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Molecular Phylogeny of Hybridizing Species from the Genus *Spartina* Schreb. (Poaceae)

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Interspecific hybridization events have been reported in the genus *Spartina* Schreb. (Poaceae), involving the east American species *Spartina alterniflora*, and including either introgression (e.g., with the western American *Spartina foliosa*) or allopolyploid speciation (e.g., with the Euro–African *Spartina maritima*). Molecular phylogenetic analysis of the genus has been undertaken in order to understand phylogenetic relationships and genetic divergence among these hybridizing species. Twelve *Spartina* species have been sequenced for two nuclear DNA regions (*ITS* of ribosomal DNA, and part of the *Waxy* gene) and one chloroplast DNA spacer (*trnT-trnL*). Separate and conditional combined phylogenetic analyses using *Cynodon dactylon* as the outgroup have been conducted. *Spartina* is composed of two lineages. The first clade includes all hexaploid species: the Euro–African *S. maritima* ($2n = 60$), the East-American *S. alterniflora* ($2n = 62$) and the West-American *S. foliosa* ($2n = 60$). *Spartina alterniflora* appears as a closely related sister species to *S. foliosa*. Although belonging to the same lineage, *Spartina maritima* appears consistently more genetically differentiated from *S. alterniflora* than *S. foliosa*. The tetraploid species *S. argentinensis* ($2n = 40$) is placed at the base of this first clade according to the *Waxy* data, but its position is not well resolved by the other sequences. The second well-supported main lineage within genus *Spartina* includes the other tetraploid American species. Significant incongruence has been encountered between the *waxy* based tree and both the *ITS* and *trnT-trnL* trees concerning the position of *S. densiflora*, suggesting a possible reticulate evolution for this species. The results agree with hybridization patterns occurring in *Spartina*: introgression involving closely related species (*S. alterniflora* and *S. foliosa*) on one hand, and alloploid speciation involving more differentiated species (*S. alterniflora* and *S. maritima*) on the other hand. © 2002 Elsevier Science (USA)

***Key Words: Spartina*; phylogeny; hybridization; *ITS*; *Waxy gene*; cp *trnT-trnL* spacer.**

INTRODUCTION

Hybridization and polyploidy are common events in plants, and they represent major evolutionary processes in the Poaceae (Stebbins, 1987). Hybridization may have several alternative consequences (Arnold, 1997), ranging from hybrids of limited adaptive value, to successful introgression involving repeated backcrosses with parental genotypes, or stabilization of a new homoploid species (Rieseberg and Wendel, 1993; Rieseberg, 1997). Ultimately, chromosome doubling of hybrids may lead to highly fertile new allopolyploid species. These different situations are encountered in the genus *Spartina* Schreb., where at least three cases of recent interspecific hybridization have been well documented, with well-known evolutionary and ecological consequences (Marchant, 1967; Raybould *et al.,* 1991; Guénégou *et al.,* 1991; Gray and Raybould, 1997; Daehler and Strong, 1997; Ayres *et al.,* 1999; Ainouche and Baumel, 2001; Baumel *et al.,* 2001).

Spartina belongs to the subfamily Chloridoideae which represents a well-supported monophyletic lineage within Poaceae (Hsiao *et al.,* 1999). Most *Spartina* species (13) are native to the New World (Mobberley, 1956), and only four taxa originate from Western Europe: *Spartina maritima* (Curtis) Fernald, *Spartina anglica* C.E. Hubb., *Spartina x townsendii* H. Groves & J. Groves, and *Spartina x neyrautii* Foucaud. *Spartina* inhabits mainly intertidal mudflats, but some species extend to coastal dunes, inland freshwater swamps, or even dry prairies in central North America. Commonly referred to as cord grasses, they are perennial, often rhizomatous, but are also caespitose or stoloniferous. As in other Chloridoideae, the base chromosome number is $x = 10$ in the genus *Spartina*, $2n$ ranging from 40 to 124 for species investigated to date (Marchant, 1968). Both polyploidy and aneuploidy are observed in this genus (Marchant, 1968).

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Opportunities for an alien species introduction resulting in interspecific hybridization have dramatically increased since the last century as a consequence of human activities. As a striking example, the smooth cord grass *Spartina alterniflora* Loiseleur ($2n = 62$), native to the East (Atlantic) coast of North America, has been introduced to both the west (Atlantic) European coast and to the Western (Pacific) North American coast, and in both regions, it hybridized with indigenous species. In the mid-1970s, *S. alterniflora* was introduced into California, where it hybridized with the native *Spartina foliosa* Trin. (Daehler and Strong, 1997). Repeated hybridizations and backcrosses resulted in numerous introgressant hybrids, which threaten the original native populations of *S. foliosa* and which modify estuary ecosystems (Ayres *et al.,* 1999). During the last century, *S. alterniflora* was accidentally introduced in the Southampton Bay, England, where it hybridized with *S. maritima* ($2n =$ 60), giving rise, in 1870, to *S. x townsendii*, a sterile hybrid (Groves and Groves, 1882). Chromosome doubling produced the allopolyploid *S. anglica* ($2n = 120$, 122, 124, according to Marchant 1968), a fertile and vigorous invasive species colonizing British (Raybould *et al.,* 1991; Thompson, 1991) and French (Guénégou and Levasseur, 1993) salt marshes and estuaries since 1890 and 1906, respectively. This species is now introduced on several continents where various local policies are designed to control the spread of this species (Hedge *et al.,* 1997).

In contrast, the parental species remain confined to a few sites along the British coast (Gray and Raybould, 1997). *Spartina alterniflora* has been also introduced at various sites in France, but it has remained localized in Western Brittany (near Brest) and in the Southern Atlantic French coast near Hendaye, where hybrids (involving the native *S. maritima*) were also reported and described as *S. x neyrautii* (Foucaud, 1894).

Thus, introductions of *S. alterniflora* and its subsequent hybridization with different indigenous species have several evolutionary, ecological, and economical implications. Above all, *S. anglica* is known as a good example of recent, sympatric, allopolyploid speciation, where hybridization led to a new successful invasive species (Ellstrand and Schierenbeck, 2000). It is among the few known cases of natural hybridization events where the parental species are known and still extant and where both time and site of hybridization are known; therefore it is an invaluable model for studying reticulate evolution in plants. The evolutionary potential of a new species is closely dependent on the available diversity contributed by the parental species and of its subsequent changes in the resultant hybrid. The former is related to the number of hybridization events involving one or several parental genotypes (Soltis and Soltis, 1999), and the latter, to the potential dynamics of the hybrid genome (Bayer, 1999; Wendel, 2000). Additionally, intergenomic diversity (i.e., fixed heterozygosity) of young allopolyploids is strongly correlated with divergence of the parental subgenomes, which are reunited in the same polyploid nucleus.

