BIODIVERSITY RESEARCH

The production of hybrids with high ecological amplitude between exotic *Spartina densiflora* and native *S. maritima* in the Iberian Peninsula

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ABSTRACT

Aim Hybridization is a common and potent mechanism of plant evolution that has the potential to be evolutionary significant in its own right, and hybrids are common between invasive and native congeneric species. Our aims were to document the existence and nature of new *Spartina* hybrids arising between introduced *Spartina densiflora* and native *S. maritima* in tidal marshes of the Iberian Peninsula, to examine the actual and potential range of hybrids in the intertidal zone, and to analyse the seed set of hybrids.

Location South-West Iberian Peninsula.

Methods Hybrids were characterized using chloroplast and nuclear DNA, and ploidy assessments. The ecological tolerance of the hybrids was studied using vegetation surveys and transplant experiments.

Results We found that both parental species have been seed parents to hybrids and that all hybrids had an additive pattern of species-specific nuclear markers consistent with F_1 hybrids. Hybrid chromosome numbers varied between $2n = $ ca. 65 and $2n = $ ca. 94–97, while *S. maritima* had $2n = $ ca. 60 and *S. densiflora* had $2n = $ ca. 70. Hybrids grew in three discrete locations along the intertidal zone but were capable of growing throughout the ranges of both parental species in transplanted gardens, and in most cases, grew better than the parental species. While the potential exists for the origination of another invasive *Spartina* hybrid species, thus far hybrid plants are not fertile, limiting their ability to invade and spread.

Main conclusions We recommend the eradication of all of the currently quite limited hybrid cordgrass and to fight the *S. densiflora* invasion in the Iberian Peninsula to prevent the origination of a new invasive allopolyploid *Spartina* species.

Keywords
Chloroplast DNA, cordgrass, Guadiana, invasion, Odiel, Piedras.

INTRODUCTION

Hybridization is a common and potent mechanism of plant evolution that has the potential to be evolutionary significant in its own right, showing a creative role in producing new lineages (Arnold, 1997). Following hybridizations, new invasive allopolyploid species have formed in several genera including *Spartina* (see Table 1 in Schierenbeck & Ellstrand, 2009). Increased invasion ability of hybrids was postulated by Ellstrand & Schierenbeck (2000) to arise because of evolutionary novelty, increased genetic variation, fixed heterosis and dumping genetic load. In addition, the colonization of newly available territories may be facilitated by interspecific gene exchanges, allowing some species to bypass colonization by seeds (Petit *et al.*, 2003), and hybridization may also facilitate the development of ecological succession at the same time that a new hybrid taxon originates (Figueroa *et al.*, 2003). While hybridization occurs naturally without human intervention, it

Table 1 Frequency of species-specific Random Amplified Poly-morphic DNA bands, identified by primer and size, for species and hybrids. Frequencies of some bands may not reach 1.0 in hybrids if the species parent was a heterozygote (band present/band absent), or if the fragment was polymorphic in the parental species, such as primer C1 band 1600

Primer	Band size (bp)	Frequency of band		
		Spartina densiflora	*S. maritima*	Hybrids
A7	550	1.0	0.0	1.0
	950	0.0	1.0	1.0
B12	1500	1.0	0.0	1.0
C1	325	0.0	1.0	0.9
	1600	0.4	0.0	0.4
C12	1200	1.0	0.0	0.8
	1500	0.0	1.0	1.0
D5	575	1.0	0.0	1.0
F10	575	1.0	0.0	1.0
	800	0.0	1.0	1.0
G2	400	0.0	1.0	1.0
	1050	0.0	1.0	1.0
G9	550	1.0	0.0	1.0
G18	550	0.0	1.0	1.0
H7	400	1.0	0.0	1.0
	650	0.0	1.0	1.0
	950	0.0	1.0	0.9

is greatly facilitated by human introductions (Abbott, 1992; Ramsey & Schemske, 1998).

In this context, human-mediated invasions of *Spartina* cordgrasses are becoming a model system for studying the outcomes of interspecific hybridizations in plants. Interspecific hybridizations between native and exotic *Spartina* have resulted in: (1) the allopolyploid species *S. anglica* C. E. Hubbard arising in the 1800s through polyploidization from *S.* × *townsendii* (Ferris *et al.*, 1997; Ayres & Strong, 2001; Baumel *et al.*, 2001); (2) sterile hybrids between *S. foliosa* Trin. and *Spartina densiflora* Brongn. (Ayres *et al.*, 2008b) and between *S. alterniflora* Loisel. and *S. maritima* (Curtis) Fernald in the United Kingdom (UK) forming *S.* × *townsendii* Groves (Marchant, 1968), and in France forming *S.* × *neyrautii* (Baumel *et al.*, 2003); and (3) introgressing hybrid swarms between *S. alterniflora* and *S. foliosa* (Ayres *et al.*, 1999). Recent investigations into the origins of *S. densiflora*, native to South America, indicate that this species has an ancient hybrid origin most likely between *S. alterniflora* and *S. arundinacea* (Fortune *et al.*, 2008). Taken together the foregoing suggests that the hexaploid clade of *Spartina* (Baumel *et al.*, 2002), formed by *S. alterniflora*, *S. foliosa* and *S. maritima*, may be particularly prone to interspecific hybridization.

Spartina hybrids, both recent and ancient, have shown high competitive ability and a wide ecological range (Kittelson & Boyd, 1997; Nieva *et al.*, 2001; Pakenham-Walsh, 2003; Ayres *et al.*, 2004; Hall *et al.*, 2006; Nehring & Hesse, 2008; Strong & Ayres, 2009). Consequently, the spread of *Spartina* hybrids may imperil native species, including the native *Spartina*, and

native habitats (Huiskes *et al.*, 1995; Ayres *et al.*, 2003, 2004; Hall *et al.*, 2006). However, few studies have experimentally examined the ecological range of *Spartina* hybrids in relation to parental species.

