1   **Signs of local adaptation by genetic selection and isolation promoted by extreme**

2   **temperature and salinity in the Mediterranean seagrass *Posidonia oceanica***

3   **Running title:** Genetic selection in an extreme environment

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Abstract

Adaptation to local conditions is known to occur in seagrasses, however, knowledge of the genetic basis underlying this phenomenon remains scarce. Here, we analyzed *Posidonia oceanica* from six sites within and around the Stagnone di Marsala, a semi-enclosed coastal lagoon where salinity and temperature exceed the generally described tolerance thresholds of the species. Sea surface temperatures (SSTs) were measured and plant samples were collected for the assessment of morphology, flowering rate and for screening genome-wide polymorphisms using double digest restriction-site-associated DNA sequencing. Results demonstrated more extreme SSTs and salinity levels inside the lagoon than the outer lagoon regions. Morphological results showed significantly fewer and shorter leaves and reduced rhizome growth of *P. oceanica* from the inner lagoon and past flowering events were recorded only for a meadow farthest away from the lagoon. Using an array of 51,329 SNPs, we revealed a clear genetic structure among the study sites and confirmed the genetic isolation and high clonality of the innermost site. Fourteen outlier loci were identified and annotated with several proteins including those relate to plant stress response, protein transport and regulators of plant-specific developmental events. Especially, five outlier loci showed maximum allele frequency at the innermost site, likely reflecting adaptation to the extreme temperature and salinity regimes, possibly due to the selection of more resistant genotypes and the progressive restriction of gene flow. Overall, this study helps us to disentangle the genetic basis of seagrass adaptation to local environmental conditions and may support future works on assisted evolution in seagrasses.

Keywords: seagrasses, ddRAD, SNPs, local adaptation, ocean warming, hypersaline.

1. Introduction

Populations, if locally adapted, tend to exhibit traits that provide advantages under local environmental conditions (Kawecki & Ebert, 2004). This has been observed in a wide range of species across terrestrial (Jackrel & Wootton, 2014; Lascoux, Glémin, & Savolainen, 2016; van Boheemen, Atwater, & Hodgins, 2019) and marine environments (Barth et al., 2017; Cayuela et al., 2020; van Oppen et al., 2018), including seagrasses (Blok, Olesen, & Krause-Jensen, 2018; Hämmerli & Reusch, 2002; King, McKeown, Smale, & Moore, 2018).

Seagrasses are marine angiosperms distributed in thousands of kilometers of the sedimentary shorelines across the sub-Artic to tropical regions (Short, Carruthers, Dennison, & Waycott, 2007). Seagrass meadows deliver numerous essential ecosystem services such as oxygen production, habitat provision, nutrient recycling, and coastal erosion prevention, among many others (Fourqurean et al., 2012; Lamb et al., 2017; Orth, Luckenbach, Marion, Moore, & Wilcox, 2006) and represent one of the most important natural carbon sinks on Earth (Fourqurean et al., 2012).

In seagrasses, signs of adaptation to local conditions have been documented for a number of species under several abiotic factors [e.g. light (Dattolo et al., 2017), water quality (Maxwell et al., 2014), nutrients (Pazzaglia et al., 2020), salinity (Tomasello et al., 2009), warming (Marín-Guirao et al., 2018), among others] and over a wide range of spatial scales [e.g. between sites of the same region (Maxwell et al., 2014), between regions (Tuya et al., 2019), along with depth gradients (Dattolo et al., 2017), latitudinal gradients (Jahnke et al., 2019; Ruocco, Jahnke, Silva, Procaccini, & Dattolo, 2022), and between seas (Nguyen et al., 2020; Pansini, La Manna, Pinna, Stipcich, & Ceccherelli, 2021; Stipcich et al., 2022)]. It is important to note that conclusions on local adaptation on seagrasses have been derived not only from population genetic data but also from the comparison of phenotypic responses to environmental stressors among populations. In general,

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seagrass populations thriving in fluctuating conditions are more capable to endure stress than those living in more stable environments (Blok et al., 2018; Hämmerli & Reusch, 2002; King et al., 2018; Pazzaglia, Reusch, Terlizzi, Marin Guirao, & Procaccini, 2021). These locally-adapted populations can provide potential materials for assisting the evolution of natural populations and for improving seagrass restoration activities (Bulleri et al., 2018; Nguyen, Ralph, Marín-Guirao, Pernice, & Procaccini, 2021; Pazzaglia et al., 2021; Tuya et al., 2019).

To date, knowledge of the genetic basis underlying local adaptation to environmental conditions in seagrasses remains scarce (but see Hughes and Stachowicz, 2004; Ruggiero et al., 2005; Tuya et al., 2021; Ruocco et al., 2022). Moreover, intraspecific variation among populations is often ignored or under-estimated when assessing specific responses of populations to their surrounding environment, as well as, when predicting potential changes in their future distribution (Hu et al., 2021; Pazzaglia et al., 2021).

The seagrass *Posidonia oceanica* is endemic to the Mediterranean Sea where it forms widespread monospecific meadows on rocks and sandy seabed and provides numerous vital ecosystem services (Campagne, Salles, Boissery, & Deter, 2015; Procaccini et al., 2003; Serra & Mazzuca, 2011). It is known that the tolerance limits of *P. oceanica* range between 33−39‰ for salinity (Sanchez-Lizaso et al., 2008) and 9−29℃ for temperature (Boudouresque & Meinesz, 1982). Stagnone di Marsala is a semi-enclosed coastal lagoon along the western coast of Sicily, Italy (Vizzini, Sarà, Michener, & Mazzola, 2002). This lagoon represents a unique area where *P. oceanica* occurs during summer under temperature and salinity conditions that far exceed the described thresholds of the species' tolerance [i.e. maximum temperature and maximum salinity recorded in some parts of the lagoon were 30℃ and 48‰ (Mazzola & Vizzini, 2005)]. By using 13 microsatellite markers together with lepidochronological analysis, Tomasello *et al.*, (2009)

86   showed that *P. oceanica* atolls in the innermost area of the lagoon exhibited lower shoot-growth

87   and were genetically isolated from the meadows outside the lagoon. This suggests a possible

88   selection of genotypes that adapted to the persistent stressful conditions inside the lagoon.

89   In an era of rapid environmental changes, the *P. oceanica* population of the Stagnone di Marsala

90   lagoon represents a natural experimental model system for investigating seagrass response to

91   future environmental conditions. Combining prior knowledge from Tomasello *et al.* (2009) and

92   the application of *state-of-the-art* approaches in genetic research represents a unique opportunity

93   to better understand the genetic basis of adaptation to extreme conditions in seagrasses. To this

94   aim, samples of *P. oceanica* were collected from two sites inside the lagoon and four sites outside

95   the lagoon [those relatively corresponded with sampling localities in Tomasello *et al.*, (2009)].

96   Measurements included sea surface temperature, plant morphology, past growth rate, past

97   flowering events, and screening of genome-wide polymorphisms using double digest restriction-

98   site associated DNA (ddRAD) for SNPs identification and detection of outlier loci (Peterson,

99   Weber, Kay, Fisher, & Hoekstra, 2012). SNP markers could provide many advantages over

100   microsatellites (as applied in Tomasello *et al.*, 2009), as they are denser and have more uniform

101   distribution within genomes making them more useful for population and mapping studies

102   (Balloux, Brunner, Lugon-Moulin, Hausser, & Goudet, 2000; Xing et al., 2005) and most

103   importantly, they allow for the detection of potential adaptive DNA polymorphisms at specific

104   functional loci that are candidates for genetic adaptation to local environmental conditions (Hung

105   et al., 2012; Lasky et al., 2015; van Oppen et al., 2018). This kind of approach (i.e. RAD

106   sequencing) has been widely applied to study evolutionary mechanisms of different marine

107   organisms (Gaither et al., 2015; Hohenlohe et al., 2010; Jahnke, Moknes, Le Moan, Martens, &

108   Jonsson, 2022; van Oppen et al., 2018) including some recent studies on seagrasses (Phair, Toonen,

109 Knapp, & von der Heyden, 2020, 2019; Ruocco et al., 2022). We hypothesize that (*i*) the high

110 levels of salinity and temperature in the interior of the lagoon have selected the most resistant

111 genotypes favouring the local adaptation of the *P. oceanica* population to these extreme conditions,

112 (*ii*) these genotypes manage to survive under conditions that exceed the thresholds of the species

113 through genetic mutations in certain functional loci and/or their high phenotypic plasticity. We

114 expected that (*1*) *P. oceanica* plants from sites inside the lagoon would show a lower level of

115 genetic variation than those from sites outside the lagoon and (*2*) these plants would differ

116 morphologically and genetically from those outside the lagoon. Morphological and genetic

117 differences would also exist between the two inside-lagoon sites.

