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Rhizobium favelukesii sp. nov., isolated from the root nodules of alfalfa (Medicago sativa L)

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Corresponding Author:	Mariano Pistorio, Ph.D. Instituto de Biotecnología y Biología Molecular, CCT-La Plata, CONICET, Departamento de Ciencias Biollógicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata La Plata, Buenes Aires ARGENTINA
First Author:	Gonzalo Torres Tejerizo
Order of Authors:	Gonzalo Torres Tejerizo
	Marco Antonio Rogel
	Ernesto Ormeño-Orrillo
	María Julia Althabegoiti
	Juliet Fernanda Nilsson
	Karsten Niehaus
	Andreas Schlüter
	Alfred Pühler, Prof.
	María Florencia Del Papa
	Antonio Lagares
	Esperanza Martínez-Romero
	Mariano Pistorio, Ph.D.
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Rhizobium favelukesii sp. nov., isolated from the root nodules of alfalfa (*Medicago sativa* L)

Gonzalo Torres Tejerizo[1,3], Marco Antonio Rogel[2], Ernesto Ormeño-Orrillo[2], María Julia Althabegoiti[1], Juliet Fernanda Nilsson[1], Karsten Niehaus[3], Andreas Schlüter[3], Alfred Pühler[3], María Florencia Del Papa[1], Antonio Lagares[1], Esperanza Martínez-Romero[2] and Mariano Pistorio[1,*]

[1]IBBM (Instituto de Biotecnología y Biología Molecular), CCT-CONICET-La Plata, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calles 47 y 115 (1900) La Plata, Argentina.

[2]Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Av. Universidad 1001, Col. Chamilpa. 62210 Cuernavaca, Morelos, México.

[3]CeBiTec, Bielefeld Universität, Bielefeld, Germany.

*Corresponding author: Mariano Pistorio

Phone: +54-221-425-0497 ext. 31

Fax: +54-221-422-3409 ext. 56

E-mail: pistorio@biol.unlp.edu.ar

Runing title: *Rhizobium favelukesii* sp. *nov.*

New Taxa - Proteobacteria

The complete genome of *Rhizobium favelukesii* strain LPU83 is available with the following accession numbers: HG916852 (chromosome), HG916853 (pLPU83a), CBYB010000001-58 (pLPU83b), HG916854 (pLPU83c) and HG916855 (pLPU83d). All sequence data were downloaded from public databases.

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ABSTRACT

Rhizobium strains LPU83^T and Or191 were isolated from root nodules of alfalfa grown in acid soils from Argentina and the USA. These two strains—both sharing the same plasmid pattern, lipopolysaccharide profile, insertion-sequence fingerprint, 16S rRNA gene sequence, and PCR-fingerprinting pattern—are different from reference strains representing defined *Rhizobium* species. On the basis of previously reported data and from new results obtained by DNA-DNA hybridization, phenotypic characterization, and phylogenetic analyses; strains LPU83^T and Or191 can be considered to represent a novel species of the genus *Rhizobium*, for which the name *Rhizobium favelukesii* sp. nov. is hereby proposed. The type strain of this species is LPU83^T (= CECT 9014^T = LMG 29160^T), for which an improved draft-genome sequence is available.

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Rhizobia—a group designation for members of the genera *Azorhizobium*, *Rhizobium*, *Ensifer (Sinorhizobium)*, *Mesorhizobium*, *Bradyrhizobium*, *Neorhizobium*, *Phyllobacterium*, *Microvirga*, *Devosia* (belonging to the Alphaproteobacteria) and *Burkholderia* and *Cupriavidus* (belonging to the Betaproteobacteria)—are soil and rhizosphere bacteria of agronomic significance because they form nitrogen-fixing symbioses with leguminous plants (Gyaneshwar *et al.*, 2011, Mousavi *et al.*, 2014, Mousavi *et al.*, 2015, Ormeño-Orrillo *et al.*, 2015, Peix *et al.*, 2014).

Alfalfa (*Medicago sativa*) is the most widely cultivated forage legume for cattle and other farm animals, encompassing about 32 million hectares worldwide (Michaud *et al.*, 1988). A particular characteristic of alfalfa is the specificity of that legume in relation to its symbiotic partners *Ensifer meliloti* and *Ensifer medicae*. Both of these bacterial species are extremely sensitive to low pHs (Glenn & Dilworth, 1994), with their growth rates decreasing and even ceasing at pH 5.5 or below (Howieson *et al.*, 1992, Reeve *et al.*, 1993). Several studies focussing on the isolation and characterization of alfalfa-nodulating rhizobia from acid soils have demonstrated the presence of another group of strains that are able to nodulate alfalfa, represented by *Rhizobium* sp. LPU83 and *Rhizobium* sp. Or191, in such soils. The members of this group are acid-tolerant and have an extended host range, as they possess the ability to nodulate *Leucaena leucocephala* and *Phaseolus vulgaris* among other legumes (Del Papa *et al.*, 1999, Eardly *et al.*, 1985, Wegener *et al.*, 2001) and are highly competitive for the nodulation of alfalfa in acid soils (Del Papa *et al.*, 2003).

To further characterize this rhizobial group, we undertook a series of experiments to finally classify these rhizobia strains using two representative strains LPU83^T and Or191. Based on the results of our polyphasic taxonomic study, both strains are considered to

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represent a novel species of the genus *Rhizobium* and should not be included in the *Rhizobium tibeticum* species as stated by Reeve *et al.* (2015).

Strain LPU83^T was isolated from a nodule of alfalfa grown in an acid soil (pH 6.08) of Castelar, Buenos Aires, Argentina (Del Papa *et al.*, 1999). This strain is able to nodulate *Medicago sativa*, *Medicago truncatula*, *Melilotus* spp., *Trigonella* spp., *Phaseolus* vulgaris and *Leucaena leucocephala*, although the biologic nitrogen fixation of strains LPU83^T and Or191 is inefficient (Del Papa *et al.*, 1999, Wegener *et al.*, 2001). LPU83 shared the same plasmid patterns, lipopolysacharide profiles, insertion-sequence fingerprints and PCR-fingerprinting patterns obtained with the ERIC primers, MBOREP1 and BOXC1 than the strain Or191 isolated from acid soils in Oregon, USA.

