Article

Phylogenetic Analyses of Rhizobia Isolated from Nodules of *Lupinus angustifolius* in Northern Tunisia Reveal *Devosia* sp. as a New Microsymbiont of Lupin Species

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Abstract: Thirty-two bacterial isolates were obtained from root nodules of *Lupinus angustifolius* growing in Northern Tunisia. Phylogenetic analyses based on *recA* and *gyrB* partial gene sequences grouped the strains into six clusters: four clusters belonged to the genus *Bradyrhizobium* (22 isolates), one to *Microvirga* (8 isolates) and one to *Devosia* (2 isolates), a genus that has not been previously reported to nodulate lupin. Representative strains of each group were further characterized. Multi-Locus Sequence Analysis (MLSA) based on *recA* and *glnII* gene sequences separated the strains within the genus *Bradyrhizobium* into four divergent clusters related to *B. canariense*, *B. liaoningense*, *B. lupini*, and *B. algeriense*, respectively. The latter might constitute a new *Bradyrhizobium* species. The strains in the *Microvirga* cluster showed high identity with *M. tunisiensis*. The *Devosia* isolates might also represent a new species within this genus. An additional phylogenetic analysis based on the symbiotic gene *nodC* affiliated the strains to symbiovars *genistearum*, *mediterraneense*, and to a possibly new symbiovar. These results altogether contributed to the existing knowledge on the genetic diversity of lupin-nodulating microsymbionts and revealed a likely new, fast-growing, salt-tolerant rhizobial species within the genus *Devosia* as a potentially useful inoculant in agricultural practices or landscape restoration.

Keywords: *Lupinus angustifolius*; lupin; rhizobium; *Devosia*; *Bradyrhizobium*; *Microvirga*; microsymbiont; biodiversity; microbiota; Tunisia

Citation: Msaddak, A.; Rey, L.; Imperial, J.; Palacios, J.M.; Mars, M.; Pueyo, J.J. Phylogenetic Analyses of Rhizobia Isolated from Nodules of *Lupinus angustifolius* in Northern Tunisia Reveal *Devosia* sp. as a New Microsymbiont of Lupin Species. *Agronomy* **2021**, *11*, 1510. <https://doi.org/10.3390/agronomy11081510>

Academic Editor: Alwyn Williams

Received: 16 June 2021

Accepted: 26 July 2021

Published: 29 July 2021

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1. Introduction

The *Leguminosae* or *Fabaceae* family includes more than 19,700 species, many of which are agronomically important [1]. The *Genistee* tribe is presently considered to include 618 species in 25 genera [2]. *Lupinus* is considered the largest *Genistee* genus in terms of species number with around 270 species [3,4]. This genus may be divided into two groups, the first of which is centered in the Mediterranean Basin and the Canary Islands [5,6] and comprises 13 Old World species with smooth and rough seeds [3,7]. The second group includes the New World species [8]. The New World species number ranges from 200 to 400 located in the Americas. Lupins are able to colonize very different environments and diverse ecosystems [9], including marginal impoverished or contaminated soils [10]. *L. angustifolius*, commonly named narrow leaf lupin and blue lupin, grows wild in agricultural lands as well as in the wetlands of coastal plains in the Northern region of Tunisia.

Lupins, partly due to their effective nitrogen-fixing symbiosis with different soil bacteria (collectively called rhizobia), have been grown since antiquity as an important

pulse crop and as a green manure. *Lupinus* species have been studied from various perspectives as indicators of phosphorus and nitrogen in soils poor in nutrients, because of their dual efficacy in fixing atmospheric nitrogen and solubilizing soil phosphates [11], phytoremediation of polluted soils [12,13] and as medicinal plants [14]. The high protein content of lupin seeds is similar to that of soybean, and lupin cultivation has been suggested as an alternative to soybean in Europe [15], as new sources are needed to meet the increasing demand for plant protein [16].

Rhizobia belong to the Alpha and Betaproteobacteria [17]. Legume-rhizobium symbioses provide a net input of nitrogen into the ecosystems. The great diversity of lupin species reflects a vast diversity of their nodulating rhizobia. *Lupinus* species are mainly nodulated by slow-growing bacteria that belong to the genus *Bradyrhizobium*. The major species are *B. canariense*, *B. japonicum* [18–21], *B. elkanii* [22], and *B. valentinum* [23]. Other fast-growing rhizobia species, such as *Ochrobactrum lupini* [24], *Mesorhizobium loti* [25], *Phyllobacterium trifolii* [26] and *Microvirga* spp. [27–29] can also effectively nodulate different *Lupinus* spp. Recently, several microsymbionts isolated from *L. albus* growing in Tunisian soils were identified as species within the genera *Agrobacterium*, *Rhizobium* and *Neorhizobium* [30].

