

Applied Soil Ecology

Deciphering the phylogenetic affiliation of rhizobial strains recommended as chickpea inoculants in Argentina --Manuscript Draft--

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Corresponding Author:	Pablo Bogino Universidad Nacional de Río Cuarto Río Cuarto, Argentina
First Author:	Emiliano Foresto
Order of Authors:	Emiliano Foresto Fiorela Nieves Santiago Revale Walter Giordano Pablo Bogino
Abstract:	Chickpea (<i>Cicer arietinum</i> L.) is globally cultivated due to its high nutritional value. As with other legumes, its success depends greatly on its inoculation with effective symbiotic rhizobial strains. Since its agricultural importance as an alternative winter crop in Argentina is very recent, there are limited phylogenetic studies on the affiliation and origin of the two strains used for its inoculation here. We attempted to define their specific identity through a multilocus sequence approach on seven housekeeping genes (phylogeny and average nucleotide identity), as well as on the basis of 16S rRNA gene sequence and <i>nodC</i> gene analysis. The strains were accurately and conclusively corroborated as <i>M. ciceri</i> and <i>M. mediterraneum</i> species, and their denominations were found to be associated to originally described chickpea-nodulating strains.
Suggested Reviewers:	Giulia Devescovi devescov@icgeb.org María Julia Estrella estrella@intech.gov.ar Encarna Velázquez evp@usal.es
Response to Reviewers:	

Mrs. Shiping Deng, Prof.

Journal Editor

E-Mail: shiping.deng@okstate.edu

Re: Revised Manuscript ID: APSOIL-D-20-00424 R2; Foresto, et al., "Deciphering the phylogenetic affiliation of rhizobial strains recommended as chickpea inoculants in Argentina".

Dear Prof. Shiping Deng,

Thank you very much for your e-mail of April 30, 2021 regarding the revision of this MS, with the attachment of comments by you and reviewer 1.

We are hereby submitting a revised MS, which has been modified in response to the editor and reviewer's comments. Our detailed, point-by-point responses to each comment are given below and are highlighted in yellow.

We are grateful for the feedback and the opportunity to revise our manuscript. We hope that you and the reviewer will find this revised version acceptable for publication in *Applied Soil Ecology*.

We look forward to hearing from you.

With best regards,

Dr. Pablo Bogino

APSOIL-D-20-00424. Response to Review.

Editor and Reviewer comments:

Editor:

Additional minor revision is needed before further consideration.

ANSWER: We have addressed the stated points.

Reviewer #1:

Manuscript Number: APSOIL-D-20-00424R1

Title: Deciphering the phylogenetic affiliation of rhizobial strains recommended as chickpea inoculants in Argentina

The authors have corrected the manuscript according to my previous comments and then I think that this work merits to be published in Applied Soil Ecology journal. Nevertheless some minor corrections should be performed before publication.

1. Lines 387-388: The parentheses in the last sentence of this figure legend are not necessary.

ANSWER: DONE.

2. Figure 2. Please delete the letter "T" after the strain name WSM1271 because this is not the type strain of *M. ciceri* and it is not correct to design a type strain for a symbiovar (this is only accepted for species and subspecies).

ANSWER: DONE. The same correction has been performed in Figure 1 and Figures of Supplementary.

3. Figure 3: As far as I know the astragalus/caraganae group has not been defined as a symbiovar. Therefore, unless authors can provide a reference defining this symbiovar, the abbreviation "sv" before "astragalus/caraganae" should be removed.

ANSWER: DONE. The abbreviation "sv" has been removed.

In this figure it lacks the type strain of *M. ciceri* UPM-Ca7 T (DQ407292.1) and taking into account that this strain also belongs to the symbiovar *ciceri*, it should be added to the nodC gene phylogenetic tree.

ANSWER: DONE. *M. ciceri* UPM-Ca7^T has been added.

Highlights.

The taxonomic status of chickpea inoculation strains in Argentina is unclear

Two inoculant-recommended strains were identified by MLSA and *16S rRNA* gene phylogeny

nodC gene analysis made it possible to define their host range

Each strain was respectively identified as *M. ciceri* and *M. mediterraneum* species

These findings are highly relevant for adequate chickpea inoculation strategies.

1 *Applied Soil Ecology*

2 **Short communication**

3

4 **Deciphering the phylogenetic affiliation of rhizobial strains**

5 **recommended as chickpea inoculants in Argentina**

6

7 **Emiliano Foresto^a, Fiorela Nieves^a, Santiago Revale^b, Walter Giordano^a, Pablo**

8 **Bogino^{a*}**

9

10 ^aInstituto de Biotecnología Ambiental y Salud (INBIAS), CONICET. Departamento de

11 Biología Molecular, Facultad de Ciencias Exactas, Físico-Químicas y Naturales.

12 Universidad Nacional de Río Cuarto (UNRC), Río Cuarto, Córdoba, Argentina.

13 ^bWellcome Centre for Human Genetics, University of Oxford, Oxford, OX3 7BN, UK.

14

15 * Author for correspondence. E-mail: pbogino@exa.unrc.edu.ar; Tel.: +54-358-467-

16 6424; Fax: +54-358-467-6232.

17

18 **Abstract:** Chickpea (*Cicer arietinum* L.) is globally cultivated due to its high nutritional value.
19 As with other legumes, its success depends greatly on its inoculation with effective symbiotic
20 rhizobial strains. Since its agricultural importance as an alternative winter crop in Argentina is
21 very recent, there are limited phylogenetic studies on the affiliation and origin of the two strains
22 used for its inoculation here. We attempted to define their specific identity through a multilocus
23 sequence approach on seven housekeeping genes (phylogeny and average nucleotide identity),
24 as well as on the basis of *16S rRNA* gene sequence and *nodC* gene analysis. The strains were
25 accurately and conclusively corroborated as *M. ciceri* and *M. mediterraneum* species, and their
26 denominations were found to be associated to originally described chickpea-nodulating strains.

