

Little effect of ecological factors and symbiotic specificity on the distribution of *Medicago* subsect. *Intertextae* (Urban) Heyn (Fabales Fabaceae) in the Mediterranean Basin

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ABSTRACT

In the Mediterranean region, *Medicago granadensis* Willd. and *M. muricoleptis* Tineo (Fabales Fabaceae) of the *Intertextae* subsection are rare and present, each one in well defined areas, whereas the others taxa, in the same subsection, *M. ciliaris* (L.) All. and *M. intertexta* (L.) Mill., are more frequent with large distribution. This kind of geographical distribution is not a coincidence; certainly different factors are the origin of this distribution. In 125 sites of different Mediterranean countries, the ecology of the four taxa was studied, 34 trapped strains in one site of *Intertextae* rhizobiums were characterized by rep-PCR and some symbiotic tests were carried out. The results confirm that *M. muricoleptis* and *M. granadensis* are endemic in the north and east of the Mediterranean, respectively; whereas, *M. ciliaris* and *M. intertexta* are widespread. Although their geographic specificity, the four taxa showed no important differences in ecological conditions. *M. muricoleptis*, given its presence in the north of the Mediterranean only, is distinguished by its precipitation requirement. By inference, the absence of *M. muricoleptis* and *M. granadensis* in some regions is not due to the absence of the symbiont. We confirm the symbiotic specificity of *Sinorhizobium medicae* (Rome, 1996) for the *Intertextae* subsection and the existence of coevolution between taxa (widespread and endemic) and rhizobia. We suppose that endemic taxa are in the process of allopatric speciation, which explains their narrow distribution in the Mediterranean Basin.

KEY WORDS

Distribution; diversity; ecology; *Intertextae*; *Medicago*; Mediterranean; symbiosis.

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INTRODUCTION

Legumes hold an important economic and social place and have a definite environmental benefit in the Mediterranean Basin, which is the area of distribution of several genera of this family, including the *Medicago* L. genus. This genus includes 86 species (Small & Jomphe, 1989) and comprises several sections and subsections, including the *Intertextae* (Urban) Heyn subsection (Small, 2011; Coulot & Rabaute, 2013). Some taxa of the *Inter-*

textae show a morphological ambiguity, which reflected in different classifications. Indeed, some authors ascribe to the subsection 4 species, namely, *Medicago ciliaris* (L.) Krock., *M. intertexta* (L.) Mill., *M. muricoleptis* Tineo and *M. granadensis* Willd. (Quézel & Santa, 1962; Lesins & Lesins, 1979; Small & Jomphe, 1989; Coulot & Rabaute, 2013) and others mention only 3 species, *M. intertexta*, *M. muricoleptis* and *M. granadensis* (Heyn, 1963; Ponert, 1973; Jauzein, 1995; Dobignard & Chatelain, 2012; Tison et al., 2014). According to

the latter authors *M. ciliaris* is either a subspecies, *M. intertexta* subsp. *ciliaris* (L.), Ponert, or a botanical variety [*M. intertexta* var. *ciliaris* (L.) Camb, Heyn] of *M. intertexta*.

The early observations on the distribution of annual *Medicago* in relation with soil factors were made by Trumble & Donald (1938) and Aitken & Davidson (1954). Besides, the first work on autoecology was carried out in Australia by Andrew & Hely (1960). Thereafter, several studies were performed, particularly in the regions of origins of *Medicago*, which allowed the definition of the ecological requirements of various species (Abdelguerfi et al., 1988; Ehrman & Cocks, 1988; Prospero et al., 1989; Ehrman & Cocks, 1990; Abdelkefi et al., 1992; Bounejmate, 1992; Bounejmate et al., 1992a; 1992b).

For the most common taxa of the *Intertextae* subsection, *M. ciliaris* and *M. intertexta*, autoecology research was carried out, but most of that research did not allow comparisons between the two taxa since they were included in a single species as indicated by Heyn (1963). The most specific study on these two taxa was carried out in Algeria (Abdelguerfi-Laouar et al., 2003) on 179 sites. Besides, *M. muricoleptis* and *M. granadensis* are poorly distributed and are reported in the literature as rare taxa. Although they belong to the same subsection as *M. ciliaris* and *M. intertexta*, they are not found in Algeria, Morocco, and Tunisia. The ecological requirements of the two taxa are not yet determined; they have only been reported in various regions (Table 1).

Other factors, besides ecological ones, symbiosis for example, may affect the distribution of legumes species. The species of rhizobia that nodulate the *Medicago* ssp. belong to the *Ensifer* (formerly *Sinorhizobium*) genus and are *S. meliloti* (Dangeart, 1926) and *S. medicae* (Rome, 1996). One of the major characteristics of the rhizobium-legume association is their host specificity. In general, a given rhizobium species can only establish an efficient symbiotic relationship with a limited number of plant partners. Similarly, legumes species can only be specific to a certain number of rhizobium species. This coevolution is often ignored in studies on the distribution and evolution of legumes taxa. The *Intertextae* subsection taxa were described as specific to *S. medicae* (Béna et al., 1998; 2005). Béna et al. (1998) suggest, for this

subsection, a recurrent loss of the capacity to form an efficient symbiosis with strains of the *S. meliloti* species; they hypothesize a punctual mutation inducing a modification of the mechanisms of recognition such as the flavonoid structure.

The rather particular geographical distribution of the four *Intertextae* subsection taxa, namely those with a wide distribution and those with a narrow distribution, needs clarification as to whether this is due to an ecological adaptation and/or to a specific taxon-rhizobium interactions; this is the objectives of this work.

MATERIAL AND METHODS

Given the taxonomic ambiguity that exists within the subsection of the *Intertextae*, we will use the term taxon instead of species for *M. ciliaris*; synonym: *M. intertexta* subsp. *ciliaris* and *M. intertexta* var. *ciliaris*, *M. intertexta*, *M. muricoleptis* and *M. granadensis* throughout the document.

Ecological data and origin of sites

The study is focused on taxa *M. ciliaris*, *M. intertexta*, *M. muricoleptis* and *M. granadensis*. The collected ecological data come from the Australian *Medicago* Genetic Resource Centre (South Australian Research and Development Institute: SARDI), a total number of 125 sites were surveyed in 17 Mediterranean countries and islands (Table 2). 13 sites of 125 are sympatric (sci and sig), one of which is sympatric to *M. intertexta* and *M. granadensis* (sig; Israel) and the other (12) are for *M. ciliaris* and *M. intertexta* (sci). All the other sites are allopatric (sc, si, sm and sg) and correspond only to a species per site among the four (Table 3).

