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The enigmatic invasive *Spartina densiflora*: A history of hybridizations in a polyploidy context

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Abstract

The aim of this study was to explore the origin of the invasive *Spartina densiflora* by analysing samples from the native region (South America) and from a recently colonized area (California). A combination of various molecular data (chloroplast and nuclear sequences, molecular fingerprint) and ploidy level estimations was used to answer the question whether the reticulate phylogenetic pattern previously detected in *S. densiflora* was restricted to California, or alternatively, whether a more ancient hybrid origin preceded formation of this species in its native area. We found that this species is heptaploid in both its native and introduced range. Identification of nuclear homeologous sequences indicate that this species has a reticulate origin in its native range, involving a lineage related to the hexaploid clade formed by *S. alterniflora*, *S. foliosa*, and *S. maritima*, and another lineage related to the sub-Antarctic endemic *S. arundinacea* that provided the chloroplast genome. The samples from California displayed similar multilocus patterns to the samples from Chile, supporting the hypothesis that this species originated on the southeast American coast (Argentina), from where it eventually spread to the west coast of South America (Chile) first and to the Northern Hemisphere (California) later.

Keywords: allopolyploidy, hybridization, reticulate evolution, *Spartina densiflora*

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Introduction

Molecular studies devoted to the understanding of population and species history have revealed that reticulate evolution is an important, previously underestimated, phenomenon in many lineages (Avise 1994; Arnold 1997). Interspecific hybridization is not only common in plants but also in animals (Rieseberg *et al*. 2006), with important evolutionary consequences such as lateral gene transfer via introgression (Rieseberg & Wendel 1993) or formation of new species via homoploid or alloploid speciation (Rieseberg 1997). By bringing together initially divergent genomes, reticulate events generate new genetic combinations and molecular interactions that play critical adaptive roles (Riddle & Birchler 2003; Rieseberg *et al*. 2007). The relationship between hybridization and invasion has received continuing attention in evolutionary ecology with many examples of rapidly spreading species of hybrid origin (Abbott 1992; Ellstrand & Schierenbeck 2000; Petit *et al*. 2003; Schierenbeck & Ainouche 2006; Ainouche *et al*. 2008).

Salt marsh species in the genus *Spartina* provide fascinating examples of invasive species that can arise from either homoploid or allopolyploid hybridization and subsequently have important ecological consequences (Ainouche *et al*. 2008). Recent hybridization events have been well-documented following introductions of the hexaploid *Spartina alterniflora* Loisel. ($2n = 62$) outside its native range, the Atlantic American coast, to the Pacific Californian coast (since the 1970s) and to the Atlantic European coast at the end of the 19th century (Ainouche *et al*. 2004a;

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Ayres *et al*. 2004). In California, recurrent hybridization with the hexaploid native *Spartina foliosa* Trin. (2*n* = 62, Ayres *et al*. 2008a) has resulted in rapidly expanding introgressant hybrid populations (Ayres *et al*. 1999, 2004, Antilla *et al*. 2000) that overgrow tidal mud flats and threaten local populations of *S. foliosa* (Ayres *et al*. 2003, 2004; 2008b).

In Western Europe, hybridization of *S. alterniflora* with the hexaploid native *Spartina maritima* (M.A. Curtis) Fern. 2*n* = 60, Marchant (1968) in the Bay of Southampton resulted in a sterile F_1 hybrid *Spartina* × *townsendii* Groves. Duplication of the hybrid genome gave rise to a fertile and invasive allododecaploid species, *Spartina anglica* C. E. Hubbard (2*n* = 120–124) that has expanded rapidly in Western Europe salt marshes and elsewhere (Raybould *et al*. 1991; Thompson 1991; Baumel *et al*. 2001; Ainouche *et al*. 2004b).

The austral cordgrass *Spartina densiflora* Brongn., native to South America, is widely distributed along the temperate marshes of Southern Brazil, Uruguay, Argentina, and patchily distributed along the Chilean coast where it was originally described (Brongniard 1929; Bortolus 2006 and references therein). This species was introduced to California, probably from Chile, during the late 18th century via solid ballast (Spicher & Josselyn 1985). It is now well-established in Humboldt Bay where it is the dominant salt marsh plant (Kittelson & Boyd 1997). It was recently found at Gray's Harbor, Washington, possibly introduced as a contaminant of oyster spat (Pfauth *et al*. 2003; Bortolus 2006). *S. densiflora*, first thought to be a variant of the native *S. foliosa,* was transplanted during the late 1970s to the San Francisco Bay for marsh restoration (Faber 2000) where it is now spreading (Ayres *et al*. 2004). Recently, hybrids between introduced *S. densiflora* and native *S. foliosa* individuals have been recorded in San Francisco Bay (Ayres *et al*. 2008a). *S. densiflora* was also accidentally introduced in Europe (Iberian Peninsula), on the southern coast of Spain where it has invaded the Gulf of Cadiz (Nieva *et al*. 2001, 2005) and it is spreading to the Algarve Province (Portugal) and to Gibraltar. The species was also introduced in North Africa at the Merja Zerga lagoon of Morocco (Fennane & Mathez 1988).

Spartina densiflora has a C4 photosynthetic metabolism, as do all *Spartina* species, and displays large ecological amplitude. It can thrive in habitats that are brackish, hypersaline, intertidal, or strictly terrestrial (Mobberley 1956; Nieva 1996; Kittelson & Boyd 1997; Clifford 2002; Nieva *et al*. 2002; Vicari *et al*. 2002). This perennial species is characterized by a caespitose growth form and unrolled leaves displaying ridges on the upper side. It expands vegetatively by short tillers or sexually via high seed production (Kittelson & Boyd 1997; Nieva *et al*. 2005; Bortolus 2006). *S. densiflora* is 'an ecosystem-engineer' (*sensu* Jones *et al*. 1994) that mediates and modifies the physical environment and the community structure in both native and invaded marshes. It commonly shapes distributional patterns of above- and below-ground organisms in sandy, muddy, and rocky bottom environments (Bortolus *et al*. 2002; Bortolus 2006).

