Rhizobium laguerreae sp. nov. nodulates *Vicia faba* on several continents

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Several fast-growing strains nodulating *Vicia faba* in Peru, Spain and Tunisia formed a cluster related to *Rhizobium leguminosarum*. The 16S rRNA gene sequences were identical to that of *R. leguminosarum* USDA 2370^T, whereas *rpoB, recA* and *atpD* gene sequences were phylogenetically distant, with sequence similarities of less than 96 %, 97 % and 94 %, respectively. DNA–DNA hybridization analysis showed a mean relatedness value of 43 % between strain FB206^T and *R. leguminosarum* USDA 2370^T. Phenotypic characteristics of the novel strains also differed from those of the closest related species of the genus *Rhizobium*. Therefore, based on genotypic and phenotypic data obtained in this study, we propose to classify this group of strains nodulating *Vicia faba* as a novel species of the genus *Rhizobium* named *Rhizobium laguerreae* sp. nov. The type strain is FB206^T (=LMG 27434^T=CECT 8280^T).

Vicia faba is a cultivated legume from tribe Viciae that constitutes an important crop in all continents (Duc *et al.*, 2010) and which fixes atmospheric nitrogen in symbiosis with fast-growing rhizobial species from the genus *Rhizobium* (Kuykendall, 2005). At the time of writing, the genus *Rhizobium* contains several species nodulating *Vicia* such as *Rhizobium leguminosarum* (Kuykendall, 2005) and *Rhizobium fabae* (Tian *et al.*, 2008) and several unclassified strains isolated from different *Vicia* species in different continents (Santillana *et al.*, 2008, Álvarez-Martínez *et al.*, 2009; Tian *et al.*, 2008, 2010; De Meyer *et al.*, 2011; Rahi *et al.*, 2012).

Abbreviations: ML, maximum-likelihood; NJ, neighbour-joining; RAPD, random amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, *atpD*, *recA*, *rpoB* and *nodC* sequences of strains FB310, FB403, FB14022, CVIII4 and PEVF08 are shown in Figs 1 and 2.

Two supplementary tables and three supplementary figures are available with the online version of this paper.

The objective of this study was to analyse the taxonomic status of several strains able to nodulate *Vicia faba* isolated in different continents in previous works (Table S1 available in IJSEM Online). Strains PEVF08 and CVIII4, isolated in Peru and Spain, respectively, formed a cluster clearly distinguishable from *R. leguminosarum* on the basis of the *recA* and *atpD* gene analysis (Santillana *et al.*, 2008; Álvarez-Martínez *et al.*, 2009). Strains FB206^T, FB310, FB403 and FB14022, isolated from effective nodules of *Vicia faba* in Tunisia, were classified into the species *R. leguminosarum* by 16S-RFLP analysis and partial sequencing of the 16S rRNA gene (Saïdi *et al.*, 2013). However the housekeeping gene analysis of these strains performed in the present work showed that they are distinguishable from *R. leguminosarum* and form a cluster together with strains PEVF08 and CVIII4.

The genetic diversity of this group of strains was assessed by random amplified polymorphic DNA (RAPD)-fingerprinting using the M13 primer as previously described (Rivas *et al.*, 2006) and comparison to the type strains from

the phylogenetically related species of the genus *Rhizobium*. The results showed different RAPD patterns for all strains of the group, which confirmed their genetic diversity (Fig. S1A). A dendrogram was reconstructed based on the matrix generated using the UPGMA method and the Dice coefficient with Bionumerics version 4.0 software (Applied Maths). The results of this analysis showed higher similarity values between strains of the proposed novel species than those found between strains of the novel species and their closest relatives (Fig. S1B).

Amplification and sequencing of the complete 16S rRNA gene of strain FB206^T was carried out according to Rivas *et al.* (2007), those of *recA* and *atpD* according to Gaunt *et al.* (2001), that of *rpoB* according to Martens *et al.* (2008) and that of *nodC* as described by Laguerre *et al.* (2001). The obtained sequences were compared with those from the GenBank database using the BLASTN program (Altschul *et al.*, 1990), and the 16S rRNA gene sequences were also compared with those from the EzTaxon-e server (Kim *et al.*, 2012). Sequences were aligned using the CLUSTAL X software (Thompson *et al.*, 1997). Evolutionary distances were calculated according to Kimura's two-parameter model (Kimura, 1980). Phylogenetic trees were inferred using the neighbour-joining (NJ) (Saitou & Nei, 1987) and maximum-likelihood (ML) (Rogers & Swofford, 1998) analyses. MEGA5 software (Tamura *et al.*, 2011) was used for all analyses.