The genus *Devosia* was defined as a result of the reclassification of *Pseudomonas riboflavin* as *Devosia riboflavina* [31]. *Devosia* bacteria are aerobic, Gram-negative, rod-shaped or oval and they do not form spores. Twenty-one validated species have been identified within the genus (<http://www.bacterio.net/devosia.html>, accessed on 16 July 2020). Some *Devosia* species have been reported to nodulate legumes [32–34]. We have previously reported that *L. angustifolius* from Tunisia was nodulated by new lineages within the *Bradyrhizobium* and *Microvirga* genera [35], but, to the best of our knowledge, there are no reports on *Devosia* symbiotic nitrogen-fixing bacteria that nodulate *Lupinus* species.

The main objective of the present paper was to further investigate and characterize the diversity of the rhizobia that nodulate *L. angustifolius* growing in different sites in Northern Tunisia.

2. Materials and Methods

2.1. Sampling Sites, Isolation of Bacterial Strains and Culture Conditions

Effective nodules were collected from *L. angustifolius* roots growing wild or in soil collected from six different locations in Northern Tunisia: *Mraissa*, *Takelsa*, *Borj Hfaied*, *Hammamet*, *Sejnane* and *Tabarka*. The ecological and climatic characterization of the sampling sites is indicated in Table 1.

Nodules were surface-sterilized with 95% ethanol for 1 min, 25% sodium hypochlorite for 3 min, then rinsed several times with sterile distilled water; they were individually crushed on sterile plates, and a loopful of nodule material was streaked onto yeast-mannitol agar (YMA) [36]. Plates were then incubated aerobically at 28 °C and colonies could be visualized after 4 to 10 days.

Single colonies were streaked on fresh YMA plates. Pure isolates were maintained on YMA plates at 4 °C for temporary preservation, or stored in 20% (*w/v*) glycerol suspensions at −80 °C. The isolates used in this study are listed in Table 1.

2.2. Nodulation and Cross-Inoculation Test

The ability of all purified isolates to nodulate their original host was assessed. The isolates were also tested for nodulation on *L. luteus*, *L. micranthus*, *L. mariae-josephae*, *Retama sphaerocarpa*, *Vigna unguiculata* and *Glycine max*. Seed germination and plant inoculation were carried out as previously described [37]. Plants were grown under bacteriologically controlled conditions in a greenhouse for 3 to 8 weeks, depending on the legume host, with a 16.0/8.0 h light/dark photoperiod at 25/23 °C, and they were watered with sterile Jensen's liquid medium once a week. Three uninoculated Leonard jars per legume were included as negative controls. Nodules were examined for number and inside color. Three replicates of each strain were used in the nodulation tests.

Table 1. Designations and geographical origins of *L. angustifolius* endosymbionts in Northern Tunisia.

Site	Number of Isolates	Isolate Code	Isolate Code Number	Soil pH	Environment	Bioclimatic Stage	Soil Texture
Takelsa	5	LanT	1, 3, 5, 6, 7	7.5	Area with orange trees	semi-arid superior	sandy
Mraissa	5	LanM	4, 5, 6, 7, 9	8.0	Area planted with cereals	semi-arid superior	sandy
Borj Hfaiedh	2	LanB	5, 6	8.5	Area with olive trees	semi-arid superior	sandy
Hammamet	3	LanH	1, 2, 5	9.0	Semiurban area, next to the sea	semi-arid superior	sandy
Sejnane	2	LanS	2, 5	7.5	Semiurban area	humid	clay
Tabarka	15	LanTb	3, 4, 5, 6, 8, 9, 11, 12, 14, 15, 16, 17, 18, 19, 20	6.5	Area planted with cereals	humid	sandy

2.3. DNA Isolation, PCR Amplifications and Sequencing

Bacterial DNA was obtained by the alkaline lysis method [38]. PCR was performed in a 25 µL volume containing DNA (5–10 ng), 2.5 µL of 10× PCR buffer containing magnesium chloride (Roche Applied Science), 10 µM of each primer, 10 mM of each dNTP, 1 µL DMSO and 1 U of Taq DNA polymerase (Roche Applied Science, Mannheim, Germany).