27

28 **Introduction**

29 Symbiotic biological nitrogen fixation (SBNF) between rhizobia and legumes (Fabaceae) is a
30 mutualistic association that enables the specific interaction between these prokaryotic and
31 eukaryotic organisms (Wang et al., 2018). This symbiosis has been studied from different
32 perspectives, including basic research into the molecular, biochemical, physiological and
33 genetic diversity of the partners, and applied research in the fields of ecology, technology and
34 agriculture. For instance, bacterial inoculants have been developed and applied as part of
35 sustainable agriculture programs (Santos et al., 2019). Legume inoculation with selected
36 rhizobal strains increases the plant's chances of obtaining assimilable nitrogen compounds. In
37 turn, this might lead to higher crop yields, while reducing the use of chemical fertilization.
38 (Oldroyd and Dixon, 2014).

39 *Mesorhizobium* is a genus comprising species that establish nitrogen-fixing symbiosis with a
40 wide variety of legume hosts (Laranjo et al., 2014), such as chickpea (*Cicer arietinum* L.). In
41 spite of the worldwide relevance of this crop as a valuable source of protein for food and forage
42 (Jukanti et al., 2012), the mesorhizobium-chickpea symbiosis remains poorly studied. In the mid
43 90s, two species were described to specifically nodulate chickpea, namely *Mesorhizobium ciceri*
44 UPM-Ca7^T (Nour et al., 1994) and *Mesorhizobium mediterraneum* UPM-Ca36^T (Nour et al.,
45 1995). Since the 2000s, rhizobia able to symbiotically associate with chickpea have been shown

46 to belong to several other species within the genus *Mesorhizobium*, including *M. amorphae*, *M.*
47 *tianshanense* (Rivas et al., 2007), *M. loti* (Laranjo et al., 2004), *M. muleiense* (Zhang et al.,
48 2017), *M. abyssinicae*, *M. shonense* (Tena et al., 2017), and *M. wenxiniae* (Zhang et al., 2018).
49 In Argentina, chickpea is increasingly positioning itself as an alternative winter crop, and
50 therefore requires special attention in all aspects. Data from the National Institute of
51 Agricultural Technology (Instituto Nacional de Tecnología Agropecuaria, INTA) states that the
52 strains used to inoculate it here were received nearly twenty years ago from the Agricultural
53 Research Service of the United States Department of Agriculture (Beltsville, Maryland, USA),
54 under the denominations *Rhizobium ciceri* USDA 3383 and *Rhizobium mediterraneum* USDA
55 3392 (INTA staff, personal communication). These strains have been renamed since as *M. ciceri*
56 R30 and *M. mediterraneum* R31, respectively, but there is no precise phylogenetic identification
57 to back this up. Previous research is limited to an initial description of the strains (Nour et al.,
58 1995, 1994) or to insufficient, indirect taxonomic classification (Zhou et al., 2013), and modern
59 methodological approaches have not been used so far to taxonomically locate them.
60 Thus, our aim was to clarify the phylogenetic identity of these two strains by using nearly the
61 full length of the *16S rRNA* gene sequence as well as the full length of seven housekeeping
62 genes analyzed through multilocus sequence analysis (MLSA). A phylogeny analysis of a partial
63 sequence of the *nodC* gene was also carried out to define host range and to further characterize
64 the symbiotic features of the strains.

65

66 **2. Materials and methods**

67

68 *2.1. Bacterial strains.* We used two rhizobial strains named R30 and R31, recommended by the
69 National Institute of Agricultural Technology (INTA) for inoculating chickpea in Argentina.

70

71 *2.2. Sequence data analyses.* The sequences of the genes featured in the present study were
72 obtained from the sequenced genomes of each strain (data not published). The following genes
73 were chosen: *16S rRNA*, *nodC*, and seven housekeeping genes (HKG) for MLSA, namely *dnaK*

74 (molecular chaperone), *glnII* (glutamine synthetase II), *gyrB* (DNA gyrase beta subunit), *recA*
75 (recombinase A), *rpoB* (RNA polymerase subunit beta), *thrC* (threonine synthase) and *truA*
76 (tRNA pseudouridine synthase). All of them were deposited in the GenBank database from the
77 National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/genbank/>)
78 and accession numbers were obtained (Table 1). The BLAST search program (NCBI) (Altschul
79 et al., 1997) was used to ascertain the identity (similarity) between the sequences of each gene,
80 as well as the closest sequence identity between genes (Table 1). Alignment of individual genes
81 (*16S rRNA*, *nodC* and HKG) and concatenated full-length HKG from the two strains with
82 sequences retrieved from the GenBank database (Supplementary Table S1) was performed with
83 the ClustalW algorithm in MEGAX software (version 10.1.7) (Kumar et al., 2018). A complete
84 deletion procedure was used to calculate distances, and phylogenetic trees were built through
85 the maximum-likelihood method. The evolutionary relationship of taxa was inferred with the
86 neighbor-joining method (Saitou and Nei, 1987). Tree topology robustness was analyzed by
87 bootstrap analysis (1000 replicates) (Felsenstein, 1985). The evolutionary distances were
88 computed through the Tamura 3-parameter method or the Kimura 2-parameter (Tamura, 1992).
89 For this study, we selected the rhizobial sequences of seven complete (full-length) HKG
90 sequences retrieved only from rhizobial strains with sequenced genomes, with the exception of
91 *M. ciceri* UPM-Ca7^T (USDA 3383^T), for which only partial sequences of those genes are
92 available. We also implemented an average nucleotide identity (ANI) calculation based on the
93 concatenated HKG (ANI_g) (De Lajudie et al., 2019). ANI calculations were made on
94 KostasLab's online ANI calculator (<http://enve-omics.ce.gatech.edu/ani/>), using both best hits
95 (one-way ANI) and reciprocal best hits (two-way ANI) between two genomic datasets (Goris et
96 al., 2007).

