Agromorphological Characterization, Cyanogenesis and Productivity of Accessions of White Clover (*Trifolium repens* L.) Collected in Northern Spain

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Abstract

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White clover (*Trifolium repens* L.) is an essential element of sustainable livestock systems in temperate climates because of its adaptability to a range of management and soil fertility conditions. The performance of 15 accessions of white clover collected in northern Spain, and of two cultivars, the medium-leaved Huia and the large-leaved California, grown as spaced plants in Galicia, and in sward plots in Asturias was compared over a period of two years. The data obtained were reduced to two principal components that cumulatively explained 92.4% of the total variance. Cluster analysis identified three groups of accessions that described 71% of the phenotypic variation among accessions. One group of five accessions collected from upland sites was characterized by low dry matter yield, low height, reduced plant spread, short petioles, small leaves and thin stolons. This group can be defined as small-leaved and of interest for gardening. Another group, composed only by the cv. California and the cv. Huia, was characterized by high plant spread and height, long wide leaves and thick petioles and stolons; dry matter yields were similar to those of the first group. The last group, which includes ten accessions collected from low and medium altitude sites, had the highest dry matter yields and intermediate morphological character between the previous groups. This group can be defined as medium-leaved and of interest for grazing and/or cutting.

Keywords: genetic resources; multivariate analysis; spaced plants; sward plots

The Mediterranean region is the center of origin of 110 of the 237 species of *Trifolium*, including white clover (*Trifolium repens* L.), distributed in temperate and subtropical climates in the northern and southern hemispheres (Zohary & Heller 1984; Ellison *et al*. 2006).

Genetic resources will play an important role as a source of novel traits and genetic variation for future white and red clover breeding programmes, building on past success from introduced germplasm (Pokorný *et al.* 2003; Jakešová *et al.* 2011).

White clover is the most common cultivated clover species worldwide (Ellison *et al.* 2006). The ability of white clover to fix nitrogen may increase the nitrogen content of the soil and partly substitute nitrogen fertilization (Frame & Newbould 1986; Allen 2008). In addition, white clover improves the nutritional value of pasture by increasing its protein and mineral contents (Reid & Strachan 1974), and also improves its digestibility and palatability (Jones & Roberts 1991). However, under conventional management, the application of nitrogenous mineral fertilizers may negatively affect clover, and alter the botanical composition of pasture (Garcia *et al*. 2005). In contrast, the lack of need for nitrogenous mineral fertilizer has a twofold effect; on one hand the development of legumes is not inhibited, and on the other, the reduced fertilizer input leads to greater profitability of exploitations (Álvarez-Iglesias *et al*. 2009).

White clover is an outcrossing, insect pollinated, allotetraploid ($2n = 4x = 32$) species with a high level of genetic diversity within natural populations (Williams 1987).

Varieties of white clover are usually classified by the size of their leaves, into small or dwarf-leaved, common or intermediate-leaved and large-leaved varieties (Muslera & Ratera 1991). The small varieties denominated as wild are well adapted to cold northern climates and are characterized by being prostrate, with small leaves, stolons and flowerheads and low yielding. They are usually recommended for gardens and for pasture for grazing cows. The common or intermediate-leaved varieties (Hollandicum-type) are characterized as more upright with larger leaves, stolons and flowerheads per plant than small clovers. One of the most commonly used varieties worldwide is Grasslands Huia or Huia, adapted to a moist temperate climate and soils of medium to high fertility, with 600 mm or more of evenly distributed rainfall and characterized by high production in spring and autumn and persistence under grazing.

The large-leaved types were originally represented by an ecotype from irrigated pastures in northern Italy, called Ladino. As presently used, the term ladino clover is incorrect (Williams 1987) because the large types of white clovers selected are quite different from the original Italian ecotype. This group includes the cultivar California. Large-leaved white clovers are less tolerant of heavy grazing, but can produce more forage than the other types. They are therefore recommended for mixtures used for harvesting or for pasture for grazing cows (Piñeiro & Pérez 1992).

According to Younie and Baars (2005), white clover can be used for management regimes ranging from continuous sheep grazing (for which small-leaved varieties are most suitable) to lax defoliation, including cutting (for which larger leaved varieties are most suitable).

There is an annual world consumption of 8000 to 10 000 t of white clover seed (Mather *et al.* 1996) and the cultivar Huia, which has dominated the market for many years is gradually being replaced by improved cultivars, particularly from the United Kingdom and New Zealand (Frame & Laidlaw 2005).

Breeding objectives in white clover include improved compatibility with grasses, increased resistance to pest and diseases, and better adaptation to drought. Success has been achieved in combining spring growth and improved cold hardiness (Rhodes & Ortega 1996).

