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Flow cytometric and cytogenetic analyses of Iberian Peninsula *Festuca* spp.

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Abstract. *Festuca* L. has an important diversification centre in the Iberian Peninsula. We used chromosome counting, fluorescence (FISH) and genomic in situ hybridization (GISH), and DNA flow cytometry (FCM) to clarify the taxonomic position of several taxa, to search for phylogenetic relationships and to assess the extent and pattern of genome variation in fescues. The chromosome number of *Festuca duriotagana* var. *barbata* is determined for the first time and new ploidy level estimations are given for *F. rothmaleri* and *F. summilusitana*. In the latter species, besides the reported decaploid level, dodecaploidy was found in some populations, which points to the existence of an unrecognized taxon. Moreover, these differences were confirmed by FCM and a high positive correlation was found with the type of substrate where *F. summilusitana* was growing. For each section, a decrease of genome size with increase of polyploidy was observed. In general, in situ hybridization techniques failed to reveal phylogenetic relationships among the selected species. In FISH, a variation in the number of rDNA sites was observed in some species. GISH results indicate that diploid *F. henriquesii* is not a progenitor of the studied polyploid species.

Keywords: Fescues; *Festuca*; flow cytometry; genome size; Iberian Peninsula; karyology; in situ hybridization; molecular cytogenetics

Introduction

Festuca L. is one of the most complex and numerous genus of Poaceae. In the Iberian Peninsula there is an important diversity centre of approximately 100 taxa (Cebolla Lozano and Rivas Ponce 2003). From these, 24 taxa distributed through six sections are recognized in the Portuguese Continental flora (Franco and Rocha Afonso 1998). These include five species (*Festuca brigantina* Markgr.-Dann., *F. duriotagana* var. *barbata* Franco & R. Afonso, *F. elegans* Boiss., *F. henriquesii* Hack. and *F. summilusitana* Franco & R. Afonso) listed in the European Community Habitats Directive 92/43/EEC–Annex II, where they are referred as species of community interest whose conservation requires the designation of special areas. Moreover, *F. brigantina* ssp. *brigantina*, *F. duriotagana* var. *barbata* and *F. henriquesii* are endemic to the Portuguese

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Continental region. The close morphological similarities among fescues and the occurrence of a high number of ecotypes/varieties that resulted from specific genetic variability and different environmental conditions, leads to difficulties in the identification and classification of species from this genus (de la Fuente et al. 1997, Huff and Palazzo 1998). Due to its high complexity, Iberian Peninsula species of *Festuca* have been the subject of intensive taxonomic and karyologic studies (e.g. de la Fuente et al. 2001, Ortúñez and de la Fuente 2004). Many of these studies used both approaches, as for this genus the number of chromosomes has been regarded as an important tool in delimitation of new species, which sometimes are not so easily separated using morphoanatomic analyses (de la Fuente and Ortúñez 2000).

Besides chromosome number, their morphology can also be an important character in the study of relationships among species (Harper et al. 2004). Fluorescence in situ hybridization (FISH) is a powerful tool that enables the visualization of specific DNA sequences on chromosomes squashed on a microscope slide. Ribosomal DNAs (rDNAs) are among the most used DNA sequences for FISH, as their sites differ between closely related species (both in number of sites and in their position), while the order of most genes remains conserved during evolution (Harper et al. 2004). FISH with rDNAs has been applied to study the phylogeny of some of the most economically important fescue species (e.g. *F. arundinacea* Schreb., *F. pratensis* Huds. and *F. arundinacea* var. *glaucescens* Boiss., Thomas et al. 1997). Also, Harper et al. (2004) used FISH to study the rDNA patterns on chromosomes of *F. scariosa* (Lag.) Asch. & Graebn. (section *Scariosae*) and four diploid species of section *Montanae* in order to search for diploid progenitors of polyploid species.

Genomic in situ hybridization (GISH) is a modified technique of FISH that, in hybrids or allopolyploid plants, enables the visualization of chromosomes of different genomes with different colours (Raina and Rani 2001). This method is based on hybridization of total genomic DNA with chromosomal DNA fixed on a microscopic slide (Schwarzacher et al. 1989). In *Festuca*, despite having been already used for phylogenetic purposes in the discrimination of ancestral progenitor genomes of allohexaploid *F. arundinacea* (Humphreys et al. 1995), most of the studies concerned hybrids of *Lolium* x *Festuca* (Festulolium) (e.g. Kopecký et al. 2005a,b; Kopecký et al. 2006; Kosmala et al. 2006).

Estimations of nuclear DNA content may provide useful information in phylogenetic relationships analyses. Furthermore, it can also provide important knowledge on the complexity of a genome (Doležel 1997). As shown by Bennett and Leitch (2005), genome size is still unknown for about 98% of angiosperm species. Until now, most of the estimations of nuclear DNA content of fescues were determined using Feulgen microdensitometry (Bennett et al. 1982, Grime and Mowforth 1982, Schifino and Winge 1983, Seal 1983, Ceccarelli et al. 1992) and only recently, flow cytometry (FCM), has been used for genome size estimations within this genus: Huff and Palazzo (1998) estimated the nuclear DNA content of 10 fine fescue species and Arumuganathan et al. (1999) estimated the genome size of *F. arundinacea* and *F. longifolia* Thuill.

The objective of this work was to study the cytology of several fescue species. We intend to determine for the first time the chromosome number of *F. duriotagana* var. *barbata*, clarify the chromosome number of *F. ampla* Hack. and confirm the ploidy level of the remaining species, especially *F. summilusitana* var. *barbata*. Also, molecular cytogenetic techniques of FISH and GISH were applied in wild species of *Festuca* to search for phylogenetic relationships, namely the progenitor character of *F. henriquesii*, the only diploid species from the West Iberian Peninsula. Finally, it was our goal to use FCM to estimate the nuclear DNA content of 14 taxa (from which 11 are first estimations) and therefore contribute to the assessment of the extent and pattern of genome size variation within this genus.

Materials and methods

Plant material. Plant and seed samples of 14 taxa of *Festuca* were collected from several field locations in Portugal (Table 1). Field collected and seed germinated plants were potted and maintained in a greenhouse at 22 ± 1°C, photoperiod of 16 h and light intensity of 530 ± 2 μmol $\cdot$ $m^{-2} \cdot s^{-1}$. Voucher specimens were collected and kept in the Herbarium of the University of Aveiro (AVE).

Chromosome preparations and counting. When available, seeds were germinated in Petri dishes on wet filter paper and the obtained seedlings were planted in 30 mm pots in the greenhouse. Well developed plantlets were transferred into a hydroponic culture with an aerated solution of 0.9 g $\cdot$ L Hydroponex (Hu-Ben, Čerčany, Czech Republic) at 25 ± 2°C. In some cases, field collected plants were transferred directly into hydropony. Mitotic metaphase spreads were prepared from roots tips according to the protocol of Masoudi-Nejad et al. (2002).

