Rhizobium acidisoli sp. nov., isolated from root nodules of *Phaseolus vulgaris* in acid soils

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Two Gram-negative, aerobic, non-motile, rod-shaped bacterial strains, FH13^T and FH23, representing a novel group of *Rhizobium* isolated from root nodules of *Phaseolus vulgaris* in Mexico, were studied by a polyphasic analysis. Phylogeny of 16S rRNA gene sequences revealed them to be members of the genus *Rhizobium* related most closely to '*Rhizobium anhuiense*' CCBAU 23252 (99.7 % similarity), *Rhizobium leguminosarum* USDA 2370^T (98.6 %), and *Rhizobium sophorae* CCBAU 03386^T and others ($\leq$98.3 %). In sequence analyses of the housekeeping genes *recA, glnII* and *atpD,* both strains formed a subclade distinct from all defined species of the genus *Rhizobium* at sequence similarities of 82.3–94.0 %, demonstrating that they represented a novel genomic species in the genus *Rhizobium*. Mean levels of DNA–DNA relatedness between the reference strain FH13^T and the type strains of related species varied between 13.0$\pm$2.0 and 52.1$\pm$1.2 %. The DNA G+C content of strain FH13^T was 63.5 mol% (T_m). The major cellular fatty acids were 16 : 0, 17 : 0 anteiso, 18 : 0, summed feature 2 (12 : 0 aldehyde/unknown 10.928) and summed feature 8 (18 : 1ω7*c*). The fatty acid 17 : 1ω5*c* was unique for this strain. Some phenotypic features, such as failure to utilize adonitol, L-arabinose, D-fructose and D-fucose, and ability to utilize D-galacturonic acid and itaconic acid as carbon source, could also be used to distinguish strain FH13^T from the type strains of related species. Based upon these results, a novel species, *Rhizobium acidisoli* sp. nov., is proposed, with FH13^T (=CCBAU 101094^T=HAMBI 3626^T=LMG 28672^T) as the type strain.

Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization; ML, maximum-likelihood; MLSA, multilocus sequence analysis; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *atpD*, *recA*, *glnII* and *nodC* gene sequences of strain FH13^T are KJ921033, KJ921069, KJ921098, KJ921080 and KJ921061, respectively. That for the whole genome sequence is PRJNA295402 (http://www.ncbi.nlm.nih.gov/bioproject/PRJNA295402).

Two supplementary tables and 11 supplementary figures are available with the online Supplementary Material.

The genus *Rhizobium* was proposed for a group of fast-growing, nodule-forming bacteria and, at the time of writing, about 85 species have been described, including the recently reported *Rhizobium sophorae*, '*Rhizobium saphorirradicis*' (Jiao *et al.*, 2015), *Rhizobium capsici* (Lin *et al.*, 2015), '*Rhizobium anhuiense*' (Zhang *et al.*, 2015) and '*Rhizobium lentis*' (Rashid *et al.*, 2015). Most have been isolated from nodules of leguminous plants or the roots of cereals as symbiotic or associated nitrogen-fixing partners. Based upon its polyphyletic characteristics, reclassification of species in this genus into the genera *Agrobacterium*,

Allorhizobium, *Neorhizobium*, *Rhizobium* and *Pararhizobium* has been suggested recently (Mousavi *et al.*, 2014, 2015).

Phaseolus vulgaris (L.), commonly known as bean or common bean, is a legume species that originated in Mexico and the Andes. It has been cultivated worldwide as a grain or vegetable crop, and forms root nodules in different regions with a wide range of symbiotic nitrogen-fixing bacteria belonging to the genera *Rhizobium*, *Ensifer* and *Burkholderia* (Talbi *et al.*, 2010; Aserse *et al.*, 2012; Mnasri *et al.*, 2012). In a previous study on the diversity of *Rhizobium* in different Mexican soils, a group of unidentified rhizobia with 22 strains related to *Rhizobium leguminosarum* was detected from acid soils based upon 16S rRNA gene sequence phylogeny (Verástegui-Valdés *et al.*, 2014). In a subsequent analysis of *recA* gene sequences, two strains (FH13^T and FH23) comprised a sublineage in the novel group differing from all recognized species within the *R. leguminosarum* complex. To clarify their exact taxonomic position, strains FH13^T and FH23 were further characterized by using a polyphasic approach, including phylogenetic analyses based on the 16S rRNA gene, housekeeping genes *atpD* (coding for ATP synthase β subunit), *glnII* (coding for glutamine synthetase II) and *recA* (coding for DNA recombinase A), symbiotic genes *nodC* (coding for *N*-acetylglucosaminyltransferase) and *nifH* (coding for nitrogenase reductase), genomic DNA–DNA hybridization (DDH), and phenotypic analyses.