Morphological variability has led to the confusing taxonomic treatment of *S. densiflora* in its native range (Mobberley 1956) as it has been variously described as *Spartina montevidensis* Arech. (Uruguay), *Spartina patagonica* Speg. (Argentina) or *Chauvinia chilensis* Steud. (Chile). Morphologically intermediate plants referred to as *Spartina longispica* St Yves in Argentina are considered to be hybrids between *S. alterniflora* and *S. densiflora* (Parodi 1919; Mobberley 1956; Cabrera 1970). The ploidy level of *S. densiflora* is not clearly established. This species seems closely related to the tetraploid species *S. arundinacea* (Thouars) Carmich. (Baumel *et al*. 2002); 2*n* = 60 was initially reported by Gerish (1979) from *S. foliosa* specimens which were later identified as *S. densiflora* (Spicher & Josselyn 1985). Recently, Ayres *et al*. (2008a) found 2*n* = 70 in *S. densiflora* samples from California, and 2*n* = 66, 94, 96 in *S. foliosa* × *S. densiflora* hybrids from San Francisco Bay. More extensive ploidy level surveys are needed in native and introduced populations.

Molecular phylogenies have revealed conflicting topologies for *S. densiflora* samples (Baumel *et al*. 2002). Chloroplast DNA (*trn*T-*trn*L spacer) and nuclear *ITS* indicated a close relationship with the tetraploid *S. arundinacea* (sub-Antarctic islands) and *S. ciliata* Brong. (Southeast America), whereas the nuclear *Waxy* gene indicated a strongly supported phylogenetic relationship with a hexaploid clade formed by *S. alterniflora*, *S. foliosa* and *S. maritima*. This conflict was interpreted as resulting from either paralogous sampling in a polyploid species or from a recent reticulate event involving the analysed sample of *S. densiflora* (collected from California). Additional analysis of *Waxy* and intensive sequence cloning (Fortune *et al*. 2007) revealed the existence of paralogous sets of *Waxy* loci in *Spartina* as a consequence of a duplication that occurred prior to the divergence of *Spartina* from other *Chloridoideae*.

Our aim was to determine whether the reticulate phylogenetic pattern previously detected in *S. densiflora* was restricted to exotic populations in California, or whether a more ancient hybrid origin preceded formation of this species in its native range. Further, we wished to verify the potential source population for the invasive population in California. To answer these questions, we analysed a set of samples from both the native range (South America) and from a recently colonized area (California) and combined various molecular data (chloroplast and nuclear sequences, molecular fingerprint) with ploidy level estimations.

Materials and methods

Plant material

We collected and analysed samples of *Spartina densiflora* from both the eastern (Argentina) and western (Chile) American

Table 1 *Spartina* species and accessions used

Taxa	Origin
S. densiflora Brogn. 1	Chiloe Island (Ancud, Chile)
S. densiflora Brogn. 2	Mar Chiquita lagoon, (Buenos Aires, Argentina)
S. densiflora Brogn. 3	Buenos Aires Province (Argentina)
S. densiflora Brogn. 4	Cerro Ayanzado 1 (Chubut, Argentina)
S. densiflora Brogn. 5	Cerro Ayanzado 2 (Chubut, Argentina)
S. densiflora Brogn. 6*	Isla de los Pajaros (Chubut, Argentina)
S. densiflora Brogn. 7	Riacho Marsh (Chubut, Argentina)
S. densiflora Brogn. 8	Isla de los Pajaros 1 (Chubut, Argentina)
S. densiflora Brogn. 9	Isla de los Pajaros 2 (Chubut, Argentina)
S. densiflora Brogn. 10	San Rafael CS, San Francisco Bay (CA, USA)
S. densiflora Brogn. 11	Tiburon BP, San Francisco Bay (CA, USA)
S. densiflora Brogn. 12	Samoa, Humboldt Bay (CA, USA)
S. densiflora Brogn. 13	Jacoby Creek, Humboldt Bay (CA, USA)
S. densiflora Brogn. 14	Mad River, Humboldt Bay (CA, USA)
S. argentinensis Parodi	Santa Fe Province (Argentina)
S. patens (Aiton) Muhl.	Shark River (NJ, USA)
S. arundinacea (Thouars) Carmich.	Amsterdam Island (Kerguelen Archipelago)
S. bakeri Merr.	Florida (USA)
S. cynosuroides (L.) Roth.	New Jersey (USA)
S. pectinata Link.	Missouri (USA)
S. alterniflora Loisel.	Landerneau (Finistère, France)
S. foliosa Trin.	Bolinas Lagoon (CA, USA)
S. maritima (Curtis) Fern.	St Armel (Morbihan, France)

*, *S. densiflora* with 'Patagonica' phenotype.

coast in the native region of the species (South America), and from the Californian coast where *S. densiflora* was introduced (Table 1). One sample (no. 6, Table 1) displayed a 'Patagonica' phenotype, one of the numerous names given to *S. densiflora* due to its important morphological variation (Mobberley 1956). Plants were maintained in the greenhouse before DNA extractions and other experiments were performed. Other representatives of the major clades previously described in *Spartina* (Baumel *et al*. 2002; Fortune *et al*. 2007) were included in the phylogenetic analyses (see below). A sample of *Cynodon dactylon* collected in Brittany (France) was included as outgroup in the phylogenetic analyses for rooting the *Spartina* trees.

Flow cytometry

The relative ploidy levels of the different accessions were determined using flow cytometry, according to methods described by Baumel *et al*. (2003) with the following modifications: In order to break cells and obtain free nuclei suspensions, fresh leaves were chopped with a razor blade in a Partec Kit DAPI solution (05.5002-CyStain UV) composed of 1 vol. nuclei extraction buffer and 4 vols staining buffer. A Partec CA II device flow cytometer (at the Agronomical Research Station INRA Le Rheu, France) with DPAC software was used to measure the fluorescence emission of these nuclear suspensions. *Pisum sativum* was used as internal standard. During the whole session of measurements, channel 300 was arbitrarily adjusted to *P. sativum*. The DAPI staining method was used for routine ploidy level estimation of several individuals per species (three independent measurements on each of the samples presented in Table 1).

Genome size was measured with propidium iodide staining (Coba de la Pena & Brown 2001) at the Service de Cytometrie, Institut des Sciences du Vegetal (Gif sur Yvette, France) using a Coulter EPICS Elite ESP flow cytometer. Leaves were chopped in Galbraith buffer with 1% triton and 1% PVP. Three internal standards were used for calibration: *Petunia hybrida* (2C = 2.85 pg), *Lycopersicon esculentum* (2C = 1.99 pg) and *Pisum sativum* (2C = 8.84 pg). Several measurements were made for each sample and only measurements with a coefficient of variation inferior to 3% were retained. Genome size was estimated for the following samples, representing different ploidy levels: *S. densiflora* (sampled in Argentina), *Spartina alterniflora* (Finistère, France), *Spartina maritima* (Morbihan, France), *Spartina foliosa* (San Francisco Bay, CA, USA), and *Spartina bakeri* Merr. (FL, USA).