For chromosome counting, chromosomes on a slide were counterstained with 1.5 μg $\cdot$ mL^1 4′, 6-diamidino-2-phenylindole (DAPI) made in Vectashield antifade solution (Vector Laboratories, Burlingame, CA, USA). Slides were evaluated with an epifluorescence Olympus AX70 microscope (Center Valley, PA, USA). Images were acquired with a Cooke SensiCam B/W camera (Eugene, OR, USA) and processed using ScionImage (Frederick, MD, USA) and Adobe Photoshop (San José, CA, USA) software.

Fluorescence in situ hybridization with rDNA probes. For FISH analyses, chromosomes of species from sections *Festuca* (*F. henriquesii*, *F. ampla* ssp. *ampla*, *F. brigantina* and *F. summilusitana*), *Aulaxyper* (*F. rubra* ssp. *litoralis* and *F. duriotagana*) var. *barbata* and *Subbulbosae* (*F. paniculata* ssp. *multispiculata*) were hybridized with biotin-labelled DNA clone pTa71 (Gerlach and Bedbrook 1979) containing a 9 kb *Eco*RI fragment of wheat ribosomal DNA and carrying the 18S-5.8S-26S cluster of ribosomal DNA genes (referred from here as 45S rDNA), and with digoxigenin (DIG) labelled probe for 5S rDNA. This probe was prepared using PCR with a pair of specific primers (RICRGAC1, RICRGAC2), which amplify 303 bp in rice (Fukui et al. 1994), using rice genomic DNA as a template. In situ hybridization was performed according to the protocol of Masoudi-Nejad et al. (2002). Sites of probe hybridization were detected by biotin-streptavidin-Cy3 conjugate (Amersham, Piscataway, NJ, USA) and fluorescein-conjugated anti-DIG antibody (Roche, Indianapolis, IN, USA). Chromosomes were counterstained and evaluated using the methodology described above.

Genomic in situ hybridization. In GISH experiments, total genomic DNA of *F. henriquesii* was labelled with biotin according to manufacturer's instructions (Biotin-Nick Translation Kit, Roche, Indianapolis, IN, USA) and used as a probe on *F. ampla* ssp. *ampla*, *F. brigantina*, *F. summilusitana*, *F. paniculata* ssp. *multispiculata*, *F. rubra* ssp. *litoralis* and *F. duriotagana* var. *barbata* chromosomes. Salmon sperm DNA was used as blocking DNA. In situ hybridization and detection was done as described for FISH. Some slides where FISH was already applied were also analyzed by GISH. For this, slides were washed according to the protocol described by Schwarzacher and Heslop-Harrison (2000).

Genome size estimations using flow cytometry. For FCM analyses of genome size, leaves of field collected or seed germinated plants were used as plant material. Nuclear suspensions were prepared according to Galbraith et al. (1983), by chopping 200 mg of leaf tissue of *Festuca* sp. and 50 mg of internal standard leaves, with a razor blade in a glass Petri dish containing 1.5 mL of Tris.$MgCl_2$ nuclear isolation buffer (200 mM TRIS, 4 mM $MgCl_2 \cdot 6H_2O$, 0.5% (v/v) and Triton X-100, pH 7.5, Pfosser et al. 1995) supplemented with 1% (m/v) polyvinylpyrrolidone 10 (PVP-10). With exception of *F. ampla*, *Pisum sativum* cv. Ctirad (2C = 9.09 pg of DNA, Doležel et al. 1998) was used as an internal reference standard for all the studied species. In *F. ampla*, *Z. mays* cv. CE-777 (2C = 5.43 pg of DNA, Doležel et al. 1998) was chosen as reference standard. In *Festuca* sp., in order to obtain a good number of nuclei per mL, the quantity of plant material and chopping intensity had to be increased. Nuclear suspension was then filtered through an 80 μm nylon filter and 50 μg $\cdot$ mL^{-1} of propidium iodide (PI, Fluka, Buchs, Switzerland) and 50 μg $\cdot$ mL^{-1} of RNAse (Fluka, Buchs, Switzerland) were added to the samples to stain the DNA and avoid staining of double stranded RNA, respectively. Samples were kept on ice and analyzed within a 10 min period in a Beckman Coulter EPICS XL (Beckman Coulter, Hialeah, FL, USA) flow cytometer equipped with a

Table 1. Scientific name and localities of sample collection of the *Festuca* species studied in this work. Voucher specimens are kept in the Herbarium of the University of Aveiro (AVE)

Species	Localities
Festuca L. subg. *Festuca*	
Section *Subbulbosae* (Nyman) Hack.	
F. paniculata (L.) Schinz & Thell. ssp. *multispiculata* Rivas Ponce & Cebolla	Bragança: Serra da Nogueira, summit with 1218 m a.s.l. between Castelinho and Nogueira, 29TPG72. Coimbra: Serra do Açor, Mata da Margaraça, 29TNE9252, c. 600 m.
Section *Festuca*	
F. henriquesii Hack.	Guarda: Serra da Estrela, near Torre, 29TPE16, c. 1970 m.
F. ampla Hack. ssp. *ampla*	Coimbra: Serra do Açor, Covanca, 29TPE0049, c. 900 m.
F. ampla Hack. ssp. *transtagana* Hack.	Faro: Foia, 29SNB33, c. 900 m.
F. brigantina Markgr.-Dann. ssp. *brigantina*	Bragança: Mosqueiro, 29TPG72, c. 1070 m.
F. summilusitana Franco & Rocha Afonso	Bragança: Serra da Nogueira, summit with 1218 m altitude between Castelinho and Nogueira, schist, 29TPG72. Vila Real: Serra do Marão, Senhora da Serra, schist, 29TNF96, c. 1415 m. Aveiro: Serra do Caramulo, Caramulinho, granite, 29TNE68, c. 1050 m. – population C1 Aveiro: Serra do Caramulo, near Cruzinha, schist, 29TNE7195, c. 1000 m. – population C2 Aveiro: Serra do Caramulo, near Monteteso, schist, 29TNE7094, c. 900 m. – population C3 Aveiro: Serra da Freita, 1 km N of Albergaria da Serra, schist, 29TNF62, c. 1000 m. Coimbra: Serra da Lousã, Castelo do Trevim, schist, 29TNE7038, c. 1200 m. – population L1 Coimbra: Serra da Lousã, Penedos de Góis, quartzite, 29TNE7439, c. 1050 m. – population L2 Coimbra: Serra da Lousã, Santo António das Neves, schist, 29TNE7137, c. 1150 m. – population L3 Coimbra: Serra da Lousã, c. 750 m S of Castelo do Trevim, schist, 29TNE7037, c. 1090 m. – population L4 Guarda: Serra da Estrela, near Torre, granite, 29TPE16, c. 1960 m. – population E1 Guarda: Serra da Estrela, between Sabugueiro and Penhas Douradas, granite, 29TPE17, c. 1200 m. – population E2