Spartina densiflora was introduced to the Southwest Iberian Peninsula centuries ago from South America (Bortolus, 2006). Since then, the species has colonized salt marshes across most of the full tidal range of the region (Nieva *et al.*, 2001). The invasion of *S. densiflora* brought it into contact with the indigenous low-marsh dominant, *S. maritima*, which has a key role as a primary colonist of intertidal mudflats (Castellanos *et al.*, 1994). Recently, atypical *Spartina* plants exhibiting a phenotype intermediate between *S. maritima* and *S. densiflora* have been discovered in estuaries sustaining co-occurring populations of both cordgrass species. The phenotypically intermediate plants had leaves longer than those of *S. maritima* but shorter than *S. densiflora*, and the ridges on their adaxial surface were more pronounced than in *S. maritima* but less than in *S. densiflora*. These distinct plants were observed at three different estuaries along the SW coast of the Iberian Peninsula.

Here, we describe the outcome of hybridization between *Spartina densiflora* and *S. maritima* in three different estuaries along the SW Iberian Peninsula. To find out whether the unusual *Spartina* plants were hybrids of *S. densiflora* and *S. maritima*, we determined chromosome numbers for ploidy assessment. We employed species-specific chloroplast DNA sequences to determine seed parentage (Ferris *et al.*, 1997) and nuclear DNA markers to document the range of hybrid types within each estuary. We conducted vegetation surveys to investigate actual range and abundance of hybrids in the intertidal zone, we assessed hybrid fitness by measuring seed set in several populations and we compared the potential range and growth of putative *S. densiflora* × *maritima* hybrids with both parental species by carrying out transplant experiments at various elevations along the intertidal zone.

METHODS

Study sites

This work was carried out at Odiel, Piedras and Guadiana Marshes on the Atlantic coast of the Southwest Iberian Peninsula (Odiel Marshes: 37°08′–37°20′N, 6°45′–7°02′W; Piedras Marshes: 37°12′–37°18′N, 7°12′–7°06′W; Guadiana Marshes: 37°10′–37°16′N, 7°28′–7°16′W) (Fig. 1). These salt marshes are natural areas protected by the Andalusian government. The semidiurnal tides have a mean range of 2.10 m and a mean spring tidal range of 2.97 m, representing 0.40–3.37 m above Spanish Hydrographic Zero (SHZ). Mean sea level is +1.85 m relative to SHZ. The area is under a Mediterranean climate with Atlantic influence.

We searched for phenotypically unusual *Spartina* plants during field surveys of every estuary along the SW coast of the Iberian Peninsula. We discovered unusual *Spartina* plants at five marshes in three different estuaries. In each of these
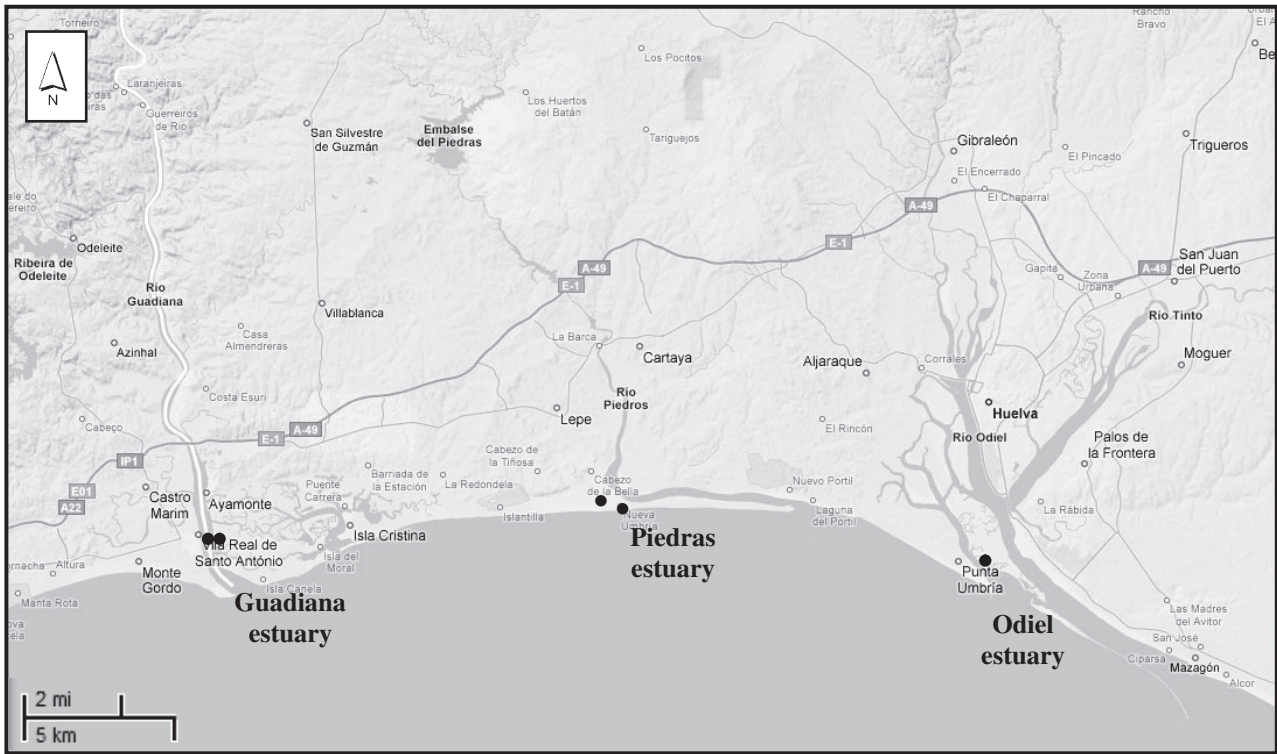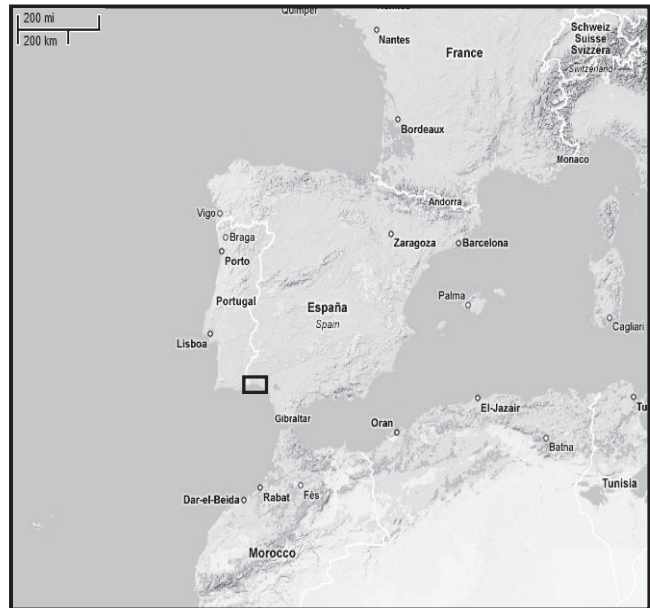
Figure 1 Map showing the location of Guadiana, Piedras and Odiel estuaries on the SW coast of Iberian Peninsula. ● sampled points for **4** genetic and chromosome analyses.

