

Sinorhizobium americanus sp. nov., a New *Sinorhizobium* Species Nodulating Native *Acacia* spp. in Mexico

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Summary

The sinorhizobia isolated from root nodules of *Acacia* species native of Mexico constitute a diverse group of bacteria on the basis of their metabolic enzyme electromorphs but share restriction patterns of the PCR products of 16S rRNA genes and a common 500 kb symbiotic plasmid. They are distinguished from other *Sinorhizobium* species by their levels of DNA-DNA hybridization and the sequence of 16S rRNA and *nifH* genes. *nolR* gene hybridization patterns were found useful to identify sinorhizobia and characterize species. A new species, *Sinorhizobium americanus*, is described and the type strain is CFNEI 156 from *Acacia acatzensis*.

Key words: taxonomy – *Rhizobium* – *Sinorhizobium* – *Acacia* – *nolR* – sym plasmid – genetic diversity – novel species

Introduction

Leguminous trees have an important role in maintaining soil fertility. *Acacia* is one of the largest genera in the Leguminosae and has a world wide distribution [51]. *Acacia* species are highly tolerant to drought, valuable as fodder and wood and are used in ambitious reforestation programs in the Sahara desert. Some *Acacia* species are considered fast growing trees and have records of good nitrogen fixation capacities with their symbiotic bacteria [3, 10].

Different symbionts such as *Mesorhizobium plurifarium* [8], *Sinorhizobium sahelense*, *S. teranga* [9], *S. kostiense*, and *S. arboris* [41] have been isolated from nodules of *Acacia senegal*, *A. laeta*, *A. aculaeta*, *A. raddiana*, *A. horrida*, *A. mollissima*, *A. seyal* and *A. tortilis*. Two biovars have been identified among *S. teranga* isolates and strains belonging to *S. teranga* bv. *acaciae* nodulate with most of the *Acacia* species mentioned. In addition, *Bradyrhizobium* species have been found in *A. mangium*, *A. decurrens*, *A. auriculiformis*, *A. mollissima*, *A. mearnsii*, and *A. dealbata* [1, 2, 35, 39]. *A. caven* in Uruguay nodulates both with fast and slow growing rhizobia but most efficiently with the fast growing ones [15]. Non-nodulating or erratic-in-nodulation *Acacia* species have been reported [14, 42].

In Australia the rhizobia species nodulating acacias seem to be determined by the environmental conditions [1]. It has been shown that best results in ecological restoration

are obtained with native bacteria as inoculants [44] and gains in nitrogen fixation of acacias in Africa were documented upon inoculation with native strains [52].

In Mexico several native *Acacia* species are encountered. While *Acacia farnesiana* is widely spread, other *Acacia* species such as *A. acatzensis*, *A. cochliacantha*, and *A. bilimekii* are endangered species in some regions due to overexploitation and forest destruction. It has been observed that the *Acacia* species with a wide distribution nodulate with more diverse bacteria than the rare *Acacia* species [53]. The rhizobia species nodulating *Acacia* spp. in Mesoamerica are unknown.

In rhizobia systematics various approaches have been used. Most commonly primary groupings are established from the analysis of total protein profiles [9], PCR-fingerprints [33], multilocus enzyme electrophoresis [58], or phenotypic characteristics [6]. Ribosomal gene sequences and DNA-DNA hybridization are performed to elucidate similarities to described species. Characteristics of symbiotic genes and host range are traits currently analyzed in the description of new species. Phylogenetic relationships among rhizobia have been recently established based on the analyses of *recA* and *atpD* gene sequences and these were in general agreement to those derived from 16S rRNA gene sequences [17]. As an additional genetic marker, we explored here the use of *nolR* restriction frag-

ment length polymorphisms (RFLPs). *nolR* is a global regulator [5] that also regulates *nod* gene expression [31] by binding *nodD* operators. *nolR* gene is located on the chromosome of *S. meliloti* and is a single copy gene [16]; *nolR* genes have been also found in other rhizobia species such as *S. fredii*, *R. leguminosarum* and *R. etli* but in *Mesorhizobium* or *Bradyrhizobium* strains *nolR* genes were not found [30].

The aim of this work was to characterize the fast growing *Sinorhizobium* strains from the *Acacia* spp. native to Mexico. Using standard approaches to define new species [21], we describe here a novel *Sinorhizobium* species for acacias in Mexico.

Materials and Methods

Strain isolation

Nodules were obtained from 7 plants of *A. farnesiana*, *A. acatensis*, *A. pennatula*, *A. macilenta* and *A. cochliacantha* in a reserve area in Sierra de Huautla in Morelos, Mexico. After surface sterilization nodules were squeezed on PY medium (5 g peptone, 3 g yeast extract and 0.6 g of calcium chloride) with 15 g agar per liter. Bacteria were incubated aerobically for 3 days at 29 °C and the isolates were purified by streaking single colonies in YM [54] supplemented with bromothymol blue 0.05% to determine morphology and production of acidity or alkalinity. Strains were maintained in 28% (vol/vol) glycerol-water solution at -20 °C.

Nodulation tests

The presumptive rhizobia were tested for nodulation on their original host. Only the isolates forming nodules were studied. The seeds from the five acacias species were sterilized by immersion in concentrated H₂SO₄ for 20 min. They were then washed five times with sterile water and placed in plates with agar, 0.8% in water, to germinate at 28–30 °C. When the roots were approximately 2 cm long, the seedlings were planted in pots (150 ml capacity) containing washed and sterilized vermiculite and 50 ml of N-free Fahraeus solution [13]. Plants were maintained at a day temperature of 30 °C, at night temperature of 20 °C and a light phase of 16 h. Germinating seeds were inoculated with approximately 10⁷ log-phase cells of each *Acacia* spp. isolate or with *Mesorhizobium plurifarium* strain LMG 11895. Control non-inoculated *Acacia* plants were included. *Phaseolus vulgaris* bean, soybean and *Leucaena leucocephala* seeds were inoculated with *R. etli* bv. phaseoli CFN42 [47] and *R. tropici* CFN299 [38] as controls and with representative *Acacia* spp. isolates.

