

# Adaptive responses along a depth and a latitudinal gradient in the endemic seagrass *Posidonia oceanica*

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1 **Adaptive responses along a depth and a latitudinal gradient in the**  
2 **endemic seagrass *Posidonia oceanica***

3

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25

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27

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29

30 **Abstract**

31 Seagrass meadows provide important ecosystem services and are critical to  
32 the survival of the associated invertebrate community. However, they are  
33 threatened worldwide by human-driven environmental change.  
34 Understanding the seagrasses' potential for adaptation is critical to assess  
35 not only their ability to persist under future global change scenarios, but also  
36 to assess the persistence of the associated communities.  
37 Here, we screened wild population of *Posidonia oceanica*, an endemic long-  
38 lived seagrass in the Mediterranean Sea, for genes that may be target of  
39 environmental selection, using an outlier and a genome-wide transcriptome  
40 analysis. We identified loci, which polymorphism or differential expression  
41 was associated with either a latitudinal or a bathymetric gradient, as well as  
42 with both gradients, in an effort to identify loci associated with temperature  
43 and light. We found candidate genes underlying growth and immunity to be  
44 divergent between populations adapted to different latitudes and/or depths,  
45 providing evidence for local adaptation. Furthermore, we found evidence of  
46 reduced gene flow among populations, including adjacent populations.  
47 Reduced gene flow, combined with low sexual recombination, small  
48 effective population size, and long generation time of *P. oceanica*, raises  
49 concerns for the long-term persistence of this species, especially in the face  
50 of rapid environmental change driven by human activities.

51

52 **Keywords**

53 outliers, transcriptome, candidate genes, adaptation, priority species,

54 Mediterranean Sea

55

## 56 **Introduction**

57 Organisms have historically responded to changes in the environment by  
58 migrating, tolerating or adapting (Hessen *et al*, 2013). However, human  
59 activities exacerbate environmental changes, imposing shifts in ecological  
60 niches that often surpass the adaptive potential of species (Hoegh-Guldberg  
61 *et al*, 2007) and lead to local extinctions (Cardinale *et al*, 2012; Smith *et al*,  
62 2007). Stress imposed by temperature changes has been associated with loss  
63 of biodiversity (Both *et al*, 2006; Corlett and Westcott, 2013; Franks *et al*,  
64 2014; Van Der Wal *et al*, 2013), with severe consequences on sessile  
65 species (Rivetti *et al*, 2014). Indeed, range reduction of the large majority of  
66 benthic marine species has been associated with global warming (Bay and  
67 Palumbi, 2014; Jueterbock *et al*, 2016; Sanford and Kelly, 2011). However,  
68 because of the complex interplay among temperature and other  
69 environmental stressors, it remains a challenge to disentangle the impact of  
70 temperature stress from the one of other environmental factors.

71         Here, we investigated local adaptation of populations of the seagrass  
72 *Posidonia oceanica* sampled along a latitudinal and a bathymetric gradient,  
73 using a genome scan and a transcriptome analysis. Temperature and light  
74 are key environmental factors varying along these gradients; in particular  
75 light is strongly associated with the bathymetric gradient. We hypothesized  
76 that a signature of local adaptation shared between the bathymetric and the  
77 latitudinal gradient reflects adaptation to temperature, while a signature of

78 local adaptation associated with the bathymetric gradient reflects adaptation  
79 to light cues. To test these hypotheses, we looked mined for outlier loci  
80 uniquely associated with the bathymetric or latitudinal gradient, as well as  
81 for shared outlier loci between gradients. Furthermore, we performed a  
82 genome-wide differential expression analysis to identify candidate genes  
83 and gene pathways associated with the bathymetric gradient.

84         Seagrasses are one among the most valuable ecosystems on earth, as  
85 they are important providers of ecosystem services and sustain many  
86 invertebrate and vertebrate communities (Costanza *et al*, 1997; Giakoumi *et*  
87 *al*, 2015). Understanding how seagrasses respond to environmental change  
88 is critical not only to assess their ability to persist to future global change,  
89 but also to assess the persistence of the associated communities. The  
90 seagrass *Posidonia oceanica* (L.) Delile is an endemic long-lived seagrass in  
91 the Mediterranean Sea. Individual shoots of this plant survive for decades  
92 (Short *et al*, 2011), and grow via horizontal rhizome elongation (1 to 10 cm  
93 per year; Marbà and Duarte, 1998). Single genotypes can persist for  
94 millennia via asexual reproduction (Arnaud-Haond *et al*, 2012; Ruggiero *et*  
95 *al*, 2002). Flowering via sexual reproduction is extremely heterogeneous at  
96 spatial and temporal scales (Diaz-Almela *et al*, 2006; Jahnke *et al*, 2015a).  
97 Moreover, the establishment success of flowers is low (Balestri and Cinelli,  
98 2003; Diaz-Almela *et al*, 2006). *P. oceanica* populations in the  
99 Mediterranean Sea show pronounced genetic structure (Arnaud-Haond *et al*,

