Signs of local adaptation by genetic selection and isolation promoted by extreme temperature and salinity in the Mediterranean seagrass Posidonia oceanica

Signs of local adaptation by genetic selection and isolation promoted by extreme temperature and salinity in the Mediterranean seagrass *Posidonia oceanica*

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,

63 seagrass populations thriving in fluctuating conditions are more capable to endure stress than those
64 living in more stable environments (Blok et al., 2018; Hämmerli & Reusch, 2002; King et al.,
65 2018; Pazzaglia, Reusch, Terlizzi, Marin Guirao, & Procaccini, 2021). These locally-adapted
66 populations can provide potential materials for assisting the evolution of natural populations and
67 for improving seagrass restoration activities (Bulleri et al., 2018; Nguyen, Ralph, Marín-Guirao,
68 Pernice, & Procaccini, 2021; Pazzaglia et al., 2021; Tuya et al., 2019).

69 To date, knowledge of the genetic basis underlying local adaptation to environmental conditions
70 in seagrasses remains scarce (but see Hughes and Stachowicz, 2004; Ruggiero et al., 2005; Tuya
71 et al., 2021; Ruocco et al., 2022). Moreover, intraspecific variation among populations is often
72 ignored or under-estimated when assessing specific responses of populations to their surrounding
73 environment, as well as, when predicting potential changes in their future distribution (Hu et al.,
74 2021; Pazzaglia et al., 2021).

75 The seagrass *Posidonia oceanica* is endemic to the Mediterranean Sea where it forms widespread
76 monospecific meadows on rocks and sandy seabed and provides numerous vital ecosystem
77 services (Campagne, Salles, Boissery, & Deter, 2015; Procaccini et al., 2003; Serra & Mazzuca,
78 2011). It is known that the tolerance limits of *P. oceanica* range between 33 – 39‰ for salinity
79 (Sanchez-Lizaso et al., 2008) and 9 – 29℃ for temperature (Boudouresque & Meinesz, 1982).
80 Stagnone di Marsala is a semi-enclosed coastal lagoon along the western coast of Sicily, Italy
81 (Vizzini, Sarà, Michener, & Mazzola, 2002). This lagoon represents a unique area where *P.*
82 *oceanica* occurs during summer under temperature and salinity conditions that far exceed the
83 described thresholds of the species' tolerance [i.e. maximum temperature and maximum salinity
84 recorded in some parts of the lagoon were 30℃ and 48‰ (Mazzola & Vizzini, 2005)]. By using
85 13 microsatellite markers together with lepidochronological analysis, Tomasello *et al.*, (2009)

showed that *P. oceanica* atolls in the innermost area of the lagoon exhibited lower shoot-growth and were genetically isolated from the meadows outside the lagoon. This suggests a possible selection of genotypes that adapted to the persistent stressful conditions inside the lagoon.

In an era of rapid environmental changes, the *P. oceanica* population of the Stagnone di Marsala lagoon represents a natural experimental model system for investigating seagrass response to future environmental conditions. Combining prior knowledge from Tomasello *et al.,* (2009) and the application of *state-of-the-art* approaches in genetic research represents a unique opportunity to better understand the genetic basis of adaptation to extreme conditions in seagrasses. To this aim, samples of *P. oceanica* were collected from two sites inside the lagoon and four sites outside the lagoon [those relatively corresponded with sampling localities in Tomasello *et al.*, (2009)]. Measurements included sea surface temperature, plant morphology, past growth rate, past flowering events, and screening of genome-wide polymorphisms using double digest restriction-site associated DNA (ddRAD) for SNPs identification and detection of outlier loci (Peterson, Weber, Kay, Fisher, & Hoekstra, 2012). SNP markers could provide many advantages over microsatellites (as applied in Tomasello *et al.*, 2009), as they are denser and have more uniform distribution within genomes making them more useful for population and mapping studies (Balloux, Brunner, Lugon-Moulin, Hausser, & Goudet, 2000; Xing et al., 2005) and most importantly, they allow for the detection of potential adaptive DNA polymorphisms at specific functional loci that are candidates for genetic adaptation to local environmental conditions (Hung et al., 2012; Lasky et al., 2015; van Oppen et al., 2018). This kind of approach (i.e. RAD sequencing) has been widely applied to study evolutionary mechanisms of different marine organisms (Gaither et al., 2015; Hohenlohe et al., 2010; Jahnke, Moknes, Le Moan, Martens, & Jonsson, 2022; van Oppen et al., 2018) including some recent studies on seagrasses (Phair, Toonen,

Knapp, & von der Heyden, 2020, 2019; Ruocco et al., 2022). We hypothesize that (*i*) the high levels of salinity and temperature in the interior of the lagoon have selected the most resistant genotypes favouring the local adaptation of the *P. oceanica* population to these extreme conditions, (*ii*) these genotypes manage to survive under conditions that exceed the thresholds of the species through genetic mutations in certain functional loci and/or their high phenotypic plasticity. We expected that (*1*) *P. oceanica* plants from sites inside the lagoon would show a lower level of genetic variation than those from sites outside the lagoon and (*2*) these plants would differ morphologically and genetically from those outside the lagoon. Morphological and genetic differences would also exist between the two inside-lagoon sites.

2. Materials and methods

2.1. Study area

The Stagnone di Marsala lagoon is a shallow area with an average depth of 1.5 m and a surface area of about 2000 ha (Vizzini et al., 2002). This basin exhibits distinct lagoon features, such as limited water exchange and slow turnover and has the highest annual variation in temperature and salinity among sites where the presence of *P. oceanica* has been reported. The lagoon can be subdivided into a northern and a southern basin with different geomorphological and environmental characteristics. The northern basin has an average depth of 1.1 m and it is connected with the open sea through a channel 400 m wide and 20 ─ 30 cm deep northwards. The annual water temperature in the northern basin ranges from minima 10.0 ─ 11.8 °C in January to maxima 29.1 – 30.0 °C in August, while salinity ranges from 32.8 ─ 48.0‰, (Sarà, Leonardi, & Mazzola, 1999; Mazzola & Vizzini, 2005; Vizzini et al., 2002). A salinity level of 51‰ has recently been recorded in the northern basin of the lagoon (Spinelli, 2018) indicating an increase in salinity level in this area.

Over-sedimentation and lack of maintenance over recent years caused the partial closure of the northern channel resulting in even more extreme environmental conditions in the inner lagoon (Calvo S., Tomasello A., *personal observation*). In this part of the basin, *P. oceanica* forms atoll-like structures (Calvo & Frada-Orestano, 1984), a rare feature of *P. oceanica* meadows observed in few other localities along the Tunisian, Turkish and Corsican coasts [see Tomasello *et al.*, (2020) for related references]. In addition, the atoll structure of the Stagnone area is in strong regression with a marked decrease in the plant's primary production recorded about 30 years ago (Calvo, Ciraolo, & Loggia, 2003; Pergent et al., 2014). The southern basin is slightly deeper (about 2 m of depth) and it is connected with the surrounding open sea through a 3000 m wide opening, in which a vast *P. oceanica* reef platform (*Plateau Récifale*) is present (Tomasello et al., 2009). Lastly, the surrounding open sea is environmentally more stable with a year-round temperature ranging from a minimum of 14.1℃ during winter to a maximum of 26.4℃ during summertime and a stable salinity level of 37‰ (Vizzini et al., 2002). Here, *P. oceanica* forms a very large meadow (Calvo et al., 2010) from the surface to about 30 m depth (Bellissimo, Sirchia, & Ruvolo, 2020), characterized by the most extensive living reef, to our knowledge, along the Mediterranean coasts (about 40 km long, Calvo S, Tomasello A, *personal observation*).