Multilocus enzyme electrophoresis (MLEE)

Cell extracts from *Acacia* spp. isolates and reference strains were prepared from 50 ml PY broth cultures grown overnight at 28 °C according to the procedure of Caballero-Mellado and Martínez-Romero [4]. The metabolic enzymes were separated by electrophoresis on starch gel and the selective staining of the enzymes was done according to the procedures described by Selander et al. [48]. Distinctive mobility variants (electromorphs) of each enzyme, numbered in order of decreasing anodal mobility were equated with alleles at the corresponding structural gene locus. Allele profiles or electrophoretic types (ETs) were equated with multilocus genotypes. The genetic distance between pairs of ETs was estimated as the proportion of dissimilar loci. Clus-

tering of ETs and a dendrogram were constructed using the un-weight pair group method with averages (UPGMA) [50]

DNA isolation and PCR amplification

Isolates were grown overnight in 5 ml of PY. Total DNA was isolated and purified using the Genomic Prep™ kit (Amersham). Primers fD1 and rD1 [60] corresponding to positions 8–27 and 1524–1540 respectively of the *E. coli* 16S rRNA sequence were used to amplify the corresponding fragments from each isolate. Conditions for amplification were 1 cycle 94 °C 3 min; 35 cycles 94 °C 1 min, 57 °C 1 min, 72 °C 2 min; and 1 cycle at 72 °C 5 min. Primers *nifH*-1 and *nifH*-2 [11] which amplify a 601 bp fragment between positions 256 and 856 of the *S. meliloti* *nifH* sequence and primers *nifH*-211f (CCA CSA CCT CCC AAA ATA CRC T) and *nifH*-781r (CGG CAC RAA GTG RAT GAG CT) corresponding to positions 211 and 781 of the *S. meliloti* *nifH* gene were used in PCR reactions to synthesize *nifH* genes, with a first cycle of 94 °C 5 min, then 30 cycles at 92 °C 1.5 min, 1.5 min at 60 °C and 2 mins at 72 °C, one last cycle at 72 °C 5 min.

Amplified 16S ribosomal DNA restriction analysis (PCR-RFLP analysis)

Subsamples (15 µl) of the PCR products were individually digested with the restriction nucleases *MspI*, *HinfI*, *HhaI*, *Sau3AI* and *RsaI* and the restriction fragments were analyzed by electrophoresis in 3% (w/v) agarose gels [58]. The isolates were grouped according to their RFLP patterns into rDNA types and compared to those of the type strains of related species.

Nucleotide sequencing

PCR products were purified using Wizard™ PCR Preps DNA purification System for Rapid Purification of DNA Fragments (Promega). PCR products were sequenced directly (16S rRNA and *nifH* PCR products) or after cloning 16S rRNA PCR products with TOPO TA Cloning Kit (Invitrogen). Almost full length sequences of the 16S rRNA genes were determined as described previously [46].

The sequences were aligned using PILEUP of Wisconsin GCG. Aligned sequences were analyzed using the Molecular Evolutionary Genetic Analysis (MEGA) Package version 1.01 [32] to produce a Jukes-Cantor distance matrix [28].

DNA-DNA reassociation, *nifH* and *nolR* gene hybridization

DNA of isolates and reference strains including unclassified *Sinorhizobium* strains related to *S. fredii*, GR06 and GRX8 from bean nodules from Spain [25, 34] were digested with the restriction endonuclease *EcoRI* for the determination of DNA-DNA hybridization according to the methods described [38]. *nifH* RFLPs were determined from Southern blots using *EcoRI* digested genomic DNA hybridized to a 600 bp *SalI* fragment of *nifH* of *R. etli* bv. phaseoli CFN42 as a probe [56]. From aligned *nolR* gene sequences of *R. leguminosarum* (AJ001934) and *S. meliloti* (X59050) primers SmnolR730F (5' GGY ATC GAT TCG KCA GG 3') and SmnolR1142R (5'GGT GCT GAG AAA GAG CCG ATT3') were designed and used to synthesize a *nolR* gene fragment of 413 bp comprising the 5' half of the gene (199 bp) and upstream sequence (214 bp) from *S. meliloti* 41 and *R. etli* bv. phaseoli CFN42. The *S. meliloti* *nolR* upstream sequence includes 117 nucleotides of a putative ORF designated Y04457 in the *S. meliloti* genome [16]. PCR products were sequenced to verify their identity and were ³²P labelled using Rediprime to be used as probes in Southern blot *EcoRI* digests. *nolR* hybridization was at 60 °C with nylon transfer membranes and first wash was at 50° with SSC 2× for 30 min, subsequent washes were at room temperature with SSC 2× and SSC 1× for 30 min each and the last one with SSC 0.5× for 15 min.

Plasmid analysis

Plasmids were visualized using a modified *Eckhardt* [12] procedure of *Hynes and McGregor* [27]. Strains were grown overnight in 5 ml of PY liquid medium, plasmid mobilities were determined in 0.7% agarose gels using plasmids of *R. etli* bv. phaseoli CFN42, *R. tropici* CFN299 [18] and *S. meliloti* 1021 [16] as references. Symbiotic plasmids were identified by Southern hybridization analysis using as a probe a 2.0 kb *EcoRI-PstI* fragment containing *nodAB* of *R. tropici* CFN299 cloned in pUC18.

