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The chloroplast genome of the hexaploid *Spartina maritima* (Poaceae, Chloridoideae): Comparative analyses and molecular dating ☆

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ABSTRACT

The history of many plant lineages is complicated by reticulate evolution with cases of hybridization often followed by genome duplication (allopolyploidy). In such a context, the inference of phylogenetic relationships and biogeographic scenarios based on molecular data is easier using haploid markers like chloroplast genome sequences. Hybridization and polyploidization occurred recurrently in the genus *Spartina* (Poaceae, Chloridoideae), as illustrated by the recent formation of the invasive allododecaploid *S. anglica* during the 19th century in Europe. Until now, only a few plastid markers were available to explore the history of this genus and their low variability limited the resolution of species relationships. We sequenced the complete chloroplast genome (plastome) of *S. maritima*, the native European parent of *S. anglica*, and compared it to the plastomes of other Poaceae. Our analysis revealed the presence of fast-evolving regions of potential taxonomic, phylogeographic and phylogenetic utility at various levels within the Poaceae family. Using secondary calibrations, we show that the tetraploid and hexaploid lineages of *Spartina* diverged 6–10 my ago, and that the two parents of the invasive allopolyploid *S. anglica* separated 2–4 my ago via long distance dispersal of the ancestor of *S. maritima* over the Atlantic Ocean. Finally, we discuss the meaning of divergence times between chloroplast genomes in the context of reticulate evolution.

1. Introduction

The chloroplast (cp) genome, or plastome, of most land plants is composed of two inverted repeats (IR) that separate a large (LSC) and a small (SSC) single copy regions (Jansen and Ruhlman, 2012; Kolodner and Tewari, 1979). It has an average size of about 150 kb, ranging from 117 kb in *Erodium Carvifolium* (Blazier et al., 2011) to 218 kb in *Pelargonium* × *hortorum* (Chumley et al., 2006), with a gene content and order relatively well conserved in angiosperms (Jansen and Ruhlman, 2012), although extensive modifications may be encountered in some lineages such as the Campanulacae, Fabaceae or Geraniaceae families (Guisinger et al., 2010; Jansen et al., 2007; Martin et al., 2014). Chloroplast coding and non-coding regions have been employed to infer plant phylogenetic relationships at various taxonomical levels (Clegg and Zurawski, 1992; Jansen et al., 2007; Moore et al., 2010; Shaw et al., 2007, 2014). Their haploid state, uniparental inheritance and general absence of recombination (Jansen et al., 2007; Moore et al., 2010) make such sequences particularly useful for phylogenetic and phylogeographic studies in the contexts of reticulate evolution (*i.e.* hybridization) and polyploidy that characterize the history of most plant lineages (Fawcett and Van de Peer, 2010; McKinnon, 2004; Wendel and Doyle, 2005).

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This study focuses on genus *Spartina* in the Poaceae family. A worldwide phylogenetic analysis of the Poaceae based on molecular and morphological data obtained from more than 12,000 grasses was recently performed (Soreng et al., 2015) and clarified the subfamily classification. *Spartina* Schreb. represents a strongly supported monophyletic clade in the Chloridoideae subfamily (tribe Zoysieae), where it was found embedded in the paraphyletic genus *Sporobolus* (Peterson et al. 2010a, 2014b). This led to the proposal to conserve *Sporobolus* against *Spartina*, at the genus level (Peterson et al., 2014c). The *Spartina* clade (syn: *Sporobolus* sect. *Spartina* Peterson et al., 2014c) is comprised of 17 species colonizing mostly coastal saltmarshes (Mobberley, 1956; Clayton et al., 2006). Recurrent hybridization and polyploidization have particularly affected this lineage, with important evolutionary and ecological consequences (Ainouche et al., 2009; Strong and Ayres, 2013). The basic chromosome number in this group is $x = 10$ (Marchant, 1967) with ploidy levels ranging from tetraploid to dodecaploid species (Ainouche et al., 2012). *Spartina* evolved in two main lineages (Baumel et al., 2002): the first one containing American tetraploid species and the second one containing two American hexaploids (*S. alterniflora* Loisel. and its sister species *S. foliosa Trin.*) and the Old-World hexaploid species *S. maritima* Curtis. These two lineages are now recognized as *Sporobolus* subsections *Spartina* and *Alterniflori* respectively (Peterson et al., 2014b). Phylogenetic relationships in the hexaploid clade were well resolved using nuclear (ITS and Waxy) and chloroplast (*trnL-trnF*, *trnT-trnL*, *rpl32-trnL*, and *rps16-trnK* spacers, *trnL*, *ndhA* and *rps16*, introns) sequences (Baumel et al., 2002; Fortune et al., 2007; Peterson et al., 2014b), whereas relationships among the tetraploids remain to be resolved. Inter-specific crosses within and between these two main lineages produced various homoploid and allopolyploid (heptaploid, nonaploid and dodecaploid) taxa (Ainouche et al., 2012). In particular, hybridization between the hexaploid species *S. alterniflora* and *S. maritima* at the end of the 19th century resulted in the formation of a new vigorous and invasive allododecaploid species *S. anglica*, which has become a textbook example of recent allopolyploid speciation and a model for studying the evolution of polyploid genomes (Ainouche and Wendel, 2013). We focus here on its hexaploid ($2n = 6x = 60$) parent *Spartina maritima*, which is distributed along the Atlantic European and South-African coasts and was repeatedly involved in hybridization events. In Europe, *S. maritima* hybridized with the introduced American hexaploid *S. alterniflora*, which provided the maternal genome (and plastome) to the resulting homoploid hybrids *S. x neyrautii* in France and *S. x townsendii* in England. The latter hybrid has given rise to the above-mentioned allododecaploid *S. anglica* by genome doubling (Baumel et al., 2003; Ferris et al., 1997). In Spain, bidirectional hybridization occurred between *S. maritima* and the introduced heptaploid *S. densiflora* resulting in individuals that inherited alternatively the plastome of *S. maritima* or of *S. densiflora* (Castillo et al., 2010).