2.2. Sample collection

On the 7th of September 2020, *P. oceanica* shoots with integer orthotropic rhizome (i.e. they were harvested until to the insertion point with their plagiotropic rhizomes) were haphazardly collected at about 1 m of depth from atolls or reefs present in six different sites (i.e. 20 ─ 30 shoots from each site). To maximize the number of genotypes collected, samples were harvested at a minimum distance of 5 m from each other. Sampling stations included (***i***) two sites inside the Stagnone di

Marsala lagoon [***North-basin*** (close to the atolls site in Tomasello *et al.,* 2009), in the northern basin of the lagoon: samples were collected from 5 different atolls with an average of 4 – 6 samples 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, 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*** (corresponds with Récif site in Tomasello *et al.,* 2009), in the southern basin of the lagoon (37°50'35"N, 12°27'29"E)] and (***ii***) four sites outside the lagoon [***OpenSea-A*** (corresponds with Plateau site in Tomasello *et al.,* 2009: 37°50'26"N, 12°26'45"E), ***OpenSea-B*** (37°48'48"N, 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)] (**Fig. 1**). Soon after collection, 96 leaf sub-samples (~10 cm; 16 samples per site) were selected for DNA extraction. Samples were gently cleaned out of epiphytes before being dried and stored with silica gel until further analysis. The rest of the collected material was kept in a cooler container filled with seawater and transported shortly to the laboratory for morphological measurements.

2.3. Sea surface temperature

Sea surface temperature (SST) data were obtained through image analysis based on satellite remote sensing data from the Sea and Land Surface Temperature Radiometer sensors installed on the Sentinel-3 mission satellites with a spatial resolution of 250 m (https://apps.sentinel-hub.com/). Data were collected from May to September for the years 2017 to 2020. Then, the data from the year 2017 was chosen because it contained the highest number of images. Selected images were analyzed using QGIS software (http://qgis.osgeo.org/) to obtain average and maximum temperatures during the May-September period for each study site.

2.4. Morphological and growth performance measurements

Two sets of biometric measures were taken including leaf biometry and dating (Pergent-Martini et al., 2005). Leaf biometry and morphological measurements were carried out on the leaf bundle as described in previous studies (Girard, 1977; Giraud, 1979). Measurements included leaf number per shoot, leaf length (cm) and shoot surface (cm^2). Dating was carried out on rhizomes by lepidochronology (Pergent, 1990), which provides a reliable estimation of their growth performance. This method is based on the analysis of the cyclic variations of the sheaths thickness along the rhizomes. In particular, starting from the basal portion towards the apex of the rhizome, the sheaths were detached from the nodes with the aid of a scalpel and arranged on a laboratory table in the sequence corresponding to their order of insertion. At the same time, their thickness was preliminarily assessed by touch by means of a slight bending in order to identify the sheath where the inversion of the thickness trend (from decreasing to increasing) occurred, corresponding to the possible finding of the relative minimum. Subsequently, a thin section was made on both the suspected sheath minimum and previous and following ones at about 10 – 12 mm from the base for confirmation or rectification by using micrometric binoculars. At this point, the rhizome was dissected transversally at the nodes corresponding to the finding of sheaths with the minimum relative thickness. In this way for each rhizome, the cyclic variation of the sheath thickness was detected to isolate rhizome segments corresponding to a one-year period, determined between each pair of sheaths of minimum relative thickness ('lepidochronological year' according to Pergent, 1990). Consequently, it was also possible to date rhizome segments corresponding to a lepidochronological year. Each lepidochronological year was dated starting from the rhizome apex (sampling year) downward and backdating the sequence of cycles with their corresponding rhizome segment. This reiterative procedure was performed until the rhizome segment connected 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).

206 **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).

The influence of geographic distance (Euclidean distance in kilometres) on genetic distance (measured as pairwise F_{ST}) was investigated using Mantel test based on Pearson's product-moment correlation with 1000 permutations. The Mantel test was done in R-studio v.1.2.5033 (R Core Team, 2018) using the package *vegan* (Oksanen et al., 2013).

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

Total genomic DNA (gDNA) was isolated from about 30 mg of dried tissue using NucleoSpin® Plant II kit (Macherey-Nagel) by following the manufacturer's instructions. Total gDNA integrity was checked through 1% agarose gel electrophoresis and total gDNA purity was determined spectrophotometrically by examining 260/230 and 260/280 nm absorbance ratios using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific). Finally, DNA concentration was accurately measured by the Qubit dsDNA BR assay kit with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

Ninety-five ddRAD-seq library construction and sequencing were conducted at IGATech (Udine, Italy) using an IGATech custom protocol, with minor modifications with respect to Peterson's double digest restriction-site associated DNA preparation (Peterson et al., 2012). To ensure the quality of sequencing outcomes, for each site, one sample was randomly selected and sequenced twice. The final number of biological replicates for each site was $n = 14$ for OpenSea-C and $n = 15$ for the other sites (i.e. North-basin, South-basin, OpenSea-A, OpenSea-B, and OpenSea-D), respectively (i.e. 89 unique samples + 6 technical replicates). In short, gDNA was double digested with both *Sph*I and *Mbo*I endonucleases (New England BioLabs). Fragmented DNA was purified with AMPureXP beads (Agencourt) and subsequently ligated with T4 DNA ligase (New England BioLabs). Samples were pooled on multiplexing batches and bead purified as before and then they were size-selected and underwent several purification steps. ddRAD-seq libraries were sequenced

with 150 cycles in paired-end mode on NovaSeq 6000 instrument following the manufacturer's instructions (Illumina, San Diego, CA).

2.7. Single nucleotide polymorphisms (SNPs) calling

Single nucleotide polymorphisms (SNPs) calling was performed *de novo* using Stacks software package v2.53 (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013). First, raw Illumina reads were demultiplexed using the *process_radtags* utility (Catchen et al., 2013). The short reads of each sample were assembled into exactly matching stacks using the *ustacks* utility (Catchen et al., 2013). The creation of the loci catalog (i.e. a set of consensus loci from all the analyzed samples) was done using *cstacks* and matching each sample against the catalog using *sstacks* and *tsv2bam* utilities (Catchen et al., 2013). *gstacks* ultility (Catchen et al., 2013) was used to pull in paired-end reads, assemble the paired-end contigs and merge them with the single-end locus, align reads to the locus and ultimately call SNPs. Finally, detected loci were filtered using the *populations* program included in Stacks v2.53 (Catchen et al., 2013), with option –R=0.75 to retain only loci that were represented in at least the 75% of the whole metapopulation and with cutoff --*max-obs-het*=0.8, to process a nucleotide site at a locus with observed heterozygosity at a maximum of 80%.

2.8. Genetic variation analysis and clonality assessment

Individual genetic variation and population differentiation was assessed by a Principal Component Analysis (PCA) using the R package *SNPRelate* (Zheng et al., 2012) and by an ADMIXTURE analysis using the software ADMIXTURE 1.3.0 (Alexander & Lange, 2011). To choose the best estimate of the number of clusters (K), the ADMIXTURE cross-validation procedure was used

with default settings. The hypothetical number of K was set from 1 to 15 then the K value with the lowest cross-validation error was chosen to use for ADMIXTURE analysis.

Clonality assessment, including genetic distance among all samples and number of distinct multilocus lineages (MLLs) for each site, was done using the R package *poppr* (Kamvar, Brooks, & Grünwald, 2015). The genetic distance limit for setting delimitation of clones was determined based on the maximum genetic distance detected between technical replicates as done in a recent study (Ruocco et al., 2022). Based on results from the clonality assessment, clones as well as technical replicates (i.e. samples sequenced twice) were removed from the dataset before all subsequent analyses including outlier detection (section 2.9). Pair-wise Weir and Cockerham F_{ST} estimates between sampling sites were calculated with VCFtools (Danecek et al., 2011). Observed (Ho) and expected (He) heterozygosity, as well as F_{IS} values across all loci for each sampling site were calculated by using the R package *hierfstat* (Goudet, 2005).