Phenotypic characterization

Two representative isolates and reference *Sinorhizobium* strains *S. fredii* USDA 205^T and *S. teranga* LMG 7834 were grown on PY agar plates and incubated for 24 h at 28 °C. Bacteria were swabbed from the plates, suspended in 10mM MgSO₄ and the inoculum density adjusted to 0.03 OD₆₀₀ and tested in Biolog GN MicroPlate for Gram-negative bacteria (MicroPlate with 95 different carbon sources) that were incubated at 29 °C

for 24 h without shaking, absorbance was measured at 570 nm on MICROPLATE-BIO-TEK-Instruments EL311.

Intrinsic antibiotics resistance was determined on PY medium amended with filter-sterilized solutions of the following antibiotics: erythromycin 300 mg l⁻¹, carbenicillin 100 mg l⁻¹, gentamicin 5 mg l⁻¹, nalidixic acid 20 mg l⁻¹ and kanamycin 500 mg l⁻¹. Ability to growth on LB (peptone 10g, yeast extract 5g, NaCl 10g l⁻¹) medium, in PY at 37 °C and MM [19] was tested.

Results

Metabolic enzyme electrophoretic analysis

Fast growing isolates from *Acacia* spp. nodules were analyzed according to their patterns of electrophoretic mobility of 13 metabolic enzymes and diverse electrophoretic types (ETs) were observed. Most of the fast growing isolates (80%, 31 strains) were found to be

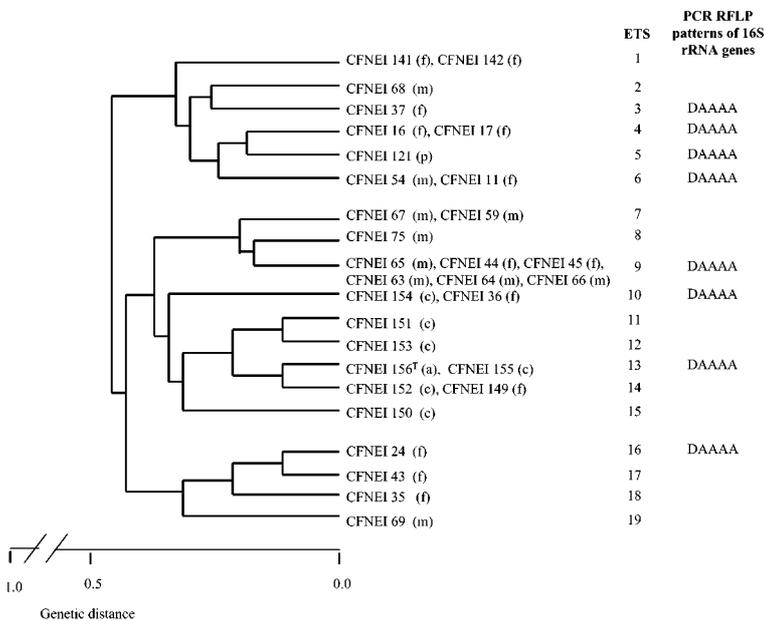


Fig. 1. Dendrogram of the relatedness of cluster I *Acacia* spp. isolates based on the electrophoretic mobility of the following metabolic enzymes analyzed for each strain: aconitase (ACO), alanine dehydrogenase (ALD), esterase (EST), glucose-6-phosphate dehydrogenase (G6P), NADP-independent glutamate dehydrogenase (GD2), hexokinase (HEX), isocitrate dehydrogenase (IDH), indophenol oxidase (IPO), malate dehydrogenase (MDH), malic enzyme (ME), phosphoglucose isomerase (PGI), phosphoglucomutase (PGM) and xanthine dehydrogenase (XDH). PCR-RFLP patterns of 16S rRNA genes. Digests with the following enzymes *HinfI*, *RsaI*, *HbaI*, *MspI*, *Sau3A1*, according to each ET. Letters in parenthesis indicate the original *Acacia* host species a = *Acacia acatensis*, c = *Acacia cochliacantha*, f = *Acacia farnesiana*, m = *Acacia macilenta* and p = *Acacia pennatula*.

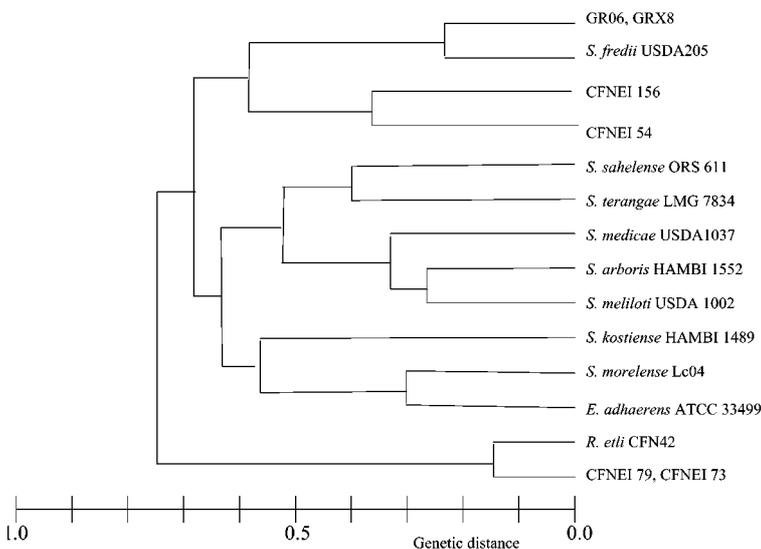


Fig. 2. Dendrogram of the relatedness of two representative *Sinorhizobium americanus* isolates (CFNEI 156 and CFNEI 54) and type strains of different *Sinorhizobium* species determined by MLEE with the following metabolic enzymes: alanine dehydrogenase (ALD), esterase (EST), glucose-6-phosphate dehydrogenase (G6P), NADP-independent glutamate dehydrogenase (GD2), isocitrate dehydrogenase (IDH), indophenol oxidase (IPO), malate dehydrogenase (MDH). Also shown are *Acacia* spp. *Rhizobium* isolates CFNEI 79 and CFNEI 73 and *R. etli* CFN42^T.

