



Genome size of grass *Festuca* mountain species from the southwestern European Pyrenees: variation, evolution, and new assessments

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Abstract

Festuca is one of the most ecologically and economically important genera of temperate grasses. Species of its type subgenus, *Festuca*, are common components of cool-seasonal pastures and are especially frequent in mountainous areas, where there are often several morphologically similar species that grow in the same or comparable habitats and sometimes live in sympatry. Nuclear DNA assessments by flow cytometry can be used to identify taxa and uncover new ploidy levels in species complexes for which new chromosome data are provided or previous chromosome counts and genome sizes are known. Holoploid (2C) values of newly studied Pyrenean *Festuca* subgen. *Festuca* sects. *Eskia*, *Festuca* and *Aulaxyper* species fall within the expected ranges for these taxonomic groups and include 2x, 4x, 6x and 8x ploidy levels. Monoploid (1Cx) genome sizes of diploids and polyploids are larger in the species of the more ancestral *F.* sect. *Eskia* group showing a decreasing trend in the species of the more recently evolved *F.* sects. *Festuca* and *Aulaxyper* lineages. 1Cx values of high polyploid *Aulaxyper* taxa are among the smallest of the three *Festuca* sections, corroborating previous findings. Our analysis provides new genome size values and inferred ploidy levels for hexaploid *F. heteromalla* and octoploid *F. trichophylla* and highlights the genomic and ecological differentiation of tetraploid *F. gautieri* subsp. *gautieri* from diploid *F. gautieri* subsp. *scoparia*.

Keywords *Festuca* · Evolution of monoploid genomes · Flow cytometry · Nuclear DNA amount · Ploidy level · Pyrenees

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Introduction

Festuca L. (Poaceae) is one of the main genera of the temperate pooid grasses, both in number of species and in ecological and economic importance, as it encompasses some of the world’s best forages, pastures and lawn species. The genus includes more than 500 species (*Festuca*; Catalán 2006), considering both broad and fine-leaved *Festuca* taxa, and up to 644 accepted species, considering fine-leaved subtribe Loliinae taxa [*Festuca* and several minor subsumed genera (Plants of the World On-line <http://www.plantsoftheworldonline.org/taxon/urn:lsid:ipni.org:names:328907-2>, as per 5th April 2023)]. Fescues are native to temperate zones and mountainous subtropical and tropical regions of the five continents (Catalán 2006; Minaya et al. 2017). Phylogenetic analyses have consistently detected two main lineages within *Festuca* and its close genera of subtribe Loliinae, the broad-leaved and the fine-leaved clades (Catalán et al. 2004, 2007; Inda et al. 2008; Minaya et al. 2017; Moreno-Aguilar et al. 2020, 2022a). Taxonomically, *Festuca* has been classified into 11 subgenera with several sections within each, according to Alexeev’s revision of world fescues (Catalán 2006;

Moreno-Aguilar et al. 2022b). Nine of those subgenera were assigned to broad-leaved *Festuca* and two to fine-leaved *Festuca*, including the largest subgenus *Festuca* (Catalán 2006; Moreno-Aguilar et al. 2022b). Subgenus *Festuca* includes sections *Eskia* Willk., *Festuca* (*F. ovina* group) and *Aulaxyper* Dumort. (*F. rubra* group) that contain taxa broadly distributed in Eurasia. Biogeographic studies have shown that the Loliinae originated in the Palearctic region and that the Mediterranean—SW Asia area has been the main center of diversification of both broad- and fine-leaved Loliinae (Inda et al. 2008, 2014; Minaya et al. 2017). The Iberian Peninsula and the Pyrenees are one of the richest regions in *Festuca* species, accounting for near 100 taxa of which 40% are endemics (Kerguélen and Plonka 1989; Devesa et al. 2013, 2020). Most of the Iberian and Pyrenean fescues grow in mountainous areas where there are often several different species living together.

Cytogenetic analyses have provided a cue for the analysis of evolutionary trends and speciation processes within subtribe Loliinae. Catalán (2006) showed the first comparative study of molecular phylogenetic inference and karyotype evolution in *Festuca*, a genus with a uniform chromosome base number of $x=7$ and ploidy levels ranging from $2x$ to $12x$. Recently, a new ploidy level of $14x$ was recorded within *F. sect. Festuca* (*F. yvesii* subsp. *summilusitana* $2n=14x=98$; Martínez-Sagarra et al. 2021). *Festuca* exhibits striking differences in monoploid genome sizes ($1Cx$) between the broad- and the fine-leaved lineages, showing a 2.5-fold range decrease in chromosome size and C-values from more ancestral broad-leaved lineages (*Drymanthele*, *Scariosa*, *Subbulbosae*) to more recently evolved fine-leaved lineages (*Festuca*, *Aulaxyper*) (Catalán 2006). The early diverging fine-leaved *Eskia* lineage reveals an intermediate karyotype pattern between the broad- and fine-leaved groups (Catalán et al. 2006). Šmarda et al. (2008) further hypothesized that the Loliinae lineage probably experienced a two-fold genome and chromosome size enlargement and a GC enrichment with respect to its close relatives, which was subsequently followed by dramatic reductions, especially in the rapidly evolving fine-leaved group. Loureiro et al. (2007) and Šmarda et al. (2008) also detected genomic downsizing in the fine-leaved Loliinae and in the polyploids, with the genomic losses being more pronounced in allopolyploids with large progenitor genomes than in autopolyploids with small progenitor genomes. Moreno-Aguilar et al. (2022a) found a large and significant correlation between the sizes of the Loliinae genomes and their respective amounts of repetitive elements (especially Retand and Angela retrotransposons). These authors also showed that changes in the Loliinae genome sizes were likely caused by gains or losses in their repetitive elements. Their analysis of the evolution of the Loliinae repeatome suggested that the large genome sizes of broad-leaved diploid Loliinae were

