Reclassification of *Rhizobium tropici* type A strains as *Rhizobium leucaenae* sp. nov.

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Rhizobium tropici is a well-studied legume symbiont characterized by high genetic stability of the symbiotic plasmid and tolerance to tropical environmental stresses such as high temperature and low soil pH. However, high phenetic and genetic variabilities among *R. tropici* strains have been largely reported, with two subgroups, designated type A and B, already defined within the species. A polyphasic study comprising multilocus sequence analysis, phenotypic and genotypic characterizations, including DNA–DNA hybridization, strongly supported the reclassification of *R. tropici* type A strains as a novel species. Type A strains formed a well-differentiated clade that grouped with *R. tropici*, *Rhizobium multihospitium*, *Rhizobium miluonense*, *Rhizobium lusitanum* and *Rhizobium rhizogenes* in the phylogenies of the 16S rRNA, *recA*, *gltA*, *rpoA*, *glnII* and *rpoB* genes. Several phenotypic traits differentiated type A strains from all related taxa. The novel species, for which the name *Rhizobium leucaenae* sp. nov. is proposed, is a broad host range rhizobium being able to establish effective root-nodule symbioses with *Leucaena leucocephala*, *Leucaena esculenta*, common beans (*Phaseolus vulgaris*) and *Gliricidia sepium*. Strain CFN 299^T (=USDA 9039^T=LMG 9517^T=CECT 4844^T=JCM 21088^T=IAM 14230^T=SEMIA 4083^T=CENA 183^T=UMR1026^T=CNPSo 141^T) is designated the type strain of *Rhizobium leucaenae* sp. nov.

A group of bacterial species, collectively known as rhizobia, can induce the formation of specific structures, named nodules, on the roots of legumes eliciting a symbiotic process in which the rhizobia fix atmospheric nitrogen and supply it to the plant. Interest in the use of rhizobia as biofertilizers in agriculture has promoted studies on their diversity and the description of a large number of rhizobial species. *Rhizobium tropici* is a broad host range rhizobial species that was isolated from *Leucaena* spp. nodules in Brazil (Hungria *et al.*, 2000; Martínez-Romero *et al.*, 1991), and from *Gliricidia sepium* (Acosta-Durán & Martínez-Romero, 2002) and *Acaciella angustissima* (Rincón-Rosales *et al.*, 2009) in Mexico. The species has also been isolated from common bean (*Phaseolus vulgaris*) nodules in several countries (Amarger *et al.*, 1994; Anyango *et al.*, 1995; Diouf *et al.*, 2000; Grange & Hungria, 2004; Pinto *et al.*, 2007). *R. tropici* has been used as an efficient inoculant for beans in the tropics, due to its high tolerance to environmentally stressful conditions and its high genetic stability (Hungria *et al.*, 2000, 2003).

R. tropici, reported in 1991 (Martínez-Romero *et al.*, 1991), was the first description of a rhizobial species to include 16S rRNA gene sequence analysis. At that time, in spite of evidence that supported the proposal of two species from

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The GenBank/EMBL/DDBJ accession numbers for the 49 sequences reported in this paper are provided in Table S1 (available in IJSEM Online).

Five supplementary tables and seven supplementary figures are available with the online version of this paper.

the nodule isolates analysed, e.g. differences in 16S rRNA gene sequences and low DNA–DNA hybridization values, only one novel species was accepted. However, two types, A and B, were recognized to account for these differences. Other species are now recognized to be close relatives of *R. tropici*, such as *Rhizobium lusitanum* (Valverde *et al.*, 2006), *Rhizobium multihospitium* (Han *et al.*, 2008), *Rhizobium miluonense* (Gu *et al.*, 2008) and *Rhizobium rhizogenes* (Hernández-Lucas *et al.*, 2004; Velázquez *et al.*, 2010). Some of these species are either more closely related to type A or to type B strains in phylogenetic trees (Han *et al.*, 2008; Valverde *et al.*, 2006; Velázquez *et al.*, 2010). Recently, it was recommended that species containing several distinct genotype clusters should be subdivided into multiple species, each corresponding to a single genotype cluster (Achtman & Wagner, 2008). In this study, we review reported differences between the two *R. tropici* groups and present new evidence to suggest that *R. tropici* type A strains belong to a novel species that is distinct from *R. tropici*.

