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Agronomic, leaf anatomy, morphology, endophyte presence and ploidy characterization of accessions of *Festuca* group *rubra* collected in northern Spain

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Abstract

Fifteen accessions of *Festuca* group *rubra* collected in northern Spain were characterized and grouped into four *Festuca* taxa on the basis of leaf anatomy, morphology and ploidy; seven were identified as *F. heteromalla*; two as *F. trichophylla* ssp. *asperifolia*; two as *F. nigrescens* ssp. *microphylla* and four as *F. rubra* ssp. *pruinosa*. All the accessions and one commercial cultivar 'Wilma' (*F. nigrescens* ssp. *nigrescens*), used as control, were established at the Mabegondo Agronomical Research Centre, A Coruña (Galicia) in a completely randomised block design with three replicates of 10 plants per accession. The plants were agronomically characterized for seven traits during 2004 and 2005. Cluster analysis was useful in identifying four clusters that described 66.5% of the phenotypic variation. Cluster 1 consisted of nine accessions with early heading, intermediate values of green colour and tolerance to crown rust in autumn and the highest seasonal growth. Cluster 2 contained two accessions with intermediate values of heading, green colour, good tolerance to crown rust in autumn and intermediate seasonal growth. Cluster 3 contained two accessions and the cultivar Wilma, which showed early heading, dark green colour, good tolerance to crown rust in autumn and spring and intermediate seasonal growth. Cluster 4 consisted of two late heading accessions with dark green colour, and the best tolerance to crown rust in autumn and intermediate seasonal growth. Thirteen of the 15 accessions (86.6%) were infected by fungal endophytes, with the degree of infection ranging from 2 to 73%.

Additional key words: endophyte, fine fescues, genetic resources, hierarchical clustering, leaf sections, multivariate analysis, turfgrass.

Resumen

Caracterización agronómica, anatomía foliar, morfología, presencia de hongos endofitos y nivel de ploidía de accesiones de *Festuca* grupo *rubra* recogidas en el Norte de España

Quince accesiones de *Festuca* grupo *rubra* recogidas en el norte de España se caracterizaron y agruparon en cuatro taxa usando caracteres de anatomía foliar, morfología y ploidía: siete de ellas como *F. heteromalla*, dos como *F. trichophylla* ssp. *asperifolia*, dos como *F. nigrescens* ssp. *microphylla* y cuatro como *F. rubra* ssp. *pruinosa*. Todas las accesiones y el cultivar 'Wilma' (*F. nigrescens* ssp. *nigrescens*) se establecieron en el Centro de Investigaciones Agrarias de Mabegondo, A Coruña (Galicia), en un diseño en bloques completos al azar con tres repeticiones de 10 plantas por accesión. Durante los años 2004 y 2005 se anotaron siete caracteres agronómicos en las plantas. Mediante la clasificación jerárquica se identificaron cuatro grupos, describiendo el 66,5% de la variación fenotípica. El grupo 1 incluyó nueve accesiones con espigado precoz, valores intermedios de color verde y de tolerancia a las royas coronadas otoñales y los mayores crecimientos estacionales. El grupo 2 comprendió dos accesiones con valores intermedios de fecha de espigado, color verde, buena tolerancia a las royas coronadas otoñales y crecimientos estacionales medios. El grupo 3 incluyó dos accesiones y el cultivar Wilma, los cuales mostraron espigado precoz, color verde oscuro, buena tolerancia a las royas coronadas otoñales y primaverales y valores intermedios de crecimientos estacionales. El grupo 4 incluyó las dos accesiones más tardías de espigado con color verde oscuro y con la mayor tolerancia a las royas coronadas de otoño y con valores intermedios de crecimientos estacionales. Trece de las 15 accesiones (86,6%) presentaron hongos endofitos (2-73%).

Palabras clave adicionales: análisis multivariante, césped, clasificación jerárquica, endofito, festucas finas, recursos genéticos, secciones foliares.

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Introduction

Fine fescue is a common term used to describe a number of morphologically similar species of *Festuca* that are agronomically important as turf. The fine fescues (*Festuca* spp.) are fine leafed, cool season turf grasses that are extremely tolerant to shade, drought, and low pH (5.5 to 6.5) (Ruemmele *et al*., 1995). Their natural low maintenance requirements and ability to produce attractive turf makes them an important turfgrass in northeastern USA and Europe. Fine fescues show poor traffic tolerance, and their use in heavily trafficked areas or athletic fields is therefore limited. They are often mixed with Kentucky blue-grass (*Poa pratensis* L.) and turf-type perennial ryegrasses for dry shaded areas (Bughrara, 2004).

Several species of fine leaf fescue show an outstanding performance due to the presence of endophytes (Clarke *et al*., 2006). This endophyte/plant symbiosis has been shown to enhance insect, disease and drought resistance not only in fine leaf fescue, but also in perennial ryegrass (*Lolium perenne* L.) and tall fescue (*F. arundinacea* Schreb.) (Funk and White, 1997). Thus, the potential biological and economic impacts of endophyte-enhanced fine fescue may be of great value to turfgrass managers and researchers. Information on endophyte infection status in germplasm is important for turf breeders for separating host-conditioned plant traits from interactions involving endophytes (Kindler *et al*., 1991).

Although there is still much to learn about the nature of this beneficial association, it is well known that endophyte-produced alkaloids play a role in enhanced resistance to turfgrass pests (Richardson *et al*., 1997). In addition, several fine fescue species infected by endophytes have shown increased resistance to dollar spot (Clarke *et al*., 2006), caused by *Sclerotinia homoecarpa*. Recent turf trials at Rutgers (USA) have demonstrated that endophyte-infected cultivars and selections of fine fescue also show increased tolerance to summer stress, producing a brighter, lusher and denser turf (Funk and White, 1997). Endophytes therefore have the potential to reduce pesticide use while maintaining healthy turf.

In northern Spain, tall fescue populations are commonly infected by endophytes. Oliveira and Castro (1997) found that 17 out of 19 accessions (i.e. 90%) contained endophyte fungus, with the degree of infection ranging from 7 to 100%.

