

# Assessment of *Posidonia oceanica* (L.) Delile conservation status by standard and putative approaches: the case study of Santa Marinella meadow (Italy, W Mediterranean)\*

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## ABSTRACT

The conservation status of the *Posidonia oceanica* meadow at Santa Marinella (Rome) was evaluated through both standard (bed density, leaf biometry, "A" coefficient, Leaf Area Index, rhizome production) and biochemical/genetic approaches (total phenol content and Random Amplified Polymorphic DNA marker). The biochemical/genetic results are in agreement with those obtained by standard approaches. The bed under study was ranked as a disturbed one, due to its low density, and high heterogeneity in leaf biometry, LAI values, "A" coefficient and primary production. This low quality ranking is confirmed by both mean phenol content in plants, quite high and scattered, and by the low genetic variability in the meadow, with a very high similarity of specimen at a local scale. Hence, these two putative approaches clearly identify the endangered conservation status of the meadow. They link plant biodiversity and ecophysiology to ecosystem 'health'. Furthermore, they are repeatable and standardizable and could be usefully introduced in meadows monitoring to check environmental quality.

**Keywords:** *Posidonia oceanica*; Genetic Variability; Environmental Indicator; RAPD; Seagrass Monitoring; Total Phenols

\*This work is dedicated to the memory of the late Professor Eugenio Fresi

## 1. INTRODUCTION

*Posidonia oceanica* (L.) Delile (*PO*) is the dominant endemic seagrass in the Mediterranean Sea, where it forms meadows which play a crucial role in coastal ecosystem dynamics. They produce high amount of oxygen and organic compounds, sustain food nets, and act as a nursery/refuge for several species. Furthermore they preserve coastal systems trapping sediments into the *matte* and reducing hydrodynamics [1]. *PO* is regarded as a key species, being listed in the Habitat Directive 92/43/EEC.

Throughout most of the Mediterranean Sea, natural processes and human activities are responsible for a widespread *PO* meadow regression [2-3]. The identification of 'new diagnostic tools' to monitor the meadows conservation status is a critical issue. Standard monitoring indicators are several [4-10]; according to Montefalcone [11] standard monitoring indicators can be classified as structural descriptors at individual level (shoot phenology and biomass), at population level (bed density and coverage) or at community level (leaves epiphytes). However, many of them suffer of a lack in sensitivity and often to obtain significant information it's necessary to use a combination/integration of indicators [12]. The identification of approaches providing early and ecologically relevant information, useful for policy and management, is still a critical issue.

Recently two putative approaches were proposed [13-15]: phenolic compounds and Random Amplified Polymorphic DNA (RAPD) markers. If conveniently modified, these well known methods can clearly identify *PO* alterations.

Phenolic compounds, as in terrestrial plants [16], can work as biochemical markers of environmental stress

(total phenol determination is an easy and rapid assay). They are a class of secondary metabolites widely distributed in terrestrial [17] and aquatic plants [18-19]. Phenolic compounds are present in roots, stems, rhizomes, leaves, flowers and fruits playing several structural and physiological roles, including the defense of plants. High phenol concentrations were found in *PO* leaves exposed to different environmental pressures: competition with the invasive seaweed *Caulerpa taxifolia* [20-21], contamination by metals [22], proximity to intensive fish aquaculture [23]. The increase of phenolic compounds represents a generic response to different environmental stress and thus can be used to screen the meadow health state. Total phenol concentration varies in leaves with season due to leaves' short lifespan [21,24] and with depth. On the opposite, levels of synthesis and accumulation of phenolic compounds are more stable in rhizomes [15,24].