To establish the phylogenetic position of the strains within the genus *Rhizobium*, DNA sequences of rhizobia were collected from the database of the National Center for Biotechnology Information (NCBI) and aligned with the Clustal module implemented by the MEGA5 software (Tamura *et al.*, 2011). The models of sequence evolution were selected with the jModelTest 2.1.7 program (Darriba *et al.*, 2012). For all the analyses (*i. e.*, the 16S rRNA, *recA-atpD-rpoB*, and *recA-atpD* concatenated genes) the model used was GTR +I+G. Maximum-likelihood trees were constructed on the basis of the selected model by means of the PhyML v3.1 software (Guindon & Gascuel, 2003). The robustness of the maximum-likelihood topologies was evaluated by bootstrap analysis (100 replicates). We employed the best of NNIs and SPRs algorithms to search the tree topology and used 100 random trees as initial tree constructions.

Supplementary Fig. 1 presents a phylogenetic tree based on the 16S rRNA gene sequence of rhizobia, from which data we chose the nearest-neighbour rhizobia to construct the tree shown in Fig. 1. The acid-tolerant alfalfa-nodulating strains LPU83^T and Or191

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clearly formed a clade with *R. tibeticum* CCBAU 85039^T. The 16S rRNA gene sequences of LPU83^T and Or191 are 100% concordant and therefore identical, while respective identities of 99.9% and 99.2% were found with *R. tibeticum* CCBAU 85039^T and *R. grahamii* CCGE 502^T. Moreover, in the GenBank-database, strains with 100% 16S rRNA genetic identity with LPU83^T and Or191 were found, four of those—*Rhizobium* sp. T136, *Rhizobium* sp. T1473, *Rhizobium* sp. T1155, and *Rhizobium* sp. T1470—having been described by Bromfield *et al.* (2010) and another one, *Rhizobium tibeticum* strain 246-1, reported by Stajkovi-Srbinovi *et al.* (2012). A well supported clade was therefore constructed consisting of LPU83^T, Or191, *R. tibeticum, R. grahamii, R. endophyticum, R. mesoamericanum,* and *R. cauense* (Fig. S1).

Three housekeeping genes—namely, *recA-atpD-rpoB*—were concatenated for tree construction to further explore the phylogenetic relationships among our two strains and other rhizobia. Firstly, a tree with several rhizobia strains was constructed (Fig. S2). We took the closest rhizobia, as shown in Fig. 2. These analyses clearly separated the LPU83^T and Or191 strains from *R. tibeticum*; nevertheless, these bacteria are still closely related. The identity among *recA, atpD* and *rpoB* genes of LPU83^T and *R. tibeticum* was 99% for each gene. For the other strains sharing 100% 16S rRNA gene identity with LPU83^T and Or191, the *rpoB* gene was not available in the GenBank database. Accordingly, to include those strains in the study, the *recA* and *atpD* genes were concatenated to construct the new phylogenetic tree that is shown in Fig. 3. Strains T136, T1155, and *R. tibeticum* 246-1 grouped with LPU83^T and Or191. Strains T1473 and T1470 were closely related to the latter group, but strain *R. tibeticum* CCBAU 85039^T was placed outside of the group. Bromfield *et al.* (2010) described that the strains T136 and T1155 featured the same plasmid pattern as Or191 is also highly relevant. Fig. 4 shows that the plasmid pattern of

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111 LPU83^T is identical to that of Or191 but clearly different from that of *R. tibeticum* CCBAU
112 85039^T.

113 The work by Stajkovi-Srbinovi *et al.* (2012) did not include either LPU83^T or Or191
114 sequences, while the DNA-DNA hybridization (DDH) between strains *R. tibeticum* 246-1
115 and *R. tibeticum* CCBAU 85039^T was lower than 70%. To advance in the positioning of
116 LPU83^T and Or191 in relation to closely related species, we performed DDH experiments
117 using Southern-blot hybridizations as described previously (Martínez-Romero *et al.*, 1991).
118 The DDH values between strains LPU83^T and Or191, were greater than 84%, indicating
119 that both were members of the same species. For all the members of the other *Rhizobium*
120 taxa tested, the DDH values were in the range of 10 to 34% with DNA of strain LPU83^T as
121 a probe (Table 1). As the DDH value obtained for LPU83^T and *R. tibeticum* CCBAU
122 85039^T was too low in comparison with the identity of the housekeeping genes *recA-atpD-*
123 *rpoB*, we performed a new DDH experiment between both strains using the methodology
124 described by Ezaki *et al.* (1989) according to a modification of the method (Cleenwerck *et*
125 *al.*, 2002, Goris *et al.*, 1998). In this case, a 62% value of DDH was obtained, confirming
126 the classification of LPU83^T as a novel species.

127 The phenotypic features of growth capabilities of the novel strains and other relevant
128 taxa were studied in PM1 BIOLOG microplates as previously described (López-López *et*
129 *al.*, 2010). LPU83^T and Or191 share the same carbon-source–utilization pattern (Table 2).
130 In order to deeper characterize the strains biochemical profile, different tests were
131 performed as of production of urease, nitrate reductase, ß-galactosidase, oxidase and
132 catalase. The complete phenotypic properties of strains LPU83^T and Or191 are given in the
133 species description below, and characteristics that differentiate those two from closely
134 related species are provided in Table 2. The table also shows how the overall growth

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specificities of those two strains clearly differentiate them from the other closely related species analysed (López-López *et al.*, 2012, López-López *et al.*, 2010). LPU83 and Or191 differed from its closest relative *R. tibeticum* CCBAU 85039^T in metabolism of L-alanine, L-alanyl glycine, L-aspartic acid , citric acid , D-glucosaminic acid , glycyl L-glutamic acid, D-malic acid , L-proline , D-psicose , pyruvic acid , monomethyl succinate , L-threonine, urease activity and sensitivity to tetracycline (5 µg/ml).

