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Diverse Bacteria Affiliated with the Genera *Microvirga*, *Phyllobacterium*, and *Bradyrhizobium* Nodulate *Lupinus micranthus* Growing in Soils of Northern Tunisia

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ABSTRACT The genetic diversity of bacterial populations nodulating *Lupinus micranthus* in five geographical sites from northern Tunisia was examined. Phylogenetic analyses of 50 isolates based on partial sequences of *recA* and *gyrB* grouped strains into seven clusters, five of which belong to the genus *Bradyrhizobium* (28 isolates), one to *Phyllobacterium* (2 isolates), and one, remarkably, to *Microvirga* (20 isolates). The largest *Bradyrhizobium* cluster (17 isolates) grouped with the *B. lupini* species, and the other five clusters were close to different recently defined *Bradyrhizobium* species. Isolates close to *Microvirga* were obtained from nodules of plants from four of the five sites sampled. We carried out an in-depth phylogenetic study with representatives of the seven clusters using sequences from housekeeping genes (*rrs*, *recA*, *glnII*, *gyrB*, and *dnaK*) and obtained consistent results. A phylogeny based on the sequence of the symbiotic gene *nodC* identified four groups, three formed by *Bradyrhizobium* isolates and one by the *Microvirga* and *Phyllobacterium* isolates. Symbiotic behaviors of the representative strains were tested, and some congruence between symbiovars and symbiotic performance was observed. These data indicate a remarkable diversity of *L. micranthus* root nodule symbionts in northern Tunisia, including strains from the *Bradyrhizobiaceae*, *Methylobacteriaceae*, and *Phyllobacteriaceae* families, in contrast with those of the rhizobial populations nodulating lupines in the Old World, including *L. micranthus* from other Mediterranean areas, which are nodulated mostly by *Bradyrhizobium* strains.

IMPORTANCE *Lupinus micranthus* is a legume broadly distributed in the Mediterranean region and plays an important role in soil fertility and vegetation coverage by fixing nitrogen and solubilizing phosphate in semiarid areas. Direct sowing to extend the distribution of this indigenous legume can contribute to the prevention of soil erosion in pre-Saharan lands of Tunisia. However, rhizobial populations associated with *L. micranthus* are poorly understood. In this context, the diversity of endosymbionts of this legume was investigated. Most *Lupinus* species are nodulated by *Bradyrhizobium* strains. This work showed that about half of the isolates from northern Tunisian soils were in fact *Bradyrhizobium* symbionts, but the other half were found unexpectedly to be bacteria within the genera *Microvirga* and *Phyllobacterium*. These unusual endosymbionts may have a great ecological relevance. Inoculation with the appropriate selected symbiotic

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bacterial partners will increase *L. micranthus* survival with consequent advantages for the environment in semiarid areas of Tunisia.

KEYWORDS *Bradyrhizobium*, *Lupinus micranthus*, *Microvirga*, *Phyllobacterium*, nodulation

Lupinus is the largest genus in the tribe Genisteae within the Fabaceae family, with around 275 species (1). Lupines are widely distributed in the Old and New Worlds, but only 15 species have been identified in the Mediterranean region (2–4). A few species from this genus have been cultivated in rotations with cereals for more than 2,000 years (5).

The endosymbiotic bacteria of cultivated lupines *L. albus*, *L. angustifolius*, and *L. luteus* belong mostly to *Bradyrhizobium* lineages (6–9). However, endosymbionts from most wild lupines are unknown or scarcely studied; among them are the *Bradyrhizobium* strains isolated from *L. albescens* native to Brazil (10) and strains from *L. mariae-josephae* from eastern Spain (11, 12). Also, *Ochrobactrum* and *Microvirga* strains have been isolated from *L. honoratus* in Argentina (13) and *L. texensis* in Texas (USA), respectively (14, 15).

L. micranthus is a wild lupine spread widely around the Mediterranean. Symbiotic and phylogenetic analyses of isolates from nodules of field-grown plants in Algeria and Spain recently showed that they all belong to the *Bradyrhizobium* genus (16). There are no reports addressing rhizobia nodulating *L. micranthus* in Tunisia, where four other *Lupinus* species have been described, namely *L. albus*, *L. angustifolius*, *L. luteus*, and *L. cosentinii* (17). This work shows an unexpected biodiversity among rhizobial isolates from *L. micranthus* in North Tunisia, including isolates belonging to *Microvirga* and *Phyllobacterium* genera.

The *Microvirga* genus comprises mainly soil bacteria, and currently, 13 species have been described (18–24). Of these species, only 4 are legume symbionts and were designated *M. lotononidis* and *M. zambiensis*, both isolated from *Listia angolensis*, *M. vignae*, nodulating *Vigna unguiculata* (21), and the already mentioned *M. lupini*, isolated from *L. texensis* (15).

The *Phyllobacterium* genus was originally described by D. H. Knösel (25) for bacteria isolated from leaf nodules of tropical ornamental plants. Thus far, this genus contains 10 species, six of which were isolated from root nodules of legumes and are designated as follows: *Phyllobacterium sophorae*, isolated from *Sophora flavescens* (26); *P. trifolii*, isolated from *Trifolium pratense* (27); *P. leguminum*, isolated from *Argyrolobium uniflorum* and *Astragalus algerianus* (28); *P. loti*, isolated from *Lotus corniculatus* (29); *P. endophyticum*, isolated from *Phaseolus vulgaris* (30); and *P. ifriqiyense*, isolated from *Astragalus algerianus* and *Lathyrus numidicus* (28). However, the capacities to induce effective nodulation on the original host plant were only demonstrated for *P. trifolii* and *P. sophorae* (26, 27). This diversity in symbiosis suggests that legume roots are the preferred habitats for species of the genus *Phyllobacterium*, more so than leaf nodules (31).

RESULTS

Isolation and diversity of *L. micranthus*-nodulating bacteria in northern Tunisia. A total of 50 rhizobial isolates were obtained from root nodules collected in April 2015 from wild *Lupinus micranthus* growing in five different areas in North Tunisia (Table 1 and Fig. 1). These isolates nodulated and fixed nitrogen with *L. micranthus* plants inoculated under bacteriologically controlled conditions. Inoculated plants were dark green, clearly different from the yellowish, smaller noninoculated control plants, and produced 3 to 10 red nodules per plant at 30 days postinoculation. When the isolates were examined in free-living conditions, different phenotypic characteristics were observed on yeast extract mannitol agar (YMA) plates. Most of the isolates (i.e., 32) produced circular, convex, smooth, and mucilaginous colonies 2 to 3 mm in diameter at 28°C; half of them appeared after 3 to 4 days postinoculation and the other half after

TABLE 1 Designations and geographical origins of *L. micranthus* endosymbionts in northern Tunisia

Sites	Coordinates	Altitude[a] (m)	Soil pH	No. of isolates	Designation	Environment	No. of isolates (group)[b]
Borj Hfaiedh	36°28′50.03″N 10°35′15.30″E	106	8.5	11	LmiB	Area with olive trees	3 (II), 2 (V), 6 (VII)
El Alia	37°11′23.26″N 10°00′19.83″E	47	8.3	4	LmiE	Area next to greenhouses	4 (VII)
Hammamet	36°23′38.98″N 10°36′57.47″E	4	9.0	9	LmiH	Semiurbanized area, next to the sea	3 (IV), 6 (VII)
Mraissa	36°45′13.00″N 10°33′14.20″E	10	8.0	10	LmiM	Area planted with cereals	3 (I), 3 (III), 4 (VII)
Takelsa	36°45′43.17″N 10°34′53.73″E	51	7.3	16	LmiT	Area planted with orange trees	14 (I), 2 (VI)

[a]Above sea level.
[b]Groups (I to VII) are defined in Fig. 1 and 2. Strain similarities: group I, *B. lupini*; group II, *B. betae*; group III, *B. valentinum*; group IV, *B. retamae*; group V, *B. diazoefficiens/B. rifense*; group VI, *Phyllobacterium* sp.; and group VII, *Microvirga* sp.

6 to 7 days. Six isolates had extremely low growth rates and required more than 7 days to generate nonmucoid colonies.