100 2007; Serra *et al*, 2010), with reduced gene flow both across latitudes  
101 (Procaccini *et al*, 2002) and depths (Migliaccio *et al*, 2005; Procaccini *et al*,  
102 2001). Low genetic diversity has been observed at regional and global scale  
103 (Arnaud-Haond *et al*, 2007; Serra *et al*, 2010). Furthermore, low genetic and  
104 genotypic diversity have been associated with local or regional extinctions  
105 (Jahnke *et al*, 2015b). These studies support predictions of *P. oceanica*  
106 functional extinction from the Mediterranean Sea under projected global  
107 warming (Jorda *et al*, 2012).

108

## 109 **Materials & Methods**

### 110 *Sampling design*

111 We collected *P. oceanica* populations from six localities in the  
112 Mediterranean Sea along a latitudinal (1,000 Km, Fig. 1) and a bathymetric  
113 gradient. Each locality was sampled at two depths (5 m and 20 – 25 m)  
114 (Table S1). Average sea surface temperature (SST) records from the last  
115 three decades were provided by Copernicus  
116 (SST\_MED\_SST\_L4\_REP\_OBSERVATIONS\_010\_021\_a\_143885878394  
117 4) (Nardelli *et al*, 2013). Using these records, we calculated average  
118 temperature differences among populations (Table S2). Historical records  
119 show differences of up to 10°C between the two sampled depths, in  
120 particular when vertical mixing is prevented by the presence of the summer  
121 thermocline (Marin-Guirao *et al*, 2016). This temperature difference is less

122 pronounced in winter. For one of the sampled locations (Stareso, Table S1),  
123 the photosynthetic active radiation (PAR) was recorded at the time of  
124 sampling (1pm), and corresponded to 430 and 92  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at 5 and 20  
125 m, respectively (Procaccini *et al*, 2017). Comparable differences in  
126 irradiance between depths can be expected at the other sampled locations.  
127 However, PAR data are not available for all locations.

128 At each site and from each depth, between 20 and 31 adult leaves of *P.*  
129 *oceanica* were sampled non-destructively (Table S1). The mean distance  
130 between samples at each site ranged between 5 and 8 m to reduce the  
131 chance of collecting identical clones (Arnaud-Haond *et al*, 2007; Serra *et al*,  
132 2010). After collection, the leaves were cleaned from epiphytes and stored  
133 in silica gel, prior to DNA extraction. All samples were genotyped at 23  
134 putatively neutral microsatellite loci, 14 of which were EST-linked (Table  
135 S3; Alberto *et al*, 2003; Arranz *et al*, 2013; Migliaccio *et al*, 2005,  
136 Procaccini and Waycott, 1998).

137 The Stareso population (Corse, 8°45'E, 42°35'N, Fig. 1) was previously  
138 used to generate a reference transcriptome for *P. oceanica* (D'Esposito *et al*,  
139 2016). Here, we used the available RNA-Seq data to identify differentially  
140 expressed genes between the shallow and deep stands of the same  
141 population. As samples were collected at two time points within the same  
142 day (12 noon and 6.30 pm), we also studied plastic response in genome-



143 wide expression in response to light by identifying differential gene  
144 expression between the samples collected at the same depth at different time  
145 points. At the time of sampling, sunrise was at 7.25 am and sunset was at  
146 6.45 pm.

147

148 *Population response to environmental change – outlier analysis*

149 DNA was extracted from 271 samples (*ca.* 20 mg of dried tissue per  
150 sample) collected from the six localities at two depths (Table S1) using the  
151 NucleoSpin® 96 Plant II kit (Macherey-Nagel), following a modified  
152 protocol optimized for a Biomek FX robotic station (Tomasello *et al.*, 2009).  
153 Genotyping was performed using an ABI Prism 3730 automated DNA  
154 sequencer (Applied Biosystems), following PCR amplification, with the  
155 following cycling: 95°C for 15min, 35 cycles of 94°C for 30 sec, 60°C for 1  
156 min 30 sec and 72°C for 1 min, with a final extension step of 60 °C for 30  
157 min. Only samples that were successfully genotyped at  $\geq 90\%$  of the loci  
158 (22/24 loci) were used for downstream analyses.

159 Although the likelihood of sampling the same genotype was low due to our  
160 sampling design, we screened for the presence of identical multilocus  
161 genotypes (MLGs) using the psex ( $F_{IS}$ ) (the probability that the repeated  
162 genotypes originate from distinct sexual reproductive events considering  
163 departures from Hardy–Weinberg equilibrium) in GenClone (Arnaud-

164 Haond and Belkhir, 2007). When identical MLGs were detected, only one  
165 was retained within each site for downstream analyses. Our data-set  
166 comprised 237 individuals (18-22 individuals per population, Table S1).