Strains FH13^T and FH23 were isolated previously (Verástegui-Valdés *et al.*, 2014) from root nodules of trap plants of *Phaseolus vulgaris* grown in acid soils using standard procedures (Vincent, 1970) on peptone yeast (PY) agar (peptone, 5 g; yeast extract, 3 g; $CaCl_2$, 0.6 g; agar, 18 g; pH 7.0) plates incubated at 28 °C. Several reference strains of closely related species were obtained, including *Rhizobium phaseoli* ATCC 14482^T, *R. leguminosarum* USDA 2370^T, *Rhizobium fabae* CCBAU 33208^T, *Rhizobium laguerreae* FB206^T, *Rhizobium pisi* DSM 30132^T, *Rhizobium etli* CFN 42^T and '*R. anhuiense*' CCBAU 23252. All the strains were maintained on PY agar at 4 °C (less than 1 month) and in PY broth supplemented with 20 % (w/v) glycerol at −80 °C for long-term storage.

Genomic DNA of each isolate was extracted from 5 ml of a tryptone yeast (TY) broth culture (peptone in PY replaced with tryptone, incubated at 28 °C for 24 h with shaking at 160 r.p.m.) by the guanidine thiocyanate method (Terefework *et al.*, 2001). Amplification and commercial sequencing of 16S rRNA, *atpD*, *recA* and *glnII* genes for isolates FH13^T and FH23 were carried out according to Vinuesa *et al.* (2005), using the corresponding primers (Table S1, available in the online Supplementary Material). The obtained sequences were compared with those in GenBank by BLAST searches (Altschul *et al.*, 1997). To establish the phylogenetic position of the novel strains within the genus *Rhizobium*, the acquired sequences were aligned with those of the type strains of *Rhizobium* species using the CLUSTAL X (2.0) program (Thompson *et al.*, 1997), and the presence of chimeric sequences was checked with the RDP Chimera Check program. Problem sequences were manually edited with SEAVIEW software (Galtier *et al.*, 1996). The program jMODELTEST 3.06 (Posada, 2008) was used to select appropriate models of sequence evolution by the Akaike information criterion. Neighbour-joining (NJ) (Saitou & Nei, 1987) and maximum-likelihood (ML) (Rogers & Swofford, 1999) phylogenies were reconstructed with MEGA 5.05 software (Tamura *et al.*, 2011) and PhyML (http://www.atgc-montpellier.fr/phyml) (Guindon & Gascuel, 2003), respectively. Node support was evaluated with bootstrap analysis using 1000 pseudoreplicates in the NJ tree and by the Shimodaira–Hasegawa-like approximate likelihood-ratio test in the ML tree (Guindon *et al.*, 2010). The corresponding sequence from *Bradyrhizobium japonicum* USDA 6^T (=LMG 6138^T) was used as an outgroup. Similarities among sequences were calculated using the MatGAT v.2.01 software (Campanella *et al.*, 2003).

The acquired 16S rRNA gene sequences of strains FH13^T and FH23 were almost identical. The phylogeny of the 16S rRNA gene (1321 bp fragment) sequences firmly identified the novel group represented by strains FH13^T and FH23 as a member of the genus *Rhizobium* based on both the NJ (Fig. 1) and the ML (Fig. S1) trees. Strains FH13^T and FH23 showed highest 16S rRNA gene sequence similarity with '*R. anhuiense*' CCBAU 23252 (99.7 %), followed by *R. leguminosarum* USDA 2370^T (98.6 %), *R. sophorae* CCBAU 03386^T, *R. phaseoli* ATCC 14482^T, *R. fabae* CCBAU 33202^T and *R. pisi* DSM 30132^T (98.3 %), *R. laguerreae* FB206^T (97.8 %) and *R. etli* CFN 42^T (97.7 %) (Table 1). All of these species are members of the recently revised genus *Rhizobium* (Mousavi *et al.*, 2014, 2015). Taking the threshold of 97 % similarity for species delineation (Graham *et al.*, 1991), all these species were closely related rhizobia and 16S rRNA gene sequence analysis was unable to differentiate them clearly.