97

98 **3. Results and Discussion**

99 Many modern sustainable agricultural schemes depend on successful SBNF in legumes and,
100 therefore, on the application of appropriate, competitive and efficient inoculants. Choosing the
101 right inoculant demands accurate knowledge about the identity of the bacterial strains used. In

102 the case of *M. ciceri* R30 and *M. mediterraneum* R31, the two strains used to inoculate chickpea
103 in Argentina, information is scarce and even the denominations themselves come from outdated,
104 ambiguous data. The species type strains originally described as chickpea-nodulating are *M.*
105 *ciceri* UPM-Ca7^T (USDA 3383^T) and *M. mediterraneum* UPM-Ca36^T (USDA 3392^T) (Nour et
106 al., 1994; Nour et al., 1995).

107 The search for the most identical sequences for each gene showed that R30 had a clearly high
108 identity with *M. ciceri* UPM-Ca7^T, *M. ciceri* CC1192 and with strains belonging to *M. ciceri* bv.
109 *biserrulae* (Table 1). *M. ciceri* CC1192 is recommended for chickpea inoculation in Australia
110 (Haskett et al., 2016) and the *M. ciceri* bv. *biserrulae* strains were defined as belonging to a new
111 biovar inside the *M. ciceri* group (Nandasena et al., 2007). In the case of R31, most of its genes
112 had high identity with *Mesorhizobium* sp. strains grouped as *M. mediterraneum* strains isolated
113 from *Cicer reticulatum* L. (a wild ancestor of chickpea) (Greenlon et al., 2019). The
114 unambiguous identification of R31 as *M. mediterraneum* arises from its 100 % identity with
115 most of the gene sequences of *M. mediterraneum* UPM-Ca36^T (Table 1).

116 Usually, the phylogenetic relationships between bacteria are initially assessed by *16S rRNA*
117 sequence analysis (Chun and Rainey, 2014), since it can differentiate strains from closely related
118 species. We conducted this analysis by comparing the chickpea-inoculant strains with several
119 *Mesorhizobium* species. R30 and R31 were grouped on the main branch of the 16S
120 mesorhizobial phylogenetic tree (Fig. 1). This main mesorhizobial group was further divided
121 into two branches, with R30 on one of them and R31 on the other. R30 was very close
122 phylogenetically to *M. ciceri* strains, *M. loti* NZP2213, *M. australicum*, *M. qingshengii*, *M.*
123 *caldicola*, and *M. sophorae*. On the other hand, R31 was found to be closest to a group that
124 includes *M. mediterraneum*, *M. ciceri* Ca181, *M. temperatum*, *M. prunedense*, *M. delmotii*
125 and *M. muleiense*. High identity of the *16S rRNA* gene sequences between *Mesorhizobium*
126 species seems to be common, given that a large number of them were grouped together through
127 this analysis with less than 98% difference between them. Still, although a difference over
128 98.7% in *16S rRNA* sequences is considered good enough to stell species apart (Yarza et al.,
129 2014), the mesorhizobial species were differentiated below (or near) this boundary and grouping

130 patterns could be distinctly defined. Indeed, R30 and R31 had 98,66% *16S rRNA* gene sequence
131 identity but were clearly separated into different groups of mesorhizobial species.

132 Despite the usefulness of the 16S marker for preliminary taxonomical identification of bacteria,
133 a MLSA of several chromosomal HKG is vastly more accurate to precisely define the
134 taxonomic position of rhizobial strains (Sannazzaro et al., 2018; De Lajudie et al., 2019; Estrella
135 et al., 2020). We selected the full length of seven protein-coding genes (*recA*, *glnII*, *dnaK*, *gyrB*,
136 *rpoB*, *thrA* and *truA*) (Pérez-Yépez et al., 2014) to increase the robustness of the phylogenetic
137 analysis, and compared them with those genes recovered from *Mesorhizobium* species whose
138 genome was sequenced and available in GenBank (NCBI). Phylogenetic trees were built for
139 both individual HKG (Supplementary Fig. S1-S7) and concatenated HKG (Fig. 2), to
140 taxonomically cluster the two strains within the *Mesorhizobium* genus. R30 was consistently
141 clustered with strains belonging to *M. ciceri* and *M. ciceri* bv. *biserrulae* species, and was also
142 close to *M. sophorae* and *M. qingshengii* in most trees. R31 showed a clear association to *M.*
143 *mediterraneum* and a consistent clustering with *M. temperatum*, *M. muleiense*, *M. prunedense*
144 and *M. thianshanense*.

145 We also calculated the average nucleotide identity of the concatenated HKG (ANIg) between
146 R30 and R31, and between each of these strains and their closest organisms in the phylogenetic
147 HKG tree as a reliable estimation of whole-genome similarity (Pérez-Yépez et al., 2014). The
148 ANIg value between R30 and R31 was only 90,68%, which is further evidence of them being
149 phylogenetically different species (Table 2). The taxonomic affiliation of R30 to the *M. ciceri*
150 species was confirmed by the 98,5-99% ANIg value obtained when comparing it with *M. ciceri*
151 UPM-Ca7^T, *M. ciceri* CC1192 and *M. ciceri* bv. *biserrulae* strains. R31 was taxonomically
152 defined as an *M. mediterraneum* species, since its ANIg value was 100% with *M.*
153 *mediterraneum* USDA 3392. It also showed a shared ANIg of 95,0-95,5% with *M. muleiense*,
154 *M. thianshanense*, *M. temperatum*, and *M. prunedense*. These values were close to the 96%
155 boundary that discriminates between species, and are in agreement with the small difference
156 described by other authors for these species (Degefu et al., 2013; Wei et al., 2009).