When observed by transmission electron microscopy, we were not able to observed flagellum in LPU83^T (Fig 5). Swimming assays were performed as described previously (Althabegoiti *et al.*, 2008). LPU83^T does not exhibit swimming motility in TY (Beringer, 1974), PY (Noel *et al.*, 1984), YEM (Vincent, 1970) and AG (Sadowsky *et al.*, 1987) with 0.3% agar in contrast of *R. tibeticum* CCBAU 85039^T (Fig 5).

The fatty-acid profiles of strains LPU83^T and Or191, as well as that of *R. tibeticum* CCBAU 85039^T, were determined with the MIDI system through the use of the TSBA5 database after incubation for 24 h on YEM agar plates; Table 3). All the strains contained fatty-acid profiles that were common to the *Rhizobium* genus—such as C16:0, C18:0 cyclo w8c, C18:1 w7c, and the second group of fatty acids that could not be separated by GLC with the MIDI system referred to as summed feature 2 (*i. e.*, |one or more C12:0 aldehyde, the unknown equivalent-chain-length species 10.928, iso-C16:1 I and C14:0 3-OH; (Tighe *et al.*, 2000). Strains LPU83^T and Or191, produced a rather similar profile and could be distinguished from *R. tibeticum* by their synthesis of a summed feature 3 (*i. e.*, C16:1 w7c, and/or iso-C15:0 2-OH) along with C18:0 3-OH.

The phenotypic, chemotaxonomic, and genotypic data from the present study indicate that strains LPU83^T and Or191 represent a novel species of the *Rhizobium* genus that can be distinguished from their nearest phylogenetic neighbours phenotypically as well as

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159 genotypically. We therefore propose to classify these bacteria as a novel species, for which
160 the name *Rhizobium favelukesii* sp. nov. is hereby proposed.
161
162 **Description of *Rhizobium favelukesii* sp. nov.**
163 *Rhizobium favelukesii* (fa.ve.lu.ke'si.i N.L. gen. n. favelukesii, of Favelukes, named in
164 honor of Prof. Dr. Gabriel Favelukes, who made valuable contributions to the development
165 of rhizobiology in South America). The species consists in aerobic, nonspore-forming,
166 Gram-negative rods that grow on TY, PY, YEM, but not on LB medium. The colonies on
167 PY-agar plates are circular and convex with regular margins, pearly and appear within 3
168 days at 28 °C. The optimal growth occurs at 28 °C and pH 7; nevertheless, the species can
169 grow at pH 4.5, but not in any media at 37 °C. *Rhizobium favelukesii* does not exhibit
170 swimming motility. The bacterium uses acetic acid, L-alanine, L-alanyl glycine, L-aspartic
171 acid, bromosuccinic acid, citric acid, fumaric acid, D-galactonic acid-c-lactone, D-
172 glucosaminic acid, L-glutamine, glycyl L-glutamic acid, *myo*-inositol, D-malic acid,
173 maltotriose, N-acetyl-3-D-mannosamine, L-proline, D-psicose, pyruvic acid, monomethyl
174 succinate, L-threonine, succinic Acid, D-galactose, D-trehalose, D-mannose, glycerol, D-
175 glucuronic acid, D-gluconic acid, L-lactic acid, formic acid, D-mannitol, D-fructose, acetic
176 acid, α-D-glucose and α-D-lactose, —but cannot use dulcitol, L-malic acid, uridine, D-
177 serine, L-arabinose, 2-aminoethanol, phenylethyl-amine, and glycyl-L-proline—as carbon
178 sources. Tests for urease, nitrate reductase, ß-galatosidase, oxidase are positive, while
179 catalase was weakly positive.The most abundant fatty acids are C18:1 w7c and summed
180 feature 2 (probably C12:0 aldehyde, the unknown equivalent-chain-length species 10.928,
181 iso-C16:1 I and C14:0 3-OH). At the molecular level, *R. favelukesii* can be differentiated

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from other species of the genus *Rhizobium* by sequence analysis of the *recA*, *atpD*, and *rpoB* genes and by DNA-DNA hybridization.

The type strain LPU83^T (= LMG 29160 ^{T} = CECT 9014^T) was isolated from an alfalfa (*Medicago sativa*) root nodule in the course of a plant-trap experiment inoculated with soil from Castelar, Buenos Aires, Argentina (soil pH 6.08). The DNA G+C content of the type strain is 59.65 mol% as determined from the 7.57 Mbp of total genomic sequence. The genome of strain LPU83^T consists of one chromosome and four plasmids.

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Fig. 1. *Maximum-likelihood phylogeny constructed from 16S rRNA gene sequences of R. favelukesii and closely related rhizobia.* Accession numbers are between curved brackets. Bootstrap values higher that 50% are shown at the nodes. The bar indicates 2 substitutions per 100 nucleotide positions.

Fig. 2. *Maximum-likelihood phylogeny constructed from concatenated recA-atpD-rpoB* genes *of R. favelukesii and closely related rhizobia.* Accession numbers are between curved brackets. Bootstrap values higher that 50% are shown at the nodes. The bar indicates 1 substitution per 100 nucleotide positions.

Fig. 3. *Maximum-likelihood phylogeny reconstructed from concatenated recA-atpD genes sequences.* Accession numbers are between curved brackets. Bootstrap values higher that 50% are shown at the nodes. The bar indicates 1 substitution per 100 nucleotide positions.

Fig. 4. *Plasmid profiles in Eckhardt-like gels.* Lane 1, LPU83^{T}; lane 2, Or191, lane 3, *R. tibeticum* CCBAU 85039^{T}.

