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Flow cytometry and GISH reveal mixed ploidy populations and *Spartina* nonaploids with genomes of *S. alterniflora* and *S. maritima* origin

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• *Background* The genus *Spartina* exhibits extensive hybridization and includes classic examples of recent speciation by allopolyploidy. In the UK there are two hexaploid species, *S. maritima* and *S. alterniflora*, as well as the homoploid hybrid *S.* × *townsendii* ($2n = 60$) and a derived allododecaploid *S. anglica* ($2n = 120, 122, 124$); the latter two are considered to have originated in Hythe, southern England at the end of the 19th century.
• *Methods* Genomic *in situ* hybridization (GISH) and flow cytometry were used to characterize the genomic composition and distribution of these species and their ploidy levels at Eling Marchwood and Hythe, both near Southampton, southern England.
• *Key Results* GISH identified approx. 60 chromosomes each of *S. maritima* and *S. alterniflora* origin in *S. anglica* and 62 chromosomes from *S. alterniflora* and 30 chromosomes from *S. maritima* in a nonaploid individual from Eling Marchwood, UK. GISH and flow cytometry also revealed that most (94 %) individuals examined at Hythe were hexaploid (the remaining two individuals (6 %) were dodedcaploid; $n = 34$), whereas hexaploid (approx. 36 % of plants), nonaploid (approx. 27 %) and dodecaploid (approx. 36 %) individuals were found at Eling Marchwood ($n = 22$).
• *Conclusions* Nonaploid individuals indicate the potential for introgression between hexaploid and dodecaploid species, complicating the picture of polyploid-induced speciation within the genus. Despite the aggressive ecological habit of *S. anglica*, it has not out-competed *S.* × *townsendii* at Hythe (homoploid hybrids at a frequency of 94 %, $n = 34$), despite >100 years of coexistence. The success of GISH opens up the potential for future studies of polyploid-induced genome restructuring in this genus.

Key words: Polyploidy, speciation, *Spartina*, nonaploid, nonaploid bridge, interspecific hybridization, GISH.

INTRODUCTION

Most or all angiosperm species are believed to have experienced at least one round of polyploidy in their ancestry (Otto and Whitton, 2000; Bowers *et al.*, 2003; Mallet, 2007; Soltis *et al.*, 2009), indicating its importance in the evolution of the flowering plants. Certainly polyploidy has been a driving force behind speciation in the genus *Spartina* (Poaceae) (Ainouche *et al.*, 2004*a*). All *Spartina* species reported to date are polyploid with a base chromosome number $x = 10$ (Marchant, 1968). This genus evolved through two main lineages that diverged approx. 2·5–3 MYA (Christin *et al.*, 2008), including tetraploid and hexaploid species (Baumel *et al.*, 2002*b*) that are most likely of hybrid origin (Fortune *et al.*, 2008). Four taxa are of interest here: the hexaploids *S. alterniflora* Loiseleur ($2n = 60, 62$) and *S. maritima* Fernald ($2n = 60$), their homoploid hybrid *S.* × *townsendii* H. Groves & J. Groves ($2n = 60, 62$) and the derived allododecaploid *S. anglica* C.E. Hubbard ($2n = 12x = 120, 122, 124$).

The allopolyploid *Spartina anglica* formed during the 19th century in Hythe, Southampton, after the introduction of *S. alterniflora*, a perennial grass native to the Atlantic coast of North America. Interspecific hybridization of *S. alterniflora* with the native British *S. maritima* (which inhabits the Atlantic coast of Africa and Europe) produced the infertile hybrid *S.* × *townsendii*, first identified in 1880 (Groves and Groves, 1880) – there are no reports of seed set as far as the authors are aware. Following this, genome duplication in the hybrid resulted in the formation of the fertile allopolyploid *S. anglica* (Marchant, 1963), a vigorous salt marsh species that has rapidly expanded in European salt marshes and has now colonized several continents (Marchants, 1967, 1968). Isozyme studies (Raybould *et al.*, 1991), followed by a range of molecular methods (Ayres and Strong, 2001; Baumel *et al.*, 2001, 2002*a*, 2003; Ainouche *et al.*, 2004*a*, *b*), have confirmed the parentage of *S. anglica* and *S.* × *townsendii*, and analysis of the plastid sequences revealed the maternal parent to be *S. alterniflora* in all European populations investigated to date (Ferris *et al.*, 1997; Baumel *et al.*, 2001).

