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RESEARCH ARTICLE

Pheno-morphological, agronomic and genetic diversity among natural populations of sulla (*Hedysarum coronarium* L.) collected in Sicily, Italy

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Abstract Sulla (*Hedysarum coronarium* L.) is a short-lived perennial forage legume that plays a key role in cereal-based systems in semi-arid Mediterranean regions, particularly in organic production and low-input oriented agriculture. In Sicily, the species is widespread both as a wild and cultivated plant. The present study assessed the phenotypic and genetic variation among natural populations of sulla collected from different environments throughout Sicily and analysed how the patterns of phenotypic diversity varied according to the environmental parameters of each collection site. Two commercial varieties and two Sicilian agro-ecotypes were also included in the study as controls. Principal components analysis (PCA) was performed on the sites using geographic, climatic, and pedological data to assess the differences in types of collection sites. PCA was also performed on the accessions (using pheno-morphological and agronomic data) to establish the importance of different traits in explaining multivariate polymorphisms. The results showed a large degree of genetic diversity (based on ISSR markers) and variability in pheno-morphological and agronomic traits. PCA did not clearly differentiate the accessions according to their habitats of origin, but in some cases accessions from the same habitat had a tendency to group together. The agronomic attributes of several populations were more pronounced than those of the controls. The observed variability may be valuable when selecting for *H. coronarium* varieties suitable for various uses (e.g., hay production, grazing, soil protection).

Keywords *Hedysarum coronarium* · Mediterranean environment · Intra-specific diversity · Forage legume · ISSR

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Introduction

Sulla (*Hedysarum coronarium* L., syn. *Sulla coronaria* [L.] Medik.), also known as sweet vetch, Italian or Spanish sainfoin, and French honeysuckle, is a short-lived perennial legume native to the Mediterranean basin (Talamucci 1998; Issolah et al. 2006), where it is extensively grown as a 2-year forage crop for grazing and/or hay or silage production. The species plays a key role in cereal-based systems of semi-arid regions, particularly in organic production and low-input oriented agriculture, and is commonly used to enhance the productivity and sustainability of farming systems (e.g., as a nitrogen supply and to

maintain soil organic matter). It is cultivated throughout the region, including in Portugal, Spain, Italy, Greece, Morocco, Algeria, and Tunisia (Flores et al. 1997; Trifi-Farah et al. 2002).

Today there is a growing interest in sulla in traditional and in non-traditional areas (particularly in New Zealand and Australia), due to its excellent adaptability to marginal and drought-prone environments (Borreani et al. 2003; Annichiarico et al. 2008); versatility as a forage crop (e.g., used for grazing, hay and silage production, and grazing associated with hay production); and good-quality forage with high protein content (Bullitta et al. 1996; Borreani et al. 2003) and moderate levels of condensed tannins (Amato et al. 2005). Moreover, it has several non-agricultural uses; for example, it is planted to protect soil (Watson 1982) and revegetate disturbed lands (Flores et al. 1997) as well as for honey production and landscape architecture (Talamucci 1998).

Sulla was domesticated in the recent past; the first data on its cultivation dates back to only the 18th century in southern Italy and Sicily (de Candolle 1883). Today, in Sicily, sulla is grown on ~50,000 ha, and mostly agro-ecotypes are used. These are sympatric with the natural populations from which they are derived, and differ from these natural populations mainly by exhibiting a more erect plant growth habit. Trifi-Farah et al. (1989) reported the same pattern (i.e., mainly sympatric agroecotypes with more erect growth) for cultivated sulla in Tunisia. However, in recent years, several varieties from areas (mainly in central Italy) in which it is easier to produce seed have been introduced to Sicily. This trend risks eroding the genetic identity of Sicilian agro-ecotypes. Moreover, considering that sulla in Sicily is also widespread in the wild and that the species is highly allogamous (Yagoubi and Chriki 2000), the introduction and utilization of allochthonous genotypes could also potentially pollute the gene pool of natural populations. On the other hand, in Italy the intra-specific diversity of wild sulla has not yet been studied, and all assessments of the genetic diversity of sulla have considered only agro-ecotypes (Monotti 1975; Porceddu and Monotti 1976; Amato et al. 1997).

Sicily has a typical Mediterranean climate with hot and dry summers and warm and wet winters. However, some climatic variation related to its altitude and distance from the coast as well as variation in pedogenetic substrate across the island, amongst other factors, have led to great eco-geographical diversity, which in turn may have driven the intra-specific differentiation of *H. coronarium*.

The aims of the present study were to evaluate the extent of pheno-morphological, agronomic, and molecular diversity in natural populations of *H. coronarium* from different environments throughout Sicily, and to analyse the relationships among the diversity patterns and environmental parameters of the collection sites.

Materials and methods

Germplasm collection and climatic characterization of the sites

Specimens from 36 natural populations of *H. coronarium* throughout Sicily were collected in June and July 2005, mainly from roadsides, abandoned agricultural lands, and semi-natural pastures (Fig. 1). At each collection site, mature pods were harvested from a minimum of 30 randomly chosen plants, over an area of 50–100 m^2. The pods were bulked and then threshed in the laboratory. This seed mixture was considered representative of the natural population sampled. Latitude, longitude, and altitude were also recorded using a portable Global Positioning System (GPS) receiver. Moreover, soil samples from the top 0.20 m were taken to analyse pH (2.5:1 in H_2O). Other climatic data were taken from the governmental report 'Climatologia della Sicilia' (Cartabellotta et al. 1998), and each collection site was matched with data from the closest weather station with similar altitude and a minimum of 30 years of consistent records. Nine climate data points from each weather station were considered: mean temperature during the growing season (September–May); mean maximum temperature of the hottest month; mean minimum temperature of the coldest month; cold period (number of months with a mean minimum temperature $<7°C$); mean rainfall during the growing season; mean rainfall in summer (June–August); rainy days per year; mean annual evapotranspiration (ETP); and the Emberger index (Iq), calculated as $[R/(T_A^2 - T_B^2)] \times 100$, where R is the mean annual rainfall, T_A is the mean maximum temperature of the hottest month, and T_B is mean minimum temperature of the coldest month (Emberger 1955).