In this context, assessing genetic divergence among the parental species is of high interest. Surprisingly, in spite of both fundamental and applied interests of spartines, little is known about their evolutionary history, which has been mostly examined on the basis of morphology (Mobberley, 1956). As part of ongoing research on the evolutionary fate of parental genomes within the invasive allopolyploid *S. anglica*, this study focuses on the potential utility of nuclear and chloroplast DNA sequences for understanding phylogenetic relationships among species of *Spartina*, with particular emphasis on species involved in the parentage of *S. anglica* (*S. alterniflora x S. maritima*).

As examining DNA sequences from several independent sources maximizes effectiveness of molecular data, particularly when tracking species rather than gene phylogeny (Maddison, 1995), three sources of data have been used in this study. We have sequenced nuclear ribosomal DNA (rDNA) internal transcribed spacers (*ITS*), part of the nuclear Granule-Bound Starch Synthase gene (*Waxy* gene), and the chloroplast DNA (cpDNA) *trnT-trnL* spacer of different *Spartina* species from the Old and New World. The *ITS* region is one of the most frequently used sequences in plant phylogenetic studies involving closely related taxa (Soltis and Soltis, 1998), due to its relatively fast evolutionary rate, and its easy amplification using universal primers (Baldwin *et al.,* 1995). Ribosomal DNA genes are present in high copy number per haploid genome, and they generally undergo concerted evolution, which limits the risk of comparing paralogous sequences. However, in taxa of hybrid origin, multiple copies may either be retained, (Buckler and Holtsford, 1996; Cronn *et al.,* 1996; Baumel *et al.,* 2001) or be subject to various evolutionary forces such as directional concerted evolution (Wendel *et al.*, 1995a) or intergenomic introgression (Wendel *et al.,* 1995b). For this reason, single copy genes are of particular interest in phylogenetic reconstruction. The *Waxy* gene is present as a single copy in the Poaceae where it has been recently used for phylogenetic purposes (Mason-Gamer *et al.,* 1998). This gene is composed of 13 translated exons separated by 12 variable introns providing potentially useful information within genera. Comparing nuclear DNA-based phylogenies with (maternally inherited) chloroplast sequence-based reconstruction is also recommended, particularly when reticulate evolution is expected in a group (Soltis and Soltis, 1998; Small *et al.,* 1998). In this paper, we have chosen to introduce sequences from the *trnT-trnL* spacer, which has been found, from previous studies (A. Baumel and M. L. Ainouche, unpublished), to be the most variable noncoding sequence heretofore investigated compared

TABLE 1

List, Chromosome Number, Origin of the Analyzed Taxa, and Genbank Accession Numbers

Taxa	2*n*	Source	Accession no.
S. alterniflora Loisel.	62[a]	South England, UK	*ITS*: AF272775 *Waxy*: AF275261 *trnT-trnL* spacer: AF275667
S. argentinensis Parodi	40[a]	Santa Fe, Argentina	*ITS*: AF372642 *Waxy*: AF372460 *trnT-trnL* spacer: AF372627
S. arundinacea (Thouars) Carmich.	40[a]	Crozet Islands	*ITS*: AF372634 *Waxy*: AF372464 *trnT-trnL* spacer: AF372631
S. bakeri Merr.	40[a]	Florida, USA	*ITS*: AF372639 *Waxy*: AF372465 *trnT-trnL* spacer: AF372632
S. ciliata Brongn.	?[c]	Buenos Aires, Argentina	*ITS*: AF372636
S. cynosuroides (L.) Roth	40[a]	New Jersey, USA	*ITS*: AF372637 *Waxy*: AF372462 *trnT-trnL* spacer: AF372630
S. densiflora Brongn.	?	California, USA	*ITS*: AF372635 *Waxy*: AF372466 *trnT-trnL* spacer: AF372629
S. foliosa Trin.	60[b]	California, USA	*ITS*: AF372641 *Waxy*: AF372459 *trnT-trnL* spacer: AF372626
S. gracilis Trin.	40[c]	Hsiao *et al.* 1999	*ITS*: AF019844
S. maritima (Curtis) Fern.	60[a]	Brittany, France	*ITS*: AF272776 *Waxy*: AF275262 trnT-trnL spacer: AF275669
S. patens (Aiton) Muhl.	40[a]	New Jersey, USA	*ITS*: AF372638 *Waxy*: AF372463 *trnT-trnL* spacer: AF372628
S. pectinata Link	40[a]	Missouri, USA	*ITS*: AF372640 *Waxy*: AF372461 *trnT-trnL* spacer: AF372625
Cynodon dactylon (L.) Pers.	?	Brittany, France	*ITS*: AF372643 *Waxy*: AF 372467 *trnT-trnL* spacer: AF372633

[a] From Marchant, 1968.
[b] From Daehler and Strong, 1977.
[c] ?, Unknown.

to *rp116* intron, *trnL* intron, and *trnL-trnF* spacer (Ferris *et al.,* 1997; Baumel *et al.,* 2001), in the *Spartina* chloroplast genome.

MATERIAL & METHODS

Plant Material

List, origin, and chromosome numbers of the 12 *Spartina* species examined in this study are presented in Table 1. Species of known hybrid origin (e.g., *S. x townsendii, S. anglica*) have been deliberately excluded from the analyses.

Most sequences have been obtained from fresh material, as several attempts to amplify DNA from herbarium accessions resulted in poor quality products, except for *ITS* of *Spartina ciliata* Brongn. (herbarium sample from Museum National d'Histoire Naturelle, Paris). DNA extracts from *Spartina pectinata* Link, *Spartina densiflora* Brongn, and *Spartina argentinensis* Parodi have been kindly provided by Travis Columbus (Rancho Santa Ana Botanical Garden, CA). Voucher specimens are deposited in the University of Rennes 1, except for the samples of *S. pectinata, S. densiflora, S. argentinensis*, and *S. ciliata* which are deposited in Rancho Santa Ana Botanical Garden (CA), and the Museum National d'Histoire Naturelle (France), respectively. For *Spartina gracilis*, we used the *ITS* sequence previously published by Hsiao *et al.* (1999) (GenBank accession presented in Table 1). Two species (*S. ciliata* and *S. gracilis*) are missing from the analyses based on *Waxy* and *trnT-trnL* spacer sequences.

