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CARACTERIZACIÓN DE RHIZOBIA QUE NODULAN *Acaciella angustissima* Y SU EMPLEO COMO INOCULANTES.

T E S I S

QUE PARA OBTENER EL GRADO ACADÉMICO DE:

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P R E S E N T A

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Índice

Resumen

Acaciella angustissima es una leguminosa arbustiva nativa del sur de México cuyos simbiontes fijadores de N_2 no se habían estudiado. Las bacterias aisladas de los nódulos de *A. angustissima* en Chiapas y Morelos fueron caracterizadas mediante la secuencia de genes cromosomales y plasmídicos, también la nodulación y competitividad fueron investigadas. Los estudios filogenéticos derivados de las secuencias del gene *rpoB* indicaron que *A. angustissima* es una leguminosa no-selectiva y es nodulada por *Sinorhizobium mexicanum, Rhizobium tropici, Mesorhizobium plurifarium* y *Agrobacterium tumefaciens,* y también, por bacterias relacionadas a *S. americanum, R. etli*, *R. gallicum,* y por una bacteria perteneciente a una especie no reportada que fue denominada como *Sinorhizobium chiapanecum.* Esta propuesta está basada en la secuencia de los genes *gyrA, nolR, recA, rpoB* y *rrs,* hibridación ADN-ADN y por las características fenotípicas. Las secuencias de los genes simbióticos *nodA* y *nifH* de *S. chiapanecum* fueron similares a las secuencias de los genes de *S. mexicanum* y ambas especies formaron un grupo bien definido junto con otros *Sinorhizobium* aislados de hospederos americanos. *S. mexicanum* ITTG $R7^T$ resultó ser una cepa altamente competitiva para ocupar nódulos y muy eficiente para promover el crecimiento de la planta *A. angustissima.* Estas propiedades simbióticas hacen de ITTG $R7^T$, una cepa con potencial para ser empleada como un biofertilizante para esta importante leguminosa arbustiva.

Palabras claves:

Acaciella angustissima, fijación de N_2, nodulación, diversidad de rhizobia, simbiosis leguminosa.

Abstract

Acaciella angustissima is a legume shrub native of southern Mexico whose symbionts N_2 fixers had not been studied. Bacteria isolated from nodules of *A. angustissima* in Chiapas and Morelos were characterized by the sequence of chromosomal and plasmid genes, also nodulation and competitiveness were investigated. The phylogenetic studies derived from *rpoB* gene sequences indicated that *A. angustissima* is a non-selective legume and is nodulated by *Sinorhizobium mexicanum*, *Rhizobium tropici*, *Mesorhizobium plurifarium* and *Agrobacterium tumefaciens*, and also 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, rpo*B, 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 sequences of symbiotic genes *nodA* and *nifH* of *S. chiapanecum* were similar to those from *S. mexicanum* and both species formed a well-defined group along with other isolated *Sinorhizobium* of American hosts. *S. mexicanum* ITTG $R7^T$ proved to be a highly competitive strain to occupy nodules and more efficient to promote plant growth in *A. angustissima*. These symbiotic properties make the ITTG $R7^T$, a strain with potential to be used as a biofertilizer for this important legume shrub.

Keywords

Acaciella angustissima, N_2 fixation, nodulation, rhizobial diversity, legume symbiosis.

I. Introducción

Acacia spp. (Leguminosae, Mimosoidea) es un género de árboles o arbustos que crecen principalmente en las regiones áridas y semiáridas de los trópicos y subtrópicos. De las más de 1200 especies de acacias conocidas, cerca de 850 son endémicas para Australia, siete para el Sur de América y 135 para África, con algunas especies presentes en Asia (Mohamed *et al*., 2000). Algunas especies del genero *Acacia* (Rico, 2001) han sido recientemente reubicadas al género *Acaciella* (Rico-Arce & Bachean, 2006). El género *Acaciella* se caracteriza por tres aspectos morfológicos, que son excepciones del género *Acacia*: plantas completamente inermes, ausencia de nectarios extraflorales y políades con 8 granos de polen. La única característica que lo mantenía dentro del género *Acacia* era el número de estambres. *Acaciella angustissima* es una de las 15 especies actualmente descritas dentro de este importante género, se distribuye ampliamente desde el sur de los Estados Unidos hasta la Argentina (Fig. 1).

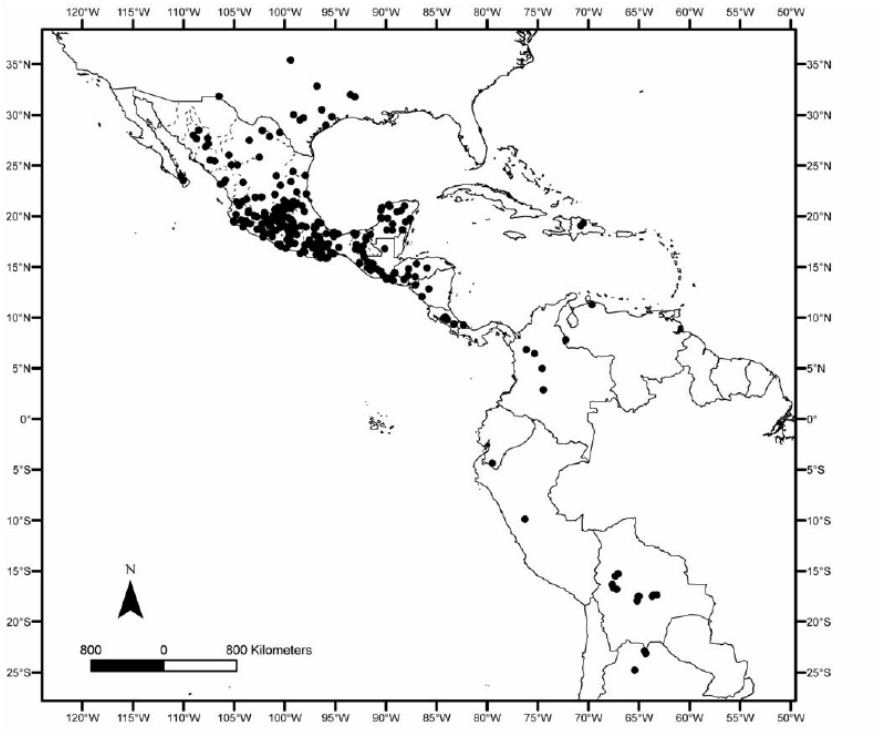

Figura 1. Rango biogeográfico de *Acaciella angustissima.*

Fuente: (Rico-Arce & Bachean, 2006)

En México, poblaciones importantes de *A. angustissima* se localizan principalmente en la costa del Pacífico, en donde ocupan un amplio espectro de hábitats, desde niveles cercanos al mar hasta los 2500 m de altura. Éstos incluyen bosque de pino-encino, bosque seco de temporal y semi-desértico. Las especies son tolerantes a un amplio espectro de tipos de suelos (Dzowela, 1994).

A. angustissima crece como un arbusto pequeño principalmente de 2-7 m de altura con un tronco corto único, produce muchas ramas y abundante follaje (Fig. 2). En su ambiente natural es encontrada sobre laderas, suelos pedregosos, cimas y en pastizales con otros arbustos (Roshetko, 2001). Recientemente, *A. angustissima* ha generado un creciente interés para su uso en sistemas de agroforestería, debido a su rápido crecimiento, capacidad de fijación de nitrógeno y a la calidad de los taninos que acumula en su corteza (Rincón *et al*., 2003). Detalles sobre la biología y ecología de *A. angustissima* y su importancia como un recurso biótico puede ser encontrado en el apéndice 7.1 de este documento.

Los árboles fijadores de nitrógeno, como *A. angustissima* forman islas de fertilidad, incrementan el contenido de materia orgánica del suelo, previenen la erosión y forman un refugio para la flora y fauna (Reyes-Reyes *et al*., 2003), como sucede en Chiapas, en donde este arbusto es el hábitat preferido del insecto *Llaveia mexicanorum* (Margarodidae), una especie en peligro de extinción, del cual se extrae una grasa que es empleada en las artesanías de la laca (Grillasca, 2007). Una de las características biológicas mas importantes de *A. angustissima* es su capacidad para establecer simbiosis con bacterias fijadoras de nitrógeno (Rincón-Rosales & Gutiérrez-Miceli, 2008). Sin embargo, existen muy pocos reportes relacionados con la diversidad de rizobia que nodulan a esta leguminosa.

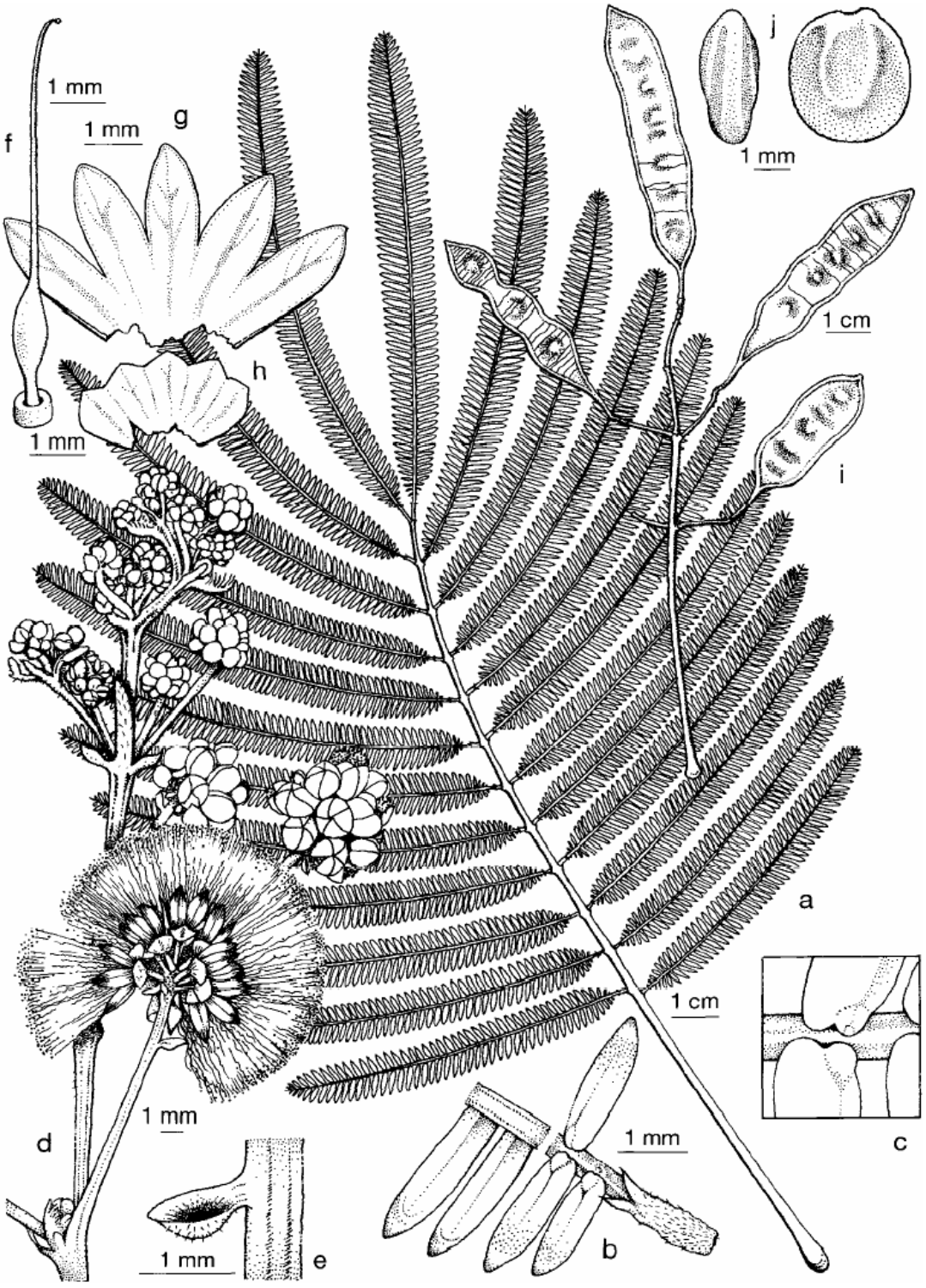

Figura 2. *Acaciella angustissima.* a, hoja; b, detalle de los folíolos y la base de la pinna; c, inserción de los folíolos; d inflorescencia; e, bráctea de la inflorescencia; f, gineceo mostrando el nectario basal; g, corola abierta; h, cáliz abierto; i, infrutescencia; j , semillas (Esquema tomado de Rico-Arce & Bachean, 2006).

A partir de arbustos que crecen en varios ecosistemas naturales en Chiapas y Morelos, México, se han aislado una cantidad importante de cepas de rizobia y se ha encontrado que *A. angustissima* es una especie promiscua y la nodulan principalmente rizobia de rápido crecimiento. La mayoría de los aislados obtenidos, tanto en Chiapas, como en Morelos, correspondieron al genero *Sinorhizobium.* Dentro de este grupo de bacterias fue identificada una nueva especie y se propuso el nombre de *Sinorhizobium mexicanum* para este linaje (Lloret *et al*., 2007). Información más detallada sobre la descripción *S. mexicanum* puede ser encontrado en el apéndice 7.2.

Se han empleado diversas pruebas en el estudio de la sistemática de rizobia. Muchos de los agrupamientos primarios normalmente se establecen mediante el análisis de perfil de proteína total (de Lajudie *et al*., 1994), huellas digitales por PCR (Laguerre *et al*., 1994), electroforesis de enzimas métabolicas (MLEE) (Wang *et al*., 1998) o por las características fenotípicas (Chen *et al*., 1988). Las secuencias del gen ribosomal y la hibridización ADN-ADN son realizadas para elucidar las similitudes a la especie descrita. Así también, las características de los genes simbióticos y el tipo de hospedero que nodulan son las pruebas actualmente analizadas en la descripción de nuevas especies (Haukka *et al*., 1998; Wang *et al*., 2002; Toledo *et al*., 2003). Las secuencias consenso ERIC y REP han sido ampliamente usadas en los estudios de ecología, genéticos y taxonómicos, así como también para la identificación de cepas de rizobia (Mostazo *et al*., 2002). Por otro lado, Lloret *et al*. (2007) reportaron que los genes *gyrA, nolR, recA, rpoB, rrs* y *ssb,* fueron útiles para identificar al nuevo linaje de *S. mexicanum* que nodula a la leguminosa nativa *A. angustissima* en los bosques tropicales de Chiapas y Morelos.

Cabe señalar que *S. mexicanum* (Lloret *et al.,* 2007) no ha sido único simbionte encontrado en los nódulos de *A. angustissima,* sino que también se aislaron otros grupos simbióticos, algunos de los cuales corresponden a especies no descritas antes. El análisis filogenético realizado con la secuencia parcial del gen cromosomal *rpoB,* que codifica para la subunidad β de la RNA polimerasa (Dahllöf *et al*., 2000; Khamis *et al*., 2003) permitió conocer que *A. angustissima* es nodulada por bacterias rizobia perteneciente al género *Rhizobium, Sinorhizobium, Agrobacterium* y *Mesorhizobium.* Este estudio fue complementado con el análisis filogenético de los genes plasmídicos *nifH* y *nodA* (Rincón-

Rosales *et al*., 2008). Los genes *nifH* y *nodA* han sido comúnmente empleados en varios estudios de la diversidad de rizobia que nodulan leguminosas arbustivas que crecen en América Latina, África (Haukka *et al*., 1998) y México (Toledo *et al*., 2003; Lloret *et al*., 2007).

En el capítulo III, de esta tesis se presenta el estudio completo sobre la diversidad de rizobia que nodulan a *A. angustissima* en dos regiones fisiográficas de México y en donde se incluye la propuesta de la nueva especie *Sinorhizobium chiapanecum.*

Considerando la importancia biológica, ecológica, social y económica que representa *A. angustissima,* se han establecido viveros en los estados de Chiapas y Morelos, con la finalidad de lograr su propagación y posterior re-introducción en su hábitat natural, mediante programas de reforestación. Durante el cultivo de esta leguminosa en suelos agrícolas se observó que se requiere de inoculantes de bacterias fijadoras de nitrógeno para lograr un buen desarrollo de las plantas. Lo anterior obligó a analizar y seleccionar las cepas más adecuadas para su empleo como inoculantes. Las cepas para ser consideradas como inoculantes deben de ser eficientes en la fijación de nitrógeno y competitivas (Hungria *et al.,* 2000; Mostazo *et al*., 2002). La competitividad entre cepas proporciona más información sobre las características de una bacteria para nodular a una planta de leguminosa. Con este tipo de ensayos, se encontró que *Rhizobium etli* fue más competitiva para la formación de nódulos que *R. tropici, R gallicum* y otras especies que se conoce nodulan a *Phaseolus vulgaris* (Martínez-Romero y Rosenblueth, 1990; Segovia *et al*., 1993; Martínez-Romero *et al*., 1998; Martínez-Romero, 2003). Asimismo, los niveles de fijación de nitrógeno es una característica deseable para seleccionar bacterias y éste fue el criterio utilizado para demostrar que las cepas *Burkholderia* son los simbiontes naturales de las plantas de *Mimosa* en América (Chen *et al*., 2005). De igual manera, tal como se indica en este trabajo, la selección de cepas para su empleo como inoculantes en *A. angustissima* se logró mediante pruebas de competencia entre cepas que representan cada uno de los géneros identificados previamente, así mismo por la evaluación del potencial de nodulación y capacidad de fijación de nitrógeno.

II. Objetivo

El objetivo de este trabajo fue caracterizar los simbiontes que nodulan a *Acaciella angustissima* en México y evaluar el potencial de nodulación de estas cepas y su capacidad de fijación de N_2 para ser empleadas como inoculantes en esta leguminosa arbustiva.

2.1. Objetivos específicos:

- Caracterizar genéticamente e identificar las bacterias rizobia que nodulan a la leguminosa arbustiva *A. angustissima* que crece en condiciones naturales en dos regiones fisiográficas de México.