	Guarda: Serra da Estrela, Poço do Inferno, granite, 29TPE27, c. 1100 m. – population E3
	Guarda: Serra da Estrela, Gouveia, between Santinha and Santiago, schist, 29TPE28, c. 1415 m. – population E4
Section *Eskia* Willk.	
F. elegans Boiss. ssp. *merinoi* (Pau) Fuente & Ortúñez	Coimbra: Serra do Açor, Cabeço da Fonte de Espinho, 29TNE9452, c. 1000 m.
	Guarda: Serra da Estrela, Manteigas, 29TPE29, c. 1100 m.
Section *Aulaxyper* Dumort.	
F. nigrescens Lam. ssp. *microphylla* (St.-Yves) Markgr.-Dann.	Coimbra: Serra do Açor, Cabeço da Fonte de Espinho, 29TNE9452, c. 1000 m.
F. rubra L. ssp. *litoralis* (G. F. W. Meyer) Auquier	Viana do Castelo: Foz do Rio Neiva, 29TNG10, c. 1 m
F. rubra L. ssp. *pruinosa* (Hack.) Piper	Viana do Castelo: near Farol de Montedor, 29TNG12, c. 2 m
F. rubra L. ssp. *rubra*	Coimbra: Serra do Açor, Casais de São Pedro, 29TPE0052, c. 1250 m.
F. rothmaleri (Litard.) Markgr.-Dann.	Coimbra: Serra do Açor, near Castanheira, 29TNE9449, c. 900 m.
	Guarda: Serra da Estrela, near Lagoa Comprida, 29TPE16, c. 1600 m.
F. duriotagana Franco & Rocha Afonso var. *barbata*	Portalegre: Barragem de Belver, 29SND87, c. 40 m.
Festuca L. subg. *Schenodorus* (P. Beauv.) Peterm.	
Section *Schenodorus*	
F. arundinacea Schreb. ssp. *mediterranea* (Hack.) K.Richt.	Coimbra: Serra do Açor, Fajão, margins of Rio Ceira, 29TNE9146, c. 475 m.

488 nm air-cooled argon-ion laser. Integral fluorescence and fluorescence pulse height and width emitted from nuclei were collected through a 645 nm dichroic long-pass filter and a 620 nm band-pass filter. Prior to analysis, the instrument was checked for linearity with fluorescent beads (Beckman Coulter). Doublets, partial nuclei, nuclei with associated cytoplasm and other debris were removed from analysis using a specific gating region defined in a linear-fluorescence (FL) pulse integral vs. FL pulse height. At least 5,000 nuclei were analyzed per sample. For each species, at least five individuals were analyzed in three different days to avoid errors due to instrumental drift.

The holoploid genome size (2C; *sensu* Greilhuber et al. 2005) of *Festuca* species was estimated according to the following formula:

$$\text{2C nuclear DNA content (pg)} = \frac{\textit{Festuca}\text{ sp. } G_0/G_1 \text{ peak mean}}{\text{reference standard } G_0/G_1 \text{ peak mean}} \times \text{nuclear DNA content of reference standard}$$

the monoploid genome size (2Cx; *sensu* Greilhuber et al. 2005) of all species was also calculated in mass values (pg) and Mbp (1 pg = 978 Mbp, Doležel et al. 2003).

Statistical analysis. For each species, within and between populations (when applicable) differences in nuclear DNA content were analyzed and compared using either a t-test or a one-way ANOVA. When necessary, a multiple comparison Tukey-Kramer test was applied to determine exactly which groups presented significantly different values. In sections *Festuca* and *Aulaxyper*, linear regression analyses were performed between mean nuclear DNA content and chromosome numbers of each species. All statistical studies were performed using SigmaStat for Windows Version 3.1 (Systat, Karnataka, India).

Results

Chromosome counting. The chromosome numbers determined in this study are presented in Table 2. With the exception of *F. summilusitana*, where besides the reported decaploid ploidy level ($2n = 10x = 70$) a new chromosome number has been found ($2n = 12x = 84$, Fig. 1A), the observed chromosome counts were generally in agreement with previously

Table 2. Observed (by chromosome counting), estimated (using the linear regression analysis) and reported chromosome numbers of the *Festuca* species studied in this work

Species	Distribution	Chromosome number (2n)			
		Observed	Estimated	Reported	Reference
F. paniculata ssp. *multispiculata*	Iberian Peninsula	28	-	28	(Devesa et al. 1990)
F. henriquesii	Portugal	14	14	14	(Ferrero and de la Fuente 1996, de la Fuente et al. 2001)
				28	(Ortúñez and de la Fuente 1995)
F. ampla ssp. *ampla*	Iberian Peninusla and North Africa	28	28	28, 42	(de la Fuente et al. 2001)
				56	(Malik and Thomas 1966)
F. ampla ssp. *transtagana*	Iberian Peninsula	-	28	-	-
F. brigantina ssp. *brigantina*	Portugal	56	56	56	(Ferrero and de la Fuent, 1996, de la Fuente et al. 2001)
				42	(Ortúñez and de la Fuente 1995)
F. summilusitana	Iberian Peninsula	70, 84	70, 84	70	(de la Fuente and Ortúñez 2000, de la Fuente et al. 2001)
F. elegans ssp. *merinoi*	Iberian Peninsula	-	-	28	(Ferrero and de la Fuente 1996, Ortúñez and de La Fuente 2004)
F. nigrescens ssp. *microphylla*	Europe	-	42	42	(de la Fuente et al. 1997)
F. rubra ssp. *litoralis*	Europe	42	42	42	(Kerguélen 1975)
F. rubra ssp. *pruinosa*	Worldwide	42	42	42	(Kerguélen 1975)
				42	(Seal 1983)
F. rubra ssp. *rubra*	Worldwide	-	56	42, 56	(Konarska 1974)
				56	(Huff and Palazzo 1998)
				42	(Devesa et al. 1990)
F. rothmaleri	Iberian Peninsula	-	42	56	(Al-Bermani et al. 1992)
F. duriotagana var. *barbata*	Portugal	70	70	-	-
F. arundinacea ssp. *mediterranea*	Worldwide	-	-	42	(Queirós 1973)

