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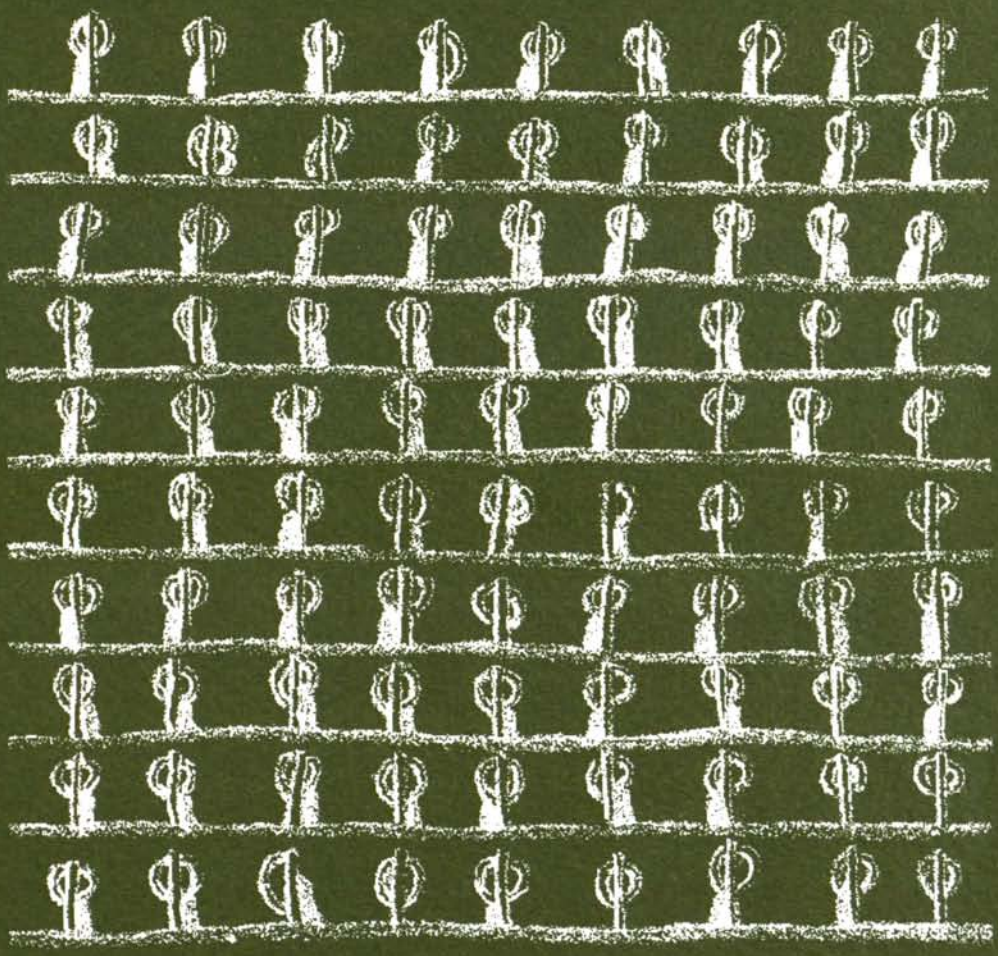

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A CONTRIBUTION TO THE STUDY OF THE DISTRIBUTION OF *MEDICAGO - SINORHIZOBIUM* SYMBIOSIS IN SARDINIA (ITALY)

G. Brundu*[***], I. Camarda*, M. Caredda**, G. Garau**, S. Maltoni**, P. Deiana**

** Dipartimento di Botanica ed Ecologia Vegetale, University of Sassari, Via F. Muroni, 25 - 07100 Sassari, Italy. ** Dipartimento di Scienze Ambientali Agrarie e Biotecnologie Agro-Alimentari. University of Sassari, Via E. De Nicola, 1 - 07100 Sassari, Italy.*

SUMMARY — The paper summarises the results of a *Medicago-Sinorhizobium* germplasm survey and collection carried out in the island of Sardinia (Italy) in 1998/99 and subsequent laboratory isolation of microbial strains, soil sample analyses and determination of *Medicago* species. According to a stratified sampling methodology, the major ecological characteristics of island's habitats were taken into account, collecting and surveying mostly in natural or semi-natural habitats (no roadside sites nor cultivated fields were sampled).

Forty-six sites, widely distributed in semi-natural representative areas of Sardinia, ranging from sea level to above 1,000 m a.s.l., were sampled and 24 were surveyed to gather additional data on species distribution. Root nodules were collected from 15 species (13 annuals) out of the total 21 *Medicago* species recorded in Sardinia. Isolation of root nodule bacteria accessions and identification of 29 strains were achived. A total number of 17 species were surveyed and mapped.

The present study gives a first contribution to the knowledge of the present distribution of the species of the genus *Medicago* in Sardinia with special concern to the species recorded or sampled during the survey and highlights the presence of *Medicago* biodiversity hot spots.

Key words: *Medicago-sinorhizobium symbiosis, Sardinia.*

INTRODUCTION

The genus Medicago L.

Medicago L. (*Leguminosae*), a predominantly Mediterranean genus, comprises a high number of species, annual herbs, herbaceous perennials and rare shrubs, many of which are markedly polymorphic. SMALL and JOMPHE (1989) in their synopsis on this genus have delimited it into 12 sections and eight subsections composed of 85 species and 18 infraspecific taxa. More recently, they have increased the total number by adding the new taxon *M. truncatula* Gaertn. f. *laxicycla* E. Small (SMALL *et. al.*, 1991) and proposed that *M. rigidula* be split into two separate species, namely *M. rigidula* e *M. rigiduloides*. Delimitation of *Medicago* from its close relatives in tribe *Trifoliae* subtribe *Trigonellinae* remains controversial (SMALL *et al.*, 1990; SMALL *et al.*, 1992) such as the debate over whether the genus *Medicago* should be considered a comparatively small one of less than 60 species, based primarily on the character of fruit coiling, or a larger one of more than 80 species.