locations, *S. maritima* occurred within a few metres of *S. densiflora*, which is a frequent occurrence in the Iberian salt marshes invaded by the South American species. Tissues from putative *Spartina* hybrids were collected for genetic, chromosome and seed set analyses from (1) a low marsh (between +2.00 and +2.20 m SHZ) dominated by *S. maritima* in Odiel Marshes where two clumps of phenotypically unusual *Spartina* were detected (37°10′50″N, 6°57′09″W); (2) a low

marsh (between +2.20 and +2.60 m SHZ) dominated by *Sarcocornia perennis* ssp. *perennis* (Miller) Scott. in Piedras Marshes with just one *Spartina* clump with unusual phenotype (37°13′17″N, 7°10′46″W); (3) a middle marsh (between +2.70 and +2.85 m SHZ) dominated by *Atriplex portulacoides* L. and densely invaded by *S. densiflora* in Piedras Marshes where just one *Spartina* clump with an unusual phenotype was detected (37°12′39″N, 7°10′12″W); (4) a low marsh (between +1.80

and +2.60 m SHZ) dominated by *S. perennis* in Guadiana Marshes where three unusual *Spartina* clumps were found (37°11′42″N, 7°24′18″W); and (5) a middle marsh (between +2.90 and +2.95 m SHZ) dominated by *S. densiflora* in Guadiana Marshes where tens to 100+ of unusual *Spartina* plants were present (37°11′43″N, 7°24′69″W) (Fig. 1).

Random Amplified Polymorphic DNA (RAPD)

RAPD primers that amplified DNA fragments specific to *Spartina maritima* and *S. densiflora* were identified from previous work (Ayres & Strong, 2001 and Ayres *et al.*, 2008b; respectively). The two species were then evaluated for band species-specificity using DNA extracted according to the protocol in Daehler *et al.* (1999) from 10 *S. densiflora* and 10 *S. maritima* individuals growing at least 10 m apart one from each other in both studied marshes in Guadiana estuary. Operon (originally Operon Technologies, Alameda, CA, USA) primers used were A7, B12, C1, C12, D5, F10, G2, G9, G18 and H7. DNA from 10 suspected hybrids (the two clumps detected in Odiel Marshes, the two detected in Piedras Marshes and six from Guadiana Marshes) was amplified using the above primers. Reaction conditions are described in Daehler *et al.* (1999).

Maternity – chloroplast DNA (cpDNA)

Using universal primers provided by Taberlet *et al.* (1991), we amplified the trnT × trnF chloroplast intergenic spacer region for all samples as in Blum *et al.* (2007). The ca. 1750 bp region was amplified in two sections; the primer pair trnA-trnB was used to recover the trnT × trnL segment, and the primer pair trnC-trnF was used to recover the trnL × trnF segment (Taberlet *et al.*, 1991). Because indels are common across the trnT × trnF region, amplicons from panels of parental *S. densiflora* and *S. maritima* were electrophoresed on 1% agarose gels to compare the size of trnT × trnL and trnL × trnF segments in each species. A ca. 380 bp indel was found in the trnT × trnL region that differentiates *S. densiflora* chloroplast lineages from *S. maritima* lineages, which was consistent with their position in the two clades described for genus *Spartina* by Baumel *et al.* (2002). Following protocols in Blum *et al.* (2007), we characterized the basis of the observed size difference by sequencing the trnT × trnF region in 10 *S. densiflora*, 10 *S. maritima* and 10 hybrids from Guadiana Marshes. Haplotypes were differentiated by base pair polymorphism and indel variation, with comparisons made to all *S. densiflora* and *S. maritima* trnT × trnL and trnL × trn F accessions available on Genbank. The size difference of trnT × trnL PCR amplicons, as scored on 1% agarose gels, was subsequently used to identify the cpDNA ancestry of putative hybrids.

Chromosome counts

Actively growing roots tips were collected from vegetatively propagated *S. densiflora*, *S. maritima* and putative hybrids

grown in the University of Leicester greenhouses, originally collected from Odiel, Piedras and Guadiana Marshes from the same clumps used for molecular analysis. Actively growing root tips were removed from the plants, pretreated with ∂ Bromo naphthalene or 0.002M 8-Hydroxyquinoline for 24 h at 4 °C h, fixed for 24 h in 3:1 ethanol: acetic acid, hydrolysed for 10 min in 5 N HCl at 20 °C and stained in 2% aceto orcein. After acid hydrolysis, the root cap was cut off and discarded, and the meristematic regions were cut off, then placed on a cleaned microscope slide in a drop of 2% orcein (Sigma certified) in 45% acetic acid and the meristematic tissue teased out into the drop of stain using fine tungsten needles. Slides were then gently heated, squashed and examined under a Zeiss Large Research microscope using the Planapo ×63 objective. Careful drawings were made to determine the chromosome count, and suitable preparations were recorded photographically.