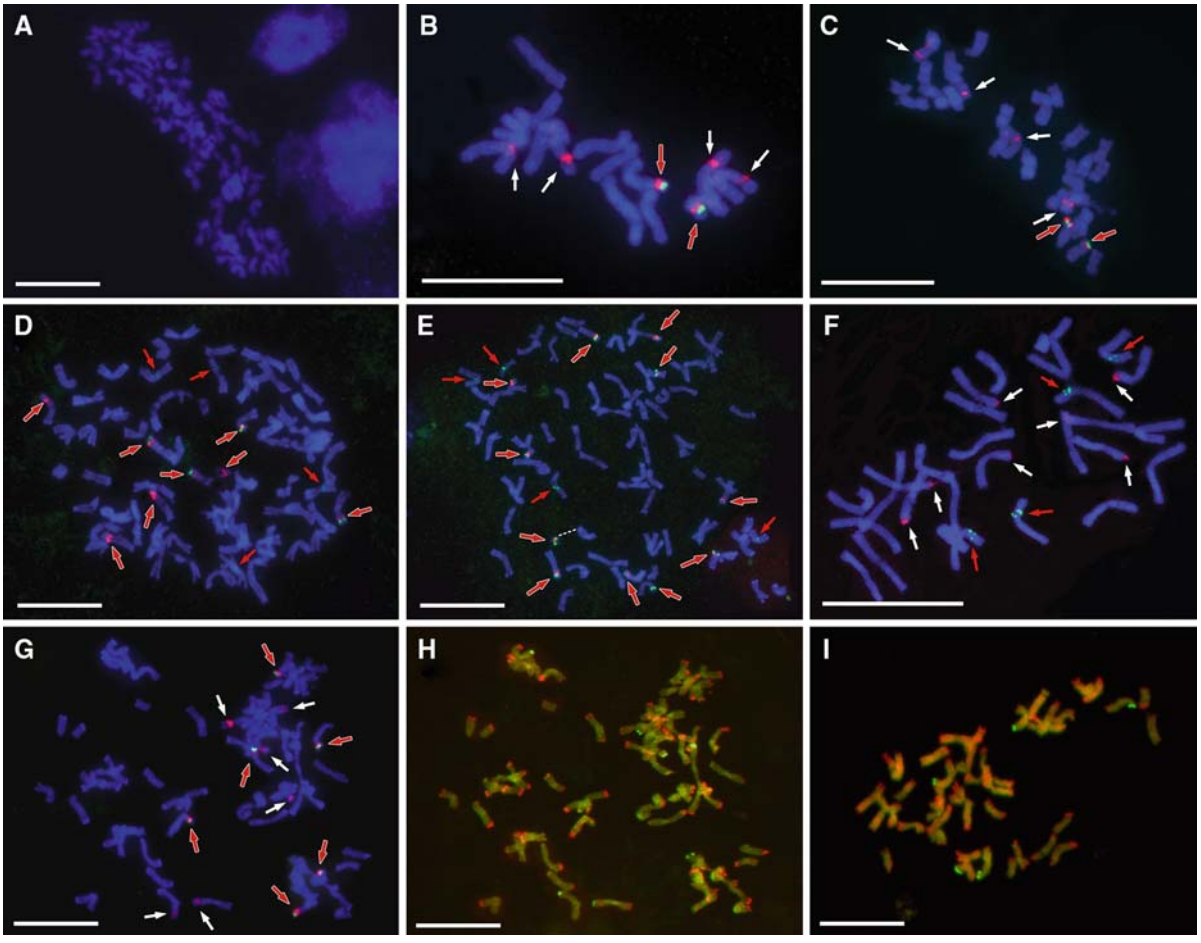

Fig. 1. Cytogenetic analysis of fescue species. **A**. Metaphase spread of *F. summilusitana* from population C1 ($2n = 12x = 84$). **B-G**. FISH on metaphase plates with probe for 45S rDNA (red color) and probe for 5S rDNA (green color) in *F. henriquesii* **B**, *F. ampla* ssp. *ampla* **C**, *F. summilusitana* from Serra do Caramulo **D**, *F. summilusitana* from Serra do Caramulo **E**, *F. paniculata* ssp. *multispiculata* **F**, *F. duriotagana* var. *barbata* **G**. White thin arrows point for chromosomes carrying 45S rDNA, red thin arrows for chromosomes with the 5S rDNA and red arrows with white contour (thicker) for chromosomes carrying both 45S and 5S rDNAs. **H-I**. GISH on mitotic metaphase plates of *F. duriotagana* var. *barbata* **H** and *F. rubra* ssp. *litoralis* **I**. Total genomic DNA of *F. henriquesii* was labeled with biotin and used as a probe (green color); salmon sperm DNA was used as block. (A-I) Chromosomes were counterstained using DAPI (blue color). (scale bar = 10 μm)

reported estimations (Table 2). A wide range of ploidy levels was observed, from 14 chromosomes in *F. henriquesii* to 70 and 84 chromosomes in *F. summilusitana*. The analyzed individuals of *F. ampla* ssp. *ampla* and *F. ampla* ssp. *transtagana* were all tetraploids ($2n = 4x = 28$). The number of chromosomes of *F. duriotagana* var. *barbata* ($2n = 10x = 70$) was counted for the first time. No B-chromosomes were observed in the studied species.

Nuclear DNA content estimations. The 2C nuclear DNA content of 14 taxa of *Festuca* was determined using FCM (Table 3). Fluorescence histograms (Fig. 2) of relative nuclear DNA content showed distinct G_0/G_1 peaks with coefficients of variation (CV) usually below 4.0% for fescue. In 83.5% of the estimations the CV value was below 3.0%, and only in 1.6% of the cases these values were above 4.0%. *Festuca* species mean holoploid

Table 3. Nuclear DNA content estimations of *Festuca* species studied in this work[a]

Species	Number of populations	Nuclear DNA content						CV (%)	n
			2C range						
		2C (pg)	Min.	Max.	Dif. Pop.	2Cx (pg)	2Cx (Mbp)[1]		
F. paniculata ssp. *multispiculata*	2	15.02 ± 0.382	14.21	15.62	n.s.	3.75	3,671	2.78	10
F. ampla ssp. *ampla*	1	8.74 ± 0.183	8.47	8.93	-	2.18	2,136	2.55	5
F. ampla ssp. *transtagana*	1	9.16 ± 0.381	8.27	9.52	-	2.29	2,239	2.96	10
F. henriquesii	1	5.86 ± 0.226	5.50	6.10	-	2.93	2,864	2.44	10
F. brigantina	1	17.08 ± 0.249	16.78	17.38	-	2.14	2,087	2.47	5
F. summilusitana	14	23.77 ± 1.584	20.50	27.19	*	2.38	2,325	2.48	129
F. elegans ssp. *merinoi*	2	12.31 ± 0.148	12.20	12.74	n.s	3.09	3,011	1.90	10
F. nigrescens ssp. *microphylla*	1	13.80 ± 0.104	13.65	13.94	-	2.30	2,249	2.11	5
F. rubra ssp. *litoralis*	1	13.19 ± 0.257	12.98	13.55	-	2.20	2,149	2.70	5
F. rubra ssp. *pruinosa*	1	12.69 ± 0.073	12.62	12.78	-	2.11	2,068	2.36	5
F. rubra ssp. *rubra*	1	17.66 ± 0.305	17.29	18.09	-	2.21	2,159	3.07	5
F. rothmaleri	2	13.66 ± 0.203	13.42	14.05	n.s.	2.28	2,227	2.13	10
F. duriotagana var. *barbata*	1	20.66 ± 0.186	20.41	20.81	-	2.07	2,021	1.90	5
F. arundinacea ssp. *mediterranea*	1	15.94 ± 0.311	15.56	16.38	-	2.67	2,598	2.30	5

