UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY
INTERDEPARTMENTAL PROGRAMME IN MICROBIOLOGY

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SYMBIOTIC AND PHYLOGENETIC CHARACTERIZATION OF RHIZOBIA THAT NODULATE THE NOVEL SPECIES OF *Lupinus* (*L. mariae-josephi*)

GRADUATION THESIS
University studies

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DIPLOMSKO DELO
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Ljubljana, 2009

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The thesis work is a completion of university studies of microbiology. The work was carried out in the laboratory of microbiology in the Department of Biotechnology, E.T.S.I. Agrónomos, Polytechnical University of Madrid, and in the facilities of CBGP, Centro de biotechnología y genómica de plantas, Madrid (Spain).
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AB The genus *Lupinus* L. is a large and diverse group of papilinoid legumes comprised of about 275 species. The lupins are among the richest plants in seed storage proteins next to soybean, what makes them of growing agronomic and ecological interest as proteins and nitrogen suppliers. *Lupinus mariae-josephi* is a novel lupin species recently discovered in the Eastern part of Spain (Valencia province). This species is unique for its natural growth conditions requiring limy alkaline soils with high concentrations of calcium carbonate to grow. Several *Lupinus mariae-josephi* root-nodulating bacteria were isolated from rhizospheric soil of Valencia using trap plants. A polyphasic approach analyzing phenotypic, symbiotic and genetic properties was used to characterize the *L. mariae-josephi* endosymbiotic bacteria. The *L. mariae-josephi* nodulating strains tested were extremely slow-growing bacteria (g > 24h) in standard yeast mannitol media. Legume cross-inoculation experiments showed that *L. mariae-josephi* plants and its symbionts had host specificity remarkably different from symbiotic bacteria of other *Lupinus* spp. from the Iberian Peninsula that characteristically grow in acid soils. Phylogenetic analysis based on 16S rRNA sequences confirmed that *L. mariae-josephi* isolates belong to the *Bradyrhizobium* genus of slow-growing bacteria, and revealed that all of them cluster in a unique monophyletic group of bradyrhizobia related to *B. elkanii*.

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IJ en
JI en/sl
AI Rod *Lupinus* L. je velika in raznolika skupina stročnic, ki obsega približno 275 vrst iz družine metuljnic. Rastline volčjega boba igrajo vedno večjo vlogo v kmetijstvu in ekologiji. Njihova semena namreč vsebujejo veliko beljakovin in dušika, podobno kot beljakovinsko bogata semena soje. Nedavno so v vzhodnih predelih Španije v provinci Valencija odkrili novo vrsto volčjega boba, imenovano *Lupinus mariae-josephi*, ki uspeva v apnenčastih in bazičnih tleh z visokimi koncentracijami kalcijevih ionov. Iz rizosfernih tal so z rastlinskimi vabami uspeli izolirati več bakterijskih sevov, ki tvorijo specifične simbiontske povezave z *L. mariae-josephi*. Namen te diplomske naloge je bil preučiti lastnosti sevov *L. mariae-josephi* z analizo fenotipskih, simbiotskih in genotipskih lastnosti. Vsi sevi so v standardnem gojišču s kvasnim ekstraktom in manitolom rasli izredno počasi (g > 24h). Križno inokulacijski testi so pokazali, da se gostiteljska specifičnost rastline *L. mariae-josephi* in njenih endosimbiontov zelo razlikuje od specifičnosti endosimbiotskih bakterij drugih vrst *Lupinus* spp., ki rastejo v kislih tleh Iberskega polotoka. Filogenetska analiza na podlagi *rrs* sekvenc je potrdila, da izolati *L. mariae-josephi* pripadajo rodu *Bradyrhizobium*, ki vključuje počasi rastoče bakterije. Hkrati pa je tudi razkrila, da ti izolati pripadajo posebni monofiletski skupini vrst rodu *Bradyrhizobium*, ter so najbolj sorodni vrsti *B. elkanii*.

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ABBREVIATIONS AND SYMBOLS

AG	L-arabinose Na-gluconate medium
aLRT	approximate likelihood-ratio test
atpD	gene coding F1 sector of membrane-bound ATP synthase
BNF	biological nitrogen fixation
dnaK	gene coding for chaperone protein HSP70
dNTPs	deoxyribonucleotide triphosphates
DMSO	dimethyl sulfoxide
E. coli	*Escherichia coli*
EPS	extracellular polysaccharides
FID	flame ionizing detector
glnII	gene coding glutamine synthetase II
GS	glutamine sythetase
GOGAT	glutamate synthase
ITS	internal transcribed spacer
LB	Luria-Bertani medium
noeI	gene involved in the methylation of the Nod factor fucose residue
nifH	gene involved in MoFe nitrogenase protein maturation
nod	genes coding nodulation proteins
nolL	gene involved in acetylation of Nod factor fucose residues
OD_{600}	optical density at wavelength 600 nm
rrs	gene coding small ribosomal subunit 16SrRNA
SSU	small subunit
UV	ultraviolet light
VIS	visible light
YMB	yeast mannitol broth

1 INTRODUCTION

Recently, a novel lupin species *Lupinus mariae-josephi* was described growing from storage seeds which were collected from the region of Valencia in the Eastern part of Spain (Pascual, 2004). The plant grows in alkaline soil with high concentrations of Ca^{2+}, whereas other lupin species of the Iberian Peninsula normally grow in acid soils (Castroviejo and Pascual, 1999). The genus *Lupinus* L. (lupins) is a large and diverse group of papilinoid legumes (family *Leguminosae*) comprising about 275 species; however, only 13 species can be found in the Old World (Aïnouche *et al.*, 2004). From the biochemical point of view, the lupins are among the richest plants in seed storage proteins, the characteristic that makes them of growing agronomic and ecological interest as proteins and nitrogen suppliers (Gladstones, 1974, *op.cit.*: Aïnouche *et al.*, 2004; Cerreti, 1983; *op.cit.*: Aïnouche *et al.*, 2004).

Legumes are widely known for their ability to establish symbiotic associations with stem and root-nodulating bacteria, collectively known as rhizobia (Menna *et al.*, 2006). However, despite the agronomic, alimental and ecological interest of *Lupinus* L., this plant has been poorly studied with respect to its symbionts. Generally, lupins have been reported to be nodulated by slow-growing rhizobia, namely species belonging to genus *Bradyrhizobium*: *B. japonicum* (Jarabo-Lorenzo *et al.*, 2003), *B. canariense* (Stępkowski *et al.*, 2005), and *B. elkanii* (Barrera *et al.*, 1997). In spite of recent progress, there is still a large number of legume isolates, especially bradyrhizobial strains, which need to be more deeply studied to define their taxonomic status which remains unclear due to the inconsistency among the results obtained by different taxonomic methods (Stępkowski *et al*., 2003; Moulin *et al*., 2004; Stępkowski *et al*., 2007).

1.1 HYPOTHESES AND OBJECTIVES

The objective of this graduation thesis was a phylogenetic and symbiotic characterization of endosymbiotic bacteria isolated from nodules of novel lupin species *Lupinus mariae-josephi*. Strains isolated from lupins have been mostly reported as slow-growing rhizobia and members of the genus *Bradyrhizobium*. It was predicted that the isolated strains could

belong to this genus, although they could differ from rhizobia nodulating *Lupinus* spp. in acid soils because *L. mariae-josephi* grows in particular natural growth conditions (alkaline pH, high concentrations of Ca^{2+}). Moreover, *L. mariae-josephi* plants do not seem closely related to other lupins described in the Iberian Peninsula.

The specificity of rhizobial strains isolated from nodules of *L. mariae-josephi* with lupins and other legume hosts was studied. Additionally, it was expected that the study of the specificity of *L. mariae-josephi* would unravel a high specificity of the host plant towards rhizobial strains. The study of the specificity of *L. mariae-josephi* was performed by using lupins from the Iberian Peninsula and related legumes and their symbionts.

2 STATE OF THE ART

2.1 BIOLOGICAL NITROGEN FIXATION

Nitrogen is an essential element of biomolecules such as amino acids, proteins, vitamins, nucleic acids and many others required for growth and reproduction of organisms. The atmospheric nitrogen (N_2) represents approximately 78% of the Earth's atmosphere and is the biggest reservoir of nitrogen. However, because of the strong triple bond between the two atoms (N≡N) it cannot be used directly by animals or plants. The only living organisms capable of reducing N_2 to a form that is accessible to plants and animals belong to domains *Bacteria* and *Archaea*. The reduction process is called biological nitrogen fixation (BNF) or diazotrophy (Young, 1992).

BNF is performed by free living diazotrophs or diazotrophs associated to other organisms and endosymbionts. Together they account for an estimated amount of 150 Tg of fixed nitrogen annually, representing ~ 90% of all of fixed N_2 in the terrestrial environments. The remaining 10% is fixed abiotically, primary by lightning. On the other hand the human activity, especially by generation of ammonium compounds for agricultural fertilizers with the Haber-Bosch process, contributes an estimated 140 Tg of additional fixed nitrogen each year which are mainly used for agricultural fertilizers (Zaharan, 1999; Gage, 2004).

The demand for fertilizers is rising proportionally to the rise of the world population but the production costs and the impact on the environment make their use problematic. BNF is, compared to chemical fertilization, more efficient, economical, and environmental friendly which makes it a good candidate for the expanded use in agriculture.

2.1.1 Nitrogenase

The enzyme complex responsible for nitrogen reduction is the nitrogenase which is irreversibly inactivated by oxygen. The reduction of N_2 to NH_3 is a highly endergonic and energy consuming reaction:

$$N_2 + 16MgATP + 8H^+ + 8e^- \xrightarrow{N2ase} 2NH_3 + H_2 + 16MgADP + 16P_i$$

Most nitrogenases are composed of two proteins: dinitrogenase reductase and dinitrogenase with an iron molybdenum cofactor (FeMo-co). Two more homologous, alternative nitrogenases can be found in nature: the V-containing nitrogenase and Fe-only nitrogenase (Rubio and Ludden, 2005).

The stoichiometry of the reaction showed above holds true only in laboratory conditions. In natural conditions up to 40 molecules of ATP can be hydrolyzed for the reduction of only one molecule N_2 (Hill, 1992). Additionally, for every reduced molecule of N_2 the nitrogenase complex produces another molecule H_2. The hydrogen production has been described as one of the major factors that affect the efficiency of symbiotic nitrogen fixation, however, some rhizobia species have developed a system of hydrogenases that allows them to recycle the generated hydrogen (Baginsky *et al.*, 2002).

3.1.2 Nitrogen fixing organisms

Nitrogen fixing microorganisms are roughly clustered in three groups: one group consists of free living microorganism such as *Klebsiella*, *Azotobacter* and *Rhodobacter* spp., the microorganisms of the second group only fix nitrogen in extracellular associations (*Azospirillum* spp., *Anabaena*, *Nostoc*) (Boddey *et al.*, 2000), and the third forms endosymbiosis with higher plants. In the latter group actinomycetes from the genus *Frankia* that are associated with wooden plants such as *Alnus* (alder) or *Casuarina* (Benson and Clawson, 2000) can be found as well as a group of rhizobia belonging to the α-proteobacteria subclass that associate with leguminous plants. The rhizobia are gram-negative soil bacteria that form nodules on the plant roots where the bacteria fix atmospheric nitrogen. Currently, six genera are widely recognized: *Rhizobium*, *Azorhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, and *Bradyrhizobium*. The taxonomy of fast-growing rhizobia has been well developed, and has led to the proposal of about thirty new species. It is a group in continuous expansion. In contrast, slow-growing rhizobia (i.e. genera *Bradyrhizobium* and *Azorhizobium*) taxonomy remains unclear due to the inconsistency among the results obtained by different taxonomic methods (Jarabo-Lorenzo *et al.*, 2003). The fast growing rhizobia are closely related. Slow-growing rhizobia

are on the contrary fairly distant in the SSU phylogeny, being no more related to the other rhizobia, or to each other, as many non-symbiotic bacteria.

Microorganism belonging to other rhizobial branches have also been found to nodulate legumes, i.e. genera *Metylobacterium* isolated from nodules of *Crotalaria* (Sy *et al.*, 2001), *Blastobacter* from *Aeschynomene indica* (van Berkum and Eardly, 2002), *Devosia* isolated from an aquatic legume *Neptunia natans* (Vanparys *et al.*, 2005), *Ochrobactrum* from *Lupinus honoratus* (Trujillo *et al.*, 2005), and *Phyllobacterium* from *Trifolium pratense* (Valverde *et al.*, 2005) (Figure 1). Phylogenetic sequence analysis of nodulation genes which are shared by all different genera, showed that they are closely related to rhizobial genes, suggesting that they were acquired by horizontal gene transfer (Sy *et al*., 2001; Trujillo *et al.*, 2005; Valverde *et al.*, 2005).

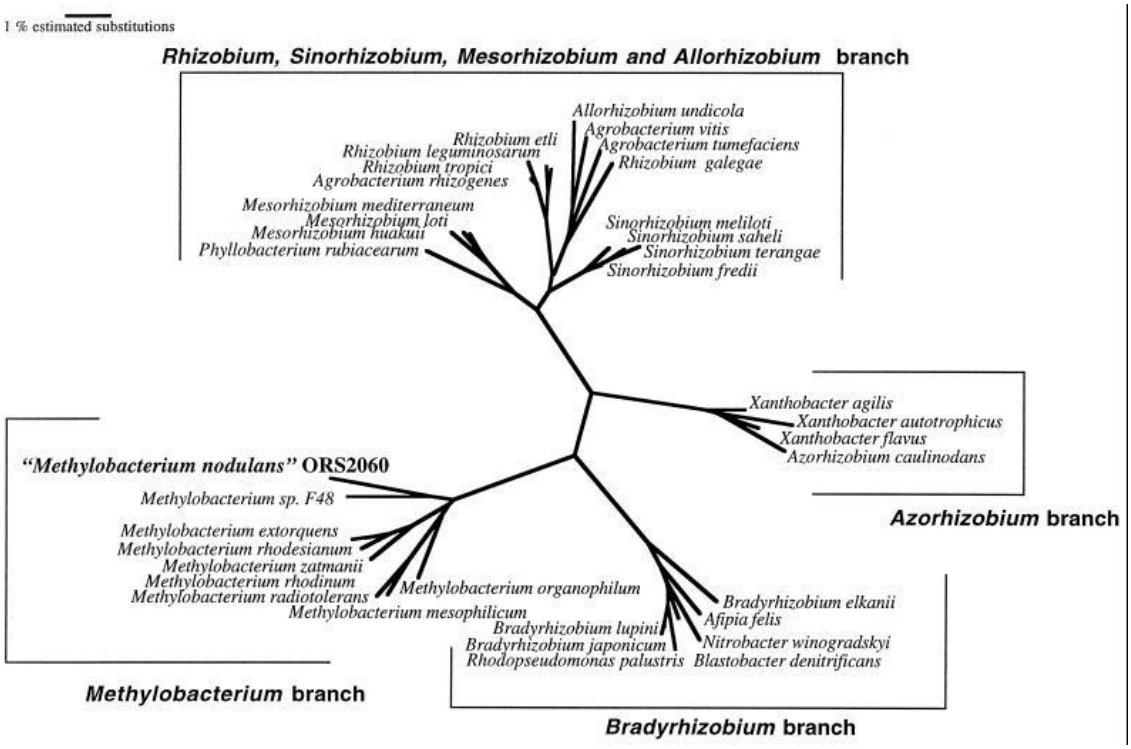

Figure 1: Unrooted phylogenetic tree showing the different rhizobial branches in the α subdivision of *Proteobacteria*. The tree was constructed by using the neighbor-joining method from almost full-length 16S rDNA sequences (Sy *et al.*, 2001: 215).

Slika 1: *Nekoreninjeno filogenetsko drevo prikazuje različne veje rizobijev znotraj skupine α proteobakterij. Drevo je bilo izdelano iz skoraj v celoti določenih 16S rDNA zaporedij z uporabo metode povezovanja sosedov (Sy in sod., 2001: 215).*

The recent identification of β-proteobacterial strains of the genus *Burkholderia* that are able to nodulate legumes changed the long-held dogma that only bacteria of the α-subdivision are symbionts of legumes (Moulin *et al.*, 2001). These strains were subsequently described as *Burkholderia tuberum*, *Burkholderia phymatum* and *Burkholderia caribensis* which nodulate tropical legumes such as *Aspalathus* and *Machaerium* (Vandamme *et al.*, 2003). In addition, strains isolated from root nodules of *Mimosa* spp. were described as *Ralstonia taiwanensis* and were also classified as β-proteobacteria (Chen *et al.*, 2001). The findings based on the sequencing of nodulation genes led to the hypothesis that β-rhizobia evolved from diazotrophs through multiple lateral *nod* gene transfers and confirmed the phylogenetic diversity of nitrogen-fixing legume β-rhizobial symbionts (Chen *et al.*, 2003). γ-proteobacteria were also found associated with legume nodules including genera like *Enterobacter* and *Escherichia*, although their characteristics and role is yet to be defined (Benhizia *et al.*, 2004).

Nitrogenase and nodulation genes are often clustered on large plasmids known as pSym (*Rhizobium*, *Sinorhizobium*, and in some species of *Mesorhizobium*) or within genomic islands referred to as symbiosis islands SIs (*Bradyrhizobium*, *M. loti*). All rhizobia are tightly related to non-symbiotic bacteria, so it is assumed that some rhizobia species developed from non-symbiotic bacteria through horizontal transfer of symbiotic genes (Wang and Martínez-Romero, 2000). Rhizobial genomes appear to be highly dynamic entities and this is particularly reflected by the presence of many insertion sequence (IS) elements, transposases, and related genes, within regions encoding symbiotic functions (MacLean *et al.*, 2007).

3.1.3 Interaction rhizobia-legume

The interaction between rhizobia and legumes results in a formation of highly specialized structures called nodules. On the basis of morphological, anatomical, and histological differences legumes' nodules are divided in two main separate types: determined and indetermined nodules. Indetermined nodules retain their meristemic activity, while determined nodules do not (Prell and Poole, 2006). The latter appear on plants of tropical

climates like soybean, bean and cowpea plants, whereas indetermined nodules are found in more temperate climate on pea, alfalfa, and clover. Development of a rhizobium-plant symbiosis involves a highly coordinated exchange of signals between the host plant and the bacteria and leads to a gradual and coordinated differentiation and adjustment of physiology and metabolism in both partners. The infection process is triggered by plant's root exudates, flavonoids and betains, to which the rhizobia respond by induction of nodulation (*nod*) genes. The *nodABC* genes are present in almost all rhizobia and are required for the synthesis of the lipo-chitooligosaccharide backbone that can be modified by various chemical groups. Detection of Nod factors by the host plant induces major developmental changes such as cortical cell division and root hair formation which are required for the entry of rhizobia into the host (Brencic and Winans, 2005).