Ecological studies

Ecological studies were carried out over a tidal gradient that included the low and the middle marshes of the Guadiana estuary, as noted earlier. The relative cover of each taxon was recorded using the point-intercept method along three parallel linear transects (243 m long) perpendicular to the tidal line in January 2005. Cover data were grouped every 50 cm. Elevation relative to SHZ was surveyed along the three transects to a resolution of 2 cm with a Leica NA 820 theodolite (Singapore); reference points were determined in relation to measurements of tidal extremes (Ranwell *et al.*, 1964).

Six sampling and transplant points were located along one of the transects at different plant zones, at elevations of 1.47, 1.82, 2.70, 2.76, 2.93 and 3.16 m relative to SHZ on November 2005. Redox potential of the sediment was determined seasonally during 2006 between 0 and 10 cm soil depths with a portable metre and electrode system (Crison pH/mV p-506). Since no differences were detected between sample days (*T*-test for depended samples, $P > 0.05$), all data were analysed together ($n = 60–120$). On August 2006, ca. 150 mL of sediment from a depth of 0–10 cm was stored in sealed containers and transported to the laboratory where pH (pH/redox Crison and its electrode M-506) and porewater electrical conductivity, as an estimation of sediment salinity (conductivity metre, Crison-522), were recorded ($n = 6–32$) at 25 °C (Castillo *et al.*, 2005). Soil water content was gravimetrically determined in August 2008 by weighing 100 g of soil before and after drying for 24 h at 80 °C to constant weight ($n = 5–10$). Every abiotic variable was recorded during low tide to minimize short-term temporal variations. The daily inundation period with spring tide (h day^{-1}) was calculated from tide tables.

Material of *S. maritima*, *S. densiflora* and putative hybrids was transplanted to each of the six locations on the transect on December 2005. Transplants were small clumps that were chosen primarily so that each had a similar amount of underground reserves (represented by rhizomes and roots) and 15 adult tillers (Castillo *et al.*, 2000). *Spartina densiflora* and

hybrid plants were obtained from an elevation of *c.* +2.90 m SHZ on the transect where both taxa are well established in a common environment. *Spartina maritima* plants were obtained from the lower part of the transect (*c.* +1.90 m SHZ) where it was growing. No attempt was made to remove local sediment adhering to the clumps. Plant material for every taxon was obtained from different and representative individuals separated at least 10 m from each other. These plants were also used for molecular and chromosome analysis. Five to ten replicated clumps of each taxon were planted at each location on the transect in a row parallel to the tidal line. Individual clumps were planted manually at a depth of 10–15 cm after manually removing the existing vegetation to avoid interspecific competition. The manual removal of colonizing plants was carried out periodically during the entire experimental period (Castillo *et al.*, 2000).

Clump mortality and tiller relative growth rate (TGR) were determined 32 months after planting for the three taxa at each location on the transect. A clump was considered dead when no green tillers remained. TGR was calculated as the net increase in number of live tillers per surviving clump after 32 months, divided by the initial number of tillers (tiller tiller^{-1} year^{-1}). Tiller height was measured from the base of the tiller to the tip of the tallest leaf for every surviving transplant in August 2008 ($n = 10$ in each clump; chosen at random among the population of mature, taller shoots without a flowering spike). The median survival time of *S. maritima* and *S. densiflora* shoots has been previously estimated at 9–15 months (Castellanos *et al.*, 1998; Nieva *et al.*, 2005), so green shoots 32 months after transplantation were considered to be new shoots having emerged after successive cycles of growth and senescence.

Seed set

The seed set for known hybrid plants at the five marshes was assessed on three mature inflorescences per plant (1–10 plants per population). Florets were considered to have a ripe seed if it could be felt when the floret was lightly pressed with a fingertip. Spikes were collected between October and November 2009 and stored at 5 °C in the dark until they were analysed. In addition, seed set for *S. maritima* and *S. densiflora* was also recorded at one population in the studied locations in Guadiana Marshes where the hybrid invasion was more extensive.

Statistical analyses

Analyses were carried out using SPSS release 12.0 (SPSS Inc., Chicago, IL, USA). Deviations were calculated as the standard error of the mean. Data were tested for normality and homogeneity of variance. General linear models were build to compare variations in plant traits (TGR and tiller height) along the transplant transect in which taxon, elevation and their interaction were the independent variables. When the *F*-test was significant ($P < 0.05$), Tukey's-*b* test was used for multiple mean comparisons between transplant points and taxa.

RESULTS

RAPDs

We found seven bands specific to *S. densiflora* and nine bands specific to *S. maritima* (Table 1). All of these bands coincided with those recorded for each *Spartina* species in previous studies (*S. densiflora*: Ayres *et al.*, 2008b; Fortune *et al.*, 2008; *S. maritima*: Ayres & Strong, 2001) proving that the Spanish samples were representative of both parental species. As was found in previous work with these two species (Ayres & Strong, 2001; Baumel *et al.*, 2001; Ayres *et al.*, 2008b; Fortune *et al.*, 2008), very little nuclear variation was found in either species. However, one polymorphic band (c. 1600 bp) in *S. densiflora* was found using primer C1. The *Spartina* plants with unusual phenotype showed an additive pattern in which plants mostly contained all species-specific markers from both parents (16 bands). Four of these putative hybrid individuals lacked one or two diagnostic fragments for *S. densiflora* or *S. maritima*.

Maternity-cpDNA

Chloroplast DNA (cpDNA) haplotype analysis demonstrated that both *S. maritima* and *S. densiflora* have acted as seed parents of hybrids in Iberian estuaries. Hybrids derived from *S. densiflora* and *S. maritima* seed parents were segregated along the tidal range in both the Piedras and Guadiana estuaries. Those hybrids having *S. maritima* as a seed parent were located at low marshes close to *S. maritima* plants, and those with *S. densiflora* as a seed parent occurred at middle marshes near *S. densiflora* plants (Table 2).

