Discriminating the ancestral progenitors of hexaploid *Festuca arundinacea* using genomic *in situ* hybridization

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The phylogeny of *Festuca arundinacea* Schreb. ($2n = 6x = 42$) was determined using GISH. Total genomic DNA of putative ancestral species was labelled with rhodamine and hybridized to chromosome preparations of hybrids involving these species and *F. arundinacea*. The degree of hybridization to chromosomes known to be homologous to the probe DNA was compared with that found simultaneously on chromosomes of the genome of *F. arundinacea*. It was concluded that the tetraploid species *Festuca arundinacea* var. *glaucescens* contributed two genomes and the diploid species *Festuca pratensis* one, to create the allohexaploid species *F. arundinacea*.

Keywords: *Festuca arundinacea, Festuca glaucescens, Festuca pratensis*, GISH, phylogeny.

Introduction

Tall fescue (*Festuca arundinacea* Schreb.) is a hexaploid ($2n = 6x = 42$), bivalent forming species with a wide range of distribution from North Africa to northern Europe (Borrill *et al.*, 1976). The tetraploid species *F. arundinacea* var. *glaucescens* Boiss ($2n = 4x = 28$) (referred to here as *F. glaucescens*) and the diploid *F. pratensis* Huds. ($2n = 2x = 14$) were proposed as two of the progenitors of *F. arundinacea* by Chandrasekharan & Thomas (1971). They based their conclusions on the evidence of close morphological similarities between the F_1 hybrid *F. glaucescens* × *F. pratensis* and tall fescue, together with data on the meiotic behaviour of hybrids and derivatives. Several authors considered that *F. pratensis* was the source of the A genome of *F. glaucescens* (Malik & Thomas, 1967; Chandrasekharan & Thomas, 1971). Others agreed that *F. pratensis* was one genome in hexaploid *F. arundinacea* but that it and *F. glaucescens* were separate genomes and together led to the formation of the hexaploid species (Sleper & Nelson, 1990; Humphreys & Ghesquière, 1994; Xu & Sleper, 1994).

The precise nature of differentiation at the molecular level between chromosomes of species within the *Lolium/Festuca* complex can be revealed through the use of *in situ* hybridization techniques (Thomas *et al.*,

1994); the chromosomes of different species can be identified by using genomic DNA probes together with excess quantities of DNA of the other species as blocker. In this report we describe a series of *in situ* hybridization studies involving three hybrids between *F. pratensis*, *F. glaucescens* and *F. arundinacea* to confirm the phylogeny of the hexaploid species.

Materials and methods

The hybrids *F. pratensis* × *F. glaucescens* and *F. pratensis* × *F. arundinacea* were produced by pollinating synthetic autotetraploid cytotypes ($2n = 4x = 28$) of *F. pratensis* with the other two species. A hexaploid hybrid combining the two haploid genomes of *F. glaucescens*, one genome of *L. multiflorum* and the three genomes of *F. arundinacea* was produced by doubling the chromosome number of the F_1 hybrid *L. multiflorum* ($2x$) × *F. glaucescens* ($4x$) and pollinating the amphiploid with *F. arundinacea* ($6x$). The hybrids therefore had the following proposed genomic constitutions.

(1) *F. pratensis* (P) × *F. glaucescens* (G) ($4x$) – PPGG;

(2) *F. pratensis* (P) ×
    F. arundinacea (PG) ($5x$) – PPPGG;

(3) *L. multiflorum* (M) × *F. glaucescens* (G) ×
    F. arundinacea (PG) ($6x$) – MPGGGG.

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All hybrids required embryo rescue. Mitoses from root tip meristems were used as substrate for *in situ* hybridization with total genomic DNA probe (TGP) from *F. pratensis* and *F. glaucescens*.

The methods of root tip preparations, DNA extraction and labelling, and *in situ* hybridization were as described by Thomas *et al.* (1994). All of the TGPs were labelled with rhodamine-4-dUTP (Fluoro-red, Amersham) after first being sheared to 200 to 1000 base pairs by suspending a microfuge tube containing the DNA in an ultrasonic bath for 6 min. All preparations were counterstained with DAPI and mounted in Vectashield antifade. They were examined using a Leitz DM microscope. DAPI and rhodamine images were photographed and superimposed on Kodak Ektachrome 400 HC slide film.