Improvements do not only concern agronomic characteristics, but also cyanogenic polymorphism (the production of hydrogen cyanide), because according to some authors, the yield and persistence of the plant are associated with moderate levels of cyanogenesis (Caradus & Williams 1989). In addition, the possible toxic effects of some cyanogenic compounds in ruminants must be taken into account in improvement programmes, and is one of the descriptors suggested by the IBPGR (1992) for white clover.

For the effective use of white clover in northern Spain, cultivars that tolerate cold winters, that start growing before spring, have a higher dry matter yield production and that are more persistent than Huia (which accounts for 80% of the white clover seeds sold in the EU) are required (Piñeiro *et al*. 2000).

As white clover is almost always grown in mixtures with grasses, it is also important to optimize its contribution to the sward, as well as its persistence and yield stability (Marshall *et al*. 2003).

In addition to the use of adapted cultivars, the implementation of suitable management techniques would be of great interest to maintain white clover in pasture, as proposed in the European Union project: Effect: European Farms for effective Clover Technology (1998–2002). This project was aimed at transferring the current knowledge on white clover, obtained in numerous research projects carried out in European centres in the last 30 years, to agricultural exploitations (Piñeiro *et al*. 2000).

The aim of the present study was to characterize 15 accessions of white clover collected in northern Spain, by their morphology, flowering and cyanogenesis, sown as spaced-plants in Galicia, and as mixed swards with perennial ryegrass in Asturias.

MATERIAL AND METHODS

Plant material. The Plant Genetic Resources Unit of the Institute of Grassland and Environmental Research (IGER), Aberystwyth, United Kingdom, and the Crop Production Area of the University of Oviedo, Spain carried out a joint collecting expedition in northern Spain between 23 June and 7 July 2003. Vegetative collections of *Lotus corniculatus*, *Trifolium pratense* and *Trifolium repens* were made at different sites in the Cantabrian Mountains, covering a broad range of altitude, agricultural management systems and ecological conditions (Oliveira *et al.* 2007).

The method of collection was to gather 25–50 separate vegetative parts per population at each site visited. Collection of vegetative material avoids bias towards sexually reproductive genotypes and is not limited to periods of seed harvest (Chorlton *et al.* 2003). The origins and accession numbers of the 15 accessions of white clover collected are listed in Table 1. A map of the distribution of the accessions of *Trifolium repens* collected in the Cantabrian Mountains is shown in Figure 1. The accessions were mainly collected from cut and/or grazed grasslands.

The samples were transported to the laboratory in Aberystwyth, and the vegetative units were cleaned and reduced to one tiller, or stolon, per unit. The populations were grown-on in separate isolation chambers under optimum conditions of light, heat, nutrients and water. The main details of the regeneration procedure are described by Chorlton *et al.* (2003). A population sample of 30 plants (genotypes) gives rise to 30 mother plant seed samples. A balanced bulk sample was made up of equal aliquots from each mother plant and one part of that bulk sample was sent to the Crop Production Area of the University of Oviedo. Seeds from these populations were stored in waterproof packages, at 0–4°C, in the Centro de Recursos Fitogenéticos Nacional (CRF), Alcalá de Henares, Madrid and in the Crop Production Area (FAO Code = ESP103) in Mieres, Asturias.

Sward plot evaluation. Biomass production of the 15 populations of white clover collected in the Cantabrian mountain range was studied during a period of two years (2006 and 2007). A field trial was established in a completely random block design with three replicates. The area used was a well-established meadow, which was ploughed and rototilled before sowing the trial. The field (43°35'N, 5°47'W) is located in Candás, Carreño (Asturias), at an altitude of about 80 m, in a plot used by the University of Oviedo. The soil in the area is sandy clay loam, pH = 6.1, with a high organic matter content (6%), high phosphorus content (47 ppm), and adequate potassium con-

Table 1. Inventory number in the Centro de Recursos Fitogenéticos (Madrid) seed bank (accession number in brackets) and origins of the 15 accessions of *Trifolium repens*

Inventory number	Province	Location	Habitat	Lat (N)	Long (W)	Alt (m a.s.l.)
NC079623 (1)	Asturias	Puertas	grassland	4319	452	347
NC079625 (3)	Asturias	Pto de la Cubilla	grassland	4259	552	1701
NC079627 (5)	Asturias	Gamoniteiro	grassland	4310	554	1500
NC079628 (6)	Asturias	Gamoniteiro	grassland	4310	555	1373
NC079629 (7)	Asturias	Pto Ventana	grassland	4303	600	1568
NC079630 (8)	Asturias	La Espina	grassland	4324	618	667
NC079631 (9)	León	La Uña	grassland	4304	508	1205
NC079633 (11)	Asturias	La Garganta	grassland	4320	700	877
NC079636 (14)	León	Portelo	abandoned	4243	659	1229
NC079637 (15)	León	Pto del Manzanal	grassland	4235	613	1228
NC079639 (17)	Asturias	Faro de Peñas	grassland	4339	550	90
NC079641 (19)	Asturias	San Roque (Tazones)	amenity	4331	516	132
NC079644 (22)	Asturias	Mieres	grassland	4216	548	186
NC079645 (23)	Asturias	Olloniego	grassland	4318	548	332
NC079646 (24)	Asturias	La Colladona	moorland	4311	536	853