Multilocus sequence analysis (MLSA) of housekeeping genes is a valuable technique for investigating taxonomic relationships (Martens *et al.*, 2008). Therefore, it was used to define the taxonomic position of the novel strains in the present study. In the NJ and ML phylogenetic trees reconstructed based on *recA* (479 bp), *glnII* (578 bp), *atpD* (479 bp) (Figs S2–S7) and concatenated *recA–glnII–atpD* (1540 bp) gene sequences (Fig. 2), strains FH13^T and FH23 formed a distinct lineage in the clade represented by *R. leguminosarum* USDA 2370^T, including also *R. fabae* CCBAU 33208^T, *R. laguerreae* FB206^T, *R. pisi* DSM 30132^T, *R. sophorae* CCBAU 03386^T and '*R. anhuiense*' CCBAU 23252. Levels of similarity in the housekeeping genes with those of the most closely related *Rhizobium* species ranged from 82.3 to 94.0 % (Table 1), indicating that strains FH13^T and FH23 represented a novel species, as similarities below 96.0 % in MLSA have been observed between several recently described

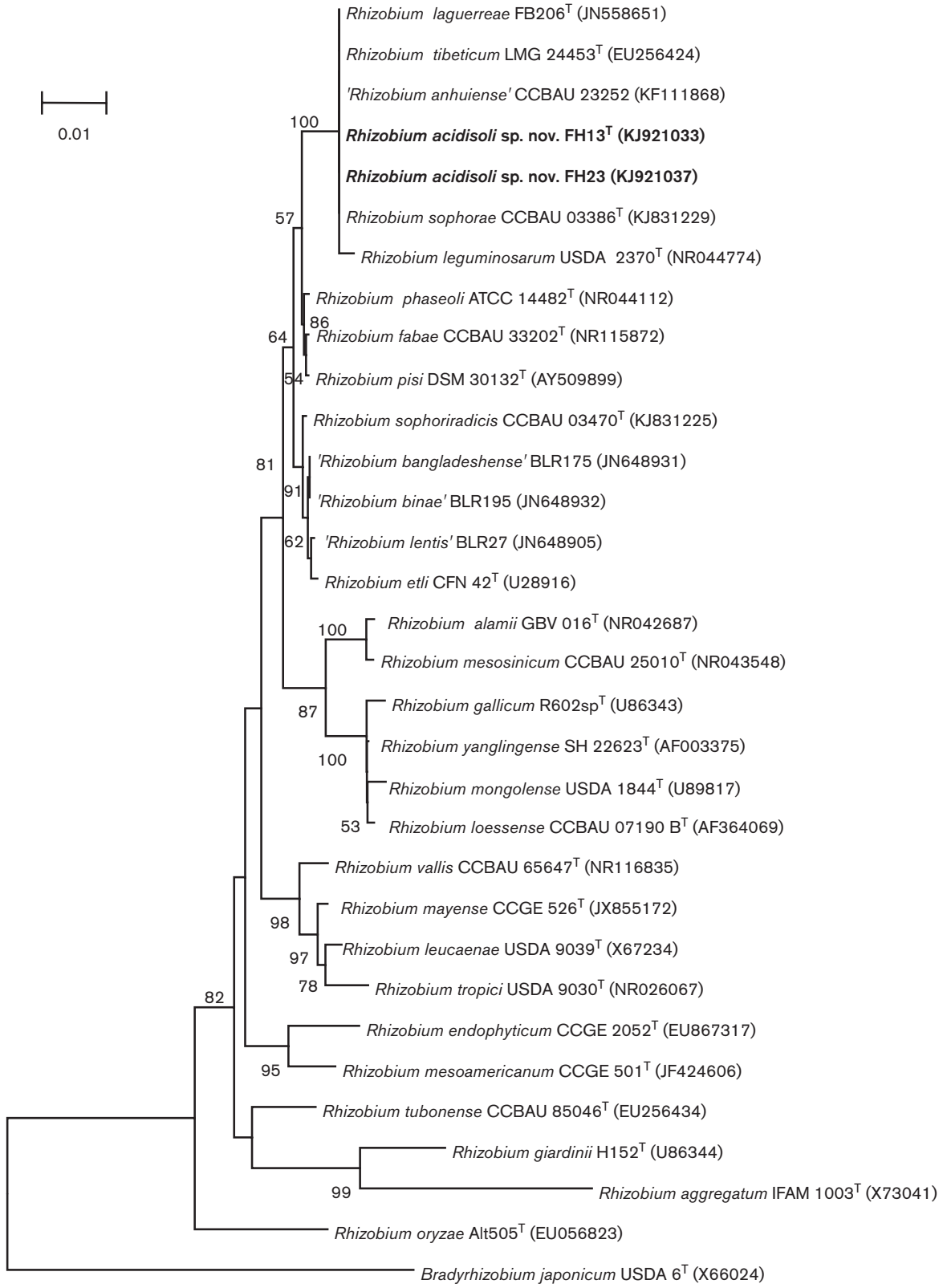

Fig. 1. NJ tree based 16S rRNA gene sequences (1321 nt) of strain FH13^T (*Rhizobium acidisoli* sp. nov.) and closely related *Rhizobium* species. Number above branches indicates bootstrap support (>50 %). *B. japonicum* LMG 6138^T was included as the outgroup. Bar, 1 % nucleotide substitution.