167 All genotyped populations were used in an outlier analysis to  
168 identify loci putatively associated with the latitudinal and depth gradients.  
169 We contrasted pairwise populations at the extreme of the latitudinal  
170 gradient. In addition, we contrasted stands of the same population sampled  
171 at 5m and 25m. Prior to this analysis, we performed a population genetic  
172 structure analysis, following Orsini *et al.* (2012). Specifically, we performed  
173 an analysis of population genetic differentiation in Arlequin3.5 with 10,000  
174 permutations (Excoffier and Lischer, 2010) and a population genetic  
175 structure analysis using a Discriminant Analysis of Principal Component  
176 (DAPC) (Jombart *et al.*, 2010) to identify the number of independent  
177 populations present in the dataset. We performed the DAPC implemented in  
178 Adegnet (Jombart, 2008) in R 3.2.2. (R Development Core Team, 2012)  
179 using eight principal components as suggested by the a-score optimization  
180 analysis. The analysis of population differentiation and the genetic  
181 discrimination identified a northern and a southern population cluster, and  
182 identified as independent populations stands of the same populations  
183 sampled at different depths (Fig. 2). This separation is also evident from the  
184 genetic differentiation analysis (Table S4). Hence, for the downstream  
185 analysis we considered populations from the same locality at different

186 depths as independent, and grouped populations along the latitudinal  
187 transect into a northern and a southern group.

188         To identify loci putatively linked to environmental factors, we used  
189 multiple pairwise population comparisons. This approach has been  
190 previously shown to reduce the number of false positives, by showing  
191 parallel patterns of locus-specific variation among replicate population  
192 comparisons (Orsini *et al*, 2012). To identify loci associated with the depth  
193 gradient, we contrasted stands at different depths of the same population,  
194 and a pool of all shallow vs all deep stands (Table S5). To identify loci  
195 associated with the latitudinal gradient, we performed pairwise population  
196 comparisons separately for the shallow and deep stands. For this analysis,  
197 we contrasted three random population pairs from the northern and the  
198 southern groups, as well as the pool of northern versus southern populations  
199 at the two depths (Table S6). With this approach, we were able to identify  
200 putative outlier loci shared between the bathymetric and latitudinal gradients  
201 as well as outlier loci unique to either gradient. The outlier analysis was  
202 conducted with Lositan (Antao *et al*, 2008) and BayeScan (Foll and  
203 Gaggiotti, 2008). To obtain Lositan results we run simulations for 50,000  
204 iterations, and used a 95% confidence interval. We performed the BayeScan  
205 analysis with default settings and used the provided R script to identify loci  
206 showing significant deviation from expectations under neutrality by plotting  
207 their posterior distribution. Using a conservative approach to reduce false

208 positives (Orsini *et al*, 2012), we plotted the number of times loci were  
209 detected as outliers in the pairwise population comparisons. To decide on  
210 the minimum threshold needed to define an outlier as a locus potentially  
211 under selection, we plotted the frequency with which every locus was  
212 detected as an outlier in pairwise analyses of population comparisons. Loci  
213 falling outside the 95% boundaries of this frequency distribution were  
214 considered ‘real’ outliers. Loci present only in comparisons from the  
215 bathymetric or latitudinal gradient are hereafter referred to as “bathymetric  
216 loci” and “latitude loci”, respectively. Loci common to the two gradients are  
217 referred to as “gradient-shared loci”.

218

219 *Association of neutral and non-neutral genetic variation with the*  
220 *environment*

221 The outlier analysis allowed us to distinguish neutral from putatively non-  
222 neutral loci. These two categories of loci were used in a correlative analysis  
223 with temperature and geographic distance. For this correlative analysis, we  
224 used the following categories of loci: 1) strictly neutral (not identified as  
225 outliers in any of the gradients); 2) gradient-shared; 3) latitude loci; and 4)  
226 bathymetric loci. On these four categories of loci we measured the  
227 partitioning of molecular variance via AMOVA with GenAlEx 6.5 (Peakall  
228 and Smouse, 2012) using 1,000 permutations. Three hierarchical levels were

229 used: a) depth (populations at two depths) or latitude (North and South  
230 populations, separately for shallow and deep stands); b) populations within  
231 groups, where the groups were either populations at the same depth or at the  
232 same latitude and; c) individuals within populations.

233 The allele frequencies of loci calculated by shallow and deep stands, as  
234 well as by northern and southern populations are listed in Table S7.