likely the result of past allopolyploidizations followed by diploidizations, while the massive repeatome and genome contractions observed in some polyploid lineages (e. g., *F. sect. Aulaxyper*, *F. subgen. Schedonorus*) were probably the consequence of large genomic rearrangements and losses in these highly hybridogenic lineages (Moreno-Aguilar et al. 2022a). Within *Festuca* subgen. *Festuca*, the two taxonomically complex and most broadly studied groups are *F. sects. Festuca* and *Aulaxyper* Dumort. (Catalán 2006; Catalán et al. 2004, 2006, 2007). A third section that contains a relatively high number of taxa distributed in mountainous areas of the circum-Mediterranean region is *F. sect. Eskia* Willk. (Torrecilla et al. 2003, 2013). Some lineages experienced complex reticulate evolutionary processes, including homoploid and heteroploid hybridizations and polyploidizations (Catalán 2006; Torrecilla et al. 2013; Marques et al. 2016). Some species of fine-leaved fescues are morphologically very similar, and their classification is still controversial.

Numerous karyological and cytogenetic studies have been conducted in *Festuca* subgen. *Festuca* in Europe (summarized in Kerguélen and Plonka 1989; Fuente and Ortúñez 1988, 2001; Fuente et al. 2001; Ortúñez and Fuente 1995, 2004; Ferrero and Fuente 1996; Loureiro et al. 2007; Šmarda et al. 2008; Devesa et al. 2013, 2020; Martínez-Sagarra et al. 2021, 2022), sometimes to identify the species or to clarify the relationships between these taxa growing in the mountains and to assign them to the species or complexes within a phylogenetic context. Nuclear DNA assessments by flow cytometry can be a useful tool to estimate the ploidy levels of the samples and to carry out an initial selection of the studied taxa, especially in areas where they coexist and in species complexes in which previous chromosome counts have already been carried out. Despite the numerous taxa treated in previous studies (<https://cvalues.science.kew.org/search/angiosperm>, accessed 5th April 2023), some of the species growing in the Pyrenees and other mountain systems have not been thoroughly surveyed yet.

This study deals with the cytogenetic analysis of taxonomically complex SW European Pyrenean *Festuca* subgen. *Festuca* plants from sections *Eskia*, *Festuca* and *Aulaxyper*. The understanding gained from genome size assessments, combined with careful observation regarding morphology and habitat, in addition to our present knowledge of the chromosome number, will contribute toward clarifying the identity of some of the more critical taxa. The aims of our research are: (i) to assess the genome size variation between and within some groups of Pyrenean fescues, and (ii) to test if the genome size assessments fit previous hypotheses on different evolutionary trends of monoploid, $1Cx$, genomes for species of each section and if they could help discover new ploidy levels and distinguish or discriminate between some related species.

Material and methods

Plant material

Plant material studied together with the localities of provenance is summarized in Table 1. The herbarium vouchers of these taxa are deposited in the herbaria of the Botanical Institute of Barcelona (BC) and of the Escuela Politécnica Superior de Huesca (University of Zaragoza).

DNA content assessment and chromosome counting

Nuclear DNA content was assessed for 102 individuals of 33 populations belonging to 14 taxa.

Fresh young leaves of the plants were co-chopped using a razor blade with an internal standard in the proportions 2:1 in 1200 μ l of LB01 buffer (Doležel et al. 1989) with 0.5% Triton X-100 and supplemented with 100 μ g/ml ribonuclease A (RNase A, Boehringer, Meylan, France) in a Petri dish. *Pisum sativum* L. ‘Express Long’ (2C = 8.37 pg; Marie and Brown 1993) was used as internal standard and was first analyzed separately in 600 μ l of LB01 buffer in order to locate the peak position of the standard in the fluorescence histogram. Nuclei were filtered through a 70- μ m nylon filter in order to eliminate cell debris before the addition of 36 μ l of propidium iodide (1 mg/ml, solution in water; Invitrogen Eugene, Oregon, USA). From one to five individuals were used per population (Table 1), and two samples of each individual collected were extracted and measured independently. Fluorescence analysis was carried out using an Epics XL flow cytometer (Coulter Corporation, Hialeah, Florida, USA) at the Centres Científics i Tecnològics de la Universitat de Barcelona, with the standard configuration as described in Garnatje et al. (2006). Acquisition was stopped at 8000 nuclei. The DNA content was calculated assuming a linear correlation between the fluorescence signals (of the stained nuclei) and DNA amount. Mean and standard deviations were calculated for 2C values of each population. The mean of the half-peak coefficient of variation (HPCV) was also calculated for both target plant and internal standard. A nonparametric Kruskal–Wallis test was performed to compare the 1Cx values of the three studied sections after a Shapiro–Wilk test of normality.