The use of leaf anatomy data to supplement the limited morphological characters in *Festuca* was first reported by Hackel (1882), and has been used extensively since then (Aiken *et al*., 1985).

Although the taxonomy of the fine fescues often proves difficult, most species used for turf can be divided into two groups: *Festuca* group *rubra* and *Festuca* group *ovina* (Huff and Palazzo, 1998). Measurable differences between these two main groups may be based on leaf blade anatomy, leaf sheath morphology and root fluorescence (Hubbard, 1984; Wilkinson and Stace, 1991). In the *Festuca* group *rubra*, the sheaths of young tiller leaves are fused into a tube extending almost to the top, and some or all tillers emerge extravaginally (rhizomes). This group includes creeping (produce creeping stolons and/or rhizomes) and noncreeping (do not produce creeping stolons and rhizomes) bunch type grasses. In the *Festuca* group *ovina*, the upper 40% (at least) of the sheaths of young tiller leaves have free, overlapping margins, and all the tillers emerge intravaginally. This group includes only noncreeping bunch type grasses with stiff leaves (Turgeon, 2004). Species characterization becomes difficult within each of the two groups. The three main species of the *F. rubra* group for use in turf are chewings fescue (*F. nigrescens* ssp. *nigrescens* and *F. nigrescens* ssp. *microphylla*), strong creeping red fescue (*F. rubra* ssp. *rubra*) and slender creeping red fescue (*F. trichophylla* ssp. *asperifolia*, *F. heteromalla*, and *F. rubra* ssp. *pruinosa*). Chewings fescue is easily separated from the two creepers on the basis of the absence of extravaginal stems (rhizomes). It is hexaploid and has 42 chromosomes (2n = 6x = 42), has low growing, dense turf and is a noncreeper, although it is deep rooted. Morphological differences between the two creepers are more difficult to distinguish, as they are based on quantitative differences in rhizome diameter and rhizome biomass production (Schmit *et al*., 1974). Strong creeping red fescue is octoploid, has 56 chromosomes (2n = 8x = 56), and has many long, spreading rhizomes as well as larger seeds. Slender creeping red fescue is hexaploid, has 42 chromosomes (2n = 6x = 42), shorter, slender rhizomes and can form

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Abbreviations used: CIAM (Centro de Investigaciones Agrarias de Mabegondo, Mabegondo Agronomical Research Centre), LSD (least-significant difference), M (mean), masl (meters above sea level), NS (non significant), PC (principal component), PCA (principal component analysis), SD (standard deviation).

compact, dense turf. Species within the *F.* group *ovina* are more difficult to distinguish.

Classification of *Festuca* species on the basis of morphological and anatomical characters alone is complicated by the variability in the morphology of particular characters (Šmarda *et al*., 2005). Terrel (1979) reported that taxonomic characterization of the morphological and anatomical structure of plants was not influenced by environmental factors. Ploidy level and chromosome determination is very important for taxon delimitation (Wilkinson and Stace, 1991). Hubbard (1984) reported a chromosome number of 56 for strong creeping red fescue and 42 for slender creeping red fescue and chewings fescue. Thus, ploidy level became a basic classification criterion for separating strong creeping red fescue from the other two species in the group *rubra*.

The objective of the present study was to characterize the morphological and genetic variability of *Festuca* group *rubra* accessions collected from northern Spain, on the basis of agronomic, leaf anatomy, morphology, endophyte presence, and ploidy data. Knowledge of such variability should provide useful in assessing the potential value of these accessions in Spanish and North American breeding programmes for turf and forage plants.

Material and methods

Plant material

Seeds of 15 accessions of *F.* group *rubra* were collected from wild populations from different sites throughout Asturias, in 2000. Each of these populations was collected as seeds from at least 50 plants taken from an ecologically homogeneous area of 100-1000 m^2. This was considered to yield a sample of seeds representative of the original panmictic population (Tyler *et al*., 1984). The origins and accession numbers of these 15 populations are listed in Table 1. The accessions were collected from environments unsuited to agricultural production, such as roadsides, paths, mountainous areas, wasteland and coastal cliffs. All the seeds were stored in the laboratory in waterproof packages, at 0-4ºC.

Table 1. Accession number and origins of the 15 accessions of *Festuca* group *rubra*

Accession	Province	Location	Habitat	Latitude	Longitude	Altitude
2	Asturias	Folgueiras	Roadside	4325N	0710W	300
3	Asturias	Armada	Wasteland	4307N	0551W	1100
4	Asturias	Villanueva de Oscos	Roadside	4318N	0659W	650
7	León	Puerto de Tarna	Wasteland	4307N	0514W	1490
8	Asturias	Tanes	Path	4315N	0525W	495
11	Asturias	Alto de Cobertoria	Wasteland	4310N	0558W	1179
13	Asturias	Paramios	Wasteland	4322N	0701W	500
14	León	Carande	Wasteland	4250N	0450W	1000
15	León	Acebedo	Wasteland	4303N	0507W	1180
16	Asturias	Sta. Eulalia de Oscos	Wasteland	4315N	0701W	560
19	Asturias	Playa de Penarronda	Cliffs	4332N	0659W	3
20	Asturias	Playa de Navia	Beach	4333N	0643W	6
21	León	La Uña	Wasteland	4303N	0507W	1250
27	Asturias	Playa de Pormenande	Cliffs	4332N	0649W	3
29	Lugo	Faro de Ribadeo	Cliffs	4333N	0702W	9

Leaf anatomy and morphology

For each accession, at least two leaf sections from two different plants were analyzed.

For analysis of the leaf anatomy, cross section of leaf blades were cut with a razor blade (Gillette superplatinum), directly on fresh material or after boiling the dry material in water, with the aim of softening it and recovering the original form. With the aim of following a standardized method, the last sheathing leaf was chosen from a new shoot, on half of a plicate leaf, according to the suggestions of Hackel (1882), Saint-Yves (1927) and Portal (1999).

The cross sections were then dyed with Fasga staining (Tolivia and Tolivia, 1987) and photomicrographs were taken with a camera (Zeiss Photomicroscope II and Nikon D 40). Photographs were taken at 40x magnification.