235         We used ArcGIS 10.1® (ESRI) to extract the sea surface  
236 temperature (SST) values for our sampling locations and measured  
237 geographic distances between sampling locations using the shortest path  
238 over the sea without crossing land. We performed a Multiple Matrix  
239 Regression with Randomization analysis (MMRR) (Wang, 2013) using R to  
240 identify correlations between genetic distance (measured as  $F_{ST}$  at both  
241 neutral and outlier loci), geographic distance, and temperature (measured as  
242 the difference between locations of averaged temperature between 1981 and  
243 2012, the period for which SST is available). MMRR uses a randomized  
244 permutation procedure to correct for possible dependency between  
245 geographic distance and environmental variables. As compared to a classic  
246 partial Mantel test, this method reduces type I errors (Wang, 2013). We used  
247 10,000 permutations to test for significant correlations among the  
248 standardized matrices of genetic distance, geographic distance and  
249 temperature for shallow populations only, as only superficial SST  
250 temperatures were available.

251 *Functional annotation of outlier loci*

252 The sequences of the outlier loci associated with the gradients were used for  
253 functional annotations after masking for low complexity regions, which  
254 improves gene homology searches. The annotation was done by blasting the  
255 sequence of the outlier loci in the NCBI databank and against the reference  
256 transcriptome of *P. oceanica* (D'Esposito *et al*, 2016) using blastx (Altschul  
257 *et al*, 1997) followed by function query in the EMBL-EBI Pfam data base  
258 (Bateman *et al*, 2004).

259

260 *Population response to environmental change – evolutionary and plastic*  
261 *differences in gene expression*

262 We used RNA-Seq data previously generated to assemble the *P. oceanica*  
263 reference transcriptome (D'Esposito *et al.*, 2016). Transcriptome data were  
264 available from the Stareso population. Specifically, two biological  
265 replicates, collected at two different times of the day (12 noon and 6.30 pm)  
266 at two depths (5 m and 20 m depth) were available. PAR at 12 noon was  
267  $350 \mu\text{mol m}^{-2} \text{s}^{-1}$  at -5 m, and  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$  at -20 m. PAR values at  
268 6.30 pm were  $30 \mu\text{mol m}^{-2} \text{s}^{-1}$  at -5 m and zero at -20 m, respectively  
269 (Procaccini *et al*, 2017).

270 We mapped the transcripts onto the reference transcriptome of *P. oceanica*  
271 using Bowtie (Langmead *et al*, 2009) and performed a differential

272 expression analysis between shallow and deep stands, as well as between  
273 sampling times at the same depth ( $P_{adj} = 0.01$ ) with DESeq2 (Love *et al*,  
274 2014). We calculated the Biological Coefficient of Variation (BCV) on the  
275 overall samples using edgeR (McCarthy *et al*, 2012). For this analysis, only  
276 genes with read counts  $> 1M$  in at least two samples were used. The  
277 clustering of samples was visualized in an MDS plot using the plotMDS  
278 function in edgeR. Genome-wide differential expression profiles were  
279 visualized with heatmaps plotted using heatmap.2 from the R package  
280 ‘*gplots*’. Raw zeta-scores were calculated from the DESeq2 normalized  
281 count using the R package ‘*recommenderlab*’.

282

## 283 **Results**

### 284 *Population response to environmental change – outlier analysis*

285 The outlier analysis identified two “gradient-shared loci” (Pooc-PC047G07  
286 and Pooc-50), four “bathymetric loci” (Pooc-PC047G07, Pooc-50, Poc-45,  
287 and Pooc-PC003H09, Table S5) and three “latitude loci” (Pooc-PC047G07,  
288 Pooc-50 and Pooc-PC045G11, Table S6).

289 Gradient-shared loci showed similarity to genes with known  
290 function. The locus Pooc-50 showed significant similarity to the pEARL11-  
291 like protein 3 (accession Q9SU33.1), a protein associated with membrane  
292 lipid transfer in *Zostera marina* (accession KMZ61949). The locus Pooc-

293 047G07 showed significant similarity to a 50S ribosomal protein L35  
294 (accession PF01632 for structural domain, P23326.1 for gene annotation),  
295 one of the large subunits of the ribosome. The frequency of the major allele  
296 at the outlier-shared loci was the same in both gradients (Table S7).  
297 The outlier PooC-PCo45G11, associated with latitude, showed significant  
298 similarity to the highly conserved eukaryotic translation initiation factor 5A-  
299 1 (accession: P69039.1), which is also a conserved domain (PTZ00328).  
300 The bathymetric locus Poc-45 did not show similarity to a gene with known  
301 function, whereas the bathymetric locus PooC-PC003H09 showed high  
302 similarity to PR-1, a plant protein associated with pathogens defence  
303 mechanisms in plants (accession: P33154.1).