157 Interestingly, no connection was observed between *16S rRNA* gene identity and ANI values for
158 R30 and R31. The *16S rRNA* gene sequences of the two strains were 98,66% identical to each
159 other, a very close percentage to the 98,7% limit used to consider strains as belonging to the
160 same species (Yarza et al., 2014). However, the 90,68% ANI_g value between them was far from
161 the >96% required to delimit species (Zhang et al., 2012), and thus the strains were confirmed
162 as different species. Thus, MLSA (HKG sequence comparison, phylogenetic trees and ANI_g) is
163 crucial to accurately establish the phylogenetic position of bacterial strains belonging to the
164 same genus, and to compensate for the lack of certainty offered by *16S rRNA* gene sequence
165 analysis in this respect.

166 As a whole, our taxonomic study confirms that R30 is a *M. ciceri* closely related to the
167 originally described UPM-Ca7^T (USDA 3383^T), and that R31 is *M. mediterraneum* UPM-Ca36^T
168 (USDA 3392^T).

169 Finally, we further explored the main biological role of SBNF to determine their symbiotic
170 positioning at the symbiovar level (Rogel et al., 2011), by analyzing the *nodC* gene encoding N-
171 acetylglucosaminyltransferase involved in the synthesis of Nod factors. The sequences between
172 the two inoculant strains were 99,4% identical (Table 1) and clustered together inside the
173 symbiovar *ciceri* (Fig. 3), which reflects their specificity towards the *Cicer arietinum* legume.
174 This demonstrates that taxonomic differences between mesorhizobial strains (such as those
175 indicated by 16S and HKG phylogeny) do not translate to differences in legume host specificity.
176 As the present study shows, strains from five *Mesorhizobium* species (*M. ciceri*, *M.*
177 *mediterraneum*, *M. huakuii*, *M. muleiense* and *M. qingshengii*) are functionally capable of
178 establishing symbiotic relationships with chickpea, a phenomenon possibly explained by lateral
179 gene transfer processes between them as an adaptive mechanism to improve environmental
180 fitness under various conditions (type of soil, contaminants, presence of new plant species or
181 cultivars, etc.) (Zhang et al., 2017).

182 Moreover, we observed that R30 and R31 have a phylogenetic relationship with a chickpea-
183 nodulating strain named Ca181 and classified as an *M. ciceri* (Yadav et al., 2013), whose
184 phylogenetic position has not been confirmed. From reports that compare rhizobia isolated from

185 chickpea nodules with different *Mesorhizobium* strains, Ca181 could be inferred to be in a
186 separate cluster from other *M. ciceri* strains (Singh et al., 2019; Yadav et al., 2013), in
187 agreement with our study. In sum, this strain is likely a different species from *M. ciceri*, but
188 more research is necessary.

189 Relevant observations were made when comparing genes from R30 and R31. While *16S rRNA*
190 and *nodC* were highly similar, with identity percentages of approximately 99%, the identity
191 percentages for the HKG were lower, ranging from 94,4% for *rpoB* to 86,5% for *truA* (Table 1).
192 Similarly, the ANI percentage of the concatenated HKG was 90,7% between the two strains
193 (Table 2). The distinct genetic and symbiotic differentiation between the strains, (Fig. 4)
194 confirms that they belong to different species, *M. ciceri* and *M. mediterraneum*, respectively.

195 These findings are highly relevant at the time of choosing strains to inoculate chickpea
196 depending on the region of cultivation. Biogeography and environmental factors appear to have
197 a strong impact both on the diversity of mesorhizobial populations and the adaptation of
198 introduced strains (Elias and Herridge, 2015; Greenlon et al., 2019; Santos et al., 2019). Studies
199 of this sort ought to be conducted in soils where chickpea is grown in Argentina, to design
200 appropriate inoculation strategies with the two strains already in use or with an as-of-yet not
201 isolated indigenous strain that might produce better results. In the meantime, strategies of co-
202 inoculation with R30 and R31 are recommended.

203

204 **4. Conclusions**

205 The present study consisted of a genetic analysis performed at the phylogenetic and functional
206 levels, which validated the taxonomic affiliation of the strains recommended as chickpea
207 inoculants in Argentina. R30 and R31 were confirmed to belong to *M. ciceri* and *M.*
208 *mediterraneum* species, respectively. With such precise data now available, better inoculation
209 strategies may be designed for the cultivation of this legume in different agro-climatic locations.

210

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218

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Table 1. Sequence identities of R30 and R31 strains.