To evaluate the diversity of the isolates, an internal sequence of *recA* was obtained. Previous work in our group showed that the analysis of *recA* sequences is a very resolutive tool for estimating the similarities among rhizobial strains and predicting their lineages and genera (16, 33, 34). An analysis of *recA* sequences using BLAST enabled the clustering of the 50 *L. micranthus*-associated isolates into 7 groups (Fig. 2). Sequences from isolates in five groups (groups I, II, III, IV, and V) matched those of the genus *Bradyrhizobium* and were similar to *B. lupini* (17 isolates), *B. betae* (3 isolates), *B. valentinum* (3 isolates), *B. retamae* (3 isolates), and *B. diazoefficiens* (2 isolates) type strains, respectively. The 2 isolates in group VI matched type strains of species from the genus *Phyllobacterium*, and the last group (group VII, Fig. 2) contained 20 isolates that appeared similar to one another. Sanger chromatograms for *recA* sequences of these isolates displayed sequence ambiguities (up to 14 in 533 nucleotides [nt]). Preliminary draft genome sequences of two of these isolates (*L. micranthus* LmiM8 and LmiE10) suggested the existence of four nonidentical *recA* copies that might explain these results (Fig. 2). The location of group VII *recA* sequences in the *recA* phylogenetic tree predicts their membership in the *Microvirga* genus. The presence of several copies of *recA* in isolates LmiM8 and LmiE10 is in line with what has been described for *M. lupini* (two copies), *M. lotononidis* (two copies), and *M.* sp. BSC39 (three copies) (35–37). Therefore, a different housekeeping gene marker, *gyrB*, commonly used with *Microvirga* species (15, 21, 22), was selected for amplicon characterization. Sequencing of *gyrB* amplicons confirmed the affiliation of the 20 isolates in group VII to the *Microvirga* genus (Fig. 3).

The isolates included in *Microvirga* and *Phyllobacterium* genera had mean generation times of approximately 5 h in tryptone yeast (TY) medium in contrast with *Bradyrhizobium* isolates, which had a mean generation time of more than 9 h (Table 2). *Microvirga* colonies were pink in color after 8 days in YMA, as has been observed for

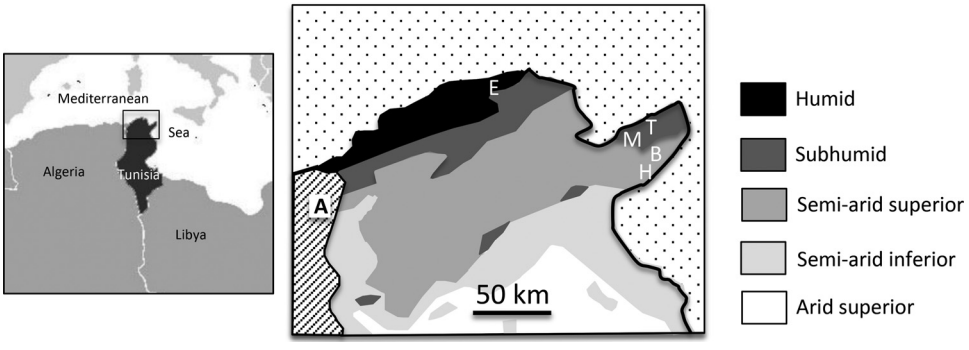

FIG 1 Sites of plant sampling in northern Tunisia. Bioclimatic areas are indicated on the right. A, Algeria; E, El Alia; M, Mraissa; T, Takelsa; B, Borj Hfaiedh; H, Hammamet. Template maps were obtained from Wikipedia (left; https://upload.wikimedia.org/wikipedia/commons/a/af/Tunisia_Locator.png) and Tunisia's National Research Institute of Water, Forests and Rural Engineering (right; http://www.inrgref.agrinet.tn/an/?p=19).

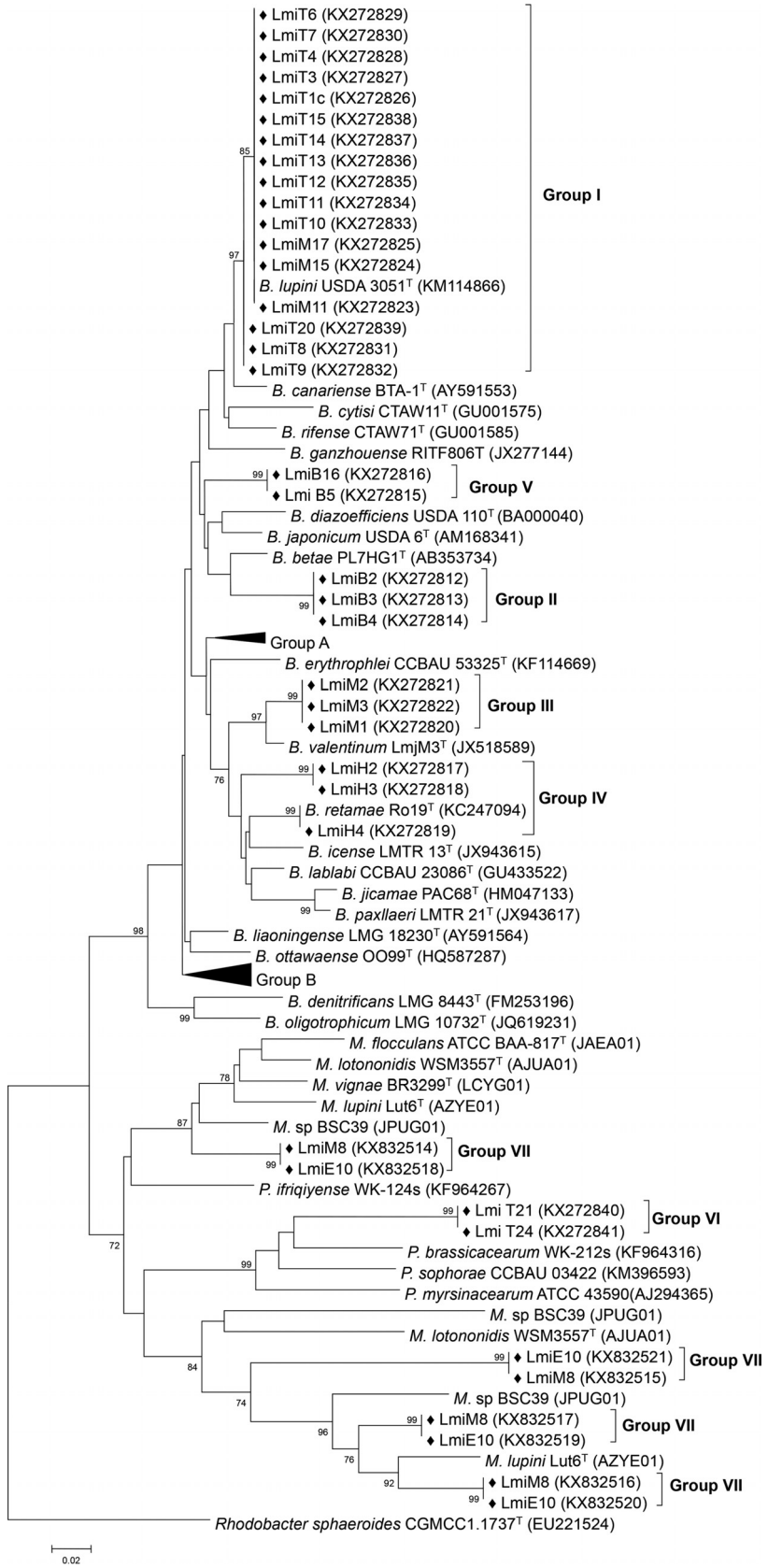

FIG 2 Neighbor-joining phylogenetic tree of Tunisian *L. micranthus*-associated (Lmi) isolates and reference strains based on *recA* (375 bp) sequences. Bootstrap values were calculated for 1,000 replications, and those greater than 70% are indicated at the internodes. Black diamonds (◆) indicate Tunisian Lmi

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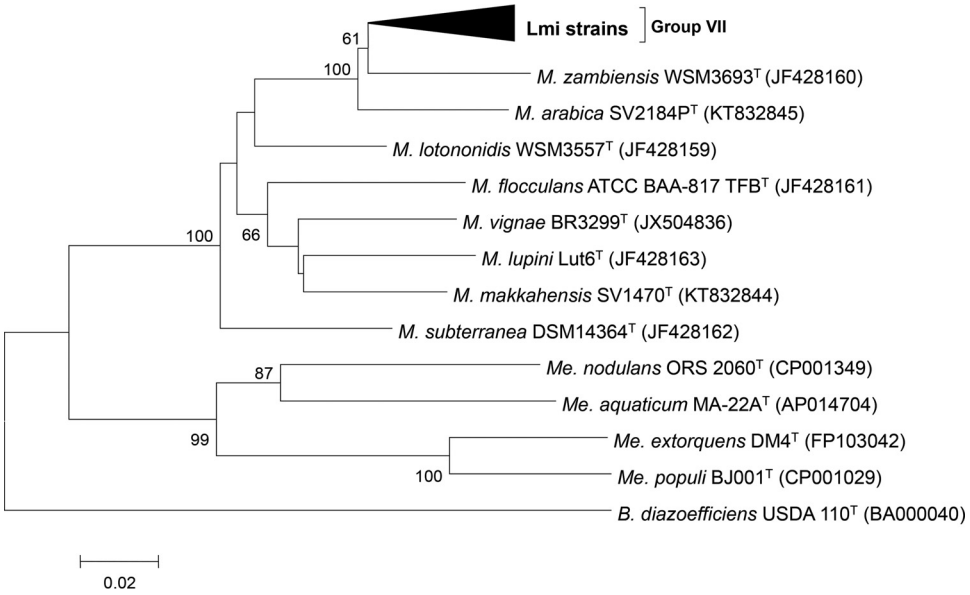