PCR amplicons of genes coding for DNA recombination and repair protein RecA (*recA*), DNA gyrase subunit B (*gyrB*), 16S rRNA (*rrs*), chaperone protein DnaK (*dnaK*), glutamine synthetase 2 (*glnII*), and N-acetylglucosaminyl transferase (*nodC*) were obtained using previously described primers and conditions [37,39]. PCR amplifications of *recA*, 16S rRNA, *glnII* and *nodC* genes were performed and amplicons were sequenced as previously reported [37]. *nifH* was amplified by using the appropriate primers with PCR conditions previously described [34].

glnII appeared to be absent in the genomes of *Microvirga* spp., as it could not be retrieved from data banks or from any sequenced *Microvirga* genomes. Hence, *dnaK* was used, and the phylogenetic tree for this group of strains was assembled using *gyrB* and *dnaK* concatenated sequences.

Amplification products were purified from the unincorporated primers and (deoxynucleosidetriphosphates (dNTPs) with the NucleoSpin® Extract II (Macherey-Nagel, Dueren, Germany) or by gel electrophoresis followed by band purification with the same kit, if necessary. Sequencing was performed externally at Secugen (Madrid, Spain) and STAB Vida (Lisbon, Portugal).

2.4. Phenotypic Characterization

Cell morphology and size after growth on YMA agar plates at 28 °C for 10 days were visualized. The growth temperature range (20–40 °C) was tested on YMA plates at pH 7.0 for 10 days. pH tolerance was determined over the pH range 4.0–12.0 at one unit intervals. The resistance to various antibiotics (ampicillin: 100 µg/mL, gentamicin: 30 µg/mL, tetracycline: 5 µg/mL, spectinomycin: 50 µg/mL, kanamycin: 50 µg/mL, streptomycin: 10 µg/mL and chloramphenicol: 10 µg/mL), as well as salt tolerance (0–5%, w/v), were assessed on YMA plates at pH = 7.0 after 10 days at 28 °C. All phenotypic analyses were performed in triplicate.

2.5. Phylogenetic Analyses

We used a polyphasic approach including genetic analyses by sequencing of house-keeping (*rrs*, *recA*, *glnII*, *gyrB* and *dnaK*) and symbiotic (*nodC* and *nifH*) genes. Sequences were compared with those in GenBank using BLASTN (<http://www.ncbi.nlm.nih.gov/blast>, accessed on 6 March 2021) and the EzBioCloud Database (<https://www.ezbiocloud.net/>, accessed on 6 March 2021), and aligned with CLUSTALW.

Phylogenetic and molecular evolutionary analyses were conducted using the MEGA7.0 software [40]. The maximum likelihood (ML) and neighbor-joining (NJ) statistical methods

and the Kimura two-parameter model were used for gene and for multilocus sequence analysis (MLSA) (combined sequences of *glnII* and *recA*; *gyrB* and *dnaK*). ML and NJ analyses provided similar results. NJ phylogenetic trees are shown in Figures. Phylogenetic trees were bootstrapped with 1000 bootstrap replications. Accession numbers are shown in the tree figures and were obtained after deposit in the Genbank sequence database.

3. Results

3.1. Isolation and Symbiotic Efficiency of *L. angustifolius*-Nodulating Bacteria from Northern Tunisia

A total of 32 rhizobial bacteria were isolated from *L. angustifolius* nodules growing naturally in six different areas in Northern Tunisia (Table 1). These isolates were tested for re-nodulation and efficient nitrogen-fixing activity by re-inoculating *L. angustifolius* seedlings and growing the plants under bacteriologically axenic conditions. All isolates were able to nodulate their original host, as corroborated by the formation of nodules that were pink inside, which is indicative of active nodules. The nodule number ranged from 3 to 10 per plant. Indirect nitrogen fixation effectiveness could be assessed by the dark green color of the leaves compared to uninoculated yellowish control plant leaves, and by visual observation of pink-red color due to the leghemoglobin presence inside the nodules.

