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Cytogenetic relationships within the Maghrebian clade of *Festuca* subgen. *Schedonorus* (Poaceae), using flow cytometry and FISH

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Abstract

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Festuca subgen. *Schedonorus* is a group of broad-leaved fescues, which can be divided into two clades: European and Maghrebian. We employed fluorescent *in situ* hybridization —FISH— with probes specific for 5S and 35S ribosomal DNA and genome size estimation using flow cytometry to shed light on the determination of possible parental genomes of polyploid species of the Maghrebian clade. Our results indicate that octoploid *F. arundinacea* subsp. *atlantigena* probably originated from crossing of the tetraploids *F. arundinacea* subsp. *fenas* —$2n = 4x = 28$— and *F. mairei* —$2n = 4x = 28$— followed by whole genome duplication. However, a large reconstruction of karyotype and genome downsizing has been revealed. Similarly, hexaploid *F. arundinacea* subsp. *corsica* presumably resulted from the interspecific hybridization of the diploid *F. pratensis* and tetraploid *F. arundinacea* subsp. *fenas.* Several scenarios on the origin of decaploid *F. arundinacea* var. *letourneuxiana* are discussed. This study contributed to our knowledge on the phylogeny of broad-leaved fescues and provided new information on the karyotypes —chromosome numbers, ploidy levels and numbers and positions of rDNA loci— using FISH and genome size estimations using flow cytometry in selected taxa of this important grass genus.

Keywords: Broad-leaved fescues, *Festuca* subgen. *Schedonorus,* FISH, genome size, rDNA.

Resumen

Ezquerro-López, D., Kopecký, D. & Inda, L.Á. 2017. Relaciones citogenéticas en el clado magrebí de *Festuca* subgen. *Schedonorus* (Poaceae), mediante la utilización de citometría de flujo y FISH. *Anales Jard. Bot. Madrid* 74(1): e052.

Festuca subgen. *Schedonorus* es un grupo de festucas de hojas anchas que se divide tradicionalmente en dos clados, uno europeo y otro magrebí. Mediante hibridación *in situ* fluorescente —FISH— con sondas específicas para las regiones ribosómicas 5S y 35S en su cariotipo y estimaciones de tamaño genómico mediante citometría de flujo se intentó determinar los posibles genomas parentales de las especies poliploides del clado magrebí. Nuestros datos indican que la especie octoploide *F. arundinacea* subsp. *atlantigena* probablemente se originó a partir del cruce de los tetraploides *F. arundinacea* subsp. *fenas* —$2n = 4x = 28$— y *F. mairei* —$2n = 4x = 28$— seguido de la duplicación del genoma. Sin embargo, también se ha detectado una reconstrucción del cariotipo y una reducción del tamaño genómico. De forma similar la especie hexaploide *F. arundinacea* subsp. *corsica* parece ser un híbrido entre el diploide *F. pratensis* y el tetraploide *F. arundinacea* subsp. *fenas.* Se discuten diversas posibilidades sobre el origen del decaploide *F. arundinacea* var. *letournexiana.* Este trabajo contribuye a conocer mejor la filogenia de las festucas de hoja ancha y proporciona nueva información sobre los cariotipos de estas especies —números cromosomáticos, niveles de ploidía y número y posición de loci de rDNA— mediante FISH y la estima de sus tamaños genómicos mediante citometría de flujo.

Palabras clave: *Festuca* subgen. *Schedonorus,* festucas de hoja ancha, FISH, tamaño genómico, rADN.

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INTRODUCTION

Subtribe Loliinae Dumort. belongs to the broad subfamily of grasses Pooideae Benth. —Poaceae Barnhart— and is formed by the genus *Festuca* L. and 10 closely related genera (Catalán, 2006; Inda & al., 2008). This large group of temperate grasses is found over most of the world —except the Antarctic region—, with Eurasia being the primary center of diversification. *Festuca* was morphologically divided into 2 main groups characterized according to the width of the leaves: the so-called "broad-leaved" and "fine-leaved" fescues (Hackel, 1882). This separation has been supported by recent molecular phylogenetic analyses (Torrecilla & Catalán, 2002; Catalán & al., 2004; Inda & al., 2008).

The *"Schedonorus -Lolium* complex" is one of the main groups within the broad-leaved fescues. This complex comprises representatives of *F.* subgen. *Schedonorus* (P. Beauv) Petern., *Lolium* L., and *Micropyropsis* Romero Zarco & Cabezudo. The complex can be divided into two clades, the "European clade" and the "Maghrebian clade", based on the geographical location of most of their respective representatives. The divergence of these two clades has been supported by molecular studies (Inda & al., 2014).

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The area of origination of the Maghrebian clade was inferred to be the western Mediterranean region, where successive divergence occurred during the early Pliocene and Pleistocene. This results in the dispersal of the clade mostly in NW Africa and SW Europe (Inda & al., 2014). The speciation of the members of this clade was presumably associated with interspecific hybridization and several steps of polyploidization (Borrill & al., 1977; Inda & al., 2014).