118 **2. Materials and methods**

119 **2.1. Study area**

120 The Stagnone di Marsala lagoon is a shallow area with an average depth of 1.5 m and a surface

121 area of about 2000 ha (Vizzini et al., 2002). This basin exhibits distinct lagoon features, such as

122 limited water exchange and slow turnover and has the highest annual variation in temperature and

123 salinity among sites where the presence of *P. oceanica* has been reported. The lagoon can be

124 subdivided into a northern and a southern basin with different geomorphological and

125 environmental characteristics. The northern basin has an average depth of 1.1 m and it is connected

126 with the open sea through a channel 400 m wide and 20−30 cm deep northwards. The annual water

127 temperature in the northern basin ranges from minima 10.0−11.8 °C in January to maxima 29.1 –

128 30.0 °C in August, while salinity ranges from 32.8−48.0‰, (Sarà, Leonardi, & Mazzola, 1999;

129 Mazzola & Vizzini, 2005; Vizzini et al., 2002). A salinity level of 51‰ has recently been recorded

130 in the northern basin of the lagoon (Spinelli, 2018) indicating an increase in salinity level in this

131 area.

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132 Over-sedimentation and lack of maintenance over recent years caused the partial closure of the

133 northern channel resulting in even more extreme environmental conditions in the inner lagoon

134 (Calvo S., Tomasello A., *personal observation*). In this part of the basin, *P. oceanica* forms atoll-

135 like structures (Calvo & Frada-Orestano, 1984), a rare feature of *P. oceanica* meadows observed

136 in few other localities along the Tunisian, Turkish and Corsican coasts [see Tomasello *et al.*, (2020)

137 for related references]. In addition, the atoll structure of the Stagnone area is in strong regression

138 with a marked decrease in the plant's primary production recorded about 30 years ago (Calvo,

139 Ciraolo, & Loggia, 2003; Pergent et al., 2014). The southern basin is slightly deeper (about 2 m of

140 depth) and it is connected with the surrounding open sea through a 3000 m wide opening, in which

141 a vast *P. oceanica* reef platform (*Plateau Récifale*) is present (Tomasello et al., 2009).. Lastly, the

142 surrounding open sea is environmentally more stable with a year-round temperature ranging from

143 a minimum of 14.1℃ during winter to a maximum of 26.4℃ during summertime and a stable

144 salinity level of 37‰ (Vizzini et al., 2002. Here, *P. oceanica* forms a very large meadow (Calvo

145 et al., 2010) from the surface to about 30 m depth (Bellissimo, Sirchia, & Ruvolo, 2020),

146 characterized by the most extensive living reef, to our knowledge, along the Mediterranean coasts

147 (about 40 km long, Calvo S, Tomasello A, *personal observation*).

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2.2. Sample collection

150 On the 7th of September 2020, *P. oceanica* shoots with integer orthotropic rhizome (i.e. they were

151 harvested until to the insertion point with their plagiotropic rhizomes) were haphazardly collected

152 at about 1 m of depth from atolls or reefs present in six different sites (i.e. 20−30 shoots from each

153 site). To maximize the number of genotypes collected, samples were harvested at a minimum

154 distance of 5 m from each other. Sampling stations included (*i*) two sites inside the Stagnone di

155 Marsala lagoon [***North-basin*** (close to the atolls site in Tomasello *et al.,* 2009), in the northern

156 basin of the lagoon: samples were collected from 5 different atolls with an average of 4−6 samples

157 per atoll (*atoll 1*: 37°52'54"N, 12°28'29"E; *atoll 2*: 37°52'49"N, 12°28'22"E; *atoll 3*: 37°52'54"N,

158 12°28'21"E; *atoll 4*: 37°52'55"N, 12°28'21"E; and *atoll 5*: 37°52'56"N, 12°28'19"E) & ***South-basin***

159 (corresponds with Récif site in Tomasello *et al.,* 2009), in the southern basin of the lagoon

160 (37°50'35"N, 12°27'29"E)] and (***ii***) four sites outside the lagoon [***OpenSea-A*** (corresponds with

161 Plateau site in Tomasello *et al.,* 2009: 37°50'26"N, 12°26'45"E), ***OpenSea-B*** (37°48'48"N,

162 12°25'53"E), ***OpenSea-C*** (37°51'27"N, 12°26'35"E), and ***OpenSea-D*** (37°53'18"N, 12°25'42"E)]

163 (**Fig. 1**). Soon after collection, 96 leaf sub-samples (∼10 cm; 16 samples per site) were selected

164 for DNA extraction. Samples were gently cleaned out of epiphytes before being dried and stored

165 with silica gel until further analysis. The rest of the collected material was kept in a cooler container

166 filled with seawater and transported shortly to the laboratory for morphological measurements.

167 **2.3. Sea surface temperature**

168 Sea surface temperature (SST) data were obtained through image analysis based on satellite remote

169 sensing data from the Sea and Land Surface Temperature Radiometer sensors installed on the

170 Sentinel-3 mission satellites with a spatial resolution of 250 m (https://apps.sentinel-hub.com/).

171 Data were collected from May to September for the years 2017 to 2020. Then, the data from the

172 year 2017 was chosen because it contained the highest number of images. Selected images were

173 analyzed using QGIS software (http://qgis.osgeo.org/) to obtain average and maximum

174 temperatures during the May-September period for each study site.

175 **2.4. Morphological and growth performance measurements**

176 Two sets of biometric measures were taken including leaf biometry and dating (Pergent-Martini

177 et al., 2005). Leaf biometry and morphological measurements were carried out on the leaf bundle

178 as described in previous studies (Girard, 1977; Giraud, 1979). Measurements included leaf number

179 per shoot, leaf length (cm) and shoot surface (cm^2). Dating was carried out on rhizomes by

180 lepidochronology (Pergent, 1990), which provides a reliable estimation of their growth

181 performance. This method is based on the analysis of the cyclic variations of the sheaths thickness

182 along the rhizomes. In particular, starting from the basal portion towards the apex of the rhizome,

183 the sheaths were detached from the nodes with the aid of a scalpel and arranged on a laboratory

184 table in the sequence corresponding to their order of insertion. At the same time, their thickness

185 was preliminarily assessed by touch by means of a slight bending in order to identify the sheath

186 where the inversion of the thickness trend (from decreasing to increasing) occurred, corresponding

187 to the possible finding of the relative minimum. Subsequently, a thin section was made on both

188 the suspected sheath minimum and previous and following ones at about $10-12$ mm from the base

189 for confirmation or rectification by using micrometric binoculars. At this point, the rhizome was

190 dissected transversally at the nodes corresponding to the finding of sheaths with the minimum

191 relative thickness. In this way for each rhizome, the cyclic variation of the sheath thickness was

192 detected to isolate rhizome segments corresponding to a one-year period, determined between each

193 pair of sheaths of minimum relative thickness ('lepidochronological year' according to Pergent,

194 1990). Consequently, it was also possible to date rhizome segments corresponding to a

195 lepidochronological year. Each lepidochronological year was dated starting from the rhizome apex

196 (sampling year) downward and backdating the sequence of cycles with their corresponding

197 rhizome segment. This reiterative procedure was performed until the rhizome segment connected

198 to the horizontal axis is reached, representing the year of shoot birth. For each annual segment the

199 elongation and the number of sheaths were determined to estimate the speed of growth and number

200 of leaves produced. Moreover for each shoot the total rhizome length, corresponding to cumulative

201 speed of growth and shoot age by counting the distance in year from the year of birth were

202 calculated as previously done elsewhere (Calvo et al., 2021; Pergent & Pergent-Martini, 1990;

203 Tomasello et al., 2016). This method also made it possible to detect past flowering occurrences by

204 finding floral stalk remains between the sheaths (Pergent, Boudouresque, Crouzet, & Meinesz,

205 1989).

2.5. Statistical analysis

207 Prior to analysis, homogeneity of variance of the response variables was tested by Levene's test

208 and Shapiro–Wilk test was used to validate data normality. As a result, data from shoot

209 morphological measurements were normally distributed, however, with prevalent unequal

210 variances. Therefore, Tamhane's T2 test [that is an all-pairs pairwise-t-test suitable for unequal

211 variances (Tamhane, 1979)] was used to check for significant differences among sampling sites

212 for shoot morphological measurements. Average speed of growth of rhizomes was plotted across

213 the lepidochronological years for visualization of the entire time series obtained in each site (Calvo

214 et al., 2006). While rhizome length was processed by using reference growth charts classification

215 step-by-step procedure reported in Tomasello et al., 2016, to bypass the known confounding effect

216 of age on rhizome growth (Tomasello et al., 2007; Vizzini et al., 2010; Tomasello et al., 2016). In

217 this case, most recent annual rhizome segments corresponding to the last 3 lepidochronological

218 years were excluded from the statistical analysis, because their growth was incomplete at the time

219 of sampling (see Tomasello et al., 2016 for further details). Data were analysed using the statistical

220 package IBM SPSS Statistics (v. 15).

