Endosymbiotic bacteria nodulating a new endemic lupine **Lupinus mariae-josephi** from alkaline soils in Eastern Spain represent a new lineage within the **Bradyrhizobium** genus*

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a b s t r a c t

Lupinus mariae-josephi is a recently described endemic Lupinus species from a small area in Eastern Spain where it thrives in soils with active lime and high pH. The L. mariae-josephi root symbionts were shown to be very slow-growing bacteria with different phenotypic and symbiotic characteristics from those of Bradyrhizobium strains nodulating other Lupinus. Their phylogenetic status was examined by multilocus sequence analyses of four housekeeping genes (16S rRNA, glnII, recA, and atpD) and showed the existence of a distinct evolutionary lineage for L. mariae-josephi that also included Bradyrhizobium jicamae. Within this lineage, the tested isolates clustered in three different sub-groups that might correspond to novel sister Bradyrhizobium species. These core gene analyses consistently showed that all the endosymbiotic bacteria isolated from other Lupinus species of the Iberian Peninsula were related to strains of the B. canariense or B. japonicum lineages and were separate from the L. mariae-josephi isolates. Phylogenetic analysis based on nodC symbiotic gene sequences showed that L. mariae-josephi bacteria also constituted a new symbiotic lineage distant from those previously defined in the genus Bradyrhizobium. In contrast, the nodC genes of isolates from other Lupinus spp. from the Iberian Peninsula were again clearly related to the B. canariense and B. japonicum bv. genistearum lineages. Speciation of L. mariae-josephi bradyrhizobia may result from the colonization of a singular habitat by their unique legume host.

Introduction

A group of Gram-negative soil bacteria, collectively known as rhizobia, are able to establish a nitrogen-fixing symbiosis with legume plants. The nitrogen (N_2) fixation process occurs inside nodules formed on the legume plant roots and has major ecological and agricultural importance. **Lupinus** is a diverse legume genus comprising over 200 species that grow in a wide range of ecological conditions and thrive in nutrient-poor acid soils and arid climates, where cultivation of more demanding crops is problematic. This ability has promoted its cultivation since antiquity as green manure and a pulse crop [13,14].

Approximately 90% of known **Lupinus** species are distributed in the New World, while only a few (10–13) are native to the Mediterranean Basin and North Africa [1,12,13]. **Lupinus** is well known as a complex and taxonomically difficult genus. Based on broad systematics and evolution studies, **Lupinus** appears as a strongly supported genus within the **Genisteae** tribe, and its members are distributed in five solid clades [1,2]. The five clades correlate with geographical distribution, and one of them includes the **Lupinus** spp. from the Old World. Within this clade, six **Lupinus** species are native of the Iberian Peninsula: the yellow lupine **L. luteus**, the narrow-leaf lupine **L. angustifolius**, the blue lupine **L. cosentinii**, **L. hispanicus**, **L. gredensis** (**L. hispanicus** subsp. **bicolor**) and **L. micranthus**, all growing mainly in acid or neutral soils (pH 5.0–7.0) where the agriculturally important species (**L. luteus** and **L. angustifolius**) are cultivated as forage [5,12,38]. Two additional species (**L.**

* Accession numbers: Nucleotide sequence data reported are available in the GenBank database under the following accession numbers: 16S rDNA, HQ233226–HQ233246; atpD, HQ233182–HQ233204 plus HQ596210 (USDA117) and FN563969; recA, HQ233141–HQ233160 plus HQ596212 (USDA117); glnII, HQ233161–HQ233182 plus HQ596211 (USDA117); nodC, HQ233205–HQ233225.

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albus and L. polyphillus) have been introduced and are now naturalized.

A new species of Lupinus, described as L. mariae-josephi by Pascual [28], is endemic to a small area in Eastern Spain [9]. Remarkably, in contrast with other Lupinus spp. from the Old World that are adapted to grow in acid soils [40,41], L. mariae-josephi thrives in chromic Luvisols with active lime and high pH (• 8.0) [9,25]. A recent taxonomic study placed L. mariae-josephi in a unique phylogenetic position within the Lupinus genus [23].

Despite the agronomic and ecological interest in lupines, the taxonomic classification and phylogenetic placement of rhizobia nodulating Lupinus spp. are still somewhat in a state of flux. Lupinus spp. are nodulated mainly by slow-growing strains classified in the genus Bradyrhizobium [3]. Despite the high genetic diversity uncovered within the Bradyrhizobium genus, only six species have been validated so far: the three soybean-nodulating species B. japonicum [20], B. elkanii [22] and B. liaoningense [51], B. canariense, isolated from endemic genistoid legumes and from the Loteae tribe in the Canary Islands [47], B. yuanmingense [52] from Lespedeza spp. in China, and B. betae from tumor-like root deformation on sugar-beet in Northern Spain [30]. Three new species, designated B. pachyrhizi and B. jicamae, isolated from the legume Pachyrhizus erosus [29], and B. iriomotense, isolated from a root outgrowth of Entada koshunensis, a legume available in Japan [18], have recently been proposed. Additionally, the photosynthetic bradyrhizobia and two other evolutionary lineages, labeled as genospecies alpha and beta, represent unnamed species that may include a greater number of bradyrhizobia isolates not yet characterized [48].

Based on their legume-host ranges and on phylogenetic analysis using housekeeping and symbiotic gene sequences, a significant heterogeneity among lupine bradyrhizobia has been unraveled. Most endosymbiotic bacteria isolated so far from Lupinus in Europe, Australia and Africa are placed in the B. japonicum or in the B. canariense lineages, and are intermingled with isolates from other Genisteae legumes and from Ornithopus spp. [19,36,37,45], whereas Lupinus spp. from the New World are mostly nodulated by strains of B. japonicum [19,36,47].

No endosymbiotic bacteria from Lupinus mariae-josephi have been previously characterized. The restricted geographical location and the adaptation to alkaline soils of this unique Lupinus species emphasize the interest in the isolation and identification of its symbiotic bacteria and in the possible implication of the symbiosis in the establishment of this lupine endemism. These studies may answer questions on the specificity and relative N_2 fixation efficiency of this symbiotic interaction, as well as on the evolutionary history and adaptation of rhizobia to environmental constraints and to the host legume. In this context, special attention has been paid to the comparison of L. mariae-josephi rhizobia with isolates obtained from nodules of other Lupinus spp. of the Iberian Peninsula, generally adapted to acid soils.