The emergence of *S. anglica* in southern Britain is reported to have paralleled the decline of native *S. maritima* along the entire southern coast of England, and now only a few populations exist on Hayling Island near Southampton and possibly

on the Isle of Wight (Raybould *et al.*, 2000). Contrasting with the rapid spread of the new allopolyploid *S. anglica*, the introduced parental species *S. alterniflora* remains confined to a few sites near Southampton (Charman, 1990). Since its emergence, *S. anglica* has gone on to colonize many parts of the world, frequently with severe ecological consequences (Hacker *et al.*, 2001; An *et al.*, 2007; Cottet *et al.*, 2007). Land reclamation is common at sites colonized by *S. anglica* and, for this reason, many national wildlife authorities have imported this pioneer species as a means of combating land loss due to rising sea levels and erosion of mud flats. These factors have combined to give this young species a diverse ecological range throughout areas of Europe, Australia and China.

Spartina anglica populations exhibit little inter-individual genetic variation (Raybould *et al.*, 1991; Ayres and Strong, 2001; Baumel *et al.*, 2001, 2002*b*). Such a lack of diversity is thought to have resulted from a genetic bottleneck at the time of formation (Ainouche *et al.*, 2004*a*). This genetic uniformity indicates a single origin or multiple origins involving similar genotypes (Ayres and Strong, 2001; Baumel *et al.*, 2001, 2003; Yannic *et al.*, 2004). In fact, where hybridization occurs, there is little genetic variation amongst the parental species. Southern England is the northern limit of the range of *S. maritima*, and populations here do not produce seeds, instead they propagate vegetatively (Yannic *et al.*, 2004). There is also little genetic variation among individuals of *S. alterniflora*, which were introduced from America, due to founder effects (Baumel *et al.*, 2003). The original population of *S.* × *townsendii*, as well as individuals of the derived neo-polyploid *S. anglica*, exhibits additivity of multilocus parental molecular markers (Ayres and Strong, 2001; Baumel *et al.*, 2001, 2002*b*). In spite of belonging to the same (hexaploid) clade, *S. maritima* and *S. alterniflora* exhibit considerable divergence (Baumel *et al.*, 2002*a*) as through diploidization processes they have retained different paralogous gene copies that arose from ancient polyploidy (Fortune *et al.*, 2008).

While no extensive structural changes have been observed in the homeologous genomes of *S.* × *townsendii* and *S. anglica* (Baumel *et al.*, 2002*b*; Ainouche *et al.*, 2004*a*, 2009; Fortune *et al.*, 2008), the hybridization step which gave rise to *S.* × *townsendii* has resulted in epigenetic alterations that have also been transmitted to the allopolyploid, *S. anglica* (Salmon *et al.*, 2005). These changes mostly affect the regions flanking transposable elements (Parisod *et al.*, 2009). Considerable changes to the transcriptome have also accompanied the formation *S.* × *townsendii* and *S. anglica* (Chelaifa *et al.*, 2010). These epigenetic changes most likely explain the phenotypic plasticity observed in natural populations of the allopolyploid (Thompson, 1990).