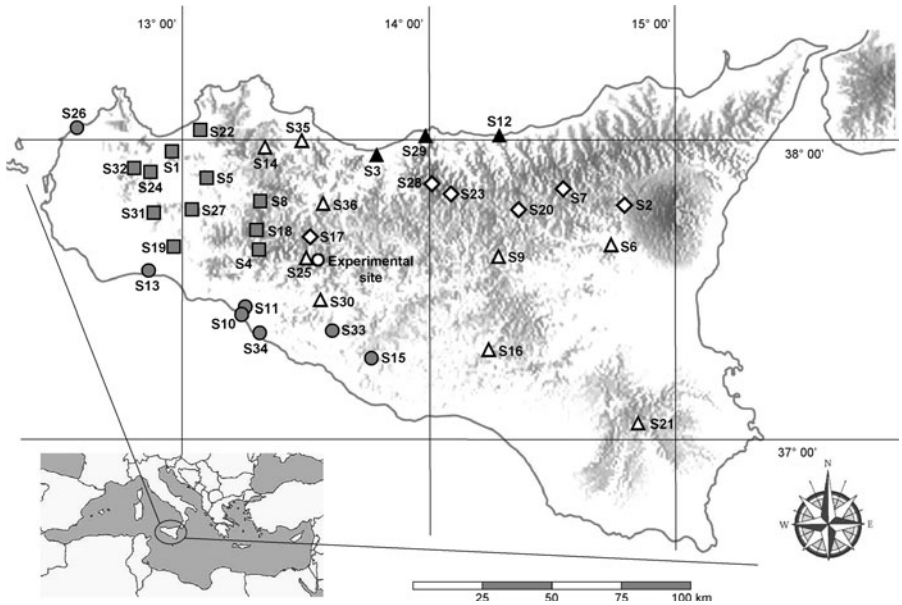

Fig. 1 Geographic locations of the 36 collection sites of natural populations of *Hedysarum coronarium* (S1–S36) in Sicily, and the experimental site. Different symbols indicate the 5 habitats identified by cluster analysis performed on collection site characteristics

Field trials: experimental site

Two field trials were performed during the 2005/2006 (1st year of the crop cycle) and 2006/2007 (2nd year) growing seasons in a hilly area of Sicily (37°30′ N, 13°31′ E, 178 m asl) on a Vertic Haploxerept soil. The topsoil (0–0.40 m) had the following characteristics: 38% clay, 25% silt, and 37% sand; pH 8.4; 1.27% organic matter and 0.85‰ total N.

The study site has a semiarid Mediterranean climate (as defined by Emberger 1955). The total rainfall at the site during the first growing season was 558 mm, which was very close to the long-term period average. During the second growing season, the total rainfall was 645 mm, 20% more than the long-term period average. Rainfall was mainly concentrated in the September–December (350 mm) period and during spring, with a peak in March (150 mm).

Pheno-morphological characterization under spaced-plant conditions

Seeds from 36 natural Sicilian populations of sulla, plus two Italian cultivars (Grimaldi and S. Omero), and two Sicilian agro-ecotypes (Gangi and Resuttano) included in the experiment as controls, were sown into 'Jiffy pots' on 29 November 2005, using 150 scarified seeds for each accession. After 6 weeks, 21 randomly chosen seedlings of each accession were transplanted to the field (7 plants per plot spaced at 1 × 1 m). A randomized block design with three replications was used. The soil was not fertilized and weeds were manually removed throughout the growing season. The trial was conducted under rain-fed conditions, although each plot was initially irrigated immediately following transplantation.

Five plants per accession and per plot were randomly selected, and the following phenological and morphological characters were recorded during the first crop cycle: days from sowing to first flower (hereafter, flowering time); plant height (measured 170 days after sowing); growth habit (1 = prostrate, 4 = erect); leaf length, leaflet number, and length and width of the terminal leaflet (all based on the leaf below the first flower); plant 'leafiness' (1 = min, 9 = max). Moreover, for each accession, an equal weight of mature seeds from each selected plant was bulked, and a representative sample was extracted to record the seed area and the seed elongation, the latter calculated as ratio of the lengths of the major and minor axes of the seed. These seed measurements were obtained on digital photographs using the image analysis software Image Tool 3.0 (UTHSCSA 2008). Mean seed weight was also determined for each sample.

Agronomic evaluation in dense stands

The 40 accessions of sulla (the 36 natural populations plus the same 4 controls included in the pheno-morphological characterization) were sown on 23

December 2005 at 280 viable seeds m^{-2} in micro-plots (four rows 2 m long and 0.25 m apart). A randomized block design with three replications was used. Plots were not fertilized and the trial was performed under rain-fed conditions. Weeds were manually removed.

