the Port-Cros National Park (France) between 1989 and 1991: Izmir, Turkey; Athens, Greece; Taranto, Italy; Ischia Island, Italy; Lavezzi, France; Port-Cros, France; Banyuls, France; Palma de Majorca, Balearic Islands, Spain; Marsa Bay, Algiers. By cluster analysis two major Mediterranean groups were distinguished, the Eastern Mediterranean Group (EMG) and the Western Mediterranean Group (WGM). This suggests that eastern and western populations of *P. oceanica* have diverged during the colonization of the Mediterranean (after near extinction of the Mediterranean biota in the Messinian period, approximately 5.6 million years ago), and have experienced little gene flow between them. Cluster analysis also indicated that previously described phenological differences among *P. oceanica* populations in

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different sectors of the Mediterranean are not mere phenotypic responses to different climatic and hydrological conditions but may well have a genetic basis.

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## 1. Introduction

Seagrasses play a major ecological role in coastal zones of tropical and temperate seas. Among them, the genus *Posidonia* is considered a relict from the early cretaceous age, when the plant was present in all seas (Den Hartog, 1970; Hemminga and Duarte, 2000). Subsequently, the genus suffered a disjunction to a bipolar distribution: today, the genus *Posidonia* survives with nine species along the southern coast of Australia (Short et al., 2001) and one species, *Posidonia oceanica* (L.) Delile, endemic to the Mediterranean. In the latter, *P. oceanica* is the dominant seagrass species, largely contributing to the biodiversity of coastal bottoms and forming a unique habitat for many organisms that live below and within its dense canopy (Boudouresque et al., 1984). *P. oceanica* meadows represent, on soft bottoms, the ecosystem with the greatest primary productivity, part of which is exported to neighbouring systems.

The observed regression of *P. oceanica* beds has been attributed to both natural, e.g., passage of long storms, *Labyrinthula* infections, sea level rise, temperature increase, and anthropogenic causes, e.g., construction along the shore-line, trawling, eutrophication (Marbà et al., 1996; Bianchi, 1997; Bianchi and Morri, 2004). Pérès (1984) even suggested that these observations of local *P. oceanica* decline are symptoms of a large-scale die-off, which could eventually decimate the species in the wider Mediterranean.

The decline of *P. oceanica* could be facilitated by lower genetic diversity, resulting from the low rate of sexual reproduction of this species, which would make it prone to disease and reduce plant vigour and its capacity to adapt to adverse conditions. *P. oceanica* is a long-living plant with a slow growth rate. Anthropogenic modifications of the coastal environment, taking place more rapidly than the capacity of the plant to adapt to such changes, are reducing its distribution area.

Investigations of genetic variability in hydrophilous angiosperms showed significant diversity at both genetic and phenotypic levels (Waycott et al., 1997). In *P. oceanica* plants from the Western Mediterranean, Procaccini et al. (1996) found a low genetic variability. Meinesz et al. (1993) found that shoots coming from different areas of the Mediterranean and transplanted at Port Cros became phenotypically similar.

Previous papers on other seagrass species, such as *Zostera marina* L. (Reusch et al., 2000) and *Posidonia australis* Hooker f. (Waycott and Samson, 1997), have described how patterns of genetic diversity are affected by other factors, including spatial structure, age and maturity of meadows. Genetics of *P. oceanica* have been investigated with different approaches. Karyotype analyses were conducted by Semroud et al. (1992) on Algerian samples, followed by Capiomont et al. (1996) who first investigated genetic variability in the plant using enzyme polymorphism. Procaccini et al. (2000) made several attempts, using microsatellite technique and more recently Raniello and Procaccini (2002) suggested