Both indel size variation and base pair polymorphism differentiated *S. densiflora* and *S. maritima* cpDNA lineages. Across the trnT × trnL, two single base pair indels and one ca. 380 bp indel differentiate the two species. One four base pair indel in the trnL × trnF region differentiated the two species.

Table 2 Chloroplast DNA (based on a ca. 380 bp indel found in the trnT × trnL region), chromosome number and seed set for *Spartina maritima* × *densiflora* hybrids at low and middle marshes in three different estuaries at SW Iberian Peninsula. The number of plants used in each location is also indicated (between parentheses when the number used for seed set was different)

Location	Habitat	# plants	Cp DNA	Chromosome number	Seed set
Odiel	Low marsh	1	*S. maritima*	ca.94	0
Piedras	Low marsh	1	*S. maritima*	ca.94	0
	Middle marsh	1	*S. densiflora*	ca.66	0
Guadiana	Low marsh	5	*S. maritima*	ca.92, ca.94	0
	Middle marsh	5 (10)	*S. densiflora*	ca.64, ca. 65	0

A total of 16 fixed base pair substitutions were found across the trnT × trnF region that differentiated *S. densiflora* from *S. maritima*.

Of the specimens sequenced, no haplotype variation was found across *Spartina densiflora* parental individuals and hybrids derived from a *S. densiflora* seed parent. The haplotype recovered among these individuals was identical to the haplotype found among *S. densiflora* and *S. densiflora* × *foliosa* hybrids (derived from an *S. densiflora* seed parent) in San Francisco Bay, California, USA (Ayres *et al.*, 2008b), as well as the haplotype recovered in native *S. densiflora* in Chile and Argentina by Fortune *et al.* (2008). The haplotype was identical to the haplotype found by Fortune *et al.* (2008) from *S. densiflora* in Humboldt Bay (California, USA), but two separate single base pair indels distinguish this haplotype from the haplotype recovered by Baumel *et al.* (2002) from a specimen sampled in California, USA.

Two haplotypes were found among *S. maritima* and hybrids derived from *S. maritima* seed parents. No base pair substitutions were found among the individuals sequenced for this study, but one single base pair indel differentiated two *S. maritima* haplotypes from other *S. maritima* and hybrids. In a broader context, two base pair substitutions and two separate single base pair indels across the trnT × trnF region distinguish the more common *S. maritima* haplotype that we found from that of a specimen taken from Brittany (France) by Baumel *et al.* (2002). A single base pair substitution in the trnL × trnF region differs between the haplotype found by Fortune *et al.* (2008) in *S. maritima* sampled from St. Armel (Morbihan, France) and the two *S. maritima* haplotypes we found. Four additional base pair substitutions in the trnL × trnF region differentiate *S. maritima* from Suffolk County (UK) from the haplotypes we discovered (Ferris *et al.*, 1997).

Chromosome counts

Spartina maritima plants had 2n = 60 chromosomes and *S. densiflora* plants showed 2n = 70 chromosomes. Hybrid plants had between ca. 64 chromosomes at low marshes and ca. 94 chromosomes at middle marshes (Table 2). These counts were always open to a variation of one or two chromosomes because of the presence of a couple of floating secondary constrictions on two *S. maritima* chromosomes.

Ecological studies

From our vegetation surveys, we found that *S. maritima* had high cover and grew mainly at lower elevations near *Sarcocornia perennis* ssp. *perennis*. On the other hand, *S. densiflora* had high cover and occurred mainly in middle and high marshes; however, it also grew, with reduced cover, in low marsh together with *Sarcocornia perennis* (Fig. 2). *Spartina* hybrids grew in three zones spanning the intertidal zone: (1) the low marsh dominated by *S. perennis* and *S. maritima*; (2) the middle marsh coinciding with the *Atriplex portulacoides* zone

and (3) the middle-high marsh where the highest relative cover of the invasive *S. densiflora* was recorded (50%). The relative cover of the *Spartina* hybrid was always lower than 20% (Fig. 2).

In the transplant experiment, sediment redox potential varied between −38 ± 11 and 205 ± 6 mV and covaried with elevation ($r = 0.94$, $P < 0.01$, $n = 6$). Sediment water content during summertime low tide showed the opposite response to redox potential along the intertidal zone, varying between 42 ± 1% at the lowest point (+1.47 m SHZ) and 4 ± 1% at the highest elevation (+3.16 m SHZ). Sediment pH (between 6.5 and 7.9) and electrical conductivity (between 7 ± 1 and 12 ± 1) did not show a clear variation pattern along the intertidal zone (Fig. 3).

All three taxa died at the lowest transplant point located at an intertidal plain below the lower distribution limit of *S. maritima*. Transplanted hybrids grew from the low marsh where *S. maritima* flourished to high marshes with *Arthrocnemum macrostachyum* (Moric.) Moris and *Suaeda vera* Forsk. Transplanted *S. maritima* did not survive at the highest elevation (+3.16 m S.H.Z.), and *S. densiflora* did not survive at the lowest elevation distribution limit of *S. maritima* (Fig. 3). Further, hybrids grew as well as or better than the parental species at every point on the intertidal gradient except for the highest transplant point where *S. densiflora* grew marginally better. Hybrids exhibited tillers taller than *S. maritima* at every elevation throughout the transplant transect and taller than *S. densiflora* at +2.93 m SHZ ($F = 201.111$, $P < 0.0001$; Tukey's-*b* test, $P < 0.05$) (Figs 3 & 4). The TGR of hybrids was greater than *S. maritima* except at the two higher elevations and greater than *S. densiflora* at the middle part of the transect (+2.70 and +2.76 m SHZ) ($F = 24.673$, $P < 0.001$; Tukey's-*b* test, $P < 0.05$) (Fig. 3).

Seed set

More than 4000 florets were assessed, and no seed was detected in any hybrid plants. The seed set for *S. densiflora* was 65% (over 1000 florets). No *S. maritima* seed was found.