Ecological data analysis

Nine (9) ecological characters were studied; they correspond to orographic (altitude and slope), climatic (annual rainfall) and edaphic characters (pH, soil texture, soil depth, soil reaction, type of drainage and land use). Analyses of variance (ANOVA) with a single factor (sites) were carried out on 76 sites whose data on rainfall, altitude, and pH were available; the 'site' factor was considered as random effect. The software used is STAT-ITCF

Species	Regions	References
<i>M. ciliaris</i> (L.) All.	- Macaronesia Madeira Island (Portugal), Canary Island (Spain)	Coulot & Rabaute, 2013 Davis, 1965-1988
	- North Africa Algeria, Egypt, Morocco, Tunisia	Dobignard & Chatelain, 2012
	- Western Asia Cyprus, Iraq, Israel, Lebanon, Western Syria, Western Turkey	Heyn, 1963 Jahandiez & Maire, 1931-1941
	- South-Eastern Europe Greece (including Crete), Italy (including Sardinia and Sicily)	Lesins & Lesins, 1979 Meikle, 1977-1985
	- South-Western Europe France (including Corsica), Portugal and Spain (including the Balearic Islands)	Mouterde, 1966 Pignatti, 1982
	- Macaronesia Canary Island (Spain)	Pottier-Alapetite, 1979-1981
	- North Africa Northern Algeria, Morocco, Tunisia	Quézel & Santa, 1962
	- South-Eastern Europe Greece, Italy (including Sardinia and Sicily)	Small & Jomphe, 1989 Small, 1981
<i>M. intertexta</i> (L.) Mill.	- South-Western Europe France (including Corsica), Portugal and the South of Spain	Small et al., 1981 Smythies, 1984-1986
	- North Africa Northern Egypt	Täckholm, 1974
	- Western Asia Israel, Lebanon, Jordan, Western Syria, Turkey	Townsend & Guest, 1966 Tutin et al., 1964-1980
	- South-Eastern Europe Southern Italy (including Sicily)	Zohary & Feinbrun-Dothan, 1966
<i>M. muricoleptis</i> Tineo	- South-Western Europe Southeast of the France near Toulon	GRIN, 2014

Table 1. Geographical distribution of the *Intertextae* subsection taxa.

(Gouet & Philippeau, 2002). For each quantitative variable, two analyses of variance were carried out; the first analysis comprised 4 levels corresponding to the presence of the taxon: (i) site of *M. ciliaris* (sc+sci), (ii) site of *M. intertexta* (si+sci), (iii) site of *M. muricoleptis* (sm) and (iv) site of *M. granadensis* (sg+sig); the second analysis (5 levels) differed from the first by the distinction of allopatric sites (12) from sympatric sites

for *M. ciliaris* and *M. intertexta*. For only those where the null hypothesis is rejected a Fisher's LSD test was calculated. The hypothesis is null when the F test (MSA/MSE) of ANOVA is greater than 1 ($p < 0.05$).

For the qualitative variables, environmental profiles were established for each taxon having more than five sites; the classes of each variable are represented in Table 4.

<i>M. ciliaris</i> : Algeria: 5.776 - 10.464 - 10.465 - 10.642 - 10.643 - 10.644 - 10.645 - 10.648 - 10.792 - 10.932- 10.933 - 10.934 - 10.935 - 10.936 - 11.571 - 11.574 - 11.575 - 11.576 - 12.547 - 12.551. Crete : 2.081. Cyprus : 23.627 - 23.629 - 23.635 - 23.636. Greece : 2.239. Israel : 2.004 - 7.687 - 7.688. Italy : 2.075 - 5.782 - 22.419 - 22.420. Morocco : 25.506 - 25.506 - 29.024. Portugal : 7.826 - 24.554 - Sardinia 26.016. Sicily : 2.067 - 2.186 -24.831 -24.844 - 24.847 - 24.849 - 24.852 - 24.853. Spain : 7.721 - 28.255 - 28.256. Syria : 22.206 - 24.022 - 24.023. Tunisia : 5.785 - 5.786 - 7.714 - 7.723 -10.637 - 18.460 - 18.463 - 18.464 - 20.104 - 21.802 - 21.803 - 21.804. Turkey : 23.932.
<i>M. intertexta</i> : Algeria: 4.010 - 10.647 - 10.649 - 10.650 - 10.651 - 10.652 - 10.659 - 10.788 - 10.791 - 11.579 - 11.580 - 11.909 - 11.910 - 11.911 - 11.912 - 15.748. Cyprus : 2.367 - 2.370 - 5.777. Egypt : 13.797 - 13.799. France : 2.044 - 2.045. Israel : 1.589 - 2.363 - 2.364 - 2.366 - 5.779. Italy : 2.071 - 2.375. Jordan : 18.084 - 19.002. Morocco : 10.662. Portugal : 2.372 - 2,377 - 5.783 - 8,323. Sardinia : 12.302 - 26.021. Sicily : 24.816 - 24.819 - 24.821 - 24.823 - 24.824. Spain : 1.590 - 2.361 - 2.362 - 28.257. Syria : 13.795 - 13.796. Tunisia : 4.008 - 4.011 - 5.489 - 7.724 - 18.465 - 18.466.
<i>M. granadensis</i> : Israel : 2.359 - 5.778 - 5.822. Syria : 13.818 - 13.819 - 22.207. Turkey : 23.928 - 23.931 - 26.380.
<i>M. muricoleptis</i> : Greece : 22.423. Italy : 15.343 - 22.422. Sicily : 24.825 - 25.026 - 25.027 - 25.002.

Table 2. Collection of SARDI.

Country	Allopatric sites				Sympatric sites		Total
	<i>M. ciliaris</i> (sc)	<i>M. intertexta</i> (si)	<i>M. muricoleptis</i> (sm)	<i>M. granadensis</i> (sg)	<i>M. ciliaris</i> - <i>M. intertexta</i> (sci)	<i>M. intertexta</i> - <i>M. granadensis</i> (sig)	
Algeria	15	11	-	-	5	-	31
Crete	1	-	-	-	-	-	1
Cyprus	4	3	-	-	-	-	7
Egypt	-	2	-	-	-	-	2
Spain	2	3	-	-	1	-	6
France	-	2	-	-	-	-	2
Greece	1	-	1	-	-	-	2
Israel	3	4	-	2	-	1	10
Italy	4	2	2	-	-	-	8
Jordan	-	2	-	-	-	-	2
Morocco	3	1	-	-	-	-	4
Portugal	1	3	-	-	1	-	5
Sardinia	1	2	-	-	-	-	3
Sicily	5	2	4	-	3	-	14
Syria	3	2	-	3	-	-	8
Tunisia	10	4	-	-	2	-	16
Turkey	1	-	-	3	-	-	4
Total	54	43	7	8	12	1	125

Table 3. Number of allopatric and sympatric sites by country.