The rhizobial strains used in this study are listed in Table S2 (available in IJSEM Online). Four Brazilian strains (BR 828, BR 10043, CPAO 29.8, 77) were chosen as representatives of a large collection of strains identified as *R. tropici* type A in previous studies (Grange & Hungria, 2004; Martínez-Romero *et al.*, 1991; Mercante *et al.*, 1998; Pinto *et al.*, 2007). The well-studied strain CFN 299^{T} from the type A subgroup was also included and proposed as the type strain of the novel species. Three previously unreported Mexican isolates (CCGE 521, CCGE 522, CCGE 523) obtained from Zacatecas, the largest common-bean-growing area in Mexico, were also included. All strains were deposited at the 'Diazotrophic and Plant Growth Promoting Bacteria Culture Collection' of Embrapa Soja (Londrina, Brazil), and at the Center for Genomic Sciences Culture Collection (Cuernavaca, Mexico). Except where specified, strains were grown on yeast extract-mannitol (YM) broth [as Vincent (1970), except with a mannitol concentration of 5 g l^{-1}] in the dark, at 28 °C. Source cultures were maintained in YM agar (YMA) at 4 °C. Stocks were prepared on YM and kept at −80 °C (in 30 % glycerol) for long-term storage.

Total genomic DNA of each strain was extracted as described before (Kaschuk *et al.*, 2006). Repetitive extragenic palindromic-PCR genomic fingerprints with the BOX-A1R primer were generated and analysed as described by Pinto *et al.* (2007). Genomic fingerprints revealed a group of four Brazilian and three Mexican strains that clustered with CFN 299^{T}, the reference type A strain. This cluster was clearly separated from *R. tropici* type B (Fig. S1, available in IJSEM Online) as well as from other rhizobial type or reference strains (data not shown). In a previous study, Brazilian type A strains CFN 299^{T}, CPAO 29.8 and 77 also clustered separately from *R. tropici* type B in a combined analysis of REP, ERIC and BOX-A1R fingerprints (Pinto *et al.*, 2007).

Besides the 16S rRNA gene phylogeny, a multilocus sequence analysis approach with five protein-coding genes was used to establish the relationships between type A strains and related taxa. Near full-length 16S rRNA gene and fragments of *recA*, *gltA*, *rpoA*, *glnII* and *rpoB* were amplified and sequenced as described previously (Ribeiro *et al.*, 2009; Rincón-Rosales *et al.*, 2009). All these genes have been used previously in studies of rhizobial diversity (Martens *et al.*, 2007; Ribeiro *et al.*, 2009; Rincón-Rosales *et al.*, 2009). Multiple sequence alignments were performed with CLUSTAL_X version 1.83 (Thompson *et al.*, 1997) and manually checked with BioEdit (Hall, 1999). Best-fit models of sequence evolution were selected for each gene, and for the concatenated set of five protein-coding genes with JModelTest 0.1.1, using the Akaike information criterion (Posada, 2008). Maximum-likelihood phylogenies were constructed with PhyML 3 using subtree pruning and regrafting moves to improve tree topology (Guindon *et al.*, 2010). Support for tree nodes was evaluated by the Shimodaira–Hasegawa-like approximate likelihood-ratio test implemented in PhyML.

R. tropici type A strains formed a well-supported clade in the 16S rRNA gene phylogeny and, together with *R. lusitanum*, *R. rhizogenes*, *R. tropici* type B, *R. multihospitium* and *R. miluonense*, constituted a group of closely related species, hereby designated the '*R. tropici* group' (Fig. 1). The 16S rRNA gene sequences of type A strains showed $\geqslant$99.8 % identity to each other and $\leqslant$99.4 % identity with other strains in the '*R. tropici* group' (Table S3, available in IJSEM Online). Willems & Collins (1993) described an insertion of 72 nucleotides in the V1 region of the 16S rRNA gene of strain CFN 299^{T}. Later, it was confirmed that the insertion was present in several type A but not in type B strains (Hungria *et al.*, 2000; van Berkum *et al.*, 1994). In this study, presence of the insertion was confirmed in the Brazilian type A strains CPAO 29.8, 77, BR 10043 and BR 828, and also in the Mexican strains CCGE521, CCGE522 and CCGE523. The insertion was absent in all available sequences from the *Rhizobium* type strains with a complete V1 region sequenced. Thus, the 72 bp insertion seems to be a characteristic of type A strains. Another genetic difference in the ribosomal operon between type A and type B strains was highlighted in a study by Pinto *et al.* (2007), in which strains CFN 299^{T}, CPAO 29.8 and 77 showed similar profiles in the amplified rRNA gene restriction analysis of the 23S rRNA and were separate from the cluster including *R. tropici* CIAT 899^{T}.

