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Phylogenetic diversity of *Bradyrhizobium* strains isolated from root nodules of *Lupinus angustifolius* grown wild in the North East of Algeria

Hanane Mellal[a], Benhizia Yacine[a], Leila Boukaous[a], Sabra Khouni[a], Ammar Benguedouar[a], Antonio Castellano-Hinojosa[b,*], Eulogio J. Bedmar[b]

[a] *Laboratoire de Biologie Moléculaire et Cellulaire, Département de Microbiologie, Université Frères Mentouri — Constantine 1, Algeria*
[b] *Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, CSIC, Granada, Spain*

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ABSTRACT

From a total of 80 bacterial strains isolated from root nodules of *Lupinus angustifolius* grown wild in the North-Eastern Algerian region of El Tarf, 64 plant host-nodulating strains clustered into 17 random amplified polymorphic DNA (RAPD) fingerprinting groups. The nearly complete 16S rRNA gene sequence from the representative strain of each group revealed they were closely related to members of the genus *Bradyrhizobium* of the Alphaproteobacteria, but their affiliation at the species level was not clear. Sequencing of the housekeeping genes *glnII* and *recA*, and their concatenated phylogenetic analysis, showed that 12 strains belong to *B. lupini*, other 2 strains affiliated with *B. diazoefficiens* and that 1 strain was closely related to *B. japonicum*. The remaining two strains showed similarity values ≤95% with *B. cytisi* and could represent new lineages within the genus *Bradyrhizobium*. Sequencing of the symbiotic *nodC* gene from 4 selected bradyrhizobial strains showed they were all similar to those of the species included in symbiovar genistearum.

Introduction

Within the Genisteae tribe of the Fabaceae, the genus *Lupinus* comprises around 275 species, most of them from diversity centers in Southern and Western North America, the Andean areas of South America in the New World and only 15 species native to the Mediterranean regions and Africa in the old World [1,2,17,24]. Legumes are well-known because of their ability to establish symbiotic associations with soil bacteria, collectively known as rhizobia, that fix atmospheric dinitrogen (N_2) within the nodules formed in the roots, and occasionally on the stems, of the plants. *Lupinus* species are nodulated predominantly by slow-growing rhizobia classified within the genus *Bradyrhizobium*, including *B. japonicum*, *B. lupini*, *B. canariense* and *B. elkanii* species [30,36,37,41]. Also, fast growing bacteria have been isolated from *Lupinus*, among them *Ochrobactrum lupini* from *L. honoratus* [38] and different strains of *Microvirga* from *L. texensis* [3] and *L. micranthus* [27,28].

Lupines are of great economical interest because their seeds have a high protein and lipid content [6]; in addition, they can contain quinolizidine alkaloids on which lupines rely for chemical defense against herbivores and microorganisms, and for competition with other plants [11,22,29,45]. They have a deep, wide root system which allows the plants to thrive in soils holding little water and nutrients, which together with their N_2-fixing capacity enable them to be used for cover crops and pasture improvement, green manure, soil stabilization and recovery of degraded soils [18]. Lupines are adapted to a range of highly divergent climatic and environmental conditions. With the exception of *L. mariae-josephae* [14], which is nodulated by *B. valentinum* [13], they rarely develop in calcareous alkaline soils with a pH higher than 6.8 [15], grow well in poor nutrient soils, are tolerant to dry conditions, salt excess, and heavy metal contamination [26,40]. Native lupine species from the Old World have traditionally been grouped into smooth-seeded and rough-seeded species [1,2], of which the smooth-seeded *L. angustifolius*, *L. albus* and *L. luteus* are the most abundant [15] and thrive in areas surrounding the Mediterranean Basin and Africa [2,5,12,17]. The Mediterranean climate is often characterized by irregular rainfall distribution and hot dry summers. In this region, most soils (about 25 million ha) are eroded and shallow, with low organic matter content, in some cases lower than the desertification index (<17 g organic matter kg^{-1}) [20,39].

* Corresponding author at: Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, CSIC P.O. Box 419. 18080-Granada, Spain.
E-mail address: ach@ugr.es (A. Castellano-Hinojosa).

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Table 1
Physicochemical properties of the soils.