2.9. Outlier SNPs identification and functional annotation

Three genome scan methods were used to identify outlier SNPs across the whole dataset. The first method was based on F_{ST} values and implemented in the program *BAYESCAN* v.2.1 (Foll, 2012; Foll & Gaggiotti, 2008). It was used with prior odds set to 100 and using a threshold of $q \leq 0.3$ and posterior probability $P>0.5$. The second method was also based on F_{ST} values and implemented in the R package *OutFLANK* (Whitlock & Lotterhos, 2015). *OutFLANK* analysis was performed using default settings and SNPs with a *p*-value less than 0.01 were considered as 'suggestive' outliers [as done in a previous study (Andrew, Jensen, Hagen, Lundregan, & Griffith, 2018)]. The last method based on multivariate analysis and implemented in the R package *pcadapt* (Luu, Bazin, & Blum, 2017) was used with default settings [that computed a test statistic based on Mahalanobis distance which is a multi-dimensional approach that measures how distant a point from the mean

(Luu et al., 2017)]. To define the correct number of principle components (PCs) to use in *pcadapt* analysis, we started with $K = 20$ PCs then $K = 3$ was selected as the most appropriate value for the analysis based on an inspection of a scree plot (Luu et al., 2017). In the last step, any SNP with a *p*-value less than 0.01 with Bonferroni correction for multiple comparisons was considered as an outlier SNP.

To reduce the likelihood of detecting false positives, a Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to identify shared and unique outliers detected from the different methods. Only SNPs that were identified as outliers by at least two methods were considered 'true' outliers. Other SNPs (either detected as outliers by only one of the three methods or not detected as outliers by neither of the methods) were classified as neutral. Subsequently, allele frequencies of the 'true' outliers among sites were computed using the R package *genepop* (Rousset, 2008).

To determine whether an outlier SNP may be included in potential coding sequences, chromosome regions of the 'true' outlier SNPs were mapped against a previously published *P. oceanica* transcriptome (Ruocco et al., 2020) by using the BLASTn algorithm (Camacho et al., 2009). Positive hits were identified if a homologous sequence was present around the SNP position with a high scoring stretches of sequence similarity of at least 70 bp with a percentage of identity greater than 85% (only the best hit was selected for each alignment). Subsequently, a sequence similarity search was carried out between *P. oceanica* contigs (i.e. corresponding to the positive hits) against UniProt protein database (downloaded in February 2022) using the BLASTx software (Camacho et al., 2009) to identify potential protein functions corresponding to outlier SNPs (only the best hits was selected for each alignment).

311 ## 3. Results

312 ### 3.1. Environmental data

313 Seawater temperature inside the lagoon was higher in comparison with the outside lagoon area
314 (**Fig. 1**). In particular, average SST of North-basin and South-basin were 8.1℃ and 3.7℃ higher,
315 respectively, than the average SST of open-sea sites (**Fig. 1**). Maximum SST of North-basin was
316 31.1℃ and South-basin was 28.7℃, while the maximum SST of the outside lagoon sites varied
317 from 23.9 to 26.1℃. In addition, while temperature variation among the four outside lagoon sites
318 was less than 2℃ (e.g., the average SSTs varied from 20.7 to 22.3℃ and the maximum SSTs varied
319 from 23.9 to 26.1℃; **Fig. 1**), both average SST and maximum SST of North-basin were 4.5℃
320 higher than those of South-basin (**Fig. 1**).

321 ### 3.2. Morphological and growth performance

322 There were significant differences among the study sites for all morphological measurements
323 (Tamhane's T2 test, $p < 0.05$; **Fig. 2, Supplementary Table S1 – 3**), being plants from North-
324 basin different from plants from the rest of the study sites. In detail, plants from North-basin had
325 on average three leaves per shoot, being significantly lower than the average number of leaves of
326 plants from the other sites (i.e. ~ 5 leaves per shoot; **Fig. 2**). Similarly, plants from North-basin
327 had shorter leaves when compared with plants from the other sampling sites (Tamhane's T2 test,
328 $p < 0.05$; **Fig. 2, Supplementary Table S2**). Consequently, shoot surface area at North-basin was
329 also significantly lower than the surface area of plants from all other sites (**Fig. 2**). In particular,
330 the shoot surface of plants from North-basin was 51% lower than the surface of plants from South-
331 basin and 58 – 67% than plants from the outside-lagoon sites (**Fig. 2**). Dating measures allowed
332 to reconstruct production of leaf number and growth performance within temporal ranges from

2006 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. S3**).

PCA results showed a strong genetic differentiation of *P. oceanica* between (***i***) the two inside-lagoon sites (North-basin & South-basin; **Fig. 4A**) versus the four outside-lagoon sites (OpenSea-A – D; **Fig. 4A**) and (***ii***) between those from inside lagoon (North-basin versus South-basin). In detail, samples from North-basin separated from all samples of the other sites along the PC1 explaining 11.1% of the total variance of the data set (**Fig. 4A**). Interestingly, samples of South-basin were divided into two distinct groups, one group differentiated from all other samples along the PC2 (that accounts for 9% of the total variance) while the other group clustered with samples from OpenSea-B – D (**Fig. 4A**).

Genetic partitioning among sites was further confirmed by results from ADMIXTURE analysis (**Fig. 4B**). First, K=9 was identified as an 'optimal K' (i.e. number of genetic clusters) as it had the lowest cross-validation error of 0.177 among other K values (**Supplementary Table S6)**. Then, with the assumption of nine genetic clusters, the clustering analysis implemented in ADMIXTURE showed clear divergences in genetic structures among sites (**Fig. 4B**). No substructure was detected at North-basin as this site was dominated by a single homogeneous genetic component (**Fig. 4B**). This structural component was also present, however in a small proportion, in all other sites (**Fig. 4B**). On the other hand, all the other sites were characterized by diversified substructures (e.g. 8 – 9 components). It is important to note that the dominant substructure differed among all sites (**Fig. 4B**).

The North-basin atolls were characterized by extremely low clonal richness ($R = 0.143$), as the 15 investigated individuals represented only 3 MLLs, while the number in other sites ranged from 8 – 10 MLLs, with an average R value of 0.6 (**Table 1** and **Supplementary Fig. S4**). In the South-basin, also located inside the lagoon, the number of MLLs (i.e. 10) was equal to or even higher 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. S4**). 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,

2020; Vargas-Yáñez et al., 2008), (*ii*) the increased frequency and intensity of marine heatwaves in the Mediterranean Sea (Darmaraki et al., 2019) and (*iii*) the gradual closure of the 400-m wide channel in the north side of the lagoon, which further contributes to limit water exchange (Calvo A., Tomasello A., *personal observation*). Likewise, a salinity level of 51‰ has been recently documented in the northern basin of the lagoon (Spinelli, 2018), where a maximum value of 48‰ was previously recorded (Mazzola & Vizzini, 2005; Tomasello et al., 2009). This pushes up the acknowledged salinity and temperature tolerance limits for *P. oceanica* (Nguyen, Bulleri, Marín-Guirao, Pernice, & Procaccini, 2021; Sandoval-Gil, Ruiz, & Marín-Guirao, 2023).

Observations carried out over two decades (from November 2000 to September 2020) reported undersized *P. oceanica* shoots growing in the northern basin of the Stagnone of Marsala lagoon (Loggia et al., 2004; Tomasello et al., 2009; Spinelli, 2018; the present study). This can be considered a sign of long-term exposure of *P. oceanica* to the extreme conditions in the area [both extreme temperature and extreme salinity (Fernández-Torquemada & Sánchez-Lizaso, 2005; Marín-Guirao, Sandoval-Gil, Bernardeau-Esteller, Ruíz, & Sánchez-Lizaso, 2013; Ruíz, Marín-Guirao, & Sandoval-Gil, 2009)]. A similar shoot size reduction has been described in another *P. oceanica* population living under salinity levels above the normal tolerance threshold of the species (Marín-Guirao, Sandoval-Gil, García-Muñoz, & Ruiz, 2017). Marín-Guirao et al., (2017) proposed that this morphological modification may serve as a stress-coping mechanism, as previously described in terrestrial plants (Lichtenthaler, 1996). Similarly, reduced sized *P. oceanica* shoots have also been documented in natural vents under strong seawater acidification (Gambi, Esposito, & Marín-Guirao, 2023). In addition, lepidochronological results also demonstrated that plants from the northern basin exhibited the slowest growth performance in comparison with other sites. This further confirms the constraints imposed by extreme

environmental conditions to which *P. oceanica* plants are undergoing in this section of the basin. Furthermore, our study continues to report a lack of flowering events inside the lagoon in the last few decades (1984 – 2004, Tomasello et al., 2009; 2007 – 2019, present study). Flowering in seagrasses has been considered an adaptive mechanism (i.e. escape through sexual reproduction) to cope with unfavourable conditions (Nguyen, Ralph, et al., 2021). Previous studies have found a positive relationship between flowering events and extreme thermal stress (Blok et al., 2018; Diaz-Almela, Marbà, & Duarte, 2007; Marín-Guirao, Entrambasaguas, Ruiz, & Procaccini, 2019; Ruiz et al., 2018). Hence, we hypothesize two possible scenarios: the extreme condition in the Stagnone di Marsala lagoon (*i*) could exceed the threshold limit for flowering induction in *P. oceanica* or (*ii*) could have selected 'less-flowering' genotypes.