Fig. 5. *Microscopical analysis of LPU83^{T}*. Aliquots of cultures were observed by transmission electron microscopy after staining with 2% (w/v) potassium phosphotungstate (pH 5.2; 2% [w/v] KOH). **A**, **C**, and **D:** Different magnifications of LPU83^{T} (no flagellum visible). **B:** No free flagella were observed in the preparations. **E** and **F**, swimming test on 0.3% agar in YEM and PY, respectively.

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Supplementary Fig. 1. *Maximum-likelihood phylogeny constructed from 16S rRNA gene sequences.* Accession numbers are between curved brackets. Bootstrap values higher that 50% are shown at the nodes. The bar indicates 5 substitutions per 100 nucleotide positions.

Supplementary Fig. 2. *Maximum-likelihood phylogeny constructed from concatenated recA-atpD-rpoB genes.* Accession numbers are between curved brackets. Bootstrap values higher that 50% are shown at the nodes. The bar indicates 5 substitutions per 100 nucleotide positions.

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337 **Table 1.** Average DNA–DNA hybridization values of *Rhizobium favelukesii* and related
338 type strains
339

	Strain	Percent ± standard error
R. favelukesii	LPU83^T	100
R. favelukesii	Or191	84 ± 4
R. tibeticum	CCBAU 85039^T	34 ± 11
R. grahamii	CCGE 502^T	16 ± 1
R. endophyticum	CCGE 2052^T	14 ± 2
R. mesoamericanum	CCGE 501^T	16 ± 4
R. phaseoli	ATCC 14482^T	10 ± 3
R. fabae	CCBAU 33202^T	14 ± 3
R. etli	CFN 42^T	12 ± 3
R. tropici	CIAT 899^T	13 ± 4
R. mongolense	USDA 1844^T	16 ± 6
R. gallicum	R602^T	10 ± 7
R. mesosinicum	CCBAU 25010^T	10 ± 8

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342 **Table 2.** Distinctive features of the growth phenotypic of the novel species in comparison
343 to that of type strains of phylogenetically related species and *R. leguminosarum*.

344

Species/Strains [a]	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Utilization as sole carbon source of [b]:							
Acetic acid	+	+	-	+	+	-	+
L-alanine	+	+	-	+	-	+	-
L-alanyl glycine	+	+	-	+	-	-	+
L-aspartic acid	+	+	+	-	-	+	-
Bromosuccinic acid	+	+	-	-	+	-	+
Citric acid	+	+	+	-	-	-	-
Fumaric acid	+	+	+	+	+	+	-
D-galactonic acid-c-lactone	+	+	-	-	+	-	+
D-glucosaminic acid	+	+	+	-	-	-	+
L-glutamine	+	+	-	+	+	+	+
Glycyl L-glutamic acid	+	+	-	+	-	-	+
Myo-Inositol	+	+	+	+	+	+	-
D-malic acid	+	+	+	+	-	-	+
Maltotriose	+	+	+	+	+	-	+
N-acetyl 3-D-mannosamine	+	+	-	+	+	+	+
L-proline	+	+	+	+	-	+	+
D-psicose	+	+	+	+	-	-	+

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Pyruvic acid	+	+	-	+	-	-	+
Monomethyl succinate	+	+	-	+	-	-	-
L-threonine	+	+	-	+	-	-	-
Resistance to tetracycline (5 mg ml)	-	-	**ND**	**ND**	+	**ND**	**ND**
Growth with/at:							
TY 37 ºC	-	-	+	**W**	-	-	**ND**
LB	-	-	-	-	-	+	**ND**
β-galactosidase	+	+	**ND**	**ND**	+	**ND**	**ND**
Ureasa	+	+	**ND**	**ND**	-	**ND**	**ND**
Oxidasa	+	+	**ND**	**ND**	+	**ND**	**ND**
Nitrato reductase	+	+	**ND**	**ND**	+	**ND**	**ND**

[a]The number above each column corresponds to the different species and/or strains analyzed: **1**, *R. favelukesii* LPU83^T; **2**, *R. favelukesii* Or191; **3**, *R. mesoamericanum* CGE 501^T; **4**, *R. grahamii* CGE 502^T; **5**, *R. tibeticum* CCBAU 85039^T; **6**, *R. endophyticum* CCGE 2052^T; **7**, *R. leguminosarum* USDA 2370^T.

[b](+), growth; (-), no growth; W, weak growth; ND, not determined.. These data were obtained in the present study.

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353 **Table 3.** Cellular fatty-acid composition (%) present in *R. favelukesii* strains LPU83^T,
354 Or191, and in *R. tibeticum* CCBAU 85039^T.
355

	R. favelukesii		*R. tibeticum*
Fatty acid	LPU83^T	Or191	CCBAU 85039^T
C15:0 2-OH	9.1	10.94	10.69
C15:0 iso 3-OH	ND	ND	ND
C16:0	1.68	2.21	3.74
C16:0 3-OH	3.65	1.86	2.55
C17:0 cyclo	ND	ND	ND
C18:0	1.26	1.26	ND
C18:0 3-OH	2.93	1.42	ND
C18:1 2-OH	4.25	3.94	3.65
C11-methyl 18:1_7*c*	ND	ND	ND
C19:0 cyclo w8*c*	1.27	1.61	2.83
C19:0 10-methyl	0.2	ND	ND
C18:1 w7c	25.27	20.2	27.72
Summed features*			
2	49.63	55.66	48.83
3	0.78	0.9	ND

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*Summed features are groups of two or more fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2 contained one or more C12:0 aldehyde, unknown equivalent chain length 10.928, iso-C16:1 I and C14:0 3-OH. Summed feature 3 contained C16:1 w7c and/or iso-C15:0 2-OH.