Next step is the binding of rhizobia to host root hairs. The weak Ca^{2+}-depending binding is mediated by a bacterial protein called rhicadhesin and followed by a tight binding with cellulose fibrils also synthesized by the bacteria. Host lectins have also been shown to play roles in rhizobial adhesion (Gage, 2004). The tip of a root hair, to which rhizobia are bound, curls back on itself, trapping the bacteria and forming the infection thread. Probably, a localized degradation of root hair wall occurs at the site of the infection. After bacteria enter a root hair, they begin to travel along an infection thread toward a developing nodule. The initiation and extension of the infection thread depends on the production of specific extracellular polysaccharides (EPS) by the bacteria. Afterwards, the bacteria are differentiated into "bacteroids" which differ from normal bacteria in size, form, and cell wall composition. Typically, cyclic glucanes, nitrogenase, and specific terminal oxidase are synthesized. Within the nodule, the plant supplies rhizobia with a carbon source in the form of dicarboxylic acids, which are then metabolized via the tricarboxylic acid cycle. In return, nitrogenase catalyzes N_2 to ammonium. However, major assimilating pathway (glutamine synthetase (GS) and glutamate synthase (GOGAT)) is repressed in bacteroids (Prell and Poole, 2006). Bacteroids rather produce amino acids such as aspartate and alanine and cycle them back to the plant for asparagine synthesis (Lodwig *et al.*, 2003).

The conditions in the nodules must be microaerofilic because of the sensibility of the nitrogenase to oxygen. Oxygen concentration is the major signal controlling the expression of *nif* (coding nitrogenase) and *fix* genes (coding membrane-bound cytochrome oxidase). The central zone of nodules is protected by a layer of internal cortical cells bonded with

glycoproteins. Nodules also synthesize large amounts of leghemoglobin that binds oxygen and holds the oxygen concentration levels to approximately 25 nM, and finally, the bacteroids produce the specific terminal oxidase cbb_3 with high affinity to oxygen (Brencic and Winans, 2005).

The transcriptomes and proteomes of bacteroids differ considerably from those of free-living cells. Overall, gene expression is largely down-regulated in bacteroids, possibly as a result of growth arrest and a stationary phase-like existence (Capela *et al.*, 2006).

3.1.4 Legumes

3.1.4.1 Family *Leguminosae*

The *Leguminosae* is one of the largest and diverse families of plants with approximately 730 genera and 19.400 species, and is of great importance in agriculture around the world. The wide use of legumes as food crops, forages, and green manure is mainly associated with their ability to establish symbiotic associations with stem and root-nodulating rhizobia (Menna *et al.*, 2006). This enables them to grow in depleted and exhausted soil. Legumes have been cultivated in Europe for many centuries (Kurlovich *et al.*, 2002).

3.1.4.2 Genus *Lupinus* L.

The genus *Lupinus* L. (lupins) is a group of papilinoid legumes including about 275 species. They are annual and perennial herbaceous species, occurring in a wide range of ecological conditions in both the Old and the New World. Over 90% of the species in the genus occur in the New World, with two main centers of species diversity in western North America (≈100 species) and the Andes (≈85 species). Only few of them (about 13) are native for the Mediterranean region and North Africa (Aïnouche *et al.*, 2004). The enormous diversity of lupins could also require a large diversity of rhizobia nodulating them (Jarabo-Lorenzo *et al.*, 2003). From the biochemical point of view, the lupins are among the richest plants in seed storage proteins (35-42%) next to soybean and are of growing agronomic and ecological interest as proteins and nitrogen suppliers (Gladstones,

1974, *op.cit.*: Aïnouche *et al.*, 2004; Cerreti, 1983; *op.cit.*: Aïnouche *et al.*, 2004). Because of an increasing demand for soybean and other protein rich aliments, lupins have been proposed as a good alternative or supplement to the demand.

Because of lupin's extensive distribution and wide ecological amplitude of its habitats it is not clear how many true species exist within *Lupinus* nor its exact origin (Kurlovich *et al.*, 2002), although it is predicted that the ancestors of lupins, and also of other *Genistae* and many other papilionoid tribes, are of Old World origin (Wink *et al.*, 1999). Taxonomic confusion exists in the literature, where numerous taxa or groups are distinguished based on only a few minor and inconsistent morphological characters. Over 1700 names have been proposed for *Lupinus* of the New World (Aïnouche and Bayer, 1999). In spite of that, the lupins have always been regarded as a natural and distinct group. According to a recent systematic review, supported by molecular techniques that include sequencing of internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA, the genus *Lupinus* L. is a monophyletic genus which is unambiguously part of the tribe *Genistae* (Bisby, 1981, *op.cit.*: Aïnouche and Bayer, 1999; Aïnouche *et al.*, 2004). The ITS phylogeny suggests rapid initial radiation of the lupins into different geographical lineages, including the eastern-western disjunction of the New World species, and the paraphylic-like diversification of the Old World (Mediterranean and North African clades), the latter group being traditionally subdivided into two groups based on their seed coat structure (smooth-seeded and rough-seeded) (Figure 2). The relative rate tests performed detect unequal rates of ITS evolution within *Lupinus*, indicating that the ITS regions violate the assumption of rate constancy among different lineages. The tests revealed unequal rates of ITS sequence evolution between annual (with rapid substitution rates) and perennial lupin species (with slower substitution rates), suggesting a role for the generation time in affecting the evolutionary history. However, no significant ITS substitution rates are evident among the most strongly supported clades and subclades (Aïnouche and Bayer, 1999; Aïnouche *et al.*, 2004). The western American lupins appear as a young and dynamic group undergoing intense processes of diversification, whereas most Old World lupins are endangered and numerous populations are threatened with extinction (Aïnouche *et al.*, 2004). According to Castroviejo and Pascual (1999) only 8 species of *Lupinus* L. grow in the Iberian Peninsula: *L. angustifolius* L., *L. luteus* L., *L. hispanicus* Boiss., *L. gredensis* Gand., *L. cosentinii* Guss., *L. micranthus* Dougl., *L. albus* L. and *L. polyphyllus* Lindl., the latter two being

domesticated species (Table 1), although *L. gredensis* Gand. could be considered a subspecies of *L. hispanicus* Boiss. (Castroviejo and Pascual, 1999).

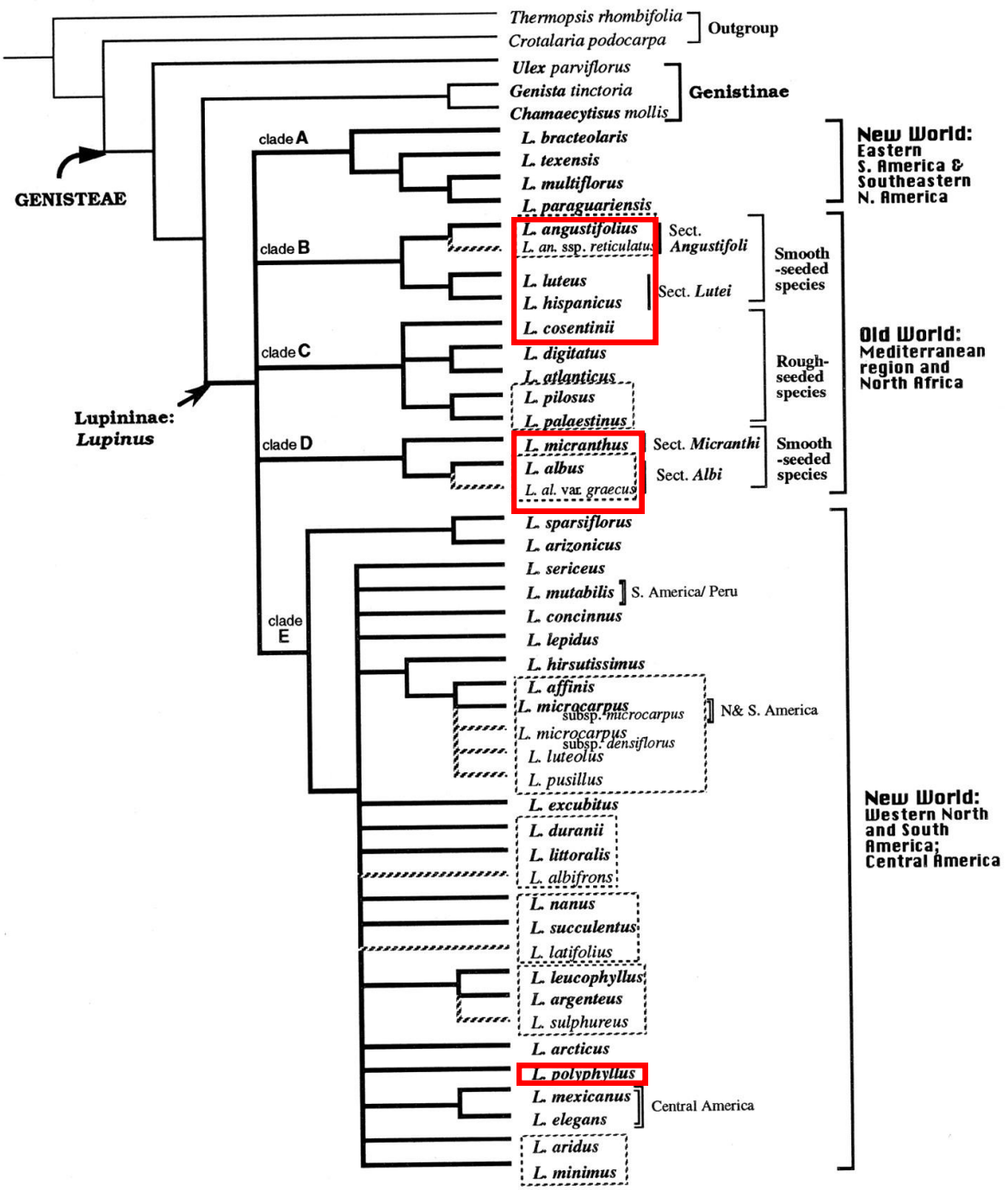

Figure 2: The strict consensus tree of 36 species of *Lupinus* and five extra-lupin taxa based on ITS sequence data. The dashed boxes contain the taxa that have identical ITS sequences and the red boxes mark species from the Iberian Peninsula (Aïnouche and Bayer, 1999: 596).

Slika 2: *Popolno skupno drevo izdelano na podlagi 36 ITS zaporedij vrst iz rodu Lupinus L. in petih zaporedij, ki ne pripadajo temu rodu. V črtkanih okvirih so označene vrste z identičnimi ITS zaporedji in v rdečih okvirih so označene vrste, ki rastejo na Iberskem polotoku (Aïnouche in Bayer, 1999: 596).*

Table 1: Morphological and geographical characteristics of lupin species found on the Iberian Peninsula (summarized from Kurlovich *et al*., 2002).
Preglednica 1: *Morfološke in geografske značilnosti vrst volčjega boba, ki se pojavljajo na Iberskem polotoku (povzeto po Kurlovich in sod., 2002)*

species *vrsta*	**height** *višina* **(cm)**	**color of corolla** *barva venčnih listov*	**length (mm) and shape of pods** *dolžina (mm) in oblika strokov*	**n° of seeds in a pod** *število semen v stroku*	**mass of a seed** *masa semena* **(mg)**	**shape and color of seeds** *oblika in barva semen*	**soil** *tip tal*	**distribution** *razširjenost*
***L. albus* L.**	30-120	white, grayish and light blue, less frequently pink blue, dark blue or violet blue *bele, sivkaste ali bledo modre barve, redkeje rožnato modre, temno ali vijolično modre*	70-180x10-20, no data *ni podatka*	3-6	30-240	square, compressed, white with a variable tinge of salmon pink, or dotted, dark brown *kvadratasta in stisnjena, bela z odtenki rožnate ali rjavimi pikami*	meadows, pasture and grassy slopes, predominantly on sandy and acid soils *travniki, pašniki in travnata pobočja, večinoma v peščenih in kislih tleh*	Mediterranean (Greece, Apennine Peninsula), Egypt, Libya, Palestine. Cultivated around the world. *Sredozemlje (Grčija, Apeninski polotok), Egipt, Libija, Palestina. Gojena širom sveta.*
***L. angustifolius* L.**	20-150	blue, violet, less frequently pink and white *modre, vijolične, redkeje rožnate ali bele barve*	35-50x7-10, oblong, slightly inflated *podolgovati, rahlo napihnjeni*	4-7	30-240	globular, smooth, variously colored *kroglaste oblike, z gladkim ovojem in različno obarvana*	meadows, among rocks, in bushes, on seaside sands and near reservoirs, along the roads and, as weed, in the field *travniki, med kamni ali grmovjem, v peščenih priobalnih tleh, ob cestah in na poljih kot plevel*	Mediterranean countries, Asia Minor, Transcaucasia and Iran. Widely cultivated in N Europe, SE USA and New Zealand. *Sredozemlje , Mala Azija, Kavkaz in Iran; gojijo jo v severni Evropi, JV ZDA in na Novi Zelandiji.*
***L. cosentinii* Guss.**	20-120	light blue from sides and yellowish in the center *svetlo modri ob straneh in rumeni v sredini*	40-55x13-16, densely villous or softly hirsute *Porasli z dlačicami ali gladki*	3-5	no data *ni podatka*	square, compressed, tubercular, light grey to more brown with black marks *kvadratasti, stisnjeni, gomoljasti, svetlo sive barve ali rjave s črnimi madeži*	coastal sandy soils *peščena priobalna tla*	Widespread in Tunis, Morocco, SW Spain, Portugal. Cultivated in Australia. *Tunizija, Maroko, JZ Španija, južna Portugalska, Korzika, Sicilija, Sardinija; gojena v Avstraliji.*

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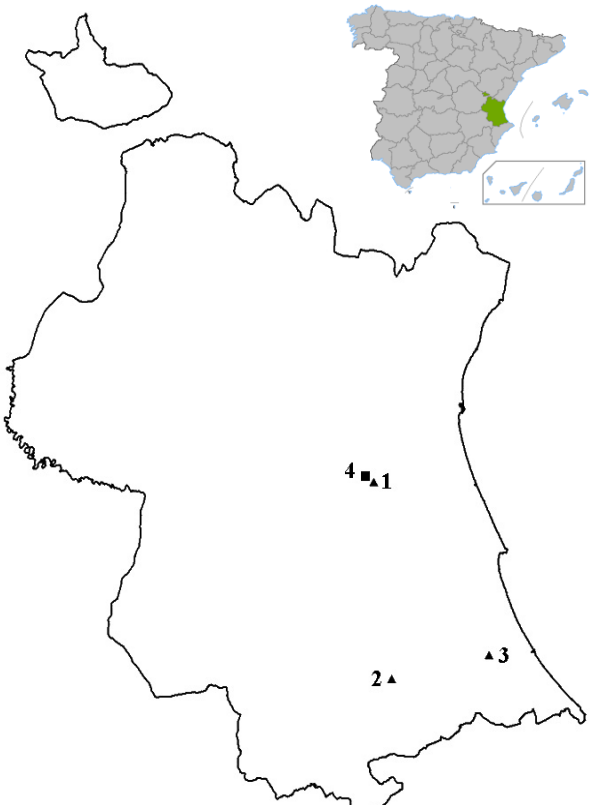
species *vrsta*	**height** *višina* **(cm)**	**color of corolla** *barva venčnih listov*	**length (mm) and shape of pods** *dolžina (mm) in oblika strokov*	**n° of seeds in a pod** *število semen v stroku*	**mass of a seed** *masa semena* **(mg)**	**shape and color of seeds** *oblika in barva semen*	**soil** *tip tal*	**distribution** *razširjenost*
***L. micranthus* Dougl.**	10-50	blue with a white spot *modra z belimi pikami*	30-50x9-12, brownish, coarsely hirsute *rjavi, grobo porasli z dlačicami*	2-5	no data *ni podatka*	lentiform, slightly square with dark brown arcs *lečaste oblike, rahlo kvadratasta, stisnjena, gladka, rjave barve*	coasts, frequently limier soils than *L. angustifolius priobalni predeli, pogosto v bolj apnenčastih tleh kot L. angustifolius*	Mediterranean *Sredozemlje*
***L. hispanicus* Boiss.**	60-70	cream-colored, violet *kremaste ali lila/vijolične barve*	40-60x6-12, flattened, hirsute *sploščeni, porasli z dlačicami*	5-7	50-150	white, olive brown or cream-colored to light reddish or greenish brown with darker brown specks *bela, olivno rjava, kremasta, svetlo rdeča, zelenorjava z*	moderately and highly acidized soils, on granite and shaly mountain slopes, sandy soils *zmerno ali zelo kisla tla, na granitnih ali gorskih pobočjih, v peščenih tleh*	Widespread in southern and central Spain *razširjena v južni in osrednji Španiji*
***L. luteus* L.**	20-80	bright goldish-yellow *zlatorumenkasta*	40-60x10-14, elongated *podolgovati*	4-6	50-150	spherical, oblate, variable coloring *sferična, različno obarvana: rožnata, rjava, rumenkasta, temno vijolična, lisasta*	mild sandy and volcanic soils in mining belts *peščena in vulkanska tla rudniških pasov*	Iberian Peninsula, Morocco, Tunisia, and Algeria, Corsica, Sardinia, Sicily. Cultivated in N Europe, Belarus. *Iberski polotok, Maroko, Tunizija, Alžirija, Korzika, Sardinija, Sicilija. Gojena v severni Evropi in Belorusiji.*
***L. polyphyllus* Lindl.**	50-150	diverse colors, often violet *različnih barv, pogosto vijolične*	flat, elongated *ploščati, podolgovati*	9(6-7)	20	oval, weakly squeezed, having brown, black or different color *ovalne, rahlo stisnjene oblike in rjave, črne ali druge barve*	fluvial detrital deposits and inundated soils of the rivers *rečne detritne usedline in poplavljena polja*	Canada, USA (Alaska, N California, W Oregon, Washington) *Kanada, ZDA (Aljaska, severna Kalifornija, zahodni Oregon, Washington)*

3.1.4.3 Novel lupin species *Lupinus mariae-josephi*

A novel lupin species was recently defined by Pascual (2004). *L. mariae-josephi* thrives in alkaline soil with high concentrations of Ca^{2+}. It does not seem closely related to any other species of the Iberian Peninsula that grow, as does the majority of *Lupinus* species, in acid soils (Castroviejo and Pascual, 1999). This gives much importance to the discovery, however, the reason for permanence in alkaline soil is not known. Later on, wild-type populations were discovered in the Valencia province (Figure 3) (Navarro *et al.*, 2006; Fos *et al.*, 2006). For the fear of *L. mariae-josephi* plants being soon extinct, a microreserve of plants has been created in Llombai (microreserve of Lloma de Tramusar). Moreover, two new ones are being created in Xátiva and Gandía (Fos *et al.*, 2006). No other geographical locations are known where *L. mariae-josephi* grows.



L. mariae-josephi is an annual plant that grows up to 30 cm in height. Its stalks and stems are villous; leaflets are oblong, naked from above and villous from below. Legumes measure 50x20 mm in size and hold 3 to 4 darkly colored seeds with light brown spots (Pascual, 2004) (Figure 4).

Figure 3: The locations of *L. mariae-josephi* in the Valencia province: already known (■) and new (▲). 1. Lloma del Tramussar (Llombai), 2. Pla de Tramussar (Xátiva), 3. El Borrell, Pla dels Tramussos (Gandía), 4. Els Castellars and Lloma Plana (Montserrat) (picture taken from Fos *et al.*, 2006: 22). The figure is modified with the picture of Spain (Valencia province, 2009).

Slika 3: *Prikaz že poznanih (■) in novih lokacij (▲) populacij vrste L. mariae-josephi v provinci Valencija:. 1. Lloma del Tramussar (Llombai), 2. Pla de Tramussar (Xátiva), 3. El Borrell, Pla dels Tramussos (Gandía), 4. Els Castellars in Lloma Plana (Montserrat) (slika iz Fos in sod., 2006: 22). Slika je bila preoblikovana s sliko Španije (Valencia province, 2009).*

a

b

c

d

Figure 4: Morphological characteristic *of L. mariae-josephi*. 2a, leaves (picture taken from Fos *et al.*, 2006: 23); 2b, seeds (Navarro *et al.*, 2006: 63); 2c, a fruit (Navarro *et al.*, 2006: 63); 2d, flowers (Fotonatura).