[a] The values are given as mean and standard deviation of the mean of the holoploid nuclear DNA content (2C in pg) of individuals of each species. The 2C range is defined by the minimum (Min.) and maximum (Max.) value obtained for each species. Differences among populations (Dif. Pop.) were analyzed using a t-test or a one-way ANOVA (n.s. – not significantly different ($P < 0.05$); * significantly different at $P < 0.05$). The monoploid nuclear DNA content (2Cx) in mass values (pg) and Mbp, the mean sample coefficient of variation of G_0/G_1 DNA peak (CV, %) and the number of analyzed individuals (n) are also provided for each species

[1] 1 pg DNA = 978 Mbp (Doležel et al. 2003)

genome sizes (2C) ranged from 5.86 pg/2C in *F. henriquesii* to 23.77 pg/2C in *F. summilusitana* (Table 3). With the exception of *F. summilusitana*, low standard deviations of 2C nuclear DNA content were obtained (< 2.5%), with no statistically significant differences being observed among populations ($P \leq 0.05$), revealing a high homogeneity of the values within each species. The analysis of the monoploid genome sizes (2Cx) revealed interesting results (Table 3): usually within each section, a decrease of the 2Cx value was observed with the increase of polyploid level (e.g. diploid species *F. henriquesii* presented a 2Cx value of 2.93 pg, while octoploid species *F. brigantina* presented a 2Cx value of 2.14 pg). Also, the 2Cx value for species with the same ploidy level seemed to differ between sections. Despite the analyses included only one species, taxa belonging to sections *Subbulbosae* and *Eskia* presented a 2Cx value of 3.75 pg and 3.09 pg, respectively, and seemed to have a higher monoploid genome size (i.e. chromosomes of a basic set are bigger, on average) than taxa belonging to sections *Festuca* and *Aulaxyper*. From these sections, *Aulaxyper* seems to have taxa with the smallest chromosomes, on average. The only taxon from section *Schenodorus*, i.e. *F. arundinacea* ssp. *mediterranea*, had a 2Cx value of 2.67 pg.

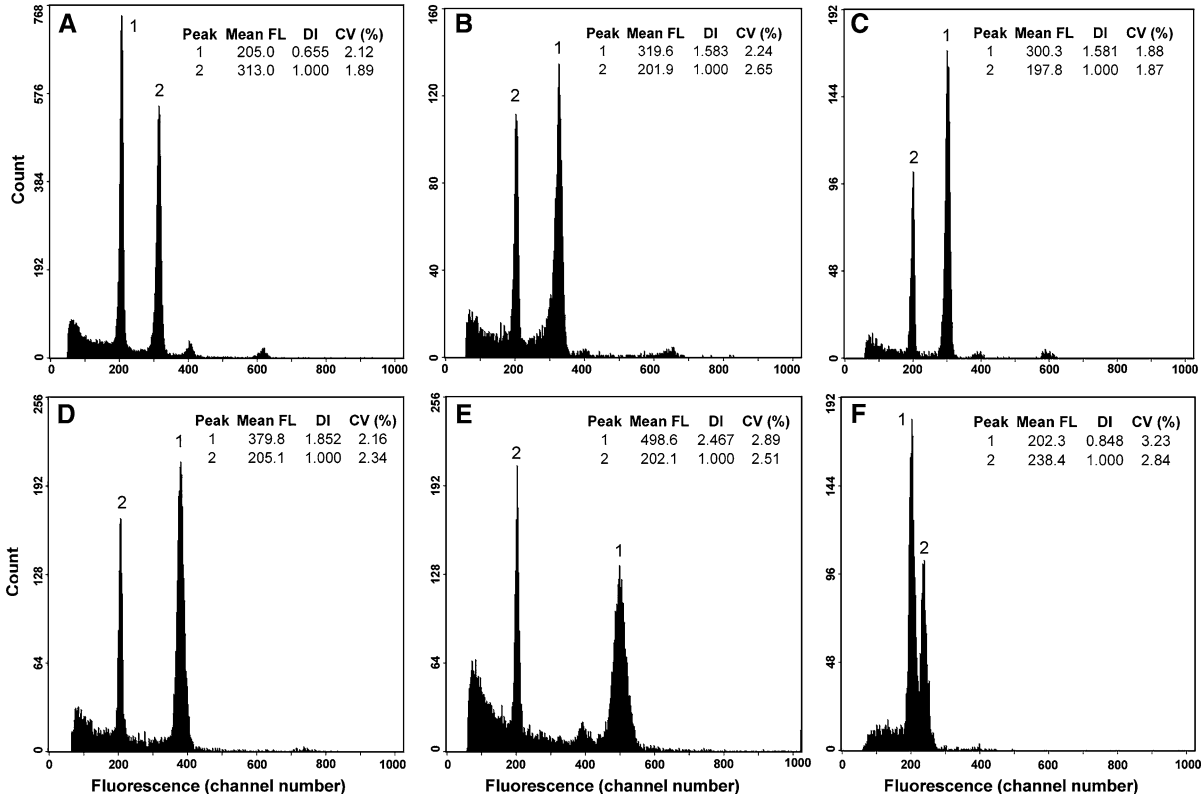

Fig. 2. Flow cytometric histograms of relative fluorescence intensity obtained after simultaneous analysis of nuclei isolated from the internal reference standard and the *Festuca* spp.: **A**. *F. henriquesii* ($2n = 2x = 14$), **B**. *F. ampla* ssp. *ampla* ($2n = 4x = 28$), **C**. *F. rothmaleri* ($2n = 6x = 42$), **D**. *F. brigantina* ($2n = 8x = 56$), **E**. *F. summilusitana* ($2n = 10x = 70$). In these histograms the following peaks are marked: 1 – nuclei at G_0/G_1 phase of sample; 2 – nuclei at G_0/G_1 phase of internal standard (*Pisum sativum* cv. Ctirad with 2C = 9.09 pg DNA in histograms A, C, D and E, and *Zea mays* cv. CE-777 with 2C = 5.43 pg DNA in histogram B). (F) Histogram obtained after simultaneous analysis of nuclei isolated from *F. summilusitana* collected in Serra do Marão (peak 1, $2n = 10x = 70$) and *F. summilusitana* from population C1 (peak 2, $2n = 12x = 84$). The mean channel number (mean FL), DNA index (DI = mean channel number of sample / mean channel number of reference standard) and coefficient of variation value (CV, %) of each peak are also given

Within sections *Festuca* and *Aulaxyper*, the observed chromosome numbers were highly positively correlated with the nuclear DNA content estimations (Fig. 3). Regression coefficients (R^2) of 0.9929 and 0.9781 were obtained for sections *Festuca* and *Aulaxyper*, respectively. Linear regression analyses (y = 3.3183x - 3.0554, section *Festuca*; y = 3.6429x - 6.6568, section *Aulaxyper*) were used to estimate the ploidy level of fescue species within each section. With exception of *F. rothmaleri*, these estimations were in agreement with our observations and with previously reported values. A tetraploid level was estimated for *F. ampla* ssp. *transtagana* ($2n = 4x = 28$).

