Molecular Ecology (2005) **14**, 1163–1175 doi: 10.1111/j.1365-294X.2005.02488.x

Genetic and epigenetic consequences of recent hybridization and polyploidy in *Spartina* (Poaceae)

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Abstract

To study the consequences of hybridization and genome duplication on polyploid genome evolution and adaptation, we used independently formed hybrids (*Spartina* × *townsendii* and *Spartina* × *neyrautii*) that originated from natural crosses between *Spartina alterniflora*, an American introduced species, and the European native *Spartina maritima*. The hybrid from England, *S.* × *townsendii*, gave rise to the invasive allopolyploid, salt-marsh species, *Spartina anglica*. Recent studies indicated that allopolyploid speciation may be associated with rapid genetic and epigenetic changes. To assess this in *Spartina*, we performed AFLP (amplified fragment length polymorphism) and MSAP (methylation sensitive amplification polymorphism) on young hybrids and the allopolyploid. By comparing the subgenomes in the hybrids and the allopolyploid to the parental species, we inferred structural changes that arose repeatedly in the two independently formed hybrids. Surprisingly, 30% of the parental methylation patterns are altered in the hybrids and the allopolyploid. This high level of epigenetic regulation might explain the morphological plasticity of *Spartina anglica* and its larger ecological amplitude. Hybridization rather than genome doubling seems to have triggered most of the methylation changes observed in *Spartina anglica*.

Keywords: AFLP, hybridization, methylation, MSAP, polyploidy

Received 4 November 2004; revision received 6 January 2005; accepted 6 January 2005

Introduction

Hybridization and polyploidy are known to be prominent processes inducing diversification and speciation in plants (Stebbins 1950; Grant 1971; Abbott 1992; Masterson 1994; Rieseberg & Wendel 2004) and they have long been considered to have important ecological consequences (Lewis 1980). Recently, an active research area has emerged from genomic approaches, permitting the exploration of the genetic and functional consequences of uniting two diverged genomes into a common nucleus. These approaches suggest various factors that might help explain the evolutionary success of allopolyploids, including recurrent hybridization involving various parental genotypes that increase the genetic diversity of the new species (Doyle *et al*. 2004; Soltis *et al*. 2004), fixed heterozygosity and genetic redundancy with independent evolution of the duplicated genes (Cronn *et al*. 1999; Senchina *et al*. 2003), and dynamic alterations of the duplicated genome at both the structural and functional levels (reviewed in Wendel 2000; Levy & Feldman 2002; Liu & Wendel 2002; Liu & Wendel 2003; Osborn *et al*. 2003). Recent studies have shown that genetic and epigenetic instabilities may affect recently duplicated genomes. In the short term, polyploid genome evolution appears often to be accompanied by rapid and biased structural changes (Song *et al*. 1995; Feldman *et al*. 1997; Liu *et al*. 1998a, b; Ozkan *et al*. 2001), accompanied by activation of transposable elements and epigenetic changes that modulate gene expression (Comai 2000; Comai *et al*. 2000; Shaked *et al*. 2001; Kashkush *et al*. 2002, 2003; Adams *et al*. 2003; He *et al*. 2003; Wang *et al*. 2004). The genetic and epigenetic changes triggered by hybridization and genome duplication may have important phenotypic consequences (Comai *et al*. 2000; Schranz & Osborn 2000, 2004; Madlung *et al*. 2002) and appear as a key feature determining the adaptive success of newly formed allopolyploid species (Wendel & Doyle 2004). Most of the

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foregoing insights have emerged from experimental model systems involving cultivated species and their relatives, with relatively less being understood about genomic responses to hybridization and polyploidization in natural populations.

The genus *Spartina* offers an excellent opportunity to analyse the short-term evolutionary consequences of allopolyploid speciation, as it contains two recent (about 150 years old) and well-documented interspecific hybridization events (Ainouche *et al*. 2004a), one of them having resulted in a particularly successful allopolyploid species, namely *Spartina anglica* (Ainouche *et al*. 2004b and references therein). The eastern North American *Spartina alterniflora* ($2n = 62$) was accidentally introduced to western Europe during the 19th century. Hybridization with the indigenous *Spartina maritima* ($2n = 60$) in Southampton Bay (England) resulted in the mostly sterile F_1 hybrid named *Spartina* × *townsendii* that is still growing vegetatively at the original site of hybridization. Chromosome doubling in the hybrid gave rise to the vigorous and fertile allopolyploid species *Spartina anglica* (Hubbard 1968) that has rapidly expanded in the western European saltmarshes (Gray *et al*. 1990; Thompson 1991; Baumel *et al*. 2001). As the parental species are hexaploid, the allopolyploid *S. anglica* ($2n = 100, 122, 124$) is dodecaploid (Marchant 1968). To our knowledge, the nature of polyploidy (autopolyploid vs. allopolyploid) has not been elucidated in either parental species (*S. maritima* and *S. alterniflora*); however, regarding the frequency of hybridization in this genus (Ainouche *et al*. 2004a), a reticulate origin of these taxa would not be surprising. *S. anglica* is a rhizomatous species with the capacity to quickly increase sediment accumulation. This feature led to its voluntary introduction for land reclamation in northern Europe, China, Australia, New Zealand, and North America. The rapid spread of the introduced plants and the subsequent ecological changes in the newly colonized areas have resulted in various unsuccessful attempts of eradication (e.g. Ranwell & Downing 1960; Hedge *et al*. 1997).

Various lines of evidence have revealed that *S. anglica* populations lack interindividual genetic diversity (Raybould *et al*. 1991a; Thompson *et al*. 1991a; Baumel *et al*. 2001), as a result of both clonal propagation and a severe genetic bottleneck at the time of its formation in Southampton Bay (Ainouche *et al*. 2004a, b). *S. anglica* has the same chloroplast genome as the maternal species *S. alterniflora* (Ferris *et al*. 1997; Baumel *et al*. 2001). The two parental species (*S. maritima* and *S. alterniflora*) lack genetic diversity in the region where they hybridized (Raybould *et al*. 1991b; Baumel *et al*. 2003; Yannic *et al*. 2004), but because their genomes are rather divergent (Baumel *et al*. 2002a), the allopolyploid contains two well-differentiated, homoeologous genomes. Moreover, it has been shown that *S. anglica* populations display significant morphological plasticity to environmental change, interpreted as playing a central role in the fitness of the

