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Nucleolar dominance arises in Spartina homoploid hybrids and persists after allopolyploidization

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SUMMARY

Allopolyploid *Spartina anglica* C.E. Hubbard (2*n* = 120–124) has become recognized as a model system of recent allopolyploid speciation. It arose by interspecific hybridization between *S. alterniflora* (2*n* = 62) introduced from North America and the native European *S. maritima* (2*n* = 60) about 150 years ago. In addition, sterile first-generation homoploid hybrids *S.* × *townsendii* and *S.* × *neyrautii* (both 2*n* = 62) are still extant. In this study, we carried out a population-level study of epigenetic silencing of 35S rDNA loci, also known as nucleolar dominance. Using molecular, genomic, and cytogenetic methods, we analyzed 75 individuals of *S. anglica* (collected from 11 French populations and 5 UK populations), 34 individuals of *S.* × *townsendii* (3 populations, all from the UK), and 2 individuals of *S.* × *neyrautii* from the south of France. We observed strong transcriptional dominance of *S. alterniflora*-inherited rDNA in all hybrid and allopolyploid individuals. The dominant rDNA units were nearly devoid of methylation at CWG sites, in contrast to those of the silenced *S. maritima*-inherited rDNA (M-loci), which exhibited hypermethylation. At the DNA level, few (2%) *S. anglica* individuals have completely lost M-loci, indicating that rDNA diploidization proceeds extremely fast in *Spartina*, and such a process may be influenced by preceding epigenetic processes. We conclude that nucleolar dominance is already present in extant homoploid hybrid lineages and is largely maintained in *S. anglica*, with occasional partial relaxation.

Keywords: ribosomal DNA, rDNA, nucleolar dominance, rDNA silencing, population epigenetics, invasive species, *Spartina anglica*.

INTRODUCTION

Nucleolar dominance refers to the epigenetic silencing of 35S ribosomal RNA (rRNA) genes (encoding 18S, 5.8S and 26S rRNA) affecting organisms with multiple rDNA loci in their genomes. It is manifested cytologically on chromosomes by secondary constrictions and entails the formation of nucleoli by nucleolar organizing regions (NORs) inherited from only one parental species of a hybrid or allopolyploid (Navashin, 1934, and reviewed by Borowska-Zuchowska et al., 2023). At the molecular level, silencing of rRNA genes is dependent on chromatin epigenetic marks, including DNA and histone methylation (reviewed by Pikaard et al., 2023). In hexaploid wheat, silencing has been correlated with structural features of the intergenic spacers (Sardana et al., 1993), that is, sequences flanking the 35S rDNA arrays (Handa et al., 2018), and cytosine methylation (Flavell et al., 1988; Tulpova et al., 2022). Furthermore, chromosome rearrangements are known to impact the activity of nucleolar organizing regions (NORs) in rye-wheat hybrids (Caperta et al., 2002), pointing to a role for broader chromosome contexts. In *Brachypodium hybridum*, which is a more ancient allopolyploid compared to wheat, strong uniparental silencing of rDNA loci occurs in nearly all populations of independent origin (Borowska-Zuchowska & Hasterok, 2017). Despite an extensive literature on nucleolar dominance in plants, few studies have addressed the question of the immediate impacts of hybridization on

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35S rDNA activity and hence nucleolar dominance and its subsequent inheritance following polyploidization. Studies of rDNA silencing in synthetic polyploid lines have suggested that it may take several generations before nucleolar dominance is established (Burns et al., 2021; Guo & Han, 2014; Joly et al., 2004; Ksiazczyk et al., 2011). In the generation of synthetic polyploid wheat, both sets of parental nucleolus organizing regions (NORs) are expressed after interspecific hybridization, but asymmetric silencing of one parental NOR was established following chromosome doubling, indicating that genome duplication rather than hybridization is the epigenetic silencing trigger (Guo & Han, 2014). In contrast, rDNA silencing was observed as early as in the F1 generation in intergeneric hybrids (Long et al., 2015; Mahelka et al., 2023). Recently formed (i.e., <200 years) natural allopolyploids, including *Cardamine schulzii* (Urbanska et al., 1997), *Senecio campestris* (Abbott & Lowe, 2004), *Tragopogon mirus*, *T. miscellus* (Soltis et al., 2004), and *Mimulus peregrinus* (Vallejo-Marín, 2012), provided opportunities to study the impact of polyploidy on homoelogous protein gene expression, yet nucleolar dominance has been investigated only in some (Matyásek et al., 2007; Zozomová-Lihová et al., 2014). However, some populations of these recently formed allopolyploids do show signs of concerted evolution of rDNAs, despite their young age (Kovarik et al., 2005; Lowe & Abbott, 1996; Zozomová-Lihová et al., 2014), which complicates interpretation of rDNA expression analyses in hybrids and polyploids.

Interspecific hybridization between Spartina *alterniflora*, *syn. Sporobolus alterniflorus* (♀, $2n = 62$) was introduced from North America and the native British *S. maritima* syn. *Sporobolus maritimus* (♂, $2n = 60$) produced the largely infertile homoploid *S.* × *townsendii* ($2n = 62$), first collected in 1870 at Hythe, Southampton, United Kingdom, and named in 1880 (Groves & Groves, 1880). The derived allopolyploid *Spartina anglica* C.E. Hubbard ($2n = 120–122$) is thought to have formed by the spontaneous duplication of chromosome sets soon after the hybridization event (Ainouche, Baumel, Salmon, & Yannic, 2004; Hubbard & Stebbings, 1968; Marchant, 1963; Muhammad & Ki, 2024) and has colonized salt marshes worldwide (Ainouche, Baumel, & Salmon, 2004; Strong & Ayres, 2013). In addition to the Hythe hybrid, parental species also hybridized in the South of France, giving rise to *S.* × *neyrautii*. However, this hybrid did not give rise to a fertile allopolyploid as far as is known. The genomic composition of the parental species has been recently elucidated (Salmon et al., 2025). These previously thought hexaploids (considering the base chromosome number of $x = 10$ from comparative cytogenetics) appear to have evolved from an ancestral diploid genome ($2n = 2x = 20$) that duplicated 9.6–24.4 million years ago (WGD1), resulting in a tetraploid ($2n = 4x = 40$) that subsequently underwent descending dysploidy that led to a new base chromosome number of 15. This diploidized genome duplicated again (WGD2) 2.1–6.2 MYA to form a tetraploid genome ($2n = 4x = 60$), which is actually a diploidized meso-octoploid. The modern *S. alterniflora* and *S. maritima* genomes thus inherited from these two successive whole genome duplication events four divergent subgenomes A1A2B1B2 (Salmon et al., 2025). The speciation event between these parental species was accompanied by chromosome restructuring that resulted in the additional chromosome pair of *S. alterniflora* ($2n = 62$) compared to *S. maritima*. As a consequence, the neopolyploid *S. anglica* genome exhibits a complex hexadecaploid ($16x$) structure: 2(A1a + A2a + B1a + B2a) + 2(A1m + A2m + B1m + B2m). The genome composition of *S.* × *townsendii* and *S.* × *neyrautii* homoploid hybrids ($8x$) would correspond to the (A1a + A2a + B1a + B2a) + (A1m + A2m + B1m + B2m) formula. At Hythe, hybrids and neopolyploids occur in sympatry at or near their place of origin (Huska et al., 2016; Renny-Byfield et al., 2010). All English and French populations of *S. anglica* studied have the same "major" multilocus genotype, which is identical with that of *S.* × *townsendii* (Baumel et al., 2001). Indeed, populations of *S. anglica* and *S.* × *townsendii* show minimal genetic variation based on additivity of nuclear and plastid markers (Ainouche, Baumel, & Salmon, 2004; Baumel, Ainouche, Kalendar, & Schulman, 2002; Raybould et al., 1991), although they exhibit extensive epigenetic differences at the level of transcription (Chelaifa et al., 2010; de Carvalho et al., 2013; Giraud, Lima, Huteau, et al., 2021) and DNA methylation (Parisod et al., 2009; Salmon et al., 2005). Retention of both parental rDNAs (intragenic transcribed spacer (ITS) and intergenic spacer (IGS) markers) has been reported in European populations of *S. anglica* (Baumel, Ainouche, Bayer, et al., 2002; Huska et al., 2016). This apparently contrasts with the study of Asian populations of *S. anglica*, where homogenization of 35S rDNA via intergenomic recombination has been observed (Muhammad & Ki, 2023).

