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Genetic structure of *Spartina* hybrids between native *Spartina maritima* and invasive *Spartina densiflora* in Southwest Europe

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Highlights

- Genetic structure of *Spartina* hybrids was determined by that of their parents.
- Hybrids were genetically more similar to *S. maritima* than to *S. densiflora*.
- Hybrids with greater genetic differentiation were more spatially separated from each other.
- Hybrids with greater genetic differentiation were present in contrasted environments.
- No correlation was found between the molecular markers and plant traits.

Abstract

Interspecific hybridization represents an evolutionary force resulting in novel genotypes. The genomic changes that occur as a result of hybridization affect both genome structure and gene expression and consequently determine hybrid phenotypes and ecology. This study provides new data on the dynamics of hybrid invasions, integrating effects of the genetic, phenotypic, geographical and environmental scenario with hybridization following invasion of a halophyte community by an exotic plant species. We analyzed the spatial genetic structure of sterile *Spartina* F1 hybrid populations established at the Gulf of Cadiz (Southwest Iberian Peninsula) and that of their parental species native *S. maritima* and invasive *S. densiflora* using nuclear DNA (Simple Sequence Repeats) and chloroplast DNA sequences. We also analyzed the relationships between the spatial genetic structure of the hybrids, their phenotypic variability and their marsh environment. The studied populations of *Spartina* hybrids were establishing hybrid zones with a spatial genetic structure inherited from both parental species. The hybrids were genetically more similar to the native than to the invasive species. The hybrid populations with greater genetic differentiation were those more spatially separated from each other and that were present in more contrasted sedimentary environments, revealing respective isolation processes by distance and by environment. The

hybrids in the Guadiana Estuary were the most genetically differentiated and with the highest transgressive behavior in terms of tiller height.

Abbreviations

AMOVA, Analysis of Molecular Variance.

ANOVA, Analysis of Variance.

cpDNA, Chloroplast DNA.

GB, Genbank.

GD, Genetic distance.

PCoA, Principal Coordinate Analysis.

PD, Phenotypic Distance.

SHZ, Spanish Hydrographic Zero.

SSRs, Simple Sequence Repeats.

Keywords: Hybridization, genetic diversity, microsatellite, genetic structure, heterosis, invasion biology

Introduction

Interspecific hybridization represents an evolutionary force (Yakimowski and Rieseberg, 2014) that promotes rapid genetic, epigenetic and phenotypic changes (Cara et al., 2013; Chen and Ni, 2006; Salmon et al., 2005). By resulting in novel genotypes, hybridization can lead to more rapid adaptive changes than through mutation (Long, 1991; Minder et al., 2007). To identify the role of hybrids in the structuring of plant communities, it is necessary to analyze their underlying population genetics background, their dispersion mechanisms and the intensity of selection (Sloop et al., 2011). Hybrid zones are considered important tools for the study of evolutionary processes between divergent populations and genetic structure at the community level (Minder et al., 2007; Sloop et al., 2011; Whitham et al., 1999).

The regional-scale genetic structure that we observe today is determined by the differences generated between ancestral colonizers (Slatkin, 1987; Sloop et al., 2011). Thus, according to the classical population genetic approach, genetic similarity among populations decreases with geographic distance, explained by the isolation by distance (Malécot, 1948; Wright, 1943) and the stepping-stone models (Kimura and Weiss, 1964). This has been directly related to the reduction of gene flow between more distant populations leading to reproductive isolation promoting genetic drift and selection (Ellstrand, 2014; Slatkin, 1987). In the particular case of introduced species, genetic drift may be emphasized as a consequence of bottlenecks leading to a general reduction of genetic variability (founder effect) in the introduced population in relation to the genetic variability of the species in its native range (Brzyski et al., 2014; Castillo et al., 2018; Dlugosch and Parker, 2008). In addition, environmental (biotic and abiotic) heterogeneity may lead to genetic differentiation between populations, mainly by selective pressure that favors local adaptation (Hübner et al., 2009; Linhart and Grant, 1996) as a consequence of isolation by environment (Wang and Bradburd, 2014). The effect that hybridization may have on the established spatial genetic structure will

depend on different factors such as the genetic variability of the parental species, the dispersion capacity of the hybrids, their fertility and backcrossing frequency with the parental species (introgression) and seedling recruitment (Abbott, 1992; Arnold, 2006; Ellstrand and Schierenbeck, 2000; Graham et al., 1995; Rieseberg et al., 2003). Thus, populations of hybrids with a marked genetic structure related to reproductive isolation and local adaptation can be found (Sloop et al., 2011), as well as others without genetic differences between populations in different geographic locations when introgression and sexual reproduction homogenize populations (Yatabe et al., 2009).

The genomic changes that occur as a result of hybridization affect both genome structure and gene expression and consequently determine hybrid phenotypes (Baack and Rieseberg, 2007). In hybrid taxa, the phenotype is the combination of different types of gene expression that can be additive (equal to the mid-parent value), dominant (equal to the value of one parent) or overdominant (higher or lower than the values of both parents) (Bassene et al., 2009). Depending on these differences in gene expression, hybridization may affect the evolutionary process in different ways, being negative if low fitness hybrids with limited ecological range are produced that can lead to reproductive isolation, or having positive effects when the new genotypes hybrids with greater fitness and large ecological amplitudes (Barton, 2001). The latter are the result of the predominance of transgressive traits (exceeding trait values of both parents) related to heterosis or hybrid vigor (Chen, 2010; Lippman and Zamir, 2007). In the last decades, there have been important advances in the knowledge of molecular mechanisms underlying gene expression of hybrids, although there is still lack of understanding of the genetic regulation of the phenotypes of hybrids (Bird et al., 2018; Tirosh et al., 2009; Yoo et al., 2014). In hybrids deriving from at least one invasive species (crossed with a native or non-native species), it has been reported that heterosis frequently increases the invasiveness of the offspring (Ellstrand, 2009; Ellstrand and Schierenbeck, 2000; Hovick

and Whitney, 2014) such as the hybrids between the native *Spartina foliosa* Trin. and the invasive *S. alterniflora* Loisel in San Francisco Bay (Ayres et al., 2004). Also, hybridization involving an invasive species usually counteracts the decrease in population genetic variability as a consequence of bottlenecks in invasions derived from a single introduction event (Ellstrand and Schierenbeck, 2000), increasing the success of the invasion in the offspring.

In the genus of polyploid grasses *Spartina* (syn. *Sporobolus* following Peterson et al., 2014) (Poaceae), there are several examples of hybrids and allopolyploids between invasive and native species which exhibit transgressive traits that confer hybrid vigor (Ainouche et al., 2009; Ayres et al., 2004; Castillo et al., 2010; Lee et al., 2016; Pakenham-Walsh et al., 2010). Along the Atlantic coast of the Gulf of Cádiz (Southwest Iberian Peninsula), populations of hybrids between the native *Spartina maritima* (Curtis) Fernald. ($2n = 6x = 60$) and the invasive from the west coast of South America *Spartina densiflora* Brongn. ($2n = 7x = 70$) have been described in at least three different estuaries: the joint estuary of Tinto and Odiel Rivers, the estuary of Piedras River and the estuary of Guadiana River. Two different hybrids have been found: *S. maritima* \times *densiflora* ($2n = 9.5x = 95$), product of the fecundation of unreduced ovules of *S. maritima* fertilized by *S. densiflora* pollen in low marshes, and *S. densiflora* \times *maritima* ($2n = 6.5x = 65$) obtained by the fecundation of regular ovules of *S. densiflora* by *S. maritima* pollen in middle marshes (Castillo et al., 2010). These reciprocal hybrids are transgressive in their tiller height, lateral expansion of their tussocks, and survival along the intertidal gradient (Castillo et al., 2010). In addition, it has been observed that they play a disruptive role in the typical zonation pattern of invaded European salt marshes, showing a high invasive potential limited by their sterility (Gallego-Tévar et al., 2018a). Castillo et al. (2010) previously described the existence, maternal origin, chromosome number and potential habitat of *Spartina* hybrids in the Gulf of Cádiz. Here, we build from

this foundation, and report novel insights derived from genetic analyses to provide greater insight on effects of the hybridization process. No previous study has analyzed the genetic structure of the invasion of the parental *S. densiflora* in the Gulf of Cádiz and whether this neophyte was introduced only once or several times. Following its abundance and distribution pattern, *S. densiflora* seems to be introduced firstly in the joint estuary of Odiel and Tinto rivers (Nieva, 1996).