Collection of rhizobium strains

In a homogeneous site in Algiers (sub-humid, soil with loamy texture and slightly alkaline pH), 8 populations, two by taxon, were planted. The populations of *M. granadensis* and *M. muricoleptis* originate from the Mediterranean collection (SARDI) and those of *M. ciliaris* and *M. intertexta* originate from an Algerian collection (ENSA d'Alger). Populations of *M. ciliaris* (C58) and *M. intertexta* (158) from a sympatric site were taken into account. On the generated seedlings, nodules were collected from which a collection of 34 strains of rhizobia was obtained (Table 5). Bacterial isolates were obtained using the crushed-nodule method (Vincent, 1970) from nodules removed from 60 days old plants. The various strains were stored in glycerol at -80 °C, after having been identified by a symbiotic test on *M. polymorpha*, in addition to the analysis of 16S rRNA sequences (Rome et al., 1996).

Extraction of DNA, Amplification by REP-PCR and method of analysis

The DNA extraction was conducted following the protocol of Abdelguerfi-Laouar (2005). The rep-PCR and BOX-PCR was completed according to the protocol of Rademaker et al. (1998). A primer for BOX-PCR (BOX A1R: 5'-CTACGGCAAGGCGACGCTGACG-3') and two primers for REP-PCR (REP 1R: 5'-IIICGICGICATCIGGC-3' and REP 2I: 5'-ICGICTTATCIGGCCTAC-3') were used (Versalovic et al., 1994). The visualization of bands was made by UV on long agarose gels at 1.5 % (0.5X TBE) at 90 V voltage.

The binary data were established on the basis of the presence (1) absence (0) of bands of REP and BOX markers. From these data, a tree of genetic distance was obtained by groupings of plant from a comprehensive comparison of characters, then by calculating the mean distances of grouped plants (NJ method: Neighbour Joining). Support for clustering was determined by bootstrap procedure applied on binary REP-BOX data (1000 replications). The software used is Dissimilarity Analysis and Representation for Windows (DARwin) (Perrier et al., 2003; Perrier & Jacquemoud-Collet, 2006).

Symbiotic Test

The two species *S. meliloti* and *S. medicae* represented by a 2011 strain and two ABS7 strains (Bekki et al., 1987) and USDA1827, respectively, were inoculated on the populations of the four taxa of the *Intertextae* subsection. For symbiotic tests we kept the same host populations that served to obtain the collection of rhizobium. The seeds were disinfected with sulphuric acid for 30 minutes and then germinated after flushing. After germination, each seed was transferred, in sterile conditions, in tubes containing the FAHRAEUS agar medium (1.5 %) and placed in a culture chamber (20 °C, 60 % relative humidity and a photoperiod of 16 h light and 8 h darkness). The inoculation was carried out after 48 h of development of the roots (0.3 ml/seedling). The notations begin after a week and end after 90 days. The experimental protocol used for the symbiotic tests is total randomization with three plants per population.

RESULTS

Frequencies and geographical distribution of taxa

The most frequent taxon of the *Intertextae* subsection in the Mediterranean region is *M. ciliaris* with a frequency of 47.8 % followed by *M. intertexta* (40.6 %) and, finally, *M. granadensis* (6.5 %) and *M. muricoleptis* (5.1 %). Of 17 Mediterranean countries and islands, Algeria has the highest frequency for *M. ciliaris* and *M. intertexta*. From figure 1, which represents the distribution of 121 sites (longitude and latitude data available), *M. muricoleptis* and *M. granadensis* are confined in two restricted and different regions: the North and the East of the Mediterranean, respectively. These two taxa are included in the area of distribution of *M. ciliaris* and *M. intertexta*.

By comparing the presence of taxa by country between the bibliographic data (Table 1) and the collection studied (Table 2, Fig. 1) similarities and differences can be noted. In the Collection SARDI, *M. ciliaris* was not collected in Egypt, Iraq, France, and Lebanon; the same is true for *M. intertexta* in Crete, Greece and Turkey. *M. muricoleptis* was not collected in France and *M. granadensis* was not col-

lected in Egypt, Jordan, and Lebanon. Contrarily to the literature, *M. ciliaris* and *M. intertexta* were collected in Israel; *M. intertexta* was collected in Egypt, Cyprus, and Jordan; these two taxa therefore exist in these countries.

Inter-taxa ecological variability

Rainfall, altitude and pH

The comparison among *M. ciliaris*, *M. intertexta*, *M. muricoleptis* and *M. granadensis* shows that, with regard to altitude and pH, few differences exist (Table 6). Only rainfall allows differentiating between the sites of *M. ciliaris*, *M. intertexta*, sympatric sites (*M. ciliaris* and *M. intertexta*) (Table 7), *M. granadensis* and the sites of *M. muricoleptis*. This latter taxon prefers the highest rainfalls that exceed 800 mm. *M. granadensis* has the lowest rainfall average of all the subsection.

To have an idea about the limits of adaptation of each taxon, minimum and maximum values of each character and each taxon were taken into considerations. *M. intertexta* shows very broad adaptation for its precipitation needs; it shows the biggest amplitude with 850 mm (Fig 2). It manages

to grow in dry conditions (150 mm, a single site in Jordan) as well as in very wet conditions (1000 mm), but given the average for this character, it is more frequent in the rainy areas. *M. granadensis* and *M. ciliaris* are the least demanding in terms of precipitation. *M. muricoleptis* is water demanding as it is only found in sites with more than 700 mm of rainfall (Fig 2). In altitude, minimum and maximum values are very close in the four taxa; they vary from 5 to 10 m for low altitudes and from 900 to 1000 m for higher values. Sympatric sites of *M. ciliaris* and *M. intertexta* show an average rainfall closer to the average for *M. intertexta* than to the average for *M. ciliaris*.

Edaphic characters (Texture, drainage, depth of soil and soil reaction)

The soil textures in the sites of the four taxa are fine; they are clayey or clay-loam. The loamy texture is specific only to *M. ciliaris*, *M. intertexta* and *M. granadensis* (Fig. 3). The *Intertextae* taxa are found both in soils with good water drainage and soils where water stagnates on the surface. Only *M. intertexta* shows 100%, in relative frequency, for the class of submerged soil (waterlogged) (Fig. 4) it is the only taxon that was not mentioned in the

