



Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm



Definition of two new symbiobars, sv. *lupini* and sv. *mediterranense*, within the genera *Bradyrhizobium* and *Phyllobacterium* efficiently nodulating *Lupinus micranthus* in Tunisia

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ARTICLE INFO

Article history:

Received 12 December 2017

Received in revised form 21 March 2018

Accepted 14 April 2018

Keywords:

Phyllobacterium

Bradyrhizobium

Lupinus micranthus

Phylogeny

Symbiobars

ABSTRACT

In this study, a polyphasic approach was used to analyze three representative strains (LmiH4, LmiM2 and LmiT21) from a collection of six previously described strains isolated in Tunisia from root nodules of *Lupinus micranthus*. The phylogenetic analysis of the concatenated *rrs*, *recA* and *glnII* genes showed that strain LmiH4 had 100% concatenated gene sequence identity with the type strain *Bradyrhizobium retamae* Ro19T. Similarly, strain LmiM2 shared 100% concatenated gene sequence identity with the species *Bradyrhizobium valentinum* LmjM3T. However, strain LmiT21 showed an identical concatenated gene sequence with reference strain *Phyllobacterium sophorae* CCBAU03422T. The *recA-glnII* concatenated protein-coding genes used produced incongruent phylogenies compared with 16S rDNA phylogeny. The *nodC* gene analysis showed that the strains were phylogenetically divergent to the *Bradyrhizobium* symbiobars defined to date, and represented two new symbiobars. Plant infection analysis revealed that the three strains showed moderate host range and symbiotic specificities.

Based on their symbiotic characteristics, we propose that the three strains isolated from *Lupinus micranthus* nodules belong to two new symbiobars, with the first denominated *lupini* within the two species *Bradyrhizobium valentinum* (type strain LmiM2) and *B. retamae* (type strain LmiH4), and the second denominated *mediterranense* within the species *P. sophorae* (type strain LmiT21).

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Lupinus is the largest genus in the tribe *Genisteae* within the *Fabaceae* family and has approximately 275 species [9]. The endosymbiotic bacteria of cultivated lupines have been deeply studied [3,20,35,46,53], while endosymbionts from most wild lupines have been scarcely studied. The *Lupinus* species have been reported to be nodulated by fast- and slow-growing rhizobia, although the latter are those more frequently isolated from this legume [1,3,37,40,50]. The slow-growing rhizobia effectively nodulating *Lupinus* represent species in the genus *Bradyrhizobium*, including *B. canariense* and *B. japonicum* [20,46,53], as well as *B. valentinum* [11] and *B. elkanii* [17]. The fast-growing bacteria that effectively nodulate *Lupinus* are *Ochrobactrum lupini* [50], *Phyl-*

lobacterium trifolii [52], and *Microvirga lupini* [2]. In addition, two studies have reported that *Lupinus* species could be nodulated by *Mesorhizobium* [43,47].

Lupinus micranthus is an Old World lupine species that belongs to the secondary gene pool of cultivated white lupin (*L. albus* L.) [44]. It is a wild legume broadly distributed in the Mediterranean region and generally grows on sandy loams, although it has also been found on coarse sands and heavier, calcareous soils [26]. It is mostly observed on mildly acidic to alkaline soils [7], in scrublands, and along roadsides [10].

In a previous study, we reported the remarkable diversity of *L. micranthus* root nodule symbionts in northern Tunisia [34]. These included strains from the *Bradyrhizobiaceae* (*Bradyrhizobium* spp.), *Methylobacteriaceae* (*Microvirga* spp.), and *Phyllobacteriaceae* (*Phyllobacterium* spp.) families that contrasted with those of the rhizobial populations nodulating lupines in the Old World, includ-

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Table 1
Percentages of the different gene sequence identities between reference *Bradyrhizobium* strains and *L. micranthus* strains (LmiM2, LmiH4).

Reference strains	LmiM2 and LmiH4 gene sequence identities					
	16S rRNA		recA		glnII	
	LmiM2	LmiH4	LmiM2	LmiH4	LmiM2	LmiH4
<i>B. rifense</i> CTAW71	97	97	90	91	88	88
<i>B. cytisi</i> CTAW11	97	97	91	91	89	88
<i>B. ganzhouense</i> RITF806	97	97	91	91	88	89
<i>B. canariense</i> BTA-1	98	98	92	92	87	88
<i>B. lupini</i> USDA 3051	98	98	79	80	87	88
<i>B. betae</i> LMG 21987	98	97	91	91	89	89
<i>B. japonicum</i> USDA 10	98	98	91	91	89	91
<i>B. diazoefficiens</i> USDA 110	98	98	90	91	88	91
<i>B. arachidis</i> CCBAU 051107	97	97	92	92	88	90
<i>B. iriomotense</i> EK05	97	97	89	89	88	87
<i>B. manausense</i> BR 3351	97	97	91	90	90	90
<i>B. liaoningense</i> LMG 18230	98	98	91	92	89	89
<i>B. ottawaense</i> OO99	98	98	89	89	88	89
<i>B. yuanmingense</i> CCBAU 10071	98	98	90	91	88	90
<i>B. daqingense</i> CCBAU 15774	98	98	90	90	87	89
<i>B. denitrificans</i> LMG 8443	97	97	88	89	87	88
<i>B. oligotrophicum</i> LMG 10732	79	79	89	89	85	86
<i>B. pachyrhizi</i> PAC48	99	99	92	93	88	91
<i>B. ferriligni</i> CCBAU 51502	98	97	93	93	89	91
<i>B. elkanii</i> USDA 76	99	99	93	94	89	91
<i>B. erythrophlei</i> CCBAU 53325	99	99	93	92	88	90
<i>B. retamae</i> Ro19	99	100	93	100	91	99
<i>B. icense</i> LMTR 13	99	99	94	95	92	97
<i>B. valentinum</i> LmjM3	99	99	97	94	93	88
<i>B. lablabi</i> CCBAU 23086	99	99	94	95	91	94
<i>B. jicamae</i> PAC68	99	99	93	93	92	94
<i>B. paxllaeri</i> LMTR21	99	99	92	94	91	94

