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Spartina versicolor Fabre: Another case of *Spartina* trans-Atlantic introduction?

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Abstract Intercontinental introductions are widespread in the genus *Spartina*, with important ecological and evolutionary consequences. The native or introduced status of *Spartina* species is then critical with regard to biodiversity assessment, especially for vulnerable Mediterranean coastline ecosystems. *Spartina versicolor* was first recorded in southern France in 1849, then successively in various places on the European and North-African Mediterranean and Atlantic coasts. This species is considered to be either a European native or an invasive species introduced from North America which has a high morphological similarity to the Atlantic American species *Spartina patens*. We performed extensive sampling of *S. versicolor* in Europe and North Africa (from natural populations and herbarium collections) and compared these samples to other European and American *Spartina* species (including *S. patens*). Chromosome counts were reported for the first time and revealed that *S. versicolor* is tetraploid ($2n = 4x = 40$). Phylogenetic analyses based on chloroplast and nuclear ribosomal DNA sequences did not reveal any molecular variation within *S. versicolor.* In this species, a single haplotype, that is identical to one haplotype of *S. patens*, was found in the four chloroplast and the nuclear ribosomal ITS regions investigated. In addition, simple sequence repeat markers were used and revealed a low level of genetic diversity within *S. versicolor*, suggesting that the introduction of *S.*

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versicolor occurred from a narrow genetic pool of *S. patens* from North America.

Keywords Cordgrass · Genetic diversity · Species status · Mediterranean · Microsatellites · Phylogeny

Introduction

Wetland habitats are among the most threatened in the Mediterranean as a consequence of intense urbanization, anthropogenic disturbance and increased number of invasive taxa (Médail and Verlaque 1997). In France, Mediterranean wetlands are among the habitats that are the most colonized by invasive species (Verlaque et al. 2002). Because surveys of biodiversity are generally poorly coordinated in the Mediterranean biodiversity hotspot (Marignani et al. 2014), inference of the native or introduced plant species status is not trivial. This status is an essential parameter for biodiversity management and conservation biology. It also represents critical information with regard to population and species evolutionary history. Establishing native status for a species in a given region is not an easy task and requires a combination of different approaches to elucidate the origin, mode of formation and biogeography of the considered taxon. The increased opportunities for long-distance human-mediated species dispersal make these researches even more complex (Kowarik 2003). In this context, molecular markers and evolutionary genetics provide important insight to trace back population, species origin and migration history (Mansion et al. 2008; Hardion et al. 2014).

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In *Spartina* (cordgrasses), recurrent intercontinental introduction events and biological invasions are particularly common and well-documented (Daehler and Strong 1996a, b; San Léon et al. 1999; Baumel et al. 2001; Sanchéz-Gullon 2001; Ayres et al. 2004; An et al. 2007; Ainouche et al. 2009; Campos et al. 2004; Lonard et al. 2010; Saarela 2012; Strong and Ayres 2013). This grass genus (Poaceae, Chloridoideae) represents a well-supported monophyletic lineage (Baumel et al. 2002; Fortune et al. 2007) closely related to some members of the paraphyletic *Sporobolus* genus and *Calamovilfa* (Peterson et al. 2014). It is composed of about 15 perennial species that have diversified mostly in the New World (Mobberley 1956). Introduction of species outside their native range over the past 150 years has accelerated diversification by facilitating hybridization with native species, introgression or speciation, resulting in several superimposed divergent genomes that coexist in the species currently found in the wild (Ainouche et al. 2012). The basic (haploid) chromosome number in *Spartina* is considered to be $x = 10$ (Marchant 1968), and all species recorded to date are polyploid, ranging from tetraploids to dodecaploids. Molecular phylogenies from nuclear and chloroplast DNA sequences have indicated that genus *Spartina* has evolved through two main lineages including tetraploid and hexaploid species respectively (Baumel et al. 2002). The tetraploid lineage is composed of species native to the New World, colonising coastal or inland salt marshes from either Northern (*Spartina patens*, *Spartina bakeri, Spartina gracilis*, *Spartina cynusoroides, Spartina pectinata*) or Southern (*Spartina ciliata, Spartina arundinacea*) hemispheres. The tetraploid *S. argentinensis* (syn. *S. spartinae*), which has a disjunct distribution in North-Central America and in South-America, is sister to the hexaploid lineage. This later clade is composed of *Spartina maritima*, *Spartina alterniflora*, and *Spartina foliosa,* all colonizing low marsh zones. *Spartina maritima,* native to the Western Europe and African Atlantic coasts, is one of the few Old World native species with recent taxa of hybrid origin and the controversial *S. versicolor* (see below). Accidental or deliberate introductions lead to various hybridization events within or between the tetraploid and hexaploid lineages (reviewed in Ainouche et al. 2012; Strong and Ayres 2013).

In Europe, introductions of the hexaploid *S. alterniflora*, native to the Atlantic American coasts and its subsequent hybridization with hexaploid *S.*

maritima led to the formation of two sterile F1 hybrids in Southern England (*S. x townsendii*) and in Southwest France (*S. x neyrautii*). Genome duplication in the British hybrid resulted in the vigorous and fertile allododecaploid *S. anglica* (Hubbard 1968; Guénégou et al. 1988; Gray et al. 1990; Gray et al. 1991). This species rapidly expanded in range and spread naturally to western European saltmarshes. It is now introduced in various continents, leading to various attempts to control or eradicate the species (e.g. Hacker et al. 2001; Cottet et al. 2007). Introduced *S. alterniflora* is progressing along the western Atlantic coasts of France and Spain (Baumel et al. 2003; Campos et al. 2004). Another introduced *Spartina* species in Europe is the native South-American heptaploid species *S. densiflora* (Fortune et al. 2008) that is invading Mediterranean saltmarshes of the Iberian peninsula (Bortolus 2006; Castillo et al. 2008), where it hybridized with the hexaploid *S. maritima* (Castillo et al. 2010).