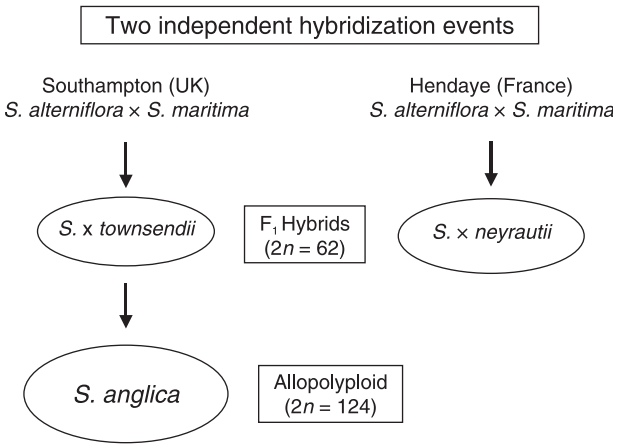

Fig. 1 Historical origin of *Spartina* × *neyrautii*, *Spartina* × *townsendii*, and *Spartina anglica*.

populations (Thompson *et al*. 1991a, b, c). *S. anglica* tolerates low-lying estuary mudflats and has greater ecological amplitude than its parents. It has been shown that this species displays enhanced physiological mechanisms that enable the species to tolerate both anoxic and polluted sediments, including increased O_2 and H_2S transport, and lower O_2 requirements (Lee 2003).

A second sterile hybrid was formed in southwest France (Basque region) at approximately the same time as *S.* × *townsendii*, and this one is named *Spartina* × *neyrautii* (Foucaud 1897). This hybrid displayed a different morphology than the British hybrid (Marchant 1977), i.e. resemblance to *S. alterniflora* with larger and fleshy leaves. Consequently, it was believed for a long time to result from the reciprocal cross, with *S. maritima* as the maternal parent (Marchant 1977). However, Baumel *et al*. (2003) recently found that remnant clones of this hybrid have the same maternal genome as *S.* × *townsendii* and *S. anglica*, which was inherited from *S. alterniflora*.

In this study, we use the recent (150 years old) hybrid–allopolyploid system represented by *S.* × *neyrautii*, *S.* × *townsendii*, and *S. anglica* (Fig. 1) to examine the early genetic and epigenetic changes that affect the maternal (*S. alterniflora*) and paternal (*S. maritima*) genomes that became reunited in the same nucleus. In addition to providing a unique opportunity to explore early evolutionary changes in natural populations, the 'experiment' of hybridization has been naturally replicated, thereby facilitating potential insights into genomic events that may be directed as opposed to stochastic. Two types of molecular analysis were conducted on the parental species, their hybrids, and the allopolyploid using genome-wide screens based on amplified fragment length polymorphism (AFLP) analysis. This fingerprinting technique involves the digestion of genomic DNA by two restriction enzymes followed by two rounds

of selective amplification on a subset of the restriction fragments. By using a combination of primers with different selective nucleotides, this method is conducive to sampling hundreds to thousands of genetic loci. In addition to revealing genetic changes, epigenetic consequences of hybridization and genome duplication were investigated using the methylation sensitive amplified polymorphism (MSAP) method (Reyna-Lopez *et al*. 1997), which employs isoschizomers displaying various sensitivities to methylation at the restriction sites. Both AFLP and MSAP procedures have been recently used successfully for the screening of genetic and epigenetic changes in hybrids or polyploids (Liu *et al*. 2001; Shaked *et al*. 2001; Madlung *et al*. 2002). Using these techniques, we asked the following specific questions: (i) Is rapid genomic change a common feature in the natural hybrid and allopolyploid *Spartina* genomes, as it is in experimentally resynthesized allopolyploid wheat (Feldman *et al*. 1997; Liu *et al*. 1998a, b; Ozkan *et al*. 2001; Shaked *et al*. 2001; Kashkush *et al*. 2002, 2003), *Brassica* (Song *et al*. 1995), and *Arabidopsis* (Comai *et al*. 2000; Lee & Chen 2001; Madlung *et al*. 2002; Wang *et al*. 2004)? (ii) Are genetic and /or epigenetic changes triggered by hybridization or by genome duplication, or both? (iii) Are the changes in *S.* × *neyrautii* and *S.* × *townsendii* that result from two independent hybridization events similar?

Materials and methods

Plant material

Five *Spartina* species were included in the study: the two parental species, *Spartina maritima* and *Spartina alterniflora*; the two hybrids, *Spartina* × *neyrautii* (Hendaye, France) and *Spartina* × *townsendii* (Hythe, England); and the allopolyploid, *Spartina anglica* (Fig. 1). The parental species were sampled in the region where the hybridization events occurred to analyse the closest possible genotypes involved in hybrid formation. *S. alterniflora* was sampled near Hythe (England) and in Hendaye (Southwest France). *S. maritima*, now extinct in Hythe, has recently been shown to lack genetic diversity in western Europe (Yannic *et al*. 2004). Therefore, the samples used in this study were collected from the western coast of France, in Hendaye and in Noirmoutiers (one individual from each population). Samples from the dodecaploid species *Spartina anglica* from Keyhaven (Southampton Bay, England) and Baie des Veys (France) were first tested in a preliminary screening, and one individual (from Keyhaven) was retained in subsequent analyses. This individual represents the 'major genotype' encountered in the western European populations of *S. anglica* (Baumel *et al*. 2001). All plants used are maintained in the greenhouse at the University of Rennes 1, and have been previously characterized using various molecular analyses (Baumel *et al*. 2001, 2002a, b, 2003).

Genomic screening methodology

DNA extraction. DNAs were prepared by the cetyltrimethyl ammonium bromide (CTAB) method (Ausubel *et al*. 1995) according to the procedure adapted for *Spartina* by Baumel *et al*. (2001). Approximately 30 mg of fresh leaves were ground in a 2% CTAB solution (1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 2% CTAB). The extract was incubated for 1 h at 65 °C, and 500 µL of chloroform were added to the supernatant for precipitation. After centrifugation, the DNA pellet was washed with 70% ethanol, and then resuspended in Tris-EDTA buffer.