Table 1. Genomic comparison of strains FH13^T and FH23 with strains of related species of the genus *Rhizobium*

ND, Not determined in this study.

	Sequence similarity (%)							
	rRNA	*recA*	*glnII*	*atpD*	MLSA	DDH relatedness (%)*	Genome ANI (%) with FH23	DNA G+C content (mol%) (T_m)
R. acidisoli sp. nov. FH13^T	100	100	100	100	100	100	ND	63.5
R. acidisoli sp. nov. FH23	100	98.9	100	98.9	99.3	95.6±2.2	100	63.6
R. phaseoli ATCC 14482^T	98.3	90.7	92.7	94.1	92.3	45.0±0.6	88.10	ND
R. leguminosarum USDA 2370^T	98.6	93.6	92.3	89.5	94	52.0±1.2	ND	ND
R. l. sv. viciae 3841	ND	ND	ND	ND	ND	ND	87.75	ND
R. l. sv. phaseoli 4292	ND	ND	ND	ND	ND	ND	88.38	ND
R. fabae CCBAU 33208^T	98.3	91.3	94.5	93.7	91.5	36.3±0.9	ND	ND
R. laguerreae FB206^T	97.8	93	87.7	87.4	92.7	43.8±3.0	ND	ND
R. pisi DSM 30132^T	98.3	93	91.3	92.8	93.1	39.1±1.4	ND	ND
R. etli CFN 42^T	97.7	90.4	93.2	86.1	92.1	31.5±2.6	87.31	ND
'*R. anhuiense*' CCBAU 23252	99.7	93.8	84.3	94.8	91.9	24.0±2.0	87.99	ND
R. sophorae CCBAU 03386^T	98.3	73.6	80.2	81.3	82.3	14.6±2.6	ND	ND
R. mongolense USDA 1844^T	95.1	82.3	78.6	83.6	83.6	ND	78.96	ND

*Measured spectrophotometrically (values are mean±SD).