304

305 *Association of neutral and non-neutral genetic variation with the*  
306 *environment*

307 The analysis of molecular variance (AMOVA) was performed on four  
308 categories of loci: 1) strictly neutral (not identified as outliers in any of the  
309 gradients); 2) gradient-shared; 3) latitude loci; and 4) bathymetric loci.  
310 Overall, the molecular variance showed different patterns for neutral and  
311 outlier loci (Table 1). The proportion of molecular variance for all four sets  
312 of loci was highest at within population level (Table 1). The molecular  
313 variance at within group level was significant for neutral loci and  
314 comparable between the depth (22%) and the latitudinal gradients (17% and



315 19% -the two depths are analysed separately, Table 1). For these loci, the  
316 among-groups variance was small yet significant (Table 1). The variance of  
317 loci associated with latitude was highest at within-population level; for this  
318 gradient, 4% of the variance of outlier loci was associated with group  
319 variation (Table 1). All molecular variance in the bathymetric outliers was  
320 explained at within-population level (Table 1).

321         The analysis of correlation between genetic distance, temperature  
322 and geographic distance conducted on the shallow populations (MMRR)  
323 showed that temperature was significantly correlated with neutral genetic  
324 variation, but not with genetic variation at the outlier loci (Table 2). This  
325 analysis also showed that geographic distance did not correlate with genetic  
326 distance among populations neither at neutral nor at outlier loci. Geographic  
327 distance correlated significantly with sea surface temperature (p-value =  
328 0.03).

329

### 330 *Population response to environmental change – evolutionary and plastic* 331 *differences in gene expression*

332 The genome-wide differential expression analysis was conducted on stands  
333 of the Stareso population. This analysis identified a total of 2,059  
334 differentially expressed genes between stands, of which 1,565 were up-  
335 regulated and 494 were down-regulated (Table S8). Variability in gene  
336 expression was high (BVC = 38%) among samples. The MDS plot

337 following this analysis revealed a clear separation between shallow and  
338 deep stands; moreover, it clustered samples from the same depth (Fig. S1).

339 A large proportion of the differentially expressed genes identified by the  
340 DESeq2 analysis belonged to five functional categories: transcription  
341 factors, metabolic genes, cell wall remodeling, and signaling pathways (Fig.  
342 3, Table S8). The remainder of the differentially expressed genes not falling  
343 in these functional categories is listed in Table S8 and includes a number of  
344 uncharacterized proteins. Generally, the two deep samples (taken at the  
345 same location but at two different times: 12 noon and 6.30pm) showed  
346 higher similarity than the two shallow samples in the number of expressed  
347 genes and the direction of change in expression (Fig. 3). Differences in  
348 genome-wide gene expression between shallow and deep stands were  
349 mainly associated with constitutive differential expression of bLHL (basic-  
350 helix-loop-helix), cell wall genes and MYB (Fig. 3 and Table S8).

351 A total of 121 transcription factors (TF) were identified in our  
352 analysis, falling into four main functional domains, WRKY, MYB, bHLH,  
353 and Ethylene-responsive genes. MYB and bHLH were largely upregulated  
354 in the shallow stand, whereas WRKY transcription factors were largely  
355 upregulated in the deep stand (Fig. 3). The transcription factors identified in  
356 our analysis have diverse biological functions ranging from disease  
357 resistance, abiotic and biotic stress response, senescence, development,

358 differentiation, and metabolism (Table S6). In particular, bHLH is a DNA  
359 binding protein involved in flavonoid biosynthesis (Hichri et al., 2011).

360 Phenylpropanoids, flavonoid biosynthesis and lignin biosynthesis  
361 coding genes were highly represented in the differential expression analysis  
362 (Fig. 3, Table S8). In the phenylpropanoid pathway we identified genes  
363 coding for cinnamic acid 4-hydroxylase (C4H) and 4-coumarate-coenzyme-  
364 A-ligase enzyme (4CL) (Table S8). In the flavonoid biosynthesis pathways,  
365 we identified genes coding for chalcone isomerase (CHI) and chalcone  
366 synthase (CHS), dihydroflavonol-4-reductase, flavanone-3-hydroxylase,  
367 leucoanthocyanidin reductase and anthocyanidin reductase. In the lignin  
368 biosynthesis pathway we identified cinnamic-acid-4-hydroxylase, 4-  
369 coumarate-CoA-ligase, Shikimate-O-hydroxycinnamoyltransferase,  
370 caffeoylshikimate esterase, caffeic acid 3-O-methyltransferase and  
371 shikimate O-hydroxycinnamoyltransferase (Table S8).

372 Most of the transcripts involved in secondary metabolism and cell  
373 wall remodeling were upregulated in the shallow population (Fig. 3, Table  
374 S8). Among the genes involved in cell wall remodeling we identified genes  
375 coding for xyloglucan endotransglucosylase (XET), expansins and cellulose  
376 synthase. Finally, genes involved in phosphorylation or de-phosphorylation  
377 were the fourth most abundant category retrieved in our analysis. A large  
378 proportion of phosphatases was upregulated in the shallow population (Fig.  
379 3).