Character	Site		
	Lake Mellah	Lake Oubéïra	Lake Tonga
GPS coordinates	36°54′06.4"N 82° 02′8.9"E	36°50′21.4"N 8°25′38.8"E	36°52′52.7"N 8°31′24.4"E
Texture	Sandy-loam 87% sand 3% silt 10% clay	Sandy 95% sand 1% silt 4% clay	Silty-clay 10% sand 45% silt 45% clay
pH_{water}	6.58	6.81	5.52
Organic matter (%)	2.58	2.10	1.87
Carbon (%)	1.29	0.87	0.75
Nitrogen (%)	0.14	0.08	0.09

L. angutifolius, common names narrow leaf lupine and blue lupine, grows wild under those conditions in the wetlands of the coastal plain in the Northeast region of El Tarf in Algeria. In this country, like in others around the Mediterranean Basin, blue lupins are of great interest in sustainable agriculture; they are also increasingly employed in both human and animal nutrition and for medicinal and cosmetic purposes [46]. However, data on the bacterial species that can be found in nodules of wild-grown *L. angutifolius* have not been reported. Therefore, the primary objective of this research was to identify the rhizobial symbionts within nodules of *L. angustifolius* plants growing in the Northeast area of the Algerian Tarf region. Sequence analysis of the housekeeping genes 16S rRNA, *glnII* and *recA* showed that the isolates belonged to *B. lupini*, *B. cytisi*, *B. japonicum* and *B. diazoefficiens*. PCR amplification was used to look for the symbiotic gene *nodC*, and it was studied by sequencing and phylogenies.

Materials and methods

Sampling sites, isolation of bacteria from nodules and culture conditions

Nodules were collected from roots of healthy *L. angustifolius* plants growing wild in the vicinity of lakes Tonga, Oubéïra and Mellah (12 plants/location) (supplementary Fig. S1). The lakes make part of a complex of wetlands located on the coastal plain near the town of El Kala (36°53′N; 8°29′E) in El Kala National Park in the North Eastern region of El Tarf (Algeria). Location of the sampling sites is shown in Fig. S1, and the main physicochemical properties of the soils are indicated in Table 1.

Nodules were surface-sterilized with 0.25% $HgCl_2$ for 5 min, rinsed thoroughly with sterile distilled water, placed independently in Petri dishes and, finally, crushed in a drop of sterile water with a sterile glass rod. The resulting suspension was streaked onto Petri dishes containing solid yeast extract-mannitol (YEM) medium [42] supplemented with 0.025 g Congo Red/L and incubated at 30 °C for 12 d. After incubation, the colony-forming units (CFUs), which represented all of the colony types that could be distinguished by microscopic observation, were chosen. After identification, rhizobial strains used in this study were routinely grown on YEM medium.

DNA isolation and PCR amplifications

Essentially, bacterial genomic DNA was isolated after growth of the cells in liquid YEM medium, collected by centrifugation in a microfuge and extracted by addition of 200 μl 0.05 M NaOH and heating at 100 °C for 4 min. Then, 200 μL Tris-HCl (pH 8.0) was added and the mixture centrifuged at 13000 rpm for 3 min. Finally, the supernatant containing the DNA was recovered and kept at −20 °C until use. The quantity of DNA was determined using a Nanodrop spectrophotometer (NanoDrop ND1000, Thermo Fisher Scientific, USA).