221 The influence of geographic distance (Euclidean distance in kilometres) on genetic distance

222 (measured as pairwise F_{ST}) was investigated using Mantel test based on Pearson's product-moment

223 correlation with 1000 permutations. The Mantel test was done in R-studio v.1.2.5033 (R Core

224 Team, 2018) using the package *vegan* (Oksanen et al., 2013).

225 **2.6. DNA extraction, ddRAD-seq library preparation and sequencing**

226 Total genomic DNA (gDNA) was isolated from about 30 mg of dried tissue using NucleoSpin®

227 Plant II kit (Macherey-Nagel) by following the manufacturer's instructions. Total gDNA integrity

228 was checked through 1% agarose gel electrophoresis and total gDNA purity was determined

229 spectrophotometrically by examining 260/230 and 260/280 nm absorbance ratios using a

230 NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific). Finally, DNA concentration

231 was accurately measured by the Qubit dsDNA BR assay kit with the Qubit 2.0 Fluorometer

232 (Thermo Fisher Scientific).

233 Ninety-five ddRAD-seq library construction and sequencing were conducted at IGATech (Udine,

234 Italy) using an IGATech custom protocol, with minor modifications with respect to Peterson's

235 double digest restriction-site associated DNA preparation (Peterson et al., 2012). To ensure the

236 quality of sequencing outcomes, for each site, one sample was randomly selected and sequenced

237 twice. The final number of biological replicates for each site was $n = 14$ for OpenSea-C and $n =$

238 15 for the other sites (i.e. North-basin, South-basin, OpenSea-A, OpenSea-B, and OpenSea-D),

239 respectively (i.e. 89 unique samples + 6 technical replicates). In short, gDNA was double digested

240 with both *Sph*I and *Mbo*I endonucleases (New England BioLabs). Fragmented DNA was purified

241 with AMPureXP beads (Agencourt) and subsequently ligated with T4 DNA ligase (New England

242 BioLabs). Samples were pooled on multiplexing batches and bead purified as before and then they

243 were size-selected and underwent several purification steps. ddRAD-seq libraries were sequenced

244 with 150 cycles in paired-end mode on NovaSeq 6000 instrument following the manufacturer's

245 instructions (Illumina, San Diego, CA).

2.7. Single nucleotide polymorphisms (SNPs) calling

247 Single nucleotide polymorphisms (SNPs) calling was performed *de novo* using Stacks software

248 package v2.53 (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013). First, raw Illumina

249 reads were demultiplexed using the *process_radtags* utility (Catchen et al., 2013). The short reads

250 of each sample were assembled into exactly matching stacks using the *ustacks* utility (Catchen et

251 al., 2013). The creation of the loci catalog (i.e. a set of consensus loci from all the analyzed

252 samples) was done using *cstacks* and matching each sample against the catalog using *sstacks* and

253 *tsv2bam* utilities (Catchen et al., 2013). *gstacks* ultility (Catchen et al., 2013) was used to pull in

254 paired-end reads, assemble the paired-end contigs and merge them with the single-end locus, align

255 reads to the locus and ultimately call SNPs. Finally, detected loci were filtered using the

256 *populations* program included in Stacks v2.53 (Catchen et al., 2013), with option –R=0.75 to retain

257 only loci that were represented in at least the 75% of the whole metapopulation and with cutoff --

258 *max-obs-het*=0.8, to process a nucleotide site at a locus with observed heterozygosity at a

259 maximum of 80%.

2.8. Genetic variation analysis and clonality assessment

261 Individual genetic variation and population differentiation was assessed by a Principal Component

262 Analysis (PCA) using the R package *SNPRelate* (Zheng et al., 2012) and by an ADMIXTURE

263 analysis using the software ADMIXTURE 1.3.0 (Alexander & Lange, 2011). To choose the best

264 estimate of the number of clusters (K), the ADMIXTURE cross-validation procedure was used

265   with default settings. The hypothetical number of K was set from 1 to 15 then the K value with the

266   lowest cross-validation error was chosen to use for ADMIXTURE analysis.

267   Clonality assessment, including genetic distance among all samples and number of distinct

268   multilocus lineages (MLLs) for each site, was done using the R package *poppr* (Kamvar, Brooks,

269   & Grünwald, 2015). The genetic distance limit for setting delimitation of clones was determined

270   based on the maximum genetic distance detected between technical replicates as done in a recent

271   study (Ruocco et al., 2022). Based on results from the clonality assessment, clones as well as

272   technical replicates (i.e. samples sequenced twice) were removed from the dataset before all

273   subsequent analyses including outlier detection (section 2.9). Pair-wise Weir and Cockerham F_{ST}

274   estimates between sampling sites were calculated with vcftools (Danecek et al., 2011). Observed

275   (Ho) and expected (He) heterozygosity, as well as F_{IS} values across all loci for each sampling site

276   were calculated by using the R package *hierfstat* (Goudet, 2005).

277   **2.9. Outlier SNPs identification and functional annotation**

278   Three genome scan methods were used to identify outlier SNPs across the whole dataset. The first

279   method was based on F_{ST} values and implemented in the program *BAYESCAN* v.2.1 (Foll, 2012;

280   Foll & Gaggiotti, 2008). It was used with prior odds set to 100 and using a threshold of $q \leq 0.3$ and

281   posterior probability P>0.5. The second method was also based on F_{ST} values and implemented in

282   the R package *OutFLANK* (Whitlock & Lotterhos, 2015). *OutFLANK* analysis was performed

283   using default settings and SNPs with a *p*-value less than 0.01 were considered as 'suggestive'

284   outliers [as done in a previous study (Andrew, Jensen, Hagen, Lundregan, & Griffith, 2018)]. The

285   last method based on multivariate analysis and implemented in the R package *pcadapt* (Luu, Bazin,

286   & Blum, 2017) was used with default settings [that computed a test statistic based on Mahalanobis

287   distance which is a multi-dimensional approach that measures how distant a point from the mean

288   (Luu et al., 2017)]. To define the correct number of principle components (PCs) to use in *pcadapt*

289   analysis, we started with $K = 20$ PCs then $K = 3$ was selected as the most appropriate value for the

290   analysis based on an inspection of a scree plot (Luu et al., 2017). In the last step, any SNP with a

291   *p*-value less than 0.01 with Bonferroni correction for multiple comparisons was considered as an

292   outlier SNP.

293   To reduce the likelihood of detecting false positives, a Venn diagram

294   (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to identify shared and unique

295   outliers detected from the different methods. Only SNPs that were identified as outliers by at least

296   two methods were considered 'true' outliers. Other SNPs (either detected as outliers by only one

297   of the three methods or not detected as outliers by neither of the methods) were classified as

298   neutral. Subsequently, allele frequencies of the 'true' outliers among sites were computed using

299   the R package *genepop* (Rousset, 2008).

300   To determine whether an outlier SNP may be included in potential coding sequences, chromosome

301   regions of the 'true' outlier SNPs were mapped against a previously published *P. oceanica*

302   transcriptome (Ruocco et al., 2020) by using the BLASTn algorithm (Camacho et al., 2009).

303   Positive hits were identified if a homologous sequence was present around the SNP position with

304   a high scoring stretches of sequence similarity of at least 70 bp with a percentage of identity greater

305   than 85% (only the best hit was selected for each alignment). Subsequently, a sequence similarity

306   search was carried out between *P. oceanica* contigs (i.e. corresponding to the positive hits) against

307   UniProt protein database (downloaded in February 2022) using the BLASTx software (Camacho

308   et al., 2009) to identify potential protein functions corresponding to outlier SNPs (only the best

309   hits was selected for each alignment).

310

3. Results

3.1. Environmental data

Seawater temperature inside the lagoon was higher in comparison with the outside lagoon area (**Fig. 1**). In particular, average SST of North-basin and South-basin were 8.1℃ and 3.7℃ higher, respectively, than the average SST of open-sea sites (**Fig. 1**). Maximum SST of North-basin was 31.1℃ and South-basin was 28.7℃, while the maximum SST of the outside lagoon sites varied from 23.9 to 26.1℃. In addition, while temperature variation among the four outside lagoon sites was less than 2℃ (e.g., the average SSTs varied from 20.7 to 22.3℃ and the maximum SSTs varied from 23.9 to 26.1℃; **Fig. 1**), both average SST and maximum SST of North-basin were 4.5℃ higher than those of South-basin (**Fig. 1**).

