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Uniformity of the nuclear and chloroplast genomes of *Spartina maritima* (Poaceae), a salt-marsh species in decline along the Western European Coast

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Spartina maritima is a salt-marsh species from European and African Atlantic coasts. In the northern range of the species (including north-west France), a rapid decline of the populations has been observed during the 20th century. In this paper, the molecular diversity of 10 populations of *S. maritima* from France has been investigated using nuclear and chloroplast DNA markers: inter-simple sequence polymorphism (ISSR), randomly amplified polymorphic DNA (RAPD), inter-retrotransposon amplified polymorphism (IRAP), and PCR-RFLP of a 5 kb long portion of chloroplast DNA. The results reveal an extremely low level of genetic variation in this species: only one nuclear marker (out of 98) was polymorphic, with the presence of two genotypes randomly distributed among the populations. The lack of genetic diversity is interpreted in light of the almost exclusive vegetative propagation of the species in its northern range, the colonization history of the populations, and the origin of *S. maritima* ($2n = 60$) in the hexaploid lineage of the genus and in the context of the management of *S. maritima* populations.
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Introduction

The salt-marsh grass, *Spartina maritima* Curtis (Poaceae) covers a wide and discontinuous range from South Africa to North Europe along the Atlantic seaboard (Mobberley, 1956). Until the 19th century it was the only *Spartina* species known on the Atlantic European coast (Marchant and Goodman, 1969). This native species has an important role as primary colonist of intertidal mud flats since it is able to trap and stabilize sediment efficiently, thus facilitating successional development (Castillo *et al*, 2000). In the beginning of the 19th century, S. *alterniflora* Loisel was introduced from the Northeast American coast by ship's ballast into Southampton water (Southern England) and the Bidassoa estuary (Southwest France). Hybridization with local *S. maritima* plants gave rise to sterile hybrids, named *Spartina* × *townsendii* and *Spartina* × *neyrautii*, in England and in France, respectively (Groves and Groves, 1881; Foucaud, 1897). Around 1890, a chromosome doubling in *S.* × *townsendii* resulted in a fertile allopolyploid species, *S. anglica*, in Southampton water (Hubbard, 1968; Gray *et al*, 1990). Both F1 hybrids are exclusively clonal species that remain localized in the site of hybridization, whereas *S. anglica* is a particularly vigorous and invasive species, which has spread both naturally and as a result of artificial plantations to become a particularly invasive species on different continents (Thompson, 1991; Guénégou and Levasseur, 1993; Gray and Raybould, 1997). Recent molecular investigations have revealed that this species displays a remarkable lack of interindividual genotypic variation (Baumel *et al*, 2001, 2002b; Ainouche *et al*, 2004a).

Spartina anglica was first recorded in France in 1906 and since then, it has rapidly spread all along the French coast. At the same time, the regression of *S. maritima* has been observed in British and northwest (Brittany) French salt marshes (Marchant, 1967; Raybould *et al*, 1991b; Guénégou and Levasseur, 1992). In Southampton water for example, all *S. maritima* plants have now disappeared (Raybould *et al*, 2000). Marchant (1967) and Des Abbayes *et al* (1971) suggested that the regression of *S. maritima* in northern Europe has been accelerated by *S. anglica* invasion that had supplanted *S. maritima* populations. However, it has also been noticed that the decay of *S. maritima* populations is also observed in sites where *S. anglica* is not present, and that the allopolyploid colonizes open bare flats rather than previously extant vegetation areas (Raybould *et al*, 1991b). Therefore, these two species may not really be competitors, and may differ in their strategy of dissemination (Baumel *et al*, 2001).