Random amplified polymorphic DNA (RAPD)-polymerase chain reactions (RAPD-PCR) were performed using primer M13 (5′-GAGGGTGGCGGTTCT-3′) according to Rivas et al. [33]. The DNA bands present in each electrophoretic lane were coded for input into a database including all the strains studied, and Jaccard's similarity coefficient was calculated to construct the distance matrix from which a dendrogram was built using the unweighted pair group with arithmetic mean (UPGMA) using the Quantity One software (Bio Rad). PCR amplifications of 16S rRNA gene fragments were carried out by using the primers 27F, 1522R and 800R [34]. The primer pairs glnII 12F and glnII 689R and recA 41F and recA 640R were used for amplification of the *glnII* and *recA* genes, respectively [43]. The primers nodCF and nodCI were used for amplification and sequencing of the *nodC* gene [25]. Amplification products were purified with the GeneJET PCR purification kit (Thermo Fisher Scientific), verified by electrophoresis in agarose gels and subjected to cycle sequencing using the same primers as for PCR amplification, with ABI Prism dye chemistry. The products were analyzed with a 3130 × l automatic sequencer at the sequencing facilities of Estación Experimental del Zaidín, CSIC, Granada, Spain. All the obtained sequences were compared with those from GenBank using the BLASTN program (http://www.ncbi.nlm.nih.gov/blast) and the EzBiocloud Database (https://www.ezbiocloud.net/) [47], and aligned using the Geneious software (Biomatters Ltd., Auckland, New Zealand). Distances were calculated according to Kimura's two-parameter model [21]. Neighbour-joining [35] and maximum likelihood [16] phylogenetic reconstructions gave similar results, therefore only maximum likelihood phylograms are presented. MEGA 7.0 was used for all the phylogenetic analyses [23]. Identity values were calculated by pairwise analysis and gaps were not considered. Accession numbers of the nucleotide sequences used in this study are shown in the figure trees.

Nodulation tests

The isolates were tested for nodulation on *L. angustifolius* and soybean (*Glycine max* L. Merr., cv. Williams). Seeds of *L. angustifolius* were surface-sterilised by immersion in 95% ethanol (v/v) for 10 s, scarified using 98% sulfuric acid for 3 min, washed several times with sterile water and left in water for 1 h. Soybean seeds were surface-sterilized by adding 96% ethanol (v/v) for 30 s, H_2O_2 15% (v/v) for 8 min, and finally washed with sterile water. Finally, the seeds were placed on Petri dishes containing 1% agar-water and allowed to germinate in the dark at 30 °C for 2–4 d. Seedlings were transferred to 500 ml flasks containing the N-free Fahraeus nutrient solution [42] supplemented with 1% (w/v) $CaCO_3$ and closed with 2-hole-perforated plastic caps, one for inserting the sprout rootlet and the other for inoculation of the seedlings, as previously described [32]. Two days old seedlings were inoculated independently with 1 mL of bradyrhizobial suspensions (~10^8 cells mL^{-1}) and kept at room temperature for 6–8 weeks under a 16.0/8.0 h light/dark photoperiod. Non-inoculated plants were used as a control. Indirect effectiveness of the nodules for nitrogen fixation was estimated by visual assay of red leghemoglobin presence in cross-sections and by the dark green intensity of the leaves compared to uninoculated control plants.

Results

16S rRNA, glnII, recA and nodC phylogenetic analyses

A total of 80 bacterial strains were isolated from root nodules of wild-grown *L. angustifolius*. Strains were named La, represent-