For each species, differences in nuclear DNA content among populations were not statistically significant (Table 3). The only exception was observed in *F. summilusitana* where two ranges of non overlapping DNA values were obtained: plants growing on schist substrate presented nuclear DNA estimations ranging from 20.50 to 24.00 pg (mean 2C value = 22.69 ± 0.617 pg of DNA) while plants from granite presented 2C values ranging from 24.02 to 27.19 pg (mean 2C value = 25.67 ± 0.754 pg of DNA) (Table 4). These

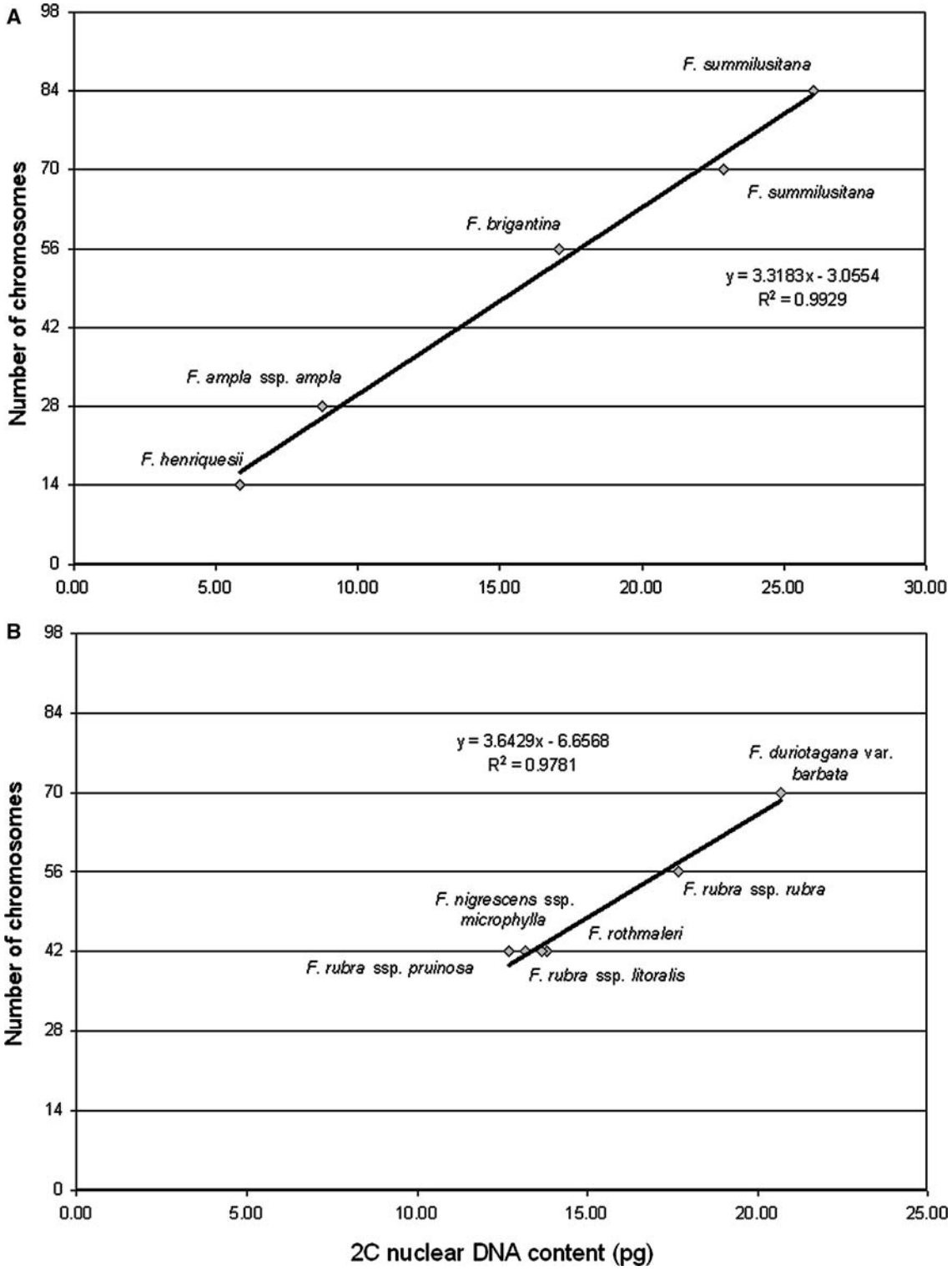

Fig. 3. Linear regression analyses between mean nuclear DNA content and chromosome number for species belonging to sections *Festuca* (**A**) and *Aulaxyper* (**B**) The linear regression equation and coefficient are provided in each graphic

Table 4. Nuclear DNA content estimations in *Festuca summilusitana*[a]

Population	Type of substrate	Nuclear DNA content			
			2C range		
		2C (pg)	Min.	Max.	n
Bragança: Serra da Nogueira	S	22.34 ± 0.431[ab]	21.80	22.89	5
Vila Real: Serra do Marão	S	22.39 ± 0.379[ab]	21.86	22.93	5
Aveiro: Serra do Caramulo – C1	G	25.42 ± 0.625[c]	24.81	26.57	6
Aveiro: Serra do Caramulo – C2	S	22.66 ± 0.286[ab]	22.30	23.06	9
Aveiro: Serra do Caramulo – C3	S	23.36 ± 0.684[b]	22.63	24.00	3
Aveiro: Serra da Freita	S	23.23 ± 0.425[b]	22.59	23.62	5
Coimbra: Serra da Lousã – L1	S	22.93 ± 0.614[ab]	21.38	23.39	9
Coimbra: Serra da Lousã – L2	S	23.06 ± 0.517[ab]	22.08	23.70	7
Coimbra: Serra da Lousã – L3	S	23.25 ± 0.444[b]	22.44	23.80	7
Coimbra: Serra da Lousã – L4	S	23.07 + 0.251[ab]	22.69	23.29	5
Guarda: Serra da Estrela – E1	G	25.37 ± 0.523[c]	24.02	26.24	30
Guarda: Serra da Estrela – E2	G	26.78 ± 0.318[d]	26.40	27.18	5
Guarda: Serra da Estrela – E3	G	26.72 ± 0.367[d]	26.35	27.19	5
Guarda: Serra da Estrela – E4	S	22.28 ± 0.561[a]	20.50	23.44	28
	S	**22.69 ± 0.617**	**20.50**	**24.00**	**83**
	G	**25.67 ± 0.754**	**24.02**	**27.19**	**46**

[a] The values are given as mean and standard deviation of the mean (SD) of the nuclear DNA content (2C in pg) for individuals of each population. Means followed by the same letters (a, b, c or d) are not significantly different at $P < 0.05$ according to the Tukey-Kramer multiple comparison test. The DNA range, defined by the minimum (Min.) and maximum (Max.) value obtained for individuals in each population and the number of analyzed individuals (n) are also provided. For each population the type of substrate where fescue individuals were growing is given (*S* – schist, *G* – granite). In bold, the mean and SD values of nuclear DNA content, the DNA range and the number of individuals according with the type of substrate are given. Please note that there are no overlapping values between individuals of each type of substrate

results were in accordance with the two observed chromosome numbers for this species ($2n = 10x = 70$ and $2n = 12x = 84$), with the lower values corresponding to individuals presenting the decaploid ploidy level and the higher ones, to dodecaploid individuals.