FIG 3 Neighbor-joining phylogenetic tree of Tunisian *L. micranthus*-associated (Lmi) isolates and reference strains based on *gyrB* (623 bp) sequences. Bootstrap values were calculated for 1,000 replications, and those greater than 60% are indicated at the internodes. Tunisian Lmi isolates with identical *gyrB* sequences were collapsed in group VII: LmiM8, LmiE10, LmiH9, LmiE9, LmiH1, LmiB17, LmiB20, LmiB18, LmiB6, LmiB21, LmiB24, LmiE5, LmiE6, LmiH5, LmiH6, LmiH8, LmiH10, LmiM7, LmiM9, and LmiM10 (accession numbers KX784068 to KX784087). Other accession numbers from the GenBank database are shown in parentheses. *B.*, *Bradyrhizobium*; *M.*, *Microvirga*; *Me.*, *Methylobacterium*.

other *Microvirg*a strains, such as *M. subterranea* (20), *M. lupini* (15), and *M. makkahensis* (22). The growth rates of bradyrhizobial isolates from groups III and IV were similar to those of *B. valentinum* and *B. retamae*, respectively, with an extra-low growth rate phenotype on YMA plates.

The *L. micranthus* nodule isolates studied were obtained from five locations in northern Tunisia, and their geographical origins are shown in Table 1 and Fig. 1. Among the seven groups identified, *Bradyrhizobium* isolates in groups II, III, and V were recovered from only one site, and the *Phyllobacterium* isolates were recovered only from nodules of plants collected in Takelsa. Moreover, in all but one of the sites, isolates belonged to at least two different groups. The exception was El Alia, where all 4 isolates were within the *Microvirga* group.

To perform a deeper multilocus phylogenetic analysis, 9 representatives of the seven clusters (with two from each of the two larger groups) were randomly selected. For *Bradyrhizobium* and *Phyllobacterium* isolates, *rrs*, *recA*, and *glnII* were chosen for analyses because they are considered robust markers for determining the phylogeny and taxonomy of rhizobia (38) (Fig. 4a). Of note, *glnII* seems to be absent from the genomes of *Microvirga* spp., since it does not appear in data banks or in any of the *Microvirga* genomes sequenced. Consistent with this, *glnII* did not appear in the draft genome sequences of the strains LmiM8 and LmiE10 obtained by our group (data not shown). Therefore, *dnaK* was used instead, and the phylogenetic tree for this group of strains was made with concatenated sequences from *gyrB*, *dnaK*, and *rrs* (Fig. 4b). These phylogenetic trees are in agreement with previous phylogenetic analyses, although

FIG 2 Legend (Continued)
isolates. Collapsed groups include group A: *B. elkanii* USDA76T, *B. ferriligni* CCBAU 51502T, *B. pachyrhizi* PAC48T, *B. viridifuturi* SEMIA 690T, *B. embrapense* CNPSo 2833T, and *B. tropiciagri* CNPSo 1112T; and group B: *B. ingae* BR 10250T, *B. iriomotense* EK05T, *B. kavangense* 14-3T, *B. manausense* BR 3351T, *B. subterraneum* 58 2-1T, *B. yuanmingense* CCBAU 10071T, *B. vignae* 7-2T, *B. daqingense* CCBAU 15774T, and *B. huanghuaihaiense* CCBAU 23303T. Group VII includes only two strains (LmiM8 and LmiE10) that contain 4 copies of *recA* (see the text for details). *B.*, *Bradyrhizobium*; *M.*, *Microvirga*; *P.*, *Phyllobacterium*. Accession numbers from GenBank and whole-genome sequencing (NCBI) databases are shown in parentheses.

TABLE 2 Symbiotic characteristics and generation times of Tunisian *Lupinus micranthus* representative isolates

Lmi isolate	Symbiovar[a]	Group[b]	Generation time (h)[d]	Nodulation phenotype with host[c]:				
				Lmi	Lan	Lmj	Vun	Mat
T21	A	VI	5.5	+	+	−	W	+
E10	A	VII	4.8	+	+	W	W	+
M8	A	VII	4.6	+	+	W	W	+
H4	B	IV	14.4	+	+	+	+	+
M2	B	III	22.3	+	W	+	+	+
B3	C	II	9.4	+	−	−	+	+
T14	*genistearum*	I	13.1	+	W	W	W	+
T3	*genistearum*	I	11.5	+	+	W	W	+
B5	*genistearum*	V	16.4	+	+	−	+	+

[a]As shown in Fig. 4.
[b]Groups (I to VII) are defined in Fig. 2, 3, and 4. Phylogenetic similarities: group I, *B. lupini*; group II, *B. betae*; group III, *B. valentinum*; group IV, *B. retamae*; group V, *B. diazoefficiens/B. rifense*; group VI, *Phyllobacterium* sp.; and group VII, *Microvirga* sp.
[c]Nodulation evaluated by color of nodules: +, red; W, white; −, no nodules. In all cases, plants with white nodules were smaller than plants with red nodules. Lmi, *L. micranthus*; Lan, *L. angustifolius*; Lmj, *L. mariae-josephae*; Vun, *Vigna unguiculata*; Mat, *Macroptilium atropurpureum*.
[d]Generation times in TY medium at 28°C.

group V appeared closer to *B. rifense* than to *B. diazoefficiens*, in contrast with the *recA* analysis. On the other hand, the representative *Bradyrhizobium* isolates were compared with *L. micranthus*-associated strains recently isolated in Algeria and Spain (16). Results shown in Fig. 4a indicate that the largest group present in soils from all three countries contains bacteria belonging to the *B. lupini/B. canariense* lineages. Group V contains isolates from Algeria and Tunisia, represented by isolates LmicT3 and LmiB5, respectively (Fig. 4a). Isolates from group IV were assigned to *B. retamae* species. The remaining groups of isolates identified in this study (group II, III, V, VI, and VII) may constitute different genospecies.

Phylogenetic analysis based on *nodC* sequence. The *nodC* gene has been used to trace host range and symbiovars within the genus *Bradyrhizobium* (32, 39). An analysis of *nodC* sequences from representatives of the different groups of Tunisian *L. micranthus*-associated isolates suggests the existence of four different symbiovars (Fig. 5); three of them include *Bradyrhizobium* isolates and one includes *Phyllobacterium* and *Microvirga*-type isolates. Most of the representative isolates were symbiovar *genistearum*, which also encompasses strains able to nodulate legumes of the Genisteae tribe, such as the type strains of *B. lupini*, *B. rifense*, *B. cytisi*, and *B. canariense*. The two remaining symbiovars of *Bradyrhizobium*-type representative isolates may correspond to new symbiovars designated symbiovar B (LmiH4 and LmiM2), close to symbiovar *retamae*, and symbiovar C (LmiB3), which is distant from other *Bradyrhizobium* symbiovars, including symbiovar *retamae* nodulating *Retama* spp., and *Lupinus mariae-josephae* (11, 40) and symbiovar *sierranevadense* nodulating *Genista versicolor* (41). The fourth symbiovar, symbiovar A, comprised *Microvirga* and *Phyllobacterium* isolates with identical *nodC* sequences that define a new (100% bootstrap support) lineage with some similarity to *nodC* sequences from *M. lupini* and *P. sophorae* (Fig. 5).

Plant nodulation tests of Tunisian *L. micranthus*-associated isolates. We examined the symbiotic characteristics of representative isolates from the seven identified *L. micranthus* groups by cross-inoculation tests using *Lupinus angustifolius*, *L. mariae-josephae*, *Vigna unguiculata*, and *Macroptilium atropurpureum* as hosts (Table 2). No differences in the sizes of plants or appearances of the nodules were found when *L. micranthus* was inoculated with any of the representative isolates. However, clear differences in nodulation and symbiotic efficiency were observed when other hosts were inoculated with the same isolates. Isolate LmiB3 failed to nodulate *L. angustifolius*, and isolates LmiM2 and LmiT14 produced white nodules unable to fix nitrogen, whereas the remaining isolates produced healthy plants (Table 2). *L. mariae-josephae* plants were efficiently nodulated only by isolates of symbiovar B (Fig. 5), while isolates