Our study demonstrates a clear genetic isolation of *P. oceanica* from inside versus outside the lagoon, especially for the individuals of the northern basin. This is in line with several previous studies showing that seagrass populations from confined environments (such as coastal lagoons) tend to exhibit some levels of genetic isolation [e.g. *Zostera marina* populations in San Quintin Bay, Mexico (Muñiz-Salazar, Talbot, Sage, Ward, & Cabello-Pasini, 2006); *P. oceanica* in the Marmara Sea (Meinesz et al., 2009) and the Stagnone di Marsala (Tomasello et al., 2009); *Halophila beccarii* populations in Cau Hai lagoon, Vietnam (Phan, De Raeymaeker, Luong, & Triest, 2017) or recently *Halophila ovalis* populations in Dongsha Island, Taiwan (Liu & Hsu, 2021)]. Additionally, we observed a reduction in the number of distinct genotypes detected (especially for the northern basin) when compared with Tomasello *et al.*, (2009). While the dissimilarity in the power of discriminating clones between the two used approaches (microsatellites versus ddRADseq) could have certainly contributed to this difference (Balloux et al., 2000; Xing et al., 2005), we cannot exclude that the continuous deterioration of the

environmental conditions (increased water temperature and salinity) inside that lagoon had caused the disappearance of some genotypes that were previously identified (Tomasello et al., 2009). It is interesting to note that while the majority of seagrass studies have shown a positive relationship between genetic diversity and the ability to endure environmental stressors of seagrass populations (Ehlers, Worm, & Reusch, 2008; Jahnke, Olsen, & Procaccini, 2015; Massa, Paulino, Serrão, Duarte, & Arnaud-Haond, 2013; Randall Hughes & Stachowicz, 2011), there are also several studies providing evidences to support the opposite (Arnaud-Haond, Marbà, Diaz-Almela, Serrão, & Duarte, 2010; Connolly et al., 2018; Diaz-Almela, Arnaud-Haond, et al., 2007). Our results showed no significant correlation between genetic distance and geographic distance (as verified by Mantel test) thus eliminating the potential effect of isolation by distance for the genetic isolation of *P. oceanica* populations inside the lagoon. Instead, the isolation is likely related to the existence of geographic barriers and/or the strong environmental filter exerted by the extreme conditions of the lagoon on possible propagules coming from the frequently blooming open sea populations (Tomasello et al., 2009 and this study). Moreover, the history of *P. oceanica* distribution in the area (the present distribution is most likely the remnant of a wider distribution present when hydrodynamic conditions inside the lagoon favored greater water exchange with the open sea) can exclude the possibility of bottleneck (and/or founder effect) happening in this area. As a result, genetic drift is also unlikely to be the cause of the genetic differentiation in the inside-lagoon populations. This is further supported by the fact that the genetic diversity of the North-basin population, in the face of observed heterozygosity (Ho), was actually comparable to most of other sites or even higher than some other sites (e.g., OpenSea-B) and this was already observed by Tomasello et al., (2009) with microsatellite markers. Together, the genetic isolation of the inner-lagoon individuals is, more likely, the result

of (1) the progressive extremization of the conditions inside the lagoon and a subsequent selection ("environmental filtering") of the more resistant genotypes, as well as (2) the progressive restriction of gene flow between patches inside and outside the lagoon.

Our study identified several outlier SNPs that may be related to *P. oceanica* survival at extreme environmental conditions, such as in the Stagnone di Marsala lagoon, but potentially also in other localities [e.g. Mar Menor lagoon, Marmara Sea (Meinesz et al., 2009)]. Below we report the main functions associated with outlier SNPs selected in our analysis.

Glutaredoxins (also known as *Thioltransferases*) are small ubiquitous redox enzymes that are involved in the response to oxidative stress through the regeneration of enzymes participating in peroxide and methionine sulfoxide reduction (Rouhier, Lemaire, & Jacquot, 2008). Plants produce ROS-scavengers (also known as antioxidants) to minimize the negative impacts of oxidative stress (Hasanuzzaman, Nahar, & Fujita, 2013; Nguyen et al., 2020; Paridah et al., 2016). In seagrasses, ROS-scavengers are an important mechanism to cope with different stressors including warming (Gu et al., 2012; Liu, Tang, Wang, Zang, & Zhou, 2016; Nguyen et al., 2020; Purnama, Hariyanto, Sri, Manuhara, & Purnobasuki, 2019; Reusch et al., 2008; Tutar, Marín-Guirao, Ruiz, & Procaccini, 2017; Winters, Nelle, Fricke, Rauch, & Reusch, 2011) and hyper-salinity (Capó et al., 2020; Marin-Guirao et al., 2011; Sandoval-Gil et al., 2023). Hence, the genetic mechanisms underlying the mediation of ROS may play a critical role in promoting the local adaptation of *P. oceanica* to extreme environmental conditions. This is consistent with previous studies highlighting the role of ROS-managing mechanisms on the local adaption of organisms to different environmental conditions [e.g. the reef-building coral *Pocillopora damicornis* with temperature and light (van Oppen et al., 2018); the brown alga *Ectocarpus siliculosus* with copper stress (Ritter et al., 2010), among others].

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

528 *WD repeat-containing protein WRAP73* is a member of the WD-repeat (WDR) protein
529 superfamily, which comprises an extremely diverse number of regulatory proteins strongly
530 conserved across eukaryotes, playing key roles in several mechanisms such as signal transduction,
531 cytoskeletal dynamics, protein trafficking, nuclear export, and RNA processing, and are especially
532 prevalent in chromatin modification and transcriptional mechanisms (van Nocker & Ludwig,
533 2003). WDR proteins are intimately involved in a variety of cellular and organismal processes,
534 including cell division, apoptosis, flowering, and meristem organization (van Nocker & Ludwig,
535 2003). In *Arabidopsis*, WD-repeat proteins have been increasingly recognized as a key regulator
536 of plant-specific developmental events (van Nocker & Ludwig, 2003). *Purine permeases* are first

known to be involved in the transport of purine nucleobase substrates, and their derivatives including phytohormones like cytokinins (Gillissen et al., 2000). Derivatives of nucleic acid bases and nucleotides play potentially important roles in cell division, senescence, and defense reactions (Gillissen et al., 2000). Moreover, recent studies have demonstrated additional roles of this protein family in the plant secondary metabolism and root cell growth (Gani, Vishwakarma, & Misra, 2021; Hildreth et al., 2011; Jelesko, 2012). *Retrotrans_gag domain-containing protein* is related to Retrotransposon gag protein (a class of transposable elements) that are commonly activated by stresses and external change in all eukaryotes, including plants (Grandbastien, 1998). *AP-5 complex subunit beta-1* is associated with AP-5 Adaptor protein complexes that facilitate the trafficking of cargo from one membrane compartment of the cell to another by recruiting other proteins to particular types of vesicles. This is important for plant growth and enable cells to communicate with the environment (Park et al., 2013). Finally, *C2 domain-containing protein* plays a role in signal transduction and membrane trafficking (Zhang & Aravind, 2010).