For each accession, dry matter yield was recorded by cutting half of each plot at 50% flowering (from 5 to 29 May 2006, depending on genotype). The susceptibility of each accession to powdery mildew (*Erysiphe polygoni* DC; 0 = absent, 7 = max) was estimated before cutting. On 20 June 2006 the same sub-plots were mowed again to evaluate plant regrowth after the first cut. The other half-plots were left uncut and were harvested at maturity (July 2006). Pods were separated from biomass and then passed through a laboratory hulling machine to assess seed production. In autumn of the second year of the crop cycle (30 November 2006), all plots were completely mowed to determine biomass production. In the subsequent spring, dry matter production was evaluated for each accession by cutting half of each plot at 50% flowering (from 19 April to 3 May 2007, depending on genotype). The other half-plots were left uncut and then harvested at maturity (July 2007) to estimate the seed yield of accessions.

At each cut, a forage sample was extracted from the biomass and separated into botanical fractions (stems, leaves, flowers, and loments). These were dried at 60°C to a constant weight. In addition, for the two cuts at flowering, samples were dried and ground to a fine powder to determine total N content using the Kjeldahl method.

Characterization by ISSR molecular markers

The analysis included all 40 accessions of *H. coronarium* used in the two field trials. Total genomic DNA was extracted from young, healthy, and fresh leaflets according to the procedure described by Phillips et al. (2001) with minor modifications. For each accession, DNA was extracted from two individual plants. The DNA concentration of each sample was quantified by measuring absorbance at 260 nm as described by Sambrook et al. (1989), and adjusted to a concentration of 25 ng/µl. A total of six inter-simple sequence repeat (ISSR) primers were used to amplify the DNA: $(ACC)_6CC$, $CC(ATG)_6$, $CCAT(GT)_7$, $GCA(AC)_7$, $GGG(AC)_7$, and $AG(CA)_8$. Polymerase chain reaction (PCR) amplification was performed according to the following conditions: a 25 µl reaction mixture containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 800 µM dNTP, 0.5 µM each primer, 1 Unit Taq polymerase, and 25 ng total cellular DNA was prepared. Amplification was performed in a 96-well GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA) under the following cycle program: initial denaturation at 94°C for 4 min, followed by 36 cycles at 94°C for 30 s (denaturation), 53–56°C (depending on primer) for 45 s (annealing), and 72°C for 120 s (extension), followed by a final extension step at 72°C for 7 min. PCR-amplified DNA fragments were separated on a 1.5% agarose gel containing 1 × TBE (45 mM Tris–borate, 1 mM EDTA) and 0.5 µg/ml aqueous solution of ethidium bromide. About 25 µl reaction product (with an adequate amount of loading buffer) was loaded and the gel was run for 7 h at 100 V. The gel was then visualized under ultraviolet light.

Statistical analysis

The main descriptive statistics were calculated for all recorded pheno-morphological and agronomic traits. In both field experiments, an analysis of variance (ANOVA procedure, SAS Institute Inc. 2002) was performed for each trait according to the experimental design to test the significance of variation among accessions. All variables corresponding to proportion were arcsine-transformed.

To assess the differences in types of collection sites, principal components analysis (PCA) was performed (PRINCOMP procedure, SAS INSTITUTE INC. 2002) on the sites using geographic (altitude), climatic (see above), and pedological (soil pH) data. The PCA was based on the correlation matrix of variables. Following Kaiser's criterion (Kaiser 1960), only those principal components showing an eigenvalue ≥ 1 were retained for cluster analysis (CLUS procedure, SAS INSTITUTE INC. 2002) adopting Euclidean distance as a measure of dissimilarity and the Ward's method as the clustering algorithm. Estimates of the number of clusters (habitats) were achieved using pseudo F and t^2 statistics (Milligan and Cooper 1985). In addition, the mean values of the geographic and pedo-climatic data were calculated for each habitat and least significant differences were computed.

PCA (based on the correlation matrix) was also performed using pheno-morphological and agronomic

data to establish the importance of different traits in explaining multivariate polymorphisms. The inclusion of strongly correlated variables can lead to weighting of the analysis in favour of those variables (Pengelly and Maass 2001). To avoid this element of distortion, correlations among all variables were calculated, and one of the variables was removed from the analysis in cases where two variables were highly correlated ($r > 0.70$).

For the molecular analysis, amplified bands from each primer were scored as present (1) or absent (0) for all of the genotypes analysed. Only bands showing consistent amplification were considered; smeared and weak bands were excluded from the analysis. Nei's (1972) genetic identity between each accession pair was determined. Matrices based on genetic and phenotypic (pheno-morphological and agronomic) distances were compared calculating the Mantel's test statistic Z, and significance was determined using 1000 permutations (routine MXCOMP of the NTSYS-pc package; Rohlf 1998).

Additional statistics were computed to estimate the level of polymorphism among the 36 natural populations studied. The number of amplified loci, percentage of polymorphic loci, average observed and effective alleles, Nei's (1973) gene diversity, and Shannon's information index (Lewontin 1972) were calculated for each primer and among all primers. The discriminant power of each primer was calculated as the sum of the Gregorius differentiation coefficients (Gregorius 1987) of each locus. All calculations and analyses were conducted using POPGENE, version 1.31 (Yeh et al. 1999).