tent (125 ppm). As the fertility was adequate, no fertilizer was added. Plots of 1.50 × 1.20 m were used for the white clover trial. Sowing was carried out on 19/10/2005, with a mixture of perennial ryegrass (*Lolium perenne* L.) tetraploid cv. Tove, at a dose of 4 kg/ha *T. repens* + 20 kg/ha *L. perenne*. Two commercial cultivars per species were used as controls: the medium-leaved Huia and the large-leaved California. A mixed stand was used, as the advantage of selecting white clover under competitive conditions has been demonstrated (Rhodes & Ortega 1996; Annicchiarico 2003). The seed mixture was placed in a small bucket and mixed with fine dry sawdust before being broadcasted by hand. This mixture was sprinkled evenly across the plot. After sowing, the surface of each plot was lightly raked to bury the seed and the whole area was then rolled with a flat roller. The plots were not irrigated or fertilized after sowing and cutting.

The plots were cut manually (with garden shears) in one term to 5 cm above ground level and then allowed to regrow to 25–30 cm (plant height). Samples were obtained from within 0.5 × 0.5 m sampling frames placed in the centre of each plot. Evaluation was carried out by grass/clover separations and oven drying (80°C during 24 h) to determine dry matter yield (DMY) of white clover and perennial ryegrass (in t/ha and year) and the percentage of clover content (CLC in % DMY). The plots were cut five times in 2006 and 2007. Harvesting was carried out on 3 May, 10 July, 6 August, 5 October and 23 December in 2006, and on 22 April, 25 June, 1 August, 8 October and 7 December in 2007.

Spaced plant characterization. The agromorphological study was established at the Agrarian Research Centre of Mabegondo (CIAM) (43°14'50''N, 8°15'45''W) in a plot near the coast (100 m a.s.l.), on a silt loam soil. The site was arranged in a completely randomized block design with three replicates of 10 plants per accession and replicate (in total 30 plants per accession). The seeds were sown in seed trays at the beginning of autumn (2007), and were transferred to the experimental plot at the end of November (2007), with a distance of 0.5 m between lines and 0.5 m between plants. Fertilizer (NPK, 8:15:15) was applied at a rate of 500 kg/ha. The soil was covered with black plastic mesh to prevent growth of weeds. The control cultivars were the same as those used in the sward plot trial.

The following ten agromorphological (IBPGR 1992) traits were evaluated during the 2-year pe-

Figure 1. Map of the distribution of the accessions of *Trifolium repens* collected in the Cantabrian Mountains

riod of the study (2008–2009): Flo, flowering date recorded at flowering, as the number of days after January 1st (a plant is in flower when three florets are showing colour and recorded twice weekly, on Tuesdays and Fridays), Alt, plant height recorded at flowering (cm), Anc, plant width recorded at flowering (cm), Lfc, central leaflet length assessed in June/July on the central leaflet of the 3rd–4th leaf from the end of a rapidly growing stolon (mm), Afc, central leaflet width, measured as Lfc (mm), Grp, petiole diameter, measured on petiole of leaf used for Lfc (mm), Lop, petiole length, measured on petiole of leaf used for Lfc (cm), Gre, stolon diameter, measured at the junction of the petiole used in Grp (mm), Ain, abundance of heads per plant assessed when all the plants of a populations are in flower (0–9 scale, where 9 is the maximum), Enf, tolerance to foliar diseases (rust and common leaf spot) at the end of spring (0–9, where 9 = maximum tolerance). Seasonal yields of the spaced plants were not evaluated because white clover is almost universally grown as mixtures with grass and as in other forage species, there are no consistent relationships between yields from spaced plants and sward plots (Rhodes 1987).

Percentage cyanogenic plants. The presence or absence of cyanogenic glucoside (HCN) was assessed on leaf material (3–4 leaves) from each individual plant, where absence was indicated by no change in the colour of picric acid paper after 24 h, and presence by a change in the colour of picric acid paper after 2 or 24 h (Corkill 1940; López Díaz *et al.* 2009). The same 30 plants (3 replicates of 10 plants) were studied per accession in the agromorphological study, including both cultivars. Two determinations were made: one in the summer of 2008 and another in the winter of 2008–2009. Distilled water (750 μl) and toluene (200 μl) were added to the fresh tissue in each test tube to destroy the membranes and thus bring the enzyme and substrate in contact with each other. The percentage of cyanogenic plants was calculated from the data obtained.