DISCUSSION

The unusual *Spartina* plants discovered in three estuaries along the SW Iberian Peninsula (Guadiana, Piedras and Odiel) proved to be hybrids between native *S. maritima* and introduced *S. densiflora* as shown by molecular fingerprinting. All the hybrids had a mostly additive pattern of nuclear DNA fragments, indicating that they inherited all species-specific fragments from both parents (Table 1). A similar mostly additive pattern of RAPD fragments has been found in first generation *S.* × *townsendii* and the allopolyploid *S. anglica* (Ayres & Strong, 2001), and in first generation *S. densiflora* × *foliosa* (Ayres *et al.*, 2008c). Lack of a diagnostic fragment in four hybrid plants could be because of heterozygosity in the parent concealed by the dominant nature of RAPD markers, polymorphism in the diagnostic band, as was seen for primer
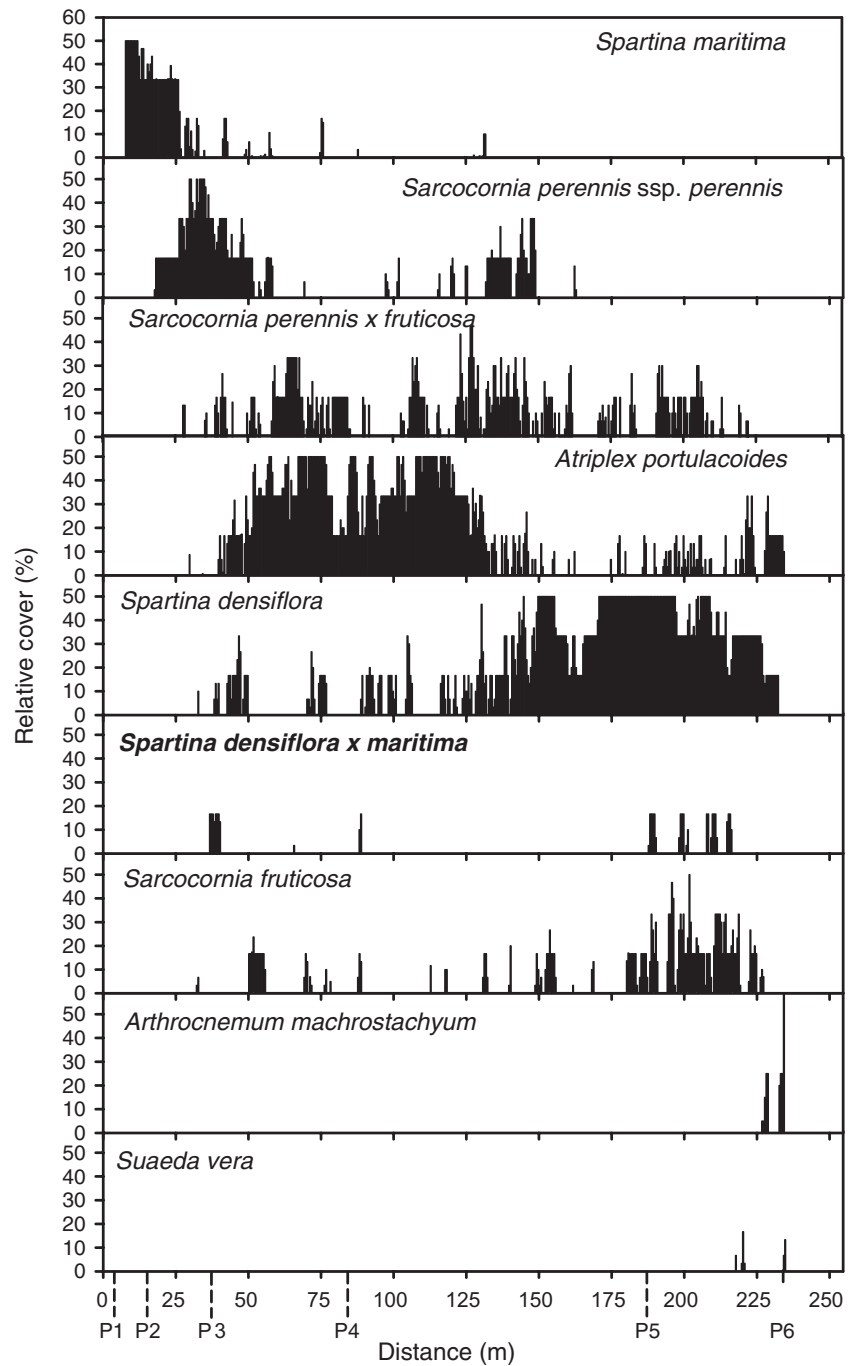
Figure 2 Mean relative percent cover of every taxa present over the intertidal zone from low to high elevations at San Bruno marsh (Guadiana river estuary, SW Iberian Peninsula) ($n = 3$ transects). Location over the intertidal zone for every transplant point (P).

C1, or fragment loss in the hybrid. In contrast, the frequencies of species-specific fragments in introgressive hybrids are variable and generally <1.0, as was seen in introgressive *S. alterniflora × foliosa* hybrids (Ayres *et al.*, 1999). Thus, we conclude that all *S. densiflora × maritima* hybrids are first generation F1s and not introgressive hybrids.