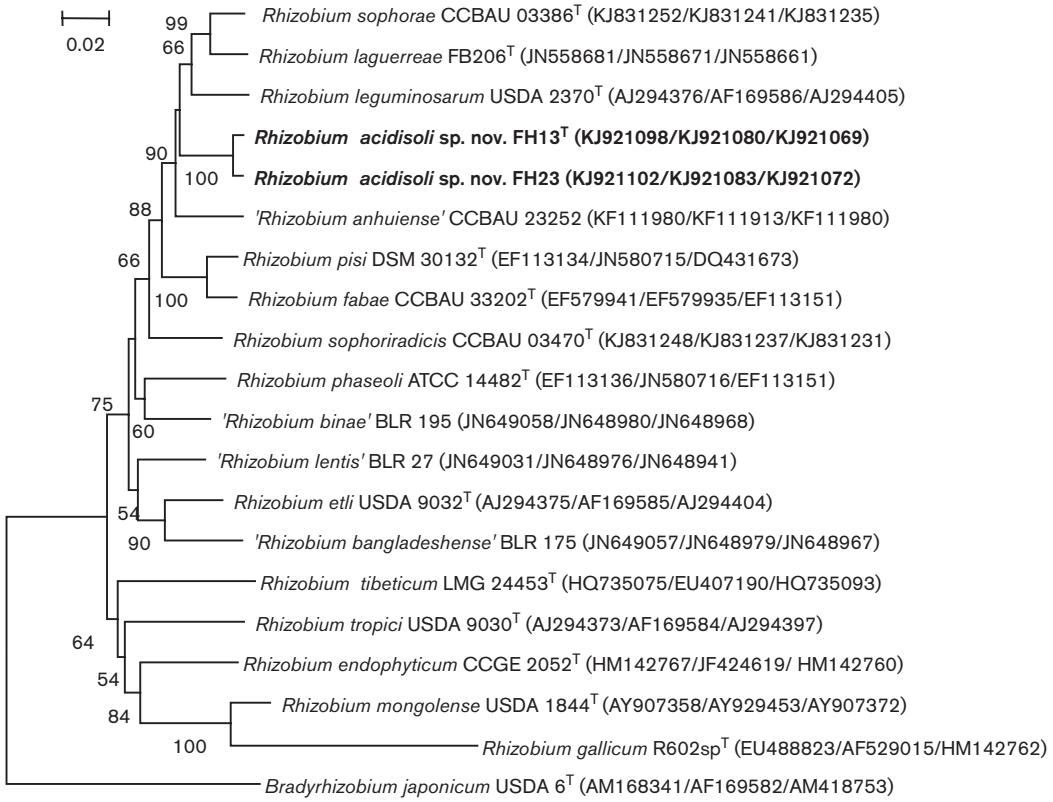

Fig. 2. Phylogenetic tree based on concatenated sequences of the housekeeping genes *recA*, *glnII* and *atpD* showing the relationships between strain FH13^T (*Rhizobium acidisoli* sp. nov.) and related *Rhizobium* species. The tree was reconstructed with the NJ method with Kimura two-parameter distances. Number above branches indicates bootstrap support (>50 %). *B. japonicum* USDA 6^T (=LMG 6138^T) was included as the outgroup. Bar, 2 % sequence divergence.

Table 2. Cellular fatty acid profiles of the novel isolates and type strains of related *Rhizobium* species

Strains: 1, FH13^T; 2, FH23; 3, *R. phaseoli* ATCC 14482^T; 4, *R. leguminosarum* USDA 2370^T; 5, *R. fabae* CCBAU 33202^T; 6, *R. laguerreae* FB206^T; 7, *R. pisi* DSM 30132^T; 8, *R. etli* CFN 42^T; 9, '*R. anhuiense*' CCBAU 23252. −, Not detected.

Fatty acid*	1	2	3	4	5	6	7	8	9
9 : 0	−	−	−	−	−	0.7	−	−	−
12 : 0 anteiso	1.0	−	−	1.3	−	−	−	−	−
12 : 0	−	0.6	0.4	−	−	0.6	−	0.4	0.3
13 : 0 anteiso	1.1	−	−	1.50	−	−	−	−	0.3
14 : 0 anteiso	1.6	−	−	2.2	−	−	−	−	0.4
14 : 0	0.9	1.6	1.8	1.4	1.0	2.1	0.5	1.7	1.1
15 : 0 iso	0.3	−	−	−	1.0	−	−	2.4	−
15 : 0 anteiso	1.8	1.2	1.1	1.9	4.4	0.6	0.5	8.5	0.9
15 : 1ω5*c*	0.2	−	−	−	−	−	−	−	−
16 : 0 N-alcohol	−	−	−	−	−	−	−	0.6	−
16 : 0 iso	−	−	−	−	0.8	−	−	1.3	−
16 : 0 anteiso	1.6	−	0.7	1.6	0.5	−	−	1.3	0.3
14 : 0 3-OH/16 : 1 iso I	−	6.4	−	−	−	−	−	−	−
16 : 0	11.9	29.4	29.0	22.6	18.9	40.2	9.3	25.4	18.6
15 : 0 2-OH	1.3	0.9	−	−	1.0	0.5	1.3	−	1.3
17 : 0 anteiso	7.8	1.0	4.4	6.9	5.0	−	−	8.8	2.1
17 : 0 cyclo	−	−	−	1.06	−	−	−	−	−
17 : 1ω8*c*	4.5	−	5.7	5.3	3.2	−	−	7.9	−
17 : 1ω5*c*	0.7	−	−	−	−	−	−	−	−
17 : 0	−	−	0.8	1.3	0.6	−	−	−	−
18 : 1 iso H	−	−	1.3	1.4	−	−	−	1.2	−
16 : 0 3-OH	2.9	2.1	1.2	−	1.8	2.0	−	0.9	2.4
18 : 0	10.8	12.9	17.1	17.1	15.1	15.3	10.3	13.6	21.6
18 : 1ω7*c* 11-methyl	2.4	2.1	1.1	2.6	4.6	2.3	12.1	−	6.3
17 : 0 iso 3-OH	0.4	−	0.8	0.5	−	0.4	−	−	−
17 : 0 2-OH	0.6	−	−	0.8	−	−	−	0.5	−
19 : 0 iso	−	−	−	−	−	−	−	−	0.5
19 : 0 cyclo ω8*c*	−	−	−	1.9	−	1.1	3.2	−	4.3
18 : 0 3-OH	−	2.2	−	−	1.8	2.0	3.0	−	2.8
20 : 2ω6,9*c*	−	−	−	−	−	−	2.8	−	−
20 : 0	0.3	−	−	0.4	−	0.3	−	−	0.3
Summed feature 2	4.5	6.4	3.8	3.4	5.6	5.4	13.7	2.2	7.9
Summed feature 3	0.9	5.1	0.5	1.0	1.0	0.7	1.0	1.4	0.5
Summed feature 7	−	−	−	1.6	−	−	−	0.6	−
Summed feature 8	40.5	34.4	30.3	22.0	33.5	25.6	40.3	21.0	27.9