a new protocol for the extraction of the DNA from old rhizome remnants. Random amplified polymorphic DNA (RAPD) technique was first applied on *P. australis* by Waycott (1995), who found 16 four-primer genotypes; Franconi et al. (1995), used 8 primers to analyse several populations of *P. oceanica* in Tyrrhenian Sea (western Mediterranean) to find both species-specific and situ-specific polymorphism. The latter, in particular, was correlated to abiotic parameters such as light, photoperiod and temperature, with significant variation in proximity to a coastal power plant. Alberto et al. (2001) using RAPD markers revealed genetic homogeneity in the seagrass *Cymodocea nodosa* (Ucria) Ascherson at its northern Atlantic limit, demonstrating that there was a lack of genetic variability among the meadows. Like Alberto et al. (2001), we apply this methodology to reveal polymorphism within and among populations of the same species. In fact, this method provides an easy and economical way to amplify DNA that may be variable among samples and results in specific banding patterns for each individual (Hadrys et al., 1992). For example, RAPD markers have revealed intra-population variability in *Z. marina* (Micheli et al., 2004) and in *P. australis* whilst previous allozyme studies found none (Waycott, 1998). Some authors used RAPD markers for resolving genets in natural and cultivated clonal plants, including seagrasses (Liu and Furnier, 1993; Gabrielsen and Brochmann, 1997; Waycott, 1998; Bush and Mulkahy, 1999; Fisher et al., 2000; Verburg et al., 2000; Auge et al., 2001).

In this paper, the RAPD technique is used to analyse the genotype of *P. oceanica* at two levels: a small-scale level, i.e., within a meadow, and a large-scale level, among different populations of the Mediterranean Sea. Genotypic diversity is analysed and compared to phenotypic variability across geographic distance.

## 2. Materials and methods

### 2.1. Study areas

The small-scale approach, by testing genetic differences within a meadow, was conducted at Monterosso al Mare, in the National Park of “Cinque Terre” (Ligurian Sea). There, the *P. oceanica* bed covers a surface of nearly 30 ha from 2 to 25 m depth and has been studied since 1991. It suffered heavy regression in 1969 because of the construction of a coastal embankment creating a low-energy shadow zone on its eastern, down current side, thus promoting a fine-grained sedimentation with the suffocation of *P. oceanica* that, in this site, was partially replaced by *C. nodosa*. Mean grain size of sediments and their mineralogical composition showed a westward decrease, from the embankment to Punta Mescio, that is consistent with the rough sea resuspension and the transportation of fine sediments by the dominant westward current and with the trend in *P. oceanica* cover (Bianchi and Peirano, 1995; Torricelli and Peirano, 1997; Cavazza et al., 2000; Peirano et al., 2001; Peirano, 2002). At present, the meadow is divided into three zones with a cover of 75–100%, 20–60% and <25% (Fig. 1). Within each zone Savini et al. (1999) found that phenological parameters (the number of leaves per shoot and the width and length of leaves) of *P. oceanica* varied in relation to both the distance from the embankment and the direction of the dominant current. In the same area 72 orthotropic and plagiotropic shoots

