

Collection, characterization and evaluation of wild *Hedysarum coronarium* L. populations from Andalusia (southern Spain)

Eva María Córdoba^{1*}, Salvador Nadal², Belén Román², Clara Isabel González-Verdejo²

¹Área de Producción Agraria, IFAPA Centro “Alameda del Obispo”, Córdoba, Spain

²Área de Mejora y Biotecnología de Cultivos, IFAPA Centro “Alameda del Obispo”, Córdoba, Spain

*Corresponding author: eva.cordoba.ext@juntadeandalucia.es

Abstract

Sulla (*Hedysarum coronarium* L.) is a short-lived perennial legume native to the Mediterranean basin. This legume plays an important role in forage production due to its high protein content, moderate levels of condensed tannins and a multifunctional agriculture. In Spain, like other countries, different factors are causing the destruction of natural ecosystems and are thus leading to a loss of genetic resources. Therefore, before these resources are irreversibly lost, we collected natural sulla populations from different Andalusian (southern Spain) environments. Additionally, several natural populations and three commercial varieties included as controls, were evaluated and compared for pheno-morphological and agronomic characteristics. Twenty-six accessions were collected, and statistical analysis indicated the presence of genetic variability within the species and between the populations for both the pheno-morphological and agronomic traits studied. The agronomic attributes of several native populations were more pronounced than or similar to the commercial accessions. In Principal Component Analysis, the first two components explained over 80% of genetic variation. Principal Component 1 was positively influenced by the internode number of the principal stem, the diameter of principal stem and the length of the principal stem. Principal Component 2 was positively influenced by the number of inflorescences and by end of flowering and negatively influenced by the leaf area. The results in this study suggest that the characteristics widely vary and have been identified as sources for use in forage or pasture.

Keywords: genetic resources; legumes; fodder crops; dry-land systems; sulla.

Abbreviations: PH- Plant height; LA- Leaf area; DM- dry matter; DMY- Dry matter yield; INPS- Internode number of principal stem; NL- Number of leaflets; EF- End of flowering; GF- Green forage, DPS- Diameter of principal stem, NI- Number of inflorescences, EAF- Start flowering; LPS- Length of principal stem; PCA- Principal component analysis; PC1- Principal component 1; PC2- Principal component 2; IFAPA- Institute of Agricultural and Fisheries Research and Training.

Introduction

After a long period of decline, there is a renewed interest in forage legumes for several economical and ecological reasons (Carlier et al., 2008). Particularly, perennial forage legumes adapted to dry and marginal environments have garnered increasing attention under climate change predictions of lower and more erratic rainfall patterns (Porqueddu et al., 2011). The main environmental advantage of legume-based forage crop husbandry is the reduction of fossil energy that is necessary to synthesize inorganic N fertilizers and reform other protein sources into useful feed for cattle. In addition, they improve soil structure and reduce soil erosion (Bilalis et al., 2009). Sulla (*Hedysarum coronarium* L., syn. *Sulla coronaria* [L.] Medik.) is a semi-perennial forage legume that is perfectly adapted to low-input, rain-fed agricultural systems (Ruisi et al., 2011), allowing it to be used in grazing, haying, ensiling (Sulas, 2005), and supplying nitrogen produced by symbiotic fixation (Sarno and Stringi, 1979). Moreover, it exhibits a good forage quality with adequate protein content (Borreani et al., 2003) and moderate levels of condensed tannins (Amato et al., 2005). These secondary metabolites prevent bloating in ruminants (Ramirez-Restrepo and Barry, 2005) and reduce methane emissions into the atmosphere from dairy cow farming (Woodward et al., 2002). Therefore, sulla could be employed as a forage crop in rotation with cereals to

help enhance the productivity and sustainability of farming systems in Andalusia, particularly in organic production and low-input agriculture. Traditionally, this species has been grown in several countries around the Western Mediterranean basin, especially in Spain, Italy and North Africa (Trifi-Farah et al., 2002). Today, Spain is facing a loss of genetic resources primarily due to both pressure from humans on natural ecological systems, leading to a destruction of numerous natural ecological systems, and the introduction and utilization of commercial varieties, predominantly Italian. Wild, spontaneous populations of sulla still thrive in Andalusia. Thus, before these resources are completely lost, they must be explored, collected, studied and conserved. Numerous studies have focused on developing improvements for evaluating and preserving this forage crop. Some of these studies applied pheno-morphological, physiological and agronomic traits to identify, characterize and evaluate different sulla collections worldwide (Flores et al., 1997; Issolah and Khalfallah, 2007; Salis et al., 2010; Ruisi et al., 2011). Conversely, the genetic diversity of *H. coronarium* has been assessed using amplified fragment length polymorphism (AFLP), restriction fragment length polymorphisms (RFLP) and inter simple sequence repeat (ISSR) (Trifi-Farah and Marrakchi, 2002; Marghali et al., 2005; Kaddour et al., 2011; Marghali et al., 2012).