The extensive and rapid spreading (via both seed production and vegetative means) of the fertile *S. anglica* has not been seen in the sterile, yet vigorous *S.* × *townsendii* (Ainouche *et al.*, 2004*a*), leading Ainouche and co-workers to propose that the more extensive ecological adaptability of *S. anglica* compared with its hexaploid progenitors is due to chromosome doubling, rather than interspecific hybridization. Previous studies have documented the distribution of the varying ploidy-levels and species within this genus (Marchant, 1967, 1968; Charman, 1990); however, there have been no recent studies using cytogenetic approaches to interpret the frequency of ploidy levels in mixed populations of *Spartina*. By extensively sampling *Spartina* populations in the area where indigenous *S. maritima* and introduced *S. alterniflora* originally hybridized, the aim here is to evaluate the relative abundance of the different ploidy levels (homoploid and allopolyploid) encountered in this region and to analyse, for the first time, these populations using molecular cytogenetic approaches.

MATERIALS AND METHODS

Plant material

Spartina individuals were taken from two locations near Southampton on the south coast of England. Twenty-two plants along a north-west to south-east transect were collected at Eling Marchwood on the 13 August 2007 (Fig. 1A). Thirty-four plants were also collected at Hythe (around 6 shoreline miles from the site at Eling Marchwood), on 6 August 2007 along a transect following a north-east to south-west direction (Fig. 1B). Samples collected on both
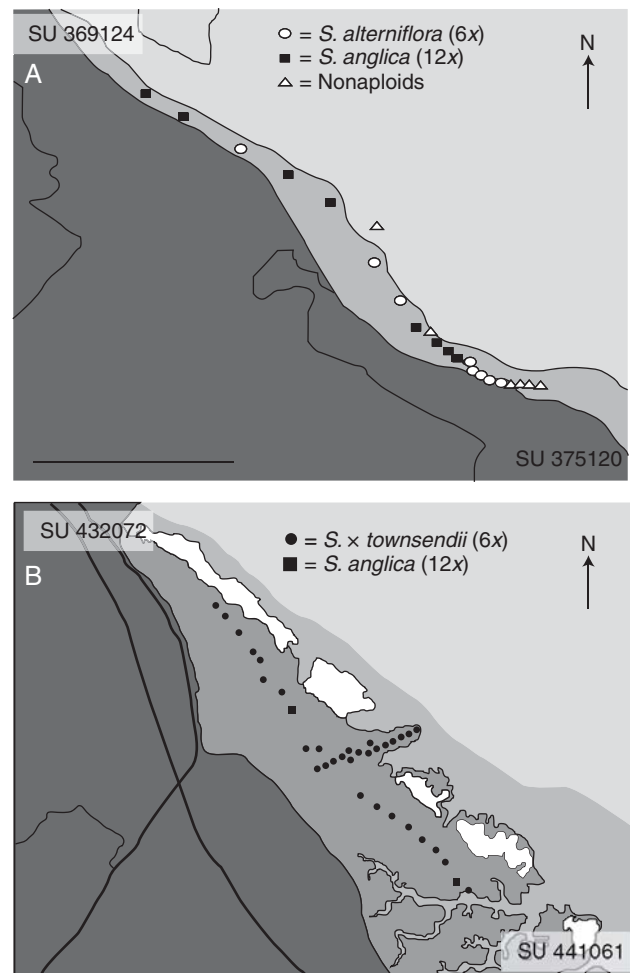

Fig. 1. Transect showing ploidy levels of plants collected at (A) Eling Marchwood and (B) Hythe. Dark shaded areas represent forest or field, medium grey areas represent tidal zone and pale grey represents water. White sections represent cockle shell deposits. Scale bar = 500 m. Ordnance survey grid references are shown at the top-left and bottom-right corners of (A) and (B).

days were returned to the laboratory for re-potting and analysis.