Fluorescence in situ hybridization. Chromosome morphology analysis using FISH was successfully performed for some of the species (*F. henriquesii*, *F. ampla* ssp. *ampla* and *F. brigantina*), where well defined patterns of rDNAs signals were obtained. For others (*F. summilusitana*, *F. rubra* ssp. *litoralis*, *F. duriotagana* var. *barbata* and *F. paniculata* ssp. *multispiculata*), the application of this technique was less successful, as a variable number of rDNA signals was obtained with doubts remaining on their exact number. For *F. henriquesii*, three pairs of chromosomes carrying 45S rDNA were found, one of which was linked with 5S rDNA (Figure 1B). In *F. ampla* ssp. *ampla*, a similar pattern to *F. henriquesii* was obtained (Fig. 1C). In *F. brigantina* four pairs of chromosomes carried both 45S and 5S rDNAs and one pair contained only the 5S rDNA locus. For these three taxa, the location and number of the signals were reproducible. In *F. summilusitana*, while one chromosome pair with 5S rDNA was consistently found, a variable number of chromosomes carrying both 45S and 5S rDNAs (8 to 11 signals, Fig. 1D and E, respectively) was observed. A similar variability was found in *F. paniculata* ssp. *multispiculata*, where two pairs of chromosomes with 5S rDNA were

constantly found, and a variable number of 6 to 8 chromosomes containing the 45S rDNA was obtained (e.g. Fig. 1F, where seven 45S rDNA signals can be observed). In most of the spreads of *F. duriotagana*, var. *barbata* three pairs of chromosomes carried 45S and 5S rDNAs and another three contained only the 45S rDNA locus (Fig. 1G). Nevertheless, in some cases, a different pattern of signals was observed: two pairs of chromosomes carrying 45S and 5S rDNAs, two pairs carrying only the 45S and another two containing only the 5S locus. In *F. rubra* ssp. *pruinosa*, whereas the most common pattern consisted on one pair of chromosomes carrying both 45S and 5S rDNAs, another pair containing 5S rDNA and two chromosome pairs carrying the 45S rDNA, in some spreads the 45S signal was only found in three chromosomes.

Genomic in situ hybridization. Genomic in situ hybridization with hybridization of total genomic DNA of *F. henriquesii* on chromosome spreads of *F. ampla* ssp. *ampla*, *F. brigantina*, *F. summilusitana*, *F. paniculata* ssp. *multispiculata*, *F. rubra* ssp. *litoralis* and *F. duriotagana*, var. *barbata* was in most cases unsuccessful. An unpredictable and unexplained homology with whole chromosomes, excluding the telomeric regions, was found when total genomic DNA of *F. henriquesii* was hybridized with *F. ampla* ssp. *ampla* chromosomes. With the exception of *F. paniculata* ssp. *multispiculata* chromosomes, to which there was no hybridization, hybridization with other species resulted in a specific number of unexpected and highly localised hybridization signals (Fig. 1H and I), with no evident hybridization in the remaining parts of the chromosomes.

Discussion

Chromosome counting. Cytologically, *Festuca* is a diversified genus that presents ploidy levels ranging from diploid ($2n = 2x = 14$) to dodecaploid ($2n = 12x = 84$), with about 74% of the species occurring as polyploids (Seal 1983). Eurasia appears as a primary diversification centre of this genus, with the occurrence of a higher percentage of species with lower ploidy levels (especially, diploid and tetraploid, Dubcovsky and Martínez 1992). Nevertheless, whereas this is clearly evident for species belonging to the Spanish flora with a predominance of the diploid level in most sections (mostly distributed in the eastern half of Iberian Peninsula) (de la Fuente and Ortúñez 2001, de la Fuente et al. 2001), in the Portuguese Continental Flora only one diploid species is recognized, i.e. *F. henriquesii*. The diploid level of this species was confirmed by our work and, as suggested for other diploid species, it seems that this taxon presents ancient and stable characters (Ferrero and de la Fuente 1996, de la Fuente et al. 2001). Ortúñez and de la Fuente (2004) suggested a correlation between ploidy level and geographical distribution, with diploid species being orophilous and having a restricted distribution in the Iberian Peninsula, whereas species of higher ploidy levels present a wider distribution area. This is the case of *F. henriquesii*, an endemic taxon from Serra da Estrela and Serra do Gerês, Peneda and Barroso (Ferrero and de la Fuente 1996). Nevertheless, species from section *Festuca* with higher ploidy levels, as *F. summilusitana*, are also restricted to a reduced area of distribution.

Two ploidy levels, tetraploid and hexaploid, are currently assigned to *F. ampla*, with the lower level corresponding to populations in the boundaries of distribution and the higher one to populations in the central area (de la Fuente et al. 2001). However, in our work we were only able to detect tetraploid individuals, despite having analyzed one population in each of the suggested areas of distribution. The chromosome number of *F. rothmaleri* estimated in our work ($2n = 6x = 42$) differed from the reported ploidy level for this species, i.e., octoploid (Al-Bermani et al. 1992). Little information on the studied specimens was provided by the authors and in light with the data provided in the study of de la Fuente et al. (2001), where hexaploid specimens of *F. ampla* were recognized as *F. rothmaleri*, it seems that the occurrence of the hexaploid level in *F. rothmaleri* should not be discarded.

Nevertheless, further studies should be done to confirm the ploidy level of this species.

The chromosome number of *F. summilusitana* has also been subject of constant updates and rectifications over the years. This species after being first identified as hexaploid (Ortúñez and de la Fuente 1995) was recently given a decaploid level by de la Fuente and Ortúñez (2000) and de la Fuente et al. (2001). In our work, two ploidy levels were obtained both by chromosome counting and flow cytometric estimation: decaploid ($2n = 2x = 70$) and dodecaploid ($2n = 2x = 84$). Whereas our results are in accordance with those of de la Fuente et al. (2001) for the populations of Serra do Marão, Serra da Nogueira and Serra da Estrela ($2n = 10x = 70$), for the last locality, in populations of granite substrate, a dodecaploid level was found. Dodecaploid populations were also found in granite substrate of Serra do Caramulo. The occurrence of such a high ploidy level in *Festuca* genus is almost new, as until now it was only reported for *F. gamisansii* ssp. *gamisansii* (Portal 1999). Nevertheless, the accuracy of this estimation has been questioned by Foggi et al. (2005).