A sample of *Cynodon dactylon* (L.) Pers. from Brittany was used as an outgroup in the phylogenetic analyses because it is closely related to *Spartina* (Hsiao *et al.,* 1999). The *ITS* sequence we obtained for this spe-

cies was identical to that previously published by Hsiao *et al.* (1999).

DNA Extraction, PCR, and Sequencing

Genomic DNA was isolated from leaves using a CTAB method previously used with *Spartina* species (Baumel *et al.,* 2001).

For all sequences, each amplification reaction (100 μl final volume) contains 50 ng template DNA, 1X incubation buffer (Appligen), 7.5 mM $MgCl_2$, 10 μM each primer, and 5 unities of Taq polymerase (Appligene). Amplification of the *ITS* region was performed using primers *ITS-1* and *ITS-4* (White *et al.,* 1990). The PCR cycling regime involved 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 48°C, and 2 min extension at 72°C.

A portion of the *Waxy* gene was amplified using the "F for" and "K bac" primers of Mason-Gamer *et al.* (1998), which allowed amplification of approximately 500 bp between exon 8 and exon 10. The PCR cycling regime involved 35 cycles of 1-min denaturation at 94°C, 1-min annealing at 65°C, and 2-min extension at 72°C.

The *trnT-trnL* spacer was amplified with primers "a" and "b" of Taberlet *et al.* (1991). The PCR cycling regime involved 30 cycles of 1-min denaturation at 94°C, 1-min annealing at 48°C, and 2-min extension at 72°C.

PCR products were checked on 1.5% agarose gels before sequencing. When no band heterogeneity was observed, purification of the PCR products was carried out with Millipore ultrafree-MC membranes. Direct sequencing from the purified PCR products was performed using the ABI prism Big Dye Terminator Cycle Sequencing Ready reaction kit (PE Biosystems, Foster City, CA) on an automated sequencer (ABI PRISM 310 genetic analyse, PE Biosystems). All fragments were sequenced in both directions. When direct sequencing of PCR products failed to yield clean sequence (e.g., *Waxy* sequence of *Cynodon dactylon*), amplified fragments were cloned prior to sequencing. In some cases (*Waxy* and *trnT-trnL* spacer amplifications of *S. densiflora*), sequence heterogeneity was detected on the agarose gel (i.e., bands of different sizes). Consequently, DNA fragments corresponding to the expected size were cut from the gel and purified with Geneclean III Kit (Bio 101, Vista, CA) following the instructions of the manufacturer. Purified fragments from agarose were then also inserted into pGEM-T (Promega) plasmid for cloning. Insert sequencing was done in both directions using T7 and Sp6 primers, following the manufacturer's instructions, and two clones were sequenced each time.

Sequences were first aligned with Clustal V (Higgins *et al.,* 1992), then refined manually in the data matrix using MacClade (Maddison and Maddison, 1992).

Phylogenetic Analyses and Sequence Divergence among Species

As some informative insertions–deletions (indels) were found in *ITS, Waxy*, and *trnT-trnL* sequences, they were coded following the recommendations of Barriel (1994), where indels are coded as discrete characters (presence/absence) in order to minimize the number of mutations. Integration of indels in the phylogenetic analyses provided more informative characters, but did not change topologies that were obtained when indels were excluded. Therefore, only analyses from the data matrix with indels coded are presented here.

Parsimony analyses were performed for each region with PAUP*4 (Swofford, 1998) using branch and bound searches including *Cynodon dactylon* as the outgroup. Constant and uninformative characters were removed from the data matrix. Support for the different clades was tested by bootstrap analysis (1000 replicates using heuristic search) and decay analysis (Bremer, 1988).

As incongruence was encountered between different gene trees, statistical significance of this incongruence was tested by the homogeneity test (HT_F as designated by Johnson and Soltis, 1998) of Farris *et al.* (1995), which is analogous to the ILD (incongruence length difference) test of Mason-Gamer and Kellogg (1996). We also used the significantly less parsimonious test (SLP_T as designated by Johnson and Soltis, 1998), analogous to the WSR (Wilcoxon signed-rank) test (Templeton, 1983). Both tests were conducted using PAUP 4* according to the procedure described in Johnson and Soltis (1998). Phylogenetic analysis combining the three data sets was then performed, after pruning taxa displaying significant heterogeneity.

RESULTS

Sequence Characteristics within Spartina

GenBank accession numbers of the different taxa used in this study are presented in Table 1. No intra-individual sequence heterogeneity has been observed for most species examined. For *S. densiflora*, bands of different size were present in the PCR products of the *Waxy* gene and the *trnT-trnL* spacer. Therefore only the band of the expected "normal" size, observed in other *Spartina* species, has been cloned and sequenced in two clones that yielded identical sequences.

Characteristics of the studied sequences of *Spartina* are summarized in Table 2. Total length of the aligned sequences in 534 bp for the *Waxy* gene, 606 bp for the *ITS* region, and 841 bp for the *trnT-trnL* spacer. The portion of the *Waxy* gene analyzed in this study is more variable among *Spartina* species than both the entire *ITS* region and *trnT-trnL* spacer. Twenty-two percent of the aligned sequence is variable in *Waxy* against 19% in the *ITS* region and 3.4% in the *trnT-trnL*

TABLE 2

Nuclear *ITS*, *Waxy*, and cpDNA *trnT-trnL* Spacer Sequence Variation within *Spartina*

Sequence characteristics	*ITS1*	*ITS2*	*5.8S*	Total *ITS*
Aligned length (nucleotides)	218	222	166	606
Mean % G+C content (and standard deviation)	55.8 (1.6)	49.9 (1.3)	52.2 (0.6)	52.7 (1.3)
Variable sites (indels excluded), and	62	50	7	119
% on total aligned length	28%	22.5%	4%	19%
Potentially informative sites	36	30	3	69
Indels	2	9	1	12
Potentially informative indels	0	6	1	7

	Exon 8	Intron 8	Exon 9	Intron 9	Exon 10	Total *Waxy*
Aligned length (nucleotides)	104	98	180	107	44	533
Mean %G+C content (and standard deviation)	59 (2.6)	46.6 (2.4)	65.8 (7)	43.3 (3.8)	60.8 (0.9)	57 (3)
Variable sites (indels excluded), and	11	32	29	41	5	118
% on total aligned length	10.5%	32.5%	16%	38%	11%	22%
Potentially informative sites	3	24	22	24	4	77
Indels	1	13	0	15	1	30
Potentially informative indels	0	10	0	12	0	22

	cpDNA *trnT-trnL* spacer
Aligned length (nucleotides)	841
Mean % G+C content (and standard deviation)	26.5 (1)
Variable sites (indels excluded), and	28
% on total aligned length	3.4%
Potentially informative sites	13
Indels	26
Potentially informative indels	8

spacer. The *Waxy* gene displays more informative sites (77) than the *ITS* region (69).

