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Agromorphological characterization and dollar spot fungus susceptibility in accessions of common bent (*Agrostis capillaris* L.) collected in northern Spain

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Abstract

Fourteen *Agrostis capillaris* accessions collected in northern Spain were characterized in a trial with a low-fertilization regime, carried out in two successive years (2004 and 2005). The commercial cultivar 'Golfin' was included in the study as a control. All accessions and the commercial cultivar were evaluated for 11 agromorphological characteristics and susceptibility to dollar spot disease, caused by *Sclerotinia homoeocarpa* F.T. Bennet. The data obtained were reduced to five principal components that cumulatively explained 85.4% of the total variance. Cluster analysis was useful in identifying four groups of accessions that described 50% of the phenotypic variation among accessions. Cluster 1 consisted of four accessions with the highest resistance to dollar spot, dark green colour, late heading date and high autumn turf quality. Cluster 2 included the two earliest heading accessions, dark green colour, intermediate tolerance to dollar spot and low autumn turf quality. Cluster 3 comprised six accessions with the latest heading date, dark green colour and low tolerance to dollar spot. Cluster 4 consisted of two accessions and the 'Golfin' cultivar, with lightest green colour, low autumn turf quality and the poorest tolerance to dollar spot. The results of this study suggest the potential value of some of these common bent accessions collected in northern Spain for turf and forage improvement.

Additional key words: *Agrostis tenuis*, artificial inoculation, browntop, colonial bentgrass, multivariate analysis, turfgrass.

Resumen

Caracterización agromorfológica y susceptibilidad al hongo «dollar spot» en accesiones de agrostis común (*Agrostis capillaris* L.) recogidas en el Norte de España

Catorce accesiones de *Agrostis capillaris* procedentes del norte de España se caracterizaron en un régimen de baja fertilización durante dos años (2004 y 2005) en un diseño de bloques completos al azar con dos repeticiones de 15 plantas por accesión (en total 30 plantas por accesión). El cultivar comercial 'Golfin' se incluyó como control en el estudio. Todas las accesiones y el cultivar 'Golfin' se evaluaron mediante 11 caracteres agromorfológicos y la susceptibilidad a la enfermedad fúngica «dollar spot» (*Sclerotinia homoeocarpa* F.T. Bennet). Los datos obtenidos se redujeron a cinco componentes principales que explicaron el 85,4% de la varianza total. Mediante una clasificación jerárquica se identificaron cuatro grupos, que describen el 50% de la variación fenotípica entre las accesiones. El grupo 1 incluyó cuatro accesiones con la mayor resistencia al «dollar spot», color verde oscuro, espigado tardío y muy buena calidad de césped en el otoño. El grupo 2 incluyó las dos accesiones más precoces de espigado, tolerancia intermedia al «dollar spot» y mala calidad de césped en el otoño. El grupo 3 comprendió seis accesiones tardías de espigado, color verde oscuro y baja tolerancia al «dollar spot». El grupo 4 incluyó dos accesiones y el cultivar 'Golfin' con color verde más claro, baja calidad otoñal de césped y la menor tolerancia al dollar spot. Los resultados de este estudio muestran el potencial de algunas de las accesiones de agrostis común procedentes del norte de España para uso en mejora de céspedes y forrajes.

Palabras clave adicionales: *Agrostis tenuis*, análisis multivariable, césped, inoculación artificial.

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Introduction

The genus *Agrostis* (bentgrass) comprises 150 to 220 species distributed around the world (Hitchcock, 1951; Bjorkmann, 1960; Watson and Dallwitz, 1992). Only three species are commonly used as turfgrasses in Spain: common bent (*Agrostis capillaris* L. syn. *Agrostis tenuis* Sibth.), velvet bentgrass (*Agrostis canina* L.) and creeping bentgrass (*Agrostis stolonifera* L. syn. *Agrostis palustris* Huds.).

Common bent ($2n = 4x = 28$, $A_1A_1A_2A_2$), known as browntop in Europe and colonial bentgrass in the United States, is a perennial, outcrossing, cool-season grass native to Europe and temperate Asia. The main characteristics of this grass are as follows: fine to very fine texture with high density, may be slightly less dense than creeping bentgrass ($2n = 4x = 28$, $A_2A_2A_3A_3$) (Jones, 1956a); colour variable, light to dark green; growth habit bunch-type or with very short stolons or rhizomes; adapted to well-drained, moderately acid soils, and sun to partial shade; will not tolerate heat or drought, and requires irrigation if rainfall is not adequate; intermediate management requirements; requires annual nitrogen fertilization of 50 to 300 kg ha^{-1}, depending on management intensity; requires frequent mowing and will tolerate low mowing heights; turf is usually maintained at mowing heights of 6 to 25 mm depending on use and site, and is prone to thatch development (Bughrara, 2004); cannot tolerate as much traffic as creeping bentgrass; disease problems similar to creeping bentgrass. The species is not widely used in northern Spain. May lack uniformity due to heterogeneity of types. It is used on golf course roughs, fairways, tees, home lawns and for erosion control (Hubbard, 1984). Seeding rate is 150 kg seed ha^{-1}. The stoloniferous creeping bentgrass is the best adapted species for use on golf course greens (Wipff and Fricker, 2001). Velvet bentgrass ($2n = 2x = 14$, A_1A_1) is a very fine-textured weakly stoloniferous grass that forms a high density turf (Jones, 1956b). It is more tolerant of shade, drought and soil acidity than other bentgrasses (Turgeon, 2005).