Amplified fragment length polymorphism (AFLP) analysis. The AFLP procedure was performed according to a standard protocol (Vos *et al*. 1995), with slight modifications (Liu *et al*. 2001). One-half microgram of genomic DNA was digested at 37 °C for 1 h. The restriction reaction contained 5 units of *Eco*RI (or *Pst*I) and 5 units of *Mse*I, 1× of Y-Tango Buffer (MBI Fermentas), in a final volume of 40 µL. Adaptors were ligated for 2 h at 37 °C. The ligation reaction contained 5 pmol of *Eco*RI (or *Pst*I) adaptors, 50 pmol of *Mse*I adaptors, 1 X DNA ligase buffer (Promega), and 1 unit of DNA ligase, which was added to the 40-µL digested solution. The digested-ligated DNA was diluted with 150 µL of H_2O. The preselective amplification by polymerase chain reaction (PCR) was carried out by using a single selective base at the 3′ end of each of the *Mse*I and *Eco*RI (or *Pst*I) primers (Table 1). Each reaction contained 10 pmol of (*Mse*I + 1) primer, 10 pmol of (*Eco*RI + 1) [or (*Pst*I + 1)] primer, 0.2 mM of each dNTPs, 1× *Taq* DNA polymerase buffer, 2 units of *Taq* DNA polymerase, and 5 µL of diluted restriction-ligation products in a final volume of 50 µL. The amplification profile was one cycle at 72 °C for 2 min, followed by 30 cycles at 94 °C for 45 s, 56 °C at 45 s, and 72 °C at 2 min and a final extension at 60 °C for 30 min. Ten microliter of this PCR was electrophoresed on 1.5% agarose gels and stained with ethidium bromide to verify the success of the preselective amplification (a DNA smear in the size range of 100–1500 bp is expected).

After successful preamplification, the PCR products were diluted 1:20 with H_2O. Selective amplification was performed with *Eco*RI (or *Pst*I) primers labelled with either 6-carboxyfluorescein (6-FAM) or 4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein (HEX), and unlabelled *Mse*I primers. The sequence of the selective primers is similar to that of the preselective primers, with the addition of two variable nucleotides at the 3′ end (Table 1). The selective amplification reaction contained 25 pmol of (*Mse*I + 3) primers, 5 pmol of (*Eco*RI + 3) [or (*Pst*I + 3)] labelled primers, 0.2 mM of each dNTPs, 1× *Taq* DNA polymerase buffer, 0.5 units of *Taq* DNA polymerase, and 2.5 µL of diluted preselective product in a final volume of 20 µL. The PCR protocol was one cycle at 94 °C for 2 min one cycle at 94 °C for 30s, 65 °C for 30s and

Table 1 AFLP and MSAP adapters and primers used in this study

		Sequences
Adaptors		
*Mse*I-adapter II		5′-GACGATGAGTCCTGAG
*Mse*I-adapter II		5′-TACTCAGGACTCAT
*Eco*RI-adapter I		5′-CTCGTAGACTGCGTACC
*Eco*RI-adapter II		5′-AATTGGTACGCAGTC
*Pst*I-adapter I		5′-CTCGTAGACTGCGTACATGCA
*Pst*I-adapter II		5′-TGTACGCAGTCTAC
*Hpa*II/*Msp*I-adapter I		5′-GATCATGAGTCCTGCT
*Hpa*II/*Msp*I-adapter I		5′-CGAGCAGGACTCATGA
Preselective Primers		
*Mse*I + C		5′-GACGATGAGTCCTGAGTAAC
*Eco*RI + A		5′-GACTGCGTACCAATTCA
*Pst*I + A		5′-GACTGCGTACATGCAGA
*Hpa*II/*Msp*I + 0		5′-ATCATGAGTCCTGCTCGG
Selective Primer combinations used in AFLP		
*Eco*RI + AAC/*Mse*I + CAA	*Pst*I + AAC/*Mse*I + CAA	
*Eco*RI + AAC/*Mse*I + CAC	*Pst*I + AAC/*Mse*I + CAC	
*Eco*RI + ACA/*Mse*I + CAA	*Pst*I + ACA/*Mse*I + CAA	
*Eco*RI + ACA/*Mse*I + CAC	*Pst*I + ACA/*Mse*I + CAC	
*Eco*RI + AGC/*Mse*I + CAA	*Pst*I + AGC/*Mse*I + CAA	
*Eco*RI + AGC/*Mse*I + CAC	*Pst*I + AGC/*Mse*I + CAC	
*Eco*RI + ACG/*Mse*I + CAA		
*Eco*RI + ACG/*Mse*I + CAC		
Selective Primer combinations used in MSAP		
*Eco*RI + AAC/*Hpa*II/*Msp*I + TCAA	*Eco*RI + AAC/*Hpa*II/*Msp*I + TCAC	
*Eco*RI + ACA/*Hpa*II/*Msp*I + TCAA	*Eco*RI + ACA/*Hpa*II/*Msp*I + TCAC	
*Eco*RI + AGC/*Hpa*II/*Msp*I + TCAA	*Eco*RI + AGC/*Hpa*II/*Msp*I + TCAC	
*Eco*RI + ACG/*Hpa*II/*Msp*I + TCAA	*Eco*RI + ACG/*Hpa*II/*Msp*I + TCAC	

72 °C for 2 min, followed by nine cycles of a 1.0 °C decrease per cycle in annealing temperature, followed by 35 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min, and a final extension at 60 °C for 30 min.

Amplification products were electrophoretically separated using a capillary system on an ABI Prism 310 DNA sequencer. AFLP fragments were analysed by visual inspection using the ABI GENESCAN ANALYSIS 2.1 software. The information (presence vs. absence of a given DNA fragment) was translated into a presence/absence data matrix. The molecular basis of any given polymorphism is usually sequence polymorphism affecting either the restriction site or nucleotides adjacent to the restriction site that prevent selective amplification. Deletions, insertions, and rearrangements may affect the presence or the size of the restriction fragments. As most of the polymorphism results from presence/absence of a priming site, AFLP markers are usually considered as dominant. Polymorphic fragments (i.e. different bands) between the parental species *S. maritima* and *S. alterniflora* were first screened, and deviation from additivity (i.e. loss of parental fragment, or appearance of new fragment) was examined in the hybrids and the allopolyploid.

