Rhizobia with different symbiotic efficiencies nodulate Acaciella angustissima in Mexico, including Sinorhizobium chiapanecum sp. nov. which has common symbiotic genes with Sinorhizobium mexicanum

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Abstract

Bacteria from nodules of the legume Acaciella angustissima native to the south of Mexico were characterized genetically and their nodulation and competitiveness were evaluated. Phylogenetic studies derived from rpoB gene sequences indicated that A. angustissima is nodulated by Sinorhizobium mexicanum, Rhizobium tropici, Mesorhizobium plurifarium and Agrobacterium tumefaciens and by bacteria related to Sinorhizobium americanum, Sinorhizobium terangae, Rhizobium etli and Rhizobium gallicum. A new lineage related to S. terangae is recognized based on the sequences of gyrA, nolR, recA, rpoB and rrs genes, DNA–DNA hybridization and phenotypic characteristics. The name for this new species is Sinorhizobium chiapanecum and its type strain is ITTG S70 T. The symbiotic genes nodA and nifH were similar to those from S. mexicanum strains, which are Acaciella symbionts as well, with nodA gene sequences grouped within a cluster of nod genes from strains that nodulate plants from the Mimosoideae subfamily of the Leguminosae. Sinorhizobium isolates were the most frequently obtained from A. angustissima nodules and were among the best strains to promote plant growth in A. angustissima and to compete in interstrain nodule competition assays. Lateral transfer of symbiotic genes is not evident among the genera that nodulate Acaciella (Rhizobium, Sinorhizobium and Mesorhizobium) but may occur among the sympatric and closely related sinorhizobia that nodulate Acaciella.

Introduction

Bacteria in the roots or the stems of legumes fix nitrogen and provide the plant with this nutrient. Symbiotic bacteria have been studied from only a small proportion of the extant legume species, and diverse genera such as Rhizobium, Sinorhizobium, Mesorhizobium, Bradyrhizobium, Devosia, Methylobacterium, Burkholderia and Cupriavidus have been reported to contain nodulating species (Young & Haukka, 1996; Sprent, 2001; Sy et al., 2001; Rivas et al., 2002; Chen et al., 2003; Vandamme & Coenye, 2004; Elliott et al., 2007a,b). Mexico is a very diverse country and occupies the fourth place in plant diversity terms (Rzedowsky, 1978) with many endemic legumes. The genus Acaciella is found mainly in Mexico and has well-supported botanical differences to be recognized as a new species different from the Acacia genus where it was formerly classified (Rico-Arce & Bachean, 2006).

Nitrogen-fixing trees and shrubs are valuable to maintain forest fertility and N₂ fixation allows their growth in infertile soils while enriching soil nitrogen. In Chiapas, Acaciella angustissima shrubs that can grow in poor soils are being used in agroforestry systems, due to their rapid growth rate, high capacity for nitrogen fixation and the quality of the tannins that accumulate in their bark (Rincón-Rosas & Gutiérrez-Miceli, 2008). Interestingly, these shrubs are

Keywords

Acaciella angustissima; N₂ fixation; nodulation; rhizobial diversity; legume symbiosis.
the preferred hosts of *Llaveia mexicanorum* (Williams & MacVean, 1995), a native homeoptera scale insect, which is used by indigenous people of Chiapas and Mesoamerica to produce a fat for traditional lacquer wood handcrafts (Grillascas, 2007). We established nurseries to propagate *A. angustissima* plants and became aware that inoculants were required to attain good plant development. This prompted us to analyze and select strains for inoculation.

One of the *sinorhizobial* groups we encountered corresponded to a new species and we proposed the name *Sinorhizobium mexicanum* for this lineage (Lloret *et al*., 2007). However, modifications in the *Sinorhizobium* genus taxonomy have occurred (Young, 2003). The bacteria belonging to *Sinorhizobium* have been transferred to the genus *Ensifer* (Young, 2003) because according to judicial rules, *Ensifer* has priority over *Sinorhizobium*. This new *Sinorhizobium* species had to be named as *Ensifer mexicanus* (Lloret *et al*., 2007) instead of *S. mexicanum*. In this work, we chose to use the former name *Sinorhizobium* as used in many recently published papers.

*Sinorhizobium mexicanum* was not the only symbiont found in *A. angustissima* nodules. The objective of this study was to characterize the other symbionts of *A. angustissima* in Mexico (including a novel *sinorhizobial* species), their interstrain nodulation competitiveness and their plant growth promotion in *A. angustissima*.

**Materials and methods**

**Sample sites**

Isolates used in this study were obtained from root nodules of *A. angustissima* collected from the Sumidero Canyon National Park in Chiapas, Mexico, and from nodulated trap plants grown in pots containing soils collected from an ecological reserve area in Sierra de Huautla in Morelos, Mexico (Supporting Information, Fig. S1). The Chiapas and Morelos collecting sites were c. 1000 km apart and both are characterized by deciduous forest vegetation (Lloret *et al*., 2007).