The Maghrebian clade involves only polyploid species, such as *F. arundinacea* subsp. *fenas* (Lag.) Bornm. —$2n = 4x = 28$—, *F. mairei* St.-Yves —$2n = 4x = 28$—, *F. arundinacea* subsp. *corsica* (Hack.) Kerguélen —$2n = 6x = 42$—, *F. arundinacea* subsp. *atlantigena* (St.-Yves) Auquier —$2n = 8x = 56$—, and *F. arundinacea* var. *letourneuxiana* (St.-Yves) Torrec. & Catalán —$2n = 10x = 70$—. On the other hand, the European clade is represented by both diploid —*F. pratensis* Huds., *F. fontqueri* St.-Yves, and *Micropyropsis tuberosa* Romero Zarco & Cabezudo— and polyploid species —tetraploid *F. pratensis* subsp. *apennina* De Not. and hexaploids *F. arundinacea* Schreb. subsp. *arundinacea* and *F. gigantea* (L) Vill.— (Borrill & al., 1977; Catalán & al., 2004; Hand & al., 2010; Inda & al., 2008, 2014).

The advent of molecular cytogenetics enabled to employ techniques such as fluorescent *in situ* hybridization —FISH— and genomic *in situ* hybridization —GISH— for phylogenetic studies. Humphreys & al. (1995) used GISH to indicate the origin of hexaploid *F. arundinacea* by interspecific hybridization of diploid *F. pratensis* and tetraploid *F. arundinacea* var. *glaucescens* Boiss. *(F. arundinacea* susbp. *fenas)*. This hybridization was followed by whole genome duplication or alternatively, merge of unreduced gametes took place. Similarly, GISH results confirmed that diploid *F. pratensis* is one of the progenitors of tetraploid *F. pratensis* subsp. *apennina* (Kopecký & al., unpubl.). This method has been also successfully used for the genome composition analysis of various interspecific hybrids including ×*Festulolium* Asch. & Graebn. (Thomas & al., 1994; Kopecký & al., 2005). On the other hand, it was not possible to distinguish parental genomes in hybrids of *Lolium perenne* L. and *Lolium multiflorum* Lam. (Kopecký & al., 2008a), as well as progenitors of polyploid fine-leaved fescues from the Iberian Peninsula including *F. ampla* Hack. subsp. *ampla, F. brigantina* (Markgr.-Dann.) Markgr.-Dann., *F. summilusitana* Franco & Rocha Afonso, and *F. duriotagana* Franco & Rocha Afonso (Loureiro & al., 1997). Similarly, FISH with probes for specific DNA sequences can be employed for evolutionary studies. The most frequently used are probes specific for 5S and 35S rDNAs. The number and position of rDNA loci have been applied in many cytomolecular evolutionary analyses including the *Schedonorus-Lolium* complex (Thomas & al., 1996, 1997; Harper & al., 2004; Lideikyte, 2008; Inda & Wolny, 2013). Besides *in situ* hybridization techniques and many genetic markers, the estimations of genome size have also been used supportive tool to indicate possible progenitors of allopolyploids and identify interspecific hybrids, where genome size of parents differ for at least 15%, such as hybrids of *Lolium multiflorum* and *F. pratensis* (Huska & al., 2016; Kopecký & al., unpubl.). The advantage of flow cytometry is the relative low-cost and high-throughput for screening thousands of such potential hybrids and allopolyploids.

Several species of *F.* subgen. *Schedonorus,* including tall fescue *(F. arundinacea)* and meadow fescue *(F. pratensis),* are widely used for forage and turf. Moreover, the ability of interspecific hybridization within *Schedonorus-Lolium* complex is recently used in grass breeding and several interspecific and intergeneric hybrids including hybrid ryegrass and ×*Festulolium* —hybrids of *Festuca* and *Lolium*— have been released and became popular among farmers (Kopecký & al., 2005, 2008a). Climatic changes call for implementation of new alleles into existing crop cultivars and thus, we can predict increasing interest in the employment of other wild species in breeding. Despite the importance of this complex, only little is known on the origination and the genomic composition of *F.* subgen. *Schedonorus* species, especially those of the Maghrebian clade. Such knowledge would be important for the targeted selection for interspecific crosses.

The aim of our study was to determine the potential progenitor genomes of polyploid fescue species of the Maghrebian clade using fluorescent *in situ* hybridization with probes specific for ribosomal DNAs —5S and 35S— and the estimation of genome size using flow cytometry and with respect to the phylogenetic framework of this group obtained in our previous studies.

MATERIAL AND METHODS

Plant material

We focused on various fescues from the Maghrebian clade. The seeds of these species were obtained from the germplasm banks Western Regional Plant Introduction Station —WRPIS—, the Agriculture Service of the United States —USDA, USA—, and Aberystwyth Genetic Resources —ABY, Aberystwyth, United Kingdom—. The species with their accessions are (Table 1): *F. mairei:* WRPIS PI-610941 —Morocco— and WRPIS PI-283312 —Morocco, cultivated in Sweden—; *F. arundinacea* subsp. *fenas:* WRPIS PI-289654 —Spain— and ABY-BN 354 —France, Rouen—; *F. arundinacea* subsp. *corsica:* ABY-BN 1175 —cultivated in Belgium, Liege—; *F. arundinacea* subsp. *atlantigena:* ABY-BN 865 and ABY-BN 807 —Morocco—; *F. arundinacea* var. *letourneuxiana:* ABY-BN 275 —Morocco— and ABY-BN 400 —Morocco.