Methylation-sensitive amplification polymorphism (MSAP) analysis. We followed the procedure employed by Liu *et al*. (2001) for allopolyploid cotton. The modified protocol substitutes for *Mse*I as the frequent cutter the isoschizomers *Hpa*II and *Msp*I, which recognize the same tetranucleotide sequence 5′-CCGG but with different sensitivities to methylation at cytosines. The rare cutter *Eco*RI is unchanged. Digestion and ligation stages were performed simultaneously and by using longer incubation times (overnight) than standard, to ensure complete digestion. The restriction-ligation reaction contained 500 ng of genomic DNA, 10 units of *Hpa*II (or *Msp*I), 10 units of *Eco*RI, 100 pmol of *Hpa*II/*Msp*I adaptor, 10 pmol of *Eco*RI adaptor, with 0.6 unit of T4 DNA ligase in a 1× T4 DNA ligase buffer, plus 0.5 M NaCl and 1 mg/mL BSA, in a total volume of 25 μL. Preselective amplification was accomplished by using [*Eco*RI + 1] and [*Hpa*II/*Msp*I + 0] primers (Table 2). Selective amplification was performed using [*Eco*RI + 3] labelled primers and [*Hpa*II/*Msp*I + 4] unlabelled primers (Table 1). Reaction components and conditions are exactly the same as described earlier for the AFLP analysis.

The methylation state of the restriction sites (CCGG) recognized by the two isoschizomers *Hpa*II and *Msp*I leads

Table 2 AFLP fragments observed in the parents (*Spartina maritima* and *Spartina alterniflora*) and in the hybrids *Spartina* × *neyrautii* and *Spartina* × *townsendii* generated by *Eco*RI-*Mse*I and *Pst*I-*Mse*I digestions (11 selective primer combinations)

		Parental fragments	Parental fragments in both hybrids	Parental fragments missing in both hybrids	Parental fragments missing in only one hybrid	
					S. × *neyrautii*	*S.* × *townsendii*
Common parental fragments		448	445 (99.3%)	2 (0.4%)	1 (0.2%)	0
Diagnostic fragments	From *S. maritima*	250	242 (96.8%)	2 (0.8%)	4 (1.6%)	2 (0.8%)
	From *S. alterniflora*	284	237 (83.4%)	30 (10.6%)	12 (4.2%)	5 (1.8%)
Total number of diagnostic fragments between parents		534	479 (89.7%)	32 (6.0%)	16 (3.0%)	*7 (1.3%)*
Novel fragments		—	7	—	10	4

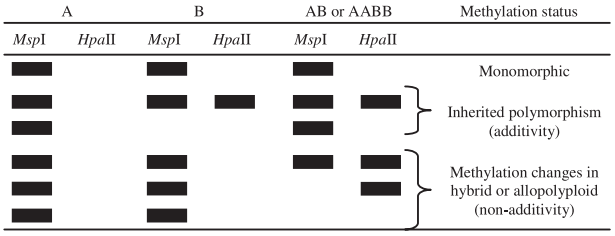

Fig. 2 MSAP patterns expected from the comparison between two parental species A and B, and their derivative hybrid (AB) or allopolyploid. These patterns represent a sample of the possible methylation states that can be observed, considering additive vs. nonadditive parental patterns in the hybrid or allopolyploid. Changes between the F_1 hybrid (AB) and the allopolyploid (AABB) are also possible but are not displayed on this scheme.

to a differential cleavage. The specificities of *Hpa*II and *Msp*I are described in the restriction enzyme database REBASE (Roberts & Macelis 2001). While *Hpa*II is sensitive to methylation of the internal cytosine at both strands, *Msp*I is sensitive to methylation of the external cytosine. Methylation of the external cytosines (on both strands) or a full methylation of both cytosines prevents cutting, which makes these two methylation patterns indistinguishable by the MSAP technique. Comparison of amplification products from *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I however, allows detection of some methylation changes. A fragment present in both digestions indicates that the corresponding CCGG site (or sites) is unmethylated. The presence of fragments in *Eco*RI + *Msp*I digestions and their absence in *Eco*RI + *Hpa*II digestions is attributed to methylation of the internal cytosines on both strands. The presence of fragments in *Eco*RI + *Hpa*II digestions and their absence in *Eco*RI + *Msp*I digestions is attributed to hemimethylation of the external cytosine. Some of the expected MSAP patterns when analysing two different species and their hybrid and allopolyploid derivative are presented in Fig. 2.

In both AFLP and MSAP procedures, replicates were performed to avoid technical bias, and patterns resulting from two independent digestions were compared for each sample. Only stable and repeatable patterns were retained for analysis.

Isolation and sequencing of differentially methylated fragments

To gain insight into the nature of the differentially methylated fragments, five polymorphic MSAP fragments (A–E) were isolated from polyacrylamide gels, reamplified by PCR, and sequenced. To accomplish this we used unlabelled selective *Hpa*II/*Msp*I primers and *Eco*RI primers that were end-labelled with radioisotope [ATP (^{32}P) end-labelling grade from ICN Radiochemicals] followed by selective amplification. Two microlitre of the reaction product was combined with 6 μL of loading buffer, incubated at 94° for 4 min, then snap cooled on ice. Samples were electrophoresed on a denaturing 6% polyacrylamide gel. Polymorphic bands were excised from the gel and the DNA was extracted by boiling in 100 μL of water for 5 min. The eluted DNA was used as template for PCR following the cycling conditions of the selective amplification. The PCR product was purified using the QIAGEN PCR Purification Kit and then directly sequenced using the *Eco*RI + A primer.

Results

Standard AFLP analysis

The total number of AFLP fragments scored in this study is 1003. By using 11 selective primer combinations (Table 1), 982 AFLP parental fragments were generated, of which 534 were unique to one or the other parent ('diagnostic fragments', Table 2) and 448 were present in both parents ('common parental fragments', Table 2). The *Spartina alterniflora* sample from Hendaye did not yield satisfactorily

repeatable results, so the *S. alterniflora* individual from Hythe was subsequently used as representative of the maternal species for both *Spartina* × *townsendii* and *Spartina* × *neyrautii*. The distribution of fragments among the progenitor species and their derived hybrids and allopolyploid are shown in Fig. 2.

Comparison of AFLP banding patterns obtained in parents and in the two natural hybrids. The hybrids shared 479 (89.7%) of the fragments discriminating the parents (Table 2). Thirty-two (6%) parental fragments are absent in both hybrids, whereas 16 missing fragments are unique to *S.* × *neyrautii* and seven are unique to *S.* × *townsendii* (Table 2). Thus, there is a shared component of nonadditivity as well as a unique one. Notably, most parental fragments not detected in the hybrids originated from the maternal parent, *S. alterniflora* (89.5% and 87.5% of the parental fragments absent in *S.* × *townsendii* and *S.* × *neyrautii*, respectively).