Pheno-morphological characterization and agronomic evaluation are important first steps towards conserving and utilizing plant genetic resources. Assessment of the genetic variability can be achieved using morphological measurements and phenotypic characterization (Greene et al., 2008). The use of pheno-morphological and agronomic variation provides complementary information to molecular marker characterization. The genetic variation in the germplasm corresponding to pheno-morphological traits can be subjected to multivariate analysis to establish the relationships among various accessions. Applying this type of analysis to germplasm collection studies allows for a better definition of the collection structure, identification of more relevant variables, detection of the relationships among the accessions, and identification of possible groups (Martínez-Calvo et al., 2008). Of the different analysis methods, the Principal Component Analysis (PCA) has been widely used in studies assessing the variability of germplasm collections that encompass many species (Martínez-Calvo et al., 2008; Nikolić et al., 2010; Khadivi-Khub et al., 2012). The objectives of this study were to collect and conserve sulla genetic resources from Andalusia, assess their pheno-morphological characteristics, and perform agronomic evaluations, all to identify useful donors for various relevant agronomic traits.

Results

Germplasm collections

From several expeditions, 26 new natural populations were collected from Andalusia (Table 1).

Pheno-morphological variability and agronomic evaluation

We analyzed the following variables for the 26 natural populations collected and 3 commercial varieties: mean values, variability ranges, variation coefficients and minimum significant differences among accessions, which are reported in Table 2. The pheno-morphological attributes exhibited considerable variability among the accessions, particularly the LA (leaf area of one leaflet from the middle of the main stem, measured as cm^2 (leaflet length x width)), NI (number of inflorescences), LPS (length of the principal stem in cm) and INPS (internode number of the principal stem), whereas the EF (days from transplantation until the end of flowering), EAF (days from transplant flowering initiation), NL (number of leaflets from the middle of the principal stem) and DPS (diameter of the principal stem) displayed little phenotypic variation. The average accession LPS was 63.9, with Carmen cv. presenting the highest value and Hc-27 the lowest. The collection was characterized by an EAF of 67.2 days and an EF of 116.5 days. The varieties Carmen, Grimaldi, Del País and natural Hc-1 and Hc-24 populations were characterized by late flowering, and Hc-12, Hc-32, Hc-3 and Hc-10 flowered the earliest. The results demonstrate that almost half the accessions exhibited a prostrate growth habit, followed by 35 % exhibiting a rosette habit and 20 % exhibiting an erect habit (Additional file 1). At the first cut, GF and DMY varied greatly among the natural populations/varieties. The forage production and dry matter yield differed significantly in the first crop cycle for *H. coronarium* accessions (Table 3). The average annual production of green forage was approximately $29.040 \text{ kg ha}^{-1}$ ($3.082 \text{ kg ha}^{-1} \text{ DM}$). The "Del País" variety yielded the highest average forage production of the different accessions.

This variety, along with the Hc-14, Hc-9, Hc-15, Hc-26, Hc-11, Hc-6, Córdoba and "Carmen" cv. accessions, yielded the driest matter, and Hc-14 exhibited the highest amount of protein content in the dry matter (Table 3). The results obtained during the second year of cultivation (Table 3) did not reveal any statistically significant differences between different varieties/natural sulla populations for green forage and DMY production. The average annual green fodder and DMY production was slightly higher than the previous year, approximately $37.537 \text{ kg ha}^{-1}$ and $3.819,6 \text{ kg ha}^{-1}$, respectively. However, the analysis revealed significant differences in the protein content in the dry matter between accessions. All accessions displayed higher protein content in the second year of cultivation, except Hc-14 and Hc-9, which decreased. In the year of the study, Hc-6 exhibited the highest amount of protein, followed by the "Córdoba" line and Hc-13 with the lowest (Table 3). The natural Hc-13 population obtained the lowest yield for both growing seasons.

Principal component analysis

The Principal Component 1 (PC1) accounted for 68.4 % of the total variation and was positively influenced by INPS, DPS and LPS. The Principal Component 2 (PC2) explained 11.5 % of the variance and was positively influenced by NI and EF and negatively influenced by LA (Table 4). Figure 1 displays the *H. coronarium* distribution, collected from different areas of Andalusia, in a biplot of the first two PCs, the vectors of the original variables, and their contribution (represented by the length of the vector) to the PCs. According to the accession positions in the planes of components 1 and 2, three groups were formed. The accessions Hc-1, Hc-25, Hc-34 and the varieties Carmen, Grimaldi and Del País, which are predominantly characterized by a large diameter, main stem length, and number of internodes, comprised the first group and represent the major contributions of component 1. The second group was composed of the accessions Hc-5, Hc-6, Hc-7, Hc-8, Hc-13, Hc-14, Hc-15, Hc-16, Hc-17, Hc-23, Hc-29, Hc-31 and Hc-33 and represented the intermediate values for the yield components. This group included component 1 members with intermediate values, and negatively contributing component 2 members were incorporated into the accessions Hc-24 and Hc-30. The accessions Hc-3, Hc-4, Hc-9, Hc-10, Hc-12, Hc-18, Hc-27 y Hc-32, i.e., the lowest contributing component 1 members, comprised the third group. This group was divided into two subgroups according to the flowering cycle. Subgroup I exhibited a long flowering cycle, and subgroup II had a short flowering cycle.