Genomic *in situ* hybridization (GISH)

Cytological preparations were made from seed embryos or root tips from pot-grown plants. Metaphases were accumulated by pre-treatment of material in saturated Gammexane® (hexachlorocyclohexane; Sigma) in water for 4 h, then fixed for 24 h in 3 : 1 absolute ethanol : glacial acetic acid and stored in 100 % ethanol at −20 °C. Plant material was squashed onto a glass slide after enzyme digestion as described in Sumner and Leitch (1999). GISH was performed using total genomic DNA probes from *S. alterniflora* (digoxigenin-11-dUTP labelled) and *S. maritima* (biotin-14-dUTP labelled) labelled by nick translation (Roche Nick Translation Kit) according to the manufacturers instructions. GISH was conducted as described in Lim *et al.* (1998) after denaturation in 70 % formamide (v/v) in 2× SSC (0·3 M sodium chloride, 0·03 M sodium citrate) at 70 °C for 2 min. The hybridization mix comprised of 50 % (v/v) formamide, 10 % (w/v) dextran sulfate, 0·1 % (w/v) sodium dodecyl sulfate in 2× SSC and 50 μg mL^{-1} of each genomic probe. After overnight hybridization at 37 °C, slides were washed in 20 % (v/v) formamide in 0·1× SSC at 42 °C at an estimated hybridization stringency of 80–85 %. Sites of probe labelling were detected with 20 μg mL^{-1} fluorescein-conjugated anti-digoxigenin IgG (Roche Biochemicals Ltd) and 5 μg mL^{-1} Cy3-conjugated streptavidin (Amersham Pharmacia Biotech), and chromosomes were counterstained with 2 μg mL^{-1} DAPI (4′,6-diamidino-2-phenylindole) in 4× SSC, mounted in Vectashield (Vector Laboratories) medium. Material was photographed with an Orca ER camera mounted to a Leica DMRA2 epifluorescent microscope. Images were processed with Improvision Openlab software (Improvision, Coventry, UK) and adjusted for colour balance, contrast and brightness uniformly.

Analysis of ploidy levels

Flow cytometry was used to screen for ploidy level using methods described in Hanson *et al.* (2005) and either a Partec PAII or a Partec CyFlow flow cytometer. *Pisum sativum* 'Minerva Maple' was used as a calibration standard for assessing ploidy levels.

RESULTS

GISH

Two *Spartina* individuals, both originally identified as *S. anglica*, and thus assumed to be dodecaploids were selected for analysis by GISH. Figure 2A and B shows a GISH-labelled root tip metaphase from a plant collected at Eling Marchwood. The approx. 120 chromosomes resolve into two groups, one comprising approx. 60 chromosomes that predominantly stained with the *S. alterniflora* probe (green) while the remaining approx. 60 chromosomes were labelled with the *S. maritima* probe (orange). The individual had a dodecaploid karyotype that could be designated for simplicity AAMM, assuming A and M represent triplicated ancestral genomes

from the hexaploid *S. alterniflora* and *S. maritima*, respectively. Frequently the short arm and/or centromere regions of the chromosomes of *S. alterniflora* origin were more heavily labelled, indicative of heterochromatic sequences (see arrowheads on prophase cell in Fig. 2C). In addition the GISH data reveal that recombination between parental sets of chromosomes is likely to be minimal (beyond the resolution of this experiment) or absent, as all chromosomes stained one colour along their whole length.

Figure 2D–F shows a GISH-labelled metaphase from another plant collected at Eling Marchwood. There were only 92 chromosomes, 30–32 fewer than expected. Of these, approx. 60 chromosomes preferentially hybridized with the *S. alterniflora* probe (green) while approx. 30 were labelled with the *S. maritima* probe (orange) (Fig. 2F). The individual was therefore considered to be a nonaploid (9x), with a genome designation of AAM.

Distribution of Spartina *ploidy levels*

The flow cytometric analysis of 56 individuals from Hythe and Marchwood revealed hexaploid, nonaploid and dodecaploid individuals. Overall, combined data from both sites showed that nonaploids were the rarest, occurring at a frequency of approx. 10·7 % (= 6 individuals), whereas dodecaploids and hexaploids occurred at higher frequencies of 17·8 % (= 10 individuals) and 71 % (= 40 individuals), respectively. However, the occurrence and distribution of the different ploidy levels and species varied markedly between the two sites.