Gene	Strain: GBAN Id % (coverage %)	Most closely related sequences (accession number) (Id %; coverage %)	
		R30	R31
<i>16S</i>	R30: MT229308 R31: MT229309 98,66 (100)	<i>M. ciceri</i> UPM-Ca7 ^T * (DQ444456) (100; 90) <i>M. ciceri</i> bv. <i>biserrulae</i> WSM 1284 (CP015064.1) (100; 100) <i>M. ciceri</i> CC1192 (CP015062.1) (100; 100)	<i>M. mediterraneum</i> UPM-Ca-36 ^T * (L38825) (99,93; 95) <i>M. sp.</i> M6A.T.Cr.TU.016.01.1.1 (CP034452.1) (100; 100) <i>M. sp.</i> AA22 (CP048406.1) (99,87; 100)
<i>recA</i>	R30: MT237340 R31: MT237341 93,58 (96)	<i>M. ciceri</i> UPM-Ca7 ^T * (AB253227.1) (100; 37) <i>M. ciceri</i> bv. <i>biserrulae</i> WSM 1284 (CP015064.1) (99,91; 100) <i>M. ciceri</i> CC1192 (CP015062.1) (99,91; 100)	<i>M. mediterraneum</i> USDA 3392 ^T (NPKI01000051.1) (100; 100) <i>M. sp.</i> M6A.T.Cr.TU.016.01.1.1 (CP034452.1) (99,91; 100)
<i>dnaK</i>	R30: MT237316 R31: MT237317 92,96 (99)	<i>M. ciceri</i> UPM-Ca7 ^T * (AB253035.1) (100; 34) <i>M. ciceri</i> bv. <i>biserrulae</i> WSM 1284 (CP015064.1) (99,62; 100) <i>M. ciceri</i> bv. <i>biserrulae</i> WSM1271 (CP002447.1) (99,62; 100)	<i>M. mediterraneum</i> USDA 3392 ^T (NPKI01000015.1) (100; 88) <i>M. sp.</i> M6A.T.Cr.TU.016.01.1.1 (CP034452.1) (98,20; 100)
<i>glnII</i>	R30: MT237326 R31: MT237327 90,95 (96)	<i>M. ciceri</i> USDA 3383 ^T (AF169580.1) (99,52; 79) <i>M. ciceri</i> CC1192 (CP015062.1) (99,91) <i>M. ciceri</i> bv. <i>biserrulae</i> WSM 1284 (CP015064.1) (99,62)	<i>M. mediterraneum</i> USDA 3392 ^T (NPKI01000041.1) (100; 100) <i>M. sp.</i> M6A.T.Cr.TU.016.01.1.1 (CP034452.1) (97,50; 100)
<i>rpoB</i>	R30: MT237344 R31: MT237345 94,44 (99)	<i>M. ciceri</i> UPM-Ca7 ^T * (EF457945.1) (98,36; 16) <i>M. ciceri</i> bv. <i>biserrulae</i> WSM 1284 (CP015064.1) (99,52; 100) <i>M. ciceri</i> CC1192 (CP015062.1) (99,37; 100)	<i>M. mediterraneum</i> USDA 3392 ^T (NPKI01000045.1) (100; 100) <i>M. sp.</i> M6A.T.Cr.TU.016.01.1.1 (CP034452.1) (99,35; 100)
<i>gyrB</i>	R30: MT237322 R31: MT237323 89,98 (98)	<i>M. ciceri</i> UPM-Ca7 ^T * (AM076342.1) (100; 19) <i>M. ciceri</i> bv. <i>biserrulae</i> WSM 1284 (CP015064.1) (99,72; 100) <i>M. ciceri</i> CC1192 (CP015062.1) (99,64; 100)	<i>M. mediterraneum</i> USDA 3392 ^T (NPKI01000015.19) (100; 100) <i>M. sp.</i> M6A.T.Cr.TU.016.01.1.1 (CP034452.1) (97,82; 100)
<i>truA</i>	R30: MT237355 R31: MT237356 86,50 (96)	<i>M. ciceri</i> USDA 3383 ^T (JX064292.1) (96,35; 42) <i>M. ciceri</i> bv. <i>biserrulae</i> WSM1271 (CP002447.1) (99,61; 100) <i>M. ciceri</i> bv. <i>biserrulae</i> WSM 1284 (CP015064.1) (99,48; 100)	<i>M. mediterraneum</i> USDA 3392 ^T (NPKI01000015.1) (100; 100) <i>M. sp.</i> M6A.T.Cr.TU.016.01.1.1 (CP034452.1) (98,54; 100)
<i>thrA</i>	R30: MT237350 R31: MT237351 86,93 (100)	<i>M. ciceri</i> USDA 3383 ^T (JX064364.1) (95,44; 53) <i>M. ciceri</i> bv. <i>biserrulae</i> WSM 1284 (CP015064.1) (99,16; 100) <i>M. ciceri</i> bv. <i>biserrulae</i> WSM1271 (CP002447.1) (99,16; 100)	<i>M. mediterraneum</i> USDA 3392 ^T (NPKI01000017.1) (100; 100) <i>M. sp.</i> M6A.T.Cr.TU.016.01.1.1 (CP034452.1) (98,02; 100)
<i>nodC</i>	R30: MT237330 R31: MT237331 99,40 (100)	<i>M. ciceri</i> UPM-Ca7 ^T * (DQ407292.1) (99,74; 57) <i>M. sp.</i> M2A.F.Ca.ET.046.03.2.1 (CP034449.1) (100; 100)*** <i>M. ciceri</i> CC1192 (CP015062.1) (99,40; 100)	<i>M. mediterraneum</i> USDA 3392 ^T (GQ167241.1) (100; 83) <i>M. sp.</i> M6A.T.Cr.TU.016.01.1.1 (CP034452.1) (99,62; 100) [#] <i>M. ciceri</i> CC1192 (CP015062.1) (99,10; 100)

346 * *M. ciceri* type strain UPM-Ca7^T is the same as USDA 3383^T, and *M. mediterraneum* type strain UPM-Ca36^T is the same as USDA 3392^T. The different
347 denominations in this table coincide with the strain denominations for the gene accession numbers available in GenBank.

348 *** Six strains similar to this (Greenlon et al., 2019) have 100% Id with R30 strain. Seven strains similar to this (Greenlon et al., 2019) have between 99,92
349 and 99,10% Id with R30 strain.

350 # 13 strains similar to this (Greenlon et al., 2019) have between 99,62 and 99,10% Id with R31 strain.

351

352

353 **Table 2.** Average nucleotide identity (ANI) percentage for the concatenated dataset of HKG

354 between *M. ciceri* R30 or *M. mediterraneum* R31 and closely related species.