The decline of *PO* can be facilitated by low genetic diversity which results from a restricted gene flow, as suggested by trinucleotide [25-26] and dinucleotide [27] microsatellites. Low genetic diversity may result in low resistance, low resilience and limited adaptability to environmental changes [13,28-29]. RAPD markers have been used to assess the pattern of genetic diversity and the genetic structure of rare and endangered plants. They provide information for the conservation of endangered plants [30]. Moreover, they have been successfully used to assess genetic diversity of other seagrasses [31-32]. RAPDs revealed both the intra-population variability in *P. australis* [33], and the high genetic homogeneity in *Cymodocea nodosa* from Northern Atlantic [34]. They have been used to compare the genetic diversity of *Cymodocea nodosa* and *P. oceanica* populations in the Mediterranean Sea [35]. Furthermore, RAPD markers revealed a decreased genetic diversity in *PO* along an anthropogenic disturbance gradient, both at small within a meadow and at Mediterranean scale [13]. Analogous trends were found in others seagrasses [36].

Hence, total phenol content in rhizome and RAPD markers in leaves are inexpensive and uncomplicated methods, potentially useful for *PO* meadows monitoring.

The aim of this study is to describe the conservation status of a specific meadow by comparing the results of phenol content and RAPDs (putative approaches) to the standard ones. The superimposition of results will confirm that the putative methods can detect alterations in the meadow and properly contribute to assess the meadow "health status".

To this end, plants from the Santa Marinella meadow (Rome, Italy, W Mediterranean), collected in Spring

2004, were analyzed to obtain the quantification of phenolic compounds and RAPD marker profile.

## 2. MATERIALS AND METHODS

### 2.1. Study Site and Sampling

The study was conducted on the *PO* meadow of Santa Marinella (Rome, Italy; **Figure 1**), a Site of Community Importance (according to Habitat Directive 92/43/EEC). This meadow, spanning from Capo Linaro to Santa Severa, for a 13.5 km coastline and covering a surface of 1,800 ha, can be considered a "pure bed", *i.e.* monospecific, with patched distribution and a regressive limit, characterized by the presence of dead *matte*. The lower limit is at -20 m depth [37]. This meadow is under anthropogenic impact, mainly intensive agriculture and land use, causing increased water turbidity and fine sediment input. The streams and watercourses flowing in Santa Marinella sea stretch are responsible of pollutant, fine sediment and nutrient input to the sea [38-40].

Sampling was carried out in late spring 2004; 30 sampling stations were randomly chosen in the central area of the meadow (about 5 ha), on a relatively homogeneous topography. The bathymetric values of the sampling area ranged from 7.5 to 13.5 m depth (see Supplemental Data for sampling site information).