Results

F. pratensis *(4x)* ×F. glaucescens *(4x)*

The tetraploid hybrid contains two homologous sets of *F. pratensis* chromosomes and the two genomes of *F. glaucescens*. The hybrid was probed with *F. pratensis* TGP: 14 chromosomes fluoresced and 14 were dull with minimal cross hybridization (Fig. 1a). This result shows that the *F. pratensis* genome is not present in *F. glaucescens*.

F. pratensis *(4x)* × F. arundinacea *(6x)*

On the assumption that there is close affinity between *F. pratensis* and one of the genomes of *F. arundinacea*, the *F. pratensis* TGP would be expected to hybridize to 21 chromosomes of the hybrid. As can be seen in Fig. 1b, 21 chromosomes do fluoresce confirming that *F. pratensis* is one of the progenitors of the hexaploid species since the third set of 7 chromosomes that fluoresce has to be one of the *F. arundinacea* genomes.

/L. multiflorum ×F. glaucescens/ *(6x)* ×F. arundinacea *(6x)*

This hybrid contains the three genomes of *F. arundinacea*, the two genomes of *F. glaucescens* and one genome of *L. multiflorum*. When the *F. glaucescens* TGP was used with *L. multiflorum* and *F. pratensis* DNA as blocker, 28 chromosomes fluoresced red and 14 chromosomes remained dull. *F. glaucescens* DNA has therefore hybridized to the *F. glaucescens* genomes and to two of those from *F. arundinacea*. This is evidence that two of the *F. arundinacea* genomes are homologous to those of *F. glaucescens*.
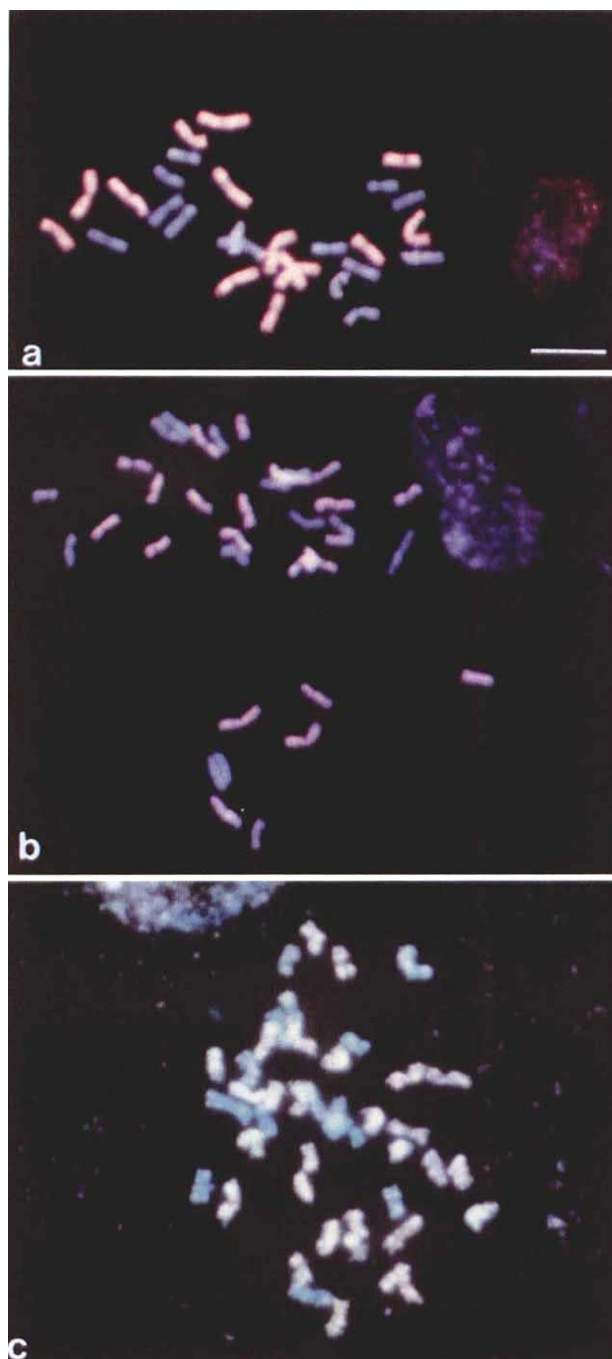

Fig. 1 Genomic *in situ* hybridization of root tip chromosomes of (a) *Festuca pratensis* (4x) × *F. glaucescens* (4x), (b) *F. pratensis* (4x) × *F. arundinacea* (6x), (c) (*Lolium multiflorum* × *F. glaucescens*) (6x) × *F. arundinacea* (6x). Bar = 10μm.