In this study, we carried out a population-level study of nucleolar dominance in *Spartina* allopolyploids. The power of the system is that it allows us to analyze the expression of parental homoelogs in the first-generation homoploid hybrids and derived allopolyploids, addressing which event—hybridization or polyploidization—is decisive for establishing nucleolar dominance. To our knowledge, this question has not been addressed in any natural system so far. Using genomic, cytogenetic, and transcriptomic methods, we obtained evidence for rapid reprogramming of parental NORs in *Spartina* homoploid hybrids and the stable inheritance of nucleolar dominance in the derived *S. anglica* allopolyploid.

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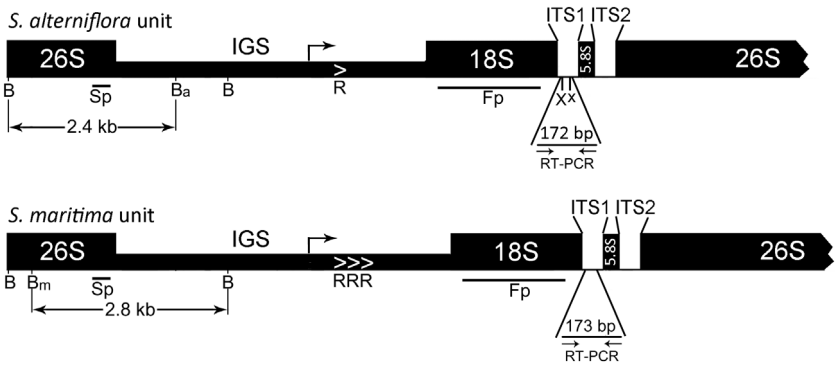


Figure 1. Schematic diagram showing the organization of the rDNA units in parental *S. alterniflora* and *S. maritima*.
The positions of locus-specific primers used in RT-PCR (arrows), restriction sites (B—BstNI, X—Xbal, and x—Xmal), and sites of probe hybridization (Sp—Southern probe, Fp—FISH probe) are indicated. For BstNI, only a subset of sites proximal to probe hybridization regions are shown (in total, there were 22 and 23 BstNI sites in the assembled *S. maritima* and *S. alterniflora* rDNA units, respectively). Ba sites are specific for the A-genome, Bm for the M-genome, while B indicated shared sites. One and three repeated units (R) were identified within the 5′-ETS of A- and M-rDNA variants, respectively.

RESULTS

Parental additivity of rDNA homoelogs and infrequent elimination of M-type loci in *Spartina* hybrids

To determine the occurrence of parental rDNAs (referring to 35S rDNA if not otherwise specified) derived from both progenitors (hereafter named "A-type" and "M-type" rDNA for *S. alterniflora* and *S. maritima*, respectively) in *Spartina* homoploid hybrids and allopolyploids, we carried out Southern blot hybridization analysis of 35S rDNA homoelogs. Compared to our previous study of genetic variation in *Spartina* (Huska et al., 2016), the number of individuals analyzed has increased from 91 to 142 and includes progenitor species and a new homoploid hybrid, *S.* × *neyrautii* (Table S1). Figure S1 shows selected representative hybridizations of BstNI-restricted DNAs from *S. anglica*, *S.* × *townsendii*, and *S.* × *neyrautii*. Both parental hybridization fragments of the expected size (Figure 1) were visualized in all hybrids and allopolyploids (Figure S1) except for two *S. anglica* individuals (both from Marchwood), which have lost the M-specific fragment (12-20A and 13-36A). The hybridization signals were counted for each sample (Table S2), and the values were statistically evaluated (Figure 2a). In *S. anglica*, the homoelogous rDNA ratio (defined as the A/A + M signal intensity) was skewed against the A-type units (40.0% mean, 37.0% median) compared to *S.* × *townsendii*, which had more balanced ratios (45% mean, 47.3% median) (Mann–Whitney two-tailed test: z score − 2.7, $P = 0.00697$). In contrast, a single individual from *S.* × *neyrautii* had a relatively high (71%) proportion of A-type units. The interindividual variability was higher in *S. anglica* (range of A-type rDNA proportion 15.0–100%) compared to *S.* × *townsendii* (20.4–65.0%). The homoelogous copy numbers were further analyzed by locus-specific qPCR in 44 DNA samples of *S. anglica* (Table S2). Overall, there was a good correlation (Pearson's coefficient, $R = 0.6834$) between ratios determined by Southern blot and q-PCR. Together, 92% of individuals of *S. anglica* and 100% of homoploid hybrids retained both parental rDNA copies, although their ratios varied within and between populations.

Next, we determined the 35S and 5S rDNA copy number in representative *Spartina* individuals using genomic Illumina reads. The total numbers of rDNA copies ranged from 923 to 3183 copies/1C for 35S rDNA and from 2627 to 7105 copies/1C for 5S rDNA (Table S3). The "standard" *S. anglica* (16-38B individual from Marchwood) had about double the amount of rDNA repeats compared to parental tetraploids. In contrast, *S. anglica* individual 13-36A (from the same population), which had lost nearly all M-type units (Figure S1), harbored a low copy number of 35S rDNA. Both "standard" 16-38B and 13-36A mutant individuals showed a similar copy number of 5S rDNA. We note that, on average, the rDNA copy numbers estimated from *in silico* data using Illumina reads were about 30% lower compared to those determined previously by slot blot hybridization (Huska et al., 2016).