## 380 **Discussion**

381 We investigated natural populations of *P. oceanica* with the aim of  
382 identifying signature of natural selection in response to key environmental  
383 factors varying along latitude and depth, such as light and temperature. Our  
384 hypothesis testing was that loci shared between the bathymetric and  
385 latitudinal gradient were candidates for adaptive responses to temperature, a  
386 factor common to the two gradients. Conversely, loci associated with the  
387 bathymetric gradient were likely associated with adaptation to different light  
388 regimes.

389 The candidate loci identified in the outlier analysis, and shared between the  
390 bathymetric and latitudinal gradient, include a ribosomal protein (Pooc-  
391 047G07, 50S ribosomal protein L35) and a lipid transfer protein 3 (Pooc-50,  
392 pEARLI1-like lipid transfer protein 3), whose functions are potentially  
393 associated with both temperature and photoperiod. L35 is a structural  
394 constituent of the ribosomes, and is among the cellular proteins  
395 differentially regulated under stressful conditions (see review Kosovà *et al*,  
396 2018). In bacteria, L35 is commonly downregulated under thermal stress  
397 (e.g. Nevarez *et al*, 2008). Although mechanisms of stress response differ  
398 among taxa, L35 may be potentially involved in stress response in *P.*  
399 *oceanica*. The protein pEARLI1-like lipid transfer protein 3 is a member of  
400 the PRP (proline-rich protein) family, which has multiple functions in  
401 plants: i) regulation of flowering time and lignin synthesis (Shi *et al*. 2010);

402 ii) protection of the plasma membrane and cell wall against low temperature  
403 (Zhang and Schläppi 2007); iii) and resistance to fungal infection. Relevant  
404 to our findings is that EARLII can be activated by temperature and changes  
405 in photoperiod (Burier and Schläppi 2004).

406 Other candidate loci associated with the bathymetric and latitudinal  
407 gradients include the eukaryotic translation initiation factor 5A-1 (Poc-  
408 PCo45G11) and a plant pathogen protein (Poc-PC003H09). Translation  
409 initiation factors have been shown to play a fundamental role in growth and  
410 development of plants by regulating cell division, cell growth, and cell death  
411 (Feng *et al.*, 2007; Hopkins *et al.*, 2008). Plant pathogenesis-related proteins,  
412 such as the PR-1 identified here, are highly expressed in plants after  
413 infections and act as an anti-fungal agent (Van Loon *et al.*, 2006).  
414 Interestingly, the translation initiation factor 5A-1 is involved in regulation  
415 of cellular processes underlying both plant development and programmed  
416 cell death (Hopkins *et al.*, 2008), and in response to heat stress (Xu *et al.*  
417 2011).

418         The number of loci used in the outlier analysis was small, hence we  
419 had limited power in identifying shared loci between gradients. A genome-  
420 wide polymorphism analysis will likely alleviate the limitations of the  
421 current study. Overall, the candidate genes identified in the current study  
422 regulate central metabolism and cell functions, such as growth and  
423 development, or are associated with pathogens response. Hence, they do not

424 have a direct link to light or temperature. However, since the link between  
425 temperature and metabolism in the context of climate change is well-  
426 recognized (Tewksbury *et al*, 2008), the candidate genes identified here may  
427 be indirectly linked to temperature. For example, cell wall hardening has  
428 been suggested to alleviate heat stress in in *Posidonia oceanica* (Marín-  
429 Guirao *et al*, 2017) as well as in other seagrass species (e.g. *Zostera marina*)  
430 (Franssen *et al*, 2014; Jueterbock *et al*, 2016). Follow up experiments under  
431 controlled laboratory conditions are required to establish a causal  
432 association between the putative candidate genes identified here and  
433 temperature.

434         We observed significant divergence in genome-wide gene  
435 expression between shallow and deep stands of the Stareso population.  
436 Furthermore, we observed a significantly larger divergence between the two  
437 shallow than between the two deep samples. Divergence in genome-wide  
438 gene expression between shallow and deep stands is likely explained by  
439 evolutionary differences in basal gene expression, whereas differences in  
440 gene expression between samples collected at the same depth suggests  
441 plastic responses to changing irradiance and temperature. Irradiance  
442 measured at the Stareso site was markedly different between the two  
443 sampling times. Recent studies support the notion that *P. oceanica* living  
444 along the bathymetric gradient have differential resilience and are locally  
445 adapted to their local environment (Dattolo *et al*, 2017; Marin-Guirao *et al*,

446 2017). Shallow populations of *P. oceanica* show faster and higher induction  
447 of heat-response gene, a potential form of local adaptation to higher  
448 temperature regimes (Marin-Guirao *et al*, 2016).