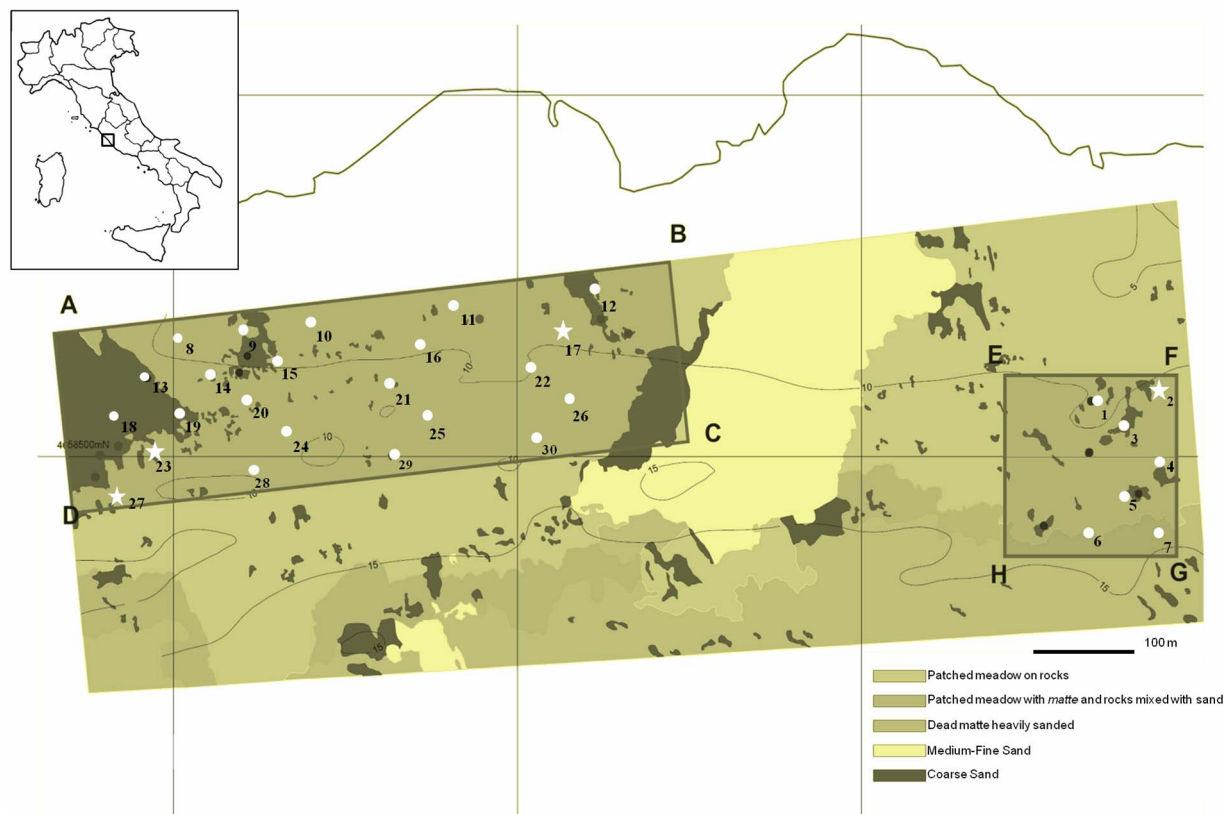
Samples were obtained by SCUBA diving. In each sampling station shoots were collected for phenological/lepidochronological analyses (15) and for phenol determination (3 orthotropic). Furthermore, in 4 selected stations (**Figure 1**), 5 orthotropic shoots per site were sampled for RAPD marker analyses.

In § 3.2 are shown some data from the Talamone meadow (Tuscany, Italy; 30 sampling sites at comparable depth), which is considered well preserved according to phenological and lepidochronological analysis [24]. All these samples were analysed in our laboratory by the same experimental protocol (unpublished data by L. Migliore, personal communication).

### 2.2. Phenological and Lepidochronological Analyses

Shoot density was evaluated *in situ* by counting the number of shoots using 40 × 40 cm standard quadrates, five measurements at each site. The value obtained is expressed as number of shoots/m<sup>2</sup>.

Number and biometry of leaving leaves (foliar shoots) were determined, on 10 shoots for sampling site, according to Giraud [41]. "A" coefficient (percentage of leaves with lost apex) and Leaf Area Index (LAI, leaf surface area per shoot, cm<sup>2</sup>/shoot) were also calculated.



**Figure 1.** Sampling area's map in the Santa Marinella meadow (Rome, Italy), showing the 30 sampling stations and the bottom characteristics. Stations where genetic analysis was performed are indicated by stars instead of dots.

Lepidochronological analysis was carried out on 5 shoots for sampling site, according to standard methods [42], in order to estimate the annual primary production of the rhizomes, expressed as mg rhizome (dry weight, d.w.) produced per shoot per year.

### 2.3. Total Phenols

Total phenol determinations were carried out in duplicate on 3 different rhizomes for each sampling site according to Migliore *et al.* [15]. Shoots were maintained under dark and stored at  $-20^{\circ}\text{C}$  until processing. Plants were first rinsed in 0.1 Triton-X (Sigma) and then in distilled water to remove epiphytes and contaminants. Phenolic compounds were extracted according to Legrand [43] modified for *PO* on 100 mg (fresh weight, f.w.) of basal, intermediate and apical sections of the rhizome. Quantification of total phenols was performed by spectrophotometry [ $\lambda = 724 \text{ nm}$ , chlorogenic acid (Sigma) as standard] using the Booker and Miller [44] method. The amount of protein, known to interfere with Folin-Ciocalteu reagent (Sigma), was determined by the Bradford assay.

