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
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 Genistae 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. trifoli*, 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. trifoli* 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...
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

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

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

aAbove sea level.
bGroups (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.

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**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.ingref.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 isolates.
other Microvirga 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). 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 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, 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*.

**FIG 2** Legend (Continued)
TABLE 2 Symbiotic characteristics and generation times of Tunisian Lupinus micranthus representative isolates

<table>
<thead>
<tr>
<th>Lmi isolate</th>
<th>Symbiovar</th>
<th>Group</th>
<th>Generation time (h)</th>
<th>Nodulation phenotype with host</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lmi</td>
</tr>
<tr>
<td>T21</td>
<td>A</td>
<td>VI</td>
<td>5.5</td>
<td>+</td>
</tr>
<tr>
<td>E10</td>
<td>A</td>
<td>VII</td>
<td>4.8</td>
<td>+</td>
</tr>
<tr>
<td>M8</td>
<td>A</td>
<td>VII</td>
<td>4.6</td>
<td>+</td>
</tr>
<tr>
<td>H4</td>
<td>B</td>
<td>IV</td>
<td>14.4</td>
<td>+</td>
</tr>
<tr>
<td>M2</td>
<td>B</td>
<td>III</td>
<td>22.3</td>
<td>+</td>
</tr>
<tr>
<td>B3</td>
<td>C</td>
<td>II</td>
<td>9.4</td>
<td>+</td>
</tr>
<tr>
<td>T14</td>
<td><em>genistearum</em></td>
<td>I</td>
<td>13.1</td>
<td>+</td>
</tr>
<tr>
<td>T3</td>
<td><em>genistearum</em></td>
<td>I</td>
<td>11.5</td>
<td>+</td>
</tr>
<tr>
<td>B5</td>
<td><em>genistearum</em></td>
<td>V</td>
<td>16.4</td>
<td>+</td>
</tr>
</tbody>
</table>

As shown in Fig. 4.


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, *Lupinus micranthus*; Lan, *Lupinus angustifolius*; Lmj, *Lupinus mariae-josephae*; Vun, *Vigna unguiculata*; Mat, *Macroptilium atropurpureum*.

Generation times in TY medium at 28°C.

*As shown in Fig. 4.*


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, *Lupinus micranthus*; Lan, *Lupinus angustifolius*; Lmj, *Lupinus mariae-josephae*; Vun, *Vigna unguiculata*; Mat, *Macroptilium atropurpureum*.

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 LmiC3 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 Genistae 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...
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 *genisteae* (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.
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 *Phyllobacterium* 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 semi-arid 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 semi-arid 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. Endo-symbiotic 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 node 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 _Macrotritium latifolium_ seedlings were surface sterilized using 95% ethanol (1 min) and 25% sodium hypochlorite (3 min). _L. mariae-josephii_ 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 × 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 STABviva (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.
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