3.2. Morphological and growth performance

There were significant differences among the study sites for all morphological measurements (Tamhane's T2 test, $p < 0.05$; **Fig. 2, Supplementary Table S1−3**), being plants from North-basin different from plants from the rest of the study sites. In detail, plants from North-basin had on average three leaves per shoot, being significantly lower than the average number of leaves of plants from the other sites (i.e. ~ 5 leaves per shoot; **Fig. 2**). Similarly, plants from North-basin had shorter leaves when compared with plants from the other sampling sites (Tamhane's T2 test, $p < 0.05$; **Fig. 2, Supplementary Table S2**). Consequently, shoot surface area at North-basin was also significantly lower than the surface area of plants from all other sites (**Fig. 2**). In particular, the shoot surface of plants from North-basin was 51% lower than the surface of plants from South-basin and 58−67% than plants from the outside-lagoon sites (**Fig. 2**). Dating measures allowed to reconstruct production of leaf number and growth performance within temporal ranges from 2006

15

to 2019 (**Supplementary Table S4**, **Fig. S1**). Shoot age varied between 1 and 12 years, with an overall average 3.5 ± 0.2 years (**Supplementary Table S4**, **Fig. S2**). The mean values per site of the reconstructed trends of speed of growth of the rhizomes and number of leaves produced ranged from 6.7 ± 0.4 to 11.5 ± 0.9 mm/shoot/year and 7.1 ± 0.1 to 7.5 ± 0.1 mm/shoot/year, respectively **Supplementary Fig. S1, Supplementary Table S4**,). Rhizome length displayed average values from 21.7 ± 3.0 and 39.5 ± 10.3 mm (**Supplementary Table S4**). Past flowering was detected only in stations 5 and 6, outside the lagoon. According to reference growth charts applied to rhizome length, different classes of growth were observed, with the value of station 1 (North-basin) falling in the lowest percentile range (**Fig. 3**).

In addition, it is worth noting that even no significant differences were detected (only except for two cases including (i) leaf number per shoot between South-basin vs. OpenSea-D and (ii) shoot surface between South-basin vs. OpenSea-C, **Fig. 2A,C**, **Supplementary Table S1,3**), it is clear that the plants from South-basin exhibited a reduction in their morphology in comparison with the plants from the outside lagoon with lower number of leaves per shoot, shorter leaf length and smaller shoot surface (**Fig. 2**).

3.3. Accuracy of genotyping, genetic diversity and differentiation

The sequencing of ddRAD libraries produced a total of 442,837,278 reads (i.e. ~4.7 million reads per sample, **Supplementary Table S5**). Subsequently, a total of 51,329 SNPs were identified across 95 *P. oceanica* samples. Genotyping correspondence between technical replicates was 96.6% on average and they clustered close to each other in the genetic distance tree obtained with *poppr* (**Supplementary Fig. S6**).

354 PCA results showed a strong genetic differentiation of *P. oceanica* between (*i*) the two inside-

355 lagoon sites (North-basin & South-basin; **Fig. 4A**) versus the four outside-lagoon sites (OpenSea-

356 A−D; **Fig. 4A**) and (*ii*) between those from inside lagoon (North-basin versus South-basin). In

357 detail, samples from North-basin separated from all samples of the other sites along the PC1

358 explaining 11.1% of the total variance of the data set (**Fig. 4A**). Interestingly, samples of South-

359 basin were divided into two distinct groups, one group differentiated from all other samples along

360 the PC2 (that accounts for 9% of the total variance) while the other group clustered with samples

361 from OpenSea-B − D (**Fig. 4A**).

362 Genetic partitioning among sites was further confirmed by results from ADMIXTURE analysis

363 (**Fig. 4B**). First, K=9 was identified as an 'optimal K' (i.e. number of genetic clusters) as it had the

364 lowest cross-validation error of 0.177 among other K values (**Supplementary Table S6**). Then,

365 with the assumption of nine genetic clusters, the clustering analysis implemented in ADMIXTURE

366 showed clear divergences in genetic structures among sites (**Fig. 4B**). No substructure was detected

367 at North-basin as this site was dominated by a single homogeneous genetic component (**Fig. 4B**).

368 This structural component was also present, however in a small proportion, in all other sites (**Fig.**

369 **4B**). On the other hand, all the other sites were characterized by diversified substructures (e.g. 8−9

370 components). It is important to note that the dominant substructure differed among all sites (**Fig.**

371 **4B**).

372 The North-basin atolls were characterized by extremely low clonal richness ($R = 0.143$), as the 15

373 investigated individuals represented only 3 MLLs, while the number in other sites ranged from

374 8−10 MLLs, with an average R value of 0.6 (**Table 1** and **Supplementary Fig. S7**). In the South-

375 basin, also located inside the lagoon, the number of MLLs (i.e., 10) was equal to or even higher

376 than that of the outside-lagoon sites (**Table 1**). Among the 6 sites, all MLLs found in North-basin,

377 South-basin and OpenSea-A were unique for each site, while among OpenSea-B−D we found

378 some shared MLLs (**Supplementary Fig. S7**). Observed (Ho) and expected (He) heterozygosity

379 ranged from 0.20 to 0.22, and from 0.11 to 0.21, respectively (**Table 1**). Expected heterozygosity

380 (He) was lower than observed heterozygosity (Ho) (excess of heterozygotes) at all study sites,

381 particularly in North-basin atolls (**Table 1**). The inbreeding coefficient (F_{IS}) was negative at all

382 sites and North-basin exhibited the lowest value (-0.889) among all (**Table 1**).

383 Global pairwise F_{ST} distances (i.e., genetic differentiation based on all SNPs after clone removals)

384 between North-basin versus other sites were roughly double of any other distances (**Table 2**),

385 suggesting a limited gene flow not only between North-basin and the outside-lagoon sites but also

386 between North-basin and South-basin ($F_{ST} = 0.227$). Among the four outside-lagoon sites,

387 OpenSea-B presents the highest F_{ST} values in all pairwise comparison between populations (**Table**

388 **2**) suggesting a limited gene flow toward the southernmost side of the whole sampling area. The

389 highest pairwise F_{ST} value was detected between OpenSea-B and North-Basin (0.34). High levels

390 of gene flow were generally observed between northern OpenSea sites (A, C and D).

391 Moreover, a Mantel test showed no significant correlation between genetic distance (measured as

392 pairwise F_{ST}) and geographic distance (measured as pairwise Euclidean distance in kilometres)

393 where r = 0.515 and *p* = 0.103.

394 **3.4. Identification and annotation of outlier SNPs**

395 For the identification of outlier SNPs, only the ones shared by at least two of the three genome-

396 scanning algorithms (*Bayescan*, *OutFLANK* and *pcadapt*) were considered. As a result, a total of

397 fourteen 'true' outlier SNPs were identified (**Fig. 4C, Supplementary Table S7**). Flanking regions

398 of all fourteen outlier SNPs showed a reliable match with *P. oceanica* transcript sequences

399 (**Supplementary Table S8**) and could be annotated with eleven different proteins by considering

400 the best hit of each SNP (**Table 3**). Among those annotated proteins, six of them are potentially

401 related to plant stress responses whilst the others are associated with several functions such as

402 purine nucleobase transmembrane transporter activity, protein transport, among others (**Table 3**).

403 Interestingly, fixed (max. allele frequency) alternative alleles were found only in North-basin and

404 OpenSea-B (**Fig. 4D6**). Especially, four SNPs with fixed alternative alleles were found exclusively

405 in North-basin including three SNPs with functions related to plant stress response (i.e. *SNP>4564*

406 *NS=81_pos98*, *SNP>145013 NS=85_pos198* and *SNP>107233 NS=81_pos235*) and one SNP

407 related to Purine nucleobase transmembrane transporter activity (i.e. *>34231 NS=78_pos44*) (**Fig.**

408 **4D**, **Table 3**). In case of OpenSea-B, among the five fixed alleles detected, there was one SNP (i.e.

409 *>126268 NS=74_pos268*) with annotated function related to plant stress response (i.e. *cell wall*

410 *modification*) (**Fig. 4D**, **Table 3**).