3.2. Phylogenetic Analysis Based on *recA* and *gyrB* Genes

The *recA* gene was amplified and sequenced for all isolates. The sequences were compared with those available in GenBank. As shown in the *recA* phylogenetic tree topology (Figure 1a), *L. angustifolius* strains are grouped in six different clades, four within the *Bradyrhizobium* genus (22 strains) and one with *Devosia* (2 strains). A sixth group, based on *gyrB* gene phylogeny, formed by *Microvirga* (8 strains) is shown in Figure 1b. The bradyrhizobia subgroups contained 17 strains affiliated to *B. lupini*, 2 strains to *B. canariense*, 1 to *B. liaoningense* (86%) and 2 strains to *B. algeriense*. At least one representative isolate of each pattern was selected for further phylogenetic analyses.

In order to define a more phylogenetically robust position of the strains, an analysis based on the concatenation of relevant housekeeping genes was performed. *recA* and *glnII* were used for *Bradyrhizobium* strains as they have been proven to be appropriate in phylogenetic studies of this genus [23,37]. *glnII* could not be identified in any *Microvirga* strains draft genome sequences. Thus, *dnaK* was used instead, and the phylogenetic tree for this group of strains was made using *gyrB* and *dnaK* concatenated sequences. The trees again distributed *L. angustifolius* nodulating strains in six distinct groups, four (I, II, III and IV) contained all the bradyrhizobia, one (V) grouped the *Devosia* strain and one (VI) comprised the *Microvirga* strains (Figure 2a,b).

Strains LanT1, LanTb3, LanM5 and LanTb18 clustered with *B. lupini* USDA3051. LanS2 and LanTb17 grouped with *B. canariense* BTA-1 and *B. liaoningense* LMG18230, respectively. LanH1 occupied a position between *B. valentinum* LmjM3 and *B. algeriense* RST89. LanTb5 was located in a taxonomic position separate from the *Bradyrhizobium* spp., which suggested that it might constitute a new species under a different genus. The phylogenetic tree based on the concatenated *gyrB* and *dnaK* sequences (Figure 2b) revealed that strains in Group VI (LanM6, LanTb9 and LanH5) grouped with *M. tunisiensis* LmiM8^T with almost 100% identity.

(a)

Figure 1. Cont.

Figure 1. Neighbor-joining phylogenetic trees based on housekeeping *recA* (a) and *gyrB* (b) gene sequences of Tunisian *L. angustifolius*-nodulating isolates in this study and reference strains. Black diamonds (◆) indicate Tunisian strains. Bootstrap values (above 70%) using 1000 replicates are indicated at branching points. Accession numbers are shown in brackets. Bars: 0.1 and 0.05 estimated substitutions, respectively.

3.3. 16S rRNA Gene Phylogeny

Phylogenetic analysis of the nearly full length sequences of 16S rRNA gene of 11 representative strains showed five large groups (Figure 3). Groups I and II of the analysis based on housekeeping genes form a single group when analyzed based on the *rrs* gene. This group contained five strains (LanTb18, LanM5, LanS2, LanT1, and LanTb3) grouped with *B. canariense* BTA-1 and *B. lupini* USDA 3051. The rest of the groups were similarly distributed. LanTb17 grouped with *B. liaoningense* LMG18230. Strain LanH1 occupied a separate position in the extra-slow bradyrhizobial clade formed by *B. valentinum* LmjM3, *B. pachyrhizi* PAC48 and *B. lablabi* CCBAU 23086, and might constitute a new species in the *Bradyrhizobium* genus. Three fast growing strains (LanM6, LanH5 and LanTb9) were identical to *Microvirga tunisiensis* LmiM8, also isolated in Northern Tunisia from *L. micranthus* [37]. The 16S rRNA sequence of LanTb5 shared 99% identity with *Devosia* sp. Dc2a-9 isolated from algal-bacterial consortia (<https://www.ncbi.nlm.nih.gov/nuccore/300068687>, accessed on 10 March 2021). Altogether, the phylogenetic analyses revealed several *Bradyrhizobium*, *Microvirga* and *Devosia* species, which had not been previously described, as microsymbionts of *L. angustifolius*.

Figure 2. Cont.

Figure 2. Neighbor-joining phylogenetic trees of Tunisian *L. angustifolius* (Lan) microsymbionts and reference strains based on concatenated gene sequences. (a) Phylogeny of the *Bradyrhizobium* isolates based on *recA* and *glnII* sequences. (b) Phylogeny of the *Microvirga* isolates based on *gyrB* and *dnaK* sequences. Filled diamonds: Tunisian Lan isolates obtained in this work. Bootstrap values calculated for 1000 replications and those greater than 50% are indicated at the internodes. GenBank accession numbers are shown in brackets.