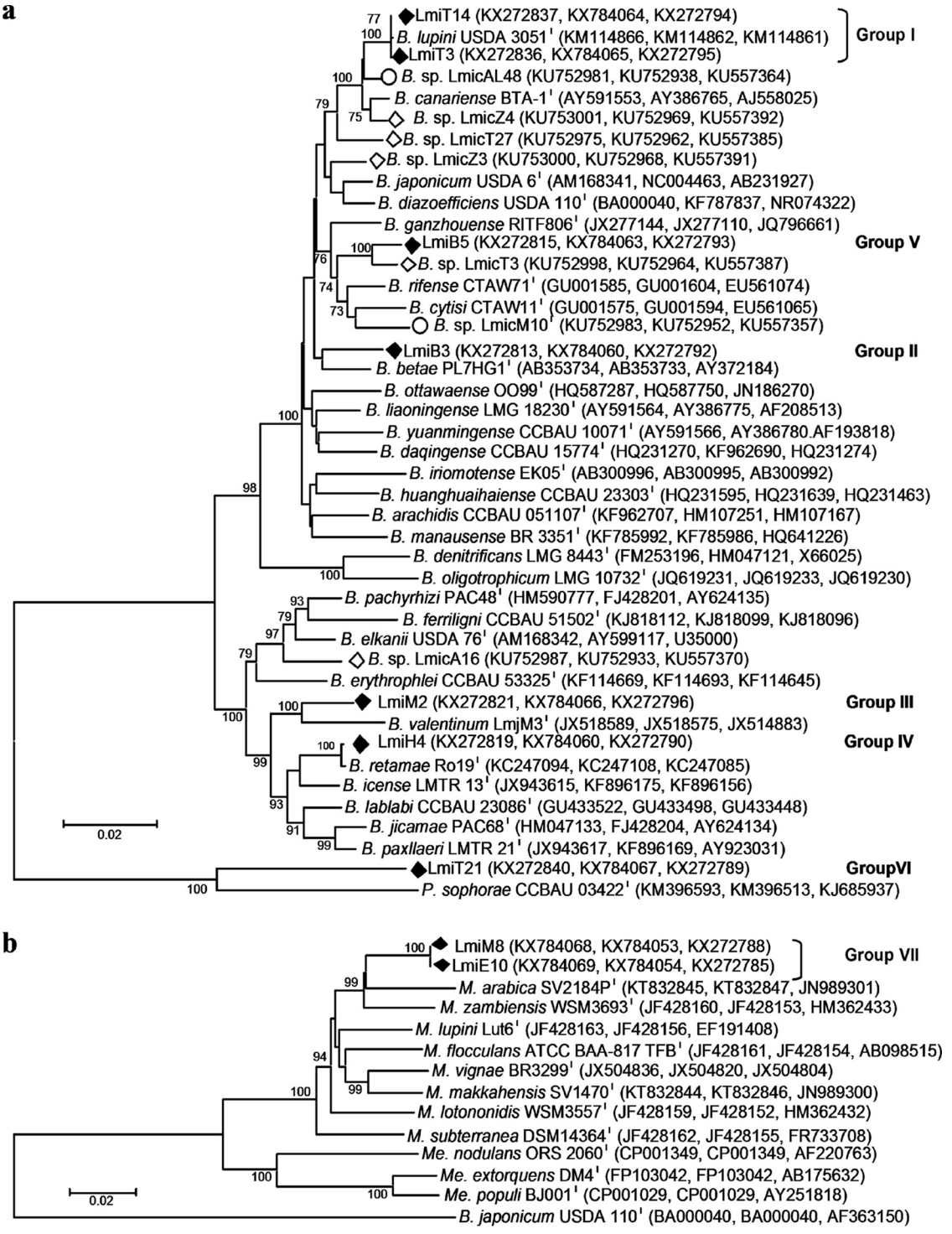

FIG 4 Neighbor-joining phylogenetic trees of Tunisian *L. micranthus*-associated (Lmi) isolates and reference strains based on concatenated sequences. (a) Phylogeny based on *recA* (372 bp), *glnII* (451 bp), and *rrs* (1209 bp) sequences. (b) Phylogeny based on *gyrB* (621 bp), *dnaK* (741 bp), and *rrs* (1303 bp) sequences. Symbols: ◆, Tunisian Lmi isolates obtained in this work; ○, Lmic isolates from Spanish soils; and ◇, Lmic isolates from Algerian soils described in reference 16. Bootstrap values were calculated for 1,000 replications, and those greater than 70% are indicated at the internodes. Accession numbers from GenBank database are shown in parentheses. *B.*, *Bradyrhizobium*; *M.*, *Microvirga*; *Me.*, *Methylobacterium*.

from other symbiovars induced no nodules or only white inefficient nodules. White inefficient nodules were also elicited in *V. unguiculata* by *Microvirga* and *Phyllobacterium* isolates grouped in symbiovar A and by *Bradyrhizobium* strains of symbiovar *genistearum* (group I), while red efficient nodules were elicited in this host by strains of

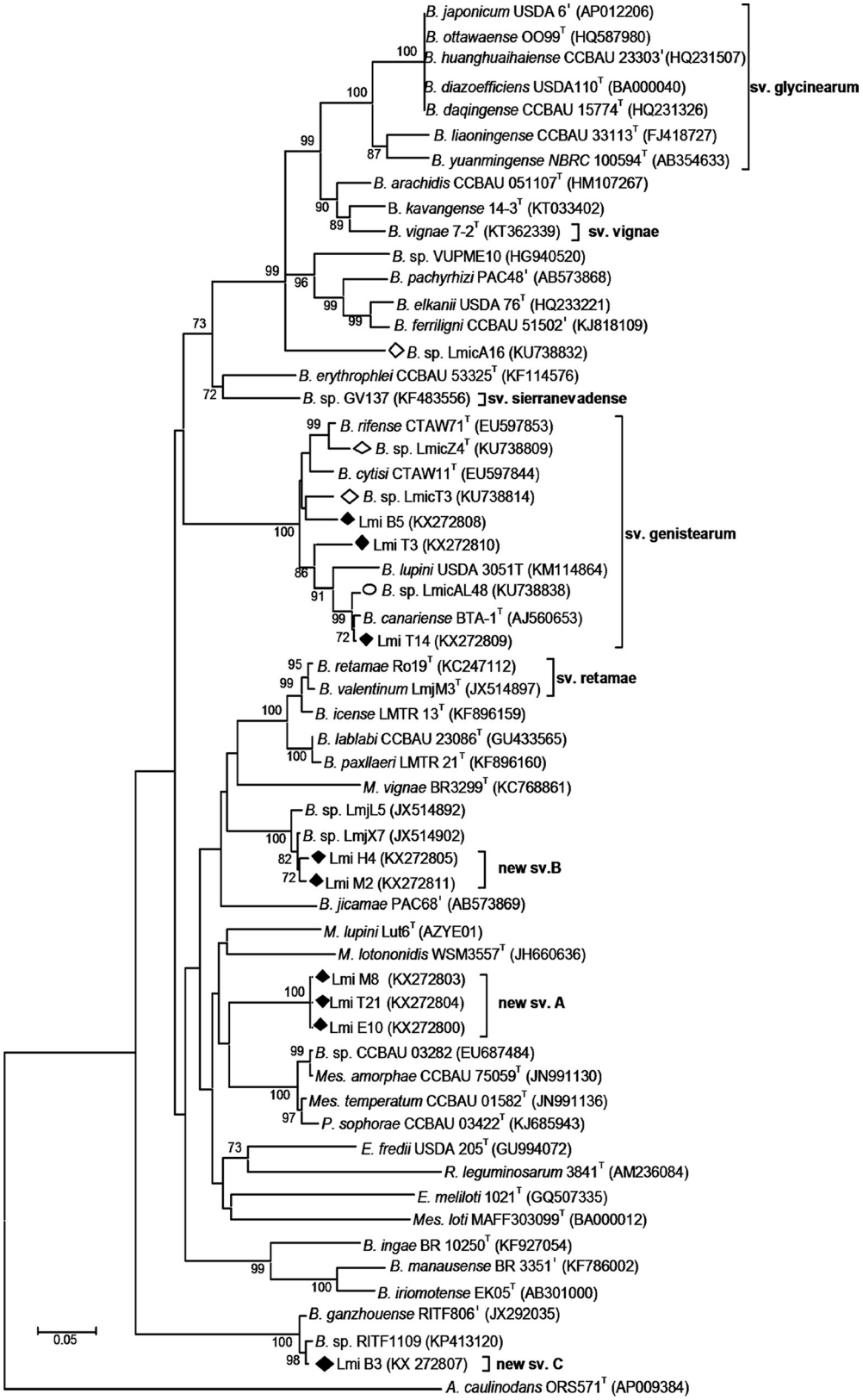

FIG 5 Neighbor-joining phylogenetic tree of Tunisian *L. micranthus*-associated (Lmi) isolates and reference strains based on *nodC* sequences. Symbols: ◆, Tunisian Lmi isolates obtained in this work; ○, Spanish Lmic isolates; and ◇, Algerian Lmic isolates described in reference 16. Bootstrap values were calculated for 1,000 replications, and those greater than 70% are indicated at the internodes. Accession numbers from the GenBank and whole-genome sequencing (NCBI) databases are shown in parentheses. *B.*, *Bradyrhizobium*, *E.*, *Ensifer*; *M.*, *Microvirga*; *Mes.*, *Mesorhizobium*; *P.*, *Phyllobacterium*; *R.*, *Rhizobium*; sv., symbiovar.