ND: Not detected

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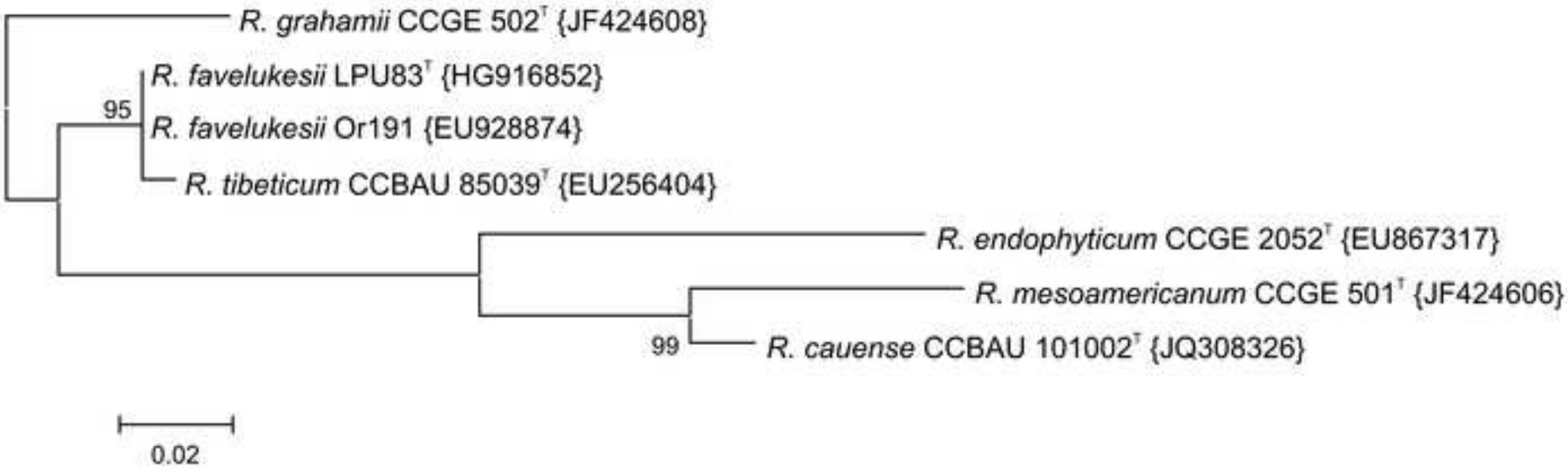

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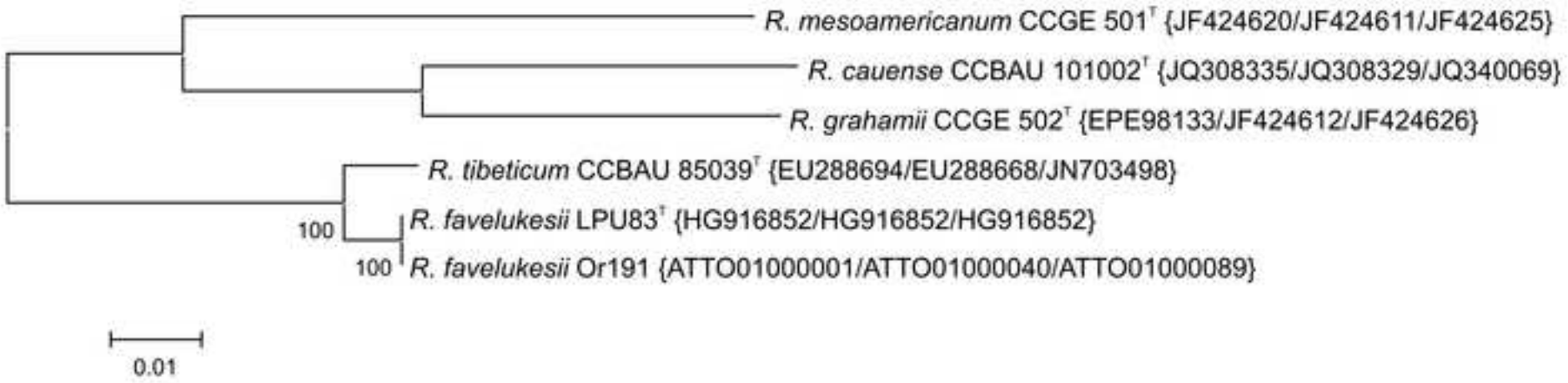

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Figure3

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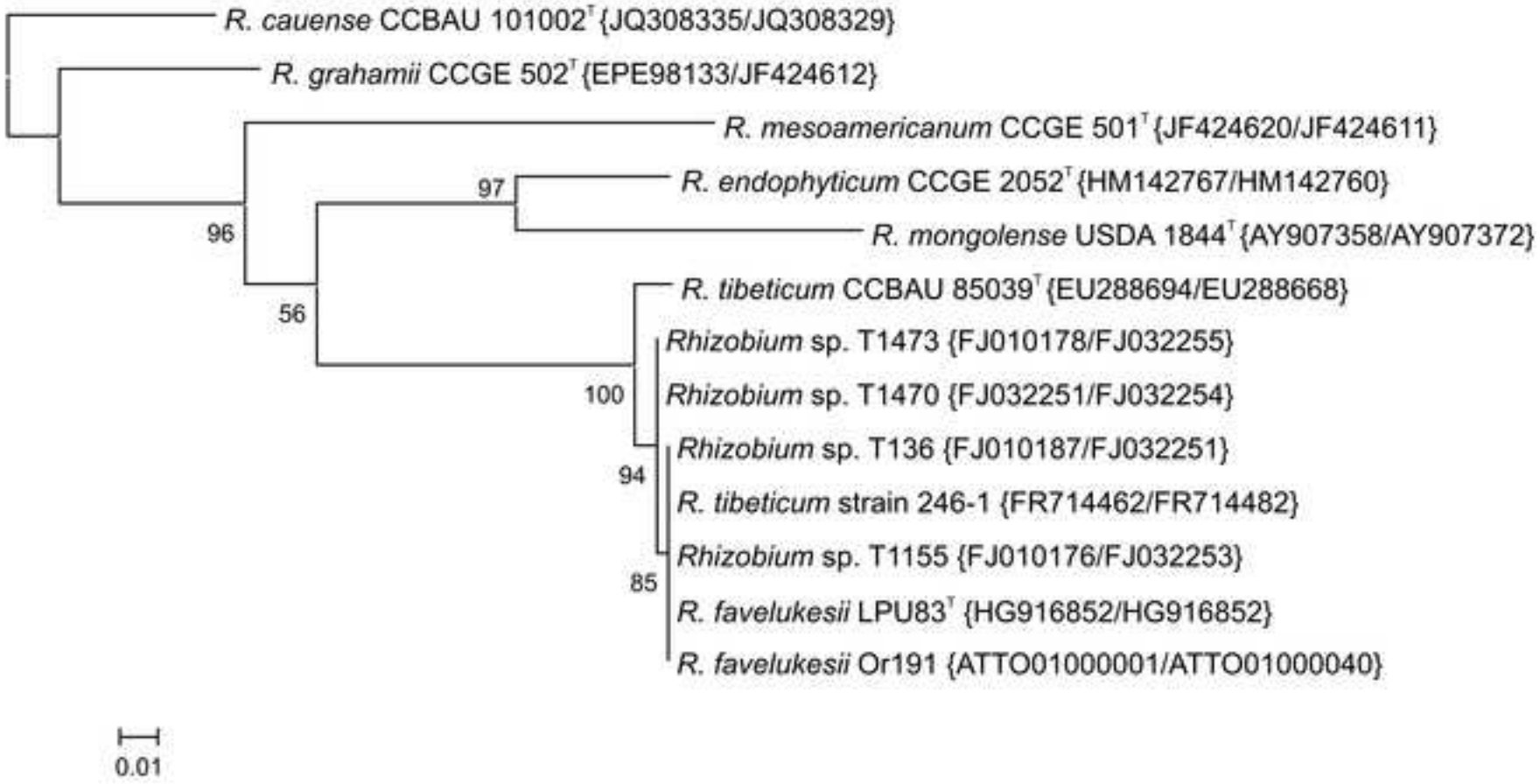