Many species of the genus have significant and wide-ranging agricultural and environmental applications, e.g. *M. sativa* L. (IRWIN *et al.*, 2001). Moreover, annual medics are of great importance in Mediterranean pastures, as well as in South-western Australian and South American rangelands. Biological nitrogen fixation, by symbiotic rhizobia within root nodules, may lessen the use of nitrogen fertilisers, thus providing a suitable tool for "biological" farming and for reducing environmental nitrogen pollution. The "self-reseeding" capability of annual medics is fea-

**** Corresponding author; gbrundu@tin.it*

ture that leads to low input agricultural techniques, but also explains medics behaviour as invasive aliens outside their natural range (OLIVIERI *et al.*, 1991). Invasiveness is especially typical of most annual species with spiny coils, although invasive legumes may often fail at colonisation attempts within habitats where mutualist partners, the specific rhizobia, are scarce (PARKER, 2001). Seed physiology might be another invasive trait, as stated by SIDHU and CAVERS (1977) for *M. lupulina*. The world-wide distribution of *M. lupulina* may also be promoted by the presence of simple and gland-tipped trichomes that provide various kinds of physical and chemical protection against herbivores, particularly insects (GOERTZEN and SMALL, 1993).

The genus Medicago L. in Sardinia

In spite of different studies and collection surveys for agricultural and rangeland management purposes on the *Medicago* species present in Sardinia, there is not any comprehensive study on the presence and distribution pattern of the species of this genus in the Island. Furthermore, historical records of local floras and collection surveys have to be carefully verified.

According to the main Flora of Italy (PIGNATTI, 1982) 21 species and 5 subspecies of the genus *Medicago* L. are present in Sardinia. In addition, DESOLE (1945) has recorded *M. solerolii* Duby on a small island along the Sardinian North-West coast and the same species was later recorded by DE MARTIS and LOI (1989) in the South of Sardinia. ABDELGUERFI and GUITTONNEAU (1989) have determined as *Medicago heterocarpa* Spach a herbarium specimen coming from the North of Sardinia, dated 1882 and they regarded it as synonymous to *M. sphaerocarpa* Bertol. var. *ovalis* Moris (1837). In a more recent paper GILLESPIE and MCCOMB (1991) have rejected the names *M. heterocarpa* Spach and *M. lesinsii* Small and suggested use of the name *M. murex* Willd. for 2n=2x=16 species and *M. sphaerocarpos* Bertol. for 2n=2x=14 species inside the highly variable *M. murex* group.

According to LESINS and LESINS (1979), SMALL and JOMPHE (1989) and the Med-Checklist (GREUTER *et al.*, 1989) the 26 taxa present in Sardinia as recorded by PIGNATTI (1982) correspond to 23, 21 and 22 taxa, respectively. If we also consider the records of Authors after Pignatti (see above) the total number of taxa reaches, 25, 24, 25 respectively (see Table 1).

For many species LESINS and LESINS (1979) refer to the varieties introduced by HEYN (1963) to account for the great variability of many species in the genus (e.g. 3 subspecies for *M. polymorpha*), while SMALL and JOMPHE (1989) have attempted to reduce the number of taxa. The "simplification" proposed by Small and Jomphe is of great operational help in identifying plant samples.

Symbiotic root nodule bacteria of Medicago sp.pl.

Rhizobial nodulation of legumes is species and strain specific. *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*; JORDAN, 1983) and especially *S. medicae* (Rome *et al.*, 1996) are the most restrictive in nodulation preference, normally nodulating only the legumes belonging to the three genera *Medicago*, *Melilotus* and *Trigonella*. The variability of these symbiotic bacteria is seemingly at least as great as *Medicago* variability. There is evidence of the presence of different strains, within both the *Sinorhizobium* species, with high specificity to different accessions in the same *Medicago* taxa (BROCKWELL *et al.*, 1988; PAFFETTI *et al.*, 1996; JEBARA *et al.*, 2001), as well as variable adaptability to different soil conditions, especially to soil pH (BENGGU and O'HARA, 1996; HOWIESON and EWING, 1986; HOWIESON *et al.*, 1993; KAWURI and O'HARA, 1998), salinity and drought, besides different degrees of nodulation ability and survival.

Previous collections of Medicago and Rhizobium germplasm in Sardinia

Sardinian *Medicago-Rhizobium* germplasm has already been taken into account in previous collecting surveys.

In 1977 FRANCIS and GILLESPIE (1977, 1981) collected seeds of *Lupinus* sp. pl. and of oth-

TABLE 1. – *The synoptic chart compares the Small and Jomphe (1989) taxonomic scheme that applies to the species present in Sardinia according to Pignatti (1982) with the nomenclature adopted by other Authors. According to Pignatti, table reports also the status (in=native, end=endemic, ext=neophyte, arc=archeophyte), the distribution in Sardinia(common, rare), the maximum elevation m a.s.l.* (in Italy).