High ploidy levels and frequent hybridization events make phylogenetic analyses in *Spartina* particularly challenging using nuclear sequences, and reinforce the need to develop additional informative chloroplast markers to decipher the complex history of this genus. However no chloroplast genome of *Spartina* have been sequenced so far and despite that Chloridoideae contain ca. 1600 species in 31 genera and exhibit a worldwide distribution (Peterson et al., 2007; Soreng et al., 2015), only one Chloridoideae plastome has been sequenced: *Neyraudia reynaudiana* (Wysocki et al., 2014), which belongs to another tribe (Triraphideae, Soreng et al., 2015). This contrasts with other Poaceae subfamilies such as the Pooideae or Bambusoideae for which twelve and eight plastomes are available respectively (NCBI Organelle Genome Resources, www.ncbi.nlm.nih.gov/genomes/ accessed on 30 November 2014). The sequencing and analysis of the plastome of *Spartina* will thus expand the current understanding of chloroplast genome evolution in Poaceae. It will also provide new genetic markers to better resolve the phylogenetic relationships in Chloridoideae (Hilu and Alice, 2001; Peterson et al., 2014a; Soreng et al., 2015), and will allow estimating divergence times of Chloridoideae lineages, including *Spartina*.

In this study, we assembled the plastid genome of *Spartina maritima* and inferred its molecular evolution by comparing its structure and gene content to those of other published Poaceae plastid genomes. We identified variable coding and non-coding regions with potential phylogenetic and taxonomic utility in Chloridoideae, and used some of them to reassess phylogenetic relationships in *Spartina*. Finally, we dated the divergence between Chloridoideae and other grass lineages and between the different *Spartina* clades, providing the first estimate of the plastome divergence times in the polyploid *Spartina* species.

2. Material and methods

2.1. Plant material and DNA isolation

Samples from *Spartina maritima* were collected at the Etel river estuary (Morbihan, France), transplanted and maintained in controlled conditions in the greenhouse at the University of Rennes 1 (France). Total genomic DNA was isolated from fresh young leaves using the extraction kit Nucleospin Plant II (Macherey Nagel), following instructions provided by the manufacturer.

For comparative and molecular dating analyses (see below), genomic DNA was also extracted from other *Spartina* species including the hexaploid *S. alterniflora* (Landerneau, Finistère, France), the following tetraploid species: *S. bakeri* (Florida, USA), *S. arundinacea* (Amsterdam island), *S. patens* (New Jersey, USA), and representatives of related lineages from the Chlorioideae subfamily: *Sporobolus heterolepis* (Iowa, USA) and *Cynodon dactylon* (Ille et Vilaine, France).

2.2. High throughput sequencing, plastome assembly and annotation

Genomic DNA of *S. maritima* was subjected to one run of pyrosequencing using a GS-FLX 454 pyrosequencer (Life Sciences – Roche) at the Environmental and Functional Genomics platform (Biogenouest, Rennes). This run generated 993,229 reads (average length: 450 bp) after removal of low quality sequences. Reads corresponding to plastid DNA were extracted using a BLASTn (*E*-value: 10^{-6}) search against the plastome sequences of 23 Poales: *Anomochloa marantoidea* (Genbank accession: NC_014062), *Bambusa emeiensis* (NC_015830), *Bambusa oldhamii* (NC_012927), *Dendrocalamus latiflorus* (NC_013088), *Ferrocalamus rimosivaginus* (NC_015831), *Indocalamus longiauritus* (NC_015803), *Phyllostachys nigra* (NC_015826), *Phyllostachys edulis* (NC_015817), *Triticum aestivum* (NC_002762), *Festuca arundinacea* (NC_011713), *Lolium perenne* (NC_009950), *Coix lacryma-jobi* (NC_013273), *Saccharum officinarum* (NC_006084 and NC_005878), *Sorghum bicolor* (NC_008602), *Zea mays* (NC_001666), *Oryza nivara* (NC_005973), *Oryza sativa japonica* (NC_001320), *Oryza sativa indica* (NC_008155), *Brachypodium distachyon* (NC_011032), *Hordeum vulgare* (NC_008590), *Typha latifolia* (NC_013823) and *Phoenix dactylifera* (NC_013991). A total of 35,976 reads were recovered and assembled using Newbler (v. 2.6, Roche. Inc.). Thirty-two contigs ranging in size from 261 to 45,711 bp were obtained. Only the contigs covered by more than 50 reads were taken into account and organized using the plastome of *Saccharum officinarum* as a reference. All the genomic regions located at the junction between two contigs were verified by Sanger sequencing. The primers were designed using Primer 3 Plus (Rozen and Skaletsky, 2000) and are provided in supplementary Table S1. Additionally, a quarter of one