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. 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 and year effects were considered to be random. The accession effects were tested against the accession × year interaction, and when found to be significant, were used in further analyses (except for tolerance to foliar diseases and dry matter yields, in which the accession × year interaction was important and the data from each year were used) (Charmet *et al.* 1989). Separation of means was performed by the least-significant difference (LSD) test.

Multivariate relationships among accessions were explored with a principal component analysis (PCA), with a correlation matrix derived from the significant characters (Varimax rotation method). Of the variables revealed by the ANOVA to be significant, the following variables were eliminated in the PCA: Flo, Ain, Enf1, Enf2, Pc and DMY1, after it was shown by the Anti-image option of the Factor analysis that the values of the diagonal elements in this matrix (measures of sampling adequacy) were lower than 0.5 and the Kaiser-Meyer-Olkin measure of sampling adequacy (KMO) was < 0.5. After elimination of these variables, the values of both the diagonal elements in the Anti-image matrix and the KMO were higher than 0.5, which is good according to Kaiser (1974). The retained variables were reduced to two independent linear combinations, i.e. the principal components (PC) of the variables with eigenvalues greater than 1, which cumulatively explained 92.4% 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 in the 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 classification tree. In order to explore differences between the clusters obtained, a table of means and standard deviations (SD) is also shown. Statistical analyses were computed with SPSS version 19 (SPSS 2011).

RESULTS

The results of analysis of variance of the 13 characters in two years are summarized in Table 2. The accession effect tested against the accession × year effect (when this interaction effect was significant)

Table 2. Mean squares from the analyses of variance of 13 traits evaluated in 15 accessions and two cultivars (Huia and California) during two years as spaced plants at Mabegondo and in sward plots at Candás

Traits	Year	Replicate	Year × replicate	Accession	Accession × year	Error
Flo	251.00^{NS}	88.92^{NS}	73.08^{NS}	7934.45***	2386.49***	461.33
Alt	381.21^{NS}	80.79^{NS}	24.09^{NS}	820.49***	239.87***	31.95
Anc	5664.79*	279.49^{NS}	4.78^{NS}	4064.21***	1082.84***	253.76
Ain	69.03^{NS}	1.80^{NS}	7.51^{NS}	194.10***	2.60^{NS}	3.62
Lfc	27.98**	0.01^{NS}	1.04^{NS}	18.70***	3.48***	0.25
Afc	26.96**	0.01^{NS}	0.28^{NS}	11.38***	2.61***	0.15
Grp	6.18*	0.01^{NS}	0.47^{NS}	7.56***	1.45***	0.08
Lop	1441.04*	8.23^{NS}	66.21^{NS}	604.52***	188.25***	17.61
Gre	11.95*	0.16^{NS}	0.22^{NS}	17.46***	1.73***	0.21
Enf	17.59^{NS}	2.45^{NS}	1.59^{NS}	62.18^{NS}	15.90***	1.90
PC	521.34^{NS}	110.91^{NS}	53.08^{NS}	2052.35***	144.29^{NS}	195.71
DMY	25.98*	9.69^{NS}	2.28^{NS}	3.96^{NS}	2.80**	0.89
CLC	5041.11**	111.72^{NS}	15.76^{NS}	20.18^{NS}	25.75^{NS}	19.38

Flo – flowering date; Alt – plant height; Anc – plant width; Ain – abundance of heads per plant; Lfc – central leaflet length; Afc – central leaflet width; Grp – petiole diameter; Lop – Petiole length; Gre – stolon diameter; Enf – tolerance to foliar diseases; PC – principal components; DMY – dry matter yield; CLC – clover content; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; $^{NS}P > 0.05$

was significant for all the characters except for the tolerance to foliar diseases, the percentage of clover and the dry matter yield. The mean values of traits and SD for the accessions are shown in Table 3, considering the mean values obtained in each year for the tolerance to foliar diseases and the dry matter yields. The LSD values at the bottom of each column represent the minimum difference between any two accessions required to ensure a probability level of at least 0.05.

The differences in flowering date between accessions were between 100 and 140 days from 1 January, and eventually from cultivar California up to 77 days. As the accession effect for the flowering date tested against the accession × year effect was significant, the ranking of the accessions did not differ from one year to another and the range of expression was only influenced by the year, due to the low temperatures (minimum temperatures < 5°C) in the first five months of 2009 in Galicia.