In this study, the spatial genetic structure of the *Spartina* hybrid populations established at the Gulf of Cadiz and that of their parental species *S. maritima* and *S. densiflora* was explored using nuclear DNA (Simple Sequence Repeats, SSRs). These co-dominant markers are potentially polymorphic and allow for genetic discrimination among populations (Baisakh et al., 2009; Gedye et al., 2012; Vieira et al., 2016) and are useful in detecting hybrids combining different parental allelic combinations (Ayres et al., 2008; Hogle and Zaremba, 2014; Sloop et al., 2011). Additionally, chloroplast (cp) DNA sequences were used to assess the maternal origin of hybrid taxa (Ferris et al., 1997; Baumel et al., 2003). We also analyzed the relationships between the spatial genetic structure of the hybrids, their phenotypic variability and their marsh environment. Hybrids and parental populations were phenotypically examined by measuring twelve vegetative morphological traits, and their sedimentary abiotic environment was characterized. This enabled us to study the genetic structure of populations of hybrids between the native *S. maritima* and the invasive *S. densiflora* in relation to their parental species. More specifically, we analyzed the relationship between genetic distances among populations and (1) their geographic distance to assess the relative contribution of gene flow and drift in the population structure and consequent isolation by distance, (2) their abiotic environmental distances to identify the influence of processes of local adaptation and isolation by environment, and (3) their phenotypic differentiation by using morphological markers to evaluate the relationship between genetic

and phenotypic distances. Considering the sterility of the studied *Spartina* hybrids, we hypothesized that the genetic structure of these hybrids would be determined by the genetic structure of the parental populations, whose genetic differentiation would increase together with the geographical distance between estuaries and with the environmental distance due to the contrasted habitat of both parental species.

Material and methods

Study sites

This study was carried out in three estuaries along the Atlantic coast of Gulf of Cádiz (Southwest Iberian Peninsula): Tinto-Odiel Estuary (37°08'–37°20'N, 6°45'–7°02'W), Piedras Estuary (37°12'–37°18'N, 7°06'–7°12'W) and Guadiana Estuary (37°10'–37°16'N, 7°16'–7°28'W) (Figure 1). These estuaries are under Mediterranean climate with Atlantic influence presenting a mean sea level of +1.85 m relative to Spanish Hydrographic Zero (SHZ) and an average semi-diurnal tidal range of 2.10 m. Their coastal marshes are characterized by a well-defined plant zonation pattern in which low marshes (+2.44 to +2.91 m SHZ) are dominated by *S. maritima*, *Salicornia ramosissima* J. Woods and *Sarcocornia perennis* (Mill.) A.J. Scott., whereas *Atriplex portulacoides* (L.) Allen, *Sarcocornia perennis x fruticosa* and *S. densiflora* are the most abundant taxa at middle marshes (+2.91 to +3.37 m SHZ), and high marshes are colonized by halophytes such as *Arthrocnemum macrostachyum* (Moric.) C. Koch, *Limoniastrum monopetalum* (L.) Boiss. and *Atriplex halimus* L. (Castellanos et al., 1994; Castillo et al., 2008a; Figueroa et al., 2003).

Plant collection

Before collecting the plant material for this study, we conducted field trips to locate tussocks of *Spartina* hybrids between *S. maritima* and *S. densiflora* following the first description by

Castillo *et al.* (2010). The hybrids were identified using the intermediate character of their adaxial crests (deeper than *S. maritima* and less than *S. densiflora*) and leaves (longer than *S. maritima* and shorter than *S. densiflora*) and the transgressive character of the tussock height (Castillo *et al.*, 2010). During these field trips, we recorded that the presence of hybrids was abundant in the Guadiana Estuary, with some hundreds of hybrids in just one marsh location known locally as ‘San Bruno’, whereas only five tussocks of hybrids were located in the Piedras Estuary and eleven were documented in the Tinto-Odiel Estuary. The collection of plant material was carried out in June-July 2016 from all known hybrid tussocks in the joint estuary of Tinto and Odiel Rivers (5 hybrids from a middle marsh close to ‘La Rábida’ monastery (37° 13' 42" N, 6° 54' 39" W), 3 hybrids from a middle marsh close to the sandspit known locally ‘La Cascajera’ (37° 11' 2" N, 6° 57' 21" W), 2 hybrids from a middle marsh at ‘Don Claudio II’ marsh (37° 11' 10" N, 6° 56' 22" W), and 1 hybrid from a low marsh at ‘Estero del Colmenar’ (37° 14' 17" N, 6° 59' 21" W)). Collections also included the only five known hybrid tussocks in the estuary of Piedras River including 1 from a low marsh area close to the road from ‘El Terrón’ seaport to ‘La Antilla’ beach (37° 13' 2" N, 7° 10' 51" W) and 4 from two different middle marsh areas (37° 13' 4" N, 7° 10' 14" W). Collections were made of 10 hybrid tussocks from ‘San Bruno’ marsh in the estuary of Guadiana River, including 5 from a low marsh area (37° 11' 41" N, 7° 24' 22" W) and 5 from a middle marsh area (37° 11' 46" N, 7° 24' 10" W) (Fig. 1). The plant material of the parental species was sampled from the closest tussocks of *S. maritima* and *S. densiflora* to the sampled hybrids (N = 18 for *S. maritima*; N = 22 for *S. densiflora*). In total, 66 individuals were collected and analyzed in this study.

Chloroplast DNA sequencing

DNA was extracted from the collected dried leaves of *S. maritima*, *S. densiflora* and their

hybrids by employing a NucleoSpin Plant Extraction Kit (Macherey-Nagel GmbH & Co, Düren, Germany). The *trnT-trnF* chloroplast DNA (Cp-DNA) region was amplified using the primer pair *a* and *b* to recover the *trnT-trnL* segment, and primer pair *c* and *f* to recover the *trnL-trnF* segment (Taberlet et al. 1991). The Polymerase Chain Reaction (PCR) was performed in a Mastercycler thermal cycler (Eppendorf AG, Hamburg, Germany) and underwent denaturation for 2 min at 94 °C, followed by 35 cycles at 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min 30 s, followed by an extension phase at 72 °C for 7 min. PCR products were purified using a NucleoSpin Gel and PCR purification kit (Macherey-Nagel GmbH & Co), and sequenced directly (on both directions) by Sanger at MacroGen Europe sequencer (Amsterdam, The Netherlands).

Simple Sequence Repeats (SSRs)

Neutral molecular markers such as SSRs are commonly used for genotyping in studies of phenotypic variations since they provide independent and complementary assessment of genetic diversity to phenotypic variation most likely submitted to selective constraints (e.g. Chapman et al., 2009). Genetic diversity was analysed at eight microsatellite (SSR) loci, using eight primer pairs (MS2, MS7, MS13, MS14, MS15, MS16, MS17 and MS18) (Table 1) designed on genomic assemblies from *Spartina maritima* and successfully employed in *S. densiflora* (Castillo et al., 2018). The protocol used for microsatellite locus amplification followed that of Baumel *et al.*, (2016) and Castillo *et al.*, (2018). DNA extracts were diluted to a concentration of 10 ng μL^{-1} and amplified using the 8 microsatellite primer pairs selected. We used FAM labelled reverse primers for PCR and amplification products were diluted to 1/30 before subsequent analysis. An aliquot of 2 μL of these diluted PCR products were mixed with 10 μL of formamide solution (975 μL formamide + 25 μL of Liz-500 size marker) and separated by electrophoresis in an ABI PRISM 16-capillary 3130xl Genetic Analyzer

(Applied Biosystems Inc., Waltham, USA). Alleles were identified and binned using GeneMapper 4.1 software (Applied Biosystems Inc., Waltham, USA).

Sedimentary variables and phenotypic plant traits

The sedimentary environment variables and phenotypic plant traits for the studied tussocks of *S. maritima*, *S. densiflora* and their hybrids were recorded at low tide during June and July 2016. Every sedimentary variable was measured at a depth of 0-5 cm in monospecific plots (ca. 50 x 50 cm) where discrete tussocks were located. Sediment redox potential (Eh) and elevation above SHZ were measured *in situ* in the field, being each measurement the mean of 3 sub-replicates. We used a Leica NA 820 theodolite (Singapore) with a resolution of 2 cm for recording elevation. Measurements were referred to m above SHZ by using tidal extremes as reference points (Ranwell et al., 1964). Sediment Eh was obtained by using a portable meter and an electrode system (Crison pH/mV p-506). On the other hand, pH of interstitial water, electrical conductivity (mS cm^{-1}) and sediment water content (%) were determined in the laboratory after collecting sediment samples in 200 ml sealed recipients. Distilled water was added to the sediment (1:1, v/v) for the determination of pH (pH/redox Crison with the electrode M-506) and double water volume was used (1:2, v/v) for the measurement of electrical conductivity (conductivity meter, Crison-522) (Curado et al., 2014). Sediment water content was measured as the difference between fresh weight (FW) and dry weight (DW) after drying ca. 100 g of sediment in a stove at 80 °C for 48 h.

