



# Phenotypic and molecular diversity of *Lupinus mariae-josephi* endosymbiotic Bacteria

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## Abstract

*Lupinus mariae-josephi* is a new *Lupinus* species recently described in a Southeastern area of Spain (Valencia) in soils of singularly high pH and active lime content. Bacteria from *L. mariae-josephi* have been isolated and correspond to 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 subgroups 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 in the *nodC* *Bradyrhizobium* tree and likely have a common unique ancestor for the symbiotic genes with nodule isolates from *Retama* spp.

At this moment two draft genome sequences belonging to a *Bradyrhizobium* isolated from *L. angustifolius* (ISLU101) and to a *Bradyrhizobium* isolated from *L. mariae-josephi* (LmjC) have been obtained. The first analysis showed that both genomes correspond to very large chromosomes (>8000 genes) with a high number of unique proteins. *nod* genes organization are highly conserved among ISLU101, LmjC and *B. japonicum* USDA110. LmjC presents a single cluster with *nod* genes from diverse origins. ISLU101 *fix* genes are found in a single cluster homologous to that of USDA110. LmjC possess a complete copy of *fix* genes homologous to that from USDA110 and an incomplete one similar to *S. meliloti*. LmjC possess TypeIII and TypeIV secretion systems. ISLU101 has a Type IV homologous to that of photosynthetic *Bradyrhizobium* BTA1, and two copies of Type VI, one homologous to that of USDA110 and the other to *R. leguminosarum*. Preliminary data indicate that ISLU101 contains a potential second replicon (~100 genes) with high homology to *Bradyrhizobium* BTA1 plasmid sequence.

Geographical distribution of *Lupinus mariae-josephi* plant in Spain



Figure 1. Map of Valencia area where *L. mariae-josephi* are located.

## Results



Figure 2. *Lupinus mariae-josephi* in its natural environment

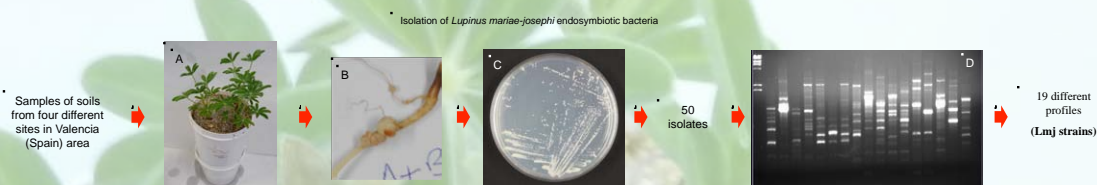


Figure 3. Isolates of *Lupinus mariae-josephi* endosymbiotic bacteria were obtained by using trap plants from soils of five spots of native plant population of Valencia province. A: Trap plants in the Leonard pots. B: example of nodule obtained from trap plants 21 days after inoculation C: appearance of LmjC isolates in YMG after 10 days. D: PCR-RAPDs profile of LmjC strains.

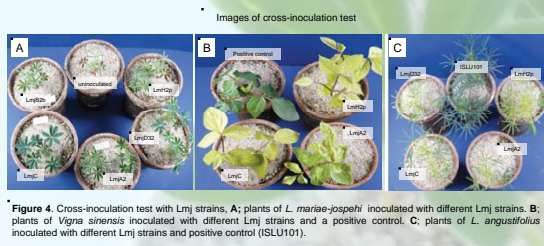


Figure 4. Cross-inoculation test with LmjC strains. A: plants of *L. mariae-josephi* inoculated with different LmjC strains. B: plants of *Vigna sinensis* inoculated with different LmjC strains and a positive control. C: plants of *L. angustifolius* inoculated with different LmjC strains and positive control (ISLU101).

Genome properties of <i>B. canariense</i> ISLU101 and <i>B. sp.</i> LmjC strains			
Genome properties	<i>Bradyrhizobium canariense</i> ISLU101	<i>Bradyrhizobium sp.</i> LmjC	<i>Bradyrhizobium japonicum</i> USDA110
Genome size	8.3 Mb	8.3 Mb	9.1 Mb
GC content	61%	59.85%	64%
Number of genes	8900	8901	8370
Percent coding	76%	75%	86%
Number of proteins	8844	8751	8317
Replications origins	2	1	1

Comparative genomics	
<i>B. japonicum</i> USDA110 (8,317 proteins)	3184
<i>B. canariense</i> ISLU101 (8,844 proteins)	889 373
<i>B. sp. lupini</i> LmjC (8,751 proteins)	3713 371 4136



Figure 5. Phylogenetic tree based on the concatenated (*glnII+recA+atpD*) genes, obtained using the Maximum Likelihood method. *L. mariae-josephi* sequences are shown in red. The sequences of endosymbiotic bacteria from other *Lupinus* species that thrives in the Iberian Peninsula are shown in green.