Chromosome counts

Actively growing root tips were collected from three vegetatively propagated *S. densiflora* plants growing in the UC Davis greenhouses, originally collected from Tomales Bay, CA. Approximately 6 mm of root tips were removed

from the plants, pretreated with 8-hydroxquinoline for 3 h, fixed for 24 h in 3:1 ethanol: acetic acid, hydrolysed for 10 min in 5 N HCl at 20 °C, and stained with Schiff's reagent. Root tips were dissected to isolate the meristematic tissue from epidermal cells and stained with 1.5% acetocarmine during squash preparation. Because of the high number of small chromosomes, these mitotic counts were verified on at least three slides per plant. Cells were viewed with an Olympus BX-51 microscope at 1000× and digitally documented with a Pixera Penguin 600ES CCD camera.

DNA extraction, amplification, cloning and sequencing

DNA was extracted from 100 mg fresh leaves with the DNeasy Plant Mini Kit (QIAGEN) according to the instructions of the manufacturer.

We analysed two nuclear regions: the *Waxy* gene coding the Granule Bound Starch Synthase (GBSSI) and the *ITS* region of the nrDNA, and two chloroplast regions: the *trn*T-*trn*L spacer, and the *trn*L-*trn*F intron and spacer. Primers used for the nuclear markers were F-For (5′-TGCGAGCTC GACAACATCATGCG-3′) and K-bac (5′-GCAGGCTCGAA GCGGCTGG-3′) from Mason-Gamer *et al*. (1998) for the *Waxy* gene and ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) for the *ITS* region (White *et al*. 1990). For the chloroplast sequences, we used primers a (5′-CATTACAAATGCGATGCTCT-3′) and b (5′-TCTACCGATTTCGCCATATC-3′), c (5′-CGAAATCGGTAG ACGCTACG-3′) and f (5′-ATTTGAACTGGTGACACGGAG-3′) for the *trn*T-*trn*L and *trn*L-*trn*F regions (Taberlet *et al*. 1991), respectively.

The *Waxy* amplifications were performed as described in Fortune *et al*. (2007). For the *ITS* and chloroplast regions, polymerase chain reactions (PCRs) were conducted in 50 μL volumes containing 5 μL 10×RedTaqPCR buffer (Sigma), 5 μL of 2 mM each dNTPs (Eurogentech), 1 μL 5 pM forward primer, 1 μL 5 pM backward primer, 2.5 U RedTaq DNA polymerase (Sigma), and 1 μL of genomic DNA solution. PCR conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 48 °C, and 2 min at 68 °C, with a final extension cycle at 68 °C for 10 min.

Waxy and *ITS* PCR products were cleaned with the Nucleospin Extract II kit (Macherey-Nagel) and cloned using the pGEM T-Easy Vector System kit (Promega) following the manufacturers' instructions. Positive clones were tested by PCR, purified with the Nucleospin Plasmid Quick Pure kit (Macherey-Nagel) and sent to Macrogen, Inc. (Seoul, South Korea) for sequencing. Five clones per species were analysed for the *ITS*. Twenty clones per accession of *S. densiflora* were sequenced for the *Waxy* gene. In polyploids, sequence heterogeneity is expected for nuclear genes that are not affected by concerted evolution; thus, the identification of homeologues (genes duplicated by polyploidy) is particularly important in the context of our study.

Sequence alignment and phylogenetic reconstruction

Sequences were corrected and manually aligned with BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Indels in the alignment were coded as proposed by Barriel (1994) in MacClade (Maddison & Maddison 1992). Phylogenetic reconstructions were performed using PAUP* version 4.0b10 (Swofford 2003). Preliminary neighbour-joining analyses were conducted followed by maximum-parsimony analyses with heuristic searches, default options and *Cynodon dactylon* as the outgroup. We used 10 000 replicates for the bootstrap (BS) analyses. Maximum-likelihood analyses were conducted with heuristic searches as described in Harrison & Langdale (2006). ModelTest 3.7 (Posada & Crandall 1998) and ModelTest WebServer (http://darwin.uvigo.es) were used to establish the model of sequence evolution with all options set to default. Model TrN + G was retained for the *Waxy* gene (unequal bases frequencies, proportion of invariable sites = 0, gamma distribution shape parameter = 0.8448) and for the *ITS* region (unequal bases frequencies, proportion of invariable sites = 0, gamma distribution shape parameter = 0.5250). Model TVM + G was retained for the *trn*T-*trn*L data set (unequal bases frequencies, proportion of invariable sites = 0, gamma distribution shape parameter = 0.3591).

Molecular fingerprinting

The goal of the fingerprint analysis was to detect species diagnostic DNA fragments that would help in identifying interspecific hybrids in the introduced and native area of *S. densiflora.* Multilocus fingerprinting was performed by analysing intersimple sequence repeat (ISSR) and randomly amplified polymorphic DNA (RAPD) fragments from *S. densiflora* on a subset of the samples presented in Table 1 (samples 1, 2, 3, 6, 7, 10, 11, 12, 13, 14). For comparative purposes, we included samples of *S. alterniflora* from the native range in Massachusetts, *S. foliosa* from San Francisco Bay, CA, an *S. alterniflora* × *foliosa* F_1 hybrid artificially created through hand-pollination by D. Ayres, and a naturally occurring F_1 hybrid between *S. densiflora* and *S. foliosa* growing at Creekside Park, San Francisco Bay, CA. By including F_1 hybrids, we can examine patterns of band transmission. Genomic DNA was amplified according to the protocol previously employed in *Spartina* (Baumel *et al*. 2001) using the following ISSR primers: SSR10 [$(AGC)_6G$]. Amplifications were performed as following: initial denaturation 94 °C 1′, 35 cycles of 94 °C 30 s, 56 °C 30 s, 68 °C 2′ and final elongation 68 °C 15′. The amplification products were run on 1.5% agarose gels stained in 5% ethidum bromide. Four RAPD primers were chosen based on results of previous studies (Ayres *et al*. 1999; Ayres *et al*. 2008a) on