449         Differentially expressed genes between depths were enriched for  
450 flavonoid biosynthesis and lignin biosynthesis coding genes. These are  
451 metabolism-linked genes responsible for a wide range of biochemical  
452 pathways providing plants with secondary metabolites (Weisshaar and  
453 Jenkins, 1998). Also enriched were genes involved in cell wall loosening.  
454 These latter genes were generally upregulated in the shallow stand, and  
455 downregulated in the deep stand, suggesting that bathymetric pressure  
456 affects the populations of *P. oceanica* studied here. Finally, differentially  
457 regulated genes between depths included lignin coding genes, the second  
458 most abundant component of plant cell-walls, as well as xyloglucan  
459 endotransglucosylase (XET), expansins and cellulose synthase, These genes  
460 are commonly associated with cell wall remodeling.

461         A clear genetic structure was observed both among stands of the  
462 same population and among populations. In addition to clear population  
463 genetic structure, we observed significant population differentiation ( $F_{ST}$ ),  
464 confirming reduced gene flow among populations. The population genetic  
465 structure was not explained by geographic distance as neither neutral nor  
466 non-neutral genetic variation were significantly correlated with geographic  
467 distance (no Isolation by Distance, IBD). These findings were in line with

468 previous studies (Arnaud-Haond *et al.*, 2007; Dattolo *et al.* 2017; Serra *et al.*,  
469 2010). Conversely, neutral genetic variation was significantly correlated  
470 with temperature. The patterns of correlation observed here can be  
471 explained by a scenario of Isolation by colonization (IBC, Orsini *et al.*,  
472 2013), determined by the genetic pattern of early colonization and fuelled  
473 by low establishment success of immigrants. This scenario is supported by  
474 observations of limited gene-flow among meadows (Arnaud-Haond *et al.*,  
475 2007; Jahnke *et al.*, 2017; Serra *et al.*, 2010) and high levels of clonal  
476 reproduction in *P. oceanica* (Arnaud-Haond *et al.*, 2012; Serra *et al.*, 2010).  
477 An IBC scenario agrees with the concept of the “founder takes all” put  
478 forward by Waters *et al.* (2013). The “founders takes all” scenario suggests  
479 that density-dependent processes are likely important in constraining  
480 dispersal in the marine environment and play a key role in determining local  
481 genetic structure, even in highly connected ecosystems that would otherwise  
482 promote panmixia (Waters *et al.*, 2013).

483           Alternatively, the observed population genetic differentiation  
484 observed in our study can be explained by endogenous selection on intrinsic  
485 genetic incompatibilities (Bierne *et al.* 2011). High genetic structure and low  
486 gene flow among populations of *P. oceanica* may be indicative of hybrid  
487 fitness depression or endogenous selection against migrants (Bierne 2002).  
488 Laboratory crosses and fitness analysis across multiple generations is  
489 required to validate this hypothesis.



490 **Conclusions**

491 The candidate genes associated with depth and/or latitude underpin central  
492 metabolic, cell remodelling and immunoregulation functions. Given the  
493 demonstrated link between metabolism and temperature, most of the  
494 assessed genes may be indirectly associated with temperature response.  
495 Moreover, some of the candidate genes associated with both gradients have  
496 been previously linked to temperature and/or light.

497         The analysis of genome-wide differential gene expression clearly  
498 identified divergence in basal expression of five protein functional  
499 categories. This divergence is likely explained by adaptation to the local  
500 environment. Evidence for reduced gene flow among populations and  
501 among stands of the same population at different depths, combined with low  
502 sexual recombination, small effective population size, and long generation  
503 time of *P. oceanica*, pose concerns for the long-term persistence of this  
504 species, especially in the face of rapid environmental change driven by  
505 human activities. These concerns are supported by a recent meta-analysis  
506 suggesting that *P. oceanica* meadows with low genetic and genotypic  
507 diversity are unable to persist in highly impacted areas (Jahnke *et al*,  
508 2015b).

509

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516 **Conflict of Interest:** The authors declare that they have no competing  
517 interests

518

519 **Data Archiving:** Microsatellites genotypes are deposited at dryad entry  
520 XXX. The reference transcriptome of *P. oceanica* is available at NCBI  
521 (Accession number:GEMD01000000). The data generated in this  
522 amanuscript are available at the NCBI Sequence Read Archive (SRA) under  
523 the accession number SRR3289754, SRR3289740, SRR3289755 and  
524 SRR3289704

525 Authors' contributions: MJ performed the population genetic analyses and  
526 wrote the first manuscript draft. DDE and ED performed the genotyping.  
527 DDE also performed RNA extraction for the transcriptomic analysis. LO  
528 (Luigi Orru') and AL performed the DE analysis and the functional gene  
529 annotation. GP and FB conceived the sampling strategy along the latitudinal  
530 gradient. FB coordinated and performed the sampling of two populations.