**Bacterial strains**

The *A. angustissima* strains analyzed in this study are listed in Table 1. Bacteria were obtained as described by Vincent (1970) using peptone yeast agar (PY) as growth medium (Toledo *et al*., 2003). Plates were incubated aerobically at 28 °C for 3 days and the isolates were purified by streaking single colonies on fresh PY plates. Single colony formation and morphology were observed in yeast extract mannitol (YEM) and PY media at 28 °C as reported by Toledo *et al*. (2003). The acid/alkaline reaction was verified by spreading the inoculum on YEM plates (pH 7.0) containing 25 μg mL⁻¹ bromothymol blue (Vincent, 1970).

| Table 1. Bacteria isolated from nodules of *Acaciella angustissima* |
|-----------------------------|----------------|
| Species and strains*        | Geographical origin |
| *Agrobacterium tumefaciens* |
| I TTG 52                    | Chiapas, Mexico    |
| I TTG 56                    | Chiapas, Mexico    |
| I TTG 59                    | Chiapas, Mexico    |
| I TTG 510                   | Chiapas, Mexico    |
| CFN ESH11                   | Morelos, Mexico    |
| CFN ESH16                   | Morelos, Mexico    |
| *Mesorhizobium plurifarium* |
| CFN ESH5                    | Morelos, Mexico    |
| CFN ESH18                   | Morelos, Mexico    |
| CFN ESH19                   | Morelos, Mexico    |
| CFN ESH22                   | Morelos, Mexico    |
| CFN ESH26                   | Morelos, Mexico    |
| *Rhizobium sp. (R. gallicum related)* |
| I TTG 511                   | Chiapas, Mexico    |
| *Rhizobium sp. (R. leguminosarum/R. etli related)* |
| CFN ESH6                    | Morelos, Mexico    |
| CFN ESH7                    | Morelos, Mexico    |
| CFN ESH34                   | Morelos, Mexico    |
| *Rhizobium tropici*         |
| CFN ESH9                    | Morelos, Mexico    |
| CFN ESH10                   | Morelos, Mexico    |
| CFN ESH23                   | Morelos, Mexico    |
| CFN ESH25                   | Morelos, Mexico    |
| CFN ESH27                   | Morelos, Mexico    |
| CFN ESH29                   | Morelos, Mexico    |
| I TTG 57                    | Chiapas, Mexico    |
| *Sinorhizobium sp. (S. americanum related)* |
| I TTG 58                    | Chiapas, Mexico    |
| *Sinorhizobium chiapanecum sp. nov.* |
| I TTG R11                   | Chiapas, Mexico    |
| I TTG S1                    | Chiapas, Mexico    |
| I TTG S568                  | Chiapas, Mexico    |
| I TTG S701                  | Chiapas, Mexico    |
| I TTG S71                   | Chiapas, Mexico    |
| *Sinorhizobium mexicanum*   |
| CFN ESH1                   | Morelos, Mexico    |
| CFN ESH2                   | Morelos, Mexico    |
| CFN ESH3                   | Morelos, Mexico    |
| CFN ESH4                   | Morelos, Mexico    |
| I TTG R4                   | Chiapas, Mexico    |
| I TTG R71                  | Chiapas, Mexico    |
| I TTG S3                   | Chiapas, Mexico    |
| I TTG S4                   | Chiapas, Mexico    |
| I TTG S5                   | Chiapas, Mexico    |
| I TTG S64                  | Chiapas, Mexico    |

*Identity according to the sequence analysis of the chromosomal gene rpoB.*

**Nodulation tests**

*Acaciella angustissima* seeds were scarified with H₂SO₄ for 15 min and surface sterilized with 1% (v/v) sodium hypochlorite for 10 min. Treated seeds were germinated on 0.8% agar–water plates and then placed in glass tubes filled with vermiculite moistened with Fahraeus medium (Fahraeus, 2008).
DNA isolation, genomic fingerprinting and DNA–DNA hybridization

Isolates were grown overnight in 2 mL PY. Total DNA was isolated and purified using the Genomic PrepTM kit (Amer sham). Enterobacterial repetitive intergenic consensus (ERIC) genomic fingerprinting was obtained by PCR using primers ERIC1R and ERIC2 as described by Versalovic et al. (1994). The fingerprints were visually analyzed after resolution of PCR products using electrophoresis in 1.5% agarose gels loaded with half the volume of the 25 µL PCR reaction. ERIC fingerprinting was used only to confirm that the isolates analyzed were not clones or siblings (Ormeno-Orrillo et al., 2006; Lloret et al., 2007). Strains showing different patterns were considered for sequencing and phylogenetic analysis. The DNA relatedness was determined using DNA–DNA hybridization experiments using 32P-labelled DNA of the newly proposed species (described below) Sinorhizobium chiananeum ITTG S70T as a probe. A filter hybridization method described previously was used (Martínez-Romero et al., 1991). The amounts of DNA were standardized using integrating gel fluorescence with the Eagle Eye II system (Stratagene). ANOVA and t-tests were performed to compare the percentage of DNA–DNA hybridization values among species and within species using angular transformation of percentage data (Knudson & Curtis, 1947; Martínez-Romero & Rosenblueth, 1990).