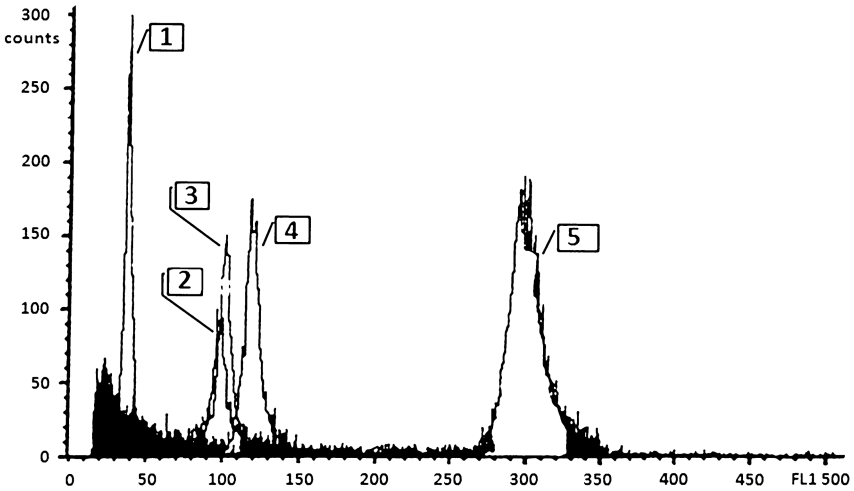

Fig. 1 Ploidy levels and genome size estimation using flow cytometry. This is a composite image of multiple runs superimposed on the basis of the internal standard. The peaks illustrate the corresponding fluorescence intensity (DAPI staining) among four *Spartina* species (1–4) and *Pisum sativum* (5) as internal standard. For clarity, G2 peaks were manually removed and only G1 peaks are displayed for each species. Genome sizes (in pg) estimated using Propidium Iodide are presented for each corresponding species.

	Species	Origin	Genome size (2C in pg)
1	*S. bakeri* (2n = 40)*	Florida USA	1.43 – 1.48 pg
2	*S. maritima* (2n = 60)*	Morbihan France	3.70 – 3.85 pg
3	*S. alterniflora* (2n = 62)*	Finistère France	4.33 – 4.36 pg
4	*S. densiflora* (2n = 70**)	Argentina	4.53 – 4.55 pg

*From Marchant 1968, **From Ayres *et al*. (2008a), and this study.

hybrids between *S. alterniflora*, *S. foliosa*, and *S. densiflora* that identified and used species-specific diagnostic DNA fragments (A2, B12, B7, D5; Operon). The amplification procedure was performed as described in Daehler *et al*. (1999), except that a Hybaid PCR Express thermocycler was used.

A neighbour-joining analysis based on the presence and absence of the amplified ISSR and RAPD markers was performed using PAUP* version 4.0b10 (Swofford 2003) with all options set to default and 10 000 BS replicates.

Results

Genome size estimation of various Spartina *samples and ploidy levels*

The results obtained using the flow cytometry measurements are presented in Fig. 1. The tetraploid species *Spartina bakeri* exhibited a mean fluorescence value of 35.16, which is similar to the values found for other tetraploid *Spartina* species measured such as *S. arundinacea* and *S. pectinata* (not shown). Genome size estimations differed slightly when performing different measurements; the mean 2C values ranged from 1.43 to 1.48 pg. Hexaploid *Spartina maritima* and *Spartina alterniflora* displayed mean fluorescence values of 98.29 and 108.14, respectively. Genome size ranged from 3.70 to 3.85 pg for *S. maritima* and from 4.33 to 4.36 pg for *S. alterniflora*. The third hexaploid species, *Spartina foliosa* from San Francisco Bay, displayed an intermediate value between *S. maritima* and *S. alterniflora* (4.05 pg, not shown). *Spartina densiflora* exhibited a higher value than the other species, with a mean fluorescent value of 123.19 in the sample from Argentina and genome size ranging from 4.53 to 4.55 pg. All other *S. densiflora* plants sampled from Argentina, as well as those collected in Chile and California, displayed similar genome size estimation. Chromosome analysis of four mitotic cells confirmed a count of $2n = 70$ for *S. densiflora*.

Chloroplast DNA sequence analysis

All the *trn*T-*trn*L sequences obtained for the different samples of *S. densiflora* were identical to the one previously found (GenBank Accession no. AF372629) by Baumel *et al*. (2002), except one sample from Samoa (Humboldt Bay, California) that displayed one nucleotide substitution. Including sequences of other Spartina species (GenBank Accession nos AF372625 to AF372633, and AF275667 to AF275669 from Baumel *et al*. 2002) resulted in an aligned matrix of 841 characters. Among these, 761 characters were constant and only 15 were parsimony informative. This alignment required the inclusion of gaps from 1 to 426 nucleotides. The 426-nucleotide gap was encountered in the sequences of all the samples of *S. densiflora* and *S. arundinacea*, which reinforces the conclusion concerning their close phylogenetic relationship (see below).

The sequences of the *trn*L-*trn*F region were identical in all the *S. densiflora* samples analysed (GenBank Accession no. EU056301). The sequences obtained for *Cynodon dactylon* (GenBank Accession no. EU056301) and different *Spartina* species (GenBank Accession nos EU056301 to EU056308)

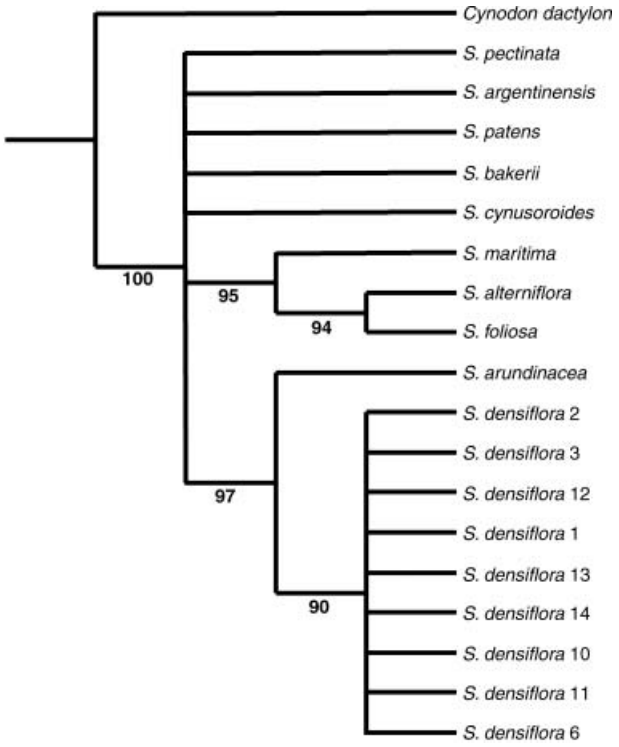

Fig. 2 Strict consensus of the 88 most parsimonious trees obtained from the *trn*T-*trn*L spacer data set. In bold: bootstrap values (10 000 replicates). Numbering of *Spartina densiflora* samples relate to Table 1.