Strain	<i>M. ciceri</i> R30	Strain	<i>M. mediterraneum</i> R31
<i>M. ciceri</i> bv. <i>biserrulae</i> WSM 1284	99,57	<i>M. mediterraneum</i> USDA 3392 ^T	100
<i>M. ciceri</i> CC1192	99,44	<i>M. ciceri</i> Ca181*	96,33
<i>M. ciceri</i> bv. <i>biserrulae</i> WSM 1271	99,39	<i>M. prunaredense</i> isolate 1	95,53
<i>M. ciceri</i> UPM-Ca7 ^T **	98,58	<i>M. temperatum</i> SDW018	95,44
<i>M. ciceri</i> Ca181*	92,37	<i>M. tianshanense</i> CGMCC 1.2546	95,03
<i>M. mediterraneum</i> R31	90,68	<i>M. muleiense</i> CGMCC 1.11022	95,01

355 Comparisons were carried out with seven concatenated genes (*recA*, *dnaK*, *rpoB*, *gyrB*, *glnII*,

356 *truA* and *thrA*, 12257 bp). * Comparison between R30 or R31 and *M. ciceri* Ca181 was

357 performed with the same dataset of concatenated genes excluding *gyrB* (9925 pb). **

358 Comparison between R30 and *M. ciceri* UPM-Ca7^T (USDA 3383^T) was performed with the

359 seven concatenated genes by using a reduced number of pb (4148 pb).

360

361

362 **Figure Legends**

363

364 **Fig. 1.** Phylogenetic tree of representative members of *Mesorhizobium* genus and recommended
365 chickpea-inoculant strains (indicated in bold) based on aligned *16S rRNA* gene sequences (1518
366 nt). The Kimura 2-parameter model was used to construct the tree. The percentage of replicate
367 trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is
368 shown next to the branches. Scale bar: 1 nt substitutions per 100 nt.

369

370 **Fig. 2.** Phylogenetic tree of representative members of *Mesorhizobium* genus and recommended
371 chickpea-inoculant strains (indicated in bold) based on concatenation of seven full-length HKG
372 (12257 positions in the final dataset). The Tamura 3-parameter model was used to construct the
373 tree. The percentage of replicate trees in which the associated taxa clustered together in the
374 bootstrap test (1000 replicates) is shown next to the branches. Scale bar: 2 nt substitutions per
375 100 nt.

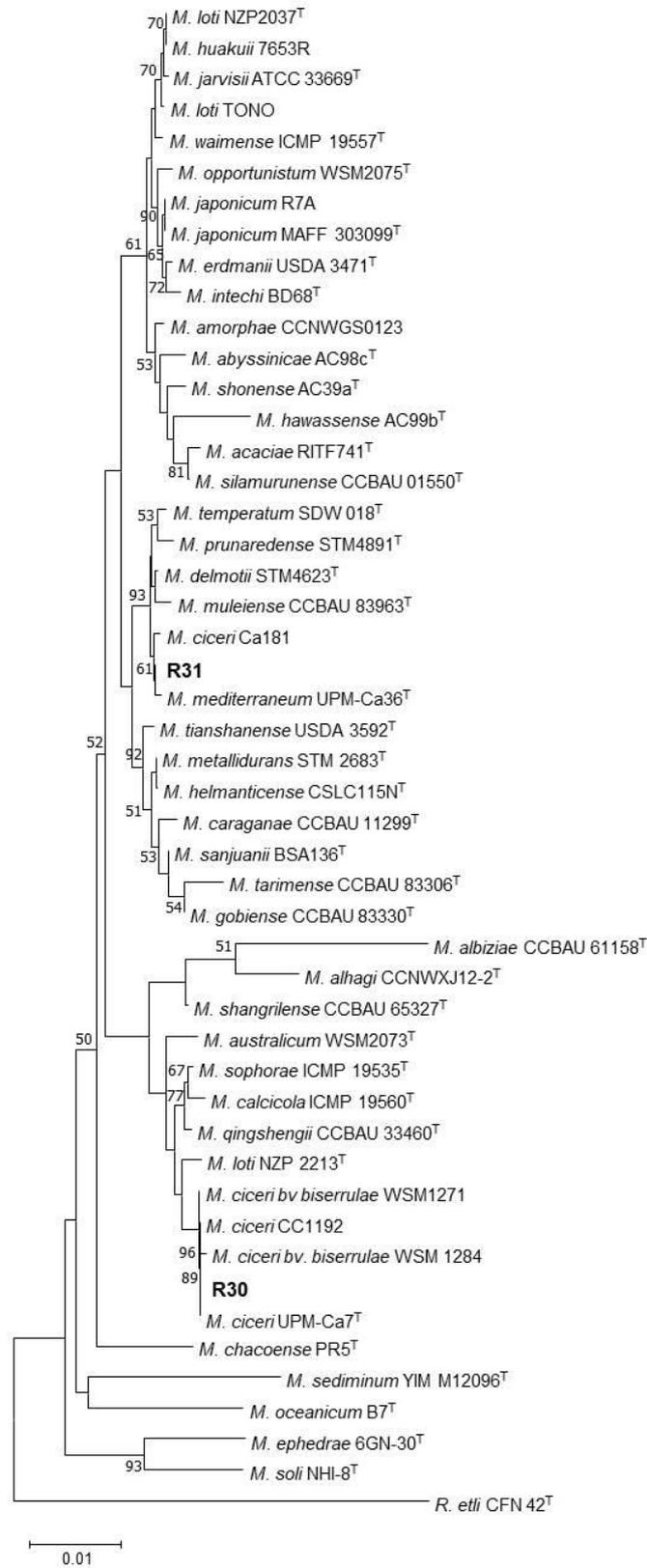
376

377 **Fig. 3.** Phylogenetic tree of representative members of *Mesorhizobium* genus and recommended
378 chickpea-inoculant strains (indicated in bold) based on partial *nodC* gene sequence (382 nt). The
379 different symbiovars (sv.) defined within the genus are shown. The Tamura 3-parameter model
380 was used to construct the tree. The percentage of replicate trees in which the associated taxa
381 clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Scale
382 bar: 5 nt substitutions per 100 nt.

383

384 **Fig. 4.** Integrated graph of genetic analysis based on closest phylogenetics for *16S rRNA* gene
385 clustering, HKG clustering or ANIg, and *nodC* gene clustering in R30 and R31. Most strain
386 denominations have been avoided to facilitate the design of the graph, and were included only
387 when clarification was needed. *It was not analyzed by MLSA. Positioning here refers to the
388 work carried out in the present study.