Chromosome counts were carried out according to the following protocol. Root tips from plants growing in pots were pretreated in distilled water for 24 h at 4 °C. This material was fixed in a solution of absolute ethanol and glacial acetic acid (3:1) for 30 min in the dark. Subsequently, the material was transferred to a new solution of absolute ethanol and glacial acetic acid (3:1) for 4 h

in the dark. After this step, the material was conserved at –20 °C in the same solution. For chromosome counts, root tip meristems were excised, hydrolyzed for 10–12 min on 1N HCl at 60 °C, washed with distilled water, stained with 1% aceto-orcein and squashed on a drop of 9:1 45% acetic acid: glycerol. Slides were observed with a Zeiss Axioplan microscope, and the best metaphase plates were photographed with an AxioCam HRm camera.

Results and discussion

Data on the genome size assessments (2C and 1Cx values), chromosome numbers (new data and previously published data), and estimated ploidy levels for the studied taxa are shown in Table 1. Some ploidy levels have been inferred from genome size values compared to genome sizes of other species of *Festuca* included in this study or analyzed by other authors (Kerguelen and Plonka 1989; Loureiro et al. 2007; Šmarda et al. 2008; Devesa et al. 2020; Martínez-Sagarra 2018; Martínez-Sagarra et al. 2021, 2022; Moreno-Aguilar et al. 2022a) [Table 1, asterisk: estimations based on the congruence with chromosome number, “ploidy level”; no asterisk: estimations based solely on genome size, “DNA-ploidy level”]. Overall, the 2C values range from 4.41 pg of diploid *F. marginata* subsp. *andresmolinae* to 16.36 pg of octoploid *F. laevigata* sensu lato, both within *F. sect. Festuca*, and monoploid (1Cx) values range from 1.85 pg of *F. nigrescens* subsp. *microphylla* (*F. sect. Aulaxyper*) to 2.92 pg of *F. gautieri* subsp. *scoparia* (*F. sect. Eския*). However, the 2C/1Cx values and inferred ploidy levels (2x, 4x, 6x, 8x) of the studied taxa vary considerably among sections (Table 1), confirming the previous findings of Catalán (2006), Šmarda et al. (2008) and Moreno-Aguilar et al. (2022a) on the decreasing genome-size trend from *F. sect. Eския*, to *F. sects. Festuca* and *Aulaxyper*, and the larger 1Cx reduction observed for polyploid genomes in *F. sect. Aulaxyper*. The nonparametric Kruskal–Wallis test shows statistically significant differences ($p < 0.0001$) between 1Cx values of the *F. sect. Eския* and those of the other two sections, *Festuca* and *Aulaxyper*. The average of HPCV (half peak coefficient of variation) of the investigated plant is 2.29 and that of the standard is 3.46, most values being below 5%, accounting for data reliability.

Festuca sect. *Eския*. Within the *F. sect. Eския* group, mean monoploid genome values (1Cx) of *F. eskia* (2.79 pg) and *F. gautieri* subsp. *scoparia* (2.85 pg) are highly similar; the two sister species constitute one of the earliest diverging lineages of the fine-leaved clade (Inda et al. 2008; Minaya et al. 2017; Moreno-Aguilar et al. 2020). 2C values for nuclear DNA amount of diploid *F. eskia* (5.45–5.69 pg; $2n = 2x = 14$; Table 1) fall within those reported in the broadly sampled study of Marques et al. (2016). This subalpine species,