The following vegetative plant traits were recorded at the same time as the collection of plant material for genetic analyses and the characterization of the sedimentary environment: number of live and dead leaves, length, base width and weight of the flag leaf (first adult leaf completely unfolded on a tiller), foliar area, the Specific Leaf Area (SLA), diameter and length of tillers and the number of leaves per tiller length, tiller density, and the

Leaf Area Index (LAI). The number of live and dead leaves, and the length, base width and weight of the flag leaf were measured in 5 tillers randomly selected from each tussock. Foliar area was calculated as a triangle using leaves width at the base and length. Then, foliar areas were used to calculate SLA ($\text{m}^2 \text{g}^{-1}$) (Garnier et al., 2001) dividing by the DW of the same leaves. The difference in leaf width between unfolded (manually) and folded (in the field) leave area was expressed in percentage to calculate leaf adaxial rolling (Premachandra et al., 1993). In addition, diameter and length of tillers and the number of leaves per tiller length (leave cm^{-1}) were recorded for the same 5 tillers per tussock. Also, the number of tillers in 10 x 10 cm plots ($n = 5$ plots) was counted for the determination of tillers density (tiller cm^{-2}). LAI ($\text{m}^2 \text{m}^{-2}$) (Jonckheere et al., 2004) was estimated as the product between the mean foliar area, the mean number of leaves per tiller and the mean tiller density of a given tussock divided by the soil occupied area by that tussock at the base of its tillers or by its aerial above-ground structures, including the area occupied by its tilted tillers.

Data analyses

Chloroplast DNA sequences of the hybrids were compared to those from *S. maritima* and *S. densiflora* collected in the three estuaries, in order to assess the maternal origin of the hybrids. These sequences are deposited in Genbank under accession numbers MK314916 to MK314943. They were compared to those previously published for *S. maritima* (GB accessions KP176438, Rousseau-Gueutin *et al.*, 2015, AF 275669, Baumel *et al.*, 2001) and *S. densiflora* (GB accession AF372629, Baumel *et al.*, 2002). Sequences were aligned using Geneious 9.1.2 software (Kearse et al., 2012) and a phylogenetic (Maximum Parsimony) analysis was performed using MEGA X software (Kumar et al., 2018)

SSR alleles were determined by comparison with the standard marker size (GeneScan-500 LIZ Size Standard), and the different “genotypes” (harbouring different allele

combinations) were scored. Since the studied *Spartina* taxa are all polyploid, allelic dosage per individual cannot be ascertained. SSRs were then treated as "dominant markers", a convenient procedure for genetic analyses in polyploids (Obbard et al., 2006). Alleles were recorded as either present or absent using the GenAlex 6.502 software (Peakall and Smouse, 2012, 2006). Up to 10 different alleles could be expected per locus since the taxa with the highest level of ploidy was *S. maritima x densiflora* ($2n = 9.5x = 95$ chromosomes) (Castillo et al., 2010). Genetic distances (GD) between individuals and populations of *S. maritima*, *S. densiflora* and their hybrids were calculated following Huff et al. (1993) and Nei (1987), respectively. The intra-population genetic diversity parameters, effective number of alleles and Shannon Information Diversity Index (Brown and Weir, 1983) were obtained for each studied population of the *Spartina* hybrids at the estuary level. In addition, Analysis of Molecular Variance (AMOVA) was used to determine the proportion of genetic differentiation within and among different hybrid populations at the estuary level. The fixation index (Φ_{PT}), analogous to Wright's (F_{ST}), was obtained following the standardization of Meirmans (2006) as an indicator of population genetic differentiation for dominant data.

Two-way analysis of variance (ANOVA) were performed to compare mean sedimentary variables and vegetative plant traits (dependent variables) for the *Spartina* hybrids and their parental species among estuaries, using taxon and estuary as grouping factors. In case of significant ANOVA, Tukey's honestly significant difference (HSD) was used as post hoc analysis. Before using the parametric tests, dependent variables were assessed for homoscedasticity using Levene's test and for normality using the Shapiro-Wilk test. When these requirements for parametric analysis were not met, transformations of type inverse (for LAI) and square root (for number of dead leaves and tiller density) were carried out. These analyses were conducted using SigmaPlot (Systat Software, San Jose, CA) version 14 for Windows. Deviations of all data were calculated as standard error of the mean (SE) and

a significance level (α) of 0.05 was applied for every analysis.

Phenotypic (PD) and sedimentary (SD) distances between plants in the field were calculated as Gower's similarity index ranging between 0 and 1 (Gower, 1971) using the package *vegan* (Oksanen et al., 2018) of R-software to obtain a pairwise dissimilarity matrix. In order to compare genetic, phenotypic, sedimentary and geographic distance matrices, different Mantel's test for Matrix Correspondence (Mantel, 1967) were conducted following the method of Smouse et al. (1986) and Smouse and Long (1992) using GenAlEx 6.502 software. Principal Coordinate Analysis (PCoA) with the SSR-based matrices of phenotypic and genetic distances, with the algorithm of Orlóci (1975), was also carried out using GenAlEx 6.502 software. Canonical Correspondence Analyses (CCA) and the Monte-Carlo permutation test were performed using the *vegan* package of R-software (R Core Team 2016). The CCA was conducted to test the significance of the relationship between the sedimentary characteristics measured at every monospecific hybrid stand and the presence/absence matrix of the different alleles of the 8 loci analyzed in individuals of each stand. The significance of the canonical correlation coefficients was evaluated with Monte-Carlo permutation tests (999 permutations).

Results

Genetic diversity

Chloroplast DNA sequences enabled the differentiation of *S. maritima* and *S. densiflora* plastomes and the determination of the maternal genome donor in hybrids. The *trnL-trnT* region exhibits diagnostic nucleotide substitutions and indels between *S. maritima* and *S. densiflora*, with a notable 384 bp deletion in *S. densiflora* (Fig. 2). There is almost no sequence variation among accessions from the same species. One *S. maritima* individual from Piedras Estuary displayed a unique 29 bp deletion, corresponding to a repeated region. This

individual was not included in subsequent genetic comparisons, as it was not involved in the parentage of the detected hybrids (see below). Six hybrids from Tinto-Odiel, 1 from Guadiana (from low marshes) and 4 from Piedras Estuaries had *S. maritima* as maternal genome donor, whereas 5 hybrids from Tinto-Odiel, 1 from Guadiana (from middle marshes) and 1 from Piedras Estuaries had *S. densiflora* as maternal parent (Fig. 2). The analyses of one hybrid individual from low marsh and another one from middle marsh in Guadiana Estuary corroborated the maternal origin of both populations previously analysed by Castillo *et al.*, (2010).

The eight SSR primer pairs amplified 1-7 different alleles per locus with an average of 5 alleles per locus and a total sum of 41 alleles. On average, the number of different alleles found per locus and individual for the parental species was 2 (1- 4) and 2 (1-6) for *S. densiflora* and *S. maritima*, respectively. At the 8 analyzed loci, 31 different alleles were found for *S. maritima* and 24 for *S. densiflora* (Table 2). According to Nei's index, the genetic distance (GD) among populations of each parent was greater between Guadiana and Tinto-Odiel (*S. densiflora*: 0.045, *S. maritima*: 0.144) and Guadiana and Piedras Estuaries (*S. densiflora*: 0.063, *S. maritima*: 0.144) than between Tinto-Odiel and Piedras Estuaries (*S. densiflora*: 0.023, *S. maritima*: 0.005). The GD between populations of both parental species in the same estuary was higher in the Guadiana Estuary (0.684) than in Tinto-Odiel (0.449) and Piedras (0.428) Estuaries.