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North European populations of *S. maritima* lack vigor; they are characterized by slow lateral extension of individuals that rarely reproduce sexually, while southern European and African plants are bigger, more robust and produce viable seeds (Marchant, 1967). The variation in vigor may result from more stressful environmental conditions encountered by plants introduced from Africa in Northern areas of Europe (Chevalier, 1923; Marchant, 1967). Moreover, *S. maritima* is usually a dominant species of the lower marsh in numerous coastal marshes and estuaries. This habitat is particularly vulnerable to global warming. Actually, a consequence of the enhanced greenhouse effect is the rise of the sea level (Titus *et al*, 1991). An average sea-level rise is predicted to be about 20 cm by 2050 (IPCC, 2001), and Van Wijnen and Bakker (2001) predicted that marshes at lowest elevation would degenerate. Consequently, it can be expected that the sea-level rise will erode the outer boundary of the salt marshes, that is the lower marsh habitat of *Spartina* species. In England, Raybould *et al* (1991b) noticed in 1986 that the few remaining populations of *S. maritima* were frequently confined to eroded, high level marshes with dense vegetation. Therefore, the severe decline of the populations should be considered a sign of a threat in the northern range of the species in Europe. Several recent publications pinpoint the importance of genetic diversity ('neutral' and 'adaptive') for population level responses to environmental change (for a review, see Fraser and Bernatchez, 2001; Hedrick, 2001). Molecular markers provide appropriate tools for the definition of conservation units based on genetic diversity and allow for the evaluation of levels of genetic variability in relation to the vegetative extent of natural populations. For a plant reproducing mainly by vegetative growth, like *S. maritima*, what appears to be a 'large' population may be actually 'small' in terms of effective population size. For conservation purposes, it is therefore of interest to estimate the levels of genetic variation in populations of *S. maritima* growing on the northern limit of the species where they have been regressing for more than a century. To our knowledge, no molecular diversity analysis has been performed to date at the DNA level in *S. maritima* populations. A previous study based on allozyme markers revealed a lack of genetic variation in British populations (Raybould *et al*, 1991b). For these reasons, the objective of the present study is to evaluate the amount and distribution of genetic variation within and between populations of *S. maritima* along the French Atlantic coast where both healthy and regressing populations occur.

Materials and methods

Population sampling

A total of 10 *S. maritima* populations were sampled during spring 2001 from Saint-Briac (Côtes d'Armor, France) to Seudre estuary (Charente Maritime, France) on the Channel and Atlantic French coast, respectively (Table 1). From 3 to 20 individuals per population were collected, forming 125 samples for analysis. Vegetative characters were used in the field to distinguish the two species. Compared to *S. maritima*, the leaves of *S. anglica* are wider, straighter, and form a broad angle with the culm, whereas the leaves of *S. maritima* form an acute angle with the culm and are readily disarticulated from the sheath (Mobberley, 1956). In Saint Briac, Saint-Armel, and Seudre, *S. maritima* grows in sympatry with *S. anglica*. In Saint Armel particularly, patches consist of mixed clones of both species. The corresponding sampled populations were then used to check for possible gene flow between the two species (see data analysis).

Individual plants were collected in the field with roots, leaves, and rhizomes to allow both DNA extraction (from leaves) and transplantation into the greenhouse. As *Spartina* species are clonal plants, at each site, we tried to maximize the distance between samples to avoid collecting from the same individual (minimum distance: 50 m in large populations and 3 m in small populations composed of scattered clones).

Molecular methods

Three PCR-based fingerprinting techniques have been used: random amplified polymorphic DNA (RAPD, Williams *et al*, 1990); inter-simple sequence repeats (ISSR, Wolfe *et al*, 1998); and inter-retrotransposon amplified polymorphism (IRAP, Kalendar *et al*, 1999). In addition, the PCR-RFLP approach was developed to detect chloroplast diversity among *S. maritima* populations.

DNA extraction

DNA was prepared by the cetyltrimethylammonium bromide (CTAB) method (Ausubel *et al*, 1995) with RNaseA treatment. 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

Table 1 List of sampled sites

Sites	*No.*	*Location*	*Population feature*	*Number of Samples*
Saint Briac	1	Côtes d'Armor	Scattered patches in the Frémur estuary, in sympatry with *S. anglica*	9
Ile Chevalier	2	Finistère	Large and continuous meadow	20
Laïta	3	Finistère	Small population along the Laïta river	10
Plouharnel	4	Morbihan	Large and continuous meadow	10
Auray	5	Morbihan	Large and continuous meadow	20
Saint Armel	6	Morbihan	Large population with mixed patches with *S. anglica*	19
Pen Bron	7	Loire-Atlantique	Large population	10
Port du Pavé	8	Vendée	One isolated patch in a harbor zone	3
Noirmoutier	9	Vendée	Large and continuous meadow	15
Seudre	10	Charente-Maritime	Small population formed of scattered patches along the Seudre river, in sympatry with *S. anglica*	9

was incubated for 1 h at 65°C, and then 500 µl of chloroform was added. After mixing and microcentrofugation, 500 µl of isopropanol was added to the supernatant for precipitation. After microcentrofugation, the genomic DNA pellet was washed with 70% ethyl alcohol, and then it was resuspended in Tris-EDTA buffer.