The 16S rRNA gene was identical in all strains from the proposed novel species (data not shown) and in *R. leguminosarum* USDA 2370^T forming a cluster which also

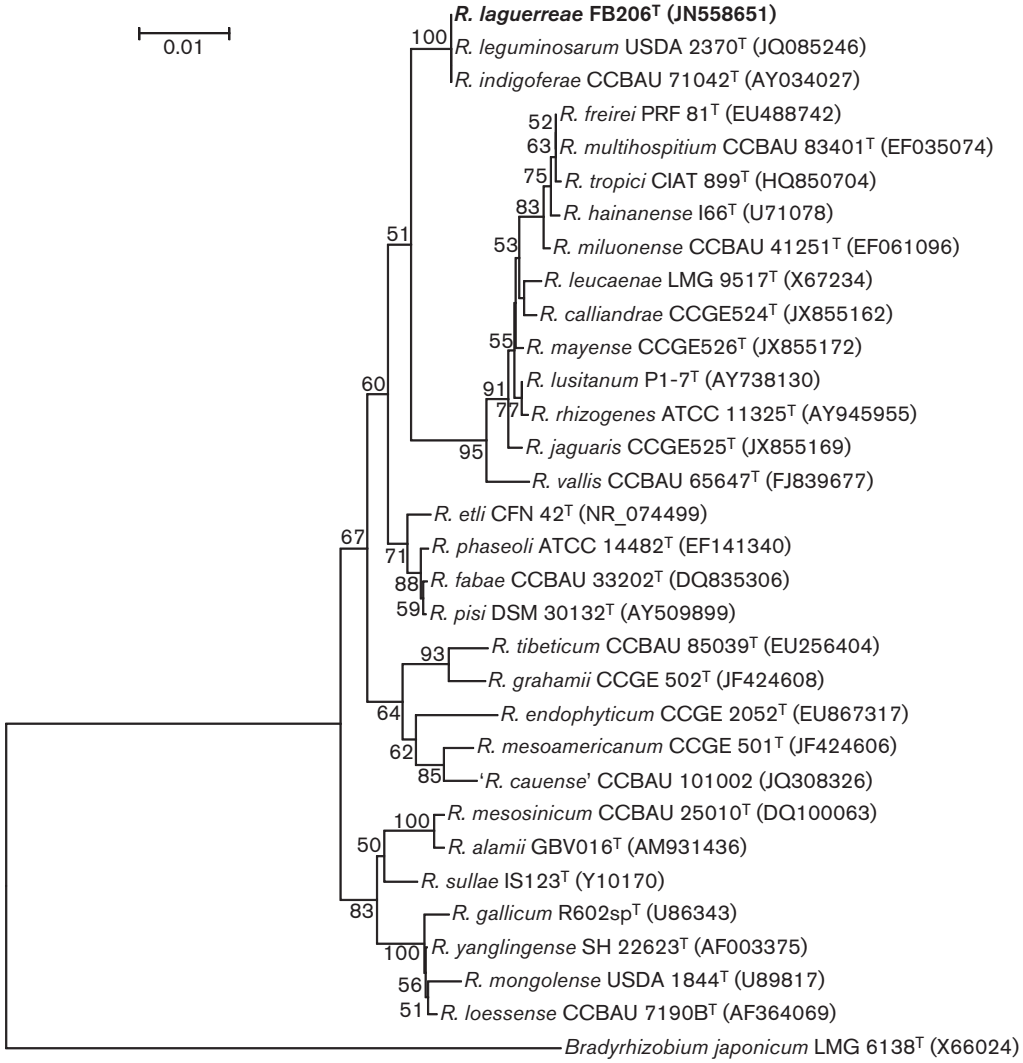

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (1317 positions) showing the relationships among *Rhizobium laguerreae* sp. nov. and closely related species of the genus *Rhizobium*. The significance of each branch is indicated by a bootstrap value (in percentage) calculated for 1000 subsets (only values $\geqslant$50 % are indicated). Bar, 1 substitution per 100 nt positions.