Results

Geographic variation and climatic differences among the collection sites

The latitude of the collection sites varied from 37°01′ N to 38°03′ N, and the longitude ranged from 12°33′ E to 14°52′ E. The lowest collection altitude was 7 m asl and the highest was 1027 m asl. More than 30% of the sulla specimens were collected at an altitude lower than 200 m and only 11% were sampled from sites above 800 m asl. The mean rainfall during the growing season ranged from 407 to 758 mm and mean summer rainfall ranged from 7 to 42 mm. The mean temperature during the growing season was between 11.3 and 20.6°C. Most populations (78%) were from the subhumid zone, while 11% were from the semi-arid zone and 11% were from the humid zone (all defined according to the Emberger 1955 classification of bio-climates). Soil pH ranged from 7.1 to 8.4; 14% of the sites had soil with a pH below 7.5, and 28% had soil with a pH above 8.0.

PCA identified three principal components (PC) with eigenvalues > 1, which together accounted for 82.6% of the total variance. PC1 explained 52.1% of the variation and was positively influenced by mean temperature during the growing season, mean temperature of the coldest month, and ETP, all of which were contrasted with cold period, altitude, rainfall during the growing season, and rainy days per year (Fig. 2). PC2 explained 20.8% of the variation and was dominated positively by Iq, rainfall during the growing season, rainy days per year, and soil pH.

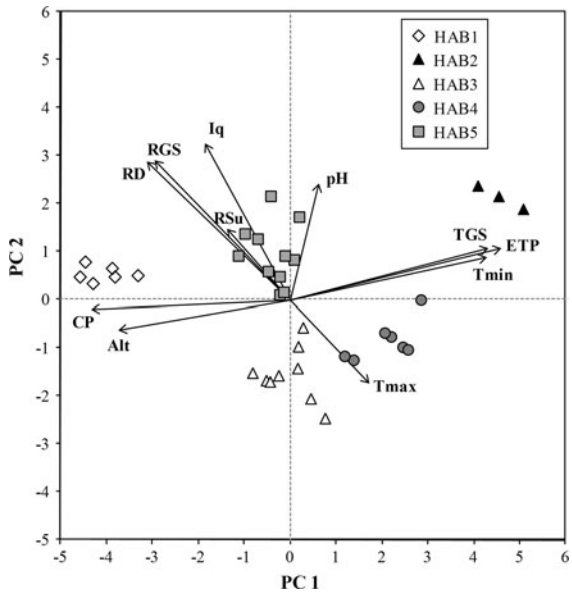

Fig. 2 Principal components (PCs) biplot of sulla germplasm collection site characteristics. The 11 arrows intersecting at (0,0) represent the original variables. The length of each vector is proportional to its contribution to the PCs. Different symbols indicate the 5 habitats identified by cluster analysis. *TGS*: mean temperature during the growing season; *Tmax*: mean maximum temperature of the hottest month; *Tmin*: mean minimum temperature of the coldest month; *RGS*: mean rainfall during the growing season; *RSu*: mean summer rainfall; *RD*: rainy days per year; *CP*: cold period; *Iq*: Emberger index; *ETP*: mean annual evapotranspiration; *Alt*: altitude; pH: soil pH

The habitats of the Sicilian *H. coronarium* accessions formed five distinct groups based on geographic and pedo-climatic data (Table 1). Habitat 1, which included six sites (from mountain areas of Sicily), plotted to the upper-left quadrant of Fig. 2 and was separated from the other habitats by having higher altitude and rainfall, and lower ETP and temperature during the growing season. Habitat 2, on the positive extreme of the PC1, included three sites located on the northern coast of Sicily, with the highest values being temperature during the growing season, minimum temperature of the coldest month, and ETP. Both Habitat 3 and Habitat 4 had negative PC2 values and formed separate clusters on the basis of temperature and rainfall. Habitat 3 included nine sites mainly located in the hilly area of the Sicilian inland with low temperatures during the growing season. Habitat 4 included seven sites, mainly located on the south-western coast of Sicily, with high temperatures during the growing season and low rainfall (in some cases <450 mm, on a yearly basis). Habitat 5 included 11 sites mainly located in the western hilly area of Sicily, characterized by having climatic conditions intermediate from those of the other habitats.

Table 1 Average geographic and climatic parameters of the 5 habitats of the collection sites identified by cluster analysis

Variables		HAB1[a] ($n = 6$)	HAB2 ($n = 3$)	HAB3 ($n = 9$)	HAB4 ($n = 7$)	HAB5 ($n = 11$)	*P*-value	l.s.d. $P < 0.05$
Mean temperature during growing season	(°C)	12.7	20.6	14.8	18.6	16.2	<0.0001	1.50
Mean max temperature of the hottest month	(°C)	28.5	29.9	31.6	30.5	31.2	<0.0001	1.03
Mean min temperature of the coldest month	(°C)	3.3	10.0	5.3	8.1	6.3	<0.0001	0.88
Mean rainfall during growing season	(mm)	702	583	543	486	649	<0.0001	66.5
Mean summer rainfall	(mm)	33	36	27	15	23	0.0002	8.3
Rain days per year	(d)	78	72	65	59	74	<0.0001	7.9
Mean ETP year	(mm)	752	955	847	904	887	<0.0001	34.7
Cold period	(n)	4.5	0.0	3.3	0.0	2.2	<0.0001	1.37
Emberger index (Iq)		92	78	59	58	72	<0.0001	8.7
Altitude	(m a.s.l.)	900	47	453	123	337	<0.0001	164.6
Soil pH		7.7	7.9	7.6	7.8	8.0	0.0079	0.3

[a] *HAB1* mountain area, *HAB2* northern coast, *HAB3* inland hilly area, *HAB4* southern coast, *HAB5* western hilly area