In summary, our study suggests that local adaptation to extreme conditions in seagrasses might be promoted by the selection of genotypes equipped to survive such adverse conditions together with a limited gene flow. The selected genotypes may be dominated by several “tolerant” genotypes with mutations (outlier SNPs) on genes with a role in different biological processes including plant stress responses (e.g. ROS-scavenging activities and cell wall modification), essential functions such as cellular transport and plant developmental events, among others. These findings provide a better understanding of the genetic basis of local adaptation in seagrasses and offer new clues in our attempt to assist the adaptation of those foundation species in the future (Bulleri et al., 2018; Nguyen, Ralph, et al., 2021). We acknowledge the difficulties of clearly distinguish the relative 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).

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Data Accessibility and Benefit-Sharing

Data Accessibility Statement

Raw sequencing data and VCF files are available on Dryad (https://doi.org/10.5061/dryad.1zcrjdfxp).

Benefit-Sharing Statement

Not applicable

Author Contributions

HMN, AT, LMG, MP, and GP conceived and designed the experiment. AT performed sample collection, biometry data analysis, integration, supervision, and interpretation. FCP performed laboratory biometry analysis and data pre-processing. SC performed data interpretation. MR and ED extracted and prepared DNA samples for the ddRAD sequencing. HMN, MR and ED conducted the bioinformatics analysis of ddRAD data and guided their interpretation. HMN wrote the first draft of the manuscript. All authors wrote and reviewed the manuscript.

Conflict of Interest

The authors declare no competing interest.

Tables and Figures

Table 1 Genetic and genotypic diversity indices of *P. oceanica* across sites. N: number of individual samples; MLLs: number of distinct Multi Locus Lineages; *R* [(G-1)/(N-1)]: clonal 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

Table 2 Global Weir and Cockerham weighted pairwise F_{ST} estimated among study sites based on 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

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	–	–	–

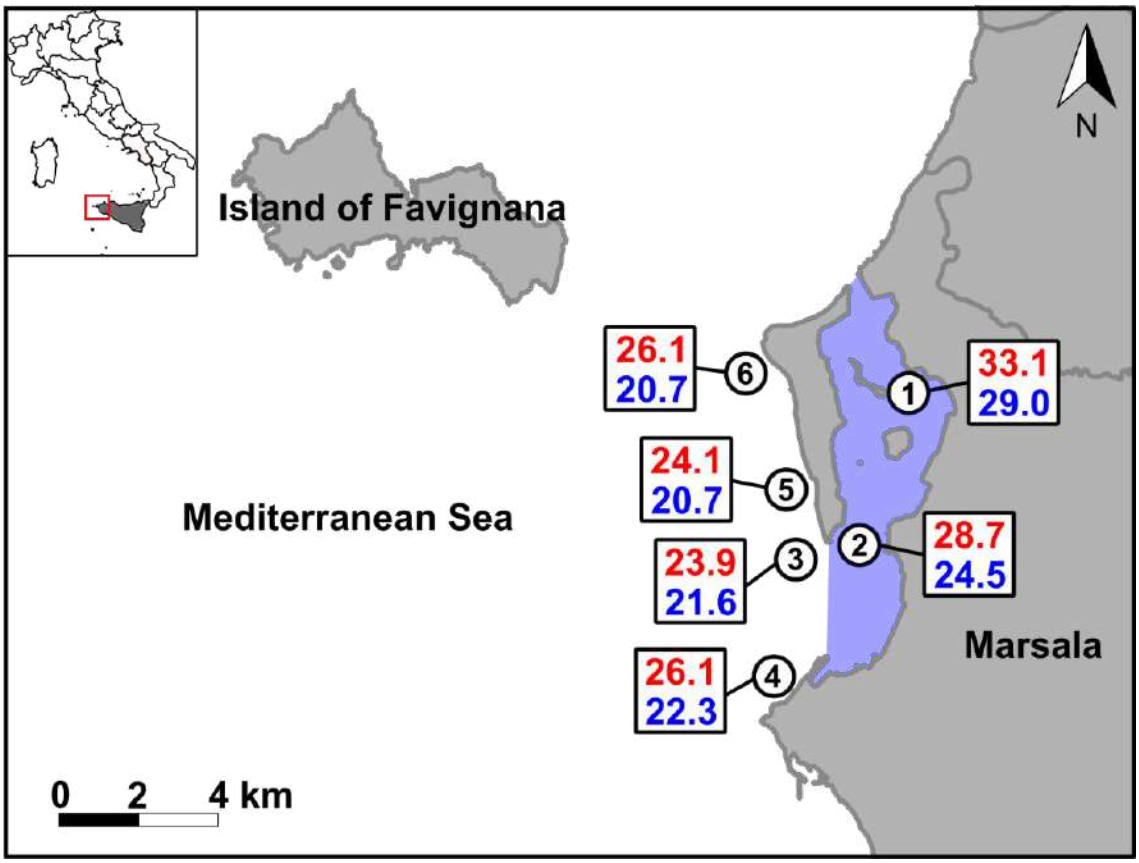


Figure 1 Sample collection sites in this study: (**1**) North-basin, (**2**) South-basin, (**3**) OpenSea-A, (**4**) OpenSea-B, (**5**) OpenSea-C, and (**6**) OpenSea-D. The Stagnone di Marsala lagoon is in light blue. The red and blue numbers indicate maximum and average sea surface temperatures (℃), respectively, at each collection site in the period May – September 2017.

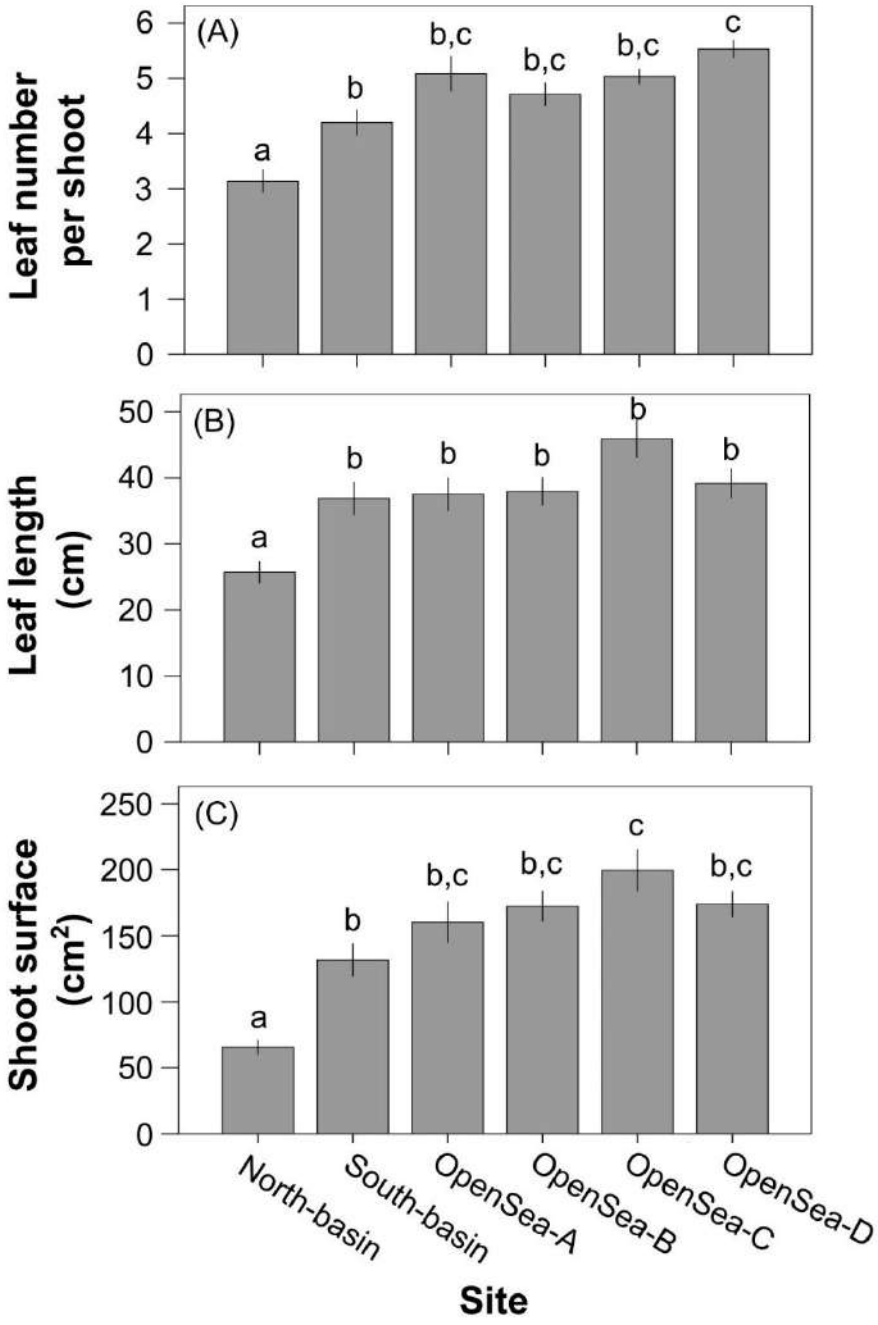


Figure 2 Leaf morphological results. Data are mean ±SE. Letters over the bars indicate results of Tamhane's T2 test (Details can be found in **Supplementary Table S1 – 3**).