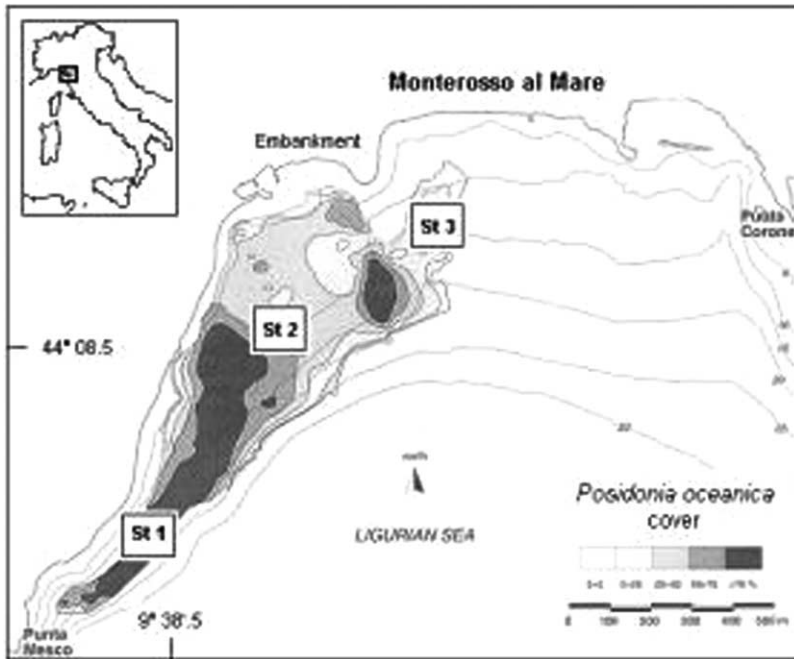


Fig. 1. Map of *P. oceanica* cover at Monterosso al Mare showing the three sampling stations and the increase of the meadow regression from Punta Mesco to the embankment (from Cavazza et al., 2000, modified).

were sampled at three stations (St. 1: Punta Mesco):  $44^{\circ}08'10''\text{N}$ ,  $9^{\circ}38'20''\text{E}$ ; St. 2: Intermediate:  $44^{\circ}08'30''\text{N}$ ,  $9^{\circ}38'40''\text{E}$ ; St. 3: Embankment:  $44^{\circ}08'35''\text{N}$ ,  $9^{\circ}33'45''\text{E}$ ) at 15 m in depth, to be analysed by RAPD technique. Sampling was carried out along a transect 40 m long, at four sites for each station taking three plants at random from each site. From each plant, both orthotropic and plagiotropic shoots were sampled in order to be sure that no relevant differences in genetic analysis occurred.

The large-scale approach, testing genetic differences between populations at basin (Mediterranean Sea) level, was conducted by analysing genetic characteristics of 11 mature, 8–10-year-old shoots (orthotropic cuttings), one for each population, from different Mediterranean sites. These had been transplanted, during 1989–1991 (Meinesz et al., 1993), in the “Posidonium” collection of “La Palud” Bay (Fig. 2), in the National Marine Park of Port-Cros (France,  $43^{\circ}01'\text{N}$   $6^{\circ}24'\text{E}$ ). The sites of origin were: Izmir, Turkey; Athens, Greece; Taranto, Italy; Ischia Island, Italy (two different meadows); Lavezzi, France; Port-Cros, France; Banyuls, France; Palma de Majorca, Balearic Islands, Spain; Marsa Bay, Algiers (two different samples).

## 2.2. Laboratory analysis

All shoots collected by SCUBA divers were washed in distilled water and stored in liquid nitrogen at  $-180^{\circ}\text{C}$ . Subsequently, extractions of genomic DNA were carried out

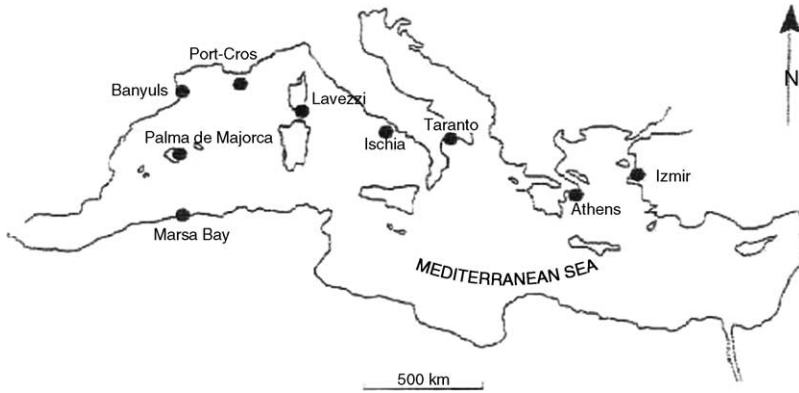


Fig. 2. Origin of Port-Cros *Posidonia oceanica* transplants across the Mediterranean.