Table 2 Range of variation, means, and standard deviation (SD) for 11 phenological and morphological traits recorded for 36 sulla Sicilian populations, 2 Sicilian agro-ecotypes, and 2 cultivars

Traits	Sicilian natural populations ($n = 36$)				Agro-ecotypes		Varieties		*P*-value
	Min	Max	Mean	SD	Gangi	Resuttano	Grimaldi	S.Omero	
Flowering time (d)	139	154	148	3.4	150	149	155	156	<0.0001
Plant height (cm)	11	57	26	11.8	58	34	29	35	<0.0001
Central leaflet length (mm)	24.9	38.0	32.1	3.89	36.9	32.9	32.4	32.9	0.0005
Central leaflet width (mm)	20.6	28.7	24.2	2.43	27.9	25.4	25.4	26.2	0.0019
Leaf length (cm)	9.1	16.4	12.7	2.08	15.8	15.0	12.4	14.9	<0.0001
Number of leaflets per leaf	6.4	9.2	7.8	0.67	9.1	8.8	8.2	8.9	<0.0001
Leafiness	4.4	6.9	5.8	0.60	5.2	6.1	5.9	6.9	0.0011
Plant growth habit	1.0	3.9	2.1	1.00	3.5	3.0	3.4	3.4	<0.0001
Seed weight (mg)	4.8	6.3	5.5	0.40	6.4	5.9	5.1	5.1	<0.0001
Seed area (mm^2)	5.3	6.6	5.9	0.33	6.0	5.8	5.6	5.4	<0.0001
Seed elongation	1.15	1.21	1.17	0.015	1.16	1.17	1.17	1.18	ns

Pheno-morphological variability and agronomic evaluation

The ANOVA results showed highly significant differences for most of the recorded traits among the 40 *H. coronarium* accessions. The mean and range of variation for each of the 11 traits observed for spaced plants are given in Table 2.

On average, the flowering time of the natural populations was 148 days after sowing (139–154 days). The two cultivars Grimaldi and S. Omero were characterized by late flowering, showing the first flower after the latest-flowering natural population.

The natural populations varied widely in plant growth habit; about 50% exhibited prostrate growth while only 11% showed erect growth. Both agro-ecotypes and both cultivars showed an erect or semi-erect growth habit.

Dry matter yield at all cuts varied greatly among the natural populations, highlighting the different responses of populations in terms of winter growth, regrowth capacity after cut, and autumnal growth after summer stasis (Table 3). Total biomass production over 2 years ranged between 1,204 and 2,607 g DM m^{-2} and some natural populations had significantly higher yields than controls. Moreover, the distribution of forage production differed between the 2 years of the crop cycle, where the first-year incidence of total biomass was between 19.7 and 39.3%.

None of the accessions was completely resistant to powdery mildew. Most natural populations appeared highly susceptible, but some showed a low level of susceptibility similar to that observed for controls.

Patterns of pheno-morphological and agronomic variation

The PCA based on both pheno-morphological and agronomic data clearly discriminated the sulla accessions. The first three components with eigenvalues greater than 1 explained 78.0% of the total variation in the variables, which is a good summary of the original data set. PC1 accounted for 44.1% of the total variation and was positively influenced by leaf length, plant height, plant growth habit, and number of leaflets per leaf. PC2 explained 18.0% of the variance; the characters with the greatest (positive) influence were leafiness, flowering time, and total biomass production (1st + 2nd year). PC3 accounted for 15.8% of the total variation and was dominated by the effects of first-year incidence on total biomass (negatively) and, positively, by powdery mildew susceptibility and total seed production (1st + 2nd year). Figure 3 shows the distribution of the *H. coronarium* accessions, collected from the habitats defined in Fig. 2, in the 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.

PCA did not clearly differentiate the accessions according to their habitats of origin, but in some cases, accessions from the same habitat had a tendency to stay together. Accessions from Habitat 1 (higher altitude and rainfall, and lower temperature) were mainly confined to the upper-left quadrant of Fig. 3. These were characterized by prostrate growth habit, small plants and leaves, and high total biomass production (Table 4), with the exception of a population that plotted to the lower right quadrant (on the extreme of PC1) near the agro-ecotype Gangi. Germplasm from Habitat 2 plotted to the negative side of PC1 and was characterized by small and light seeds and low powdery mildew susceptibility. Accessions sampled from the hilly area of the Sicilian inland (Habitat 3) were scattered among all quadrants, showing wide phenotypic and agronomic variability and reflecting the diversity of their collection sites in terms of environmental characteristics. Accessions sampled from the southern coast of Sicily (Habitat 4) were mainly scattered throughout the lower-left quadrant and were characterized by early phenology, high powdery mildew susceptibility, low leafiness, and very low biomass production. Material from Habitat 5 plotted to the positive side of PC1. These were characterized by late flowering, great plant and leaf dimensions, and high biomass production. The varieties Grimaldi and S. Omero and the agro-ecotype Resuttano showed similar characteristics to those observed for the accessions from Habitat 5.