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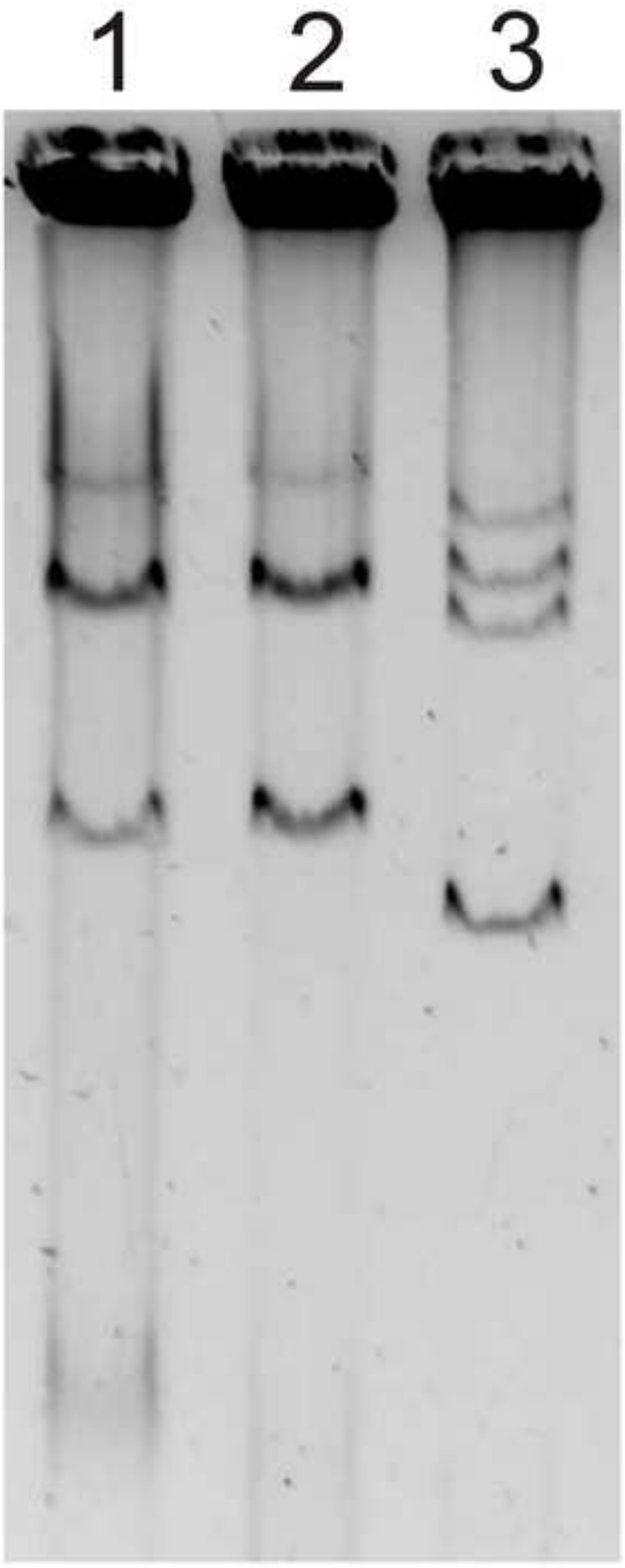