Slika 4: *Morfološke karakteristike rastlinske vrste L. mariae-josephi. 2a, listi (slika iz Fos in sod., 2006: 23); 2b, semena (Navarro in sod., 2006: 63); 2c, strok (Navarro in sod., 2006: 62); 2d, cvetovi (Fotonatura).*

3.1.5 Lupin nodulating bacteria

Despite the agronomic, alimental, and ecological interest of *Lupinus*, this plant has been poorly studied with respect to its symbionts. Cross-inoculation studies have shown that lupins share a common rhizobial pool with other legumes in the tribe *Genistae* and are effectively nodulated by rhizobia isolated from serradella (*Ornithopus* L., tribe *Loteae*) and by rhizobia isolated from *Lotus* L., *Anthyllis* L., and *Phaseolus* L. genera (Stępkowski *et al.*, 2007). Generally, lupins have been reported to be nodulated by fast- and slow-growing rhizobia, although the latter are those more frequently isolated from this legume.

Lupin is a promiscuous host legume that is nodulated by rhizobia with very different chromosomal genotypes, which usually belong to several slow-growing species of *Bradyrhizobium*: *B. japonicum* (Jarabo-Lorenzo *et al.*, 2003), *B. canariense* (Stępkowski *et al.*, 2005) and *B. elkanii* (Barrera *et al.*, 1997). However, fast-growing species from other genera have been found to nodulate lupins: *Ochrobactrum lupini* (Trujillo *et al.*, 2005) and *Phyllobacterium trifolii* (Valverde *et al.*, 2005). There is definitive specificity between *Bradyrhizobium* strains and lupin lines, since significant variation in lupin dry root weight has been reported when inoculated with different bradyrhizobial strains (Robinson *et al.*, 2000). Therefore, it is crucial that specific lupin cultivar and bradyrhizobial strain combinations are identified (Menna *et al.*, 2006).

3.1.5.1 Genus *Bradyrhizobium*

Genus *Bradyrhizobium* normally forms nodules on tropical or subtropical plants (*Glycine, Vigna*). The bacteria grow slowly (g ≈ 6-10h) and can produce a considerable amount of extracellular polysaccharides and alkalinize the growth medium (Jordan, 1982). They also possess many antibiotic resistance genes (Vincent, 1970). Only few species of *Bradyrhizobium* are widely recognized, some of them also nodulate soybeans, including *B. japonicum* (Jordan, 1982), *B. liaoningense* (Xu *et al.*, 1995), and *B. elkanii (*Kuykendall *et al.*, 1992). Three new *Bradyrhizobium* species have been admitted recently: *B. yuanmingense* isolated from *Lespedeza* (Yao *et al.*, 2002) with very broad geographic and host ranges including soybean (Vinuesa *et al.*, 2008), the acid-tolerant *B. canariense* from

lupins of the Canary Islands (Vinuesa *et al.*, 2005), and *B. betae*, isolated from tumor-like root deformations of sugar beet *Beta vulgaris* (Rivas *et al.*, 2004). In spite of this progress, there is still a large number of legume isolates, especially bradyrhizobial strains, which need to be more deeply studied to define their taxonomic status (Jarabo-Lorenzo *et al.*, 2003).

3.1.6 Phylogenetic analysis of rhizobia

Definition of new bacterial genera and species, and the description of evolutionary relationships have been mainly inferred from 16S SSU gene sequences (Woese, 1987) since rRNA is one of the most conserved genes in bacterial cells. For this reason, genes that encode the rRNA (rDNA) are suitable for identifying a new taxonomic group, calculating related groups, and estimating rates of species divergence.

Based on an estimation showing that 16S rDNA genes evolve at a rate of 1% per 50 million years (Ochman *et al.*, 1999) it was proposed that different rhizobial lineages have diverged before the appearance of legumes (Turner and Young, 2000). Therefore, bacterial nodulation capacity may have been acquired in a single lineage after diversification of bacteria which can explain the limited DNA-DNA homology between some lupin isolates and *B. japonicum* strains that possess very similar 16S rDNA sequences (Barrera *et al.*, 1997) and the incongruity between phylogenies of nodulation (*nodC, nodZ, nodA, noeI, nolL* and *nifH*) and housekeeping genes (*atpD, dnaK, glnII, recA,* and *rrs*). This invoke the hypothesis of multiple lateral transfer of symbiotic loci among rhizobia lineages (Jarabo-Lorenzo *et al.*, 2003; Stępkowski *et al.*, 2003; Moulin *et al.*, 2004; Stępkowski *et al.*, 2007). In addition, it was recently reported that some photosynthetic bradyrhizobial strains can nodulate in the absence of conventional *nod* genes (Giraud *et al.*, 2007). Therefore, nodulation genes may only suggest a common origin of symbiotic genes but not the complete evolution and diversification of rhizobia.

3 MATERIALS AND METHODS

3.1 BIOLOGICAL MATERIAL

3.1.1 Bacterial strains

In this work we used various bacterial strains isolated from nodules of different lupin species that grow in the acid soils of the Iberian Peninsula and other legumes. Five strains of *Lupinus mariae-josephi* plant were selected for investigation: A2, B2b, C, Db2 and H2p.

Table 2: List of used bacterial strains.
Preglednica 2: *Seznam uporabljenih bakterijskih sevov.*

bacterium ***bakterija***	**host plant** ***gostiteljska rastlina***	**geographical source** ***geografski izvor***	**source** ***vir***
B. japonicum			
752	*Glycine max* L.	unknown *neznan*	GB[1]
804	*G. max* L.	unknown *neznan*	BBRC[2]
B. elkanii			
ISJ94 (USDA76)	*G. max* L.	California (USA) *Kalifornija (ZDA)*	Temprano [3]
ISJ98 (USDA117)	*G. max* L.	Mississippi (USA) *Mississippi (ZDA)*	Temprano
ISJ99 (USDA275)	*G. max* L.	India *Indija*	Temprano
***Bradyrhizobium* sp.**			
A2	*Lupinus mariae-josephi*	Llombai (Valencia, Spain) *Llombai (Valencija, Španija)*	Ruiz-Argüeso[4]
B2b	*L. mariae-josephi*	Llombai (Valencia, Spain) *Llombai (Valencija, Španija)*	Ruiz-Argüeso
C	*L. mariae-josephi*	Llombai (Valencia, Spain) *Llombai (Valencija, Španija)*	Ruiz-Argüeso
DB2	*L. mariae-josephi*	Llombai (Valencia, Spain) *Llombai (Valencija, Španija)*	Ruiz-Argüeso
H2p	*L. mariae-josephi*	Llombai (Valencia, Spain) *Llombai (Valencija, Španija)*	Ruiz-Argüeso

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[4] E.T.S.I.A. Escuela Técnica Superior de Ingenieros Agrónomos, Madrid, Spain *(Španija)*

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897	*L. angustifolius* L.	unknown *neznan*	ISF[5]
874	*Lupinus angustifolius* L.	unknown *neznan*	IMAC[6]
ISLU8	*Lupinus luteus* L.	San José de la Rinconada (Seville, Spain) *San José de la Rinconada (Sevilja, Španija)*	Temprano
ISLU12	*Lupinus cosentinii* Guss.	Pilas (Seville, Spain) *Pilas (Sevilja, Španija)*	Temprano
ISLU14	*Lupinus micranthus* Dougl.	Pilas (Seville, Spain) *Pilas (Sevilja, Španija)*	Temprano
ISLU15	*L. luteus* L.	Pilas (Seville, Spain) *Pilas (Sevilja, Španija)*	Temprano
ISLU16	*Ornithopus compressus* L.	Pilas (Seville, Spain) *Pilas (Sevilja, Španija)*	Temprano
ISLU21	*Lupinus hispanicus* Boiss.	Navahondilla (Ávila, Spain) *Navahondilla (Ávila, Španija)*	Temprano
ISLU22	*L. angustifolius* L.	El Pedroso (Seville, Spain) *El Pedroso (Sevilja, Španija)*	Temprano
ISLU38	*L. angustifolius* L.	Cañaveral (Cáceres, Spain) *Cañaveral (Cáceres, Španija)*	Temprano
ISLU40	*L. hispanicus* Boiss.	Béjar (Salamanca, Spain) *Béjar (Salamanca, Španija)*	Temprano
ISLU41	*L. hispanicus* Boiss.	San Bartolomé (Ávila, Spain) *San Bartolomé (Ávila, Španija)*	Temprano
ISLU90	*L. angustifolius* L.	Badajoz (Spain) *Badajoz (Španija)*	Temprano
ISLU101	*L. angustifolius* L.	Despeñaperros (Jaén, Spain) *Despeñaperros (Jaén, Španija)*	Temprano
ISLU122	*L. micranthus* Dougl.	Almonte (Huelva, Spain) *Almonte (Huelva, Španija)*	Temprano
ISLU127	*O. compressus* L.	Monterrubio (Salamanca, Spain) *Monterrubio (Salamanca, Španija)*	Temprano
ISLU203	*Lupinus albus* L.	Temuco (Chile) *Temuco (Čile)*	Temprano
924	*Vigna* Savi sp.	unknown *neznan*	Nitragin[7]
835	*Cicer arietinum* L.	Cañameros (Cáceres, Spain) *Cañameros (Cáceres, Španija)*	Ruiz-Argüeso
861	*Ornithopus sativus* L.	Madrid (Spain) *Madrid (Španija)*	Ruiz-Argüeso

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[6] Department of Microbiology, University of Agricultural Science, Uppsala, Sweden *(Švedska)*

[7] Nitragin Argentina SA, Buenos Aires, Argentina

3.1.2 Plant material

Several legume species were used as host plants – mainly lupins (*Lupinus mariae-josephi*, *L. albus* cv. Multolupa, *L. angustifolius* cv. Uniharvest, *L. cosentinii* var. eregulla, *L. hispanicus* ec. Cazalla, *L. luteus* cv. Aurea, *L. micranthus* ec. Gibraleón, and *L. gredensis*), yellow serradella (*Ornithopus compressus* cv. 5599), cowpea (*Vigna unguiculata* cv. unguiculata) and chickpea (*Cicer arietinum*).

3.2 ANTIBIOTICS, ENZYMES, REACTANTS

The enzyme Taq polymerase of 5000 U ml^{-1} was provided by GE Healthcare and was used in accordance with the manufacturer's instructions.
The antibiotic ampicillin was provided by Sigma Chemical Co. (St. Louis, Missouri, USA) The solution was prepared and stored following the instructions by Sambrook and Russell (2001). When required the final concentration was 100 μg ml^{-1}.

3.3 MEDIA AND GROWTH CONDITIONS

Rhizobial strains were cultivated at 28°C in:

- YMB medium (Vincent, 1970): for general maintainment of strains, determining growth in flasks, bioscreening, and for DNA extraction (yeast extract 0.4 g l^{-1}, mannitol 10 g l^{-1}, NaCl 0.1 g l^{-1}, K_2HPO_4 0.5 g l^{-1}, $MgSO_4$ 0.2 g l^{-1}, pH was adjusted to 6.8 or as wished).

- AG medium (Sadowsky *et al.*, 1987): for maintainment of strains (HEPE 0.13 g l^{-1}, MES 0.11 g l^{-1}, $FeCl_3 \cdot 6H_2O$ 0.0067 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.18 g l^{-1}, $CaCl_2 \cdot 2H_2O$ 0.013 g l^{-1}, Na_2SO_4 0.25 g l^{-1}, NH_4Cl 0.32 g l^{-1}, Na_2HPO_4 0.125 g l^{-1}, L-arabinose 1.0 g l^{-1}, Na-gluconate 1.0 g l^{-1}, yeast extract 1.0 g l^{-1}, pH was adjusted to 6.9).

Strains of *Escherichia coli* were cultivated at 37 °C in:

- LB medium (Luria-Bertani) (Miller, 1972): yeast extract 5 g l^{-1}, tryptone 10 g l^{-1}, NaCl 5 g l^{-1}.

All the media were prepared with distilled water and sterilized at 115 °C for 20 minutes. Agar (15 g l^{-1}) was added to the medium when needed to prepare agar plates.

For the prolonged conservation of bacteria cultures in exponential growth phase were stored and saved in microcentrifuge tubes with 20% of glycerol at -70 °C and -40 °C.

3.4 MICROSCOPY

The samples of rhizobial strains were observed with an optical microscope Zeiss Axophot (Carl Zeiss AG, Oberkochen, Germany) in bright field under an oil immersion objective lens with a magnification value of 100x. The actual magnification was 1000x.
The samples were taken from AG medium agar plates and stained on microscope slides with crystal violet in order to achieve sufficient contrast.

3.5 *rrs* GENE AMPLIFICATION AND SEQUENCING

3.5.1 Genomic DNA isolation

Genomic DNA was extracted from colonies grown on agar-based YMB medium. The cells were transferred to a 1.5 ml microcentrifuge tube with a toothpick, lysed with 0.25% SDS and 0.05 M NaOH and incubated for 15 minutes at 85-90 °C. After vortexing the samples, 100 µl of water was added into the microcentrifuge tube and vortexed again. The samples were centrifuged for 5 minutes at 13 000 rpm and after that the supernatant was collected.

3.5.2 *rrs* gene amplification by PCR

The amplification of *rrs* gene by the polymerase chain reaction (PCR) was conducted according to the proceedings of GE Healthcare. All primers used were synthesized by Sigma-Aldrich®.

The reaction was carried out with primers 41f (5'-GCTCAGATTGAACGCTGGCG-3') and 1488f (5'-CGGTTACCTTGTTACGACTTCACC-3') (Herrera-Cervera *et al.*, 1999). Each reaction contained the following substances in a final volume of 25 µl: 1 µl of genomic DNA (the actual mass of DNA varied between 10-100 ng depending on the strain, 10x diluted if necessary), 1 µl of DMSO, 0,5 µl of dNTPs (10 nM of each dNTP), 1 µl of 5' oligonucleotide (10 pmol μl^{-1}), 1 µl of 3' oligonucleotide (10 pmol μl^{-1}), 2.5 µl of Taq buffer (with $MgCl_2$), 0.4 µl of Taq polymerase (5 U μl^{-1}), 18 µl of sterile water. The temperature program was: 2 min at 94 °C, 10 cycles of denaturation (40 s at 94 °C), annealing (1 min at 60 °C, lowering 1 °C each cycle – »touch down«) and extension (2 min at 72 °C), 25 cycles of denaturation (40 s at 94 °C), annealing (1 min at 50 °C) and extension (2 min at 72 °C) and final extension at 72 °C (Herrera-Cervera *et al.*, 1999).

3.5.3 Cloning of DNA fragments and plasmid transformation in *E. coli*

The DNA fragments containing the *rrs* gene were cloned in the TOPO® vector (Invitrogen) according to the manufacturer's instructions. The resulting plasmids were transformed into competent One Shot® *E. coli* cells. The transformed cells were selected on LB medium with ampicillin and X-Gal to perform a blue white screening.

3.5.4 Plasmid DNA isolation

Plasmid DNA extraction from *E. coli* cells was followed by the protocol of the alkaline lysis method described by Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981) with modifications of Sambrook and Russell (2001). For the small-scale preparation (minipreparation), a volume of 1.5 ml of *E. coli* cells in exponential growth phase (10^9 cells ml^{-1}) was used. The precipitated plasmid DNA was suspended in sterile water.

The presence and orientation of successfully cloned fragments was analyzed by restriction analysis using *Eco*RI enzyme and subsequent agarose gel electrophoresis.

3.5.5 DNA electrophoresis in agarose gel

The electrophoresis in 1% agarose gels stained with ethidium bromide were used for evaluating total isolated DNA, PCR products, and restriction fragments in TBE or TAE buffers (Sambrook and Russell, 2001). The samples (vol. 18 µl) were mixed with 2 µl of loading buffer (60% glycerol, 20 mM EDTA, 0.25% bromphenol blue and 20 units of ribonuclease A per ml). The size of fragments was estimated by comparison of the electrophoretic mobility of DNA fragments of bacteriophage λ (Roche, Basilea, Switzerland) restricted with *Hind*III and a commercial DNA marker Ladder Plus (MBI Fermentas, Vilnius, Lithuania).

3.5.6 DNA purification and sequencing

Successfully cloned fragments in TOPO® vectors were purified from a plasmid DNA sample using GENECLEAN® *Turbo* (MP Biomedicals, Southern Carolina, USA) in accordance with manufacturer's instructions.
Fragment sequencing was carried out by Secugen (Madrid, Spain) using universal primers T7 (5'-GTAATACGACTCACTATAGGGC-3') and M13 reverse primer (5'-CAGAAACAGCTATGAC-3')

3.6 PHYLOGENETIC TREE CONSTRUCTION

For the sequences assembly the programs Sequencher (Codes Gene Corporation, Ann Arbor, Michigan, USA) and Chromas (Technelysium Pty Ltd, Austria) were used. Phylogenetic trees construction was performed using a webpage Phylogeny.fr (Dereeper *et al.*, 2008). Sequences of the new isolates and related bacteria were aligned using multiple alignment software MUSCLE (Edgar, 2004) and Gblocks (Castresana, 2000) to improve

the alignment. The distances were calculated according to Kimura's two-parameter method (Kimura, 1980) and the phylogenetic trees were inferred using the maximum likelihood method (Guidon and Gascuel, 2003). Usual bootstrapping procedure was replaced by a new confidence index, an approximate likelihood-ratio test (aLRT) (Anisimova and Gascuel, 2006). Trees were viewed with the TreeDyn program (Chevenet *et al.*, 2006) and additionally with the MEGA2 package (Kumar *et al.*, 2001).

3.7 BACTERIAL GROWTH ANALYSIS

3.7.1 Bacterial growth in flasks

To determine the generation times of bacteria at different pH values, the strains isolated from nodules of *L. mariae-josephi* were cultivated in YMB batch cultures at 28 °C for ten days. The optical density (OD) value of each sample (vol. 1 ml) was measured spectrophotometrically using a UV/VIS spectrophotometer (Ultrospec III, Amersham Pharmacia Biotech AB, Uppsala, Sweden) at 600 nm every 24 hours.

3.7.2 Bioscreen

YMB batch cultures inoculated with different strains were tested for bacterial growth rate at different pHs by bioscreen. From each strain culture, two repetitions (vol. 200 μl) were transferred to wells of a Costar plate (Bio-Rad Laboratories, Inc., California, USA). The filled plates were placed in the bioscreen for analysis. The optical density was measured at 28 °C every 2 hours for five days at 600 nm wavelength.

3.8 PREPARATION OF LEONARD JAR UNITS

Leonard units were prepared as previously described (Leyva *et al.*, 1987). Briefly, a gauze wick linked the plant roots with the nutritive solution. The pots were filled with vermiculite, an inert substrate, and placed on a tin can where the nutritive solution was

later added. Finally, pots were covered with aluminum foil and sterilized at 120 °C during 2 hours.

The Leonard nutritive solution (Leonard, 1944) was routinely prepared in 15 l containers with sterile and deionized water. The nutrients were taken from stock solutions: 37.5 ml K_2HPO_4 (concentration in stock solution: 69.6 g l^{-1}), 37.5 ml KCl (29.8 g l^{-1}), 37.5 ml $MgSO_4 \cdot 7H2O$ (98.6 g l^{-1}), 7.5 ml of oligoelements (0.078 g l^{-1} $CuSO_4 \cdot 5H_2O$, 0.22 gl^{-1} $ZnSO_4 \cdot H_2O$, 2.03 g l^{-1} $MnSO_4 \cdot 7H_2O$, 0.01 g l^{-1} $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.43 g l^{-1} H_3BO_3), 15 ml ferric citrate (1.79 g l^{-1}).