The correlation coefficients (or factor loadings) for the eight significant traits and the first two PCs (eigenvalues greater than 1) are shown in Table 4. The first PC was positively correlated with plant height, petiole length and diameter, stolon diameter, and leaflet width and length. The trait that was positively correlated with the second component (and thus behaved independently from those associated with the first component) was the dry matter yield in the second year. The percentage variance in an observed variable that is accounted for by the retained components is also shown in this table.

The projection of the accessions on the plot of components 1–2 (92.4% of the total variance explained) is shown in Figure 2. Accessions on the extreme right-hand side of the plot (e.g. 30) showed the highest values for most agromorphological traits. Accession 3 on the extreme left-hand side of the same plot presented the lowest values of the majority of agromorphological traits. In the upper part of the plot, Acc. 19 showed the highest values for the dry matter yield in the second year of evaluation. At the bottom of the plot, Acc. 8 showed the lowest dry matter yield.

Hierarchical clustering analysis performed on the first two PCs provides the dendrogram shown in Figure 3. Each time two accessions are joined by a vertical line, a subcluster or cluster is created. The dendrogram also shows the values of the distance coefficients for each step or vertical line.

This suggests that the cut-off point should be at the three cluster level. The partition shows a

Table 3. Two-year means (standard deviation in brackets) for 15 traits in 15 accessions and two cultivars (Huia and California) during two years as spaced plants at Mabegondo and in sward plots at Candás