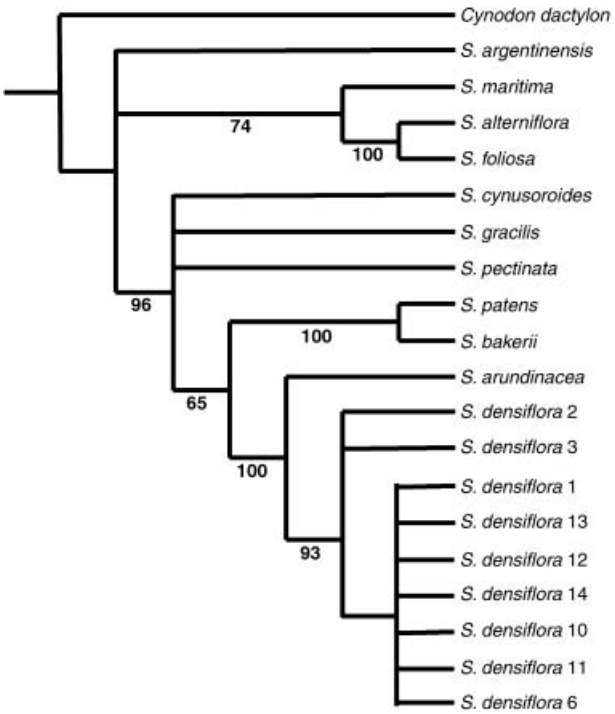

Fig. 3 Strict consensus of the 20 most parsimonious trees obtained from the *ITS* data set. In bold: bootstrap values (10 000 replicates). Numbering of *Spartina densiflora* samples relate to Table 1.

provided 958 aligned characters of which 890 were constant and 10 parsimony-informative. The *trn*T-*trn*L chloroplast data set was more informative than the *trn*L-*trn*F data set in which phylogenetic relationships between our taxa of interest were completely unresolved and where no sister species to *S. densiflora* could be identified. In the two chloroplast DNA regions analysed, all the *S. densiflora* samples with different geographical origins exhibited the same sequence. The combination of the two chloroplast data sets significantly decreased the resolution of the trees obtained; thus, we present the phylogenetic analysis based on the *trn*T-*trn*L data set only (Fig. 2).

This analysis resulted in 88 most parsimonious trees (length = 86; CI = 0.965; RI = 0.935). In the strict consensus tree (Fig. 2), the phylogenetic relationships between the different *Spartina* species were congruent with earlier work (Baumel *et al*. 2002). All *S. densiflora* sequences were sister to *S. arundinacea* (BS 97%). The maximum-likelihood analysis confirmed this result (not shown).

Nuclear DNA analyses

ITS region. The five cloned sequences per sample exhibited the same *ITS* sequence as those previously obtained by direct sequencing of amplified products (GenBank Accession nos AF372634 to AF372643, Baumel *et al*. 2002), indicating that the nrDNA repeats are most likely homogenized by concerted evolution in the polyploid *Spartina* species. The *ITS* region provided an alignment of 611 nucleotides with gaps ranging from one to six nucleotides. The data matrix contained 409 constant characters and 77 parsimony-informative characters. The maximum-parsimony method returned 20 equally parsimonious trees (length = 275; CI = 0.865; RI = 0.848). The strict consensus tree (Fig. 3) was congruent with previous results from Baumel *et al*. (2002). All the accessions of *S. densiflora* from Argentina displayed the same sequence. The samples from Chile and California were identical and differed from the Argentina samples by one substitution. The sample from Argentina with the 'Patagonica' phenotype (no. 6, Table 1) exhibited the same sequence as the samples from California and Chile. All the *S. densiflora* sequences came out as sister to *S. arundinacea* (BS 100%). The maximum-likelihood analysis (not shown) confirmed this result.

Waxy gene. Molecular cloning performed on the *S. densiflora* samples from Argentina, Chile and California (Humboldt Bay, HB; San Francisco Bay, SFB) resulted in nine more or less different *Waxy* sequences (see below). These sequences were aligned together with previously cloned *Waxy* sequences of *Spartina* (Fortune *et al*. 2007) and resulted in a data matrix of 575 characters of which 380 are constant and 85 parsimony-informative. A maximum-parsimony analysis