For practical reasons, the solution and water were sterilized in 2 l containers and later combined. Just before watering, 5.16 g of $CaSO_4$, that rendered the concentration 0.34 g l^{-1} in the final volume of the nutritive solution, was added.

3.9 PLANT GROWTH IN BACTERIOLOGICALLY CONTROLLED CONDITIONS

3.9.1 Germination of sterile seeds

Plant seeds of *L. mariae-josephi*, *L. luteus* cv. Aurea, *L. cosentinii* var. eregulla, *L. angustifolius* cv. Uniharvest, *L. micranthus* ec. Gibraleón and *L. hispanicus* ec. Cazalla were mechanically scarified with a scalpel and later on sterilized by soaking the seeds in alcohol (1 min) and 20% sodium hypochlorite (3 min). Finally, the seeds were washed in water 10 times (1 min). Other seeds were sterilized following the same protocol without being scarified. Then the seeds were germinated for several days at 28 °C on water-agar medium (1% agar). *Ornithopus compressus* cv. 5599 seeds were first incubated at 4 °C for two days and after at 28 °C also on water-agar medium.

3.9.2 Plant growth

In summary, plant growth was carried out as described by Leyva *et al.* (1987).

Once the seeds were germinated, they were planted into Leonard jars: every seed was inoculated with 4 ml of the corresponding bacterial culture grown in YMB medium at 28

°C from 6 to 10 days. These plants were grown for four or five weeks in a growth chamber with 16h/day illumination and day/night temperature cycle (25 °C/18 °C).

3.10 NITROGENASE ACTIVITY

The nitrogenase activity in nodules was determined by acetylene reduction technique as described by Ruiz-Argüeso *et al.* (1978). After examination of the root system, we could roughly determine which nodules are active, less active or non-active by their color. N_2 fixing activity of nodules was roughly estimated by the color of the inside of the nodules (red, reddish, white). Then, nodulated root fragments were placed into 18 ml or 25 ml vials that were hermetically fitted with silicone rubber septa and 0.5 ml of acetylene (produced by hydrolysis of calcium carbide) was injected into each vial at room temperature. Two samples of 0.5 ml were extracted from the vials after 30 minutes and 1 hour, respectively, using a syringe that was pricked onto a rubber cap until it was injected into a Shimadzu gas chromatograph (model GC-8A) with a flame ionizing detector (FID) and nitrogen as the carrier gas. The temperature of the column and detector was set at 100 °C and 150 °C, respectively. The flame burns the gas samples within the carrier gas, forming ions and electrons. The electrical conductivity is then proportionate to the concentration of charged particles within the gas. The quantity of gases in the samples was determined by height of the peaks comparing it to a standard ethylene sample (0.19 μmol). The nodule nitrogenase activity was expressed in μmol of ethylene per gram of fresh nodules' mass and per hour.

4 RESULTS

Rhizobia able to nodulate *L. mariae-josephi* were isolated from the rizospheric soils of Llombai, a mountainous region of Valencia province (Spain), by the plant-trap methodology. Five strains (Table 2) were selected for this work out of 15 isolated that have been purified and checked for nodulation of *L. mariae-josephi* under controlled conditions. Additionally, rhizobia nodulating lupins from the Iberian Peninsula and other legumes (Table 2) were also used in this work.

4.1 MORPHOLOGY OF BACTERIA ISOLATED FROM NODULES OF *L. mariae-josephi*

The morphological characteristics of strains isolated from nodules of *L. mariae-josephi* were investigated by comparing growth on YMB plates of the *L. mariae-josephi* strains and the well studied *Bradyrhizobium japonicum* USDA110.

The growth of strains of *L. mariae-josephi* on YMB medium was very slow and remarkably slower than of the *B. japonicum*, suggesting that they belonged to the genus *Bradyrhizobium*. Colonies of strain H2p were not detected before 9 days of incubation at 28 °C on YMB plates. Colonies were almost imperceptible, cream in color, and not mucoid. Meanwhile, *B. japonicum* USDA110 formed visible small, round, cream in color, and mucoid colonies with diameter of approximately 2-3 mm (Figure 5) after 5 days of incubation on YMB plates.

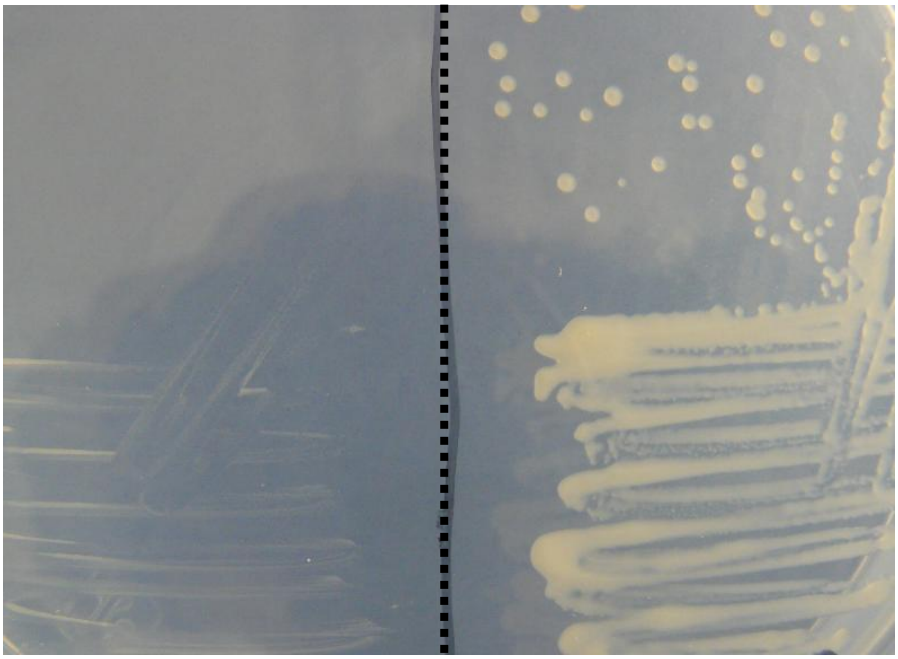


Figure 5: Comparison of growth and morphology of colonies after 9 days of incubation on YMB medium at 28 °C between H2p strain (left) and *B. japonicum* USDA 110 (right). Other strains of *L. mariae-josephi* (A2, B2b, C, and Db2) showed similar characteristics as H2p.

Slika 5: *Primerjava rasti in morfologije kolonij med sevoma H2p (levo) in B. japonicum USDA110 (desno) po 9 dneh inkubacije na YMB gojišču na 28 °C. Ostali sevi izolirani iz nodulov L. mariae-josephi (A2, B2b, C, Db2) so kazali podobne značilnosti kot H2p.*

Additionally, we compared the cell morphology of five strains isolated from nodules of *L. mariae-josephi* with various strains isolated from nodules of legumes, such as ISLU101, ISLU122, and *B. japonicum* USDA110 that nodulate *L. angustifolius*, *L. hispanicus*, and *Glycine max*, respectively.

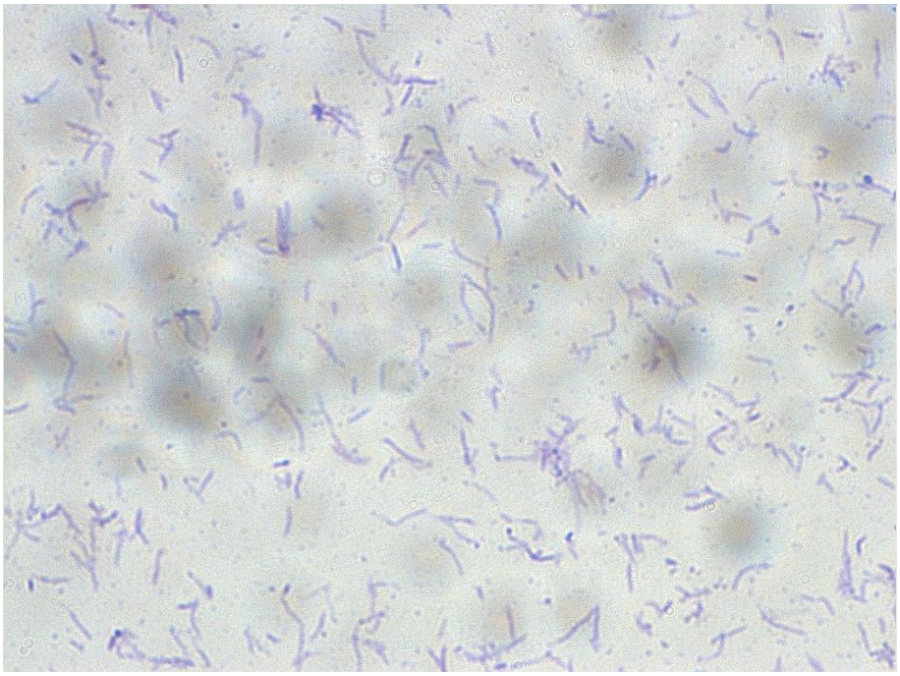


Figure 6: Microscopic presentation of A2 strain grown in AG medium at 28 °C for 1 day. The cells were observed with an optical microscope in bright field under the magnification 1000x.
Slika 6: *Pogled celic seva A2 pod mikroskopom. Bakterije smo inkubirali en dan pri 28°C na AG gojišču in jih opazovali pod optičnim mikroskopom v svetlem polju ter pod povečavo 1000x.*

All five strains isolated from nodules of *L. mariae-josephi* and other previously mentioned strains had similar morphology, short rods frequently forming short chains (Figure 6).

4.2 GENERATION TIMES OF BACTERIA ISOLATED FROM NODULES OF *L. mariae-josephi*

We studied the generation times of *L. mariae-josephi* strains at different pH values in comparison with rhizobia strains isolated from nodules of lupin growing in acid soils and with *Bradyrhizobium japonicum* USDA110 which is the type strain of the slow-growing group of rhizobia (g ≈ 6-10h). The optical density (OD) of batch cultures was measured every two hours at 600 nm wavelength by bioscreen. The strains used were isolates from nodules of *L. mariae-josephi* (B2b, C, and H2p), from *L. angustifolius* grown in Seville (Spain) (strain ISLU101) and *Bradyrhizobium japonicum* strain USDA110.

4.2.1 Bioscreen

Growth curves are presented in Figures 7-10 and generation times are summarized in Table 3. Generally, the shortest generation times corresponded to pH 6.8 which is an optimal pH for most bradyrhizobial strains. At this pH value the generation time for rhizobia of *L. mariae-josephi* was 2 days and approximately 1 day for ISLU101 strain and *B. japonicum* USDA110 (Figure 8). Similar generation times were measured at pH 8 with the exception of strain B2b which grew much slower ($g \approx 6$ days) (Figure 9).

B. japonicum USDA110 and ISLU101 showed a wide tolerance range to high and low pH values. *B. japonicum* USDA110 was the most tolerant to pH 9 and no differences were observed at this pH among strains B2b, C, H2p and ISLU101. The most remarkable result was the sensitivity of *L. mariae-josephi* strains to acid pH being unable to grow at pH 5. It is also noteworthy that strain B2b that holds the highest generation time, grows much slower compared to C and H2p as the pH is higher. The difference is statistically relevant at pH 8 and partially at pH 9 (compared to strain C) (Figure 11). This is relevant since the alkaline pH of the soil where *L. mariae-josephi* grows may have been a selective factor for its symbionts.

The generation times were also estimated by measuring OD of batch cultures grown in flasks as described in Materials and methods. No significant differences were found regarding the relative results obtained by the bioscreen procedure, although the measured values were higher.

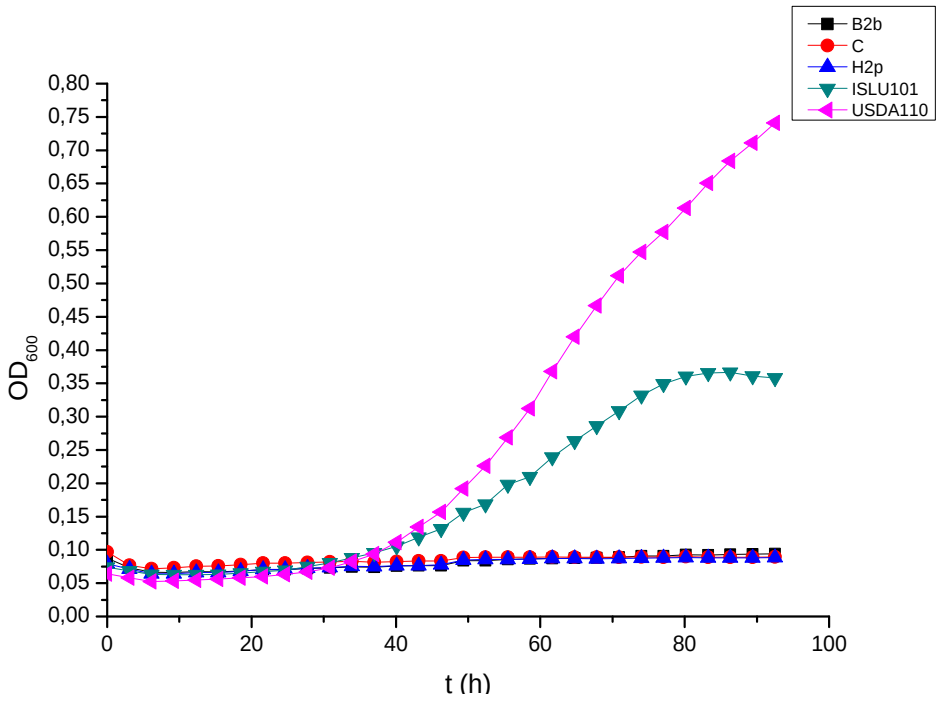


Figure 8: Growth curves of various rhizobial strains in a YMB medium at pH 5.0 as determined by bioscreen.

Slika 7: *Rastne krivulje različnih sevov rizobijev v tekočem YMB gojišču pri pH 5,0, kot smo jih določili z metodo bioscreen.*

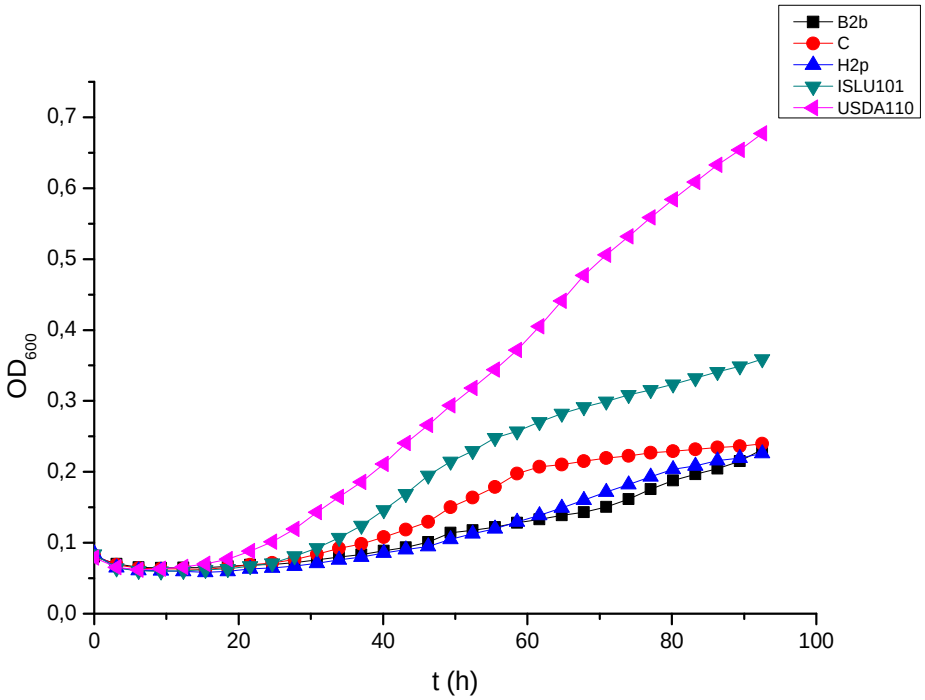


Figure 7: Growth curves of various rhizobial strains in a YMB medium at pH 6.8 as determined by bioscreen.

Slika 8: *Rastne krivulje različnih sevov rizobijev v tekočem YMB gojišču pri pH 6,8, kot smo jih določili z metodo bioscreen.*

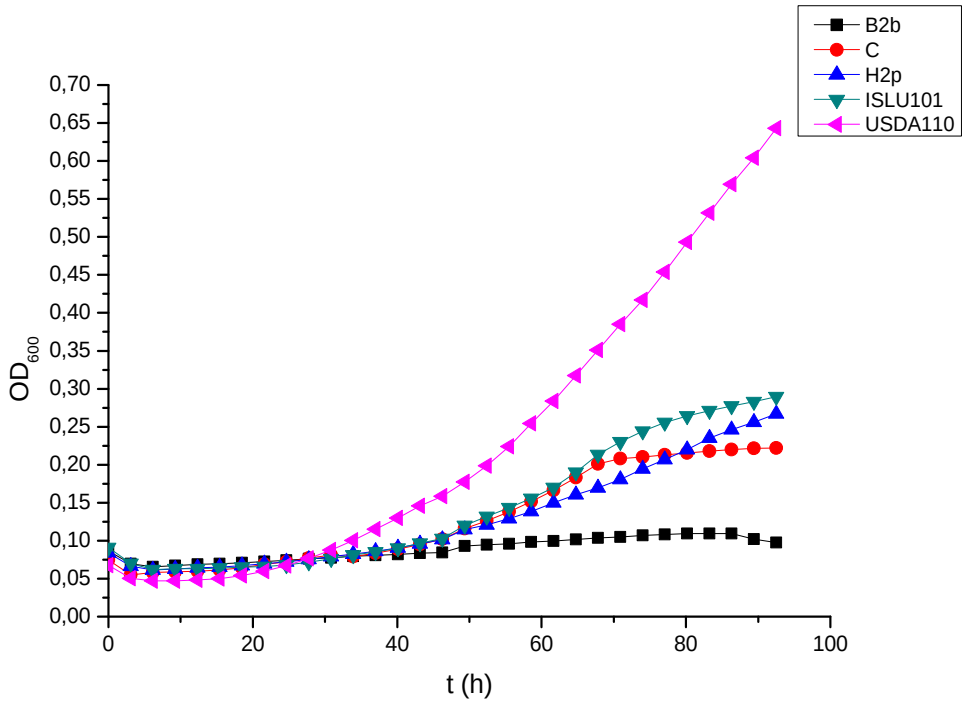


Figure 10: Growth curves of various rhizobial strains in a YMB medium at pH 8.0 as determined by bioscreen.