included *R. fabae* LMG 23997^T, *Rhizobium pisi* DSM 30132^T, *Rhizobium phaseoli* ATCC 14482^T and *Rhizobium etli* CFN 42^T after NJ (Figs 1 and S2) and ML (data not shown) analyses. Complete identity in the 16S rRNA gene sequences has been found in other species of the genus *Rhizobium* such as between the recently described species *Rhizobium freirei* and *Rhizobium multihospitium* (Dall'Agnol *et al.*, 2013). These species are distinguishable by housekeeping gene analysis, which complements that of the 16S rRNA gene in taxonomic studies at the species level (Tindall *et al.*, 2010).

In the genus *Rhizobium* the housekeeping genes *recA* and *atpD* have been sequenced in all species and they are very useful to differentiate among closely related species within the *R. leguminosarum* phylogenetic group (Ramírez-Bahena *et al.*, 2008). The *rpoB* gene, sequenced in all species from this phylogenetic group, has been included together with *recA* and *atpD* in MLSA schemes in recent species descriptions of members of the genus *Rhizobium* (Dall'Agnol *et al.*, 2013; Rincón-Rosales *et al.*, 2013) allowing the differentiation between those with 100 % sequence similarity in the 16S rRNA genes (Dall'Agnol *et al.*, 2013). The sequence similarity values of *rpoB, recA* and *atpD* genes among the studied strains ranged from 100 % to 98 %, 100 % to 97 % and from 100 % to 99 %, respectively. The concatenated gene sequences analysis placed the strains in a cluster related to *R. leguminosarum, Rhizobium indigoferae*, *R. pisi* and *R. fabae* (Fig. 2). The most closely related species to *R. laguerreae* sp. nov. were *R. leguminosarum* and *R. indigoferae* with less than 96 %, 97 % and 94 % sequence similarity in *rpoB, recA* and *atpD* genes, respectively. These values are similar to those found among some species of the genus *Rhizobium* such as *R. freirei*,