In the western Mediterranean, damp depressions in dune habitats are colonized by *Spartina versicolor* Fabre (Fig. 1) that grows also in brackish marshes in the Atlantic Coast of the Southwest Iberian Peninsula. This species, also named *Spartina juncea* or *Spartina durieui* (Chevalier 1923; Saint-Yves 1932), had a controversial taxonomic status. It was initially recorded almost simultaneously in several Mediterranean places: first in Southern France near Agde (Fabre 1849), then in Italy (Parlatore 1848–1850), Algeria (Cosson and Maisonneuve 1867), and Portugal (Daveau 1897). In 1901, Neyraut detected this taxon on the Southwest French Atlantic coast, near Arcachon (Coste 1906). Since then, *S. versicolor* has established all along the western Mediterranean coasts: in Corsica (Jeanmonod and Burdet 1989) as well as on the Atlantic and Mediterranean coasts of the Iberian Peninsula (Sanchéz-Gullón 2001). *S. versicolor* was considered as either a native Mediterranean plant (e.g. Sanchéz-Gullón 2001; Giuliano and Stanisci 2010; Tison et al. 2014a, b), or an invasive species introduced from America (Sanz Elorza et al. 2004; Tison and de Foucault 2014). Based on morphological similarities, Mobberley (1956) considered *S. versicolor* as synonymous to *S. patens*, assuming that the Mediterranean populations were introduced from the Atlantic North American coast where *S. patens* is abundant in high marsh and dunes. Recent studies on salt marshes along the Spanish Atlantic coast have underlined the presence of *S. patens* in Europe (San Leon 1999; Page et al. 2010) and renewed interest in the status of *S. versicolor*. Prieto et al. (2011) examined three *S. versicolor* individuals from northern Spain (Asturias) using Internal transcribed Spacer (ITS) sequences of nuclear ribosomal DNA genes, and compared these sequences to those initially published in genus *Spartina* by Baumel et al. (2002) and Ferris et al. (unpublished). These individuals exhibited similar ITS sequence to S. *patens*, which led these authors to suggest that *S. versicolor* should be considered as *S. patens*. But it cannot be excluded that a native Mediterranean *Spartina* species exists besides the introduction of *S. patens* in Spain. Cases of cryptic invasion have already been documented in the recent history of *Spartina* (e.g. Bortolus et al. 2015) and can be difficult to detect (Valtuena et al. 2011).

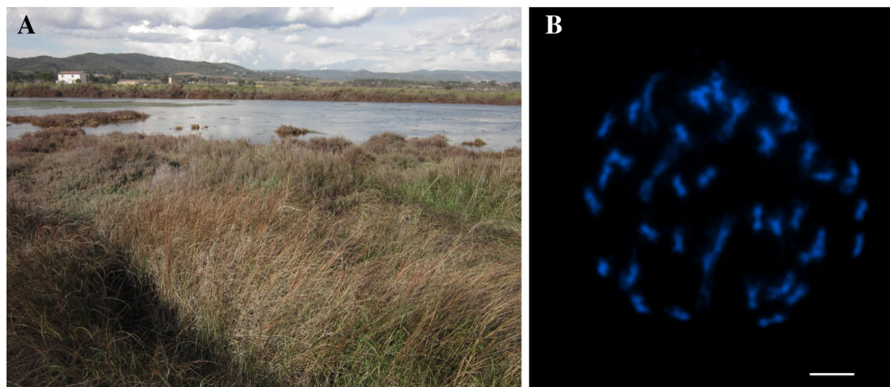


Fig. 1 *Spartina versicolor* **a** vigorous population from Vieux Salins (Hyères, France) **b** mitotic chromosomes counterstained with DAPI (2n = 40), bar represents 5 µm

In this study, we aim at answering the following questions: Is *S. versicolor* Fabre from Europe conspecific with *S. patens* from North America and if so is European *S. patens* another case of trans-Atlantic introduction into Europe? As highlighted above, worldwide invasions of *Spartina* are common phenomena and the Mediterranean coast may be one of the various places where American *Spartina* have settled after dispersal by ships during the 18th or 19th centuries. Gaining insight into the native or introduced status of *S. versicolor,* to its relationship with other *Spartina* species will be of critical importance in order to better understand the biogeography and diversification of *Spartina* species, as well as to determine the conservation priority level and management policy of *S. versicolor* in the Old World. To answer these questions, populations of *S. versicolor* sampled from various Mediterranean and Atlantic sites in Europe and North-Africa (including reference types from herbaria) are analyzed using cytogenetic and molecular (microsatellite, nuclear and chloroplast DNA sequences) data, and compared to North-American *Spartina* species.

Materials and methods

Plant material

Fifty-seven individuals of *S. versicolor* were sampled from numerous Mediterranean sites (n = 47) and from herbarium collections (n = 10) (Table S1). The analyzed samples include specimens from various populations in France (including Corsica), Italy, Portugal, Spain and Algeria as well as the first *S. versicolor* plants from Agde (France) discovered by Fabre (1849). Eight *Spartina patens* samples were obtained from the Atlantic North American coast (Table S1). Representatives from eight other *Spartina* species (*S. argentinensis*, *S. alterniflora*, *S. arundinacea*, *S. bakeri*, *S. densiflora*, *S. foliosa*, *S. maritima* and *S. pectinata*) and from *Sporobolus cryptandrus* were additionally introduced in phylogenetic analyses.