3.4. NodC Gene Phylogeny

Partial *nodC* sequences were obtained from only six bradyrhizobial strains. Five were in groups I and II defined in Figure 1 and lined up with symbiovar *genistearum* formed by strains able to nodulate legumes in the *Genisteeae* tribe, such as *B. lupini*, *B. rifense*, *B. cytisi* and *B. canariense* type strains (Figure 4). LanH1, in group IV of Figure 1, might correspond to a new symbiovar designated new sv. A, which is distant from other *Bradyrhizobium* symbiovars and close to symbiovar *retamae*, defined with strains nodulating *Retama* spp., and *L. mariae-josephae* [41]. *nodC* sequences from *Microvirga* strains (LanM6, LanTb9 LanH5) were identical to that of *Microvirga tunisiensis* LmiM8 belonging to the new *mediterraneense* symbiovar (Figure 4) [42]. The LmiM8 strain was an effective endosymbiont isolated from *L. micranthus*.

Figure 3. Neighbor-joining phylogenetic tree based on 16S rRNA sequences of Tunisian isolates and reference strains. Black diamonds (◆) indicate Tunisian strains. Bootstrap values using 1000 replicates are indicated at branching points. Bar 0.02 estimated substitutions.

Figure 4. Neighbor-joining phylogenetic tree based on *nodC* sequences (620 bp) of Tunisian *L. angustifolius* endosymbionts and reference strains: Tunisian Lan (◆) isolates obtained in this work. Bootstrap values calculated for 1000 replications and those greater than 70% are indicated at the internodes. Accession numbers from GenBank and NCBI databases are shown in brackets. Bar: 0.05 estimated substitutions. *M.*, *Mesorhizobium*; *B.*, *S.*, *Sinorhizobium*, *Bradyrhizobium*, *R.*, *Rhizobium*.

Amplification of *nodC* and *nodA* could not be obtained for strains LanTb17 and LanTb5. *nifH* gene sequencing affiliated LanTb17 (MZ343378) to *Bradyrhizobium japonicum* CCBAU43129 with 97% identity. LanTb5 (MZ369158) *nifH* gene was aligned to *Rhizobium tropici* CIAT 899 with 78% identity. These results might indicate that these novel *L. angustifolius* microsymbionts might have acquired their *nif* genes from phylogenetically distant sources through lateral transfer.

3.5. Host Range of Rhizobial Strains Isolated from *L. angustifolius* Nodules

The symbiotic specificity of *L. angustifolius*-nodulating rhizobia was investigated in plant cross-inoculation assays under axenic conditions. The selected strains were tested for nodulation with their original host and six additional legumes (*L. luteus*, *L. micranthus*, *L. mariae-josephae*, *Retama sphaerocarpa*, *Vigna unguiculata* and *Glycine max*) (Table 2). All tested strains were able to nodulate *L. angustifolius*, *L. luteus* and *L. micranthus*, with the exception of LanTb17, which only nodulated its original host (Table 2). LanT1, LanM5, LanTb3, LanTb18 and LanS2 strains formed white nodules with *V. unguiculata*. *Microvirga* sp. strains induced the formation of oversized non-effective nodules in *L. mariae-josephae* roots. LanS2, Tb3, M5 and LanTb5 were unable to form root nodules in *L. mariae-josephae* roots. LanH1 affiliated to extra-slow-growing *Bradyrhizobium* species, such as *B. valentinum*, which was originally isolated from *L. mariae-josephae*, and formed effective nodules with *L. mariae-josephae*. All tested strains were unable to form nodules on soybean, but formed white nodules on *R. sphaerocarpa* roots (Table 2). Overall, the nodulation analyses revealed that *L. angustifolius* endosymbionts present a diverse host range and symbiotic specificity.

Table 2. Legume host-range analysis of representative rhizobial strains isolated from *L. angustifolius* nodules.