PCR amplification and gene sequencing

An internal fragment of the chromosomal genes gyrA, nolR, recA, rpoB and 16S rRNA gene (rrs), and the symbiotic genes nifH and nodA were amplified using standard PCRs. Primers and annealing temperatures used for gyrA, nolR, recA, rrs, rpoB and nifH genes were performed as described in Lloret et al. (2007) and by Haukka et al. (1998) for nodA. Before sequencing, the amplification mixture was purified using the PCR product purification system of Roche™. The sequences generated were deposited in the GenBank public database and their accession numbers were included in the phylogenetic trees.

Phylogenetic analysis

The protein-coding sequences were aligned using the program CLUSTAL W (Thompson et al., 1994) and then aligned based on codons using DAMBE v4.2.13 (Xia & Xie, 2001). The alignments were edited with MOLEDIT v5 (Hall, 1999). The best-fit evolutive models for each set of sequences were selected by the AKAIKE information criterion implemented in the MODELLER v3.06 (Posada & Buckley, 2004). rpoB gene sequences were analyzed using the TrN+1+Γ model of evolution based on an alignment of 642 nucleotides from positions 3262 to 3903; nodA using the GTR+1+Γ model with 522 nucleotides from positions 67 to 588; nifH with the TrN+1+Γ model of evolution based on 474 nucleotides from positions 313 to 787; and for the gyrA, recA and nolR genes the model of evolution and alignment positions were as reported by Lloret et al. (2007). These positions were based on the rpoB, nolR, nodA and nifH genes of Sinorhizobium meliloti 1021 and recA and gyrA of Agrobacterium tumefaciens C58. The phylogenetic trees were inferred with the maximum-likelihood (ML) method using the program PHYML v2.4.4 (Guindon & Gascuel, 2003) considering the θ-parameter for the Gamma distribution and the proportion of invariable sites estimated by the program. For the inference of the rrs phylogenetic tree, Sinorhizobium type strains were analyzed by the neighbor-joining method (NJ) (Saitou & Nei, 1987) implemented in MEGA v3.1 (Kumar et al., 2004) using the TrN+G model with the θ-parameter for the Gamma distribution estimated with MODELLER. The rrs phylogenetic tree was constructed using an alignment of 1417 nucleotides from positions 28 to 1444 with respect to the rrs gene of S. meliloti 1021. The topology robustness was estimated by a nonparametric bootstrap test using 100 pseudoreplicates for ML and 1000 for NJ.

Competition assays

The nodulation capacity was evaluated in competition assays of S. mexicanum ITTG R7T or S. chiananeum ITTG S70T against one randomly selected strain from each of the bacterial groups identified previously by rpoB gene sequence analysis. Twenty-one treatments resulted from the 12 combination mixtures plus each of the eight single strains as positive nodulation controls, and the negative control (uninoculated plants). Four replicates of inoculated plants were used per treatment. The plant growth conditions were as mentioned above for nodulation tests. The competitiveness was evaluated by the number of nodules obtained from each member of the mixture with respect to the total number of nodules.
nODULES. The identity of the reisolated strains was determined by plasmid patterns using the Eckhardt procedure (Eckhardt, 1978). The variation in nodule number was analyzed statistically by ANOVA using SAS software (SAS Institute Inc., 1989), followed by comparison of means by Tukey’s test ($P < 0.05$).

**Plant inoculation assays**

The strains with the best nodulation capacity and high competitiveness were used as inoculants. Germinated seedlings of *A. angustissima* were planted in vermiculite tubes with Fahraeus medium (Fahraeus, 1957) and inoculated as described above. Plants without inoculum, with or without 30 mg KNO$_3$-N per plant, served as control (Hungria et al., 2001). Six replicate tubes were used per treatment and these were arranged in a completely randomized design. The plants were grown in a climate chamber at 28°C for 90 days. At harvest, the shoot height, shoot dry weight, root dry weight and nodule number were determined, and total shoot nitrogen was assayed using the Kjeldahl method (Bremner & Mulvaney, 1982). The effect of the inoculation was analyzed statistically by ANOVA, followed by comparison of means using Tukey’s test ($P < 0.05$).

**Results**

**Strain identity, diversity and phylogeny**

A total of 94 strains were obtained from *A. angustissima* root nodules in Chiapas and Morelos that were confirmed to form nodules in the original host. Thirty-eight strains that represented the different ERIC-PCR electrophoretic patterns were used for PCR amplification and sequencing. The taxonomic position of the selected strains from *A. angustissima* was determined according to the phylogenetic analysis performed with partial sequences of the chromosomal gene *rpoB*, which encodes the β-subunit of RNA polymerase (Fig. 1).