Chromosome counts

The chromosome number of *S. versicolor* was determined on mitotic chromosomes obtained from two plants collected in France (Vieux Salins and Saint-Louis du Rhone). Mitotic chromosomes were observed on metaphasic cells isolated from root tips. The roots tips of 0.5–1.5 cm length were treated with 0.04 % 8-hydroxiquinoline for 2 h at 4 °C in the dark followed by 2 h at room temperature to accumulate metaphases, then fixed in ethanol-acetic acid (3:1, *v/v*) for 12 h at 4 °C and stored in ethanol 70 % at −20 °C. After washing in 0.01 M enzyme buffer (citric acid-sodium citrate pH 4.5) for 15 min, the roots were digested in a solution of 5 % Onozuka R-10 cellulase (Sigma) and 1 % Y23 pectolyase (Sigma) at 37 °C for 30 min. The root tips were then washed with distilled water for 30 min. Root tips transferred on a slide were squashed in a drop of 3:1 ethanol-acetic acid fixation solution. After air-drying, slides were stained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured using a CoolSnap HQ camera (Photometrics, Tucson, Ariz) on an Axioplan 2 microscope (Zeiss, Oberkochen, Germany) and analysed using MetaVue™ (Universal Imaging Corporation, Downington, PA).

DNA isolation, PCR amplification and DNA sequencing

Genomic DNA was isolated from 100 mg of fresh (or 30 mg of herbarium) leaves from each individual using the NucleoSpin® Plant II Kit (Macherey–Nagel), following instructions provided by the manufacturer. DNA concentrations were estimated using the Nanodrop Spectrophotometer ND 1000 (Thermo Fischer Scientific).

Four chloroplast and ten nuclear regions were amplified. Chloroplast sequences were chosen among the most variable intergenic regions identified in Poaceae (Rousseau-Gueutin et al. 2015) or within *Spartina* (Blum et al. 2007; Kim et al. 2013): it included the *ndh*C-*trn*V, *pet*A-*psb*J (primers designed from Rousseau-Gueutin et al. 2015), and the *trn*L-*trn*F and *trn*T-*trn*L intergenic regions (Taberlet et al. 1991). Nuclear regions included Internal transcribed Spacers (*ITS*) of nuclear ribosomal genes (rDNA) (White et al. 1990) and nine microsatellite markers (SSR 6, 40, 44, 72, 109, 122, 161, 172, 188) identified from *S. maritima* Bacterial Artificial Chromosome end sequences (Ferreira de Carvalho et al. 2013). The primer sequences used in this study are indicated in Table S2.

Amplifications of chloroplast and ITS regions were carried out using the high fidelity KOD polymerase (Toyobo, Novagen) in a total volume of 50 µl. The reaction mix included 1X of KOD buffer, 1.5 mM MgSO4, 0.2 mM dNTP, 0.3 µM of each primer, 0.02 U of KOD polymerase, and 20 ng template DNA. Cycling conditions were 94 °C for 2 min, followed by 32 rounds of 94 °C for 20 s, 59.5 °C for 10 s and an extension at 70 °C for 15 s. Chloroplast and nuclear (ITS) PCR products were purified using the PCR Clean-up Gel extraction kit (Macherey–Nagel) and the purified products were sent to Macrogen Europe (Amsterdam, Netherlands) for direct sequencing. Long PCR products (from the *trn*T-*trn*L, *trn*L-*trn*F and *ndh*C-*trn*V regions) were sequenced from both sides. Sequences were cleaned and verified visually on the chromatograms (no double peaks observed).

Simple Sequence Repeat (SSR) detection was performed for 60 samples (52 Old Word *S. versicolor* and 8 New World *S. patens*) using 9 microsatellite loci. Amplifications of all microsatellites were performed in 20 µL that contained 10 ng DNA, 4 µL of 5X buffer, 4 mM MgCl2, 0.2 mM dNTPs, 0.4 µM of each primer, and 0.4 units of *Taq* polymerase (Q-Biogen) in a PTC-200 Gradient Thermal Cycler (MJ Research), following touchdown PCR protocols (Migliore et al. 2013). The 5′ ends of the forward primers were labelled with PET or NED. The fluorescently labelled PCR products were diluted (1/40) and were separated by capillary electrophoresis, with a 500 bp size standard (LIZ500), using an ABI Prism® 3730xl (Applied Biosystems) automatic sequencer. Alleles were sized using PEAK SCANNER 1.0 software (Applied Biosystems). Genotyping (PCR and electrophoresis) was repeated for 8 samples to verify the reproducibility of the peak patterns.

Phylogenetic analyses

The data matrices generated for individual or concatenated chloroplast regions as well as the nuclear ITS matrix were obtained after aligning all sequences using Geneious (Drummond et al. 2010) and adjusting them manually. These matrices were first subjected to phylogenetic analyses using maximum parsimony. Sequence data were analyzed using PAUP* v4.0b10 (Swofford 2001) with heuristic search and the default search options. The phylogenetic analyses were performed using sequences from *Sporobolus cryptandrus*, a closely related species to *Spartina* (Peterson et al. 2014) as outgroup. Bootstrap analyses were performed with 1000 replicates (Felsenstein 1985). In addition, these data matrices were subjected to Maximum Likelihood phylogenetic analyses. The best-fitted model of sequence evolution for each region (individual or concatenated) was determined by using JModeltest (Posada 2008) implemented in MEGA 5.0 (Tamura et al. 2011). Maximum likelihood analyses were then performed for each matrix using PhyML (Guindon and Gascuel 2003), with 1000 replicates of bootstrap.

Microsatellite analyses

Out of the nine SSR loci, six were considered to be reliable and were subsequently analyzed. After comparisons of replicates, all dubious peaks were removed. Since most genotypes have at least three alleles in this polyploid species (see below), SSR markers were analyzed as binary data and the matrix of allele size was converted into presence/absence data matrix. Genalex 6.51 software (Peakall and Smouse 2006) was used to search for matching genotypes, bearing evidence for identical clones. The search for matching genotype was repeated accounting for one, two or three allelic errors. Allelic accumulation curves were performed (specaccum function, *vegan* R package, Oksanen et al. 2013) to match allelic richness of *S. versicolor* and *S. patens*.