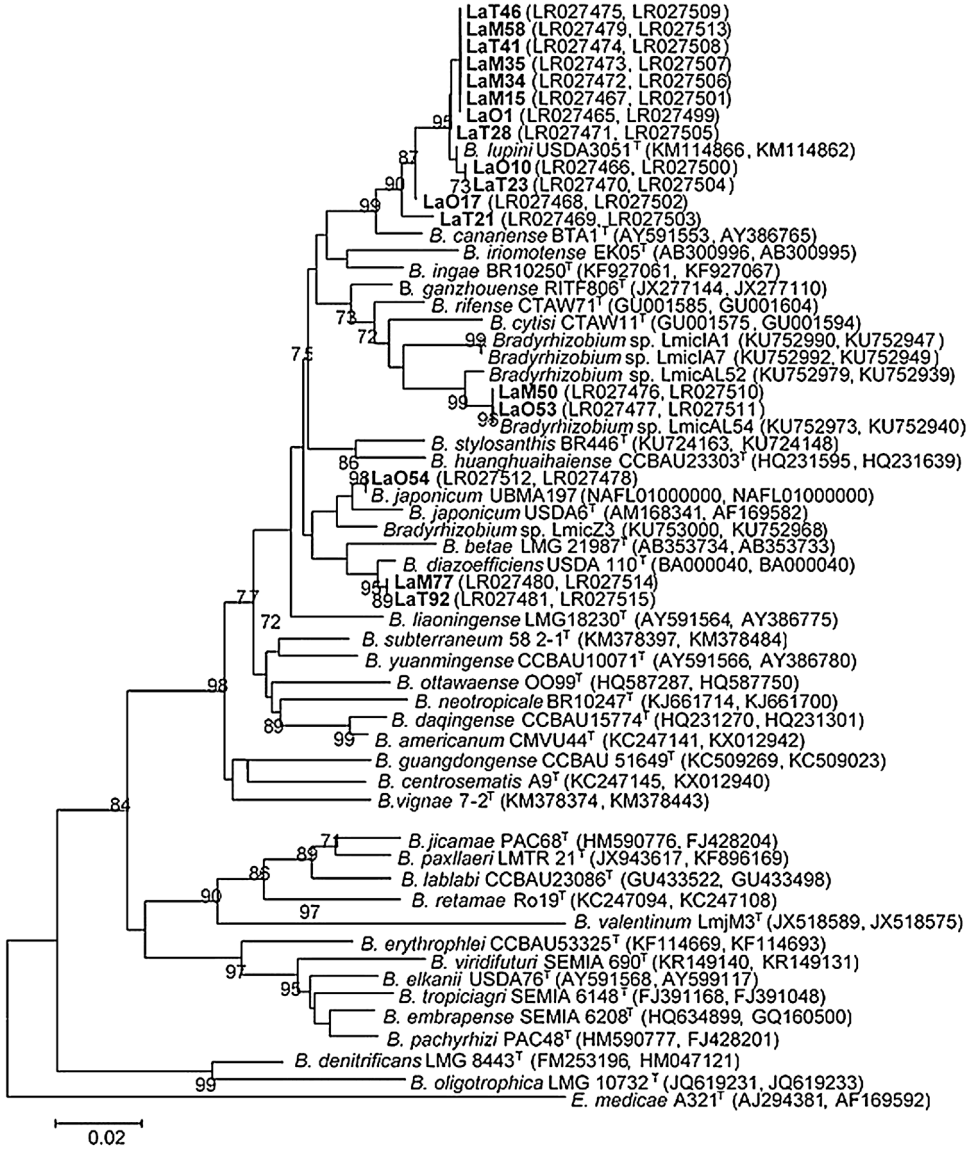

Fig. 1. ML phylogenetic tree based on concatenated partial *recA* + *glnII* sequences of strains from nodules of wild-grown *L. angustifolius* and phylogenetically related species within the genus *Bradyrhizobium*. The analysis was based on 870 nucleotides. Isolates are denoted in bold. Bootstrap values are indicated as percentages derived from 1000 replications. Values lower than 70 are not shown. Bar, 2 nucleotides substitution per 100 nucleotides. The tree is rooted with *Ensifer medicae* A321^T.

ing *L. angustifolius* followed by the letters either M, T or O for the strains isolated from lakes Mellah, Tonga or Oubéïra, respectively. Out of the 80 isolates, only 64 formed effective nodules when used to inoculate *L. angustifolius*, the original host plant from which they were isolated; further characterization of the remaining 16 strains was not pursued in this study. After RAPD-PCR fingerprinting, the 64 isolates were represented by 17 different profiles at 80% similarity (Table 2). The nearly complete sequence of the 16S rRNA gene from a representative strain of each RAPD group showed that all of them were members of the genus *Bradyrhizobium* within the family Bradyrhizobiaceae of the Alphaproteobacteria. The phylogenetic tree (supplementary Fig. S2) inferred from the 16S rRNA gene sequences revealed that the strains LaT92 and LaM77 showed 99.5 and 99.4% identity with *B. diazoefficiens* USDA 110^T, respectively, the strains LaM50 and LaO53 had 99.6 and 99.4% identity with *B. cytisi* CTAW11^T/*B. rifense* CTAW71^T, respectively, the strain LaM54 shared 99.9% identity with *B. daqingense* CCBAU 15774^T, and that the remaining 12 strains, LaO1, LaO10, LaO17, LaT21, LaT23, LaT28, LaT41, LaT46, LaM15, LaM34, LaM35 and LaM58 showed identity values higher than 99.5% with *B. lupini* USDA 3051^T. PCR amplification of the *glnII* and *recA* genes yielded single DNA bands of approximately 0.7 and 0.6 kb that were sequenced, respectively. A concatenated phylogenetic tree based on the obtained *recA* and *glnII* sequences (Fig. 1) revealed that strains LaO1, LaO10, LaM15, LaO17, LaT21, LaT23, LaT28, LaM34, LaM35, LaT41, LaT46 and LaM58 grouped with *B. lupini* USDA 3051^T with identity values higher than 98.2%, that strains LaM50 and LaO53 affiliated with the *B. cytisi* CTAW11^T lineage with which they shared 94.9% identity values, that LaO54 was closely related to *B. japonicum* USDA 6^T with 98.2% identity, and that strains LaM77 and LaT92 clustered with *B. diazoefficiens* USDA 110^T with 97.0% identity. Phylogenetic trees based on individual *glnII* (supplementary Fig. S3) and *recA* sequences (supplementary Fig. S4) gave similar results to those obtained for the concatenated tree, with no further insights into the affiliation of the isolates. Utilization of the primer pair nodCF/nodCI resulted in amplification (approximately 0.6 kb) of the *nodC* gene from strains LaM15, LaO53, LaO54 and LaM77 that were chosen as the representative strains for each of the corresponding clusters they were grouped. A phylogenetic tree (Fig. 2) showing the relationship between the *nodC* genes from those strains and other