In addition to missing parental fragments, novel AFLP bands were detected in the two hybrids (10 in *S.* × *neyrautii*, four in *S.* × *townsendii*), and an additional seven novel fragments were detected in both hybrids (Table 2). However, of the 10 novel fragments observed in *S.* × *neyrautii* when considering *S. alterniflora* from Hythe as its maternal parent, four are detected in *S. alterniflora* from Hendaye (data not shown). Thus, at least some portion of the novel fragments observed in *S.* × *townsendii* and *S.* × *neyrautii* reflect parental polymorphism detected between the genotypes of *S. alterniflora* from Hendaye and from Southampton.

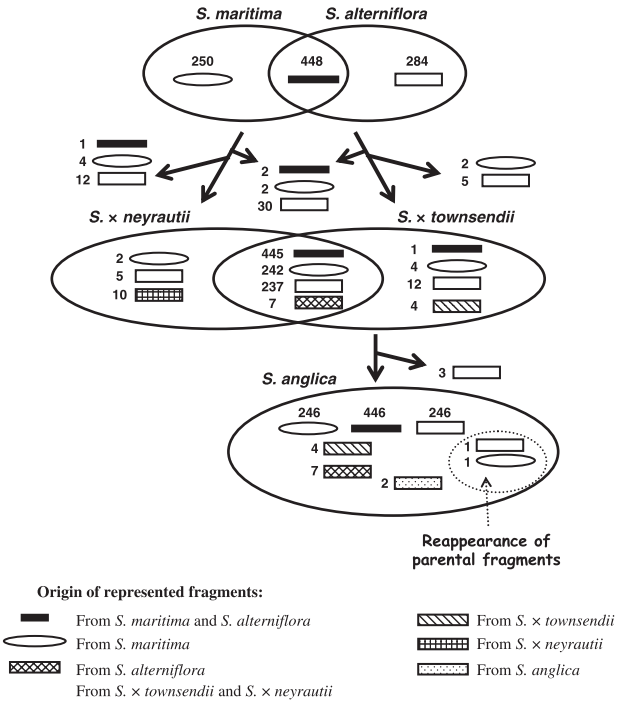

Fig. 3 AFLP fragments observed in parental species *Spartina maritima* and *Spartina alterniflora*, their hybrids *Spartina* × *neyrautii* and *Spartina* × *townsendii*, and the natural allopolyploid, *Spartina anglica* shared fragments are represented in overlapping ovals, whereas lost fragments are indicated outside. Symbols used for each fragment indicate the taxonomic specificity of the fragments.

Comparison of AFLP banding patterns among parents, the hybrid and its derivative polyploid. *Spartina anglica* exhibits 492 of the 495 AFLP fragments (99.4%) that discriminate *S. alterniflora* and *S. maritima* and that are present in the hybrid, *S.* × *townsendii*, from which the allopolyploid derives (Fig. 3). The three fragments absent in *S. anglica* match the pattern of *S. alterniflora*. All 11 of the novel AFLP fragments in *S.* × *townsendii* (noted in the previous discussion) were also detected in *S. anglica*. The allopolyploid displays two novel AFLP fragments as well as two additional parental fragments that were not detected in *S.* × *townsendii* (Fig. 3). Reappearance of parental fragments in allopolyploids has previously been reported in wheat and was explained by methylation changes of the *Eco*RI site (Shaked *et al*. 2001).

Methylation-sensitive AFLP analysis

Comparison of nonmethylated banding patterns observed in the parents and in the two natural hybrids. By using seven selective primer combinations for the MSAP analysis (Table 1), 346 nonmethylated parental fragments were generated, of which 224 (64.7%) were unique to one or the other parent (Table 3). Both hybrids exhibit deviation from additivity as well as their own unique patterns, as was observed in the AFLP analysis. The hybrids shared 82.6% of the fragments diagnostic for the two parents. However, 23 expected parental fragments are absent from both hybrids (10.6%), while 16 additional parental fragments (3.6%) are missing in one or the other of the two hybrids (eight in each; see Table 3). This absence of parental fragments is biased toward those derived from the maternal parent *Spartina alterniflora*, as observed in the standard AFLP analysis. Novel fragments were also detected in the two hybrids: six in *S.* × *neyrautii*, two in *S.* × *townsendii*, and three shared between both hybrids (Table 3).

Comparison of nonmethylated banding patterns observed in the parents, the hybrid and its derived polyploid. The banding pattern of *S. anglica* is a composite of 224 of the 228 MSAP nonmethylated fragments (98.2%) that are diagnostic of its two parents and are also present in the hybrid *S.* × *townsendii* (Fig. 4). Three parental fragments were absent in *S. anglica*, two from *S. alterniflora*, and one from *S. maritima*. Five of the nine new nonmethylated fragments observed in the hybrid *S.* × *townsendii* are present in *S. anglica*. There are also three new nonmethylated fragments and five parental fragments (three from *S. maritima* and two from *S. alterniflora*) in *S. anglica* that are absent *in S.* × *townsendii* (Fig. 4).

Table 3 Nonmethylated MSAP fragments observed in the parents (*Spartina maritima* and *Spartina alterniflora*) in the hybrids *Spartina* × *neyrautii* and *Spartina* × *townsendii* generated by *Eco*RI–*Hpa*II and *Eco*RI–*Msp*I digestions (seven selective primer combinations)

		Parental fragments	Parental fragments in both hybrids	Parental fragments missing in both hybrids	Parental fragments missing in only one hybrid	
					S. × *neyrautii*	*S.* × *townsendii*
Common parental fragments		122	119 (97.5%)	1 (0.8%)	1 (0.8%)	*0*
Diagnostic fragments	From *S. maritima*	120	109 (90.8%)	5 (4.2%)	5 (4.2%)	1 (0.8%)
	From *S. alterniflora*	104	76 (73.1%)	18 (17.3%)	3 (2.8%)	7 (6.7%)
Total number of diagnostic fragments between parents		224	185 (82.6%)	23 (10.6%)	8 (3.6%)	*8 (3.6%)*
Novel fragments		—	3	—	6	2