At Hythe the vast majority of plants analysed (i.e. 32 of the 34 plants = 94 %) were hexaploid and all were identified morphologically as the homoploid hybrid *S. × townsendii* (Fig. 1B). The occurrence of the other hexaploid species *S. maritima* or *S. alterniflora* could be ruled out on morphological grounds. The remaining two individuals were dodecaploids corresponding to *S. anglica*.

In contrast, at Eling Marchwood three ploidy levels were observed in the 22 plants analysed (Fig. 1A). Eight individuals (36·4 %) identified as *S. alterniflora* were shown to be hexaploid while six plants (27·2 %) showing considerable morphological diversity ranging between *S. anglica* and *S. alterniflora* phenotypes were found to be nonaploid similar to the findings of Marchant (1967, 1968). The remaining eight plants (36·4 %), which were morphologically identified as *S. anglica*, were dodecaploid. Individuals of different ploidy levels were intermixed and in close association along the transect, although there was a tendency for nonaploids to be more abundant towards the south-east end of the transect (Fig. 1A).

Because there was some variability in the position of the peaks in the flow histograms between individuals at the same ploidy level, the aim was to determine the likelihood that the samples were taken from a population with a continuous distribution of genome sizes. A computer program was developed in Mathematica 5·0 (Wolfram Illinois) to simulate sampling from a continuous population of 1000 individuals with the same mean and standard deviation (mean = 10·709, s.d. = 3·247, n = 56) of the collective genome size estimates of all 56 plants analysed. After 1000 randomizations (each time taking 56 samples) only 22 of the resulting distributions had
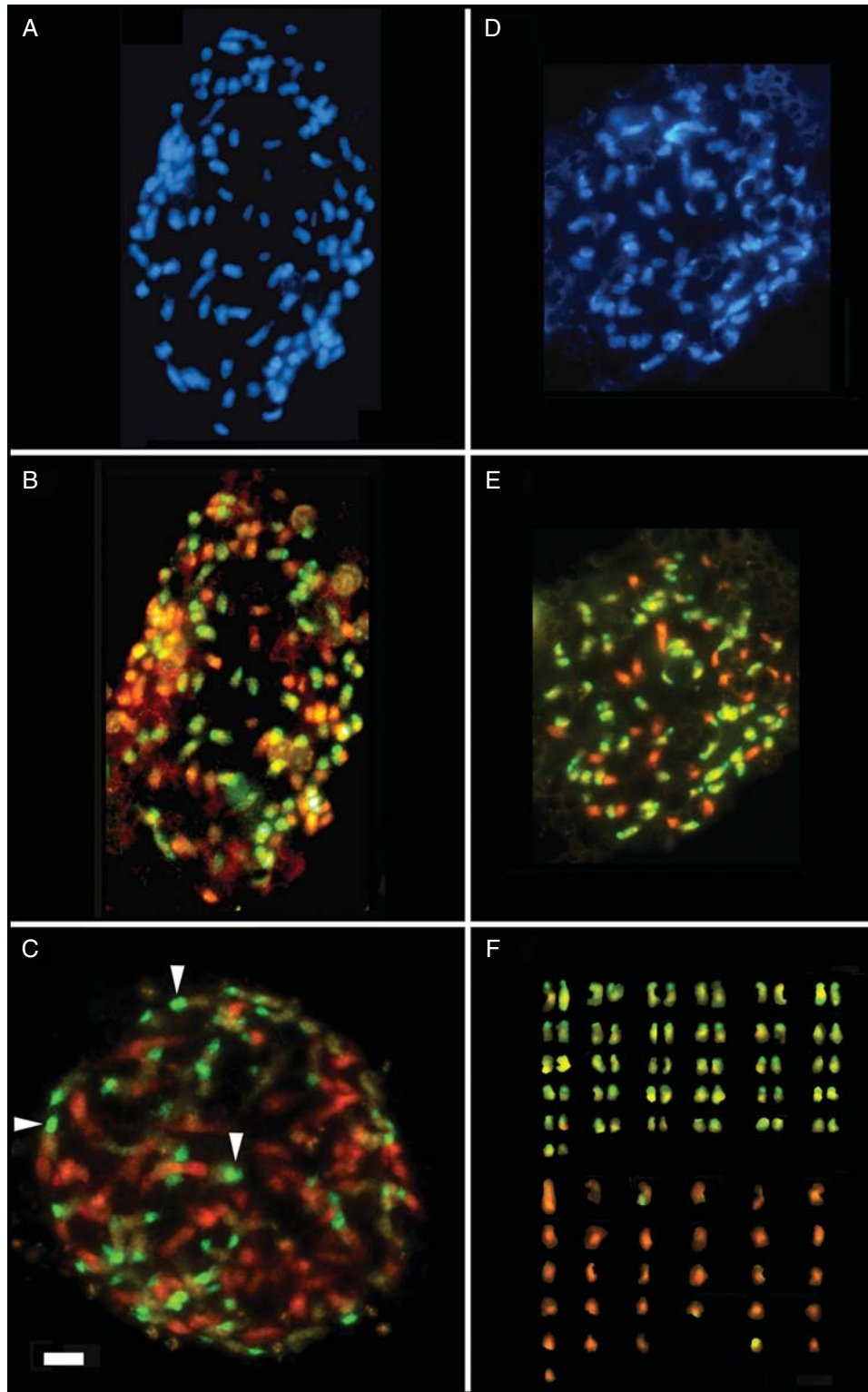