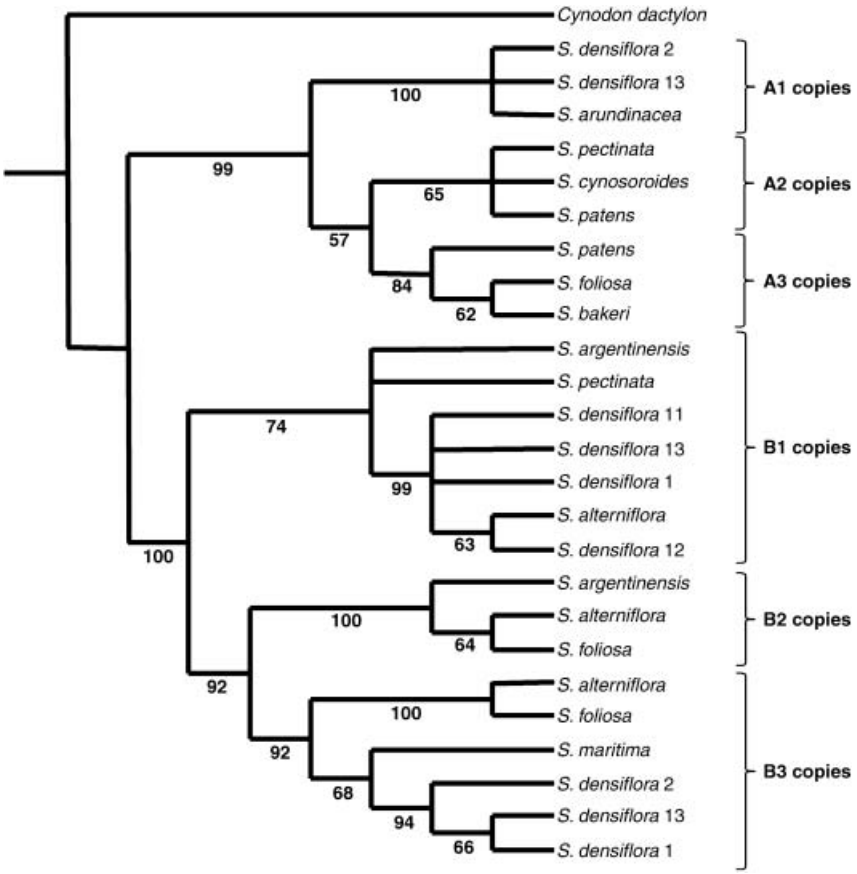

Fig. 4 Strict consensus of the four most parsimonious trees obtained from the *Waxy* data set. In bold: bootstrap values (10 000 replicates). Numbering of *Spartina densiflora* samples relate to Table 1.

performed on this data set returned four equally parsimonious trees (length = 235; CI = 0.923; RI = 0.960, Fig. 4). The Maximum Likelihood analysis displayed the same topology as the strict consensus of the four most parsimonious trees (not shown).

Based on the strict consensus tree (Fig. 4), two highly divergent, well-supported gene lineages were identified, corresponding to two *Waxy* paralogues, A (BS 99%) and B (BS 100%), as described by Fortune *et al*. (2007).

In the A *Waxy* lineage, three clades were encountered (A1, A2 and A3). Within the A1 clade, sequences of *S. densiflora* from Argentina (2) and California (HB, 13) grouped together with the sequence of *S. arundinacea* (BS 100%). The A2 clade (BS 65%) was composed of the sequences of *S. pectinata*, *S. cynusoroides* and *S. patens*. Within the A3 clade (BS 84%), another copy of *S. patens* was sister to sequences of *S. bakerii* and *S. foliosa*. The A2 and A3 sequences of the tetraploid *S. patens* may represent homeologous *Waxy* A copies. Only one sequence type is encountered in the A clade for the other polyploid species; this may be the result of incomplete *Waxy* copy amplification or alternatively differential loss of homeologous copies in these species.

In the *Waxy* B lineage, three clades could be distinguished. The B1 clade (BS 74%) included a sequence of *Spartina argentinensis*, a sequence of *S. pectinata*, and a well-supported (99% bootstrap) subclade comprising sequences of *S. densiflora* from California (11, 13) and Chile (1), and *S. alterniflora*. The B2 clade (BS 100%) was composed of a copy of *S. argentinensis* as sister to identical *S. alterniflora* and *S. foliosa* sequences. The B3 clade (BS 92%) included two subclades. In one clade (BS 100%), a copy of *S. alterniflora* was sister to a copy of *S. foliosa*. In the other clade (BS 68%), a copy of *S. maritima* was sister to sequences of *S. densiflora* from Argentina (2), Chile (1) and California (13), the latter two being more closely related. In this B gene lineage, three divergent, homeologous sequences (B1, B2, B3) were identified in *S. alterniflora*, whereas two (B2, B3) homeologues were found in *S. foliosa* and only one (B3) was present in *S. maritima*. Thus, not all hexaploid species have retained three *Waxy* homeologues. Two different (B1 and B2), homeologous sequences were identified in *S. argentinensis*. One of these homeologues (B2) was closely related to those of *S. foliosa* and *S. alterniflora*. The two (B1, B3) homeologous copies that were encountered in the samples of *S. densiflora* were closely related the *S. alterniflora* and *S. maritima* B sequences, respectively, whereas in the A clade, the copy of *S. densiflora* was placed in a polytomy with that of *S. arundinacea*. One South American (2) and one Californian (13) *S. densiflora* plants were represented in both *Waxy* gene lineages. All *S. densiflora* subclades (A1, B1, and B3) contained specimens from both South-America and California. A few nucleotide changes

(0–3 substitutions) differentiated the orthologous sequences of the *S. densiflora* samples from Argentina, Chile and California which were almost identical to sequences previously reported for this species (A1: DQ534507, B1: DQ534503, B3: DQ534506, Fortune *et al*. 2007).

Molecular fingerprinting

Twenty-one and 19 polymorphic DNA markers were obtained by the ISSR and RAPD analyses, respectively. Table 2 displays the patterns observed for the RAPD markers. Two bands were specific to *S. foliosa* (A2575, D51100), two bands were specific to *S. alterniflora* (B7550, D5600), and six bands were specific to *S. densiflora* (B12350, B121250, B7700, B71200, D5550, D5800). Five bands were common to both *S. alterniflora* and *foliosa,* but not found in *S. densiflora* (B12550, B121100, B7650, B71100, D5300). Two bands were specific to subsets of *S. densiflora* (A2900 found in Humboldt Bay, B12750 found in all non-Argentinean *S. densiflora*). A single band was common to *S. alterniflora* and *S. densiflora* (A21400) and a single band was common to *S. densiflora* and *S. foliosa* (B7800).

Each individual was easily characterized to species or hybrid based on presence or absence of species specific bands. The F_1 hybrids, *S. densiflora* × *S. foliosa* and *S. alterniflora* × *S. foliosa*, showed an additive banding pattern of bands specific to their respective parent species. This additivity was not observed in any of the *S. densiflora* samples from Argentina, Chile or California, indicating that these samples did not result from recent hybridization involving the hexaploid *S. alterniflora* or *S. foliosa*.

The neighbour-joining analysis based on both ISSR and RAPD data (Fig. 5) indicated a clear divergence between the populations of *S. densiflora* and the two hexaploid species, *S. alterniflora* and *S. foliosa*. The samples from California appeared more similar to the sample collected in Chile than those from Argentina. All *S. densiflora* individuals from Argentina grouped together.

Discussion

Our molecular investigations of *Spartina densiflora* samples from different locations in South and North America provide new insights into the polyploid nature and origin of this enigmatic species and shed light on the role of hybridization in generating invasive species.