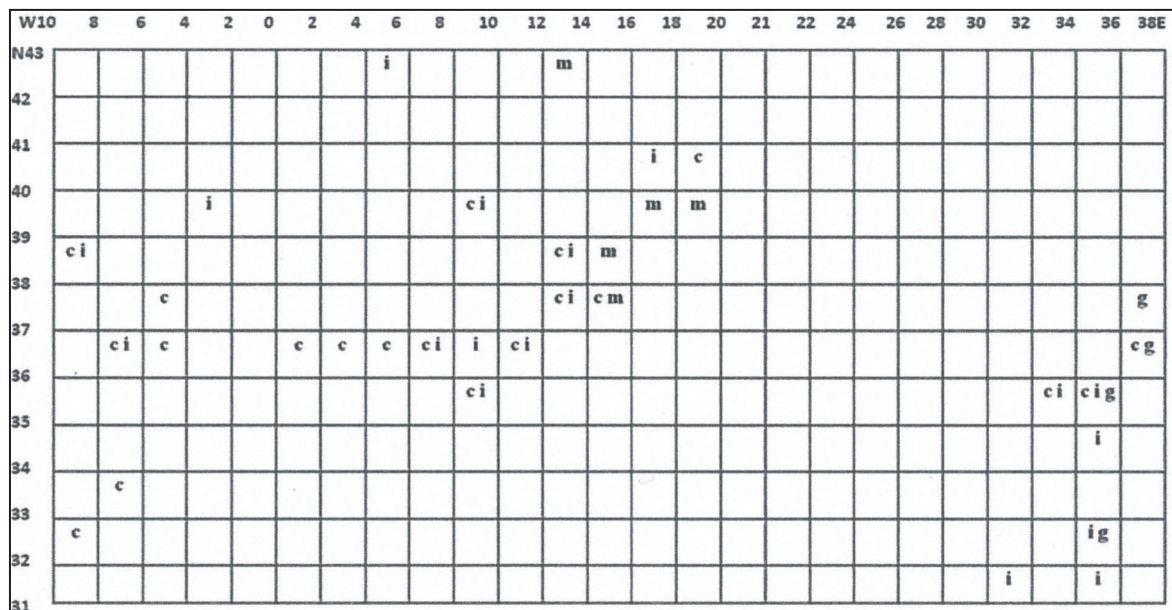


Figure 1. Geographical distribution of *Intertextae* subsection taxa (121 sites) by longitude and latitude. The rectangle is 1 x 2 degrees [1 line: North Latitude (N: 31 to 42); 2 column: East Longitude (E: 0 to 38) and West (W: 0 to 10)]. Abbreviations : c, *M. ciliaris*; i, *M. intertexta*; g, *M. granadensis*; m, *M. muricoleptis*.

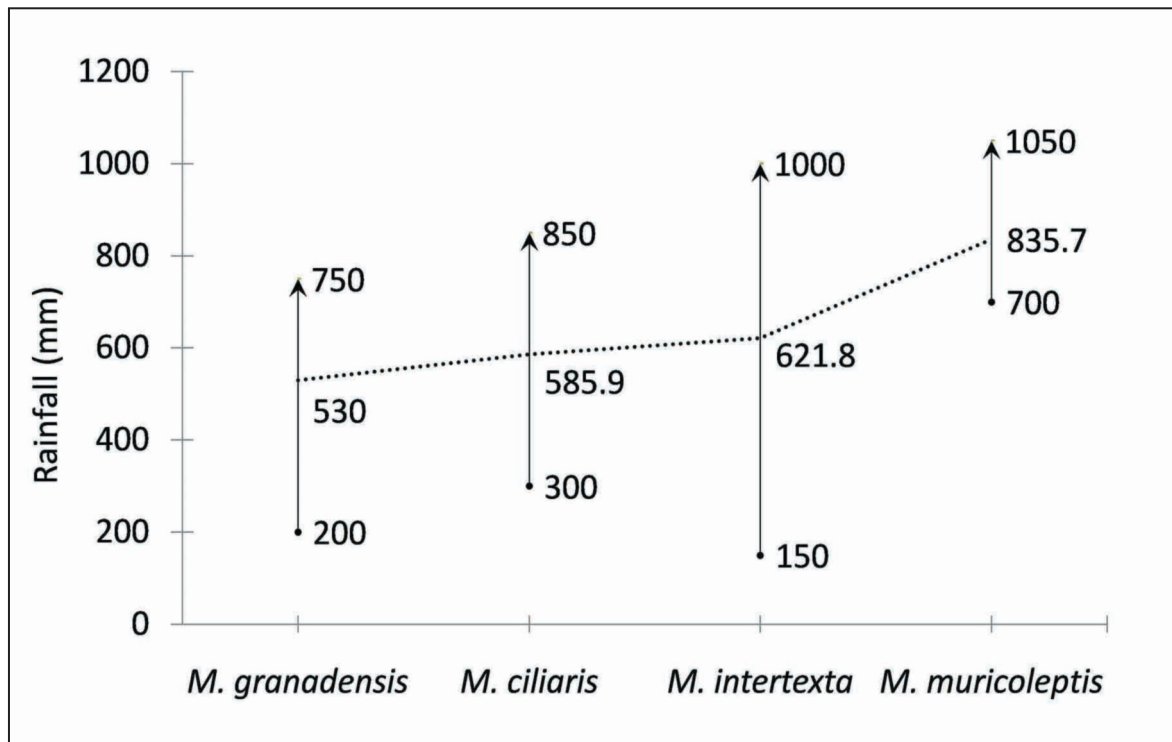


Figure 2. Minimum, average, and maximum values of annual average rainfalls by taxon.

class with more than 40 cm of depth (Fig. 5). *M. ciliaris* and *M. intertexta* can develop in soils that are not very deep (10 cm). *M. muricoleptis* and *M. granadensis* prefer deep soils.