In hybrid plants, an average of 3 (0-6) different alleles per locus and individual was obtained, with a total of 36 different alleles for the 8 surveyed loci. All the alleles observed in the parental species were also present in different proportions in the hybrids, except one absent allele of *S. densiflora* (MS18-3) and three of *S. maritima* (MS14-4, MS15-7, MS18-7). Thirteen alleles of the hybrids were unique in *S. maritima*, 8 alleles were exclusive of *S. densiflora* and 15 alleles of the hybrids were found in both parental species. This high number

of *S. maritima* alleles compared to *S. densiflora* was maintained in both *S. maritima* \times *densiflora* (13 unique alleles of *S. maritima* and 7 of *S. densiflora*) and *S. densiflora* \times *maritima* (11 of *S. maritima* and 8 of *S. densiflora*) hybrids, although the maternal proportion increased for *S. maritima* \times *densiflora* (Table 2). As for the parental species, the greatest GD for the hybrids according to the Nei's index, was found between the Guadiana and Tinto-Odiel (0.167) and between Guadiana and Piedras (0.125) Estuaries; while the GD between Tinto-Odiel and Piedras Estuaries was lower (0.028). Five unique alleles were found in Guadiana Estuary (MS14-1 and MS18-2 of *S. maritima* and the hybrids, MS15-7 of *S. maritima* and MS18-3 and MS18-4 of *S. densiflora*), 2 unique alleles were recorded in Tinto-Odiel Estuary (MS2-2 of *S. maritima* and the hybrids and MS18-7 of *S. maritima*), and 1 unique allele was found in Piedras Estuary (MS14-4 of *S. maritima*) (Table 2). Genetic differentiation of *Spartina* hybrids at the estuary level ($\Phi_{PT} = 0.547$) was higher among populations (55%) than within populations (45%) (Table 3).

These genetic differences are represented in the PCoA of GD that allowed distinguishing the parental species along the PC-2 axis and positioning the hybrids in the center, closer to *S. maritima* than to *S. densiflora*. The PC-1 axis separated individuals from different estuaries, with *Spartina* populations from the Guadiana Estuary showing negative values and those of the Tinto-Odiel and Piedras Estuaries with positive values (Fig. 3A).

As expected, the hybrids showed higher genetic diversity (I) than their parental species at all estuaries, except at Piedras Estuary where it was similar for the hybrids and *S. densiflora*. In the Guadiana Estuary, the hybrids displayed a genetic diversity 2.00 times higher than *S. densiflora* and 2.50 times than *S. maritima*, while in both Tinto-Odiel and Piedras Estuaries it was 1.25 and 1.50 higher, respectively. The intrapopulation genetic diversity of the hybrids in the Guadiana Estuary was twice that of Tinto-Odiel and Piedras Estuaries. Despite having higher total number of alleles, *S. maritima* exhibited lower inter-

individual genetic diversity than *S. densiflora* (Table 4).

Ecology and phenotypic diversity of the hybrids

The Guadiana Estuary had lower sediment salinity ($17.1 \pm 1.3 \text{ mS cm}^{-1}$) and elevation ($2.62 \pm 0.04 \text{ m}$) for every taxon than interstitial sediment salinity and elevation measured in Tinto-Odiel ($24.8 \pm 1.3 \text{ mS cm}^{-1}$ and $2.96 \pm 0.05 \text{ m}$, respectively) and Piedras Estuaries ($22.9 \pm 1.6 \text{ mS cm}^{-1}$ and $3.08 \pm 0.05 \text{ m}$, respectively). Moreover, sediments from the Tinto-Odiel Estuary exhibited lower redox potential ($-9 \pm 16 \text{ mV}$) than the other estuaries (Guadiana: $100 \pm 16 \text{ mV}$, Piedras: $79 \pm 19 \text{ mV}$). The Tinto-Odiel Estuary also had higher sediment pH (6.9 ± 0.1) than the Guadiana (6.7 ± 0.1) and Piedras (6.4 ± 0.1) sites. On the other hand, the recorded sedimentary variables did not change significantly between *Spartina* taxa within estuaries, except for sediment characteristics in the lower elevation occupied by *S. maritima* in the Tinto-Odiel Estuary as compared to areas occupied by the other studied taxa (Table 5; see Table 6 for statistical tests).

The vegetative phenotypes of the *Spartina* hybrids were characterized by some traits similar to one or to both parental species, others that were intermediate between the parental species, and some transgressive traits that were superior to both parents. Thus, SLA (except for hybrid individuals from the Piedras Estuary), the number of leaves and the leaf width for the hybrids were similar to those of *S. maritima* and greater than the values recorded for *S. densiflora*. In contrast, the tiller diameter of hybrids only for the Guadiana Estuary, and the number of dead leaves and the foliar area for all measured hybrids were similar to those of *S. densiflora* and greater than those of *S. maritima*. In addition, the number of leaves per tiller length for the hybrids was similar to this metric for *S. densiflora* and 2.5 times lower than data recorded for *S. maritima*. In contrast, leaf length, leaf rolling and tiller density for the hybrids were intermediate between the lower values of *S. maritima* and the higher values of *S.*

densiflora. Tiller length for the hybrids at Guadiana Estuary (49.8 ± 2.6 cm) and almost LAI for every estuary (P -value = 0.072) exceeded those of both parental species (*S. maritima*: 13.5 ± 3.7 cm, *S. densiflora*: 29.6 ± 2.6 cm) (see Table 6 for statistical tests).

In general, *Spartina* plants of parents and hybrids from the Guadiana Estuary had 16% shorter leaves than leaves measured in the other estuaries. Plants from the Guadiana Estuary for every taxon showed also 71% higher tiller densities, 17% lower numbers of leaves and 16% smaller leaf areas than those of Piedras Estuary. Plants from the Piedras Estuary exhibited 34% lower LAI than Tinto-Odiel Estuary and 16% greater leaf width than the other two estuaries. *Spartina maritima* growing at Tinto-Odiel Estuary showed greater SLA than at Piedras, wider tillers than at Guadiana Estuary, and taller tillers and fewer leaves per tiller length than both estuaries (Table 5; see Table 6 for statistical tests).

The phenotypic difference between both parental species and their hybrids is shown in the graphic representation of the PD that separated *S. maritima* from the other taxa along the PC-2 axis. *Spartina densiflora* and the hybrids appeared mixed, with a slight tendency to separate *S. densiflora* in the opposite direction to *S. maritima* along the PC-2 axis. Instead of the difference reported above, the combination of the recorded phenotypic plant traits did not distinguish the populations from the three estuaries for any taxon (Fig. 3B).

The Mantel tests showed that the PD of the *Spartina* hybrids was independent of their sedimentary, genetic and geographic distances. PD between hybrids tended to increase with their GD, however this relationship was not significant (P -value = 0.060) (Fig. 4A). On the other hand, GD between hybrids increased with their sedimentary and geographic distances (Fig. 4B, C).

A total proportion of 85.8% of the total variance of the data linking sedimentary variables and alleles presence for every hybrid individual was explained by the first two axes of the CCA (Fig. 5). Axis 1 was strong and negatively correlated with sediment conductivity

and marsh elevation, and Axis 2 was negatively correlated with sediment pH, and sediment water content correlated negatively with Axis 1 and positively with Axis 2 (Fig. 5; Supplementary material 1). Axes 1 and 2 were significant at $P < 0.001$, as reported by the Monte-Carlo permutation test (Supplementary material 1). All the hybrids from Guadiana Estuary and one from Piedras Estuary (H-P1) were positively correlated with Axis 1, while the rest of the hybrids from Piedras Estuary and every hybrid from Tinto-Odiel Estuary were negatively correlated with the primary axis. Alleles MS7.2, 7.4, 13.1 and 16.3 were associated negatively with Axis 1, whereas a group of 10 alleles were associated positively with Axis 1 (Fig. 5; Table S1, Supplementary material). Axis 2 was positively correlated with 2 hybrid individuals from Guadiana, 2 from Piedras and 1 from Tinto-Odiel Estuary, and negatively correlated with 1 hybrid from Guadiana, 1 from Piedras and 5 from Tinto-Odiel Estuary. Three alleles (MS7.2, 7.3 and 13.1) were positively related to Axis 2, whereas the allele MS16.3 was negatively related to this axis (Fig. 5; Table S1, Supplementary material).

Discussion

Our results show that the genetic structure of sterile *Spartina* F1 hybrids between the native *S. maritima* and invasive *S. densiflora* along the coast of the Gulf of Cadiz (Southwest Iberian Peninsula) was mainly determined by the genetic structure of the parents, the hybrids being genetically more similar to the native than to the invasive species.