The contribution of parental homoelogs to the total number of 35S rDNA copies was determined in *S. anglica* and *S.* × *townsendii* individuals by haplotype analysis of the internal transcribed spacer 1 (ITS1). To do this, we selected a subregion containing several fixed substitutions specific for each parent (Figure S2). The aligned parental ITS1 reads were well separated into two clades comprising sequences from *S. alterniflora* and *S. maritima*. The *S. maritima* clade was more heterogeneous than that of *S. alterniflora* (Figure S3E), consistent with previous observations of ITS diversity (Muhammad & Ki, 2022). It contained two subvariants, "A" and "T," differing in the A4042T substitution (coordinates are according to the GenBank KT874468 accession). Two well-supported clades were also revealed in each individual of *S. anglica* and *S.* ×

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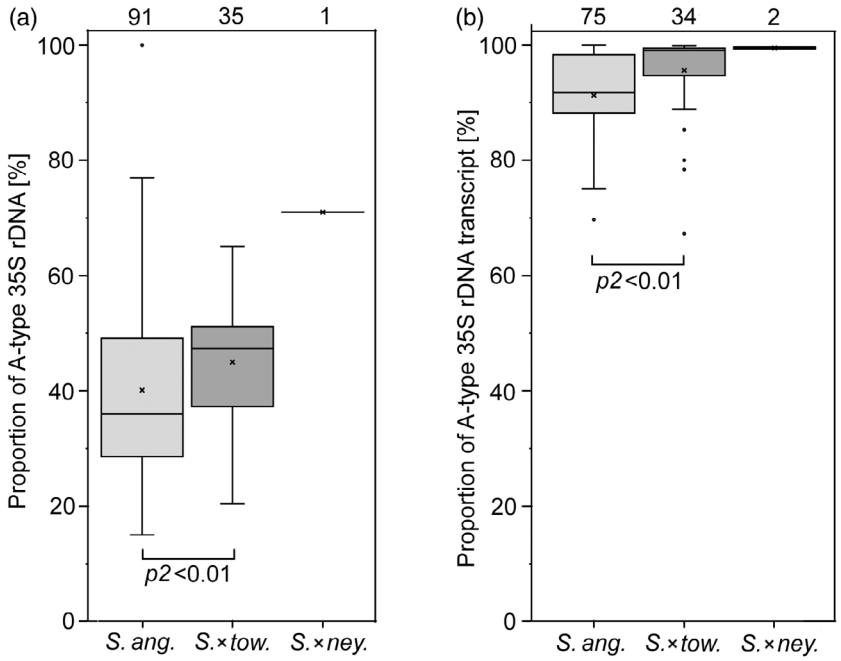


Figure 2. Statistical analysis of *Spartina* rDNA genomic abundance and expression.
Box plots showing genomic abundance (a) and homoeologous rDNA expression (b) in *S. anglica* (*S. ang.*), *S.* × *townsendii* (*S.* × *tow.*), and *S.* × *neyrautii* (*S.* × *ney.*). Y-axes represent the proportion of A-type units out of the total (A + M) units. The number of samples analyzed is shown above each box, and the box plots display the top (third) and bottom (first) quartiles, median values (lines in boxes correspond to medians; "×" is a mean, with whiskers reflecting 1.5 × interquartile range away from the first/third quartile and outliers individually plotted). A non-parametric Mann–Whitney test was used for comparisons between the groups. The data were obtained from the Southern blot hybridization (a) and RT-qPCR analyses (b) (Table S2).

Table 1 Transcriptomic and genomic analyses of rDNA homoelogs in *Spartina* hybrids and allopolyploids

				A-type		M-type	
Species	Sample	GenBank archive	*N*[a]	Count	Percentage	Count	Percentage
S. anglica	RNA	SRR4011104	64	59	92.2	5	7.8
	DNA	SRR10224432	100	48	48.0	52	52.0
S. × *townsendii*	RNA	SRR4011100	51	48	94.1	3	5.9
	DNA	SRR10224433	101	43	42.6	58	57.4
S. × *neyrautii*	RNA	SRR4011094	56	56	100.0	0	0.0
	DNA		nd	nd	nd	nd	nd

[a]The numbers correspond to reads used in phylogenetic analyses of ITS haplotypes forming distinct clades (Figure S3).

townsendii (Figures S3a,c). The "A"-clade comprised reads from *S. anglica*/*S.* × *townsendii* and the *S. alterniflora* consensus. A slightly more diversified "M"-clade comprised ITS sequences from *S. anglica*/*S.* × *townsendii* and the *S. maritima* consensus. The proportion of rDNA homoelogs to total rDNA is shown in Table 1.

Dominant A-type rDNA transcription in *S. anglica* and homoploid hybrids

To determine the expression levels of homoelogous rDNA loci, we carried out RT-qPCR analysis of 75 *S. anglica* individuals (43 from French and 32 from British populations), 34 *S.* × *townsendii*, and two *S.* × *neyrautii* individuals (Table S1), and representative results are shown in Figure 3. It is evident that the contribution of A-type transcripts to total rRNA transcripts (A/A + M ratio) was high, as most rRNAs were dominated by those transcribed from the A-subgenome. Statistical evaluations of the RT-qPCR data (Table S2) are shown as box plot graphs (Figure 2b). The median of A-type transcript proportions was 91.8, 99.0, and 99.5% for *S. anglica*, *S.* × *townsendii*, and *S.* × *neyrautii*, respectively. Thus, the contribution of A-type transcripts to total rRNA transcripts was lower in *S. anglica* compared to both homoploid hybrids (Mann–Whitney

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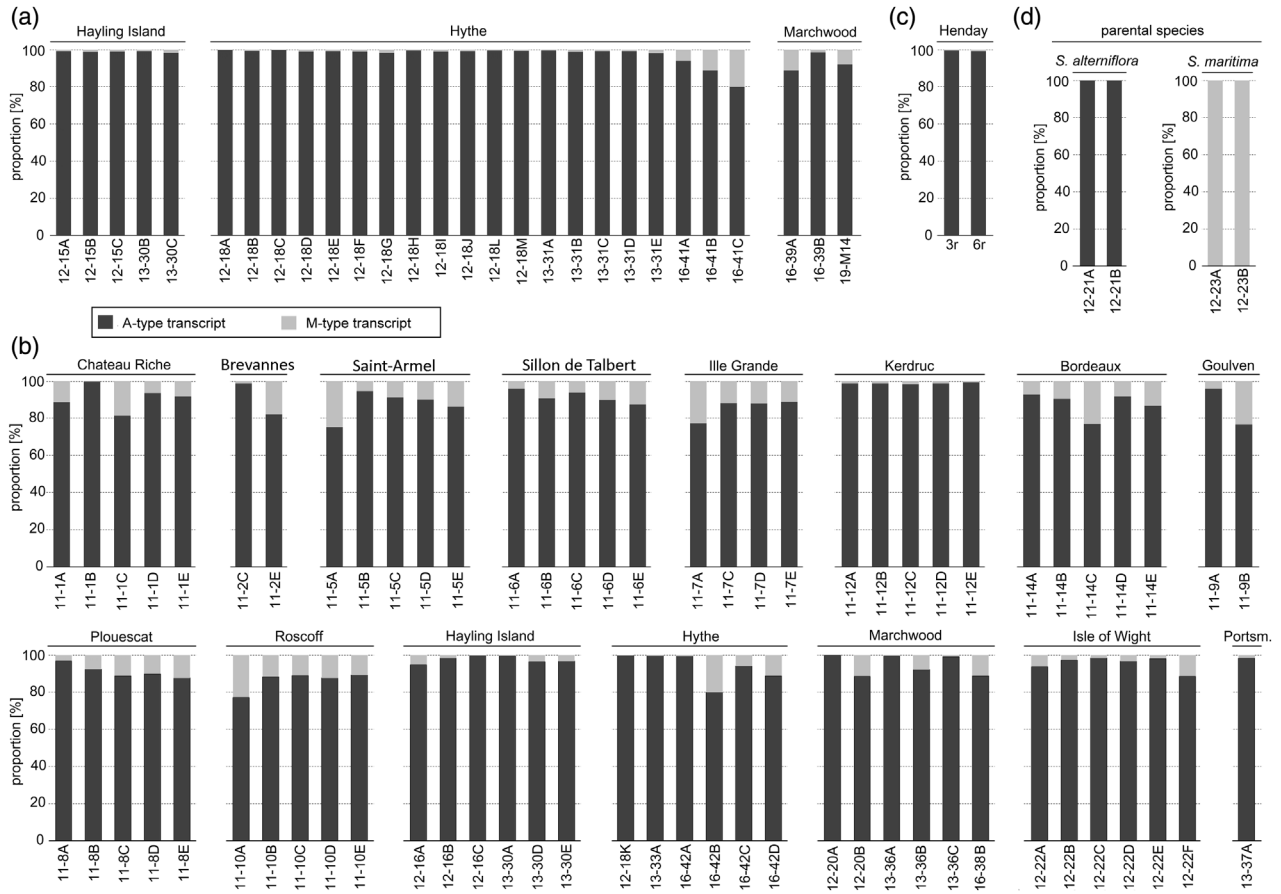