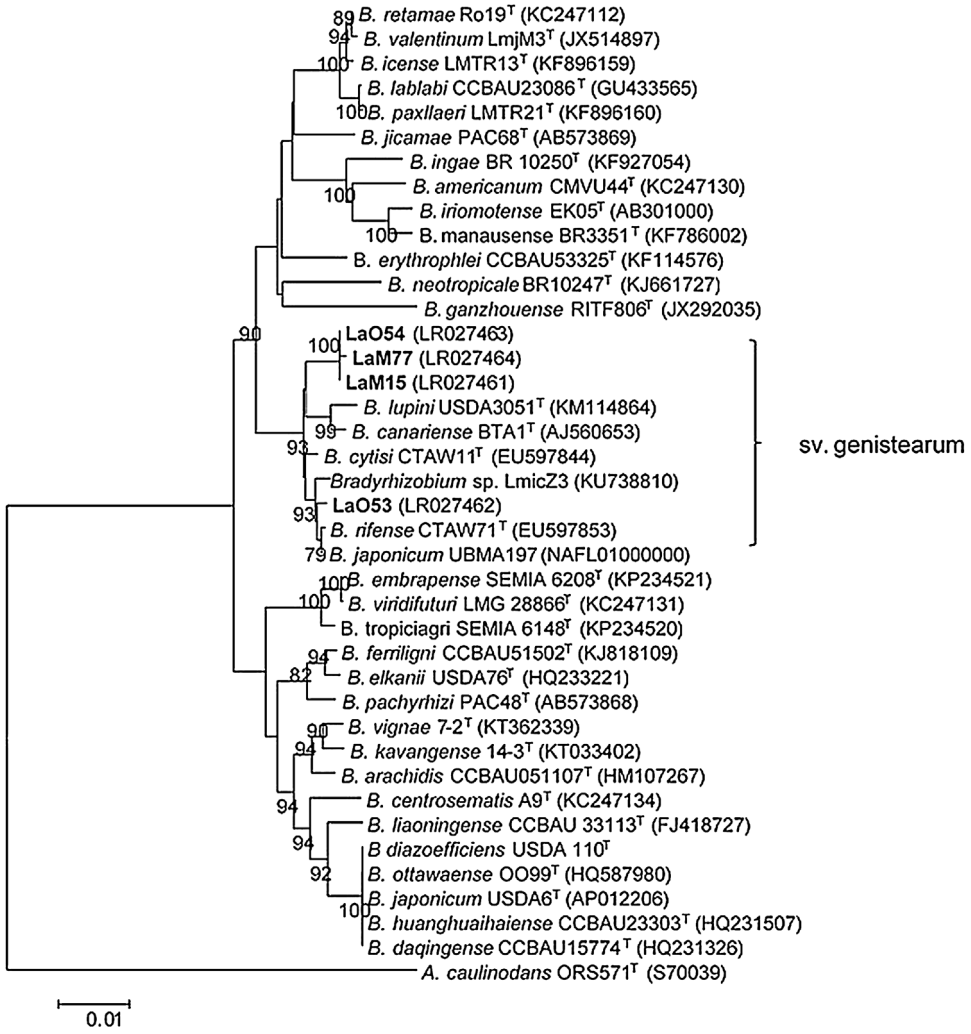

Fig. 2. ML phylogenetic tree based on *nodC* sequences of strains from nodules of wild-grown *L. angustifolius* and phylogenetically related species within the genus *Bradyrhizobium*. *Bradyrhizobium* symbiovars are also shown. The analysis was based on 580 nucleotides. Isolates are denoted in bold. Bootstrap values are indicated as percentages derived from 1000 replications. Values lower than 70 are not shown. Bar, 1 nucleotide substitution per 100 nucleotides. The tree is rooted with *Azorhizobium caulinodans* ORS571^T.

Table 2
Identification of bradyrhizobial strains from root nodules of wild-grown L. angustifolius. Strains in bold were chosen as the representative strains of each RAPD-PCR group.

Strain	RAPD-PCR pattern	Closest relative genus on basis of 16S rRNA gene
LaO1, LaO74	I	*Bradyrhizobium*
LaO10, LaO11, LaO49, LaO12	II	*Bradyrhizobium*
LaM15, LaO19	III	*Bradyrhizobium*
LaO17, LaM48, LaO51	IV	*Bradyrhizobium*
LaT21, LaO16, LaM2	V	*Bradyrhizobium*
LaT23, LaT40, LaT47, LaT22, LaT20	VI	*Bradyrhizobium*
LaT28, LaT71, LaT99, LaT27	VII	*Bradyrhizobium*
LaM34, LaM8, LaT18, LaM32	VIII	*Bradyrhizobium*
LaM35, LaM45, LaM13, LaM3	IX	*Bradyrhizobium*
LaT41, LaT26, LaM63, LaM69	X	*Bradyrhizobium*
LaT46, LaO29, LaM43, LaM57	XI	*Bradyrhizobium*
LaM50, LaM88, LaM38, LaM39	XII	*Bradyrhizobium*
LaO53, LaO79, LaO82, LaO85, LaM36	XIII	*Bradyrhizobium*
LaO54, LaO6, LaT33, LaM55	XIV	*Bradyrhizobium*
LaM58, LaM9	XV	*Bradyrhizobium*
LaM77, LaT25, LaO62	XVI	*Bradyrhizobium*
LaT92, LaT60, LaO30, LaT24, LaM72, LaM90, LaT14	XVII	*Bradyrhizobium*