	Flora d'Italia			Flora d'Italia	Med Checklist	Monography	Monography
	El	**Fr**	**Status**	**Pignatti**	**Greuter**	**Small & Jomphe**	**Lesins & Lesins**
M. aculeata Willd.	*600*	*C*	*IN*	X	*M. doliata* Car.	*M. doliata* Car.	*M. doliata* Car.
M. arabica (L.) Hudson	*600*	*C*	*IN*	X	X	X	X
M. arborea L.	*300*	*R*	*EXT*	X	X	X	X
M. ciliaris (L.) All.	*300*	*C*	*IN*	X	X	X	*M. ciliaris* (L.) Krocker
M. heterocarpa Spach				*M. murex* Willd.	[*M. lesinsii* Small]	[*M. lesinsii* Small]	
M. hispida Gaertner	*1000*	*C*	*IN*	X	*M. polymorpha* L.	*M. polymorpha* L.	*M. polymorpha* L.
M. intertexta (L.) Miller	*600*	*C*	*IN*	X	X	X	X
M. intertexta (L.) Miller var. *tubercolata* Moris				X			
M. italica (Miller) Fiori				*M. tornata* (L.) Miller	X + *M. italica* subsp. *tornata* (L.) Emb. *et* M.	X	*M. tornata* (L.) Miller
M. litoralis Rohde	*600*	*C*	*IN*	X	*M. litoralis* Loisel.	*M. litoralis* Rohde ex Lois.	*M. litoralis* Rohde ex Lois.
M. lupulina L.	*2000*	*C*	*IN*	X	X	X	X
M. lupulina L. var. *cupaniana* (Guss.) Boiss.		*C*	*IN*	X			X
M. marina L.	*0*	*C*	*IN*	X	X	X	X
M. minima (L.) Bartal.	*1600*	*C*	*IN*	X	*M. minima* (L.) L.	X	X
M. minima (L.) Bartal. var. *recta* (Willd.) Burnat				X			*M. minima* (L.) Bartal. var. *minima* Heyn
M. monspeliaca (L.) Trautv.				*T. monspeliaca* L.	X		
M. murex Willd.	*600*	*C*	*IN*	X	X	X	X
M. orbicularis (L.) Bartal.	*1300*	*C*	*IN*	X	X	X	X
M. orbicularis (L.) Bartal. var. *biancae* (Tod.) Urb.				X			
M. praecox DC	*300*	*R*	*IN*	X	X	X	X
M. praecox DC var. *pontificialis* (Gennari) Urb.		*R*	*END*	X			
M. rigidula (L.) All.	*1200*	*C*	*IN*	X	X	X	X
M. rotata Boisser			*EXT*	[X]	[X]	[X]	[X]
M. rugosa Desr.	*800*	*C*	*IN*	X	*M. rugosa* Desr. in Lam.	X	X
M. sativa L. subsp. *sativa*	*1900*	*C*	*ARC*	X	X	X	X
M. scutellata (L.) Miller	*600*	*C*	*IN*	X	X	X	X
M. soleirolii Duby	*100*	*R*	*EXT*	[X]	[X]	[X]	[X]
M. sphaerocarpos Bertol.				*M. murex* Willd.			
M. tenoreana Ser.	*1900*	*R*	*IN*	X	*M. tenoreana* DC	X	*M. tenoreana* Ser. in DC
M. tornata (L.) Miller	*0*	*R*	*IN*	X	*M. italica* (Miller) Fiori subsp. *tornata* (L.) Emb. *et* M.	*M. italica* (Miller) Steud. *ex* F.	X
M. truncatula Gaertner	*850*	*C*	*IN*	X	X	X	X
M. tuberculata (Retz.) Willd.	*600*	*R*	*IN*	X	X	*M. turbinata* (L.) All.	*M. turbinata* (L.) All.
Total number of taxa				**26 [28]**	**22 [25]**	**21 [24]**	**23 [25]**

[] = Non recorded by Pignatti (1982) for Sardinia

er forage species, from acid and sandy soils, in order to derive plant materials specifically adapted to South-Western-Australian soils. Thirteen species of annual medics (*M. polymorpha, M. arabica, M. littoralis M. truncatula, M. minima, M. orbicularis, M. murex, M. praecox, M. rigidula, M. rotata, M. rugosa, M. tornata, M. turbinata*) from 162 collecting sites were sampled. The survey was part of a broader Mediterranean collection programme that covered Italy, Portugal, Spain, Turkey, Greece, Crete. The collected germplasm was used for a breeding programme that ended with the release of the *M. murex* cv "Zodiac". The record of *M. rotata* should be carefully verified, being the species normally present only in the Eastern part of the Mediterranean basin.

PIANO *et al.* (1982) collected germplasm of the following 8 species in 208 sites in Sardinia: *M. orbicularis* (21% of the collection sites), *M. truncatula* (11%), *M. murex* (21%), *M. turbinata* (7%), *M. minima* (9%), *M. polymorpha* (54%), *M. arabica* (36%), *M. intertexta* (6%) stating the role of soil type and pH on species distribution.

In 1989 root nodule and legumes of the species *M. polymorpha* (mainly *M. polymorpha* L. var. *vulgaris* Shin.) were collected by PORQUEDDU *et al.* (1992) on 49 sites, differing considerably for altitude (from 10 to 1,040 m a.s.l.), edaphic conditions and substrate (pH range from 4.9 to 8.8). The accessions from mountain districts were more likely to be determined as *M. polymorpha* var. *vulgaris* Shin. and those from coastal districts and lower altitudes as *M. polymorpha* var. *polymorpha* Heyn (LOI *et al.*,1995). According to these authors, a difference in distribution such as that may support HEYN'S (1963) contention that there are different varieties and suggest that they may have ecological significance. A more detailed study on 10 accessions of the previous collection was performed by LOI *et al.* (1993) to asses the diversity of responses to winter temperature and soil pH and the relationship between genotype response and environmental traits of the site of collection. Genotypes of *M. polymorpha* collected from high altitude sites were more plastic in response to temperature, while no strong relationship was found between the degree of tolerance to acid conditions in the nodulation phase and the pH of the site of collection suggesting that general adaptation to acidity may be a characteristic of certain species rather than of ecotypes.

In 1993, HOWIESON and LOI (1994) sampled legumes and nodules of *M. arabica, M. sphaerocarpa, M. aculeata, M. murex, M. polymorpha, M. rigidula*, on 24 collecting sites, mainly located on acid substrates of North Central Sardinia. *M. polymorpha* was collected from sites with pH range of 5.5-8.5.

In 1997 BULLITTA *et al.* (1998) collected different pasture species on 20 sites on the island of the Asinara National Park, in North-West Sardinia. Six annual medics were sampled: *M. aculeata , M. arabica, M. littoralis M. orbicularis, M. polymorpha, M. truncatula* and 24 bacterial strains were isolated.

The nomenclature cited quotes the names of the medics as referred in the single publications.

The aim of the present study is to make a contribution to the knowledge of the distribution-pattern of *Medicago-sinorhizobium* symbiosis in the island of Sardinia, to collect annual and perennial *Medicago* L. species and their relative symbiotic bacteria and to create a sinorhizobial collection derived from different species collected in different Sardinian sites. To single out *Medicago-sinorhizobium* distribution patterns adapted to specific pedo-climatic conditions by analysing the general environmental parameters of each site of origin, such as pluviometry, mean temperature, soil characteristics (e.g. pH and organic matter), land use. The collected bacterial strains, after laboratory and field evaluation, may be used as inoculum for legumes in pasture improvement programmes and restoration of degraded sites of particular environmental interest (e.g. National Parks, Protected Areas *ex* Dir. n. 43/92/EEC "Habitat" *etc.*).