Sclerotinia homoeocarpa F.T. Bennett causes dollar spot, a leaf disease of closely mowed turf throughout the world (Vargas, 1994; Bonos *et al*., 2003). Symptoms appear as bleached spots about the size of a silver dollar coin that may overlap to produce large irregular areas of sunken dead turfgrass (Walsh *et al*., 1999). Prolonged high humidity in the turfgrass canopy is required for fungal growth. Different strains of dollar spot fungi initiate growth under different temperatures; thus disease may develop from late spring to late autumn (Turgeon, 2005). Colonial and velvet bentgrass cultivars are less susceptible to dollar spot isolates than creeping bentgrass cultivars (Chakraborty *et al*., 2006).

Dollar spot management is highly dependent on cultural practices and applications of fungicide (Williams *et al*., 1996; Chakraborty *et al*., 2006). Development of bentgrass cultivars that show resistance to dollar spot would reduce the costs and environmental impacts of fungicide applications. Breeding programmes for dollar spot resistance are mainly based on materials collected from old golf course turfs (Casler and Duncan, 2003). Other possible sources of dollar spot resistance originate from introductions of *Agrostis* species.

Creeping bentgrass, the premier turf used on golf putting greens in cool-season areas, is considered to be highly susceptible to dollar spot. Although there is evidence for genetic resistance to dollar spot in some clones of creeping bentgrass (Vincelli *et al*., 1997; Bonos *et al*., 2003), there are no resistant cultivars available at present (Vargas, 1994). Use of the disease resistance of colonial bentgrass to improve disease resistance in other bentgrass species by interspecific hybridization and genome introgression is a promising approach (Belanguer *et al*., 2004).

A diverse genetic background provides a supply of allelic variation that can be applied to create new and favourable unique gene combinations. Two different types of procedures are available to evaluate diversity among plant genotypes: investigation of morphological traits, and fingerprint profiles of DNA markers. Morphological characteristics can be used to estimate genetic distances, and to examine morphological divergence in large collections of plant genotypes (Lasa *et al*., 2001; Oliveira *et al*., 2008). Estimates of genetic distance based on DNA marker polymorphisms provide a unique capacity to describe genetic diversity because of the abundant polymorphisms and the fact that these are independent of environmental effects. To conserve and study the existing genetic resources of colonial

Abbreviations used: AFLP (amplified fragment length polymorphisms), bp (base pairs), CBS (CBS Fungal Biodiversity Centre, Utrecht, the Netherlands), CIAM (Centro de Investigaciones Agrarias de Mabegondo, A Coruña, Spain), CRF (Centro de Recursos Fitogenéticos, Madrid, Spain), LSD (least-significant difference), m.a.s.l (metres above sea level), NS (non significant), PC (principal component), PCA (principal component analysis), PCR (polymerase chain reaction), SD (standard deviation), WARD (increase in sum of squares hierarchical clustering method).

bentgrass, accessions from northern Spain destined for use in breeding programmes have previously been investigated by use of amplified fragment length polymorphism (AFLP) markers (Zhao *et al*., 2006).

Spain is one of the centres of origin of the colonial bentgrass species, and is well suited for exploration aimed at diversifying the existing germplasm collection (Crossa *et al*., 1994). Some colonial bentgrass accessions have been collected and conserved in northern Spain. However, the relationships among these materials are not well known, and little information is available regarding levels of agromorphological diversity and dollar spot susceptibility among populations of colonial bentgrass from northern Spain. Assessment of such diversity may contribute to eliminating undesirable duplications in the germplasm collection and to increase the efficiency of agronomical research and breeding efforts.

The objective of the present study was to characterize the diversity of *Agrostis capillaris* accessions collected in northern Spain, on the basis of agromorphological and dollar spot susceptibility data. Knowledge of such variability should be useful for assessing the potential value of these accessions for Spanish and North American turf and forage breeding programmes.

Material and methods

Plant material

Seeds of 14 accessions of *A. capillaris* were collected from grasslands in northern Spain, in 2000. Each of these populations was collected as seeds from at least 50 plants taken from an ecologically homogeneous area of 100-1,000 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 the 14 populations are listed in Table 1. The accessions were collected from grasslands, wasteland and paths. The grasslands had a long history of agricultural management, but no recent record of ploughing and reseeding with modern forage varieties. All the seeds were stored in waterproof packages, at 0-4°C, in the CRF (Centro de Recursos Fitogenéticos Nacional, in Alcalá de Henares, Madrid) and the CIAM [Centro de Investigaciones Agrarias de Mabegondo (FAO Code = ESP119) in A Coruña, Galicia].

Plant characterization

After the seeds were cleaned they were germinated in a peat soil mixture, in September 2002, and maintained under greenhouse conditions for 6 months. The morphological study was established on the «Casero» farm in Carreño (43° 35' N, 5° 47' W, 80 m.a.s.l.) on an Inceptisol soil in a plot used by the University of Oviedo. The site was arranged in a completely randomized block design with two replicates of 15 plants per accession and replicate (in total 30 plants per accession). Plants were transplanted to the field in March 2003 (50 cm apart). The commercial cultivar 'Golfin' (*A. capillaris*) was included as a control.