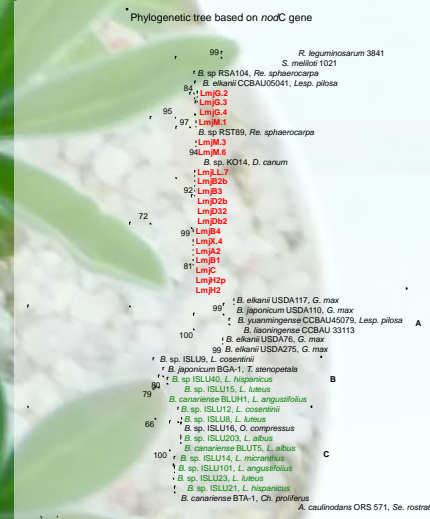


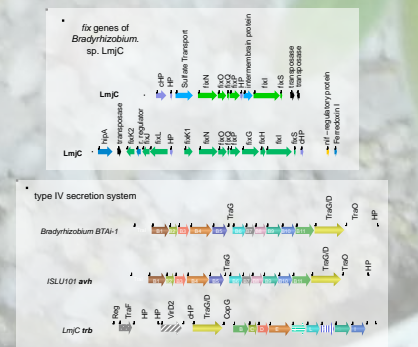
Figure 6. Phylogenetic analysis based on the *nodC* gene: A: *B. japonicum* glycinisrum lineage, B: *B. canariense* lineage, C: *B. japonicum* genistinarum lineage. LmjC sequences are indicated in red, in green isolates from other *Lupinus* spp. of the Iberian Peninsula.

## Symbiotic genes of LmjC and ISLU101 strains

strains	<i>nod</i> genes	<i>fix</i> genes	<i>nif</i> genes
<i>Bradyrhizobium sp.</i> LmjC	similar to <i>nod</i> genes of different species ( <i>Mesorhizobium</i> , <i>Methylobacterium</i> , etc)	Two clusters, one similar to <i>B. japonicum</i> USDA110 <i>fix</i> genes, the other similar to <i>S. meliloti</i> <i>fix</i> genes	Similar to that from <i>B. japonicum</i> USDA110
<i>B. canariense</i> ISLU101	Similar to <i>nod</i> genes of <i>B. japonicum</i> USDA110	Similar to <i>fix</i> genes of <i>B. japonicum</i> USDA110	Similar to <i>nif</i> genes of <i>B. japonicum</i> USDA110

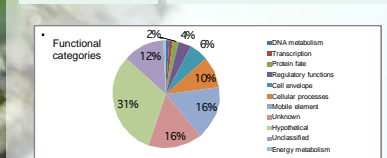
## Secretion systems

strains	Secretion systems
<i>Bradyrhizobium sp.</i> LmjC	SSIII similar to SSIII of USDA110
<i>B. canariense</i> ISLU101	SSIV unique
	SSVI 1 <sup>st</sup> copy similar to the one of <i>B. japonicum</i> USDA110 2 <sup>nd</sup> copy similar to the one of from <i>R. leguminosarum</i>



## *B. canariense* ISLU101 has a plasmid (pISLU101)

pISLU101 properties	Strain	pISLU101 similarities
Estimated size	<i>B. japonicum</i> USDA110	10.47%
Number of genes	<i>Bradyrhizobium sp.</i> BTA1	44.76%
Percent coding	Others 18 strains	47.14%



## Conclusions

- Endosymbiotic bacteria isolated from *Lupinus mariae-josephi* group together in a new clade within the *Bradyrhizobium* genus, and they are well differentiated from the currently named species and, singularly, from the nodule isolates from other lupins of the Iberian Peninsula.
- *B. canariense* ISLU101 and *B. sp. lupini* LmjC genomes have a very large chromosome with a high number of unique proteins, like the rest of *Bradyrhizobium* genomes sequenced to date.
- *nod* and *nif* genes are highly conserved among ISLU101, LmjC and USDA110. LmjC presents a *nod* cluster with genes from diverse origins.
- ISLU101 *fix* genes are in a single cluster homologous to the one of the USDA110. LmjC possesses a complete set of *fix* genes homologous to those from USDA110 and an incomplete cluster similar to *S. meliloti*.
- LmjC possesses Type III and Type IV secretion systems. ISLU101 has a T4SS homologous to one from BTA1, and two copies of T6SS, one homologous to the one of USDA110 and the other to *R. leguminosarum*.
- Preliminary data indicate that ISLU101 contains a potential second replicon (~100 genes) with high similarity to *Bradyrhizobium* BTA1 plasmid sequence.

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