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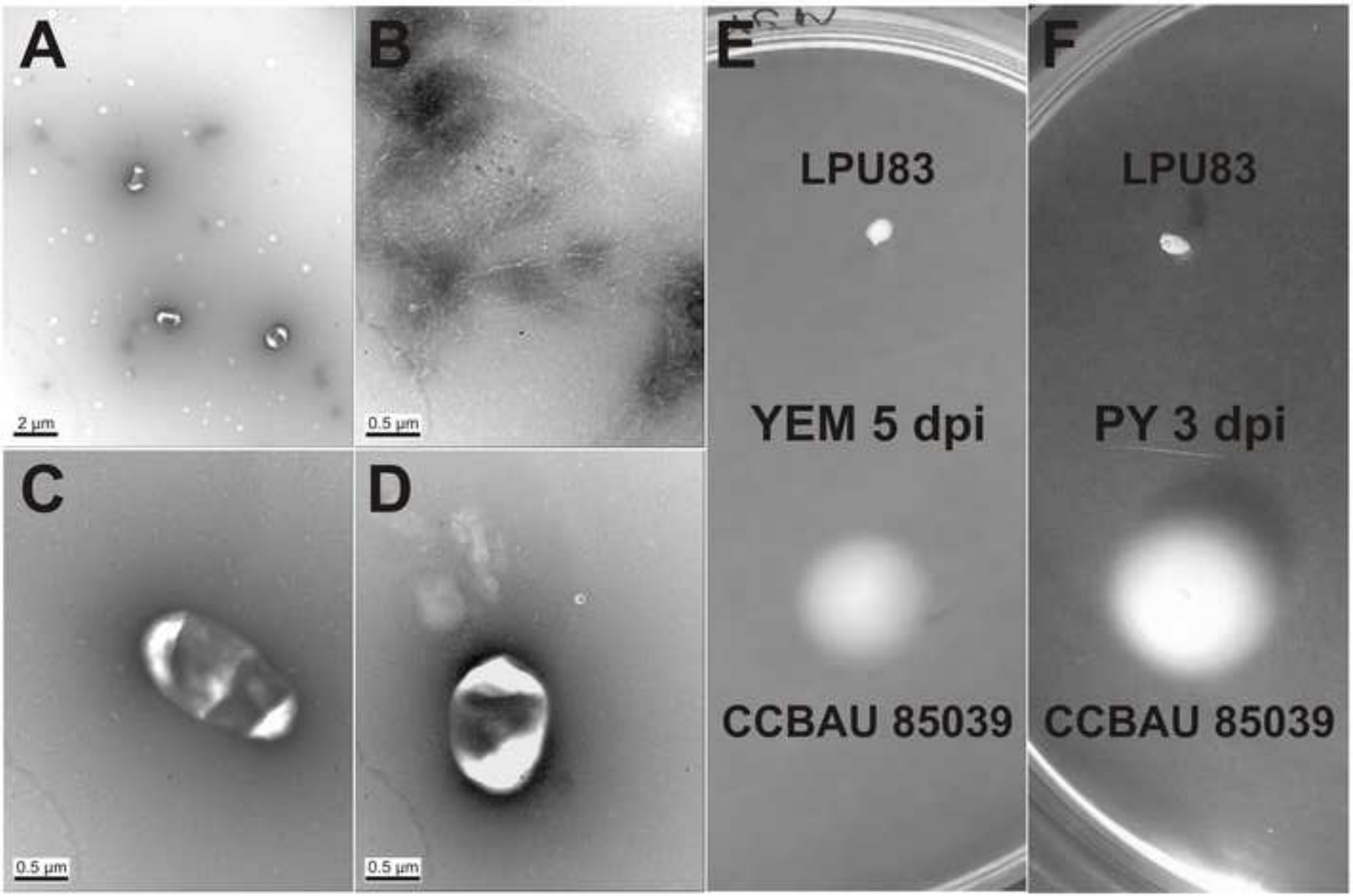

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Supplementary Figures

Rhizobium favelukesii sp. nov., isolated from the root nodules of alfalfa (*Medicago sativa* L)

Gonzalo Torres Tejerizo[1,3], Marco Antonio Rogel[2], Ernesto Ormeño-Orrillo[2], María Julia Althabegoiti[1], Juliet Fernanda Nilsson[1], Karsten Niehaus[3], Andreas Schlüter[3], Alfred Pühler[3], María Florencia Del Papa[1], Antonio Lagares[1], Esperanza Martínez-Romero[2] and Mariano Pistorio[1,*]

[1]IBBM (Instituto de Biotecnología y Biología Molecular), CCT-CONICET-La Plata, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calles 47 y 115 (1900) La Plata, Argentina.

[2]Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Av. Universidad s/n, Col. Chamilpa. 62210 Cuernavaca, Morelos, Mexico.

[3]CeBiTec, Bielefeld Universität, Bielefeld, Germany.