Ploidy level of native and introduced S. densiflora *populations*

The flow cytometry analyses reveal that most native and introduced populations of *S. densiflora* have higher genome size than the other species known to be tetraploid or hexaploid. All the samples we have analysed display a higher DNA content than the hexaploid *Spartina* species, *S. maritima*, *S. alterniflora* and *S. foliosa*, and contradicts some speculations about the possible hexaploidy of *S. densiflora* (Spicher & Josselyn 1985). Genome size variation may result from several genomic changes, including rapid accumulation or elimination of repetitive DNA involving transposable elements, structural rearrangement, segmental duplication or whole genome duplication (polyploidy) (Bennetzen *et al*. 2005; Piegu *et al*. 2006). In this case, our results confirm the chromosome number $2n = 70$ found here and reported by Ayres *et al*. (2008a). As all the accessions of *S. densiflora* analysed in this study exhibit the same genome size, it is reasonable to consider this species as heptaploid over all the range examined.

Gene phylogenies and origin of S. densiflora

The combination of cytoplasmic and nuclear DNA markers has been particularly informative in revealing the origin of *S. densiflora*. The chloroplast DNA phylogenies indicate that all *S. densiflora* samples derive from a lineage closely related to *Spartina arundinacea*, endemic from sub-Antarctic islands. The two nuclear sequences analysed (*ITS* and *Waxy*)

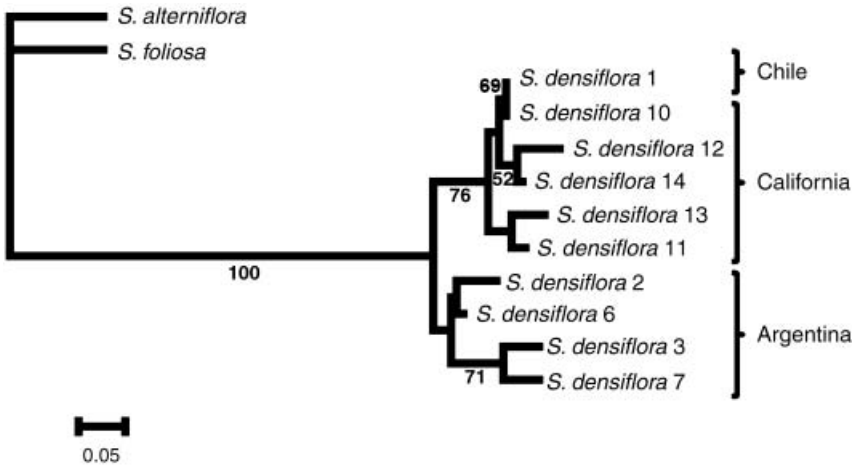

Fig. 5 Neighbour-joining tree based upon RAPD and ISSR data. In bold: bootstrap values (10 000 replicates).

Table 2 RAPD-banding patterns in *Spartina densiflora* samples

	A2575	A2900	A21400	B12350	B12550	B12750	B121100	B121250	B7550	B7650	B7700	B7800	B71100	B71200	D5300	D5550	D5600	D5800	D51100
alt	0	0	1	0	1	0	1	0	**1**	1	0	0	1	0	1	0	**1**	0	0
alt × folio	**1**	0	1	0	1	0	1	0	**1**	1	0	1	1	0	1	0	**1**	0	**1**
folio	**1**	0	0	0	1	0	1	0	0	1	0	1	1	0	1	0	0	0	**1**
densi × folio	0	0	1	1	1	1	1	0,5	0	1	1	1	1	1	1	1	0	1	**1**
densi 10	0	0	1	1	0	1	0	1	0	0	1	1	0	1	0	1	0	1	0
densi 11	0	0	1	1	0	1	0	1	0	0	1	1	0	1	0	1	0	1	0
densi 12	0	1	1	1	0	1	0	1	0	0	1	1	0	1	0	1	0	1	0
densi 14	0	1	1	1	0	1	0	1	0	0	1	1	0	1	0	1	0	1	0
densi 13	0	1	1	1	0	1	0	1	0	0	1	1	0	1	0	1	0	1	0
densi 1	0	0	1	1	0	1	0	1	0	0	1	1	0	1	0	1	0	1	0
densi 2	0	0	1	1	0	0	0	1	0	1	1	1	0	1	0	1	0	1	0
densi 3	0	0	1	1	0	0	0	1	0	0	1	1	0	1	0	1	0	1	0
densi 6	0	0	1	1	0	0	0	1	0	0	1	1	0	1	0	1	0	1	0
densi 7	0	0	1	1	0	0	0	1	0	0	1	1	0	1	0	1	0	1	0

S. foliosa specific
S. alterniflora specific
S. densiflora specific
Shared by *S. alterniflora* and *S. foliosa*

alt, *S. alterniflora*; alt × folio, *S. alterniflora* × *S. foliosa* hybrid; folio, *S. foliosa*; densi, *S. densiflora*; densi × folio, *S. densiflora* × *S. foliosa* hybrid.

provide complementary information: All the cloned repetitive nuclear rDNA sequences (*ITS*) examined in the different accessions display the same sequence, a sequence that is sister to *S. arundinacea*. This suggests that the rDNA repeats have been homogenized by concerted evolution toward this parental repeat type.

Three divergent *Waxy* sequence types were isolated from *S. densiflora* (Fig. 4), which phylogenetically resolve in a manner consistent with the hypothesis of two *Waxy* paralogues (A and B) in genus *Spartina* (Fortune *et al*. 2007). Two sequence types (B1 and B3) of *S. densiflora* are sister to *S. maritima* and *S. alterniflora*, respectively. The third sequence type fall into clade A and is sister to *S. arundinacea*. Thus, the B1 and B3 copies may represent homeologous genes (i.e. orthologous genes duplicated by polyploidy). The number of *Waxy* copies encountered was not correlated with the ploidy level of *S. densiflora* where up to seven homeologues may be expected at each orthologous locus in an allopolyploidy context. This discrepancy may be due to either differential copy loss of each of the *Waxy* paralogues that can affect duplicated genes (Small & Wendel 2000; Fortune *et al*. 2007) or to biased sequence sampling of the heterogeneous amplification products. In either case, the placement of the obtained copies in the two *Waxy* clades is highly informative: orthologous genes (i.e. within clade A or within clade B) trace species histories, and it appears that in these cases, the *Waxy* copies cloned in *S. densiflora* tell different stories in each of the paraloguous clades A and B. In clade B, the two homeologues indicate a close phylogenetic relationship with the hexaploid lineage (*S. maritima*, *S. alterniflora*, *S. foliosa*), whereas in clade A, the *Waxy* copy of *S. densiflora* points to a sister relationship with the tetraploid *S. arundinacea*. This latter placement agrees with the phylogenetic relationship exhibited by nuclear *ITS* and chloroplast sequences (Baumel *et al*. 2002; this study). On 20cloned sequences for *S. arundinacea*, only one 'A' sequence type was obtained. No sequences of *S. arundinacea* are represented in clade B, which suggests a probable loss of the corresponding sequences. Thus, the B1 copies of *S. densiflora* appear more related to the hexaploid *S. alterniflora* than to the tetraploid *Spartina patens* which is not congruent with the chloroplast and *ITS* data.