The largest percentage of isolates found at both sites corresponded to *S. mexicanum* (26.3%) while the lowest corresponded to bacteria related to *S. americanum* and *Rhizobium gallicum*, both with 2.6%. A new lineage related to *S. mexicanum* and *Sinorhizobium terangae* was isolated only in Chiapas while only the strains related to *Rhizobium etli* and *Mesorhizobium plurifarium* were found in Morelos. The largest percentage of the isolates in Chiapas corresponded to *S. mexicanum* (33.3%) and in Morelos *Rhizobium tropici* (30.0%).

The *Sinorhizobium* sp. strain ITTG S70$^T$ *rrs* gene was found to be different from all sequences available in the GenBank database and had 99% identity to its closest relative *S. mexicanum*. The phylogenetic tree with the sequences of *rrs* genes (Fig. 2) and the phylogenetic trees with *rpoB* (Fig. 1), *gyrA*, *nolR* and *recA* genes (Fig. 3) were constructed including all of the type strains of *Sinorhizobium* species. The *recA* gene has been used previously in rhizobial phylogenetic studies (Gaunt et al., 2001; Vinuesa et al., 2005); *gyrA*, *recA*, *nolR* and *rpoB* were used previously to describe a new *Sinorhizobium* species (Lloret et al., 2007). *gyrA* encodes the alpha-subunit of DNA gyrase, *nolR* encodes a transcriptional regulator (Chen et al., 2000, 2005) and *recA* encodes the recombination protein RecA. In all phylogenetic trees, the position of strain ITTG S70$^T$ as a different lineage within the *Sinorhizobium* genus was well supported. Strains ITTG R11, ITTG S68 and ITTG S71 had sequences identical to those from ITTG S70$^T$ that was chosen to represent this new lineage.

Total DNA from the strain ITTG S70$^T$ showed low hybridization with the strains belonging to *S. mexicanum* (<42%) and *S. terangae* (<56%), while hybridization to three strains from its own group, ITTG S68, ITTG S71 and ITTG R11 (>74%), was higher than the limit proposed for new species (70%, Stackebrandt et al., 2002) (Table S2). DNA–DNA hybridization differences between *S. chiapanecum* strains and the closest species, *S. mexicanum* and *S. terangae*, were statistically significant with $P < 0.05$ and $P < 0.10$, respectively. It remains to be established whether similar plasmids in *S. terangae* and *S. chiapanecum* account for part of the DNA hybridization obtained. Description of species should be based on chromosomal and not plasmidic characteristics (Martínez-Romero & Jarvis, 1993). Also, phenotypic differences distinguishing *S. chiapanecum*, *S. terangae* and *S. mexicanum* are presented as Table S1.

The DNA–DNA hybridization, the phylogenetic position and phenotypic characteristics support that this *Sinorhizobium* lineage corresponds to a new species within the genus *Sinorhizobium*, and the proposed name is *S. chiapanecum* because it was isolated in Chiapas.

The *nifH* and *nodA* phylogenetic trees are shown in Figs 4 and 5, respectively. Symbiotic genes from the different rhizobia isolated from *A. angustissima* had affiliations with the corresponding genes from species in the genera *Rhizobium, Sinorhizobium* and *Mesorhizobium*. The phylogenies of these two symbiotic genes were incongruent with the phylogeny obtained with the chromosomal gene *rpoB*. The *nodA* sequences from *Rhizobium* sp. strains CFN ESH6 and CFN ESH34 (related to *R. etli*) isolated from *A. angustissima* were similar to the *nodA* gene of *Rhizobium giardinii* H152T isolated from common bean in France, but with the *nifH* gene analysis these strains were found to be related to the *nifH* gene of *R. etli* bv. *mimosae* Mim2 isolated from *Mimosa affinis* in Mexico. The *nodA* and *nifH* genes of *R. tropici* strains CFN ESH23, CFN ESH25, CFN ESH10, CFN ESH29 and CFN ESH9 were related but not identical to *nodA* and *nifH* gene sequences from *R. tropici* CFN299 isolated from *P. vulgaris* in Mexico. The symbiotic gene sequences of *S. chiapanecum* and *S. mexicanum* isolated...
from *A. angustissima* clustered together and were related to a different and well-supported group that included mainly sequences from *Sinorhizobium* isolated from American legumes, among them, the strains *Sinorhizobium* sp. BR827 and BR816 from *L. leucocephala* in Brazil and *S. americanum* CFN EI156 isolated from *Acacia acutifolia* in Mexico. The nodA and nifH gene sequences from *S. terangae*, the closest relative of *S. mexicanum* according to *rpoB* gene sequences,
grouped in a far distant cluster. *Mesorhizobium plurifarium* isolated from *A. angustissima* has nodA and nifH gene sequences similar to several *Mesorhizobium* species isolated from American and African hosts, mainly with the strains *Mesorhizobium* sp. DWO366 isolated from *Acacia polyaquanta* in Kenya and *Mesorhizobium* sp. INPA78b isolated from *L. leucocephala* in Brazil.