Fɪɢ. 2. Root tip metaphases (A, B, D–F) and prophase nucleus (C) after DAPI staining (blue fluorescence) (A, D) and GISH (B, C, E, F) using digoxigenin-labelled probes of *S. alterniflora* genomic DNA (green, detected using FITC-conjugated anti-digoxigenin IgG) and biotin-labelled probes of *S. maritima* genomic DNA (orange, detected using Cy3-conjugated streptavidin). Shown in (A–C) are cells from an AAMM dodecaploid individual with approx. 120 chromosomes, approx. 60 of which are of *S. maritima* origin and approx. 60 of *S. alterniflora* origin. Note, the intense signal in the short arm of chromosomes (arrowheads) of *S. maritima* origin in the prophase (C). This is indicative of heterochromatic sequences (also seen in E and F). The same metaphase of an AAM nonaploid individual with 92 chromosomes is shown in (D–F). The AAM karyotype (F) has chromosomes organized by size and parental genome. Due to their similar morphologies and staining patterns, chromosomes may not be in natural pairs. Scale bar = 5 μm.

discontinuities that were similar to the real data. The real data is therefore likely to be discontinuous and structured around integer ploidy levels ($P = 0.022$).

DISCUSSION

Spartina is one of the classic models of recent polyploidy-induced speciation. *Spartina anglica* is thought to have been formed like many natural polyploids via genome doubling of an interspecific hybrid, typically the route used to make synthetic polyploid crops. The current consensus is that polyploid formation frequently arises in nature via unreduced gamete formation. The mean frequency of unreduced gametes, based on studies across many eukaryote taxa, including angiosperms, is approx. 5 % (Ramsey and Schemske, 1998), although their frequency is affected by genetic and environmental factors. Recently, the first gene, *AtPS1*, directly associated with diploid gamete formation was characterized in *Arabidopsis thaliana*. Mutations in this gene were shown to cause misalignment of the spindle in meiosis II, resulting in astonishingly high levels of diploid gametes (up to 65 %) (d'Erfurth *et al.*, 2008).