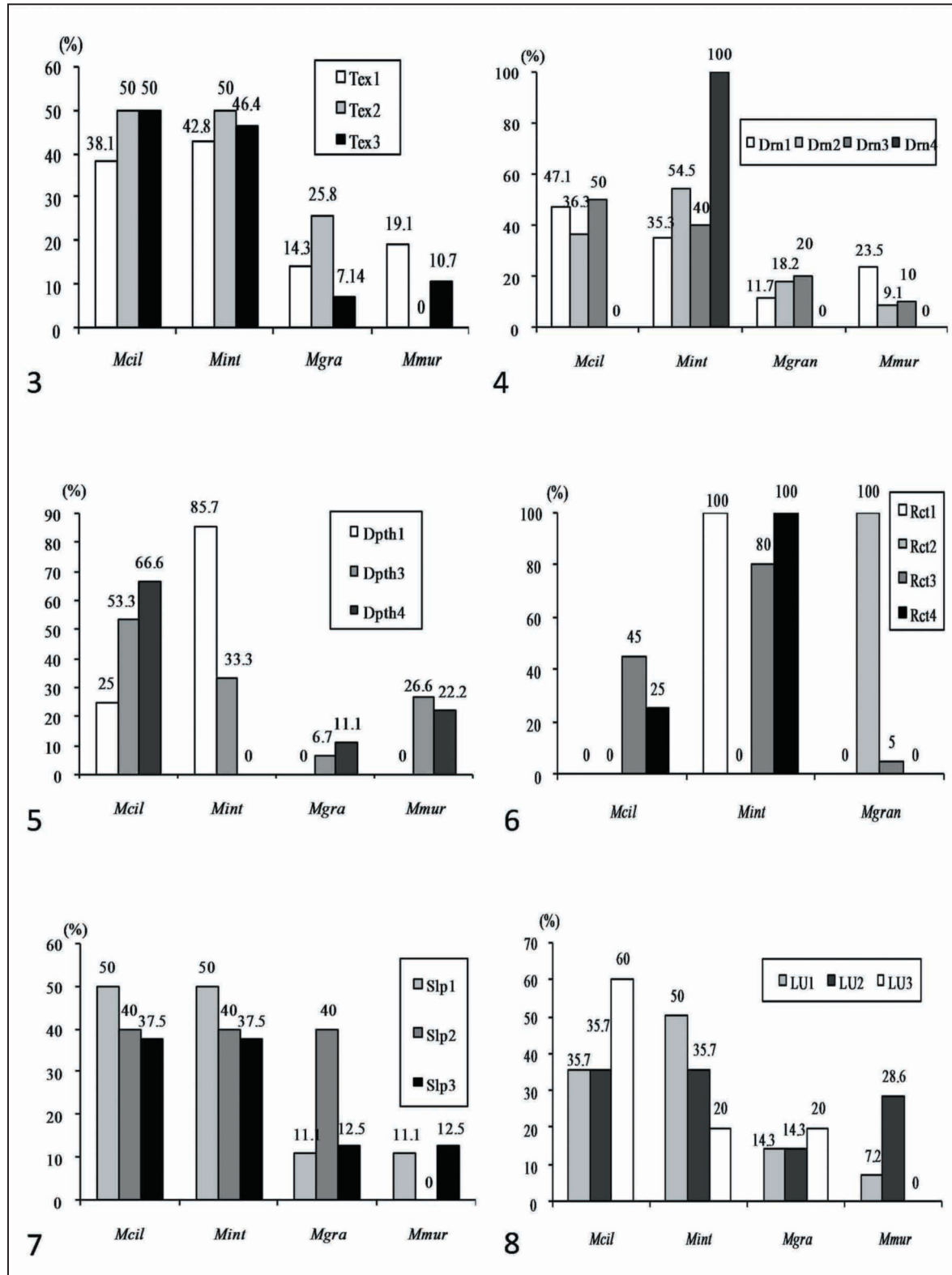
M. granadensis does not seem to withstand soil salinity considering its total absence in this type of soil (Fig. 6). Owing to the absence of data on soil reaction, *M. muricoleptis* was not studied. Although this data is qualitative, ecological profiles confirm the resistance of *M. ciliaris* and *M. intertexta* to salts as they are present in various sites qualified as salty. Although the variance analysis did not highlight significant differences for the pH, from ecological profiles of the soil reaction character (Fig. 6) there are preferences depending on the taxon. All of the three taxa *M. ciliaris*, *M. intertexta* and *M. granadensis* grow on alkaline soils. *M. granadensis* is the only one present in sites with neutral soils and *M. intertexta* confirms its presence on acid soils. Acid soils, with a pH of 5, characterize only a few sites of *M. ciliaris* and *M. intertexta*. By contrast, neutral and alkaline soils are suitable for the four taxa of the subsection.

Slope and Land Use

M. ciliaris and *M. intertexta* prefer flat grounds in contrast to *M. granadensis*, which prefers sloping grounds (16-30%). *M. muricoleptis* is intermediary with a preference for flat to undulating ground (Fig. 7). The four taxa of the *Intertextae* subsection are found in cultivated and grazed soils and only *M. ciliaris*, *M. intertexta* and *M. granadensis* are found on roadsides (Fig. 8). *M. ciliaris* and *M. granadensis* are more frequent on roadsides and *M. intertexta* is more frequent in cultivated lands. Unlike other taxa, *M. muricoleptis* is found more often in pastures.

Symbiotic responses

The endemic taxa, *M. muricoleptis* and *M. granadensis*, just as the taxa of *M. intertexta* and *M. ciliaris*, are exclusively nodulated, in Algeria. The inoculation of 34 trapped strains of rhizobia on *M. polymorpha* showed an efficient nodulation Nod+/Fix+ on all tested plants. This result confirms that all collection of rhizobium is *S. medicae*.



Figures 3–8. Ecological profiles indicate the relative frequency of the taxon in the different classes (for class limits see Table 4). Figure 3: soil texture. Figure 4: soil drainage. Figure 5: depth. Figure 6: soil reaction. Figure 7: slope. Figure 8: land use.

The inoculation of the reference strain 2011 *S. meliloti* shows that on *M. truncatula* (A17) all the plants nodulate and are efficient. No taxon of the *Intertextae* nodulated efficiently with this strain. *M. ciliaris* and *M. intertexta* do not show any nodule. *M. ciliaris* offers the highest percentage of bumps

and *M. granadensis* shows the highest percentage of Nod-. In *M. granadensis* and *M. muricoleptis* non efficient nodules were formed on a small number of plants (Fig. 9). The strain ABS7, obtained from *M. ciliaris*, nodulates efficiently, without exception, all the plants of the *Intertextae* subsection.

Variables	Abbreviation	Class codes	Classes
Texture	Tex	Tex1-Tex3	Clay, loamy, loamy-clay
Drainage	Drn	Drn1-Drn4	Good soil drainage, free flow, Stagnation in surface, Submersion
Depth (cm)	Dpth	Dpth1-Dpth3	0-10, 20-40 and +40 cm
Soil Reaction	Rct	Rct1-Rct4	Acidic, Neutral, Alkaline, Saline
Slope (%)	Slp	Slp1-Slp3	0-3 % Flat, 3-8 % hilly, 16-30 % sloping
Land Use	LU	LU1-LU3	Crops, grazing, roadside

Table 4. Upper Limits of qualitative variable classes used for ecological profiles.

Taxon	Population	Code	Altitude (m)	Rainfall (mm)	Origin	Rhizobia Strains
<i>M. ciliaris</i>	DZ.C242	242	980	450	Algeria	3 Strains: DZ.c242.G3.1 ; DZ.c242.G3.2 ; DZ.c242.G3.3
<i>M. ciliaris</i>	DZ.C58	58	860	649	Algeria	3 Strains: DZ.c58.G1.2, DZ.c58.G1.4, DZ.c58.G1.5
<i>M. intertexta</i>	DZ.I331	331	880	1053	Algeria	5 Strains: DZ.i331.G2.1; DZ.i331.G2.2; DZ.i331.G2.3 ; DZ.i331.G2.4 ; DZ.i331.G2.5
<i>M. intertexta</i>	DZ.I58	58	860	649	Algeria	5 Strains: DZ.i58.G2.1, DZ.i58.G2.2, DZ.i58.G2.3, DZ.i58.G2.4 DZ.i58.G2.5
<i>M. granadensis</i>	DZ.GAUS98	5.822	200	550	Israel	5 Strains: DZ.GAUS98.1.1, DZ.GAUS98.1.2, DZ.GAUS98.1.3, DZ.GAUS98.1.4, DZ.GAUS98.1.5,
<i>M. granadensis</i>	DZ.GAUS105	23.928	950	600	Turkey	5 Strains: DZ.GAUS105.1.1, DZ.GAUS105.1.2, DZ.GAUS105.1.3, DZ.GAUS105.1.4, DZ.GAUS105.1.5
<i>M. muricoleptis</i>	DZ.MAUS.110	24.825	630	800	Sicily	5 Strains: DZ.MAUS.110.1.1, DZ.MAUS.110.1.2, DZ.MAUS.110.1.3, DZ.MAUS.110.1.4, DZ.MAUS.110.1.5
<i>M. muricoleptis</i>	DZ.MAUS.107	25.028	5	700	Italy	3 Strains: DZ.MAUS.107.1.1 DZ.MAUS.107.1.3, DZ.MAUS.107.1.4

Table 5. Collection of 34 rhizobia of *Intertextae* obtained by trapping.