following the protocol suggested by Dellaporta et al. (1983). The PCR conditions were similar to those described by Echt et al. (1992) with some modifications involving reaction buffer (100 mM Tris–HCl, 500 mM KCl and 25 mM MgCl<sub>2</sub>). Amplification reactions were carried out in a thermal cycler (Perkin-Elmer/Cetus) with the following temperature program: 2 min denaturation cycle at 94 °C and, subsequently, 40 cycles of 30 s at 37 °C annealing temperature and 2 min at 72 °C synthesis temperature. For each DNA (1 µg/µL) amplification, the AmpliTaq DNA Polymerase (0.2 µg/µL) (Applied Biosystems) was used and 11 different easily repeated oligonucleotides (10 mM) were chosen for their capacity for discriminating bands and scoring them as present/absent (Franconi et al., 1995; Waycott, 1995). The sequences of the primers (5'–3') utilized are reported in Table 1.

The amplification products were separated by gel electrophoresis (agarose 1.4%), then the samples were stained with Ethidium bromide and placed under UV light and photographed (Polaroid 667). All the electrophoresis runs were repeated in the same experimental conditions, with the aim of verifying the reproducibility of the method. Reproducibility and repeatability of amplification products were tested for each primer used in the experiments.

Table 1  
Sequences of primers, relative GC contents and melting temperatures ( $T_m$ )

| Primer | Sequence         | Percentage GC | $T_m$ (°C) |
|--------|------------------|---------------|------------|
| BY11   | 5'-ATCCACTGCA-3' | 50            | 30         |
| BY13   | 5'-CCTTGACGCA-3' | 60            | 32         |
| BY15   | 5'-CTCACCGTCC-3' | 70            | 34         |
| BY12   | 5'-GGTCGCAGGC-3' | 80            | 36         |
| DN4    | 5'-GTCGTGCTAT-3' | 50            | 30         |
| DN5    | 5'-CCGACGGCAA-3' | 70            | 33         |
| DN6    | 5'-TGGACCGGTG-3' | 70            | 34         |
| UBC22  | 5'-AAGCCTCCCC-3' | 70            | 34         |
| UBC24  | 5'-GGGTGAACCG-3' | 70            | 34         |
| UBC26  | 5'-CGCCCCAGT-3'  | 80            | 36         |
| UBC28  | 5'-GCTGGGCCGA-3' | 80            | 36         |

Genetic data analysis focussed on the number of molecular fragments for each specimen examined, both monomorphic and polymorphic, following Waycott (1995).

### 2.3. Statistical analysis

Cluster analysis (UPGMA) of the similarity indices was carried out using NT-SYS software (Rohlf, 1993). The method was used to determine similarities among samples. A pairwise distance matrix was computed based on Nei's coefficient of similarity (Nei and Li, 1979). A cophenetic value matrix was also produced from the tree matrix using the COPH program from NT-SYS-pc software. This matrix was then employed to check the validity of fit of cluster analysis by comparing it to the original pairwise distance matrix using Mantel test (MXCOMP program from NT-SYS pc). The cophenetic correlations found are statistically significant and their value ( $r$ ) can be used as a measure of validity of fit for a cluster analysis.

Fragment sizes of RAPD were estimated from the gel by comparison with a 1 Kb ladder marker. The bands were recorded as present (1) or absent (0) and assembled in a data matrix table. Then similarity coefficients (Dice index) were obtained (Simqual data matrix, NT-SYS-pc) and their average and standard deviation were calculated by Excel program.

## 3. Results

### 3.1. Small-scale approach

Samples collected at Monterosso al Mare showed that the percentage of polymorphism, obtained through the calculation of amplified polymorphic and monomorphic molecules, was 80% at St. 1 (Punta Mesco), 69% at St. 2 (Intermediate) and 66% at St. 3 (Embankment). Primers DN5 and DN6 revealed high polymorphism, with values of 77.3 and 76.0%, respectively. Primer BY12 also generated a high number of polymorphic fragments (66.2%), distinguishing samples on the basis of the total of molecular products amplified. Primer BY12 amplified the greatest interval in terms of products reaching 3.0 Kb, the greatest molecular weight obtained from the Monterosso al Mare meadow. All the 11 primers gave high reproducibility of electrophoresis pattern in the signal intensity and in the same number of bands for each different analysis performed.