*Summed feature 2, 12 : 0 aldehyde/unknown ECL 10.928; summed feature 3, 16 : 1ω7*c*/16 : 1ω6*c*; summed feature 7, 19 : 1ω7*c*/19 : 1ω6*c*; summed feature 8, 18 : 1ω7*c*.

Rhizobium species and related species (Jiao *et al.*, 2015; Lin *et al.*, 2015; Rashid *et al.*, 2015; Zhang *et al.*, 2015).

DDH has been used as the gold standard for numerical circumscription of genospecies in the classification of prokaryotes (Roselló-Morá *et al.*, 2011). In the present study, genomic DNA from strains FH13^T and FH23 and the reference strains was extracted following the protocols described by Marmur (1961). The DNA G+C contents of strains FH13^T and FH23 were determined using the thermal denaturation method (T_m) with DNA of *Escherichia coli* K-12 as a standard (Marmur & Doty, 1962). DNA–DNA relatedness was determined using the spectrophotometric method (De Ley *et al.*, 1970). DDH techniques are based on an attempt to make raw comparisons of whole genomes between different organisms in order to calculate their overall genomic similarities, and it has been suggested as a standard technique for the description of novel species (Graham *et al.*, 1991). The level of DNA–DNA relatedness between strains FH13^T and FH23 was 95 %, confirming that they are members of the same genomic species, while strain FH13^T showed 13–52 % relatedness with the reference type strains of closely related *Rhizobium* species (Table 1), values below the threshold of 70 % for species delineation (Wayne *et al.*, 1987).

To further confirm the genetic relationships between the novel group and the related species, the average nucleotide

identity (ANI) between the genomes was calculated. The genome of strain FH23 was sequenced by Novogene using the Illumina Miseq platform. Five related genome sequences (Table 1) were downloaded from the GenBank database, while the genome sequence of '*R. anhuiense*' CCBAU 23252 was provided by Dr J. P. W. Young. The Legacy BLAST kit v2.2.26 (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/release/2.2.26/) along with the software Jspecies v1.2.1 (Richter & Rosselló-Móra, 2009) was used for ANI calculation. Each of the seven different bacterial genomes was compared against the others in bidirectional assays. ANI produced by BLAST (ANIb) was chosen for the test. The ANI values between strain FH23 representing the novel group and the type or reference strains for *R. leguminosarum* (sv. phaseoli 4292/sv. viciae 3841), *R. phaseoli* ch24_10, '*R. anhuiense*' CCBAU 23252, *R. etli* CFN 42^T and *Rhizobium mongolense* USDA 1844^T were 88.37/87.75, 88.10, 87.99, 87.31 and 78.96 %, respectively (Table 1). These values were consistent with the phylogenetic relationships revealed by MLSA (Fig. 1) and lower than the species threshold of 95 % (Richter & Rosselló-Móra, 2009), indicating that the novel group represents a distinct species as reported recently in the description of defined species (Rashid *et al.*, 2015; Zhang *et al.*, 2015). Strains FH13^T and FH23 had DNA G+C contents of 63.5 and 63.6 mol%, which are within the range reported for the genus *Rhizobium* (Ramírez-Bahena *et al.*, 2008).

Fatty acid profile is commonly used for describing and characterizing novel rhizobial species (Quan *et al.*, 2005). For cellular fatty acid determination, cells of strain FH13^T and the reference strains were grown at 28 °C on yeast mannitol agar (YMA) for 3 days. Cellular fatty acids were obtained from cells by extraction and methylation according to the MIDI method (Sasser, 1990) and then were identified and quantified with the MIDI System software (ver. 6.0, Aerobe RTSBA6 method and RTSBA6 database) (Tighe *et al.*, 2000). A total of 23 and 14 fatty acids were detected from strains FH13^T and FH23, respectively, while 11–22 were detected from the seven reference strains. The presence of 14 : 0, 15 : 0 anteiso, summed feature 2 and summed feature 3 as minor components (0.5–7.9 %, except 13.7 % for summed feature 2 for *R. pisi* DSM 30132^T), and of 16 : 0, 18 : 0 and summed feature 8 as major components (10.8–40.5 %, except 9.3 % for 16 : 0 for *R. pisi* DSM 30132^T) were common for all nine strains. Only the trace components 15 : 1ω5*c* (0.2 %) and 17 : 1ω5*c* (0.7 %) for FH13^T and the minor component 14 : 0 3-OH/16 : 1 iso I for FH23 (6.4 %) were detected as unique fatty acids for the novel isolates (Table 2). The results demonstrated that the fatty acid profiles had little value for distinguishing these closely related *Rhizobium* species.