- Realizar el estudio filogenético y de la diversidad de las cepas rizobia aisladas de nódulos de *A. angustissima* empleando el gen cromosomal *rpoB* y los genes plasmídicos *nifH* y *nodA*.

- Seleccionar cepas rizobia eficientes y competitivas para ser empleadas como inoculantes en *A. angustissima.*

III. Rhizobia with different symbiotic efficiencies nodulate *Acaciella angustissima* in Mexico including *Sinorhizobium chiapanecum* sp. nov. that has common symbiotic genes with *S. mexicanum.*

RESEARCH ARTICLE

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

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Acaciella angustissima; N_2 fixation; nodulation; rhizobial diversity; legume symbiosis.

Abstract

Bacteria from nodules of the legume *Acaciella angustissima* native to the south of México 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 *A. angustissima* (*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). México is a mega diverse country and occupies the fourth place in plant diversity (Rzedowsky, 1978) with many endemic legumes. The genus *Acaciella* is found mainly in México and has well-supported botanical differences recognized as being 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 N_2 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-Rosales & Gutiérrez-Miceli, 2008). Interestingly, these shrubs are 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 (Grillasca, 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* (Lapage *et al.*, 1992). 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 México (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, México, and from nodulated trap plants grown in pots containing soils collected from an ecological reserve area in Sierra de Huautla in Morelos, México (supporting 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	
ITTG S2	Chiapas, México
ITTG S6	Chiapas, México
ITTG S9	Chiapas, México
ITTG S10	Chiapas, México
CFN ESH11	Morelos, México
CFN ESH16	Morelos, México
Mesorhizobium plurifarium	
CFN ESH5	Morelos, México
CFN ESH18	Morelos, México
CFN ESH19	Morelos, México
CFN ESH22	Morelos, México
CFN ESH26	Morelos, México
Rhizobium sp. (*R. gallicum* related)	
ITTG S11	Chiapas, México
Rhizobium sp. (*R. leguminosarum*/*R. etli* related)	
CFN ESH6	Morelos, México
CFN ESH7	Morelos, México
CFN ESH34	Morelos, México
Rhizobium tropici	
CFN ESH9	Morelos, México
CFN ESH10	Morelos, México
CFN ESH23	Morelos, México
CFN ESH25	Morelos, México
CFN ESH27	Morelos, México
CFN ESH29	Morelos, México
ITTG S7	Chiapas, México
Sinorhizobium sp. (*S. americanum* related)	
ITTG S8	Chiapas, México
Sinorhizobium chiapanecum sp. nov.	
ITTG R11	Chiapas, México
ITTG S1	Chiapas, México
ITTG S68	Chiapas, México
ITTG S70^T	Chiapas, México
ITTG S71	Chiapas, México
Sinorhizobium mexicanum	
CFN ESH1	Morelos, México
CFN ESH2	Morelos, México
CFN ESH3	Morelos, México
CFN ESH4	Morelos, México
ITTG R4	Chiapas, México
ITTG R7^T	Chiapas, México
ITTG S3	Chiapas, México
ITTG S4	Chiapas, México
ITTG S5	Chiapas, México
ITTG S64	Chiapas, México

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

Nodulation tests

Acaciella angustissima seeds were scarified with H_2SO_4 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,

1957). Inoculation tests were also performed with *Acacia farnesiana*, *Leucaena leucocephala* and *Phaseolus vulgaris* cv. Negro Jamapa as described (Lloret *et al.*, 2007). Bacteria for inoculation were grown in individual PY plates and suspended in 1 mL sterile distilled water, serially diluted and absorbance was determined at $A_{600\,nm}$. Final cell numbers were determined by plating on PY medium to count CFUs. Approximately 10^6 bacteria mL^{-1} were added to each plant rootlet and the plants were grown in a plant growth chamber at 28 °C (Räsänen *et al.*, 2001). A negative control with uninoculated seedlings was included. After 30 days, surface-disinfected nodules were harvested and crushed in PY plates and single colonies were picked up and reinoculated for their authentication. Bacteria were conserved in 65% glycerol–PY broth and stored at −80 °C. Working cultures were maintained on YEM slants at 4 °C (Vincent, 1970).

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 Prep™ kit (Amersham). 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 be confirm that the isolates analyzed were not clones or siblings (Ormeño-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 ^{32}P-labelled DNA of the newly proposed species (described later) *Sinorhizobium chiapanecum* ITTG S70^T 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 (Knudsen & 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 BIOEDIT v5 (Hall, 1999). The best-fit evolutive models for each set of sequences were selected by the AKAIKE information criterion implemented in the MODELTEST v3.06 (Posada & Buckley, 2004). *rpoB* gene sequences were analyzed using the TrN+I+Γ model of evolution based on an alignment of 642 nucleotides from positions 3262 to 3903; *nodA* using the GTR+I+Γ model with 522 nucleotides from positions 67 to 588; *nifH* with the TrN+I+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 α-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 α-parameter for the Gamma distribution estimated with MODELTEST. 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 R7^T or *S. chiapanecum* ITTG S70^T 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. 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), *gyr*A, *nol*R and *rec*A 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 a-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* $H152^T$ 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 México. 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 México. The symbiotic gene sequences of *S. chiapanecum* and *S. mexicanum* isolated

Fig. 1. Phylogenetic tree estimated using the ML method with partial sequences of the chromosomal protein encoding gene *rpoB* using the PHYML program. The alignment length was 642 nucleotides from positions 3262 to 3903 of the *rpoB* gene of *Sinorhizobium meliloti* 1021. Only bootstrap values ≥50% are shown. Type strains are indicated by superscript T. The *Acaciella angustissima* strains are shown in bold. Only haplotypes were included in each terminal branch. The accession numbers for the sequences are indicated within parenthesis. Those generated in this work are shown in bold.

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 acatlensis* in México. The *nodA* and *nifH* gene sequences from *S. terangae*, the closest relative of *S. mexicanum* according to *rpoB* gene sequences,

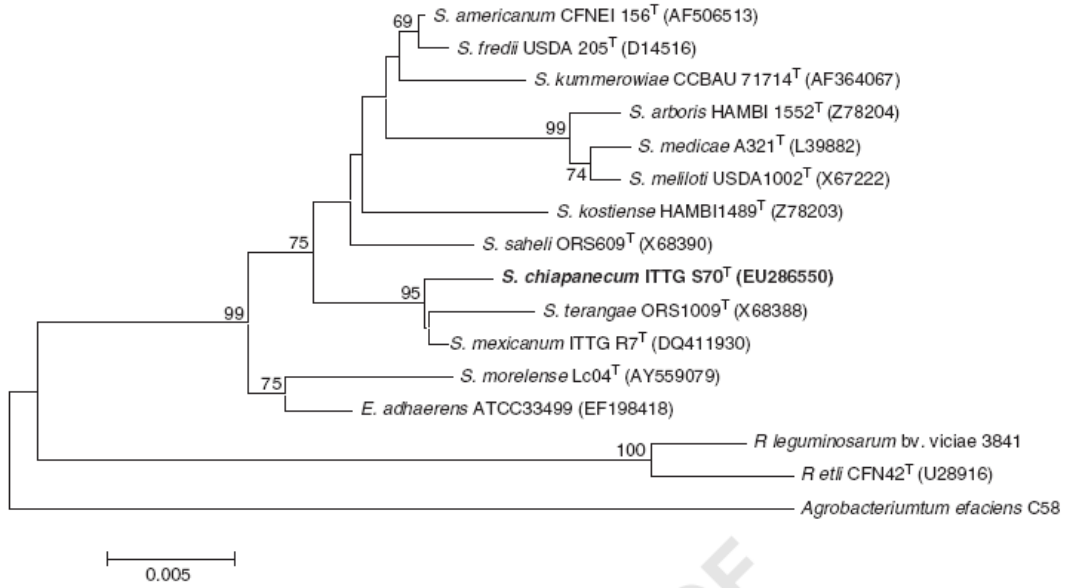

Fig. 2. Phylogenetic tree estimated by the NJ method with the sequences of *rrs* genes using the Tamura Nei model considering gamma (α-parameter = 0.9271). The alignment length was 1416 nucleotides, from positions 28 to 1444 of the *rrs* gene of *Sinorhizobium meliloti* 1021. Only bootstrap values $\geq 50\%$ are shown. Type strains are indicated by superscript T. *Sinorhizobium chiapanecum* is shown in bold.

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 polyacantha* 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^T, ITTG S70^T, CFN ERSH34, CFN ERSH5 and ITTG S7 showed the best nodulation capacity and high competitiveness. *Sinorhizobium mexicanum* strain ITTG R7^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^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^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. gallicum*) 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^T and *S. chiapanecum* ITTG S70^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_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^T and *S. chiapanecum* ITTG S70^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^T showed a significantly higher total shoot nitrogen compared with other treatments ($P < 0.05$). ITTG R7^T was

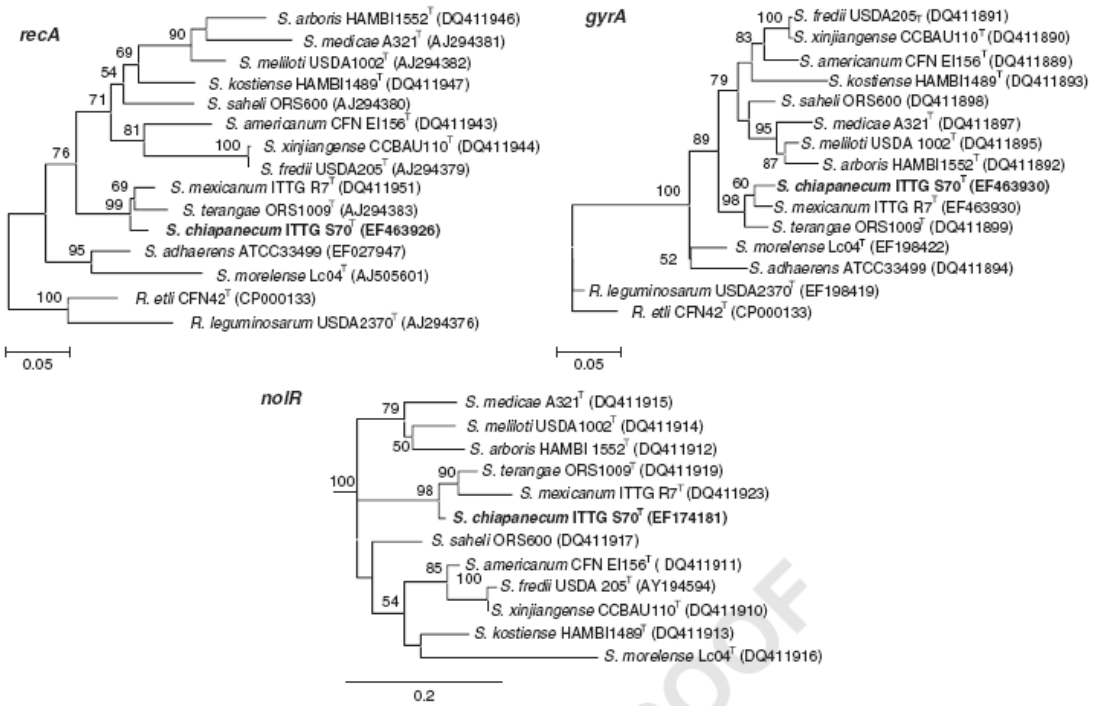

Fig. 3. ML phylogenetic trees of partial sequences of the chromosomal protein encoding genes *gyrA*, *recA* and *nolR*. Type strains are indicated by superscript T. Only bootstrap values > 50% are shown. The accession numbers for the sequences are indicated within parenthesis. Those generated in this work for *Sinorhizobium chiapanecum* are shown in bold. The branches corresponding to the outgroup sequences *Rhizobium leguminosarum* USDA 2370 and *Rhizobium etli* CFN42 for the *nolR* tree are not shown because they were too divergent from the *Sinorhizobium* sequences.

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 México 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^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 S70^T showed low hybridization values with the strains belonging to *S. terangae* ORS1009^T (< 48%) and with *S. mexicanum* ITTGR7^T (< 33%). This species can be differentiated from other described *Sinorhizobium* species on the basis of the phylogenetic analysis of the chromosomal genes *rrs*, *gyrA*, *recA*, *rpoB* and *nolR*. The type strain ITTG S70^T was isolated from nodules of *A. angustissima* collected in the Sumidero Canyon National Park, Chiapas, México. *Sinorhizobium chiapanecum* ITTG S70^T nodulated and fixed nitrogen in *A. angustissima, Acaciella cochliancantha, Acaciella farnesiana, Acaciella pennatula, Dolichos lablab, P. vulgaris, L. leucocephala* and *Lysiloma acapulcensis* and tolerated salinity and acidity (data not shown). ITTG S70^T has characteristics of the species.

Discussion

Tropical forests in México 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 México 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 México in relation to the

Fig. 4. Phylogenetic trees estimated using the ML method with partial sequences of the symbiotic protein encoding *nifH* gene using the PHYML program. The alignment length was 474 nucleotides from positions 313 to 786 of the *nifH* gene with respect to the *nifH* gene encoded on the pSymA of *Sinorhizobium meliloti* 1021. Only bootstrap values ≥50% are shown. Type strains are indicated by superscript T. The *Acaciella angustissima* strains are shown in bold. The accession numbers for the sequences are indicated within parenthesis. Those generated in this work are shown in bold.

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 contrast to acacias, no bradyrhizobia or *Betaproteobacteria* strains were found nodulating this legume. *Acaciella angustissima* was among the legume hosts of Latin American origin that formed nitrogen-fixing nodules with the African sinorhizobial strains *Sinorhizobium arboris* HAMBI 1552^T,

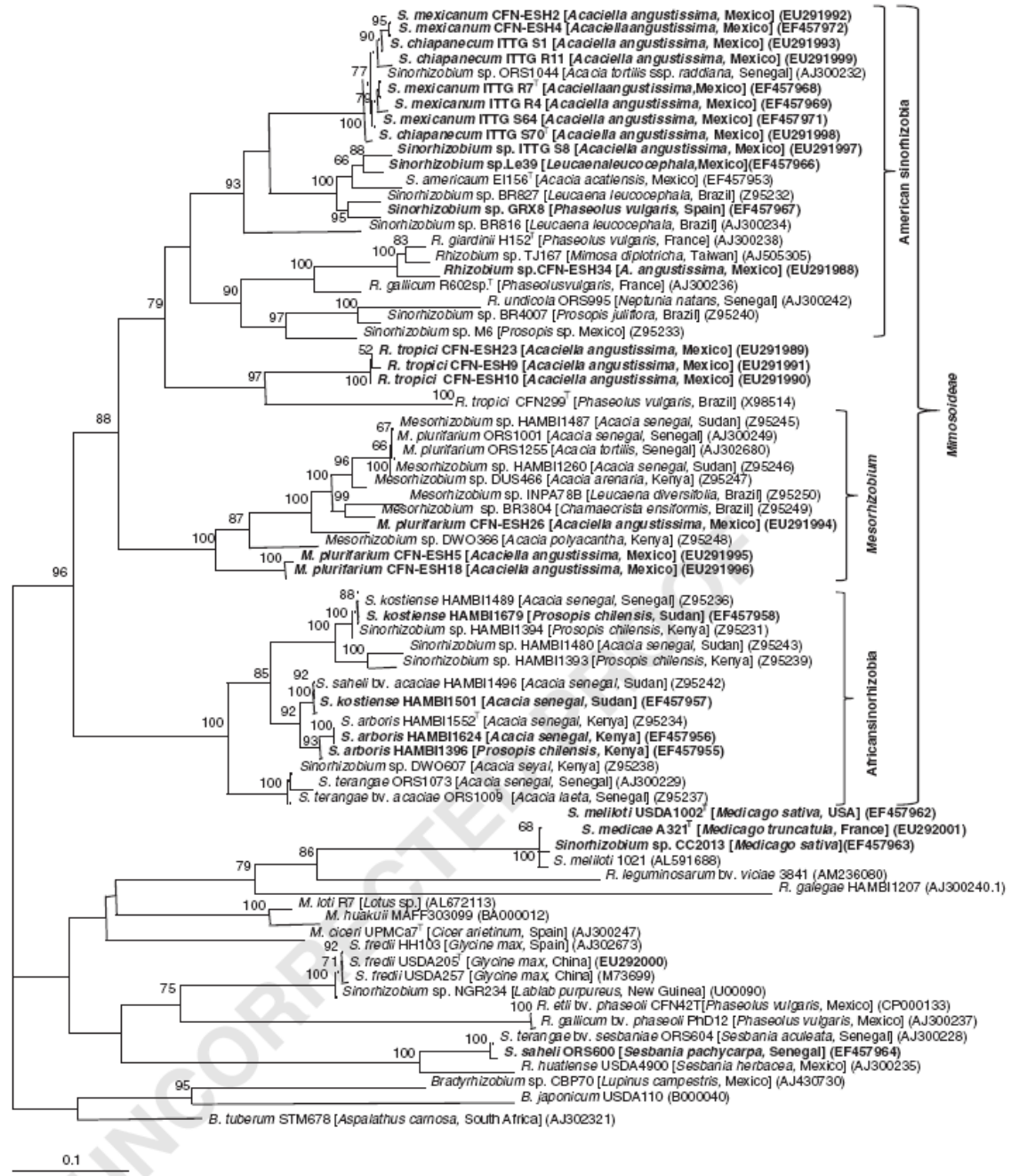

Fig. 5. Phylogenetic trees estimated using the ML method with partial sequences of the symbiotic protein encoding the *nodA* gene using the PHYML program. The alignment length was 522 nucleotides from positions 67 to 588 of the *nodA* gene with respect to the *nodA* encoded on the pSymA of *Sinorhizobium meliloti* 1021. Only bootstrap values ≥ 50% are shown. Type strains are indicated by superscript T. The *Acaciella angustissima* strains are shown in bold. Only haplotypes were included in each terminal branch. The accession numbers for the sequences are indicated within parenthesis. Those generated in this work are shown in bold.