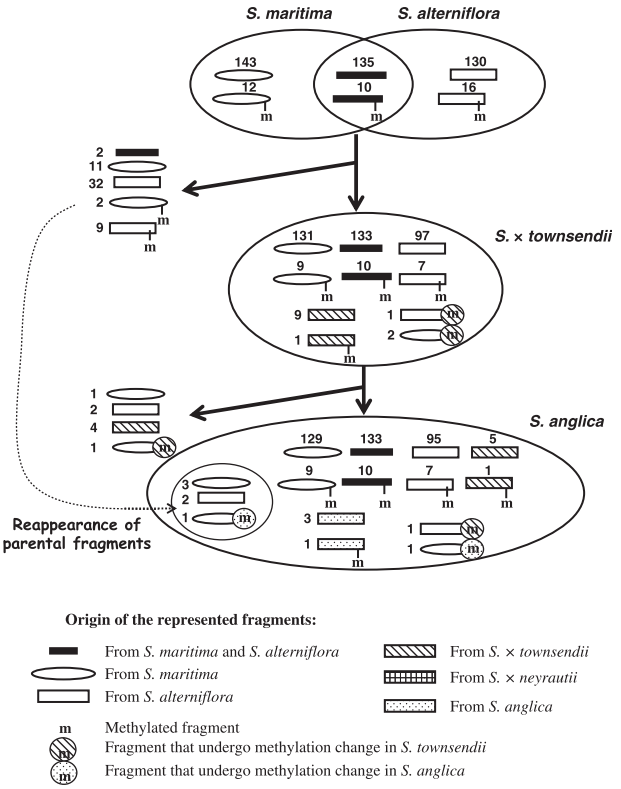

Fig. 4 MSAP fragments observed in the parental species *Spartina maritima* and *Spartina alterniflora*, their hybrids *Spartina* × *neyrautii* and *Spartina* × *townsendii*, and the natural allopolyploid, *Spartina anglica*.

MSAP fragments that experienced methylation changes in the hybrid(s) and/or in the allopolyploid. The proportion of methylated sites was estimated to be 8.9% in *S. alterniflora* and 7.3% in *S. maritima*. These two species shared 10 methylated fragments, while 12 and 16 methylated fragments discriminate them, respectively (Fig. 4). The hybrids exhibit the same patterns for 84.1% of their methylated fragments (Table 4). Additivity of the methylated parental patterns is demonstrated in only 52.3% of the methylated fragments between the two hybrids. We observed that 22.7% of the parental methylated fragments are absent in the two hybrids (Table 4). The same methylation alteration in the hybrids is observed for four fragments (Table 4), which could mean that a parental nonmethylated fragment is methylated in the hybrids or that a parental hemi-methylated fragment is methylated at the internal cytosines in the hybrids. A new methylated fragment appears in *S.* × *neyrautii* and in *S.* × *townsendii* (Table 4). Six methylated fragments differentiate the two hybrids: two parental methylated fragments are absent in *S.* × *neyrautii*; three parental methylated fragments experience methylation changes in *S.* × *neyrautii*; and one parental methylated fragment is absent in *S.* × *neyrautii* and has another methylation state in *S.* × *townsendii* (Table 4).

Spartina × *townsendii* and *Spartina anglica* exhibit the same methylation state for 71.4% of the methylated fragments, comprising 27 fragments that are additive in the hybrid and the polyploid and 13 fragments that are absent or hypermethylated in both species (Table 5). Interestingly, nearly one-quarter (23.2%) of the parental methylated fragments underwent the same changes in the hybrid and the allopolyploid: 10 parental fragments are absent in the hybrid and the allopolyploid and three parental, methylated fragments experienced methylation changes in the hybrid and the allopolyploid. Four parental methylated fragments are either differentially altered in the hybrid and the allopolyploid or absent in the hybrid or the allopolyploid and altered in the hybrid or the allopolyploid (Table 5). Seventeen (13 + 4) parental fragments are affected by methylation changes in *S.* × *townsendii* and/or in *S. anglica*, 10 and seven originating from *S. alterniflora* and *S. maritime*, respectively (Table 5). Two new methylated fragments appeared, one in the hybrid as well as the allopolyploid and the other only in *S. anglica* (Table 5). These new fragments may correspond to the appearance of a new methylated restriction site or to a

Table 4 Fate of parental methylated fragments in the hybrids *S.* × *townsendii* (T) and *S.* × *neyrautii* (N) (seven selective primer combinations)

	Same patterns in the both hybrids				Different patterns in the two hybrids		
Parental methylation patterns	Additivity	Absences	Methylation alteration	New methylated fragment	Absent in N	Methylation alteration in N	Absent in N and methylation alteration in T
Monomorphic (9)	9	0	0	0	0	0	0
Polymorphic between parents (44)	23 (52.3%)	10 (22.7%)	4 (9.1%)	1 (2.3%)	2 (4.5%)	3 (6.8%)	1 (2.3%)
	38 (86.4%)				6 (13.6%)		

Table 5 Methylation patterns in *Spartina maritima* and *Spartina alterniflora*, in the hybrid *Spartina* × *townsendii*, and in the allopolyploid *Spartina anglica* (eight selective primer combinations)

			Spartina maritima		*Spartina alterniflora*		*Spartina* × *townsendii*		*Spartina anglica*			
Methylation patterns			*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	Number of sites	Total (%)
Monomorphic		M1	+	−	+	−	+	−	+	−	4	10 (17.9)
		M2	−	+	−	+	−	+	−	+	6	
Polymorphic between parents	Additivity in both hybrid and polyploid	A1	−	−	+	−	+	−	+	−	2	27 (48.2)
		A2	+	−	−	−	+	−	+	−	2	
		A3	−	+	−	−	−	+	−	+	7	
		A4	−	−	−	+	−	+	−	+	5	
		A5	−	+	+	+	+	+	+	+	3	
		A6	+	+	+	−	+	+	+	+	5	
		A7	+	+	−	+	+	+	+	+	3	
	Absent in both hybrid and polyploid	B1	−	−	−	+	−	−	−	−	6	13 (23.2)
		B2	−	−	+	−	−	−	−	−	3	
		B3	+	−	−	−	−	−	−	−	1	
	Methylation alteration in the hybrid and the allopolyploid	C1	−	−	+	+	−	+	−	+	1	
		C2	−	+	+	+	−	+	−	+	2	
	Absent in hybrid and methylation alteration in allopolyploid	D1	+	−	−	−	−	−	−	+	1	4 (7.1)
	Methylation alteration in hybrid and absent in allopolyploid	D2	−	+	−	−	+	−	−	−	1	
		D3	+	+	−	−	−	+	−	−	1	
	Methylation alteration in allopolyploid only	D4	+	+	−	−	+	+	+	−	1	
Absent in parents	Present in both hybrid and polyploid	E1	−	−	−	−	−	+	−	+	1	2 (3.6)
	Present in allopolyploid only	E2	−	−	−	−	−	−	−	+	1	

methylation alteration of parental fragments that were undetectable by the methylation-sensitive enzymes (methylation of the external cytosines or full methylation of the cytosines at the parental restriction site).