Accessions	Flo	Alt	Anc	Ain	Lfc	Afc	Grp	Lop	Gre	Enf1	Enf2	PC	DMY1	DMY2	CLC
1	121.8	9.0	55.4	6.9	1.7	1.3	1.1	7.1	2.4	8.9	8.3	96.7	8.9	7.0	13.9
	(28.7)	(4.5)	(8.2)	(1.8)	(0.1)	(0.1)	(0.1)	(0.5)	(0.1)	(0.6)	(1.8)	(8.2)	(1.3)	(0.7)	(8.6)
3	121.3	5.2	30.3	2.4	0.7	0.6	0.6	2.0	1.5	8.7	9.0	46.0	6.0	6.7	12.8
	(25.7)	(1.9)	(5.7)	(1.8)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.7)	(0.5)	(18.1)	(0.1)	(0.1)	(7.1)
5	102.0	8.1	42.3	2.9	1.4	1.1	1.0	4.5	2.2	7.6	8.9	76.6	7.7	6.4	13.6
	(18.8)	(2.1)	(6.4)	(1.5)	(0.1)	(0.1)	(0.1)	(0.4)	(0.1)	(2.5)	(0.6)	(14.4)	(0.1)	(0.5)	(4.7)
6	102.1	6.3	40.6	4.1	0.8	0.7	0.7	2.6	1.5	8.6	9.0	60.7	5.9	5.9	11.7
	(19.7)	(1.9)	(6.6)	(2.3)	(0.1)	(0.1)	(0.1)	(0.2)	(0.1)	(0.8)	(0.7)	(13.2)	(0.5)	(0.4)	(8,1)
7	117.9	6.1	40.1	3.4	1.0	0.8	0.8	2.7	1.9	9.0	8.8	39.3	8.2	5.9	13.6
	(19.1)	(1.4)	(6.0)	(2.4)	(0.1)	(0.1)	(0.1)	(0.2)	(0.1)	(0.6)	(0.9)	(12.2)	(2.2)	(0.7)	(10.2)
8	106.3	5.8	37.8	1.9	1.0	0.8	0.8	3.1	1.8	9.0	8.0	61.5	7.4	5.8	13.4
	(21.7)	(1.8)	(8.7)	(1.4)	(0.1)	(0,1)	(0.1)	(0.3)	(0.1)	(1.5)	(1.5)	(26.2)	(1.1)	(0.1)	(9.9)
9	115.1	12.1	50.4	7.2	2.3	1.9	1.4	11.5	2.9	8.5	8.2	64.3	6.7	6.4	13.6
	(23.9)	(6.0)	(9.1)	(1.8)	(0.1)	(0.1)	(0.1)	(0.8)	(0.1)	(1.2)	(2.0)	(23.4)	(2.4)	(0.4)	(9.6)
11	110.1	14.8	59.7	5.2	2.2	1.7	1.4	10.1	2.8	8.6	8.1	91.7	8.0	5.4	14.4
	(17.2)	(4.5)	(9.5)	(1.9)	(0.1)	(0.1)	(0.1)	(0.8)	(0.1)	(1.1)	(1.7)	(9.8)	(0.1)	(0.6)	(5.6)
14	108.2	17.4	63.1	7.8	3.3	2.6	2.0	20.3	3.6	9.0	6.1	96.7	8.0	7.1	18.7
	(18.1)	(6.2)	(8.3)	(1.5)	(0.2)	(0.1)	(0.1)	(1.4)	(0.1)	(0,6)	(1.8)	(5.2)	(0.2)	(0.3)	(8.8)
15	124.6	12.2	68.2	5.9	2.2	1.7	1.3	12.0	2.5	9.0	6.9	73.9	7.4	6.9	16.4
	(26.7)	(4.9)	(7.1)	(2.2)	(0.1)	(0.1)	(0.1)	(1.1)	(0.1)	(0.8)	(2.4)	(10.8)	(1.2)	(0.8)	(9.8)
17	115.6	14.6	68.3	8.5	2.3	1.9	1.5	11.9	2.8	9.0	9.0	98.3	9.6	7.2	16.2
	(25.9)	(4.2)	(8.6)	(1.1)	(0.1)	(0.1)	(0.1)	(0.6)	(0.1)	(0.5)	(0.4)	(4.1)	(1.2)	(0.8)	(11.2)
19	119.0	8.9	57.8	7.8	1.7	1.3	1.1	7.3	2.3	8.8	7.9	89.2	8.4	7.6	17.9
	(21.7)	(4.1)	(9.1)	(1.8)	(0.1)	(0.1)	(0.1)	(0.9)	(0.1)	(0.8)	(1.9)	(13.7)	(1.5)	(0.3)	(9.9)
22	110.5	10.8	63.1	6.3	2.4	1.9	1.7	12.6	3.3	8.8	8.9	100.0	8.5	7.0	15.4
	(25.2)	(4.8)	(8.7)	(2.0)	(0.1)	(0.1)	(0.1)	(0.8)	(0.1)	(0.7)	(0.8)	(0.0)	(2.5)	(0.2)	(12.1)
23	119.2	9.9	56.2	7.0	1.4	1.1	0.9	5.6	2.1	9.0	6.6	88.3	8.2	7.3	13.5
	(25.0)	(3.8)	(7.5)	(2.1)	(0.1)	(0.1)	(0.1)	(0.4)	(0.1)	(0.5)	(2.2)	(13.3)	(1.9)	(0.3)	(7.8)
24	99.7	10.9	57.9	3.2	1.7	1.4	1.3	6.7	2.7	8.7	9.0	68.3	5.4	6.4	16.0
	(19.5)	(5.3)	(7.7)	(2.0)	(0.1)	(0.1)	(0.1)	(0.7)	(0.1)	(1.2)	(0.7)	(12.5)	(1.8)	(0.3)	(8.7)
Huia	99.6	17.5	57.8	7.7	2.4	1.7	1.6	10.1	3.1	4.3	6.9	84.6	8.9	6.1	14.9
	(17.9)	(5.1)	(7.8)	(1.6)	(0.1)	(0.1)	(0.1)	(0.6)	(0.1)	(2.4)	(2.8)	(10.7)	(1.1)	(0.8)	(10.9)
California	71.3	18.8	65.4	6.2	3.6	2.9	2.5	15.0	4.1	4.1	3.8	82.9	9.7	6.2	14.3
	(6.5)	(3.3)	(9.9)	(2.6)	(0.1)	(0.1)	(0.1)	(1.1)	(0.1)	(1.4)	(2.2)	(8.9)	(1.4)	(0.6)	(7.0)
LSD (P = 0.05)	9.5	2.5	7.2	0.8	0.2	0.2	0.1	1.8	0.2	1.0	1.1	16.3	2.1	1.0	–

Flo – flowering date; Alt – plant height; Anc – plant width; Ain – abundance of heads per plant; Lfc – central leaflet length; Afc – central leaflet width; Grp – petiole diameter; Lop – Petiole length; Gre – stolon diameter; Enf – tolerance to foliar diseases; PC – principal components; DMY – dry matter yield; CLC – clover content; LSD = least significant differences at 5% level

Table 4. Correlation coefficients for the eight statistically significant traits and the first two principal components (92.4% of the total variance explained) and communality estimates from a principal component analysis of the correlation matrix of the traits

Traits	Component 1	Component 2	Communalities
Alt	0.927	0.152	0.882
Anc	0.775	0.552	0.905
Lfc	0.978	0.165	0.984
Afc	0.975	0.163	0.977
Grp	0.987	0.030	0.975
Lop	0.817	0.275	0.743
Gre	0.979	0.071	0.963
DMY2	0.068	0.981	0.966

Alt – plant height; Anc – plant width; Lfc – central leaflet length; Afc – central leaflet width; Grp – petiole diameter; Lop – Petiole length; Gre – stolon diameter; DMY – dry matter yield