Although *S. maritima* individuals exhibited more different alleles (31) than *S. densiflora* (24) at the 8 investigated loci, their genetic diversity among tussocks from the same estuary was the lowest of the studied taxa. This low inter-individual variability of *S. maritima* is consistent with its low sexual reproduction (Castellanos et al., 1994, 1998) and previous molecular investigations in Europe (Yannic et al., 2004). It also agrees with its high levels of local adaptation to stressful and stable low marshes (Castellanos et al., 1994; Castillo

et al., 2008b; Contreras-Cruzado et al., 2017). *S. densiflora*, with hybrid origin (Fortune et al., 2008), has a higher ploidy level (7x) than *S. maritima* (6x), which most likely increases genetic diversity (Petit et al., 1999; Soltis and Soltis, 2000). In contrast, *S. maritima* exhibited greater interpopulation genetic differentiation between estuaries than *S. densiflora*. The weak population structure of *S. densiflora* agrees with its invasive status and associated bottlenecks resulting from founding effects (Brzyski et al., 2014; Dlugosch and Parker, 2008). Additionally, establishment of these invading populations is more recent than the native populations of *S. maritima* that can survive for decades, or even centuries, in non-successional marshes (Castellanos et al., 1998), allowing natural selection, neutral mutations and genetic isolation to act longer on these native populations. Moreover, the genetic isolation between *S. maritima* populations may be stronger than between *S. densiflora* populations due to their lower sexual reproduction. *S. densiflora* produces many viable seeds (Kittelson and Milton, 1997; Nieva et al., 2001) that can be transported by tides and currents floating on water (Xiao et al., 2016) and by birds (Vivian-Smith and Stiles, 1994). Invasive *S. densiflora* introduced along the Atlantic coast of North America also showed low genetic differentiation among populations (Castillo et al., 2018).

Our genetic analyses using cpDNA and nuclear SSR markers confirmed the hybrid origin of every tussock identified as such in the field and the reciprocal crosses that occurred in the Guadiana, Piedras and Tinto-Odiel Estuaries. In relation to the 8 SSR loci analyzed, most of the alleles present in the parental species were also present in their hybrids (27 out of 31 of *S. maritima* alleles and 22 out of 24 of *S. densiflora* alleles), while the 48% of the alleles of *S. maritima* and 63% of *S. densiflora* were shared between both parental species. Consequently, the hybrids contained greater number of different alleles than both parental species separately. Hybridization is expected to increase the number of alleles at all loci (Long, 1991) while non-inherited alleles may be associated to changes occurred during

hybridization such as chromosomal rearrangement, gene loss or changes in genome size (Baack and Rieseberg, 2007), but we cannot reject possible technical bias in detecting some alleles. Thus, the hybrids showed higher inter-individual genetic diversity within populations than their parents, except in the Piedras Estuary population. The studied hybrids are sterile, each hybrid tussock being the product of independent hybridization events (Castillo et al., 2010). Gene flow within populations tends to homogenize the genetic differences between individuals (Lenormand, 2002; Slatkin, 1987), which does not occur in these sterile hybrids as it could occur in the parental species, especially in *S. densiflora* as mentioned above. Moreover, the fact that hybrids whose seed parent was *S. maritima* inherited all its genetic material from unreduced ovules (Castillo et al., 2010) could explain that, globally, hybrids inherited more alleles from *S. maritima* than from *S. densiflora*. Nonetheless, the maternal origin of the hybrids has not induced a scenario in which all the individuals of the same hybrids would globally share more alleles with the seed parent than with the pollen parent.

Both parental species and their hybrids showed clear spatial genetic structure. The genetic structure of the hybrids was revealed by their higher genetic differentiation among populations (55%) than within populations (45%) at the estuary level, which was the consequence of the genetic structure of their parental species. Thus, the genetic structure of the hybrids reflected local crossing between parental species, which may be related to short distance dispersal of their pollen (Davis et al., 2004), followed by restricted seed dispersal. The tussocks from the Guadiana Estuary for every taxon were clearly differentiated from those sampled in the Piedras and Tinto-Odiel Estuaries, which are located closer to each other along the coast. Thus, the spatial genetic structure of the hybrids was directly related to geographic distances among populations. The total or partial reproductive isolation by distance predicts the increase of genetic differentiation with geographic distance due to reduction or lack of gene flow between populations (Baack et al., 2015; Wright, 1943). Sloop

et al. (2011) observed that hybrids between the native *Spartina foliosa* and the invasive *Spartina alterniflora* in San Francisco Bay also showed a marked genetic structure due to limitations in seeds and pollen dispersal. The GD of the hybrids analyzed in our study was also directly related to the differences in their sedimentary environments among marsh habitats that may have imposed selective pressures on the parental species, favoring isolation by environmental processes (Wang and Bradburd, 2014). Thus, for example, *S. maritima* growing in low marshes starts its flowering earlier in the year than *S. densiflora* (B. Gallego-Tévar, personal observation). Similar observations were made in wild barley where the spatial population structure was determined not only by geographic isolation, but also by differences in environmental conditions such as elevation, temperature and rainfall (Hübner et al., 2009). In our case, plants from Guadiana Estuary were colonizing in sediments with lower conductivities and at lower elevations than in Piedras and Tinto-Odiel Estuaries. Our results demonstrated a relationship between alleles and the sedimentary environment. Some alleles recorded only for hybrids from Guadiana Estuary were associated with relatively low sediment salinities. In contrast, specific alleles of hybrids from Tinto-Odiel and Piedras Estuary were associated with higher salinities, higher elevations, and with more acidic sediments.

The hybrids from Guadiana Estuary were the only hybrids showing transgressive tiller height, while the hybrids in Piedras and Tinto-Odiel Estuaries were taller than *S. maritima* but not different from *S. densiflora*. This was probably related to the recorded greater genetic differentiation between the parental species in the Guadiana Estuary, as greater genetic distances between parents have been frequently related to the development of heterosis leading hybrid vigor in their hybrids (Ali et al., 1995; Krystkowiak et al., 2009; Pandey et al., 2018; Reif et al., 2003; Stelkens and Seehausen, 2009). Along with differences in parental genotypes among populations, sedimentary environment differences in Guadiana Estuary

(lower conductivity and elevation) may also contribute to the heterosis shown by the hybrids. Different studies in hybrids such as those on the genera *Ipomopsis* (Campbell and Waser, 2007), *Artemisia* (McArthur et al., 1998) and *Iris* (Arnold et al., 2012) proved that the phenotype and the fitness of hybrids can be strongly influenced by the environment in which they develop (reviewed in Arnold and Martin, 2010). Gallego-Tévar et al. (2018a) described the studied *Spartina* hybrids as showing very low spatial interaction with neighboring halophytes in the Guadiana Estuary. In this population, the hybrids grew taller than their parental species, and were displacing native species and even the invasive parental *S. densiflora*. These observations suggest the superior invasive behavior of these hybrids. Their high competitive ability coupled with sterility-limited ability to disperse, have resulted in a marked decrease in the spatial coincidence (two taxa growing together) of the *Spartina* hybrids with other halophytes from 62% in 2003 to 6% in 2016 though their relative cover has remained constant (Gallego-Tévar, unpublished field data).

The three taxa of *Spartina* had shorter leaves in Guadiana than in the other estuaries and higher tiller density and smaller number and area of leaves than in the Piedras Estuary. But also, all taxa exhibited greater width of leaves in Piedras Estuary, where the lower pH was registered, with respect to the other estuaries and lower LAI than in Tinto-Odiel. Although the influence of the genotype cannot be ruled out, these differences among the three estuaries could be marked by the environment since both *Spartina maritima* and *S. densiflora* are species with high reported phenotypic plasticity in contrasted environments (Castillo et al., 2016, 2014, 2005). In fact, *S. maritima* presented taller tillers in Tinto-Odiel Estuary where the sediment redox potential was lower, which has been previously described in the species (Castillo et al., 2005). A common garden experiment including all sampled hybrid populations would help to resolve the extent to which the lack of correlation between GD and PD was due phenotypic plasticity related to clear environmental differences between estuaries

and habitats.