389

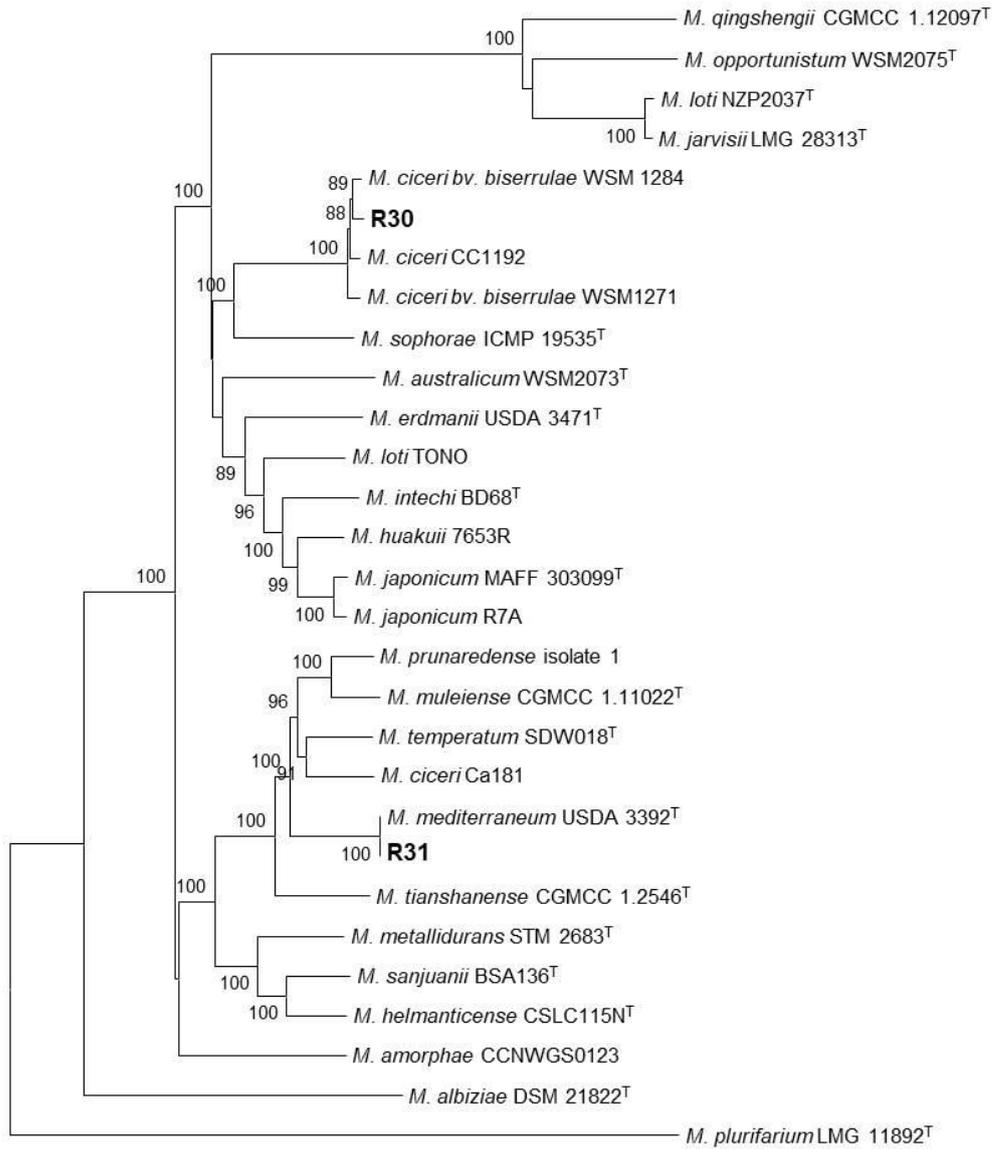


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Fig. 1.

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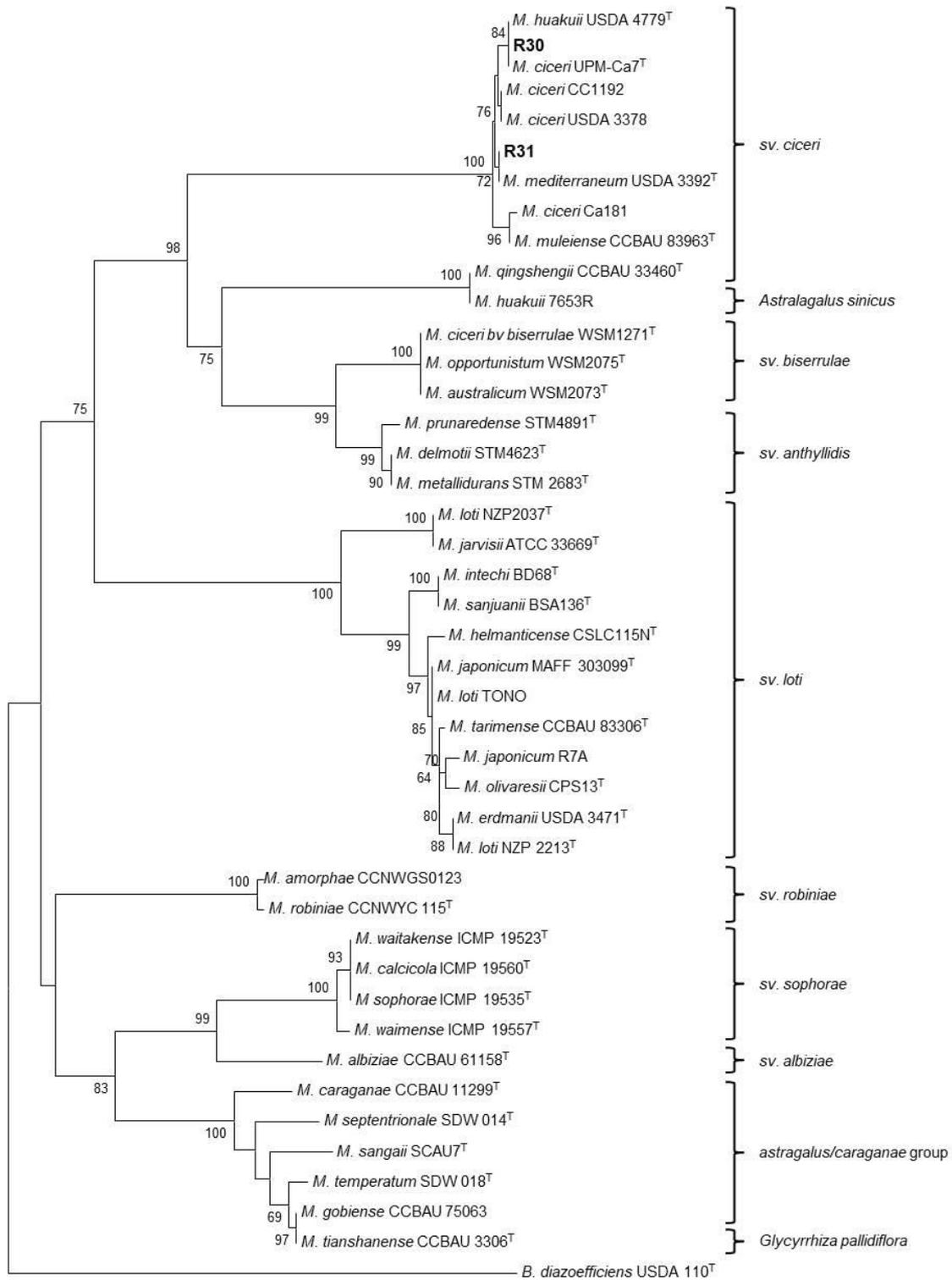
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Fig. 2.