Chromosome preparations

Caryopses samples of the cultivars were germinated in Petri dishes on wet filter paper and seedlings were planted in pots in the greenhouse. Mitotic metaphase spreads have been prepared from root tips according to Jenkins & Hasterok (2007) and Valladolid & al. (2004). The caryopses without paleas and lemmas were germinated on filter paper moistened with water for 4 days at 20-22 °C in darkness. Once geminated, the seeds were immersed in ice cold water for 24 h. They were then fixed in a mixture of methanol and glacial acetic acid 3:1 —v/v— for 4 h at room temperature and stored at -20 °C until use. The root tips were cut —1 cm— and washed several times with 0.01 M citrate

Table 1. Summary of the number of chromosomes (2*n*), holoploid and monoploid genome size, and the numbers of 5S and 35S rDNA loci in various species of *Festuca* subgen. *Schedonorus* [a, Kopecký & al. (2010); b, Thomas & al. (1997), Lideikyte & al. (2008), and Ksiazczyk & al. (2010)].

	Chromosome number and ploidy level	1C (pg)	1Cx (pg)	No. of 5S rDNA loci	No. of 35S rDNA loci
F. pratensis	2*n* = 2*x* = 14	3.25 ± 0.04[a]	3.25	2[b]	2[b]
F. mairei (Morocco WRPIS PI-610941 & WRPIS PI-283312)	2*n* = 4*x* = 28	5.02 ± 0.08	2.51	4	6
F. arundinacea subsp. *fenas* (Spain WRPIS PI-289654 and France ABY-BN 354)	2*n* = 4*x* = 28	5.24 ± 0.07	2.62	4	6
F. arundinacea subsp. *corsica* (ABY-BN1175)	2*n* = 6*x* = 42	8.31 ± 0.09	2.77	6	6
F. arundinacea	2*n* = 6*x* = 42	8.73 ± 0.04[a]	2.91	6[b]	4[b]
F. arundinacea subsp. *atlantigena* (unknown origin ABY-BN 865 and Morocco ABY-BN 807)	2*n* = 8*x* = 56	8.11 ± 0.15	2.02	12	10
F. arundinacea var. *letourneuxiana* (Morocco ABY-BN 275 & ABY-BN 400)	2*n* = 10*x* = 70	9.85 ± 0.04	1.97	16	12

buffer —pH 4.8—. The cell wall was digested by incubating the samples in an enzyme mixture —4% pectinase from *Aspergillus niger,* 1% cellulase (Onozuka R-10), and 1% cellulase from *Trichoderma viride* in 10 mM citrate buffer pH 4.8— for 2 h at 37 °C. Thereafter, the squashing of root meristem tissue has been done in a drop of 45% acetic acid. The slides were kept on dry ice for at least 30 min. The coverslips were then removed and the mixture of absolute ethanol and glacial acetic acid —3:1 by volume— was poured over the slides followed by the immersion of the slides in absolute ethanol for 30 min.

Fluorescent in situ *hybridization (FISH) with rDNA probes*

FISH has been done according to Jenkins and Hasterok (2007) with minor modifications. DNA clone pTa794 containing 5S rDNA from *Triticum aestivum* L. was labelled by Nick translation with digoxigenin-dUTP —Roche— and the clone containing 35S rDNA region from *Arabidopsis thaliana* L. was labelled by Nick translation with tetramethyl-rhodamine-dUTP —Roche.

The slides were pretreated with RNase A —100 mg RNAse A, Sigma R-5503/ml of 10 mM Tris-HCl, and 15 mM NaCl— added to each slide for 75 min/37 °C in humid chamber followed by the fixation of the slides with 1% formaldehyde in PBS —Sodium Phosphate Buffered Saline pH 7— for 10 min/RT. The hybridization mixture involves 20 µl 100% deionized formamide, 8 µl 50% dextran sulfate —w/v—, 4 µl 20x SSC, 2 µl 10% SDS —w/v—, 1 µl salmon sperm DNA, 2.5 µl 5S rDNA probe, and 2.5 µl 35S rDNA probe. Hybridization mixture was denatured at 75 °C for 10 min. Thereafter, the mixture has been placed on slide with chromosome squashes and covered with coverslip. The slides were denatured at 72 °C for 4:30 min and were kept in humid chamber for hybridization —16 h/37 °C—. After stringency washing, the sites of hybridization have been detected by the Anti-DIG-FITC conjugate —Roche—. Chromosomes were counterstained with 0.2 mg/ml 4',6-diamidino-2-phenylindole —DAPI— in the Vectashield antifade solution —Vector Laboratories—. Slides were evaluated with Motic BA410 fluorescent microscope. Micrografx Picture Publisher and Adobe Photoshop software were used for processing color pictures.