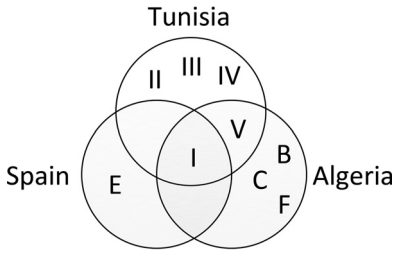

FIG 6 Venn diagram showing the *Bradyrhizobium* groups of *L. micranthus*-associated isolates shared by soils from different countries. Tunisian groups were defined in Fig. 2, and Spanish and Algerian groups were described by Bourebaba et al. (16). Group I in Tunisia corresponds to clusters A of Spain and Algeria, and group V corresponds to cluster D of Algeria.

Bradyrhizobium group V (symbiovar *genistearum*) and symbiovars B and C. Finally, *M. atropurpureum* plants were effectively nodulated by all *L. micranthus*-associated isolates (Table 2).

DISCUSSION

We performed a phylogenetic analysis of isolates from nodules of wild *L. micranthus* growing in five locations in northern Tunisia. The analysis showed a remarkable diversity of symbiotic bacteria, including isolates belonging to three genera, *Bradyrhizobium*, *Microvirga*, and *Phyllobacterium*, from three different families within the order *Rhizobiales*, namely *Bradyrhizobiaceae*, *Methylobacteriaceae*, and *Phyllobacteriaceae*, respectively. In previous studies, a clear predominance of *Bradyrhizobium* lineages was described for most *Lupinus* species (6, 7, 9, 12, 42). In lupine species from the Old World, *B. canariense* and *B. japonicum* are the two dominant rhizobial species in root nodules (8, 9). One exception is the *B. valentinum* species, a microsymbiont of *L. mariae-josephae*, a particular endemism of central-eastern Spain that grows in alkaline soils (11). In this study, most of the root nodule isolates (56%) were ascribed to the *Bradyrhizobium* genus, but the remaining 44% were, notably, fast-growing nonbradyrhizobia. This outcome contrasts with that from a recent study of *L. micranthus* symbiosis that identified all nodule isolates from Algeria and Spain soils as belonging to the genus *Bradyrhizobium*, mainly *B. lupini/B. canariense* (16). Similarly, most of the Tunisian bradyrhizobial isolates also belonged to the *B. lupini/B. canariense* lineage. A closer comparison of the five Tunisian *Bradyrhizobium* groups (groups I to V) with the five *Bradyrhizobium* groups from Algeria and the two groups from Spain (16) suggests the existence of specific phylogenetic groups for different geographical locations, as well as a greater diversity of these bradyrhizobia in northern Africa than in Spain (Fig. 6).

Unexpectedly, a large fraction of the northern Tunisia *L. micranthus* root nodule isolates were fast-growing *Rhizobiales* from the *Microvirga* (20 isolates) and *Phyllobacterium* (2 isolates) genera. Although a large number of lupine root nodule isolates from around the world has been characterized (6, 7, 9, 10, 12, 16, 42), only one example of nodulation by *Microvirga* has been reported, namely *M. lupini* from the North American *L. texensis* species (14, 37). This appears to be a highly specific symbiosis, since no other *Lupinus* sp. has been reported that can form effective nitrogen-fixing nodules with *M. lupini*. Of the 13 *Microvirga* species currently described, only four are able to establish root nodule symbioses with different legumes, namely *M. lupini* with *L. texensis* in Texas-northeastern Mexico (15), *M. zambiensis* and *M. lotononidis* with *Listia angolensis* in Zambia and Angola, respectively (15), and *M. vignae* with *Vigna unguiculata* in Brazil (21). The limited and haphazard geographical distribution of *Microvirga* symbionts suggests that Tunisian *Microvirga* isolates from *L. micranthus* might have recently acquired their symbiotic capacity, perhaps by horizontal transfer from other symbionts, a phenomenon that has been evidenced in different systems (43–45). The *Microvirga* isolates from *L. micranthus* may constitute a new genospecies different from *M. zambiensis/M. arabica*, their closer symbiotic relatives (Fig. 4b). It should be emphasized that *M. zambiensis* fixes nitrogen in association with the South African legume *Listia*

angolensis of the tribe Crotalarieae, sister to the tribe Genisteae, a large and diverse group that largely occurs in Africa (46).

A second remarkable result from this work is our finding that *Phyllobacterium*-related bacteria effectively nodulate lupines. *Phyllobacterium* strains have been identified in different environments (31), mostly as plant-associated bacteria (28, 30, 47, 48). To date, the *Phyllobacterium* genus contains 10 species, but only two, *P. trifolii* and *P. sophorae*, are able to induce effective nodules on their host legumes, *Trifolium pratense* and *Sophora flavescens*, respectively (26, 27). *P. trifolii* was also reported to induce white inefficient nodules in *Lupinus albus* (27). Strains of *P. leguminum* and *P. ifriqiyense*, isolated from nodules of *Astragalus algerianus* and *Lathyrus numidicus*, respectively, and growing in the infra-arid zone of southern Tunisia, were unable to induce effective nodules in any of the plants tested (28).

Soil pH can be an important factor determining the presence of different rhizobial species associated with one plant. Rodríguez-Echeverría et al. (49) studied the endosymbionts of *Retama sphaerocarpa* in acidic and alkaline soils. Like *L. micranthus*, *R. sphaerocarpa* is widely distributed in the Mediterranean and is nodulated by *B. canariense* only in soils with a pH below 7.0. In contrast, *B. retamae* appeared in nodules of plants growing in soils with a pH higher than 7.5. Similarly, in this work, most strains of the *B. lupini/B. canariense* lineage (group I) were isolated from neutral or mildly alkaline soils (Takelsa, pH 7.3, and Mraissa, pH 8.0), and *Phyllobacteriu*m strains (group VI) were identified only in Takelsa (Table 1). In contrast, *Microvirga* strains were found in all soils with a pH of 8 to 9 and were absent only in Takelsa. Thus, *Microvirga* strains appear to have a preference for alkaline soils, similar to that of the remaining *Bradyrhizobium* groups (II, III, IV and V) nodulating *L. micranthus*. In this respect, it is worth noting that groups III and IV are similar to *B. valentinum* and *B. retamae*, respectively, and these have been associated with alkaline soils (12, 34, 49). Overall, the available evidence suggests that symbiotic bacteria with certain genotypes are adapted to particular ecosystems where soil pH might play an important role. This assertion is consistent with the higher bradyrhizobial diversity observed in *L. micranthus* nodules from Tunisian (neutral or alkaline) soils versus Algerian and Spanish (acidic) soils (16).

The phylogeny of symbiotic *nod* genes has been frequently related to symbiotic performances of rhizobial strains (9, 42, 50, 51). Here, a large diversity of *nodC* sequences was observed within *L. micranthus* endosymbionts, which clustered into four symbiovars.

(i) Symbiovar *genistearum* was described by Vinuesa et al. (45) and includes many strains nodulating a large number of plants from the tribe Genisteae, including the genera *Lupinus*, *Cytisus*, *Retama*, *Chamaecytisus*, and *Spartium*, growing in Africa, Europe, and America (12, 33, 39, 40, 45). The majority of *L. micranthus*-associated isolates related to *B. lupini* (group I), and those similar to *B. cytisi/B. rifense* lineages (group V) belonged to this symbiovar. This is in line with the finding that most symbionts of *Cytisus triflorus* from Morocco and Algeria, as well as most isolates from *L. micranthus* from Algeria and Spain, belong to *B. canariense/B. lupini* and *B. cytisi/B. rifense* lineages and to symbiovar *genistearum* (16, 33, 52). Importantly, these microorganisms may be dominant in neutral or acidic soils.

(ii) Besides bradyrhizobial *L. micranthus*-associated isolates, symbiovar B included strains *Bradyrhizobium* sp. LmjX7 and *Bradyrhizobium* sp. LmjL5 isolated from nodules of *L. mariae-josephae* (34). Interestingly, *L. mariae-josephae* elicited only red effective nodules with strains of symbiovar B, while other symbiovars either did not produce nodules or produced white and inefficient nodules (Table 2). These results indicate that *L. mariae-josephae* and *L. micranthus* can be nodulated by a common *Bradyrhizobium* lineage.

(iii) Isolate LmiB3 appears to represent a new genospecies (close to *B. betae*) and a new symbiovar, far from other strains isolated from lupines or from other Genisteae legumes. In fact, the closest *nodC* sequences corresponded to *Bradyrhizobium* strains associated with *Acacia melanoxylon* grown in South China (53). At present, it is not clear

why isolates from geographically distant plants as diverse as *Lupinus* and *Acacia* have *nodC* genes that are so similar.

