

# Phenotypic and phylogenetic characterization of endosymbiotic bacteria from *Lupinus mariae-josephi*

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## Summary

*Lupinus mariae-josephi* is a *Lupinus* species that thrives in a Southeastern area of Spain (Valencia) in soils of singularly high pH and active lime content. It is nodulated by extra-slow growing bacteria symbiotically and phylogenetically distant to endosymbiotic strains nodulating other *Lupinus* sp. native of the Iberian Peninsula and adapted to growth in acid soils. Cross-inoculation experiments revealed that the *L. mariae-josephi* endosymbiotic bacteria are unable to nodulate or efficiently fix nitrogen with well-known *Lupinus* spp. Their species affiliation was examined by a multilocus sequence analysis of four housekeeping genes (16S rDNA, *glnII*, *recA*, *atpD*) and the symbiotic *nodC* gene. Single and concatenated phylogenetic analyses of these genes consistently revealed that *L. mariae-josephi* endosymbiotic bacteria belong to a clade, within the *Bradyrhizobium* genus, highly differentiated from the *Bradyrhizobium* clade that includes currently named *Bradyrhizobium* species as well as the endosymbiotic bacteria from *Lupinus* species tested in this study. Within this new clade the *L. mariae-josephi* bacteria nested in several subgroup that may correspond to novel sister species. The phylogenetic analysis based on the *nodC* gene showed that *L. mariae-josephi* endosymbiotic bacteria define a novel branch of the *nodC* *Bradyrhizobium* tree and likely have a common unique ancestor for the symbiotic genes with nodule isolates from *Retama* spp.

## Introduction

Based on their legume-host ranges and on phylogenetic analysis, a significant heterogeneity among bradyrhizobia nodulating *Lupinus* spp. has been unraveled in the last few years. Most isolates have been related to the *B. canariense* and the *B. japonicum* lineages (Jarabo-Lorenzo *et al.*, 2003; Vinuesa *et al.*, 2005; Stepkowsky *et al.*, 2007). *Lupinus mariae-josephi*, a recently described species of *Lupinus* (Pascual, 2004), thrives in soils of high pH and active lime content in a southeastern area of Spain (Valencia), and it is endangered due to its reduced habitat.

## Materials and Methods

Isolates of *L. mariae-josephi* endosymbiotic bacteria were obtained using trap plants from soils of five spots of native plant population of Llombai area (Valencia). Phylogenetic trees were generated by Neighbor-joining (NJ) and Maximum-likelihood (ML) methods employing the MEGA 4.1 and PAUP software and the phylogenetic web server at [www.phylogeny.fr](http://www.phylogeny.fr).

## Results and Discussion

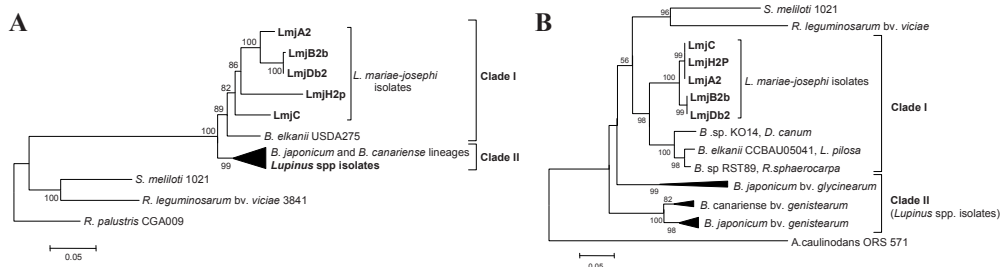
*L. mariae-josephi* is nodulated by extra-slow growing bacteria with symbiotic and phylogenetic characteristics singularly different from *Bradyrhizobium* strains nodulating other *Lupinus* sp. native of the Iberian Peninsula and adapted to grow in acid soils. Cross-inoculation experiments performed with five representative isolates (Table) showed that *L. mariae-josephi* strains do not nodulate or efficiently fix N<sub>2</sub> with other *Lupinus* spp. Their species affiliation was examined by a multilocus sequence analysis of four housekeeping genes (16S rDNA, *glnII*, *recA*, *atpD*) and the symbiotic *nodC* gene. Single and concatenated

(*glnII+recA* and *glnII+recA+atpD*) analysis consistently revealed that *L. mariae-josephi* endosymbiotic bacteria are members of the *Bradyrhizobium* genus and belong to a single evolutionary clade (Clade I) highly differentiated from the *Bradyrhizobium* Clade II that includes currently named *Bradyrhizobium* species and, singularly, all the endosymbiotic bacteria from *Lupinus* species adapted to acid soils in the Iberian Peninsula.

**Table.** Legume host-range analysis of representative isolates of *Lupinus mariae-josephi* endosymbiotic bacteria strains.

Strains	LmjA2	LmjB2b	LmjC	LmjD2b	LmjH2p
<b>Legume hosts</b>	Nodulation + NF	Nodulation + NF	Nodulation + NF	Nodulation + NF	Nodulation + NF
<i>L. mariae-josephi</i>	Yes + high	Yes + high	Yes + high	Yes + high	Yes + high
<i>L. angustifolius</i>	No	No	No	No	No
<i>L. luteus</i>	No	No	No	No	No
<i>L. micranthus</i>	Yes + poor	No	Yes + poor	No	Yes + poor
<i>L. hispanicus</i>	No	No	No	No	No
<i>L. cosentinii</i>	Yes + poor	Yes + poor	Yes + high	No	Yes + poor
<i>L. gredensis</i>	No	No	No	No	No
<i>L. albus</i>	Yes + poor	Yes + poor	Yes + high	Yes + poor	Yes + poor
<i>M. atropurpureum</i>	No	No	Yes + poor	ND	No
<i>O. compressus</i>	No	No	No	ND	No

The combined results from these analyses show that the tested *L. mariae-josephi* isolates nest in three sub-groups that might correspond to novel sister *Bradyrhizobium* species.



**Figure.** NJ trees showing the phylogenetic relationship of *L. mariae-josephi* isolates with isolates from *Lupinus* spp. and rhizobial reference strains based on concatenated *glnII+recA+atpD* genes (A) and on symbiotic *nodC* gene (B).

The 16S rDNA topology tree showed that the Clade I also includes bradyrhizobia isolates from *Retama* spp. (Boulila *et al.*, 2009) and *Phaseolus lunatus* (Ormeño-Orrillo *et al.*, 2006) as well as *B. elkanii*, *B. pachyrhizi* and *B. jicamae* species. The phylogenetic analysis based on the *nodC* gene showed that all *L. mariae-josephi* endosymbiotic bacteria studied defined a novel branch of the *Bradyrhizobium* tree. In contrast, the symbiotic genes of isolates from other *Lupinus* spp. of the Iberian Peninsula are clearly related with the *B. canariense* lineage. The allopatric speciation of *L. mariae-josephi* bradyrhizobia may have its origin in the colonization by its singular legume host of a singular habitat, such as the basic and high calcium carbonate soils of Valencia area.

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