Sites	Factors	Rainfall		Altitude		pH	
		Number of sites	Average (mm)	Number of sites	Average (m)	Number of sites	Average
<i>M. ciliaris</i> (sc+sci)		34	585.88 b	34	278.24	16	7.98
<i>M. intertexta</i> (si+sci)		30	621.83 b	30	249.50	8	7.73
<i>M. granadensis</i> (sg)		5	530.00 b	5	398.00	4	7.90
<i>M. muricoleptis</i> (sm)		7	835.71 a	7	333.57	3	7.17
Meaning		-	* *	-	ns	-	ns

LSD = 1.996

Table 6. Comparison between environmental factors of the sites of *Medicago ciliaris*, *M. intertexta*, *M. granadensis* and *M. muricoleptis*. The letters a and b: indicate average groups. P(t), t-test on the groups of average [***: P < 0.001; **: P < 0.01; *: P < 0.05; n.s: P > 0.05].

Sites	Factors	Rainfall		Altitude		pH	
		Number of sites	Average (mm)	Number of sites	Average (m)	Number. of sites	Average
<i>M. ciliaris</i> (sc)		22	571.82 b	22	288.41	13	7.84
<i>M. intertexta</i> (si)		18	628.61 b	18	242.78	5	7.20
<i>M. granadensis</i> (sg)		5	530.00 b	5	398.00	4	7.90
<i>M. muricoleptis</i> (sm)		7	835.71 a	7	333.57	3	7.17
Mixed <i>M. ci-M. int</i> (sm)		12	611.67 b	12	259.58	3	8.60
Meaning		-	* *	-	ns	-	ns

LSD = 1.98

Table 7. Comparison between environmental factors (3) of sites with *Medicago ciliaris* alone, *M. intertexta* alone, *M. granadensis*, *M. muricoleptis* and mixed sites with *M. ciliaris* and *M. intertexta*. The letters a and b: indicate average groups. P(t), t-test on the groups of average [***: P < 0.001; **: P < 0.01; *: P < 0.05; n.s: P > 0.05].

By contrast, USDA1827 (obtained from *M. truncatula*) nodulates neither *M. muricoleptis* nor *M. granadensis*. It nodulates one population of each taxon of *M. intertexta* and *M. ciliaris*. Indeed, the USDA1827 is specific within the species *M. truncatula*, for it nodulates some populations and not others.

Genetic diversity of the trapped rhizobia

The electrophoresis of rep-PCR and BOX-PCR revealed 114 bands (we only took intense bands) of which 45 are monomorphic and the remainder is made up of polymorphic bands. Polymorphism is more frequent in *M. granadensis* strains with 55.5%

of polymorphic bands, followed by *M. intertexta* strains (50%); it is less than 25% in the other taxa. We did not find specific bands that differentiate the strains of the 4 taxa. Genetic distances (Fig. 10) show a structuring of the strains of *S. medicae* by forming 2 different groups. The first group (G1) corresponds to the strains that nodulate *M. ciliaris* and *M. intertexta* and the second group (G2) is the one trapped in *M. granadensis* and *M. muricoleptis*. There are 2 strains of *M. granadensis* of the population DZ.GAUS98, which come together with those of *M. intertexta*. Symbiotic specificity is clearly established among widespread taxa and endemic ones.

DISCUSSION

Geographic distribution

In the Mediterranean Basin, the four taxa, which have a close relationship (Rose et al. 1988), show different types of distributions: (i) endemic and rare represented by *M. granadensis* and *M. muricoleptis*, (ii) widespread and common represented by *M. ciliaris* and *M. intertexta*. These results confirm those obtained by several authors (Table 1). The new data is that *M. ciliaris* and *M. intertexta* are present in other countries that were never mentioned before in the literature. *M. ciliaris* is present in Israel and *M. intertexta* in Israel, Egypt, Cyprus, and Jordan. *M. ciliaris* is the most frequent in the Mediterranean basin and particularly in Algeria. In this country, this taxon is ranked fifth (163 sites/564) (Abdelguerfi, 2002).

Contrary to *M. ciliaris*, in the south west of the Mediterranean *M. intertexta* is rare; it is absent in the Northwest of Algeria and in the arid areas (Abdelguerfi-Laouar et al., 2003). *M. muricoleptis* is present in very restricted areas, Toulon in France and Sicily in Italy. Nevertheless (according to Coulot & Rabaute, 2013), the presence of *M. muricoleptis* in Toulon (France) is accidental, endemic in the north Mediterranean, from the south of Italy to Greece and *M. granadensis* is more confined to western Asia, endemic in the East of the Mediterranean and only adventitious in Europe and Chile. Contrary to what mentioned by Small et al. (1999), *M. granadensis* coexists with *M. intertexta* as the two taxa were found together on a site.

Ecological requirements

M. muricoleptis is the most demanding water of the *Intertextae*. Its geographic distribution corresponds perfectly with this need. In Morocco, *M. intertexta* and *M. ciliaris* were found only in the areas with high rainfall and are confined to the areas with mild winter and summer (Bounejmate et al., 1992a, 1992b; Bounejmate, 1996). This does not reflect their requirement in the Mediterranean basin.

The *Intertextae* is known for its preference for heavy and humid soils (Small et al., 1999). In Algeria, *M. ciliaris*, *M. intertexta*, *M. rigidula* and *M. scutellata* prefer heavy soils and *M. ciliaris* grows particularly on marly soils (Abdelguerfi, 2002). Considering the specificity of the texture of the *Intertextae*, the taxa should withstand soils with poor water drainage. Indeed, the results show that the taxa are found both in soils with good water drainage and soils where water stagnates on the surface. *M. intertexta* is the most adapted to hydromorphic soils, is considered one of the most tolerant to waterlogging of the Annual *Medicago* genus (Francis & Poole, 1973). The waterlogging and salinity are associated characters in the Mediterranean basin (Kepner et al., 2005). The tolerance of plants to this condition is due to a combination of anatomical, physiological and morphological adaptations (Predepea-Javahar, 2012). Only *M. ciliaris* and *M. intertexta* are present in salt soil. Therefore we may conclude that these two taxa are adapted to salinity. Indeed, genotypes originating from salty soils are often considered as tolerant, that is the conclusion of Ben Salah et al. (2010) when they compared under saline conditions (100 mmol of NaCl) two pure lines originating from two different sites, one rich in salt, and the other poor.