Molecular diversity

Six ISSR primers were used to evaluate the 40 *H. coronarium* accessions. Limiting the analysis to the 36 natural Sicilian populations, 51 well-resolved bands were observed (Table 5). The amplified fragments ranged from 150 bp [primer $AG(CA)_8$] to 1.6 Kb [primer $CCAT(GT)_7$] in size. The number of

Table 3 Range of variation, means, and standard deviation (SD) for 15 agronomic traits recorded for 36 sulla Sicilian populations, 2 Sicilian agro-ecotypes, and 2 cultivars

Traits	Sicilian natural populations ($n = 36$)				Agro-ecotypes		Varieties		*P*-value
	Min	Max	Mean	SD	Gangi	Resuttano	Grimaldi	S.Omero	
1st year									
Spring cut									
Biomass production (g DM m^{-2})	188	798	411	122.3	570	406	795	880	<0.0001
Leaves content (% on DM tot)	44.1	79.7	65.1	8.43	52.4	56.2	56.5	47.4	0.0007
Nitrogen content (g kg^{-1} DM)	22.1	29.7	25.9	1.93	25.2	25.0	26.5	24.8	0.0001
Powdery mildew susceptibility	1.3	6.0	4.0	1.31	2.0	3.7	2.0	1.3	<0.0001
Regrowth									
Biomass production (g DM m^{-2})	3	230	105	65.0	163	99	124	29	<0.0001
Leaves content (% on DM tot)	0.0	97.9	67.1	33.23	69.1	84.3	77.7	87.7	<0.0001
2nd year									
Autumn cut									
Biomass production (g DM m^{-2})	258	739	510	132.7	467	599	381	381	<0.0001
Leaves content (% on DM tot)	74.4	94.1	85.3	5.73	88.5	75.2	94.0	96.8	<0.0001
Spring cut									
Biomass production (g DM m^{-2})	473	1,433	971	215.8	1,262	1,160	1,107	956	<0.0001
Leaves content (% on DM tot)	18.0	30.9	23.5	3.15	27.0	22.4	25.2	23.6	<0.0001
Nitrogen content (g kg^{-1} DM)	18.7	25.7	21.8	0.18	19.0	21.9	19.3	19.6	<0.0001
1st + 2nd year									
Total biomass production 1st + 2nd year (g DM m^{-2})	1,204	2,607	1,997	332.4	2,463	2,264	2,408	2,247	<0.0001
1st year incidence (% on total biomass)	19.7	39.3	26.0	4.00	29.8	22.3	38.2	40.5	<0.0001
Seed production									
1st year (g m^{-2})	6.3	178.6	38.6	33.30	174.1	61.2	118.8	71.3	<0.0001
2nd year (g m^{-2})	3.6	52.3	15.4	10.34	34.7	31.1	26.8	27.3	<0.0001

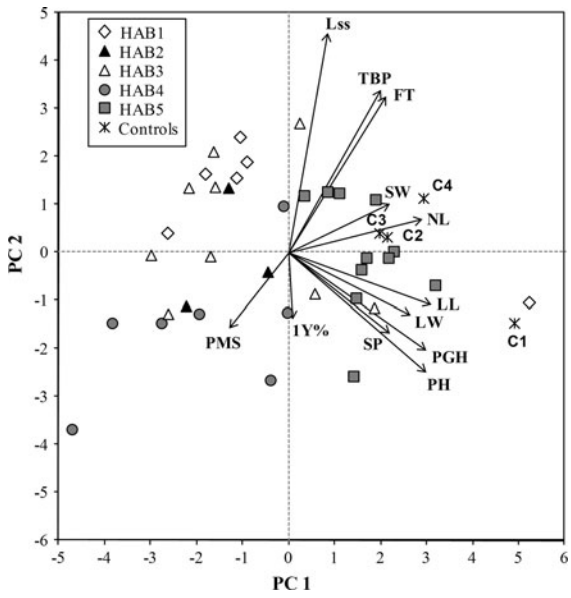

Fig. 3 Principal components (PCs) biplot of sulla pheno-morphological and agronomic germplasm traits. The arrows intersecting at (0,0) represent the original variables. The length of each vector is proportional to its contribution to the PCs. Different symbols indicate the 5 habitats identified by cluster analysis performed on collection site characteristics. *FT*: flowering time; *PH*: plant height; *PGH*: plant growth habit; *LL*: leaf length; *LW*: width of terminal leaflet; *Lss*: leafiness; *SW*: seed weight; *NL*: number of leaflets per leaf; *PMS*: powdery mildew susceptibility; *TBP*: total biomass production (1st + 2nd year); 1Y%: first year incidence on total biomass production; *SP*: total seed production (1st + 2nd year). *C1*: Agroecotype Gangi; *C2*: Agroecotype Resuttano; *C3*: cv Grimaldi; *C4*: cv S. Omero

ISSR bands obtained with each primer varied from 4 [primer $CC(ATG)_6$] to 14 [primer $AG(CA)_8$], with an average of 8.5 bands per primer. Out of the 51 amplified bands, 39 (PPL = 76.5%) were polymorphic. The number of polymorphic markers detected with each primer ranged from 1 [primer $CC(ATG)_6$] to 11 [primer $AG(CA)_8$]. The total differentiation coefficient was on average equal to 1.67 ± 1.01. The highest value was obtained using the primer $(ACC)_6CC$ ($\delta = 2.86$), while the lowest was obtained using the primer $CC(ATG)_6$ ($\delta = 0.44$). The average number of observed and effective alleles was 1.75 ± 0.44 and 1.29 ± 0.32, respectively, with the highest values identified with the primer $(ACC)_6CC$ (1.90 and 1.49) and the lowest with the primer $CC(ATG)_6$ (1.25 and 1.20). Average Nei's gene diversity was 0.18 ± 0.18, with the highest value identified with the primer $(ACC)_6CC$ (0.29) and the lowest with the primer $CC(ATG)_6$ (0.11). Average Shannon's information index was high (0.29 ± 0.25), and the highest value was obtained with the primer $(ACC)_6CC$ (0.44) and the lowest value was obtained with the primer $CC(ATG)_6$ (0.16).