Holoploid genome size estimation using flow cytometry

The leaves of adult plants growing in pots were used for flow cytometry analyses of genome size estimation. Nuclear suspensions were prepared from 200 mg leaves of *Festuca* and 200 mg leaves of internal standard. We used either *Pisum sativum* L. 'Ctirad' —1C = 4.55 pg of DNA— or *Secale cereale* L. 'Dankovske' —1C = 8.09 pg DNA— as standards (Dolezel & al., 1998). Leaves in 500 µl Otto I reagent (Otto, 1992) were chopped by razor blade on a Petri dish. The suspension was filtered using 50 µm pore nylon filters and 1000 µl of Otto II reagent —with propidium iodide— was added. Samples were analyzed using a CyFlow Space SYSMEX. At least 5,000 nuclei were analyzed per sample. Each sample was analyzed three times during different days. Only measurements with coefficient of variation —CV— below 3.5% have been considered in our study. 5 plants from each population were analyzed.

RESULTS AND DISCUSSION

Cytogenetic observations using FISH with rDNA probes

Chromosome number has been determined for all the genotypes (Table 1). We confirmed tetraploidy in *F. mairei* and *F. arundinacea* subsp. *fenas* —2*n* = 4*x* = 28—, octoploidy in *F. arundinacea* subsp. *atlantigena* —2*n* = 8*x* = 56—, and decaploidy in *F. arundinacea* var. *letourneuxiana* —2*n* = 10*x* = 70—. We detected hexaploidy —2*n* = 6*x* = 42— in the accession ABY-BN 1175 of *F. arundinacea* subsp. *corsica.*

FISH resulted in the detection of 4 5S rDNA loci located proximally and 6 35S rDNA loci present in telomeric or subtelomeric chromosome regions in both populations of *Festuca arundinacea* subsp. *fenas* —2*n* = 4*x* = 28—. Not a single chromosome carried both 5S and 35S rDNA loci (Fig. 1a). 4 5S rDNA and 6 35S rDNA loci have been detected in a Moroccan population of *F. mairei* —2*n* = 4*x* = 28—, all being present on different chromosomes (Fig. 1b). The other population, PI-283312, showed slightly different results. We detected the same number of 5S and 35S rDNA loci, but one chromosome carried