Table 2. Nodule occupancy by strains of *Sinorhizobium mexicanum* ITTG R7^T and *Sinorhizobium chiapanecum* ITTG S70^T 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^h		
A. tumefaciens ITTG S2	0.5 (±1.0)^{gh}		
M. plurifarium CFN ESH5	8.0 (±1.6)^{bcde}		
Rhizobium sp. ITTG S11	2.0 (±0.8)^{fgh}		
Rhizobium sp. CFN ESH34	10.5 (±2.5)^{abc}		
R. tropici ITTG S7	4.0 (±1.6)^{defgh}		
Sinorhizobium sp. ITTG S8	2.25 (±2.1)^{fgh}		
S. chiapanecum ITTG S70^T	10.0 (±1.6)^{abc}		
S. mexicanum ITTG R7^T	14.0 (±3.7)^a		
S. chiapanecum ITTG S70^T+*A. tumefaciens* ITTG S2	4.5 (±1.3)^{defgh}	61 (18)†	39
S. chiapanecum ITTG S70^T+*M. plurifarium* CFN ESH5	4.5 (±2.6)^{defgh}	33 (18)	67
S. chiapanecum ITTG S70^T+*Rhizobium* sp. ITTG S11	4.25 (±1.7)^{defgh}	82 (17)	18
S. chiapanecum ITTG S70^T+*Rhizobium* sp. CFN ESH34	3.25 (±1.9)^{efgh}	23 (13)	77
S. chiapanecum ITTG S70^T+*R. tropici* ITTG S7	2.5 (±1.3)^{fgh}	80 (10)	20
S. chiapanecum ITTG S70^T+*Sinorhizobium* sp. ITTG S8	3.75 (±2.1)^{defgh}	67 (15)	33
S. mexicanum ITTG R7^T+*A. tumefaciens* ITTG S2	6.0 (±1.4)^{cdef}	75 (24)	25
S. mexicanum ITTG R7^T+*M. plurifarium* CFN ESH5	8.75 (±5.1)^{abcd}	89 (35)	11
S. mexicanum ITTG R7^T+*Rhizobium* sp. ITTG S11	5.5 (±1.9)^{cdefg}	100 (22)	0
S. mexicanum ITTG R7^T+*Rhizobium* sp. CFN ESH34	12.0 (±1.4)^{ab}	71 (48)	29
S. mexicanum ITTG R7 ^{T}+*R. tropici* ITTG S7	3.75 (±1.3)^{defgh}	67 (15)	33
S. mexicanum ITTG R7^T+*Sinorhizobium* sp. ITTG S8	6.5 (±3.4)^{cdef}	65 (26)	35

*Mean values of four replicates. The means followed by the same letter are not significantly different ($P < 0.05$).
†In parenthesis, total number of nodules analyzed.

Table 3. Effect of inoculation by the strains with high competitivity 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 c*	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^T	24.8 a	112.9 a	44.0 a	5.3 a	101.6 b
S. mexicanum ITTG R7^T	25.1 a	134.7 a	52.6 a	5.8 a	158.9 a
KNO_3-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 ($P < 0.05$).

Sinorhizobium kostiense HAMBI 1489^T and *S. terangae* bv. *acaciae* ORS 1058 (Räsänen *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) encompasses a set of diverging strains. In this study, *M. plurifarium* strains were found in *A. angustissima* only in Morelos. In México, *M. plurifarium* were found nodulating *Sesbania* (*Papilioniodeae*) trees (Wang *et al.*, 1999) and *L. leucocephala* plants grown in Morelos soils. *Acaciella* 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 *nodA* 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 *Mimosoidea* 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 *Acaciella*.

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. polyacantha, Acacia nilotica* and *S. sesban* (Khbaya *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* represented an equivalent or a larger fraction of nodule isolates compared with the other species encountered. *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 annus*) and found that these strains are not tumorogenic (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. chaipanecum* nodulating *Acaciella* have the same *nodA* 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. leucephala* in México (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. mediterranense and *S. americanum* to those from *S. mexicanum* and *S. chiapanecum*, we consider that *A. angustissima* symbionts would not correspond to biovar mediterranense because the isolates that we found to be closely related to biovar mediterranense 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.

Statement

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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 chiapanecum* strain ITTG S70^T and related reference strains.

Table S2. Levels of total DNA-DNA relatedness as percent of hybridization of *Sinorhizobium chiapanecum* strain ITTG S70^T isolated of *A. angustissima* with the *S. mexicanum* and *S. terangae* strains.

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IV. Discusión

La flora de México es reconocida por su riqueza en especies. Tal diversidad es consecuencia de la extensa gama de ambientes que caracterizan el territorio del país que es centro de evolución de muchos linajes vegetales (Rzedowsky, 1978). Suponemos que la diversidad de plantas y condiciones ambientales también tiene como consecuencia una gran diversidad de bacterias asociadas a plantas nativas. De leguminosas nativas de México se han obtenido y caracterizado nuevas especies de rizobia (ver Apéndice 7.3). *Acaciella angustissima* es una leguminosa arbustiva versátil, que crece en suelos áridos y en sitios secos que no son adecuados para muchos cultivos vegetales. Hay interés en usar esta especie en sistemas de agroforestería debido a su rápido crecimiento y alta capacidad de fijación de nitrógeno (Roshetto, 2001). Los arbustos de *A. angustissima* que crecen en las regiones áridas y semi-áridas fijan entre 80-100 kg N ha^{-1} $año^{-1}$, superando a otras leguminosas arbustivas que en promedio fijan de 20-40 kg N ha^{-1} $año^{-1}$ (Dzowela, 1994), esta capacidad de fijación está en función de las bacterias rizobia, con las que establece simbiosis. El análisis filogenético de las secuencias parciales del gen *rpoB* de las cepas rizobia aisladas de *A. angustissima* en Chiapas y Morelos, México reveló que esta leguminosa es nodulada por los géneros *Rhizobium, Mesorhizobium, Sinorhizobium* y *Agrobacterium.* De esta manera, encontramos que el mayor porcentaje de aislados en ambos sitios correspondió a *Sinorhizobium mexicanum* (Lloret *et al.,* 2007), una nueva especie de sinorhizobia relacionada con la especie Africana *S. terangae.* En otros trabajos se ha reportado que las acacias son noduladas por *Sinorhizobium terangae* bv. acaciae (de Lajudie *et al.,* 1994), *S. kostiense* (Nick *et al.,* 1999)*, S. americanum* (Toledo *et al.,* 2003)*, Mesorhizobium plurifarium* (de Lajudie *et al.,* 1998) y *Bradyrhizobium* spp. (Lafay y Burdon, 2001). Lo anterior indica que las acacias son leguminosas hospederas muy promiscuas. Es conocido que las especies de rizobia dominantes pueden ser preferentemente encontradas en las leguminosas promiscuas y que éstas tienen la ventaja de encontrar simbiontes en nichos nuevos y adaptarse a áreas geográficas diferentes (Moreira *et al.,* 1998). *Acaciella angustissima* tiene un amplio rango de distribución desde el sur de los Estados Unidos a Bolivia, Colombia, Perú, Ecuador y Argentina (Rico-Arce y Bachean, 2006). En México, esta leguminosa puede ser localizada en distintas regiones geográficas,

que se caracterizan por tener varios tipos de vegetación, climas y suelos, así como diferentes altitudes. Se ha observado que las especies de *Acacia* que tienen una amplia distribución presentan nódulos formados por una mayor diversidad de bacterias rizobia, en comparación con aquellas especies de *Acacia* raras (Thrall *et al.,* 2000). En Australia los rizobia que nodulan acacias parecen estar determinadas por las condiciones ambientales (Barnett y Catt, 1991). En Chiapas, los arbustos de *A. angustissima* crecen en suelos poco profundos, secos, de naturaleza alcalina, y con deficiencia en fósforo y nitrógeno. El clima que predomina en esta región es de tipo cálido húmedo y las plantas por lo general están ubicadas a una altitud menor a los 1000 m (Rincón-Rosales y Gutiérrez-Miceli, 2008). Por el contrario, en Morelos, las plantas crecen en suelos ligeramente ácidos, medianamente profundos, ricos en materia orgánica y con un buen contenido de potasio (Toledo, 2003). La altitud media de este sitio es de 1200 m y el clima predominante es templado-húmedo (García, 1973). Para ponderar nuestros resultados es importante considerar que las condiciones climáticas y edáficas que presentan estos sitios, actúan como factores abióticos limitantes que definen la diversidad de los rhizobia que establecen simbiosis con esta leguminosa. También es importante señalar que los sitios de muestreo corresponden a áreas naturales protegidas, poco impactada por los humanos, que pudiera haber permitido el aislamiento geográfico de los microsimbiontes a través de un largo periodo de tiempo. Así pues, lo anterior permite comprender algunos de nuestros hallazgos en este trabajo, ya que, por ejemplo; *Sinorhizobium chiapanecum* fue aislado solo en Chiapas, mientras que las cepas relacionadas a *Rhizobium etli* y *Mesorhizobium plurifarium* fueron encontradas solo en Morelos. En México, cepas de *M. plurifarium* han sido aislada de plantas de *Leucaena leucocephala* que crecen en suelos de Morelos, (Wang *et al.,* 1999).

S. chiapanecum corresponde a una nueva especie, que se diferencia de otras especies de *Sinorhizobium* con base en los resultados de la hibridación ADN-ADN, la secuencia de los genes cromosomales *rpoB*, *rrs, gyrA, nolR y recA,* y también por las características fenotípicas. Esta serie de pruebas son las recomendadas por los comités de bacteriología, para la descripción de nuevas especies (Stackebrandt *et al*., 2002).

El análisis filogenético de las secuencias de los genes simbióticos *nifH* y *nodA* fue también empleado para la caracterización genética de los rhizobia aislados de *A. angustissima* considerando que la historia evolutiva de los genes simbióticos depende de su

interacción con el ambiente y debe ser interpretada de forma independiente a la de los genes cromosomales (Sullivan *et al.,* 1998), ya que los genes *nifH* y *nodA* revelan las historias evolutivas de la simbiosis (la fijación de nitrógeno y nodulación). En este trabajo se observó que las secuencias de los genes *nifH* y *nodA* de los aislados de esta leguminosa, tuvieron relación con los correspondientes genes de las especies de los géneros *Rhizobium, Sinorhizobium* y *Mesorhizobium.* Con este análisis genético se encontró que los genes *nodA* y *nifH* de *S. chiapanecum* sp. nov. fueron similares a aquellos de las cepas de *S. mexicanum* y que ambas especies formaron un grupo bien definido junto con otras especies de *Sinorhizobium* aisladas de hospederos americanos. Todas las secuencias de las cepas *Sinorhizobium* aisladas de los nódulos de *A. angustissima* resultaron diferentes a la secuencia de los genes *nodA y nifH* de especies de *Sinorhizobium* aisladas de hospederos africanos, incluyendo la especie *S. terangae,* que de acuerdo a la filogenia del gen cromosomal *rpoB,* resultó ser el pariente más cercano de *S. mexicanum.*

Sinorhizobium mexicanum y S. chiapanecum fueron los aislados obtenidos mas frecuentemente de esta leguminosa y la capacidad de nodulación de estas cepas fue evaluada en pruebas de competencia con las cepas de los diferentes géneros identificados; esto, con la finalidad de seleccionar cepas más efectivas para su empleo como inoculantes. La competencia entre cepas es una prueba que permite obtener información sobre la eficiencia de una bacteria para nodular a una planta de leguminosa cuando dos o más cepas son probadas juntas en los ensayos de nodulación. De esta manera, se determinó que la cepa *S. mexicanum* ITTG $R7^T$ es altamente competitiva y eficiente para nodular a *Acaciella angustissima* y que puede ser empleada como un inoculante para promover un mayor crecimiento de las plantas. Un efecto positivo se ha reportado en varias especies de *Acacia* y de otras leguminosas arbustivas cuando fueron inoculadas con rizobia nativas seleccionadas (Njiti y Galiana, 1996; Räsänen *et al*., 2001; Diabate *et al.,* 2005). Se evaluó el espectro de hospedero de la cepa ITTG $R7^T$ y se encontró que puede nodular a las leguminosas *A. cochliacantha, Acacia farnesiana, Acacia pennatula, Dolichos lablab, Leucaena leucocephala, Lysiloma acapulcensis* y *Phaseolus vulgaris*. Asimismo esta cepa tolera salinidad y acidez. Estas características y las anteriormente señaladas, hacen que *S. mexicanum* ITTG $R7^T$ sea una cepa ideal para ser usada como un biofertilizante.

V. Conclusiones

- La leguminosa arbustiva *Acaciella angustissima* es una especie promiscua que puede ser nodulada por varios de los géneros alfa-proteobacteria conocidos.

- El análisis filogenético derivados de las secuencias del gen cromosomal *rpoB* permitió definir que esta leguminosa es nodulada por *Sinorhizobium mexicanum, Rhizobium tropici, Mesorhizobium plurifarium* y *Agrobacterium tumefaciens*, y por bacterias relacionadas a *S. americanum, Rhizobium etli*, *R. leguminosarum* y *R. gallicum*.

- El porcentaje más grande de aislados encontrado en ambos sitios correspondió a *S. mexicanum* (26.3 %), mientras que el más bajo correspondió a la bacteria relacionada a *S. americanum* (*S.* sp. A) y *R. gallicum* (*R.* sp. G) ambos con 2.6 %.

- Una nueva especie de sinorhizobia que nodula a esta leguminosa fue propuesta como *Sinorhizobium chiapanecum* ITTG S70^T y fue aislada solo en Chiapas.

- *S. chiapanecum* sp. nov. fue diferenciada de otras especies de *Sinorhizobium* con base en el análisis filogenético de los genes cromosomales *rpoB* y *rrs.* El ADN total de la cepa ITTG S70^T mostró valores bajos de hibridación con las cepas pertenecientes a *S. terangae* ORS1009^T (<48%) y con *S. mexicanum* ITTGR7^T (<33%).

- La filogenia de los genes simbióticos *nodA* y *nifH* fueron incongruentes con la filogenia obtenida con la del gen *rpoB.* Los genes *nodA* y *nifH* de *S. chiapanecum* fueron similares a aquellos de las cepas de *S. mexicanum*, quienes también son simbiontes de *Acaciella*.

- *S. mexicanum* y *S. chiapanecum* fueron de los aislados más frecuentemente obtenidos de *A. angustissima* y de acuerdo a la secuencia de los genes simbióticos *nifH* y *nodA* formaron un grupo bien definido junto con otras especies de *Sinorhizobium* aisladas de hospederos americanos.

- *S. mexicanum* ITTG R7^T es una cepa altamente competitiva y efectiva que promueve un mejor crecimiento de las plantas de *A. angustissima.* Por lo tanto puede ser recomendada para ser usada como un biofertilizante.

VI. Referencias adicionales

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VII. Apéndice

7.1. Biological characteristics of *Acaciella angustissima* (Mill) Britton & Rose in its natural habitat and assessment of its bark potential in Chiapas, México.

CARACTERÍSTICAS BIOLÓGICAS DE *Acaciella angustissima* (Mill.) Britton & Rose EN SU HABITAT NATURAL Y EVALUACION DE SU POTENCIAL CORTICAL EN CHIAPAS, MÉXICO

BIOLOGICAL CHARACTERISTICS OF *Acaciella angustissima* (Mill.) Britton & Rose IN ITS NATURAL HABITAT AND ASSESSMENT OF ITS BARK POTENTIAL IN CHIAPAS, MÉXICO

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Resumen

En Chiapas, México, una amplia variedad de plantas tienen potencial para uso industrial. Timbre (*Acaciella angustissima*) es una leguminosa arbustiva que acumula taninos en su corteza, y se usa para curtir pieles. En la Depresión Central y en la Meseta Comiteca se seleccionaron ocho sitios para estudiar sus características biológicas y su hábitat, así como evaluar su rendimiento cortical. Los resultados indicaron que *A. angustissima* es una leguminosa muy versátil que crece en diferentes tipos de suelos, en zonas con temperaturas, precipitaciones y altitudes variables. Los arbustos estudiados no presentaron diferencias morfológicas, pero las plantas que crecen en la Meseta Comiteca tuvieron mayor altura que las de la Depresión Central. El sitio experimental Lomas presentó la mayor densidad de arbustos (15 390 plantas ha^{-1}), observándose que 100% de la población estaba conformada por plantas adultas y que registraron el mayor rendimiento cortical en 4 años (176.0 t corteza ha^{-1}) en comparación con los otros sitios estudiados. Sin embargo, la variación en la densidad poblacional del Timbre indica que no hay condiciones para su aprovechamiento. Por tanto, es necesario implementar un programa de reforestación para el aprovechamiento racional de este recurso biótico.

Palabras clave: *Acaciella angustissima*, densidad poblacional, rendimiento cortical, Timbre.

Introducción

Acaciella angustissima es un miembro de la familia leguminosae, que tiene su origen en Belice, América Central (Rico-Arce y Bachean, 2006). En México es conocida como Cantemo, Guajillo y Timbre (Roshetko, 2001). Hay interés en usar *A. angustissima* en sistemas de agroforesteria debido a su rápido crecimiento, capacidad de fijación de nitrógeno y a la calidad de los taninos que acumula en su corteza (Rincón *et al*., 2003). Los árboles fijadores de nitrógeno, como *A. angustissima*,

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Abstract

A wide variety of plants in Chiapas, Mexico, have potential for industrial use. *Acaciella angustissima*, whose local name is Timbre, is a leguminous shrub that accumulates tannins in its bark, which is used to tan leather. In the Central Depression and the Comitec Plateau eight sites were selected to study its biological characteristics and its habitat and to assess bark yield. The results indicated that *A. angustissima* is a very versatile legume that grows in different types of soils, in areas with varying temperatures, rainfall and altitudes. The shrubs studied were not morphologically different, but the specimens that grow on the Comitec Plateau are taller than those of the Central Depression. In the Lomas experimental site shrub population was denser (15 390 plants ha^{-1}) than in the other sites and 100% of this population was composed of adult plants. The highest bark yield was recorded at 4 years of age (176.0 t bark ha^{-1}) at this site. However, the variation in population density indicates that there are no conditions for its use. Therefore, it is necessary to implement a reforestation program to assure rational use of this natural resource.