Chromosome counts and the lack of set seed imply that the interspecific hybrid has not undergone chromosomal doubling to form a new allopolyploid species. Hybrid counts of 2n = ca. 64, ca. 65, ca. 66 are consistent with a single reduced chromosome complement from each species as *S. maritima*

$n = 30$ and *S. densiflora* $n = 35$. These hybrids were found only in middle marshes with *S. densiflora* as maternal species. In addition, plants with near-non-aploid (9x) counts of 2n = ca. 94 (base chromosome number being $n = 10$ in this group) were the types encountered at low marshes where *S. maritima* was the maternal species. These non-aploids could have resulted from the union of an unreduced gamete of *S. maritima* (=60) with a reduced gamete from *S. densiflora* (=35) or from backcrosses between unreduced gametes of the hybrids (=65; unreduced gametes are likely following their irregular meiosis) and reduced gametes of *S. maritima* (=30).
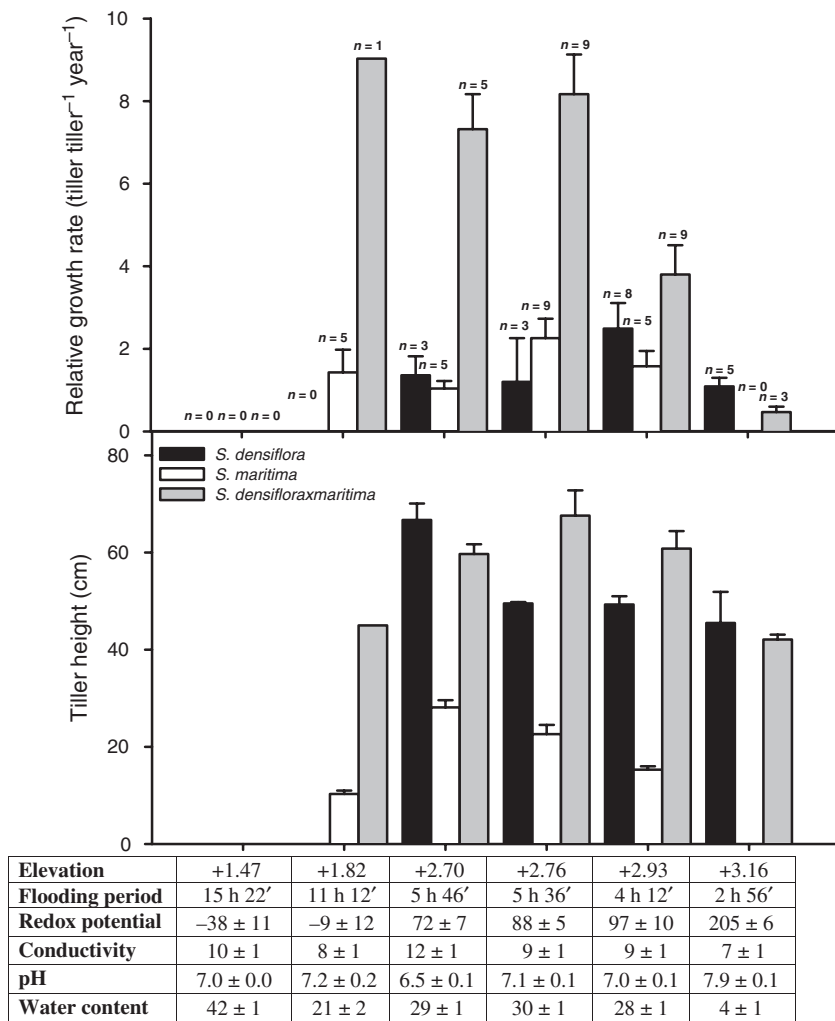
Figure 3 Tiller relative growth rate (tiller tiller^{-1} year^{-1}) and tiller height (cm) ($n = 0$–9 surviving clumps) for invasive *Spartina densiflora*, native *Spartina maritima* and hybrid *Spartina densiflora* × *maritima* on a transplant transect along the intertidal zone in Guadiana Marshes (SW Iberian Peninsula) according to elevation over Spanish Hydrographic Zero, daily flooding period with spring tide (h day^{-1}), redox potential (mV; $n = 60$–120), sediment electrical conductivity (mS cm^{-1}; $n = 6$–32), pH ($n = 6$–32) and water content of the sediments between 0–10 cm depth (%; $n = 10$–20). Values are mean ± standard error mean.

Elevation	+1.47	+1.82	+2.70	+2.76	+2.93	+3.16
Flooding period	15 h 22′	11 h 12′	5 h 46′	5 h 36′	4 h 12′	2 h 56′
Redox potential	−38 ± 11	−9 ± 12	72 ± 7	88 ± 5	97 ± 10	205 ± 6
Conductivity	10 ± 1	8 ± 1	12 ± 1	9 ± 1	9 ± 1	7 ± 1
pH	7.0 ± 0.0	7.2 ± 0.2	6.5 ± 0.1	7.1 ± 0.1	7.0 ± 0.1	7.9 ± 0.1
Water content	42 ± 1	21 ± 2	29 ± 1	30 ± 1	28 ± 1	4 ± 1

The scattered geographical distribution of hybrid plants, together with the absence of seeds, clearly indicates that multiple, independent hybridization events occurred that gave rise to these plants. This was confirmed by chloroplast and nuclear DNA evidence; both species have been seed parents of hybrids at two of the three estuaries, and some hybrids vary slightly in nuclear fingerprinting patterns. This nuclear variation is likely because of variation in the parental species, as was seen in *S. densiflora* for the polymorphic C1-1600 bp band, or hidden heterozygosity as in C1-320 bp and C12-1200 bp, or loss of the parental fragment in the hybrid. Because of the dominant nature of RAPD phenotypes, heterozygosity in the parent species is undetectable.

Our ecological studies demonstrated that *S. densiflora* × *maritima* hybrids exhibit ecological amplitudes greater than either parental species. Hybrids were able to survive in lower elevations in the intertidal zone where *S. maritima* naturally grows and in high marshes where *S. densiflora* invades (Fig. 3). Abiotic conditions of high soil water and low redox potential, because of long inundation period (>11 h), which excludes *S. densiflora* (Castillo *et al.*, 2000), and reduced water content, which excludes *S. maritima*, were

well tolerated by hybrids in the absence of competition. In addition, hybrids exhibited much higher TGR than either parental species throughout most of the intertidal zone, and hybrids always had taller tillers than *S. maritima*. Thus, *S. maritima* x *densiflora* hybrids showed transgressive traits, such as taller tillers and a higher abiotic tolerance in the intertidal zone, which may result from the advantage of heterosis or hybrid vigour (Comai, 2005). These results agree with those recorded previously for other invasive and transgressive plant and animal hybrids taxa in relation to heterosis (Vila & D'Antonio, 1998; Facon *et al.*, 2005; Drake, 2006; Rieseberg *et al.*, 2007; Schierenbeck & Ellstrand, 2009). A few works have shown that hybrids among disparate source populations, such as *Raphanus raphanistrum* × *sativus* or *Helianthus annus annus* × *debilis*, had a higher performance in novel environments than their parental species (Campbell *et al.*, 2006; Whitney *et al.*, 2006).