ing *L. micranthus* from other Mediterranean areas nodulated mostly by *Bradyrhizobium* strains [5,11,17,20,46,53].

Therefore, the present study aimed to investigate in detail the symbiotic characteristics of three novel strains (LmiH4, LmiM2, and LmiT21) from a collection of six particular strains, four of which belonged to *Bradyrhizobium* sp. and the others to *Phyllobacterium* spp. Based on their phenotypic, genotypic (*rrs*, *recA*, *glnII*) and symbiotic characteristics (*nodC* phylogeny and nodulation phenotype on different hosts), we propose that the three representative strains belong to two new symbiovars, the first named *lupini* (including the strains *Bradyrhizobium retamae* LmiH4 and *Bradyrhizobium valentinum* LmiM2), and the second named *mediterraneense* (representing strain *Phyllobacterium sophorae* LmiT21).

The *rrs*, *recA*, *glnII* and *nodC* gene sequences of the LmiH4, LmiM2, and LmiT21 strains were obtained in a previous study [34]. Fragments for the 16S rDNA, *recA*, *glnII* and *nodC* genes obtained from PCR products were checked for sequencing quality after getting the sequencing results. Sequences were assembled with Geneious Pro 5.6.5 and, together with relevant sequences obtained from the GenBank database, were aligned using ClustalW [6]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA6 [48]. The maximum likelihood (ML) method and the Kimura two-parameter model were used for different genes and multilocus sequence analysis (MLSA) (combined sequences of *rrs*, *glnII* and *recA*). Phylogenetic trees were bootstrapped with 1000 bootstrap replications. Selected gene sequences obtained in this study were deposited in the GenBank sequence database, and their accession numbers are shown on the trees.

Since rhizobia rRNA genes may occasionally undergo possible horizontal gene transfer or recombination, variable mutation rates and simple stochastic variation [12,16,54], and may not always accurately reflect prokaryotic phylogeny [25,31,56,58], the concatenation of housekeeping gene sequences for *glnII*, *recA*, and *rrs* was performed. The alignment of the *rrs*, *recA* and *glnII* concatenated sequences generated 2032 nucleotides, and the sequences were used to construct a tree (Fig. 1A and B). The phylogenetic

analysis confirmed the placement of the two strains LmiH4 and LmiM2 into the *Bradyrhizobium* group and that of strain LmiT21 into the *Phyllobacterium* group.

The concatenated gene sequence identity values were higher than 99% for strain LmiH4 and type strain *B. retamae* Ro19^T isolated from root nodules of *Retama monosperma* and *Retama sphaerocarpa* in Spain and Morocco [18]. Similarly, strain LmiM2 shared 100% concatenated gene sequence identity with the species *B. valentinum* LmjM3^T isolated from *Lupinus mariae-josephae* nodules in Spain [11] (Fig. 1A). Several previous studies have shown that lupines in the Old World, including Mediterranean areas, were mainly nodulated by *Bradyrhizobium* strains [5,11,17,20,46,53].

For the *Phyllobacterium* group, strain LmiT21 had an identical concatenated sequence to that of *P. sophorae* CCBAU03422^T, isolated recently from root nodules of *Sophora flavescens* in China [21], with a high bootstrap support (100%) (Fig. 1B). At the time of writing, the genus *Phyllobacterium*, described by Knösel [24], contains ten validated species: *Phyllobacterium myrsinacearum* isolated from leaf nodules of *Myrsinaceae* [32], *Phyllobacterium catacumbae* isolated from a volcanic rock [22], *Phyllobacterium trifolii* isolated from a *Trifolium repense* nodule [52], four species isolated from the rhizoplane of different plants, *P. brassicacearum*, *P. bourgognense*, *P. leguminum* and *P. ifriqiyense* [29], *P. endophyticum* isolated from nodules of *Phaseolus vulgaris* [14], *Phyllobacterium sophorae* isolated recently from root nodules of *Sophora flavescens* [21], and *P. loti*, a recently described species, isolated from nodules of *Lotus corniculatus* [41].

To create a consistent and robust position for the two strains LmiT21 and LmiM3, and for a better understanding of the length of the branches between LmiM2 and the type strain *B. valentinum* LmjM3^T, as well as between strain LmiT21 and the type strain *P. sophorae* CCBAU03422^T, the percentages of the different gene sequence identities were determined (Tables 1 and 2). No *glnII* sequences were available in the databases for the *Phyllobacterium* type strains, except for *P. sophorae* CCBAU03422^T.

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