Figure 3. Population-level analysis of rDNA expression in *Spartina* hybrids and polyploids and their parents.
Samples included RNA from *S.* × *townsendii* (a), *S. anglica* (b), *S.* × *neyrautii* (c), and parental *S. alterniflora* and *S. maritima* (d). Stacked graphs were constructed using the RT-qPCR values (Table S2). The proportions of A- and M-type transcripts are indicated in dark and light gray bars, respectively.

two-tailed test: z score − 3.65, $P = 0.0003$). Interindividual variability was negligible in homoploids, while *S. anglica* showed higher variability, but the difference was not significant (Leven's test, f-ratio value 0.37909, $P = 0.5394$). Nevertheless, there were some differences between the populations. For example, individuals from the Roscoff (North Brittany) population showed on average 13.7% M-type transcripts (out of total transcripts), while the Kerdruc (South Brittany) population had a significantly lower contribution from the M-type (1.2%, t-test, $N = 10$, $P = 0.002$).

To validate the RT-qPCR results, we carried out RT-CAPS analysis on selected samples. We used selective XbaI or XmaI restriction enzymes to digest PCR (end-point) products obtained from amplification of the ITS1 region (Figure S4). The RT-CAPS of *S. anglica* and *S.* × *townsendii* yielded mostly A-specific fragments, while those of the M-type were missing or faint. In contrast, genomic CAPS yielded both parental fragments of comparable intensity. Thus, the dominant expression of rDNA of *S. alterniflora* origin in *Spartina* hybrids was confirmed by RT-CAPS.

Next, we analyzed rDNA transcriptomes of *S. anglica* from Marchwood (UK), *S.* × *townsendii* from Hythe (UK), *S.* × *neyrautii* from Henday (France), *S. alterniflora* from Hythe (UK), and *S. maritima* from the Isle of Wight (UK) (Figure S3b,d). In *S. anglica* and both homoploid hybrids, >90% of ITS1 transcripts originated from the *S. alterniflora* genome (Table 1).

Low methylation levels of *S. alterniflora* rDNA units contrast with high methylation levels of *S. maritima* units

Because of the known relationship between DNA methylation and silencing, we investigated the methylation status of A- and M-type rDNA units in progenitor genomes and derived hybrids and allopolyploids. We employed digestion of genomic DNAs with methylation-sensitive and -insensitive restriction enzymes in combination with Southern blot hybridization (Figure 4). The BstNI/PspGI isoschizomere pair is known to cut the CCWGG site, but PspGI cleavage is blocked by methylation of the second cytosine (C) (CmCWGG), while BstNI is insensitive to C methylation. Digestion of DNA from parental *S. alterniflora*

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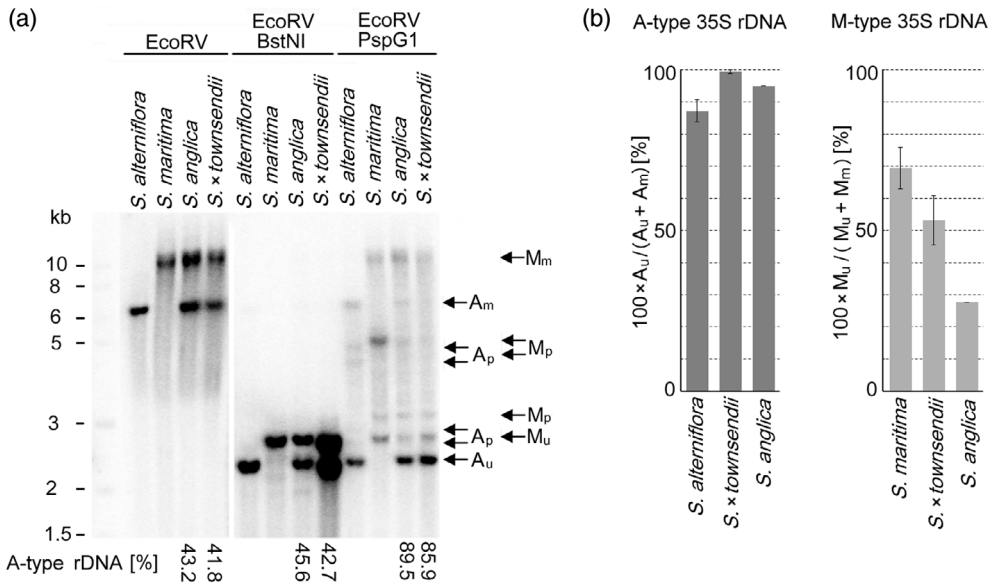


Figure 4. Cytosine methylation analysis of rDNA homoelogs in parental species and derived *S.* × *townsendii* and *S. anglica*. The methylation status of CWG sites was inspected at the BstNI/PspGI restriction sites by Southern blot hybridization.
(a) The 26S rDNA probe hybridized to genomic DNAs digested with EcoRV alone (left panel) and in combination with methylation-insensitive BstNI (middle) or its methylation-sensitive PspGI (right) isoschizomere. In the EcoRV/PspGI profiles, three types of fragments were distinguished: unmethylated (Au and Mu), partially methylated (Ap and Mp), and completely methylated (Am and Mm) fragments. The high-molecular-weight Am/Mm fragments are of similar size to those digested with EcoRV alone; the low-molecular-weight Au/Mu fragments had the same size as those of EcoRV/BstNI.
(b) Quantification of restriction fragments by phosphorimaging. The data were collected from (a) and other blots. The amount of unmethylated (presumably active) units was calculated as the ratio between Au/(Au + Am) and Mu/(Mu + Mm) fragments.

with methylation-sensitive PspGI enzyme resulted in a dominant 2.4 kb band (Au) corresponding to non-methylated units (Figure 4a). The additional relatively minor bands were of higher molecular weights, corresponding to partially (Ap) and fully (Am) methylated rDNAs. Digestion of parental *S. maritima* with PspGI resulted in a series of methylated fragments (Mp and Mm) and a relatively weak non-methylated 2.8 kb (Mu) fragment. In *S.* × *townsendii* and *S. anglica*, the probe hybridized strongly to the Au and weakly to the Mu fragments. In addition, a series of high molecular weight bands were visualized, predominantly being of the M-parent origin (Mp, Mm). There were no or only weak Am fragments visualized in hybrids. Phosphorimaging allowed us to quantify the level of undermethylated parental units in the genome (Figure 4b). The proportion of unmethylated Au fragments (relative to the sum of Au and Am fragments) increased in both hybrids compared to parental *S. alterniflora*. In contrast, the proportion of unmethylated Mu fragments decreased in hybrids compared to progenitor *S. maritima*.

