**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 2370T, 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 FB206T and *R. leguminosarum* USDA 2370T. 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 FB206T (=LMG 27434T = CECT 8280T).

**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.

**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).

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 FB206T, 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

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**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 ≥50% are indicated). Bar, 1 substitution per 100 nt positions.
included R. fabae LMG 23997T, Rhizobium pisi DSM 30132T, Rhizobium phaseoli ATCC 14482T and Rhizobium etli CFN 42T 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](image_url). 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 ≥ 50 % are indicated). Bar, 1 substitutions per 100 nt positions.

**S. Saidi and others**
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 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 2370T and R. indigofereae CCBAU 71042T, 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 was 60.3 mol%, which is within the range reported for other species of the genus Rhizobium (Ramirez-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 (Ramirez-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 MgSO4, 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 MgSO4·7H2O 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-Martinez 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’re.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).

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-glucosidas, α- and β-galactosidas, α-glucosaminidas, α- and β-fucosidas, α- and β-mannosidas, α- and β-xilosidas, α- and β-arabinosidas, α-rhamnosidas, and acid and alkaline phosphatases using chromogenic para-nitrophenyl substrates. The production of β-galactosaminidas and β-cellobioas is variable. The production of galacturonidas, glucuronidas, lactosidas and α- and β-maltosidas is negative in most of strains. The production of indole, arginine dehydrodase and gelatinase is negative in API 20NE tests. Glucose, L-arabinose, mannose, mannotol, 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, mannotol, glucose, salicin, melibiose, L-fucose, L-sorbos, L-arabinos, L-histidine, 3-hydroxybutyrte and L-proline is positive, but assimilation of itaconate, suberate, malonate, L-alanine, glycogone, 3-hydroxybenzoate, L-serine, propionate, caprate, valerate and citrate is negative; variable results are found in the case

**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 2370T; 3. *R. indigofereae* CCBAU 71042T; 4. *R. pisi* DSM 30132T; 5. *R. fabae* LMG 23997T. All data are from this study. +, Positive; −, negative; w, weakly positive; PNP, para-nitrophenyl.

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<td>Hydrolysis of:</td>
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<td>PNP-α-maltopyranoside</td>
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<td>PNP-β-β-maltopyranoside</td>
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<td>Growth in 0.8% NaCl</td>
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<td>Acid production from (72 h):</td>
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<td>l-Ribose</td>
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<td>l-Rhamnose</td>
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<td>Assimilation of (API 32GN):</td>
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<td>l-Alanine</td>
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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, polymixin B, oxitetracycline, cefuroxime and gentamicin; weakly sensitive to neomycin and erythromycin; and resistant to penicillin and claxocillin. Resistance to ampicillin is variable. All strains form effective nodules in *Vicia faba*.

The type strain FB206T (=LMG 27434T=CECT 8280T) 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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