In Morocco, Bounejmate et al. (1992a; 1992b; 1994) and Bounejmate (1996) found the two taxa in soils low in phosphorous, hydromorphic and salty. According to Abdelkrim (1995), *M. ciliaris* is found in fallows and idle lands with soils that are colluvial, heavy, mostly marly and clayey, poorly drained and more or less salty. *M. ciliaris* is classified as glycophyte and found in association with halophyte species (Abdelly et al., 2006; Merabet et al., 2006; Barret-Lennard & Setter, 2010). Other research confirmed the adaptation of these taxa to salinity (Greenway & Andrew, 1962; Lachaal et al., 1995; Chérifi et al., 2011).

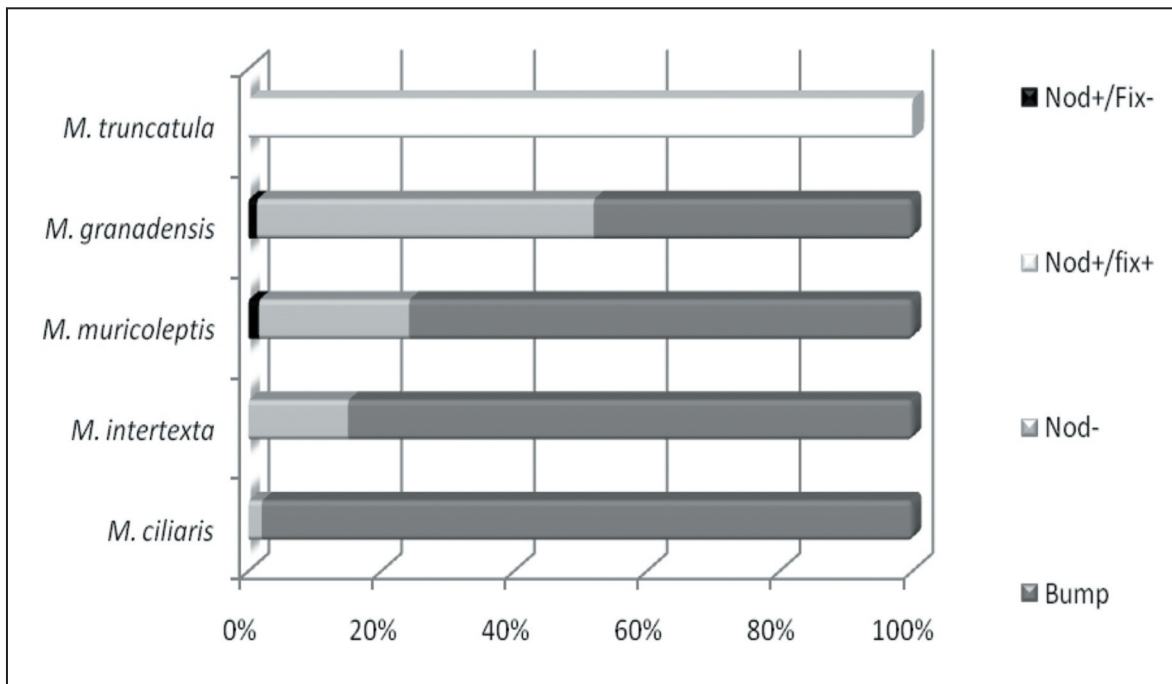


Figure 9. Symbiotic responses to the inoculation of the reference strain 2011 *S. meliloti* in the taxa of the Intertextae subsection. Abbreviations : Nod +, nodulating phenotype; Nod + /fix+, nodulating phenotype-fixer; Nod-, non nodulating phenotype; Bump, non nodulating root bulge.

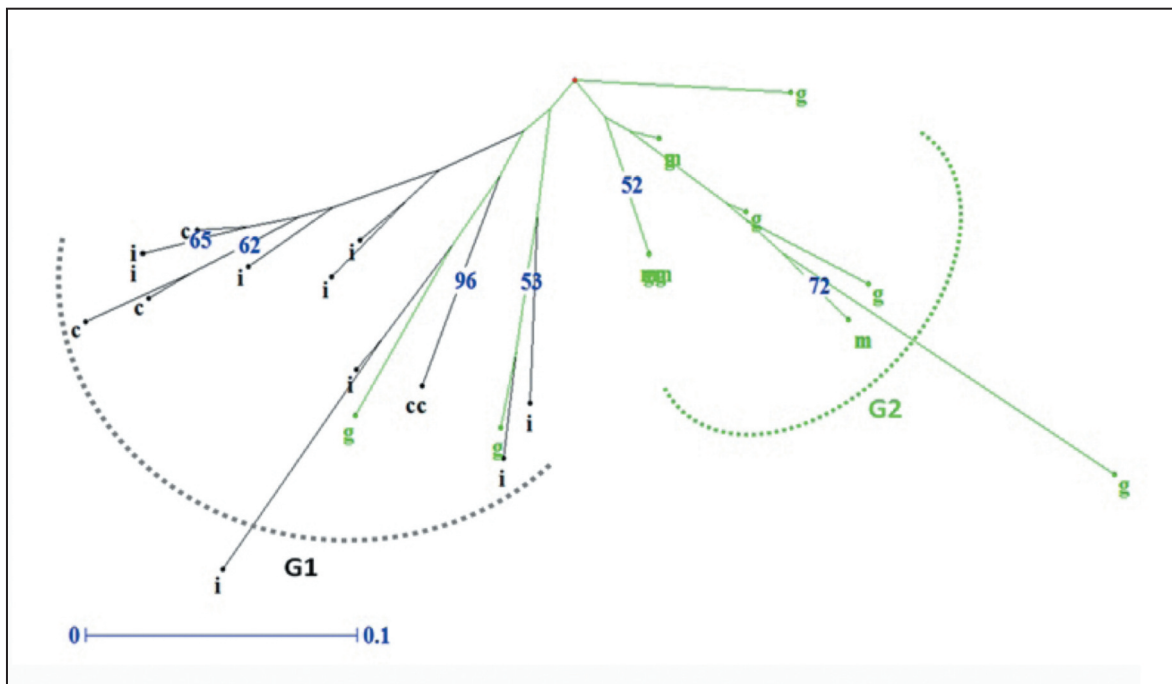


Figure 10. Un-weighted neighbour-joining tree based on the simple matching dissimilarity matrix of REP and BOX markers across the 34 strains of rhizobia trapped in a common site to all taxa in the subsection *Intertextae*. The numbers on the tips indicate bootstrap values (expressed in percentages) and are shown for all clusters with >50 % bootstrap support. Abbreviations: c, *M. ciliaris*; i, *M. intertexta*; m, *M. muricoleptis*; g, *M. granadensis*.

Furthermore, some studies have shown that the frequency of *M. intertexta* diminishes when the pressure of pasture increases (Piano, 1987). Piano et al. (1991) noted that *M. ciliaris* and *M. intertexta* are more frequent in cultivated lands.