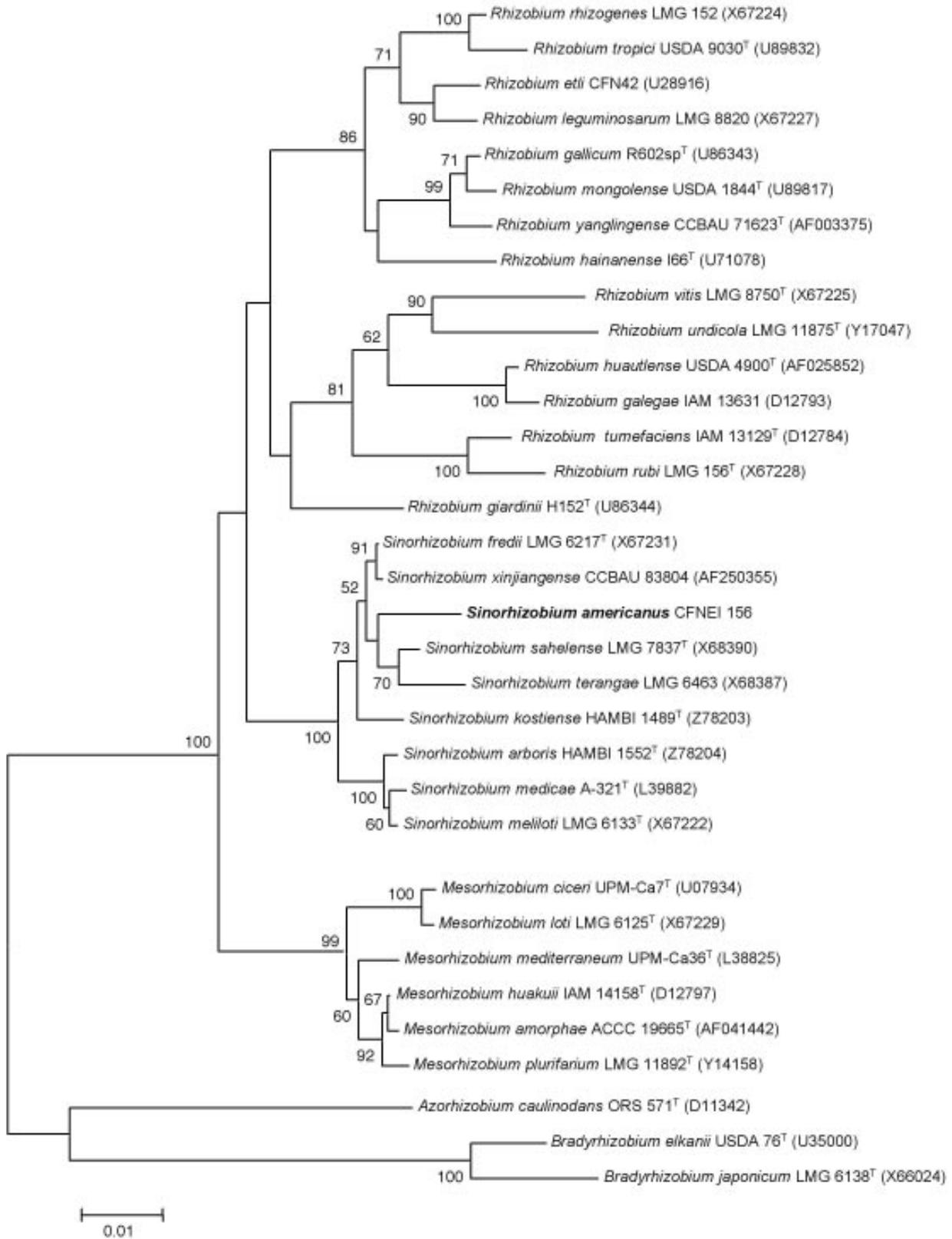


Fig. 3. Phylogenetic tree of 16S rRNA gene sequences. The 16S rRNA gene sequence obtained from CFNEI 156 has been deposited in geneBank under accession number AF506513. The sequence of the recently described *Sinorhizobium kummerowiae* has been included [59].

closely related corresponding to 19 different electrophoretic types grouped at a genetic distance of less than 0.5 and all were assigned to cluster I (Fig. 1). On average there were 1.68 strains per ET, which indicates a fairly diverse group. Cluster I comprised isolates from *Acacia farnesiana*, *A. pennatula*, *A. macilenta* and *A. cochliacantha*. There was no correlation between ETs and original plant host species and some isolates obtained from different *Acacia* species, for example from *A. farnesiana* and *A. macilenta* or *A. farnesiana* and *A. cochliacantha* were found to belong to the same ET (Fig. 1). Further comparison to the known *Sinorhizobium* species

showed that two representative isolates from cluster I (CFNEI 156 from ET13 and CFNEI 54 from ET6) grouped with *Sinorhizobium* species with 7 metabolic enzymes analyzed while a minority of *Acacia* spp. isolates (CFNEI 79 and CFNEI 73) that were not included in cluster I grouped with *R. etli* type strain CFN42 by MLEE (Fig. 2). In agreement CFNEI 79 had a *nifH* gene sequence that resembles that of *R. etli* biovar *mimosae* and 16S rRNA genes corresponding to *Rhizobium* spp. (not shown). Slow growing bacteria were occupants of around 40% of the *Acacia* spp. nodules but were not characterized.