(iv) Finally, the new symbiovar A, formed by *Microvirga* and *Phyllobacterium* strains, displayed similarities to *Phyllobacterium* strains isolated from root nodules of *Sophora flavescens* grown in Changzhi County in northern China (26) and with *Mesorhizobium* and *Bradyrhizobium* strains from root nodules of *Caragana* spp. from arid and semiarid alkaline deserts, also in northern China (54). Since *Microvirga* strains have also been found in arid soils from other areas (22), the capacity to proliferate in arid soils may be an important feature of the symbiosis between *Microvirga* and *L. micranthus* in the semiarid soils of Tunisia.

In conclusion, our study evidenced a wide biodiversity among the symbiotic bacteria isolated from *L. micranthus* nodules from northern Tunisia, including three genera, *Bradyrhizobium* and the unusual fast-growing *Microvirga* and *Phyllobacterium*. Our data and those from other sources suggest that the edaphoclimatic factors might be important for determining the occurrence of different *L. micranthus* microsymbionts. Given that *L. micranthus* is widely distributed in the Mediterranean basin, further studies from different edaphoclimatic areas might aid in gaining an understanding of the biology and specificity of this symbiosis.

MATERIALS AND METHODS

Isolation of bacterial strains from *Lupinus micranthus* nodules and culture conditions. Endosymbiotic bacteria were isolated from field root nodules sampled from *L. micranthus* plants growing in five different locations in northern Tunisia separated by up to 120 km, namely Borj Hfaiedh, Hammamet, El Alia, Mraissa, and Takelsa (Table 1 and Fig. 1). Nodules were surface disinfected with 95% ethanol (1 min) and sodium hypochlorite (3 min) from a 1:4 dilution of commercial bleach. They were then rinsed 10 times with sterile distilled water and individually crushed on sterile plates. A loopful of the nodule suspension was streaked onto yeast extract mannitol agar (YMA) plates (55). Plates were incubated at 28°C until colonies were visualized, usually after 4 to 10 days. Single colonies were picked and checked for purity by repeated streaking on yeast extract mannitol medium and then maintained at 4°C. Isolates were maintained for long-term storage in yeast mannitol broth (YMB) containing 20% glycerol (vol/vol) at −80°C. The numbers, designations, and geographical origins of the isolates used in this study are listed in Table 1. All strains included in this study were checked for their ability to effectively renodulate *L. micranthus*.

Sizes and morphologies of colonies were assessed on YMA plates after 7 to 10 days of growth at 28°C. The generation times were determined by measuring the optical density at 600 nm (OD_{600}) every 2 h in a 50-ml TY culture grown in a 250-ml Erlenmeyer flask incubated at 28°C with shaking (140 rpm). The starting OD_{600} for each of the cultures was 0.05.

Nodulation and cross-inoculation experiments. The abilities of all purified isolates to renodulate their original hosts were demonstrated. *L. micranthus*, *L. angustifolius*, *Vigna unguiculata*, and *Macroptilium atropurpureum* seedlings were surface sterilized using 95% ethanol (1 min) and 25% sodium hypochlorite (3 min). *L. mariae-josephae* seeds required a scarification step by cutting the seed coat with a razor blade. Leonard jar units with 2 seedlings were inoculated with 1 ml of rhizobial suspension (10^9 cells ml^{-1}). Plants were grown under bacteriologically controlled conditions in a greenhouse (16/8 h day/night at 25/23°C) and watered with sterile Jensen's liquid medium once per week for 3 to 8 weeks depending on the legume host. Uninoculated plants were included as negative controls.

DNA isolation and PCR amplification. Total genomic DNA was isolated from a cell pellet from 2 ml of YMB culture by a simplified alkaline lysis procedure. The pellet was suspended in 20 μl of lysis solution (0.1 N NaOH and 0.5% SDS) and placed in hot water at 80 to 90°C for 15 min. The suspension was mixed with 100 μl of sterile distilled water and centrifuged at 13,000 $\times$ *g* for 10 min. Alternatively, DNA was isolated using DNeasy blood and tissue kit columns (Qiagen, Ltd.). PCR amplicons of genes coding for DNA recombination and repair protein RecA (*recA*), DNA gyrase subunit B (*gyrB*), 16S rRNA (*rrs*), glutamine synthetase 2 (*glnII*), chaperone protein DnaK (*dnaK*), and *N*-acetylglucosaminyltransferase (*nodC*) were obtained with primers and conditions previously described for *recA*, *rrs*, *glnII*, and *nodC* (12, 16) and for *gyrB* and *dnaK* (21). Amplicons were sequenced according to the procedures described by Sánchez-Cañizares et al. (12) and were used for phylogenetic analysis. Unincorporated primers and deoxynucleoside triphosphates (dNTPs) were removed from PCR products with the NucleoSpin Extract II (Macherey-Nagel) or, when needed, by gel electrophoresis followed by band purification with the same kit. Sequencing was performed at STABvida (Lisbon, Portugal) and sequences were edited and assembled with Geneious Pro 5.6.7 software (Biomatters, Ltd., Auckland, New Zealand).

Sequence and phylogenetic analysis. Alignments of *rrs* sequences were performed using SINA service from the SILVA database (http://www.arb-silva.de/aligner) (56). Other sequences were aligned using CLUSTALW (57). The phylogenetic and molecular evolutionary analyses were carried out with MEGA 6.06 (58). The neighbor-joining statistical method and the Kimura two-parameter model (59) were used for all genes. Phylogenetic trees were subjected to 1,000 bootstrap replications, and preferred topologies were plotted.

Accession number(s). GenBank accession numbers and whole-genome sequencing codes for the sequences obtained in this study are included in the corresponding trees in Fig. 2 (KX832514 to KX832521 and KX272812 to KX272841), Fig. 3 (KX784068 to KX784087), Fig. 4 (KX784060 to KX784067 and KX272785 to KX272796), and Fig. 5 (KX272800 to KX272811).