RAPD procedure

Based on the use of a single short arbitrarily designed primer, RAPDs (Williams *et al*, 1990) are now commonly used for estimating genetic variation at the intraspecific level. Genomic DNA was amplified using five 10-mer random oligonucleotide primers (Operon Technologies): Op-C2, Op-C5, Op-C9, Op-C10, and Op-C20. These primers have been selected from among a set that released clear and reproducible electrophoresis patterns in a previous study on *Spartina* (Baumel *et al*, 2001).

Each PCR volume of 20 µl for RAPD contained 40 ng of genomic DNA, 1 × incubation *Taq* Buffer (Sigma), 1.1 mM of $MgCl_2$, 0.2 mM dNTP, 0.2 µM Operon primer, and 1.25 U of red *Taq* polymerase (Sigma). Amplification reactions were performed in a Techne thermal cycler programmed as follows: 1 min at 94°C followed by 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C. An additional cycle of 15 min at 72°C was used for final extension. Negative controls without DNA template were run with each experiment.

ISSR procedure

ISSRs represent sequences bracketed by microsatellite (SSRs) sites situated on two opposite DNA strands (Gupta *et al*, 1994). They are amplified by PCR using a single-primer designed from a di- or trinucleotide repeat with a random anchoring sequence of one to three nucleotides. ISSRs have been shown to be useful for molecular fingerprinting in natural populations (Wolfe *et al*, 1998; Esselman *et al*, 1999) or even to display somaclonal variation (Leroy *et al*, 2000). In this study, the following SSR primers were used: $(AGC)_6C$, $(TGC)_6G$, $(GTG)_7C$, $(CTC)_6G$, and $(CT)_9G$.

Amplifications for the ISSR procedure were conducted as following: Each PCR volume of 20 µl contains 1 × incubation *Taq* Buffer (Sigma), 1.1 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM each primer, 1 U of red *Taq* polymerase (Sigma), and 40 ng DNA template. Amplifications were performed in a PCR-express (Hybaid) thermal cycler using the following program: 2 min at 94°C followed by 35 cycles of 45 s at 94°C, 45 s at 52°C for $(CT)_9G$ or 58°C for the other primers, 2 min at 72°C, and terminated by 10 min of final extension at 72°C. Negative controls without DNA template were also run with each experiment.

IRAP procedure

The IRAP provides PCR-based markers depending on the position of given long terminal repeats (LTRs) of retrotransposons within the genome. The IRAP markers are generated by the proximity of two LTRs, using outward-facing primers annealing to LTR target sequences (Kalendar *et al*, 1999). The characteristics of retrotransposons (high mutation rate, dispersion over the whole genome, insertion and recombination) and the wide distribution of their activity in the plant kingdom make them particularly suitable for fingerprinting (Kalendar *et al*, 1999). This technique allows detecting the insertion polymorphism of retroelements in the genome. IRAP has been previously used in population genetics in the genus *Hordeum* (Kalendar *et al*, 1999) and later, successfully adapted to the genus *Spartina* (Baumel *et al*, 2002b). Interretrotransposon fragments were generated using the 'BAGGY' LTR primer (TCG TCG CCG GCG TCA TCT CC), kindly provided by R Kalendar and A Schulman (Plant genomics laboratory, University of Helsinki). PCR conditions for IRAP were similar to those of ISSR, except that a 59°C annealing temperature was used.

Electrophoresis procedures for ISSR, RAPD, and IRAP

After PCR amplification, products were stored at 4°C until electrophoresis. Amplified products were electrophoresed in 1.5% agarose gels in 0.5 × TBE buffer. Gels were stained in a 5% ethidium bromide solution for 30 min, then washed in water for 10 min and photographed under UV light.

Analysis of the rbcL-retA region by PCR-RFLP procedure

A chloroplast DNA region of 5 kb flanked by the *rbcL* and the *retA* genes was amplified. This region contains approximately 2500 bp of noncoding DNA. Sequences of the primers (*rbcL* ATG TCA CCA CAA ACA GAG ACT AAA GCA AGT; *retA*: CCG GCC GGT AGC TTC TCG AGG ATT TTC) were kindly provided by K Schierenbeck (California Sate University, Chico, CA, USA). RFLP was examined by using nine restriction enzymes (*Alu*I, *Bsh*1236I, *Eco*RV, *Hae*III, *Hin*6I, *Hinf*I, *Mbo*I, *Msp*I, *Rsa*I) on 10 individuals representing the 10 populations of *S. maritima* (Table 1).