411 **4. Discussion**

412 The Stagnone di Marsala is a semi-enclosed coastal lagoon, strongly isolated from the surrounding

413 open sea with a clear cline in environmental conditions especially in summer months, between the

414 northern (i.e. more confined side of the lagoon) and the southern part (i.e. more open to exchanges

415 with the open sea) (Tomasello et al., 2009; Vizzini et al., 2002). This is due to the limited water

416 exchange within the lagoon and across the major mouth (open southward to the open sea) together

417 with the existence of very shallow waters throughout the whole water body (La Loggia et al.,

418 2004). In this study, we observed a maximum summer SST of 33.1℃ that far exceeded the value

419 reported in a previous study (i.e. 30℃) (Tomasello et al., 2009). The occurrence of such extreme

420 high values observed in the northern basin may be the result of three possible, non-exclusive,

421 factors including (*i*) the gradual warming of the Mediterranean Sea(Pastor, Valiente, & Khodayar,

422 2020; Vargas-Yáñez et al., 2008), (*ii*) the increased frequency and intensity of marine heatwaves

423 in the Mediterranean Sea (Darmaraki et al., 2019) and (*iii*) the gradual closure of the 400-m wide

424 channel in the north side of the lagoon, which further contributes to limit water exchange (Calvo

425 A., Tomasello A., *personal observation*). Likewise, a salinity level of 51‰ has been recently

426 documented in the northern basin of the lagoon (Spinelli, 2018), where a maximum value of 48‰

427 was previously recorded (Mazzola & Vizzini, 2005; Tomasello et al., 2009). This pushes up the

428 acknowledged salinity and temperature tolerance limits for *P. oceanica* (Nguyen, Bulleri, Marín-

429 Guirao, Pernice, & Procaccini, 2021; Sandoval-Gil, Ruiz, & Marín-Guirao, 2023).

430 Observations carried out over two decades (from November 2000 to September 2020) reported

431 undersized *P. oceanica* shoots growing in the northern basin of the Stagnone of Marsala lagoon

432 (Loggia et al., 2004; Tomasello et al., 2009; Spinelli, 2018; the present study). This can be

433 considered a sign of long-term exposure of *P. oceanica* to the extreme conditions in the area [both

434 extreme temperature and extreme salinity (Fernández-Torquemada & Sánchez-Lizaso, 2005;

435 Marín-Guirao, Sandoval-Gil, Bernardeau-Esteller, Ruíz, & Sánchez-Lizaso, 2013; Ruíz, Marín-

436 Guirao, & Sandoval-Gil, 2009)]. A similar shoot size reduction has been described in another *P.*

437 *oceanica* population living under salinity levels above the normal tolerance threshold of the species

438 (Marín-Guirao, Sandoval-Gil, García-Muñoz, & Ruiz, 2017). Marín-Guirao et al., (2017)

439 proposed that this morphological modification may serve as a stress-coping mechanism, as

440 previously described in terrestrial plants (Lichtenthaler, 1996). Similarly, reduced sized *P.*

441 *oceanica* shoots have also been documented in natural vents under strong seawater acidification

442 (Gambi, Esposito, & Marín-Guirao, 2023). In addition, lepidochronological results also

443 demonstrated that plants from the northern basin exhibited the slowest growth performance in

444 comparison with other sites. This further confirms the constraints imposed by extreme

20

445　environmental conditions to which *P. oceanica* plants are undergoing in this section of the basin.

446　Furthermore, our study continues to report a lack of flowering events inside the lagoon in the last

447　few decades (1984-2004, Tomasello et al., 2009; 2007-2019, present study). Flowering in

448　seagrasses has been considered an adaptive mechanism (i.e. escape through sexual reproduction)

449　to cope with unfavourable conditions (Nguyen, Ralph, et al., 2021). Previous studies have found a

450　positive relationship between flowering events and extreme thermal stress (Blok et al., 2018; Diaz-

451　Almela, Marbà, & Duarte, 2007; Marín-Guirao, Entrambasaguas, Ruiz, & Procaccini, 2019; Ruiz

452　et al., 2018). Hence, we hypothesize two possible scenarios: the extreme condition in the Stagnone

453　di Marsala lagoon (*i*) could exceed the threshold limit for flowering induction in *P. oceanica* or

454　(*ii*) could have selected 'less-flowering' genotypes.

455　Our study demonstrates a clear genetic isolation of *P. oceanica* from inside versus outside the

456　lagoon, especially for the individuals of the northern basin. This is in line with several previous

457　studies showing that seagrass populations from confined environments (such as coastal lagoons)

458　tend to exhibit some levels of genetic isolation [e.g. *Zostera marina* populations in San Quintin

459　Bay, Mexico (Muñiz-Salazar, Talbot, Sage, Ward, & Cabello-Pasini, 2006); *P. oceanica* in the

460　Marmara Sea (Meinesz et al., 2009) and the Stagnone di Marsala (Tomasello et al., 2009);

461　*Halophila beccarii* populations in Cau Hai lagoon, Vietnam (Phan, De Raeymaeker, Luong, &

462　Triest, 2017) or recently *Halophila ovalis* populations in Dongsha Island, Taiwan (Liu & Hsu,

463　2021)]. Additionally, we observed a reduction in the number of distinct genotypes detected

464　(especially for the northern basin) when compared with Tomasello *et al.*, (2009). While the

465　dissimilarity in the power of discriminating clones between the two used approaches

466　(microsatellites versus ddRADseq) could have certainly contributed to this difference (Balloux et

467　al., 2000; Xing et al., 2005), we cannot exclude that the continuous deterioration of the

468 environmental conditions (increased water temperature and salinity) inside that lagoon had

469 caused the disappearance of some genotypes that were previously identified (Tomasello et al.,

470 2009). It is interesting to note that while the majority of seagrass studies have shown a positive

471 relationship between genetic diversity and the ability to endure environmental stressors of

472 seagrass populations (Ehlers, Worm, & Reusch, 2008; Jahnke, Olsen, & Procaccini, 2015;

473 Massa, Paulino, Serrão, Duarte, & Arnaud-Haond, 2013; Randall Hughes & Stachowicz, 2011),

474 there are also several studies providing evidences to support the opposite (Arnaud-Haond,

475 Marbà, Diaz-Almela, Serrão, & Duarte, 2010; Connolly et al., 2018; Diaz-Almela, Arnaud-

476 Haond, et al., 2007). Our results showed no significant correlation between genetic distance and

477 geographic distance (as verified by Mantel test) thus eliminating the potential effect of isolation

478 by distance for the genetic isolation of *P. oceanica* populations inside the lagoon. Instead, the

479 isolation is likely related to the existence of geographic barriers and/or the strong environmental

480 filter exerted by the extreme conditions of the lagoon on possible propagules coming from the

481 frequently blooming open sea populations (Tomasello et al., 2009 and this study). Moreover, the

482 history of *P. oceanica* distribution in the area (the present distribution is most likely the remnant

483 of a wider distribution present when hydrodynamic conditions inside the lagoon favored greater

484 water exchange with the open sea) can exclude the possibility of bottleneck (and/or founder

485 effect) happening in this area. As a result, genetic drift is also unlikely to be the cause of the

486 genetic differentiation in the inside-lagoon populations. This is further supported by the fact that

487 the genetic diversity of the North-basin population, in the face of observed heterozygosity (Ho),

488 was actually comparable to most of other sites or even higher than some other sites (e.g.,

489 OpenSea-B) and this was already observed by Tomasello et al., (2009) with microsatellite

490 markers. Together, the genetic isolation of the inner-lagoon individuals is, more likely, the result

491   of (1) the progressive extremization of the conditions inside the lagoon and a subsequent

492   selection ("environmental filtering") of the more resistant genotypes, as well as (2) the

493   progressive restriction of gene flow between patches inside and outside the lagoon.

494   Our study identified several outlier SNPs that may be related to *P. oceanica* survival at extreme

495   environmental conditions, such as in the Stagnone di Marsala lagoon, but potentially also in

496   other localities [e.g. Mar Menor lagoon, Marmara Sea (Meinesz et al., 2009)]. Below we report

497   the main functions associated with outlier SNPs selected in our analysis.

498   *Glutaredoxins* (also known as *Thioltransferases*) are small ubiquitous redox enzymes that are

499   involved in the response to oxidative stress through the regeneration of enzymes participating in

500   peroxide and methionine sulfoxide reduction (Rouhier, Lemaire, & Jacquot, 2008). Plants produce

501   ROS-scavengers (also known as antioxidants) to minimize the negative impacts of oxidative stress

502   (Hasanuzzaman, Nahar, & Fujita, 2013; Nguyen et al., 2020; Paridah et al., 2016). In seagrasses,

503   ROS-scavengers are an important mechanism to cope with different stressors including warming

504   (Gu et al., 2012; Liu, Tang, Wang, Zang, & Zhou, 2016; Nguyen et al., 2020; Purnama, Hariyanto,

505   Sri, Manuhara, & Purnobasuki, 2019; Reusch et al., 2008; Tutar, Marín-Guirao, Ruiz, &

506   Procaccini, 2017; Winters, Nelle, Fricke, Rauch, & Reusch, 2011) and hyper-salinity (Capó et al.,

507   2020; Marin-Guirao et al., 2011; Sandoval-Gil et al., 2023). Hence, the genetic mechanisms

508   underlying the mediation of ROS may play a critical role in promoting the local adaptation of *P.