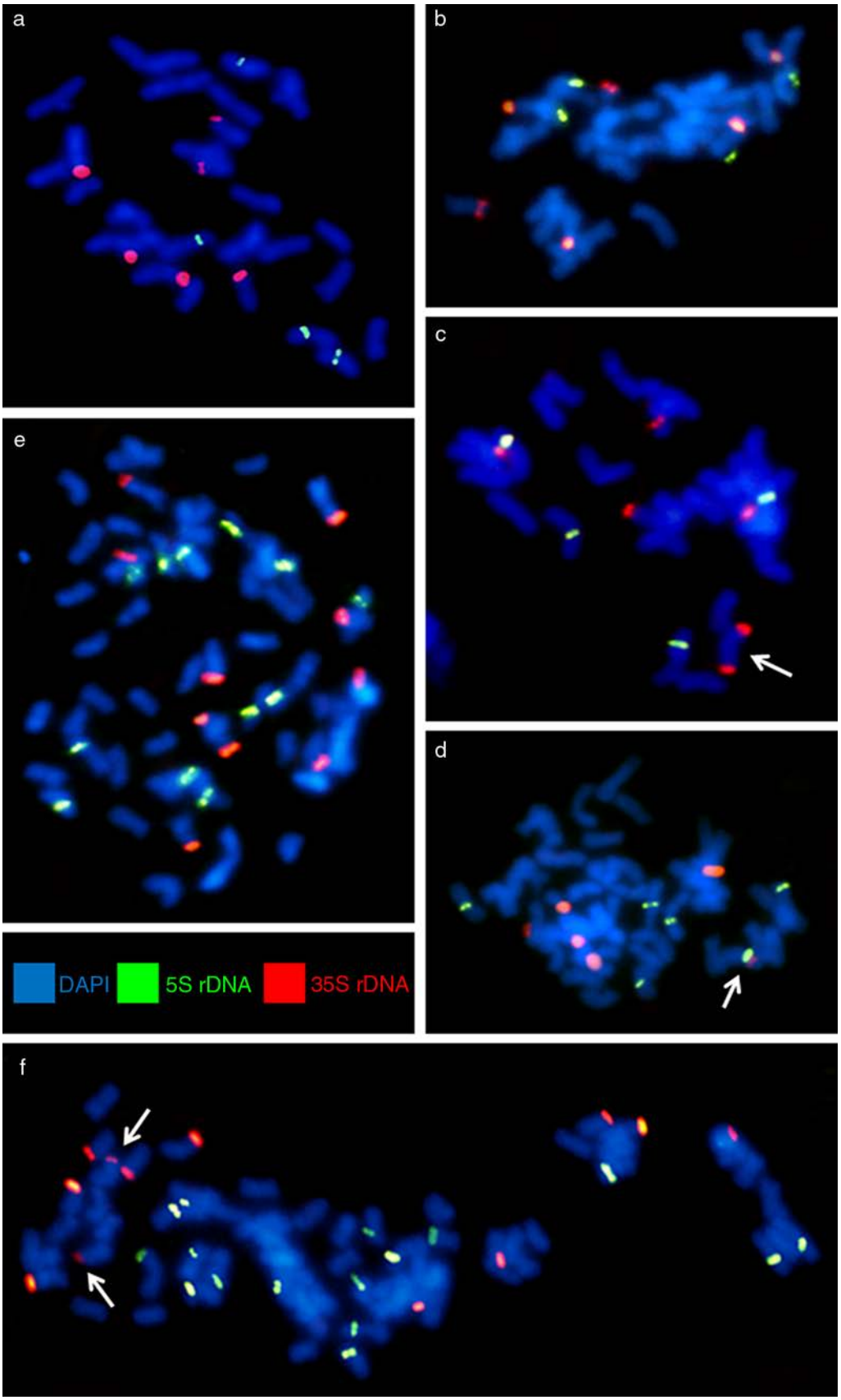

Fig. 1. Cytogenetic analysis of fescue species; FISH on metaphase plates of: **a,** *Festuca arundinacea* subsp. *fenas;* **b, c,** *F. mairei;* **d,** *F. arundinacea* subsp. *corsica;* **e,** *F. arundinacea* subsp. *atlantigena;* **f,** *F. arundinacea* var. *letourneuxiana*. [Probes for 35S rDNA in red color and for 5S rDNA in green color; chromosomes were counterstained using DAPI (blue color).]

2 signals of 35SrDNA in telomeric regions of both chromosome arms indicating non-homologous recombination (Fig. 1c, marked by an arrow). Our results correlated with those of Thomas & al. (1997). They revealed that *F. mairei* and *F. arundinacea* subsp. *glaucescens (F. arundinacea* subsp. *fenas)* have the same numbers and pattern of 5S and 35S rDNA loci. However, we found the variation in the distribution of 35S rDNA loci between 2 populations of *F. mairei*. This was not surprising, as the variation in the number of rDNA loci has been also reported in the other members of the complex, like *F. pratensis* and *Lolium perenne* by Ksiazczyk & al. (2010).

The distribution and pattern of rDNAs in hexaploid *F. arundinacea* subsp. *corsica,* octoploid *F. arundinacea* subsp. *atlantigena,* and decaploid *F. arundinacea* var. *letourneuxiana* have been identified for the first time. In *F. arundinacea* subsp. *corsica* —$2n = 6x = 42$—, 6 5S rDNA loci and 6 loci of 35S rDNA have been detected (Fig. 1d). Surprisingly, one chromosome carried both 5S and 35S rDNA loci (Fig. 1d, indicated by an arrow), located close to each other. All 5S rDNA loci have been found in pericentromeric regions. In the closely related hexaploid *F. arundinacea* subsp. *arundinacea,* Thomas & al. (1997) confirmed *F. pratensis* and *F. arundinacea* subsp. *glaucescens* as progenitors of the hexaploid *F. arundinacea* subsp. *arundinacea* based on the number and position of rDNA loci. However, the authors found differences in the number and distribution of rDNA loci in *F. arundinacea* subsp. *glaucescens* and in the genome of *F. arundinacea* subsp. *glaucescens* that are present in the hexaploid *F. arundinacea* subsp. *arundinacea.* This suggests some genome modifications either in *F. arundinacea* subsp. *glaucescens* or in *F. arundinacea* subsp. *arundinacea*. Loss of rDNA loci inherited from diploid progenitors was reported in other allopolyploids (Vaughan & al., 1993; Leggett & Markhand, 1995).

We detected 12 loci of 5S rDNA and 10 loci of 35S rDNA in both populations of *F. arundinacea* subsp. *atlantigena* —$2n = 8x = 56$—. All the loci were detected on different chromosomes (Fig. 1e). 35S rDNA loci were located distally and 5S rDNA loci have been found in centromeric or pericentromeric regions. Similarly, no variation in the numbers and positions of rDNA loci has been detected between 2 populations of decaploid *F. arundinacea* var. *letourneuxiana* —$2n = 10x = 70$—. Plants from both populations carried 16 loci of 5S rDNA preferentially located in proximal chromosome regions (Fig. 1f). In total, 12 loci of 35S rDNA, of which 2 are minor loci —indicated by arrows—, have been detected in telomeric or subtelomeric regions. All rDNA loci were present on different chromosomes.

Genome size estimations

The estimation of genome size by flow cytometry revealed consistent results among populations of individual species (Table 1). The holoploid genome sizes —1C— in tetraploids ranged from 4.98-5.06 pg in *F. mairei* to 5.20-5.28 pg in *F. arundinacea* subsp. *fenas.* In *F. arundinacea* subsp. *atlantigena* and *F. arundinacea* var. *letourneuxiana,* the holoploid genome sizes were estimated to be 8.07-8.14 pg (Fig. 2a) and 9.85 pg, respectively. Hexaploid *F. arundinacea* subsp. *corsica* had the holoploid genome 1C = 8.31 pg (Fig. 2b).