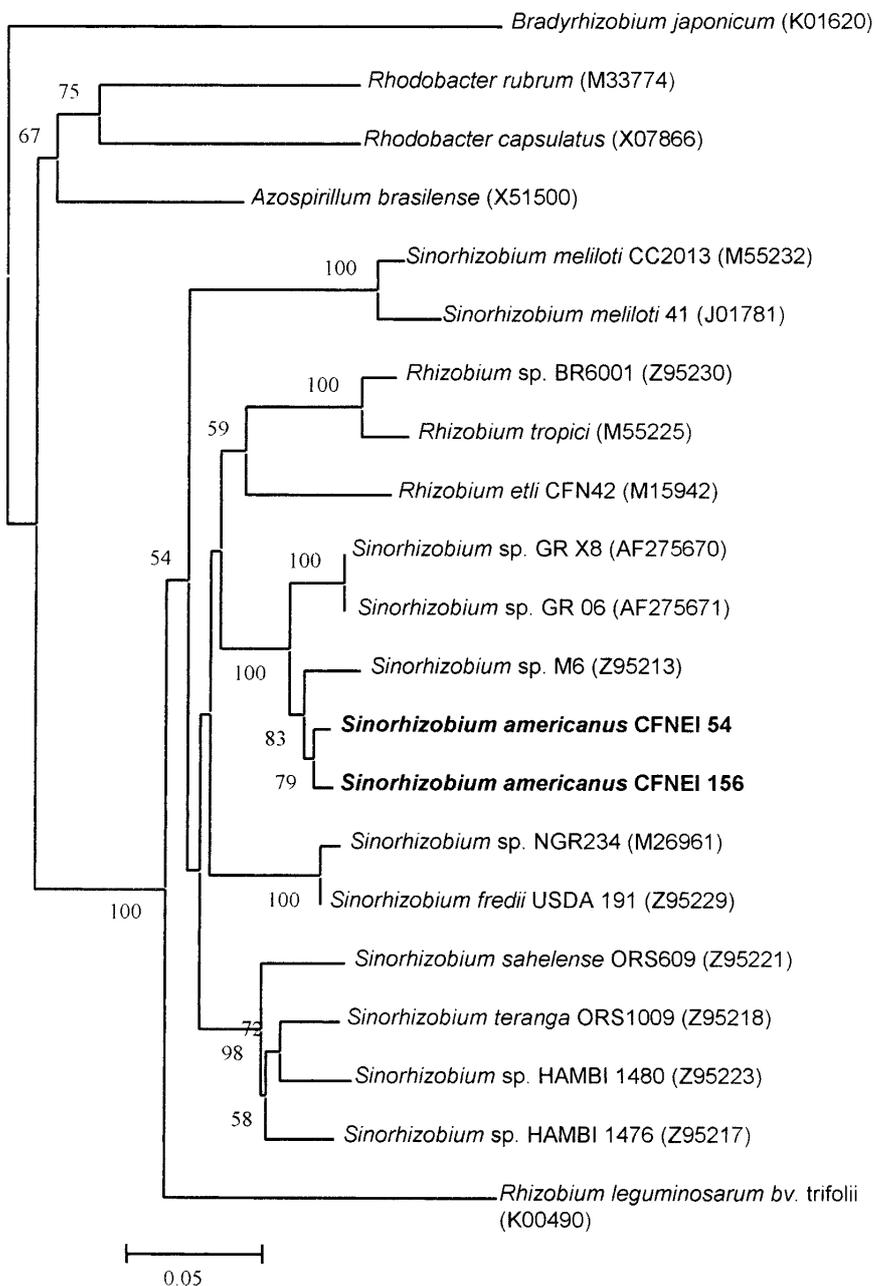


Fig. 4. Tree of partial *nifH* gene sequences. The *nifH* gene sequences obtained corresponding to nucleotides 50 to 839 (strain CFNEI 156) and 50 to 846 (strain CFNEI 54) of the *S. meliloti* sequence (NC_003037) represent 81 and 83% of the gene and have been deposited in geneBank under accession numbers AF506514 and AF506515.

Intrinsic antibiotic resistance

All cluster I isolates were resistant to nalidixic acid and most were sensitive to kanamycin. All strains were sensitive to carbenicilline, gentamicin and erythromycin.

Amplified 16S rRNA gene restriction patterns

Selected cluster I isolates (Fig. 1) had restriction patterns of 16S rRNA genes identical to those of *Sinorhizobium fredii* USDA 205^T (pattern DAAAA) with the enzymes tested.

Genomic DNA hybridization

Total DNA from cluster I isolates CFNEI 156 and CFNEI 54 hybridized at low levels (less than 15%) to the total DNA of the *Sinorhizobium* species *S. meliloti*, *S. medicae*, *S. kostiense*, *S. arboris*, *S. terangae* and *S. sahelense*; hybridization to *S. fredii* DNA was higher, ranging from 25–30% (Table 1). When *S. fredii* total DNA was used as a probe, hybridization values over 30% to CFNEI 156 and CFNEI 54 were obtained. In the hybridization experiment we included some *S. fredii* related strains, GR06 and GRX8 [25, 34] whose taxonomic affiliation has not been defined. Hybridization values obtained with these averaged 36% with CFNEI 156 and CFNEI 54. Levels of hybridization were over 82% with the cluster I isolates tested. Hybridization results with other different *Rhizobium* species were less than 20% (not shown).

16S rRNA and *nifH* gene sequences

According to the trees derived from almost complete 16S rRNA gene sequences from CFNEI 156, this strain represents a novel branch of rRNA sequence in the sinorhizobia (Fig. 3). Partial *nifH* gene sequence from CFNEI 156 and CFNEI 54 allowed their distinction from other species of rhizobia as well. In the trees derived from *nifH* sequences (Fig. 4), CFNEI 156 and CFNEI 54 were placed near M6, a *Sinorhizobium* sp. isolated from *Prosopis* in Mexico [23].