FISH analysis of rDNA

In the parental species *S. maritima* and *S. alterniflora*, previous FISH investigations revealed the presence of one pair of 45S signals (Boutte et al., 2016; Giraud, Lima, Rousseau-Gueutin, et al., 2021). In order to determine the number of rDNA loci and their chromatin structure in the hybrid and the neo-allopolyploid, we carried out fluorescence *in situ* hybridization (FISH) analysis of *S. anglica* and *S.* × *townsendii*, both sampled in the British populations. Figure 5 and Figure S5 show the 18S and 5S rDNA probes hybridized to metaphase chromosomes. In the 16-38B *S. anglica* individual from Marchwood (UK), each of the 18S and 5S probes hybridized to four sites. The 18S signal intensities were relatively balanced in contrast to 5S rDNA, which showed two strong and two weak signals. In the 13-36A *S. anglica* individual from the same population, the 18S probe hybridized to two sites only, indicating deletion of one rDNA locus. In the hybrid, also from Marchwood, the 18S and 5S rDNA probes hybridized to three and six sites, respectively. In *S.* × *townsendii* from Hythe (16-41B individual), the 18S hybridized to two sites of comparable fluorescence intensity. The 5S probe (red) hybridized to two sites, one being strong and one weak.

Because of the known relationship between chromatin decondensation and rDNA expression (Borowska-Zuchowska et al., 2021; Pontes et al., 2003), we analyzed interphase nuclei using rDNA-FISH (Figure 6; Figure S6). In the *S. anglica* interphase, the 18S probe hybridized to four sites, two of which showed extensive decondensation (arrows) and two were fully condensed. All four rDNA signals occurred in the proximity of the nucleolus. In the

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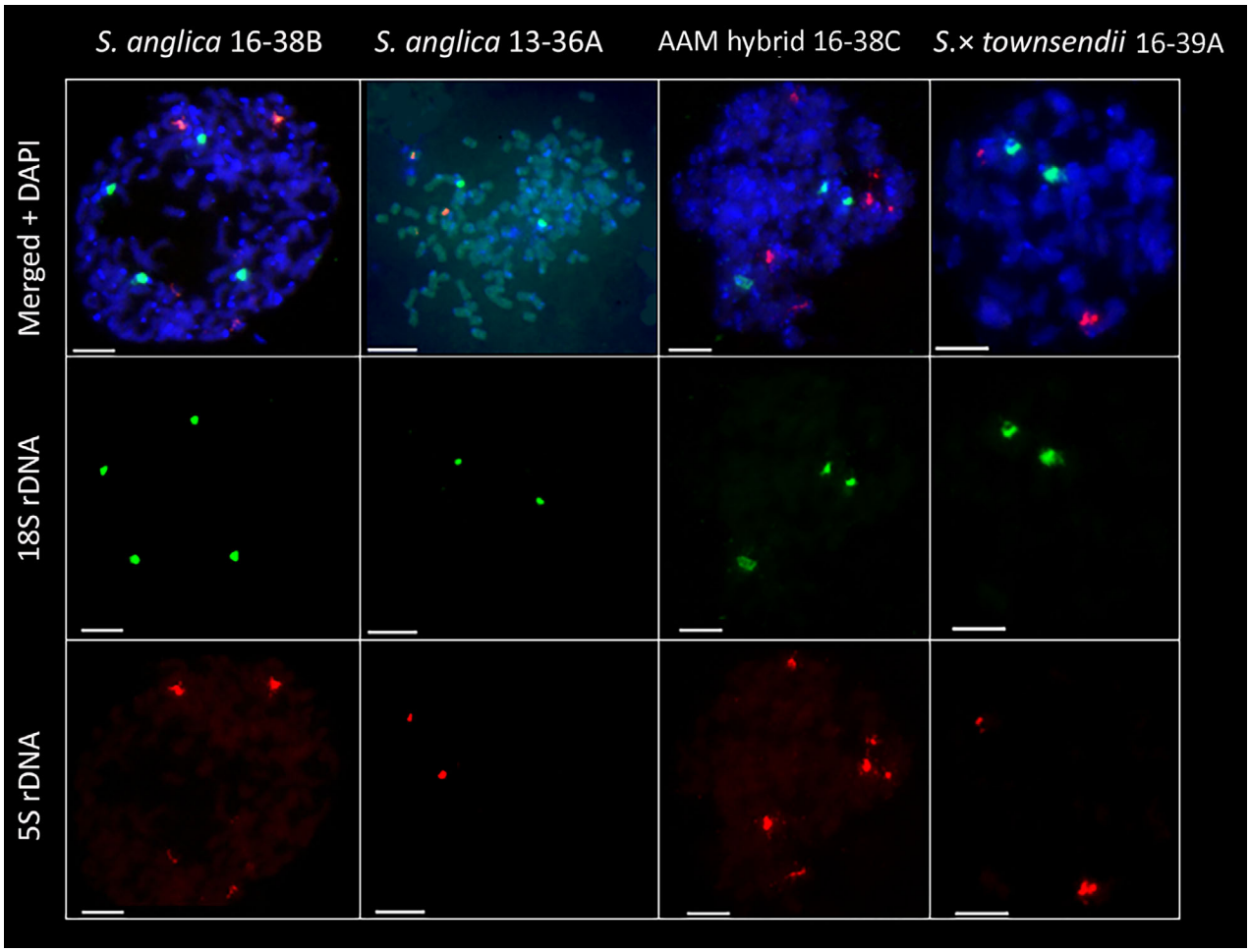


Figure 5. FISH analysis of rDNA loci in metaphase chromosomes.
Root tip chromosomes were obtained from *Spartina* hybrids and polyploids. The 18S rDNA sites are visualized in green, 5S rDNA in red, and chromatin in blue (DAPI) fluorescence. The bar represents 5 μm.

S. × *townsendii* interphase (16-41B individual), only one of the two rDNA loci showed decondensation (arrow). Stretches of partially condensed and fully decondensed chromatin were visible across the NOR. In the nonaploid hybrid interphase, two rDNA sites showed extensive decondensation (arrows), while the third was regularly condensed. Although the origin of decondensed loci is currently unknown, we infer that they are mainly composed of the A-type units.

DISCUSSION

S. anglica represents a recently formed allopolyploid species that has expanded in range tremendously since its origin less than 150 years ago. The first-generation homoploid hybrids from the time of species origin are also extant, making the *Spartina* system ideal for addressing the question of the immediate impacts of hybridization and polyploidy on rDNA expression. Through the analysis of hundreds of individuals, evidence was obtained for the early occurrence of nucleolar dominance in both homoploid hybrids and *S. anglica*.