509   oceanica* to extreme environmental conditions. This is consistent with previous studies

510   highlighting the role of ROS-managing mechanisms on the local adaption of organisms to different

511   environmental conditions [e.g. the reef-building coral *Pocillopora damicornis* with temperature

512   and light (van Oppen et al., 2018); the brown alga *Ectocarpus siliculosus* with copper stress (Ritter

513   et al., 2010), among others].

Protein serine/threonine kinase has a wide range of functions in plants including response to stressful environmental conditions and defense responses (Hardie, 1999). *Leucine-rich repeat extensin-like protein 3* are both related to cell wall modification (Draeger et al., 2015). Their involvement in plant stress response has been highlighted in terrestrial plants (Yang et al., 2006; Zwiazek, 1991) and in seagrasses (Franssen et al., 2011, 2014; Gu et al., 2012; Houston, Tucker, Chowdhury, Shirley, & Little, 2016; Jueterbock et al., 2016; Marín-Guirao et al., 2017). Indeed, cell wall modification may directly relate the substantial downsizing of *P. oceanica* plants, as observed at the northern basin of the Stagnone di Marsala (La loggia et al., 2004; Tomasello et al., 2009, this study) and potentially at the channel mouth of the Mar Menor lagoon (Marín-Guirao et al., 2017). The *α-amylase inhibitor* (*AAI protein*) is a plant lipid transfer protein (LTP). In *Arabidopsis*, LTPs are involved in the response to different environmental stressors (e.g. drought and freezing) (Guo, Yang, Zhang, & Yang, 2013). It is noteworthy that among the five outlier SNPs with maximum allele frequency in individuals from the northern basin, three of them with functions related to plant response to environmental stressors, were exclusively found in this site.

WD repeat-containing protein WRAP73 is a member of the WD-repeat (WDR) protein superfamily, which comprises an extremely diverse number of regulatory proteins strongly conserved across eukaryotes, playing key roles in several mechanisms such as signal transduction, cytoskeletal dynamics, protein trafficking, nuclear export, and RNA processing, and are especially prevalent in chromatin modification and transcriptional mechanisms (van Nocker & Ludwig, 2003). WDR proteins are intimately involved in a variety of cellular and organismal processes, including cell division, apoptosis, flowering, and meristem organization (van Nocker & Ludwig, 2003). In *Arabidopsis*, WD-repeat proteins have been increasingly recognized as a key regulator of plant-specific developmental events (van Nocker & Ludwig, 2003). *Purine permeases* are first

537 known to be involved in the transport of purine nucleobase substrates, and their derivatives

538 including phytohormones like cytokinins (Gillissen et al., 2000). Derivatives of nucleic acid bases

539 and nucleotides play potentially important roles in cell division, senescence, and defense reactions

540 (Gillissen et al., 2000). Moreover, recent studies have demonstrated additional roles of this protein

541 family in the plant secondary metabolism and root cell growth (Gani, Vishwakarma, & Misra,

542 2021; Hildreth et al., 2011; Jelesko, 2012). *Retrotrans_gag domain-containing protein* is related

543 to Retrotransposon gag protein (a class of transposable elements) that are commonly activated by

544 stresses and external change in all eukaryotes, including plants (Grandbastien, 1998). *AP-5*

545 *complex subunit beta-1* is associated with AP-5 Adaptor protein complexes that facilitate the

546 trafficking of cargo from one membrane compartment of the cell to another by recruiting other

547 proteins to particular types of vesicles. This is important for plant growth and enable cells to

548 communicate with the environment (Park et al., 2013). Finally, *C2 domain-containing protein*

549 plays a role in signal transduction and membrane trafficking (Zhang & Aravind, 2010).

550 In summary, our study suggests that local adaptation to extreme conditions in seagrasses might be

551 promoted by the selection of genotypes equipped to survive such adverse conditions together with

552 a limited gene flow. The selected genotypes may be dominated by several "tolerant" genotypes

553 with mutations (outlier SNPs) on genes with a role in different biological processes including plant

554 stress responses (e.g. ROS-scavenging activities and cell wall modification), essential functions

555 such as cellular transport and plant developmental events, among others. These findings provide a

556 better understanding of the genetic basis of local adaptation in seagrasses and offer new clues in

557 our attempt to assist the adaptation of those foundation species in the future (Bulleri et al., 2018;

558 Nguyen, Ralph, et al., 2021). We acknowledge the difficulties of clearly distinguish the relative

559 contribution of phenotypic plasticity versus local adaptation in our study. However, it is possible

560 that the simultaneous presence of phenotypic plasticity and local genetic selection in the inner-

561 lagoon *P. oceanica* populations had contributed to the observed phenomenon as demonstrated in

562 previous studies on marine and freshwater organisms (Bedulina, Zimmer, & Timofeyev, 2010;

563 Jensen et al., 2008; Pulgar, Bozinovic, & Ojeda, 2005; Yampolsky, Schaer, & Ebert, 2014).

564 **Acknowledgments**

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1015   3054.1991.tb02940.x

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Data Accessibility and Benefit-Sharing

1018   *Data Accessibility Statement*

1019   Raw sequencing data and VCF files will be deposited and made publicly available on DataDryad

1020   upon acceptance

1021   *Benefit-Sharing Statement*

1022   Not applicable

1023   **Author Contributions**

1024   HMN, AT, LMG, MP, and GP conceived and designed the experiment. AT performed sample

1025   collection, biometry data analysis, integration, supervision, and interpretation. FCP performed

1026   laboratory biometry analysis and data pre-processing. SC performed data interpretation. MR and

1027   ED extracted and prepared DNA samples for the ddRAD sequencing. HMN, MR and ED

1028   conducted the bioinformatics analysis of ddRAD data and guided their interpretation. HMN

1029   wrote the first draft of the manuscript. All authors wrote and reviewed the manuscript.

1030   **Conflict of Interest**

1031   The authors declare no competing interest.

1032   **Tables and Figures**

1033   **Table 1** Genetic and genotypic diversity indices of *P. oceanica* across sites. N: number of
1034   individual samples; MLLs: number of distinct Multi Locus Lineages; *R* [(G-1)/(N-1)]: clonal
1035   diversity; Ho: observed heterozygosity; He: expected heterozygosity; F_{IS}: inbreeding coefficient.

Site	N	MLLs	*R*	Ho	He	F_{IS}
North-basin	15	3	0.143	0.211	0.109	-0.889
South-basin	15	10	0.642	0.215	0.189	-0.108
OpenSea-A	15	9	0.571	0.220	0.212	-0.041
OpenSea-B	15	10	0.642	0.195	0.159	-0.130
OpenSea-C	14	8	0.538	0.208	0.191	-0.083
OpenSea-D	15	10	0.642	0.215	0.207	-0.036

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1041   **Table 2** Global Weir and Cockerham weighted pairwise F_{ST} estimated among study sites based on
1042   all 51,329 SNPs.