Table 1 *Festuca* samples included in the present study

Taxon	Locality, collectors, date of collection and coordinates	N	2C (pg) ± SD ^a	1Cx (pg)	1Cx (Mbp) ^b	Ploidy level	2n	Chromosome data source
<i>Festuca</i> L. subgenus <i>Festuca</i> Sect. <i>Eskia</i> Willk.								
<i>F. eskia</i> Ramond ex DC.	Spain: Aragon, Huesca, Sallent de Gállego, <i>Zabalgogezakoa</i> and <i>Catalán</i> , Jul 2014; 42°47'57.1"N 0°24'51.7"W	2	5.45 ± 0.28 [♦]	2.73	2666	2x [*]	14	Marques et al. (2016)
<i>F. eskia</i> Ramond ex DC.	Spain: Aragon, Huesca, Sallent de Gállego, Formigal, <i>Zabalgogezakoa</i> and <i>Catalán</i> , Jul 2014; 42°48'11.4"N 0°24'55.5"W	1	5.690	2.85	2782	2x [*]	14	Marques et al. (2016)
<i>F. eskia</i> Ramond ex DC.	Spain: Aragon, Huesca, Benasque, Baños de Benasque, <i>Zabalgogezakoa</i> and <i>Catalán</i> , Jul 2014; 42°39'39.69"N 0°35'08.58"E	1	5.590	2.80	2734	2x [*]	14	Marques et al. (2016)
<i>F. gautieri</i> (Hack.) K.Richt. subsp. <i>gautieri</i>	Spain: Catalonia, Toses, Collada de Toses, <i>Garnatje</i> , Aug 2013; 42°20'49"N 1°58'42"E	5	10.53 ± 0.32 [♦]	2.63	2575	4x [*]	28	This study
<i>F. gautieri</i> (Hack.) K.Richt. subsp. <i>gautieri</i>	Spain: Catalonia, Fogars de Monclús, Montseny, <i>Garnatje</i> and <i>Vallès</i> , Aug 2013; 41°46'41"N 2°26'02"E	5	10.58 ± 0.38	2.65	2587	4x [*]	28	This study
<i>F. gautieri</i> (Hack.) K.Richt. subsp. <i>gautieri</i>	France: Pyrénées Orientales, Vallcebollera, Puigmal, piste forestière 3, <i>Garnatje</i> and <i>Tenas</i> , Aug 2013; 42°22'51"N 2°02'58"E	4	10.3 ± 0.21	2.58	2518	4x [*]	28	This study
<i>F. gautieri</i> (Hack.) K.Richt. subsp. <i>gautieri</i>	France: Pyrénées Orientales, Vallcebollera, Puigmal, piste forestière 4, <i>Garnatje</i> and <i>Tenas</i> , Aug 2013; 42°23'02"N 2°02'57"E	5	10.86 ± 0.46 [♦]	2.72	2655	4x [*]	28	This study
<i>F. gautieri</i> (Hack.) K.Richt. subsp. <i>gautieri</i>	France: Pyrénées Orientales, Vallcebollera, Puigmal, piste forestière 2, <i>Garnatje</i> and <i>Tenas</i> , Aug 2013; 42°22'57"N 2°03'11"E	4	10.86 ± 0.2	2.72	2655	4x [*]	28	This study
<i>F. gautieri</i> subsp. <i>scoparia</i> (A.Kern. & Hack.) Kerguelen	Spain: Catalonia, Vall de Boí, Boí 1, <i>Garnatje</i> and <i>Luque</i> , Aug 2013; 42°30'31"N 0°52'49"E	1	5.500	2.75	2690	2x [*]	14	Marques et al. (2016)
<i>F. gautieri</i> subsp. <i>scoparia</i> (A.Kern. & Hack.) Kerguelen	Spain: Catalonia, Vall de Boí, Boí 2, <i>Garnatje</i> and <i>Luque</i> , -Aug 2013; 42°29'52"N 0°51'46"E	5	5.75 ± 0.14	2.88	2812	2x [*]	14	Marques et al. (2016)
<i>F. gautieri</i> subsp. <i>scoparia</i> (A.Kern. & Hack.) Kerguelen	Spain: Catalonia, Vall de Boí, Cavallers, <i>Garnatje</i> and <i>Luque</i> , Aug 2013; 42°34'49"N 0°51'18"E	5	5.75 ± 0.27	2.88	2812	2x [*]	14	Marques et al. (2016)

Table 1 (continued)