In the *ITS* region, less polymorphism (4% variable sites) is found in *5.8S* than in *ITS 1* and *ITS 2* (28 and 22.5%, respectively). Indels are more frequent in *Waxy* than in the *ITS* region and they are more phylogenetically informative. Intron 9 is the most variable part of the *waxy* gene with 41(38%) variable sites and 15 indels. Exon 8 and exon 10 of the *Waxy* gene are less polymorphic (10.5 and 11%, respectively), than exon 9 (16%). Exon 8 displays 11 variable sites (for 104 aligned sites), of which eight affect the third codon, one in the second codon position, and two in the first codon position. Moreover, we found that *S. arundinacea* is the only species possessing a 3-bp insertion in this coding region, resulting in two amino acid changes in the protein. In exon 9, 28 variable sites are encountered (for 180 aligned sites) among the compared *Spartina* species. Fifteen of these sites are located in the third codon position, whereas four and nine sites are situated on the second and first codon positions, respectively. Of the 44 bp sequenced in exon 10, five variable sites are found, of which three and two are first and second codon positions, respectively. Then, within *Spartina* itself, excluding polymorphisms introduced by gaps, 44 sites (13.4%) are variable in the coding region examined (328 aligned sites), of which more than half (59%) are third position mutations (26 sites). An additional 25 and 15.9% are first and second positions (11 and seven sites, respectively). Twenty-two (48%) of these substitutions were nonsynonymous overall in the coding region examined within the *Waxy* gene. We also examined pairwise divergence sequence between *Spartina* species (matrix not presented). For ITS, pairwise distances (uncorrected *P*) ranged from 0.00337 (between *S. alterniflora* and *S. foliosa*) to 0.10324 (between *S. alterniflora* and *S. cynosuroides*). For *Waxy*, pairwise distances ranged from 0.00193 (between *S. foliosa* and *S. alterniflora*) to 0.15274 (between *S. arundinacea* and *S. argentinensis*). For the trnT-trnL sequences, pairwise distances ranged from 0.00127 (between *S. alterniflora* and *S. foliosa*) to 0.01784 (between *S. foliosa* and *S. patens*). These ranges of variation are significantly more important for both nuclear (*ITS* and *Waxy*) sequences than for chloroplast trnT-trnL sequences.

Phylogenetic Analysis of ITS Sequences

Phylogenetic analysis of *ITS* region included 12 *Spartina* species. Branch and bound search (constant and uninformative characters excluded) generated 10 equally most parsimonious trees of 300 steps (CI =

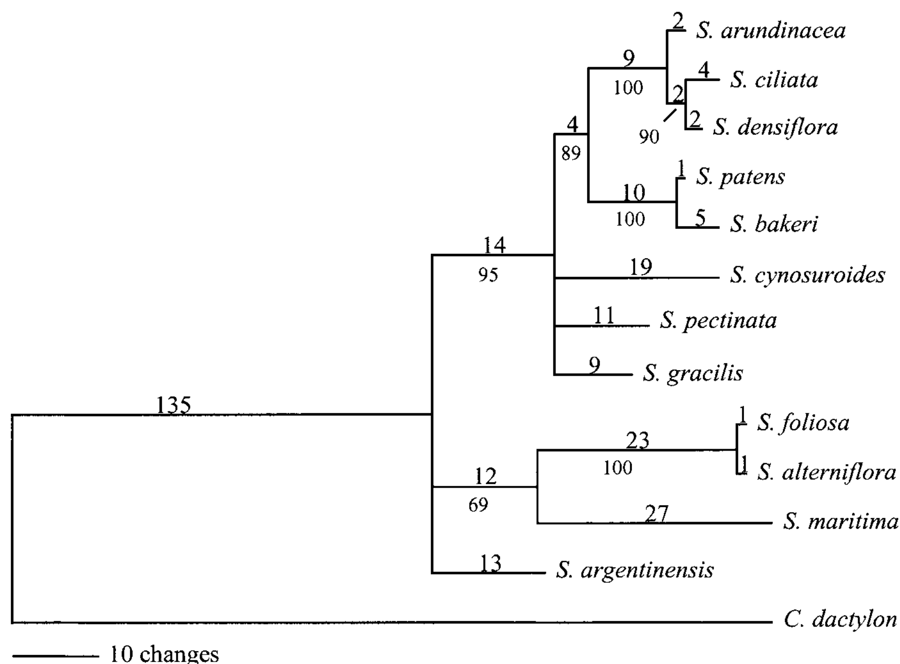

FIG. 1. *ITS* phylogeny of *Spartina*. Strict consensus of 10 equally most parsimonious trees (tree length = 300, CI = 0.870, RI = 0.775). The number of changes is indicated above the branches and the bootstrap percentages (1000 replicates) are indicated below.