Cluster analysis confirmed that similarity between samples decreased from Station 3, Embankment, to Station 1, Punta Mesco (Fig. 3). It is also evident that genetic variability is more pronounced among stations, than within samples from the same site, which are nearer geographically and genetically. The average of all similarity coefficients among the samples was  $0.66 \pm 0.03$ . Fingerprintings and Cluster analysis confirmed the absence of genetic differences between plagiotropic and orthotropic rhizomes of the same plants collected at Monterosso a Mare.

The Mantel test comparing Nei's distance and cophenetic matrices had a good and statistically significant correlation with a value of  $r = 0.88$  as a measure of validity (goodness) of fit for cluster analysis.

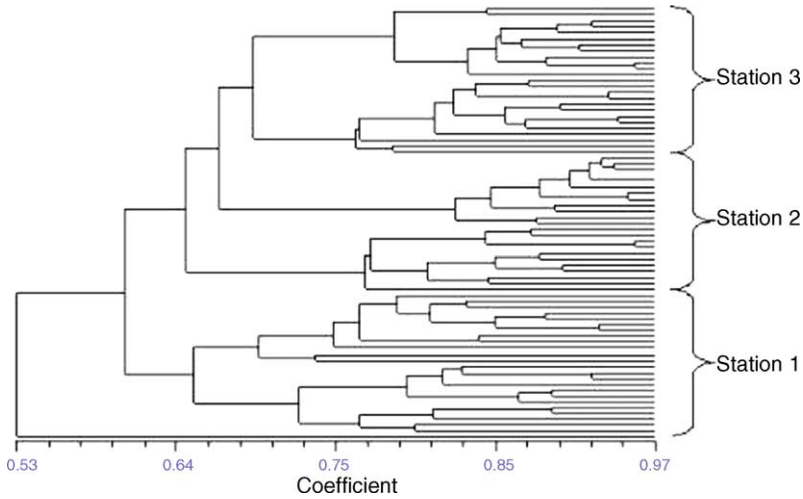


Fig. 3. UPGMA phenogram constructed from matrix of RAPD-based genetic distances among populations of *P. oceanica* at Monterosso bay.

### 3.2. Large-scale approach

Using the similar index by Nei and Li (1979), genomic fingerprintings of *P. oceanica* transplanted to Port-Cros marine park revealed several molecular fragments from each plant, varying in size from 0.2 to 3.0 Kb and a variable number of fragments between 3 and 16. Together, the 11 primers generated a total of 168 bands, of which 66% were polymorphic. A similarity matrix for all samples was calculated from PCR-generated banding patterns. Similarity coefficients range between 0.69 and 0.92. The average of all coefficients was  $0.816 \pm 0.017$ .

Primers UB26 and DN5 produced the most complex electrophoretic profiles, generating the greatest number of fragments, whilst BY12 and UB22 showed the greatest interval in terms of products, from 0.2 to 3 Kb and from 0.3 to 3 Kb, respectively. Finally, primer BY15 proved to be the best at discriminating the populations analysed, with greater polymorphism (87% of polymorphic fragments).

Also in the case of Port-Cros, all the 11 primers gave high reproducibility of electrophoresis pattern in the signal intensity and in the same number of bands for each different analysis performed.

DNA fragments generated by the RAPD marker were used to generate a phenetic tree (Fig. 4). Cluster analyses revealed the patterns of genetic distance in relation to physical distance (geographical position) and genetic variability for the 11 populations of the Mediterranean.