Slika 9: *Rastne krivulje različnih sevov rizobijev v tekočem YMB gojišču pri pH 8,0, kot smo jih določili z metodo bioscreen.*

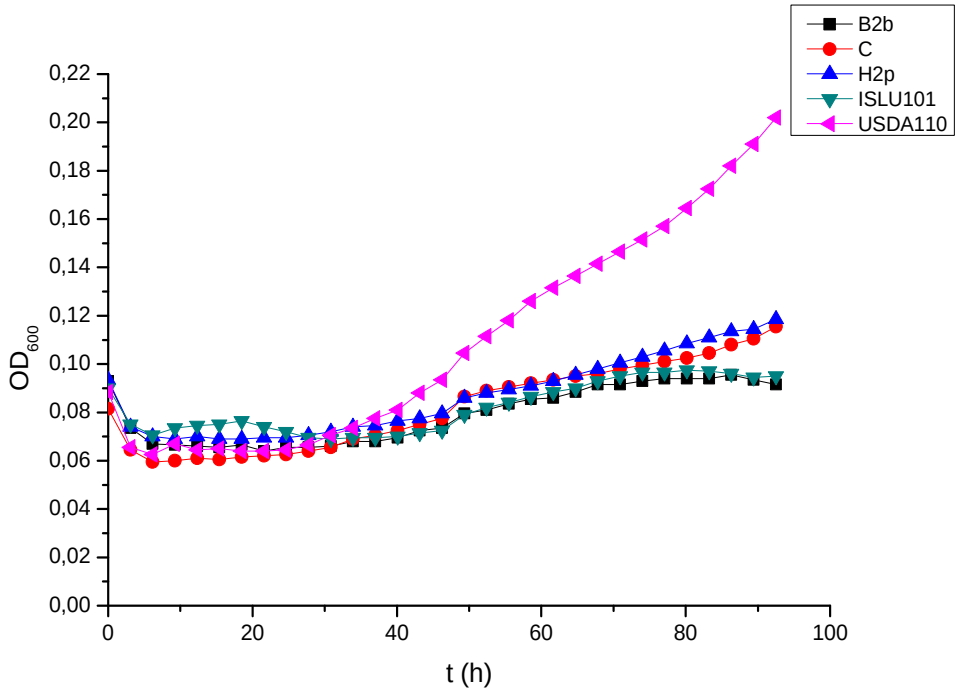


Figure 9: Growth curves of various rhizobial strains in a YMB medium at pH 9.0 as determined by bioscreen.

Slika 10: *Rastne krivulje različnih sevov rizobijev v tekočem YMB gojišču pri pH 9,0, kot smo jih določili z metodo bioscreen.*

Table 3: Generation times [h] of different rhizobial strains (*v.s.* Table 2) as measured by bioscreen. Strains were cultivated in YMB medium at 28 ºC for 5 days.
Preglednica 3: *Generacijski časi [h] različnih sevov rizobijev (glej Tabelo 2) izmerjeni z metodo bioscreen. Seve smo inkubirali 5 dni v tekočem YMB gojišču pri 28 °C.*

strains / *sevi* / pH	B2b	C	H2p	ISLU101	USDA110
5.0	>120	>120	>120	29	20
6.8	48	39	42	30	23
8.0	>120	39	41	36	22
9.0	>120	95	>120	>120	52

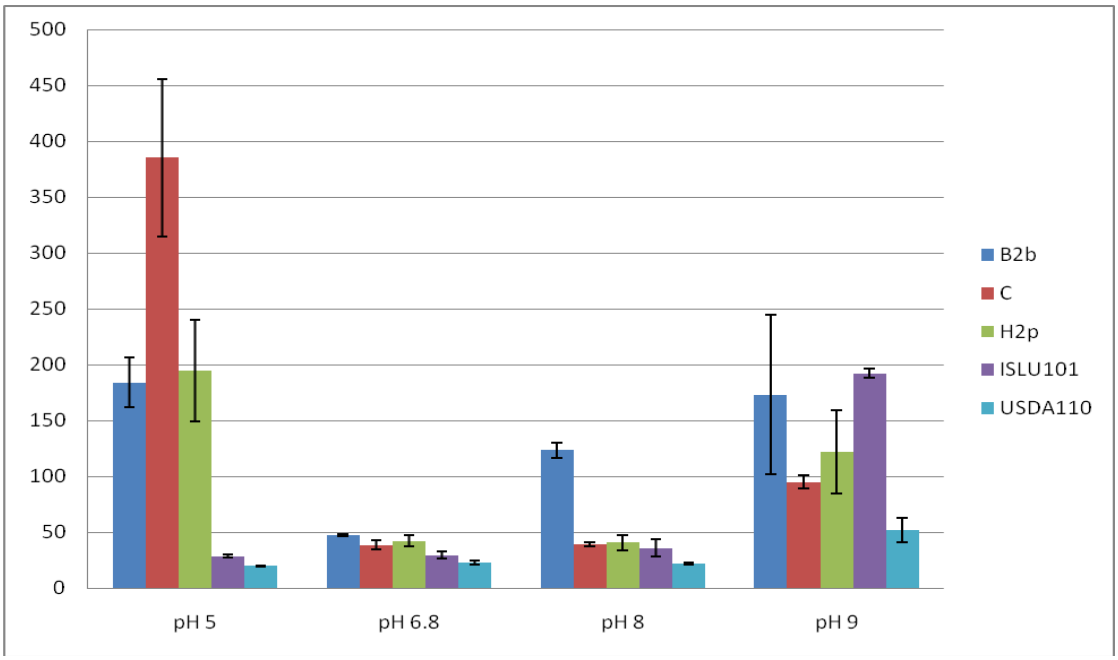


Figure 11: Generation times [h] of tested rhizobial strains at different pH values with standard deviations demonstarted. Strains were cultivated in YMB medium at 28 ºC for 5 days.
Slika 11: *Generacijski časi [h] sevov rizobijev pri različnih pH vrednostih s prikazanimi standardnimi deviacijami. Seve smo inkubirali 5 dni v tekočem YMB gojišču pri 28 °C.*

4.3 SYMBIOTIC CHARACTERIZATION

Characterization of specific interactions between *Lupinus mariae-josephi* and endosymbiotic bacteria was performed by cross-inoculation assays. The experiments were carried out in two different ways. First, the specificity of strains isolated from nodules of *L. mariae-josephi* towards other lupins from the Iberian Peninsula and other legumes, such as species of genera *Ornithopus, Glycine, Vigna,* and *Cicer*, was tested. Second, the specificity of *L. mariae-josephi* to be nodulated by rhizobial strains isolated from nodules of lupins of the Iberian Peninsula and other legume species was also investigated.
As positive controls we made inoculation tests with the strains used in this work and their original host plants (*v.s.* Table 2).

4.3.1 Specificity of bacteria isolated from nodules of *L. mariae-josephi*

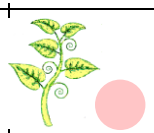
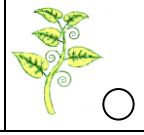
In order to test the specificity of strains isolated from *L. mariae-josephi* 11 legume species, mainly *Lupinus* spp. from the Iberian Peninsula (Table 4), were used as host legume plants.

The results of these cross-inoculation experiments for five *L. mariae-josephi* strains are summarized in Table 4 and presented in Figures 12-17. The most remarkable result is that *L. mariae-josephi* isolates are unable to nodulate *L. angustifolius, L. cosentinii, L. gredensis, L. hispanicus, L. micranthus,* and *L. luteus* species that thrive in acid soils. Only few, small and white like-nodules or eventual plant tumors were observed in some plants. As positive control *L. mariae-josephi* was efficiently nodulated with high rates of N_2 fixation by the five strains of *L. mariae-josephi* tested (Figure 12). It is noteworthy that *L. micranthus* was nodulated by the strain H2p, although the plants appeared indigent and undersized in spite of some nitrogenase activity of the nodules. In contrast, *L. albus*, a lupin species that was introduced in the Iberian Peninsula and later on domesticated, was efficiently nodulated by all strains of *L. mariae-josephi*, although its N_2 fixation rates are lower than those with *L. mariae-josephi* as host plant.

The level of nodulation was evaluated by the appearance of the plant that was green and tall when nodulation was effective and small and yellow if there were no nodules or were ineffective. We could also infer the nitrogenase activity by the appearance and the exterior ansd interior color of nodules. Red and bulky nodules normally had high nitrogenase activity, whereas white and small had none or low.

Table 4: Specificity of strains isolated from nodules of *L. mariae-josephi* towards lupins and other legumes. The level of nodulation was evaluated by the appearance of the plant (green, greenish, yellow or reddish; tall or undersized), appearance and number of nodules (red, big, and numerous; pink, big and numerous; pink, small, and scarce; white, small and few ○). Nitrogenase activity was expressed in µmol of ethylene per gram of fresh nodules' mass and per hour.

Preglednica 4: *Specifičnost sevov izoliranih iz nodulov rastline L. mariae-josephi za vrste volčjega boba in druge vrste metuljnic. Raven nodulacije je bila ocenjena z izgledom rastline (zelena, zelenkasta, rumena ali rdečkasta; visoka ali pritlikava), izgledom in številom nodulov (rdeči, veliki in številni; rožnati, veliki in številni; rožnati, majhni in redki; beli, majhni in maloštevilni ○). Nitrogenazna aktivnost je izražena v enotah µmol etilena na gram sveže mase nodulov in na uro.*

strains ***sevi*** / **host plant** ***gostiteljska rastlina***	**A2**		**B2b**		**C**		**Db2**		**H2p**	
	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA[1]	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA
L. mariae-josephi		10.2		14.6		14.1		9.3		16.3
L. albus cv. Multolupa		4.8		2.9		6.7		3.6		3.4
L. angustifolius cv. Uniharvest		ND[2]		ND		ND		ND		ND
L. cosentinii var. eregulla		ND	**X**[3]	ND	**X**	ND		0		ND

to be contd.
se nadaljuje

[1] NA, nitrogenase activity, *nitrogenazna aktivnost*

[2] ND, not determined, *podatek ni bil določen*

[3] **X**, the plant did not grow, *rastlina ni rasla*

contd.
nadaljevanje

strains ***sevi*** / **host plant** ***gostiteljska rastlina***	***A2***		***B2b***		***C***		***Db2***		***H2p***	
	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA
L. hispanicus ec. Cazalla	X	ND		ND		0	X	ND		0
L. luteus cv. Aurea		ND		ND		0		ND		0
L. micranthus ec. Gibraleón		ND		ND	X	ND		ND		4.4
L. gredensis		ND		ND		0		ND		0
Ornithopus compressus cv. 5599	X	ND		ND		ND	X	ND	X	ND
Vigna unguiculata cv. unguiculata		0	§[4]	ND		0	§	ND	§	ND
Cicer arietinum	§	ND	§	ND	§	ND		ND	§	ND

[4] §, the plant was not planted, *rastline nismo posadili*

4.3.1.1 *Lupinus mariae-josephi* plant growth

The best growth of *L. mariae-josephi* plants was observed when they were inoculated with their own symbiotic strains (Figure 12). As negative control plants that were not inoculated were used. The growth of plants inoculated with C strain is especially eye catching, and a detailed comparison of roots from C inoculated plants and control plants is shown in Figure 13.

Figure 12: Comparison of plant growth of *L. mariae-josephi* inoculated with its own endosymbiotic strains and the non-inoculated control (top from left to right: A2, negative control, B2b; bottom from left to right: C, Db2, H2p)

Slika 12: *Primerjava rasti L. mariae-josephi ob inokulaciji z lastnimi endosimbiotskimi sevi (zgoraj od leve proti desni: A2, negativna kontrola, B2b; spodaj od leve proti desni: C, Db2, H2p)*

Figure 13: A detailed comparison of *L. mariae-josephi* roots from plants inoculated with its own endosymbiotic strain C (left) and from not inoculated plants (right).

Slika 13: *Podroben prikaz korenin rastline L. mariae-josephi, ki je tvorila gomoljčke s sevom C (levo), in s koreninami rastline, ki ni bila inokulirana (desno).*

4.3.1.2 *Lupinus albus* L. plant growth

L. albus L. was the only *Lupinus* species tested that was able to form active and numerous nodules with rhizobia isolated from nodules of *L. mariae-josephi*. In certain cases however the appearance of plants inoculated with *L. mariae-josephi* strains could not be distinguished from the non-inoculated control. As expected, the best growth was noted when plants were inoculated with their own strain ISLU203 (Figure 14).

In Figure 15 a detailed comparison between roots from plants inoculated with ISLU203 and A2, respectively, is shown. A difference in the openness of the leaves can also be observed.

Figure 14: Plant growth of *L. albus* with various strains (from top down and from left to right: A2 (*L. mariae-josephi* isolate) , ISLU203 (*L. albus* isolate), B2b, C, Db2, H2p (all *L. mariae-josephi* isolates), negative control).

Slika 14: *Rast rastlin L. albus z različnimi sevi (od zgoraj navzdol in od leve proti desni: A2 (izolat L. mariae-josephi); ISLU203 (izolat L. albus), B2b, C, Db2, H2p (izolati L. mariae-josephi); negativna kontrola).*

Figure 15: A detailed comparison of *L. albus* roots from plants inoculated with ISLU203 (*L. albus* isolate) and A2 (*L. mariae-josephi* isolate), respectively.

Slika 15: *Podroben prikaz korenin L. albus ob inokulaciji s sevom ISLU203 (izolat L. albus) oz. A2 (izolat L. mariae-josephi).*

4.3.1.3 *Lupinus luteus* L. plant growth

L. luteus was a clear example of the inefficiency of strains isolated from nodules of *L. mariae-josephi* to fix nitrogen with heterologous *Lupinus* species. As shown in Figure 16 the plants were small and yellow, and could not be distinguished from the non-inoculated control plants. The *L. luteus* inoculated with *L. mariae-josephi* strains produced no nodules (Figure 17). However, when *L. luteus* were inoculated with strain 874 from *L. angustifolius* big and green plants were produced.

Figure 16: Results of the inoculation of *L. luteus* with various strains (from top down and from left to right: A2 (*L. mariae-josephi* isolate), 874 (*L. angustifolius* isolate), B2b, C, Db2, H2p (all *L. mariae-josephi* isolates), negative control).

Slika 16: *Rezultati inokulacije rastlin L. luteus z različnimi sevi (od zgoraj navzdol in od leve proti desni: A2 (izolat L. mariae-josephi), 874 (izolat L. angustifolius), B2b, C, Db2, H2p (izolati L. mariae-josephi), negativna kontrola).*

Figure 17: A detailed comparison of *L. luteus* roots when inoculated with 874 (*L. angustifolius* isolate) and A2 (*L. mariae-josephi* isolate), respectively.
Slika 17: *Podrobna primerjava korenin L. luteus ob inokulaciji s sevom 874 (izolat L. angustifolius) oz. A2 (izolat L. mariae-josephi)*

4.3.2 Specificity of *Lupinus mariae-josephi*

In a second type of cross-inoculation assays to study the specificity of *L. mariae-josephi*, plants of this species were inoculated with isolates from nodules of lupins grown in acids soils of the Iberian Peninsula and other legumes. The results are shown in Table 5 and in Figures 18-20.

L. mariae-josephi plants formed nodules with all isolates from *Lupinus* spp. of the Iberian Peninsula (ISLU strains) but the nodules formed were inactive or had low nitrogen fixation activity. This was also concluded from the appearance of plants that were always small and yellow (Table 5). An exception was strain 874 isolated from nodules of *L. angustifolius* which induced the formation of active nodules in *L. mariae-josephi*. Furthermore, *L. mariae-josephi* did form numerous, red, and most probably very active nodules with the strain 861, isolated from *Ornithopus sativus* Brot. and additionally, less active nodules with ISLU16, also an isolate from nodules of yellow serradella (*O. compressus* L.).

Positive controls were always included in the test inoculating the *Lupinus* spp. that were the original hosts of the rhizobial strains used. As expected, all the strains formed numerous and active nodules. The results are presented in Table 6.

In the following figures a comparison between differently inoculated *L. mariae-josephi* plants is shown. Most of the plants grew poorly, were light green color, small, and indistinguishable from the non-inoculated negative control (Figure 18 and 19). In most cases (ISLU strains, strain 897) they formed few reddish or white nodules without any or low activity. In Figure 20 a detailed comparison of the root systems inoculated with ISLU strains is shown.

Figure 18: Comparison of *L. mariae-josephi* plants inoculated with (from left to right): C (*L. mariae-josephi* isolate), 835 (*Cicer arietinum* isolate), negative control, and 874 (*L. angustifolius* isolate).

Slika 18: *Primerjava rastlin L. mariae-josephi ob inokulaciji z različnimi sevi (od leve proti desni): C (izolat L. mariae-josephi), 835 (izolat Cicer arietinum), negativna kontrola in 874 (izolat L. angustifolius).*

Figure 20: Comparison of *L. mariae-josephi* plants inoculated with (from top down and from left to right): ISLU8 (*L. luteus* isolate), negative control, ISLU12 (*L. cosentinii* isolate), ISLU16 (*O. compressus* isolate), ISLU40 (*L. hispanicus* isolate).

Slika 19: *Primerjava rastlin L. mariae-josephi ob inokulaciji z (od zgoraj navzdol in od leve proti desni): ISLU8 (izolat L. luteus), negativna kontrola, ISLU12 (izolat L. cosentinii), ISLU16 (izolat O. compressus), ISLU40 (izolat L. hispanicus).*

Figure 19: A detailed comparison of *L. mariae-josephi* roots from plants that were inoculated with (from left to right): ISLU8 (*L. luteus* isolate), ISLU12 (*L. cosentinii* isolate), ISLU16 (*O. compressus* isolate), and ISLU40 (*L. hispanicus* isolate).

Slika 20: *Podroben prikaz koreninskega sistema L. mariae-josephi ob inokulaciji z (z leve proti desni): ISLU8 (L. luteus izolat.), ISLU12 (L. cosentinii izolat), ISLU16 (O. compressus izolat) in ISLU40 (L. hispanicus izolat).*

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Graduation Thesis. Univ. of Ljubljana. Biotechnical Faculty. Interdep. Programme in Microbiology, 2009

Table 5: Specificity of *L. mariae-josephi* towards its own strains and strains isolated from nodules of lupins from Iberian acid soils and other legumes. For symbol explanation *v.s.* Table 4.

Preglednica 5: *Specifičnost rastlinske vrste L. mariae-josephi za lastne seve in seve izolirane iz gomoljčkov vrst volčjega boba, ki rastejo v kislih tleh Iberskega polotoka, ter drugih vrst metuljnic. Za razlago simbolov glej Tabelo 4.*

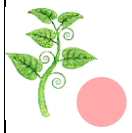
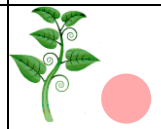
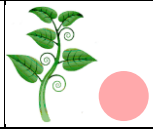
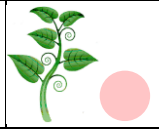
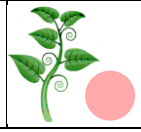
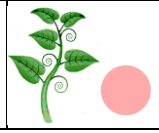
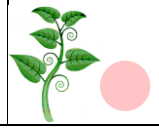
strains ***sevi*** / **host plant** ***gostiteljska rastlina***	**A2**		**B2b**		**C**		**Db2**		**H2p**	
	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA
L. mariae-josephi		10.2		14.6		14.1		9.3		16.3
	ISLU8		**ISLU12**		**ISLU16**		**ISLU40**		**ISLU101**	
		0		0		1.3		0		0.2
	ISLU122		**ISLU203**		**874**		**861**		**924**	
		1.0		0.9		7.9		ND		ND
	835		**897**		**752**		**804**			
		ND		ND		ND		ND		

Table 6: Analysis of symbiosis control formed by rhizobial strains used in Table 5 with legumes from where they were isolated. For symbol explanation *v.s.* Table 4.