Characters measured were: presence or absence of rhizomes, presence or absence of abaxial protrusions, the outline of abaxial surface in leaf sections, leaf width (mm), leaf depth (mm), number of veins, number of ribs and number of sclerenchyma bundles.

For delimitation of taxa, the classification keys of Kerguélen and Plonka (1989) and Portal (1999) were adopted.

Ploidy determination

Mitotic chromosome counts were made on root-tips taken from seedlings at the 4-5 tiller stage, by the orceine-acetic staining method. Cytological investigations were performed on at least two individual genotypes from the 16 accessions.

Plants were grown in the greenhouse. Plant roots were collected at 10:00 am. Four-five roots of 0.5-1 cm long were cut from each plant and pre-treated in ice-cold distilled water for 24 h; fixed in carnoy's solution 3:1 (95% v/v absolute ethanol:glacial acetic acid) and stored at 4ºC until use.

Before staining in orceine-acetic solution, the root-tips were washed in distilled water, in a watch glass or Petri dish. They were then dried with absorbent paper and placed on a microscope slide with 1 N HCl and of orceine-acetic acid (1:9). They were warmed for 5 min on an alcohol lamp and then left for 15 min covered with a Petri dish. They were then warmed for another 5 min. The root-tips were removed from one microscope slide to another, a drop of 45% glacial acetic acid added and the tips were then squashed with a cover glass.

Slides were viewed and chromosomes counted under an optical microscope to determine the number of chromosomes in the root-tip cell.

Chromosomes were photographed in mitotic metaphase with a simple-lens reflex camera attached to an Olympus (BX-51) microscope system at 1250x magnification.

Plant characterization

The agronomic study was established at the CIAM (Centro de Investigaciones Agrarias de Mabegondo, in A Coruña, Galicia) (43º 35' N, 5º 47' W, 90 masl, near the coast, on an inceptisol soil type). The trial was arranged in a completely randomized block design with three replicates of 10 plants per accession. Plants were transplanted to the field in March 2003, 50 cm apart. One commercial cultivar 'Wilma' (*F. nigrescens* ssp. *nigrescens*) was also included as a control.

The soil was a clay loam and had an initial pH of 5.8. It was rotovated and amended with lime at 2 Mg ha^{-1} and fertilised the year of the establishment with 32 kg N ha^{-1}, 40 kg P_2O_5 ha^{-1} and 40 kg K_2O ha^{-1}.

The study was conducted in a location in full sun, with no maintenance fertilization. The site did not receive any additional fertilizer or irrigation during the course of the study. The plants were maintained at mowing height of 5 cm, with a rotary mower. Clippings were removed after each cut.

Seven characters were observed: colour (1=light green, 9=dark green); autumn growth (1=low, 9=high); susceptibility to crown rust (*Puccinia coronata*) in autumn (1=high, 9=low), spring growth (1=low, 9=high); susceptibility to crown rust in spring (1=high, 9=low); heading date (as the number of days after January) and summer growth (1=low, 9=high).

The endophyte content in the 16 accessions was determined in 50 seeds per accession after cleaning the seeds collected, using the procedure described by Latch *et al*. (1987). The percentage of seeds with presence of endophyte was recorded as percentage infection of the accession.

Statistical analysis

Although the seven agronomic characters listed above were recorded on discrete scales (1-9), the distri-

bution of errors fitted a Gaussian distribution and therefore no data transformation was needed.

Analysis of variance of the agronomic data was first performed yearly and once the similarity of errors in each year was checked, a combined analysis was carried out for both two years. The effects year, replicate, accession, and the interactions between them were considered. The replicate effect was considered random. Separation of accessions was performed by the least-significant difference (LSD) test.

Multivariate relationships among accessions were revealed with a principal component analysis (PCA), with a correlation matrix derived from the significant characters. The original variables were reduced to three independent linear combinations, principal components (PC) of the variables, with eigenvalues greater than 1 that cumulatively explained 89.3% of the total variance.

These PCs were used as the input for an agglomerative hierarchical cluster analysis to detect groups of similar agronomic types. The squared Euclidean distance was the measure of distance, and the Ward's clustering algorithm was the method used to combine accessions into clusters (groups). Such a method can group similarly performing accessions into clusters that minimize the within-cluster variance. A partition was chosen from the view of the tree of classification. In order to examine differences between the clusters obtained, a table of means, standard deviations (SD) and the results of a one-way ANOVA (F tests) is also shown. Separation of clusters was performed by the Duncan test. Statistical analyses were computed with SPSS version 14 (SPSS, 2005).

Results

Leaf anatomy and morphology

Morphological characters and leaf section data of the 15 accessions and the control Wilma are summarized in Table 2.

In order to identify the different *Festuca* taxa (Table 3) the presence or absence of rhizomes was initially considered. Two taxa were differentiated in plants without rhizomes: 1) *F. nigrescens* ssp. *nigrescens* with an average leaf depth greater than 0.7 mm. The control ('Wilma') belongs to this species; 2) *F. nigrescens* ssp. *microphylla* with an average leaf

Table 2. Summary of average leaf cross section data and morphology

Accession	Rhizomes [1]	Abaxial protrusions [1]	Leaf section in outline	Leaf width (mm)	Leaf depth (mm)	No. of veins	No. of ribs	No. bundles of abaxial sclerenchyma
2	+	-	Keeled	0.36	1.19	7	5	7
3	+	-	Keeled	0.40	1.03	7	4	7
4	+	-	Keeled	0.32	0.70	7	4	7
7	+	+	Polygonal	0.31	0.74	6	3	5
8	+	+	Polygonal	0.32	0.67	5	3	5
11	+	-	Keeled	0.34	0.81	7	5	8
13	+	-	Keeled	0.42	1.02	8	6	7
14	+	-	Keeled	0.38	0.76	8	5	6
15	+	-	Keeled	0.32	0.80	8	5	7
16	-	-	Polygonal	0.32	0.63	5	3	7
19	+	-	Elliptical	0.43	0.80	6	3	8
20	+	-	Elliptical	0.40	0.80	7	3	5
21	-	+	Polygonal	0.29	0.63	5	3	7
27	+	-	Elliptical	0.36	0.65	5	3	7
29	+	-	Elliptical	0.38	0.73	5	3	7
Wilma	-	-	Polygonal	0.32	0.74	5	3	7

[1] +: presence, -: absence

Table 3. Principal characters distinguishing the five taxa identified within the genus *Festuca* group *rubra*. Minimum and maximum values are shown in brackets.