### 2.4. Genetic Analysis

Genetic analysis was carried out in duplicate on 5

plants from the 4 selected station. The plants were washed in distilled water and the young leaves stored in liquid nitrogen at  $-180^{\circ}\text{C}$  until processing. Extraction of genomic DNA was carried out according to the protocol of Dellaporta *et al.* [45]. The PCR amplification was performed (Perkin Elmer 2400) using 10 primers; sequences are reported in table 1. The primers (10 mM) were chosen for their capacity for discriminating bands and scoring them as present/absent; they gave high reproducibility of electrophoresis pattern in both the signal intensity and the number of bands.

All the experiments, including the PCR amplification and electrophoresis conditions, were carried out according to Micheli *et al.* [13].

### 2.5. Statistical Analysis

One-way ANOVA test was utilized to evaluate differences in total phenol content between Talamone and Santa Marinella meadows and among rhizome sections from the two sampling sites. Parametric hypotheses were tested.

Box-plots were utilized to show the distribution of bed density, leaf biometry and phenol content data; the box contains 50% data (the extremes of each box are the Q1

and Q3, 1st and 3rd quartiles), the internal horizontal segment represents the median of the distribution (Q2 value, 2nd quartile), “whiskers” range from the lowest to the highest value.

All the RAPD data were elaborated using NT-SYS-pc (Numerical Taxonomy and Multivariate Analysis System) computer package. 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. The Nei's coefficients of similarity between each pair of samples were used to construct a dendrogram using the unweighted pair group method with arithmetic averages (UPGMA).

### 3. RESULTS AND DISCUSSION

#### 3.1. Standard Monitoring Approaches

The absolute density throughout the Santa Marinella meadow is reported in **Figure 2A** and Supplemental Data; the mean value for the meadow is 342.8 leaves shoots/m<sup>2</sup>. According to the classification proposed by Pergent *et al.* [46] and modified by Buia *et al.* [47], this meadow is considered a “disturbed bed”.

The leaf biometry (*i.e.* number, length and width) was also recorded (**Figure 2B-D**) to calculate LAI values per shoot (see Supplemental Data for the complete data set). LAIs range from 120 to 68.6 and they probably represent the highest values all the year round, as LAI is maxi-

mum at the end of spring and a minimum in late autumn or at the beginning of winter [48]. Santa Marinella LAI values are comparable to those found in other Tyrrhenian meadows [49-51].