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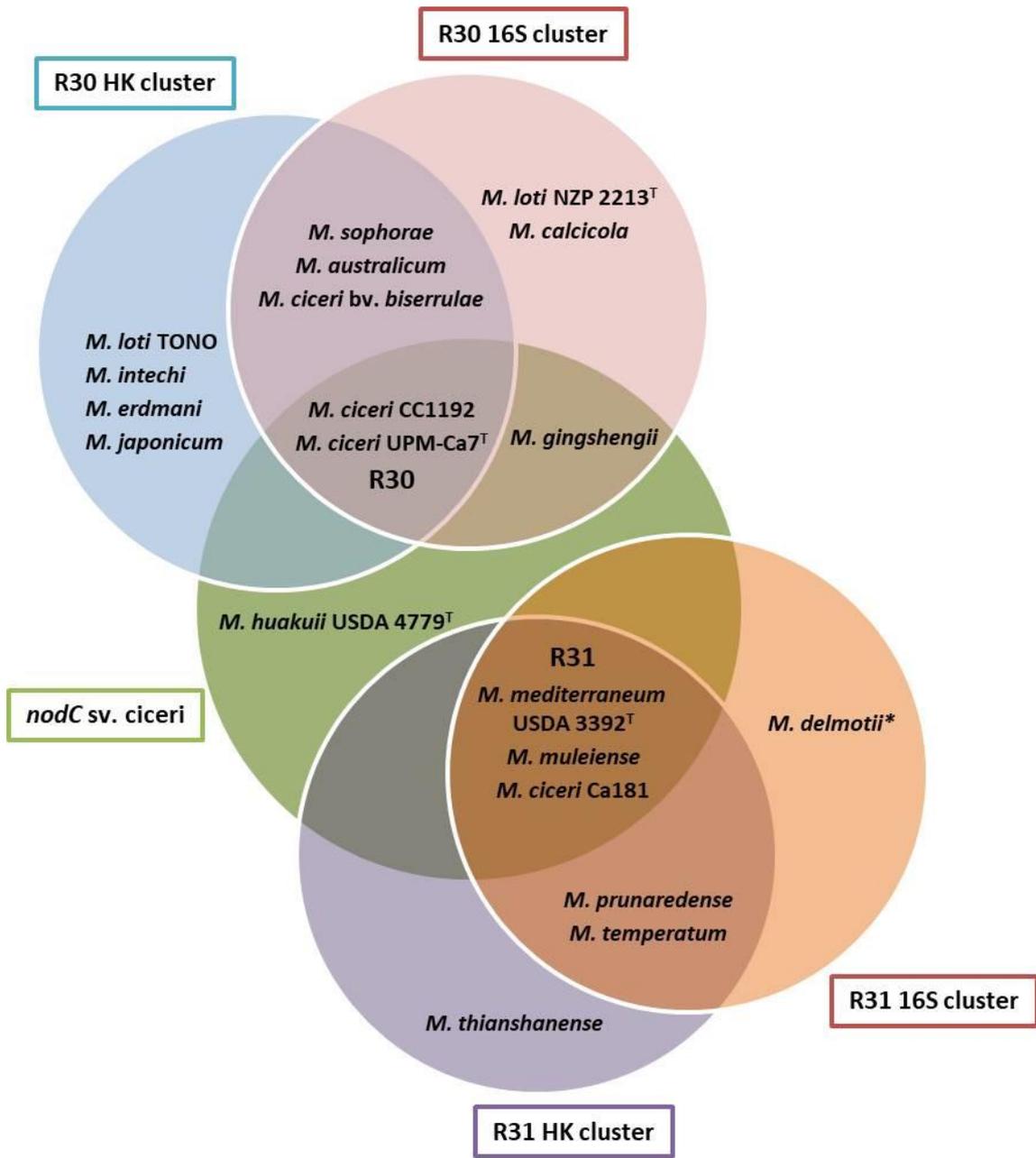


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Fig. 3.



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Fig. 4

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



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