The research and collection survey intentionally differed from previous studies be-

cause: (i) the germplasm collected and the records about species distribution does not refer to a single *Medicago* species but to most of the annual/perennial medics present in the island, taking into account also some species that have never been collected/studied before (e.g. *M. marina*); (ii) collection sites where there was more or less strong evidence of human habitat modification/degradation were avoided; (iii) the collection aimed to investigate the "natural" distribution pattern, the ecological behaviour of species and their symbiosis, and was not therefore concerned with a specific agricultural purpose.

MATERIALS AND METHODS

Survey and collection site distribution

The collection was carried out in Sardinia in March-May 1998. It (39-41° Lat. N, 8-10° Long W, Italy) comprises some 24,000 km^2, with soils very variable in origin, composition and depth. ARU *et al.* (1991) have described 35 Landscape Units with different associations of dominant soils and inclusions and, consequently, soil capability classes. Although the climate of Sardinia is typically Mediterranean, annual rainfall is not uniform over the island and within years, with an average of 350-400 mm on the Eastern South coast in contrast to an average of 1,000-1,300 mm in the central mountains.

The collection was followed by laboratory isolation of root nodule bacteria, identification of *Sinorhizobium* strains infective on *Medicago polymorpha*, analysis of soil samples and determination of herbarium specimens of *Medicago* sp pl. according to SMALL and JOMPHE'S (1989) identification guide reported in their monograph on the genus and according to their following papers and studies (SMALL *et al.*, 1990; 1991; 1992; 1999). The *Medicago* species/plant samples were determined, when possible, in the field or, afterwards, in the laboratory.

During following surveys (May-August 1998/99) in the same sites, the presence of other *Medicago* species not collected was recorded. A second network of sites was surveyed in order to have additional data on *Medicago* species distribution.

Sampling and surveying were stratified according to major ecological characteristics such as geological groups and soils, landform, altitude, vegetation types, climatic parameters, land-uses that occur within the Island of Sardinia and its main surrounding islets (ZONNEVELD, 1988; MADRAU, 1997). We collected in mostly natural or semi-natural habitats, in order to sample and record the highest number of native (non-cultivated) *Medicago* species and root nodules. Naturalised populations of *M. sativa* and root nodules were also collected, yet avoiding roadsides, although the species is frequently present in this man-made habitat with many other *Medicago* species. More attention was oriented in sampling peculiar environmental sites (National Parks, Sites of European Concern *ex* Dir. n. 43/92/EEC "Habitat" *etc.*).

The Regional Soil Map (1:250,000 - USDA Soil Taxonomy classification) was used as the main reference while planning the field activities at landscape scale (ARU *et al.*, 1991) and allocating sampling sites. The Universal Transverse Mercator coordinates (according to the European Datum fixed in 1950) of the sites were quoted from the IGMI National Map (Italian Military Geographic Institute, scale 1:25,000) or measured by GPS (Global Positioning System) and then stored in a GIS (Geographical Information System) software as point features (Arc View, ESRI).

Plant, root nodule and soil collection

Each sample was composed of the whole plant (shoot and root system) and ca. 1 dm^3 of the soil surrounding the root zone, extracted after digging a circular hole around the plant, taking care not to collect when different species of *Medicago* or other legumes were growing in too close vicinity. After laboratory manipulation, the plants were stored as herbarium specimens. The old legume, often present and still attached to the root system of annual species, was a useful tool for

identification, especially when plant phenology was not advanced enough for accurate species identification. Taking differences due to altitude into account, the March-May period in Sardinia is generally good for collecting samples with vital rhizobia and advanced enough plant phenology for determination.

Isolation of root nodule bacteria

Microbiological assays concerned the isolation of bacteria from the root nodules of *Medicago* plants collected in the field and, subsequently, from root nodules of the *Medicago* axenic plants used for the nodulation test (AA.VV., 1986). Eight to ten intact nodules per plant were obtained by cutting the roots, previously cleaned under running water, 1-2 mm above the point of attachment of the nodule, with sterile blades. The nodules were surface sterilised by immersion in 95% ethanol solution for approximately 1 minute, then transferred to a 3% hydrogen peroxide solution for 2-4 minutes, depending on the dimension of the nodules, and then washed five times in distilled sterile water. The nodules were then crushed in 1 ml of sterile water using a flamed glass rod and various dilutions (1/10, 1/100 and 1/1,000) of the suspension were streaked on YMA (Yeast Mannitol Agar) plates (Vincent 1970) containing 0.01 g l^{-1} of Congo red, and incubated in the dark at 27 °C for 2-5 days, till colonies appeared. When necessary, the colonies were purified.

The bacterial cultures which showed typical rhizobial morphology, as described by Jordan in Bergey's Manual of Systematic Bacteriology (JORDAN, 1983), and little or no Congo red absorption on YMA, were stored at –80 °C in ultra-freezer after incubation in YM broth at 27 °C for 2 days, centrifugation at 4,000 rpm for 15 min, and suspension of cultures in Eppendorfs containing YM broth and 15% glycerol.

Nodulation tests

All isolates were tested before being included in the *Medicago*-nodulating rhizobial collection. Confirmation is dependent on demonstration of nodule forming ability on a test host legume under bacteriologically controlled conditions (JORDAN, 1983).

For this aim non-scarified seeds of *Medicago polymorpha* cv "Anglona" (a cultivar released by the National Research Council of Sassari and obtained from Sardinian germplasm collected in 1989) were surface sterilised in a 95% ethanol solution for 2-3 min and in a 3% hydrogen peroxide solution for 3 min. After five washes with sterile distilled water, the seeds were left to soak for half an hour, scarified with pincers and then left to germinate on water soaked blotting paper for about 2 days at 24 °C, in the dark.

The seedlings were then aseptically transferred in 15 x 2 cm glass tubes containing sterile plant nutrient media (NORRIS and DATE, 1976) with 6 g l^{-1} agar. The plants were grown for 8 days in daylight and then inoculated with 1 ml rhizobial suspension, obtained by incubating each strain in YM broth at 27 °C for 48 hours. Foil was used to protect the roots from direct sunlight. Plants were grown for one to two months, checking roots twice a week to evaluate nodulation.