Materials and methods

Rhizobia isolation and cultural conditions

Rhizobia strains used in this study are listed in Table 1. Lupinus mariae-josephi endosymbiotic bacteria were isolated from nodules induced in L. mariae-josephi trap plants by bacteria present in the rhizospheric soil samples. These soil samples were collected in five different locations (A, B, C, D, H) in the Llombai area (Monte Aleuda, Micro-reserve "Lloma del Tramusar", geographic location coordinates 30SYJ1055, 240 m above sea level). This area is geographically located in the province of Valencia, Eastern Spain, and is characterized by chromic Luvisol soils (FAO Chromic Luvisols or "terra rossa") with high pH (7.96) that contain active lime and 12% calcium carbonate. Trap plants were cultivated for about 6 weeks in Leonard jars containing Jensen's solution [34]. Each jar was filled with a mixture (1:1) of soil and autoclaved vermiculite, and two plants were grown from axenically germinated seedlings.

Seedlings were obtained from scarified and surface-sterilized seeds (95% ethanol for 1 minute, 25% sodium hypochlorite for 3 minutes, rinsed 10 times and maintained in sterile water for 1 h). The seeds were then germinated on agar plates at 20 °C for 3 days. Jars without soil were used as negative nodulation controls. Bacteria were isolated from nodules and purified on yeast mannitol (YM [46]) or arabinose gluconate (AG [6]) agar plates following a standard technique, and then checked for their ability to nodulate L. mariae-josephi plants cultivated under bacteriologically controlled conditions (sterile Leonard jars containing vermiculite and Jensen's solution, pH 7.0). Confirmed strains were grown on YMA at 28 °C and maintained at 4 °C, or at • 80 °C in 20% glycerol for long-term storage.

Phenotypic characterization

Colony size and slime production were assessed on YM agar plates after 6 days of growth at 28 °C. Mean generation time was determined in YM or AG broth (pH 6.8, 200 rpm orbital shaking) by spectrophotometry (600 nm). Tolerance to low and high pH was estimated by following growth in YMB buffered with either 100 mM sodium citrate/citric acid (pH 4 and 5) or 50 mM Tris HCl/Tris Base (pH 8 and 9).

Cross-inoculation experiments

Cross-inoculation experiments were carried out by testing the capacity of L. mariae-josephi isolates to nodulate 14 different legume hosts and, conversely, by testing rhizobial endosymbionts isolated from these legume hosts for their ability to nodulate L. mariae-josephi plants. The experiments were carried out in sterile Leonard jars prepared as described above containing 2–5-day-old seedlings of the legume hosts tested. Rhizobial suspensions (2 mL, 10^8–10^9 cells mL^{-1}) were added to Leonard jars. Plants were grown at 28–32 °C in plant growth chambers for 3–8 weeks, depending on the legume host, and they were then examined for number, inside color and the nitrogen fixation activity of nodules. The latter was estimated by the acetylene reduction test, as previously described [31]. For each legume host–rhizobial strain combination, three replicates were used, and at least two non-inoculated controls were included per legume host.

DNA amplification

DNA was isolated from 10-day bacterial cultures grown in YMB using DNeasy Blood & Tissue Kit columns (QIAGEN Ltd.). Purified DNA (1 • L, 5–10 ng DNA) was used as template for PCR amplifications. PCR was carried out in a 25 • L volume containing 2.5 • L 10• PCR buffer with magnesium chloride (Roche Applied Science), 10 mM of each dNTP, 10 • M of each primer, 1 • L DMSO and 2.5 U Taq DNA polymerase (Roche Applied Science). Conditions for PCR were those of Herrera-Cervera et al. (16S rRNA) [15], Gaunt et al. (recA and atpD), [11] Turner and Young (glnII) [44] and Sarita et al. (nodC) [33]. Unincorporated primers and dNTPs were removed from PCR products with the Qiaquick PCR purification kit (Qiagen) or, when needed, by gel electrophoresis followed by band purification with the Qiaquick gel extraction kit (Qiagen).

Sequence analysis and phylogenetic tree construction

Partial DNA sequences for housekeeping genes 16S rRNA, glnII, recA and atpD, and symbiotic gene nodC from L. mariae-josephi (Lmj) isolates were determined. These genes were also sequenced from

Table 1
Lupinus mariae-josephi isolates, Bradyrhizobium and fast-growing rhizobia strains used in this study.

Strain	Legume host	Origin	Source or Reference[a]
LmjA2	L. mariae-josephi	Llombai A, Valencia (Spain)	This study
LmjB1, LmjB1b, LmjB2b, LmjB3, LmjB4, LmjB4a, LmjB4b	L. mariae-josephi	Llombai B, Valencia (Spain)	This study
LmjC	L. mariae-josephi	Llombai C, Valencia (Spain)	This study
LmjDb2, LmjD32	L. mariae-josephi	Llombai D, Valencia (Spain)	This study
LmjH2, LmjH2p	L. mariae-josephi	Llombai H, Valencia (Spain)	This study
ISLU22	L. angustifolius	Sevilla (Spain)	IFAPA
ISLU101	L. angustifolius	Jaén (Spain)	IFAPA
ISLU8, ISLU15, ISLU78	L. luteus	Sevilla (Spain)	IFAPA
ISLU9, ISLU12	L. cosentinii	Sevilla (Spain)	IFAPA
ISLU13, ISLU14, ISLU122	L. micranthus	Sevilla (Spain)	IFAPA
ISLU21	L. hispanicus	Ávila (Spain)	IFAPA
ISLU40	L. hispanicus	Salamanca (Spain)	IFAPA
ISLU27	L. albus	Sevilla (Spain)	IFAPA
ISLU203	L. albus	Temuco (Chile)	IFAPA
ISLU16	Ornithopus compressus	Sevilla (Spain)	IFAPA
UPM861	Ornithopus sativus	Madrid (Spain)	UPM
UPM835	Cicer arietinum	Cáceres (Spain)	UPM
UPM924	Vigna sinensis	USA	Nitragin
B. elkanii			
USDA8	Glycine max	Virginia (USA)	USDA
USDA76^T	Glycine max	California (USA)	USDA
USDA117	Glycine max	Mississippi (USA)	USDA
USDA275	Glycine max	India	USDA
B. japonicum USDA110	Glycine max	Florida (USA)	USDA
Rhizobium sp. NGR234	Lablab purpureus	Papua New Guinea	[42]
Mesorhizobium loti TONO	Lotus japonicus	Japan	[21]