**Nodulation and nodule occupancy in competition assays**

Nodule occupancy evaluated from interstrain competition assays is shown in Table 2. The strains ITTG R7, ITTG S7, CFN ERS34, CFN ERS5 and ITTG S7 showed the best nodulation capacity and high competitiveness.
Sinorhizobium mexicanum strain ITTG R7\textsuperscript{T} always had a greater occupancy of the nodules than the respective competing strain, ranging from 65% when combined with Sinorhizobium sp. ITTG S8 to 100% when combined with Rhizobium sp. ITTG S11. The S. chiapanecum strain ITTG S70\textsuperscript{T} did not always have a greater occupancy of the nodules than the competing strain, although this strain and S. mexicanum ITTG R7 were highly effective in inoculation assays with A. angustissima (Table 3). Mesorhizobium plurifarium CFN ESH5 and Rhizobium sp. CFN ESH34 had a greater occupancy than ITTG S70\textsuperscript{T} (67% and 77%), respectively. Significantly lower numbers of nodules were obtained with M. plurifarium CFN ESH5, R. tropici ITTG S7, Sinorhizobium sp. ITTG S8 (related to S. americanum), Rhizobium sp. ITTG S11 (related to R. gallicicum) and A. tumefaciens ITTG S2, with the latter showing the lowest number of nodules and very low nitrogen fixation. All reisolated strains showed colony morphology and plasmid patterns identical to the original inoculated strains (data not shown).

**Plant growth, nodulation and nitrogen fixation of A. angustissima inoculated with selected strains**

The inoculation using the selected rhizobia strains had a significant effect on the growth of A. angustissima (Table 3). Rhizobium sp. CFN ESH34, S. mexicanum ITTG R7\textsuperscript{T} and S. chiapanecum ITTG S70\textsuperscript{T} had a positive effect on shoot height, shoot dry weight and root dry weight compared with the uninoculated control plants and those with added KNO\textsubscript{3}. Plants inoculated with these strains were on average 8.3 cm taller and weighted 109 mg more than noninoculated plants 90 days postinoculation. The number of nodules obtained with S. mexicanum ITTG R7\textsuperscript{T} and S. chiapanecum ITTG S70\textsuperscript{T} was significantly different ($P < 0.05$) compared with the rest of the treatments. None of the noninoculated plants formed nodules. The plants inoculated with ITTG R7\textsuperscript{T} showed a significantly higher total shoot nitrogen compared with other treatments ($P < 0.05$). ITTG R7\textsuperscript{T} was found to be the most effective strain in terms of plant growth promotion as indicated by total plant nitrogen content.

**Characteristics of S. chiapanecum sp. nov.**

Sinorhizobium chiapanecum (chia,pa,ne’cum. N.L. neut. adj. chiapanecum of Chiapas, the name of a state in Mexico where the bacterium was isolated). Gram-negative, aerobic, motile and nonspore-forming rods. Strains are fast growing and acid producers in YEM medium. The generation time for ITTG S70\textsuperscript{T} in YEM broth is 2.33 h at 28 °C. Colonies on PY or YEM are circular, pearly, slightly translucent and
produce copious amounts of polysaccharides. Colonies are normally more than 2–4 mm in diameter within 2 days of incubation at 28°C. The strains are resistant to nalidixic acid (120 μg mL⁻¹) but not to carbenicillin (20 μg mL⁻¹), ampicillin (10 μg mL⁻¹) or chloramphenicol (10 μg mL⁻¹). They grow in media containing 0.5%, 1.0% and 2.0% NaCl but not with 3.0% NaCl. Total DNA from strain ITTG S70T showed low hybridization values with the strains belonging to Sinorhizobium chiapanecum ITTG S70 (EF463930), S. medicae A321 (DQ411937), S. meliloti USDA1002T (AJ294381), S. arabis HMBI1552T (DQ411946), S. morelense Lc04 (EF198422), S. adhaerens ATCC33499 (EF027947), S. terangae ORS1009T (DQ411944), S. mexicanum ITTG R7T (DQ411951), S. xinjiangense CCBAU1110T (DQ411944), S. fredii USDA205T (AJ294379), S. arboris HMBI1552T (DQ411947), S. saheli ORS600 (DQ411938), S. kostiense HMBI1489T (DQ411943), S. terangae ORS1009T (DQ411943), S. arboris (DQ411925), S. terangae ORS1009T (DQ411943), S. saheli ORS600 (DQ411938), S. kostiense HMBI1489T (DQ411943).
Fig. 4. Phylogenetic tree estimated using the ML method with partial sequences of the symbiotic protein encoding\textit{nifH} gene using the \textsc{phyml} program. The alignment length was 474 nucleotides from positions 313 to 786 of the \textit{nifH} gene with respect to the \textit{nifH} gene encoded on the\textit{psymA} of \textit{Sinorhizobium melloti} 1021. Only bootstrap values $\geq 50\%$ are shown. Type strains are indicated by superscript\text{T}. The \textit{Acacia} angustissima strains are shown in bold. The accession numbers for the sequences are indicated within parentheses. Those generated in this work are shown in bold.