Each PCR volume of 100 µl contained 40 ng of genomic DNA, 10 µl of incubation buffer (Sigma), 1.1 mM $MgCl_2$, 2 mM dNTP, 50 µM each primer, and 5 U of red *Taq* polymerase (Sigma). Reactions were placed in a Techne thermal cycler programmed as follows: 4 min at 94°C followed by 28 cycles of 45 s at 94°C, 1 min and 15 s at 52°C, and 9 min and 30 s at 65°C. Negative controls without DNA template were run with each experiment.

Restriction reactions (20 µl) contained 1 × incubation buffer, 10 µl of PCR product, and 5 U restriction enzyme. After 3 h at 37°C, restriction reactions were run on 2% agarose gels in TBE buffer and fragments were visualized following ethidium bromide staining.

Data analysis

Repeatability of the banding patterns and polymorphism were tested for each primer. Ambiguous and nonreproducible bands were not included in the analysis.

Molecular variation in *S. maritima* was analyzed by scoring polymorphic bands in the presence/absence of data matrix. Potential hybrid genotypes between *S. maritima* and *S. anglica* were looked for in mixed populations by comparing the obtained phenotypes to previously identified genotypes of *S. anglica* (Baumel *et al*, 2001, 2002a; A Baumel unpublished data).

Results

The five RAPD primers generated 50 repeatable markers in *S. maritima*. No polymorphism was encountered for any fragments among the 125 samples analyzed.

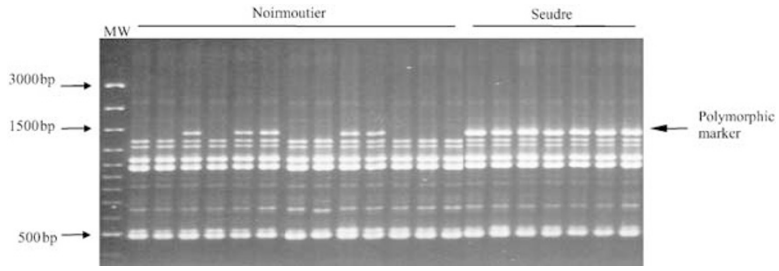

Figure 1 ISSR banding patterns obtained with $(TGC)_6G$ primer. Lane 1, DNA ladder, lanes 2–11, *S. maritima* population from Noirmoutier. The arrow on the right indicates the polymorphic band.

Table 2 Frequency of the two genotypes encountered in *S. maritima*. Genotype no. 1: absence of the 1450 bp $(TGC)_6G$ band. Genotype no. 2: presence of the 1450 bp $(TGC)_6G$ band

Sites	*N*	*Genotype no. 1*	*Genotype no. 2*
Saint Briac	9	9	0
Ile Chevalier	20	2	18
Laïta	10	10	0
Plouharnel	10	10	0
Auray	20	20	0
Saint Armel	19	13	6
Pen Bron	10	10	0
Noirmoutier	15	9	6
Port du Pavé	3	1	2
Seudre	9	0	9
Total	125	84 (67%)	41 (33%)

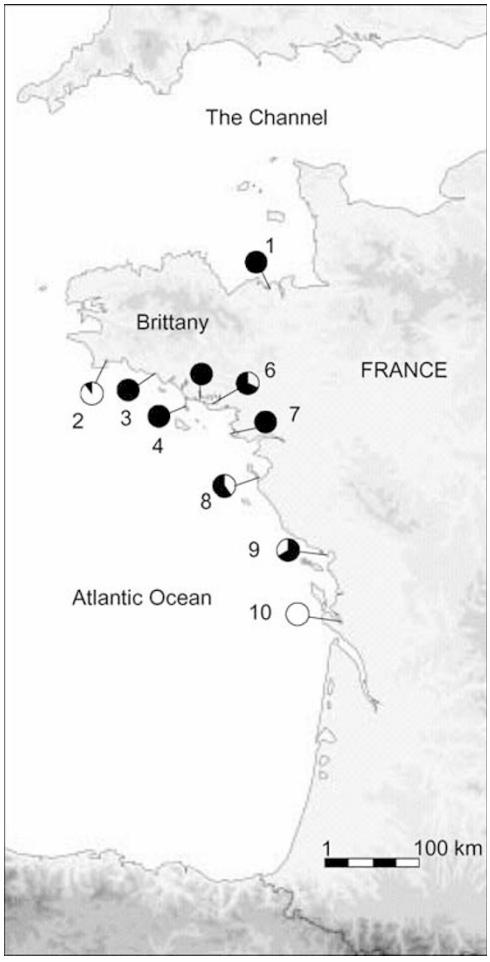

Figure 2 Geographic distribution of the two *S. maritima* genotypes encountered in the sampling area. ● Genotype no. 1: absence of the 1450 bp $(TGC)_6G$ band. ○ Genotype no. 2: presence of the 1450 bp $(TGC)_6G$ band.