Retention of parental rDNA loci in *Spartina* allopolyploids, substantial intra- and interpopulation variation in homoelog ratios, and rare locus loss

Southern blot hybridization analysis of more than 100 *S. anglica* and homoploid individuals from different populations revealed retention of parental rDNA homoelogs, which is consistent with previous studies obtained on fewer samples (Baumel et al., 2001; Huska et al., 2016). Nevertheless, there was a large variation in parental rRNA gene ratios between and within populations. Generally, the A-type 35S rRNA units were less represented than those of the M-type, which may reflect the fact that *S. alterniflora* parent (maternal genome donor) had a lower 35S rDNA copy number than *S. maritima* (paternal genome donor)

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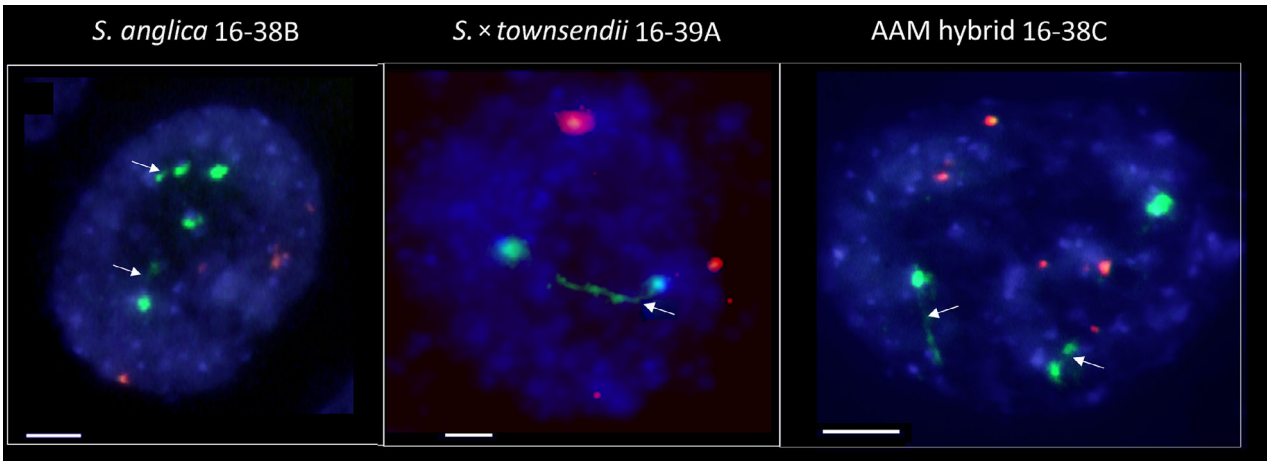


Figure 6. FISH analysis of interphase nuclei for the indicated *Spartina* individuals.
Decondensed 35S rDNA loci are indicated by arrows. The fluorescent colors are the same as in Figure 5. The bar represents 5 μm.

(Table S3). The *S. maritima* rDNA also had higher intragenomic diversity than that of *S. alterniflora*, evidenced by the presence of two ITS1 (Figure S3) and ETS (Boutte et al., 2016) variants that likely map to a single locus. These variants were apparently faithfully transmitted to hybrids and allopolyploids. There were rare individuals where the A-type units outnumbered those of the M-type. Perhaps, the most dramatic genetic event was a complete loss of the M-type rDNA loci in two *S. anglica* individuals, both derived from the same clone in Marchwood (UK). These genotypes have been repeatedly sampled over the years, and one individual has been vegetatively propagated in a greenhouse for several years, suggesting that rDNA elimination did not influence plant viability. In contrast to an Asian population of *S. anglica* (Muhammad & Ki, 2023), we did not observe recombinant ITS sequences, which may imply that interlocus homogenization might have affected Asian and not European populations of *S. anglica*. Also, GISH analysis of *S.* × *townsendii* (Figure S7) and *S. anglica* (Figure S8) failed to reveal intergenomic translocations, corroborating initial GISH experiments in a *Spartina* backcross hybrid between *S. anglica* and *S. alterniflora* (Renny-Byfield et al., 2010). It seems that subgenomes tend to retain considerable genetic intactness in *Spartina* recent hybrids, and intergenomic exchanges are rare, if any. This apparently differs from Festuca-Lolium (Poaceae) hybrids, where instant nucleolar dominance occurred in association with subgenome dominance (Mahelka et al., 2023).

Uniparental silencing of rDNA is established during interspecies hybridization and maintained in subsequent polyploidization events

In monocots, most of the data linked with nucleolar dominance has come from research on wheat (*Triticum aestivum*) and *Brachypodium* (*Poaceae*) allopolyploids (Borowska-Zuchowska et al., 2023). However, both wheat and *B. hybridum* are much older allopolyploids (thousands of years), and the immediate impacts of allopolyploidy cannot be assessed. Our population analysis of recently formed *Spartina* hybrids and allopolyploids demonstrated that nucleolar dominance is established early following interspecies hybridization in the first-generation hybrids. The direction of dominance was biased towards the maternal (*S. alterniflora*) parent, and subsequent polyploidization had little or no effect on transcription patterns established in F1 hybrids. Independently formed hybrids *S.* × *townsendii* (UK) and *S.* × *neyrautii* (France) showed similar expression patterns, indicating directionality of rDNA silencing. In some *S. anglica* lineages (e.g., 11-5A individual), we noted a relatively high contribution (up to 25%) of the M-type transcripts, suggesting partial activation of silent loci following genome duplication. This may corroborate previous observations of decreased maternal (A-type) expression dominance and an increased number of transgressively expressed genes in *S. anglica* compared to *S.* × *townsendii* (Chelaifa et al., 2010). Partial upregulation of M-type units in *S. anglica* may also be related to the organization of rDNA in the cell nucleus. In *S. anglica* and *S.* × *townsendii*, both active and inactive loci tend to associate with the nucleolus (Figure 6; Figure S7) by analogy with parental rDNAs in wheat (Leitch et al., 1992). In contrast to wheat, we did not observe blocks of rDNA heterochromatin inside *S. anglica* nucleoli. In conclusion, despite minor upregulation of silent loci in a few cases, the results are consistent with the hypothesis that nucleolar dominance is fairly stable within and across populations of *S. anglica* and the first-generation hybrids.

In allopolyploids, nucleolar dominance has been suggested to be driven by gene dosage effects, occurring in allotetraploids where rRNA gene copy numbers are double

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those of their diploid parents (Borowska-Zuchowska et al., 2023). However, both *S.* × *townsendii* and *S.* × *neyrautii* hybrids contain comparable numbers of rRNA genes as their parents (Table S3). Hence, nucleolar dominance is unlikely to be explained by gene dosage in these *Spartina* hybrids. In wheat and *Brachypodium*, silencing of rDNA units has been correlated with their DNA methylation and chromatin hypercondensation (Borowska-Zuchowska & Hasterok, 2017; Flavell et al., 1988; Guo & Han, 2014; Tulpova et al., 2022). In synthetic lines of wheat, epigenetic silencing preceded their elimination (Guo & Han, 2014). In line with these observations, the inactive M-loci exhibited high levels of methylation in both *S.* × *townsendii* and *S. anglica*. In contrast, the A-type units became less methylated in hybrids, suggesting that both loci undergo epigenetic changes in hybrids. Perhaps, contrasting methylation levels of rDNAs already existing in parental genomes may explain the expression dominance of the *S. alterniflora*-inherited units in hybrids.

Evolutionary consequences of rDNA silencing

The question arises: what may be the short- and long-term evolutionary trajectories of rDNA silencing?