Taxon	Locality, collectors, date of collection and coordinates	N	2C (pg) ± SD ^a	1Cx (pg)	1Cx (Mbp) ^b	Ploidy level	2n	Chromosome data source
<i>F. gautieri</i> subsp. <i>scoparia</i> (A.Kern. & Hack.) Kerguelén	Spain: Catalonia, Pont de Suert, Llevata, <i>Garnatje</i> and <i>Luque</i> , Aug 2013; 42°22'14"N 0°48'33"E	5	5.76 ± 0.16	2.88	2817	2x*	14	Marques et al. (2016)
<i>F. gautieri</i> subsp. <i>scoparia</i> (A.Kern. & Hack.) Kerguelén	Spain: Aragon, Huesca, Sallent de Gállego, <i>Catalán</i> , Aug 2014; 42°46'13.39"N 0°21'49.00"W	5	5.61 ± 0.15 [♦]	2.81	2743	2x*	14	Marques et al. (2016)
<i>F. gautieri</i> subsp. <i>scoparia</i> (A.Kern. & Hack.) Kerguelén	Spain: Catalonia, Gavet de la Conca, Montsec 2, <i>Garnatje</i> and <i>Luque</i> , Aug 2013; 42°02'12"N 1°00'01"E	2	5.72 ± 0.04 [♦]	2.86	2797	2x*	14	Marques et al. (2016)
<i>F. gautieri</i> subsp. <i>scoparia</i> (A.Kern. & Hack.) Kerguelén	Spain: Aragon, Huesca, Benasque, <i>Zabalgoatzkoa</i> and <i>Catalán</i> , Jul 2014; 42°39'39.69"N 0°35'08.58"E	3	5.84 ± 0.08	2.92	2856	2x*	14	Marques et al. (2016)
Sect. <i>Festuca</i>								
<i>F. airoides</i> Lam.	France: Pyrénées Orientales, Vallcebol-lera, Coma Morena, <i>Garnatje</i> and <i>Tenas</i> , Aug 2013; 42°21'25"N 2°01'23"E	4	4.45 ± 0.06	2.23	2176	2x	14	Šmarda et al. (2008) and Devesa et al. (2020)
<i>F. inops</i> De Not.	Spain: Catalonia, Monistrol de Montserrat, <i>Montserrat</i> , <i>Garnatje</i> and <i>Luque</i> , Aug 2013; 41°36'41"N 1°48'48"E, BC990686	3	9.06 ± 0.52	2.27	2215	4x	28	Fuente et al. (2001) (sub <i>F. gracilior</i>); Devesa et al. (2020)
<i>F. laevigata</i> Gaudin s.l.	Spain: Catalonia, Vall de Boí, <i>Garnatje</i> and <i>Luque</i> , Aug 2013; 42°30'31"N 0°52'49"E, BC990674	1	16.24	2.03	1985	8x	56	Kerguelén and Plonka (1989)
<i>F. laevigata</i> Gaudin s.l.	Spain: Catalonia, Vall de Boí, Cavallers, <i>Garnatje</i> and <i>Luque</i> , Aug 2013; 42°34'49"N 0°51'18"E, BC990681	5	15.83 ± 1.07	1.98	1935	8x	56	Kerguelén and Plonka (1989)
<i>F. laevigata</i> Gaudin s.l.	Spain: Aragon, Huesca, Benasque, <i>Zabalgoatzkoa</i> and <i>Catalán</i> , Jul 2014; 42°39'39.69"N 0°35'08.58"E	2	15.58 ± 0.32	1.95	1905	8x	56	Kerguelén and Plonka (1989)
<i>F. laevigata</i> Gaudin s.l.	Spain: Aragon, Huesca, Olvena, <i>Zabalgoatzkoa</i> and <i>Catalán</i> , Jul 2014; 42°06'19.05"N 0°17'18.85"E	1	16.360	2.05	2000	8x	56	Kerguelén and Plonka (1989)
<i>F. ochroleuca</i> Timb.-Lagr. subsp. <i>bigorronensis</i> (St.-Yves) Kerguelén	Spain: Aragon, Huesca, Formigal, <i>Zabalgoatzkoa</i> and <i>Catalán</i> , Jul 2014; 42°48'11.4"N 0°24'55.5"W, BC905790	1	12.120	2.02	1976	6x		
<i>F. ochroleuca</i> Timb.-Lagr. subsp. <i>bigorronensis</i> (St.-Yves) Kerguelén	Spain: Aragon, Huesca, Formigal, <i>Zabalgoatzkoa</i> and <i>Catalán</i> , Jul 2014; 42°48'11.4"N 0°24'55.5"W, BC990676	4	12.16 ± 0.05	2.03	1982	6x		

Table 1 (continued)

Taxon	Locality, collectors, date of collection and coordinates	N	2C (pg) ± SD ^a	1Cx (pg)	1Cx (Mbp) ^b	Ploidy level	2n	Chromosome data source
<i>F. marginata</i> (Hack.) K. Richt. subsp. <i>andresmolinae</i> Fuente & Ortúñez	Spain: Catalonia, Lleida, Gavet de la Conca, Montsec 2, Garnatje and Luque, Aug 2013; 42°02'12"N 1°00'01"E, BC990684	4	4.41 ± 0.13 [♦]	2.21	2156	2x	14	Fuente and Ortúñez (1993) and Devesa et al. (2020)
<i>F. yvesii</i> Semmen & Pau subsp. <i>yvesii</i>	Spain: Catalonia, Fogars de Montclús, Montseny, Garnatje and Vallès, Aug 2013; 41°46'41"N 2°26'02"E	5	11.89 ± 0.57	1.98	1938	6x	42	Kerguelen (1975) and Kerguelen and Plonka (1989)
Sect. <i>Aulaxyper</i> Dumort.								
<i>F. heteromalla</i> Pourr.	Spain: Aragon, Huesca, Formigal, <i>Zabalgoizeakoa</i> and <i>Catalán</i> , Jul 2014, University of Zaragoza herbarium; 42°47'56.4"N 0°23'59.2"W	2	13.61 ± 0.08	2.27	2218	6x	42	Kerguelen and Plonka (1989) (sub <i>F. heteromalla</i> s.l., p. 259)
<i>F. nigrescens</i> Lam. subsp. <i>nigrescens</i>	Spain: Aragon, Huesca, Formigal, <i>Zabalgoizeakoa</i> and <i>Catalán</i> , Jul 2014; 42°48'11.4"N 0°24'55.5"W	2	12.22 ± 0.79	2.04	1992	6x	42	Kerguelen and Plonka (1989) and Šmarda et al. (2008)
<i>F. nigrescens</i> Lam. subsp. <i>nigrescens</i>	Spain: Aragon, Huesca, Benasque, Baños de Benasque, <i>Zabalgoizeakoa</i> and <i>Catalán</i> , Jul 2014; 42°39'39.69"N 0°35'08.58"E, BC990678	2	12.07 ± 0.05	2.01	1967	6x	42	Kerguelen and Plonka (1989) and Šmarda et al. (2008)
<i>F. nigrescens</i> subsp. <i>microphylla</i> (St.-Yves ex Coste) Markgr.-Dann.	Spain: Catalonia, Vall de Boí, Boí 1, <i>Garnatje</i> and <i>Luque</i> , Aug 2013; 42°30'31"N 0°52'49"E, BC990683	5	11.65 ± 0.43	1.94	1899	6x	42	Kerguelen and Plonka (1989) and Fuente et al. (1997)
<i>F. nigrescens</i> subsp. <i>microphylla</i> (St.-Yves ex Coste) Markgr.-Dann.	Spain: Catalonia, Vall de Boí, Boí 2, <i>Garnatje</i> and <i>Luque</i> , Aug 2013; 42°29'52"N 0°51'46"E, BC990677	2	11.10 ± 0.30	1.85	1809	6x	42	Kerguelen and Plonka (1989) and Fuente et al. (1997)
<i>F. nigrescens</i> subsp. <i>microphylla</i> (St.-Yves ex Coste) Markgr.-Dann.	France: Pyrénées Orientales, Valleeboitera, Puigmal, piste forestière 2, <i>Garnatje</i> and <i>Tenas</i> , Aug 2013; 42°22'57"N 2°03'11"E	2	12.48 ± 0.20	2.08	2034	6x	42	Kerguelen and Plonka (1989) and Fuente et al. (1997)
<i>F. rivularis</i> Boiss.	Spain: Aragon: Huesca, Formigal, <i>Zabalgoizeakoa</i> and <i>Catalán</i> , Jul 2014; 42°47'56.4"N 0°23'59.2"W	2	5.56 ± 0.05	2.78	2719	2x	14	Kerguelen (1975), Kerguelen and Plonka (1989) and Devesa et al. (2020)
<i>F. trichophylla</i> (Ducros, ex Gaudin) K.Richt.	Spain: Aragon: Huesca, Caldearenas, Monrepos, <i>Zabalgoizeakoa</i> and <i>Catalán</i> , Jul 2014; 42°23'41.34"N 0°22'34.40"W, BC 906497	2	16.23 ± 0.35	2.03	1984	8x	56	Šmarda et al. (2008) (sub <i>F. juncea</i>)