Each test was carried out by inoculating 4 plants for each strain. Non-inoculated controls were also cultivated.

Soil analyses

The soil samples were stored at 4 °C before chemical analysis. The determination for each collection site of pH, total organic matter and humic fractions (fulvic acids, humic acids and non-humified compounds) was performed on soil samples collected at the root level.

After two days of air desiccation, each soil sample was sifted with a 2 mm diameter sieve. Determination of pH was carried out with the potentiometric method of soil suspensions, obtained by stirring 10 g of soil with 25 ml of distilled water in a beaker, after 24 hours settling. The pH was determinated on a Basic 20 Crison pH meter.

Total organic matter was determined using Walkley and Black's (1934) volumetric method. The humic fractions, *i.e.* fulvic acids

(FA), humic acids (HA) and non-humified compounds (NH) were determined after CIAVATTA *et al.* (1990). Humification index, HI = NH / (HA + FA), and the degree of humification DH (%) = [(HA + FA) / TEC]x100 were also determined.

RESULTS AND DISCUSSION

A total number of 70 (46 + 24) sites were taken into account, widely distributed in Sardinia as shown in Fig. 1 and Tab. 2. Forty-six sites were more detailed analysed, being the sites used for the *Medicago-Rhizobium* germplasm collection, while additional 24 sites were only surveyed to gather further data on species distribution without collecting samples (e.g. to preserve rare species such as *M. intertexta* that has been found only in one site).

Table two reports sites distribution according to Land Unit (soil), type of vegetation physiognomy or land-use, altitude, UTM coordinates referred to the European Datum (ED50). Sites are distributed over 18 different land units. These land units differ greatly in terms of soils, land uses, elevation, soil water balance. The pH range is 5.14 – 8.48, organic matter content range is 0.34 – 24.53, N range 0.02-1.23 (Tab. 3).

The distribution of the sites is plotted in Figure 1, with reference to the used coordinate system, collection sites are marked with a circle (o), while survey sites are marked with a cross (x).

Nodules were collected from 15 species (*M. arabica, M. ciliaris, M. italica, M. littoralis, M. lupulina, M. marina, M. minima, M. murex, M. orbicularis, M. polymorpha, M. praecox, M. rigidula, M. rugosa, M. sativa, M. truncatula*). Thirteen of the 15 sampled species are annu-

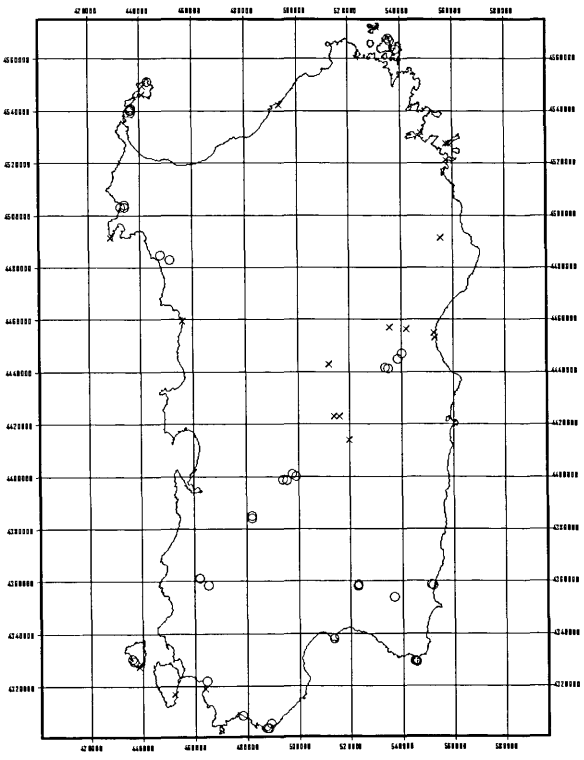

FIG. 1. – The distribution of the 46 collection (o) and 24 survey (x) sites in Sardinia, with reference to the used coordinate system (UTM ED50).

als. More precisely we sampled 14 native species out of the 21 native *Medicago* species (*M. arborea* L., *M. soleirolii* Duby and *M. sativa* L. subsp. *sativa* are considered aliens to Sardinian flora). Nodules were collected from the 100% of the perennial native species of Sardinia. We observed the presence of several nodules in all samples collected from March to May.

Although the microbiological data has yet to be collectively analysed, some qualitative trends can be identified. One hundred and twenty-five bacterial colonies with sinorhizobial morphology were isolated from plant nodules of different *Medicago* species sampled in the 46 collecting sites. Seventy-four percent of them were isolated from four plant species and precisely from *M. littoralis* (13.6%), *M. murex* (21.6%), *M. polymorpha* (23.2%) and *M. truncatula* (16%). Nodulation tests confirmed 29 strains as belonging to the *Sinorhizobium medicae* species, as they were capable of inducing nitrogen fixing nodules in the *Medicago polymorpha* host plants (Rome *et al.*, 1996) and they were included in the final microbial collection.

The relative high number of negative isolates may reflect the high presence in the nodules of saprophytic non-rhizobial bacteria, or the specifity of the symbiotic relationship between *S. medicae* strains and *M. polymorpha*. Moreover only one cultivar of the species *M. polymorpha* was used as host plant for the confirmation of isolates coming from such different taxa. The use in inoculation trials of *M. sativa* will definitely enable us to identify the *S. melioti* isolates and to compare the distribution of the two bacterial species.

Evaluating the collected data and considering that isolates which originate from such distant and diverse locations are unlikely to be genetically identical, the common ability of *S. medicae* strains to nodulate does not seem to be related to soil pH, pedological or climatic parameters and plant of origin. Nodulating strains weren't collected in soils with particular pH, organic content or altitude. In particular, strains extracted from *Medicago polymorpha* did not nodulate more than strains extracted from other species. This might confirm the strain specificity for *Medicago* subspeciespreviously reported by other Authors

The limited number of *S. medicae* isolates that responded positively to nodulation trials might be related to the difficulties that bacterial cultures experience in artificial conditions.

During the survey we collected useful information on the distribution of 17 *Medicago* species in Sardinia.