As another chemical character for bacterial taxonomy, polar lipids were extracted according to the method of Minnikin *et al.* (1984) and identified after separation with two-dimensional TLC followed by spraying of the chromatogram with appropriate detection reagents (Collins & Jones, 1980). Compared with the reference strains, the main polar lipids of strain FH13^T were phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), diphosphatidylglycerol (DPG= CL, cardiolipin) and a unknown compound (UN) (Fig. S8). This composition was similar to that of the related species (Rozahon *et al.*, 2014; Zhang *et al.*, 2014).

Although symbiotic characteristics are not considered for species differentiation, the phylogenetic relationships of the symbiotic genes were important for estimating the symbiovar in the rhizobia. Therefore, the *nifH* and *nodC* genes were amplified from strain FH23 using the primer pair nifHF/nifHI and the method described by Laguerre *et al.* (2001) and primer pair nodC540/nodC1160 (Sarita *et al.*, 2005), respectively. The method for phylogenetic analysis of these two genes was the same as for the 16S rRNA gene, and NJ phylogenetic trees were reconstructed based on the nucleotide sequences. The nodulation test was performed with the same method as used for rhizobial trapping (Verástegui-Valdés *et al.*, 2014), but the vermiculite/peat moss/soil (2 : 2 : 1, v/v) mixture was sterilized and each seedling was inoculated with 0.1 ml (approx. 10^7 c.f.u.) of a 24 h old PY broth culture.

The *nodC* (596 bp) and *nifH* (450 bp) genes were amplified and sequenced successfully for the representative isolate FH23. The *nodC* phylogenetic tree showed that the novel strain formed a clade together with the *P. vulgaris*-nodulating rhizobia designated symbiovar (sv.) *phaseoli* (Fig. S9). The close relationships between isolate FH23 and *R. phaseoli* ATCC 14482^T and *R. sophoriradicis* CCBAU 03740^T for the symbiotic genes was also revealed in the *nifH* phylogenetic tree (Fig. S10). Note that no nodule was observed on the bean seedlings of cv. Flor de Mayo grown in the sterilized acid or alkaline soils inoculated with strain FH23. This unexpected nodulation failure implied that some unknown biological factors may be necessary for the nodulation of the strains in this novel group.

Colony morphology was observed on well-separated colonies grown on YMA for 2–3 days at 28 °C. Motility was determined by observing growth of cells in test tubes containing semisolid YMA medium with 0.5 % agar after 3 days of incubation at 28 °C (Cowan & Steel, 1965). For carbon source utilization, strains FH13^T and FH23 and *R. phaseoli* ATCC 14482^T, *R. leguminosarum* USDA 2370^T, *R. fabae* CCBAU 33208^T, *R. laguerreae* FB206^T, *R. pisi* DSM 30132^T, *R. etli* CFN 42^T and '*R. anhuiense*' CCBAU 23252 were tested by using GN2 MicroPlates (Biolog) according to the manufacturer's instructions. The temperature range for growth was determined on YMA plates incubated at different temperatures (4, 10, 20, 28, 37 °C). The range of pH for growth was determined using YMA medium of pH 4.0–11.0 (at increments of 1 pH unit), adjusted by using 1 M HCl or 1 M NaOH after autoclaving. The salinity range for growth was determined on plates containing 25 ml YMA medium supplied with

0–5 % (w/v) NaCl (at increments of 0.5 %), with incubation at 28 °C for 3 days. Resistance to antibiotics was determined on YMA plates supplemented with 5, 10, 50 and 100 mg ml^{-1} of tetracycline hydrochloride, neomycin sulfate, kanamycin sulfate and gentamicin sulfate, incubated at 28 °C for 3 days. The following parameters also were included for phenotypic characterization, using the methods described by Cowan & Steel (1965): activities of catalase, cytochrome oxidase and urease; H_2S production; Tween 20, Tween 80 and starch hydrolysis; Voges–Proskaüer reaction; citrate assimilation; and nitrate and nitrite reduction. Cell size and appearance were visualized using a Hitachi S-3400 scanning electron microscope, with the sample prepared using the protocol described by Jiao *et al.* (2015).