Preglednica 6: *Rezultati simbiotskega odnosa med rizobiji (Tabela 5) in stročnicami, iz katerih so bili izolirani. Za razlago simbolov glej Tabelo 4.*

host plant ***gostiteljska rastlina***	*L. albus* cv. Multolupa		*L. angustifolius* cv. Uniharvest		*L. cosentinii* var. eregulla					
strains ***sevi***	**ISLU203**		**ISLU101**		**ISLU12**		**874**		**897**	
	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA	appearance of host plant and nodules *izgled gostiteljske rastline in nodulov*	NA
		9.3		1.1	**X**	ND	**X**	ND		ND
host plant ***gostiteljska rastlina***	*L. luteus* cv. Aurea				*L. gredensis*		*Ornithopus compressus* cv. 5599			
strains ***sevi***	**874**		**897**		**ISLU101**		**ISLU40**		**861**	
		6.4		ND		5.8	**X**	ND		ND
host plant ***gostiteljska rastlina***	*L. micranthus* ec. Gibraleón		*L. hispanicus* ec. Cazalla		*Vigna unguiculata* cv. Unguiculata		*Cicer arietinum* L.			
strains ***sevi***	**ISLU122**		**ISLU40**		**924**		**835**			
	X	ND	**X**	ND		11.9		3.9		

4.4 PHLYLOGENETIC CHARACTERIZATION OF RHIZOBIA ISOLATED FROM NODULES OF *L. mariae-josephi*

The phylogenetic characterizations of strains isolated from nodules of *L. mariae-josephi* were achieved by sequence analysis of the *rrs* genes that code a small ribosomal subunit 16S rRNA. This gene that is present in all bacteria, has an important function and is highly conserved. Consequently, it is generally accepted that *rrs* sequences reflect the evolution of microorganisms and therefore, are widely used for phylogenetic analyses to unravel evolutionary relationships.

The *rrs* genes were amplified by PCR with 41f and 1448r primers (Herrera-Cervera, 2001) and later on sequenced. All PCR products obtained had uniform lengths, indicating that the amplified genes represent only one single type of rRNA operon. As a first approach to the identification of the selected isolates the *rrs* sequences were compared.

Figure 21 shows the phylogenetic tree based on the *rrs* sequences of five *L. mariae-josephi* isolates and of strains from genera *Rhizobium, Mesorhizobium, Sinorhizobium, Phyllobacterium, Bradyrhizobium, Methylobacterium*, and *Bacillus licheniformis* K10 as an outgroup. All sequences, except the *L. mariae-josephi* isolates and certain *B. elkanii* strains, were obtained from the GenBank database. The accession numbers for the 16S rRNA gene sequences used in this work are provided in Figure 21.

In this phylogenetic tree it can be observed that:

i) The rhizobia strains that nodulate *L. mariae-josephi* were placed near other species of the genus *Bradyrhizobium* (*B. canariense, B. betae, B. japonicum, B. liaoningense, B. yuanmingense*, and *B. elkanii*) with which they formed a monophyletic taxonomic unit (100% confidence index).

ii) The resulting phylogenetic tree showed a formation of two clades. The main clade grouped all sequences of isolates of the genera *Rhizobium*, *Sinorhizobium, Mesorhizobium*, and *Phyllobacterium* (all of them corresponding to fast-growing rhizobia bacteria). Rhizobia isolated from nodules of *L. mariae-josephi* and all *Bradyrhizobium* species formed a second clade with *Methylobacterium nodulans* (slow growing rhizobia).

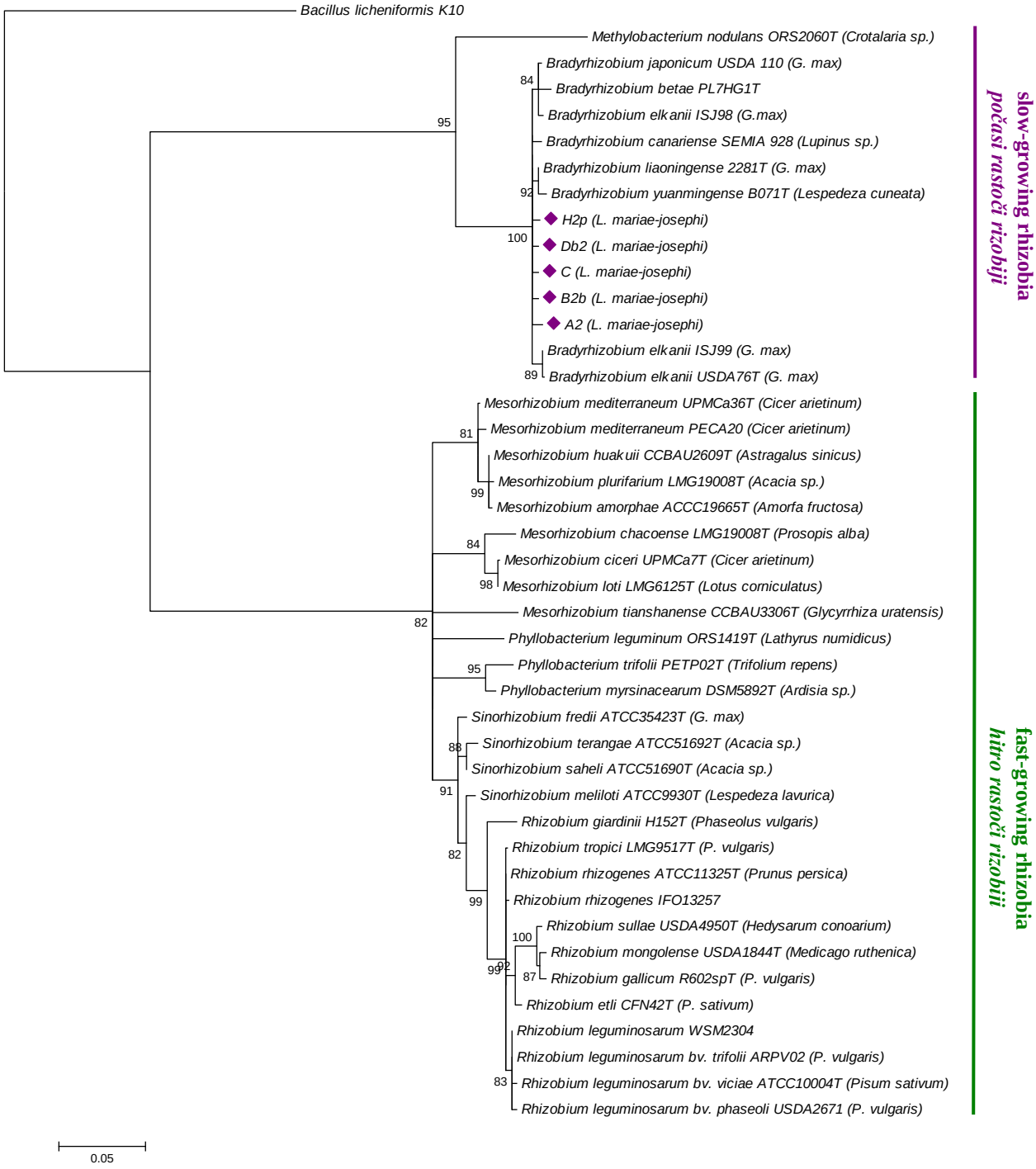


Figure 21: Phylogenetic tree generated by the maximum-likelihood method using *rrs* gene sequences of various rhizobia. The numbers next to the branches correspond to the percentage of confidence index aLRT. Legume host names are shown in brackets and those followed by a T correspond to the type strain of rhizobial species. The sequences A2, B2b, C, Db2, H2p (marked with◆), and *Bradyrhizobium elkanii* strains ISJ98, and ISJ99 were obtained in this project, others were obtained from GenBank database: *Mesorhizobium haukii* CCBAU3306 ^{T} (D12797), *M. amorphae* ACCC19665^{T} (AF041442), *M. plurifarium* LMG11892^{T} (Y14158), *M. mediterraneum* UPMCa36^{T} (AY195844), *M. loti* LMG6125^{T} (X67229), *M. ciceri* UPMCa7^{T} (U07934), *M. chacoense* LMG19008^{T} (AJ278249), *M. tianshanense* CCBAU3306^{T} (U71079), *M. mediterraneum* PECA20 (AY195844), *Sinorhizobium meliloti* ATCC9930 (X67222), *S.*

fredii ATCC35423^T (D14516), *S. saheli* ATCC51690 (X68388), *R. leguminosarum* bv. viciae ATCC10004^T (U29386), *R. etli* CFN42^T (U28916), *R. leguminosarum* bv. phaseoli USDA2671 (U29388), *R. leguminosarum* bv. trifolii ARPV02 (AY50990), *R. leguminosarum* WSM2304 (DQ838519), *R. tropici* LMG9517^T (X67234), *R. rhizogenes* ATCC11325^T (AY206687), *R. rhizogenes* IFO13257 (D14501), *R. rubi* ATCC1335^T (D14503), *R. mongolense* USDA1844T (U89817), *Phyllobacterium myrsinacearum* DSM5892^T (D12789), *P. trifolii* PETP02^T (AY786080), *P. leguminum* ORS1419^T (AY785323), *Bradyrhizobium canariense* SEMIA928 (FJ390904.1), *B. betae* PL7HG1T^T (AY372184), *B. japonicum* USDA110 (NC_004463), *B. liaoningense* 2281^T (AF363132), *B. yuanmingense* B071^T (AF193818), *B. elkanii* USDA76^T (U35000), and *Bacillus licheniformis* (DQ351930).

Slika 21: *Filogenetsko drevo izdelano iz zaporedij rrs genov različnih vrst rizobijev. Uporabili smo metodo največjega verjetja. Vrednosti indeksa zaupanja aLRT so navedeni poleg razvejitev. Črka T označuje, da je označen sev tipski sev, v oklepajih pa je navedena izvirna gostiteljska rastlina. Zaporedja sevov A2, B2b, C, Db2, H2p (ti so označeni z ◆) ter Bradyrhizobium elkanii ISJ94, ISJ98 in ISJ99 smo določili sami, ostala smo pridobili v podatkovni bazi GenBank: Mesorhizobium haukii CCBAU3306 ^{T} (D12797), M. amorphae ACCC19665^T (AF041442), M. plurifarium LMG11892^T (Y14158), M. mediterraneum UPMCa36^T (AY195844), M. loti LMG6125^T (X67229), M. ciceri UPMCa7^T (U07934), M. chacoense LMG19008^T (AJ278249), M. tianshanense CCBAU3306^T (U71079), M. mediterraneum PECA20 (AY195844), Sinorhizobium meliloti ATCC9930 (X67222), S. fredii ATCC35423^T (D14516), S. saheli ATCC51690 (X68388), R. leguminosarum bv. viciae ATCC10004^T (U29386), R. etli CFN42^T (U28916), R. leguminosarum bv. phaseoli USDA2671 (U29388), R. leguminosarum bv. trifolii ARPV02 (AY50990), R. leguminosarum WSM2304 (DQ838519), R. tropici LMG9517^T (X67234), R. rhizogenes ATCC11325^T (AY206687), R. rhizogenes IFO13257 (D14501), R. rubi ATCC1335^T (D14503), R. mongolense USDA1844^T (U89817), Phyllobacterium myrsinacearum DSM5892^T (D12789), P. trifolii PETP02^T (AY786080), P. leguminum ORS1419^T (AY785323), Bradyrhizobium canariense SEMIA928 (FJ390904.1), B. betae PL7HG1T^T (AY372184), B. japonicum USDA110 (NC_004463), B. liaoningense 2281^T (AF363132), B. yuanmingense B071^T (AF193818), B. elkanii USDA76^T (U35000) in Bacillus licheniformis (DQ351930).*

Based on these observations it was concluded that the strains isolated from the nodules of *L. mariae-josephi* belong to the genus *Bradyrhizobium* that includes slow-growing rhizobia. This is congruent with the long generation times for these bacteria. Clearly, the *Bradyrhizobium* group of strains formed a separated cluster from other species of the family *Rhizobiaceae*. The results described above induced to carry out a more detailed analysis of the phylogenetic positions of the strains isolated from *L. mariae-josephi*. For this sequences of various rhizobial species and strains, most of them from lupins grown on acid soil (i.e. ISLU strains) of the Iberian Peninsula and from soybean, were used (Figure 22). In order to emphasize that the five *L. mariae-josephi* strains were distant from the fast-growing rhizobia, species *Rhizobium leguminosarum* bv. viciae, *Sinorhizobium meliloti*, and *Mesorhizobium loti* were also included. Species *Bacillus licheniformis* K10 was included as an outgroup.

From the phylogenetic tree, presented in Figure 22, it can be concluded:

i) Almost all isolates from *Lupinus* spp. of the Iberian Peninsula (ISLU) were clustered within the *B. canariense* species.

ii) *Bradyrhizobium* species *B. lioningense, B. yuanmingense, B. japonicum, B. elkanii* and *B. betae* were clustered together (84% confidence index). Surprisingly, this cluster included strains ISLU203 (isolated from *L. albus*), ISLU127 (isolated from *O. compressus*) and ISLU15 (isolated from *L. luteus*).

iii) The more relevant result was that all isolates from *L. mariae-josephi* clustered separately from the other two identified above, close to certain *B. elkanii* strains [ISJ94 (USDA76) and ISJ99 (USDA275)]. Because of the low resolution of the tree this conclusion could be dubious, however, these results were confirmed by analysis of other housekeeping genes.

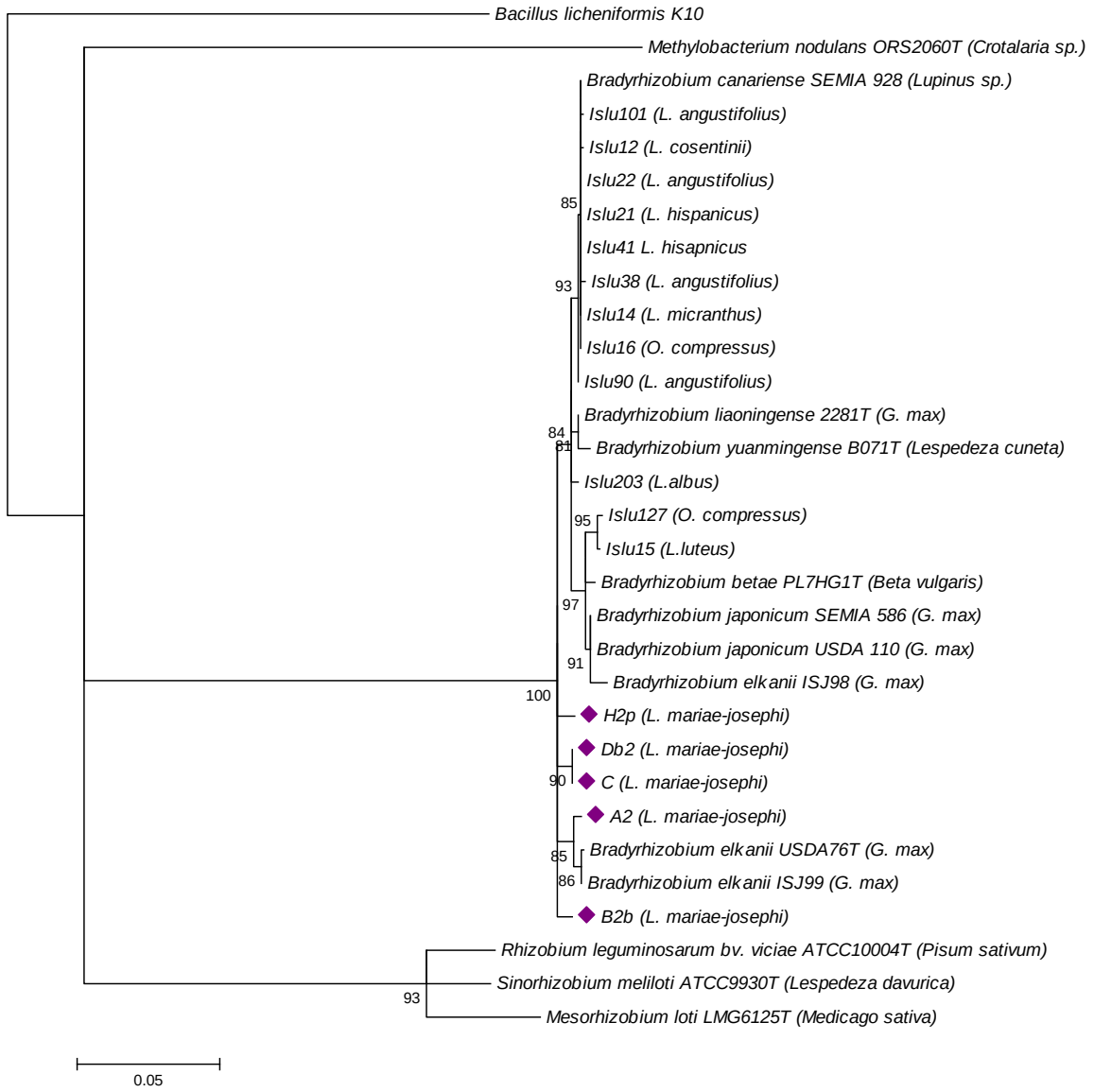


Figure 22: Phylogenetic tree generated by the maximum-likelihood method using *rrs* gene sequences from different rhizobia and bradyrhizobia strains. The numbers next to the branches correspond to the percentage of confidence index aLRT. Legume host names are shown in brackets and those followed by a T correspond to the type strain of rhizobial species T. Sequences of A2, B2b, C, Db2, H2p (marked with◆**)**, of all ISLU strains and of *Bradyrhizobium elkanii* strains ISJ94 (USDA76), ISJ98 (USDA117) and ISJ99 (USDA275) were obtained in this work, others were obtained from GenBank (*v.s.* Figure 21).

Slika 22: *Filogenetsko drevo izdelano iz zaporedij rrs genov različnih vrst rizobijev in sevov rodu Bradyrhizobium. Uporabili smo metodo največjega verjetja. Vrednosti indeksa zaupanja aLRT so navedeni poleg razvejitev. Črka T označuje, da je označen sev tipski sev in v oklepajih je navedena izvirna gostiteljska rastlina. Zaporedja sevov A2, B2b, C, Db2, H2p (ti so označeni z ◆**)**, vseh ISLU sevov ter Bradyrhizobium elkanii ISJ94, ISJ98 in ISJ99 smo določili tekom te diplomske naloge, ostala smo poiskali v podatkovni bazi GenBank (glej sliko 21).*

5 DISCUSSION AND CONCLUSIONS

A novel lupin species, *L. mariae-josephi* from the Llombai region of Valencia (Spain), was recently described by Pascual (2004). This species is able to grow in alkaline soil with high concentrations of Ca^{2+}. It does not seem closely related to any other of the eight species that can be found in Iberian Peninsula. Apparently, it does not grow in acid soils which is common for the majority of *Lupinus* L. species (Castroviejo and Pascual, 1999). By phenotypical characteristics, i.e. number of leaves and color of the seeds, it does not seem to differ very much from some other already described species in the Iberian Peninsula (such as *L. micranthus*). However, its particularity to grow in alkaline soil is uncommon.
Phylogenetic position of *L. mariae-josephi* among other lupin species have been studied by a research group in the University of Rennes, France, using genetic and molecular analyses mainly sequencing internal transcribed spacer (ITS). They compared *L. mariae-josephi* with other lupin species of the Old and New World and concluded that *L. mariae-josephi* is a unique and an exceptional species (Aïnouche, personal communication).