0.870; RI = 0.775). The resulting strict consensus tree (Fig. 1) shows that genus *Spartina* is a monophyletic group separated-from the outgroup by 135 changes. Even when introducing additional outgroup species (e.g., *Sporobolus indicus*, Chloridoideae) from GenBank (AF019841), *Spartina* remains monophyletic (results not shown). *Spartina* is split into two main clades and a single species lineage (*S. argentinensis*); the first clade, including *S. alterniflora, S. foliosa*, and *S. maritima*, is supported by 12 synapomorphies (Fig. 1), a 69% bootstrap value, and a decay value of 2. *Spartina maritima* displays 27 autapomorphies, and *S. alterniflora* and *S. foliosa* appear as weakly differentiated (by two changes) sister species, supported by 23 synapomorphies. The second clade is well supported (95% bootstrap, decay value = 6) by 14 synapomorphies, and contains eight species: *S. gracilis, S. pectinata, S. cynosuroides, S. patens, S. bakeri, S. densiflora, S. ciliata*, and *S. arundicacea*. Within this clade, *S. densiflora, S. ciliata*, and *S. arundinacea* form a well-supported monophyletic group (100% bootstrap; nine synapomorphies, decay value = 8), sister to *S. patens* and *S. bakeri* which form a monophyletic group with 10 synapomorphies (100% bootstrap, decay value = 10). The position of *S. argentinensis* is not well resolved by the *ITS* phylogeny, as it appears as a single species lineage in the basal trichotomy (Fig. 1). In four of the 10 most parsimonious trees (not shown), *S. argentinensis* belongs to the first clade, whereas it appears in the second clade in two other equally parsimonious trees. However, in four other parsimonious trees, *S. argentinensis* is positioned at the base of the genus, as sister of the two other clades.

Phylogenetic Analysis of the Waxy Gene

Phylogenetic analysis of the *Waxy* gene have been conducted on the same sample set as for ITS, excluding *S. gracilis* and *S. ciliata* (10 species analyzed).

Parsimony analysis based on *Waxy* sequences yielded three equally parsimonious trees (263 steps, CI = 0.919, RI = 0.923). The resulting strict consensus tree (Fig. 2) shows two strongly supported lineages within *Spartina*. As previously found with *ITS* data, the first clade includes *S. foliosa* and *S. alterniflora* as sister species differing in this case by only one change, *S. maritima* as sister of the former two species, but *S. densiflora, S. argentinensis*, and *S. maritima* form an evolutionary grade at the base of this clade. As with *ITS* data, the position of *S. argentinensis* was not clearly resolved, whereas in the *Waxy* phylogeny, it is unambiguously included in the "*foliosa-alterniflora-maritima*" clade, forming a monophyletic group supported by 20 synapomorphies (100% bootstrap, decay value = 16). The position of *S. densiflora* sister to this first clade is strongly supported (29 synapomorphies, 99% bootstrap, decay value = 13), and it is strikingly incongruent with ITS phylogeny (Fig. 1), where this species was unambiguously included in the other (second) clade. The second clade, a trichotomy, is supported by 33 synapomorphies (100% bootstrap value, decay value = 18). It is composed of *S. arundinacea, S. bakeri*, (as sister species), *S. cynosuroides*, and *S. pec-*

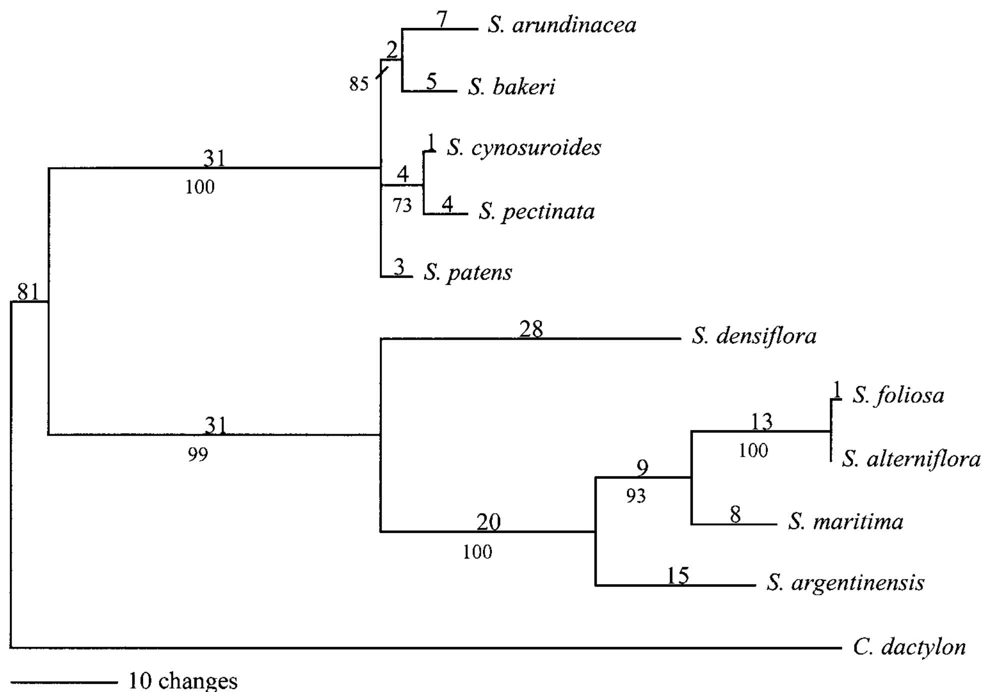

FIG. 2. *Waxy* phylogeny of *Spartina*. Strict consensus of three equally most parsimonious trees (tree length = 263, CI = 0.919, RI = 0.923). The number of changes is indicated above the branches and the bootstrap percentages (1000 replicates) are indicated below.

tinata (as sister species) and *S. patens* as an unresolved, single species lineage within this clade. This species appears alternatively as sister to the "*pectinata-cynosuroides*" clade, or at the base of this second clade, in the three equally most parsimonious trees (not shown) obtained with *Waxy* data.

Phylogenetic Analysis of the Chloroplast trnT-trnL Intergenic Spacer

The phylogenetic analysis of the *trnT-trnL* spacer has been conducted on the same set as for the *Waxy* gene (10 species).

CpDNA *trnT-trnL* spacer is notably less variable among *Spartina* species than nuclear sequences. Less phylogenetic resolution is obtained with these data. Length of this spacer ranges between 757 and 797 bp, except for *S. densiflora* and *S. arundinacea*, which share a large deletion (426 bp) compared to the other *Spartina* species. Consequently, we performed three different phylogenetic analyses: The first one included all the variable sites, with the indel coded 1/0 and the deleted sites noted as "?" (missing data) following the recommendations of Barriel (1994). This procedure allows for the incorporation of information provided by the variation within the inserted portion. The second analysis was based on a data matrix where the 426 sites corresponding to the indel were removed, and replaced by only one character (1/0 for insertion/deletion, respectively). A third analysis was performed after pruning the two species presenting the deletion (i.e., *S. densiflora* and *S. arundinacea*) from the matrix.