Discussion

One of the initial steps in a breeding program with defined plant ideotypes and primary characters of interest is to determine the amount of variation for these characters, which are present within a large number of materials, to define the valuable populations for further evaluation. Here, we present a preliminary study of the pheno-morphological and agronomic variability present in a germplasm collection of *H. coronarium*. A wide range of diversity was observed among the investigated genotypes for all tested traits. The flowering time can critically impact pasture management in seasonal, highly unpredictable environments, such as most Mediterranean-type ecosystems (Monks et al., 2010). EAF, expressed as the number of days from transplantation to the development of the first flower, was approximately 33 days.

Table 1. Characteristics of the originating environment of twenty-six natural *Hedysarum coronarium* L. populations in Andalusia (southern Spain).

Accessions code	Latitude	Longitude	Altitude (m)	Ecological environment	Harvest year
Hc-1	N 37°23'55,5"	W 05°35'33,1"	80	Tomejil (Carmona)	2004/05
Hc-3	N 36°58'29,7"	W 05°51'37,1"	69	Villamartín-Cádiz (Cruce de las Cabezas)	2004/05
Hc-4	N 36°52'43,8"	W 05°48'08,9"	47	A393 km 14. Espera-Arcos	2004/05
Hc-5	N 36°48'42,1"	W 05°48'54,9"	107	Paterna-Medina Sidónea	2004/05
Hc-6	N 36°28'30,1"	W 05°55'11,8"	118	Paterna-Medina Sidónea	2004/05
Hc-7	N 36°26'55,6"	W 05°55'13,9"	170	Medina Sidónea output to Barbate	2004/05
Hc-8	N 36°16'14,6"	W 05°56'15,1"	5	Medina Sidónea-Vejer	2004/05
Hc-9	N 36°39'39,7"	W 06°21'32,7"	32	A491 km 11	2004/05
Hc-10	N 36°36'53,3"	W 06°15'59,6"	30	A491. Rota Base-Puerto Sta. María	2004/05
Hc-12	N 36°27'22"	W 05°57'42,5"	70	"Cañada Real"	2004/05
Hc-13	N 36°27'53,7"	W 05°53'58,6"	128	A381 Medina Sidónea-Jerez	2004/05
Hc-14	N 36°36'8,9"	W 06°02'56,7"	65	A381 km 7	2004/05
Hc-15	N 36°41'23,8"	W 06°09'15,6"	7	Variant of Jerez, opposite the Hospital.	2004/05
Hc-16	N 36°43'46,3"	W 06°09'06,6"	24	"Rancho Merced" , Canal de las ochenta	2006/07
Hc-17	N 36°12'40,2"	W 05°32'37,9"	26	Road direction to "The Barrios"	2006/07
Hc-18	N 36°11'37,5"	W 05°32'53,6"	51	Forest Road direction to "Fasciana"	2006/07
Hc-23	N 36°05'42,0"	W 05°46'22,5"	39	On top of Roman ruins-Bolonia	2006/07
Hc-24	N 36°08'57,5"	W 05°49'56"	0	Ditch direction to Zarzuela-Zahara.	2006/07
Hc-25	N 36°08'49"	W 05°51'27,3"	18	Population Coast, ,military zone	2006/07
Hc-27	N 36°38'22,8"	W 05°26'45,6"	357	A-373, left margin	2006/07
Hc-29	N 36°32'04,0"	W 05°35'10,7"	497	Ca-3331, left margin	2006/07
Hc-30	N 36°22'38"	W 05°39'06,2"	35	Service road "Los Barrios-Jerez".	2006/07
Hc-31	N 36°27'00,1"	W 05°44'53,7"	63	Service road "Park Alcalá de los Gazules"	2006/07
Hc-32	N 36°29'06,5"	W 05°45'52,9"	101	Alcalá de los Gazules-San José del Valle.	2006/07
Hc-33	N 36°51'15,2"	W 06°10'00,1"	15	Road coming Trebujena.	2006/07
Hc-34	N 36° 71'83"	W 06°15'37"	24	"Rancho de la Merced"	2006/07