Table 1. Inventory number in the Centro de Recursos Fitogenéticos (Madrid) seed bank (accession number in brackets) and origins of the 14 accessions of *Agrostis capillaris*

Inventory number	Zhao *et al.*[1]	Province	Location	Habitat	Lat	Long	Alt
NC074777 (2)	1265	Asturias	Pousadoiro	Grassland	4318N	0703W	800
NC074778 (3)	1266	Asturias	Sta Eulalia de Oscos	Grassland	4315N	0701W	560
NC074779 (4)	1267	Asturias	Milladoira	Wasteland	4315N	0701W	560
NC074780 (5)	1268	Asturias	Sueiro	Grassland	4332N	0654W	140
NC074781 (6)	1269	Asturias	La Roda	Grassland	4332N	0658W	100
NC074782 (7)	1270	Asturias	El Franco	Grassland	4332N	0652W	150
NC074783 (8)	1271	Asturias	Pilando	Grassland	4333N	0647W	120
NC074785 (10)	1273	Asturias	Miudes	Grassland	4331N	0647W	90
NC074786 (11)	1274	Asturias	Navia	Path	4333N	0640W	90
NC074787 (12)	1275	Lugo	Fonsagrada	Grassland	4309N	0701W	900
NC074788 (13)	1276	Asturias	Restrepo	Grassland	4325N	0759W	600
NC074790 (15)	1278	Asturias	Viavelez	Grassland	4334N	0650W	50
NC074797 (22)	1285	León	Riaño	Grassland	4250N	0450W	1,000
NC074798 (23)	1286	Asturias	Mieres	Grassland	4310N	0550W	200

[1] Correspondence between the inventory numbers used in the CRF seed bank and the codes used by Zhao *et al.* (2006).

The site received the same amount of fertilizer —a total of 10 g N m^{-2} per year, equivalent to a low-fertilization regime— throughout the two years of study. The plants were maintained at a height of 5 cm with a rotary mower. Clippings were removed after each cut.

All the accessions used in this study were classified as *A. capillaris* according to the following characteristics: flat basal leaves, rolled vernation; membranous, truncate ligule; auricles absent; narrow, oblique collar; loose and branched panicle; palea > 1/3 the length of the lemma; peduncles length of the spikelets or shorter; branches at the lower node of the panicle with spikelets located only in the upper half or third; glumes with spines only in the upper half of the keel, palea bifida (Romero *et al*., 1998; Turgeon, 2005).

The following 11 morphological (UPOV, 1990; NTEP, 1998) characters were evaluated during the 2-year period of the study (2004-2005): Lw, leaf width (1 = narrow, 9 = broad); Gh, growth habit (1 = erect, 9 = prostrate); Co, colour (1 = light green, 9 = dark green); Aq, autumn quality (1 = very poor turf quality, 9 = outstanding turf quality); Wq, winter quality (1 = very poor quality, 9 = outstanding turf quality); Fll, flag leaf length (cm); Flw, flag leaf width (mm), Lls, length of longest stem, included inflorescence (cm); Li, length inflorescence (cm); Lui, length upper internode (cm) and Hd, heading date (as the number of days after January 1st).

Pathogen isolation and identification

Symptomatic perennial ryegrass leaf blades were collected from the «Las Caldas» golf course in Oviedo (Asturias). Fresh leaves were cut from diseased plants showing symptoms of dollar spot and immediately placed on ice (inside a plastic bag). The leaves were sent to the Plant Physiology Area, Faculty of Sciences, University of A Coruña (Galicia) for processing. Each leaf was cut into pieces of approximately 5 cm long, and placed in a small beaker with 1% (v/v) sodium hypochlorite for 30 s to sterilize the surface, then rinsed twice in sterile water, placed on potato dextrose agar (PDA), and incubated at 25°C for 5 days (Bonos *et al*., 2003).

In this study, DNA was isolated from different plant and fungal material: leaves of symptomatic perennial ryegrass turf, leaves of asymptomatic perennial ryegrass turf, fungal mycelia isolated from the symptomatic perennial ryegrass turf, and also from a reference *S. homoeocarpa* sample received from the CBS Fungal Biodiversity Centre, Utrecht, the Netherlands (CBS 510.89), according to the CTAB method of Edwards *et al*. (1991). The leaves were cut into small pieces (< 5 mm long) and ground to a fine powder with liquid nitrogen in a mortar. The sample was mixed with 1 mL CTAB buffer (2% CTAB in 100 mM Tris-HCl pH 8 with 1.4 M NaCl, 20 mM EDTA, 0.08% PVP and 0.01% β-mercaptoethanol) and disrupted in a rotor-stator homogenizer (30-60 s). The lysate was heated for 30 min at 65°C and after cooling a mixture of phenol: chloroform: isoamylic alcohol 25:24:1 was added (500 µL), and the suspension was centrifuged for 4 min at 8,000 rpm. One volume (500 µL) of chloroform: isoamylic alcohol 24:1 was added (twice) to the upper phase, and this was centrifuged under the same conditions. The upper phase was added to isopropanol (600 µL) and incubated at room temperature for 20 min. The sample was then centrifuged for 5 min at 13,000 rpm and the pellet washed with 0.2 M sodium acetate in 76% ethanol and centrifuged again for 5 min at 13,000 rpm. The pellet was washed with 70% ethanol and centrifuged as described above. The resultant pellet was dried at room temperature and diluted in 50 µL of autoclaved bidistilled water.

A set of two 22 and 26-bp-long oligomers ITS1-F 5'-CTTGGTCATTTAGAGGAAGTAA-3' universal primer (Gardes and Bruns, 1993) and PITS2SHc-R 5'GTGTTACTACGTTCAGGACCCAGCGG-3' were used as specific primers. DNA molecular weight marker (Roche, 100 bp-2,642 bp) was used to calculate the sizes of the PCR products.