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representative isolates of **Lupinus** spp. adapted to growth in acid soils from the Iberian Peninsula, one isolate from serradella and two **B. elkanii** strains. Sequences obtained from the PCR products were assembled with Sequencher (Gene Code Corporation, Ann Arbor, MI, USA). When available in the GenBank/EMBL databases, sequences of the same genes from fast-growing strains of rhizobia, and reference or recently isolated strains of **Bradyrhizobium** were used for sequence comparisons. Multiple alignments were computed with MUSCLE 3.7 [8], and manually corrected using the Se-Al application (http://tree.bio.ed.ac.uk/software/seal/). The www.phylogeny.fr web server [7] was used for maximum likelihood (ML) phylogenetic analyses, and the bioinformatics software PAUP* (version 4.0b10 [50]) for neighbor-joining (NJ) phylogenies. Phylogenetic trees were built using both the ML and NJ methods. Consistent results were obtained by both sequence analysis methods (data not shown), and only ML trees are presented. All phylogenetic trees were visualized with the MEGA 4.1 package [39].

Results

L. mariae-josephi nodule isolates (named Lmj) were obtained using trap plants and soil samples from five sites in the Llombai area (Valencia, Eastern Spain), where wild plant populations of **L. mariae-josephi** were recently found [9,25]. No more than 10 nodules were obtained per plant. Thirteen out of 75 isolates were selected at random. These isolates were tested for induction of nodulation and effective nitrogen fixation capacity by re-inoculating **L. mariae-josephi** seedlings and growing the plants under bacteriologically controlled conditions. In this test, the selected isolates induced red nodules and generated healthy plants with dark-green leaves.

The Lmj isolates were extremely slow-growing bacteria with mean generation times of >20 h in YMB or • 15 h in AGB. Colonies were not mucoid and had a size of <2 mm diameter after 6 days on YMA plates. This extremely slow growth rate complicated phenotypic characterization. Differences in YMB growth generation times were noted among isolates, which could be divided into a faster group (>20 h), represented by isolates LmjC, and LmjH2p, and a slower group (>30 h), represented by isolates LmjDb2 and LmjB2b. In contrast, the mean generation times of isolate ISLU101 from **L. angustifolius** and of isolates from other **Lupinus** spp. growing in acid soils, as well as strains **B. japonicum** USDA110, **B. canariense** ISLU16 and **B. elkanii** USDA275, were less than 10 h, and colonies were mucoid and >4 mm diameter after 6 days on YMA plates. **L. mariae-josephi** isolates were unable to grow at pH 5.0, in contrast to the **B. japonicum** USDA110 strain and isolate ISLU101 from **L. angustifolius**. However, all strains were able to grow at pH 8.0. These results are summarized in supplementary Table S1.

Plant infectivity analysis

The symbiotic specificity of rhizobia nodulating the novel **Lupinus mariae-josephi** species was investigated in plant cross-inoculation assays. In a first assay, a set of five Lmj isolates, representative of each soil sample, was examined for infectivity and N_2 fixation with seven **Lupinus** spp. growing in acid soils in the Iberian Peninsula and six other legume species, most of them reported to be nodulated by lupine rhizobia (Table 2). In a second test, 12 isolates from these **Lupinus** spp. of the Iberian Peninsula, and several related **Bradyrhizobium** strains were also tested for nodulation and N_2 fixation efficiency with **L. mariae-josephi** as the host (Table 3).

The five **L. mariae-josephi** isolates examined exhibited similar symbiotic behavior with most of the legume hosts tested (Table 2). As a control, the five strains nodulated and efficiently fixed N_2 with their cognate host **L. mariae-josephi.** Remarkably, no clear nodulation by any of the five strains was observed with **L. angustifolius**, **L. luteus**, **L. hispanicus**, and **L. gredensis**. Apart from strain LmjDb2, the Lmj isolates tested nodulated **L. cosentinii**, although nodules exhibited low acetylene reduction rates. **L. micranthus** was nodulated only by isolates LmjA2, LmjC and LmjH2p. In contrast, **L. albus** was profusely nodulated by all Lmj isolates examined, consistent with the previously reported promiscuous nodulation character of **L. albus** [43]. It is also noteworthy that only isolate LmjC was found to efficiently nodulate **Macroptilium atropurpureum**.

Table 2
Legume host-range analysis of representative Lupinus mariae-josephi rhizobia isolates.[a]

Legume hosts	Isolates									
	LmjA2		LmjB2b		LmjC		LmjDb2		LmjH2p	
	Nodulation	NF	Nodulation	NF	Nodulation	NF	Nodulation	NF	Nodulation	NF
L. mariae-josephi	•	9.9 ± 1.7	•	11.2 ± 2.6	•	14.8 ± 2.9	•	9.2 ± 2.1		15.2 ± 1.8
L. angustifolius	X		X		X		X		X	
L. luteus	X		X		X		X		X	
L. micranthus	■	1.5 ± 0.0	X		□	3.0 ± 0.8	X		■	3.7 ± 0.8
L. hispanicus	X		X		X		X		X	
L. cosentinii	■	1.3 ± 0.3	□	1.2 ± 0.4	□	9.4 ± 1.4	X		□	2.3 ± 0.7
L. gredensis	X		X		X		X		X	
L. albus	■	4.0 ± 1.3	□	2.8 ± 1.4	□	5.8 ± 1.3	□	3.5 ± 2.0	□	2.8 ± 1.1
Glycine max	X		X		X		X		X	
Vigna sinensis	•	0.0	•	0.0	•	0.0	•	0.0	•	0.0
Lotus corniculatus	X		X		X		ND		ND	
Ornithopus sp.	X		X		X		ND		X	
Cicer arietinum	X		X		X		X		X	
Macroptilium atropurpureum	•	0.0	•	0.0	□	3.9 ± 0.0	ND		•	0.0

[a] Nodulation was evaluated by the number and inside colour of nodules: (•) red, (□) reddish, (•) white, (X) no nodules, (ND) not determined. Nitrogen fixation (NF) was determined by the acetylene reduction test and expressed as • moles of acetylene reduced • (h • g of nodules)• ^{1} ± standard deviation.