The Mantel test comparing Nei's distance and cophenetic matrices had a good and statistically significant correlation with a value of  $r = 0.79$  as a measure of goodness of fit for cluster analysis. Cluster analysis shows the sample divisions into two distinct groups: the Eastern Mediterranean Group (EMG), represented by Izmir (R1), Athens (R4) and Taranto (R5), and the western Mediterranean Group (WMG). The latter, following a

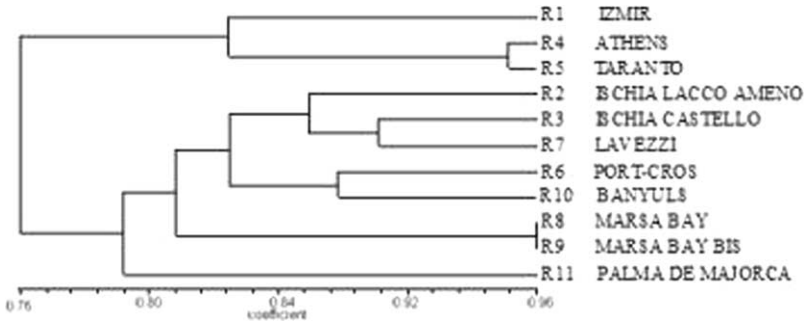


Fig. 4. UPGMA phenogram constructed from matrix of RAPD-based genetic distances among 11 populations of *P. oceanica* transplanted at Port-Cros from different Mediterranean localities.

longitudinal gradient from west to east, gathers three sub-groups: the Balearic (Palma de Majorca) sub-group (R11), the Algerian (Marsa Bay) sub-group (R8 and R9), and the Tyrrhenian-Provençal sub-group formed by Ischia Island (R2 and R3), Lavezzi (R7), Port-Cros (R6) and Banyuls (R10).

#### 4. Discussion

RAPD molecular markers allowed us to detect polymorphism in *Posidonia oceanica* populations. Genetic results of Monterosso al Mare population showed an order in genetic distance that clearly reflected the distance from the embankment (Fig. 3). The percentage of polymorphism sharply diminished approaching the coastal embankment. The observed genetic diversity trend was consistent with previous trends found in phenological (Savini et al., 1999) and sedimentological studies conducted on the Monterosso al Mare meadow (Cavazza et al., 2000). Considering the reduction in cover of living *P. oceanica* (Moreno et al., 2001) and its partial replacement by the more tolerant *C. nodosa* (Montefalcone et al., 2005), the distance gradient between Punta Mescio and the embankment can be interpreted as an anthropogenic stress gradient. The direct relation between the anthropogenic impact caused by the embankment and the meadow regression is also supported by the fact that the meadow, being situated in a marine protected area, is not affected by other types of impact and that depth and distance from the shore of the collecting stations are the same. This provides strong suggestive evidence that anthropogenic stress can reduce genetic diversity over short distances within a single meadow of *P. oceanica*. Stands with healthy plants having the highest number of leaves per shoot from Punta Mescio also showed the greatest genetic variability. In agreement with findings of Waycott (1995) on *P. australis*, the genetic data of *P. oceanica* of Monterosso al Mare indicated a lower level of polymorphism at St. 3, embankment, the part of the seagrass bed that was subjected to the greatest stress, with a lower number of leaves per shoot in both plagio- and orthotropic shoots (Savini et al., 1999). Randall et al. (1994) showed that populations of *Z. marina* from a highly disturbed habitat exhibit much lower genetic diversity than a population from an undisturbed site, demonstrating a decline of

genetic variability under physical disturbance and water quality deterioration. In our investigation we confirmed these effects, in agreement with Battaglia (1996) and Adam et al. (1994), who had already proposed genetic analysis as a tool for environmental monitoring.

Analyses conducted on the Port-Cros collection indicated that primers are potentially useful in discriminating populations of *P. oceanica* of different geographic origin. Primer DN5, for example, revealed particular differences in Athens and Marsa Bay populations, generating electrophoretic profiles that differ from all other populations, with values on the Nei index (1979) of 0.45–0.95 and 0.56–0.96, respectively. These findings were consistent to phenological data, Algerian samples maintaining larger dimensions than the other transplanted cuttings (Meinesz et al., 1993).

The primers allowed the identification of geographically close populations. Primer DN6 displayed exclusive bands in the populations of Taranto and Athens, with high molecular weights (1.8 and 2.0 Kb). By contrast, electrophoretic profiles of samples from two meadows off Ischia were quite homogeneous.