The concentration of DNA from infected plants was calculated spectrophotometrically (Varian Cary 3E UV-Visible Spectrophotometer). The amount of DNA used for a PCR reaction was 40 ng in 20 µL of reaction mixture, except for the amplification in *Lolium perenne*, which required a nested PCR in the same conditions to visualize the amplicon on agarose gel. The reaction mixture contained Reddy Mix (ABgene, Epsom, UK) and primers at a final concentration of 0.75 µM (IST1-F) and 0.5 µM (PITS2SHc-R). The thermal cycling conditions were: initial denaturation at 95°C for 95 s, followed by 30 cycles at 94°C for 30 s, 52°C for 40 s and 72°C for 2 min. A final extension step at 72°C for 10 min was added. Amplification reactions were run on a 1% agarose gel and visualized on a UV transilluminator (GelDoc XR, BioRad).

To assess the identity of the amplicon from the samples of *S. homoeocarpa* pathogen isolated from

the *L. perenne* host, an agarose gel was run and the different bands obtained were cut from the gel, extracted and purified with a gel extraction kit (QIAquick Gel Extraction kit, QIAGEN, USA). The purified fragments were sequenced by BigDye Terminator v3.1 Ready Reaction Cycle Sequencing and the Genetic Analyser 3130xl (Applied Biosystems). The sequences obtained were processed with Clustal W2.

Inoculum production

The isolate of *S. homoeocarpa* obtained on symptomatic perennial ryegrass was grown on sterilized Kentucky bluegrass (*Poa pratensis* L) seed for inoculation. Two hundred grams of Kentucky bluegrass seed were autoclaved for 15 min at 151°C. Seventy-five millilitres of dH_2O were added to the sterilized seed in an Erlenmeyer flask, and left overnight. Single isolates of *S. homoeocarpa* growing on PDA were cut into 1×1 cm pieces and transferred to the flask. Isolates were grown separately in flasks for approximately 3 wk at room temperature and then dried on newspaper for 3 days. The dried inoculum was subsequently used to inoculate the plants for evaluation of dollar spot sensivity.

Growth chamber experiment

Evaluation of dollar spot susceptibility was carried out in a growth chamber (Mieres Campus, University of Oviedo), adjusted to a 12 h photoperiod (13,000 lux) and temperature ranging from 22 to 27°C, which is optimal for maximum pathogenicity (Walsh *et al*., 1999) on 23 January 2009.

Seeds of each accession and the control ('Golfin') were germinated in the greenhouse on trays containing a peat soil mixture. Thirty plants of each accession plus the control were transferred into pots (1 plant per pot) in November 2008. After 8 weeks in the greenhouse, the plants were placed in the climatic chamber for a 3 day acclimation period prior to inoculation of the pathogen. Equal amounts of inoculum (1 g) were placed in the centre of each pot. The trays were filled with water and placed inside plastic bags to maintain high humidity and optimize the severity of the disease.

The plants were visually inspected 3 weeks after the inoculation and were scored for resistance and susceptibility, on a scale of 1 to 9, where 1 = plant completely brown from disease and 9 = no symptoms (Chakraborty *et al*., 2006). Susceptible plants were classified by size of the infection area, as characterized by increased area of brown lesions and widespread development of mycelia. The rating scale was approximately linear with respect to the percentage of diseased tissue, so that a mean rating of 4.5 would represent approximately 50% diseased leaf tissue.

The pathogenic isolates were re-isolated on potato dextrose agar after the evaluation, and their identity was confirmed by PCR.

Statistical analysis

Normality of the residuals was tested by the Shapiro-Wilk (W) test, and the homogeneity of residual variances was tested by Levene's test (SPSS, 2006). 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 years. The effects of year, replicate, accession, and the interactions between them were considered. The replicate effect was considered to be random. Separation of means was performed by the least-significant difference (LSD) test.

For evaluation of disease, analysis of variance was applied to the data obtained in the 3rd week after inoculation, considering the effects of replication and accession.

Multivariate relationships among accessions were explored with a principal component analysis (PCA), with a correlation matrix derived from the significant characters. The original variables were reduced to five independent linear combinations, principal components (PC) of the variables with eigenvalues greater than 1, which cumulatively explained 85.4% of the total variance.

The PCs were used as the input for an agglomerative hierarchical cluster analysis to detect groups of similar agronomic type. The squared Euclidean distance was used as a measure of distance, and Ward's clustering algorithm (increase in sum of squares hierarchical clustering method) was used to combine accessions into clusters. This method can group accessions into clusters that minimize the within-cluster variance. A partition was chosen from the view of the tree of classification. In order to explore 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 15 (SPSS, 2006).

Results

Pathogen identification

In order to assess the nature of the pathogen infecting the perennial ryegrass turf in the golf course, a PCR was performed on the diseased plants, and produced a 500-bp-band, which correspond to the amplicon size obtained from the sample of *S. homoeocarpa* obtained in the golf course. Furthermore, the sample of asymptomatic perennial ryegrass did not amplify.

When the bands (amplified regions) obtained were sequenced, the results showed that the DNA sequences from the three samples (*Sclerotinia homoeocarpa* CBS 510.89, pathogen isolated from symptomatic perennial ryegrass and symptomatic perennial ryegrass turf) were identical. All the sequences matched the one of S. *homoeocarpa* (accession number GQ386985) from the NCBI database (http://www.ncbi.nlm.nih.gov).