Soybeans (**Glycine max**) and chickpeas (**Cicer arietinum**) were not nodulated by Lmj isolates and only tumor-like nodules with no N_2 fixation capacity were observed with cowpeas (**Vigna sinensis**), **Lotus corniculatus**, and serradella (**Ornithopus compressus**).

In the second test, the symbiotic response of **L. mariae-josephi** as a host for rhizobial isolates from **Lupinus** spp. of the Iberian Peninsula was tested using their cognate **Lupinus** spp. as control legume hosts (Table 3). All the isolates from **L. angustifolius**, **L. luteus**, **L. cosentinii**, **L. micranthus**, **L. hispanicus** and **L. albus** induced inefficient nitrogen-fixing nodules, although differences were observed among isolates from the same lupine host. The lowest (or nil) nitrogen fixation capacity was observed with certain **L. angustifolius**, **L. cosentinii** and **L. hispanicus** isolates. On the other hand, **L. mariae-josephi** was not nodulated by soybean (either **B. japonicum** or **B. elkanii**), cowpea or chickpea strains. In contrast, the serradella strains tested induced the formation of poorly effective nitrogen fixing nodules with **L. mariae-josephi** plants.

Overall, plant infectivity analyses revealed that the **L. mariae-josephi** endosymbiotic bacteria were remarkably selective for nodulation of **Lupinus** spp. and were unable to nodulate or fix nitrogen with soybeans, serradella, cowpeas or **L. corniculatus**. In contrast, **L. mariae-josephi** was nodulated, albeit inefficiently, by most of the endosymbiotic bacteria isolated from other **Lupinus** spp. thriving in acid soils of the Iberian Peninsula.

Phylogeny of the 16S rRNA gene

The current phylogenetic classification of rhizobia is mainly based on 16S rRNA gene sequences, and therefore phylogenetic analysis based on this gene was used as a first approach for the

Table 3
Symbiotic response of Lupinus mariae-josephi to different Bradyrhizobium strains.[a]

Strains	Behavior with the original legume host			L. mariae-josephi host	
	Host	Nodulation	NF	Nodulation	NF
H2p	L. mariae-josephi			•	15.2 ± 1.8
ISLU101	L. angustifolius	•	4.6 ± 0.7	•	0.0
ISLU22	L. angustifolius	•	7.7 ± 2.0	■	0.4 ± 0.2
ISLU8	L. luteus	•	6.9 ± 1.1	•	0.4 ± 0.1
ISLU78	L. luteus	•	4.8 ± 0.2	■	0.7 ± 0.1
ISLU12	L. cosentinii	ND	ND	•	0.0
ISLU9	L. cosentinii	ND	ND	■	2.3 ± 0.1
ISLU13	L. micranthus	•	5.5 ± 2.0	■	0.2 ± 0.1
ISLU122	L. micranthus	•	3.2 ± 0.6	■	0.6 ± 0.3
ISLU21	L. hispanicus	•	7.5 ± 0.1	■	2.1 ± 1.1
ISLU40	L. hispanicus	•	4.0 ± 0.9	•	0.0
ISLU27	L. albus	•	2.9 ± 0.7	■	0.5 ± 0.4
ISLU203	L. albus	•	9.3 ± 1.3	■	0.4 ± 0.5
USDA8	G. max	•	4.9 ± 1.1	X	
USDA275	G. max	•	2.5 ± 0.6	X	
USDA110	G. max	•	4.5 ± 1.3	X	
ISLU16	O. compressus	•	3.9 ± 1.6	■	0.8 ± 0.4
UPM861	O. compressus	•	8.0 ± 0.9	■	1.2 ± 0.1
UPM924	V. sinensis	•	11.9 ± 2.4	•	0.0
UPM835	C. arietinum	•	3.7 ± 0.4	X	
TONO	L. corniculatus	•	6.4 ± 0.5	X	
NGR234	M. atropurpureum	•	11.2 ± 1.7	X	

[a] Footnote as in Table 2.