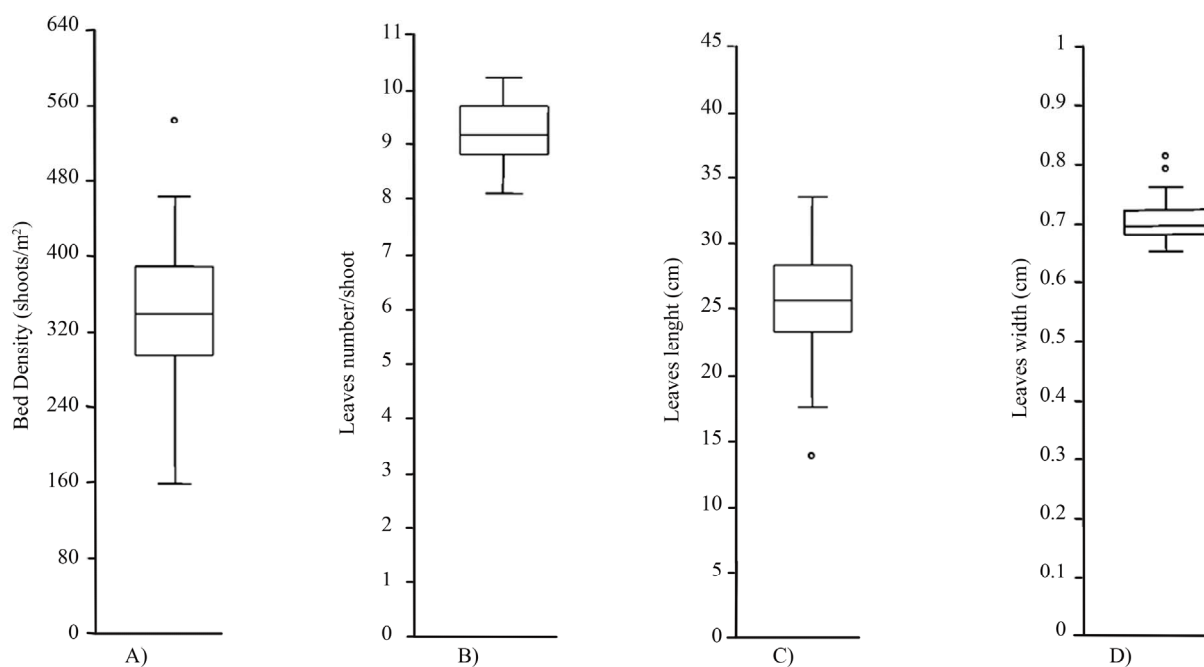
The “A” coefficient shows heterogeneous values between stations (see Supplemental Data), from a minimum of 25% to a maximum of 80%, suggesting a high spatial heterogeneity in mechanical stress or grazing in the meadow. Both “A” coefficient ( $r = -0.63$ ;  $p < 0.01$ ) and LAI/shoot ( $r = -0.56$ ;  $p < 0.02$ ) seems negatively related to depth, although the range of bathymetric values is narrow (**Figure 3A and B**).

The mean value of the rhizome production is 41.3 mg (d. w.)/shoot/yr, ranging from 26 to 59 (see Supplemental Data); values are lower than those registered in meadows with same morphological characteristics [39] and no relationship with depth was found.

#### 3.2. Putative Approach I: Phenols Content

The total phenol mean content in the entire rhizome is 18.7 mg/g (f. w.), ranging from 8.8 to 30.2 mg/g (**Figure 4A**). In all rhizomes a decrease in total phenol concentration from the apical to the basal section is found; differences are statistically significant (ANOVA,  $F = 38.1$ ,  $p < 0.01$ ; **Figure 4B**).

These data have been compared with total phenol content, quantified in plants from the Talamone (Tuscany, Italy; see § 2.1) meadow. In Talamone's plants the total phenol mean content is 5.62 mg/g f. w. ( $n = 72$ ; SE 0.15)



**Figure 2.** Density (A) and leaves biometry (B-D) reported as box-plots. The box contains 50% data, the horizontal segment represents the median, ‘whiskers’ range from the lowest to the highest recorded value.

and a decreasing apical-basal gradient was found. According to phenological and lepidochronological analysis Talamone meadow was judged as “well preserved” [39]. Phenol content in Santa Marinella plants is significantly higher and more scattered than in Talamone plants (Figure 4) as regard both the entire rhizome and the three sections.

The high phenol values can be related to the endangered plant health status in Santa Marinella meadow and, being the total phenols analysis repeatable, inexpensive and uncomplicated method, it could be useful introduced in the set of test to state the conservation of *PO* meadows.

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### 3.2. Putative approach II: RAPD markers

Ten RAPD primers generated a total of 111 bands, with fragments ranging in size from 150 to 300 bp. 55 of the 111 bands were polymorphic among the 20 individuals; the overall percentage of polymorphism in the Santa Marinella meadow was 61.1%. The percentage of polymorphic bands in each station varies from 6% to 11.6%, demonstrating a low variability in the specimens. Primer BY15 generated the highest number of polymorphic fragments (82.4%), distinguishing samples on the basis of the total of molecular products amplified. Primers DN5, BY12 and UB28 also revealed high polymorphism (ranging from 54.6% to 66.7%).