Sequencing of polymorphic MSAP fragments

Five MSAP fragments that displayed nonadditivity (e.g. methylation alteration) in the hybrids and/or the allopolyploid were gel isolated, reamplified, and sequenced. However BLAST (BLASTN and BLASTX) searches did not reveal consistent similarities with recognizable genes.

Discussion

We employed a genome-wide screening approach to explore genetic and epigenetic changes associated with hybridization and polyploidization in two recently formed natural hybrids and one ecologically successful allopolyploid species of *Spartina*. AFLP data indicate that parental genome additivity is the rule in the allopolyploid *Spartina anglica*, as suggested from previous investigations based on various markers (Baumel *et al*. 2002b; Ainouche *et al*. 2004a). This species inherited all the fragments of *Spartina* × *townsendii*, which itself contains more than 99% of the polymorphic fragments discriminating the parental species *Spartina maritima* and *Spartina alterniflora*.

Notwithstanding the general additivity of the polyploid with respect to its two progenitors, some fragment loss associated with hybridization and genome doubling was observed. Most 'missing' AFLP bands in *S.* × *townsendii* and *S. anglica* originated from the maternal parent, *S. alterniflora*. Notably, this same result is encountered in the other hybrid, *Spartina* × *neyrautii* that formed independently in southwest France. This absence of parental fragments in the hybrids and the allopolyploid could result from several causes. First, as AFLP markers are dominant, and given that *S. alterniflora* exhibits more AFLP fragments than *S. maritima*, this difference might reflect segregating heterozygosity in the former parent, which could explain the absence of the corresponding fragment in the hybrids. Second, in its native area, *S. alterniflora* exhibits more genetic diversity (Antilla *et al*. 2000) than *S. maritima* in Europe (Yannic *et al*. 2004), raising the possibility that the 'missing' bands represent nothing more than unsampled parental polymorphism. Although this species underwent important founder effects during its successive introductions in western Europe, at least two different genotypes have been implicated in hybridization with *S. maritima* (Baumel *et al*. 2003). These data corroborate the partial results we obtained for the *S. alterniflora* individual from Hendaye, which appears to be the closest genotype involved in the parentage of *Spartina* × *neyrautii*.

A third explanation for missing AFLP fragments is sequence elimination in the hybrid genomes, in this case mostly affecting the maternal genome. Sequence elimination has been reported in synthetic allopolyploid *Brassica* (Song *et al*. 1995), but this was directed against the paternal nuclear genome, which led to the hypothesis of nucleo-cytoplasmic incompatibilities in response to hybridization events (Song *et al*. 1995). In *Spartina*, the explanation of sequence elimination is reinforced by the fact that fragment loss is observed in the two hybrids, suggesting that two independent hybridizations generated the same structural response. To the extent that our results reflect sequence elimination in the hybrids, the study compliments results observed in wheat F_1 hybrids and allopolyploids, where nonrandom, reproducible sequence elimination phenomena have been reported (Feldman *et al*. 1997; Liu *et al*. 1998a; Ozkan *et al*. 2001; Shaked *et al*. 2001). However, in this case both hybridization and genome duplication trigger structural changes, while *Spartina anglica* displays the same AFLP patterns as *S.* × *townsendii*. It is clear that hybridization and polyploidy seem to generate a range of possible responses that vary among genera; Liu *et al*. (2001), for example, using a similar approach (AFLP) as in the present study, document a case of structural 'genomic stasis' in experimentally resynthesized allopolyploid *Gossypium*.

Genomic changes may result from either DNA mutations or epigenetic alterations (i.e. heritable expression changes that do not result from nucleotide sequence change) that increase the functional plasticity of the newly formed lineage when facing environmental fluctuations. Epigenetic phenomena encompass a vast array of mechanisms, such as DNA methylation, histone modifications or chromatin remodelling (Martienssen & Colot 2001; Liu & Wendel 2003) that play important role in various processes in plant development (Steimer *et al*. 2004), in the control of flowering time (Simpson 2004), and that have subsequently important consequences for plant evolution (Kalisz & Purugganan 2004). In the present study, we focused only on methylation changes, using the MSAP approach. Our data demonstrate that the hybrids have lost more unmethylated parental fragments (14% for *S.* × *neyrautii* and *S.* × *townsendii*) than in the AFLP analysis (9% for *S.* × *neyrautii* and 7.3% in *S.* × *townsendii*). This could be related to either structural changes such as those detected by standard AFLP analysis (mutations in the restriction sites) or from methylation changes undetected by MSAP (e.g. hypermethylation). Although we can only speculate about the relative importance of these two potential sources of fragment loss, the reappearance in the allopolyploid of unmethylated parental fragments that are absent in the hybrid *S.* × *townsendii* strongly suggest fluctuations of the methylation state among the parents, the hybrid, and the allopolyploid. This may explain the higher number of missing parental fragments observed in the MSAP analysis compared to the AFLP analysis.

The two hybrids, *S.* × *neyrautii* and *S.* × *townsendii*, shared the same MSAP methylation patterns for 86% of the

methylated fragments, yet only 52% of their methylated fragments were additive compared to the parental patterns. This result indicates a high level of methylation alteration in response to hybridization. The same missing MSAP bands and methylation alterations are observed in 34% of the methylated fragment in both hybrids, which indicate that methylation changes are also reproducible after two independent hybridization events involving the same parental species.

Genotypes of the parents of *S.* × *neyrautii* at Hendaye differ little from those of *S.* × *townsendii* at Southampton (Baumel *et al*. 2003; Yannic *et al*. 2004), and the two hybrids themselves are very similar genetically (Raybould *et al*. 1990; Baumel *et al*. 2003). From the present study, 37 AFLP, 24 nonmethylated MSAP and six methylated MSAP markers distinguish *S.* × *neyrautii* and *S.* × *townsendii* from one another. These hybrids exhibit remarkable morphological differences (Marchant 1977; Hubbard *et al*. 1978), with *S.* × *neyrautii* exhibiting morphological similarity to *S. alterniflora*, whereas *S.* × *townsendii* is morphologically more intermediate with respect to *S. alterniflora* and *S. maritima*. Thus, the genetic and the epigenetic changes following hybridization appear to have generated major phenotypic effects.