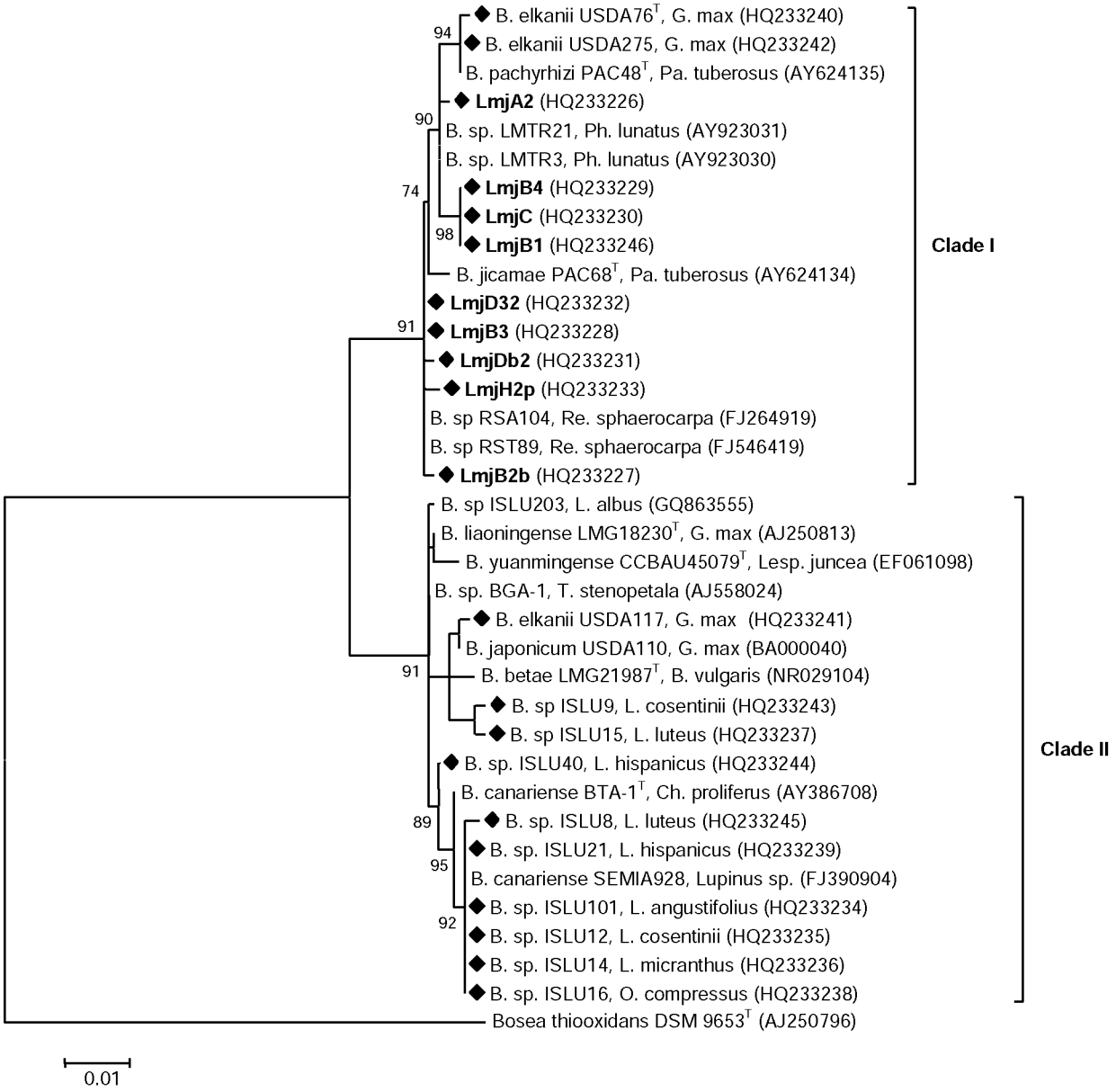

Fig. 1. Maximum likelihood phylogenetic tree showing relationships of nodule isolates from L. mariae-josephi and other symbiotic bacterial strains based on nearly complete 16S rRNA gene sequences (1400 bp). Strains designated Lmj have been isolated from L. mariae-josephi nodules in this study, and are shown in boldface type. Bootstrap values (greater than 70%) were calculated for 1000 subsets and are indicated at the relevant nodes. Isolates of L. mariae-josephi and strains indicated with diamonds (•) were sequenced in this study. Legume hosts follow strain names. Accession numbers from GenBank are shown in brackets. The scale bar shows the number of substitutions per site. Abbreviations: A, Azorhizobium; B, Bradyrhizobium; R, Rhizobium; S, Sinorhizobium; L, Lupinus; O, Ornithopus; Re, Retama; Lesp, Lespedeza; G, Glycine; M, Medicago; P, Pisum; D, Desmodium; Ch, Chamaecytisus; Se, Sesbania; T, Teline; Ph, Phaseolus; Pa, Pachyrhizus.

identification of Lmj nodule isolates. Nearly full length sequences of the 16S rRNA (• 1400 bp) were obtained from nine field nodule isolates of **L. mariae-josephi** and other rhizobial isolates. **Bosea thioxidans** [7] was used as an outgroup (Fig. 1).

The 16S rRNA phylogenetic tree showed that all the Lmj isolates tested (highlighted by bold typeface) belonged to the genus **Bradyrhizobium** in a unique clade (Clade I) that also included isolates from **Retama** spp. from Algeria [4], **B. elkanii** strains USDA76 and USDA275, isolates from Lima bean (**Phaseolus lunatus**) from Peru [26], as well as the species **B. jicamae** and **B. pachyrhizi** [29]. Clade I was highly divergent (• 91% ML bootstrap support) from a second clade (Clade II) that included well-defined and named species of **Bradyrhizobium**, including **B. japonicum** and **B. canariense** [47] and, remarkably, all the isolates from **Lupinus** spp. growing in acid soils in the Iberian Peninsula.

Within Clade I, Lmj isolates were assembled in three phylogenetically distinct subgroups (Fig. 1). Lmj isolates LmjB2b, LmjH2p, LmjDb2, LmjB3 and LmjD32 were closer to isolates from **Retama sphaerocarpa**, and isolate A2 was related to **Bradyrhizobium** sp. LMTR21 and **Bradyrhizobium** sp. LMTR3 strains from **Phaseolus lunatus**. Lmj isolates LmjC, LmjB1 and LmjB4 formed a strongly supported monophyletic group that may define a new **Bradyrhizobium** genospecies. LmjC differed from LmjA2 and LmjB2b in 8 and 14 nucleotides, respectively.

Most of the bradyrhizobia isolates from **Lupinus** spp. (**L. angustifolius**, **L. luteus**, **L. hispanicus**, **L. cosentinii** and **L. micranthus**) growing in acid soils in different geographical locations of the Iberian Peninsula, as well as isolates from **Ornithopus** sp., were related to **B. canariense** reference strain BTA-1, whereas some isolates, such as ISLU9 from **L. cosentinii**, grouped with the **B. japonicum** lineage.

Strains ISLU101, ISLU8 and ISLU21, isolated from **L. angustifolius**, **L. luteus** and **L. hispanicus**, respectively, were also found to be close to **B. canariense** strains based on restriction patterns of amplified 16S rRNA [37]. Isolate ISLU203 from **L. albus** growing in Temuco (Chile) was related to **B. japonicum** according to the 16S rRNA gene analysis, thus confirming previous reports [19,45].

In summary, analysis of 16S rRNA gene sequences showed that **L. mariae-josephi** endosymbiotic bacteria belonged to a phylogenetically distinct **Bradyrhizobium** clade separate to that of nodule isolates from all the other Iberian Peninsula **Lupinus** species.

Phylogeny based on housekeeping genes glnII, recA and atpD

Since the resolution of 16S rRNA analysis is too low for **Bradyrhizobium** species assignment [10,30], a phylogenetic analysis was performed based on the relevant housekeeping genes **glnII**, **recA** and **atpD** in order to define a more robust phylogenetic position of the **L. mariae-josephi** rhizobia. PCR amplifications resulted in DNA fragments with average length of **ca.** 893 bp for **glnII**, 599 bp for **recA** and 527 bp for **atpD**. The amplified genes of isolates from other **Lupinus** spp. from the Iberian Peninsula were also sequenced. Phylogenetic analyses were performed with these sequences and the corresponding sequences from previously studied strains [19,47] and from recently identified strains of **Bradyrhizobium** [4,26].