Monoploid genome size —1Cx— decreases with increasing ploidy level (Table 1). Tetraploid subspecies —*F. mairei* and *F. arundinacea* subsp. *fenas*— displayed a monoploid genome size in range of 2.5-2.7 pg. Hexaploid population of *F. arundinacea* subsp. *corsica* has similar monoploid genome size as tetraploids —1Cx = 2.7 pg—. Monoploid genome size of octoploid *F. arundinacea* subsp. *atlantigena* has been estimated to 2.0 pg and in decaploid *F. arundinacea* var. *letourneuxiana*, 1Cx = 1.9 pg has been estimated.

The genome size of an allopolyploid could be expected as the sum of the genome sizes of its parents. This has been found in allopolyploids of the genera *Triticum* L., *Arachis* L., *Glycine* Willd., and *Allium* L. (Rees & Walters, 1965; Hammatt & al., 1991; Sing & al., 1996; Ohri & al., 1998). However, genome downsizing following polyploidy has been detected in other species including *Brassica* L. and *Nicotiana* L. allopolyploids (Ozkan & al., 2003). The loss of DNA after polyploidization has been frequently evidenced (Leitch & Bennett, 2004). Thus, the genome downsizing evidenced in our study is not exceptional (Fig. 3). Loureiro & al. (2007) also found a regression in monoploid genome size with increasing ploidy level in fine-leaved

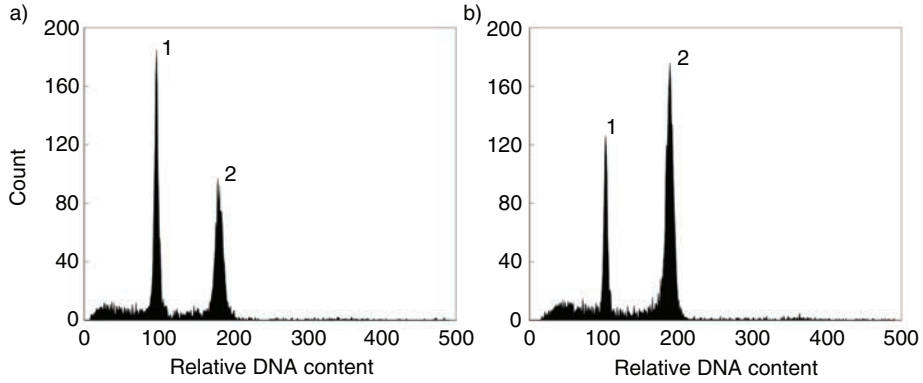

Fig. 2. Genome size estimation of fescue species using flow cytometry; histogram of relative fluorescence intensity obtained after simultaneous analysis of nuclei isolated from: **a,** *Pisum sativum* 'Ctirad' (1C = 4.55 pg, as an internal reference standard; G0/G1 nuclei, peak 1) and *F. arundinacea* subsp. *atlantigena* (G0/G1 nuclei, peak 2); **b,** *Pisum sativum* 'Ctirad' (1C = 4.55 pg, as an internal reference standard; G0/G1 nuclei, peak 1) and *F. arundinacea* subsp. *corsica* ABY-BN1175 (G0/G1 nuclei, peak 2).