Therefore, all *S. maritima* samples share the same RAPD pattern. Similarly, the BAGGY LTR primer used for IRAP generated six repeatable and monomorphic markers. RFLP analysis of the plastid *rbcL-retA* region yielded 68 invariant restriction sites, showing that all sampled individuals share the same cpDNA haplotype with respect to the restriction sites surveyed.

The only molecular variation observed was detected using ISSR analysis, and in this case, only one of 42 repeatable markers was polymorphic. The repeatability of this polymorphism generated by the $(TGC)_6G$ primer was confirmed by new DNA extractions and new PCRs. The presence or absence of this approximately 1450 bp band (Figure 1) differentiates two genotypes. Genotype no. 1 lacks the band and represents 67% of the samples (Table 2), whereas the genotype 2 (which has the band) represents 33% of the samples. Five of 10 populations are composed exclusively of genotype no. 1; four populations have both genotypes, and only one population is fixed for genotype 2. The two genotypes are present throughout the area sampled and their distribution is not geographically structured (Figure 2).

In summary, our analysis of nuclear and chloroplast DNA variation reveals only two clones on the French coast, differing only by the presence/absence of one (ISSR) nuclear DNA fragment that is present in 33% and absent in 67% of the 125 individuals analyzed.

Discussion

Our study reveals remarkable genetic uniformity in French populations of *S. maritima*. This uniformity was unexpected considering the physiognomy of the populations investigated, ranging from small populations with scattered plants to large populations forming extensive meadows. No chloroplast DNA diversity was detected and only two nuclear genotypes were encountered throughout the entire collecting area. These genotypes are very similar as they are differentiated by only one DNA fragment out of 98 amplified. Although based on different sample sets, similarly low levels of genetic diversity were also reported by Raybould *et al* (1991b) in English populations analyzed by isozyme markers. Their

results show that *S. maritima* in the UK is mostly represented by one ubiquitous genotype, with rare variants for two enzyme systems. Thus, the level of genetic diversity detected in *S. maritima* with PCR-based DNA markers does not differ from that determined with isozymes. This situation is in sharp contrast with studies on other species, where allozymes often underestimate genetic diversity in populations and species and where additional variation has been detected as soon as other markers were employed (eg Piquot *et al*, 1996; Esselman *et al*, 1999).

The absence of cpDNA variation and the presence of only two very similar nuclear genotypes in French *S. maritima* populations can be interpreted in light of the historical biogeography and evolution of this species in the genus *Spartina*. The European coasts represent the northern limit of the distribution area of *S. maritima*, which extends from the UK to the Cape of Good Hope in South Africa (Mobberley, 1956). How did *S. maritima* occupy a wide and discontinuous range from South Africa to North Europe? This raises the question of the origin of *S. maritima* within the hexaploid lineage of the *Spartina* species (Baumel *et al*, 2002a). This lineage is composed of the Afro-European Atlantic *S. maritima* ($2n = 60$), and the two sister species, the East American *S. alterniflora* ($2n = 62$) and the West American *S. foliosa* ($2n = 60$). These three species have nonoverlapping native distribution ranges but they maintain sufficient genetic relatedness to hybridize (Baumel *et al*, 2002a; Ainouche *et al*, 2004b). Since *S. maritima* is basal in this clade according to the phylogeny of genus *Spartina* (Baumel *et al*, 2002a), one can hypothesize that current populations of *S. maritima* actually represent a relic of an ancestral hexaploid species with a more widespread distribution. Another, less likely, possibility is that *S. maritima* was introduced from America to the eastern Atlantic coast and subsequently vanished from the New World.