Consistent with the 16S rRNA phylogenetic tree, analyses based on the single and concatenated genes **glnII**, **recA** and **atpD** again grouped the **L. mariae-josephi** isolates in two different clades (Clade I and Clade II) within the **Bradyrhizobium** genus. In the concatenated analysis (Fig. 2), Clade I included all the Lmj isolates tested, which formed a new lineage (99% bootstrap support) that also included **B. jicamae** PAC68 [29]. Clade I was phylogenetically highly distinct from Clade II (90% bootstrap support) that included all named **Bradyrhizobium** species and all the isolates from other **Lupinus** spp. from the Iberian Peninsula. The topology of the new lineage was well resolved and revealed that all the Lmj isolates, shown in bold type in the tree, were found in three subgroups designated subgroup **a** (isolates LmjDb2, LmjB2b, LmjB3, LmjD32 and LmjA2), subgroup **b** (isolate LmjH2p) and subgroup **c** (LmjB4a, LmjB1, LmjC and LmjB4). Isolate LmjA2 showed the highest grade of divergence within subgroup **a**.

According to both ML and NJ methods, all isolates from other **Lupinus** spp. of the Iberian Peninsula grouped in Clade II of the **Bradyrhizobium** genus. Most of the ISLU isolates nested with the **B. canariense** reference strain BTA-1 [49]. However, ISLU40, isolated from **L. hispanicus**, and ISLU203, isolated from **L. albus** in Chile, grouped with the **B. japonicum** bv. **genistearum** lineage (type strain BGA-1) [47,48]. It is also worth noting that isolate ISLU9 from **L. cosentinii**, clearly diverged from the **Lupinus** spp. isolates included in the **B. canariense** and **B. japonicum** lineages.

Phylogenetic analyses based on the single genes **glnII**, **recA** and **atpD** (Supplementary Figs. S1, S2 and S3) were essentially congruent with those based on the concatenated genes, with some minor differences. Thus, isolates from **Retama sphaerocarpa** from Northern Algeria were shown to be phylogenetically close to **L. mariae-josephi** isolates in the **glnII** and **recA** analyses (Figs. S1 and S2). Also, **B. elkanii** strains USDA76 and USDA275 were not included in Clade I but in Clade II in the **recA** and **atpD** gene phylogenies (Figs. S2 and S3).

Phylogeny of the nodC gene

NodC is a protein required for synthesis of the rhizobial nodulation factors involved in legume infection signaling. All the **L. mariae-josephi** isolates showed very similar **nodC** sequences that defined a highly supported (99% bootstrap support) monophyletic lineage (Fig. 3) with no close relationship to **nodC** sequences from other **Bradyrhizobium** lineages [45,47]. In contrast, isolates from all the other **Lupinus** spp. from the Iberian Peninsula and from **Ornithopus** spp. were recovered in **B. canariense** bv. **genistearum** or in the **B. japonicum** bv. **genistearum** lineages, and they were distant from strains nodulating **Glycine** (**B. japonicum** bv. **glycinearum**) [19,45,47]. As an example, the **nodC** sequences of ISLU101, ISLU21, ISLU23, ISLU12 and ISLU14, isolated from **L angustifolius**, **L. hispanicus**, **L. luteus**, **L. cosentinii**, and **L. micrantus**, respectively, only showed a 78-80% nucleotide sequence similarity to isolate LmjC. It is noteworthy that **nodC** sequence analysis (Fig. 3) clustered **B. elkanii** strains USDA76 and USDA275 together with strains nodulating **Glycine** (100% bootstrap support).

Discussion

Lupinus is a diverse genus that grows in a wide range of ecological conditions, including nutrient-poor arid soils and extreme climates, where cultivation of more demanding crops is problematic [14,17]. It is generally accepted that **Lupinus** spp. are legumes adapted to growth in acid soils [14,41]. In contrast, the novel **Lupinus mariae-josephi** species [23,28] thrives in soils with active lime and high pH, which are primary factors that restrict the growth of commercial lupines, although different levels of tolerance to alkaline soils have been observed [41]. Among the **Lupinus** spp. native to the Iberian Peninsula, **L. cosentinii** is the most tolerant to basic soils [5]. Based on the singular adaptation of **L. mariae-josephi** to a basic soil it was of interest to characterize the endosymbiotic bacteria of this new **Lupinus** species.

Lupinus spp. are nodulated mainly by slow-growing strains classified in the genus **Bradyrhizobium** [3]. However, studies over the last few years have revealed a high genetic diversity among **Bradyrhizobium** strains isolated from **Lupinus** found on different continents and ecosystems, as well as from agricultural and wild plants. A general conclusion from these studies is that **Lupinus** spp. endosymbiotic bacteria are related to the **B. japonicum** and **B. canariense** species [19,35–37,45,47–49].

Data obtained in this study showed that **L. mariae-josephi** symbionts were very slow-growing bacteria assigned to the **Bradyrhizobium** genus with phenotypic and phylogenetic characteristics clearly different from other members of the genus. The extra-slow-growing behavior of **L. mariae-josephi** bacteria made their characterization difficult. Extra-slow growth has previously been described, among others, for certain isolates from Lima beans (**Phaseolus lunatus**) from Peru [26] and retama (**Retama raetam** and **R. sphaerocarpa**) from Northern Algeria [4]. Despite the basic nature of the soils where **L. mariae-josephi** thrives, its nodule symbionts were not remarkably tolerant to alkaline pH in YM broth, as compared to other **Bradyrhizobium** species, but were distinctly more sensitive to acid pH. In this regard, it is outstanding (Table 2) that **L. mariae-josephi** endosymbiotic bacteria are unable to nodulate **Lupinus** spp. that require acid soils, such as **L. angustifolius**, **L. luteus**, **L. hispanicus** and **L. gredensis** [16], in contrast with their capacity to nodulate species able to tolerate basic soils such **as L. cosentinii** and **L. albus** [5]. This behavior may suggest an effect of soil on the nodulation specificity of **L. mariae-josephi** bacteria and it is relevant since most, if not all, the **Lupinus** spp. from the Old World also require acid soils [14]. However, it is more likely that the **Lupinus** spp. host is the determinant factor for specificity, and the capacity of **L. mariae-josephi** bacteria to nodulate **L. albus** and **L. cosentinii** is more congruent with the previously reported promiscuity of these species towards diverse lupine endosymbiotic bacteria [37,43].