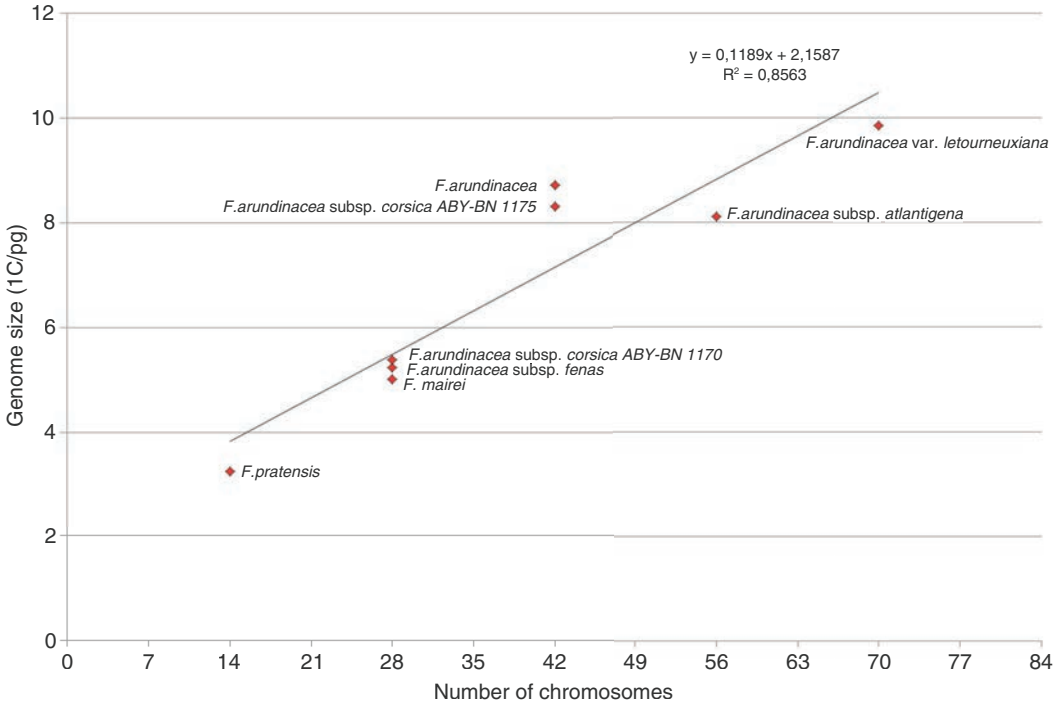

Fig. 3. Linear regression analysis between mean nuclear DNA content and chromosome number for various species of *Festuca* subgen. *Schedonorus*.

fescues. Highly polyploid species revealed significantly lower monoploid genome size compare to the diploids and tetraploids. Similarly, Šmarda & al. (2008) reported that hexaploid *F. arundinacea* subsp. *arundinacea* underwent the reduction of its genome after speciation from the cross of *F. pratensis* and *F. arundinacea* subsp. *glaucescens.* Several mechanisms including the elimination of non-coding DNA sequences during polyploid formation and the massive karyotype reconstruction including the deletions and non-homologous recombinations in the first generations are probably key factors in genome size reduction (Shaked & al., 2001).

In search of the ancestry of polyploid species

There are only two tetraploids among Maghrebian broad-leaved fescues, *F. arundinacea* subsp. *fenas (F. arundinacea* subsp. *glaucescens)* and *F. mairei.* Using FISH, we revealed that both species have the same numbers and similar positions of rDNA loci and thus, are probably closely related. It may indicate that both species originated from the cross of the same parents. This is consistent with previous reports (Thomas & al., 1997; Catalán & al., 2004; Inda & al. 2008). Based on the karyotypic analysis and the presence and distribution of secondary constrictions, Malik & Thomas (1966) suggested that *F. mairei* and *F. arundinacea* subsp. *glaucescens* share one common genome. This has been supported by molecular sequence data of chloroplast and mitochondrial DNA and ITS by Hand & al. (2010). Despite the close relationship of these species revealed by our FISH results supported by the phylogenetic study of Hand & al. (2010), we found difference in genome sizes of both species. This may indicate that they differ by at least one parental species. Alternatively, they share the same progenitors, but massive genome reconstruction of one of the species took place during its evolution, presumably genome downsizing of *F. mairei.*

Our FISH analysis of *F. arundinacea* subsp. *corsica* revealed that this species has highly similar number and position of rDNA loci as another hexaploid, *F. arundinacea* subsp. *arundinacea* from the European clade. This indicates that both hexaploids may have the same progenitors. Molecular analysis of ITS and GISH revealed that *F. arundinacea* subsp. *arundinacea* originated from the cross of maternal *F. arundinacea* subsp. *glaucescens* and paternal *F. pratensis* (Humphreys & al., 1994; Charmet & al., 1997). Similarly, phylogenetic analysis using sequence data of ITS and plastid *trn*T-L and *trn*L-F regions placed *F. arundinacea* subsp. *corsica* in the Maghrebian clade and indicated its close relationship with *F. arundinacea* subsp. *fenas* (Inda & al., 2014). Thus the later taxon could be one of the progenitors of *F. arundinacea* subsp. *corsica.* Based on the numbers and positions of rDNA loci revealed in this study and by Thomas & al. (1997), *F. pratensis* could be the second progenitor of *F. arundinacea* subsp. *corsica.* On the other hand, both species significantly differ in genome size —8.73 pg/1C in *F. arundinacea* vs. 8.31 pg/1C in *F. arundinacea* subsp. *corsica*; see Kopecký & al. (2010) and this study— and thus, *F. arundinacea* subsp. *corsica* genome probably underwent partial downsizing in larger extent than that assumed for *F. arundinacea.*

Apart from genome size changes, chromosome reshuffling probably took place after the speciation of both taxa. Non-reciprocal translocation indicated by the presence of single chromosome carrying both 5S and 35S rDNA loci has been found in *F. arundinacea* subsp. *corsica* (Fig. 1d). Similarly, structural changes of chromosomes after allopolyploidization have been detected in the hexaploid

F. arundinacea subsp. *arundinacea.* Thomas & al. (1997) reported 4 loci of 35S rDNA in the hexaploid *F. arundinacea,* 2 of which belong to *F. pratensis* and 2 to *F. arundinacea* subsp. *glaucescens.* However, they found 4 loci of 35S rDNA in *F. arundinacea* subsp. *glaucescens,* and thus, 2 loci have been lost during the evolution of the hexaploid *F. arundinacea* subsp. *arundinacea.*