	F. heteromalla	***F. trichophylla* ssp. *asperifolia***	***F. rubra* ssp. *pruinosa***	***F. nigrescens* ssp. *microphylla***	***F. nigrescens* ssp. *nigrescens***
Accessions	2, 3, 4, 11, 13, 14, 15	7, 8	19, 20, 27, 29	16, 21	Wilma
Rhizomes	presence	presence	presence	absence	absence
Abaxial protusions	absence	presence	absence	absence	absence
Leaf section in outline	Keeled	Polygonal	Elliptical	Polygonal	Polygonal
Leaf width (mm)	0.36 (0.32-0.42)	0.31 (0.31-0.32)	0.39 (0.36-0.43)	0.30 (0.29-0.32)	0.32 (0.31-0.33)
Leaf depth (mm)	0.90 (0.70-1.19)	0.71 (0.67-0.74)	0.74 (0.65-0.80)	0.63 (0.62-0.64)	0.74 (0.73-0.75)
No. of veins	7-8	5-6	5-7	5	5
No. of ribs	4-6	3	3	3	3
No. bundles of abaxial sclerenchyma	6-8	5	5-8	7	7
Chromosome number (2n)	42	42	42	42	42

depth lesser than 0.7 mm. Acc. 21 and 16 belong to this species.

Plants with rhizomes and keeled leaf section belong to taxon *F. heteromalla* (Acc. 2, 3, 4, 11, 13, 14 and 15). Plants with rhizomes, and in which the leaf section was not keeled, but had protrusions on the abaxial surface, were identified as *F. trichophylla* ssp. *asperifolia* (Acc. 7 and 8). Plants with the absence of protrusions on the abaxial surface were identified as *F. rubra* ssp. *pruinosa* (Acc. 19, 20, 27 and 29).

Table 4. Mean squares of the analyses of variance for the accessions characterised during two years for seven traits: Co, colour (1=light green, 9=dark green); Ag, autumn growth (1=low, 9=high); Ars, rust susceptibility in autumn (1=high, 9=low); Spg, spring growth (1=low, 9=high); Sprs, rust susceptibility in spring (1=high, 9=low); Hd, heading date (as the number of days after January the first); Sg, summer growth (1=low, 9=high) in the fifteen accessions and one control (Wilma).

Traits	**Mean squares**					
	Year	**Replicate**	**Year*Replicate**	**Accession**	**Accession*Year**	**Error**
Co	7.05NS	154.04NS	16.71**	58.72***	3.14NS	3.59
Ag	198.59NS	132.05NS	82.95***	31.20***	20.10***	4.32
Ars	282.81**	3.56NS	2.72NS	54.80***	14.46***	4.45
Spg	5.81NS	55.09NS	30.89***	13.41***	10.10***	3.26
Sprs	47.19NS	9.71NS	12.98*	13.81***	16.53***	4.14
Hd	22543.45**	5398.47*	109.05NS	4242.29***	384.76NS	300.33
Sg	0.91NS	23.85NS	2.77NS	24.20***	8.60NS	4.38

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, NS = $p > 0.05$

Table 5. Two-year means (SD between parentheses) for Co, colour (1=light green, 9=dark green); Ag, autumn growth (1=low, 9=high); Ars, rust susceptibility in autumn (1=high, 9=low); Spg, spring growth (1=low, 9=high); Sprs, rust susceptibility in spring (1=high, 9=low); Hd, heading date (as the number of days after January the first); Sg, summer growth (1=low, 9=high), Endo, endophyte presence (%) in the fifteen accessions and one control (Wilma).

Accessions	Co	Ag	Ars	Spg	Sprs	Hd	Sg	Endo
2	5.3	6.2	4.4	6.1	5.0	94.4	6.5	49
	(1.5)	(2.1)	(2.3)	(1.7)	(1.8)	(15.0)	(1.9)	
3	5.1	5.0	6.3	5.1	4.7	111.0	5.5	26
	(2.5)	(2.4)	(2.2)	(1.8)	(1.8)	(19.4)	(2.7)	
4	5.7	6.1	5.5	6.3	5.7	98.0	6.5	64
	(1.7)	(1.9)	(2.4)	(1.7)	(2.2)	(17.6)	(2.1)	
7	5.3	5.1	5.3	5.6	4.8	97.7	5.5	6
	(1.5)	(1.8)	(2.3)	(1.8)	(2.1)	(14.7)	(1.7)	
8	5.0	6.0	6.2	6.2	6.0	95.9	5.6	55
	(1.5)	(2.9)	(2.2)	(2.5)	(1.8)	(14.5)	(2.4)	
11	5.5	6.0	4.0	6.3	5.4	101.3	6.0	27
	(1.8)	(1.5)	(1.8)	(1.2)	(2.1)	(14.9)	(1.8)	
13	5.7	6.3	5.2	6.1	5.5	100.2	6.1	30
	(1.8)	(2.2)	(2.7)	(1.8)	(1.8)	(14.3)	(1.9)	
14	6.9	4.5	5.1	5.5	5.2	99.9	4.5	17
	(2.3)	(2.8)	(2.1)	(2.4)	(2.3)	(17.3)	(2.9)	
15	5.3	6.7	4.6	6.6	5.7	100.6	6.5	64
	(1.8)	(1.9)	(2.2)	(1.6)	(2.1)	(15.3)	(2.0)	
16	4.7	7.0	5.6	6.8	5.8	107.6	7.0	43
	(1.9)	(1.9)	(2.4)	(1.8)	(2.4)	(18.9)	(1.6)	
19	6.6	5.8	6.2	5.9	5.4	112.0	6.2	0
	(2.4)	(2.5)	(2.6)	(2.0)	(2.4)	(27.7)	(2.0)	
20	8.3	4.6	5.5	5.8	6.3	89.9	4.7	0
	(1.7)	(2.9)	(2.4)	(2.1)	(2.2)	(14.4)	(2.3)	
21	7.3	5.4	6.9	5.7	6.4	125.0	5.7	2
	(1.9)	(2.7)	(1.9)	(2.2)	(1.9)	(22.2)	(2.2)	
27	5.8	5.9	6.0	6.3	6.2	112.6	6.2	46
	(2.5)	(2.0)	(2.2)	(1.6)	(2.5)	(19.9)	(1.9)	
29	7.0	4.9	7.8	5.1	5.7	110.0	5.6	73
	(2.5)	(2.6)	(1.3)	(1.9)	(1.9)	(21.2)	(2.0)	
Wilma	7.2	5.0	4.3	5.5	5.5	98.6	6.2	0
	(1.8)	(1.8)	(1.8)	(1.6)	(2.1)	(21.9)	(1.8)	
Ftest	16.3***	7.2***	12.3***	4.1***	3.3***	14.1***	5.5***	
LSD (p =0.05)	0.8	0.8	0.8	0.7	0.8	6.8	0.7	