Nucleotide similarities between type A strains and other species of the '*R. tropici* group' were less than 94.0, 98.7, 97.2, 93.9 and 94.4 % for the *recA*, *gltA*, *rpoA*, *glnII* and *rpoB* genes, respectively. Except for *R. multihospitium* (*recA*) and *R. lusitanum* (*gltA*), similarities among type A strains were higher than between this group and the other species (Table S3). Type A sequences formed well-supported clades that differed from those containing type B and other species sequences in all single gene phylogenies (Figs S3–S7). In the phylogeny constructed with the

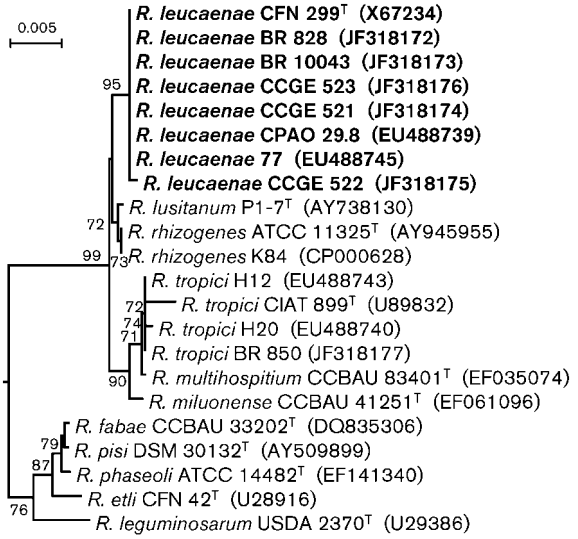

Fig. 1. Part of a maximum-likelihood phylogeny of the 16S rRNA gene showing the relationships between *Rhizobium leucaenae* strains (in bold) and other type or reference strains from closely related species (the complete phylogeny containing a larger number of reference sequences is available as Fig. S2). GenBank accession numbers are shown in parentheses. Only node supports higher than 70 % are shown. Bar, 5 nt substitutions per 1000 nt.

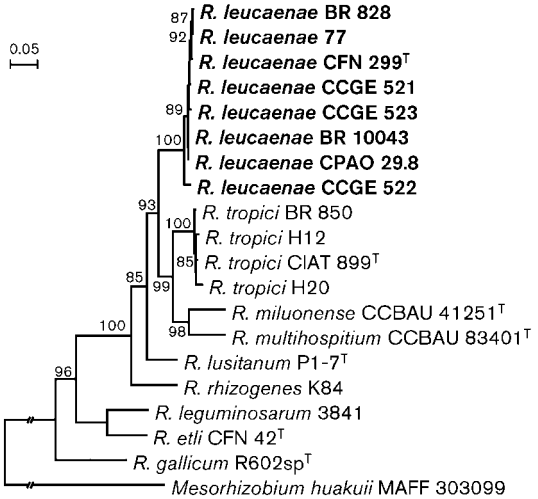

Fig. 2. Maximum-likelihood phylogeny of five concatenated protein-coding genes (*recA*+*gltA*+*rpoA*+*glnII*+*rpoB*) showing the relationships between *Rhizobium leucaenae* strains and other type or reference strains from closely related species. GenBank accession numbers for each gene and strain are given in Table S1. Only node supports higher than 80 % are shown. Bar, 5 nt substitutions per 100 nt.

concatenated alignments (Fig. 2), the type A strains formed a sister clade to a highly supported group containing *R. tropici* type B, *R. multihospitium* and *R. miluonense*. *R. lusitanum* occupied an intermediate position between the above species and *R. rhizogenes*, with the latter being the basal species in the '*R. tropici* group'.

DNA–DNA hybridizations were used to determine the DNA relatedness among type A strains and between this group and the type species of related taxa. A previously described filter hybridization methodology (Martínez-Romero *et al.*, 1991) using ^{32}P-labelled DNA of strain CFN 299^{T} as a probe was used. Type A strains shared high levels of DNA–DNA relatedness ($\geqslant$78.8 %). Strain CFN 299^{T} showed 45.5, 37.5, 38.2, 33.2 and 28.7 % hybridization values with *R. lusitanum* P1-7^{T}, *R. miluonense* CCBAU 41251^{T}, *R. tropici* CIAT 899^{T}, *R. multihospitium* CCBAU 83401^{T} and *R. rhizogenes* IAM 13570^{T}, respectively. These data are consistent with the previously reported 39 % DNA–DNA hybridization value between CFN 299^{T} and CIAT 899^{T} (Martínez-Romero *et al.*, 1991), which is below the threshold for species definition (Coenye *et al.*, 2005; Vandamme *et al.*, 1996). Other studies have also shown low DNA–DNA hybridization values between CFN 299^{T} and strains belonging to the '*R. tropici* group' (Gu *et al.*, 2008; Han *et al.*, 2008; Valverde *et al.*, 2006). All these data support the differentiation of type A strains from all the species in the '*R. tropici* group'.