Structure of genetic diversity was analyzed using multivariate analyses of *ade4* and *adegenet* packages of R (Dray and Dufour 2007; Jombart 2008). Principal coordinate analysis (PcoA, dudi.pco function, *ade4* R package) was based on Jaccard distances (Jaccard 1901) computed on SSR presence/absence (dist.binary function, *ade4* R package). For matching samples having identical genotypes (clones), only one genotype was kept in analyses based on individual genotypes and these were identified as “clones” on the PcoA plot. A discriminant analysis was conducted on the correlation of allele presence/absence to distinguish genetic groups according to the clustering procedure designed by Jombart et al. (2010) (DAPC analysis, *adegenet* R package). Finally, allele frequencies within these groups were used to compute Nei distances (Nei 1972) and to build a Neighbor Joining network to explore relationships between DAPC

genetic groups (nj function, *ape* R package, Paradis et al. 2004).

Results

Chromosome counts revealed that in both analyzed populations, *S. versicolor* individuals have 2n = 40 chromosomes (Fig. 1b), indicating that this taxon is a tetraploid species.

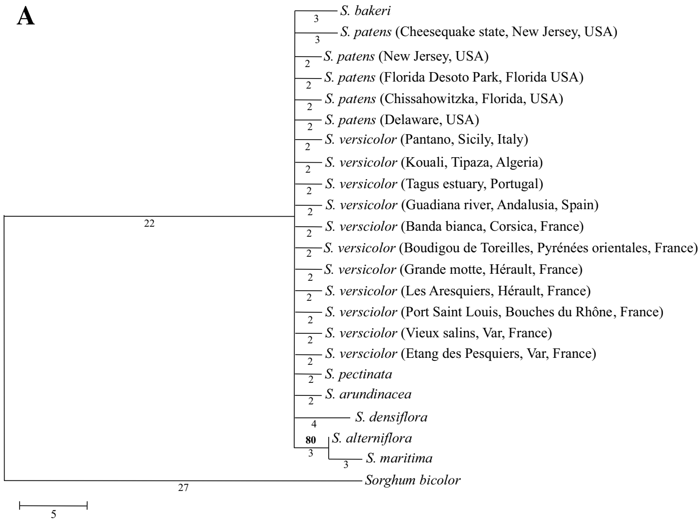
No sequence heterogeneity was observed in either chloroplast or nuclear (*ITS*) sequences. Intra-genomic polymorphism might be expected in polyploid nuclear genomes, but the *ITS* regions (belonging to the rDNA gene family) seem to have undergone concerted evolution as previously found in other *Spartina* species (Baumel et al. 2002; Boutte et al. 2015). Maximum Parsimony (MP) analyses were performed using the *ndh*C-*trn*V (601 bp), *pet*A-*psb*J (470 bp), *trn*L-*trn*F (577 bp), *trn*T-*trn*L (631 bp), chloroplast concatenated (2278 bp) or nuclear ribosomal ITS (457 bp) matrices. These analyses resulted in 23, 34, 20, 23, 3 and 19 equal most parsimonious trees. In these analyses, *S. versicolor, S. patens* and *S. bakeri* always belonged to the same clade (Fig. 2). For the most resolved tree corresponding to the ITS regions, these three species belong to a well-supported clade (99 %) and are as positioned as a sister clade to *S. arundincacea* and *S. densiflora* (100 % bootstrap support). The sequences obtained from all *S. versicolor* (including the sequences obtained by Prieto et al. 2011) and *S. patens* samples were identical, apart from a single substitution observed in one accession of *S. patens* (Cheesequake state Park, Florida) for the *ndh*C-*trn*V region. *S. versicolor* and *S. patens* are closely related to *S. bakeri*, presenting only two substitutions (one for the *ndh*C-*trn*V and one for the ITS regions). Since similar tree topologies were obtained using Maximum Likelihood, only the MP trees are presented here.

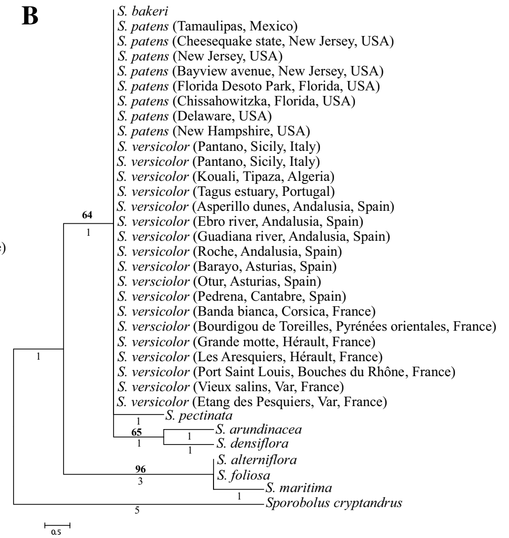
Thirty-seven alleles were recorded over the six SSR loci. All SSR genotypes were heterozygous with mostly 3 or 4 alleles per locus. Within *S. versicolor*, search for matching genotypes revealed 35 genotypes among 52 samples, 31 being unique and 4 being repeated from 4 to 11 times. These identical genotypes are referred as "clones". Accounting for one, two or three allele errors we found 41, 44 and 50 matching genotypes in *S. versicolor*, whereas no matching

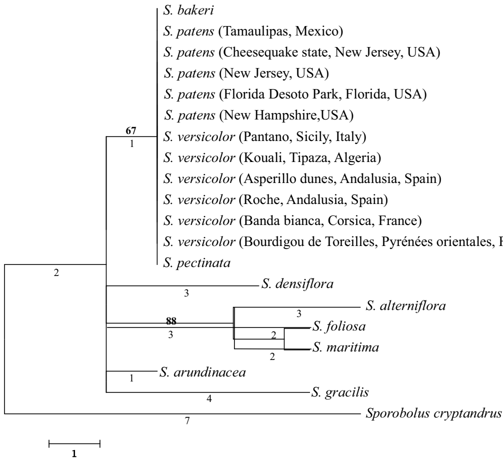
Fig. 2 Molecular phylogeny of *Spartina* based on chloroplast ▶ (**a** *ndh*C-*trn*V: 601 bp; **b** *pet*A-*psb*J: 470 bp, **c** *trn*L-*trn*F: 577 bp, **d** *trn*T-*trn*L: 631 bp, **e** concatenated sequences: 2278 bp) or **f** nuclear ribosomal ITS sequences (457 bp) using the maximum parsimony method. For each phylogeny, one of the equally parsimonious trees that is topologically identical to the 50 % majority-rule consensus tree (*pet*A-*psb*J: 34 equally parsimonious trees; concatenated chloroplast sequences: three equally parsimonious trees; ITS: 19 equally parsimonious trees) or the 50 % majority-rule consensus tree (*ndh*C-*trn*V: 23 parsimonious trees; *trn*L-*trn*F: 20 parsimonious trees) is presented. The bootstrap percentages (1000 replicates) are shown in bold above the branches and the number of changes is indicated below. The tree is rooted using either *Sporobolus cryptandrus* or *Sorghum bicolor*