The collected data on *Medicago* distribution patterns confirm that many species of the genus easily adapt to different soil types (in terms of pH, organic matter content, soil water balance *etc.*) and land uses, and spread even in degraded habitats due to their greater adaptability, to the presence of spiny coils and to human intervention [e.g. *M. polymorpha* L., *M. arabica* (L.) Hudson, *M. truncatula* Gaertn.]. *Medicago polymorpha* has been found on pH range of 5.91-8.37, this is in accordance to its known tolerance to acid conditions in the nodulation phase (Ewing and Robson, 1990; Howieson and Ewing, 1986, 1989). It is the most abundant species in Sardinia with a distribution pattern similar to other Mediterranean countries and islands (Francis and Katznelson, 1977; Adem, 1989; Prosperi *et al.*, 1989; Abdelguerfi *et al.*, 1988). It is a common ruderal species and it grows in various habitats and land uses, but usually, prefers open spaces.

Even if very abundant and probably expanding their ranges near main island settlements, road network and agricultural areas, *M. polymorpha*, *M. truncatula* and *M. arabica* can be considered as patchily distributed at different scales. This is in good accord with the patchy nature of the different habitats that are present in Sardinia, with the presence of barriers, diverse frequency and intensity of grazing pressure, patchy and unpredictable disturbances both natural and anthropogenic (e.g. fire regimes), traditional land uses, climatic variability.

TABLE 2. – *List of collection (c) and surveying (o) sites in Sardinia.*

N.	Soil	Vegetation phisiognomy / Land Use	O/C	Altitude (m a.s.l.)		UTM-E	UTM-N	Tot n. Taxa
1	D15	Rangeland with sparse shrubs	O/C	350	32T	447580	4484520	5
2	D15	Rangeland with sparse trees of cork oak	O/C	450	32T	451160	4482830	2
3	G23	Herbaceous rangeland	O/C	90	32S	481950	4384900	2
4	G23	Rangeland with sparse shrubs	O/C	95	32S	482050	4384000	1
5	M33	Sand dunes	O/C	10	32S	544800	4329500	1
6	C9	Coastal shrubland	O/C	40	32S	545200	4329250	1
7	I26	Abandoned field	O/C	10	32S	544700	4329400	4
8	A1	Coastal garigue	O/C	55	32S	513500	4338000	2
9	C10	Clearings inside wood	O/C	800	32S	523100	4358100	3
10	C10	Clearings inside wood	O/C	800	32S	523150	4358150	1
11	C10	Clearings inside wood	O/C	800	32S	523200	4358600	1
12	C10	Clearings inside wood	O/C	800	32S	523200	4358400	1
13	C10	Grassland at riparian site	O/C	450	32S	537100	4353900	1
14	C10	Grassland at riparian site	O/C	450	32S	537150	4353950	0
15	I26	Retrodunal grassland	O/C	5	32S	551400	4358600	1
16	M33	Sand dunes	O/C	2	32S	551800	4358200	2
17	E19	Rangeland with sparse trees and bushes	O/C	530	32S	497800	4400800	3
18	E19	Rangeland with sparse trees and bushes	O/C	530	32S	499050	4399950	2
19	G22	Rangeland with sparse leaf shedder oaks	O/C	250	32S	494200	4398800	2
20	G22	Rangeland with sparse leaf shedder oaks	O/C	250	32S	495600	4398500	3
21	M33	Coastal shrubland	O/C	20	32T	536500	4566700	3
22	C8	Coastal shrubland	O/C	20	32T	536400	4566600	2
23	C8	Coastal shrubland	O/C	20	32T	535800	4567750	1
24	C8	Coastal garigue	O/C	35	32T	538500	4563500	1
25	M33	Stabilised sand dunes	O/C	2	32S	487900	4303800	2
26	M33	Stabilised sand dunes	O/C	2	32S	487900	4303850	3
27	M33	Retrodunal grassland	O/C	5	32S	487400	4303950	4
28	C9	Coastal garigue	O/C	5	32S	487350	4303900	3
29	I28	Abandoned field	O/C	4	32S	489000	4305500	3
30	B4	Coatal shrubland	O/C	100	32S	478200	4308800	4
31	N34	Halophilous grassland	O/C	4	32S	464500	4322000	1
32	M33	Sand dunes	O/C	5	32S	435900	4330250	2
33	D15	Coastal garigue	O/C	8	32S	436500	4329500	4
34	B4	Holm oak wood at riparian site	O/C	340	32S	465600	4358300	3
35	Mine Pool	Mine sedimentation pool	O/C	450	32S	462200	4361150	1
36	A1	Coastal garigue	O/C	25	32T	432600	4502950	3
37	I27	Clearings inside Pinus wood	O/C	30	32T	434100	4502900	4
38	M33	Grassland at lake banks	O/C	45	32T	434200	4503800	4
39	A1	Garigue and rock outcrops	O/C	1300	32T	535400	4441150	4
40	B4	Holm oak wood	O/C	916	32T	534100	4441500	2
41	A1	Garigue	O/C	950	32T	539000	4444800	3
42	A1	Grassland at archeological site	O/C	835	32T	540400	4447000	1
43	M33	Sand dunes	O/C	5	32T	436900	4540700	2
44	M33	Stabilised sand dunes	O/C	2	32T	436800	4540750	4
45	C9	Coastal shrubland	O/C	2	32T	436800	4540200	2
46	C9	Coastal shrubland	O/C	2	32T	436600	4539800	3
47	M33	Sand dunes	O/C	2	32T	443100	4551000	1
48	B4	Clearings in holm oak wood	O	8	32T	441600	4549500	3
49	B4	Halophilous grassland	O	10	32T	441200	4546200	1
50	A2	River bed	O	2	32T	553200	4453100	1
51	A1	Coastal shrubland	O	50	32T	553000	4455000	2
52	B5	Clearings in holm oak wood	O	900	32S	516400	4423150	3
53	M33	Sand dunes	O	3	32T	557830	4527420	2