bradyrhizobial species revealed that all 4 strains belonged to symbiovar genistearum of the genus *Bradyrhizobium* with identities higher than 91.9%.

Plant nodulation tests

The bradyrhizobial strains LaM15, LaO53, LaO54 and LaM77 identified in this study produced effective symbiosis with their original host and did not form nodules on soybean. Visible differences in plant growth parameters of the plants were not observed.

Discussion

In this study we report on the isolation and identification of bradyrhizobial strains from root nodules of *L. angustifolius* grown in the natural environment of the Algerian North Eastern region of El Tarf, which is characterized by high thermal amplitudes and low rainfall. Soil data (Table 1) revealed a light texture and very low levels of C and N, which confirms severe ecological conditions for living organisms. RAPD fingerprinting was used to group the 64 effective strains, which showed 17 different patterns (Table 1). This technique has been used to cluster bacteria at the subspecies or strains level and has proved to be a powerful tool for studies of

microbial ecology and evolution [31] and references therein]. Out of the 64 bradyrhizobial strains, 20, 19 and 25 were isolated from root nodules of plants grown near the lakes Tonga, Oubéïra and Mellah, respectively. Since these three lakes have very similar environmental conditions, the results suggested no biogeographical differences among the isolates.

The nearly complete sequence of the 16S rRNA gene of a representative isolate of each RAPD group showed that all the 17 strains were members of the genus *Bradyrhizobium* (Fig. S2). Fast-growing strains capable of nodulating *L. angustifolius* were not found.