Localities, number of individuals analyzed (N), genome size [Haploid 2C (pg) and Monoploid 1Cx (pg and Mbp)] values and inferred ploidy levels (asterisk: estimations based on the congruence with chromosome number, "ploidy level"; no asterisk: estimations based solely on genome size, "DNA-ploidy level") are indicated for each population. BC, Botanical Institute of Barcelona herbarium; University of Zaragoza herbarium. (diamond: 2C (pg) estimations with HPCV (half peak coefficient of variation) > 5 but with similar values to those with HPCV < 3)

^a2C nuclear content (mean value ± SD of individuals measured). ^b 1 pg = 978 Mbp (Doležel et al. 2003). Publications of chromosome data source are indicated in References

endemic to the Pyrenees and the Cantabrian mountains, is only known to be diploid, though it participated in the origin of two recent homoploid hybrids after its interspecific crossings with its close *F. gautieri* subsp. *scoparia* ($F. \times \text{picoeuropeana}$ Nava) and *F. quadriflora* ($F. \times \text{souliei}$ St-Yves) in different parts of its distribution range (Torrecilla et al. 2013; Marques et al. 2016). Genome size values of *F. gautieri* correspond to two ploidy levels, which have been corroborated by chromosome counting (Table 1). 2C values of diploid *F. gautieri* subsp. *scoparia* (5.50–5.84 pg; $2n = 2x = 14$) also overlap with those reported in the large study of Marques et al. (2016); by contrast, 2C values of tetraploid *F. gautieri* subsp. *gautieri* (10.30–10.86 pg, $2n = 4x = 28$; Table 1) are given here for the first time. Subalpine *F. gautieri* subsp. *gautieri* shows a restricted distribution on both sides of the Eastern Pyrenees (France: Canigó, Puigmal; Spain: Ribes de Freser (Núria, Toses)), and Montseny growing only on siliceous soils (Küpfer 1974; Kerguélen and Plonka 1989; this study), by contrast subalpine *F. gautieri* subsp. *scoparia* is largely distributed on mountain ranges of the eastern Iberian Peninsula and the Pyrenees, and grows preferentially on calcareous substrates, but also in cation-rich granites (Catalán et al. 2013; Torrecilla et al. 2013; Marques et al. 2016). Kerguélen and Plonka (1989) differentiated the two *F. gautieri* taxa based on the larger sizes of spikelets and lemmas of tetraploid *F. gautieri* subsp. *gautieri* compared to those of diploid *F. gautieri* subsp. *scoparia*, detecting a common feature of larger reproductive structures in polyploids than in their progenitor diploids also observed in other fescues (Catalán 2006). However, Fuente and Ortúñez (1988), Fuente et al. (2001) and Devesa et al. (2020) did not segregate them, as they could not separate them morphologically. Our study has shown that tetraploid *F. gautieri* subsp. *gautieri* occupies a slightly broader range in the Eastern Pyrenees than previously known (Montseny) and that its strong siliceous ecological niche does not overlap with the calcicolous or cation-rich niche of its diploid *F. gautieri* subsp. *scoparia* conspecific taxon. The almost duplicated genome size of *F. gautieri* subsp. *gautieri* 4x with respect to that of *F. gautieri* subsp. *scoparia* 2x (Table 1) and their close morphology may suggest that the tetraploid taxon may have originated from the diploid taxon recently and has not yet experienced severe genome losses. In addition, the mean monoploid genome value (1Cx) of the tetraploid (2.67 pg) is roughly similar to that of the diploid, fitting the proposed evolutionary scenario of a potential autopolyploid origin for these types of *Festuca* polyploids (Šmarda et al. 2008). Further investigations would be necessary, however, to corroborate the kind of polyploid nature of the tetraploid *F. gautieri* subsp. *gautieri* and the morphological differentiation of the two taxa.