Key words: *Acaciella angustissima*, population density, bark yield, timbre.

Introduction

Acaciella angustissima, a member of the legume family, originated in Belice, Central America (Rico-Arce and Bachean, 2006). In México it is known as Cantemo, Guajillo and Timbre (Roshetko, 2001). There is interest in using *A. angustissima* in agroforestry systems because of its rapid growth, its capacity for fixing nitrogen, and the quality of the tannins that accumulate in its bark (Rincón *et al*., 2003). Nitrogen fixing shrub, such as *A. angustissima*, form islands of fertility: they increase the content of soil organic matter, prevent erosion, and provide refuge for flora and fauna (Reyes-Reyes *et al*., 2003). Over use, overgrazing, forest fires and cultivation have reduced the distribution and density of these trees and has caused a drastic reduction in

forman islas de fertilidad, incrementan el contenido de materia orgánica del suelo, previenen la erosión y forman un refugio para la flora y fauna (Reyes-Reyes *et al*., 2003). El sobreuso, sobrepastoreo, los incendios forestales y el cultivo de la tierra han reducido la distribución y la densidad de estos árboles y ha causado una disminución drástica en la fertilidad del suelo. *A. angustissima* puede encontrarse en las regiones áridas y semiáridas de México (Dzowela, 1994), y en Chiapas principalmente en la Depresión Central y en la Meseta Comiteca (Rzedowski, 1983) donde se usa en el curtido artesanal de pieles. Sin embargo, poco se conoce acerca de su distribución y abundancia, sus características biológicas, su hábitat y el aprovechamiento de su corteza. Por tanto, el objetivo de este trabajo fue realizar una caracterización biológica de *A. angustissima* en su hábitat natural, cuantificar sus poblaciones actuales y determinar su rendimiento cortical. La hipótesis fue que las poblaciones de Timbre en Chiapas no presentan las características fenológicas adecuadas para la extracción y aprovechamiento de sus cortezas.

Materiales y Métodos

La investigación se hizo de enero de 1999 a diciembre de 2003. Se seleccionaron ocho sitios en dos regiones fisiográficas del Estado de Chiapas, México, donde hay uso artesanal de *A. angustissima* para curtir pieles. En la Depresión Central se eligieron los sitios: Sumidero (Tuxtla Gutiérrez), Cruz ancha (Berriozábal), Piedra Blanca (Ocozocoautla de Espinosa) y Cañada (Suchiapa); en la Meseta Comiteca: Quija (Comitán de Domínguez), Nubes (Las Margaritas), Timbral (Trinitaria) y Lomas (Tzimol) (Figura 1). La ubicación geográfica y la altitud de los sitios se realizo con un equipo GPS, marca Sportrak Magellan 210G. Los datos de temperatura y precipitación se obtuvieron de los centros

soil fertility. *A. angustissima* can be found in arid and semi-arid regions of México (Dzowela, 1994), and in Chiapas, principally in the Central Depression and the Comitec Plateau (Rzedowski, 1978), where it is used by artisans to tan skins. However, little is known about its distribution and abundance, its biological characteristics, its habitat or the use of its bark. Thus, this study was conducted to characterize *A. angustissima* biologically in its native habitat, quantify current populations, and determine bark yield.

Materials and Methods

The study was conducted from January 1999 to December 2003. Eight sites were selected in two physiographic regions of the State of Chiapas, México, where *A. angustissima* is used by craftsmen to tan skins. In the Central Depression the following sites were selected: Sumidero (Tuxtla Gutiérrez), Cruz Ancha (Berriozábal), Piedra Blanca (Ocozocoautla de Espinosa) and Cañada (Suchiapa); Quija (Comitán de Domínguez), Nubes (Las Margaritas), Timbral (Trinitaria) and Lomas (Tzimol) on the Comitec Plateau (Figure 1). Geographic location and altitude of the sites were determined with GPS equipment, Sportrak Magellan 210G. Data on temperature and rainfall were obtained from the weather stations of the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) located in these two regions. Soil samples were collected and sent to the INIFAP Laboratorio Nacional de Fertilidad de Suelos y Nutrición Vegetal (Guanajuato, México) for physicochemical analysis. Land use at each site was determined by interviews with farmers or ejido authorities.

At each site the biological aspects of *A. angustissima* shrubs studied were morphology of leaves, flowers, fruits and seeds; these data were used to identify of the species. Samples of the specimens of timbre collected in the field were sent to the herbarium of the Instituto de Biología, Universidad Nacional Autónoma de México, for identification and taxonomic classification. Height of 10 specimens selected randomly at each site was also measured.

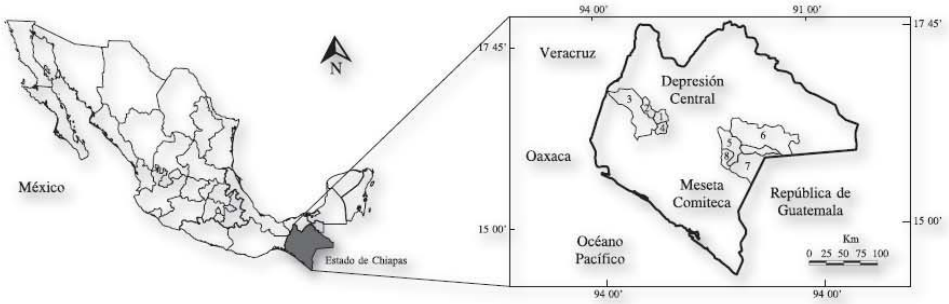

Figura 1. Ubicación de los sitios experimentales donde se estudiaron las características de *Acaciella angustissima*. I. Depresión Central (DC): 1) Sumidero (Tuxtla Gutiérrez); 2) Cruz ancha (Berriozabal); 3) Piedra blanca (Ocozocoautla); 4) Cañada (Suchiapa). II. Meseta Comiteca (MC): 5) Quija (Comitán); 6) Nubes (Las Margaritas); 7) Timbral (Trinitaria); 8) Lomas (Tzimol).

Figure 1. Location of the experimental sites where characteristics of *Acaciella angustissima* were studied. I. Central Depression (DC); 1) Sumidero (Tuxtla Gutierrez); 2) Cruz Ancha (Berriozabal); 3) Piedra Blanca (Ocozocoautla); 4) Cañada (Suchiapa). II. Comitec Plateau (MC): 5) Quija (Comitán); 6) Nubes (Las Margaritas); 7) Timbral (Trinitaria); 8) Lomas (Tzimol).

climatológicos del Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP) en estas dos regiones. Se recolectaron muestras de suelo y se enviaron al Laboratorio Nacional de Fertilidad de Suelos y Nutrición Vegetal del INIFAP (Guanajuato, México) para un análisis fisicoquímico. El uso de suelo en cada sitio se conoció mediante entrevistas directas con los campesinos o comisariados ejidales.

En cada sitio se estudiaron los aspectos biológicos de los arbustos de *A. angustissima*: morfología de las hojas, flores, frutos y semillas; estos datos se usaron para identificar la especie. Muestras de ejemplares de Timbre recolectados en campo se enviaron al Herbario del Instituto de Biología de la Universidad Nacional Autónoma de México para su identificación y clasificación taxonómica. También se determinó la altura de los arbustos eligiendo al azar 10 ejemplares en cada sitio de muestreo. Durante cuatro años consecutivos se estudiaron las etapas fenológicas del Timbre para conocer la temporada de floración y fructificación, así como el momento en que ocurre la germinación de las semillas. También se estudiaron las características de su hábitat y las plagas que atacan a esta leguminosa. La identificación de las plagas se logró con el apoyo de entomólogos del Instituto de Historia Natural y Ecología de Chiapas. La densidad de población de *A. angustissima* se determinó mediante la técnica puntos en cuadrante (Zhang, 2007): 1) en cada sitio se trazó un transecto de 200 m en dirección Norte-Sur y se ubicaron 10 puntos sobre esta línea separados a 20 m; 2) a la mitad de la línea principal se trazó un segundo transecto de 200 m Este-Oeste manteniendo el mismo número de puntos y distancias de separación; 3) con los datos (número de puntos, distancia del punto central a la planta y cobertura del árbol) se calcularon las variables: área media, densidad total, densidad relativa, dominancia relativa, frecuencia relativa, frecuencia, densidad y el valor de importancia (Franco, 1989).

La abundancia de *A. angustissima* en su hábitat se determinó por la edad de los arbustos. Las plantas se dividieron en jóvenes y adultas con base en la altura de planta, diámetro basal, grosor de la corteza y la cobertura. Las plantas con una altura media menor que 1.5 m, diámetro de tallo menor que 3 cm, grosor de corteza menor que 2.5 mm y el diámetro del follaje menor que 1.5 m se consideraron de entre uno y dos años de edad y se clasificaron como jóvenes, mientras que las de una altura media mayor que 2 m, diámetro de tallo entre 5 y 6 cm, grosor de la corteza mayor que 6 mm y diámetro del follaje entre 2 a 3 m tenían entre cuatro y cinco años de edad se consideraron plantas adultas (Roshetko, 2001). El rendimiento cortical en cuatro años (kg ha^{-1}) se calculó con base al número de plantas adultas multiplicado por el rendimiento promedio individual (kg $planta^{-1}$). El rendimiento individual se calculó *in situ* con base en la experiencia de los recolectores de corteza de Timbre en la región y la de otros colectores de acacias en África y Australia (Roshetko, 2001), lo cual se verificó calculando el volumen total de la planta. El volumen se calculó usando la ecuación del cono truncado adaptada para acacias y otras leguminosas tropicales (Cole *et al.*, 1996). Todos los análisis estadísticos se hicieron con el programa SAS (SAS Institute Inc., 1989).

During four consecutive years, phenological stages of timbre were studied to determine flowering and fruiting seasons and to observe the time of germination. Characteristics of its habitat and the pests that attack this legume were also studied. Identification of pests was achieved with the support of the Instituto de Historia Natural y Ecología of Chiapas. *A. angustissima* population density was determined with the points in quadrants technique (Zhang, 2007): 1) each site was transected by a 200 m line drawn from north to south and 10 points on this line were located every 20 m; 2) at the middle of the main line a second 200 m line was drawn from east to west with the same number of points separated by the same distance; 3) with the data (number of points, distance from the central point to the shrub and crown cover) the variables mean area, total density, relative density, relative dominance, relative frequency, frequency, density and importance value were calculated (Franco, 1989).

Abundance of *A. angustissima* was determined by age of the shrubs. Plants were divided into young and adult plants based on plant height, base diameter, bark thickness and cover. Plants with mean height below 1.5 m, stem diameter less than 3 cm, bark thickness less than 2.5 mm and foliage diameter less than 1.5 m were considered to be between one and two years old and classified as young. Those that had a mean height above 2 m, stem diameter between 5 and 6 cm, bark thickness greater than 6 mm and foliage diameter between 2 and 3 m were 4 to 6 years old and considered adult (Roshetko, 2001). Bark yield in four years (kg ha^{-1}) was calculated by multiplying number of adult plants by average individual yield (kg $plant^{-1}$). Individual yield was calculated in situ based on the experience of timbre bark gatherers of the region and other gatherers of acacias in Africa and Australia (Roshetko, 2001); this was verified calculating the total volume of the plant. Volume was calculated using the frustum cone equation adapted for acacias and other tropical legumes (Cole *et al.*, 1996). SAS software (SAS Institute Inc., 1989) was used for all the statistical analyses.

Results and Discussion

Characteristics of the experimental sites

The experimental sites located in the Central Depression are characterized by a hot humid (Aw) climate with summer rains; mean annual temperature fluctuates from 23.0 to 26.5 °C and annual rainfall is 802 to 956 mm. According to García (1973), this type of climate is common in tropical regions of México. Altitude varies from 440 m in Cañada to 1166 m in Sumidero (Table 1). The soils in these places are shallow (less than 15 cm), rocky, and slightly alkaline (pH 7.0 to 7.4); slopes are moderate (less than 30%) and water retention capacity is low. Also, there are important quantities of Ca^{2+}, little organic matter and low contents of N, P and K (Table 2). According to the Soil Survey Staff classification system (1999), these soils are of the aridisol type and are nutrient poor and very susceptible to erosion. On the Comitec Plateau

Resultados y Discusión

Características de los sitios experimentales

Los sitios experimentales ubicados en la Depresión Central se caracterizaron por presentar un clima cálido húmedo (Aw) con lluvias en verano, la temperatura media anual fluctuó de 23.0 a 26.5 °C y la precipitación anual de 802 a 956 mm. Según García (1973), este tipo de clima es común en regiones tropicales de México. La altitud varía de 440 m en La Cañada a 1166 m en El Sumidero (Cuadro 1). Los suelos en estos lugares son poco profundos (menos de 15 cm), pedregosos, con pendientes moderadas (menos de 30%), con baja capacidad para retener agua, ligeramente alcalinos (pH 7.0 a 7.4). Así mismo tienen cantidades importantes de Ca^{2+}, poca materia orgánica y bajos niveles N, P y K (Cuadro 2). De acuerdo con el sistema de clasificación del Soil Survey Staff (1999), estos suelos corresponden al tipo de los aridisoles que son pobres en nutrientes y muy susceptibles a la erosión. En la Meseta Comiteca los sitios experimentales presentaron un clima del tipo cálido subhúmedo (ACw) a

the experimental sites have a hot subhumid climate (ACw) to humid temperate (Cw), with mean annual temperatures from 17.3 to 20.1 °C and average annual rainfall of 1000 mm. Altitude varies from 1380 m in Lomas to 1660 m in Quija. The soils of Quija and Timbral are shallow (less than 30 cm), black to dark brown, clay texture, well-drained, slightly acid, rich in organic matter and high N, P and K contents. According to the classification of the Soil Survey Staff (1999), these soils are vertisols. The soils in Nubes and Lomas are cambisols: black to dark brown, 20 cm deep, rich in organic matter (more than 7%) and N, P and K.

In terms of land use, in Sumidero trees grow under undisturbed natural conditions (ecological reserve). In the sites Cruz Ancha, Piedra Blanca and Cañada, *A. angustissima* populations are found in association with corn. In the Comitec Plateau the experimental sites have undergone major disturbance due to the practice of rainfed agriculture.

Biological and ecological characteristics of *Acaciella angustissima*

A. angustissima in the experimental sites is a shrub, or bush. In the Central Depression the plants had an

Cuadro 1. Localización, condiciones climatológicas y uso del suelo de los sitios experimentales usados para estudiar *Acaciella angustissima* en Chiapas.
Table 1. Location, climate and land use of the experimental sites used for the study of *Acaciella angustissima* in Chiapas.

Sitio experimental, Municipio (localidad)	Coordenadas geográficas[†]	Tipo de Suelo[¶]	Uso del suelo	Clima[§]	Temperatura y lluvia anual	Altitud (m)
Sumidero Tuxtla Gutiérrez (DC)	16° 48' 24" N 93° 04' 56" O	Aridisol	Reserva ecológica	A(w)	26.5 °C, 950 mm	1166
Cruz ancha Berriozabal (DC)[†]	16° 47' 54" N 93° 16' 22" O	Aridisol	Agricultura, maíz	A(w)	23.0 °C, 956 mm	900
Piedra blanca Ocozocoautla (DC)	16° 45' 37" N 93° 22' 20" O	Aridisol	Agricultura, maíz	A(w)	23.8 °C, 802 mm	820
Cañada Suchiapa (DC)	16° 37' 30" N 93° 06' 00" O	Aridisol	Agricultura, maíz	A(w)	24.4 °C, 956 mm	440
Quija Comitán de Domínguez (MC)	16° 15' 37" N 92° 08' 57" O	Vértisol	Agricultura, maíz y frijol	ACw	19.4 °C, 982 mm	1660
Nubes Las Margaritas (MC)	16° 18' 04" N 91° 59' 01" O	Cambisol	Agricultura, maíz	C(w)	17.3 °C, 1025 mm	1520
Timbral Trinitaria (MC)	16° 06' 59" N 92° 03' 02" O	Vertisol	Agricultura, maíz y frijol	ACw	20.1 °C, 1020 mm	1540
Lomas Tzimol (MC)	16° 11' N 92° 12' O	Cambisol	Agricultura, maíz y frijol	C(w)	17.6 °C, 975 mm	1380

[†] DC = Depresión Central, MC=Meseta Comiteca.
[¶] Clasificación de suelos USDA (Soil Survey Staff, 1999).
[§] Clima A(w) = clima calido húmedo con lluvias en verano; ACw = clima calido sub-húmedo; C(w) = templado sub-húmedo.

Cuadro 2. Características del suelo en los ocho sitios experimentales usados para estudiar *Acaciella angustissima* en Chiapas.
Table 2. Soil characteristics at the eight experimental sites used for the study of *Acaciella angustissima* in Chiapas, Mexico.