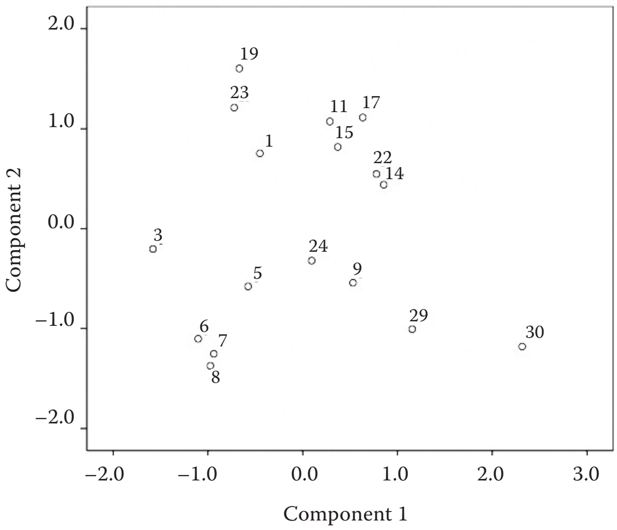

Figure 2. Plot of the principal component analysis of traits with projection of the accession number; component 1 as *x* axis and component 2 as *y* axis (92.4% of the total variance explained); accessions are numbered from 1 to 24; 29 – cv. Huia, 30 – cv. California

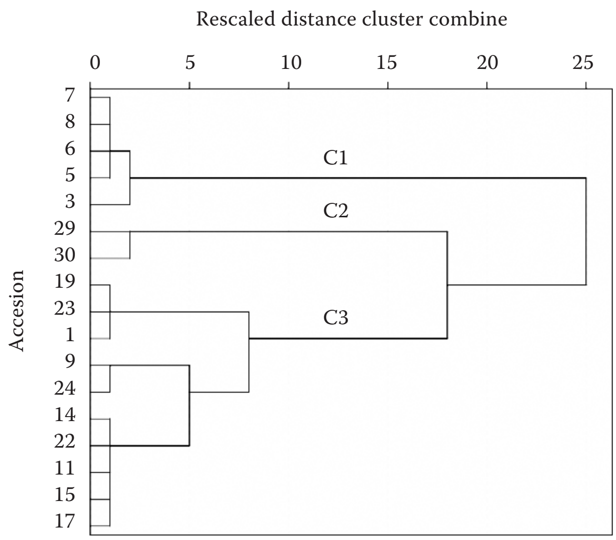

Figure 3. Dendrogram based on the results of the hierarchical cluster analysis by the Ward method (71% of the variance among accessions); C1, C2 and C3 mean Cluster 1, 2 and 3, respectively; accessions are numbered from 1 to 24; 29 – cv. Huia, 30 – cv. California

between-clusters-variance/total variance ratio of 71%. 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).

Means and standard deviations were calculated for each cluster for all traits (Table 5). One group of accessions (Cluster 1), collected from upland sites, was characterized by low height and plant spread, short petioles, small leaves, thin stolons and low dry matter yield. This group can be defined as small-leaved.

Another group (Cluster 2), composed only by the cv. California and the cv. Huia, was characterized by tall plants, large spread, long wide leaves, thick petioles and stolons and with similar dry matter yields as Cluster 1.

The last group (Cluster 3), which includes ten accessions, has intermediate-sized leaves, the highest

Table 5. Average means (standard error in brackets) for the clusters for the eight statistically significant traits

Traits	Cluster 1 (*n* = 5)	Cluster 2 (*n* = 2)	Cluster 3 (*n* = 10)	ANOVA *F* (2, 14) ratios
Alt	6.3^c (1.0)	18.1^a (1.6)	12.1^b (0.7)	20.8***
Anc	38.2^b (2.4)	61.6^a (3.8)	60.0^a (1.7)	29.6***
Lfc	1.0^c (0.2)	2.9^a (0.3)	1.9^b (0.1)	21.0***
Afc	0.8^c (0.1)	2.2^a (0.2)	1.6^b (0.1)	15.8***
Grp	0.7^c (0.1)	2.0^a (0.2)	1.3^b (0.1)	19.2***
Lop	2.9^b (1.1)	10.9^a (1.7)	8.4^a (0.8)	10.8***
Gre	1.8^c (0.2)	3.5^a (0.3)	2.7^b (0.1)	15.7***
DMY2	6.1^b (0.2)	6.2^b (0.2)	7.0^a (0.1)	12.0***

Alt – plant height; Anc – plant width; Lfc – central leaflet length; Afc – central leaflet width; Grp – petiole diameter; Lop – Petiole length; Gre – stolon diameter; DMY – dry matter yield; *n* = number of accessions; ***$P < 0.001$; means followed by different letters in the row are significantly different at the 0.05 level according to Duncan test; Cluster 1 – accessions 3, 5, 6, 7 and 8; Cluster 2 – cultivars Huia and California; Cluster 3 – accessions 1, 9, 11, 14, 15, 17, 19, 22, 23 and 24

dry matter yields, and morphological characters intermediate between the other two clusters.