UPGMA cluster analysis (Figure 5) confirmed that similarity between samples is very high (the lowest genetic similarity being 0.82). The average of all similarity coefficients among the samples is  $0.87 \pm 0.03$ . Moreover, samples from the same station always cluster in a

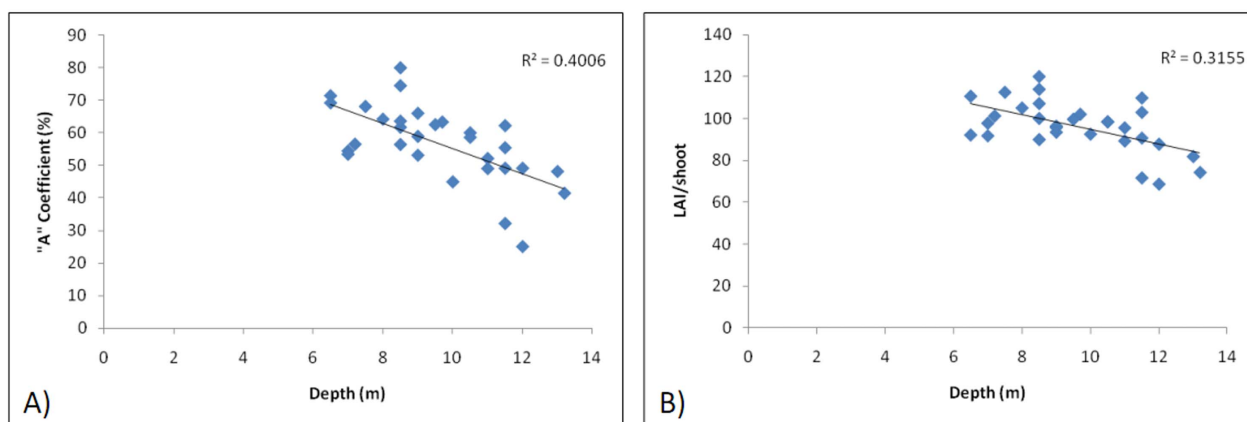


Figure 3. “A” Coefficient A) and LAI/shoot; B) variation with depth (m).