Sitio experimental	Densidad aparente (g cm^{-3})	Textura[†]	pH	Materia orgánica (g kg^{-1})	N total (g kg^{-1})	K	P	Ca	Mg	CIC
						Disponible (mg kg^{-1})		Total (mg kg^{-1})		
Sumidero	0.95	L-A	7.1	29.0	2.18	32.6	1.25	11.0	32.1	7.21
Cruz ancha	0.94	L-A	7.1	23.1	2.21	26.4	0.72	0.6	40.0	4.97
Piedra blanca	0.90	L-A	7.4	22.0	2.01	28.0	0.57	0.5	23.5	3.21
Cañada	1.20	L-A	7.0	20.8	0.81	25.5	0.68	18.6	24.5	4.07
Quija	1.01	Ar	6.8	71.0	3.72	48.5	2.18	12.9	2.5	2.30
Nubes	1.87	A-Ar	6.1	75.0	4.30	52.0	2.54	12.0	1.8	1.68
Timbral	1.87	Ar	6.0	96.0	4.42	41.5	2.48	12.9	2.5	2.15
Lomas	0.98	A-Ar	6.8	78.9	6.68	49.5	3.34	15.7	3.2	3.15

[†] Clasificación de la textura del suelo (Soil Survey Staff, 1999): L-A = limo-arcilloso, Ar = arcilloso y Ar-A = arcillo-arenoso.

templado húmedo (Cw), con una temperatura media anual de 17.3 a 20.1 °C y una precipitación promedio anual de 1000 mm. La altitud varía de 1380 m en Lomas a 1660 m en Quija. Los suelos en Quija y Timbral son poco profundos (menos de 30 cm), de color negro a café oscuro, de textura arcillosa, con buen drenaje, ligeramente ácidos, ricos en materia orgánica y con grandes cantidades de N, P y K. De acuerdo con el sistema de clasificación del Soil Survey Staff (1999), estos suelos corresponden al tipo de los vertisoles. Los suelos en Nubes y Lomas son del tipo cambisoles, negros a café oscuros, y con 20 cm de profundidad, ricos en materia orgánica (mayor de 7%) y con grandes cantidades de N, P y K.

En relación con el uso del suelo, en Sumidero los árboles crecen en condiciones naturales (reserva ecológica) sin ninguna perturbación. En los sitios Cruz Ancha, Piedra Blanca y Cañada, las poblaciones de *A. angustissima* se encontraron en asociación con cultivos de maíz. En la Meseta comiteca los sitios experimentales presentaron disturbios importantes debido a la práctica de la agricultura de temporal.

average height of 5.0 m and several thin stems, while on the Comitec Plateau they are more than 10 m tall and have a single trunk. No further notable differences in other morphological characteristics were observed between the bushes of the two regions. Leaves are asymmetric with a midrib (8-20 cm long) and 8-12 pairs of pinnae. Inflorescences were present in short clusters with white heads (2.0 cm diameter), which turn orange when they dry. Pods are dehiscent, oblong, 3.6 cm long and 6.9 mm wide, with sinuous edges, initially green, turning dark reddish brown when they mature. Seeds are 2.9-3.2 mm long, 2.5-3.0 mm wide and 1.7-2.0 mm thick, subspherical and dark brown. Timbre flowering begins at the end of summer, mainly in September, and lasts 30 to 45 d. Fruit set begins in November and continues into January. On the basis of the morphological characteristics observed in the timbre specimens, this type of acacia belongs to the genus *A. angustissima* var. *angustissima*, according to the acacia taxonomy proposed by Rico-Arce and Bachean (2006).

A. angustissima populations in the Central Depression are located mainly on hillsides, rocky slopes and grasslands with other shrubs (*Leucaena collinsii*,

Cuadro 3. Características de *Acaciella angustissima* en los sitios experimentales en Chiapas.
Table 3. Characteristics of *Acaciella angustissima* in the Chiapas experimental sites.

Sitio experimental	Cantidad de plantas	Área media (m^2)	Densidad		Dominancia		Frecuencia		Valor de importancia
			Absoluta	Relativa (%)	Absoluta	Relativa (%)	Absoluta	Relativa (%)	
Sumidero	64	1.11	0.724	80	0.816	76	1.0	65	221.77
Cruz ancha	19	1.69	0.281	48	0.571	39	0.9	39	125.27
Piedra blanca	32	7.20	0.056	40	0.140	74	0.7	30	144.86
Cañada	16	4.43	0.090	40	1.373	45	0.7	26	110.91
Quija	47	4.18	0.140	59	0.135	54	0.9	49	161.51
Nubes	38	3.20	0.175	56	0.190	51	0.8	54	160.37
Timbral	34	0.69	1.234	85	3.081	85	1.0	63	232.38
Lomas	36	0.58	1.529	90	3.751	88	1.0	77	255.24

Características biológicas y ecológicas de *Acaciella angustissima*

El hábito de crecimiento de *A. angustissima* en los sitios experimentales es arbustivo. Los arbustos en la Depresión Central tuvieron una altura promedio de 5.0 m con varios tallos delgados, y los de la Meseta Comiteca una altura mayor a 10 m con un tronco único. En relación a otras características morfológicas, los arbustos de Timbre no presentaron diferencias notables entre las regiones. Las hojas son asimétricas con una nervadura central (8-20 cm longitud) y con 8-12 pares de pinnas. Presentaron inflorescencias en racimos cortos, con cabezuelas blanquecinas (2.0 cm diámetro) que se tornan de color naranja cuando se secan. Las vainas son dehiscentes, oblongas, 3-6 cm de longitud y 6-9 mm de ancho, con márgenes sinuosos, inicialmente verdes, volviéndose de color café-marrón cuando maduran. Semillas 2.9-3.2 mm de largo, 2.5-3.0 mm de ancho y 1.7-2.0 mm de grosor, subesféricas, pardo oscuras. La floración del Timbre comienza a finales del verano, principalmente en septiembre y dura entre 30 y 45 d. La formación de frutos comienza en noviembre y se extiende hasta la primavera. La maduración de las vainas comienza a finales de diciembre y termina a principios de enero. Con base en las características morfológicas encontradas en los ejemplares de Timbre estudiados, este tipo de acacia pertenece al género *A. angustissima* var. *angustissima*, según la taxonomia de acacias propuesta por Rico-Arce y Bachean (2006).

Las poblaciones de *A. angustissima* en la Depresión Central se localizaron principalmente en laderas, pendientes rocosas y en pastizales con otros arbustos (*Leucaena collinsii*, *Byrsonimia crassifolia*, *Tecoma stan*, *Lysiloma acapulcensis* y *Acacia farnesiana*). En la Meseta Comiteca los arbustos de Timbre se localizaron en suelos poco pedregosos, laderas y bosques de tipo pino-encino, donde comparte hábitat con *Pinus oocarpa* y *Quercus acatenangensis*. En Chiapas, *A. angustissima* tolera un clima templado y suelos ácidos con buen drenaje; también resiste periodos de sequía, posiblemente debido a su raíz profunda. En los sitios de tierra baja (440 m), como en Cañada, el Timbre florece pero produce pocas semillas, mientras que a mayor altitud (1660 m) como en Quija, las plantas presentan una mayor producción de semillas. Virginia y Jarrel (1983) encontraron que la distribución y abundancia de las especies de *Acacia* y *Prosopis* en zonas áridas en Sonora, México, están limitadas principalmente por la altitud y la composición química de los suelos. En relación a las plagas, arbustos adultos de *A. angustissima* fueron atacados por insectos que dañan su corteza. La hembra del insecto *Coccus axin* (Margarodidae) afecta los tallos y ramas de plantas jóvenes, principalmente en la Cañada y Cruz Ancha. También se encontraron semillas dañadas por escarabajos (Coleoptera: Bruchidae) en todos los sitios estudiados. El daño causado por insectos hace a los árboles más vulnerables a los fuertes vientos de febrero y marzo. Nuevas plantas emergen de los arbustos caídos y, con el inicio de la temporada de lluvias (en mayo), crecen rápidamente y con vigor; esto parece ser una importante estrategia de propagación y supervivencia. Este comportamiento también fue reportado en *A. hindsii*, la cual es atacada por hormigas del género *Pseudomyrmes*, que causan la caída de arbustos viejos y el crecimiento de nuevas plantas en la costa del Pacífico de México (Raine *et al.*, 2002). En nuestro estudio se observó que semillas de timbre caídas en enero y febrero comienzan a germinar en julio (120-150 d después) cuando el suelo está todavía húmedo. Muy pocas semillas son capaces de germinar ya que la cubierta de la semilla es muy dura, haciendo difícil la absorción de agua y nutrientes (Rincón et al., 2003). Las plántulas recientemente germinadas tienen hojas compuestas bipinnadas con forma de tubérculo. La raíz principal está modificada y tiene forma de tubérculo, y las raíces secundarias son delgadas con 2 a 4 pequeños nódulos esféricos de color rojo oscuro.

Population characteristics

In our study, timbre populations were observed in the Lomas site at an altitude of 1380 m. Here, plant density (15 390 plants ha^{-1}) was higher than in the Piedra Blanca site where altitude is 820 m and density was 517 plants ha^{-1}. Räsänen (2002) reported that fires started intentionally (because of land use), climate, altitude, the nature of the soil (nutrients); the presence of pests (defoliators and de-barkers) are the main factors that regulate growth and density of acacia and other bushy legume populations that grow in desert regions and in the ecosystems of the Mexican

(Margarodidae) afectó los tallos y ramas de plantas jóvenes, principalmente en los sitios Cañada y Cruz ancha. También se encontró insectos escarabajos (Coleoptera: Bruchidae) dañando las semillas en todos los sitios evaluados. Los daños causados por los insectos hacen más vulnerables a los árboles viejos a los fuertes vientos que se presentan en febrero y marzo. De los arbustos derribados emergen nuevas plantas y con el inicio de la estación lluviosa (en mayo) crecen rápida y vigorosamente; al parecer esta es una manera importante de propagación de *A. angustissima* y una estrategia para su sobrevivencia. Este comportamiento también se ha reportado en *A. hindsii* que es atacado por las hormigas del genero *Pseudomyrmes* ocasionando la caída de arbustos viejos y el rebrote de nuevas plantas en ecosistemas de la costa del Pacifico Mexicano (Raine *et al.*, 2002). En este trabajo se observó que las semillas de Timbre que se depositaron en el suelo en enero y febrero comienzan a germinar en los primeros días de julio (120-150 d después de su caída), cuando el suelo está todavía húmedo. Muy pocas semillas logran germinar, cuya testa o cubierta es muy dura y dificulta el paso del agua y nutrientes (Rincón *et al.*, 2003). Las plántulas recién germinadas presentaron hojas compuestas dobles bipinnadas, sin filodio, con una raíz principal modificada en forma de tubérculo, con raíces secundarias delgadas que presentaban 2 a 4 nódulos esféricos pequeños de color rojo oscuro.

Características de las poblaciones

En este estudio se observó que las poblaciones de Timbre en el sitio Lomas, a una altitud de 1380 m, registró una mayor densidad de plantas (15 390 ha^{-1}), en comparación con el sitio Piedra Blanca donde la altitud es 820 m y la densidad fue 517 plantas ha^{-1}. Räsänen, (2002) reportó que los incendios (derivados del uso del suelo), el clima, la altitud, la naturaleza del suelo (nutrientes) y la presencia de plagas (defoliadores y descortezadores) son los principales factores que regulan el crecimiento y densidad de las poblaciones de acacias y otras leguminosas arbustivas que crecen en zonas desérticas y en los ecosistemas del altiplano mexicano. El análisis de correlación entre la densidad de plantas de *A. angustissima* de cada sitio contra las otras variables de tipo climatológico, edafológico y con las relacionadas al uso del suelo, indicaron que la densidad de población de *A. angustissima* en Chiapas está relacionada positivamente con la altitud ($r=0.456$; $p\leq0.05$), precipitación pluvial media ($r=0.880$; $p\leq0.01$), cantidad de nitrógeno ($r=0.727$; $p\leq0.05$), fósforo ($r=0.677$; $p\leq0.05$) y con la materia orgánica (0.591; $p\leq0.05$). En este caso, la densidad high plateau. The correlation analysis between *A. angustissima* plant density of each site with other climate, edaphic and land use variables indicated that *A. angustissima* population density in Chiapas correlates positively with altitude ($r=0.456$; $p\leq0.05$), mean rainfall ($r=0.88$; $p\leq0.01$), and amount of nitrogen ($r=0.727$; $p\leq0.05$), phosphorus ($r=0.677$; $p\leq0.05$), and organic matter ($r=0.591$; $p\leq0.05$). The highest density occurred at sites above 1000 m where a sub-humid temperate climate predominates with average annual rainfall of 1000 mm and mean annual temperature of 18.5 °C and where soils have adequate amounts o N, P and K.

Age distribution of plants

At the eight experimental sites there were significant differences ($p\leq0.05$) between percentages of adult and young plants. The highest number of adult plants was found in Lomas and the lowest in Piedra Blanca; in the other sites adult plants predominated (Table 4). Composition, diversity and structure of vascular plants are important indicators of the health and stability of an ecosystem. They are also the source of primary production and a determining element of the habitat of other organisms (Graya and Azuma, 2005). The proportions of young and adult plants found in the eight experimental sites indicate the degree of stability of the *A. angustissima* populations. The traditional cropping system (slash and burn) and forest fires affect the abundance of the timbre populations in Chiapas. The sites where mono-cropping (corn) is found and affected by forest fires showed a high proportion of young plants (shoots), but in the sites with little human activity, adult plants dominate. Forest fires damage recently germinated seedlings, but new plants can regenerate from old burnt plants. This is a typical characteristic of acacias (Räsänen, 2002).

A. angustissima bark yield

Bark yield was significantly different among all of the experimental sites ($p\leq0.05$). The highest potential yields were recorded in the Lomas site and the lowest in the Cañada site (Table 5). These results are logical since in this site the highest values in tree size, base area and population density were recorded.

Conclusions

The information obtained in this study permitted evaluation of *A. angustissima* populations in the State of Chiapas. In spite of the interesting biological and ecological characteristics of this legume, conditions for

más alta de plantas ocurrió en los sitios con una altitud mayor de 1000 m, donde predomina el clima templado sub-húmedo, una precipitación pluvial promedio de 1000 mm, temperatura anual de 18.5 °C y suelos con cantidades adecuadas de materia orgánica, N, P y K.

Distribución de las plantas de acuerdo con la edad

En los ocho sitios experimentales hubo diferencias significativas entre los porcentajes de plantas adultas y jóvenes ($p \leq 0.05$). La cantidad de plantas adultas más grandes se encontraron en Lomas y la más baja en Piedra blanca. En los demás sitios evaluados también predominaron las plantas adultas (Cuadro 4). La composición, diversidad y estructura de las plantas vasculares son indicadores importantes de la salud y estabilidad de un ecosistema; además son la fuente de la producción primaria y un determinante fundamental del hábitat de otros organismos (Graya y Azuma, 2005). Las proporciones diferentes de plantas jóvenes y maduras encontradas en los ocho sitios experimentales indican el grado de la estabilidad de las poblaciones de *A. angustissima*. El sistema tradicional de cultivo (roza-tumba-quema) y los incendios afectan la abundancia de las poblaciones del Timbre en Chiapas. Los sitios con presencia de monocultivos (maíz) y afectados por los incendios mostraron una alta proporción de plantas jóvenes (rebrotes), pero en los sitios con poca actividad humana las plantas adultas dominaron. Los incendios dañan las plántulas recién germinadas, pero pueden regenerarse de los troncos de las plantas viejas quemadas. Esta es una característica típica de las acacias (Räsänen, 2002).

Cuadro 4. Densidad de planta, plantas adultas y jóvenes de *Acaciella angustissima* en ocho sitios experimentales en Chiapas.

Table 4. *Acaciella angustissima* plant density, adult plants and young plants in eight experimental sites, Chiapas, Mexico.

Sitio experimental	Densidad de planta	Planta adulta[¶]	Planta joven[§]
		(ha^{-1})	
Sumidero	7112.8 c[†]	3848.3 c	3264.5 b
Cruz ancha	2983.0 d	2454.0 d	529.0 c
Piedra blanca	517.0 f	480.5 f	36.5 d
Cañada	957.5 ef	745.0 f	212.5 cd
Quija	1457.5 e	896.5 ef	561.0 c
Nubes	1743.0 e	1211.5 e	531.5 c
Timbral	12 670.0 b	7537.0 b	5133.0 a
Lomas	15 390.0 a	15 390.0 a	0.0 d
DMS ($p \leq 0.05$)	809.89	447.14	442.29

[†] Medias con letras diferentes son estadísticamente diferentes (Tukey; $p \leq 0.05$) y los valores corresponden al promedio de 4 repeticiones.

[¶] Plantas adultas con altura promedio mayor que 2 m o más pequeñas, tallo con 5-6 de diámetro, 6 mm de grosor de corteza y 2 a 3 m diámetro de follaje de aproximadamente 4 a 5 años.

[§] Plantas jóvenes con altura promedio de 1.5 m o más pequeñas, tallo con 3 cm de diámetro, 2.5 mm de grosor de corteza y 1.4 m diámetro de follaje de aproximadamente 1 a 2 años.

Rendimiento cortical de *A. angustissima*

El rendimiento cortical fue significativamente diferente para todos los sitios experimentales ($p \leq 0.05$). Los rendimientos potenciales más altos se registraron en el sitio Lomas y los más bajos en el sitio la Cañada (Cuadro 5). Estos resultados son lógicos ya que en este sitio se registraron los valores más altos del tamaño de los árboles, área basal y densidad poblacional.

Conclusiones

La información obtenida permitió evaluar la situación de las poblaciones de *A. angustissima* en el Estado de Chiapas. A pesar de las interesantes características biológicas y ecológicas que presenta esta leguminosa, no hay condiciones para su aprovechamiento industrial. Los datos indicaron una densidad muy baja de plantas adultas aprovechables. Sin embargo, en Lomas y Timbral es posible mantener un aprovechamiento artesanal de sus cortezas, pero de una manera sostenible. Por tanto, es necesario implementar un programa de reforestación para el uso y aprovechamiento racional de este recurso biótico.

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its industrial use do not exist. The data indicated very low density of usable adult plants. However, in Lomas and Timbral it is possible to sustain use of the bark by artisans if a program of reforestation is implemented to assure rational use of this biotic resource.

—End of the English version—

Cuadro 5. Características y rendimiento cortical de *Acaciella angustissima* en ocho sitios experimentales en Chiapas-México.
Table 5. *Acaciella angustissima* characteristics and bark yield at eight experimental sites in Chiapas, Mexico.