N.	Soil	Vegetation phisiognomy / Land Use	O/C	Altitude (m a.s.l.)		UTM-E	UTM-N	Tot n. Taxa
54	C8	Coastal shrubland	O	50	32T	558550	4527460	4
55	D15	Coastal shrubland	O	50	32T	456460	4459400	1
56	A1	Garigue	O	1000	32T	536000	4457000	8
57	A1	Garigue	O	200	32T	542500	4456500	5
58	I26	Sand dunes	O	10	32T	557780	4521140	2
59	A1	Coastal garigue	O	80	32T	429050	4491500	4
60	C8	River bed	O	30	32T	493600	4542300	6
61	A1	Garigue	O	1050	32T	514400	4423100	6
62	D16	Coastal garigue	O	8	32S	438984	4327798	1
63	N34	Coastal shrubland	O	15	32S	439500	4328900	1
64	M33	Sand dunes	O	2	32S	452338	4317037	1
65	N34	Halophilous grassland	O	10	32S	464200	4319100	2
66	C10	Rangeland with sparse bushes	O	500	32T	512600	4442900	4
67	C9	Coastal shrubland	O	15	32T	548300	4531750	3
68	A1	Garigue	O	900	32T	555750	4491300	4
69	B3	Coastal shrubland	O	10	32T	531500	4572150	1
70	B4	Garigue	O	750	32T	520050	4414000	1

M. arabica is often associated with *M. polymorpha*, yet it seems to prefer grassy, moist places and relatively more shaded unlike most other *Medicago* species, but, as already remarked, it is found in a variety of habitats and land uses.

On the contrary, other species are greatly site/habitat dependent and, consequently, it not expanding but narrowing their range, being more threatened by anthropisation and habitat fragmentation/degradation: e.g. *M. marina* L. (rare), *M. intertexta* (L.) Miller (very rare), *M. ciliaris* (L.) All. (locally frequent) and *M. rugosa* Desr. (locally frequent).

M. marina L. grows exclusively on seashores, very close to the sea, usually in loose sand with pH > 7.5 and it is the only *Medicago* of such habitat requirement, though seashore soils of more solid consistency and lower pH may be inhabited by other species, such as *M. littoralis* (common) and *M. italica* (rare). *M. marina* therefore endangered by tourism, cleaning of seashores, competition with exotic invasive species often used as ornamental along the coast (e.g. *Carpobrotus* sp. pl., *Cortaderia* sp. pl), trampling and, sometimes, even by grazing.

M. lupulina prefers moister soils and cooler temperatures than the expressly annual *Medicago* species; in warmer arid sites within the region (e.g. Asinara island in the Sardinian North-West coast) it occurs at higher altitudes where moisture and temperature are more suitable for it. It has always been found in clearings in holm oak (*Quercus ilex* L.) woods or fissured limestone and dolomite Mesozoic rock outcrops, archaeological sites. Seems to be usually annual and only biennial to perennial (rare) at higher altitudes, *i.e.* above 1,000 m a.s.l.

M. rugosa requires heavy clay soils with pH > 7 and this may be partly responsible for its sporadic occurrence. Also spineless pods with only a few seeds may not be advantageous for a wider distribution on the island and in rangelands.

M. ciliaris is found mainly in heavy loamy soils (pH 7-8), in most soils and only at low altitudes (< 300 m a.s.l.) and in the warmer sites. It may be common at the borders of wetlands and it is therefore threatened by habitat fragmentation and soil reclamation for agricultural purposes.

M. sativa is a very common ruderal species in Sardinia, even if patchy distributed, and it is mainly present along road network. It may be regarded as an archeophyte and the processes of naturalisation and expansion in habitats different from the roadside network are locally present but very rare. On the contrary its perennial habit, the high pod and

TABLE 3. –

N.	Soil	pH	SO	N
1	D15	7.10	2.06	0.10
2	D15	6.15	10.94	0.55
3	G23	7.00	1.91	0.09
4	G23	6.78	5.57	0.28
5	M33	7.74	3.08	0.15
6	C9	8.48	0.62	0.03
7	I26	7.64	1.69	0.09
8	A1	8.28	3.24	0.16
9	C10	6.69	6.68	0.33
10	C10	8.05	0.79	0.04
11	C10	6.10	3.87	0.19
12	C10	8.01	1.32	0.07
13	C10	6.55	1.90	0.10
14	C10	6.25	6.51	0.33
15	I26	7.91	0.34	0.02
16	M33	7.32	0.51	0.03
17	E19	5.91	10.89	0.54
18	E19	6.41	22.01	1.10
19	G22	6.07	9.40	0.47
20	G22	7.40	3.41	0.17
21	M33	7.70	0.85	0.04
22	C8	6.43	5.55	0.28
23	C8	7.33	11.64	0.58
24	C8	5.92	24.53	1,23
25	M33	7.98	0.65	0.03
26	M33	7.67	1.94	0.10
27	M33	6.93	0.51	0.03
28	C9	6.46	4.54	0.23
29	I28	8.37	0.82	0.04
30	B4	7.00	4.38	0.22
31	N34	7.59	3.89	0.19
32	M33	8.39	0.54	0.02
33	D15	6.77	3.70	0.18
34	B4	7.89	1.23	0.06
35	Mine Pool	7.49	2.49	0.12
36	A1	7.93	2.87	0.15
37	I27	8.23	0.52	0.03
38	M33	7.90	1.01	0.05
39	A1	7.58	8.13	0.41
40	B4	6.25	8.36	0.42
41	A1	7.21	7.98	0.40
42	A1	7.15	4.35	0.23
43	M33	8.10	0.50	0.03
44	M33	7.67	3.55	0.18
45	C9	5.14	9.45	0.47
46	C9	5.39	1.07	0.05

seed production, the propagule pressure due to agricultural uses as forage species and the tolerance to mowing and grazing give this species the possibility to persist along roadsides. This tolerance increases the competition ability in comparison to other species that are less resistant to the periodical interventions of road cleanings (normally performed by cutting-mowing or with controlled fire, that are normally required by law during summer as wild fires prevention method).

M. minima and *M. praecox* grow mainly in dry soils, *M. praecox* seems to be less frequent and to grow only at lower altitudes and higher pH values in comparison to *M. minima*.