Discussion

The evidence obtained in this study of phylogenetic relationships based on *in situ* hybridization confirms previous conclusions that the hexaploid species

evolved from a hybrid involving *F. glaucescens* and *F. pratensis* (Chandrasekharan & Thomas, 1971; Sleper & Nelson, 1990). Total genomic probes can be used to distinguish between chromosomes of different species within the *Lolium/Festuca* complex, relying on species-specific repetitive DNA sequences. Perez-Vicente *et al.* (1992) isolated species-specific DNA sequences from *L. multiflorum* and *F. arundinacea*. Some similarities were found between *F. arundinacea* and *F. pratensis* but these similarities did not extend to *L. multiflorum*. The precise comparison based on the structure of DNA therefore rules out the involvement of the rye-grass genome in the evolution of tall fescue.

The results of the *in situ* hybridization confirm the conclusions of the earlier reports that *F. arundinacea* is derived from a hybrid between the diploid species *F. pratensis* and the tetraploid *F. glaucescens*. Any uncertainty whether *F. pratensis* had contributed a genome to *F. glaucescens* was resolved; the hybridization of *F. pratensis* TGP only to the *F. pratensis* and not the *F. glaucescens* chromosomes in the tetraploid hybrid between the two species was unequivocal proof that *F. pratensis* was not a progenitor of *F. glaucescens*.

A large proportion of the genome of higher plants consists of repetitive DNA and the mechanism by which this DNA has accumulated, i.e. by cycles of mutations and amplifications, seems to have ensured that much of this DNA is species-specific (Flavell *et al.* 1980). Genomic *in situ* hybridization relies on the specificity of the repetitive DNA and appears to be very effective in making comparisons between the related genomes of the *Lolium/Festuca* complex. The substrate for the GISH in each case was a mitotic preparation of a F_1 hybrid that involved the species used as probe. This provided an internal control because a known number of chromosomes were fully homologous with the probe. It enabled a direct comparison to be made between the degree of hybridization to the chromosomes of the present day putative progenitors and those of the hexaploid. In some cells there appeared to be some differentiation, that is, the signal was brighter on some chromosomes than others. This may indicate changes to the DNA of *F. arundinacea* subsequent to the formation of the initial hybrid, or that the accessions of *F. pratensis* and *F. glaucescens* used were not fully homologous with the genotype of the progenitors.

Cytogenetic analysis based on predictive mathematical formulae to quantify relationships between genomes of different species had indicated that the rye-grass genome was more closely related to one of the tall fescue genomes than the other two (Kleijer, 1984). This was also demonstrated by Humphreys & Ghesquière (1994) who set up pentaploid hybrids of *L. multi-*florum (4x) × *F. arundinacea* in which different alleles at the *PGI/2* locus marked the two homologous chromosomes of ryegrass and the three homologous tall fescue chromosomes. They were able to show that one of the fescue alleles recombined at a greater frequency than the other two in backcrosses to a diploid *L. multiflorum*. In view of the data presented in this paper it would be reasonable to assume that this would be the allele marking the genome derived from *F. pratensis*.

The distribution of fescue species has been recorded by Borrill *et al.* (1976) who found that the tetraploid species *F. glaucescens* is confined mainly to the southern European mountains and probably extends to central and eastern Europe. The diploid species *F. pratensis* is distributed throughout the climatic regions of oceanic north-west Europe and the transitional oceanic/continental zone of central Europe. *F. arundinacea* is as ubiquitous as the diploid *F. pratensis* except that it is less common in the colder winter conditions of northern Europe and the drier regions of northern Italy. *F. pratensis* is also more prevalent at higher altitudes than *F. arundinacea*. The comparison of the distribution of the three fescue species is interesting, because the tetraploid species is restricted and probably adapted to the conditions in those specific regions, but with the acquisition of the *F. pratensis* genome it became more adaptable as the hexaploid species and was able to spread throughout most of the regions occupied by *F. pratensis*. This is very similar to the effect of the D genome in the evolution of bread wheat (Zohary & Feldman, 1962). The distribution of the relevant species would also indicate that central Europe is the probable region where *F. arundinacea* evolved as a product of the natural hybridization of the 2x and 4x species; this conclusion would be in accord with the designation of this region as the centre of diversity of temperate grass species (Hartley & Williams, 1956; Borrill, 1972).

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