Sitio experimental	AP[¶] (cm)	DM[§] (cm)	VOL[Þ] (cm^3/ha)	REND[ø] (kg)	RENDI[††] (kg ha^{-1} 4 $años^{-1}$)
Sumidero	159.3 de[†]	5.3 ef	1365.0 d	1.91 f	7350.2 c
Cruz ancha	123.5 e	5.3 ef	996.8 d	0.55 h	1349.7 e
Piedra blanca	198.7 d	6.8 de	2485.9 d	2.15 e	1033.0 e
Cañada	151.0 e	4.7 f	888.2 d	0.67 g	499.1 e
Quija	261.5 c	9.7 c	7128.8 c	5.58 c	5002.4 d
Nubes	196.5 d	7.6 d	3039.0 cd	4.17 d	5051.9 d
Timbral	318.0 b	13.6 b	16095.4 b	9.54 b	71902.9 b
Lomas	403.0 a	15.9 a	27150.4 a	11.44 a	176061.6 a
DMS ($p < 0.05$)	44.96	1.61	4144.9	0.051	1201.89

[†] Medias con letras iguales no son estadísticamente diferentes (Tukey; $p \leq 0.05$) y los valores corresponden al promedio de cuatro repeticiones.
[¶] AP: altura de planta.
[§] DM: diámetro basal.
[Þ] VOL = volumen calculado usando formula del cono truncado adaptado para acacias y otras arbustos leguminosos: $[(1/3)(pi)(r^2)(AP)]$(Cole *et al.*, 1996).
[ø] REND = rendimiento calculado *in situ*, con base en la experiencia de los colectores de corteza de "Timbre" en la región y la de otros colectores de acacias en África y Australia (Roshetko, 2001).
[††] RENDI = rendimiento; la edad en la que *A. angustissima* se considera una planta adulta es 4 años y es la etapa fenológica para recolectar y aprovechar sus cortezas (Roshetko, 2001).

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7.2. *Ensifer mexicanus* sp. nov. a new species nodulating *Acacia angustissima* (Mill.) Kuntze in México.

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Ensifer mexicanus sp. nov. a new species nodulating *Acacia angustissima* (Mill.) Kuntze in Mexico

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Abstract

A new lineage of *Ensifer* nodulating the American legume *Acacia angustissima* in the tropical forest of Chiapas and Morelos, Mexico is described. Bacteria were identified as *Ensifer* with *ssb* or *nolR* specific primers. Phylogenetic analysis with partial sequences of the five chromosomal genes *gyrA*, *nolR*, *recA*, *rpoB* and *rrs* revealed that this new lineage is related to African *Ensifer terangae*. The results of total DNA–DNA hybridization and selected phenotypic tests among the *A. angustissima* strains and *E. terangae* indicated that they belong to different species. The phylogeny with the symbiotic *nifH* gene also separates this group as a different clade but with close affinities to bacteria belonging to the genus *Ensifer* isolated from American hosts. ITTG R7^T (= CFN ER1001, HAMBI 2910, CIP 109033, ATCC BAA-1312, DSM18446) is the type strain of a new species for which the name *Ensifer mexicanus* sp. nov. is proposed.

Keywords: Phylogeny; Taxonomy; Systematics; Legume symbiont; Nitrogen fixation; *Acacia angustissima*; *Sinorhizobium*; *Ensifer*, sp. nov

Introduction

Several genera within the α-Proteobacteria [43,49,65] as well as a few genera of β-Proteobacteria [5,32] induce the formation of nodules and fix nitrogen in the roots and rarely in the stems of leguminous plants, providing nitrogen that supports plant growth. Among these, the α-Proteobacterial genus *Sinorhizobium,* now renamed as *Ensifer* (because of the joining of *Sinorhizobium* and *Ensifer* genera into the same genus [63,67]), includes over 10 species isolated from a wide range of legume hosts.

Acacia is one of the largest genera of the Leguminosae and underwent successive radiations in the Southern continents with Australia being the largest diversification center and the tropics of the Americas among the oldest. *Acacia angustissima* has a broad geographical distribution ranging from the Southern United States to Costa Rica. It is considered a promising tree species to restore eroded areas.

Acacias have been reported to be nodulated by *Ensifer terangae* bv. acaciae, *Ensifer saheli* bv. acaciae

Abbreviations: PCR: Polymerase chain reaction; rep-PCR: repetitive extragenic palindromic PCR, ERIC: Enterobacterial repetitive intergenic consensus, MLEE: Multilocus enzyme electrophoresis

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[6], *Ensifer arboris* [33], *Ensifer kostiense* [33], *Ensifer americanus* [53], *Mesorhizobium plurifarium* [7] and *Bradyrhizobium* spp. [24]. The rhizobia nodulating *A. angustissima* were unknown. In the course of a study on the diversity of *A. angustissima* symbionts from two areas of Mexico, new bacteria belonging to the genus *Ensifer* were identified. It is the aim of this work to describe this lineage as a new *Ensifer* species mainly based on the analyses of genotypic traits.

Materials and methods

Bacterial isolation, cultural conditions and plant inoculation

Bacterial isolates were obtained from naturally occurring *A. angustissima* root nodules collected in Tuxtla Gutiérrez and the Sumidero Canyon National Park in Chiapas, Mexico or from nodules harvested from young *A. angustissima* seedlings used as trap plants after their inoculation with soil samples collected from an ecological reserve area in Sierra de Huautla in Morelos, Mexico (Fig. S1 and Table 1). Chiapas and Morelos collecting sites are far away from each other (approximately 1000 km) and both have deciduous forest vegetation type. The bacteria were grown in peptone-yeast (PY) extract medium [53] or yeast extract-mannitol (YEM) medium [56] at 28 °C and further purified to single colonies. Pure cultures were authenticated by their capacity to nodulate *A. angustissima* in pots with Fahraeus solution [11] as previously described [53]. Nitrogen fixation was evaluated with the acetylene reduction assay as described previously by Martínez et al. [28]. Inoculation tests were also performed with *Phaseolus vulgaris* (common bean), *Acacia cochliacantha* and *Leucaena leucocephala*.

DNA isolation and genomic fingerprinting

Genomic DNA was isolated from overnight bacterial cultures grown in PY using the Genomic Prep™ kit (Amersham-Pharmacia). ERIC genomic fingerprinting were obtained by rep-PCR using primers ERIC1R and ERIC2 as described by Versalovic et al. [55] Reactions were carried out in 25 µl final volumes with 1 × polymerase buffer, 1 U *Taq* polymerase (Invitrogen), 20 pmol of each primer and 7.5 mM $MgCl_2$. The

Table 1. Original hosts and geographical origins of the *E. mexicanus* and reference strains

Reference strains	Isolation host	Geographical origin	Reference/source
E. adhaerens ATCC 33212^T	—	Pennsylvania, USA	[1]
E. adhaerens ATCC 33499	—	Pennsylvania, USA	[1]
Sinorhizobium morelense Lc04^T	*Leucaena leucocephala*	Morelos, Mexico	[59]
E. arboris HAMBI 1552^T	*Prosopis chilensis*	Sudan, Africa	[33]
E. fredii USDA 205^T	*Glycine soja*	Henan, China	[45]
E. kostiense HAMBI 1489^T	*Acacia senegal*	Sudan, Africa	[33]
E. kummerowiae CCBAU 71714^T	*Kummerowia stipulacea*	Shaanxi, China	[60]
E. medicae A321^T	*Medicago truncatula*	Aude, France	[39]
E. meliloti USDA 1002^T	*Medicago sativa*	Virginia, E.U.A.	[20,66]
E. saheli ORS 609^T	*Sesbania cannabina*	Senegal, Africa	[6]
E. saheli ORS 600	*Sesbania pachycarpa*	Senegal, Africa	[6]
E. terangae ORS 1009^T	*Acacia laeta*	Senegal, Africa	[6]
E. terangae ORS 1073	*Acacia senegal*	Senegal, Africa	[6]
E. terangae ORS 19	*Sesbania cannabina*	Senegal, Africa	[6]
E. terangae ORS 604	*Sesbania aculeata*	Senegal, Africa	[6]
E. xinjiangense CCBAU 110^T	*Glycine max*	Xinjiang, China	[4]
R. leguminosarum USDA 2370^T	*Pisum sativum*		[20,66]
R. etli CFN42	*Phaseolus vulgaris*	Guanajuato, Mexico	[47]
Acacia angustissima strains			
ITTG R4	*A. angustissima*	TG[a], Chiapas, Mexico	This study
ITTG R7^T	*A. angustissima*	TG, Chiapas, Mexico	This study
CFN ESH1	*A. angustissima*	HM[b], Morelos, Mexico	This study
CFN ESH3	*A. angustissima*	HM, Morelos, Mexico	This study
CFN ESH4	*A. angustissima*	HM, Morelos, Mexico	This study
ITTG S4	*A. angustissima*	SC[c], Chiapas, Mexico	This study
ITTG S64	*A. angustissima*	SC, Chiapas, Mexico	This study

[a]TM – Tuxtla Gutiérrez.
[b]HM – Huautla Mountains.
[c]SC – Sumidero Canyon.

fingerprints were visually analyzed after separation of PCR products by electrophoresis in 1.5% agarose gels loaded with half volume of the PCR reaction. Only isolates showing different patterns were considered for sequencing and phylogenetic analysis in order to be certain that strains analyzed were not clones or siblings.

DNA hybridization

The DNA relatedness was determined by DNA–DNA hybridization experiments using ^{32}P labeled DNA of strain ITTG R7 as a probe. A filter hybridization method previously described was used [29]. The amounts of DNA were standardized by integrating gel fluorescence with the Eagle Eye II system (Stratagene).

PCR amplification and gene sequencing

Internal fragments of the protein coding chromosomal genes *rpoB*, *gyrA*, *recA* *nolR* and *ssb,* and the symbiotic plasmid-borne *nifH* gene were amplified by standard PCR reactions with 1% DMSO. Primers and annealing temperatures are described in Table S1 and in [9,13,22,61]. The PCR products were purified and sequenced (except for *ssb*). The sequences generated, were deposited in the Genbank public database and their accession numbers were included in the phylogenetic trees or Table S2.

Phylogenetic analysis

The protein-coding nucleotide sequences were aligned based on codons using the program DAMBE v4.2.13 [64] which implements CLUSTAL W [52], and edited with BioEdit 5 [17]. Molecular phylogenies were reconstructed with the maximum-likelihood (ML) method using PhyML 2.4.4 [16]. The best-fit model for each set of sequences was selected by the Akaike information criterion implemented in MODELTEST 3.06 [37]. The gamma parameter and the proportion of invariable sites were estimated with PhyML. The topology robustness was estimated by nonparametric bootstrap tests using 500 pseudoreplicates. The phylogenetic tree with the *rrs* sequences from the *Ensifer* type strains was constructed by the neighbor-joining (NJ) method [41] implemented in MEGA v3.1 [23] using the TN + G model and a bootstrap test using 1000 pseudoreplicates. Intragenic recombination was checked on each alignment of the chromosomal protein coding genes using the recombination detection prediction tests implemented in the RDP2 program [27], which combines the RDP [26], GeneConv [44], Bootscan [42], MaxChi [31], Chimaera [36], and SiScan [14] programs.

Identity confidence intervals

Towards defining large databases and robust molecular phylogenies in *Ensifer*, we have partially sequenced the *gyrA* and *nolR* genes of at least two different strains for each reported species. Each set of sequences was aligned and identities were determined for all pair of combinations. The intra and inter-species nucleotide identity were determined, variance was calculated and the 99% confidence intervals were established.

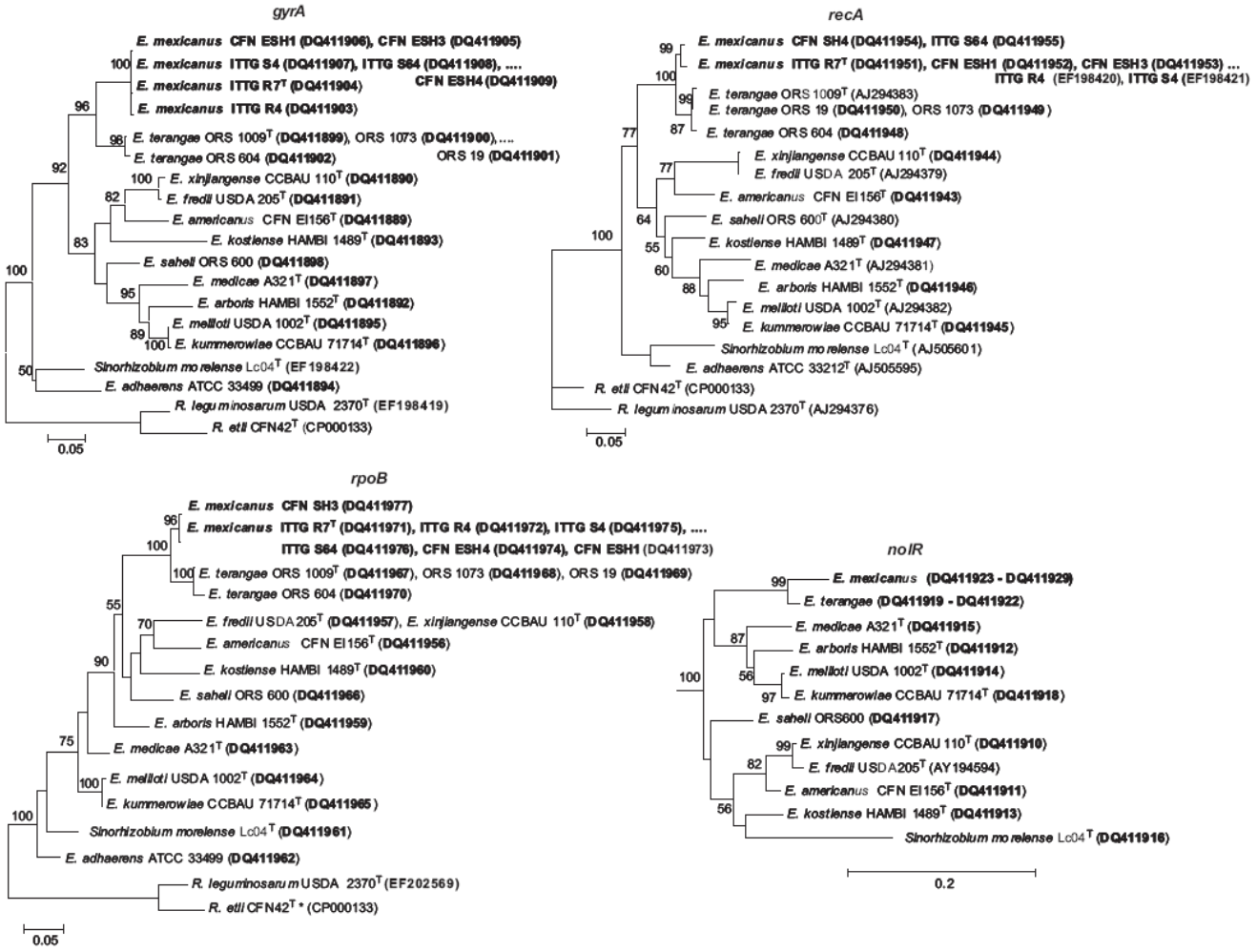
Multilocus enzyme electrophoresis (MLEE)

Cell extracts were prepared from 50 ml PY broth cultures grown overnight at 28 °C as described by Segovia et al. [46]. Protein separation on starch gels and staining of the enzymes were performed according to the procedures described by Selander et al. [48]. The following metabolic enzymes were evaluated at least three times for each isolate: indophenol oxidase, isocitrate dehydrogenase, malate dehydrogenase, aconitase, phosphoglucomutase, glucose 6-phosphate dehydrogenase, hexokinase, and α-esterases. The genetic distance between each pair of ETs was estimated as the proportion of loci at which dissimilar alleles occurred [48]. Clustering from a matrix of pairwise genetic distances was performed by using the NJ method [41].