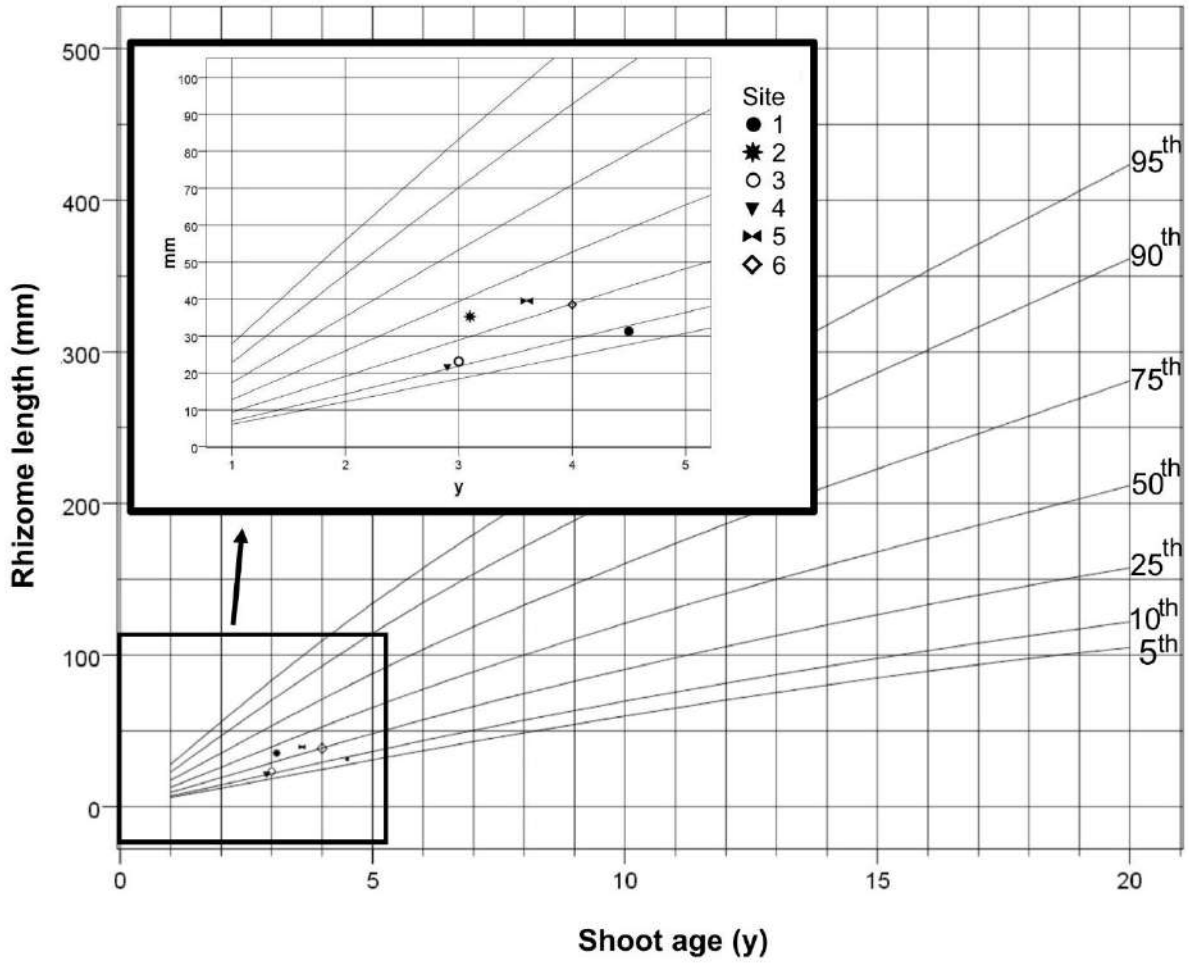


Figure 3 Growth performance measurements plotted on reference growth charts (Tomasello *et al*., 2016). (**1**) North-basin, (**2**) South-basin, (**3**) OpenSea-A, (**4**) OpenSea-B, (**5**) OpenSea-C, and (**6**) OpenSea-D. The distribution of rhizome length and shoot age averaged in each station reported 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.