genotypes were found among the 8 samples of *S. patens* even accounting for 3 allele errors. Accumulation curve (Fig. 3) accounting for the unequal sampling between *S. versicolor* (n = 52) and S. *patens* (n = 8) revealed higher allelic diversity in *S. patens* than in *S. versicolor:* i.e. for 6 samples 32 alleles were encountered in *S. patens* against 26 in *S. versicolor* (Fig. 3).

The PcoA analysis based on Jaccard distances computed between individual genotypes (Fig. 4) revealed that the main structure is due to differentiation between 6 out of 8 *S. patens* genotypes. The other 2 *S. patens* samples are more similar to *S. versicolor* genotypes. Herbarium specimens are scattered among *S. versicolor* genotypes, except one (collected in Carnon, France, in 1880 by Jouve) that is grouped with *S. patens* (H6, Fig. 4). The herbarium specimen collected by Fabre in (1849) in Agde has one of the genotypes recorded on many individuals ("matching genotypes" see Materials and methods) and identified as clone "b" (Fig. 4). This clone is found in France, Basque area, Italy and Sicily. According to the DAPC analysis (Fig. 4) and NJ network (Fig. 5), the SSR genotypes were optimally clustered in six groups. The genetic cluster number 4 (represented in green, Figs. 5, 6) was composed of European samples from France (natural populations and most herbarium samples), Italy, Corsica, and North Spain. The individuals from the southern Iberic Peninsula (south of Spain and Portugal) and Algeria are grouped in two (1 and 6) closely related clusters. Three genetic clusters could be distinguished in the American samples of *S. patens*: (1) Mexico-Florida-Delaware (2) New Jersey and (3) New-Jersey Hampshire. The herbarium sample from France sampled in Carnon (Herault) was assigned in a group with *S. patens* (Mexico-Florida-Delaware group).


A
S. bakeri
S. patens (Cheesequake state, New Jersey, USA)
S. patens (New Jersey, USA)
S. patens (Florida Desoto Park, Florida USA)
S. patens (Chissahowitzka, Florida, USA)
S. patens (Delaware, USA)
S. versicolor (Pantano, Sicily, Italy)
S. versicolor (Kouali, Tipaza, Algeria)
S. versicolor (Tagus estuary, Portugal)
S. versicolor (Guadiana river, Andalusia, Spain)
S. versciolor (Banda bianca, Corsica, France)
S. versicolor (Boudigou de Toreilles, Pyrénées orientales, France)
S. versicolor (Grande motte, Hérault, France)
S. versicolor (Les Aresquiers, Hérault, France)
S. versciolor (Port Saint Louis, Bouches du Rhône, France)
S. versciolor (Vieux salins, Var, France)
S. versciolor (Etang des Pesquiers, Var, France)
S. pectinata
S. arundinacea
S. densiflora
S. alterniflora
S. maritima
Sorghum bicolor
5


B
S. bakeri
S. patens (Tamaulipas, Mexico)
S. patens (Cheesequake state, New Jersey, USA)
S. patens (New Jersey, USA)
S. patens (Bayview avenue, New Jersey, USA)
S. patens (Florida Desoto Park, Florida, USA)
S. patens (Chissahowitzka, Florida, USA)
S. patens (Delaware, USA)
S. patens (New Hampshire, USA)
S. versicolor (Pantano, Sicily, Italy)
S. versicolor (Pantano, Sicily, Italy)
S. versicolor (Kouali, Tipaza, Algeria)
S. versicolor (Tagus estuary, Portugal)
S. versicolor (Asperillo dunes, Andalusia, Spain)
S. versicolor (Ebro river, Andalusia, Spain)
S. versicolor (Guadiana river, Andalusia, Spain)
S. versicolor (Roche, Andalusia, Spain)
S. versicolor (Barayo, Asturias, Spain)
S. versciolor (Otur, Asturias, Spain)
S. versicolor (Pedrena, Cantabre, Spain)
S. versicolor (Banda bianca, Corsica, France)
S. versciolor (Bourdigou de Toreilles, Pyrénées orientales, France)
S. versicolor (Grande motte, Hérault, France)
S. versicolor (Les Aresquiers, Hérault, France)
S. versicolor (Port Saint Louis, Bouches du Rhône, France)
S. versicolor (Vieux salins, Var, France)
S. versicolor (Etang des Pesquiers, Var, France)
S. pectinata
S. arundinacea
S. densiflora
S. alterniflora
S. foliosa
S. maritima
Sporobolus cryptandrus
0.5