Plasmid profiles

The modified Eckhardt analysis revealed that plasmids of diverse sizes including megaplasmids are found in the *Acacia* spp. isolates tested (Fig. 5). All randomly selected cluster I isolates had a 500 kb symbiotic plasmid detected by the hybridization of Eckhardt gels to *R. tropici nodAB* genes (not shown).

Host range

All cluster I isolates were found to nodulate their original hosts. Some strains were also tested for nodulation in *A. farnesiana* and *A. macilenta* and they were found to nodulate in these other species as well. Young nodules in *Acacia* were spherical, around 1 mm in diameter and elongated when older, and were observable at 30 days post inoculation. The number of nodules formed depended on host and strain. All of the randomly selected cluster I isolates (including CFNEA156 and CFNEI 54) induced nodulation on *Leucaena leucocephala* and on *Phaseolus vulgaris* but not on soybean. *Mesorhizobium plurifarum* strain LMG 11895 [8] that nodulates several *Acacia* species did not form nodules on the Mexican acacias tested indicating that African and Mexican *Acacia* spp. have

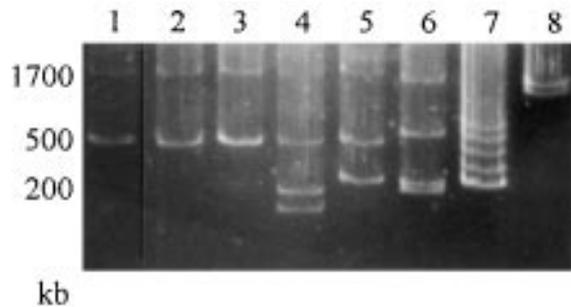


Fig. 5. Plasmid profiles of *Acacia* isolates and reference strains. Lane 1) CFNEI 54; 2) CFNEI 65; 3) CFNEI 156; 4) CFNEI 68; 5) CFNEI 73; 6) *R. tropici* CFN299; 7) *R. etli* CFN42; 8) *S. meliloti* 1021.

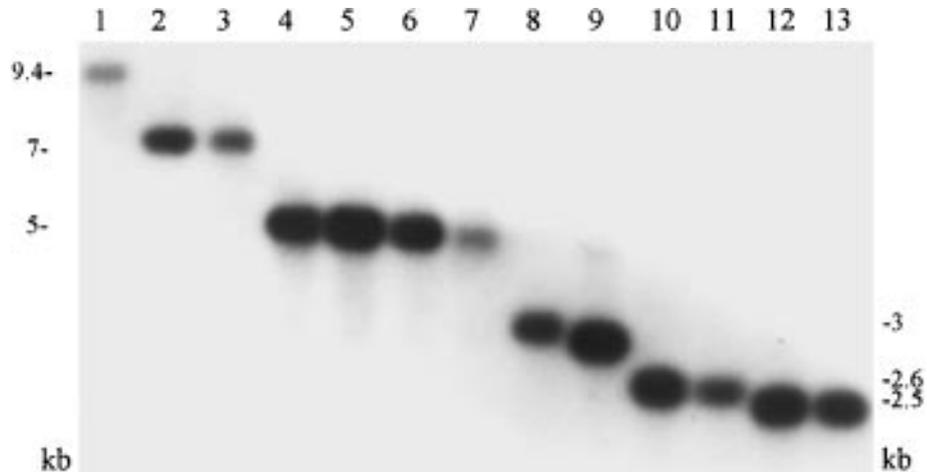


Fig. 6. Southern blot *nolR* gene hybridization patterns of *EcoRI* DNA digests of different species, lane 1) *Ensifer adhaerens* ATCC33499; 2) *S. medicae* USDA1037; 3) *Sinorhizobium* sp. NGR234; 4) *S. meliloti* 41; 5) *S. meliloti* 1021; 6) *S. kostiense* HAMB1 1489; 7) *S. morelense* Lc04; 8) *S. sahelense* ORS 611; 9) *S. arboris* HAMB1 1552; 10) *S. xinjiangense* CCBAU110; 11) *S. fredii* USDA 205^T; 12) *Sinorhizobium americanus* CFNEI 156; 13) *S. americanus* CFNEI 54.

Table 1. Levels of total DNA-DNA relatedness as percent of hybridization of two *Acacia* nodule isolates and *S. fredii* USDA 205 with the different strains listed.

	DNA relatedness with:		
	CFNEI 54	CFNEI 156	<i>S. fredii</i> USDA 205
<i>Sinorhizobium americanus</i>			
CFNEI 54	100.0	82.3	32.1
CFNEI 156	92.0	99.0	33.3
CFNEI 11		89.2	
CFNEI 16		88.0	
CFNEI 17		99.0	
CFNEI 24		85.6	
CFNEI 59		98.7	
CFNEI 75		88.9	
CFNEI 150		93.0	
CFNEI 154		86.6	
<i>Sinorhizobium</i> isolates and species			
GR06	44.5	33.1	
GRX8	38.1	34.0	56.1
<i>S. terangae</i> LMG 7834	9.5	8.3	5.2
<i>S. sahelense</i> ORS 611	2.0	2.8	2.5
<i>S. arboris</i> HAMBI 1552	9.6	10.1	1.9
<i>S. medicae</i> USDA 1037	7.7		10.8
<i>S. kostiense</i> HAMBI 1489	9.6	9.9	20.8
<i>S. fredii</i> USDA 205 ^T	25.8	30.8	100.0
<i>S. meliloti</i> USDA 1002 ^T	15.8	13.2	47.9

**Fig. 7.** Micrograph of *Sinorhizobium americanus* strain CFNEI 156.

diverged in the symbiotic specificities. *S. fredii* USDA 205^T did not nodulate *Acacia farnesiana*.