Nei's genetic identity (I) between each accession pair was determined and mean I was 0.86 ± 0.04, ranging from 0.72 to 0.96). A Mantel's test comparing the two matrices (genetic and phenotypic distances) resulted in a very low normalized Mantel's statistic Z value (matrix correlation $r = 0.07$), with a probability random $Z \geq$ observed $Z = 0.26$.

Discussion

There were highly significant inter-population differences among natural sulla populations in Sicily for most of the recorded pheno-morphological and agronomic traits.

Flowering time, expressed as the number of days from sowing to first flower, ranged from 139 to 154 days. Issolah and Khalfallah (2007) reported a range of 16 days for this phenological trait in a study carried out in Algeria on 14 natural populations of *H. coronarium*. Flores et al. (1997) found a greater range (23 days) evaluating 62 accessions mainly from Spain. The two varieties Grimaldi and S. Omero flowered significantly later than all natural Sicilian populations, suggesting that this phenological trait was highly regarded by breeders. In fact, the optimum developmental stage at which to harvest sulla for hay is at full flowering (Leto et al. 2002), and in the Mediterranean region, late flowering certainly represents an advantage, because it maximizes yield by increasing the growing season, and it localizes the cutting period to late spring and thus reduces the risk of rain during the field-drying process. However, because sulla in the Mediterranean produces seeds as the soil begins to dry out, late-flowering genotypes in natural populations would experience relatively drier conditions during seed formation and frequently suffer increased water stress, compared to early-flowering genotypes (Blum and Lehrer 1973).

Some natural populations had a prostrate growth habit, while others exhibited erect (or semi-erect) growth similar to that of the agro-ecotypes and cultivars included in the study. Such variability represents a source of intra-specific diversity that

Table 4 Means of the pheno-morphological and agronomic traits of 36 sulla Sicilian populations from different habitats

Traits	HAB1 ($n = 6$)[a]	HAB2 ($n = 3$)	HAB3 ($n = 9$)	HAB4 ($n = 7$)	HAB5 ($n = 11$)	*P*-value	l.s.d. $P < 0.05$
Pheno-morphological characterization							
Flowering time (d)	149	148	147	145	150	0.026	3.6
Plant height (cm)	22	22	20	26	35	0.047	12.8
Central leaflet length (mm)	28.3	30.8	31.4	31.5	35.6	0.001	3.65
Central leaflet width (mm)	22.0	23.4	24.2	23.6	26.1	0.006	2.45
Leaf length (cm)	11.7	11.6	11.9	11.8	14.8	0.001	1.94
Number of leaflets per leaf	8.2	7.6	7.6	7.2	8.3	0.003	0.65
Leafiness	5.9	5.7	5.9	5.1	6.0	0.025	0.64
Plant growth habit	1.6	2.0	1.6	2.0	2.9	0.013	1.00
Seed weight (mg)	5.7	5.1	5.4	5.3	5.8	0.016	0.42
Seed area (mm^2)	6.1	5.5	5.9	5.7	5.9	0.048	0.36
Seed elongation	1.2	1.2	1.2	1.2	1.2	0.688	ns
Agronomic evaluation							
1st year—spring cut							
Biomass production (g DM m^{-2})	496	354	433	277	446	0.005	121.8
Leaves content (% on DM tot)	62.8	56.1	69.9	58.4	69.2	0.003	8.27
Nitrogen content (g kg^{-1} DM)	2.5	2.7	2.5	2.7	2.6	0.093	ns
Powdery mildew susceptibility	3.2	2.4	4.4	4.9	3.9	0.020	1.38
1st year—regrowth							
Biomass production (g DM m^{-2})	88	94	89	156	98	0.238	ns
Leaves content (% on DM tot)	40.2	84.2	67.3	67.8	76.4	0.233	ns
2nd year—Autumn cut							
Biomass production (g DM m^{-2})	467	337	533	455	597	0.010	135.8
Leaves content (% on DM tot)	89.2	90.1	86.4	84.8	81.2	0.018	5.98
2nd year—spring cut							
Biomass production (g DM m^{-2})	1194	931	979	770	984	0.006	217.3
Leaves content (% on DM tot)	22.5	22.2	24.9	23.1	23.6	0.578	ns
Nitrogen content (g kg^{-1} DM)	2.1	2.2	2.2	2.3	2.1	0.275	ns
1st + 2nd year							
Total biomass production (g DM m^{-2})	2,245	1,716	2,034	1,658	2,125	0.002	320.5
1st year incidence (%)	25.9	26.1	26.0	26.9	25.5	0.976	ns
Seed production							
1st year (g m^{-2})	49.4	41.7	25.2	44.0	39.4	0.701	ns
2nd year (g m^{-2})	19.8	5.9	11.8	9.2	22.4	0.007	10.46

[a] In brackets, the number of sites included in the habitat

could be exploited when selecting for *H. coronarium* varieties for various uses (e.g., hay production, grazing, soil protection).