Plasmid content

A modified Eckhardt procedure [10,19] was used to visualize the plasmid patterns. The symbiotic plasmids

Fig. 1. Maximum likelihood phylogenetic trees of partial sequences of the chromosomal protein encoding genes. *gyrA* (528 nt, positions 1111–1638 of the *gyrA* gene of *A. tumefaciens* C58); *recA* (450 nt, positions 109–558 of the *recA* gene of *A. tumefaciens* C58); *rpoB* (672 nt, positions 3232–3903 of the *rpoB* gene of *E. meliloti* 1021); and *nolR* (267 nt, positions 25–291 of the *nolR* gene of *E. meliloti* 1021). Only bootstrap values ⩾50% are shown. Type strains are indicated by superscript T. The *E. mexicanus* strains are shown in bold. Strains with identical sequences are included in the same terminal branch. The accession numbers for the sequences are indicated within parenthesis. Those generated in this work are shown in bold. In the *nolR* tree, the *E. mexicanus* and *E. terangae* strain names were omitted because within each species the sequences were identical. The branches corresponding to the outgroup sequences *R. leguminosarum* USDA 2370 and *R. etli* CFN42 for the *nolR* tree are not shown because they were too divergent from the *Ensifer* sequences.


gyrA
E. mexicanus CFN ESH1 (DQ411906), CFN ESH3 (DQ411905)
E. mexicanus ITTG S4 (DQ411907), ITTG S64 (DQ411908), ...
E. mexicanus ITTG R7T (DQ411904)
CFN ESH4 (DQ411909)
E. mexicanus ITTG R4 (DQ411903)
E. terangae ORS 1009T (DQ411899), ORS 1073 (DQ411900),....
E. terangae ORS 604 (DQ411902)
ORS 19 (DQ411901)
E. xinjiangense CCBAU 110T (DQ411890)
E. fredii USDA 205T (DQ411891)
E. americanus CFN EI156T (DQ411889)
E. kostiense HAMBI 1489T (DQ411893)
E. saheli ORS 600 (DQ411898)
E. medicae A321T (DQ411897)
E. arboris HAMBI 1552T (DQ411892)
E. meliloti USDA 1002T (DQ411895)
E. kummerowiae CCBAU 71714T (DQ411896)
Sinorhizobium morelense Lc04T (EF198422)
E. adhaerens ATCC 33499 (DQ411894)
R. leguminosarum USDA 2370T (EF198419)
R. etli CFN42T (CP000133)
0.05
recA
E. mexicanus CFN SH4 (DQ411954), ITTG S64 (DQ411955)
E. mexicanus ITTG R7T (DQ411951), CFN ESH1 (DQ411952), CFN ESH3 (DQ411953) ...
ITTG R4 (EF198420), ITTG S4 (EF198421)
E. terangae ORS 1009T (AJ294383)
E. terangae ORS 19 (DQ411950), ORS 1073 (DQ411949)
E. terangae ORS 604 (DQ411948)
E. xinjiangense CCBAU 110T (DQ411944)
E. fredii USDA 205T (AJ294379)
E. americanus CFN EI156T (DQ411943)
E. sahelI ORS 600T (AJ294380)
E. kostiense HAMBI 1489T (DQ411947)
E. medicae A321T (AJ294381)
E. arboris HAMBI 1552T (DQ411946)
E. meliloti USDA 1002T (AJ294382)
E. kummerowiae CCBAU 71714T (DQ411945)
Sinorhizobium morelense Lc04T (AJ505601)
E. adhaerens ATCC 33212T (AJ505595)
R. etli CFN42T (CP000133)
R. leguminosarum USDA 2370T (AJ294376)
0.05
rpoB
E. mexicanus CFN SH3 (DQ411977)
E. mexicanus ITTG R7T (DQ411971), ITTG R4 (DQ411972), ITTG S4 (DQ411975), ...
ITTG S64 (DQ411976), CFN ESH4 (DQ411974), CFN ESH1 (DQ411973)
E. terangae ORS 1009T (DQ411967), ORS 1073 (DQ411968), ORS 19 (DQ411969)
E. terangae ORS 604 (DQ411970)
E. fredii USDA 205T (DQ411957), E. xinjiangense CCBAU 110T (DQ411958)
E. americanus CFN EI156T (DQ411956)
E. kostiense HAMBI 1489T (DQ411960)
E. sahelI ORS 600 (DQ411966)
E. arboris HAMBI 1552T (DQ411959)
E. medicae A321T (DQ411963)
E. meliloti USDA 1002T (DQ411964)
E. kummerowiae CCBAU 71714T (DQ411965)
Sinorhizobium morelense Lc04T (DQ411961)
E. adhaerens ATCC 33499 (DQ411962)
R. leguminosarum USDA 2370T (EF202569)
R. etli CFN42T * (CP000133)
0.05
noIR
E. mexicanus (DQ411923 - DQ411929)
E. terangae (DQ411919 - DQ411922)
E. medicae A321T (DQ411915)
E. arboris HAMBI 1552T (DQ411912)
E. meliloti USDA 1002T (DQ411914)
E. kummerowiae CCBAU 71714T (DQ411918)
E. sahelI ORS600 (DQ411917)
E. xinjiangense CCBAU 110T (DQ411910)
E. fredii USDA205T (AY194594)
E. americanus CFN EI156T (DQ411911)
E. kostiense HAMBI 1489T (DQ411913)
Sinorhizobium morelense Lc04T (DQ411916)
0.2

were identified using a PCR amplified *nifH* fragment from strain ITTGR7 as a probe as previously described [58].

Phenotypic features

Phenotypic features were analyzed as previously described [59]. Resistance to antibiotics was determined by spotting approximately 10^4 cells on PY agar supplemented with the appropriate antibiotic using 10 μg ml^{-1} of chloramphenicol, gentamicin, neomycin and streptomycin; 120 μg ml^{-1} of nalidixic acid, and 20 μg ml^{-1} of carbenicillin. Carbon source utilization was performed with the API 50 CHB/E tests (bioMérieux) according to the manufacturer instructions.

Results and discussion

Chromosomal protein-coding genes phylogenetic analyses

The bacteria isolated from *A. angustissima* nodules were identified as belonging to the genus *Ensifer* if PCR products were obtained with the *ssb* (339 bp) or *nolR* (291 bp) specific primers for the *Ensifer* genus (Table S1). They represented around 40% of the total isolates recovered from *A. angustissima* nodules. A preliminary phylogenetic tree generated with the sequences of the *rpoB* gene allowed the recognition of a putative new *Ensifer* lineage (data not shown). This lineage represented around 53% of the total *Ensifer* isolates recovered from *A. angustissima* nodules and showed seven different ERIC patterns (Fig. S2) corresponding to each strain listed in Table 1.

The description of the putative new lineage was based mainly on the analyses of partial sequences of the protein coding chromosomal genes *rpoB*, which encodes the β-subunit of RNA polymerase; *gyrA*, encoding the α-subunit of DNA gyrase; *recA* which encodes the DNA strand exchange and recombination protein, and *nolR*, encoding a *nod* gene regulator that regulates over 100 genes as well [2,3]. The *recA* gene has been used previously in rhizobial phylogenetic studies [13,57]. The best-fit models of evolution choosed were GTR+I+G, TN+G, GTR+I and TN+I+G for *gyrA, nolR, recA* and *rpoB*, respectively. In the phylogenetic trees obtained (Fig. 1), the *A. angustissima* strains constituted a consistent group, different from all described *Ensifer* species. We designate this cluster as *Ensifer mexicanus*. Its closest relative is *E. terangae*. The nodes that split both lineages have high bootstrap support values (>94%) for all genes, a strong evidence that they constitute sister taxa. No recombination of segments was detected between *E. mexicanus* and *E. terangae*, supporting that they belong to different species.

Identity confidence intervals for the chromosomal genes *gyrA* and *nolR*

Identity ranks are an indication of how much a gene sequence variates within a species. However, ranks do not provide a confidence interval to which a probability can be assigned. If the confidence interval is determined for a certain probability, then statistical tests can be used to establish if two samples belong to the same or to different populations and if a value belongs to the interval or not. Using the 99% confidence intervals for intra and inter-species nucleotide identity determined for the chromosomal genes *gyrA* and *nolR* as a reference, the strains of *E. mexicanus* were compared to *E. terangae* strains and among themselves (Table 2, Fig. 2). *Ensifer* strains belonging to the same species had higher average nucleotide identity than that calculated among species. When compared among themselves, *E. mexicanus* strains fall in the same species interval (arrow 2), whereas compared to *E. terangae* their identities are within the different species intervals (arrow 1), supporting the separation of both groups into two different species. *E. fredii* was excluded of the analysis because the strain *E. fredii* USDA 257 was not close to *E. fredii* USDA 205.

DNA–DNA homology and *rrs* phylogenetic analysis

According to the ad hoc committee for the re-evaluation of the species definition in bacteriology all species descriptions should include, besides the percent DNA–DNA homology, the almost complete sequence of the 16S rDNA gene (*rrs*) [51]. Total DNA from strain ITTG R7 showed low hybridization values with the four strains of *E. terangae* (<31%), while hybridization to four strains from its own group, ITTG R4, ITTG ES4, ITTG ES64 and CFN SH4 was >68%. These results indicate that *E. mexicanus* and *E. terangae* are different species. Both lineages were isolated from tropical forests, which are considered to be speciation cradles [38]. The results from DNA–DNA hybridization seem to indicate that *E. terangae* and *E. mexicanus* differ largely in gene content, these differences may have accumulated during their geographical isolation and ecological adaptations to hosts and local conditions. The differences in gene content seem to occur faster than the accumulation of nucleotide substitutions in the sequences of coding genes that seems to be derived from the genome of the ancestor of *E. terangae* and *E. mexicanus*.

The analysis of the *rrs* gene is not considered very useful to delineate rhizobial species because the high similarity between the species from the same genus [50,57], which could frequently be larger than 97%, exceeding the limit considered for new prokaryotic

Table 2. Inter- and intra-*Ensifer* species probability distribution of identity means and average identities of the new lineage compared to *E. terangae*

Gene	Identity 99% confidence interval		Average identity	
	Between different *Ensifer* species	Within each *Ensifer* species	Between *E. mexicanus* and *E. terangae*	Within *E. mexicanus*
gyrA	0.828–0.938	0.950–1.000	0.933	0.988
nolR	0.820–0.959	0.968–1.000	0.947	1.000

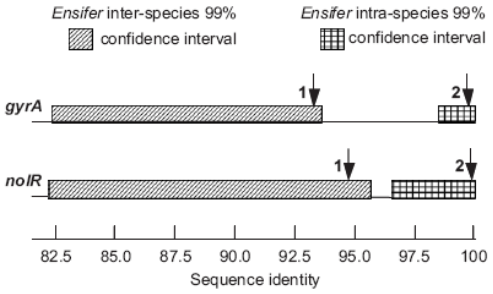


Fig. 2. 99% confidence intervals of identity means within and between *Ensifer* species. Arrows indicate the average identities value: (1) new species isolates compared to *Ensifer terangae* and (2) new species isolates compared among themselves. The confidence intervals and identities average values are shown in Table 2.

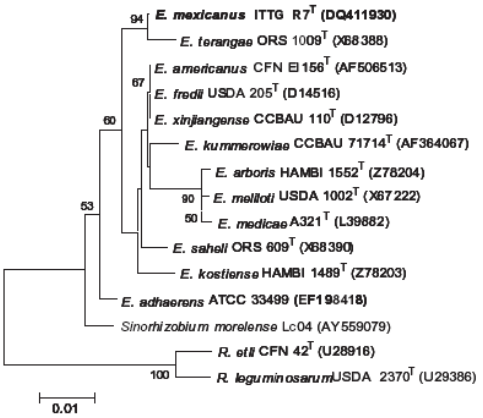


Fig. 3. Neighbor-joining phylogenetic tree of almost complete *rrs* sequences (1417 nt, from position 28 to 1444 of the *E. meliloti* 1021 *rrs* gene). Only bootstrap values $\geqslant 50\%$ are shown. Type strains are indicated by superscript T. *E. mexicanus* is shown in bold. The accession numbers for the sequences are indicated within parenthesis. Those generated in this work are shown in bold.

species description [40]. Additionally, cases of mosaicism in this gene have been reported [65]. Nevertheless, the ITTG R7^T *rrs* gene was found to be different from all sequences available in the Genbank database. The tree topology for *rrs*, shown in Fig. 3 confirmed the close relationship between *E. mexicanus* and *E. terangae* and allowed the differentiation of the new species.

MLEE analysis

The groups obtained from MLEE genetic distances are shown in Fig. S3. Each species, represented by the type strain, corresponded to a single ET. *E. mexicanus* is separated at more than 0.5 from any of the other reported species. Two highly related ETs were obtained with *E. mexicanus* strains tested and one ET was obtained for the four *E. terangae* strains tested. *E. mexicanus* and *E. terangae* were clearly separated (at 0.78 genetic distance). With this MLEE analysis as with total protein patterns, a congruency with phylogenetic based relationships is not observed [6–8,21,33,54], but these methods are very useful to recognize and support groupings.

Plasmid content and symbiotic plasmids identification

The content and size of plasmids are important features in *Rhizobium* and *Ensifer* species characterization [30]. A diversity of plasmid patterns was observed in *A. angustissima* strains, including megaplasmids slightly larger than the *E. meliloti* 1021 megaplasmids (>1.6 Mbp) (Fig. S4A). The symbiotic plasmids were identified using a PCR amplified *nifH* fragment from strain ITTG R7 as a probe. Plasmids with different sizes ranging from 300 to 600 kbp hybridized with the probe (Fig. S4B). The hybridization band was faint in CFN ESH1. No hybridization was detected with *E. meliloti* 1021 to the *E. mexicanus nifH* probe under the stringent hybridization conditions used.

nifH phylogenetic analysis

Since symbiosis is an important feature for rhizobia, the phylogeny of the *nifH* gene that encodes for the nitrogenase reductase enzyme responsible for nitrogen fixation was analyzed with the best-fit model GTR + G. The *nifH* phylogeny (Fig. 4) was incongruent with the phylogenies obtained with chromosomal genes as has been previously reported for other rhizobia [18,25,53,62]. The sequences from *A. angustissima*

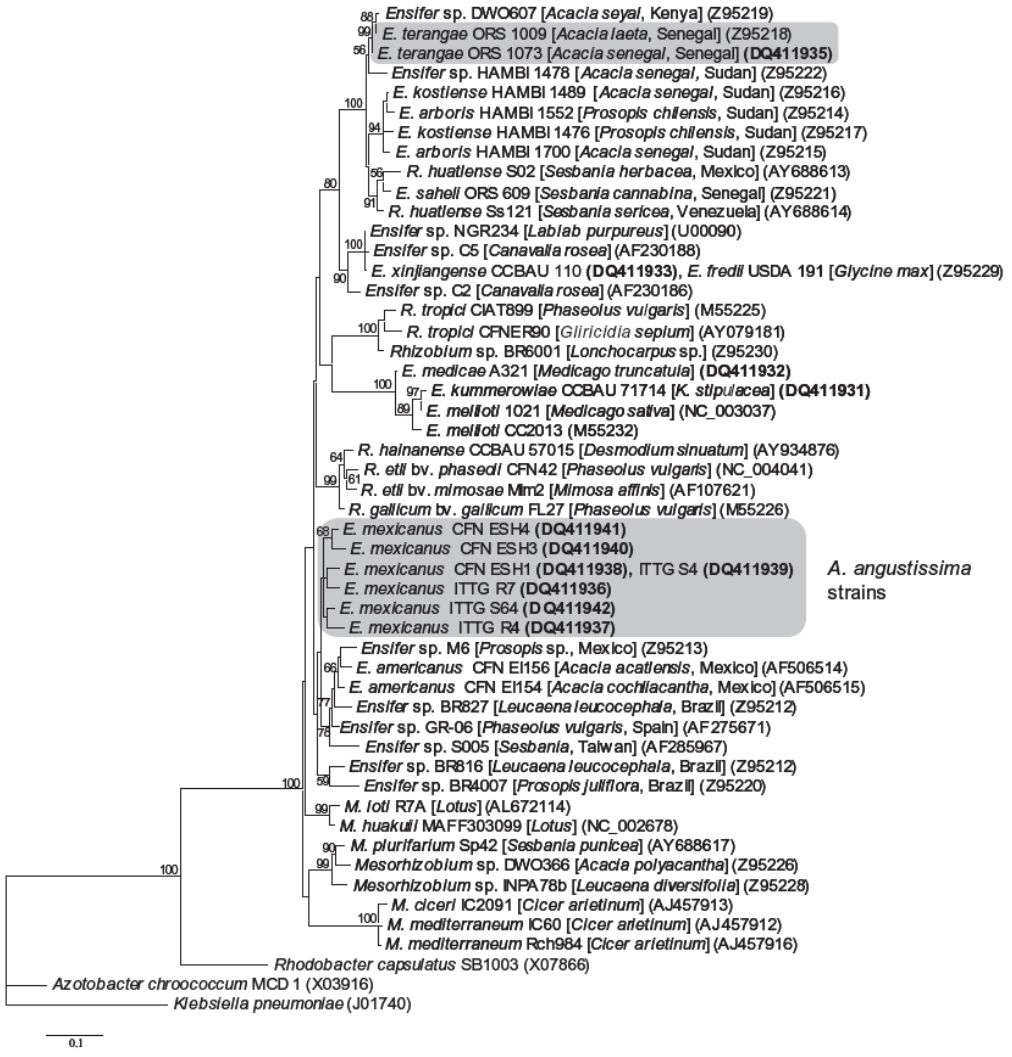


Fig. 4. Maximum likelihood phylogenetic tree of partial *nifH* gene sequences from the *Acacia angustissima* strains and representative rhizobia. Alignment length includes 475 nt from positions 313 to 787 of the corresponding gene from *E. meliloti* 1021. Only bootstrap values $\geqslant 50\%$ are shown. The isolation hosts, and in some cases the geographical origins, are indicated within brackets. The strains with identical sequence are placed in the same terminal branch. The sequences from *E. mexicanus* and *E. terangae* are highlighted. The accession numbers for the sequences are indicated within parenthesis. Those generated in this work are shown in bold.

strains clustered in a separate but not well supported group (< 50% bootstrap value) and were included in a large but also not well supported group which included mainly sequences from symbionts of American legumes. The sequences from *E. terangae*, the closest relative of *E. mexicanus* by the chromosomal gene phylogenetic criterion, grouped in a different cluster together with sequences from bacteria belonging to the genus *Ensifer* isolated from African hosts, indicating the different evolutionary histories of symbiotic and chromosomal genes. Plasmids seem to be prone to lateral transfer and genetic recombination [15,34] and mosaicism has been the outstanding characteristic of the plasmids that have been completely sequenced [12,15]. A fast divergence of symbiotic plasmids and of the genes therein may be granted. African and American sinorhizobia (now *Ensifer*) seem to have a large history of divergence based on the analysis of symbiotic gene sequences as previously noticed by other authors [18,53].

Host range

In addition to nodulating their original host legume, the *A. angustissima* strains nodulated and fixed nitrogen in *A. cochliacantha* and *P. vulgaris* (common bean) with the exception of strain ITTG R4 that nodulated but did not fix nitrogen in *P. vulgaris*. ITTG S64 was not tested. The ITTG R7^T strain was tested in *L. leucocephala* and soybean. Effective nodules were obtained in *L. leucocephala* and no nodulation occurred in soybean. The African *E. terangae* bv. acaciae strains ORS 1009^T and ORS 1073 were found to nodulate *A. angustissima* but only ORS1009^T fixed nitrogen. No nodulation was obtained with *E. terangae* bv. sesbaniae strains ORS19 and ORS604 in *A. angustissima*.