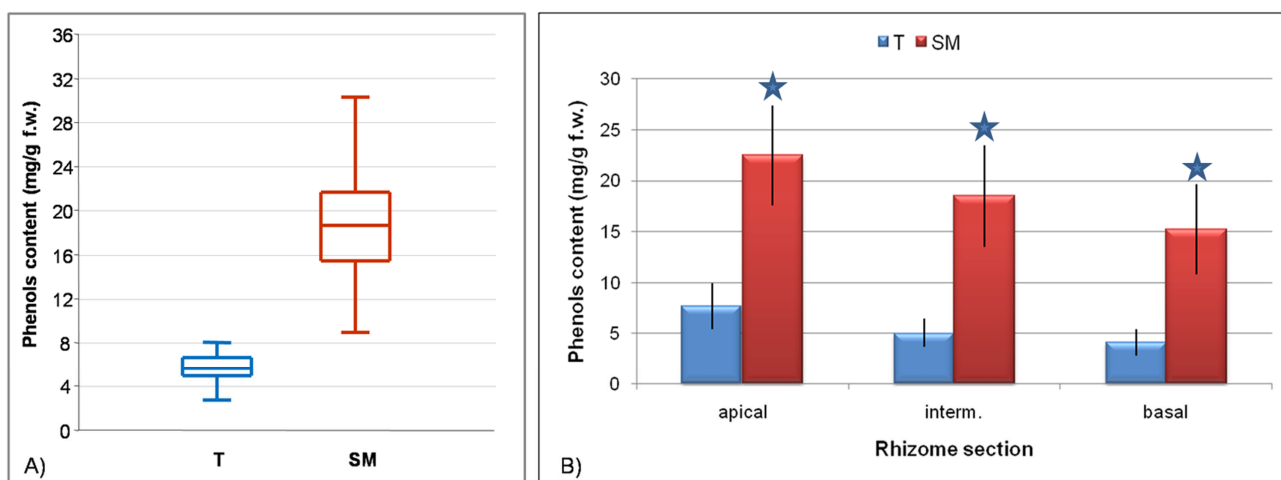
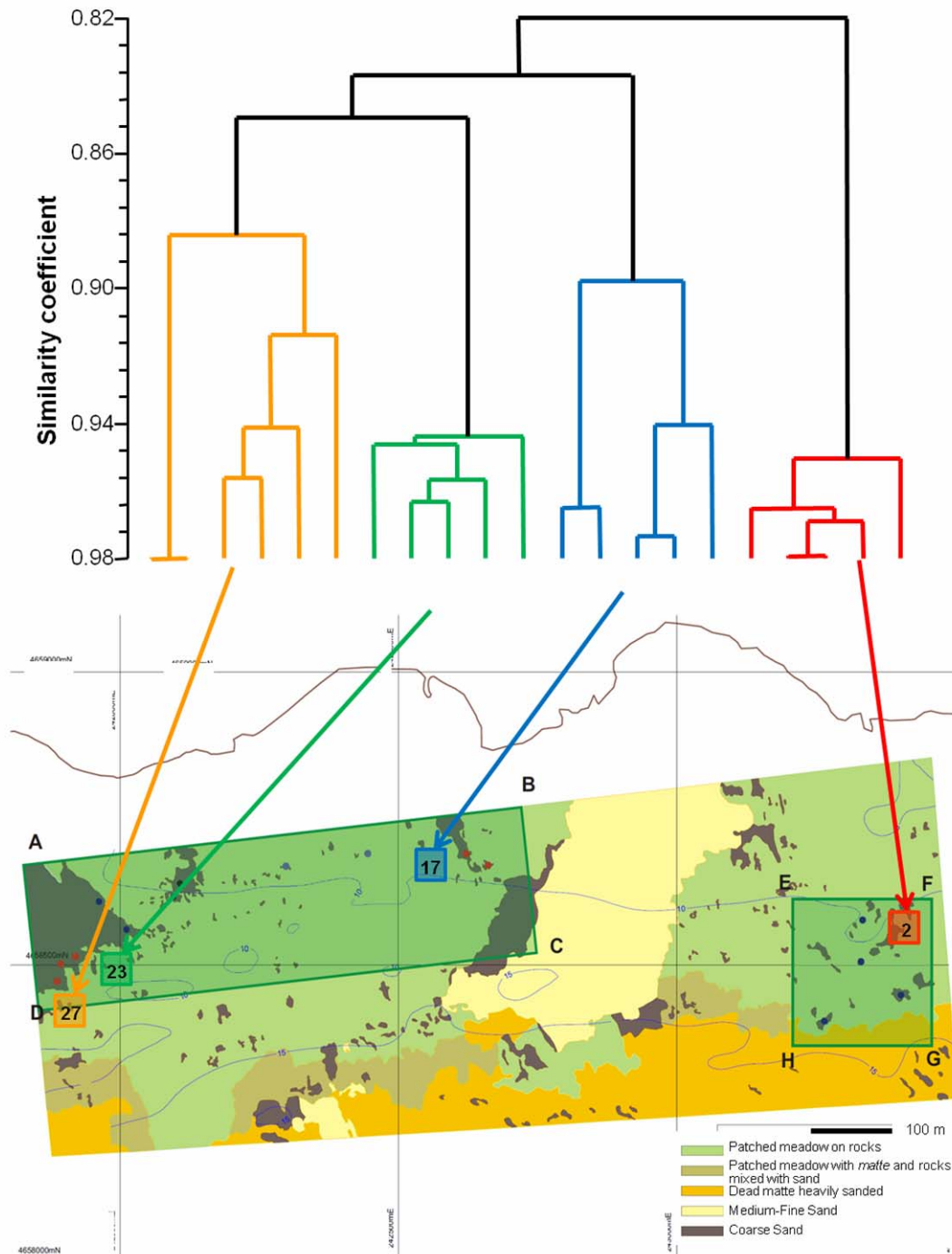