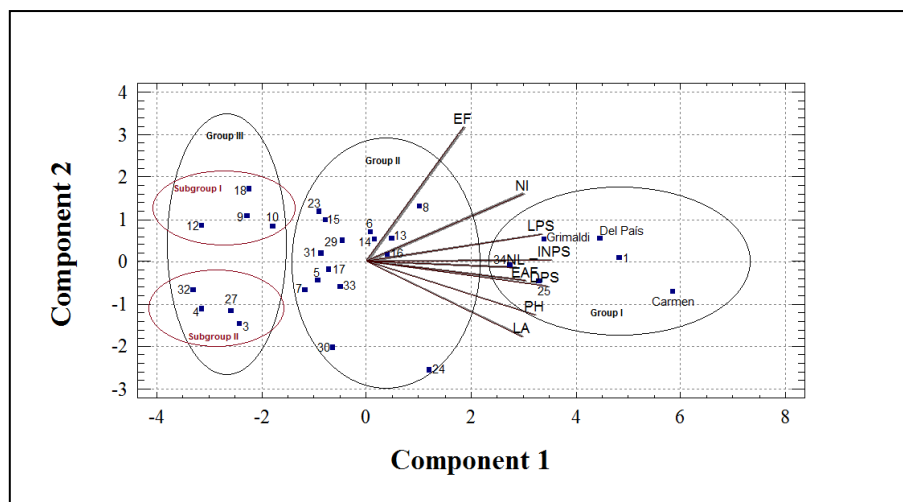


Fig 1. Distribution of *Hedysarum coronarium* accessions as analyzed principal components based on quantitative variables. The arrows intersecting at (0,0) represent the original variables (EAF, start flowering; EF, end of flowering; PH, plant height; LPS, length of principal stem; INPS, internode number of principal stem; LA, leaf area; NL, number of leaflets; DPS, diameter of principal stem and NI, number of inflorescences). The length of each vector is proportional to its contribution to the PCs.

Ruisi et al. (2011) presented a 15-day range for this trait in a study performed in Sicily with 36 natural sulla populations. Issolah and Khalfallah (2007) observed a 16-day range in Algeria with 14 natural populations, and Flores et al. (1997) observed a 23-day range studying 62 accessions that were mainly Spanish. Therefore, the greater range for this previously observed trait indicates that our genotypes harbor more variability, *a priori*, for flowering initiation than other studied germplasm collections. The two cultivars Carmen and Del País flowered later, indicating that this phenology trait could be considered by breeders, as in the case of the cultivar Grimaldi (Bonciarelli and Monotti, 1976). In addition, the accessions Hc-1, Hc-24 and Hc-25 also flowered late, having

adapted to our environmental conditions, and could potentially be selected under this approach, thus constituting one of the most important traits for breeding this species. In the Mediterranean, late flowering represents an advantage because it maximizes yield by increasing the growing season. Consequently, the cutting period is localized to late spring and thus reduces the risk of rain during the field-drying process (Ruisi et al., 2011). Therefore, we can conclude that developing late-flowering cultivars could be advantageous in future breeding programs for this species. The Hc-12 accession exhibits the longest flowering cycle (69 days), followed by Hc-10, and may be considered eligible for

Table 2. Descriptive statistics and the variability of pheno-morphological value differences evaluated in this study and collected from the sulla germplasm collection at Andalusia (southern Spain).