Growth characteristics

Cluster I isolate CFNEI 156 was able to grow on the following sole carbon sources: L-arabinose, D-fructose, D-galactose, α -D-glucuronic acid, glucuronamide and glucose-6-phosphate, as were *S. fredii* strain USDA 205^T

and *S. terangae* strain LMG 7834. Cluster I isolates could be differentiated from their closest relative, *Sinorhizobium fredii*, by using L-alanine, L-threonine and glucose-1-phosphate, which were not used by *S. fredii* USDA 205^T. L-phenylalanine was used by CFNEI 156 but not by *S. fredii* USDA 205, even though it was used as a carbon source by other *S. fredii* strains [9]. *E. adhaerens*, *S. morelense* and *S. meliloti* strains grow in LB, while all cluster I isolates did not. They do not grow at pH 4.5 but they grow at pH 8 and they grow at 37 °C. Colonies of cluster I isolates were creamy and pearly on PY and white and wet on YM medium and they were acid-producers.

nolR and *nifH* gene hybridization patterns

With the 5' fragment of *nolR* and its upstream region as a probe, bands of different sizes were observed in hybridization experiments with the different *Sinorhizobium* species tested (Fig. 6). A hybridization band was also observed in *Ensifer adhaerens* as well. It is remarkable that with the hybridization conditions used, no bands were obtained in any of *Rhizobium* species tested, *R. etli* bv. phaseoli CFN42, *R. leguminosarum* USDA 2154, *R. tropici* CFN299, *R. hainanense* I66, *R. mongolense* USDA 1844, *R. gallicum* FL27, *R. galegae* HAMBI 540, *R. huautlense* SO2, and *Allorhizobium undicola* LMG 11875 (now reclassified as *Rhizobium undicola*, Young et al. [62]) with the *S. meliloti nolR* probe described. With the same *Rhizobium* species hybridizing bands were revealed with the *R. etli* bv. phaseoli CFN42 *nolR* PCR product (not shown). *Acacia* isolates CFNEAI 156 and CFNEAI 54 had identical *nolR* hybridization bands of around 2kb, of a slightly smaller size than that of *S. fredii* (Fig. 3). Additionally CFNEI 156 and CFNEI 54 had identical *nifH* hybridization bands of around 6 kb in *EcoRI* digested DNA (not shown). Seventeen cluster I isolates showed only one *nifH* hybridization band in *EcoRI* digestions by Southern hybridization indicating that they have only one *nifH* copy.

Discussion

From the nodules of different *Acacia* species native to Mexico, we identified a group of related bacteria first referred to as cluster I isolates. Cluster I isolates seem to be the predominant species in *Acacia* spp. nodules in the sampled areas of Mexico. Strains of cluster I are related at less than 0.5 of the genetic distance defined by multilocus enzyme analysis; 0.5 has been detected as a threshold to delineate species [40, 49]. Cluster I isolates have common characteristics of colony morphology, antibiotic resistance, PCR-RFLP patterns of 16S rRNA genes and they have a 500 kb symbiotic plasmid. Two strains, CFNEI 156 and CFNEI 54, selected by having different electrophoretic types and different host origins, showed identical PCR-RFLP 16S rRNA gene patterns and very similar *nifH* gene partial sequences, high levels of total DNA hybridization, and identical hybridization patterns of *nolR* and *nifH* genes.

Cluster I is distinguished from the described *Sinorhizobium* species by their metabolic enzyme patterns (Fig. 2), their levels of DNA-DNA hybridization (Table 1), their 16S rRNA and *nifH* gene sequences. Considering that the sampling site of *Acacia* nodules is a reserve area little impacted by humans, we suspect that cluster I strains have been geographically isolated from the Asian and African species for a long time, as suggested before for Latin American and African sinorhizobia [23]. Except for *S. morelense* [57], not a single *Sinorhizobium* species was previously described from the symbionts of legumes native to the Americas. *S. morelense* strains were obtained from a minority of *Leucaena* nodules and were found to be highly resistant to antibiotics such as ampicillin (1000 mg l⁻¹) and kanamycin (500 mg l⁻¹) but sensitive to nalidixic acid and closely related to *Ensifer adhaerens*. The symbiotic plasmid of *S. morelense* was found to be unstable [57]. *S. morelense* is clearly different from cluster I isolates by their sequence of 16S rRNA genes, their metabolic enzyme patterns, their growth in LB, their sensitivity to nalidixic acid and their symbiotic plasmid instability.

Taking in consideration all results presented we propose a novel *Sinorhizobium* species for Mexican *Acacia* spp. nodule symbiotic cluster I bacteria and to name it as *Sinorhizobium americanus*.

Which markers may more accurately reflect the genetic relationships inside sinorhizobia? Multiple copies of ribosomal genes not only have the risk of having allele variation, as has been shown in *S. sahelense* and a *Sinorhizobium* spp. from *Acacia* [22] but also are subject to multiple recombination events or gene conversion mechanisms that occur in multiple copy genes [45]. Thus we consider that an ancestral relationship of *S. fredii* and *S. americanus* is more clearly reflected from DNA-DNA hybridization studies and confirmed by MLEE patterns but anyhow revealed by PCR-RFLP of the 16S rRNA genes. *Sinorhizobium americanus* isolates seemed more related to *S. fredii* and GR06 and GRX8 strains than to the other *Sinorhizobium* species. Strain GR06 and GRX8 belong to a group of *S. fredii*-like isolates from Spain obtained from *Phaseolus vulgaris* bean nodules that were incapable of nodulating soybean [25, 34]. *S. fredii*-like isolates have been also obtained from *Leucaena leucocephala* in Mexico [55] and in Brazil [9]. Some isolates from *Acacia* spp. in Morocco were found to be closely related to *S. fredii* [29]. *S. fredii* was originally isolated in China from soybean plants. The taxonomic status of the *S. fredii*-like isolates obtained outside of China is still questionable since similarities have been detected on the basis of 16S rRNA gene RFLPs or sequence or protein patterns but no DNA-DNA hybridizations have been performed. It may be speculated that a broadly distributed *Sinorhizobium* ancestor of *S. fredii* and *S. americanus* diverged locally, perhaps even recombining with sympatric bacteria (see below) becoming better adapted to local conditions and hosts.