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, NS = $p > 0.05$. LSD = Least significant differences at 5% level. The LSD values at the bottom of each column represent the minimum difference between any two accessions necessary to be 95% confident that the difference is not attributable to chance.

Ploidy determination

The chromosome number was 2n = 6x = 42 for all the accessions and the commercial cultivar.

Agronomic characterization and endophyte presence

The results of variance analysis of the seven traits and two years are summarized in Table 4. For all the characters, the accession effect was statistically significant.

The mean values of traits and SD for the accessions are shown in Table 5.

Of the 15 accessions evaluated, none had high levels of endophyte infection (> 75% seeds infected), in 13 accessions around 86.6% of the seeds were infected with endophytes, 10 were moderately infected (25% to 75%), three had low levels of infection (< 25%), and two accessions and the cv. Wilma did not have any infected seeds (Table 5).

The correlation coefficients for the seven traits and the first three PCs are shown in Table 6. The first PC was mainly negatively correlated with leaf colour in autumn, and positively with seasonal growth (autumn, spring and summer growth). The traits that were positively correlated with the second component (and thus behaved independently from those associated with the first component) were heading date and susceptibility to crown rust in autumn. The third PC was positively correlated with susceptibility to crown rust in spring.

The projection of the accessions on the plot of components 1-2 is shown in Figure 1. Accessions at the extreme right of the plot (e.g. Acc. 16) show high seasonal growth and light green colour. Acc. 14 and 20 on the extreme left of the same plot present low seasonal growths and dark green colour. On the upper part of the plot, Acc. 21 and 29 show late heading and a low susceptibility to crown rust in autumn. At the bottom of the plot, accessions show early heading and a high susceptibility to crown rust in autumn.

Hierarchical clustering analysis performed on the first three PCs provides the dendrogram shown in Figure 2. This suggests that the cut-off point should be at the four cluster level. The partition shows a between-clusters-variance/total variance ratio of 66.5%, which is quite high for such a low number of clusters. This means that the clusters differ in the multidimensional space.

Analysis of variance was carried out by considering the effect number of cluster and significant statistical differences were detected among clusters for all traits (Table 7). Cluster 1 consisted of nine accessions (2, 4, 8, 11, 13, 15, 16, 19, and 27) with early heading, intermediate green colour, intermediate values of tolerance to crown rust in autumn, low tolerance to crown rust in spring, and the highest seasonal growth. Cluster 2 contained two accessions (3 and 7) with intermediate values of heading, green colour, tolerance to crown rust in autumn, low values of tolerance to crown rust in spring, and intermediate seasonal growth. Cluster 3 contained two accessions (14, 20) and the cv. Wilma, which showed early heading, dark green colour, good tolerance to crown rust in autumn and spring and intermediate seasonal growth. Cluster 4 consisted of the two accessions (21 and 29) showing late heading, dark green colour, and the best tolerance to crown rust in autumn and intermediate seasonal growth. The two accessions of cluster 3 and the two of the cluster 4 show values as high as Wilma for traits of interest. Since the turf market requires mainly dark green colours, low growing and crown rust tolerant cultivars, the populations of these clusters appear to be the most valuable material for turf improvement.

Table 6. Correlation coefficients for seven agronomic traits and the first three principal components from a principal component analysis of the correlation matrix of the traits (Varimax rotation method)

Traits	Component 1	Component 2	Component 3
Co	-0.758	0.040	0.510
Ag	0.984	-0.059	0.078
Ars	-0.241	0.864	0.173
Spg	0.845	-0.320	0.363
Sprs	0.078	0.186	0.962
Hd	0.094	0.919	0.043
Sg	0.911	0.062	-0.046

Discussion

In all of the accessions used in this study the sheaths of the young tiller leaves were fused into a tube extending almost to the top, the number of sclerenchyma bundles was greater than three and the tillers emerged intravaginally and in some cases extravaginally (rhizomes). These characteristics are typical of the red fescue group, which includes creeping and noncreeping bunch type grasses (Turgeon, 2004).

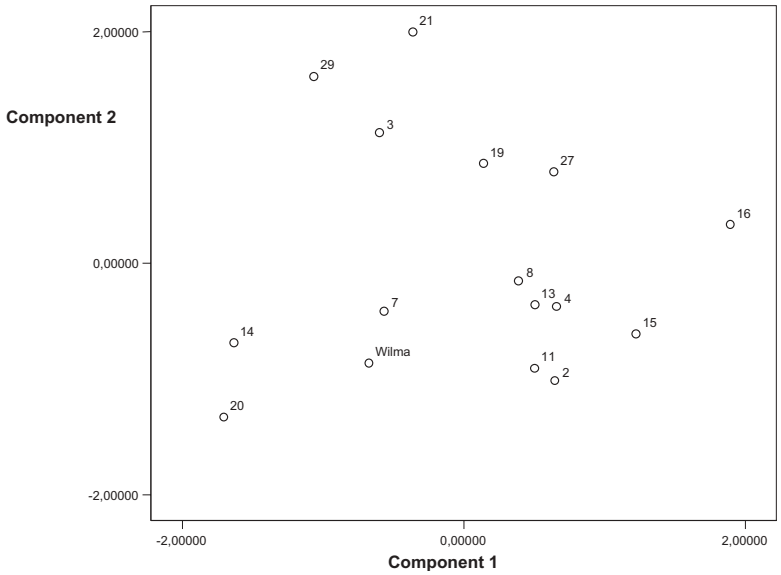

Figure 1. Plot of the principal component analysis carried out on seven traits with projection of the accession number. Component 1 as *x* axis, component 2 as *y* axis (72.2% of the total variance explained).