Accessions code	Start flowering (days)	End flowering (days)	Height (cm)	Length of principal item (cm)	Number of internode	Leaf area (cm ²)	Number of leaflets	Diameter of principal stem (cm)	Number of inflorescences
<i>Hc-1</i>	78.2±1.7ab	122±0a	52±6.5ab	89.2±5.6ab	16.2±0.6a	6.7±0.9abc	9.7±0.2abcd	2.4±0.1ab	46.2±3.3ab
<i>Hc-3</i>	53.5±1.4cde	108±0 ab	32.7±2.6bcdefg	43.2±5.3b	6±0e	5±0.9abc	8±0.4bcd	1.7±0.1abc	25.2±2.5abc
<i>Hc-4</i>	61.2±5.2 abcde	108±0b	22.5±1.5defg	41.5±3.8b	6.7±0.4de	3.2±0.4c	8.2±1bcd	1.6±0.1bc	15.7±6.7c
<i>Hc-5</i>	61.2±5.2 abcde	116.2±3.4 ab	29.7±2cdefg	56.7±8.4ab	7.5±0.5cde	5.3±1abc	8.2±0.6bcd	2±0.2abc	23.7±2.3abc
<i>Hc-6</i>	72.5±2.5abcd	122±0a	28.2±4.2cdefg	63.7±4.8ab	9±0.4bcde	4.7±1.1abc	9.5±0.5abcd	1.8±0.1abc	25.7±2.1abc
<i>Hc-7</i>	66.7±9.4 abcde	114±2.9a	24±4defg	64.2±16.5ab	9.2±2bcde	5.1±1.1abc	8.2±0.6bcd	1.7±0.1abc	17.5±4.6bc
<i>Hc-8</i>	69.7±4.2abcde	122±0a	31.5±4cdefg	79.5±4.7ab	10.5±0.6abcde	4.4±0.5abc	8.5±0.5abcd	2±0.1abc	38.7±6.5abc
<i>Hc-9</i>	66.5±3.5 abcde	118.5±3.5a	21±0.7efg	45.2±7.7b	7.5±0.8cde	3.2±0.4c	7.7±0.2cd	1.5±0c	29.6±4.3abc
<i>Hc-10</i>	54.7±4.2bcde	122±0a	23±6.1defg	43.7±2.3b	6.7±0.4de	4.4±0.9abc	8.7±0.4abcd	1.9±0.1abc	22.5±1.5abc
<i>Hc-12</i>	46±2.3e	115±4ab	14.2±1.4g	42.7±2.6b	5.7±0.7e	3.2±0.6c	8.5±0.5abcd	1.7±0.1abc	30.2±3.6abc
<i>Hc-13</i>	70±0abcde	119.7±2.2ab	26±2.9defg	77±9.9ab	9±0.8bcde	5.5±0.7abc	8.5±0.5abcd	2±0.1abc	32.5±2.3abc
<i>Hc-14</i>	63.7±6.9 abcde	116.2±3.4 ab	28.5±4.3cdefg	76.5±19.9ab	9.5±2.3bcde	4±0.9bc	9.2±0.4abcd	1.9±0.1abc	34.6±5.8abc
<i>Hc-15</i>	66.2±5.4 abcde	117.5±2.5 ab	21.2±2.1defg	56±7.3ab	8.5±1.1bcde	3.5±0.4c	9.5±0.5abcd	1.7±0abc	35.5±3.3abc
<i>Hc-16</i>	65±7.2 abcde	115±4 ab	29.7±3cdefg	76.7±10.1ab	10±1.7abcde	4.2±0.5abc	9.2±0.7abcd	2±0.2abc	33.3±10.2abc
<i>Hc-17</i>	72.5±2.5abcd	112.7±3.3 ab	27.2±2.2defg	56.2±7ab	9.7±0.7abcde	4.8±0.7abc	7.2±0.4d	1.7±0.1abc	36.7±8.7abc
<i>Hc-18</i>	61.2±5.2 abcde	122±0a	17.5±1.8fg	48.2±8.4b	7.7±0.9bcde	3±0.6c	7.7±0.2cd	1.6±0.1bc	28±4.8abc
<i>Hc-23</i>	69±4.9 abcde	119.7±2.2 ab	24.5±2.7defg	63.5±9.3ab	9.2±0.7bcde	3±0.4c	8.5±0.9abcd	1.7±0.1abc	29±3.6abc
<i>Hc-24</i>	79.7±5.1a	108±0b	34.7±2abcdef	67.2±5.4ab	10.5±1abcde	7.5±1.6abc	9±1.2abcd	2.1±0.2abc	25±8abc
<i>Hc-25</i>	76.7±4.1abc	114±2.9 ab	35±7.4abcdef	84.5±10.9ab	13±1.5abcd	5.4±0.5abc	10.7±0.4abc	2.5±0.1a	43±11.8abc
<i>Hc-27</i>	67±5 abcde	110.5±1.4 ab	25±3.3defg	39.7±5b	6.2±1.3e	4.5±0.7abc	7.7±0.6cd	1.6±0.1bc	18.6±1.4abc
<i>Hc-29</i>	65.6±2.6 abcde	113.8±2.2 ab	18.8±1.2fg	63.2±11.4ab	9.6±1.2bcde	3.5±0.5c	10.2±0.6abcd	1.8±0abc	34.7±7abc
<i>Hc-30</i>	67.7±6 abcde	108±0b	40.5±3abcd	41.5±5.9b	9.5±0.6bcde	4.9±0.3abc	9±0.5abcd	1.9±0.1abc	23.5±8.5abc
<i>Hc-31</i>	59.5±3.5 abcde	112.7±3.3 ab	24.5±2.4defg	73.2±10.4ab	9±0.7bcde	3.7±0.3bc	8.7±0.7abcd	1.8±0.1abc	31.7±5abc
<i>Hc-32</i>	52±2.3de	111.5±3.5 ab	17.2±2.1fg	40.2±3.1b	7±1de	3.5±0.4c	8.7±0.7abcd	1.8±0.1abc	11.3±1.8c
<i>Hc-33</i>	63.7±3.6 abcde	113±0 ab	24.2±2.1defg	66±8.3ab	8.5±0.2bcde	5±0.7abc	9.7±0.2abcd	1.9±0.1abc	24.6±12.6abc
<i>Hc-34</i>	74.7±4.1abcd	122±0a	35±2.6abcdef	68.7±9.7ab	11.5±1abcde	7.3±1.1abc	10.5±0.2abcd	2.3±0.1abc	35.5±2.8abc
<i>Carmen cv.</i>	78.2±2.8ab	122±0a	52.7±0.8a	100.7±14.5a	14.2±2.1ab	8.9±1.6a	11.7±0.8 ^a	2.5±0.3a	42±6.1abc
<i>Grimaldi cv.</i>	77.5±2.5abc	122±0a	39.7±5abcde	76.5±8.5ab	11.7±1.3abcde	6.4±0.1abc	10.5±0.5abcd	2.3±0.1abc	47±1ab
<i>Del Paí cv.</i>	78.2±2.8ab	122±0a	47.2±2.9abc	99.2±7.5 ^a	14±1.3abc	6.3±1.1abc	9.7±0.6abcd	2.4±0.2ab	48.5±4.4a
Average±se	67.2±1	116.5±0.5	29.7±1	63.9±2.1	9.5±0.2	4.9±0.2	9.1±0.1	1.9±0	31.2±1.2
C.V. (%) ^b	13.1	3.8	23.67	28.82	25.28	35.21	13.62	15.76	34.51

^a Averages with the letter in common are not significantly different at P<0.05 (Tukey's Test). ^b Variation coefficient expressed in percentage.