Under the hybridization conditions used, the *nolR* 5' gene fragment and upstream sequence seems to be a good taxonomic marker to easily identify sinorhizobia and could even be used in colony hybridization to rapidly

recognize a large number of strains. It remains to be established if the marker is monomorphic in each species, but at least in *S. americanus* and *S. meliloti* this seems to be the case. Two *S. meliloti* strains were found to have an identical physical map of the *nolR* region and almost identical nucleotide sequence of *nolR* gene (366 bp) and neighbor regions (around 450 bp). Only one nucleotide difference was found in the *nolR* coding sequence from two different *S. meliloti* strains [7]. Furthermore, 14 different *S. meliloti* strains had an identical *EcoRI* hybridization band of 4.4 kb [31] which is in agreement with the fragment we observed in *S. meliloti*. As a collateral result, the fact that *nolR* hybridized to *Ensifer adhaerens* (Fig. 6) constitutes additional evidence for considering it as a member of the *Sinorhizobium* genus, as it has been proposed [46, 61].

A few strains from *Prosopis* and from *Leucaena* from Latin America that corresponded to the genus *Sinorhizobium* on the basis of their 16S rRNA sequences had such different *nif* and *nod* genes from those found in the African *Sinorhizobium* that the authors considered that "sinorhizobia have had a long history of separate evolution on *Prosopis* spp. and other Latin American hosts on the one hand and on African hosts, including *Acacia* spp., on the other hand" but they also recognized that "this hypothesis needs to be tested by more extensive sampling of Latin American strains" [23]. Interestingly, the partial *nifH* gene sequence determined from the *Sinorhizobium americanus* isolates CFNEI 156 and CFNEI 54 had the largest similarity to the *nifH* gene from strain M6, a Mexican *Sinorhizobium* isolate from *Prosopis* reported by Haukka et al. [23]. Further analysis of the dendrograms based on *nifH* gene sequences ([23] and Fig. 4) suggests that *nifH* genes of the American *Sinorhizobium* isolates are related to *Rhizobium nif* genes. Such similarity might have been caused by lateral gene transfer with recombination of *nifH* occurring among *Rhizobium* and *Sinorhizobium* strains from the Americas. The suggestion of the existence of gene flow among *Rhizobium* and *Sinorhizobium* means that *Sinorhizobium* and *Rhizobium* have not constituted genetically isolated entities but instead that they might have exchanged genetic material perhaps symbiotic plasmids. Sym plasmid transfer has been deduced to occur among *Sinorhizobium* and *Rhizobium* [34] and among *Sinorhizobium terangae* and *S. sahelense* [37]. In the laboratory sym plasmids have been transferred from *Rhizobium* species to *Sinorhizobium* spp. and to *Ensifer adhaerens* strains [46]. *E. adhaerens* has been reclassified as a *Sinorhizobium* bacterium [61].

In addition to nodulating *Acacia*, *Sinorhizobium americanus* isolates also nodulated *Leucaena leucocephala* and *Phaseolus vulgaris* beans. A previous report showed that strain GRH2 from *Acacia cyanophylla* nodulated beans [36] and the reverse was also true, with the *R. etli* bv. phaseoli reference strain CFN42 nodulating *A. farnesiana* (this work and L. Martínez, unpublished). The structural similarity of the Nod factors produced by some *Acacia* and *Phaseolus* strains [36, 37, 43] may explain the cross nodulation obtained when testing *Acacia* and *Phaseolus* as host plants.

Description of *Sinorhizobium americanus*

Aerobic gram-negative, non-spore-forming rods (Fig. 7) isolated from *Acacia* nodules and able to nodulate *Acacia* spp., *Leucaena leucocephala* and *Phaseolus vulgaris*. Members of the species grow in PY, YM and in MM but not in LB medium. They grow at 37 °C, they do not grow at pH 4.5 or 5 but they grow at pH 8. Colonies are creamy and pearly on PY and white and wet on YM with copious exopolysaccharides. Colonies are visible after three days in the described media. Strains are resistant to nalidixic acid 20 mg l⁻¹ but not to carbenicillin 100 mg l⁻¹ nor to erythromycin 300 mg l⁻¹ or gentamicin 5 mg l⁻¹. They grow in media containing 0.5 and 1% NaCl but not with 2% NaCl. *S. americanus* strains have a distinct SSU-rRNA gene sequence and low levels of DNA-DNA hybridization with the type strains from other *Sinorhizobium* species. A symbiotic plasmid of 500 kb is characteristic of the species as well as particular *nolR* hybridization patterns. The type strain is CFNEI 156^T. CFNEI 156^T have been deposited as ATCC BAA-532 and DSM 15007 and reference strain CFNEI 54 as ATCC BAA-533 and DSM 15008.

Description of type strain CFNEI 156T

It has the characteristics described above for the species. CFNEI 156 is able to grow on the following sole carbon sources: L-arabinose, D-fructose, D-galactose, α-D-glucuronic acid, glucuronamide and glucose-6-phosphate and can be differentiated from its closest relative *Sinorhizobium fredii* by using L-alanine, L-threonine and glucose-1-phosphate.

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