In the genus *Festuca* leaf anatomy characters are essential for differentiating taxa and may be very useful for separating the different species at any time of the year without having to wait for the flowering stage.

The distribution and above all the ecology of the material studied are also very important and can help in distinguishing some species of the genus, since they are sometimes linked to a particular habitat. For example, *F. rubra* ssp. *pruinosa* appears exclusively on the coast, which distinguishes it from other non coastal species. Other species such as *F. heteromalla* can occupy a variety of habitats, including roadsides, paths, mountainous areas, wasteland, grasslands etc.

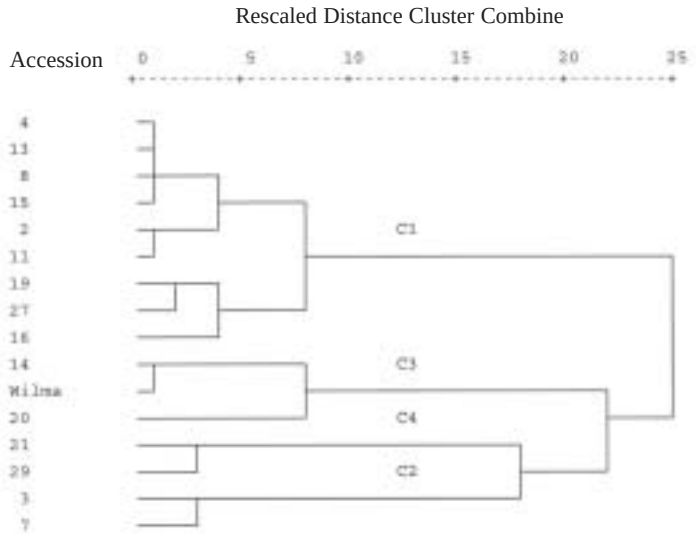

Figure 2. Dendrogram based on the results of the hierarchical cluster analysis by the Ward method (66.5% of the variance among accessions). C1, C2, C3 and C4 mean Cluster 1, 2, 3, and 4 respectively. Each time two accessions are joined by a vertical line, a subcluster or cluster is created. The dendrogram also presents the values of the distance coefficients for each step or vertical line.

Table 7. Between-cluster differences for colour (1=light green, 9=dark green); Ag, autumn growth (1=low, 9=high); Ars, rust susceptibility in autumn (1=high, 9=low); Spg, spring growth (1=low, 9=high); Sprs, rust susceptibility in spring (1=high, 9=low); Hd, heading date (as the number of days after January the first); Sg, summer growth (1=low, 9=high) traits. M = mean, SD = standard deviation, n = number of accessions.

Trait	Cluster 1 (n = 9)	Cluster 2 (n = 2)	Cluster 3 (n = 3)	Cluster 4 (n = 2)	Anova F ratios
	M (SD)	M (SD)	M (SD)	M (SD)	F (3, 12)
Co	5.51b (0.55)	5.22b (0.18)	7.48a (0.71)	7.15a (0.19)	14.35***
Ag	6.24a (0.38)	5.04b (0.02)	4.72b (0.30)	5.13b (0.33)	19.40***
Ars	5.31b (0.80)	5.82b (0.71)	4.95b (0.61)	7.39a (0.60)	5.04*
Spg	6.30a (0.26)	5.36b (0.35)	5.61b (0.20)	5.40b (0.39)	11.84**
Sprs	5.65a (0.38)	4.77b (0.06)	5.69a (0.59)	6.06a (0.52)	3.47*
Hd	102.52b (6.68)	104.40ab (9.39)	96.11b (5.44)	117.52a (10.63)	3.67*
Sg	6.31a (0.37)	5.46ab (0.05)	5.12b (0.91)	5.64ab (0.11)	5.57*

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, NS = $p > 0.05$. Means followed by different letters in the row were significantly different at the 0.05 level according to Duncan test. Cluster 1 includes accessions: 2, 4, 8, 11, 13, 15, 16, 19, 27, Cluster 2, accessions: 3, 7, Cluster 3, accessions: 14, 20 and cultivar Wilma and Cluster 4, accessions: 21 and 29.

On the basis of these characteristics five species of *Festuca* belonging to the group *rubra* have been identified:

-*Festuca nigrescens* ssp. *nigrescens*. Located in mesophyllus *Molinio-Arrhenatheretea* and wet *Nardus* pastures on acid and basic soils. It was cited by Díaz *et al*. (1994), in Asturias, in *Nardetea strictae* pastures (dense pastures developed on deep acid soils subjected to temporal variations in soil moisture). De la Fuente *et al*. (1997) also cited this subspecies in *Molinio-Arrhenatheretea* pastures (Eurosiberian as well as Mediterranean communities of dense cover typical of deep soils and with variable soil moisture and fertility, in most cases depending on the intensity of management, harvesting and grazing).

-*Festuca nigrescens* ssp. *microphylla*. This subspecies is found on mesophyllus *Molinio-Arrhenatheretea* and wet *Nardus* pastures, and prefers acid soils. Portal (1999) reported that it is sometimes found at roadsides or on disturbed land without any other vegetation, and mentioned its use as a component of seed mixes for man-made habitats (roadsides, entrances, picnic sites, etc.). It is therefore possible to find this subspecies in many different locations. Díaz *et al*. (1994) cited this subspecies in Asturias in wet *Nardus* pastures; De la Fuente *et al.* (1997) located it in pastures developed in deep soils without temporal soil moisture deficit and with Eurosiberian and Mediterranean distribution (*Festuco-Brometea*).