The origin and genome constitution of the octoploid *F. arundinacea* subsp. *atlantigena* is unclear. This species is closely related to *F. mairei* and also to *F. arundinacea* subsp. *fenas.* Thus it is possible that *F. arundinacea* subsp. *atlantigena* could have evolved by interspecific hybridization of these species either followed by whole genome duplication or alternatively, using unreduced gametes. This hypothesis is supported by the development of artificial hybrids of *F. mairei* and *F. arundinacea* subsp. *fenas,* which were fertile and morphologically close to *F. arundinacea* subsp. *atlantigena* (Chandrasekharan & Thomas, 1971). Similarly, Charmet & al. (1997) proposed *F. mairei* and *F. arundinacea* subsp. *glaucescens* as progenitors of *F. arundinacea* subsp. *atlantigena* and *F. arundinacea* var. *letourneuxiana.*

However, numbers of rDNA loci found in *F. arundinacea* subsp. *atlantigena* in this study are not a sum of rDNA loci detected in *F. mairei* and *F. arundinacea* subsp. *fenas*

Fig. 4. Hypothetical scenario on the origin of polyploid fescues from Marghrebian clade.

(Thomas & al., 1997). Thus, if these 2 species are really progenitors of *F. arundinacea* subsp. *atlantigena,* chromosome rearrangements including deletion of 2 35S rDNA loci and amplification/duplication of 4 5S rDNA loci likely took place. Moreover, the sum of genome sizes of both species highly exceeds the genome size of octoploid. Thus, if hypothesis on the origin of *F. arundinacea* subsp. *atlantigena* from the cross of *F. mairei* and *F. arundinacea* subsp. *fenas* is valid, a massive genome downsizing had to occur during the evolution of this species.

There are several scenarios for the potential speciation of the decaploid *F. arundinacea* var. *letourneuxiana.* Borrill (1972) speculated that it originated from the cross of *F. arundinacea* subsp. *arundinacea* (6*x*) and *F. mairei* (4*x*) followed by whole genome duplication. However, positions and number of rDNA loci —16 and 12 loci of 5S and 35S rDNA, respectively— in *F. arundinacea* var. *letourneuxiana* are highly different from the sum of both potential parents. Moreover, this scenario requires downsizing for over 30% of the genome after potential allopolyploidization, based on our genome size estimations.

Alternatively, *F. arundinacea* var. *letourneuxiana* could be a product of interspecific hybridization of the octoploid *F. arundinacea* subsp. *atlantigena* and the diploid *F. pratensis* followed by whole genome duplication. In this case, only the amplification of 2 5S rDNA loci would be necessary with only minor reduction of genome size. However, *F. pratensis* possesses 2 loci of 35S rDNA located proximally in the short arm of chromosome 3F (Kopecký & al., 2008b) and we did not identify such positioning of 35S rDNA in *F. arundinacea* var. *letourneuxiana* in this study.

The close phylogenetic relationship of *F. arundinacea* subsp. *atlantigena* and *F. arundinacea* var. *letourneuxiana* indicates that the former one has been involved in the origination of the latter. Moreover, the molecular analyses of cpDNA and mtDNA sequences suggest that *F. arundinacea* subsp. *atlantigena* serves as maternal progenitor (Hand & al., 2010; Inda & al., 2014). Based on the above mentioned findings and our FISH and genome size results, we hypothesize that this decaploid originated from the merge of an unreduced gamete —egg cell— of *F. arundinacea* subsp. *atlantigena* with a reduced gamete —pollen— of either *F. mairei* or *F. arundinacea* subsp. *fenas,* more presumably the former one. Such scenario would require amplification/duplication of 2 5S rDNA loci and the loss of 1 —odd— 35S rDNA loci. The sum of both potential progenitor genome sizes is almost equal to the genome size estimated for *F. arundinacea* var. *letourneuxiana* —10.62pg/1C vs. 9.85pg/1C—. Alternatively, a currently unknown or extinct diploid parent could be the missing 2*x* parent of the decaploid *F. arundinacea* var. *letourneuxiana.*

Concluding remarks

Our study provided new information on the genome size and the numbers and distributions of rDNA loci of broad-leaved fescues from Maghrebian clade and improved the knowledge on the phylogeny of this group of grasses —a hypothetical scenario on the evolution of the group is summarized in Fig. 4—. We found that *F. arundinacea* subsp. *corsica* presumably resulted from the interspecific hybridization of the diploid *F. pratensis* and the tetraploid *F. arundinacea* subsp. *fenas* and thus, has probably the same progenitors as *F. arundinacea* subsp. *arundinacea* from the European clade. The origin of the octoploid *F. arundinacea* subsp. *atlantigena* remains unclear, however, it probably originated from crossing of the tetraploids *Festuca arundinacea* subsp. *fenas* —2*n* = 4*x* = 28— and *Festuca mairei* —2*n* = 4*x* = 28— followed by whole genome duplication and massive genome reconstruction. The most probable scenario on the origin of the decaploid *F. arundinacea* var. *letourneuxiana* involves merge of an unreduced gamete of the octoploid *F. arundinacea* subsp. *atlantigena* and a reduced gamete of tetraploid fescue, presumably *F. mairei.*

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