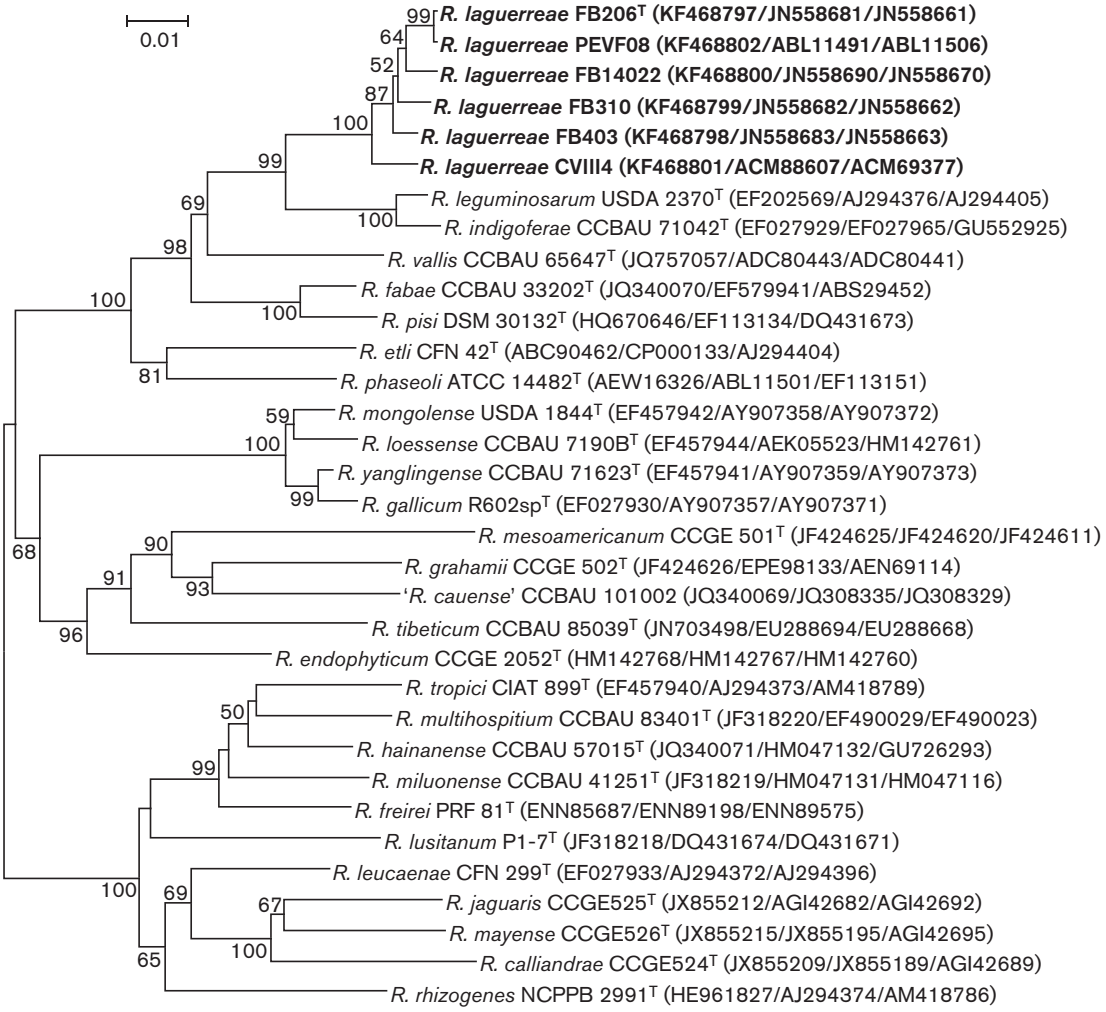

Fig. 2. Neighbour-joining phylogenetic tree based on partial concatenated sequences of *rpoB, recA* and *atpD* genes (by this order and with a total 1410 positions) showing the relationships among *Rhizobium laguerreae* sp. nov. and related species of the genus *Rhizobium*. The significance of each branch is indicated by a bootstrap value (in percentage) calculated for 1000 subsets (only values $\geqslant$50 % are indicated). Bar, 1 substitutions per 100 nt positions.

R. multihospitium, *R. miluonense*, *R. hainanense* and *R. tropici* (Fig. 2).

DNA–DNA hybridization experiments were carried out as reported previously (Ezaki *et al.*, 1989; Willems *et al.*, 2001). Strains FB206^T and CVIII4, which were isolated in different continents, presented different RAPD patterns and were the most divergent strains within the novel species according to the housekeeping gene sequences, showed a mean DNA–DNA relatedness value of 82 % (±10 %) confirming they belonged to the same species (Table S2). These two strains were also hybridized with *R. leguminosarum* USDA 2370^T and *R. indigoferae* CCBAU 71042^T, the closest relatives according to the housekeeping gene analysis, and showed values ⩽50 % in all cases (Table S2). Since this percentage is below the 70 % threshold value of DNA–DNA relatedness for definition of bacterial species (Wayne *et al.*, 1987), the group of strains analysed in this work should be assigned to a novel species.

DNA for analysis of DNA base composition was prepared according to Chun & Goodfellow (1995). The mol% G+C content of the DNA was determined using the thermal denaturation method (Mandel & Marmur, 1968) The DNA G+C content of strain FB206^T was 60.3 mol%, which is within the range reported for other species of the genus *Rhizobium* (Ramírez-Bahena *et al.*, 2008; Tian *et al.*, 2008).

Phenotypic characterization was performed in this study using the same tests and methodologies reported in the paper on reclassification of *R. leguminosarum* (Ramírez-Bahena *et al.*, 2008). Moreover in the present work, the API ID32GN kit was used according to the manufacturer's instructions (bioMérieux) with the addition of $MgSO_4$, which is not included in the media supplied with the kit, but which improves the growth of rhizobia in this system. For this, 100 µl of a sterile aqueous solution of 20 g $MgSO_4$ l^{-1} was added to each ampoule before inoculation. The type strains of closely related species of the genus *Rhizobium* were included in the phenotypic study as reference strains. Phenotypic characteristics of the novel species are reported in the species description and the differences with respect to the closest related species of the genus *Rhizobium* are recorded in Table 1.

Although symbiotic characteristics are not considered for taxonomic purposes, we have confirmed in this work that all strains belong to the symbiovar viciae (Fig. S3) as was previously reported for strains PEVF08 and CVIII4 (Santillana *et al.*, 2008; Álvarez-Martínez *et al.*, 2009).