A PCR was also performed to check whether the symptoms found in *A. capillaris* plants were caused by *S. homoeocarpa*. We obtained a single 500-bp band in symptomatic *A. capillaris* plants, which correspond to the amplicon size obtained from the initial sample of *S. homoeocarpa* obtained in the golf course. Furthermore, the sample of asymptomatic *A. capillaris* plants did not amplify. Moreover, the amplified regions were sequenced; obtaining the same nucleotidic sequence in the initial sample obtained from the golf course, in the diseased plants after inoculation and in the reference sample of *S. homoeocarpa* (CBS 510.89). All of the sequences matched the one of *S. homoeocarpa* (accession number GQ386985) from the NCBI database.

Agronomic characterization

The results of analysis of variance of the 12 traits (11 agromorphological plus dollar spot susceptibility traits) in two years are summarized in Table 2. The accession effect was statistically significant for all the characters. The mean values of traits and SD for the accessions are shown in Table 3.

Morphological characters across the colonial bentgrass accessions were highly variable, including a 13-day range in heading date and a 17.8-cm range in length of the longest stem with inflorescence. A highly significant correlation between flag leaf length and inflorescence length was observed ($r = 0.93$, $p < 0.01$).

The correlation coefficients for the 12 traits and the first five PCs are shown in Table 4. The first PC was positively correlated with heading date and negatively correlated with flag leaf width and length upper internode. The traits that were positively correlated with the second component (and thus behaved indepen-

Table 2. Mean squares of the analyses of variance of 11 agronomic traits evaluated in 15 accessions during two years

Traits	Year	Replicate	Year*Replicate	Accession	Accession*Year	Error
Lw	0.32NS	84.02NS	0.69NS	25.50***	0.11NS	0.41
Gh	1.21NS	63.47NS	7.29**	14.80***	18.61***	0.99
Co	1.28NS	13.44NS	0.11NS	29.97***	0.09NS	0.29
Aq	1.69NS	8.03NS	38.03***	49.47***	5.94***	2.29
Wq	363.50NS	13.93NS	4.55NS	45.96***	16.49NS	2.09
Fll	0.01NS	0.96NS	0.12NS	278.68***	0.17NS	4.51
Flw	0.44NS	0.01NS	0.02NS	53.87***	0.03NS	1.61
Lls	99.07NS	4.61NS	5.02NS	1258.48***	104.68NS	55.36
Li	0.01NS	123.58*	0.24NS	539.27***	0.44NS	7.04
Lui	1.22NS	3.91NS	0.39NS	209.72**	0.12NS	3.02
Hd	1.52NS	49.47NS	3.87NS	1244.98***	0.19NS	12.27
Dss		8.00NS		114.11***		2.19

Lw: leaf width. Gh: growth habit. Co: colour. Aq: autumn quality. Wq: winter quality. Fll: flag leaf length. Flw: flag leaf width. Lls: length of longest stem, included inflorescence. Li: length inflorescence. Lui: length upper internode. Hd: heading date and one disease susceptibility traits. Dss: dollar spot susceptibility in the fourteen accessions and one control (Golfin). *** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$. NS: $p > 0.05$.

Table 3. Two-year means (SD in brackets) for 11 agronomic and one disease susceptibility traits in the fourteen accessions and one control (Golfin)