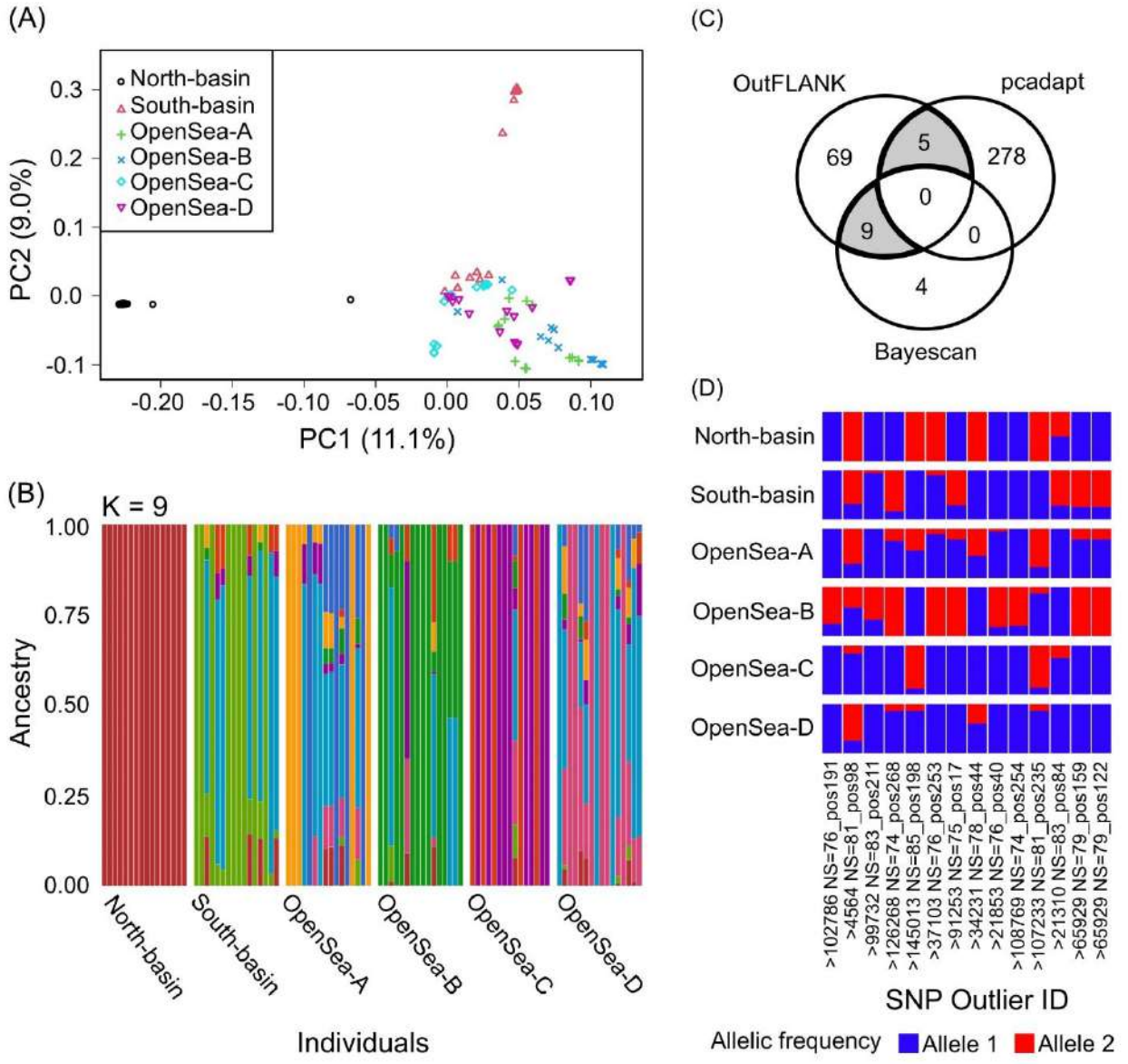
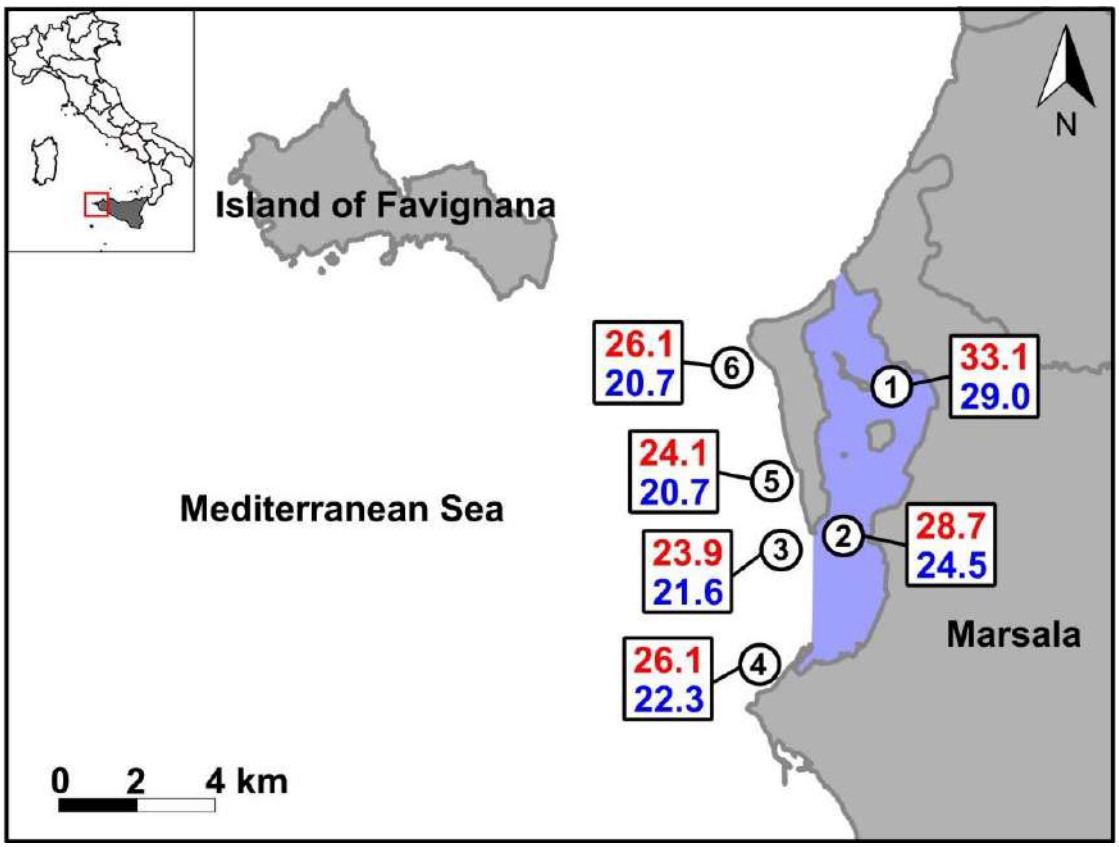
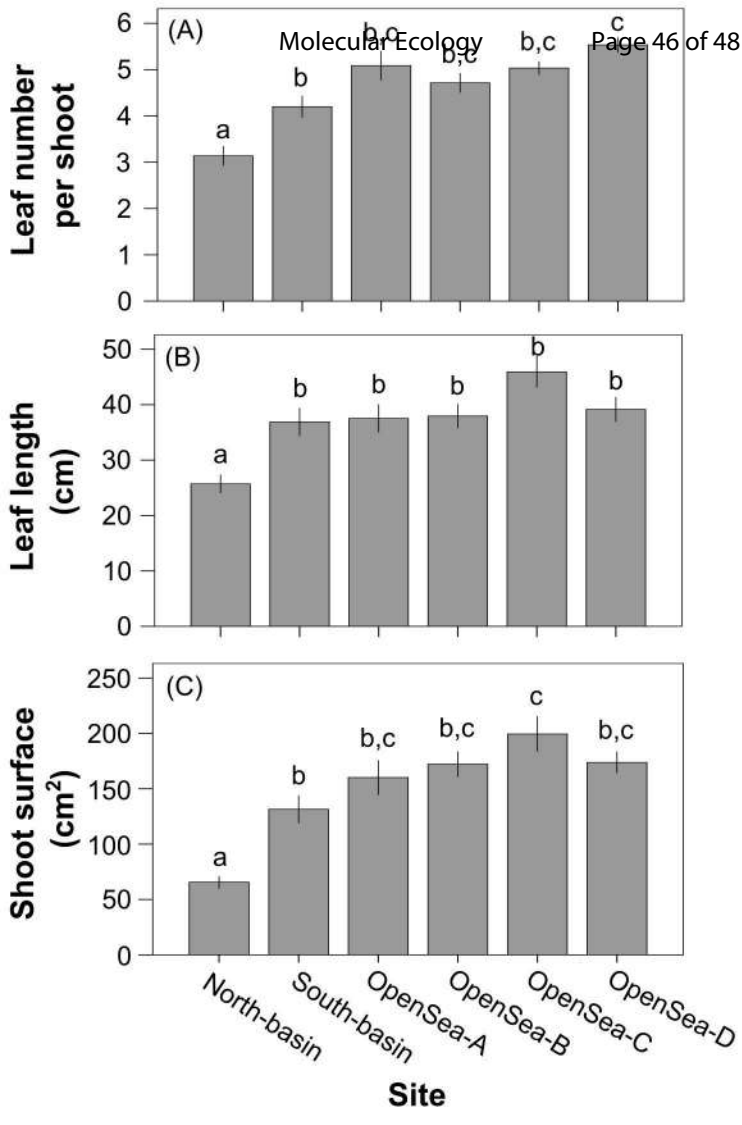
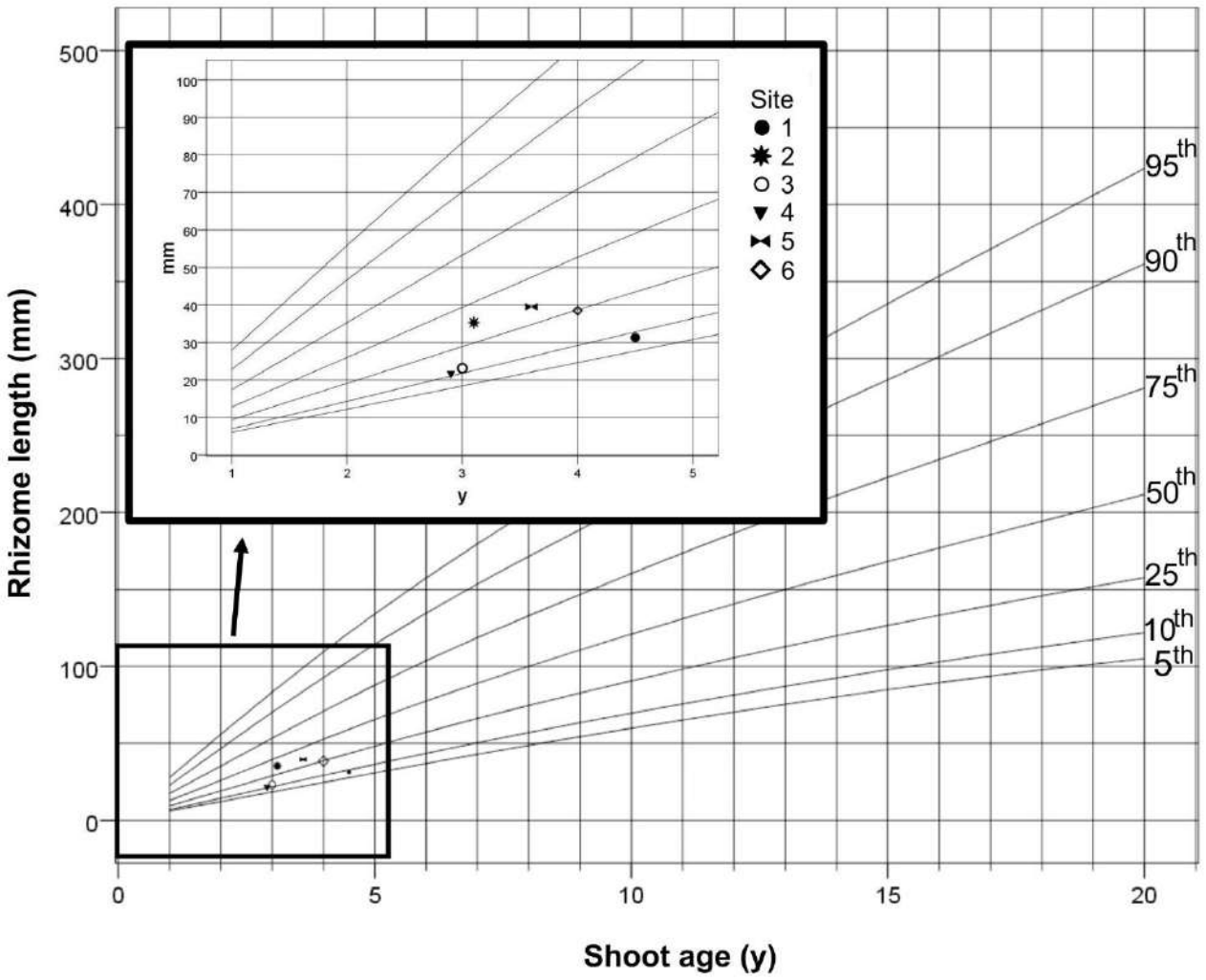
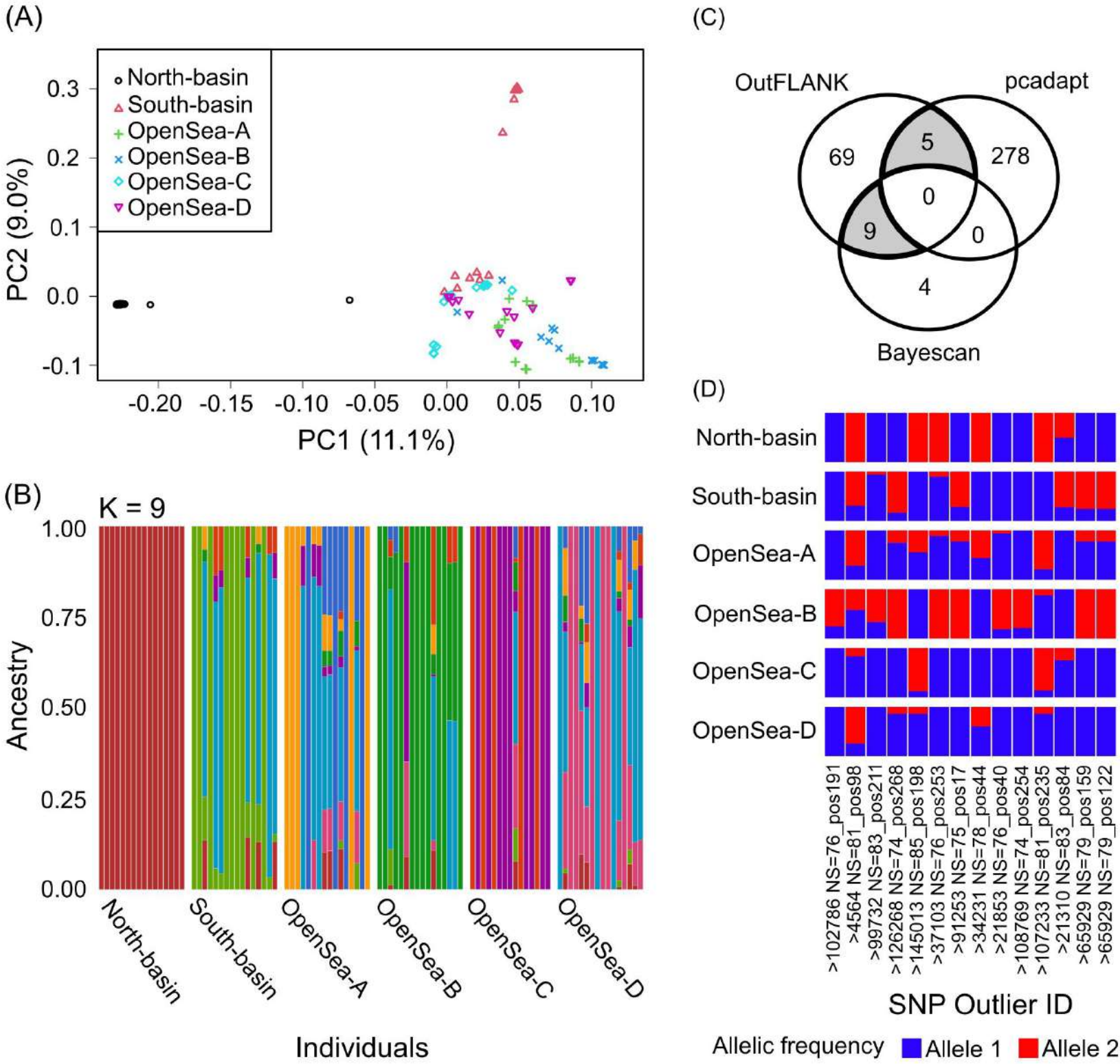