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REFERENCES

1. Cristofolini G. 1997. The biodiversity of the *Leguminosae-Genisteae* and its genesis. Lagascalia 19:121–128.
2. Ainouche AK, Bayer RJ. 1999. Phylogenetic relationships in *Lupinus* (*Fabaceae: Papilionoideae*) based on internal transcribed spacer sequences (ITS) of nuclear ribosomal DNA. Am J Bot 86:590–607. https://doi.org/10.2307/2656820.
3. Drummond CS, Eastwood RJ, Miotto STS, Hughes CE. 2012. Multiple continental radiations and correlates of diversification in *Lupinus* (*Leguminosae*): testing for key innovation with incomplete taxon sampling. Syst Biol 61:443–460. https://doi.org/10.1093/sysbio/syr126.
4. Mahé F, Pascual H, Coriton O, Huteau V, Navarro Perris A, Misset M-T, Aınouche A. 2011. New data and phylogenetic placement of the enigmatic Old World lupin: *Lupinus mariae-josephi* H. Pascual. Genet Resour Crop Evol 58:101–114. https://doi.org/10.1007/s10722-010-9580-6.
5. Gladstones JS. 1988. Distribution, origin, taxonomy, history and importance, p 1–37. *In* Gladstones JS, Atkins CA, Hamblin J (ed), *Lupins* as crop plants: biology, production and utilization. CAB International, Wallingford, United Kingdom.
6. Barrera LL, Trujillo ME, Goodfellow M, García FJ, Hernández-Lucas I, Dávila G, van Berkum P, Martínez-Romero E. 1997. Biodiversity of bradyrhizobia nodulating *Lupinus* spp. Int J Syst Bacteriol 47:1086–1091. https://doi.org/10.1099/00207713-47-4-1086.
7. Jarabo-Lorenzo A, Pérez-Galdona R, Donate-Correa J, Rivas R, Velázquez E, Hernández M, Temprano F, Martínez-Molina E, Ruiz-Argüeso T, León-Barrios M. 2003. Genetic diversity of bradyrhizobial populations from diverse geographic origins that nodulate *Lupinus* spp. and *Ornithopus* spp. Syst Appl Microbiol 26:611–623. https://doi.org/10.1078/072320203770865927.
8. Stępkowski T, Żak M, Moulin L, Króliczak J, Golińska B, Narożna D, Safronova VI, Mądrzak CJ. 2011. *Bradyrhizobium canariense* and *Bradyrhizobium japonicum* are the two dominant rhizobium species in root nodules of lupin and serradella plants growing in Europe. Syst Appl Microbiol 34:368–375. https://doi.org/10.1016/j.syapm.2011.03.002.
9. Velázquez E, Valverde A, Rivas R, Gomis V, Peix A, Gantois I, Igual JM, León-Barrios M, Willems A, Mateos PF, Martínez-Molina E. 2010. Strains nodulating *Lupinus albus* on different continents belong to several new chromosomal and symbiotic lineages within *Bradyrhizobium*. Antonie Van Leeuwenhoek 97:363–376. https://doi.org/10.1007/s10482-010-9415-7.
10. Granada CE, Beneduzi A, Lisboa BB, Turchetto-Zolet AC, Vargas LK, Passaglia LM. 2015. Multilocus sequence analysis reveals taxonomic differences among *Bradyrhizobium* sp. symbionts of *Lupinus albescens* plants growing in arenized and non-arenized areas. Syst Appl Microbiol 38:323–329. https://doi.org/10.1016/j.syapm.2015.03.009.
11. Durán D, Rey L, Navarro A, Busquets A, Imperial J, Ruiz-Argüeso T. 2014. *Bradyrhizobium valentinum* sp. nov., isolated from effective nodules of *Lupinus mariae-josephae*, a lupine endemic of basic-lime soils in Eastern Spain. Syst Appl Microbiol 37:336–341. https://doi.org/10.1016/j.syapm.2014.05.002.
12. Sánchez-Cañizares C, Rey L, Durán D, Temprano F, Sánchez-Jiménez P, Navarro A, Polajnar M, Imperial J, Ruiz-Argüeso T. 2011. Endosymbiotic bacteria nodulating a new endemic lupine *Lupinus mariae-josephi* from alkaline soils in Eastern Spain represent a new lineage within the *Bradyrhizobium* genus. Syst Appl Microbiol 34:207–215. https://doi.org/10.1016/j.syapm.2010.11.020.
13. Trujillo ME, Willems A, Abril A, Planchuelo AM, Rivas R, Ludena D, Mateos PF, Martínez-Molina E, Velázquez E. 2005. Nodulation of *Lupinus albus* by strains of *Ochrobactrum lupini* sp. nov. Appl Environ Microbiol 71:1318–1327. https://doi.org/10.1128/AEM.71.3.1318-1327.2005.
14. Andam CP, Parker MA. 2007. Novel alphaproteobacterial root nodule symbiont associated with *Lupinus texensis*. Appl Environ Microbiol 73:5687–5691. https://doi.org/10.1128/AEM.01413-07.
15. Ardley JK, Parker MA, De Meyer SE, Trengove RD, O'Hara GW, Reeve WG, Yates RJ, Dilworth MJ, Willems A, Howieson JG. 2012. *Microvirga lupini* sp. nov., *Microvirga lotononidis* sp. nov. and *Microvirga zambiensis* sp. nov are alphaproteobacterial root-nodule bacteria that specifically nodulate and fix nitrogen with geographically and taxonomically separate legume hosts. Int J Syst Evol Microbiol 62:2579–2588. https://doi.org/10.1099/ijs.0.035097-0.
16. Bourebaba Y, Durán D, Boulila F, Ahnia H, Boulila A, Temprano F, Palacios JM, Imperial J, Ruiz-Argüeso T, Rey L. 2016. Diversity of *Bradyrhizobium* strains nodulating *Lupinus micranthus* on both sides of the western Mediterranean: Algeria and Spain. Syst Appl Microbiol 39:266–274. https://doi.org/10.1016/j.syapm.2016.04.006.
17. Pottier-Alapetite G. 1979. Flore de la Tunisie: angiospermes-dicotylédones, p 299–302. (In French.) Ministère de l'Enseignement supérieur et de la Recherche scientifique et ministère de l'Agriculture, Paris, France.
18. Amin A, Ahmed I, Habib N, Abbas S, Hasan F, Xiao M, Hozzein WN, Li WJ. 2016. *Microvirga pakistanensis* sp. nov., a novel bacterium isolated from desert soil of Cholistan, Pakistan. Arch Microbiol 198:933–939. https://doi.org/10.1007/s00203-016-1251-3.
19. Caputo A, Lagier JC, Azza S, Robert C, Mouelhi D, Fournier PE, Raoult D. 2016. *Microvirga massiliensis* sp. nov., the human commensal with the largest genome. Microbiologyopen 5:307–322. https://doi.org/10.1002/mbo3.329.
20. Kanso S, Patel BK. 2003. *Microvirga subterranea* gen. nov., sp. nov., a moderate thermophile from a deep subsurface Australian thermal aquifer. Int J Syst Evol Microbiol 53:401–406. https://doi.org/10.1099/ijs.0.02348-0.
21. Radl V, Simões-Araújo JL, Leite J, Passos SR, Martins LMV, Xavier GR, Rumjanek NG, Baldani JI, Zilli JE. 2014. *Microvirga vignae* sp. nov., a root nodule symbiotic bacterium isolated from *cowpea* grown in semi-arid Brazil. Int J Syst Evol Microbiol 64:725–730. https://doi.org/10.1099/ijs.0.053082-0.
22. Veyisoglu A, Tatar D, Saygin H, Inan K, Cetin D, Guven K, Tuncer M, Sahin N. 2016. *Microvirga makkahensis* sp. nov., and *Microvirga arabica* sp. nov., isolated from sandy arid soil. Antonie Van Leeuwenhoek 109:287–296. https://doi.org/10.1007/s10482-015-0631-z.
23. Weon H-Y, Kwon S-W, Son J-A, Jo E-H, Kim S-J, Kim Y-S, Kim B-Y, Ka J-O. 2010. Description of *Microvirga aerophila* sp.nov. and *Microvirga aerilata* sp. nov., isolated from air, reclassification of *Balneimonas flocculans* Takeda et al. 2004 as *Microvirga flocculans* comb. nov. and emended description of the genus *Microvirga*. Int J Syst Evol Microbiol 60:2596–2600. https://doi.org/10.1099/ijs.0.018770-0.
24. Zhang J, Song F, Xin YH, Zhang J, Fang C. 2009. *Microvirga guangxiensis* sp. nov., a novel alpha proteobacterium from soil, and emended description of the genus *Microvirga*. Int J Syst Evol Microbiol 59:1997–2001. https://doi.org/10.1099/ijs.0.007997-0.
25. Knösel DH. 1962. Prufung von Bakterien auf Fahigkeit zur Sternbildung. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg II Abt 116:79–100.
26. Jiao YS, Yan H, Ji ZJ, Liu YH, Sui XH, Zhang XX, Wang ET, Chen WX, Chen