The two groups identified, the Eastern Mediterranean Group and the Western Mediterranean Group (WMG), lead us to hypothesise a differentiation related to the inner biogeographic subdivisions of the Mediterranean Sea (Bianchi and Morri, 2000). The Eastern Mediterranean Group, including all the samples located in the Eastern Mediterranean Sea, is characterised by a greater genetic variability. Eastern Mediterranean populations are also known to show different phenotypic characteristics, plants from the Aegean Sea being said to have smaller leaves (Panayotidis and Simboura, 1989). Many Mediterranean marine organisms are known to reach smaller sizes in the eastern basin than in the western (Morri et al., 2000). The case of *P. oceanica* suggests that phenological differences may be related to genetics, and not only to the warmer and more oligotrophic conditions in the eastern basin, as usually stated.

Samples from Taranto showed greater genetic similarity to those from Athens and Izmir than to the two distinct meadows of Ischia Island. This contrasts with the classical view of Pérès and Picard (1964) that included the biota of the Gulf of Taranto in the same biogeographic sector of the Tyrrhenian Sea. Costagliola et al. (2004) found a major genetic break between the populations of the fish *Thalassoma pavo* living in the Aegean Sea and those living to the west of the Peloponnesus, rather than at the Sicily Strait that should in theory be the biogeographic transition zone between western and eastern Mediterranean biota (Bianchi et al., 2002). These contrasting views are of particular interest, considering the relatively recent colonization of the Mediterranean from the Atlantic, after near extinction of the Mediterranean biota in the Messinian period, approximately 5.6 million years ago (Bianchi and Morri, 2000). It has been said that the Strait of Sicily acted as a filter to the recolonisation of the eastern Mediterranean, thus inducing major differences in the composition of the biota with respect to the western basin. An alternative view is that the biotic differences between western and eastern Mediterranean are due more to differences in temperature regime (a physiological barrier), and to the physical barrier made by the sill of the Strait of Sicily (Bianchi, in press). This may have profound implications for future scenarios: for example, predicted sea-warming will easily move isotherms but cannot change the morphology of straits.

The genetic results correlated with the different primary production values suggested a high efficiency of the Marsa-Bay and Athens plants. These phenological differences have normally been linked to the climatic and oceanographic characteristics of the different sectors of the Mediterranean Sea. The subdivision into sub-groups of our Western Mediterranean Group, for instance, is in accordance with the general flow pattern of the waters in the Western Mediterranean (Hopkins, 1985). Once again, our study suggests that these differences may lay over a genetic basis and are not simply the results of the action of climatic and hydrological factors on the phenotype. The Algerian population of *P. oceanica*, for instance, did not only have wider leaves but also exhibited genetic differences related to larger chromosomes, the position of satellite and centromery (Semroud et al., 1992; Semroud, 1993).

Of course our results are based on the analysis of single shoots from a common garden at Port Cros and, since these samples are transplanted shoots we must take into consideration that patterns of genetic diversity of seagrasses are influenced by other factors, such as spatial structure, age and maturity of the meadows, which all affect the recruitment potential of seedlings, pollen and vegetative propagules (Waycott and Samson, 1997). Our sampling design did not allow for a direct comparison of genetic variability at the two geographic scales we investigated; we noted, however, that the major divisions occur around 0.8 similarity. Indeed the two relevant UPGMA phenograms show that this similarity within the Monterosso al Mare meadow is mostly of the same order of magnitude as the lowest similarities between western and eastern Mediterranean samples. May this indicate that anthropogenic stress can induce genetic variations comparable in size to those deriving from the million-year long colonisation of the Mediterranean Sea? We are not able at the present stage of knowledge to provide an answer to this question. According to Amzallag (1999) environmental stress is an important factor that may induce genome variability of various types, from our study we conclude that the approach based on RAPD analysis can be applied to the study of widely different topics including the evaluation of the anthropogenic role in reducing intraspecific biodiversity and the biogeographical history of the whole Mediterranean.

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