When all phenotypical traits were taken into account, genetic, geographical and sedimentary environmental distances in hybrid populations among estuaries did not relate to their vegetative phenotypic distance, although PD and GD were close to positive correlation ($P = 0.060$). When polygenic inheritance of traits is operating, correlation between GD and PD decreases as the number of loci involved in the regulation of the phenotypic traits increases (Burstin and Charcosset, 1997; Lefebvre et al., 2001). In hybrid taxa genotype-phenotype association is even more complex since gene expression can be non-additive (Bassene et al., 2009; Meyer et al., 2007) and the molecular mechanisms that regulate gene expression are not completely identified (Bird et al., 2018; Tirosh et al., 2009; Yoo et al., 2014). In *Spartina*, hybridization and allopolyploidy were shown to be accompanied by substantial non-additive parental expression patterns in both controlled (Chelaifa et al., 2010) (Chelaifa et al. 2010) and natural conditions (Ferreira de Carvalho et al., 2017). In the hybrids studied here, both our results and previous study (Gallego-Tévar et al., 2018b) indicate phenotypic patterns reflecting parental dominance, additivity and over-dominance, making complex the genotype-phenotype relationship.

Conclusions

Populations of sterile *Spartina* hybrids between the native *S. maritima* and the invasive *S. densiflora* together with their parental species are establishing hybrid zones (Hewitt, 2008), and are developing a spatial genetic structure inherited from both parental species in the Gulf of Cadiz (Southwest Iberian Peninsula). The hybrid populations with greater genetic differentiation are those more spatially separated from each other and that are present in more contrasted sedimentary environments, revealing respective isolation processes by distance and by environment likely originating from their parental species. The hybrids in the Guadiana

Estuary were the most genetically differentiated and with the highest transgressive behavior in terms of tiller height. However, the relationship between the population genetic structure of these hybrids and their phenotype is complex and no correlation pattern was found between the molecular markers and the set of recorded vegetative morphological traits. This study provides new data on the dynamics of hybrid invasions, integrating effects of the genetic, phenotypic, geographical and environmental scenario with hybridization following invasion of a halophyte community by an exotic plant species.

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Table 1

Nucleotide sequences, of the selected 8 microsatellite primer pairs and their corresponding repeated motifs (as detected in *S. maritima* genomic assemblies) analyzed in three populations of *S. maritima*, *S. densiflora* and their hybrids in Southwest Iberian Peninsula.

Locus	Forward primer sequence	Reverse primer sequence	SSR
MS2	ATATTCCGATCCCCTCCTTG	TTCGATCGGTCATGTTTTGA	(AAAG) _n
MS7	CAGAATCACCATCATCAGCG	TTCCATTTTTTCAGGGTGAGC	(TGGCAG) _n
MS13	CTTGACCGCAACCAGTATGA	CCCAGGGCAATGGTTATACA	(TTCT) _n
MS14	TGAGTTTGAGTTCACGGTTCA	ATGTGATGCCATTTCCACAA	(AAAG) _n
MS15	TGCATTGCAGCAAGAGAATC	CGCTAGCTGATCCTGGAAAC	(GATG) _n
MS16	GGGACACGGGATAGGAAAGT	CCGCCGTGCAATTATTTATC	(GTGGA) _n
MS17	TTTGTTTCAGCTTCAGCATGG	TTCTTGCAGTCGTTCTGTGC	(GAAA) _n
MS18	TCTTATGGACCCCTTGCAGT	CATCCGATTGGCGTAAGATT	(TGATA) _n

Table 2

Allele occurrence at 8 microsatellite loci in three populations of *Spartina maritima*, *S. densiflora* and their hybrids in three different estuaries of the Gulf of Cadiz (Southwest Iberian Peninsula), TIOD, Tinto and Odiel Estuary; PI, Piedras Estuary; GU, Guadiana Estuary.

		MS 2			MS7				MS13							MS14			
		1	2	3	1	2	3	4	1	2	3	4	5	6	7	1	2	3	4
TIOD	<i>S. densiflora</i>	1	0	0	1	0.3	0	0	0.1	0.1	0	1	0.3	0	0	0	0.8	1	0
	<i>S. maritima</i>	0.1	0.1	1	0	0	1	0.1	0.4	0.1	0.1	1	0.1	0.4	1	0	0	1	0
	Hybrid	0.6	0.1	1	1	0	1	0	0	0	0	1	0	0	1	0	0.5	1	0
PI	<i>S. densiflora</i>	1	0	0.4	1	0.2	0	0	0.4	0	0	1	0	0	0	0	1	1	0
	<i>S. maritima</i>	0	0	1	0	0	1	0.2	0.6	0	0	1	0	0.6	1	0	0	1	0.2

	Hybrid	1	0	1	1	0.4	1	0.2	0.4	0	0	1	0	0	1	0	0.8	1	0				
GU	<i>S. densiflora</i>	1	0	0	1	0.2	0	0	0	0.9	0	1	0	0	0	0	0.9	1	0				
	<i>S. maritima</i>	0	0	1	0	0	1	0.2	0	1	0.8	1	1	1	0.8	0.8	0	1	0				
	Hybrid	1	0	1	1	0	1	0	0	1	0.8	0.9	1	0.9	0.9	0.6	0.9	1	0				
		MS15							MS16				MS17				MS18						
		1	2	3	4	5	6	7	1	2	3	4	1	2	3	4	1	2	3	4	5	6	7
TIOD	<i>S. densiflora</i>	0	1	0.4	1	0.8	0	0	0	1	0.1	1	0.3	0.1	1	0	1	0	0	0.3	0.9	0	0
	<i>S. maritima</i>	1	1	0.8	0	0	1	0	1	0	0	1	0	1	1	0.1	0	0	0	0	0	1	0.1
	Hybrid	0.8	1	0.5	0.4	0.6	0.6	0	0.9	0.8	0.5	0.9	0	1	0.8	0	0	0	0	0	0	1	0
PI	<i>S. densiflora</i>	0	1	0.8	0.8	0.8	0	0	0	1	0.4	1	0.2	0	1	0	1	0	0	0	0	0	0
	<i>S. maritima</i>	1	1	1	0	0	1	0	1	0	0	1	0	1	1	0	0	0	0	0	0.2	1	0
	Hybrid	1	1	0.8	0.4	0.4	1	0	1	1	0.2	1	0	1	1	0	0	0	0	0	0	1	0
GU	<i>S. densiflora</i>	0	1	0.9	0.7	0.8	0	0	0	1	0.3	1	1	0	1	0	1	0	0.1	0.4	1	0	0
	<i>S. maritima</i>	1	1	1	0.4	0	1	0.2	1	0	0.2	1	0	1	1	1	0	1	0	0	0	1	0
	Hybrid	1	1	1	0.2	0.8	1	0	1	0.9	0	1	0.8	1	1	0.8	0.9	0.9	0	0.1	0.5	1	0

Table 3

Analysis of Molecular Variance (AMOVA) illustrating the proportion of genetic variation distributed within and among populations of hybrids between *Spartina maritima* and *S. densiflora* in three estuaries of the Gulf of Cadiz, Southwest Iberian Peninsula ($\Phi_{PT} = 0.547$, $P < 0.001$).

Source	df	SS	MS	Estimated variance	Percentage
Among populations	2	48.884	24.442	2.687	55%
Within populations	23	51.155	2.224	2.224	45%

Total 25 100.038 4.911 100%

Table 4

Number of different alleles (Na), number of effective alleles (Ne), Shannon's Information Index (I) for populations of *Spartina maritima*, *S. densiflora* and their hybrids from three different estuaries of the Gulf of Cadiz (Southwest Iberian Peninsula). Values are mean \pm SE.

	Tinto-Odiel Estuary			Piedras Estuary			Guadiana Estuary		
	<i>S. maritima</i>	<i>S. densiflora</i>	Hybrids	<i>S. maritima</i>	<i>S. densiflora</i>	Hybrids	<i>S. maritima</i>	<i>S. densiflora</i>	Hybrids
Na	0.854 \pm 0.129	0.780 \pm 0.133	0.780 \pm 0.128	0.585 \pm 0.110	0.634 \pm 0.125	0.780 \pm 0.118	0.780 \pm 0.113	0.707 \pm 0.127	1.146 \pm 0.124
Ne	1.075 \pm 0.029	1.117 \pm 0.041	1.174 \pm 0.053	1.059 \pm 0.031	1.122 \pm 0.044	1.111 \pm 0.040	1.102 \pm 0.042	1.141 \pm 0.047	1.296 \pm 0.063
I	0.089 \pm 0.026	0.114 \pm 0.033	0.142 \pm 0.041	0.057 \pm 0.026	0.106 \pm 0.035	0.102 \pm 0.034	0.088 \pm 0.032	0.120 \pm 0.037	0.234 \pm 0.048
N	8	8	11	5	5	5	5	9	10

Table 5

Phenotypic traits and sedimentary variables for three populations of *Spartina maritima*, *S. densiflora* and their hybrids in three estuaries of the Gulf of Cadiz (Southwest Iberian Peninsula), TIOD, Tinto and Odiel Estuary; PI, Piedras Estuary; GU, Guadiana Estuary (values are mean \pm SE).