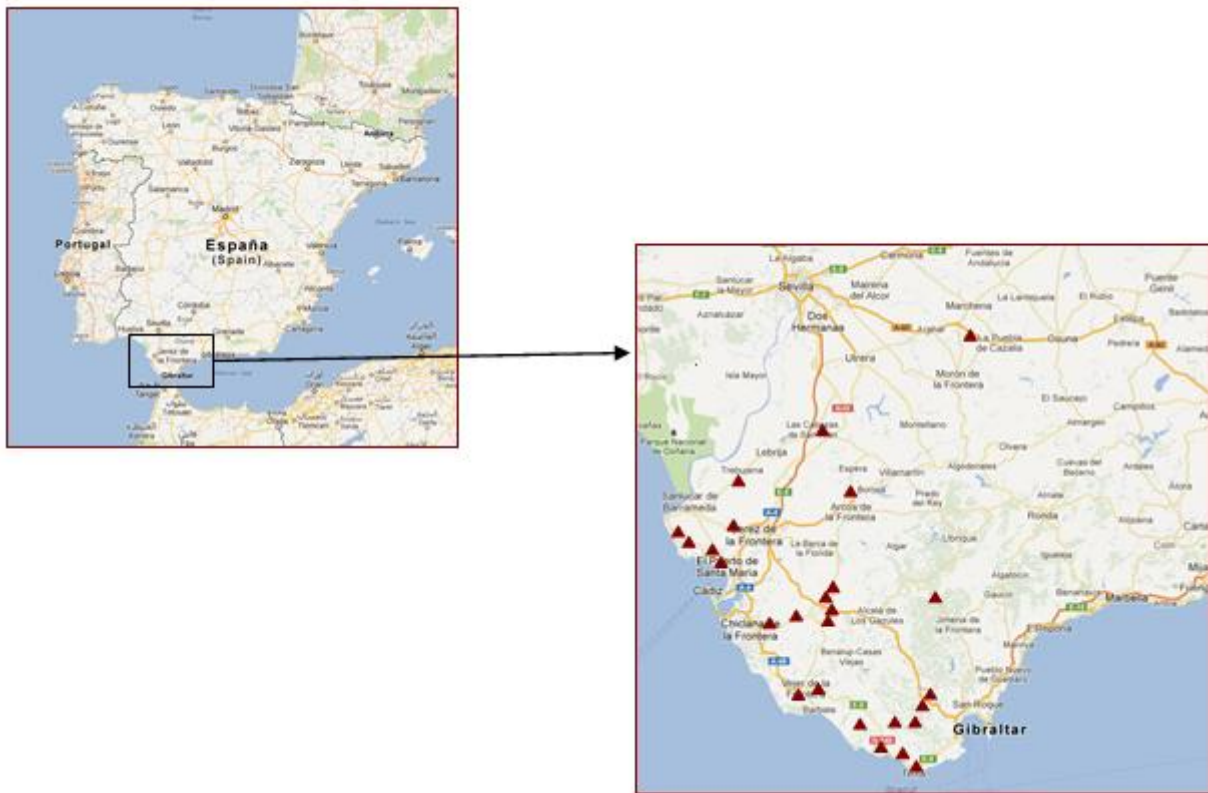


Fig 2. The distribution of natural *Hedysarum coronarium* L. population collecting sites in Andalusia. The triangles indicate the sites of natural sulla population samplings.

growth in natural pastures since it may ensure the natural regeneration of the meadow. Some natural populations exhibited erect or rosette growth, similar to that of the commercial cultivars included in the study, whereas others displayed a prostrate growth habit. This variability represents a source of intra-population diversity that could be exploited to select sulla populations for different uses, such as green forage or hay production, grazing, and soil protection. The average forage production values were approximately 29.000 kg ha⁻¹ and 3.000 kg ha⁻¹ DM. Similar results were obtained by Ruisi et al. (2011) who evaluated the production of 36 natural sulla populations from Sicily. Annicchiarico et al. (2008) yielded mean values of 4.000 kg ha⁻¹ DM which was similar to our results as well. Conversely, Montes (1994) obtained a production of 18.500 kg ha⁻¹ GF and 2.771 kg ha⁻¹ DM in the first year of cultivation in the experiment conducted in Cádiz (Andalusia). The DMY was similar to what we observed, but forage production was lower, approximately 10.500 kg ha⁻¹, possibly due to a later harvest. In the second crop cycle, the evaluated parameters increased to 37.537 kg ha⁻¹ GF and 3.8196 kg ha⁻¹ DM, resulting in an increase of approximately 22.5% and 24%, respectively, over the first year. Montes (1994) obtained yields of 28.487 kg ha⁻¹ GF and 6.352 kg ha⁻¹ DM for two cuts that compared with those obtained in our assay, the DMY was 66% higher, and forage production was lower and the results were similar to our first year observations. The highest DM yields could have been conditioned by more cuts, i.e., the results demonstrated by Montes (1994) were the yields obtained from two harvests in the same year. Moreover, Lombardi et al. (2000) evaluated two Italian cultivars and two ecotypes in Tuscany (Italy) and obtained similar results to ours, demonstrating an average DM of 3.500 kg ha⁻¹. Multivariate analysis and concrete PCA