Branch and bound searches based on the first analysis (including all species and all variable sites) resulted in 21 equally most parsimonious trees of 122 steps, CI = 0.774 and RI = 0.816 (Fig. 3). Only two

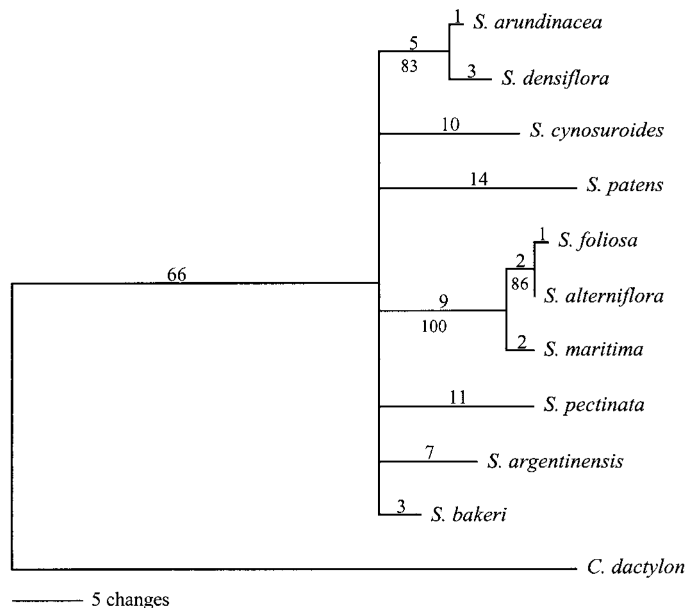

FIG. 3. *TrnT-trnL spacer* phylogeny of *Spartina*. Strict consensus of 50 equally most parsimonious trees (tree length = 126, CI = 0.921, RI = 0.756). The number of changes is indicated above the branches and the bootstrap percentages (1000 replicates) are indicated below.

TABLE 3

Statistical Tests of Incongruence for Pairwise Comparisons of Three Molecular Data Sets

Comparison data/tree	Inspection	SLP_T, *P* value	HT_F, *P* value
ITS-waxy			
All taxa	Differences for *S. densiflora* and *S. bakeri*	0.0001*	0.001*
Without *S. densiflora*	Differences for *S. bakeri*	0.0016*	0.037*
Without *S. densiflora* and *S. bakeri*	Minor differences	0.31	0.714
Waxy-ITS			
All taxa	Differences for *S. densiflora* and *S. bakeri*	0.0001*	0.001*
Without *S. densiflora*	Differences for *S. bakeri*	0.2568	0.037*
Without *S. densiflora* and *S. bakeri*	Minor differences	0.6547	0.714
CpDNA-ITS	No difference	0.4795	0.235
ITS-cpDNA	No difference	1	0.235
CpDNA-waxy	Difference for *S. densiflora*	0.0348*	0.001*
Waxy-cpDNA	Difference for *S. densiflora*	0.0001*	0.001*

Note. In each case, data of one set were constrained by the consensus tree of the alternative data set. Asterisks * indicate significant difference at $P < 0.05$.

clades are well supported: the "*foliosa-alterniflora-maritima*" clade (nine synapomorphies, 100% bootstrap, decay value = 6), which was also well supported in the previous analyses based on nuclear sequences, and the "*densiflora-arundinacea*" clade (five synapomorphies, 91% bootstrap, decay value = 1). *Spartina densiflora* has been positioned in different clades in the nuclear *ITS* and *Waxy* phylogeny. Chloroplast data are in agreement with the *ITS* phylogeny where *S. densiflora* and *S. arundinacea* also belong to the same monophyletic group. This relatedness is reinforced by the large deletion shared by the two species. Relationships among the other *Spartina* species are not resolved by *trnT-trnL* data. Removing either the 426 sites of the large indel (second analysis) or the two species sharing the deletion (third analysis) did not change the topology obtained from the first analysis (trees not shown).

Congruence between Data Sets and Combined Analysis

Comparison by eye of the topologies provided by the three analyzed sequences indicates that both soft and hard incongruences (Seelanan *et al.,* 1997) exist between the different gene trees. Incongruence was statistically tested on data sets containing the same taxa (Table 3). When considering all taxa, *ITS* and *Waxy* are significantly incongruent ($SLP_T = 0.0001$ in both directions and $HT_F = 0.001$) and *Waxy* is incongruent with cpDNA *trnT-trnL* spacer ($SLP_T = 0.0001$ and 0.0348, $HT_F = 0.001$). The incongruence between *Waxy* and the cpDNA *trnT-trnL* spacer is caused by different positions of *S. densiflora*. In contrast, the *ITS* and cpDNA *trnT-trnL* spacer topologies are statistically congruent.

By successively deleting from the analysis the taxa suspected to be responsible for the incongruence, and recalculation of both tests, it appears that *S. densiflora* and *S. bakeri* caused significant incongruence between the *ITS* and *Waxy* topologies (Table 3). Some differences remained between both topologies after pruning these two taxa, but the incongruence was not statistically significant ($SLP_T = 0.31$ and 0.6547, $HT_F = 0.714$). This is essentially due to the weak resolution for *S. argentinensis* at the base of the first clade in the ITS phylogeny, as mentioned previously.

According to the conditional combination approach (Johnson and Soltis, 1998), it is recommended that all taxa significantly responsible for incongruence should be pruned from the data matrix before performing the combined analysis. However, we decided to delete only *S. densiflora* from combined analysis, as it shifts from the first main clade to the second one when comparing *Waxy* to *ITS* phylogenies. In contrast, *S. bakeri* remains in the same major clade (i.e., the second one). Moreover, the SLP_T value for the *Waxy* data constrained by *ITS* without *S. densiflora* (0.2568) shows that this incongruence is insignificant. As sequences of *S. gracilis* and *S. ciliata* were not available for the *Waxy* gene and the *TrnT-trnL* spacer, we also deleted these species from the combined analysis.