-*Festuca heteromalla*. Present in acid and basic soils, on roadsides and meadows. It is cited in Asturias by Díaz *et al*. (1994) as representative of the *Arction lappae*. These are perennial nitrofilous communities on deep soils with variable moisture, mainly in the Eurosiberian Region but also in the Mediterranean area in the humid seasons.

-*Festuca trichophylla* ssp. *asperifolia*. Present on acid or basic soils at between 300 and 2000 m altitude on wet meadows, forest edges, rocks, etc. This subspecies has been cited in Asturias by Díaz *et al*. (1994) in *Nardetea strictae* (dense pastures developed on deep acid soils), *Calluno-Ulicetea* (communities, generally, of dense cover, on acid soils with raw humus) and *Ononido-Rosmarinetea* (open scrubland of small height developed on basic soils usually without upper horizon, mainly in the Mediterranean area also in the Eurosiberian region).

-*Festuca rubra* ssp. *pruinosa*. This subspecies is found exclusively in coastal areas such as coastal cliffs and fixed dunes and is indifferent to soil nature. Díaz *et al*. (1994) cited it in Asturias in *Arthrocnemetea fruticosi* (communities established by dominant halophytes that occupy saline soils and with variable soil moisture content).

Portal (1999) mentioned that this subspecies is sometimes used in man-made habitats due to its creeping ability; it tends to sod in quickly and tolerates treading and drought.

The chromosome counts observed in the present study were consistent with those reported by Hubbard

(1984) and Kerguélen and Plonka (1989) for the taxa identified by means of leaf anatomy.

The proportion of fine fescues accessions infected by endophytes (86.6%) was similar to that reported by Oliveira and Castro (1997) in tall fescue populations from northern Spain (90%) but the percentage of infected seeds was lower (2-73%) than reported in the latter study (7-100%).

The high proportion of fine fescue accessions infected by endophytes suggests that grass breeders may be more likely to select endophyte-infected plants in this area. However, to obtain the benefits of endophytes, at least 25% of the seeds in a given sample probably need to contain endophytes (Da Costa *et al*., 1998). It is important to point out that analysis of seeds for endophytes only determines their presence, not their viability, and further examination of plant tissue is needed to determine the viability of the endophytes.

Initial screening of the germplasm under field characterization is subject to yearly variations in climate. Results obtained must be interpreted in light of this and the screening is likely to be of value only for breeding programmes in similar environments to the screening environment (Tyler *et al*., 1987).

Single-plant characterization is of limited value, as seasonal yields are not consistently related to sward yields (Lazenby and Rogers, 1964). Their main use is to determine flowering characteristics, of which heading date is probably the most important character for determining the growth rate. The advantage of single-plant characterization is that initial screening can be done with only a few seeds. Characterization of forage grasses through multivariate analysis methods has been used carried out by many authors (Hayward *et al*., 1982; Charmet *et al*., 1989; Oliveira and González, 2000). A similar method was used in the present study. The objective of spaced plant characterization was to define an index of agronomic value for each accession from the traits of interest in order to find a stable classification of the accessions.

The scores for each accession were considered in the PCA as an alternative to the Smith-Hazel selection index, as recommended by Charmet *et al*. (1989). In this study, the first PC was positively correlated with seasonal growth and negatively with colour. The scores for Acc. 20, 14 and 29 were higher than for the control (Wilma) and showed a dark green colour and the lowest seasonal growth. The second PC was positively correlated with the heading date and with the tolerance to crown rust in autumn. The scores for Acc. 21, 29 and 14 were higher than the control (Wilma) and showed late heading dates and high tolerance to crown rust in autumn. Acc. 14 (*F. heteromalla*) and 29 (*F. rubra* ssp. *pruinosa*) combined a dark green colour, low seasonal growth and good tolerance to crown rust in autumn. On the basis of these results, northern Spanish low-maintenance turfgrass breeding programmes for *Festuca* group *rubra*, should focus on these accessions. It is important to mention that Acc. 29 belongs to *F. rubra* ssp. *pruinosa* (halophyte characteristics) and was collected near Ribadeo light-house. This material which has shown to be suitable as turfgrass may be of interest in the development and use of salt-tolerant germplasm, in order to reduce the vulnerability of current cultivars to increasing levels of salt in many turf soils (Brede and Sun, 1995). Further evaluation and breeding will be required before these accessions are available for turf use.

Morphological characterization of accessions is beneficial for evaluation of the agronomic aptitude and for plant breeding research (Rao *et al*., 1996). To overcome problems such as the interference of environmental factors with the agronomical and leaf anatomy characters used in classification (De Nova *et al*., 2006), several amplified fragment length polymorphism (AFLP) markers will be used to classify these species of *Festuca*. The results of this study suggest the possibility of using germplasm resources for turf and forage production.

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References

AIKEN S.G., DARBYSHIRE S.J., LEFKOVITCH L.P., 1985. Restricted taxonomic value of leaf sections in Canadian narrow-leaved *Festuca* (*Poaceae*). Can J Bot 63, 995-1007.

BREDE A.D., SUN S., 1995. Diversity of turfgrass germplasm in the Asian Pacific Rim countries and potential for reducing genetic vulnerability. Crop Sci 35, 317- 321.

BUGHRARA S.S., 2004. Turfgrass species and cultivar selection. MSU Extension Bulletin E-2912. Michigan, USA. 4 pp.

CHARMET G., BION A., BALFOURIER F., 1989. Agronomic evaluation of perennial ryegrass wild populations from Ireland for use in French plant breeding programmes. Agronomie 9, 985-991. doi:10.1051/agro:19891007.

CLARKE B.B., WHITE J.F. Jr., HURLEY R.H., TORRES M.S., SUN S., HUFF D.R., 2006. Endophyte-mediated suppression of Dollar spot disease in fine fescues. Plant Dis 90(8), 994-998. doi:10.1094/PD-90-0994.

DA COSTA M., BHANDARI B., CARSON J., JOHNSON-CICALESE J., MEYER W., 1998. Incidence of endophytic fungi in seed of cultivars and selections in the 1988 national fine fescue test. Proc New Jersey Turfgrass Expo. Atlantic City, New Jersey, USA. December 8-10. Vol. 30. 6 pp.