In this research project we pursued a symbiotic and phylogenetic characterization of rhizobia isolated from nodules of *L. mariae-josephi*. Finally, their growth rates and macro- and microscopic characteristics were described. The specificity of the rhizobia isolated from nodules of this novel species towards lupins found in the Iberian Peninsula and other legumes was investigated, followed by the examination of specificity of the host plant *L. mariae-josephi* towards different strains isolated from nodules of various species of lupins and other legumes. Finally, the phylogenetic positions of *L. mariae-josephi* strains were investigated by constructing a phylogenetic tree using 16S rRNA gene sequences.

5.1 MORPHOLOGICAL ANALYSIS OF RHIZOBIA ISOLATED FROM *Lupinus mariae-josephi*

In order to compare morphological characteristics of strains isolated from nodules of *L. mariae-josephi* and isolates from nodules of legumes, the morphology of colonies, slime production, and cell morphology were compared among these strains.

The comparison of growth on the YMB agar plates between rhizobia isolated from *L. mariae-josephi* and *Bradyrhizobium japonicum* USDA110 showed that after 9 days of incubation at 28 °C the colonies formed by strain H2p were almost imperceptible, whereas *B. japonicum* USDA110 formed mucoid and white colonies measuring 2-3 mm in diameter after 5 days of incubation (Figure 5). *L. mariae-josephi* strains generally grew slower on YMB medium than other strains used in this project. The growth of *L. mariae-josephi* was faster on AG medium containing gluconate and arabinose.

The colonies formed by *L. mariae-josephi* isolates were not mucoid. This could be because the media conditions (mannitol as the nutrient) do not favor the production of mucus or because these bacteria have different regulation of exopolysaccharides production or even use a different biosynthetic pathway.

Moreover, we compared the morphology of rhizobial cells with an optical microscope in bright field with crystal violet staining. All rhizobia from nodules of *L. mariae-josephi* were similar in their morphology - short rods, often forming short chains (Figure 6). This cell morphology was also similar to other isolates from nodules of Iberian Peninsula lupins and the well known *Bradyrhizobium japonicum* USDA110. However, some cells appeared to have a capsule (e.g. ISLU101), although additional staining would be needed to confirm this feature.

In conclusion, rhizobia from nodules of *L. mariae-josephi* differ from *B. japonicum* in various macromorphological characteristics, mainly the lack of exopolysacharides production and the slow growth in the standard YMB medium that contains mannitol as carbon source. However, microscopic characteristics do not reflect obvious differences among rhizobial cells that were examined.

5.2 GENERATION TIMES OF STRAINS ISOLATED FROM *Lupinus mariae-josephi*

By measuring the growth rates of strains isolated from nodules of *L. mariae-josephi* we were able to confirm that these strains may belong to the genus *Bradyrhizobium* which is considered a group of slow-growing rhizobia ($g \approx 6$-10h). To this aim, the results with one

rhizobial strain isolated from nodules of *L. angustifolius* growing in acid soils as well as *B. japonicum* USDA110 were compared.

Bioscreen results showed that the optimal pH value for growth of tested *L. mariae-josephi* strains (B2b, C, H2p) was 6.8 (g ≈ 2 days), which is also an optimal pH value for bradyrhizobial species. Generally, the mean generation time of *L. mariae-josephi* at pH 6.8 was higher than for strains USDA110 (g ≈ 15 h) and ISLU101 (g ≈ 20 h). It is noteworthy that strain B2b that held the highest generation time grew slower compared to C and H2p also as the pH was higher. The difference is statistically relevant at pH 8 and partially at pH 9 (Figure 11). This is relevant since the alkaline pH of the soil where *L. mariae-josephi* grows may have been a selective factor for its symbionts. However, in contrast to *B. japonicum* USDA110 and ISLU101 strains, *L. mariae-josephi* tested strains did not grow at low pH (pH 5). In general, USDA110 and ISLU101 seem to have a broad spectrum of tolerance to pH values with average generation times of 1-2 days and 2 days, respectively.

The generation times were also measured by cultivating the strains in batch cultures in flasks and then determining the OD in cuvettes. The relative results were similar to the results by bioscreen as all *L. mariae-josephi* strains grew slower than other tested strains. However, the absolute values of generation times were higher and finally not presented.

Bioscreening methodology seems to be a more reliable method than cultivation in flasks as it allowed more frequent and apparently also more accurate measurements. When cuvettes were used there were greater chances for contamination of the batch culture and inaccuracies due to specks on cuvettes, flocculation of the batch cultures or human errors. Furthermore, we did not use repetitions. Intriguingly, bioscreen also gave unusual results because the mean generation time for *B. japonicum* was more than 20 hours. This is not consistent with studies that report generation time of typical slow-growing rhizobia of 6 to 10 hours (Vincent, 1974).

In conclusion, the results obtained by bioscreen or the flasks methods showed that rhizobia isolated from nodules of *L. mariae-josephi* grew slower than other rhizobia strains tested and were less tolerant to acid pHs in their growth rates and mostly lagged behind at all pH values. Due to the peculiar growth conditions of the host in alkaline soils a preference for

higher pH values could be expected. However, their preference to grow better in alkaline media was not demonstrated.

5.3 SYMBIOTIC CHARACTERIZATION OF RHIZOBIA THAT NODULATE *Lupinus mariae-josephi*

Cross-inoculation tests showed high specificity not only of the rhizobia isolated from nodules of *L. mariae-josephi* but also of the host plant itself.

When testing the specificity of the strains isolated from *L. mariae-josephi* only the host plant formed abundant and red nodules. Nodules are red because they synthesize large amounts of leghemoglobin that binds oxygen and lowers the oxygen concentration levels to a minimum. Based on their color we could estimate the nitrogen fixing activity of the nodules. Reddish or white nodules were normally less active than red ones. Additionally, we could infer effective nodulation by the appearance of the plants with regard to the non-inoculated control. However, some *L. mariae-josephi* plants grew rather poorly in spite of formation of active nodules on the root system. This could be ascribed to inappropriate conditions for plant growth in the standard culture conditions. Furthermore, the plant that grew better (inoculated with the strain C) also had nodules with one of the highest nitrogenase activity in comparison with the rest of *L. mariae-josephi* strains.

The activity of nodules was tested by the reduction of acetylene assay measured by gas chromatography. This assay is based on the nitrogenase-catalyzed reduction of C_2H_2 to ethylene C_2H_4. The advantage of the technique includes facility for large number of *in situ* assays, economy, simplicity, and sensitivity of the analysis. The technique is 10^3 times more sensitive than ^{15}N tracer technique which is an alternative in determining the nitrogenase activity.

On the other hand, the *L. mariae-josephi* rhizobia strains were unable to nodulate lupins of the Iberian Peninsula: *L. angustifolius*, *L. cosentinii*, *L. hispanicus*, *L. luteus*, and *L. gredensis.* Less active nodules were formed on *L. micranthus* by H2p strain, also a native lupin of the Iberian Peninsula, although this activity was not reflected in the host plant which was yellow and small. This could be due to inability of the plant to assimilate the

nitrogen reduced compounds because of specific metabolic changes that occur in bacteroids to attain an effective NH_3 assimilation (Lodwig *et al.*, 2003).
Furthermore, the *L. mariae-josephi* strains did not form nodules with *Ornithopus compressus* (serradella). That species has been used in the past as a substitutive host plant for bacteria isolated from lupin nodules. Today its role as a substitutive plant is decreasing because it has been shown not to be effective in all cases which was also the case in this study.

L. albus L. was the only host plant widely nodulated by *L. mariae-josephi* strains. However, the nitrogenase activity of the nodules was lower than in the nodules of *L. mariae-josephi* and the plants were not as green. *L. albus* is a domesticated lupin species growing in acid soils of the Iberian Peninsula and has already been known as a more promiscuous plant with less specificity towards rhizobia strains. However, it is interesting to find similar specificity towards the same rhizobia strains between two lupin plant species growing in completely different natural conditions.

The soil in the Llombai region, where *L. mariae-josephi* was originally discovered, was described in detail by Fos *et al.* (2006). It is slightly alkaline lime soil with low carbonate levels (12.0%) and organic matter. In contrast, lupins of the Iberian Peninsula usually grow in sandy loam and with acid to neutral pH values ($5.0 < pH < 7.0$) and low $CaCO_3$ content. It was shown that commercial lupins grow poorly in neutral fine-textured or alkaline soils (Tang *et al.*, 1993, Tang and Robson, 1995). Until now, also other *Lupinus* species (*L. cosentinii* Guss.) have been reported to prosper in limy alkaline soil (pH 7.5-8.4) with high concentrations of $CaCO_3$. In addition, certain populations of *L. angustifolius* L. and *L. micranthus* Dougl. have occasionally appeared in soils with high carbonate concentrations and pH values (Castroviejo and Pascual, 1999; Fos *et al.*, 2006).

5.4 SPECIFICITY OF *L. mariae-josephi* TO FORM NODULES

As described above *L. mariae-josephi* plants formed active and numerous nodules with their own symbionts. However, when the specificity towards strains isolated from other

lupins' nodules (ISLU strains, 897 strain) was tested, it was found that few reddish or white were formed in most cases. Curiously, *L. angustifolius* strain 874 formed numerous, small and white nodules that had high nitrogenase activity. However, the plant was small and indigent. This could be due to similar symbiotic relationship as described above for *L. micranthus* L. in symbiosis with H2p strain.

L. mariae-josephi was effectively nodulated by a strain 861 and at a smaller scale with ISLU16, isolated from nodules of *Ornithopus sativus* Brot. and *O. compressus* L., respectively. Serradella strains have been reported before to be effective symbionts with lupin species (Eckhardt *et al.*, 1931). Lupin and serradella bradyrhizobia originating from Europe also form separate clades based on nodulation genes. This led to an induction of bradyrhizobia infecting legumes of the *Genistae* and *Lotae* tribes in a new biovariety, genistearum (Stępkowski *et al.*, 2005).

5.5 PHYLOGENETIC CHARACTERIZATION OF RHIZOBIA THAT NODULATE *Lupinus mariae-josephi*

We could observe the existence of two clades of rhizobia based on the 16S rRNA sequences (Figure 21). The major clade grouped all isolates of the genera *Rhizobium, Sinorhizobium, Mesorhizobium,* and *Phyllobacterium* (i.e. fast-growing rhizobia). The second, minor clade included species of the genera *Bradyrhizobium* and *Methylobacterium*. Bradyrhizobial species (*B. canariense, B. betae, B. japonicum, B. liaoningense, B. elkanii*, and *B. yuanmingense*) formed a monophyletic group which included the *L. mariae-josephi* isolates. These results were also supported also by the phylogenetic analysis of other housekeeping genes (*recA*, *glnII*, and *atpD*) (Sánchez Jiménez, 2009).

The genus *Bradyrhizobium* involves mostly slow-growing bacteria that form determinate nodules in members of the *Genistae* tribe but also includes species that can nodulate soybeans (*B. japonicum, B. elkanii, B. liaoningense,* and *B. yuanmingense*). Even though these species are evolutionary close to the rhizobia of *L. mariae-josephi* these strains do not nodulate soybeans (Sánchez Jiménez, 2009).

On the more detailed phylogenetic tree on Figure 22 we observed that the majority of the strains isolated from nodules of lupins growing in acid soils in the Iberian Peninsula (ISLU

strains) clustered with *B. canariense*, but separately from the group of isolates of *L. mariae-josephi.* All *L. mariae-josephi* isolates did not group with any of the *Bradyrhizobium* species included, except A2 strain that grouped with *B. elkanii* strains [ISJ99 (USDA275), ISJ94 (USDA76)] that nodulate soybean. The phylogenetic proximity of all *L. mariae-josephi* strains to *B. elkanii* was additionally confirmed by analyses of other housekeeping genes mentioned above (Sánchez Jiménez, 2009). From these results we can only suggest that isolates from nodules of *L. mariae-josephi* represent a new species because they do not cluster with any included species in the phylogenetic tree. However, a more detailed study should be performed to suggest that these isolates are in fact a new species.

B. elkanii was first proposed as a new species by Kuykendall *et al.* (1992). This species can nodulate soybean and is phylogenetically different from *B. japonicum*. *B. elkanii* strains [ISJ99 (USDA275), ISJ94 (USDA76)] are phylogenetically positioned close to the rhizobia of *L. mariae-josephi* but some *B. elkanii* strains such as ISJ98 group also close to *B. japonicum*. This different phylogenetic location of other *B. elkanii* strains suggests that this species is not a monophyletic group.

Complete 16S rDNA sequence analysis is a rapid technique but because of small sequence divergence among *Bradyrhizobium* strains it offers little scope for assessing diversity among these closely related strains (Willems *et al.*, 2001). Therefore, *rrs* phylogeny is usually supplemented with sequencing spacer regions between 16S and 23S rDNA, other housekeeping (*atpD, dnaK, glnII, recA*) and nodulation (*nodC, nodZ, nodA, noeI, nolL, nifH*) genes. All of these gene markers have been used by other workers for phylogenetic reconstructions of *Rhizobiaceae* (Gaunt *et al.*, 2001; Laguerre *et al.*, 2001; Willems *et al.*, 2001; Moulin *et al.*, 2004 Stępkowski *et al.*, 2005; Stępkowski *et al.*, 2007). However, incongruities between phylogenies of nodulation and housekeeping genes are not uncommon and give support to the hypothesis of multiple lateral transfer of symbiotic loci among *Bradyrhizobium* and other genera lineages (Jarabo-Lorenzo *et al.*, 2003; Stępkowski *et al.*, 2003; Moulin *et al.*, 2004; Stępkowski *et al.*, 2007). Phylogenetic analysis of *nodC* genes of strains of *L. mariae-josephi* supported this hypothesis. The *nodC* sequences from selected isolates formed an independent group from all other strains

isolated from lupins and other legumes, including *B. elkanii* strains, and grouped closely to strains of the genus *Mesorhizobium* (Sánchez Jiménez, 2009).

A more detailed understanding of the evolutionary and ecological relationships among rhizobia can be achieved through analysis of several gene sequences. Vinuesa *et al.* (2008) and Rivas *et al.* (2009) have described a multilocus sequence analysis as an adequate method for bacterial molecular systematics stating that no single gene tree is likely to be a good approximation of a species phylogeny. The most useful genes for molecular-clock studies are genes subjected to uniform selective pressure such as pseudogenes and genes that are central to metabolism (Turner and Young, 2000; Gaunt *et al.*, 2001; Janczarek *et al.*, 2009).

6 SUMMARY

6.1 SUMMARY

Biological nitrogen fixation (BNF) is performed by free living diazotrophs or diazotrophs associated to other organisms and endosymbionts that account for an estimated amount of 150 Tg of fixed nitrogen annually, representing ~ 90% of all fixed N_2 in the terrestrial environments (Zaharan, 1999). Nitrogen fixation is a reaction that reduces the atmospheric N_2 to NH_3 that is accessible to plants and animals. BNF is therefore considered as an efficient, economical, and environmental friendly natural fertilizing system.

The rhizobia are endosymbiotic gram-negative soil bacteria that form nodules on the plant roots where the bacteria fix atmospheric nitrogen. Currently six genera are widely accepted among rhizobia, *Rhizobium, Allorhizobium, Sinorhizobium, Mesorhizobium, Azorhizobium,* and *Bradyrhizobium*. However, taxonomy of the latter two genera remains unclear due to several contradictions by using different taxonomic methods (Jarabo-Lorenzo *et al.*, 2003).

The genus *Lupinus* L. is a large and diverse group of papilinoid legumes comprised of about 275 species belonging to the large family *Leguminosae.* From the biochemical point of view, the lupins are among the richest plants in seed storage proteins (35-42%) what makes them of growing agronomic and ecological interest as proteins and nitrogen suppliers (Gladstones, 1974, *op.cit.*: Aïnouche *et al.*, 2004; Cerreti, 1983; *op.cit.*: Aïnouche *et al.*, 2004). According to Castroviejo and Pascual (1999) only 8 species of *Lupinus* grow in the Iberian Peninsula (Table 1). Recently, a novel lupin species, *L. mariae-josephi* from the Llombai region of Valencia (Spain), was described by Pascual (2004). This species grows in limy alkaline soils with high concentrations of Ca^{2+} and it was proved by molecular analyses that *L. mariae-josephi* is a unique and an exceptional species (Aïnouche, personal communication).

Lupin is a promiscuous host legume that is nodulated by rhizobia with different chromosomal genotypes which usually belong to several slow-growing species of *Bradyrhizobium*: *B. japonicum* (Jarabo-Lorenzo *et al.*, 2003), *B. canariense* (Stępkowski *et al.*, 2005) and *B. elkanii* (Barrera *et al.*, 1997). However, high specificity has been observed between *Bradyrhizobium* strains and lupin lines (Robinson *et al.*, 2000; Menna *et*

al., 2006). Bacteria of the genus *Bradyrhizobium* grow slowly (g ≈ 6-10 h) and can produce a considerable amount of extracellular polysaccharides.

Hitherto, the definition of new bacterial genera and species and the description of evolutionary relationships have been mainly inferred from 16S SSU gene sequences (Woese, 1987). In order to study phylogenies of rhizobia other housekeeping and nodulation genes have also been used. However, comparison of the phylogenetic relationships established with housekeeping (*atpD, dnaK, glnII, recA, rrs*) and nodulation (*nodC, nodZ, nodA, noeI, nolL, nifH*) genes shows various incongruities between phylogenetic analyses which are most probably a consequence of lateral gene transfer of nodulation genes (Jarabo-Lorenzo *et al.*, 2003; Stępkowski *et al*., 2003; Moulin *et al*., 2004; Stępkowski *et al*., 2007).

The objective of this graduation thesis pursued a phenotypic, phylogenetic and symbiotic characterization of endosymbiotic bacteria isolated from nodules of novel lupin species *Lupinus mariae-josephi* by molecular and cross-inoculation techniques. We predicted that the isolated strains belonged to the genus *Bradyrhizobium*, although they could differ from other species of this genus because of the particular natural growth conditions of *L. mariae-josephi* (alkaline pH, high concentrations of Ca^{2+}).

L. mariae-josephi strains grew very slowly on YMB plates, forming almost imperceptible cream colonies after 9 days at 28 °C, suggesting that they belonged to the *Bradyrhizobium* genus. However, in this medium, most of bradyrhizobia are mucoid, but *L. mariae-josephi* strains were not. In contrast, no obvious differences were observed among *L. mariae-josephi* strains and *B. japonicum* by a simple microscopic analysis.

Growth results showed that rhizobia isolated from nodules of *L. mariae-josephi* had longer generation times (g ≈ 2 days) times than *B. japonicum* USDA110 (g = 15h). Due to the rather unusual growth conditions of *L. mariae-josephi* in alkaline soils with high Ca^{2+} concentrations, a preference for higher pH values was assumed for *L. mariae-josephi* isolates. However, the isolates grew best at pH 6.8, similarly to other bradyrhizobial species, but they did not tolerate acid pH values and had a limited tolerance spectrum in contrast to *B. japonicum* USDA110.

Generation time of B2b strain was the shortest of all *L. mariae-josephi* strains and other tested strains. With increasing pH values B2b lagged behind even more. This is relevant

since the alkaline pH of the soil where *L. mariae-josephi* grows may have been a selective factor for its symbionts.