C
S. bakeri
S. patens (Tamaulipas, Mexico)
S. patens (Cheesequake state, New Jersey, USA)
S. patens (New Jersey, USA)
S. patens (Florida Desoto Park, Florida, USA)
S. patens (New Hampshire,USA)
S. versicolor (Pantano, Sicily, Italy)
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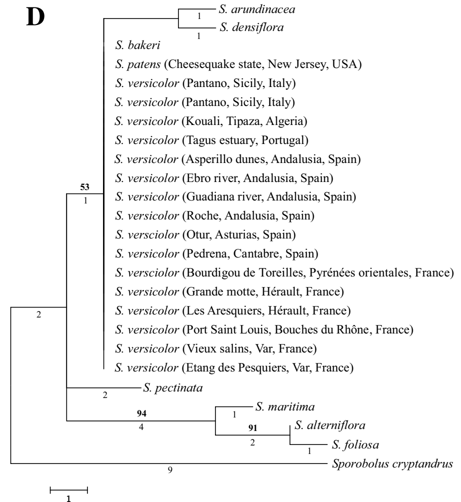
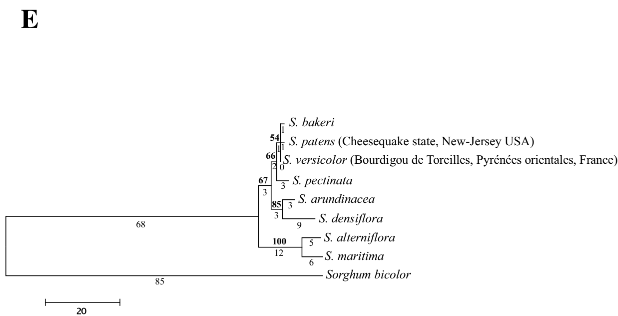
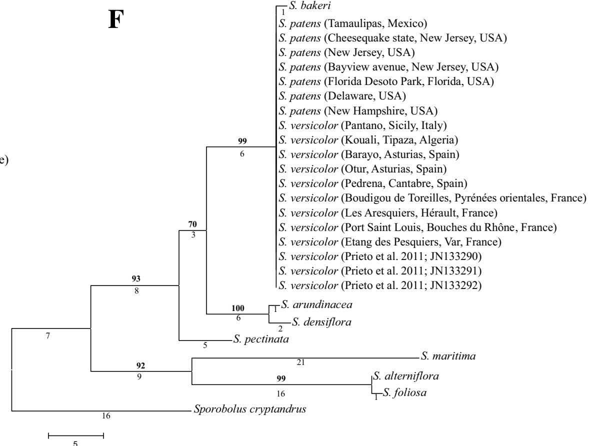
S. foliosa
S. maritima
S. arundinacea
S. gracilis
Sporobolus cryptandrus
1


D
S. arundinacea
S. densiflora
S. bakeri
S. patens (Cheesequake state, New Jersey, USA)
S. versicolor (Pantano, Sicily, Italy)
S. versicolor (Pantano, Sicily, Italy)
S. versicolor (Kouali, Tipaza, Algeria)
S. versicolor (Tagus estuary, Portugal)
S. versicolor (Asperillo dunes, Andalusia, Spain)
S. versicolor (Ebro river, Andalusia, Spain)
S. versicolor (Guadiana river, Andalusia, Spain)
S. versicolor (Roche, Andalusia, Spain)
S. versciolor (Otur, Asturias, Spain)
S. versicolor (Pedrena, Cantabre, Spain)
S. versciolor (Bourdigou de Toreilles, Pyrénées orientales, France)
S. versicolor (Grande motte, Hérault, France)
S. versicolor (Les Aresquiers, Hérault, France)
S. versicolor (Port Saint Louis, Bouches du Rhône, France)
S. versicolor (Vieux salins, Var, France)
S. versicolor (Etang des Pesquiers, Var, France)
S. pectinata
S. maritima
S. alterniflora
S. foliosa
Sporobolus cryptandrus
1


E
S. bakeri
S. patens (Cheesequake state, New-Jersey USA)
S. versicolor (Bourdigou de Toreilles, Pyrénées orientales, France)
S. pectinata
S. arundinacea
S. densiflora
S. alterniflora
S. maritima
Sorghum bicolor
20


F
S. bakeri
S. patens (Tamaulipas, Mexico)
S. patens (Cheesequake state, New Jersey, USA)
S. patens (New Jersey, USA)
S. patens (Bayview avenue, New Jersey, USA)
S. patens (Florida Desoto Park, Florida, USA)
S. patens (Delaware, USA)
S. patens (New Hampshire, USA)
S. versicolor (Pantano, Sicily, Italy)
S. versicolor (Kouali, Tipaza, Algeria)
S. versicolor (Barayo, Asturias, Spain)
S. versicolor (Otur, Asturias, Spain)
S. versicolor (Pedrena, Cantabre, Spain)
S. versicolor (Boudigou de Toreilles, Pyrénées orientales, France)
S. versicolor (Les Aresquiers, Hérault, France)
S. versicolor (Port Saint Louis, Bouches du Rhône, France)
S. versicolor (Etang des Pesquiers, Var, France)
S. versicolor (Prieto et al. 2011; JN133290)
S. versicolor (Prieto et al. 2011; JN133291)
S. versicolor (Prieto et al. 2011; JN133292)
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S. pectinata
S. maritima
S. alterniflora
S. foliosa
Sporobolus cryptandrus
5

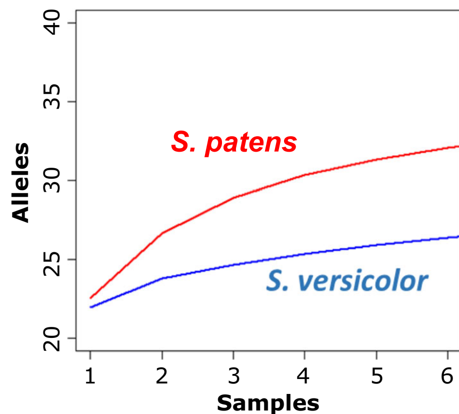

Fig. 3 Rarefaction curves of allelic diversity for the seven SSR loci

The *S. patens* cluster being the most similar to *S. versicolor* (NJ network Fig. 6) is observed all along the Atlantic North American coast from Mexico to Delaware. The cluster 4 which has most of the *S.versicolor* samples, and the herbarium sample collected by Fabre, are the most similar to *S. patens* according to the NJ network (Fig. 6).