DE LA FUENTE V., ORTÚÑEZ E., FERRERO L.M., 1997. Contribución al género *Festuca* L. (*Poaceae*) en el País Vasco y Sistema Ibérico septentrional (Península Ibérica). Itinera Geobotánica 10, 317-351. [In Spanish].

DE NOVA P.J., MONTE J.G., SOLER C., 2006. Genetic relationships within and among Iberian fescues (*Festuca* L.) based on PCR-amplified markers. Genome 49, 1170-1183. doi:10.1139/G06-077.

DÍAZ T.E., FERNÁNDEZ J.A., NAVA H.S., FERNÁNDEZ M.A., 1994. Catálogo de la flora vascular de Asturias. Itinera Geobotánica 8, 529-600. [In Spanish].

FUNK C.R., WHITE J.F. Jr., 1997. Use of natural and transformed endophytes for turf improvement. In: *Neotyphodium*/Grass interactions (Bacon C.W. and Hill N.S., eds). Ed. Plenum Press, NY, USA. pp. 229-239.

HACKEL E., 1882. Monographia *Festucarum europaearum*. Fischer T., Kassel, Berlin, Germany. 216 pp.

HAYWARD M.D., DE LACEY I.H., TYLER B.F., DRAKE D.W., 1982. The application of pattern analysis for the recognition of adaptation in a collection of *Lolium multiflorum* populations. Euphytica 31, 383-396.

HUBBARD C.E., 1984. Grasses: a guide to their structure, identification, uses and distribution in the British Isles. Penguin Books, Middle-sex, London, 476 pp.

HUFF D.R., PALAZZO A.J., 1998. Fine fescue species determination by laser flow cytometry. Crop Sci 38, 445-450.

KERGUÉLEN M., PLONKA F., 1989. Les *Festuc*a de la Flore de France (Corse comprise). Bull Soc Bot Centre-Ouest 10, 1-368. [In French].

KINDLER S.D., BREEN J.P., SPRINGER T.L., 1991. Reproduction and damage by Russian wheat aphid (*Homoptera*: *Aphididae*) as influenced by fungal endophytes and cool season turfgrasses. J Econ Entomol 84, 685-692.

LATCH G.C.M., POTTER L.R., TYLER B.R., 1987. Incidence of endophytes in seeds from collections of *Lolium* and *Festuca* species. Ann Appl Biol 111, 59-64. doi:10.1111/j.1744-7348.1987.tb01433.x.

LAZENBY A., ROGERS H.H., 1964. Selection criteria in grass breeding. II. Effect on *Lolium perenne* of differences in population density, variety and available moisture. J Agric Sci Camb 62, 285-298.

OLIVEIRA J.A., CASTRO V., 1997. Incidence and viability of *Acremonium* endophytes in tall fescue accessions from North Spain. Genet Resour Crop Evol 44, 519-522. doi:10.1023/A:1008620725857.

OLIVEIRA J.A., GONZÁLEZ A., 2000. Recursos fitogenéticos de raigrás inglés europeos: valor agronómico en condiciones de bajo mantenimiento. Invest Agrar: Prod Prot Veg 15(1-2), 67-78. [In Spanish].

PORTAL R., 1999. Festuca de France. Pers. Ed, Vals-près-Le Puy, France. 371 pp. [In French].

RAO S.A., RAO K.E.P., MENGESHA M.H., REDDY V.G., 1996. Morphological diversity in sorghum germplasm from India. Genet Resour Crop Evol 43, 559-567.doi:10.1007/BF00138832.

RICHARDSON M.D., FREEMAN G.W., MEYER W.A., REDDY P.V., WHITE J.F. Jr., 1997. Endophytes from fine fescues of Europe and North America. Int Turfgrass Soc Res J 8, 913-918.

RUEMMELE B.A., BRILMAN L.A., HUFF D.R., 1995. Fine fescue germplasm diversity and vulnerability. Crop Sci 35, 313-316.

SAINT-YVES A., 1927. Tentamen. Claves analyticae festucarum veteris orbis (subgen. *Eu-Festucarum*). Revue Bretonne de Botanique Pure et Apliquée 2, 1-124.

SCHMIT R.M., DUELL R.W., FUNK C.R., 1974. Isolation barriers and self-compatibility in selected fine fescues. Proc. 2nd Intnl. Turfgrass Res. Conf. Blacksburg, VA. ASA and CSSA, Madison, Wisconsin, USA. July 19-21. pp. 9-17.

ŠMARDA P., MÜLLER J., VRÁNA J., KOČI K., 2005. Ploidy level variability of some Central European fescues (*Festuca* subg. *Festuca*, *Poaceae*). Biologia Bratislava 60(1), 25-36.

SPSS, 2005. SPSS for windows, vers 14.0. SPSS Inc. 1989-2005.

TERREL E.E., 1979. Taxonomy, morphology and phylogeny. In: Tall fescue (Buckner R.C., Bush L.P., eds). ASA, CSSA, and SSSA, Madison, Wisconsin, USA. pp. 31-39.

TOLIVIA D., TOLIVIA J., 1987. Fasga: a new polychromatic method for simultaneous and differential staining of plant tissues. J Microsc 148(1), 113-117.

TURGEON A.J., 2004. Turfgrass management. 7th Ed. Pearson Prentice Hall, New Jersy, USA. 415 pp.

TYLER B.F., CHORLTON K.H., THOMAS I.D., 1984. Characterization of collected *Lolium perenne* populations. Report of the Welsh Plant Breeding Station, 1983, Aberysthwyth, UK. pp. 29-32.

TYLER B.F., CHORLTON K.H., THOMAS I.D., 1987. Preliminary screening of forage grasses. Collection, characteri-

zation and utilization of genetic resources of temperate forage grass and clover. IBPGR Training Courses: Lecture series 1. Chapter 2. IBPGR, Rome, Italy. pp. 13-17.

WILKINSON M.J., STACE C.A., 1991. A new taxonomic treatment of the *Festuca ovina* L. aggregate (*Poaceae*) in the British Isles. Bot J Linn Soc 106, 347-397.