*Corresponding author: Mariano Pistorio

Phone: +54-221-425-0497 ext. 31

Fax: +54-221-422-3409 ext. 56

E-mail: pistorio@biol.unlp.edu.ar

Runing title: *Rhizobium favelukesii* sp. *nov.*

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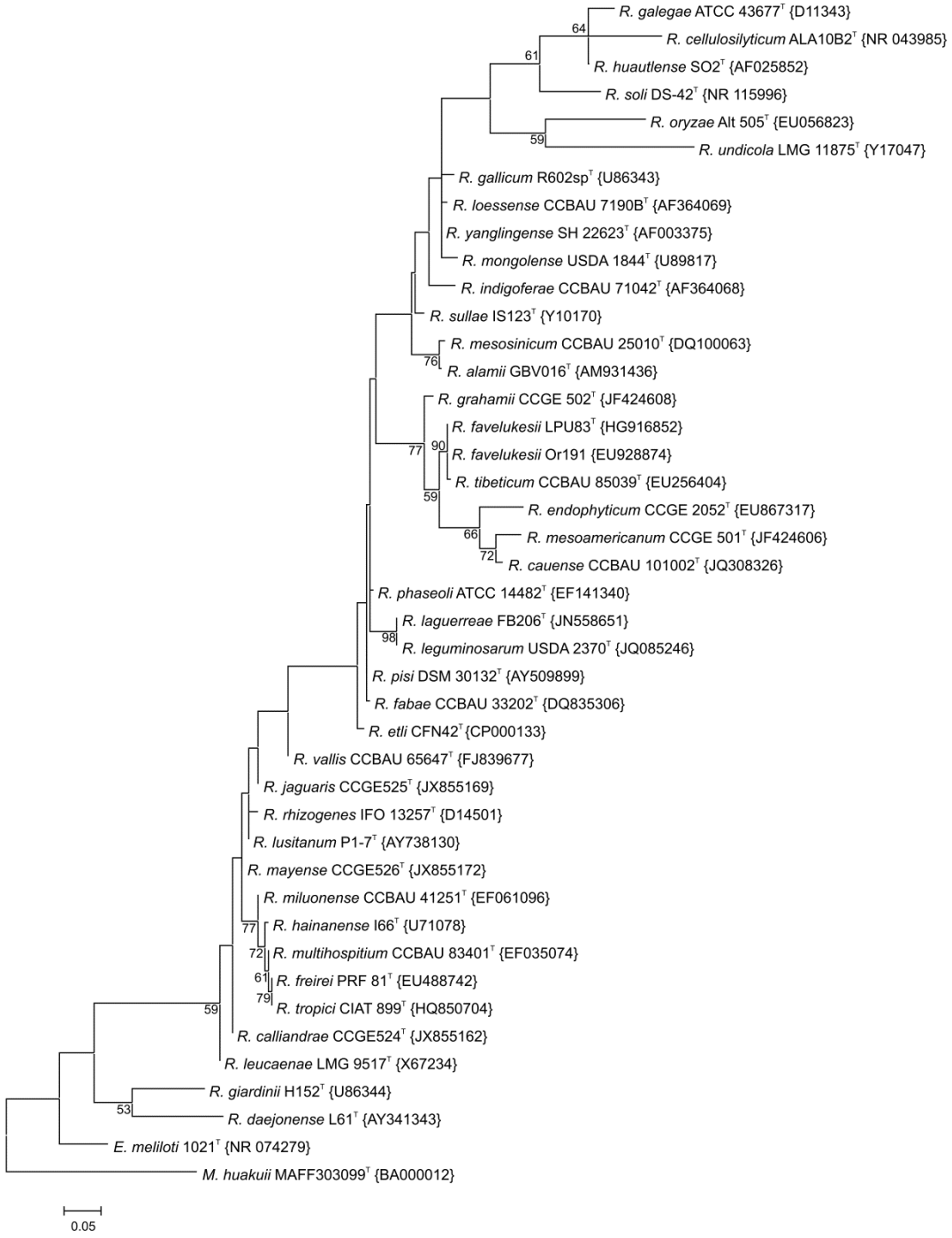

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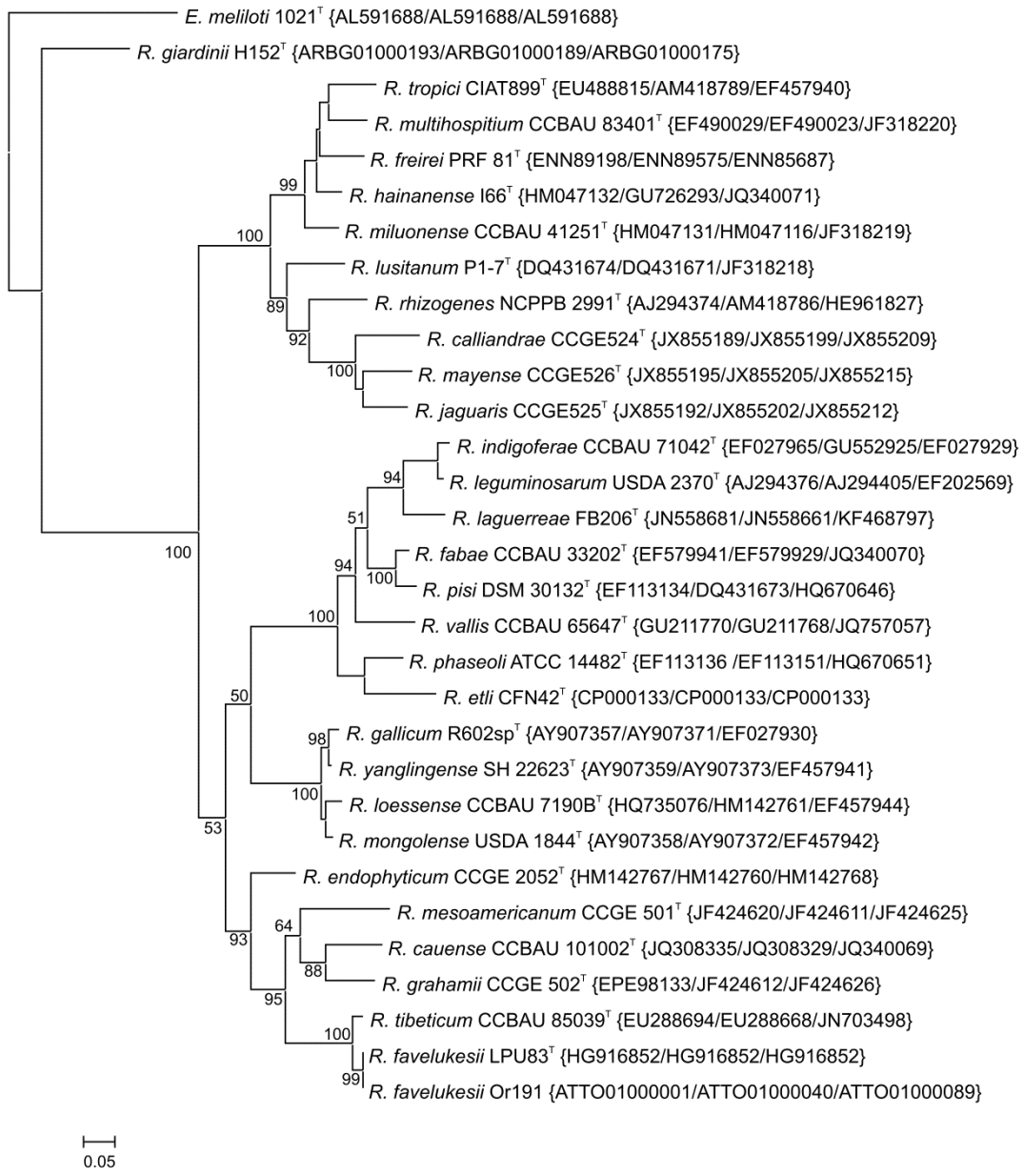

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