531 SM and GP conceived the transcriptomic work. GP conceived the study and  
532 coordinated the experimental work. LO (Luisa Orsini) coordinated data  
533 analysis and manuscript writing. All authors contributed to manuscript  
534 editing.

535

536 Supplementary information is available on *Heredity*'s website

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- 765

766 **Table 1 Molecular variance.**

767 Analysis molecular variance (AMOVA) for neutral and outlier loci. The  
 768 variance is partitioned in the following levels: i) among groups, where  
 769 groups are either populations along the depth or the latitudinal gradient; ii)  
 770 within groups, where the groups are either populations at the same depth or  
 771 at the same latitude and; iii) individuals within populations. Significant  
 772 values ( $P < 0.001$ ) are in italics (10,000 permutations).

	<b>Hierarchical level</b>	<b>among groups</b>	<b>within groups</b>	<b>within pops</b>
<b>Neutral loci</b>	depth	0%	<i>22%</i>	<i>78%</i>
	latitude shallow	<i>6%</i>	<i>17%</i>	<i>77%</i>
	latitude deep	<i>9%</i>	<i>19%</i>	<i>72%</i>
<b>Gradient shared loci</b>	depth	0%	0%	100%
	latitude shallow	0%	0%	100%
	latitude deep	0%	0%	100%
<b>Latitude loci</b>	depth	0%	<i>4%</i>	<i>96%</i>
	latitude shallow	0%	<i>4%</i>	<i>96%</i>
	latitude deep	0%	<i>4%</i>	<i>96%</i>
<b>Bathymetric loci</b>	depth	0%	1%	<i>99%</i>
	latitude shallow	0%	0%	<i>100%</i>
	latitude deep	0%	1%	<i>99%</i>

773

774

775 **Table 2 Correlation among genetic, environmental and geographic**  
 776 **variation.**

777 Multiple matrix regression with randomization analysis (MMRR) showing  
 778 correlations between geographic distance (measured between sampling  
 779 locations using the shortest path over the sea without crossing land), genetic  
 780 distance (measured as  $F_{ST}$  at microsatellite loci) and environmental distance  
 781 (measured as the difference between locations of averaged temperature  
 782 between 1981 and 2012). Significant P-values are in italics.

		<b>Intercept</b>	<b>Geographic distance</b>	<b>Temperature</b>
<b><math>F_{ST}</math> neutral</b>	<b>Coefficients</b>	-0.157	0.116	0.541
	<b>t-statistic</b>	-0.570	0.467	1.809
	<b>p-value</b>	0.817	0.669	<i>0.003</i>
<b><math>F_{ST}</math> gradient shared loci</b>	<b>Coefficients</b>	-0.422	-0.020	-0.016
	<b>t-statistic</b>	-17.164	-0.891	-0.597
	<b>p-value</b>	0.427	0.387	0.102
<b><math>F_{ST}</math> latitude loci</b>	<b>Coefficients</b>	0.484	-0.049	-0.360
	<b>t-statistic</b>	1.105	-0.123	-0.758
	<b>p-value</b>	0.157	0.869	0.305
<b><math>F_{ST}</math> bathymetric loci</b>	<b>Coefficients</b>	-0.290	-0.452	0.498
	<b>t-statistic</b>	-0.615	-1.064	0.975
	<b>p-value</b>	0.479	0.280	0.328

783

784

785 **Titles and legends to figures**

786

787 **Figure 1. *Posidonia oceanica* sampling.**

788 *Posidonia oceanica* was sampled from six geographic locations in the  
789 Mediterranean Sea along a latitudinal transect of 1,000 Km at two different  
790 depths (5 m and 20-25 m).

791

792 **Figure 2. Population structure analysis.**

793 Discriminant Analysis of Principal Component (DAPC) displaying the total  
794 set of 12 *Posidonia oceanica* populations, including shallow and deep  
795 stands. The optimal number of principal components found for the analysis  
796 was eight. Populations from the same geographic location sampled at  
797 different depths are labelled with D (deep) and S (shallow). All populations  
798 are uniquely color-coded. N/S identifies the boundary between northern and  
799 southern populations.

800

801 **Figure 3. Gene expression analysis.**

802 Heatmaps of differentially expressed genes (DEseq  $p$ -adj < 0.01) between  
803 the deep and the shallow stands of the Stareso population, grouped in  
804 functional categories. a) bHLH (basic/helix-loop-helix), b) cell wall, c)  
805 kinases, d) MYB, e) phosphatases, f) secondary metabolism, g) WRKY. S1  
806 and S2 - shallow stand sampled at 12 noon and 6.30pm, respectively; D1

807 and D2 - deep stand sampled at 12 noon and 6.30pm, respectively. Raw  
808 zeta-scores were calculated from the DESeq2 normalized counts.  
809