Discussion

Our results reveal that *S. versicolor* is a tetraploid species with 40 chromosomes, as found in *S. patens* (Marchant 1968) and no European or North-African populations analyzed can be differentiated genetically from North American *S. patens* samples as they exhibit similar rDNA *ITS* and cpDNA sequences. In his monograph of *Spartina*, Mobberley (1956) stressed the morphological similarities between these two taxa, although several phenotypes were described for *S. patens* (Mobberley 1956). Our results support the hypothesis that all European and African populations of *S. versicolor* are in fact North American *S. patens* introduced before or at the beginning of the nineteenth-century.

Although some microsatellite variation was detected between *S. patens* and *S. versicolor*, only few genotype differences were observed. Genetic differences regarding microsatellite alleles would most likely result from intraspecific genetic diversity in *S. patens-versicolor* populations; this is supported by genetic similarities between some North-American *S. patens* and *S. versicolor* samples as can be seen on PcoA results (Fig. 4). The introduction origin could be in the areas covered by the genetic cluster 4 (Fig. 5), *i.e.* France, North Spain or Italy because the corresponding genotype is the most similar to *S. patens* in the NJ network (Fig. 6). This pattern is also clear in the PcoA analysis (Fig. 4). The *S. versicolor* samples from Portugal and South Spain, or Algeria are either derived from this introduction or resulted from a second introduction.

Spartina patens is a highly variable rhizomatous species, exhibiting high ecological amplitude along the Atlantic coast of North America from Canada to Central America, colonizing high salt marsh zones, beaches and sand dunes, with variable seed set (Silander and Antonovics 1979). Allozyme studies in native *S. patens* populations (Silander 1984) revealed important polymorphism with a decreased role of vegetative reproduction from dune to marsh habitats. In contrast microsatellite genotyping revealed reduced genetic diversity in *S. versicolor* compared to North American *S. patens* samples (Fig. 3), which is consistent with a genetic bottleneck following introduction in Europe together with the predominant clonal propagation of the introduced plants. Indeed we found 17 matching SSR genotypes within *S. versicolor* but this number increased to 41 when accounting for one allele error indicating that genetic variation within *S. versicolor* could be mainly of somaclonal variation. Preliminary surveys in *S. versicolor* populations revealed sterile pollen (R. Amirouche, unpublished data), which is in agreement with the observation that this taxon rarely produces seeds (Fabre 1849; San Leon et al. 1999; Tison et al. 2014; our personal observations). Further sampling and phylogeographic analyses are needed in the native region of *S. patens* to better document the history of this taxon and to identify the precise populations that were introduced in the Mediterranean. Although various studies have documented distribution, ecology and plasticity of *S. patens* in North America (e.g. Frasco and Good 1982; Burdick and Mendelssohn 1987; Burdick et al. 1989; Foote and Reynolds 1997; Lonard et al. 2010), very few studies have documented genetic diversity in *S. patens* (e.g. Wu 2012) and there is a great need to develop DNA-based analyses at the genome level in the native range of this species, which plays an important ecological role, preventing coastal erosion and being used in dune restoration.

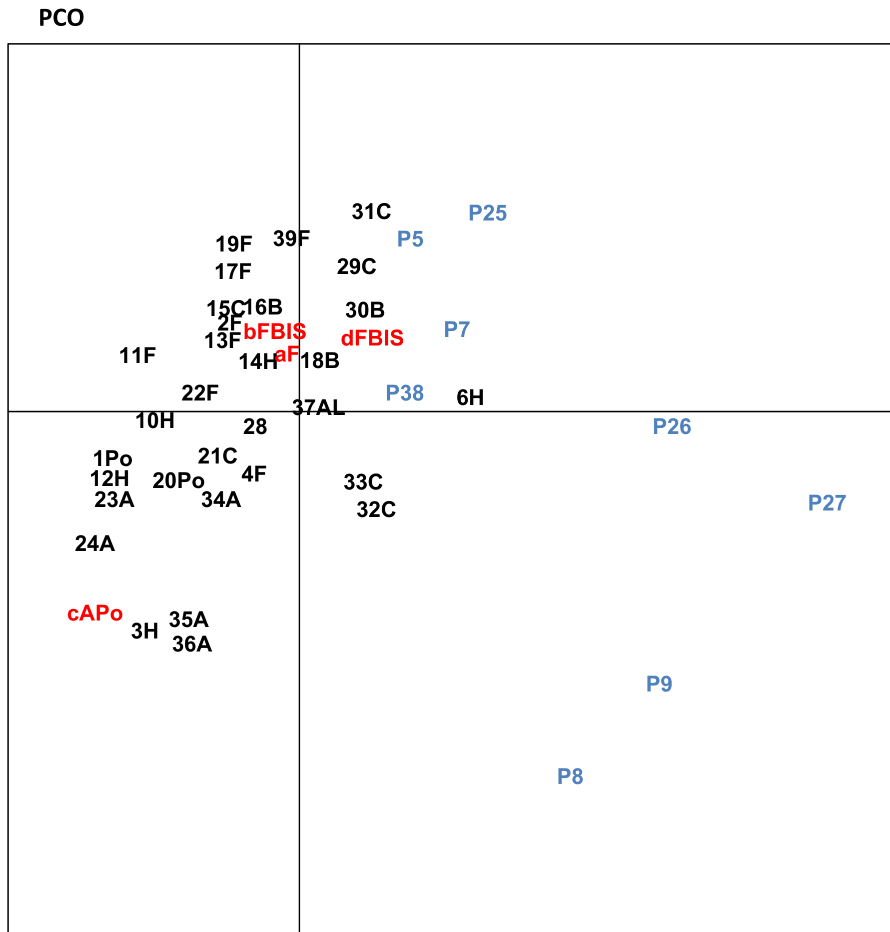

Fig. 4 Principal coordinates analysis (PcoA) of Jaccard distance based on individual SSR genotypes. *Upper-case letters* indicates the geographical origin of the sample (*A* Andalusia, *AL* Algeria, *B* Basque area, *C* Corsica, *F* France, *H* Herbarium, *I* Italy, *Po* Portugal, *S* Sicily), except for "*P*" that corresponds to *S. patens* genotypes. *Lower-case letters* indicates one of the four clonal genotypes (i.e. identical genotypes among several individuals). The herbarium specimen collected by Fabre corresponds to clone "*b*"

Another finding of our molecular phylogenetic analyses is the very close relationship between *S. patens* and *S. bakeri* which exhibit nearly identical (one nucleotide substitution in *ITS*) nuclear and chloroplast DNA sequences, confirming previous phylogenetic studies (Baumel et al. 2002; Fortune et al. 2007). These two species are hardly separable morphologically, apart from the lack of rhizome in *S. bakeri*, and its preferential freshwater habitat (Mobberley 1956). Thus, the retention of *S. bakeri* as a separate species might be questioned and would deserve further investigation.