In the current study, nonaploids were identified amongst the Eling Marchwood population, occurring at a frequency of 27.2 %. Using GISH the genomic composition of one nonaploid was shown to be AAM, comprised of two triplicated genomes ($6x = 60$) derived from *S. alterniflora* (AA) and one ($3x = 30$) from *S. maritima* (M; Fig. 2D–F). Marchant (1967, 1968) reported the occurrence of $2n =$ approx. 90 and $2n = 76$ plants at Hythe, although no such ploidy levels were encountered in the present survey at this site.

The nonaploid plants discovered at Eling Marchwood could have arisen in several possible ways. (*a*) A likely route of formation could involve a cross between regular (reduced) hexaploid gamete ($6x = $ AM) of the dodecaploid *S. anglica* (AAMM) and a regular gamete ($3x = $ A) of *S. alterniflora* (AA). This hypothesis is reinforced by the current distribution of plants, where the nonaploid plants are found with *S. alterniflora* and *S. anglica*. This was also hypothesized by Marchant (1968) to explain the occurrence of the $2n = 90$ plants found at Hythe. However, the nonaploid plants may have formed when distribution patterns were different and alternative hypotheses cannot be ruled out. (*b*) Given the relatively high frequency of unreduced gametes in many angiosperm species, nonaploids may have arisen via an unreduced gamete from *S. alterniflora* hybridizing with a normal gamete from *S. maritima*. This could have occurred if these species grew sympatrically, perhaps at the time when *S. × townsendii* itself formed (Marchant, 1967, 1968). (*c*) Another possibility involves an unreduced gamete from *S. × townsendii* crossing with *S. alterniflora*, which could have occurred at Hythe, followed by dispersal of the nonaploid hybrids to Eling Marchwood.

Currently, it is not possible to distinguish between these hypotheses because the nonaploids could have formed at any time from the introduction of *S. alterniflora* to the present day. Indeed, Marchant (1967, 1968) has documented a period of around 20 years when *S. alterniflora* and *S. maritima* co-existed at Hythe (the last record of *S. maritima* at this locality being in 1900) before the formation of the allopolyploid *S. anglica*,

leaving ample time for polyploidy event(s), as described above, to give rise to the nonaploids. It is possible that the nonaploids observed at Eling Marchwood are derived from those previously described by Marchant (1967, 1968). Certainly the distribution of plants can be influenced by plant mobility through the movement of plant fragments or seeds, which in *Spartina* can float (Huiskes *et al.*, 1995). Alternatively, the nonaploids recorded here may have been formed independently, as frequently observed in recurrently formed hybrids and allopolyploids (Soltis and Soltis, 2009).

Further studies are needed to determine whether other nonaploid cytotypes exist, such as AMM, produced by, for example, a cross between *S. anglica* and *S. maritima*. Morphologically intermediate plants are encountered in southern Brittany (France) where intermixed populations of *S. anglica* and *S. maritima* plants do occur, but the molecular analyses performed to date have failed to detect hybrids (G. Allard and M.-T. Misset, Rennes University, unpubl. res.). The possibility of backcrossing between *S. anglica* and its parental species revealed here represents a potentially significant source of genetic diversity in the otherwise genetically depauperate populations of *S. anglica.*

Backcrosses involving polyploids and their diploid progenitors are commonly reported in natural populations. In *Chamerion angustifolium*, triploids in mixed ploidy populations ($2x$, $3x$, $4x$) provide a means of tetraploid formation, via uniting unreduced triploid gametes ($3n$) with normal gametes (n). This route to polyploid formation may enable tetraploids to overcome any frequency-dependent mating disadvantages (Husband, 2004). Using computer modelling Husband and Schemske (2000) showed that the presence of so called triploid-bridges could facilitate the maintenance and fixation of tetraploid individuals at the expense of both triploids and diploids. Ayres *et al.* (2008) speculated that triploid bridges may have played a role in the evolution and recurrent formation of *S. anglica*. Thus, the *Spartina* nonaploids identified here might potentially act in such a way by providing a bridge to the formation of new ploidy levels, although no such higher ploidy levels, such as $18x$ individuals, have been recorded to date either in the literature or in this survey.