allows visualizing the differences among the individuals and identifying possible groups and relationships among individuals and variables (Martínez-Calvo et al., 2008). This technique was used to group the *H. coronarium* accessions and quantify the importance of traits from different plants for grouping accessions. As described above, the first two components accounted for approximately 80% of the total variation. DPS, INPS and LPS highly correlated with the first component. Similar results were obtained by Flores et al. (1997), who performed a general analysis of all trials included in their study to demonstrate that DPS and LA were two of the most prominent traits used for grouping and separating sulla accessions. Additionally, Figier et al. (1978) reported that the LPS was the most important character for separating the studied sulla populations. In view of results of both studies to characterize genetic resources sulla, the main stem diameter and length should be included in the analysis, where these two traits alone yield more than 50% of the variability. Moreover, Ruisi et al. (2011) reported that the sum of the leaf length and plant height, plant growth habit, and the number of leaflets per leaf were responsible for approximately 44.1 % of the total variation, and Issolah and Khalfallah (2007) demonstrated that the number of leaflets, leaves and ramifications per plant were the most important variables. The biplot diagrams can be used to select genotypes that might exhibit favorable trait combinations for breeding (Firincioglu et al., 2009) The analysis presented in Figure 1, which represents the ordination of accessions along the axes of the first and second principal components, indicates that accessions in the first group are characterized by a higher yield, representing a major vegetative feature. Commercial varieties are grouped in this area with some accessions from Andalusia (*Hc-1*, *Hc-25* and *Hc-34*). These

Table 3. Average values and coefficients of variation (CV) of the production of green forage (GF), dry matter yield (DMY) and total nitrogen content in dry matter of six accessions, one breeding line and two cultivars of sulla in the first and second crop cycle.

Variety/populations	1st year of the crop cycle			2nd year of the crop cycle		
	GF (Kg/ ha ⁻¹)	DMY (kg ha ⁻¹)	N content (kg ha ⁻¹ DM)	GF (Kg ha ⁻¹)	DMY (kg ha ⁻¹)	N content (kg ha ⁻¹ DM)
<i>Hc-14</i>	35665ab	3782.6a	843.25a	35348a	3548.8a	760.4bc
<i>Hc-9</i>	33866abc	3728.5a	832.95ab	33173a	3398.3a	798.6bc
<i>Hc-15</i>	27917cd	3113.1a	764.68abc	36907a	3768.7a	824.21abc
<i>Hc-26</i>	29753abcd	3063.7a	729.25abc	37661a	3884a	808.12abc
<i>Hc-11</i>	25908d	3058.1a	655.95bc	40827a	3963.8a	788.21bc
<i>Hc-6</i>	27353cd	2834.2a	660.07bc	41893a	4173.4a	1015.4a
<i>Hc-13</i>	13942e	1529.5b	428.89d	33863a	3398a	699.2c
<i>Córdoba</i>	30278abcd	3092.3a	639.48c	44782a	4578a	934.12ab
<i>Del País cv.</i>	37321a	3764.8a	796.17abc	36470a	3861.3a	870.3abc
<i>Carmen cv.</i>	28394bcd	2855.9a	731.39abc	34478a	3622a	759bc
Average	29040	3082.3	708.21	37537	3819.6	821.22
C.V. (%) ^a	9.95	12.12	9.56	11.75	13.39	8.59
LSD (5%) ^b	7693.6	1002.5	181.58	12908	1496.5	208.07

Averages with the letter in common are not significantly different at P<0.05 (Tukey's Test). ^a Variation coefficient expressed in percentage

^b LSD least significant difference (P<0.05)

Table 4. Estimates of variance, accumulated variances and weighting coefficients (autovectors) for the first two principal components (PC) for nine characters evaluated on 29 sulla accessions.

Total variance explained	PC1	PC2
Eigenvalues	6.15	1.04
Accumulated variance (%)	68.41	79.97
Traits		
Internode number	0.384	0.008
Leaf area (cm)	0.324	-0.413
Height (cm)	0.349	-0.292
End of flowering	0.203	0.741
Number of leaflets	0.312	-0.033
Diameter of principal stem (cm)	0.372	-0.132
Start flowering	0.329	-0.106
Number of inflorescences	0.326	0.374
Length of principal stem (cm)	0.363	0.152

latter accessions could be selected for forage due to their higher biomass. Taken together, these results suggest that these accessions were derived from commercial varieties and, therefore, exhibit similar characteristics that group them together. The second group was formed based on intermediate traits, which can be selected for use as forage or pasture. Finally, the third group could be employed in the pasture because the size of these accessions is lower, possibly because they were part of the grassland. Genetic selection has led to smaller-sized plants and a trailing growth habit, which likely adapted from one type of grazing. This group differed among accessions with large flowering cycles and reduced flowering cycles. This trait has been shown to be influenced by the place of accession origin.