Cross-inoculation tests showed high specificity not only of the rhizobia isolated from nodules of *L. mariae-josephi* but also of the host plant itself.
When testing the specificity of the *L. mariae-josephi* strains only the original host plant formed abundant, bulky, and red active nodules. *L. albus* was the only host plant in addition to *L. mariae-josephi* that was able to effectively form active nodules with *L. mariae-josephi* strains.
Cross-inoculation tests of *L. mariae-josephi* with strains isolated from nodules of other Iberian lupins and related legume species showed that *L. mariae-josephi* in general did not form or formed small and white nodules without or with low nitrogen-fixing activity. This novel species was successfully nodulated only by two strains in addition to its own: strain 861, and in smaller degree strain ISLU101, both isolated from nodules of *Ornithopus.*

The phylogenetic tree based on the *rrs* sequences from the strains isolated from nodules of *L. mariae-josephi* and different lupins and related plants and sequences from the database GenBank, showed a formation of two clades (Figure 20). The major clade grouped all isolates of the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Phyllobacterium* (i.e. fast-growing rhizobia). The second, minor clade included species of the genera *Bradyrhizobium* and *Methylobacterium*. Bradyrhizobial species (*B. canariense*, *B. betae*, *B. japonicum*, *B. liaoningense*, *B. elkanii*, and *B. yuanmingense)* formed a monophyletic group which included the selected isolates from *L. mariae-josephi*. This group was supported also by phylogenetic analyses of other housekeeping genes (*recA*, *glnII*, and *atpD*) (Sánchez Jiménez, 2009). The more detailed phylogenetic tree (Figure 21), that included more rhizobia sequences from the genus *Bradyrhizobium*, showed that the majority of the strains isolated from nodules of lupins from the Iberian acid soils (ISLU strains) clustered with *B. canariense* and separately from *L. mariae-josephi* isolates. Three *B. elkanii* strains formed a small cluster with A2 strain but not with other *L. mariae-josephi* strains. The phylogenetic proximity of all *L. mariae-josephi* strains to *B. elkanii* was additionally confirmed by other housekeeping genes (*atpD, recA, glnII*) (Sánchez Jiménez, 2009).

6.2 POVZETEK

Dušik je pomemben sestavni del biomolekul kot so beljakovine, vitamini, nukleinske kisline in mnoge druge, ki so pomembne za rast in razvoj organizmov. Atmosferski dušik (N_2) sestavlja približno 78 % atmosfere in tako predstavlja največji rezervoar dušika na Zemlji. Kljub temu pa ga živali in rastline niso zmožne neposredno koristiti zaradi močne trojne vezi med dvema atomoma (N≡N). Edini živeči organizmi, ki so zmožni reducirati N_2 v obliko, ki je dostopna rastlinam in živalim, sodijo v domeni *Bacteria* in *Archaea*. Ta proces imenujemo biološka fiksacija dušika (BNF) ali diazotrofija (Young, 1992),
BNF je proces, ki ga izvajajo prosto živeči mikroorganizmi ali mikroorganizmi v simbiontskih asociacijah in endosimbionti, ki na leto vežejo okoli 150 ton atmosferskega dušika. Približno enake količine vezanega dušika prispeva kemični proces Haber-Bosch, ki se v glavnem uporablja za proizvodnjo gnojil (Zaharan, 1999; Gage, 2004). V nasprotju z umetnim gnojenjem, BNF velja za učinkovit, ekonomičen in okolju prijazen naravni proces gnojenja prsti.

Mikroorganizme, ki vežejo dušik, v grobem razdelimo v tri skupine: prvo predstavljajo prosto živeči mikroorganizmi kot *Klebsiella, Azotobacter* in *Rhodobacter* spp., drugo mikroorganizmi, ki vežejo dušik v zunajceličnih asociacijah (*Azospirillum* spp., *Anabaena*, *Nostoc*) (Boddey in sod., 2000), ter tretjo endosimbionti z višjimi rastlinami. V zadnjo skupino spadajo aktinomicete iz rodu *Frankia* in skupina rizobijev (α-proteobakterije), ki so endosimbionti stročnic. Rizobiji so endosimbiontske po gramu negativne talne bakterije in trenutno uvrščajo mednje šest rodov: *Rhizobium*, *Allorhizobium, Sinorhizobium, Mesorhizobium, Azorhizobium* in *Bradyrhizobium*. Kljub temu taksonomija zadnjih dveh omenjenih rodov ostaja nejasna zaradi mnogih nasprotujočih si podatkov pridobljenih z različnimi taksonomskimi metodami (Jarabo-Lorenzo in sod., 2003).

Družina *Leguminosae* je ena izmed največjih in najbolj raznolikih rastlinskih družin, ki jo sestavlja približno 730 rodov in 19.400 vrst, njeni predstavniki pa so tudi zelo uporabni v kmetijstvu. Zaradi njihove sposobnosti vzpostavitve simbioze z rizobiji lahko rastejo v

revnih in izčrpanih tleh, uporabni pa so v prehrani ljudi in živali ter kot naravno gnojilo (Menna in sod., 2006).

Rod *Lupinus* L. je velika in raznolika skupina vrst volčjega boba, ki obsega približno 275 vrst. Semena rastline volčjega boba imajo eno izmed najvišjih vsebnosti beljakovin (35-42 %), zaradi česar igrajo vedno večjo vlogo tako v agronomiji kot ekologiji (Gladstones, 1974, cit. po: Aïnouche in sod., 2004; Cerreti, 1983; cit. po: Aïnouche in sod., 2004). V taksonomiji tega rodu vlada precejšna zmeda, saj so številne skupine oblikovane le na podlagi manjših in nedoslednih morfoloških lastnosti. Sistematika rodu *Lupinus* L. danes temelji na molekularnih tehnikah kot je sekvenciranje ITS (*ang.* internal transcribed spacer) DNK sekvenc. Ta je pokazala, da je rod *Lupinus* L. monofiletska skupina in nedvomno del tribusa *Gensitae* (Bisby, 1981, cit po.: Aïnouche in Bayer, 1999; Aïnouche in sod., 2004).

Castroviejo in Pascual (1999) navajata le 8 vrst rodu *Lupinus* L., ki rastejo na Iberskem polotoku (Tabela 1). Nedavno pa je Pascual (2004) opisal novo vrsto volčjega boba iz regije Llombai, v provinci Valencija (Španija). *Lupinus mariae-josephi* raste v apnenčastih in bazičnih tleh z visokimi koncentracijami Ca^{2+} in je na podlagi molekularnih analiz edinstvena ter izjemna vrsta volčjega boba (Aïnouche, osebni vir).

Vrste volčjega boba lahko nodulirajo več vrst rizobijev z različnimi genotipi, ki so navadno del skupine počasi rastočih bakterij iz rodu *Bradyrhizobium*: *B. japonicum* (Jarabo-Lorenzo in sod., 2003), *B. canariense* (Stępkowski in sod., 2005) in *B. elkanii* (Barrera in sod., 1997). Kljub temu je bila opažena visoka specifičnost med sevi rodu *Bradyrhizobium* in linijami volčjega boba (Robinson in sod., 2000; Menna in sod., 2006). Bakterije rodu *Bradyrhizobium* rastejo zelo počasi (g ≈ 6-10h) in lahko izločajo velike količine ekstracelularnih polisaharidov. Do danes je bilo sprejetih le šest vrst v rod *Bradyrhizobium*: *B. japonicum* (Jordan, 1982), *B. liaoningense* (Xu in sod., 1995), *B. elkanii* (Kuykendall in sod., 1992), B. yuanmingense (Yao in sod., 2002), *B. canariense* (Vinuesa in sod., 2005) in *Bradyrhizobium betae* (Rivas in sod., 2004).

Določitev novih bakterijskih rodov in vrst ter opis evolucijskih odnosov v glavnem še danes slonita na analizi sekvence gena majhne ribosomske podenote 16S rRNA (Woese, 1987). Poleg tega lahko filogenijo rizobijev in ostalih bakterij preučujemo s sekvenciranjem ostalih vzdrževalnih genov ali genov za nodulacijo. Primerjava med analizami filogenetskih odnosov z vzdrževalnimi geni (*atpD, dnaK, glnII, recA, rrs*) in

geni za nodulacijo (*nodC, nodZ, nodA, noeI, nolL, nifH*) pa kljub temu kaže na neskladnosti, ki so najverjetneje posledica lateralnega prenosa genov za nodulacijo (Jarabo-Lorenzo in sod., 2003; Stępkowski in sod., 2003; Moulin in sod., 2004; Stępkowski in sod., 2007).

Cilj te diplomske naloge je bil opis simbioze in filogenetskih položajev endosimbiontskih bakterij izoliranih iz gomoljčkov novo opisane vrste volčjega boba *Lupinus mariae-josephi*. V ta namen smo uporabili križno inokulacijske teste in molekularne tehnike. Predvidevali smo, da izolirani sevi spadajo v rod *Bradyrhizobium*, ki pa bi se lahko kljub temu razlikovali od ostalih vrst tega rodu zaradi nenavadnih pogojev rasti v bazičnih tleh z visokimi koncentracijami Ca^{2+}. Da bi natančno preučili njihovo raznolikost, smo preučevali njihove fenotipske in simbiotske lastnosti ter izvedli filogenetsko analizo njihovih evolucijskih odnosov.

Sevi izolirani iz nodulov *L. mariae-josephi* so na YMB gojišču rasli zelo počasi, saj je sev H2p po 9 dneh inkubacije pri 28 °C na YMB gojišču oblikoval komaj zaznavne kolonije, zaradi česar smo predvidevali, da pripadajo rodu *Bradyrhizobium*. Kolonije so bile kremaste barve in niso bile sluzave (Slika 5). Ostali sevi *L. mariae-josephi* so imeli podobne morfološke lastnosti. Dejstvu, da celice niso izločale sluzi, bi lahko botrovala sestava gojišča, ki ni dovoljevala produkcije sluzi, drugačen regulacijski sistem ali uporaba drugačne biosintetske poti. Kljub temu se te makromorfološke razlike niso odražale na mikroskopskih lastnostih, saj so vse celice rizobijev pod mikroskopom izgledale enako (Slika 6).

Z merjenjem hitrosti rasti smo hoteli potrditi, ali bi lahko sevi izolirani iz nodulov *L. mariae-josephi* pripadali rodu *Bradyrhizobium*, v katerega so vključene bakterije z daljšimi generacijskimi časi ($g \approx 6$-10h).

Rezultati merjenja hitrosti rasti so pokazali, da so vsi sevi *L. mariae-josephi* imeli daljše generacijske čase v primerjavi z ostalimi testiranimi sevi, prav tako pa so zaostajali v rasti pri kateri koli pH vrednosti. Zaradi nenavadnih naravnih razmer rasti (bazičen pH) bi lahko pričakovali boljšo rast pri višjih pH vrednostih. Kljub temu so sevi *L. mariae-josephi* najhitreje rasli pri pH 6,8 kot tudi ostali sevi rodu *Bradyrhizobium*. Vendar pa so za razliko od sevov *B. japonicum* USDA110 in ISLU101 slabo rasli pri nizkih pH vrednostih (pH 5)

in na sploh imeli ozko območje tolerance do odstopanj od optimalne pH vrednosti. Sev B2b je imel izmed vseh sevov najdaljši generacijski čas in je rasel veliko počasneje kot seva C in H2p tudi ob povišanih vrednostih pH. Ta razlika v rasti je bila statistično signifikantna pri pH 8 in deloma pri pH 9 (v primerjavi s sevom C). Iz tega lahko predvidevamo, da bazičen pH tal, v katerih uspeva *L. mariae-josephi*, vpliva na simbionte kot selektivni faktor.

B. japonicum USDA110 in ISLU101 seva sta izkazala široko območje tolerance tako na visoke kot nizke vrednosti pH. Pri pH 9 je najbolje rasel sev *B. japonicum* USDA110, medtem ko med ostalimi sevi (B2b, C, H2p in ISLU101) ni bilo opaziti velikih razlik.

Med drugim smo merili hitrost rasti tudi z merjenjem optične gostote kultur, ki smo jih gojili v erlenmajericah pri enakih pogoji. Relativni rezultati se niso razlikovali, saj so vsi sevi *L. mariae-josephi* rasli počasneje od ostalih sevov, vendar so bile vse vrednosti generacijskih časov daljše.

Rezultati križno inokulacijskih testov so pokazali ne samo visoko specifičnost rizobijev izoliranih iz nodulov *L. mariae-josephi*, ampak tudi same gostiteljske rastline.

Po inkoulaciji različnih vrst rastlin z rizobiji *L. mariae-josephi*, je slednja tvorila številne, obilne in aktivne rdeče nodule. Poleg *L. mariae-josephi* je le vrsta *L. albus* L. uspešno oblikovala aktivne nodule. Tako imata očitno ti dve rastlinski vrsti, kljub povsem drugačnim naravnim pogojem rasti, podobno specifičnost za preučevane seve *L. mariae-josephi*. V manjši meri je sev H2p oblikoval aktivne nodule na koreninah *L. micranthus* Dougl., vendar je rastlina kljub temu ni dobro rasla. Vzrok temu bi lahko bil, da rastlina ni sposobna prevzeti nastalih reduciranih dušikovih spojin zaradi mnogi sprememb v metabolizmu bakteroidov po vzpostavitvi simbiotskega odnosa (Lodwing in sod., 2003)

Specifičnost *L. mariae-josephi* smo preverjali z inokulacijo rastlin s sevi izoliranimi iz nodulov različnih vrst volčjega boba Iberskega polotoka in drugih vrst metuljnic. Pri tem rastline niso oblikovale nodulov ali pa so bili ti majhni in beli ter so imeli nizko nitrogenazno aktivnost (ISLU sevi, sev 897). Razen z lastnimi sevi je bila *L. mariae-josephi* bolj ali manj uspešno nodulirana le še z dvema sevoma: s sevom 861, ki je bil izoliran iz gomoljčkov španske detelje (*Ornithopus sativus* Brot.), in v manjši meri s sevom ISLU101, izolatom iz nodulov *O. compressus* L. Sevi izolirani iz nodulov rastlin

rodu *Ornithopus* L. so že poznani po tem, da uspešno nodulirajo mnoge vrste volčjega boba (Eckhardt in sod., 1931). Vrste bakterijskega rodu *Bradyrhizobium*, ki izvirajo iz Evrope in nodulirajo vrste volčjega boba ter španske detelje, na podlagi genov za nodulacijo oblikujejo posebno filogenetsko gručo. Na podlagi tega so za vrste rodu *Bradyrhizobium*, ki nodulirajo stročnice tribusov *Genistae* in *Lotae*, imenovali novo biovarieteto - genistearum (Stępkowski in sod., 2005).
Sev 874, izoliran iz gomoljčkov *L. angustifolius* L., je sicer oblikoval visoko aktivne nodule, ki pa so bili majhni in beli, prav tako pa je bila rastlina majhne rasti. Vzrok temu je verjetno enak kot prej opisan pri interakciji rastline *L. micranthus* Dougl. in seva H2p.

Na filogenetskem drevesu izdelanem na podlagi zaporedij *rrs* genov sta se oblikovali dve gruči (Slika 21). Večja gruča je vsebovala vse vključene izolate iz rodov *Rhizobium, Sinorhizobium, Mesorhizobium* in *Phyllobacterium* (tj. hitro rastoče bakterije). Manjša gruča je vključevala vrste rodov *Bradyrhizobium* in *Methylobacterium*. Vrste rodu *Bradyrhizobium* (*B. canariense, B. betae, B. japonicum, B. liaoningense, B. elkanii* in *B. yuanmingense*) so oblikovale monofiletsko skupino skupaj s sevi izoliranimi iz nodulov *L. mariae-josephi*. Oblikovanje te skupine je podprla tudi filogenetska analiza ostalih vzdrževalnih genov (*recA, glnII* in *atpD*) (Sánchez Jiménez, 2009).
Na podrobnejšem filogenetskem drevesu, v katerega smo vključili več zaporedij iz rodu *Bradyrhizobium*, je večina ISLU sevov (ti so bili izolirani iz nodulov več vrst volčjega boba, ki raste v kislih tleh Iberskega polotoka) oblikovala skupino z vrsto *B. canariense* in so se tako oddaljili od sevov *L. mariae-josephi*. Trije sevi vrste *B. elkanii* so oblikovali manjšo skupino le s sevom A2. Kljub temu je dodatna analiza z vzdrževalnimi geni (*atpD, recA, glnII*) potrdila, da je *B. elkanii* filogenetsko najbližja vrsta vsem sevom *L. mariae-josephi* (Sánchez Jiménez, 2009).

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ANNEXES

Annex A: Sequences of *rrs* genes determined during this work.

>A2 (1437 bp)
GCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGGGCGTAGCAATACGTCAGCGGCAGAC
GGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAACAACCCAGGGAAACTTGGGCTAATACCGGATAAG
CCCTTACGGGGAAAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAGCTAGTTGGTGAGGTAATGGCTCA
CCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAG
GCCCTAGGGTTGTAAAGCTCTTTTGTGCGGGAAGATAATGACGGTACCGCAAGAATAAGCCCCGGCTAACTTC
GTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGGTGCGTAGGCG
GGTCTTTAAGTCAGGGGTGAAATCCTGGAGCTCAACTCCAGAACTGCCTTTGATACTGAAGATCTTGAGTTCG
GGAGAGGTGAGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCAAGAACACCAGTGGCGAAGGCG
GCTCACTGGCCCGATACTGACGCTGAGGCACGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCCAGCCGTTAGTGGGTTTACTCAC
TAGTGGCGCAGCTAACGCTTTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGCAGAACCTTACCAGCCCTTGACA
TCCCGGTCGCGGACTCCAGAGACGGAGTTCTTCAGTTCGGCTGGACCGGAGACAGGTGCTGCATGGCTGTCGT
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCCGTCCTTA
GTTGCTACCATTCAGTTGAGCACTCTAAGGAGACTGCCGGTGATAAGCCGCGAGGAAGGTGGGGATGACGTCA
AGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGCGGTGTCAATGGGATGCTAAGGGGCGA
CCCTTCGCAAATCTCAAAAAGCCGTCTCAGTTCGGATTGGGCTCTGCAACTCGAGCCCATGAAGTTGGAATCG
CTAGTAATCGTGGATCAGCACGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGG
GAGTTGGTTTTACCTGAAGACGGTGCGCTAACCGAAAGGGGGCAGCCGGCCACGGTAGGGTCAGCGACTGGGG
TGAAGTCGTAACAAGGTAACCG

>B2b (1437 bp)
GCTCAGATTGAACGTTGGCGGCAGGCTTAACACATGCAAGTCGAGCGGGCGTAGCAATACGTCAGCGGCAGAC
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>C (1437 bp)
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GCCCTAGGGTTGTAAAGCTCTTTTGTGCGGGAAGACAATGACGGTACCGCAAGAATAAGCCCCGGCTAACTTC
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>Db2 (1437 bp)
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>H2p (1437 bp)
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>Islu12 (1484 bp)
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>Islu14 (1346 bp)
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>Islu15 (1347 bp)
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GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGCAGAACCTTACCAGCCCTTGACAT
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>Islu16 (1341 bp)
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>Islu21 (1352 bp)
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>Islu22 (1351 bp)
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>Islu38 (1343 bp)
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>Islu41 (1340 bp)
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>Islu90 (1356 bp)
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>Islu101 (1350 bp)
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>Islu127 (1324 bp)
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>Islu203 (1343 bp)
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GCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTTGTGCGGGAAGATAATGACGGTACCGC
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>Bradyrhizobium elkanii ISJ94 (1340 bp)
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CGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTTGTGCGGGAAGATAATGACGGTACCGCAAGAATA
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TGCGCTAACCGAAAGGGGGCAGCCGG

>Bradyrhizobium elkanii ISJ98 (1340 bp)
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>Bradyrhizobium elkanii ISJ99 (1353 bp)
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