On a shorter time scale, Chevalier (1923) suggested that the species is only native to South Africa and NW Africa from Mauritania to Morocco and from Gibraltar to the Loire estuary, and that it was introduced by shipping to more northern localities. Thus, a drastic founder effect is a possible explanation for the widespread genotypic uniformity. To examine this hypothesis, it would be interesting to evaluate the amount of genetic diversity throughout the distribution area of *S. maritima*. A preliminary analysis (G Yannic and A Baumel unpublished data) revealed that both genotypes (no. 1 and no. 2) are also observed in one sample from Portugal and Spain, respectively, and that genotype no. 1 is also present in Mauritania. These observations would suggest that both genotypes 1 and 2 were present in *S. maritima* prior to its progression along the French coast and were maintained through strict clonal spread. In the current state of our investigations, no other genotypes were scored in the southern range of the species, but the small sampling size in this area precludes any conclusion about the founder hypothesis.

A second related issue lies in the fact that *S. martima* rarely produces seeds in northern Europe. In French populations, we have observed normal flowering and fertile pollen but no seed production or seedling germination (Baumel, personal observation). Castellanos *et al* (1994) also reported the absence of germination in Spanish populations of *S. maritima*. The marginal situation of the populations analyzed here is relevant to the lack of sexual reproduction, a common feature of populations at the geographic margins of a species range (Eckert, 2002). Although seed production and sexual recruitment may often be limited by biotic and abiotic environmental factors in marginal populations, genetic factors, including change in ploidy or mutation, may also play a role in causing reduced sexual fertility (Eckert, 2002). For example, Eckert *et al* (1999) show evidence for loss of sexual capacity in highly clonal populations of *Decodon verticillatus*, providing strong support to the hypothesis that complex traits like sex are degraded by mutation when they no longer increase fitness. Then, the genetic uniformity in *S. maritima* observed here could be explained by a complex interaction between ancient founder effects and more recent loss of sexual capacity. Both issues will be addressed in further analysis of the species on a broader range.

Comparison of *S. maritima* to its most related species, *S. alterniflora* (Baumel *et al*, 2002a), reveals that the population genetics of these two species is very different (Ainouche *et al*, 2004b). The East-American *S. alterniflora* has been introduced to the Pacific coast of the US where it is now an invasive weed, most notably in the San Francisco Bay area (Daehler and Strong, 1997) and in Willapa Bay (Aberle 1993 *in* Daehler and Strong, 1997). In both sites, as well as on the eastern coast, the species shows a high level of nuclear and chloroplast diversity (Stiller and Denton 1995; Anttila *et al*, 2000; Perkins *et al*, 2002).

Despite the low level of genetic variation revealed in the present study, our results have interesting implications considering the origins of the recent hybrid and allopolyploid *Spartina* species. Chloroplast DNA markers revealed that *S. maritima* is the paternal parent of the sterile hybrid *S.* × *townsendii* and the allopolyploid *S. anglica* (Ferris *et al*, 1997; Baumel *et al*, 2001). Populations of *S. anglica* are mainly composed of one 'major' multilocus genotype, resulting from strict additivity of the nuclear genomes from *S. maritima* and *S. alterniflora* (Baumel *et al*, 2001). The unique *vs* multiple origin of the allopolyploid has been questioned (Raybould *et al*, 1991a, b; Baumel *et al*, 2001; Ainouche *et al*, 2004a). The results of the present study show that if multiple hybridization events occurred, they would have involved the same *S. maritima* genotype, identified here as the *S. maritima* 'genotype 1'. *S. maritima* and *S. alterniflora* have been involved in another hybridization event in Southern France (Foucaud, 1897), where the molecular characterization of the resulting sterile hybrid, *S.* × *neyrautii* (Baumel *et al*, 2003), indicates that *S. maritima* was the paternal genome donor (as for *S.* × *townsendii* and *S. anglica*, Ferris *et al*, 1997; Baumel *et al*, 2001). The genotype of *S. maritima* involved in the hybridization (in Spain) was 'genotype no. 2', as identified here.

As Raybould *et al* (1991b) argue, conservation of *S. maritima* may best be achieved by the protection of the larger populations. In the light of our results, it may be concluded that the French populations represent only one conservation unit. However, further surveys of phenotypic and quantitative genetic variation are needed for conservation purposes (Fraser and Bernatchez, 2001). Considering that *S. maritima* is exposed to marine erosion

and that it is characterized by very low variation, it may be predicted that this species will be very sensitive to future changes affecting the littoral environment. Therefore, numerous populations living in different ecological situations should be preserved all along the geographical range of the species. Finally, this study demonstrates that any French population could be chosen as planting stock for restoration of salt marshes in northern Europe.

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