The combined analysis based on the nine *Spartina* species (excluding *S. densiflora, S. gracilis*, and *S. ciliata*) and both nuclear and chloroplast data yielded two most parsimonious trees of 641 steps (CI = 0.903, RI = 0.855). The strict consensus tree (Fig. 4) shows that the two main clades of *Spartina* are well supported (100% bootstrap values, decay values = 80 and 84, respectively) with 47 and 53 synapomorphies, respectively. *Spartina argentinensis* is at the base of the first clade, as sister species of the "*foliosa–alterniflora–maritima*" clade, which is supported by 26 synapomorphies (100% bootstrap, decay index = 80). It is interesting to note the lengths of the terminal branch of *S. maritima* (40 changes) and of the "*alterniflora-foliosa*" clade (35

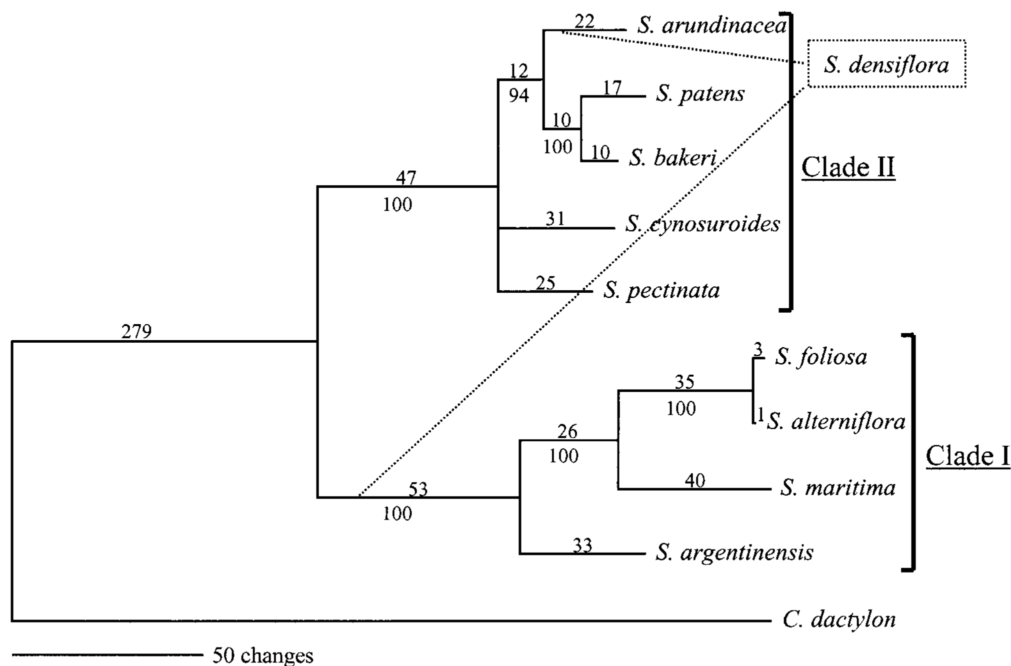

FIG. 4. Phylogeny of *Spartina*, rDNA *ITS*, *waxy*, and cpDNA *trnT-trnL spacer*, *S. densiflora* excluded: strict consensus of two most parsimonious trees (tree length = 641, CI = 0.903, RI = 0.855). The number of changes is indicated above the branches and the bootstrap percentages (1000 replicates) are indicated below. Placement of *S. densiflora* is indicated by a dotted line due to incongruence between the three data sets. Clade I and Clade II are the principal lineages of the genus *Spartina*.

changes), contrasting with the weak divergence between *S. alterniflora* and *S. foliosa* which differ by only four changes. In the second clade (clade II, Fig. 4), *S. arundinacea, S. bakeri*, and *S. patens* form a monophyletic group, the latter being sister species. Positions of *S. pectinata* and *S. cynosuroides* are not well resolved, resulting in a polytomy at the base of this second clade. The placement of *S. densiflora*, which is alternatively positioned in the first or the second clade by *Waxy* or *ITS-trnT-trnL* data, respectively, is indicated on Fig. 4.

DISCUSSION

This molecular phylogeny of *Spartina* species based on two nuclear sequences (i.e., part of the single copy *Waxy* gene, and the repetitive *ITS* region of *rDNA* genes) and one chloroplast sequence (*trnT-trnL* spacer) reveals that nuclear sequences are more variable (six times) and more informative than the chloroplast one. Moreover, the *Waxy* gene provided more informative variable sites (1.15 times) than *ITS*. Most information provided by the *Waxy* gene comes from the high rate of changes in introns. This contrasting situation between chloroplast and nuclear sequences regarding their respective evolutionary rate has often been observed in plants (e.g. Small *et al.,* 1998). This highlights again the need for investigating new rapidly evolving nuclear markers for phylogenetic reconstruction at the genus and species levels in plants (Mason-Gamer *et al.,* 1998).

Our results provide strong evidence for existence of two lineages in the genus (Fig. 4). The first clade includes all hexaploid species: the Euro–African *S. maritima* ($2n = 60$), the East American *S. alterniflora* ($2n = 62$), and the West American *S. foliosa* ($2n = 60$), which is consistent with morphological affinities previously emphasized by Mobberley (1956). Our results show that these species share a (probably hexaploid) common ancestor, and within this group, *S. alterniflora* appears as a closely related sister species to *S. foliosa*, from which it differs by only four nucleotide changes over the entire 1981 bps sequenced from the nuclear and the chloroplast genomes. This is in agreement with findings of Ferris *et al.* (2000) who noticed that *S. alterniflora* and *S. foliosa* are closely related. *Spartina alterniflora* and *S. foliosa* have nonoverlapping natural distributions, and the former was only introduced to the Western Pacific coast of North America 25 years ago (Daehler and Strong, 1997). Hybridization between *S. alterniflora* and *S. foliosa* and backcrossing with the parents then occurred repeatedly in both directions, threatening genetic integrity of the native species *S. foliosa* (Antilla *et al.,* 2000). Altogether, these data raise the question of the taxonomic rank of these two weakly genetically differentiated, nonreproductively isolated taxa, occurring primarily in

distinct geographic areas. Although belonging to the same lineage, *S. maritima* appears consistently more genetically differentiated from *S. alterniflora* than *S. foliosa*, as a consequence of a probable longer time of divergence and geographic separation on different continents. Genetic divergence between *S. maritima* and *S. alterniflora* is also supported by multilocus fingerprints (Baumel *et al.,* 2001). However, this differentiation did not prevent hybridization between the two species, subsequent to introduction of the latter into Western Europe. Hybridization events occurred at least twice independently in Europe: in Southern England, forming the sterile hybrid *S. x townsendii*, and in southwest France, resulting in the sterile hybrid *S. x neyrautii*. The sterility of these hybrids on one hand, and the fertility of hybrids between *S. alterniflora* and *S. foliosa* on the other hand make sense with respect to our findings on genetic divergence of the three species involved in hybridization. Only chromosome doubling of *S. x townsendii* restored fertility in the allopolyploid *S. anglica*, now a successful invasive species colonizing salt marshes and estuaries in Western Europe.