Total dry matter yield varied widely among accessions, and some natural populations had significantly greater yield than controls. Biomass production in both years was higher in later-flowering populations, as observed by Di Giorgio et al. (2009) and Graziano et al. (2010) in other forage legumes. Several natural populations had greater autumn dry matter yield than the control varieties. This highlights the possibility of selecting new varieties characterized by high regrowth after summer stasis, providing an opportunity for early grazing, and thus helping fill

Table 5 Genetic parameters of the 36 sulla Sicilian populations according to each ISSR primer

Primer	Sequence	Ta[a] (°C)	NAL	PPL (%)	MinLS (bp)	MaxLS (bp)	$\Sigma\delta$	n_a	n_e	h	H_{sp}
ENEA12	$CCAT(GT)_7$	54	11	81.8	250	1,600	1.66	1.82	1.23	0.15	0.26
ENEA13	$GCA(AC)_7$	53	5	60.0	250	900	0.74	1.60	1.26	0.15	0.24
ENEA14	$GGG(AC)_7$	53	7	85.7	250	650	1.49	1.86	1.34	0.22	0.34
ENEA47	$AG(CA)_8$	53	14	78.6	150	1,500	2.82	1.71	1.21	0.15	0.24
ENEA34	$(ACC)_6CC$	56	10	90.0	250	680	2.86	1.90	1.49	0.29	0.44
ENEA36	$CC(ATG)_6$	56	4	25.0	630	750	0.44	1.25	1.20	0.11	0.16
Total	–	–	51	76.5	–	–	–	1.75 ± 0.44	1.29 ± 0.32	0.18 ± 0.18	0.29 ± 0.25

[a] Ta = temperature of annealing; NAL = number of amplified loci; PPL = percentage of polymorphic loci; MinLS = minimum locus size; MaxLS = maximum locus size; $\Sigma\delta$ = sum of differentiation coefficients per locus; n_a = average of observed alleles; n_e = average of effective alleles; h = Nei's gene diversity; H_{sp} = Shannon's information index

the lack of grazing resources typical in Mediterranean environments during this time.

No plants were totally resistant to powdery mildew, although some natural populations showed a low level of susceptibility similar to that observed for control varieties. All previous studies on sulla have highlighted a high susceptibility of the Sicilian germplasm to this fungal infection (Porceddu and Monotti 1976; Amato et al. 1997). Furthermore, powdery mildew susceptibility was greater for accessions collected from environments characterized by scarce rainfall.

The PCA performed on pheno-morphological and agronomic traits was useful for identifying the most important traits associated with variation among the Sicilian sulla accessions that were clearly discriminated. However, PCA did not clearly differentiate accessions according to their habitats of origin. The great pedo-climatic variability observed among the habitats of the collection sites has presumably resulted in ideal conditions for the intra-specific differentiation of *H. coronarium*, but other factors such as the extreme geographic proximity of some sites (although very different in altitude and climatic parameters) could have confounded the relationships among the pheno-morphological traits and the pedo-climatic parameters of the collection sites. Furthermore, it is noteworthy that in Sicily, sulla is widespread both as a wild and cultivated plant, and that these forms grow in close proximity to each other; moreover, cultivated forms, frequently moved throughout Sicily (e.g., via seed exchange among farmers and among external markets), may be accidentally introduced into natural and semi-natural ecosystems. Therefore, under some conditions, gene flow may occur, considering the allogamous mating system of the species. If so, this could have altered the genetic composition of some wild populations, thereby making the relationships among the pheno-morphological traits and the environmental parameters of the collection sites less clear. Nevertheless, in some cases, accessions from the same habitat showed a tendency to group together in the PCA analysis, and the variance among habitats (assessed relative to the variance among accessions within a habitat) was significant for several traits.

Molecular analysis revealed the existence of genetic diversity among natural populations, therefore confirming the results of the phenotypic analysis. All of the genetic diversity parameters were high, in accordance with the findings of Marghali et al. (2005) and Trifi-Farah and Marrakchi (2000, 2002), who studied natural populations of *H. coronarium* in Tunisia using RFLP and AFLP markers.

The overall correspondence between the two distance matrices based on phenotypic and genetic data was very low ($r = 0.07$); this could be the result of the low number of individuals used in this study for molecular characterization (two per population). However, several studies on different species have reported low correlations between genetic and morphological distances (Vollmann et al. 2005; Johnson et al. 2007; Khan et al. 2009). One reason for this is that markers such as ISSRs detect polymorphisms in coding and, most often, in non-coding regions of DNA that are randomly distributed over the whole genome. Therefore, the genetic diversity estimated is not necessarily associated with phenotypic variation.

Another reason for the low correlation between phenotypic and marker-based estimates of diversity is that plants that appear similar based on phenotypic traits are not necessarily genetically similar, and different gene pools can develop similar phenotypes, as reported by Khan et al. (2009).

Some of the ISSR primers, used on sulla for the first time in the present study, had high discriminating power. Many produced clear, highly informative patterns. In particular, the primers $(ACC)_6CC$ and $AG(CA)_8$ had very high total differentiation coefficients (2.86 and 2.82, respectively) and the primers $GGG(AC)_7$ ($\delta = 1.49$) and $CCAT(GT)_7$ ($\delta = 1.66$) were also very useful, showing high percentages of polymorphic loci (85.7 and 81.8, respectively). Therefore, these ISSR primers can be considered new and useful tools for the molecular analysis of sulla.

In conclusion, the present study documented large genetic and phenotypic variability among natural Sicilian populations of *H. coronarium*. The relationships among pheno-morphological traits and environmental parameters of the collection sites were not clear. From a breeding point of view, the observed diversity could be a great advantage when searching for *H. coronarium* material to exploit for its several potential uses in semi-arid Mediterranean environments. Moreover, some of the Sicilian populations had biomass yields that were significantly higher than the control varieties, which are currently available on the market.

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