The DNA G+C contents of three type A strains were estimated according to Moreira *et al.* (2011). *Vibrio furnissii* CAIM 518^{T} was included as a control; the reported DNA G+C content for this strain is 50.4 mol% (Brenner *et al.*, 1983). The analyses were performed at least twice using six replicates in each experiment. The DNA G+C contents of strains CFN299^{T}, CPAO 29.8 and CCGE 522 were 64.1, 63.5 and 63.6 mol%, respectively, which are within the range reported for *Rhizobium* species. The fatty acid profile of the representative type A strain CFN 299^{T}, grown for 48 h on YMA (Vincent, 1970) plates, was determined with the MIDI system using the TSBA6 database. The results obtained (Table S5, available in IJSEM Online) further confirmed the affiliation to the *Rhizobium* genus as common fatty acids found in this genus (Tighe *et al.*, 2000) were also observed in CFN 299^{T}.

Morpho-physiological characterization of the strains included evaluation of colony morphology, acid/alkaline production in YMA, tolerance to various pH and temperature conditions, growth in Luria–Bertani (LB) and peptone-yeast extract (PY) without Ca media, as described previously (Hungria *et al.*, 2000; Martínez-Romero *et al.*, 1991). Growth on selected carbon and nitrogen sources was evaluated as described previously (Martínez-Romero *et al.*, 1991). In addition, bacteria were evaluated for the capacity to utilize the 49 carbohydrates included in the API 50CH kit (bioMérieux) as specified by the manufacturer, using YM-minus-mannitol as the basal medium. Resistance to chloramphenicol (50 μg ml^{-1}) and kanamycin sulfate (5 μg ml^{-1}) in YMA, and to captan (50 μg ml^{-1}) and thiram (25 μg ml^{-1}) in PY agar plates was also evaluated. Captan and thiram are seed-dressing

Table 1. Distinctive phenotypic features of *Rhizobium leucaenae* sp. nov. and phylogenetically related species

Taxa: 1, *R. leucaenae* (former *R. tropici* type A); 2, *R. tropici* (former *R. tropici* type B); 3, *R. lusitanum*; 4, *R. multihospitium*; 5, *R. miluonense*; 6, *R. rhizogenes*. +, Growth, −, no growth; W, weak growth; ND, not determined. Data obtained in this study or compiled from the original studies describing the species, or from Amarger *et al.* (1997), Bouzar *et al.* (1993), and Sawada & Ieki (1992). Only characteristics with consistent data between studies were included.

Characteristic	1	2	3	4	5	6
Utilization as sole carbon source						
Sodium acetate	−	−	+	−	−	−
D-Amygdalin	+	−	+	+	+	+
DL-Arginine	−	−	−	+	+	−
DL-Aspartic acid	−	−	+	+	−	−
Erythritol	−	+	+	+	+	+
Sodium formate	−	−	−	+	−	−
Glycine	−	−	+	−	−	−
Inulin	−	−	+	−	−	−
Malate	−	+	+	+	+	ND
Melezitose	−	−	+	−	−	+
DL-Proline	−	−	+	+	−	−
Sodium pyruvate	+	+	+	+	+	−
Sorbose	+	−	+	+	+	−
Utilization as sole nitrogen source						
D-Threonine	−	−	−	+	−	−
L-Threonine	−	−	−	+	+	−
Growth in/at:						
PY without Ca	−	+	+	+	+	+
LB	−	+	+	+	−	−
37 °C	+	+	+	+	+	−
40 °C	W	+	−	−	−	−
1 % NaCl	−	+	W	+	−	−
pH 4	W	+	−	+	−	ND
Resistance to (μg ml^{-1}):						
Chloramphenicol (50)	−	+	W	+	−	−
Kanamycin sulfate (5)	−	−	W	+	−	+

fungicides commonly used in agriculture and may influence the survival of seed-applied rhizobial inoculants.