The tree was drawn using the \textsc{figtree} software. 

\section*{to S. terangae ORS1009$^1$ (< 48\%) and with S. mexicanum ITTGR7$^2$ (< 33\%). This species can be differentiated from other described \textit{Sinorhizobium} species on the basis of the phylogenetic analysis of the chromosomal genes \textit{rrs}, \textit{gyrA}, \textit{recA}, \textit{rpoB} and \textit{nolR}. The type strain ITTG S70$^1$ was isolated from nodules of \textit{A. angustissima} collected in the Sumidero Canyon National Park, Chiapas, Mexico. \textit{Sinorhizobium chiapanecum} ITTG S70$^2$ nodulated and fixed nitrogen in a. \textit{angustissima}, \textit{Acacia cochlbiancantha}, \textit{Acacia farnesiana}, \textit{Acacia pennatula}, \textit{Dolichos lablab}, \textit{P. vulgaris}, \textit{L. leucocephala} and \textit{Lysiloma acapulcensis} and tolerated salinity and acidity (data not shown). ITTG S70$^2$ has characteristics of the species.}
Fig. 5. Phylogenetic tree estimated using the ML method with partial sequences of the symbiotic protein encoding the nodA gene using the PHYML program. The alignment length was 522 nucleotides from positions 67 to 588 of the nodA gene with respect to the nodA encoded on the pSymA of Sinorhizobium meliloti 1021. Only bootstrap values ≥50% are shown. Type strains are indicated by superscript T. The Acacia angustissima strains are shown in bold. The accession numbers for the sequences are indicated within parentheses. Those generated in this work are shown in bold. Host and geographical origin are in parentheses.
Discussion

Tropical forests in Mexico harbor many endemic plants and a high richness of species (Rzedowsky, 1978). Forests have abiotic and biotic characteristics that allow such diversity to exist. Plant speciation in Mexico seems to be driven by geographical isolation due to the complex topography of the country. The tropics have a large diversity of rhizobia (Wang et al., 1999; Mohamed et al., 2000; Räsänen et al., 2001; Toledo et al., 2003; Wolde-Meskel et al., 2004). Sinorhizobia seem to have radiated in Mexico in relation to the geographical isolation and diversity of climates, conditions and plants (Toledo et al., 2003; Lloret et al., 2007). The sinorhizobia-nodulating legumes in Africa and in the Americas are considered to have had a long period of diverging evolution (Haukka et al., 1998; Toledo et al., 2003; Lloret et al., 2007). Our results showed that A. angustissima was preferentially nodulated by closely related members of the Alphaproteobacteria, especially sinorhizobia. Differences in symbiotic efficiency and competitiveness were found among the isolates, with S. mexicanum and S. chiapanecum strains being highly effective symbionts and good competitors. In

Table 2. Nodule occupancy by strains of Sinorhizobium mexicanum ITTG R7 and Sinorhizobium chiapanecum ITTG S70 and the coinoculated bacteria in competition assays in Acaciella angustissima

| Treatments                                           | Nodule number (per plant) (± SD)* | Nodule occupancy (%) by  |
|------------------------------------------------------|-----------------------------------|--------------------------|
|                                                      |                                   | First strain of the combination | Second strain of the combination |
| Uninoculated                                         | 0                                 | 61 (18)                  | 39                         |
| A. tumefaciens ITTG S2                               | 4.5 (± 1.3)t                          | 33 (18)                  | 67                         |
| M. plurifarium CFN ESH5                             | 4.25 (± 1.7)t                           | 82 (17)                  | 18                         |
| Rhizobium sp. ITTG S11                               | 3.25 (± 1.9)t                           | 23 (13)                  | 77                         |
| Rhizobium sp. CFN ESH34                              | 2.5 (± 1.3)t                            | 80 (10)                  | 20                         |
| R. tropici ITTG S7                                  | 3.75 (± 2.1)t                           | 67 (15)                  | 33                         |
| Sinorhizobium sp. CFN ESH34                         | 6.0 (± 1.4)                          | 75 (24)                  | 25                         |
| S. mexicanum ITTG R7+A. tumefaciens ITTG S2          | 8.75 (± 5.1)                          | 89 (35)                  | 11                         |
| S. mexicanum ITTG R7+M. plurifarium CFN ESH5         | 5.5 (± 1.9)                           | 100 (22)                 | 0                          |
| S. mexicanum ITTG R7+Rhizobium sp. ITTG S11          | 12.0 (± 1.4)                          | 71 (48)                  | 29                         |
| S. mexicanum ITTG R7+Rhizobium sp. CFN ESH34         | 3.75 (± 1.3)                          | 67 (15)                  | 33                         |
| S. mexicanum ITTG R7+Sinorhizobium sp. ITTG S8      | 6.5 (± 3.4)                           | 65 (26)                  | 35                         |

*Mean values of four replicates. The means followed by the same letter are not significantly different (P < 0.05).