M. orbucularis, *M. murex* and *M. rigidula* distribution in Sardinia can be described as species locally common. *M. murex* seem to be more adapted to very different soils, while *M. orbicularis* prefers lower altitudes and pH values in comparison to *M. rigidula*.

M. turbinata has been observed only at Monte Albo (site n. 68 in Table 2) were it was also recorded in the past by CAMARDA (1984) as a common species. Although not recorded in other sites during the survey, it might be present in similar limestone habitats in Sardinia (and landscape units), that are indeed quite rare and difficult to scout, requiring really time consuming surveys by foot.

M. intertexta is described (LESINS and LESINS 1979) as a species that grows mainly in heavy moist soils, in more mesic fertile habitats in comparison to the other annual medics. During the survey it has been found in only one site (site n. 55, Table 2), that is really too little information about habitat requirements. In any case the Sardinian site fits the above description being localised in the D15 Land Unit (Landscapes on acid effusive rocks, with Lithic Xerorthents soils) along the West coastline.

M. doliata has not been found during the present survey although there are some historical records, e.g; CAMARDA *et al.* (1993) for the South of Sardinia, where it is indicated as locally common in a local florulas. The other species that have not be collected or observed during the survey are *M. arborea*, *M. scutellata*, *M. tenoreana*, *M. lesinsii*, *M. rotata*, *M. soleirolii*. Apart from *M. arborea* and *M. rotata* that are undoubtedly exotic to Sardinian flora, the presence of the other species or their historical records must be very careful analysed, e.g. *M. soleirolii* can be easily con-

fused with *M. italica* and some records of *M. scutellata* might be related to very variable specimens of *M. orbicularis*.

We would remark that many species, such as *M. polymorpha, M. minima, M. arabica, M. truncatula, M. littoralis*, show a high degree of morphological and phenological diversity, which might confirm and be related to different performances described in literature in terms of phytomass and seed production, root systems, suitability for and resilience to grazing and browsing, content of medicagenic acid glycosides (JURZYSTA *et al.*, 1988), hardseedness (PORQUEDDU *et al.*, 1996), heteroblastic development (DAMERVAL and CHAKASS, 1985), capability of soil restoration from heavy metals. For many of the above species it was possible, during the survey, to recognise most of the different forms indicated by HEYN (1963, 1970) and URBAN (1873), e.g. for *M. minima* on the basis of length of the spines, shape of the pods, plant hairiness, for *M. polymorpha* and *M. littoralis*. Also the presence/absence and size of anthocyanin-colored patch and white flecks in the leaves may be quite variable.

Mountainous limestone-dolomite (Paleozoic-Mesozoic) areas can be regarded, in narrower sense, as hot-spot areas *sensu* MYERS (1986; 1989) inside the Mediterranean global hot-spot area (MITTERMEIER *et al.*, 1999), with regard to medics distribution in Sardinia (A1, A2 in Table 2). In these areas there is normally the highest *Medicago* species richness and they face exceptional degrees of threat due to overgrazing, fires, soil erosion. The soils present in these landscape units, and often patchily distributed with rock outcrops, are Lithic and Typic Xerorthents, Lithic and Typic Rhodoxeralfs, Lithic and Typic Xerocrepts. Soils may be very variable in depth and normally neutral, saturated. A similar situation, even if with a lower species richness can be remarked for sandy seashores, wetlands, dry coastal areas and Sardinian islets, rangelands with sparse trees on acid effusive rocks land units.

The general variability of the materials examined, the adaptation to a wide range of different habitats, the high habitat selectivity of some species, must be a warning that the introduction of exotic *Medicago-Sinorhizobium* germplasm in rangeland ecosystems or other semi-natural habitats, may lead to a harmful reduction of total biodiversity. Interventions for rangeland improvement should always be carefully assessed and performed using native materials, above all when they are planned in sensitive environmental areas such as Natural Parks or Sites of European Concern (*ex* Dir. n. 92/43/EEC "Habitat") or *Medicago* hot-spot areas. The use of selected competitive autochthonous materials, rather than genetically modified organisms is desirable.

There can be genetic exchange between rhizobial strains in soil, as demonstrated by the identification of strain recombinants of *R. leguminosarum* biovar. *trifolii* nodulating *T. ambiguum* and *T. repens* usually (ELLIOT *et al.* ,1996). *Rhizobium* symbiotic DNA located on a Sym plasmid (VAN RHIJN and VANDERLEYDEN, 1995), can be transferred between strains or even be lost, so that non-nodulating phenotypes may appear (HARRISON *et al.*, 1988). Besides, recombination of chromosomal DNA may occur between closely related bacteria strains (RONSON, 1995). Genetic exchange of symbiotic DNA in the rhizobial soil population may suggest the problematic introduction of selected, non-indigenous strains in an environment containing indigenous rhizobia.

On the other hand, the recombination between indigenous and inoculated strains may lessen the effectiveness and persistence of the introduced rhizobial populations.

The conservation of plant and microbial genetic resources is one of the main objectives of the Reg. n. 94/1467/EU. Its first article stresses the importance of genetic plant resources and in particular forage plants, plants belonging to native flora with possible farming applications, and micro-organisms. The co-ordination and promotion of collecting activities, conservation, evaluation

and utilisation of plant and microbial genetic resources represent an essential aid in achieving the main priorities of European Agricultural Policies.

Agriculture in relation to biodiversity is a critical issue, since it is the largest single human activity impacting on global diversity. Modernisation of agricultural practices over the past few decades has played a significant role in the rapid decline in biodiversity.

This study adds some useful ecological information on most of the annual and perennial species of the genus *Medicago* in Sardinian and the symbiotic rhizobia and constitutes the starting point for a more detailed analysis of the biodiversity and physiological characteristics of the strains included in the final *Sinorhizobium* collection and of *Medicago-Sinorhizobium* symbiosis in Sardinia.

There is yet limited information and more accurate surveys are required for some species and for some part of the island (e.g. limestone areas, coastal areas, small islets). Future monitoring and geo-coding of historical data and herbarium samples will give a great help in the study of the genus distribution in the island of Sardinia. Monitoring provides data that lead to better understanding of long-term changes, which in turn contributes to more effective management of endangered species.

The data might be updated and improved in the future being stored in GIS format.

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