Therefore, based on the phenotypic and genotypic characteristics we classify the studied strains as representatives of a novel species of the genus *Rhizobium*, for which the name *Rhizobium laguerreae* sp. nov. is proposed.

Description of *Rhizobium laguerreae* sp. nov.

Rhizobium laguerreae (la.gue′rre.ae. N.L. gen. fem. n. *laguerreae* of Laguerre, to honour the recently deceased French rhizobiologist Gisèle Laguerre, who made a great contribution to research on rhizobia).

Table 1. Phenotypic differences among *Rhizobium laguerreae* sp. nov. and the type strains of closely related species of the genus *Rhizobium*

Taxa: 1, *Rhizobium laguerreae* sp. nov. (data from all strains including the type strain); 2, *R. leguminosarum* USDA 2370^T; 3, *R. indigoferae* CCBAU 71042^T; 4, *R. pisi* DSM 30132^T; 5, *R. fabae* LMG 23997^T. All data are from this study. +, Positive; −, negative; W, weakly positive; PNP, *para*-nitrophenyl.

Characteristic	1	2	3	4	5
Hydrolysis of:					
PNP-α-maltopyranoside	−	−	−	+	+
PNP-β-maltopyranoside	−	−	−	W	+
Growth in 0.8 % NaCl	−	+	−	+	−
Acid production from (72 h):					
L-Ribose	−	+	+	+	+
L-Rhamnose	+	+	+	+	−
Trehalose	−	+	+	+	+
Maltose	−	+	+	+	+
Sucrose	W	−	−	−	+
Assimilation of (API 32GN):					
Citrate	−	+	+	+	+
L-Alanine	−	−	+	−	W

Cells are Gram-negative rods as for other species of the genus. Colonies are small and pearl-white on YMA at 28 °C, which is the optimal growth temperature. The optimum pH for growth is pH 7–7.5. Growth is observed at 10–37 °C and pH 6–8, but not at 40 °C, pH 5 or in the presence of 0.8–1 % (w/v) NaCl. Nitrate reduction is negative. Positive result for production of β-galactosidase, urease and aesculin hydrolysis in the API 20NE system. Positive result for production of α- and β-glucosidases, α- and β-galactosidases, β-glucosaminidases, α- and β-fucosidases, α- and β-mannosidases, α- and β-xylosidases, α- and β-arabinosidases, α-rhamnosidases, and acid and alkaline phosphatases using chromogenic *para*-nitrophenyl substrates. The production of β-galactosaminidases and β-cellobiases is variable. The production of galacturonidases, glucuronidases, lactosidases and α- and β-maltosidases is negative in most of strains. The production of indole, arginine dehydrolase and gelatinase is negative in API 20NE tests. Glucose, L-arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose and malate were assimilated in the API 20NE system after 7 days of incubation, but gluconate, caproate, adipate, citrate and phenylacetate were not. In the API 32GN system after 7 days of incubation, the assimilation of L-rhamnose, *N*-acetylglucosamine, L-ribose, inositol, sucrose, maltose, mannitol, glucose, salicin, melibiose, L-fucose, L-sorbose, L-arabinose, L-histidine, 3-hydroxybutyrate and L-proline is positive, but assimilation of itaconate, suberate, malonate, L-alanine, glycogen, 3-hydroxybenzoate, L-serine, propionate, caprate, valerate and citrate is negative; variable results are found in the case

of acetate, lactate, 2- and 5-ketogluconate and 4-hydroxybenzoate. Acid production from L-rhamnose was positive after 72 h of incubation, but negative from L-ribose, trehalose and maltose. Acid production from glucose and sucrose is weak. Sensitive to ciprofloxacin, polymyxin B, oxitetracycline, cefuroxime and gentamicin; weakly sensitive to neomycin and erythromycin; and resistant to penicillin and cloxacillin. Resistance to ampicillin is variable. All strains form effective nodules in *Vicia faba*.

The type strain FB206^T (=LMG 27434^T=CECT 8280^T) was isolated from effective nodules of *Vicia faba* in Tunisia. The DNA G+C content of the type strain is 60.3 mol%. Additional strains of the species are FB310, FB403 and FB14022, isolated in Tunisia, and PEVF08 and CVIII4, isolated in Peru and Spain, respectively.

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