	North-basin	South-basin	OpenSea-A	OpenSea-B	OpenSea-C
North-basin					
South-basin	0.227				
OpenSea-A	0.180	0.119			
OpenSea-B	0.341	0.213	0.132		
OpenSea-C	0.203	0.145	0.082	0.199	
OpenSea-D	0.198	0.120	0.029	0.167	0.098

1043

Table 3 List of known annotated functions for the 14 true outliers from the UniProt database (Details about BLASTn and BLASTx results can be found in **Supplementary Table S6**). Annotations potentially associated with plant stress response are in grey background. − means no proteins annotated.

| SNP_Outlier_ID	Top BLASTx hit (UniProt)	Accession number	Related function
>102786 NS=76_ pos191	Glutaredoxin domain-containing protein	A0A1E5W751	Glutathione oxidoreductase activity
>4564 NS=81_ pos98	Receptor-like serine/threonine-protein kinase	A0A2P6Q381	Protein serine/threonine kinase activity
>99732 NS=83_ pos211	Protein kinase domain-containing protein	A0A251RZQ7	Protein serine/threonine kinase activity
>126268 NS=74_ pos268	Leucine-rich repeat extensin-like protein 3	A0A6P6UM88	Cell wall and growth modification
>145013 NS=85_ pos198	LRRNT_2 domain-containing protein	A0A5N6MZW6	Cell wall and growth modification
>37103 NS=76_ pos253	C2 domain-containing protein	A0A444DYZ0	Signal transduction and membrane trafficking
>91253 NS=75_ pos17	AP-5 complex subunit beta-1	A0A067JTT7	Protein transport
>34231 NS=78_ pos44	Probable purine permease	A0A540NHL2	Purine nucleobase transmembrane transporter activity
>21853 NS=76_ pos40	WD repeat-containing protein WRAP73	A0A3S3N7C1	Regulators of plant-specific developmental events
>108769 NS=74_ pos254	Retrotrans_gag domain-containing protein	A0A7J7G4T9	Retrotransposon
>107233 NS=81_ pos235	AAI domain-containing protein	A0A0D9WSI5	Plant lipid transfer protein
>21310 NS=83_ pos84	−	−	−
>65929 NS=79_ pos159	−	−	−
>65929 NS=79_ pos122	−	−	−
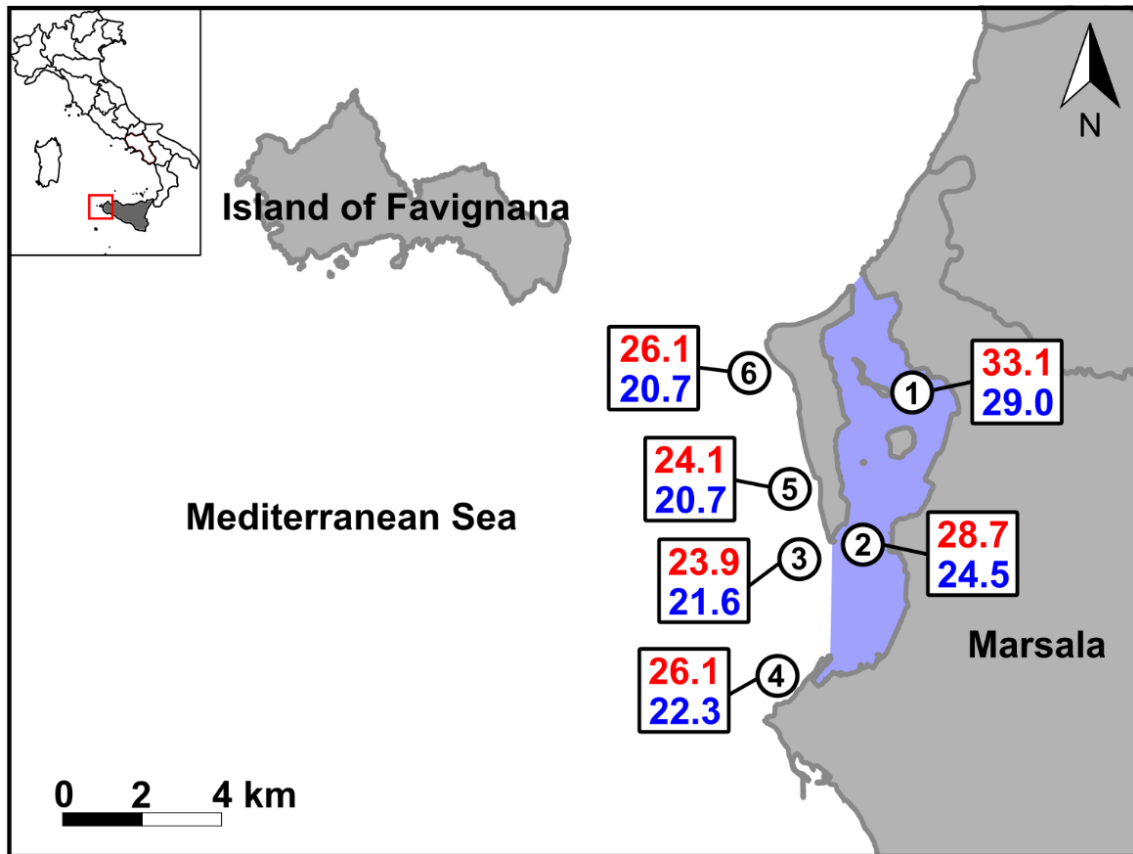
Figure 1 Sample collection sites in this study: (**1**) North-basin, (**2**) South-basin, (**3**) OpenSea-A, (**4**) OpenSea-B, (**5**) OpenSea-C, and (**6**) OpenSea-D. The Stagnone di Marsala lagoon is in light blue. The red and blue numbers indicate maximum and average sea surface temperatures (℃), respectively, at each collection site in the period May−September 2017.
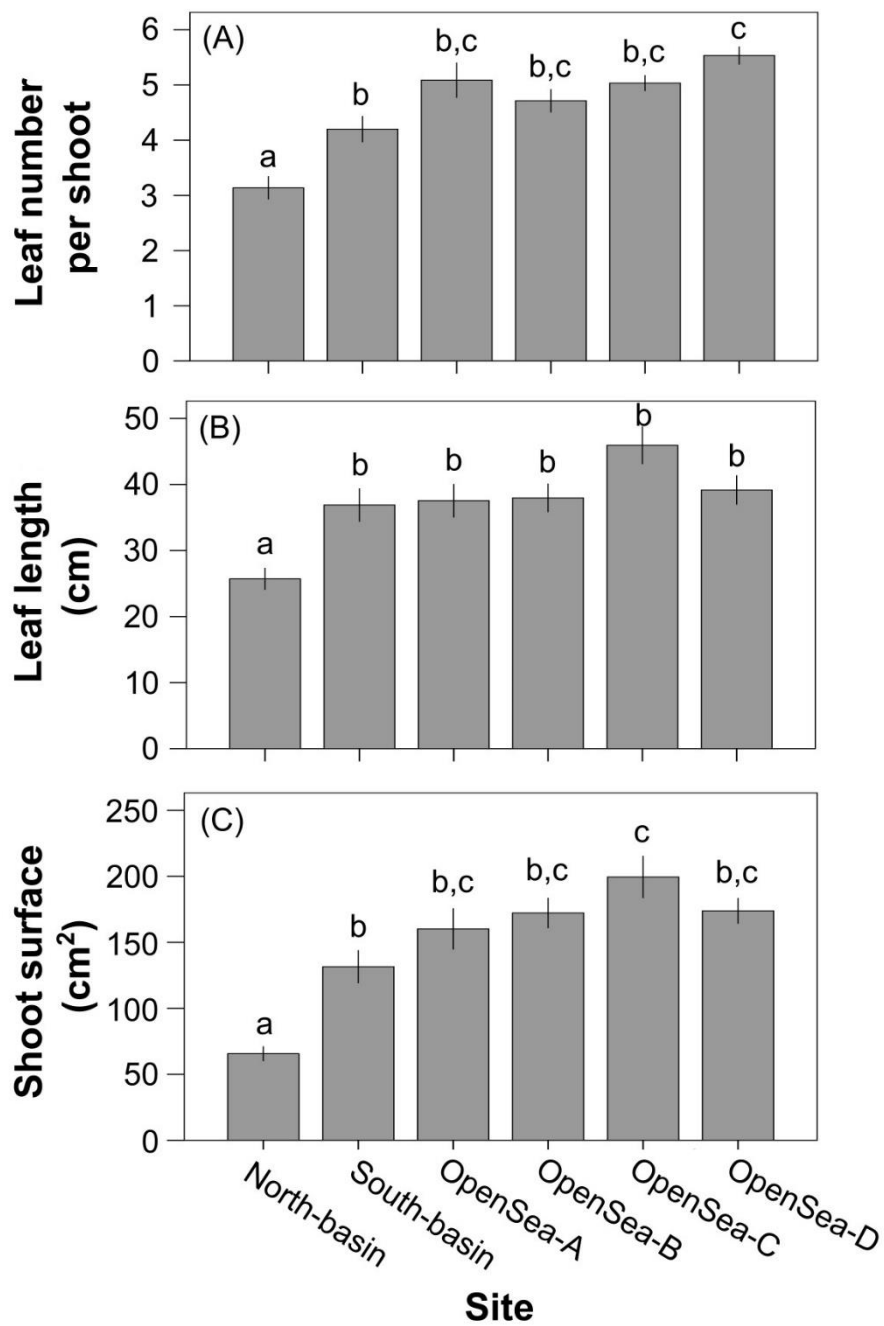
Figure 2 Leaf morphological results. Data are mean ±SE. Letters over the bars indicate results of Tamhane's T2 test (Details can be found in **Supplementary Table S1−3**).