Festuca sect. *Festuca*. Monoploid genome values (1Cx) of the *F.* sect. *Festuca* species show similar values for the

diploids *F. airoides* (2.23 pg) and *F. marginata* subsp. *andresmolinae* (2.21 pg); by contrast, those of the polyploids vary from values close to those of the diploids, like that of tetraploid *F. inops* (2.27 pg), to others slightly lower, like those of hexaploid *F. yvesii* subsp. *yvesii* (1.98 pg), octoploid *F. laevigata* (1.95–2.05 pg), and hexaploid *F. ochroleuca* Timb.-Lagr. subsp. *bigorronensis* (St.-Yves) Kerguélen (2.02 pg) (Table 1). The genome size of Prepyrenean *F. marginata* subsp. *andresmolinae* matched its reported chromosome number of $2n = 2x = 14$ (Fuente and Ortúñez 1993; Devesa et al. 2020). Alpine *F. airoides* has been the subject of some taxonomic debate in relation to its status alongside *F. supina* Schur, and its morphological similarity to *F. niphobia* (St.-Yves) Kerguélen, which may grow sympatrically in some parts of the Eastern Pyrenees (Kerguélen and Plonka 1989). Devesa et al. (2020) amalgamated these taxa into *F. airoides* s.l. differentiating two subspecies, *F. airoides* subsp. *airoides* 2x (including *F. supina* auct. pyr. non-Schur 1866) and subsp. *moliniieri* (Litard.) Mart.-Segarra & Devesa 2x, 4x (including *F. niphobia*) in the Eastern Pyrenees, the second taxon being more robust and more broadly distributed than the former. Our *F. airoides* sample fits the morphology of the typical subspecies; its 2C and 1Cx values are in agreement with those reported by Šmarda et al. (2008) for French and Bulgarian samples. The 2C values of *F. inops* correspond to a tetraploid taxon (Table 1). Šmarda et al. (2008) only detected the diploid level in Italian and French populations of this species; however, Devesa et al. (2020) subsumed *F. gracillior* (Hack.) Markgr.-Dann. under *F. inops* subsp. *inops*. Although Kerguelen and Plonka (1989) considered *F. gracillior* to be a diploid species in France, Martínez-Sagarra (2018) and Devesa et al. (2020) also detected tetraploids in *F. inops* subsp. *inops* in Spain; our genome size value of this taxon from Montserrat corroborates the findings of the latter authors.

The presence of *F. laevigata* Gaudin has not been recognized from within the Iberian Peninsula by Devesa et al. (2020), though it was recognized within the Pyrenees by Kerguélen and Plonka (1989) and Foggi and Müller (2009). Devesa et al. (2020) synonymised *F. laevigata* auct. p.p. non-Gaudin to *F. yvesii* subsp. *yvesii*. However, according to Arndt (2005) individuals showing preferentially an unbroken sclerenchyma ring and belonging to Pyrenean populations generally referred to *F. cagiriensis* Timb.-Lagr., could be united within a wider concept of *F. laevigata*. Following the classification system of Kerguélen and Plonka (1989), based on leaf blade anatomy and other morphometric traits, we have identified samples of Pyrenean and Prepyrenean populations as pertaining to *F. laevigata* s.l. and *F. yvesii* susp. *yvesii* (Table 1). We emphasize *F. laevigata* sensu lato because most populations from Spain correspond most closely to *F. cagiriensis*. Genome size values of Italian *F. laevigata* populations corresponded to octoploids (18.65 pg;