		Host Plant						
		<i>L. angusti- folius</i>	<i>L. luteus</i>	<i>L. micranthus</i>	<i>L. mariae- josephae</i>	<i>Retama sphaerocarpa</i>	<i>Vigna unguiculata</i>	<i>Glycine max.</i>
Strain	PG							
LanT1	I	+	+	+	+W	+W	+W	-
LanM5	I	+	+	+	-	+W	+W	-
LanTb3	I	+	+	+	-	+W	+W	-
LanTb18	I	+	+	+	+W	+W	+W	-
LanS2	II	+	+	+	-	+W	+W	-
LanTb17	III	+	-	-	-	-	-	-
LanH1	IV	+	+	+	+	+	-	-
LanTb5	V	+	+	+	-	+W	-	-
LanM6	VI	+	+	+	+W	+W	-	-
LanH5	VI	+	+	+	+W	+W	-	-
LanTb9	VI	+	+	+	+W	+W	-	-

PG: Phylogenetic groups identified in Figure 1. Color of nodules: (+) red, (+W) white, (-) no nodules.

3.6. Phenotypic Analyses of Narrow-Leaf Lupin-Nodulating Strains

The rhizobial isolates presented a broad biodiversity in their growth characteristics. Ten isolates could be classified as fast-growing (*Devosia* and *Microvirga*) and twenty-two of them as slow-growing rhizobia (*Bradyrhizobium*). The phenotypic traits of the 11 representative strains are summarized in Table 3. All strains were able to grow in optimal growth conditions (28 °C, pH 6.8 and <0.01% NaCl). Cells were rod shaped, Gram negative and aerobic. The *Bradyrhizobium* isolates were slow growers with mean generation times >15 h in YMB. Colonies produced in YMA were translucent, gummy and circular. They presented convex smooth margins, and their sizes were 2–3 mm in diameter after 6–7 days in YMA medium at 28 °C. LmiH1 was an extra-slow grower (generation time in YMB > 30 h) that produced punctiform, non-mucoid colonies (<1 mm) after 10 to 11 days

in YMA plates. Fast growers showed mean generation times of 4–7 h in TY. They also grew well on YMA plates. After 8 days at 28 °C, *Microvirga* colonies were 2–3 mm in diameter, convex, smooth, mucilaginous pink, and circular. The effect of temperature, salinity, pH and some antibiotics, used to quantify and qualify stress tolerance, showed a degree of heterogeneity (Table 3). All the *Microvirga* strains grew well between 20 and 37 °C, at pH 4–12, and on media containing 0.1; 0.5 and 1% salt. These strains were very sensitive to all antibiotics used (kanamycin (50 µg/mL), ampicillin (100 µg/mL), gentamicin (30 µg/mL), spectinomycin (50 µg/mL), tetracycline (5 µg/mL), streptomycin (10 µg/mL). The *Bradyrhizobium* strains grew at temperatures between 20 and 28 °C, at pH range 4 to 11 and on media containing 0.1% salt, with only strains LanS2, LanTb17 and LanH1 growing at temperatures between 20 and 37 °C. These strains were sensitive to kanamycin, gentamicin, spectinomycin, and streptomycin and were less resistant to ampicillin and tetracycline, except for strain LanTb17, which showed a good resistance to kanamycin, ampicillin and chloramphenicol. The *Devosia* LanTb5 strain grew well between 20 and 28 °C, at pH 4–11, and tolerated 1% salt added to the medium. This strain was sensitive to ampicillin, gentamicin, tetracycline, and chloramphenicol. LanTb5 could grow in presence of spectinomycin, kanamycin and streptomycin.

Table 3. Phenotypic characteristics of representative strains isolated from *L. angustifolius* growing in Northern Tunisia.

Strain	PG	GT (h)	T Range (°C)	pH Range	Salt Tolerance (NaCl %)				Antibiotics Resistance						
					0.1	0.5	1	2	Amp	Gen	Tet	Spe	Kan	Str	Chl
LanT1	I	10	20–28	4–11	+	-	-	-	+	-	+	-	-	-	+
LanM5	I	9	20–28	4–11	+	-	-	-	+	-	+	-	-	-	+
LanTb3	I	9.5	20–28	4–11	+	-	-	-	+	-	+	-	-	-	+
LanTb18	I	8.7	20–28	4–11	+	-	-	-	+	-	+	-	-	-	+
LanS2	II	9.3	20–37	4–11	+	+/-	-	-	-	-	-/+	-	-	-	-/+
LanTb17	III	8	20–37	4–12	+	+/-	-	-	+	-	+	-/+	+	-	+
LanH1	IV	18	20–37	4–11	+	+/-	-	-	-	-	-/+	-	-	-	-/+
LanTb5	V	4	20–28	4–11	+	+	+/-	-	-	-	-	+	+	+	-
LanM6	VI	4.6	20–37	4–12	+	+	+/-	-	-	-	-	-	-	-	-
LanH5	VI	4.6	20–37	4–12	+	+	+/-	-	-	-	-	-	-	-	-
LanTb9	VI	4.6	20–37	4–12	+	+	+/-	-	-	-	-	-	-	-	-