Accessions	Lw	Gh	Co	Aq	Wq	Fll	Flw	Lls	Li	Lui	Hd	Dss
2	6.9 (0.2)	4.1 (1.0)	6.9 (0.2)	5.8 (1.9)	6.3 (1.4)	5.7 (1.3)	4.4 (1.6)	70.5 (9.1)	8.7 (1.8)	10.1 (1.8)	154.1 (7.2)	7.3 (1.3)
3	6.8 (0.4)	4.4 (1.9)	6.0 (0.9)	5.6 (1.6)	5.9 (1.8)	11.4 (3.2)	2.9 (0.7)	67.4 (7.9)	14.7 (2.8)	11.7 (1.8)	159.0 (2.6)	7.7 (1.4)
4	6.9 (0.4)	3.0 (1.4)	6.8 (0.4)	6.7 (1.7)	7.5 (1.3)	7.6 (1.8)	3.4 (0.9)	64.7 (5.2)	12.4 (2.0)	10.8 (1.2)	160.8 (1.7)	6.6 (1.4)
5	6.0 (0.9)	3.2 (0.6)	6.9 (0.2)	6.6 (1.3)	7.4 (1.9)	7.1 (1.4)	2.9 (0.7)	61.0 (3.7)	10.5 (1.9)	12.7 (1.3)	159.5 (1.7)	6.3 (1.8)
6	5.9 (0.9)	3.9 (1.0)	5.9 (0.9)	7.1 (0.9)	6.3 (2.7)	8.4 (1.8)	3.1 (0.9)	61.8 (6.4)	11.9 (2.3)	10.3 (1.7)	160.9 (1.6)	2.9 (1.2)
7	5.9 (0.9)	3.9 (1.6)	6.9 (0.2)	4.2 (1.5)	4.7 (1.1)	9.6 (2.2)	5.5 (1.8)	59.5 (5.9)	13.2 (4.4)	15.2 (1.5)	147.3 (1.8)	3.2 (1.5)
8	5.0 (0.2)	3.9 (1.0)	6.9 (0.2)	6.9 (1.4)	6.8 (1.1)	6.7 (1.6)	3.1 (0.9)	62.1 (8.9)	11.6 (2.5)	11.2 (1.8)	158.1 (2.2)	4.1 (1.6)
10	6.0 (0.9)	3.4 (1.0)	5.0 (0.3)	6.3 (1.9)	6.7 (1.5)	5.5 (1.7)	4.9 (2.2)	61.5 (9.9)	9.1 (1.9)	10.3 (1.9)	154.1 (7.2)	3.2 (1.7)
11	6.0 (0.9)	3.8 (1.0)	6.9 (0.2)	6.6 (1.0)	7.1 (1.9)	12.1 (3.5)	4.7 (1.3)	64.4 (6.6)	19.9 (4.3)	13.6 (2.4)	148.6 (2.2)	6.3 (1.3)
12	6.9 (0.5)	4.2 (1.1)	6.8 (0.4)	6.1 (1.6)	6.0 (2.1)	9.3 (1.6)	4.1 (0.8)	64.1 (6.4)	14.5 (2.1)	8.9 (1.9)	159.5 (2.3)	8.1 (1.3)
13	6.9 (0.2)	3.1 (0.5)	6.9 (0.2)	6.0 (1.4)	5.9 (1.6)	10.4 (2.2)	3.9 (0.8)	74.1 (6.2)	14.7 (2.3)	12.1 (1.7)	160.8 (1.6)	6.1 (1.4)
15	5.9 (0.9)	4.1 (1.0)	6.0 (0.9)	6.7 (1.3)	7.7 (1.4)	6.8 (2.0)	3.1 (0.7)	69.3 (8.8)	11.4 (2.8)	8.1 (1.7)	160.0 (1.9)	4.4 (1.4)
22	5.1 (0.2)	3.4 (0.9)	6.0 (0.9)	5.5 (2.1)	6.8 (0.9)	6.3 (1.1)	5.3 (2.0)	61.8 (11.6)	8.8 (2.2)	10.8 (1.1)	152.1 (5.3)	5.7 (1.6)
23	5.9 (0.9)	4.6 (0.8)	6.9 (0.2)	6.5 (1.3)	6.4 (1.6)	5.5 (1.0)	2.6 (0.6)	56.3 (6.3)	9.3 (1.4)	9.2 (1.2)	158.9 (2.7)	1.9 (1.6)
Golfin	6.9 (0.2)	3.2 (1.6)	5.0 (0.3)	4.1 (1.7)	4.9 (1.5)	9.7 (3.1)	4.0 (1.0)	62.7 (5.3)	13.6 (2.8)	9.6 (1.9)	159.9 (1.8)	3.5 (1.6)
Ftest	62.6***	14.8***	101.8***	21.6***	21.9***	61.8***	33.4***	22.7***	76.6***	69.5***	101.5***	52.1***
LSD ($p = 0.05$)	0.8	0.4	0.8	0.5	0.5	0.7	0.5	2.9	1.1	0.7	1.2	0.9

*** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$. 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.

dently from those associated with the first component) were the length of inflorescence and flag leaf length. The third PC was positively correlated with winter and autumn turf quality. The fourth PC was positively correlated with dollar spot resistance, length of longest stem including inflorescence and leaf width. The last fifth PC was positively correlated with growth habit and colour.

The projection of the accessions on the plot of components 1-2 is shown in Figure 1. Hierarchical clustering analysis performed on the first five 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 50%. Generally, the sum of the variation accounted for by the partition should be at least 50% of the total variation, and more is even better (Huff, 2001).

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 5). Cluster 1 consisted of four accessions (2, 3, 12 and 13) with late heading, high autumn turf quality,

Table 4. Correlation coefficients for 11 agronomic and one disease susceptibility traits and the first five principal components from a principal component analysis of the correlation matrix of the traits (Varimax rotation method)

Traits	Component 1	Component 2	Component 3	Component 4	Component 5
Lw	0.337	0.209	–0.445	0.676	–0.020
Gh	0.153	–0.070	–0.129	–0.137	0.900
Co	–0.252	0.231	0.445	0.238	0.610
Aq	0.348	–0.064	0.862	–0.021	0.105
Wq	0.111	–0.220	0.905	0.074	–0.115
Fll	–0.042	0.916	–0.265	0.231	–0.001
Flw	–0.833	–0.051	–0.349	0.080	–0.265
Lls	0.121	0.090	0.024	0.844	–0.116
Li	–0.044	0.923	–0.043	0.195	0.034
Lui	–0.675	0.582	0.036	–0.104	–0.010
Hd	0.921	–0.065	0.120	0.167	–0.129
Dss	–0.114	0.177	0.184	0.858	0.085