The tetraploid species *S. argentinensis* ($2n = 40$) is placed at the base of this first "*maritima–alterniflora–foliosa*" clade (Fig. 4), indicating that the tetraploid *S. argentinensis* and the hexaploid *Spartina* species share a relatively recent common ancestor. Mobberley (1956) considered this species as synonymous with *Spartina spartinae* Trin., having a disjunct distribution in North–Central America and in South America. In North and Central America, the species ranges from Florida to Texas, Mexico, and Costa Rica, and in South America, the species is limited to the inland regions of Northern Argentina and Paraguay (Mobberley, 1956). In our analyses, *S. argentinensis* was represented by a single sample from Argentina, and it would be interesting to include other samples representing the northern range of this species in future investigations.

The second well-supported main lineage within *Spartina* revealed by our study (Figs. 1, 2, and 4) includes the other tetraploid American species *S. ciliata, S. arundinacea, S. pectinata, S. cynosuroides, S. patens, S. bakeri*, and *S. gracilis. Spartina patens* appears as the sister of *S. bakeri*. Both species are distributed along the east North American coast, and display morphological similarities. *Spartina bakeri* is usually distinguished by its particular vegetative habit, lacking rhizomes. *Spartina arundinacea* is sister to *S. bakeri* and *S. patens*.

The position of *S. densiflora* is interesting, as it differs for the two nuclear sequences which place this species in either the first or the second clade (Figs. 1 and 2). Both relationships are strongly supported: *ITS* data indicate that within the second clade, *S. densiflora* is closely related to *S. ciliata* and *S. arundinacea* (Fig. 1). This is also well supported by chloroplast DNA data, as *S. densiflora* and *S. arundinacea* share a 426-bp deletion in the *trnT-trnL* spacer (Fig. 3). Congruence between *ITS* and cpDNA for *S. densiflora* agrees with a general observation (Kellogg *et al.,* 1996) that ITS sequences often suggest relationships similar to those based on morphology or chloroplast genes. All these three species are distributed in the Southern Hemisphere. *Spartina densiflora* has a large distribution in South America (Brazil, Argentina, Chile), and it was introduced into California during the 19th century (Spicher and Josselyn, 1985). *Spartina ciliata* is limited to the east coast of South America (Brazil and Argentina), whereas *S. arundinacea* occurs on two distant island groups in the South Atlantic and Indian Oceans. Mobberley (1956) highlighted morphological relationships between *S. arundinacea* and *S. ciliata*. This author showed that *S. densiflora* displayed consistent morphological variation from its northern to southern range, affecting spike number and size, and noted that some confusion could occur with depauperate forms of *S. spartinae* (= *S. argentinensis*).

In contrast to the relationships suggested by *ITS* and cpCDNA, *Waxy* data (Fig. 2) place *S. densiflora* unambiguously at the base of the first clade (with *S. alterniflora, S. foliosa, S. maritima*, and *S. argentinensis*). In order to examine which part of the *Waxy* gene is in conflict with *ITS* data, separate analyses were performed with each intron and exon, and each provided the same result. This indicates that there is no conflict within the *Waxy* sequenced data, as previously encountered in some other Poaceae (Mason-Gamer *et al.,* 1998). Strong incongruence between different sequence data sets is more likely to reveal evolutionary processes such as hybridization and introgression, recombination, or lineage sorting (Wendel and Doyle, 1998). Interestingly, several attempts to amplify the *Waxy* gene of *S. densiflora* (using different PCR conditions) yielded three bands (one main band of the same size as encountered in other species, and two secondary light bands), which may indicate sequence heterogeneity in this species. Sequence heterogeneity may result from either gene duplication or hybridization. The two (identical) cloned sequences we have used in our phylogenetic analysis, even though corresponding to the typical size encountered in other *Spartina* species, might then be affected by paralogous sampling. However, if duplication occurred only in *S. densiflora*, it is rather surprising that this supposed paralogous copy is so well connected to the first clade. Another explanation would be a hybrid origin of the analyzed taxon, followed by recombination affecting the *Waxy* gene. This is more likely to affect low-copy number genes such as *Waxy*, than repetitive rDNA sequences that are subject to concerted evolution. As mentioned above, *S. densiflora* originates from South America, but the sample we have analyzed was collected in California where it may have come into contact with native (*S. foliosa*) and other introduced (*S. alterniflora*) species. However, if

the hybridization event was recent, even repetitive *ITS* sequences would not have been homogenized by concerted evolution, as observed in the hybrid species *S. x townsendii* and in the allopolyploid *S. anglica* which still retain both parental *ITS* and *Waxy* sequences (Baumel *et al.,* 2001). Additional sampling in both native and recently colonized areas of *S. densiflora*, as well as a larger cloning procedure (for the screening of sequence heterogeneity), are needed to explore this question.

In conclusion, this study provides an historical framework allowing us to understand patterns of evolution in *Spartina. Spartina alterniflora*, which is involved in interspecific hybridizations does not hybridize with sympatric east American species, as a consequence of the important divergence between the two main lineages shown by this study. Species that hybridize (namely *S. alterniflora* with *S. foliosa* and *S. maritima*), belong to the same lineage, but display different levels of genetic divergence, affecting evolutionary consequences of the hybridization events; *Spartina alterniflora*, and *S. foliosa*, which are weakly differentiated, hybridize and backcross frequently in California (Antilla *et al.,* 2000). In contrast, this study reveals that *S. alterniflora* and *S. maritima* contain two well-differentiated and divergent genomes. These provide the resulting allopolyploid *S. anglica* which contains the genetic diversity of the two parental homeologue genomes reunited in the same nucleus (Baumel *et al.,* 2001). This divergence may explain the rare occurrence of hybridization events between the parental species in Western Europe resulting in the unique origin of *S. anglica* (Raybould *et al.* 1991; Baumel *et al.,* 2001). As a successful invasive species now having a worldwide distribution, *S. anglica* demonstrates again that even rare hybridization events may have important evolutionary outcomes. Moreover, incongruences between gene trees revealed by this study indicate that reticulate events might be more frequent than previously suspected within *Spartina*.

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