All together these data suggest a polyphyletic origin of *S. densiflora* involving a tetraploid lineage (*S. arundinacea*) and a member of the hexaploid *Spartina* lineage. This species appears to be composed of divergent genomes, a feature also shared by the other rapidly expanding allopolyploid species, *Spartina anglica* (Ainouche *et al*. 2004b). Thus, the invasive *S. densiflora* represents an additional example of reticulate evolution in the genus *Spartina.* Hybridization results in a genomic shock that is increasingly recognized as playing a central role in the plasticity and evolutionary success of allopolyploid genomes (Salmon *et al*. 2005; Albertin *et al*. 2006). In *Spartina*, hybridization at both homoploid and polyploid levels has generated notorious invaders with important ecological consequences on the salt marsh communities (Ainouche *et al*. 2008).

Evidence for superimposed reticulate events in the history of S. densiflora *populations in their native and introduced range*

Baumel *et al*. (2002) first noted the conflicting topologies between the chloroplast *ITS* phylogenies and *Waxy* data in a sample of *S. densiflora* collected in California. They interpreted this conflict as a result of possible hybridization involving hexaploid species in the sampled region (California) although Humboldt Bay, the source of their *S. densiflora* sample, contains neither *S. foliosa* nor *S. alterniflora* plants. In parallel, in San Francisco Bay where the native hexaploid *S. foliosa* and the introduced *S. alterniflora* co-occur with introduced plants of *S. densiflora*, Ayres and coworkers discovered plants that appeared to be hybrids between *S. foliosa* and *S. densiflora* (Ayres *et al*. 2008a). The question then arose as to whether the phylogenetic conflict mentioned above was a result of recent hybridization events following the expansion of this invasive species, or rather the result of a hybrid origin in its native range. While previous work (based on direct sequencing of *Waxy* PCR products) found that *S. densiflora* from Argentina displayed one sequence similar to that of *S. arundinacea* (Ainouche *et al*. 2004a), the present screening of 20 clones per accession of *S. densiflora* showed that the samples from both California and Argentina contain three *Waxy* copies that are related to either the hexaploid taxa or to the tetraploid *S. arundinacea*. In the present phylogenetic analyses, we took care to exclude the recent *S. foliosa* × *densiflora* hybrids discovered by Ayres and collaborators in San Francisco Bay. The polymorphic, multilocus, species-specific markers used here confirm that the plants from both Humboldt Bay and San Francisco Bay display a similar pattern to 'original' plants from Chile, and do not exhibit an interspecific F_1-hybrid pattern like the recent *S. foliosa* × *densiflora* hybrids. Our results taken together strongly indicate that, in addition to hybridization following the recent introduction (1970s) of *S. densiflora* to the San Francisco Bay marshes, this species has a reticulate origin in its native range.

The heptaploid genome of *S. densiflora* could have been formed in the native region by interspecific hybridization, through the union of an unreduced tetraploid gamete and a reduced triploid gamete from a hexaploid species. Our analyses point to the lineage represented by *S. arundinacea* as a tetraploid candidate parent; the candidate parental hexaploid species could be *S. alterniflora* that occurs along the South American coast. Interestingly, the possible hybridization between *S. alterniflora* and *S. densiflora* was early hypothesized in South America where plants of intermediate morphology were described by taxonomists (i.e. *S. longispica* Parodi 1919; Mobberley 1956). Further studies involving

extensive population sampling in this region are needed to explore these hypotheses.

Invasion routes of S. densiflora *from its centre of origin*

Spartina densiflora now occupies vast coastal areas in both hemispheres, resulting from the high invasive potential of the species (Bortolus 2006; Mateo-Naranjos *et al*. 2007). The greater abundance, broader phenotypic variation, and wider spectrum of habitats occupied by *S. densiflora* along the native South American east coastline compared to other populations (Parodi 1919; Mobberley 1956; Bortolus 2006) provide ecological evidence to support the hypothesis that the species originated at the east coast, and spread to the west coast of South American and from there to the west coast of North America (Chile and USA, respectively, Bortolus 2006). This scenario is fully supported by our molecular analyses where multilocus DNA markers indicate a close relationship between the samples from California and Chile, which are distinct from the samples from Argentina. Nevertheless, only one sample from Chile was analysed and displayed some differences with the Californian samples. More sampling is needed to ascertain if the differences observed result from independent evolution in California and Chile or to the lack of sampling of Chilean populations. Additional investigations within and among populations of South America using variable intraspecific molecular markers will aid in our understanding of the introduction routes for this species to other places, e.g. Spain.

From an ecological perspective, this is particularly important because the invasiveness of an exotic species can depend on the specific population, and in one notable case, the specific genotype that is introduced to a given region (Schierenbeck & Ainouche 2006). An example of this comes from the invasive genotype of *Phragmites australis* that was introduced from Europe to the USA. Although *P. australis* is cosmopolitan, researchers found a population with an exotic genotype in the USA that seemed to be much more invasive than the native genotypes (Saltonstall 2002). The contrasting invasiveness reported for *S. densiflora* in different regions worldwide suggests a similar situation (Bortolus 2006). These examples show how much organisms may differ in their biology, ecology and evolution, although they are considered belonging to the same species. These examples and our study show the need for constant reliable taxonomic checking through updated methods and perspectives (Bortolus 2007) and the importance of genealogical approaches to better understand the historical and evolutionary context of species expansion.

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Dr P. Fortune completed his PhD in 2007 on the evolution of genes duplicated by polyploidy and the history of invasive *Spartina* species. Dr K. Schierenbeck studies hybridization between native and non-native taxa and contributed to this work during a sabbatical at University of Rennes. Dr D. Ayres is concerned with the evolution of *Spartina* populations from California. Dr A. Bortolus works on the ecology of salt-marsh communities from Argentina. O. Catrice and Dr S. Brown work on genome size estimations. Dr M. Ainouche works on allopolyploid speciation and the evolutionary consequences of hybridization and genome duplication in *Spartina*.