All type A strains analysed in this study showed similar reactions in the morpho-physiological tests. Known traits distinguishing type A from type B strains first reported by Martínez-Romero *et al.* (1991) and further described by Hungria *et al.* (2000) and Pinto *et al.* (2007), such as colony morphology, antibiotic-resistance patterns, growth on LB or PY without Ca were confirmed (Table 1). *R. tropici* type B strains can tolerate 40 °C, whereas type A strains grow very poorly at this temperature. Novel differences between the two groups were found in this study. Both strain types produce acid in YMA medium but only colonies from type A strains acquire a yellow colour when the medium is supplemented with bromothymol blue. Besides being more sensitive to several antibiotics than type B strains (Martínez-Romero *et al.*, 1991), type A strains are also more sensitive to the fungicides captan and thiram (Table S4, available in IJSEM Online). Additionally, 15 out of the 49 tests in the API 50CH kit differentiated the strain types (Table S4).

In the description of *R. tropici* as a novel species, type A and B strains formed well separated groups from data derived from multilocus enzyme electrophoresis analysis with eight metabolic enzymes (Martínez-Romero *et al.*, 1991). The groups were separated at a genetic distance over 0.5, which was used as the limit to distinguish species (Musser *et al.*, 1987; Selander *et al.*, 1985). Further analysis with glutamine synthetase II isoenzymes clearly distinguished type A and type B strains (Taboada *et al.*, 1996). Megaplasmids of similar size (over 1700 kb) were observed in both type A and type B strains but they were found to be subgroup-specific, indicating that type A and B strains belonged to different taxa (Geniaux *et al.*, 1995). Such megaplasmids may correspond to chromids (E. Ormeño-Orrillo and others, unpublished results) as defined by Harrison *et al.* (2010), and should have taxonomic value in contrast to conjugative plasmids. Other differences, reported elsewhere, include a Hup (uptake hydrogenase)-positive phenotype of type A strains, whereas only a few type B strains showed this characteristic (van Berkum *et al.*, 1994).

All differences in genetic and phenotypic properties between the type A and B strains of *R. tropici* reported in this and previous studies suggest that type A strains are members of a distinct species, for which the name *Rhizobium leucaenae* is proposed. This novel species can also be differentiated from other species of the '*R. tropici* group' by sequence analysis, as mentioned previously, and by the phenotypic traits presented in Table 1.

Based on data reported in previous works, *R. leucaenae* is present in several regions of Brazil (Grange & Hungria, 2004; Martínez-Romero *et al.*, 1991; Mercante *et al.*, 1998; Pinto *et al.*, 2007). Although these regions encompass a variety of ecosystems, *R. leucaenae* occurs abundantly in the Cerrados savannah, which occupies about 25 % of Brazil. In that region, *R. leucaenae* represented 79 % and 15 % of the rhizobial population obtained using *Leucaena leucocephala* and common bean as trap hosts, respectively (Mercante *et al.*, 1998). *R. leucaenae* has also been found in the state of Veracruz, Mexico, in nodules of *Gliricidia sepium* (Acosta-Durán & Martínez-Romero, 2002). In Zacatecas, the largest bean-growing area in Mexico, a low proportion (less than 10 %) of the nodule bacterial isolates from beans were identified as *R. leucaenae* based on analysis of 16S rRNA or *rpoB* gene sequences (our own unpublished results).

Description of *Rhizobium leucaenae* sp. nov.

Rhizobium leucaenae (leu.cae′nae. N.L. gen. n. *leucaenae* of *Leucaena*, referring to the isolation source of many strains of this species, root nodules of *Leucaena*).

Gram-negative, aerobic, non-spore-forming rods. Colonies on YMA medium are circular, flat, white, opaque, dry, with

low to moderate production of mucus and usually 2 to 4 mm in diameter within 2 to 3 days of incubation at 28 °C. Strains acidify the YMA medium after 3 days. Can tolerate 37 °C and grow weakly at pH 4; however, optimum growth occurs at pH 5 to 7 and at 25 to 28 °C. Strains do not grow in LB medium or PY minus Ca and are sensitive to chloramphenicol (50 μg ml^{-1}) and kanamycin (5 μg ml^{-1}). As sources of carbon, they use D-arabinose, D-arabitol, cellobiose, D-fructose, D-galactose, D-glucose, sucrose, gluconate, maltose, D-lyxose, D-mannose, D-ribose, glycerol, L-arabitol, L-fucose, L-sorbose, mannitol and xylitol. Strains induce the formation of root nodules and fix N_2 with *L. leucocephala*, *Leucaena esculenta*, *G. sepium* and *Phaseolus vulgaris*.

The type strain is CFN 299^T (=USDA 9039^T=LMG 9517^T=CECT 4844^T=JCM 21088^T=IAM 14230^T=SEMIA 4083^T=CENA 183^T=UMR1026^T=CNPSo 141^T), isolated from an effective nodule of *Phaseolus vulgaris* in Brazil. The DNA G+C content of strain CFN 299^T is 64.1 mol%.

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