The chromosome number of *F. duriotagana* var. *barbata* was first determined in this work ($2n = 10x = 70$) and although such a high ploidy level has already been reported for another species of this section (*F. nevadensis*; Galland 1988), it can be an important character to clarify the taxonomic position of this taxon. Some authors suggested the inclusion of individuals of *F. duriotagana* on the plastic variability of *F. ampla* (section *Festuca*; Anonymous 2006), while others had no doubt on classifying it as a separate taxon of section *Aulaxyper* (Al-Bermani et al. 1992, Cebolla Lozano and Rivas Ponce 2003). The chromosome number determined in this work seems to sustain the latter hypothesis, as such a high number of chromosomes have never been found for individuals of *F. ampla*.

Nuclear DNA content estimations. Flow cytometry was only sporadically used for estimating the genome size of fescue species (Huff and Palazzo 1998, Arumuganathan et al. 1999). Huff and Palazzo (1998) studied the nuclear DNA content of ten fescue species belonging to sections *Festuca* and *Aulaxyper* and also obtained a positive correlation between DNA contents and chromosome numbers. In species of the same section and for each ploidy level, mean DNA content estimations obtained by Huff and Palazzo (1998) were generally lower (up to 17%) than those obtained in our work. Differences in nuclear DNA estimations among laboratories are common more than expected (Doležel et al. 1998) and can be justified by the use of different methodologies and reference standards (e.g. Huff and Palazzo 1998, used chicken red blood cells with 2.33 pg/2C of DNA as reference standard). In a recent study by Šmarda and Bureš (2006) intraspecific DNA content variability in *F. pallens* has been documented with authors suggesting that the differences in relative genome size were correlated with geographical coordinates. Differences in the amount of DNA content among populations were also observed by Huff and Palazzo (1998). These authors suggested that B-chromosomes could be responsible for some of the reported variation. With exception of *F. summilusitana*, our data was highly homogeneous among populations. The supposed intraspecific variability observed in *F. summilusitana* was seen to be related with the occurrence of two ploidy levels in this species. The apparent preference of each "conspecific" ploidy race to a particular type of substrate (granite and schist) and the importance of the chromosome number in species delimitation within this genus suggests a possible separation of *F. summulistana* in two independent taxa. Morphoanatomic studies, necessary to confirm this hypothesis are already under development in our laboratory.

The analysis of the monoploid genome size revealed that usually, species with higher ploidy levels presented a reduction in DNA amount relative to species with lower ploidy levels. A similar observation was obtained by Seal (1983) who measured the nuclear DNA content of several *Festuca* species using

Feulgen densitometry. Recently, the loss of DNA after polyploid formation, i.e., genome downsizing, was suggested as a widespread phenomenon of considerable biological significance (Leitch and Bennett 2004). Possible explanations for this phenomenon include the elimination of non-coding DNA sequences during polyploid formation (e.g. Shaked et al. 2001). Seal (1983) also compared for each ploidy level the mean DNA contents within each section and observed differences among taxa from four sections. As in our work, this author observed that species from section *Schenodorus* presented higher mean DNA content than species from section *Aulaxyper*. Despite the interesting results obtained here regarding genome downsizing in polyploid species of *Festuca* and the remarkable nuclear DNA content differences obtained among sections of this genus, an extensive survey is still required to fully understand the patterns of genome size evolution in this genus.

Fluorescence in situ hybridization. To date only three diploid species have been identified as progenitors of polyploid fescue species. *F. pratensis*, as a progenitor of hexaploid *F. arundinacea*, was the first species to be identified, by means of RFLPs (Xu and Sleper 1994) and GISH (Humphreys et al. 1995). Recently, Harper et al. (2004) used FISH to confirm the ancestor character of *F. scariosa* and suggested that *F. altissima* was a good candidate for some polyploid species from section *Schenodorus*. The ancient and paleoendemic character of the diploid species *F. henriquesii*, suggested that it could be a good candidate as an ancestor of some polyploid species from section *Festuca*, where it is included. Indeed, FISH analyses seemed to point out in that direction as the number and distribution of rDNA sites were very similar between *F. henriquesii* and *F. ampla*. In species with higher ploidy levels, i.e., *F. brigantina* and *F. summilusitana*, this was not so evident, as the two pairs of chromosomes carrying the 45S rDNAs found in *F. henriquesii* were absent. The pattern of rDNA sites was similar between *F. brigantina* and *F. summilusitana*, with the only exceptions being some *F. summilusitana* individuals that presented a higher and variable number of chromosomes carrying both 45S and 5S rDNAs. Variation in the number and position of rDNA sites has been already observed in plants, but most of the works reported changes in the position of 45S and 5S rDNAs, and not in their number (e.g. Schubert and Wobus 1985). Doubts remained on whether this variation was real or if it was due to technical problems of the FISH procedure. Even so, it was evident that species from *Festuca* section presented different rDNA patterns from those of *Subbulbosae* and *Aulaxyper*. Chromosomes of *F. paniculata* ssp. *multispiculata* were clearly larger than those of other species which is in accordance with the FCM data. The number and distribution of rDNA sites was also very different from any other species, as no chromosomes carrying both rDNAs were observed. A variable number of 45S rDNAs was observed, with most of the genotypes being heterozygous with an extra seventh site on one chromosome. An odd number of 45S rDNA was also observed in *lolium perenne* and *L. rigidum* var. *rigidum*, but doubts remained on the nature of these additional sites (Thomas et al. 1996). Some variation on the number of rDNA sites was also observed in *F. rubra* ssp. *litoralis* and *F. duriotagana* var. *barbata*. This and the high number of chromosomes present in some of the species made the analysis and interpretation of the results a difficult task, and one could consider that in this case the application of FISH methodology to wild species of *Festuca* has not been as successful as expected. Nevertheless, this method has been of utmost importance in phylogeny studies of some fescue species (Thomas et al. 1997) and it was already applied with success in wild diploid species from section *Montanae* (Harper et al. 2004).

Genomic in situ hybridization. The application of genomic in situ hybridization to study possible phylogenetic relationships in wild fescue species revealed some unexpected

results. The unpredictable and unexplained homology with whole chromosomes found when total genomic DNA of *F. henriquesii* was hybridized with *F. ampla* ssp. *ampla* chromosomes may well be an artifact. With the exception of *F. paniculata* ssp. *multispiculata* chromosomes, to which there was no hybridization, hybridization with other species resulted in a specific pattern of highly localised hybridization signals, possibly related to heterochromatin. Takahashi et al. (1999) also obtained highly localized signals on maize chromosomes, when genomic DNA from four wild relatives was used as a probe. The lack of hybridization in *F. paniculata* ssp. *multispiculata* seems to confirm the previously established separation of these two species in separate and distant sections. Despite of some uncertainties, it seems that either the studied fescue species evolved so much that there are very few homologous zones remaining between them and *F. henriquesii*, or that this species is not an ancestral progenitor of the studied polyploid *Festuca* species. Further phylogenetic studies should focus on the remaining diploid species present in the Iberian Peninsula.

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