Table 3. Effect of inoculation by the strains with high competitiveness and nodulation capacity on the growth, nodulation and nitrogen fixation of Acaciella angustissima

| Strains                                          | Shoot height (cm) | Shoot dry weight (mg) | Root dry weight (mg) | Nodule number | Total shoot N (mg per plant) |
|--------------------------------------------------|-------------------|-----------------------|----------------------|---------------|-----------------------------|
| Uninoculated                                     | 15.0 cm*           | 76.1 b                 | 46.3 b               | 0 b           | 30.4 c                      |
| M. plurifarium CFN ESH5                          | 16.5 bc            | 95.0 b                 | 40.3 a               | 2.1 b         | 39.9 c                      |
| Rhizobium sp. CFN ESH34                          | 20.1 b             | 101.0 b                | 44.9 a               | 2.3 b         | 51.5 c                      |
| R. tropici ITTG S7                               | 17.0 bc            | 96.4 b                 | 42.9 a               | 2.3 b         | 44.3 c                      |
| S. chiapanecum ITTG S70                          | 24.8 a             | 112.9 a                | 44.0 a               | 5.3 a         | 101.6 b                     |
| S. mexicanum ITTG R7                             | 25.1 a             | 134.7 a                | 52.6 a               | 5.8 a         | 158.9 a                     |
| KNO3-N (30 mg per plant)                         | 15.3 c             | 56.1 c                 | 25.3 b               | 0 b           | 22.4 c                      |

*Mean values of six replicates. The means followed by the same letter are not significantly different (P < 0.05).
contrast to acacias, no bradyrhizobia or Betaproteobacteria strains were found nodulating this legume. *Acaciaella angustissima* was among the legume hosts of Latin American origin that formed nitrogen-fixing nodules with the African sinorhizobial strains *Sinorhizobium arboris* HAMBI 1552, *Sinorhizobium kostienii* 1489 and *S. terangae* bv. *acaciae* ORS 1058 (Rásänen et al., 2001). Tropical legumes seem to have a mild specificity when associating with nodulating bacteria (Moreira et al., 1998), although under natural conditions predominant rhizobial species may be preferentially encountered in promiscuous plants (Bala & Giller, 2001; Bala et al., 2003; Martínez-Romero, 2003) as shown here.

*Mesorhizobium plurifarium* strains originally isolated from *Acacia senegal* (de Lajudie et al., 1998) encompass a set of diverging strains. In this study, *M. plurifarium* strains were found in *A. angustissima* only in Morelos. In Mexico, *M. plurifarium* were found nodulating *Sesbania (Papilionoideae)* trees (Wang et al., 1999) and *Leucacephala* plants grown in Morelos soils. *Acaciaella and Leucaena* belong to the Mimosoideae subfamily of the Leguminosae. Plant traps with soils from Morelos were used to collect the bacteria and it has been shown that by doing so a larger diversity of bacteria may be obtained nodulating a single legume (Hungria et al., 2001), and so we predicted that *Mesorhizobium* strains were the less adapted to nodulate *A. angustissima*. This turned out to be true.

We found seven isolates of *Rhizobium* similar to *R. tropici* type A, with *nod* genes more closely related (but not identical) to *nodA* of *R. tropici* than to other *nod* genes. *Rhizobium tropici* strains are common in tropical soils and nodulate some trees from the Mimosoideae subfamily of the *Leguminosae* such as *L. leucocephala* (Martínez-Romero et al., 1991) as well as *A. angustissima* (not shown).

*Rhizobium etli* is commonly isolated from *P. vulgaris* (Segovia et al., 1993), but has also been isolated from other shrub legumes in Kenya (Odee et al., 2002). Biovars that refer to host specificity have been described in *R. etli*. Nodulation of *Mimosoideae* plants such as *M. affinis* and *Leucaena* spp. is the characteristic of biovar mimosae (Wang et al., 1999). It is probable that the *Rhizobium* sp. strains (related to *R. etli* and *R. leguminosarum*) from *A. angustissima* correspond to biovar mimosae. Strain ITTG S11 was found to be related to *R. gallicum* (Amarger et al., 1997). *Rhizobium gallicum* bv. *gallicum* was isolated from common bean and can also nodulate *L. leucocephala* (Amarger et al., 1997; Silva et al., 2005) and other species from the Mimosoideae subfamily of the *Leguminosae* (Zurdo-Piñeiro et al., 2004) but it was not known that it nodulated *Acaciaella*.