	<i>Spartina maritima</i>			<i>Spartina densiflora</i>			<i>Spartina</i> hybrids			
	TIOD	PI	GU	TIOD	PI	GU	TIOD	PI	GU	
Phenotypic traits	Number of leaves	5 \pm 0.3	6 \pm 0.4	4 \pm 0.4	3 \pm 0.3	4 \pm 0.4	3 \pm 0.3	4 \pm 0.3	5 \pm 0.4	4 \pm 0.3
	Number of dead leaves	0.3 \pm 0.4	1 \pm 0.5	0.3 \pm 0.5	3 \pm 0.4	2 \pm 0.5	2 \pm 0.4	2 \pm 0.4	3 \pm 0.5	4 \pm 0.4
	Leaves per tiller (cm ⁻¹)	0.17 \pm 0.02	0.30 \pm 0.02	0.31 \pm 0.02	0.08 \pm 0.02	0.12 \pm 0.02	0.11 \pm 0.01	0.11 \pm 0.02	0.11 \pm 0.02	0.09 \pm 0.01
	Leaf width (cm)	0.6 \pm 0.03	0.8 \pm 0.04	0.6 \pm 0.04	0.5 \pm 0.03	0.6 \pm 0.04	0.5 \pm 0.03	0.6 \pm 0.03	0.7 \pm 0.04	0.7 \pm 0.03
	Leaf length (cm)	17.4 \pm 2.3	19.3 \pm 2.7	13.66 \pm 2.7	39.1 \pm 2.3	38.8 \pm 2.7	29.7 \pm 1.9	27.7 \pm 2.3	27.8 \pm 2.7	28.8 \pm 1.9
	Leaf area (cm ²)	11.5 \pm 1.9	15.4 \pm 2.2	7.9 \pm 2.2	20.5 \pm 1.9	22.2 \pm 2.2	16.3 \pm 1.6	17.8 \pm 1.9	20.5 \pm 2.2	19.9 \pm 1.6
	SLA (m ² ·g ⁻¹)	0.013 \pm 0.001	0.009 \pm 0.001	0.011 \pm 0.001	0.007 \pm 0.001	0.009 \pm 0.001	0.008 \pm 0.001	0.012 \pm 0.001	0.010 \pm 0.001	0.010 \pm 0.001
	Leaf Rolling (%)	4 \pm 5	0 \pm 0	7 \pm 5	38 \pm 5	31 \pm 6	40 \pm 4	14 \pm 5	11 \pm 6	25 \pm 4
	Tiller diameter (mm)	3.5 \pm 0.2	3.8 \pm 0.2	3.0 \pm 0.2	3.5 \pm 0.2	3.2 \pm 0.2	3.2 \pm 0.1	3.3 \pm 0.2	3.7 \pm 0.2	3.6 \pm 0.1
	Tiller length (cm)	31.4 \pm 3.1	19.6 \pm 3.7	13.5 \pm 3.7	37.7 \pm 3.1	33.9 \pm 3.7	29.6 \pm 2.6	42.2 \pm 3.1	43.5 \pm 3.7	49.8 \pm 2.6
	Tillers density (cm ⁻²)	0.15 \pm 0.07	0.06 \pm 0.08	0.19 \pm 0.08	0.49 \pm 0.07	0.37 \pm 0.08	0.54 \pm 0.06	0.27 \pm 0.07	0.23 \pm 0.08	0.38 \pm 0.06
LAI	4.72 \pm 1.58	1.84 \pm 1.58	3.04 \pm 1.58	6.11 \pm 1.340	5.26 \pm 1.58	2.29 \pm 1.12	7.71 \pm 1.33	5.22 \pm 1.58	6.29 \pm 1.12	
Sedimentary variables	pH	7.0 \pm 0.1	6.5 \pm 0.2	6.6 \pm 0.1	7.0 \pm .1	6.4 \pm 0.2	6.7 \pm 0.2	7.0 \pm 0.1	6.4 \pm 0.2	6.7 \pm 0.1
	Conductivity (mS cm ⁻¹)	23.0 \pm 2.3	19.5 \pm 2.7	13.8 \pm 2.7	27.2 \pm 2.3	22.9 \pm 2.3	19.2 \pm 2.0	24.2 \pm 3.1	26.3 \pm 2.4	18.2 \pm 1.9
	Water content (%)	58 \pm 5	54 \pm 7	48 \pm 7	52 \pm 5	48 \pm 7	52 \pm 5	44 \pm 5	62 \pm 6	48 \pm 5
	Redox potential (mV)	- 65 \pm 28	109 \pm 33	85 \pm 33	16 \pm 33	59 \pm 33	143 \pm 25	21 \pm 28	69 \pm 33	73 \pm 23

Elevation (m above SHZ)	2.41 ± 0.08	3.02 ± 0.09	2.52 ± 0.09	3.22 ± 0.08	3.20 ± 0.09	2.67 ± 0.07	3.23 ± 0.03	3.04 ± 0.08	2.68 ± 0.03
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Table 6

F-statistic and *P*-values of two-way ANOVAs for phenotypic traits and sedimentary factors of populations from three different estuaries (Tinto-Odiel, Piedras and Guadiana Estuaries) and taxa (*Spartina maritima*, *S. densiflora* and their hybrids) as fixed factors, and their corresponding interactions. Significant differences are marked in bold.

	Taxa	Estuary	Taxa x Estuary	
Phenotypic traits	Number of leaves	F_{2,52} = 16.141; P < 0.001	F_{2,52} = 3.789; P < 0.05	F _{4,52} = 1.292; P = 0.285
	Number of dead leaves	F_{2,52} = 39.658; P < 0.001	F _{2,52} = 1.318; P = 0.276	F _{4,52} = 1.664; P = 0.172
	Leaves per tiller (cm ⁻¹)	F_{2,52} = 72.866; P < 0.001	F_{2,52} = 9.811; P < 0.001	F_{4,52} = 6.129; P < 0.001
	Leaf width (cm)	F_{2,52} = 18.629; P < 0.001	F_{2,52} = 8.040; P < 0.001	F _{4,52} = 2.360; P = 0.065
	Leaf length (cm)	F_{2,52} = 44.320; P < 0.001	F_{2,52} = 3.387; P < 0.05	F _{4,52} = 2.013; P = 0.106
	Leaf area (cm ²)	F_{2,52} = 13.546; P < 0.001	F _{2,52} = 2.318; P = 0.109	F_{4,52} = 3.418; P < 0.05
	SLA (m ² ·g ⁻¹)	F_{2,52} = 15.398; P < 0.001	F_{2,52} = 4.147; P < 0.05	F _{4,52} = 1.366; P = 0.259
	Leaf rolling (%)	F_{2,52} = 28.105; P < 0.001	F _{2,52} = 2.339; P = 0.101	F _{4,52} = 0.235; P = 0.917
Tiller diameter (mm)	F _{2,52} = 1.558; P < 0.221	F_{2,52} = 3.462; P < 0.05	F_{4,52} = 2.784; P < 0.05	

Sedimentary factors	Tiller length (cm)	$F_{2,52} = 37.133; P < 0.001$	$F_{2,52} = 3.177; P < 0.05$	$F_{4,52} = 4.407; P < 0.01$
	Tillers density (cm ⁻²)	$F_{2,52} = 18.753; P < 0.001$	$F_{2,52} = 4.244; P < 0.05$	$F_{4,52} = 0.371; P = 0.828$
	LAI	$F_{2,52} = 2.771; P = 0.072$	$F_{2,52} = 4.437; P < 0.05$	$F_{4,52} = 1.618; P = 0.184$
	pH	$F_{2,51} = 0.0259; P = 0.974$	$F_{2,51} = 12.403; P < 0.001$	$F_{4,51} = 0.205; P = 0.935$
	Conductivity (mS cm ⁻¹)	$F_{2,51} = 2.863; P = 0.066$	$F_{2,51} = 9.348; P < 0.001$	$F_{4,51} = 0.514; P = 0.726$
	Water content (%)	$F_{2,51} = 0.151; P = 0.860$	$F_{2,51} = 0.673; P = 0.514$	$F_{4,51} = 1.356; P = 0.262$
	Redox potential (mV)	$F_{2,51} = 0.761; P = 0.472$	$F_{2,51} = 12.827; P < 0.001$	$F_{4,51} = 2.188; P = 0.083$
	Elevation (m above SHZ)	$F_{2,51} = 16.070; P < 0.001$	$F_{2,51} = 28.913; P < 0.001$	$F_{4,51} = 6.626; P < 0.001$

Figure legends

Fig. 1. Map of the Gulf of Cadiz (Southwest Iberian Peninsula) with the three estuaries sampled for hybrids between *Spartina maritima* and *S. densiflora*: Tinto-Odiel, Piedras and Guadiana Estuaries. Symbols mark sampling points.