Phylogenetic analyses based on 16S rRNA and on the housekeeping genes **glnII**, **recA** and **atpD**, congruently grouped all the tested **L. mariae-josephi** isolates in a new strongly supported clade (Clade I) within the **Bradyrhizobium** genus. This clade was phylogenetically unrelated to all the isolates from other **Lupinus** spp. tested in

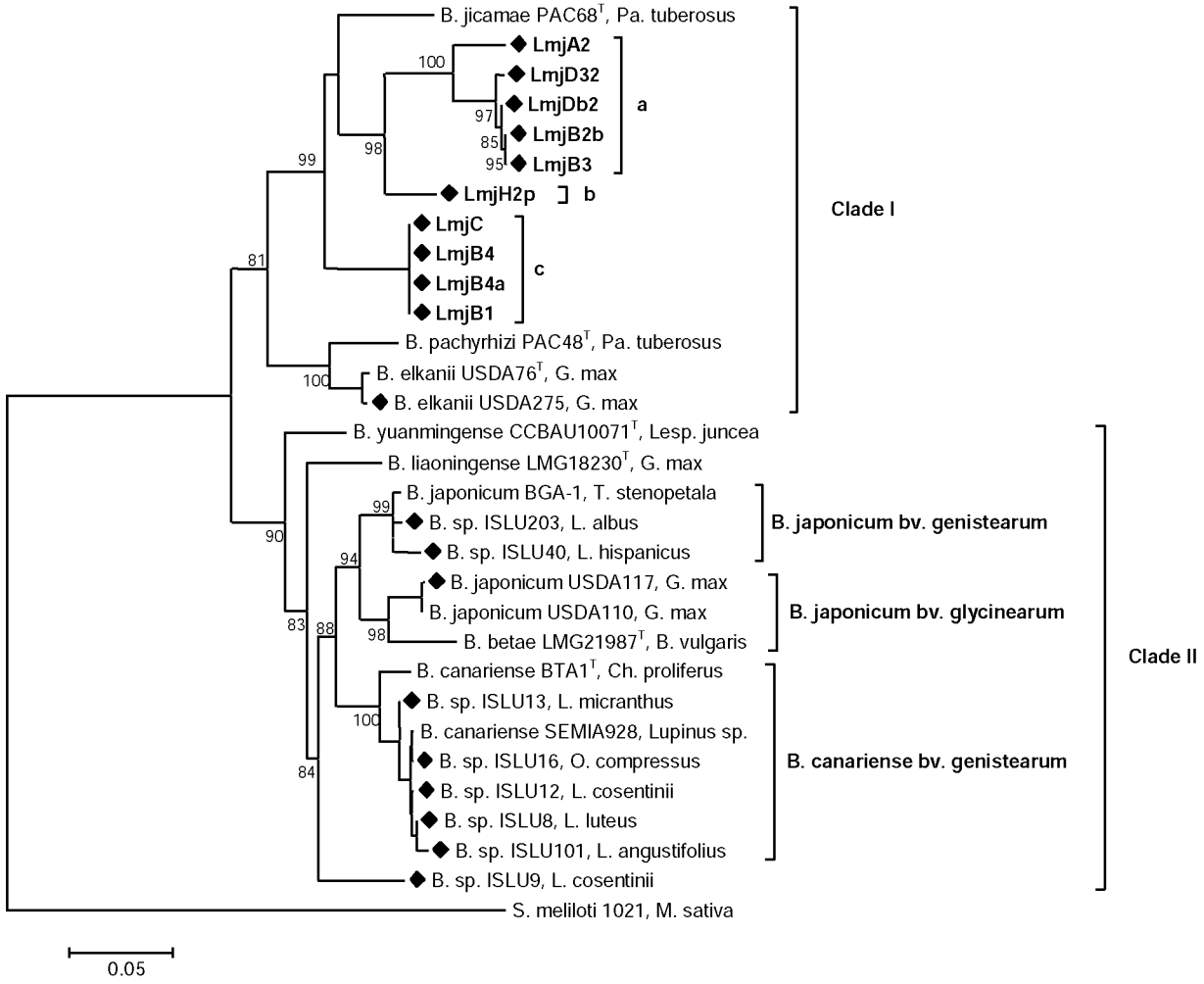

Fig. 2. Maximum likelihood phylogenetic tree based on the alignment of a 1399 bp concatenated nucleotide sequence of glnII (522 bp), recA (434 bp) and atpD (443 bp). ML bootstrap support values (• 80% over 1000 replicates) are indicated at the relevant nodes. Isolates of L. mariae-josephi are shown in boldface type. Accession numbers from GenBank are indicated in Supplementary Figs. S1, S2 and S3. Legume hosts follow the strain names. Isolates of L. mariae-josephi and strains indicated with diamonds (•) were sequenced in this study. The scale bar shows the number of substitutions per site. Letters 'a', 'b' and 'c' reflect different groups of L. mariae-josephi isolates. Abbreviations are defined in the legend for Fig. 1.

this study, as well as previously reported isolates from other **Lupinus** spp. [19,36,37,45,47]. Surprisingly, nodule symbionts of **Retama sphaerocarpa** from Northern Algeria [4] were phylogenetically close to **L. mariae-josephi** isolates, as supported by the phylogenetic analysis of **recA** and **glnII** gene sequences (Supplementary Figs. S1 and S2). However, additional data are needed to understand the meaning of this phylogenetic proximity. In contrast to bradyrhizobia from **Retama sphaerocarpa** from Northern Algeria, nodule isolates from **R. sphaerocarpa** growing in semiarid areas of Central Spain were related to **B. canariense** (Clade II) [32].

The phylogenetic tree based on the concatenated **glnII** + **recA** + **atpD** gene sequence grouped **L. mariae-josephi** isolates, together with **B. jicamae**, in a monophyletic cluster within Clade I (Fig. 2). Within this cluster, **L. mariae-josephi** isolates shared the common phenotypic trait of being able to nodulate and fix N_2 with this unique lupine [23,28] in a peculiar alkaline-high calcium content habitat, and, therefore, may constitute a new evolutionary lineage within the **Bradyrhizobium** genus. Three distinct, well-supported groups of **L. mariae-josephi** isolates could be differentiated and may represent at least three unnamed genospecies (Fig. 2). This distinction could also be observed in the single **glnII**, **recA** and **atpD** gene trees, although isolates LmjA2 and LmjH2p were differently resolved in the **recA** tree. The type isolates, LmjB2b, LmjH2p and LmjC, representing each of the three groups (**a**, **b** and **c**), also differed in symbiotic properties, such as the nodulation ability of **L. micranthus** and **M. atropurpureum**, or their nitrogen fixation efficiency (Table 2). Additional isolates and detailed phenotypic and genetic studies will be required to clearly define new species from these potential new sister genospecies of Lmj bacteria. ML phylogenetic analysis of **nodC** gene sequences also supported that **L. mariae-josephi** isolates likely represent a new, distinct evolutionary lineage. Although analysis of more symbiotic genes is needed, the high similarity among **L. mariae-josephi** isolates based on **nodC** is congruent with their host specificity range (unable to nodulate **Lupinus** spp. adapted to acid soils such as **L. angustifolius** or **L. luteus**), and again suggests that it is the plant host that determines endosymbiotic bacterial selection.