WF. 2015. *Phyllobacterium sophorae* sp. nov., a symbiotic bacterium isolated from root nodules of *Sophora flavescens*. Int J Syst Evol Microbiol 65:399–406. https://doi.org/10.1099/ijs.0.067017-0.
27. Valverde A, Velázquez E, Fernández-Santos F, Vizcaíno N, Rivas R, Mateos PF, Igual JM, Willems A. 2005. *Phyllobacterium trifolii* sp. nov., nodulating *Trifolium* and *Lupinus* in Spanish soils. Int J Syst Evol Microbiol 55:1985–1989. https://doi.org/10.1099/ijs.0.63551-0.
28. Mantelin S, Fischer-Le Saux M, Zakhia F, Béna G, Bonneau S, Jeder H, de Lajudie P, Cleyet-Marel JC. 2006. Emended description of the genus *Phyllobacterium* and description of four novel species associated with plant roots: *Phyllobacterium bourgognense* sp. nov., *Phyllobacterium ifriqiyense* sp. nov., *Phyllobacterium leguminum* sp. nov. and *Phyllobacterium brassicacearum* sp nov. Int J Syst Evol Microbiol 56:827–839. https://doi.org/10.1099/ijs.0.63911-0.
29. Sánchez M, Ramírez-Bahena MH, Peix A, Lorite M, Sanjuán J, Velázquez E, Monza J. 2014. *Phyllobacterium loti* sp. nov. isolated from nodules of *Lotus corniculatus*. Int J Syst Evol Microbiol 64:781–786. https://doi.org/10.1099/ijs.0.052993-0.
30. Flores-Félix JD, Carro L, Velázquez E, Valverde A, Cerda-Castillo E, García-Fraile P, Rivas R. 2013. *Phyllobacterium endophyticum* sp. nov., isolated from nodules of *Phaseolus vulgaris*. Int J Syst Evol Microbiol 63:821–826. https://doi.org/10.1099/ijs.0.038497-0.
31. Mergaert J, Cnockaert MC, Swings J. 2002. *Phyllobacterium myrsinacearum* (subjective synonym *Phyllobacterium rubiacearum*) emend. Int J Syst Evol Microbiol 52:1821–1823. https://doi.org/10.1099/00207713-52-5-1821.
32. Rogel MA, Ormeño-Orrillo E, Martínez-Romero E. 2011. Symbiovars in rhizobia reflect bacterial adaptation to legumes. Syst Appl Microbiol 34:96–104. https://doi.org/10.1016/j.syapm.2010.11.015.
33. Ahnia H, Boulila F, Boulila A, Boucheffa K, Durán D, Bourebaba Y, Salmi A, Imperial J, Ruiz-Argüeso T, Rey L. 2014. *Cytisus villosus* from northeastern Algeria is nodulated by genetically diverse *Bradyrhizobium* strains. Antonie Van Leeuwenhoek 105:1121–1129. https://doi.org/10.1007/s10482-014-0173-9.
34. Durán D, Rey L, Sánchez-Cañizares C, Navarro A, Imperial J, Ruiz-Argüeso T. 2013. Genetic diversity of indigenous rhizobial symbionts of the *Lupinus mariae-josephae* endemism from alkaline-limed soils within its area of distribution in eastern Spain. Syst Appl Microbiol 36:128–136. https://doi.org/10.1016/j.syapm.2012.10.008.
35. Bailey AC, Kellom M, Poret-Peterson AT, Noonan K, Hartnett HE, Raymond J. 2014. Draft genome sequence of *Microvirga* sp. strain BSC39, isolated from biological soil crust of Moab, Utah. Genome Announc 2:e01197-14. https://doi.org/10.1128/genomeA.01197-14.
36. Reeve W, Ardley J, Tian R, Meyer S, Terpolilli J, Melino V, Tiwari R, Yates R, O'Hara G, Howieson J, Ninawi M, Zhang X, Bruce D, Detter C, Tapia R, Han C, Wei CL, Huntemann M, Han J, Chen IM, Mavromatis K, Markowitz V, Szeto E, Ivanova N, Pagani I, Pati A, Goodwin L, Woyke T, Kyrpides N. 2013. Genome sequence of the *Listia angolensis* microsymbiont *Microvirga lotononidis* strain WSM3557^T. Stand Genomic Sci 9:540–550. https://doi.org/10.4056/sigs.4548266.
37. Reeve W, Parker M, Tian R, Goodwin L, Teshima H, Tapia R, Han C, Han J, Liolios K, Huntemann M, Pati A, Woyke T, Mavromatis K, Markowitz V, Ivanova N, Kyrpides N. 2014. Genome sequence of *Microvirga lupini* strain LUT6(T), a novel *Lupinus* alphaproteobacterial microsymbiont from Texas. Stand Genomic Sci 9:1159–1167. https://doi.org/10.4056/sigs.5249382.
38. Zhang YM, Tian CF, Sui XH, Chen WF, Chen WX. 2012. Robust markers reflecting phylogeny and taxonomy of rhizobia. PLoS One 7:e44936. https://doi.org/10.1371/journal.pone.0044936.
39. Peix A, Ramírez-Bahena MH, Velázquez E, Bedmar EJ. 2015. Bacterial associations with legumes. CRC Crit Rev Plant Sci 34:17–42. https://doi.org/10.1080/07352689.2014.897899.
40. Guerrouj K, Ruíz-Díez B, Chahboune R, Ramírez-Bahena MH, Abdelmoumen H, Quinones MA, El Idrissi MM, Velázquez E, Fernández-Pascual M, Bedmar EJ, Peix A. 2013. Definition of a novel symbiovar (sv. *retamae*) within *Bradyrhizobium retamae* sp. nov., nodulating *Retama sphaerocarpa* and *Retama monosperma*. Syst Appl Microbiol 36:218–223. https://doi.org/10.1016/j.syapm.2013.03.001.
41. Cobo-Díaz JF, Martínez-Hidalgo P, Fernández-González AJ, Martínez-Molina E, Toro N, Velázquez E, Fernández-López M. 2014. The endemic *Genista versicolor* from Sierra Nevada National Park in Spain is nodulated by putative new *Bradyrhizobium* species and a novel symbiovar (*sierranevadense*). Syst Appl Microbiol 37:177–185. https://doi.org/10.1016/j.syapm.2013.09.008.
42. Stępkowski T, Hughes CE, Law IJ, Markiewicz Ł, Gurda D, Chlebicka A, Moulin L. 2007. Diversification of lupine *Bradyrhizobium* strains: evidence from nodulation gene trees. Appl Environ Microbiol 73:3254–3264. https://doi.org/10.1128/AEM.02125-06.
43. Menna P, Hungria M. 2011. Phylogeny of nodulation and nitrogen-fixation genes in *Bradyrhizobium*: supporting evidence for the theory of monophyletic origin, and spread and maintenance by both horizontal and vertical transfer. Int J Syst Evol Microbiol 61:3052–3067. https://doi.org/10.1099/ijs.0.028803-0.
44. Moulin L, Béna G, Boivin-Masson C, Stępkowski T. 2004. Phylogenetic analyses of symbiotic nodulation genes support vertical and lateral gene co-transfer within the *Bradyrhizobium* genus. Mol Phylogenet Evol 30:720–732. https://doi.org/10.1016/S1055-7903(03)00255-0.
45. Vinuesa P, León-Barrios M, Silva C, Willems A, Jarabo-Lorenzo A, Pérez Galdona R, Werner D, Martínez-Romero E. 2005. *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates endemic *genistoid* legumes (*Papilionoideae: Genisteae*) from the Canary Islands, along with *Bradyrhizobium japonicum* bv. *genistearum*, *Bradyrhizobium* genospecies alpha and *Bradyrhizobium* genospecies beta. Int J Syst Evol Microbiol 55:569–575. https://doi.org/10.1099/ijs.0.63292-0.
46. Boatwright JS, Marianne MR, Wink M, Morozova T, van Wyk BE. 2008. Phylogenetic relationships of tribe *Crotalarieae* (*Fabaceae*) inferred from DNA sequences and morphology. Syst Bot 33:752–761. https://doi.org/10.1600/036364408786500271.
47. Bromfield ESP, Tambong JT, Cloutier S, Prévost D, Laguerre G, van Berkum P, Thi TVT, Assabgui R, Barran LR. 2010. *Ensifer*, *Phyllobacterium* and *Rhizobium* species occupy nodules of *Medicago sativa* (alfalfa) and *Melilotus alba* (sweet clover) grown at a Canadian site without a history of cultivation. Microbiology 156:505–520. https://doi.org/10.1099/mic.0.034058-0.
48. Rasolomampianina R, Bailly X, Fetiarison R, Rabevohitra R, Béna G, Ramaroson L, Raherimandimby M, Moulin L, De Lajudie P, Dreyfus B, Avarre JC. 2005. Nitrogen-fixing nodules from rose wood legume trees (*Dalbergia* spp.) endemic to Madagascar host seven different genera belonging to α- and β-*Proteobacteria*. Mol Ecol 14:4135–4146. https://doi.org/10.1111/j.1365-294X.2005.02730.x.
49. Rodríguez-Echeverría S, Moreno S, Bedmar EJ. 2014. Genetic diversity of root nodulating bacteria associated with *Retama sphaerocarpa* in sites with different soil and environmental conditions. Syst Appl Microbiol 37:305–310. https://doi.org/10.1016/j.syapm.2013.11.003.
50. Kalita M, Stępkowski T, Łotocka B, Małek W. 2006. Phylogeny of nodulation genes and symbiotic properties of *Genista tinctoria* bradyrhizobia. Arch Microbiol 186:87–97. https://doi.org/10.1007/s00203-006-0124-6.
51. Parker MA. 2012. Legumes select symbiosis island sequence variants in *Bradyrhizobium*. Mol Ecol 21:1769–1778. https://doi.org/10.1111/j.1365-294X.2012.05497.x.
52. Chahboune R, Barrijal S, Moreno S, Bedmar EJ. 2011. Characterization of *Bradyrhizobium* species isolated from root nodules of *Cytisus villosus* grown in Morocco. Syst Appl Microbiol 34:440–445. https://doi.org/10.1016/j.syapm.2011.04.008.
53. Lu JK, Dou YJ, Zhu YJ, Wang SK, Sui XH, Kang LH. 2014. *Bradyrhizobium ganzhouense* sp. nov., an effective symbiotic bacterium isolated from *Acacia melanoxylon* R. Br. nodules. Int J Syst Evol Microbiol 64:1900–1905. https://doi.org/10.1099/ijs.0.056564-0.
54. Li M, Li Y, Chen WF, Sui XH, Li Y, Li Y, Wang ET, Chen WX. 2012. Genetic diversity, community structure and distribution of rhizobia in the root nodules of *Caragana* spp. from arid and semi-arid alkaline deserts, in the north of China. Syst Appl Microbiol 35:239–245. https://doi.org/10.1016/j.syapm.2012.02.004.
55. Vincent JM. 1970. A manual for the practical study of root nodule bacteria. International biological programme, number 15. Blackwell Scientific, Oxford, United Kingdom.
56. Pruesse E, Peplies J, Glockner FO. 2012. SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. Bioinformatics 28:1823–1829. https://doi.org/10.1093/bioinformatics/bts252.
57. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31:3497–3500. https://doi.org/10.1093/nar/gkg500.
58. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30:2725–2729. https://doi.org/10.1093/molbev/mst197.
59. Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120. https://doi.org/10.1007/BF01731581.