Colonies of strains FH13^T and FH23 were circular, smooth, convex and white after 3 days of incubation on YMA medium. Cells were Gram-negative, motile, rod-shaped, 1.2–2.4 μm in length and 0.5–0.8 μm in diameter (Fig. S11). Strain FH13^T was able to grow at 20–37 °C and at pH 6.0–10.0, but was unable to grow on YMA supplemented with more than 1 % (w/v) NaCl. Positive results were obtained for oxidase, urease, hydrolysis of Tween 20 and nitrite reduction, but negative results for starch hydrolysis, catalase, Tween 80 hydrolysis, nitrate reduction, growth on nutrient agar, citrate assimilation, H_2S production and Voges–Proskaüer reaction. Substrate assimilation could be used to distinguish strain FH13^T from the reference strains *R. phaseoli* ATCC 14482^T, *R. leguminosarum* USDA 2370^T, *R. fabae* CCBAU 33208^T, *R. laguerreae* FB206^T, *R. pisi* DSM 30132^T, *R. etli* CFN 42^T and '*R. anhuiense*' CCBAU 23252 based on 75 features, whereas 21 were common to all the strains tested; further details are given in the species description and in Table S2. Strain FH13^T was resistant to neomycin sulfate at 5, 10 and 50 μg ml^{-1}, and to gentamicin sulfate at 5 and 10 μg ml^{-1}, but it was sensitive to tetracycline hydrochloride and kanamycin sulfate at 5 μg ml^{-1}. Comparisons of antibiotic resistance for FH13^T and the reference strains are also provided in Table S2.

Based on the results obtained, strains FH13^T and FH23 should be considered as members of a novel *Rhizobium* species that is genotypically and phenotypically differentiated from closely related species via morphology, biochemical characterization, DDH and fatty acid profiles and by phylogenetic comparison of 16S rRNA and housekeeping genes. The name *Rhizobium acidisoli* sp. nov. is proposed for this novel species.

Description of *Rhizobium acidisoli* sp. nov.

Rhizobium acidisoli (a.ci.di.so′li. L. adj. *acidus* acidic; L. n. *solum* soil; N.L. gen. n. *acidisoli* of acidic soil).

Cells are Gram-negative, aerobic, non-motile and rod-shaped (1.20–2.44 × 0.47–0.78 μm). Colonies are circular, smooth, convex and white after 3 days of incubation on YMA medium at 28 °C, which is the optimum temperature. Grows at 20–37 °C and at pH 5.0 (rarely at pH 4.5) to pH 10.0, but cannot grow on YMA supplemented with more than 1 % (w/v) NaCl. Oxidase, urease, hydrolysis of Tween 20 and nitrite reduction are positive. Starch hydrolysis, catalase, Tween 80 hydrolysis, nitrate reduction, growth on nutrient agar, citrate assimilation, H_2S production and Voges–Proskaüer reaction are negative. Can use D-arabitol, cellobiose, i-erythritol, D-galactose, gentobiose, *myo*-inositol, D-mannitol, melibiose, raffinose, D-sorbitol, sucrose, trehalose, turanose, xylitol, monomethyl succinate, acetic acid, citric acid, D-gluconic acid, D-glucosaminic acid, itaconic acid, α-ketovaleric acid, bromosuccinic acid, glucuronamide, D-alanine, L-alanyl glycine, L-aspartic acid, L-glutamic acid, glycyl L-glutamic acid and DL-α-glycerol phosphate as carbon sources; glycogen, methyl β-D-glucoside, methyl pyruvate, formic acid, D-glucuronic acid, γ-hydroxybutyric acid, α-ketobutyric acid, D-saccharic acid, glycyl L-aspartic acid, L-histidine, L-pyroglutamic acid, L-threonine, uronic acid and thymide are utilized weakly as sole carbon source. The major cellular fatty acids are 16 : 0, 17 : 0 anteiso, 18 : 0, summed feature 2 (12 : 0 aldehyde/unknown ECL 10.9) and summed feature 8 (18 : 1ω7c).

The type strain, FH13^T (=CCBAU 101094^T=HAMBI 3626^T=LMG 28672^T), was isolated from root nodule of *Phaseolus vulgaris* grown in acid soil from Acaxochitlán, Hidalgo, México. The DNA G+C content of the type strain is 63.5 mol% (T_m).

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