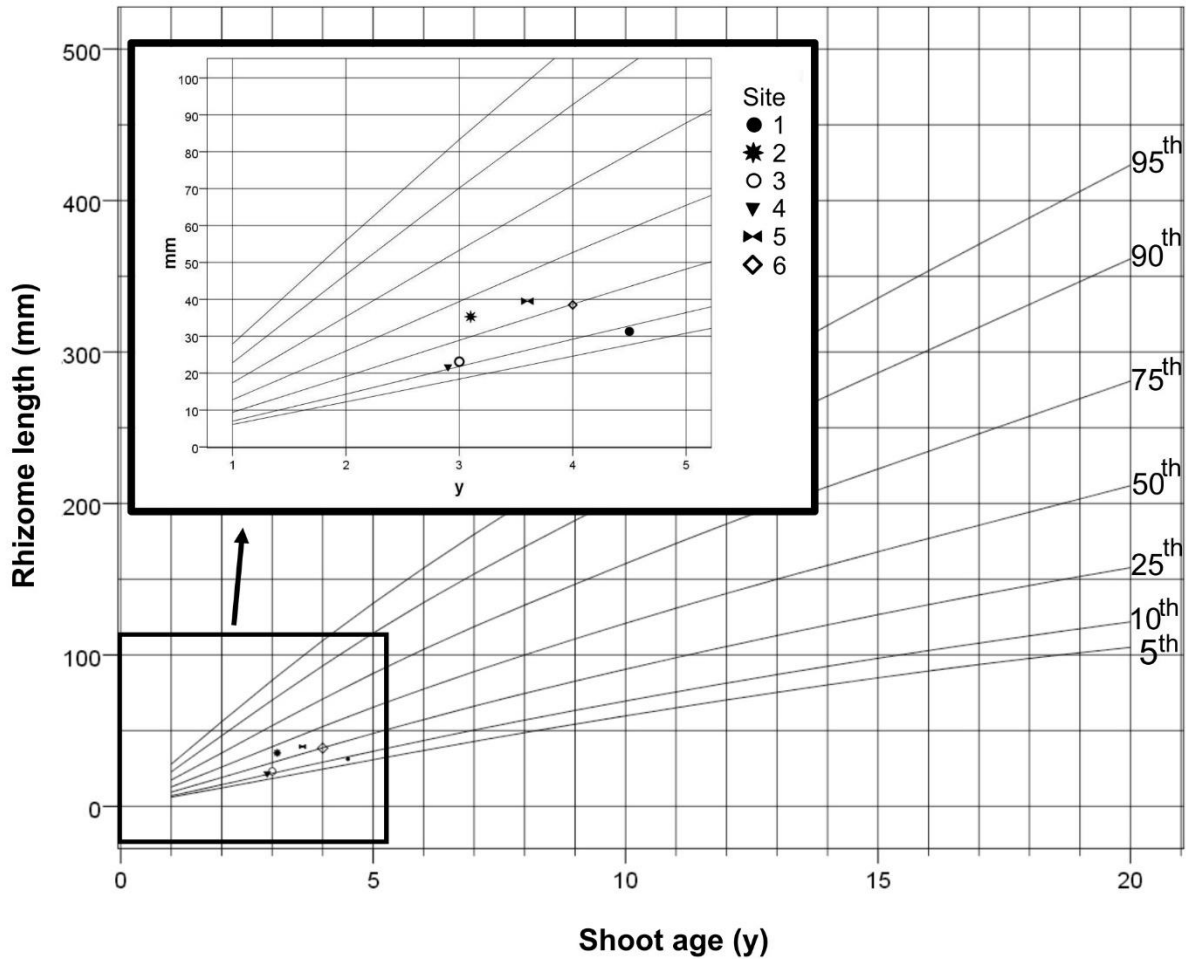**Figure 3** Growth performance measurements plotted on reference growth charts (Tomasello *et al.*, 2016). (**1**) North-basin, (**2**) South-basin, (**3**) OpenSea-A, (**4**) OpenSea-B, (**5**) OpenSea-C, and (**6**) OpenSea-D. The distribution of rhizome length and shoot age averaged in each station reported in table1 are compared with the expected percentile curves at different ages. The position of the stations within percentile ranges can best be seen in the enlarged graph.
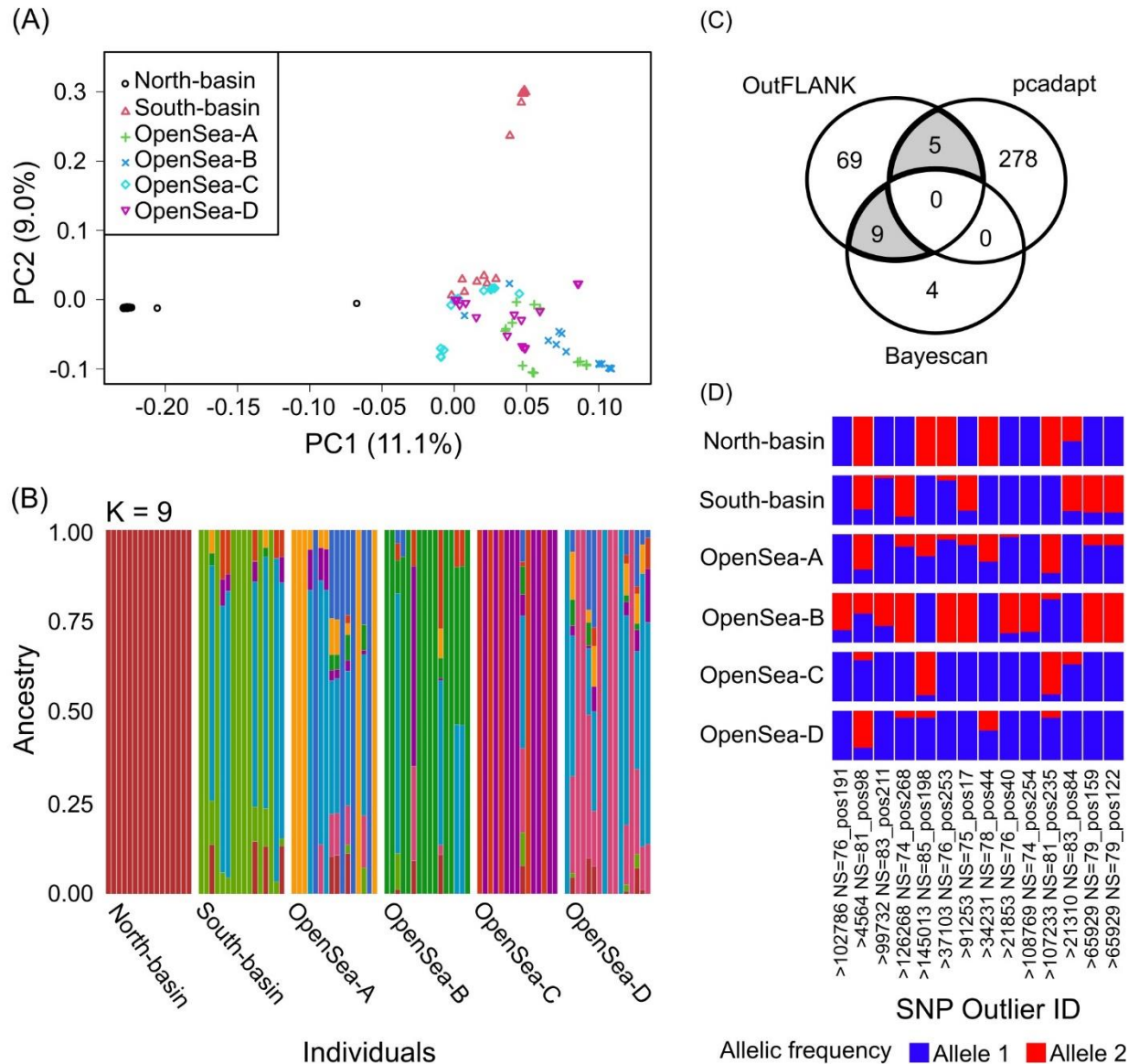
Figure 4 Results of genetic analyses for 95 *P. oceanica* samples based on all 51,329 SNPs. (A) PCA results; (B) ADMIXTURE results for K=9 with *P. oceanica* individuals on the x-axis (sorted by site) and assignment probability on the y-axis; (C) Venn diagram presents shared and unique outlier SNPs detected by the three algorithms; and (D) Graphical depiction of allele frequencies of the 14 outlier SNPs identified by at least two methods (Allele 1: Reference allele; Allele 2: Alternative allele). Details can be found in **Supplementary Table S9**.