DISCUSSION

The species of forage legumes that are most frequent and abundant in the grasslands and meadows in Asturias are *Trifolium repens*, *Trifolium pratense* and *Lotus corniculatus* (Álvarez-García 1980).

According to Díaz and Fernández (1994) and Díaz *et al.* (1994), *T. repens* is included in the class *Molinio-Arrhenatheretea*, order *Arrhenatheretalia*, within plant communities denominated as xerophytic grasslands and meadows. These communities are excellent as grazed and/or mowed 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. This type of pasture can provide fodder at a rate of 2.5 to 10 t/ha and year of dry matter (Rodriguez & Argamenteria 1995; Oliveira & Afif 2010).

The allogamous breeding system of white clover contributes to a high level of diversity for morphological traits, and ecological differentiation has led to distinct white clover ecotypes across a range of geographical gradients (Caradus & Woodfield 1997). Diverse morphology is associated with variation in habitat (Davies & Young 1967). Thus, populations from the Mediterraneam region have larger leaf size and thus a higher yielding potential than populations of white clover from northern Europe (Davies & Young 1967; Williams 1987).

All accessions and cultivars studied were cyanogenic within a range of 39.3 to 100%, with a higher frequency of cyanogenic plants at low and medium altitudes. Numerous ecological surveys of the distribution of the frequency of cyanogenic clover demonstrated an inverse relationship between the frequency of cyanogenesis and altitude (Daday 1954; Boersma *et al.* 1983; Till 1987; Noitsakis & Jacquard 1992; Pederson *et al.* 1996).

The dry matter yield values recorded in the present study were similar to other published data on herbage production measured under low-input and organic farming conditions (Jones *et al.* 1996; Frame *et al.* 1998). For the purposes of plant breeding, dry matter yield is a very important character, although the resulting classification will be influenced by the trial site, as it measures adaptation (Caradus & Williams 1989).

The significant accession × year interaction for the dry matter yield (DMY) in Asturias may be due to the fact that the second year was much drier (mean precipitation 676 mm in 2007 compared with 820 mm in 2006) and colder (mean temperature 13.8°C in 2007 compared with 14.4°C in 2006). Also, the significant accession × year interaction for the tolerance to foliar diseases (Enf) in Galicia may be due to the climatic conditions in both years. Although the total precipitation was similar in both years (1375 and 1373 mm respectively), the rainfall recorded in the first six months of 2008 was 200 mm higher than that recorded in the same period in 2009. As regards the temperature, the first five months of 2009 were exceptionally cold, and the minimum temperature did not exceed 5°C until May.

The lack of significance of the percentage of white clover (CLC) may have been due to experimental error, as the coefficient of variation was high (> 12%). This may be partly due to inadequate field plot technique. Experimental error can be reduced by increasing plot size and the number of replicates (Thomas & Abou-El Fittouh 1968).

By grouping phenotypically similar accessions, multivariate clustering techniques reduce the number of accessions that need to be included in germplasm evaluation for plant improvement programmes, and thus provide plant breeders with points of entry into large collections (Frankel 1986). With the aim of revealing other sources of polymorphism, this study should be continued with genetic markers such as chloroplast and nuclear sequence data.

In the present study, the number of different groups for germplasm evaluation could be reduced to three, but in fact one of the clusters (Cluster 2) is composed by the two cultivars used as checks. Of the other two groups obtained (Cluster 1 and 3), Cluster 1 was composed of accessions collected from upland sites (average altitude 1361.8 m a.s.l.), which are small-leaved and can be recommended for gardening.

Cluster 3, composed of accessions collected at medium and low altitude sites, was characterized by medium-sized leaves and can be recommended for cutting and rotational grazing by sheep and/or cattle. Despite having intermediate-sized leaves, the biomass production of these accessions was higher than that of the cultivars in the second year of evaluation, as reported in other studies (Juntila *et al.* 1990; Aasmo-Finne *et al.* 2000) showing that morphological variation has been identified within and between populations from these mophological groups.

The results of the study enabled us to identify some promising accessions, such as accession 19, which had the highest dry matter yield in the second year (7.6 t/ha), higher than the best available cultivars (6.2 t/ha).

Further information on growth, under grazing, in mixed swards of grass and white clover will be useful for determining which populations in this cluster will provide useful agronomic germplasm for different livestock management systems. As in other cross-pollinated forage crops, the synthetic cultivar method, which consists of one to three generations of random mating of a limited number of genotypes selected on the basis of high general combining ability, will be used to produce a cultivar adapted to northern Spain.

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