Figure 4 Results of genetic analyses for 95 *P. oceanica* samples based on all 51,329 SNPs. (A) PCA results; (B) ADMIXTURE results for K=9 with *P. oceanica* individuals on the x-axis (sorted by site) and assignment probability on the y-axis; (C) Venn diagram presents shared and unique outlier SNPs detected 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**.


Island of Favignana
Mediterranean Sea
Marsala
N
0 2 4 km
26.1
20.7
6
33.1
29.0
1
24.1
20.7
5
28.7
24.5
2
23.9
21.6
3
26.1
22.3
4


(A)
Leaf number per shoot
0
1
2
3
4
5
6
a
b
b,c
b,c
b,c
c
(B)
Leaf length (cm)
0
10
20
30
40
50
a
b
b
b
b
b
(C)
Shoot surface (cm²)
0
50
100
150
200
250
a
b
b,c
b,c
c
b,c
North-basin
South-basin
OpenSea-A
OpenSea-B
OpenSea-C
OpenSea-D
Site


Rhizome length (mm)
Shoot age (y)
0
100
200
300
400
500
0
5
10
15
20
95th
90th
75th
50th
25th
10th
5th
Site
1
2
3
4
5
6
mm
y


(A)
North-basin
South-basin
OpenSea-A
OpenSea-B
OpenSea-C
OpenSea-D
PC2 (9.0%)
PC1 (11.1%)
(B)
K = 9
Ancestry
Individuals
(C)
OutFLANK
pcadapt
Bayescan
69
5
278
0
9
0
4
(D)
SNP Outlier ID
Allelic frequency
Allele 1
Allele 2