In contrast to the distribution of Lmj isolates in three different groups (**a**, **b** and **c**) based on the phylogenetic analyses of housekeeping genes (Fig. 2 and Supplementary Figs. S1, S2 and S3), only two clearly different (86–94% bootstrap support) groups of **nodC** sequences were identified within the symbiotic lineage of Lmj isolates (Fig. 3). Singularly, cross-inoculation experiments showed symbiotic differences between the two groups (Table 2). The first genotype, that included isolates LmjA2, LmjC and LmjH2p, was able

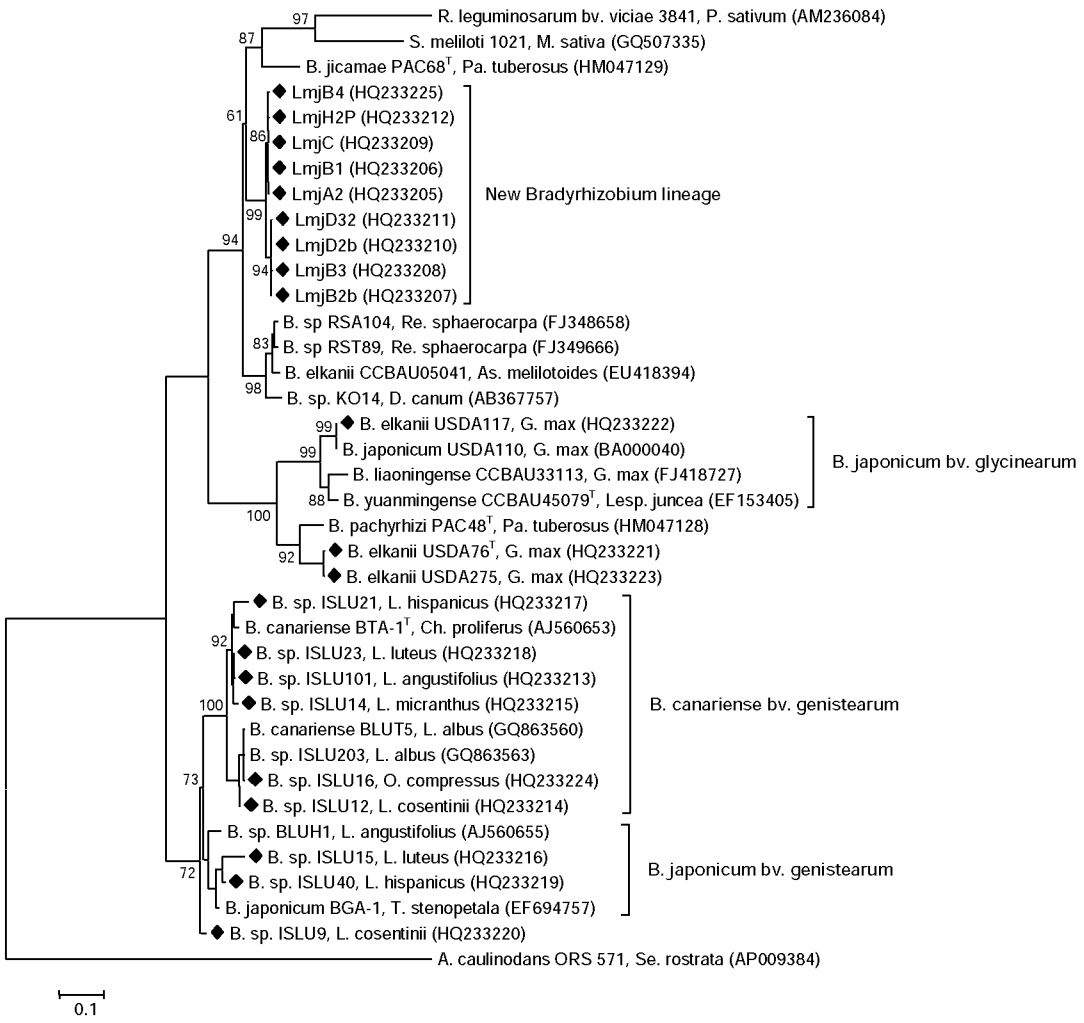

Fig. 3. Maximum likelihood phylogenetic tree based on partial nodC sequences (455 bp). Isolates designated Lmj have been isolated from L. mariae-josephi nodules in this study and are shown in boldface type. Bootstrap values (• 60% over 1000 replicates) are indicated at the relevant nodes. Legume hosts follow the strain names. Accession numbers from GenBank are shown in brackets. Isolates of L. mariae-josephi and strains indicated with diamonds (•) were sequenced in this study. The scale bar shows the number of substitutions per site. Abbreviations are defined in the legend for Fig. 1.

to nodulate **L. micranthus**, while isolates from the second genotype (LmjB2b, LmjDb2) were unable to do so. Conflicting phylogenies between core and symbiotic genes may be explained by lateral transfer of symbiotic genes among rhizobia, which is a process that has been widely documented [24,27,47,53].

Possible structural or genetic differences between the **L. mariae-josephi**/Lmj bacteria symbiosis and other lupine symbioses are unknown and will require additional symbiotic and genetic studies. Nevertheless, it can be concluded that a potential allopatric isolation of **L. mariae-josephi** in the "terra rossa" alkaline soils of Eastern Spain has selected a singular population of endosymbiotic bacteria, which are phenotypically and phylogenetically different from bacteria nodulating **Lupinus** spp. thriving in acid soils of the Iberian Peninsula, such as **L. angustifolius** or **L. luteus.**

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2010.11.020.

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