PG: Phylogenetic groups identified in Figure 1. GT: Generation Time-, no growth; +/-, weak growth (10–30% compared to the control in YMA); +, good growth (similar to the control) Amp: ampicillin (100 µg/mL); Gen: gentamicin (30 µg/mL); Tet: tetracycline (5 µg/mL); Spe: spectinomycin (50 µg/mL); Kan: kanamycin (50 µg/mL), Str: streptomycin (10 µg/mL) and Chl: chloramphenicol (10 µg/mL). Values represent the average of two experiments with three replicates each time.

4. Discussion

In this study, the isolation, identification and phenotypic characterization of rhizobial bacteria isolated from effective root nodules of *L. angustifolius* plants growing in Northern Tunisia is reported. All isolated strains were effective endosymbionts of their original host, as evidenced by the formation of red–pink nodules and the leaves' dark green color. Nodule numbers ranged from 3 to 10 per plant

Phylogenetic analyses revealed high genetic diversity levels among the *L. angustifolius* nodule isolates. Previous reports also described considerable genetic diversity among rhizobia that nodulate *Lupinus* by using diverse methodological approaches, such as M13 random amplified polymorphic DNA-PCR (RAPD-PCR) [20,21,23]. High diversity was shown by phylogenetic, symbiotic and phenotypic analyses. Some rhizobia assigned to the *Bradyrhizobium* genus were slow-growing bacteria, while some isolates were fast-growing and were assigned to the *Microvirga* and, unexpectedly, for the first time to our knowledge, to the *Devosia* genus. It is not unusual that legumes nodulated by slow-growing

Bradyrhizobium species are also efficiently nodulated by different fast-growing rhizobial species [43,44].

The housekeeping genes *glnII/recA* and *gyrB/dnaK* were used because of their proven performance as molecular markers, either individually or in combination, for assessing the evolutionary genetics of *Bradyrhizobium* and *Microvirga* species, as described in previous works [28,45]. Phylogenetic analyses based on the housekeeping genes *glnII/recA* and *gyrB/dnaK* congruently placed some tested isolates in a new strongly supported clade within the *Bradyrhizobium* (Group III) and *Devosia* (Group V) genera. The concatenated tree of the representative strain showed that four strains affiliated with *B. lupini* USDA3051, one strain grouped with *B. canariense* BTA-1, one strain clustered together with *B. liaoningense* LMG18230, one strain occupied a separate position between *B. valentinum* LmjM3 and *B. algeriense* RST89, and could constitute a new species within the genus *Bradyrhizobium*. Three strains were included in the *Microvirga* genus and, surprisingly, one strain was related to the genus *Devosia*, never described before as a lupin microsymbiont. The concatenated phylogenetic trees constructed confirmed the results obtained by the single sequence of *glnII*, *recA*, *gyrB* and *dnaK* genes. The distribution of some strains separated from the existing type species might be indicative of novel species. Nonetheless, further polyphasic studies to precisely characterize and classify them are required.

The genus *Devosia* has been reported to establish nitrogen-fixing symbiosis with the aquatic legume *Neptunia natans* growing in India [34,46]. To our knowledge, no *Devosia* strain has previously been described as a lupin symbiont. In this study, two strains (LanTb4 and LanTb5) closely clustered with *Devosia* species. These isolates were clearly separated from other *Devosia* spp. and might constitute a new species under this genus. In previous works, *Lupinus* spp. has been reported to be nodulated by fast-growing bacteria affiliated to *Mesorhizobium*, *Allorhizobium*, *Rhizobium* and *Sinorhizobium* [24,27,30,47–49]. However, in general, annual lupins are nodulated by slow-growing *Bradyrhizobium* sp. rhizobia [21,50–52]. This is in agreement with our results, as approximately 60% of our isolates belonged to different *Bradyrhizobium* species.