Symbiotic response of *M. granadensis* and *M. muricoleptis* in an exotic site

The various populations of *M. granadensis* and *M. muricoleptis* nodulated efficiently in the Algerian common site. The fact that *M. granadensis* and *M. muricoleptis* nodulated efficiently with rhizobia natives of Algeria, shows that their absence is not related to a restrictive coevolution plant-rhizobium in this region. On some species, particularly cultivated ones, a clear evolution was noted between the species and its symbiote and one of the more interesting examples is the pea, which may form an efficient symbiosis with strains of rhizobium from its site of origin in Turkey, but not with strains from other parts of this country (Lie et al., 1987).

Taxonomic determination of the trapped collection of *Rhizobia*

M. muricoleptis, *M. granadensis*, *M. intertexta* and *M. ciliaris* are exclusively nodulated by the strains of *S. medicae*, which confirms the results of Béna et al. (1998) on the *Intertextae* subsection and the results of Zribi et al. (2007) on *M. ciliaris*. Indeed, in the trapping site, there were different species of *Medicago* that were only nodulated by *S. meliloti* including species of the *Melilotus* Mill. genus. This confirms the specificity between the subsection of the *Intertextae* and *S. medicae*. A recent study (Epstein et al., 2012), comparing the two genomes of *S. medicae* and *S. meliloti*, showed that horizontal exchanges were almost exclusively of plasmid genes and that the divergence between the two species resulted from episodes of recent selection pressures.

Maybe it is not very correct to believe that this specificity, whether for *M. polymorpha* or the subsection of the *Intertextae*, is related only to the host. Indeed, other mechanisms affect the chemical structure of the synthesized Nod factors and therefore prevent the nodulation (Van Rhijin & Vanderleyden, 1995; Dénarié et al., 1996; Long, 1996). Alfalfa, for

example, is only nodulated in the presence of sulphated Nod factors. This specificity mechanism is based on the presence or absence, depending on bacterial strains, of some genes that control the synthesis of specific substitutes or their transfer on the common skeleton. Other than genetic factors, exogenous factors can influence the symbiosis either for the choice of rhizobia that nodulate the plant or for their efficiency.

According to Garan et al. (2005), *S. medicae* nodulate more the species of *Medicago* that are adapted to acid soils; by contrast, *M. meliloti* forms a more frequent symbiosis with those that grow on alkaline to neutral soils. This result does not apply to the 4 taxa of the *Intertextae* subsection, since they rather prefer alkaline to neutral soils.

Symbiotic specificity

The symbiotic response is not limited to the presence or absence of nodules. Others structures can exist and appear visually on the roots in the form of more or less developed protuberances. Generally, these forms do not grow bigger in length, in fact, their development stops quickly. The study of the effect of the Nod factors of *Rhizobium meliloti* (equivalent to *S. meliloti*) on cultivated alfalfa revealed several structures, such as nodules and other protuberances (Grosjean & Huguet, 1997).

Therefore, the symbiotic response other than the presence absence of efficient nodules cannot be dissociated from genetic variations of the host plant. In this sense, the Nod- of *M. muricoleptis* and *M. granadensis* induced by *S. meliloti* shows the difference of these taxa relative to *M. ciliaris* and *M. intertexta*.

Furthermore, the strain USDA1827 shows a specificity of inter- and intra-species host and it is not the only one. The population ES103 of *M. polymorpha* achieves an efficient symbiosis with ABS7m, while the population F34003 does not form any nodule with this same strain (Béna et al., 1998).

Genetic relation between rhizobium and taxa

According to Martínez -Romero (2009), plant-rhizobium coevolution is more oriented by the plant

than by the rhizobium; it is the plant that must constantly select the strains with which it gets in symbiosis. This result shows that the taxa acquired genetic differentiation. In Tunisia, the results of molecular and enzymatic analysis did not show strains-species specificity in indigenous strains of *M. sativa* cv. Gabbès, *M. scutellata* cv. Kelson, *M. truncatula* and *M. ciliaris* (Jebara, 2001). Although *M. muricoleptis* and *M. granadensis* do not share the same areas of distribution, they select genetically close strains of rhizobium that are different from the ones of indigenous taxa, *M. ciliaris* and *M. intertexta*.

These results indicate cospeciation between the rhizobia and the endemic and widespread taxa. Cospeciation between the rhizobia and leguminous plants is not clearly established. Some authors indicate that there is little concordance between the phylogeny of bacteria and the phylogeny of legumes plants (Doyle, 1998; Béna et al., 2005); others, on the contrary, find links between both partners (Ueda et al., 1995; Wernegreen & Riley, 1999; Laguerre et al., 2001).

What is the effect of the two factors on the geographical distribution of taxa?

When analyzing ecological data, it appears that there are no conditions specific to either taxon. Some variations were observed among taxa but are not discriminating. Consequently, *M. muricoleptis* and *M. granadensis* are not specific to some habitats and the limit of their dispersion is not ecological. According to Griggs (1940), climatic and edaphic factors are not the primary causes of the rarity of a species, but the primary cause is competitiveness. Other biotic factors may come into play; we have seen that symbiosis does not seem to be a factor that limits the dispersion of endemic species since they have grown and nodulated in Algeria where they are naturally absent. Indeed, from a study on the effect of symbiosis on the expansion of the *Medicago* genus (Béna et al., 2005), it was concluded that symbiotic specificity can influence the distribution of the species of the *Medicago* genus, but not to the point of influencing their level of endemism.

In this case, *M. muricoleptis* and *M. granadensis* show local endemism that generally results from geographic and genetic isolation. The geographical distribution of the 4 taxa may result from allopatric

speciation where the populations are first separated geographically, which provokes a net decrease or a stop of the genetic exchanges, and the progressive accumulation of differences among the populations.

According to the phylogenetic results, that indicate that *M. ciliaris* and *M. intertexta* evolved from a common lineage and that *M. intertexta* evolved independently and gave rise to *M. muricoleptis* and *M. granadensis* (Rose et al., 1988), we may assume that *M. muricoleptis* and *M. granadensis* are young species (neoendemism) (Willis, 1922; Stebbins, 1980) compared with *M. intertexta* and *M. ciliaris* and that over time they could extend to other areas. As mentioned by Lamotte (1994), in allopatric speciation, populations may be confronted to different environments allowing or provoking differential adaptations.

CONCLUSION

Ecological factors did not explain why phylogenetically close taxa belonging to the same subsection have different geographical distributions, one narrow and one wide. The ecological requirements of taxa are more or less similar demonstrating that this is not the factor limiting the distribution of *M. muricoleptis* and *M. granadensis* in the region of the Mediterranean. Either the symbiosis is not a factor limiting the presence of these taxa which perform an efficient symbiosis in an area where are naturally absent. As against, endemic and widespread taxa show selectivity in symbiont (rhizobia strains) which confirms their differences. Other factors, such as genetic pool of taxa, should be investigated to clarify the distribution of the *Intertextae*.

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