Because *Bradyrhizobium* species have highly conserved 16S rRNA gene sequences, the analyses of various core genes have been used to elucidate the taxonomic affiliations among them. In this study, the housekeeping genes *glnII* and *recA* were selected because they gave the best relative performance when used as molecular markers, either individually or in combination, for assessing the evolutionary genetics of *Bradyrhizobium* species [44]. Individual (supplementary Figs. S3 and S4) and concatenated (Fig. 1) *glnII* and *recA* gene phylogenies showed that 12 strains (70.6%) affiliated with *B. lupini* USDA3051^T, 2 strains (11.8%) grouped with *B. cytisi* CTAW11^T, other 2 strains (11.8%) clustered with *B. diazoefficiens* USDA 110^T, and that 1 strain (5.9%) was closely related to *B. japonicum* USDA6^T. *Bradyrhizobium* has been isolated from 30 out of 33 lupine species, which makes the bacterium to be the predominant genus nodulating *Lupinus* [37] and references therein]. Our results agree with those by Bourebaba et al. [5] which show that most strains isolated from *L. micranthus* grown wild in Algeria and Spain belonged to the *B. lupini/B. canariense* lineage. Also, Msaddak et al. [27] concluded that *L. luteus* root nodule symbionts in Northern Tunisia are mostly strains within the *B. canariense/B. lupini* group. All these findings reinforce the idea that *B. lupini* and *B. canariense* are common symbionts in regions with a high diversity of Genisteae such as occurs at both sides of the Mediterranean Sea [37].

The strains LaM50 and LaO53 clustered with *B. cytisi* CTAW11^T, a species first isolated from *Cytisus villosus* growing in Morocco and Spain [7,8] and also found in nodules of *L. micranthus* from soils in Algeria and Spain [5]. It is noteworthy that the strains LaM50 and LaO53 grouped with those isolated from plants grown in Algeria but not with those from Spain (Fig. 1), which lends support to the suggestion of a specific geographical origin for *B. cytisi* strains [5]. The similarity values ≤95% in the *glnII* and *recA* sequences between *B. cytisi* CTAW11^T and strains LaM50 and LaO53 suggest they may form different lineages within the genus *Bradyrhizobium*.

Following the paper by Barrera et al. [4], other authors showed that *Bradyrhizobium* isolates of Genisteae have phylogenetic affinity with *B. japonicum* and that this bacterium has been found in nodules of *L. angustifolius* grown in Australia, Poland, South Africa and Spain [37]. In this sense, the strain LaO54 in this study clustered with the *B. japonicum* strains LmicZ3 and UBMA197 isolated from root nodules of *L. micranthus* [5] and *L. angustifolius* [9] grown in North East Africa, respectively. Based on genotypic and phenotypic evidence, the reclassification of former *B. japonicum* group Ia strains into a novel species named *B. diazoefficiens* was proposed by Delamuta et al. [10]. This species includes several strains, the great majority isolated from soybean; here we show that it can also be found within nodules of wild-grown *L. angustifolius* plants in North East Africa.

The phylogenetic analysis of the symbiotic *nodC* gene of strains LaM15, LaO53, LaO54 and LaM77 revealed they grouped with sequences (Fig. 2) that define the symbiovar genistearum described by Vinuesa et al. [43] including strains nodulating *Lupinus*, *Retama*, *Cytisus* and *Chamaecytisus* in Africa, America and Europe [5,7,8,19]. The *nodC* gene of UBMA197 and LmicZ3 also clustered within the symbiovar genistearum (Fig. 2) and the identity between the aminoacidic sequences of LaO54 and UBMA197 and LmicZ3 was of 90.8% and 90.3%, respectively. These results agree with those which show that *B. japonicum* strains isolated from lupines belong to the symbiovar genistearum [4,9,37,43][4,9,37,43 and references therein]; this, in turn, indicate that strains LaM15, LaO53, LaO54 and LaM77 have acquired the *nodC* gene of the symbiovar genistearum. It is more likely that multiple horizontal transfers could be responsible for *B. japonicum* nodulating member of the Genisteae. After nodule isolation, the strains isolated in this study were able to establish new effective symbiosis with their host plant and none of them nodulated soybeans. These results confirm affiliation of the *Lupinus*-isolated strains within the symbiovar genistearum and that they are true symbionts of *L. angustifolius*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2019.01.003.

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