Figure 4. Mean total phenol content in the entire rhizome A) and in each rhizome section; B), bars indicate standard deviations), recorded in both Santa Marinella (SM) and Talamone (T) meadows. Significant differences between Santa Marinella and Talamone were found both in the entire rhizome ( $F = 656.6$ ;  $p < 0.01$ ) and among sections (indicated by a star; ★ =  $p < 0.01$ ).



**Figure 2.** UPGMA phenogram constructed from matrix of RAPD-based genetic distances among *PO* plants from the Santa Marinella meadow. It is worth to note that each cluster gathers individuals from the same area of the meadow.

group (Figure 5). The Mantel test, comparing Nei's distance and cophenetic matrices, revealed a strong and statistically significant correlation between genetic and geographic distance ( $r = 0.9$ ,  $p \ll 0.01$ ).

The genetic analysis conducted in Santa Marinella meadow demonstrates a low variability in each specimen resulting in a high similarity value within the population (0.87). This result suggests a restricted gene flow within

the population, *i.e.* the predominance of the clonal growth. The 0.87 similarity value is much higher than the one found for the Monterosso al Mare meadow (0.66) [13], where natural and anthropogenic pressures were low (being part of the bed inside the ‘Cinque Terre’ National Park, Liguria, Italy) [52-53]. The value is even higher than the one found at the Mediterranean basin scale (0.81) [13].

These results confirm that RAPDs, as the total phenols, give sound informations on the meadow. The approach is repeatable and uncomplicated, and it could be useful introduced in the set of test to state the conservation of *PO* meadows.

#### 4. CONCLUSIONS

In this study we demonstrate that the measure of total phenols and RAPD diversity (putative approaches) in *PO*, give the same picture of the meadow conservation as the classical measure of density, leaf biometry, LAI and rhizome production. According to standard indicators, the Santa Marinella meadow is defined “disturbed bed”, under regression, showing high spatial heterogeneity and low productivity; likewise, the high levels of total phenols identify the endangered conservation status of the meadow, showing to be a possible biomarkers of environmental quality. Additional research will be necessary to state the level of phenol concentration in different meadows under different environmental conditions and to define thresholds for classifying the different level of perturbation. Furthermore, a similar protocol was successfully applied on another seagrass (*Zostera noltii*), opening interesting perspectives on the application of the phenol content on a large number of marine phanerogames. The endangered conservation status of the meadow is also identified by the low genetic variability (as RAPD markers). Although genetic variability is low in some other cases [35] and can depend on different processes, this measure can properly contribute to assess the meadow conservation.

The two putative approaches should be used together with the standard techniques to better depict the conservation status of the meadows. This is in agreement with the epidemiological approach, *i.e.* the use of various lines of evidence independently, validated by weight of evidence [54-55]. This epidemiological approach has been successfully applied in estuarine ecosystems, to evaluate that the changes produced in a community structure were due to environmental pressure and not to natural variability [56].

Furthermore, this picture of the Santa Marinella meadow (2004 samples) represents a baseline for future comparison, to state possible changes occurring in the Santa Marinella meadow as a response of the continuous

anthropogenic pressure in the area.

These results highlight the interest on both the two putative tools, which are able to link plant ecophysiology and biodiversity to ecosystem ‘health’. The most remarkable feature of these approaches is their feasibility and unexpensiveness that make easy the introduction in meadows monitoring. Furthermore, we wish to apply the two putative approaches on other *PO* meadows and other seagrasses to validate the methods for broader application; after such studies the proposed methodologies might be recommended for seagrass meadow monitoring.

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