Šmarda et al. 2008) although chromosome counts of Kerguélen and Plonka (1989) indicated the putative existence of additional hexaploids. The mean 2C values of our *F. laevigata* samples from the Central and Eastern Pyrenees and Prepyrenees (16.0 pg, Table 1) are between those expected for octoploids (~ 18 pg) and hexaploids (12–13 (14) pg) (Šmarda et al. 2008). Considering the hypothesized reduction in the genome size of the fine-leaved fescues high polyploids, those values may correspond to octoploids, thus allowing us to infer a mean monoploid genome 1Cx value of 1.95–2.0 pg for them. Interestingly, our *F. yvesii* subsp. *yvesii* sample from the Eastern Prepyrenees presents a genome size value of 11.89 pg, intermediate between that expected for hexaploids and tetraploids (9–9.5 pg) (Šmarda et al. 2008) and octoploids (17.7–18.5 pg) (Martínez-Sagarra 2018). Both octoploid and hexaploid populations have previously been detected for this species in the Pyrenees (Kerguélen and Plonka 1989; Martínez-Sagarra 2018; Devesa et al. 2020); therefore, the 2C value of our Prepyrenean sample would probably correspond to a hexaploid, with an inferred small monoploid 1Cx value of 1.98 pg (Table 1). The presence in this same region of the hexaploid *F. glauca* Vill., which grows in siliceous terrain, mainly on the Catalan coast but penetrating inland as, for example, in the Alberes, may well help to explain the nature of the apparently introgressive, hexaploid populations of this species that occur within Catalonia. In fact, when cultivated, plants from near the summit of Montseny become indistinguishable from the coastal plants referred to as *F. glauca*. *F. ochroleuca* subsp. *bigorronensis* is reported to be a tetraploid taxon (Kerguélen and Plonka 1989). Our genome size values (12.12–12.16 pg) correspond to a hexaploid, however. These high Tena Valley populations have been variously interpreted, since they are often not clearly identifiable with other populations which bear the characteristic striated and often pubescent distal culm portion, although their morphological and ecological features allow us to classify them as *F. ochroleuca* subsp. *bigorronensis*.

Festuca sect. *Aulaxyper*. The large variability of nuclear DNA contents found in the studied species of *F. sect. Aulaxyper* has allowed us to identify new ploidy levels (Table 1) and to confirm the dramatic reduction in monoploid 1Cx values from diploids to high polyploids (Šmarda et al. 2008; Moreno-Aguilar et al. 2022a). 2C values for *F. rivularis* (5.56 pg), the only known continental Europe diploid representative of this lineage (Díaz-Perez et al. 2008), are provided here for the first time, allowing us to infer a monoploid genome value of 2.78 pg, close to those of species of the more ancestral *F. sect. Eския* lineage (Table 1). By contrast, 1Cx values of the *F. sect. Aulaxyper* polyploids are between one-third and one-fourth smaller than that of *F. rivularis*. Genome sizes of hexaploids *F. nigrescens* subsp. *nigrescens* (12.07–12.22 pg) and subsp. *microphylla* (11.10–12.48 pg)

and their mean monoploid genome (1Cx) values (2.01–2.02 and 1.85–2.08 pg, respectively) are similar to each other and equivalent though slightly smaller than those reported by Šmarda et al. (2008) for French, Czech and Italian populations of these taxa. Devesa et al. (2020) did not differentiate the two close subspecies in the Iberian Peninsula though they have been recognized by other authors (Kerguélen and Plonka 1989; Šmarda et al. 2008). The leaf morphology of our Formigal samples is like *F. bartherei* Timb.-Lagr., a taxon now considered to be a mere form of *F. nigrescens* subsp. *nigrescens*. A species growing in the same subalpine peat bog population as *F. rivularis* has been identified as *F. heteromalla* Pourr.; this plant shows hexaploid 2C values of 13.61 pg and a monoploid genome size of 2.27 pg (Table 1). The chromosome counts and genome size values obtained so far for French and Austrian populations of *F. heteromalla* indicate that it is an octoploid species (Kerguélen and Plonka 1989; Šmarda et al. 2008), whereas Devesa et al. (2020) did not recognize the species and subordinated it to hexaploid *F. rubra* L. subsp. *rubra*. However, the morphology of the plant, and its mountain marsh ecology, clearly differentiate it from that of typical nemoral to prairie-dwelling *F. rubra* subsp. *rubra*, corresponding to that of a hexaploid *F. heteromalla*-type species, as indicated previously by Kerguélen and Plonka (1989). Our data confirm this new ploidy level and provide the first genome size value for 6x *F. heteromalla*. The largest genome size of the studied *F. sect. Aulaxyper* species is that of *F. trichophylla* (16.23 pg; Table 1). Šmarda et al. (2008) 2C values of Italian populations of *F. trichophylla* subsp. *asperifolia* (13.1 pg) and subsp. *trichophylla* (13.2 pg) corresponded to hexaploid individuals although Kerguélen and Plonka (1989) and Al-Bermani et al. (1992) indicated an additional decaploid ploidy level for *F. trichophylla* subsp. *asperifolia* and Devesa et al. (2020) a decaploid ploidy level for both subspecies. Our 2C data correspond to an octoploid genome within *F. sect. Aulaxyper*, similar to that reported for other octoploid taxa of this lineage (e.g., *F. rubra* subsp. *junceae* 8x, 16.97 pg; Šmarda et al. 2008) thus being the first record of an octoploid ploidy level within *F. trichophylla* s.l. A monoploid genome (1Cx) value of 2.03 pg calculated for this octoploid species (Table 1) is similar to those inferred for hexaploids *F. heteromalla* and *F. nigrescens* subsp. *nigrescens* and *microphylla*, supporting the extreme reduction and downsizing of monoploid genomes in high *F. sect. Aulaxyper* polyploids, compared to diploid *F. rivularis*, and their potential allopolyploid origins (Šmarda et al. 2008; Moreno-Aguilar et al. 2022a).

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Declarations

Conflict of interest The authors declare no competing interests.

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