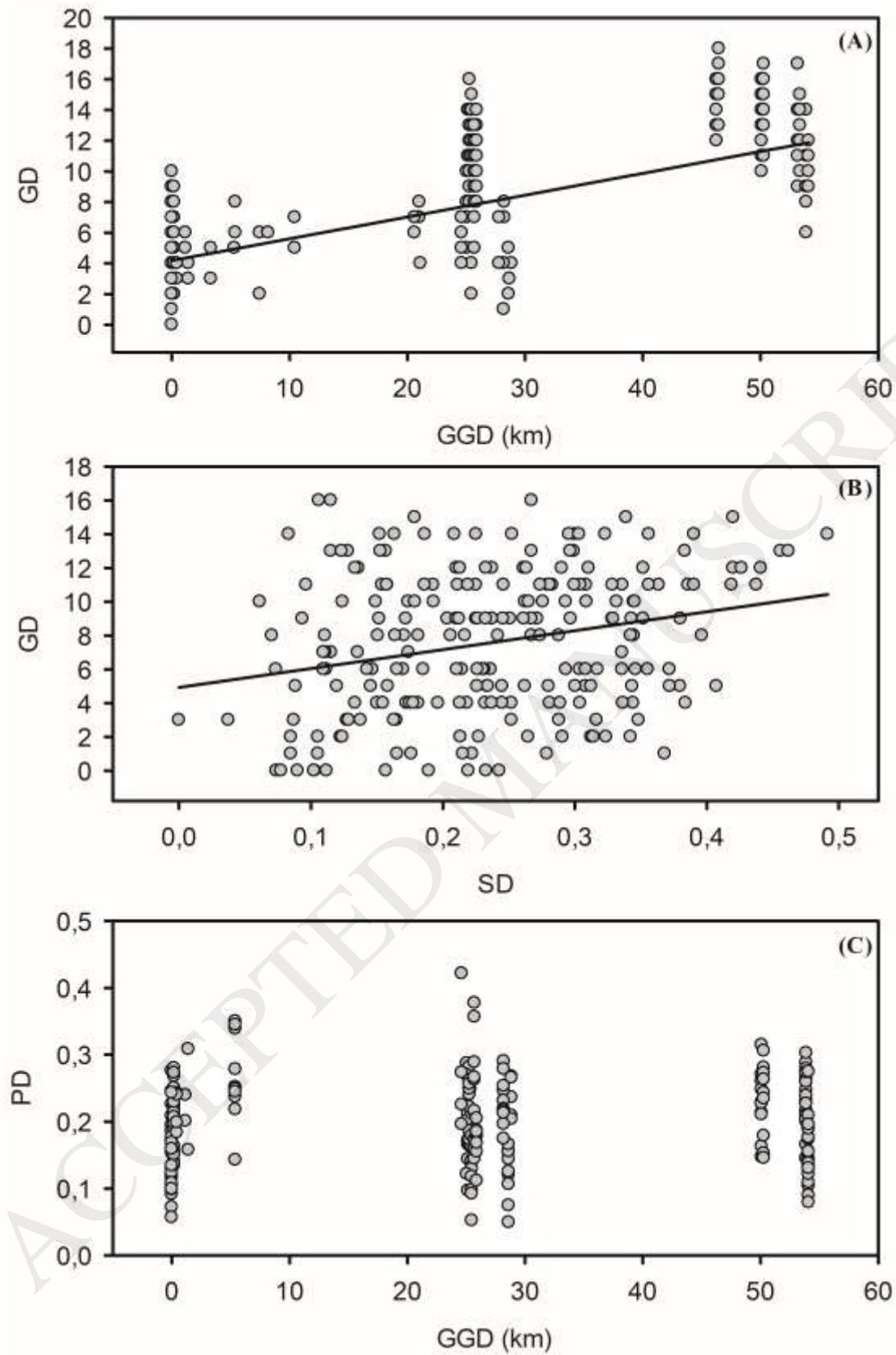
Fig. 2. Phylogenetic analysis (Maximum Parsimony) of the intergenic trnT-trnL sequences from *S. densiflora*, *S. maritima* and their hybrids (745 aligned nucleotide sites). The following accessions from Genbank were included in the analysis for comparison: KP176438 and AF275669 for *S. maritima* and AF372629 for *S. densiflora*. All positions containing gaps and missing data were eliminated. Two informative indels have been coded (as present/absent additional character states): one deletion of 384 pb characteristic of the *S. densiflora* haplotype and its corresponding insertion characteristic of the *S. maritima* haplotype; and the other indel at one nucleotide site where the *S. densiflora* haplotype has an additional nucleotide (G) that is absent (gap) from the *S. maritima* haplotype. Differential character states of eight parsimony informative characters (Pi1 to 7) which distinguish the two reference haplotypes are indicated on the respective branches. Five are represented by substitutions and two by indels of 384 bp (Pi⁴) and 1 bp (Pi⁷). One out of 10 most parsimonious trees (length = 8) is shown. The consistency index is (1,0), the retention index is (1,0). Bootstrap % values (500 replicates) are indicated.

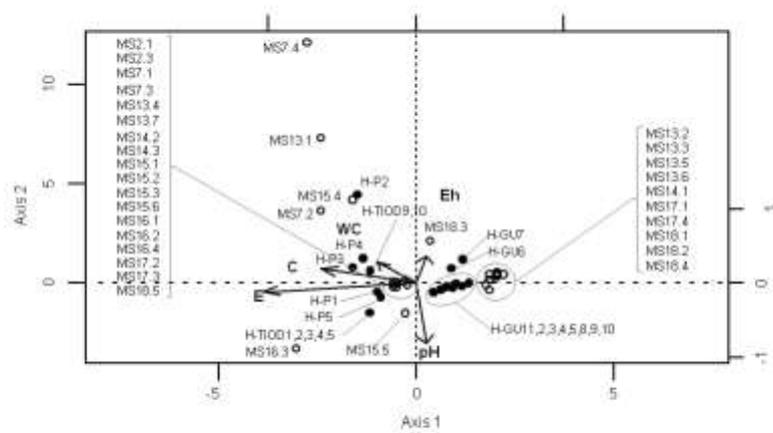
Fig. 3. Principal Coordinates Analysis (PCoA) of of *Spartina maritima* (Sm, black symbols), *S. densiflora* (Sd, white symbols) individuals and their hybrids (H, gray symbols) based on (A) Huff genetic distance using 8 SSRs loci, and (B) on Gower dissimilarity index using 12 phenotypic plant traits for 66 clumps from three different estuaries on the Gulf of Cadiz

(Southwest Iberian Peninsula): TIOD (squares), Tinto and Odiel estuary; PI (triangles), Piedras estuary; GU (circles), Guadiana estuary.

Fig. 4. Mantel tests between genetic (GD), phenotypic (PD), geographic (GGD) and sedimentary (SD) distances for hybrids between *Spartina maritima* and *S. densiflora* from the Gulf of Cadiz (Southwest Iberian Peninsula) (N = 22-26). Regression equations: (A) $y = 0.1421x + 4.1831$, $R^2 = 0.461$, P -value = 0.010, (B) $y = 11.202x + 4.9277$, $R^2 = 0.066$, P -value = 0.010; (C) P -value = 0.060.

Fig. 5. Ordination diagram of a Canonical Correspondence Analysis (CCA) with hybrid individuals (black circles), alleles of the 8 SSR loci analyzed (open circles) and sedimentary variables (arrows). SSR loci are listed in Table 2. Sedimentary variables: WC, water content; C, electrical conductivity; Eh, redox potential; E, elevation.





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