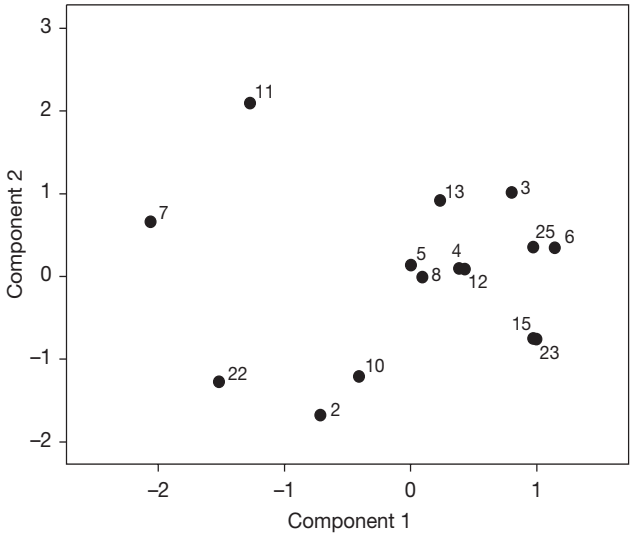

Figure 1. Plot of the principal component analysis of seven traits with projection of the accession number. Component 1 as *x* axis, component 2 as *y* axis (50% of the total variance explained). Accessions are from number 1 to 23 and cv 'Golfin' is number 25. Accessions at the extreme right of the plot (*e.g.* Acc. 6) showed the latest heading date and narrowest flag leaf. Acc. 7 on the extreme left of the same plot presented the earliest heading date and the widest flag leaf. On the upper part of the plot, Acc. 11 showed the longest inflorescence and flag leaf. At the bottom of the plot, Acc. 2 showed the shortest inflorescence and flag leaf.

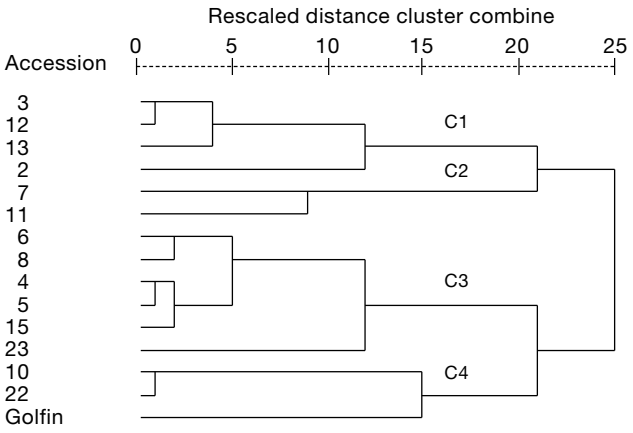

Figure 2. Dendrogram based on the results of the hierarchical cluster analysis by the Ward method (50% of the variance among accessions). C1, C2, C3 and C4 mean Cluster 1, 2, 3, and 4 respectively. Each time two accessions are joined by a vertical line, a subcluster or cluster is created. The dendrogram also presents the values of the distance coefficients for each step or vertical line.

high flag leaf and inflorescence length, dark green colour, and the greatest resistance to dollar spot. Cluster 2 comprised two accessions (7, 11) which displayed early heading, dark green colour, low autumn turf quality, intermediate tolerance to dollar spot, high flag leaf, inflorescence and upper internode length. Cluster 3 included six accessions (4, 5, 6, 8, 15 and 23) with late heading, dark green colour and high autumn turf quality and low tolerance to dollar spot. Cluster 4 comprised the two accessions (10, 22) and the cv. 'Golfin', which displayed late heading, light green colour, the poorest tolerance to dollar spot, the widest flag leaf and low autumn turf quality. Since the turf market requires mainly dark green colours, high turf

Table 5. Between-cluster differences for 11 agronomic and one disease susceptibility traits in the fourteen accessions and one control (Golfin)

Trait	Cluster 1 (n = 4) M (SD)	Cluster 2 (n = 2) M (SD)	Cluster 3 (n = 6) M (SD)	Cluster 4 (n = 3) M (SD)	ANOVA F ratios F (3, 11)
Lw	6.91 (0.05)	6.00 (0.03)	5.97 (0.58)	6.01 (0.94)	2.64NS
Gh	3.94 (0.55)	3.89 (0.08)	3.79 (0.59)	3.31 (0.10)	1.05NS
Co	6.68^{a} (0.44)	6.94^{a} (0.01)	6.61^{a} (0.48)	5.34^{b} (0.59)	6.69**
Aq	5.88^{ab} (0.24)	5.42^{b} (1.68)	6.78^{a} (0.24)	5.30^{b} (1.12)	3.66*
Wq	6.03 (0.19)	5.92 (1.72)	7.02 (0.60)	6.15 (1.09)	1.79NS
Fll	9.22^{ab} (2.45)	10.86^{a} (1.74)	7.03^{b} (0.96)	7.17^{b} (2.20)	3.07*
Flw	3.85^{b} (0.63)	5.14^{a} (0.54)	3.04^{b} (0.29)	4.77^{a} (0.65)	13.01***
Lls	69.03 (4.28)	61.95 (3.46)	62.53 (4.30)	62.02 (0.65)	3.07NS
Li	13.16^{ab} (2.96)	16.57^{a} (4.74)	11.21^{b} (1.13)	10.50^{b} (2.68)	2.97*
Lui	10.69^{b} (1.48)	14.40^{a} (1.16)	10.39^{b} (1.61)	10.27^{b} (0.62)	4.64*
Hd	158.38^{a} (2.92)	147.93^{b} (0.91)	159.71^{a} (1.10)	155.37^{a} (4.10)	12.44***
Dss	7.30^{a} (0.87)	4.77^{ab} (2.21)	4.37^{b} (1.84)	4.15^{b} (1.38)	3.35*

M: mean. SD: standard deviation. n: number of accessions. *** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$, NS: $p > 0.05$. Means followed by different letters in the row are significantly different at the 0.05 level according to Duncan test. Cluster 1 includes accessions: 2, 3, 12, 13; Cluster 2, accessions: 7, 11; Cluster 3, accessions: 4, 5, 6, 8, 15, 23, and Cluster 4, accessions: 10, 22 and the Golfin cultivar.

quality and dollar spot tolerant cultivars, the populations in cluster 1 appear to be the most valuable material for turfgrass breeding and improvement programmes.