While our transplant experiments indicated that hybrids can grow well along the entire tidal range between *S. maritima* and *S. densiflora*, our transect studies showed their distribution is currently limited to three discrete areas, and their cover is less than half that of the parental species

Figure 4 *Spartina maritima × densiflora* hybrids (black arrows) growing close to *S. maritima* (white arrows). Both were transplanted at the same time at +2.76 m Spanish Hydrographic Zero.

(Fig. 2). These observations suggest that invasion by hybrids has likely just begun, and that hybrids do not yet occupy a full range of possible locations in these estuaries. Further, the limited extent of hybrids is likely because of a lack of seed production, as the spread of hybrids may only occur by asexual reproduction by rhizome fragments and extravaginal tillers that originate from the nodes of senescent ramets that we observed here and as has been observed in *S. densiflora* (Nieva *et al.*, 2001).

Based on observations of other *Spartina* invasions involving hybridization, it is possible that a fertile new allopolyploid species might form via chromosomal doubling (2N = 130), providing a full complement of chromosomes from both parental species, may emerge in the future. Also, a triploid bridge could represent another means by which allopolyploid taxa could originate (Ramsey & Schemske, 1998; Ayres *et al.*, 2008c). Indeed this is still a possible mechanism for the production of the allopolyploid *S. anglica*, since Marchant (1968) described the presence of 9x backcrosses in Southampton Water as did Renny-Byfield *et al.* (2010).

The relatively high ecological tolerances and high tiller growth rates and heights observed among *S. densiflora × maritima* hybrids suggest that hybrids may out-compete native flora, since *S. densiflora* with a narrower ecological tolerance and lower growth rates than the hybrids is a highly successful competitor in Spanish marshes (Nieva *et al.*, 2001). In addition, hybrid seedlings have established, and adult plants have flourished within intact plant communities; that is, a disturbed, competition-free site is not required for the hybrid to succeed. The invasion by new hybrids would be particularly accelerated if they acquire the ability to set seed. Witnessing such an event would be informative from an evolutionary perspective, but the formation of a new allopolyploid species would be a serious threat to the

conservation of European marshes (Ferris *et al.*, 1997; Ayres & Strong, 2001; Baumel *et al.*, 2001). Similarly, selection favouring novel hybrid genotypes could subsequently lead to the rapid evolution of traits that accelerate invasiveness (Ellstrand & Schierenbeck, 2000; Schierenbeck & Ellstrand, 2009; Sloop *et al.*, 2009).

The participation of *S. maritima* in the formation of the hybrids, both as maternal and paternal parent, showed that the pollen and ovaries of this species are able to pollinate and be pollinated. Natural populations of *S. maritima* have failed to reproduce sexually in Europe and the UK in multiple studies (Marchant & Goodman, 1969; Cooper, 1993; Castellanos *et al.*, 1998). Self-incompatibility and/or high inbreeding depression, as has been shown for other *Spartina* species (Sloop *et al.*, 2009), may predispose genetically depauperate *S. maritima* (Ayres & Strong, 2001; Baumel *et al.*, 2001; Yannic *et al.*, 2004) to interspecific hybridization, a phenomena possibly shared by other genetically depauperate *Spartina* species including *S. densiflora* (Fortune *et al.*, 2008) and *S. foliosa* (Blum *et al.*, 2004; Sloop *et al.*, 2005). If the hexaploid clade of *Spartina*, formed by *S. alterniflora*, *S. foliosa* and *S. maritima* [and sometimes *S. densiflora* (Fortune *et al.*, 2008)], is prone to interspecific hybridization, then care should be taken to forestall the origination of invasive new species of *Spartina* by preventing the introduction of a member of this clade into the range of another species in the clade (e.g. introducing *S. maritima* along the US Pacific coast, or *S. foliosa* into European salt marshes).

Hybridization has been a natural mechanism for the origination of new species in the genus *Spartina* (Fortune *et al.*, 2008) and in other plant genera such as *Salsola* (Ayres *et al.*, 2008a), *Tragopogon*, *Senecio* and others (see Table 1 in Schierenbeck & Ellstrand (2009)). However, we agree with Pyšek *et al.* (2004) that when a new hybrid species arises as a

result of the introduction of a non-native species (or two species), it would be inconsistent to call the new species a 'native' as it would not be present in the region without human intervention and therefore the new species should be considered another negative outcome of species introduction. In this context, our specific recommendations for the Iberian Peninsula, based upon our findings and work made on other *Spartina* invasions involving hybridization, are that steps be taken to eradicate *S. densiflora × maritima* hybrids as soon as possible because of its high ecological amplitude and the possibility of the formation of a new and invasive allopolyploid species. In addition, it is also important for the conservation of Iberian salt marshes to fight the invasion of *S. densiflora* since it out-competes native species and may form invasive hybrids where it is in contact with *S. maritima*. Presently, eradication of *S. densiflora × maritima* hybrids is an achievable goal since the distribution and the relative abundance of the hybrids are still quite limited.

ACKNOWLEDGEMENTS

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BIOSKETCH

Jesús M. Castillo is a specialist in salt marsh ecology. He has worked mainly in South-West Iberian Peninsula, publishing different papers about ecological succession, plant zonation, ecological restoration and processes related with the invasion of *Spartina densiflora*, an alien cordgrass in European marshes.

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