Each parental species, *S. maritima* and *S. alterniflora*, is derived from a meso-octoploid ancestor and has diverged 2.6–6.8 MYA (Salmon et al., 2025). Despite their polyploid origin and complex genomic composition (A1A2B1B2), each has only a single rDNA locus, suggesting that rDNA diploidization has occurred at some point in this evolutionary trajectory. Of note, diploidization of rDNA was observed in the 1.4 myrs-old lineage of *B. hybridum* (Poaceae) allotetraploid (Trunova et al., 2024), indicating that diploidization of rDNA occurs within comparable time frames. From the work here, we observed that, in the recently formed *S. anglica* (within the last 150 years), rDNA diploidization might have already started, since we have observed a few individuals that have completely lost the M-type locus, regularly silenced by epigenetic processes in other individuals. We note also that intralocus homogenization has also been observed in some Asian *S. anglica* populations (Muhammad & Ki, 2023). Thus, divergence of parental copy variation and copy number loss associated with rDNA diploidization may occur very fast and far faster than a slow molecular clock measured over millions of years.

MATERIALS AND METHODS

Sampling and plant material

Nineteen sites were sampled for *Spartina* plants along the European Atlantic coast (Table S1), including five English and 14 French sites. The UK populations were repeatedly sampled between 2012 and 2021; the French populations were sampled in 2011. In the United Kingdom, sampling included two sites (Marchwood and Hythe) in Southampton Water, the presumed site of species origin, plus sites on the Isle of Wight and Hayling Island and in Portsmouth (Huska et al., 2016). In France, sampling covered a large part of the Atlantic coast range of *S. anglica* and of *S.* × *neyrautii*, with *S. anglica* ranging from Normandy (Brevans) across North and South Brittany and the Bordeaux area. At the collection sites, fresh green leaves were cut into three to four pieces, c. 5 cm long, and immediately put into the RNAlater solution in 4 ml plastic cryotubes. In total, 0.5–1.0 g of leaf tissue for each sample was stored in RNAlater®. Two to six clones were collected from each population. As *S.* × *townsendii* cannot be easily distinguished from *S. anglica* using morphological characters (Thompson et al., 1991), ploidy levels of the plants collected from the British localities were checked by flow cytometry (Huska et al., 2016). For FISH (see below), individual clones were propagated either in pots with soil or hydroponics. However, in greenhouse conditions, only a few plants survived for 2 years. Nevertheless, a single *S. anglica* and four *S.* × *townsendii* individuals from the Southampton Water populations have continued to grow successfully in a greenhouse (Czech Academy of Science) for over 10 years since their collection.

Southern blot hybridization

Total genomic DNA was extracted from leaves stored in RNAlater solution (Ambion, Austin, TX, USA) using a cetyltrimethylammonium bromide method (Doyle, 1991). Before homogenization in liquid nitrogen, the RNAlater solution was removed by washing the leaf pieces in distilled water. About 200 mg of leaf was used for extraction. Southern blot hybridization followed the protocol described in Kovarik et al. (2005). For the homoelogous rDNA analysis, genomic DNA was digested with BstNI, targeting a polymorphic site in the IGS of *S. alterniflora* and *S. maritima* (Figure 1). For the methylation analyses, DNAs were first predigested with EcoRV, and then, the resulting fragments were digested with BstNI/PspGI restriction enzymes. rDNA probes were labeled, and the hybridization signals were visualized and quantified as described in Huska et al. (2016). The probe was a 220-bp long 26S rRNA gene fragment from *Nicotiana tabacum* (Lim et al., 2000).

Quantitative PCR

Total RNA was isolated from leaves stored in RNAlater using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Purified RNA was treated with TURBOTM DNase (Ambion, Austin, TX, USA) to remove traces of DNA. Concentrations of RNA were checked by spectrophotometry at 260 nm (NanoDrop). cDNA synthesis was performed in 40 μl volumes containing 2 μg RNA, 4 pmol random primers (N9), and 200 U reverse transcriptase (Superscript II RNaseH, Invitrogen, Paisley, UK) according to the manufacturer's instructions. Two sets of locus-specific primers were designed to discriminate between the M- and A-genome ITS1 sequences. Relatively high (c. 14%) ITS1 divergence allowed the design of homoelog-specific primers (Figure S2) to amplify a central c. 170-bp long region of ITS1. The forward (P1) and reverse (P2) primers were P1_mar: 5′-TCATCCATGGCATCGGGTGGC-3′, P1_alt: 5′-TCATCCATGCCGTCGGGTGAT-3′, P2_mar: 5′-CAGCATCGCAACGCATGGCTC-3 and P2_alt: 5′-GCACCGCAGGCCATGGAAT-3′.

Quantitative PCR was carried out in a 20-μl reaction volume containing 3 μl of cDNA template or a dilution of standard, 1 μl of each 10 μM primer, and 10 μl of the 2× Fast Start SYBR Green Master Mix (Roche, Germany). The amplification conditions were as follows: 1 cycle at 95°C for 7 min; 40 cycles at 95°C for 20 sec; 57°C for 20 sec; 72°C for 30 sec; 95°C for 15 sec; 60°C for 1 min; and 95°C for 15 sec. PCR was carried out on the Applied

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Biosystems 7300 Real-Time PCR cycler. There was a linear relationship between the cycle threshold (Ct) value and the amount of standard clones in a broad range of concentrations (Huska et al., 2016). The efficiency of amplification between *S. maritima* and *S. alterniflora* cDNAs was the same. For genomic quantitative PCR, 10 ng genomic DNA per reaction was used as a template. The ratio between the A (*S. alterniflora*) and M (*S. maritima*) rRNA transcripts was calculated from the formula: 2 − (CtA − CtM)2^{−(Ct_A − Ct_M)}2 − (CtA − CtM), where "Ct" is cycle threshold and "2^" power of two. The proportion of homoelogous transcripts was expressed as a percentage of total rRNA. Three technical replicates were performed for each sample, and Ct values were expressed as a mean. Differences between the parallel samples were generally less than 0.2 cycle.

RT-CAPS and genomic CAPS analysis

Primers amplifying the 3′ terminus of the 18S gene, the whole ITS1, and the 5′ portion of the 5.8S rRNA gene (Kovarik et al., 2005) were used to analyze restriction polymorphisms in the ITS1 region. The products of endpoint RT-PCR reactions were digested with selective XbaI or XmaI restriction enzymes and separated on 2% agarose gels. Fragments were visualized by ethidium bromide fluorescence.

Fluorescence *in situ* hybridization

Chromosome spreads from root tips were prepared according to Jenkins and Hasterok (2007) with slight modifications. Meristematic cells were isolated from primary roots of plants grown in soil or hydroponics. Their division in root tips was synchronized in ice-cold water for 24 h. Roots were fixed in 3:1 ethanol:acetic acid. Prior to chromosome spreading, roots were soaked in 1% acetocarmine for at least 3 h and treated with 2% HCl for 10 min. Enzymatic treatment of roots with 20% pectinase and 2% cellulase "Onozuka R-10" (Serva) was performed for 3 h at 37°C. Resulting protoplasts were then squashed under a cover slip in 40% acetic acid and frozen in liquid nitrogen. After the removal of acetic acid, the slides with chromosome spreads were then treated with RNase A (100 μg ml^{-1}) and pepsin (50 μg ml^{-1}) as described in Lunerova et al. (2017) prior to probe application and fluorescent *in situ* hybridization (FISH).