Most methylation changes observed in *S. anglica* appeared or were initiated in the hybrid *Spartina* × *townsendii*. This observation is consistent with the hypothesis suggesting that epigenetic changes are triggered by the merger of two differentiated genomes rather than by genome doubling (Madlung *et al*. 2002). In contrast, both hybridization and genome duplication led to novel methylation patterns in wheat (Shaked *et al*. 2001).

The proportion of methylation changes appears to be variable among the hybrid/allopolyploid plant systems analysed to date using MSAP. In cotton, MSAP fragment additivity was observed in nearly all cases (Liu *et al*. 2001). Methylation changes affected 4.1% of fragments in a rice experimental F_1 hybrid (Xiong *et al*. 1999), with a slightly higher (6.9%) percentage in wheat F_1 hybrids; the latter were maintained in the allopolyploids, and an additional 4.4% of fragments experienced methylation changes in the wheat allopolyploids (Shaked *et al*. 2001). In the experimentally resynthesized allotetraploid *Arabidopsis suecica*, methylation changes representing 8.3% of the fragments were reported by Madlung *et al*. (2002). The percentages for *Spartina* represent the highest rate of methylation changes reported to date following hybridization and polyploidization, affecting nearly 30% of parental fragments. Moreover, it appears that the parental origin of the genomes (*i.e.* from *S. maritima* vs. *S. alterniflora*) did not direct these changes, unlike in wheat where 10 of 11 bands showing methylation alteration in the F_1 hybrid and the allopolyploid were from the genome of the same parental species (Shaked *et al*. 2001). When comparing our results to other studies, it is interesting to note that *S. anglica* represents a higher ploidy (12×) level system than the tetraploid or hexaploid models cited previously. Little is known about the multiple genomic composition of the hexaploid parental species *S. alterniflora* and *S. maritima*, and this question needs to be explored further.

Variation in methylation status at the intraspecific, population level needs more research and appears to be an important question to explore. Preliminary observations involving comparisons between the population of Keyhaven (UK) analysed here and a population sampled in Baie des Veys (France) indicated that interpopulation variation in methylation patterns may be encountered (A. Salmon, unpublished). In allopolyploid *Gossypium hirsutum*, consistent methylation polymorphism (67% of methylated fragments) was encountered among various accessions (A. Keyte & J. F. Wendel personal communication). Comparison of MSAP patterns in different ecotypes of *Arabidopsis thaliana* revealed 35–43% differential methylation patterns (Cervera *et al*. 2002). Natural variation in methylation levels at rDNA loci was found in nucleolar organizing regions (NOR) in *A. thaliana* (Riddle & Richards 2002) and a natural variation in nucleolar dominance was reported in newly synthesized and natural allotetraploids of *Arabidopsis suecica* (Pontes *et al*. 2003). Continuous variation exists in the degree of radial symmetry in nature of *Linaria vulgaris* flowers, which is related to methylation/demethylation processes of the *CYCLOIDEA* gene (Cubas *et al*. 1999). If methylation affects ecologically important genes, response to selection will be different because of this potential source of phenotypic variation.

In general, methylation repatterning of a gene may have consequences such as gene silencing and activation or repression of transposable elements (Liu & Wendel 2003). There have been several studies where DNA methylation changes related to polyploid genome expression have been demonstrated. In *Brassica* (Chen & Pickaard 1997a; b) and *Arabidopsis* (Chen *et al*. 1998; Lawrence *et al*. 2004), the nucleolar dominance phenomenon was found to be related to allopolyploid formation and subsequent DNA methylation and histone modifications. The first cDNA-AFLP fingerprinting approaches on synthetic and natural allotetraploid *Arabidopsis suecica* (Comai *et al*. 2000; Lee & Chen 2001; Madlung *et al*. 2002) demonstrated that allopolyploid formation leads to rapid epigenetic gene silencing that can be reversed by chemical demethylation. Kashkush *et al*. (2002) found the same process of epigenetic gene silencing related to methylation changes in newly synthesized polyploid wheat. A recent study using a RNAi strategy to repress the expression of the *DDM1* (decreased DNA methylation 1) and *MET1* (methytransferase 1) genes, elegantly showed that two protein-coding genes silenced after polyploid formation in synthetic *Arabidopsis* allotetraploids were reactivated in the *ddm*1-RNA_i and *met*1-RNA_i mutant plants (Wang *et al*. 2004). Although no dramatic methylation changes have been found in resynthesized allotetraploid

cotton (Liu *et al*. 2001), Adams *et al*. (2003) showed differential expression patterns between homeologous genes in different tissues, suggesting that subfunctionalization was consequent to allopolyploid formation. In recently formed *Tragopogon* allopolyploids, cDNA investigations have recently revealed that 5% of the genes have been silenced and that 4% exhibit novel gene expression (Soltis *et al*. 2004).

Although functional divergence of duplicated genes could be a major feature of palaeopolyploids (Blanc & Wolfe 2004) and lead to a selective advantage during long-term evolution, functional diversification per se may be less relevant to the immediate success of recently formed allopolyploids (Osborn *et al*. 2003). Allopolyploidy clearly had immediate effects in *S. anglica*, as indicated by its ecological success (Thompson 1991). Although the species underwent a severe genetic bottleneck at the time of its formation in England (Ainouche *et al*. 2004b), and despite its low level of interindividual genetic variation (Baumel *et al*. 2001, 2002a, b), considerable morphological variability is encountered among native populations (Thompson *et al*. 1991a, b, c). Heterosis and dosage effects in duplicated genomes are likely to increase the metabolic plasticity of the duplicated genes, thereby affecting the fitness of the newly formed species (Riddle & Birchler 2003). *S. anglica* is known for having rapidly invaded habitats previously unoccupied by its parental species. This species displays higher physiological tolerance (Lee 2003) that may facilitate colonization of extensively flooded zones of the low marshes. To deepen our understanding of the molecular basis of the larger ecological amplitude of the allopolyploid, investigations of gene expression changes may help identify the functions that are differently regulated following hybridization and gene duplication.

Acknowledgements

We thank Alex Baumel, Ryan Percifield and Keith Adams for valuable discussion, comments on the manuscript, and technical assistance with AFLP analysis and sequencing. This research was funded by the NSF (National Science Foundation USA), and CNRS (Centre National de la recherche Scientifique, France) collaborative project 12978.

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