The phylogeny of the important symbiotic gene *nodC* is well correlated with legume host range but independent of the rhizobial taxonomic position [53–56]. The *nodC* gene has been used as a molecular marker related to host specificity and range, and used to define *Bradyrhizobium* symbiovars [41,45,57]. The *nodC* tree revealed that the majority *Bradyrhizobium* strains clearly grouped with sequences within the *genistearum* symbiovar, which has been previously described for strains that nodulate species in the *Genisteae* tribe, including rhizobia nodulating *Cytisus*, *Lupinus* and *Retama* in Europe, America and Africa [41,45,58]. The sequences of *Microvirga* strains LanM6 and LanH5 grouped with sequences characteristic of the *mediterraneense* symbiovar. This type of *nodC* sequence was previously described for *M. tunisiensis* LmiM8 nodulating *L. micranthus* [42]. The remaining *Bradyrhizobium*-type representative isolate, LanH1, might correspond to a new symbiovar, as it was placed distant from other *Bradyrhizobium* symbiovars defined to date. Sanger chromatograms for *nifH* sequences of LanTb5 strain displayed sequence ambiguities. This may be explained by the presence of more than one copy [59].

Phenotypic tests have been used to study the diversity of rhizobial bacteria and to assess their tolerance under stress conditions [60,61]. The first macroscopic test showed that the morphology of the bradyrhizobial strains in the present work was similar to those previously reported [23,60]. The colonies formed by the *Microvirga* strains were pinkish in color, similar to those of *M. lupini* [28] and *M. vigna* [39]. pH, salinity and extreme temperatures are abiotic stresses for nodulation of agricultural legumes in Mediterranean areas. The isolates exhibited differences in their response to abiotic stress (Table 3). All isolates grew at pH 4, in agreement with reports of other strains isolated from *Lupinus* spp. showing resistance to acid soils [62]. Most strains were also tolerant to alkaline conditions (pH 11), as previously reported for rhizobia that nodulate *Prosopis juliflora* growing in alkaline soil [63]. All strains were sensitive to salinity, as they were not able to grow when NaCl concentration was higher than 0.5%. Conversely, *Microvirga* strains were able to

grow in with the presence of 1% sodium chloride. These results are also corroborated by those reported for *Microvirga vignae* [39]. Slow-growing rhizobia in the *Bradyrhizobium* genus have been reported to be more sensitive to salt stress than fast-growing rhizobia isolated from the same soils [61,64]. All strains in the present study grew at the optimum temperature of 28 °C, in contrast with a previous report that stated that the optimum temperature for *M. lupini* growth was 39 °C [28]. The effect of temperature, salinity and pH, used to qualify and quantify stresses tolerance, showed a degree of heterogeneity even when they were isolated from the same site (Table 3).

In conclusion, the isolates in this work showed a wide phenotypic and genotypic diversity. Significant heterogeneity among lupin endosymbionts was unraveled based on phylogenetic analysis using housekeeping and symbiotic gene sequences, and on their legume-host ranges. The study revealed the presence of rhizobia belonging to the three divergent genera *Bradyrhizobium*, *Microvirga* and *Devosia*. To our knowledge, this is the first report on the Alphaproteobacterial genus *Devosia* as a nodule-forming species in *Lupinus* spp. A practical aspect of the ability of lupin species to trap diverse rhizobial genotypes is that they might display important differences in their capacities to fix nitrogen depending on edaphoclimatic conditions [65,66]. The potentially new *Bradyrhizobium* and *Devosia* species reported here require further taxonomic characterization for confirmation and formal description. Finally, this work provides an array of native rhizobia that might have practical application as biofertilizers and/or inoculants with native legumes, including shrubs, in the restoration or revegetation of arid and semiarid Mediterranean areas.

Author Contributions: Conceptualization, A.M., L.R., J.I., J.M.P., M.M. and J.J.P.; methodology, A.M.; validation, A.M., L.R. and J.J.P.; formal analysis, A.M.; investigation, A.M.; resources, L.R., J.I., J.M.P., M.M. and J.J.P.; data curation, A.M.; writing—original draft preparation, A.M.; writing—review and editing, A.M., L.R., J.I., J.M.P., M.M. and J.J.P.; visualization, A.M. and J.J.P.; supervision, L.R., M.M. and J.J.P.; project administration, L.R., M.M. and J.J.P.; funding acquisition, J.M.P., M.M. and J.J.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of High Education and Research Development, Tunisia, and by the Agencia Estatal de Investigación, AEI, Spain, grant numbers AGL2017-88381-R and RTI2018-094985-B-I00. The APC was funded in part by CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

Data Availability Statement: All sequences are available from the GenBank database.

Conflicts of Interest: The authors declare no conflict of interest.

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