Our study contributes to the worldwide research performed on *Spartina* invasion (Strong and Ayres 2013), bearing support for future issues dealing with Mediterranean and Atlantic coastline invasions by *Spartina*. Indeed if *S. versicolor/patens* is scattered on the Mediterranean coast and in Southwest Iberian Peninsula (Gulf of Cádiz) where its habitats are small and rare, it is widespread in salt marshes of Gallicean estuaries on the Atlantic Spanish coast (San Leon et al. 1999; "green" genotypes in Figs. 5, 6) probably due to wetter climatic conditions and lower salinities than in the Mediterranean and in the Gulf of Cádiz. In The Gulf of Cádiz, *S. versicolor* is restricted to a few isolated populations on coastal dunes and brackish marshes where it accumulates high biomass levels but without spreading to large areas, probably due to its

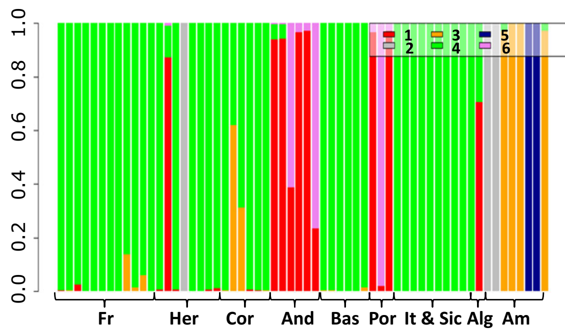

Fig. 5 Assignment of individual SSR genotypes to genetic clusters obtained from a discriminant analysis of principal components (*DAPC*). The *X axis* corresponds to the genotypes, which are labeled according to their geographical origin: France (*Fr*), Herbarium (*Herb*), Corse (*Cor*), Andalusia (*And*), Basque area (*Bas*), Portugal (*Por*), Italy (*It*), Sicily (*Sci*), Algeria (*Alg*) and *S. patens* from America (*Am*). The *Y axis* indicates the probability of assignment to genetic clusters after DAPC

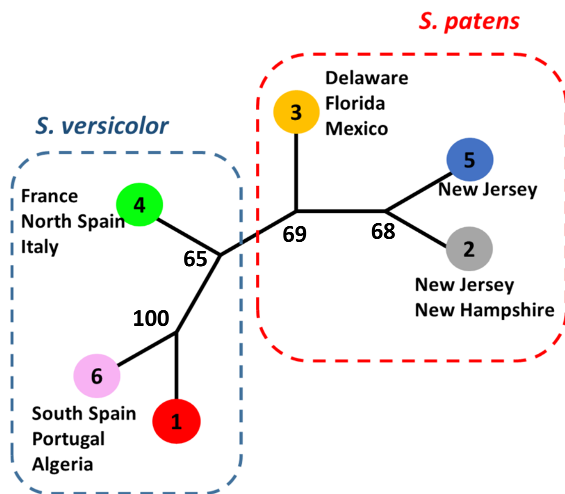

Fig. 6 Neighbor Joining network based on Nei genetic distances computed from allelic frequencies within the genetic groups obtained by the DAPC. Similar colors as for DAPC (Fig. 5) were used. *Bootstrap values* are indicated below the branches

very low seed set (Castillo, personal observation). *Spartina patens* has developed physiological mechanisms that make it able to tolerate variable salt and drought stress levels in both salt marsh and dune environments (Casolo et al. 2014). It can therefore invade new habitats if it is dispersed. Concerning recent expansion (since the twentieth century), *S. patens* was observed recently in the British Isles where one patch is known since 2005 on the Sussex coast (Stace 2010; Hounsome 2013); it was also introduced in China (An et al. 2007) and on the North American Pacific coast (Daehler and Strong 1996a; Ayres et al. 2004), where attempts to control and eradicate it are being conducted. In the Mediterranean region, *S. versicolor* appears to have expanded eastward (e.g. Italy: Valsecchi 1962; Bertacci and Lombardi 2014). In addition to our sampled site in North Africa (Kouali, near Algiers), *S. versicolor* was recorded on the East Algerian Coast near el Kala and Annaba (Cosson and Maisonneuve 1867). Updated surveys are therefore needed in Europe and North Africa to detect eventually new areas of spread.

Beyond the occurrence and spread of *S. versicolor*, conservationists and coastal managers must be aware that *Spartina* species are prone to hybridization, with either native or other introduced species (Strong and Ayres 2013) and further investigations are needed regarding the potential hybridization of *S. versicolor* with native (e.g. *S. maritima*) and invasive *Spartina* species (e.g. *S. densiflora*) in the places where they co-occur. Hybridization is known to play a key role in the *Spartina* invasion process; specifically in Spain where another recent case of *Spartina* introduction is documented, involving *S. densiflora* which became an invasive species competing with the native *S. maritima,* with which it also hybridized (Castillo et al. 2008, 2010).

In conclusion, our study provides new insights into the worldwide spread of *Spartina* and on the introduced origin of *S. versicolor*, which pinpoints new cases of introductions in Mediterranean wetlands, reinforcing the need to clarify the systematics, taxonomy and evolutionary history of introduced plants.

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