In addition, species of *Agrobacterium* were also found in this study. Bala & Giller (2001) reported that the legumes *Acacia auriculiformis*, *L. leucocephala*, *Gliricidia sepium*, *P. vulgaris* and *Sesbania sesban* formed effective nodules with one or more isolates that resembled *A. tumefaciens*. *Agrobacterium* strains have been isolated previously from nodules of *Acacia mellifera*, *A. polycantha*, *Acacia nilotica* and *S. sesban* (Khubya et al., 1998; de Lajudie et al., 1999) and shrubs growing in the semi-arid and arid climates of northwestern China (Tan et al., 1999). Odee et al. (2002) indicated that agrobacteria were often found in association with root nodules as a co-occupant with rhizobia. The *Agrobacterium* strains described here were capable of forming nodules on *A. angustissima*, but the nitrogen fixation was very low. *Agrobacterium* sp. ITTG S2 (similar to *A. tumefaciens*) showed a low level of competitiveness when inoculated in competition assays. Recently, some *Agrobacterium* strains were found to be capable of forming tumors on plants as well as nodulating (Rivas et al., 2004). In additional experiments, we evaluated the pathogenicity of the strains ITTG S2, ITTG S6 and ITTG S10 (all similar to *A. tumefaciens*) on sunflower plants (*Helianthus annuus*) and found that these strains are not tumorigenic (not shown).

We showed that the phylogenies of the symbiotic genes were incongruent with the chromosomal genes as has been reported previously (Haukka et al., 1998; Wernegreen & Riley, 1999; Laguerre et al., 2001; Toledo et al., 2003; Lloret et al., 2007). Symbiotic genes on elements such as plasmids and symbiotic islands are prone to lateral gene transfer (Sullivan & Ronson, 1998; Ochman & Moran, 2001) and may be selected by hosts (Ueda et al., 1995; Haukka et al., 1998; Wernegreen & Riley, 1999), as observed here, because the two species *S. mexicanum* and *S. chiaipanecum* nodulating *Acaciaella* have the same *nod* genes. The three main groups described based on *nod* gene sequences (Haukka et al., 1998) corresponding to African and Latin–American sinorhizobia and some *Mesorhizobium* spp. were observed in the trees presented here with several more sequences included (Fig. 5). A large group was distinguished that corresponds to *nod* genes of symbionts with the capacity to nodulate many plants from the Mimosoideae subfamily of the *Leguminosae* (Fig. 5); it is worth noticing that within this group, *R. giardinii* and *R. gallicum* *nod* gene sequences were included.

*Sinorhizobium* sp. ITTG S8, a strain related to *S. americanum*, clustered in the *rpoB* tree with some American strains isolated from *L. leucocephala* in Mexico (Wang et al., 1999) and with strain BR816 (van Rhijn et al., 1994) from Brazil. This group constitutes a sister clade to *S. americanum* and could have been identified as belonging to the same species, but unpublished DNA–DNA hybridization results from our lab showed that BR816 was not a member of *S. americanum*. A new biovar has been proposed (mediterranense) (Mnasri et al., 2007) to account for sinorhizobia closely related to *Sinorhizobium fredii* and with specificity for *L. leucocephala* and *P. vulgaris*. This biovar includes strain BR816 and some other strains that, despite being closely related to *S. fredii*, do not form nodules on soybean. In
spite of the close relatedness of the symbiotic genes of bv. mediterraneum and S. americanum to those from S. mexicanum and S. chiapanecum, we consider that A. angustissima symbionts would not correspond to biovar mediterraneum because the isolates that we found to be closely related to biovar mediterraneum were not efficient to nodulate A. angustissima, comprised only 2.6% of the original isolates and were outcompeted by S. mexicanum or S. chiapanecum.

Within the enlarged set of sequence data presented here, we observed that the nodA gene sequence from Rhizobium huautlense (not reported previously) forms a clade with other Sinorhizobium species nodulating Sesbania (Fig. 5), indicating the strong specificity for Sesbania nodulation and evidencing lateral transfer of symbiotic genes between Rhizobium and Sinorhizobium. The genetic coherence among symbiotic and chromosomal genes has been considered to be characteristic of rhizobia nodulating wild legumes (Wernegreen & Riley, 1999), but S. chiapanecum and S. mexicanum as well as R. huautlense and sinorhizobia from biovar sesbaniae, all from noncultivated hosts, do not follow this observation and show evidence of horizontal transfer of symbiotic genes.

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Authors’ contribution

R.R.-R. and L.L. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Map of Mexico showing the location of field collection sites in Chiapas and Morelos.
Table S1. Phenotypic characteristics of Sinorhizobium chiaianum strain ITTG S70 and related reference strains.
Table S2. Levels of total DNA-DNA relatedness as percent of hybridization of Sinorhizobium chiaianum strain ITTG S70 isolated of A. angustissima with the S. mexicanum and S. terangae strains.

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