Materials and methods

Plant material

Seeds of 26 *H. coronarium* accessions were collected from natural populations throughout Andalusia (southern Spain) during two expeditions. Three cultivars, called "Grimaldi", "Carmen" and "Del País", were also included as controls. The natural populations were picked mainly from roadsides, abandoned agricultural lands, and natural pastures. The distribution of the sites sampled is shown in Fig. 2. The

collecting strategy used was conditioned by species mating systems, which have previously been described as predominantly allogamous (Louati-Namouchi et al., 2000). The strategy recommended for vegetative sampling of cross-pollinated pasture species suggests a minimum sample size of 30 plants to be collected from a wide range of sites. Thus, we collected 50 individuals from each population, collecting the maximum seed per individual (Sackville Hamilton and Chorlton, 1995). Samples were collected from random positions across each site with a minimum distance between each sample to ensure that individual plants were not sampled more than once. For each sample, the latitude, longitude, and altitude were recorded using a portable Global Positioning System (GPS) receiver along with the ecological environment and harvest year (Table 1). The harvested mature pods were bulked and threshed in the laboratory. This seed mixture was considered representative of the natural population sampled. All seed samples used in our experiments were stored at low temperature (8 °C) in a cold chamber at the IFAPA Centre "Alameda del Obispo".

Experimental site and material planting

The first field trial was established based on phenomorphological characterizations. The experiment was performed at the IFAPA Centre "Alameda del Obispo" at

Córdoba (37° 51'42" N and 4° 48' W) on Typic Xerofluvent soil (SSS, 1975) during the 2007/2008 season. Seeds from 26 natural populations and three commercial control varieties were sown in October 2007 under greenhouse conditions. Plastic multipots, consisting of 100 pots (4x4x4 cm) containing peat and sand mixture, were used. Ten days following sowing, 5 seedlings were randomly chosen for each accession and transplanted 0.5 m apart in 1.5-m rows under drip irrigation and open-field, low-mesh tunnels. A simple classification design was used (IPGRI, 2001). The second field experiment was established at the IFAPA Centre "Rancho de la Merced" (36° 71' 83" N and 6° 15' W) located in Jerez de la Frontera (Cádiz) and characterized by clay loam soil and dry conditions. In this experiment agronomic traits were evaluated during two seasons (2009/2010 and 2010/2011, 1st and 2nd crop cycle, respectively). The total rainfall during these years was 1,050.6 and 434.1 mm, respectively. Ten sula accessions, seven natural populations plus two commercial varieties and one breeding line under development, "Córdoba", were sown on October 26, 2009. The experimental design was a complete randomized block with three replications. The experimental unit consisted of fourteen rows of 500 seeds/plot (8x3 m²).

Agronomic and pheno-morphological data collection

Twenty-six natural populations and three varieties were characterized for the following pheno-morphological traits during the first crop cycle: PH, LPS, INPS, LA, NL, DPS, NI, EAF and EF. The first five traits were measured during early flowering. A qualitative descriptor, the frequency of plants with a particular growth habit (rosette, prostrate or erect), was assessed. We used descriptors previously proposed for other forage legume species and those best adapted to our own species, as a specific listing for *H. coronarium* does not exist. Agronomic characteristics were recorded for *Hc-14*, *Hc-9*, *Hc-15*, *Hc-26*, *Hc-11*, *Hc-6*, *Hc-13*, "Córdoba", "Del País" and "Carmen". For each accession, GF and DMY were assessed in 2010 and 2011 on a harvest area of 2.4 m². DMY, representative of the overall fresh weight, was determined for each sample of at least 1 kg by the proportion of weeds and the amount of dry matter after oven drying the sula subsamples. Harvesting was performed when the plots reached 50% flowering. At each cut, a forage sample was extracted from the biomass and dried and ground to a fine powder to determine total N content using the official established method. This analysis was conducted by the Agricultural Regional Laboratory of Córdoba.

Statistical analysis

The main descriptive statistics were conducted for all recorded pheno-morphological and agronomic traits. In both field experiments, an analysis of variance (ANOVA) was performed for each trait according to the experimental design (a simple classification and a complete randomized block) to assess the significance of variation among all accessions. Each pheno-morphological trait was subjected to a one-way analysis of variance at a significant level of $P \leq 0.05$ (Tukey's test). An average value for each pheno-morphological trait and accession was obtained during the years of study. The quantitative trait value was standardized and subjected to a PCA, and the eigenvalues and relative variance proportions explained by each trait were calculated. The first two principal components, CP1 and CP2, were used to generate a two-dimensional graphical display.

Conclusion

The pheno-morphological and agronomic diversity observed among the sula accessions suggest a high genetic potential, which could be used to provide valuable materials for breeding programs aimed at sula improvement for Mediterranean environments. Conversely, this study provides a foundation for developing characterization and conservation strategies for the sula diversity present in different areas of Andalusia

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