Discussion

The species of the genus *Agrostis* cited by Díaz *et al*. (1994) in Asturias are: *A. capillaris* L., *A. castellana* Boiss. & Reuter, *A. curtisii* Kerguélen., *A. duriei* Boiss. & Reuter ex Willk., *A.* ×*fouilladei* P. Fourn, *A. hesperica* Romero García, Blanca & Morales Torre, *A.* × *mubeckki fouillade* (*A. stolonifera* × *A. capillaris*), *A. schleicheri* Jordan & Verlot Seslerietalia, *A. stolonifera* L. var. *pseudopugens* (Langue) Kerguélen, *A. stolonifera* L.var. *scabriglumis* (Boiss. & Reuter) C.E. Hubbard, *A. stolonifera* L.var. *stolonifera*, *A. tileni* Nieto Feliner & Castroviejo.

As one of the native areas of origin of the colonial bentgrass species, Spain is a vast reservoir for germplasm collection and exploitation (Ruemmele, 2003). According to Díaz and Fernández (1994) and Díaz *et al*. (1994), *A. capillaris* is included in class *Molinio-Arrhenatheretea*, order *Arrhenatheretalia*, within plant communities denominated as xerophytic grasslands and meadows. These communities are excellent as mowed and/or grazed grasslands suitable for use in farming. They are found in Eurosiberian mesophytic grasslands that penetrate the areas of heaviest rainfall and occupy soils that do not completely dry out in summer.

Single-plant characterization is of limited value, as seasonal yields are not consistently related to sward yields (Lazenby and Rogers, 1964). Its main use is to determine flowering characteristics, of which heading date is probably the most important character for determining 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 carried out by many authors (Hayward *et al*., 1982; Charmet *et al*., 1989; Oliveira and González, 2000; Oliveira *et al*., 2008). 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, populations included in cluster 1 (Acc. 2, 3, 12 and 13) combined dark green colour, late heading, high inflorescence and flag leaf length, high turf quality and good tolerance to dollar spot. On the basis of these results, northern Spanish turfgrass breeding programmes for *A. capillaris* should focus on these accessions.

Morphological characterization of accessions is beneficial for both evaluation of the agronomic aptitude and for plant breeding research (Rao *et al*., 1996). However, its use has been limited and it not always easy to obtain consistent results in different growing conditions and stages. To overcome problems such as the interference of environmental factors with the agromorphological characters used in classification, several AFLP markers were used (Zhao *et al*., 2006) to classify these accessions of *A. capillaris* with other USDA germplasms. Spanish accession NC074782 (Sp1270 in Zhao *et al*., 2006) was separated from the other Spanish colonial bentgrass accessions by use of ten *Eco*RI/*Mse*I and six *Pst*I/*Mse*I AFLP primer combinations. This accession belongs to cluster 3 in the present study and combined a dark green colour, early heading, high inflorescence and flag leaf length, low autumn turf quality and poor tolerance to dollar spot (mean value of 3.2).

Forage and turfgrass species are usually outcrossing, a characteristic that increases diversity of the genetic base (Renganayaki *et al.,* 2001). However, a moderate level of genetic diversity among colonial bentgrass accessions from northern Spain was observed. The moderate diversity of germplasm collections may encourage breeders to explore materials from different geographic origins to broaden the genetic base. Also, highly similar genetic backgrounds were found in some of the accessions collected in northern Spain, which is common in phenotype-characterized germplasm collections that may contain a certain amount of redundant accessions. Elimination of the redundant accessions based on molecular markers and selection of desired morphological traits used in elite cultivar development are two main objectives in any bentgrass breeding programme.

Warnke (1995) evaluated 31 cultivars of *A. tenuis* and *A. stolonifera* for resistance to *S. homoecarpa* in a growth chamber. Ninety-six percent of the plants in the study showed little or no resistance to dollar spot under these conditions. Bonos *et al.* (2003) obtained broad-sense heritability estimates of 0.56 on a single plant basis and 0.90 on an 11-plant clonal mean basis and stated that improvement in dollar spot resistance in creeping bentgrass should be possible.

Studies of host genotypes and pathogen isolates of dollar spot have shown a general lack of host genotype × pathogen isolate interaction (Chakraborty *et al*., 2006), but there are some results that suggest some race specificity for host resistance to this disease (Casler *et al*., 2007), and the importance of evaluation of each disease across a wide range of environmental conditions and pathogen isolates.

In the present study, with the controlled screening procedure described herein, we identified four accessions (2, 3, 12 and 13) of colonial bentgrasses (*A. capillaris*) belonging to cluster 1, with a high resistance to dollar spot (mean value of 7.3). The commercial colonial bentgrass cultivar 'Golfin' was found to be susceptible (mean value of 3.5). Given that 'Golfin' is a rustic cultivar used on golf course roughs that require low maintenance and even appearance, and that the accessions belonging to cluster 1 displayed better turf qualities than 'Golfin', this indicates their good potential for use as low maintenance fine turf.

On the basis of these results, the plants in these four accessions with the highest disease indices were selected and are being crossed to generate a random mating population for evaluation and selection. The objective is to develop elite clones of colonial bentgrass with good resistance to dollar spot and superior turf quality for potential release to private companies for use in breeding new varieties of colonial bentgrass or to improve the dollar spot resistance of creeping bentgrass.

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