For FISH, the probes used were 18S rDNA (a cloned gene from tomato, GenBank # X51576.1) labeled with SpectrumGreen dUTP (Abbott Molecular, IL, USA) and 5S rDNA (an *Artemisia tridentata* S4 clone, GenBank # JX101915.1) labeled with Cy3-labeled dUTP (GE Healthcare, Chalfont, St. Giles, England). For genomic *in situ* hybridization (GISH), the genomic DNAs from *S. alterniflora*, labeled with SpectrumGreen dUTP, and *S. maritima*, labeled with Cy3-labeled dUTP, were used as probes. For FISH, the hybridization mixture (30 μl per slide) contained 100 ng of each 18S probe and 5S probe, 40% formamide, and 50% dextran sulfate in 2 × SSC. For GISH, the hybridization mixture (40 μL per slide) contained 100 ng of each genomic DNA probe, 500 ng of blocking genomic DNA from each *S. alterniflora* and *S. maritima*, 40% formamide, 10% dextran sulfate, 0.125% SDS, and 1 μg of shredded salmon sperm DNA in 1 × SSC. The hybridization mixture was heated up (10 min/75°C), cooled down on ice (5 min), pipetted onto each slide, and covered with a plastic coverslip. The slides were incubated in a humid chamber at 75°C/5 min, 65°C/2 min, 55°C/2 min, 45°C/2 min, and 37°C for 17 h and then washed 2 × 5 min in 2 × SSC at 42°C, 2 × 5 min in 0.1 × SSC at 42°C, 2 × 5 min in 2 × SSC at 42°C, 5 min in 2 × SSC at RT, 7 min in 4 × SSC/0.1% Tween 20 at RT with a final brief wash in PBS. The chromosomes were counterstained in Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA) containing 1.5 μg ml^{-1} 4′,6-diamidino-2′,6′-phenylindole. Fluorescence images were captured using an Olympus AX 70 fluorescence microscope equipped with a digital camera. Images were analyzed and processed using ISIS software (MetaSystems, Altlussheim, Germany).

Transcriptomic and genomic analyses

Raw RNA-seq data or genomic reads were quality controlled using FastQC and trimmed with Trim Galore/0.6.2 using default settings. Two million (one million pairs) clean reads were mapped to the *S. maritima* 35S rDNA unit (GenBank KT874468, KT874483.1, KT874488.1 (Boutte et al., 2016)). Mapped reads were used as an input for a single nucleotide variation (SNV) calling (Table S3). The reference *S. maritima* and *S. alterniflora* ITS1 consensus sequences were obtained from clustering analysis of genomic Illumina reads (Novak et al., 2013) of *S. alterniflora* (sequence read archive SRR10224435) and *S. maritima* (SRR10224434). In each species, ITS1-specific clusters comprised more than 500 reads. The *S. alterniflora* complete unit, including most of the IGS, was assembled into a single contig.

To extract variable ITS subregions for the phylogenetic analysis, BLAST libraries were generated from mapped reads for each sample. The libraries were BLASTed against the reference ITS1 subregion from the *S. maritima* rDNA unit. Reads were then extracted from BLAST files using a command in CLC and trimmed, keeping only long (>50 nt) sequences. If necessary, reads were subsampled randomly to about 50 reads/species for the phylogenetic analysis. Reads were renamed (Galaxy server tool), adding species-specific codes. Multiple alignments containing sequences from a given allopolyploid plus a reference sequence of each diploid progenitor were carried out, and a phylogenetic NJ tree was constructed. All phylogenetic calculations were run using the Jukes-Cantor model and 100 replicates. Reads from clades were extracted (command "Extract sequence list"), realigned, and counted. Rare (<5% of input reads), highly mutated sequences falling out of the main clades were not counted.

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AUTHOR CONTRIBUTIONS

AKo, MA, and ARL conceived the experiments; AKu, RM, IJL, JFC, and DH performed the experiments; AKo wrote the manuscript; and JFC, IJL, AS, ARL, and MA modified the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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DATA AVAILABILITY STATEMENT

The raw genomic data and transcriptome data have been deposited in the NCBI (https://www.ncbi.nlm.nih.gov/bioproject/) Sequence Read Archive within the Bioprojects no. PRJNA575642 (Chromosome evolution in invasive *Spartina* plants) and PRJNA338100 (*Spartina maritima, Spartina alterniflora, Spartina* × *townsendii, Spartina* × *neyrautii*, and *Spartina anglica* raw sequence reads).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Proportions of A-rDNA (dark gray) and M-rDNA (light gray) in *Spartina anglica* (a), *S.* × *neyrautii* (b), and *S.* × *townsendii* (c) determined by Southern blot hybridization. Genomic DNAs were digested with BstNI and hybridized with the 26S rDNA probe. BstNI has a unique restriction site in (i) the *S. alterniflora* IGS downstream from the 26S rRNA gene and (ii) the *S. maritima* 26S rRNA gene, resulting in DNA fragments of sizes specific for both progenitors (Figure 1). The intensity of radioactive signals was counted by phosphorimaging, and the abundance of homoelogous A- and M-type rDNA was expressed as an A/(A + M) ratio in the graphs below the blots. All corresponding values are summarized in Table S2 and statistically evaluated in Figure 2(a).

Figure S2. Alignment of the *S. maritima* and *S. alterniflora* ITS1 regions. Brown arrows are primers used in an RT-qPCR analysis. Blue arrow: a 55-bp subregion used as a query in BLAST analyses. Brown triangle: polymorphic site in the M-type ITS1 discriminating between the "A" and "T" variants. Restriction sites for the XbaI and XmaI enzymes used in CAPS experiments are highlighted in gray.

Figure S3. Phylogeny NJ trees composed from genomic (a, c, e) and transcriptomic (b, d) ITS1 reads. The number of reads in each "A and M" clade is given in Table 1.

Figure S4. Proportions of A- and M-homoelogs in rRNA, determined by cDNA-CAPS (lanes R), and rDNA, determined by gDNA-CAPS (lanes D) analyses. (a, c) Schematic representation of the amplified 18S-ITS1-5.8S region showing the position of primers (arrows), targets for diagnostic enzymes XbaI (a) and XmaI (c), and expected sizes of DNA fragments. (b, d) The endpoint amplification products from French populations of *S. anglica*, *S.* × *townsendii, S.* × *neyrautii, S. alterniflora*, and *S. maritima* were digested with the XbaI (b) or XmaI (d) restriction enzyme having a specific recognition site in *S. alterniflora* and not *S. maritima* ITS1.

Figure S5. Additional FISH analyses of metaphase chromosomes of *S. anglica* and *S.* × *townsendii*.

Figure S6. Additional FISH analyses of *S.* × *townsendii* interphase nuclei.

Figure S7. Genomic in situ hybridization (GISH) of metaphase chromosomes from the *S.* × *townsendii* 16-39B individual. The genomic DNA probes were derived from *S. maritima* (red) and *S. alterniflora* (green).

Figure S8. Genomic in situ hybridization (GISH) of metaphase chromosomes from the *S. anglica* 13-36A individual. The genomic DNA probes were derived from *S. maritima* (red) and *S. alterniflora* (green). Note that the parental genomes retain chromosomal identity. No compensatory aneuploidy seen in other recently formed allopolyploids (Lim et al., 2008) has been observed.

Table S1. List of species, population IDs, and type of analyses carried out in this study.

Table S2. Datasets of the qPCR and Southern blot analyses. The expression and genomic representation of homoelogous rRNA genes are calculated as a percentage of the A-units of the total rDNA units (A + M-genome). (a) Homoploid first-generation hybrids, *S.* × *townsendii* and *S.* × *neyrautii*. (b) *S. anglica*.

Table S3. 18S and 5S rDNA copy number calculated from genome proportions determined from Illumina reads.

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