Spartina individuals at all three ploidy levels occurred in close proximity at Eling Marchwood, raising the possibility of introgressive hybridization between them. Hybrids involving *S. alterniflora* (introduced) and *S. foliosa* (native) have arisen in California (Ayres *et al.*, 1999) where they compete with the native plants. In addition, interspecific hybridization is reported in this area between the hexaploid *S. foliosa* and the introduced alloheptaploid *S. densiflora* (Ayres *et al.*, 2008). Furthermore *S. densiflora* originated in South America following hybridization between a hexaploid species (likely to be *S. alterniflora*) and a tetraploid species related to *S. arundinacea*, also from the southern hemisphere (Fortune *et al.*, 2008). Backcrossing of the F_1 *S. densiflora* × *S. foliosa* hybrids ($2n = 66$) has resulted in individuals with $2n = 94$–96, exhibiting either '*foliosa*' or '*alterniflora*' plastid sequence types (Ayres *et al.*, 2008). These studies, as well as data presented here, indicate the extensive reticulate evolution in the genus.

Transects at Eling and Hythe showed dodecaploids at a frequency of approx. 36 % and 6 %, respectively (Fig. 1A and B). This is in contrast to the estimated abundance of *S. anglica* by

Charman (1990), who claimed that the expansion of *S. anglica* had been concomitant with the reduction of the F_1 homoploid hybrid and parental species across the entire southern coast of Britain. It is clear from the data presented here that this broad generalization is likely to be incorrect. At Hythe, only two out of 34 individuals were *S. anglica* plants and, instead, the site was dominated by *S. × townsendii*, which comprised the remaining 32 individuals (94 %) of the sample. Furthermore, while *S. anglica* was more common at Eling Marchwood (36·4 %); it was not the dominant species as *S. alterniflora* also occurred with similar frequency (i.e. 36·4 %), with the remaining individuals being nonaploids (27·2 %). Perhaps *S. anglica* has experienced a decline in abundance in the years since Charman's study, as *S. anglica* 'die-back' resulting from age-related decline of vigour has been reported in some other sites in Britain (Gray, 2004). Such a process is not without precedence in newly formed allopolyploids as *Senecio eboracensis*, an allotetraploid that formed within the last approx. 40 years, is now virtually extinct even though it initially expanded its numbers greatly following its formation (Abbott and Lowe, 2004).

In *Spartina* all studies to date have failed to identify significant genetic changes following either hybridization or genome doubling (Ainouche *et al.*, 2004a). However, evidence of significant epigenetic reprogramming was recorded in *S. × townsendii* (Salmon *et al.*, 2005), that mostly affects the regions flanking transposable elements (Parisod *et al.*, 2009). *Spartina anglica* has inherited almost all these changes. Moreover, gene expression changes are exhibited following both hybridization and polyploidy (Chelaifa *et al.*, 2010). Perhaps epigenetic and regulatory changes triggered by interspecific hybridization, rather than those occurring subsequent to polyploidy, explain why *S. × townsendii* fares well at its site of origin in the face of competition from *S. anglica*. In addition it is likely that *S. × townsendii* has failed to spread so rapidly because of its infertility.

In conclusion, the results reveal that, in spite of fertility and invasive behaviour, the allopolyploid *S. anglica* has not outcompeted the sterile homoploid hybrid *S. × townsendii* at the hybridization site. This study also demonstrates the potential of GISH to characterize the genome composition of different ploidy levels in *Spartina*, including nonaploids. This in turn can be used to assess the possible recurrent formation of polyploids with varying ploidy levels within this group. Furthermore the present studies demonstrate that the history of hybridization and speciation in this complex is more complicated than previously thought with the potential for even more complexity than is already apparent.

ACKNOWLEDGEMENTS

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