Distinctive phenotypic features of *E. mexicanus*

E. mexicanus could be distinguished by at least one characteristic from all the described species of *Ensifer* (Table S3). The *E. mexicanus* strains were resistant to 20 μg ml^{-1} of carbenicillin contrary to *E. terangae* strains, which were sensitive. Additionally, it was observed that the old colonies of *E. terangae* turned brownish probably due to melanin production, while those of *E. mexicanus* did not. Carbon source utilization was tested only for *E. mexicanus* and its closest phylogenetic relative *E. terangae*. Both species showed similar responses to most of the carbon sources included in the API 50 CHB/E kit, but some compounds allowed to distinguish between them (Table S4). *E. mexicanus* was able to assimilate L-sorbose in contrast to *E. terangae*. The opposite was observed with 2-ketogluconate. The majority of *E. terangae* strains grew on D-melezitose, while *E. mexicanus* could not use this sugar. L-xylose was used by all *E. terangae* strains whereas only one *E. mexicanus* strain used it.

In summary, the phylogenetic and statistical analyses performed allowed the distinction of the new lineage from all described *Ensifer* species. Additionally, the DNA–DNA hybridization results and the MLEE analysis clearly distinguished them from their closest relative, *E. terangae*. These evidences support that the new lineage isolated from *A. angustissma* corresponds to a new species. We propose the name *Ensifer mexicanus* as these strains were isolated in Mexico. However, we recognize that this species could have a broader geographical distribution as has been shown for other rhizobial species [35,57].

Synonymy of the genera *Sinorhizobium* and *Ensifer*

The synonymy of the genera *Sinorhizobium* and *Ensifer* was proposed [63]. The genus *Ensifer* Casida 1982 [1] took priority over *Sinorhizobium* Chen et al. 1988 [4] because the former was described before and the name *Sinorhizobium* was rejected according to the Bacteriological Code [67]. In spite of this, *Ensifer adhaerens*, seem to be the most distant species in the genus (Figs. 1 and 3). We consider that the taxonomy of these bacteria still needs revision and the genus *Ensifer* could split into *Sinorhizobium* and *Ensifer*.

Description of *Ensifer mexicanus* sp. nov.

Ensifer mexicanus sp. nov. (me.xi.ca'nus. N.L. masc. adj. *mexicanus* pertaining to Mexico, where the strains were isolated). Aerobic, gram-negative, motile, non-spore-forming rods. They grow in PY and YEM medium forming circular, gummy, white to cream colored, slightly translucent and mucilaginous colonies of 2–4 mm diameter appearing after 2 days of incubation at 28 °C. The generation time for ITTG R7^T in YEM broth at 28 °C was 2.05 h. Acid is produced in YEM medium. In general, they can effectively nodulate *A. angustissima*, *A. cochliacantha*, *L. leucocephala* and *P. vulgaris*. The seven strains from this species can be differentiated from *E. terangae*, their closest relative, by DNA–DNA hybridization, by MLEE analysis, by their tolerance to 20 μg ml^{-1} carbenicillin and by the utilization of L-sorbose as a sole carbon source as indicated in the text. Additionally, their old colonies do not turn brown as *E. terangae* does. This species can be differentiated from all described *Ensifer* species on the basis of the phylogenetic analysis of the chromosomal genes *rrs, gyrA, recA, rpoB* and *nolR*, and by MLEE analysis. The type strain, ITTG R7^T (= CFN ER1001, HAMBI 2910, CIP 109033, ATCC BAA-1312, DSM18446) was isolated from nodules of *A. angustissima* (Mill.) Kuntz collected in Tuxtla Gutiérrez,

Chiapas, Mexico. It has the characteristics of the species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.syapm.2006.12.002.

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7.3. Diversidad genética de bacterias mutualistas de plantas.

DIVERSIDAD GENÉTICA DE BACTERIAS MUTUALISTAS DE PLANTAS

Martínez-Romero, E.[1]; Lloret, L.[1]; Ormeño-Orrillo, E.[1]; Vinuesa, P.[1]; Rosenblueth, M.[1]; Rincón, R.[2]; Rogel, M.A.[1]; Silva, C.[1]; Martínez-Romero, J.[1]; Toledo, I.[1]; López, A.[1]

RESUMEN

México es un país con una gran diversidad vegetal, animal y también microbiana. Esta última es muchas veces olvidada o desdeñada pero es esencial para la supervivencia de todas las demás especies. Ha sido gracias a los nuevos enfoques moleculares que se ha podido revelar una diversidad bacteriana insospechada y se han clasificado certeramente a las bacterias. Algunas de las bacterias contribuyen al crecimiento de las plantas y de estas bacterias hemos reconocido algunas nuevas especies. En el estudio de las bacterias endófitas (en el interior) de plantas como plátano, caña de azúcar y maíz identificamos una nueva especie de *Klebsiella*, muy cercana a *K. pneumoniae*. A esta nueva especie la nombramos *K. variicola*, que significa de muchos sitios porque se obtuvo de una amplia variedad de plantas pero también de pacientes humanos en hospitales pediátricos. No recomendamos su uso en agricultura a pesar de que estas bacterias promueven el crecimiento vegetal y son fijadoras de nitrógeno. *Rhizobium* y bacterias de géneros relacionados son capaces de formar nódulos (pequeñas biofábricas de fertilizante nitrogenado) en las raíces de las plantas leguminosas, como el frijol (*Phaseolus vulgaris*) o árboles de leguminosas como *Leucaena, Sesbania* o *Acacia*. Algunas especies de estos géneros son nativas de México y sus simbiontes no habían sido caracterizados. Reconocimos entre ellos nuevas especies, como *Rhizobium etli, R. tropici* y *Sinorhizobium americanum*, que han sido de utilidad tanto para estudios moleculares como para aplicaciones en agricultura o en proyectos de recuperación ecológica en áreas taladas. El uso de estas bacterias sustituye a los fertilizantes químicos nitrogenados y permite a las plantas crecer en suelos pobres de nitrógeno. Por otro lado, el reconocimiento adecuado de las especies nos ha permitido revelar que con la tala de los bosques de los Tuxtlas en México se pierden especies de *Bradyrhizobium* nativas de la selva. Existe aún un gran desconocimiento de las especies de bacterias asociadas a plantas.

PALABRAS CLAVE: agricultura, filogenias, *Klebsiella*, leguminosas, *Rhizobium*.

SUMMARY

GENETIC DIVERSITY OF MUTUALISTIC BACTERIA FROM PLANTS

Mexico is a country with a large diversity of plants, animals and bacteria. Bacterial diversity is not always considered in biodiversity studies but it is key to the survival of all other species. Molecular approaches have revealed an enormous and unsuspected bacterial diversity and have allowed an accurate classification of bacteria. We have discovered some new species among bacteria that promote plant growth. Studying endophytic (inside plants) bacteria from banana, sugarcane and maize, we identified a new *Klebsiella* species, closely related to *K. pneumoniae*. We named this new species *K. variicola*, meaning "different places" because it was isolated from a large variety of plants and also from human patients in pediatric hospitals. Even though these bacteria promote plant growth and fix nitrogen, we do not recommend their use in agricultural fields. *Rhizobium* and bacteria from related genera are nitrogen fixing bacteria that form nodules (small biological factories of N fertilizers) in the roots of legumes such as *Phaseolus vulgaris* (beans) or trees like *Leucaena, Sesbania* and

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Acacia. Some species of these genera are native to Mexico and their symbionts had not been previously analyzed. We discovered new species as *Rhizobium etli*, *Rhizobium tropici* and *Sinorhizobium americanum*, that have become useful not only for molecular studies but also for applications in agriculture or in reforestation projects in tilled areas. The use of these bacteria substitutes chemical nitrogen fertilizers and allows plants to grow in nitrogen poor soils. The adequate identification of species has allowed us to reveal that the tillage of Tuxtlas forest in Mexico causes loss of *Bradyrhizobium* species native to the forest. There is still an enormous lack of knowledge of bacterial species associated with plants.

KEY WORDS: agriculture, *Klebsiella*, legumes, phylogenies, *Rhizobium*.

INTRODUCCIÓN

Las plantas surgieron entre el sol y el suelo con sus microorganismos. En el suelo se encuentra una de las diversidades más altas de bacterias, entre 10^4-10^5 o más especies diferentes por gramo de suelo con 10^{12} bacterias totales por gramo. Seguramente plantas y microorganismos han coevolucionado durante cientos de millones de años. Las relaciones que se establecieron entre plantas y bacterias son de lo más diversas, destacándose las interacciones mutualistas y las patogénicas.

El objetivo de este trabajo es la descripción de la diversidad genética de bacterias benéficas de plantas que han sido estudiadas en nuestro laboratorio. Se incluirán tanto bacterias que son endofiticas (dentro de las plantas) como aquellas capaces de formar nódulos en las raíces de plantas como las leguminosas.

Endófitos

La caña de azúcar en Brasil (Boddey *et al.*, 1995) parece obtener un aporte significativo de nitrógeno que proviene de bacterias fijadoras de nitrógeno capaces de convertir el N gaseoso de la atmósfera en amonio, el cual es utilizado por las plantas. Del interior de plantas de caña de azúcar de Brasil se han aislado diferentes especies de bacterias (James & Olivares, 1997; James *et al*. 1997, Olivares *et al*., 1997; Reis *et al.,* 1994). En México aislamos cepas de la especie *Gluconacetobacter diazotrophicus* de plantas de caña de azúcar que se encontraban en campos agrícolas donde no se utilizan altas concentraciones de fertilizantes nitrogenados (Caballero Mellado y Martínez-Romero, 1994). La diversidad genética de estas bacterias fue más reducida que la encontrada en Brasil (Caballero-Mellado *et al.*, 1995) en donde se emplean normalmente bajas cantidades de fertilizantes nitrogenados. Encontramos también que la colonización de estas bacterias en el invernadero se ve disminuida cuando las plantas crecen en presencia de fertilizantes nitrogenados (Fuentes-Ramírez *et al.*, 1999). Un efecto similar del fertilizante sobre la colonización de bacterias fijadoras de nitrógeno se demostró posteriormente en arroz (Tan *et al.,* 2003). Encontramos *Gluconacetobacter diazotrophicus* y una nueva especie de *Gluconacetobacter* (Fuentes-Ramírez *et al.,* 2001) asociadas también con café, pero en la rizósfera principalmente. Investigadores en un laboratorio de E. E. U. U. reportaron que *G. diazotrophicus* es capaz de proporcionar N a plántulas de caña de azúcar y promover su crecimiento (Sevilla *et al*., 2001), tal vez por su capacidad de producir hormonas vegetales.

Del interior de diferentes tejidos de la planta de plátano aislamos diversas bacterias fijadoras de nitrógeno que promueven el crecimiento de las plantas generadas a partir de cultivos de tejidos. La bacteria más abundante en condiciones cultivables fue *Enterobacter* (Martínez *et al.*, 2003). Los estudios filogenéticos que hemos realizado con estas bacterias indican que se trata de un nuevo género cercano a *Enterobacter* (Rosenblueth *et al*., no publicado). Además aislamos un grupo considerable de bacterias pertenecientes al género *Klebsiella*. Estas bacterias fueron aisladas también de plantas de maíz, de caña de azúcar y de arroz. Sorprendentemente, las bacterias de *Klebsiella* obtenidas de las plantas fueron indistinguibles de bacterias aisladas en hospitales pediátricos de pacientes humanos enfermos. Estas bacterias se aíslan con menor frecuencia de casos clínicos que *Klebsiella pneumoniae* y representan una nueva especie, a la que nombramos *K. variicola* (Rosenblueth *et al*., 2004), cercanamente relacionada con *K. pneumoniae*. No recomendamos su uso en agricultura (Lloret *et al*., 2004) ya que pensamos que los contagios humanos provienen de reservorios naturales como las plantas (Martínez *et al*., 2004). Las bacterias de *K. variicola* de pacientes fueron todas fijadoras de nitrógeno y poseen niveles más bajos de resistencia a antibióticos que las cepas clínicas de *K. pneumoniae* (Rosenblueth *et al.*, 2004; Martínez *et al.,* 2004).

En la agricultura tradicional en México y en Perú se cultiva el frijol asociado al maíz. Supusimos que en estas condiciones las bacterias podrían compartirse entre ambas plantas, por lo que buscamos cepas de *Rhizobium etli* (que es el simbionte más común de frijol que forma nódulos fijadores de N) en las plantas de maíz. En cultivos en asociación todas las plantas de maíz contenían *R. etli*, mien-

tras que en monocultivos de maíz sólo un porcentaje bajo (20%) tenían *R. etli* (Gutiérrez-Zamora & Martínez-Romero, 2001). En la rizósfera del maíz encontramos 10^{6-8} bacterias de *R. etli* por gramo seco de suelo y 10^{3-4} por gramo de tejido fresco de raíz en el interior como endófitos. Las bacterias aisladas de maíz son capaces de re-colonizar un gran número de razas y variedades criollas de maíz y también de promover su crecimiento (Gutiérrez-Zamora & Martínez-Romero, 2001). El análisis mediante patrones de mobilidad electroforética de enzimas metabólicas y perfiles de plásmidos de poblaciones de *R. etli* de diferentes nichos: suelo, nódulos, rizósfera y del interior de raíces y tallos, reveló una diferenciación de las poblaciones (Rosenblueth & Martínez-Romero, 2004), lo que sugirió que existen clonas más adaptadas para colonizar el interior de la planta de maíz. Actualmente estamos estudiando las diferencias en el contenido genómico de una cepa de *R. etli* que es muy buena colonizadora de maíz en comparación con una cepa de *R. etli* que no coloniza eficientemente. Además, estudiamos los genes de *R. etli* que se expresan en presencia de maíz (Rosenblueth *et al*., no publicado). *Rhizobium* se ha utilizado en la agricultura por más de 100 años y no se considera que represente un riesgo para la salud. Dada la gran capacidad competitiva de algunas cepas *de R. etli* para colonizar maíz, éstas se pudieron utilizar para desplazar poblaciones de bacterias de *Burkholderia cepacia* que colonizan maíz en Europa (Chiarini *et al.*, 2000). Las cepas de *B. cepacia* se consideran un riesgo para la salud humana ya que causan infecciones (aun letales) en pacientes con fibrosis quística.

Bacterias nodulantes

Las leguminosas constituyen una de las familias más grandes de plantas. Tal vez parte de su éxito se debe a su capacidad de establecer simbiosis con bacterias fijadoras de nitrógeno. Una revisión de las poblaciones de rizobios nativas de México se publicó recientemente (Martínez-Romero, 2001). En México existe un gran número de leguminosas nativas, entre ellas destacan las plantas del género *Phaseolus* (*P. vulgaris, P. coccineus, P. lunatus* y otras 47 especies). *P. vulgaris*, el frijol común, fue domesticado en Mesoamérica y en otros dos sitios en Sudamérica (Gepts & Debouck, 1991). Las especies que se aíslan principalmente de los nódulos de frijol son *Rhizobium etli* en México (Piñero *et al*., 1988; Segovia *et al*., 1991, 1993; Silva *et al.*, 2003) y *R. tropici* en suelos ácidos en Sudamérica (Martínez-Romero *et al.*, 1991), aunque también hemos aislado *R. gallicum* bv. *gallicum* (Silva *et al.,* 2005), el cual es también simbionte de *P. coccineus*. En contraste, *P. lunatus*, el frijol Lima, establece simbiosis con diferentes linajes de *Bradyrhizobium* (Ormeño-Orrillo *et al.*, 2005), algunos de crecimiento extra lento y con *Bradyrhizobium yuanmingense* (Ormeño-Orrillo *et al.*, 2005; Vinuesa *et al.,* 2005b), además hemos encontrado un nuevo linaje que representa una nueva especie de *Bradyrhizobium* (Ormeño-Orrillo *et al*., no publicado).

Uno de los géneros más amplios de leguminosas es el de las acacias. Las acacias son altamente resistentes a la sequía con raíces que llegan a 35 m de profundidad y son noduladas por varias especies de rizobios (de Lajudie *et al*., 1994; 1998; Nick *et al.*, 1999). Describimos una nueva especie de *Sinorhizobium*, *S. americanum*, aislada de los nódulos de varias especies de acacias nativas de México (Toledo *et al*., 2003). *S. americanum* está cercanamente relacionado a *S. fredii* que nodula soya en China. *S. americanum* no nodula soya y *S. fredii* no nodula *Acacia*, lo que indica que hay una co-evolución de estas bacterias con sus hospederos. De otra especie de *Acacia, A. angustissima* que crece en las selvas de Chiapas y de Morelos hemos aislado y estamos caracterizando un nuevo linaje de sinorhizobia, cercanamente relacionado con *S. terangae*, que es simbionte de diferentes especies de *Acacia* en África.

En los nódulos de *Sesbania* en México encontramos *R. huautlense* que es capaz de nodular en condiciones de inundación (Wang *et al*., 1998). En campos inundados en Venezuela también encontramos *R. huautlense* en otras especies de *Sesbania* (Vinuesa *et al*., 2005a).

De la selva tropical de los Tuxtlas en Veracruz hemos reconocido linajes nuevos de *Bradyrhizobium*, uno de los cuales se pierde con el cambio de uso de suelo, que ocasiona también otros cambios drásticos en las poblaciones de rizobios (Ormeño-Orrillo *et al*., enviado). El conocimiento que hemos obtenido de los simbiontes de diversos árboles de leguminosas incluyendo *Acacia* (Toledo *et al.*, 2003), *Gliricidia* (Acosta *et al*., 2002) y *Leucaena* (Wang *et al*., 1999) nos ha permitido iniciar proyectos de restauración ecológica en los que introducimos leguminosas nativas y sus bacterias en áreas taladas.

La identificación y las relaciones entre las bacterias las hemos establecido con base en estudios filogenéticos considerando las secuencias de genes que codifican proteínas y también de los genes ribosomales. Estos enfoques moleculares han permitido revelar la gran diversidad genética de bacterias asociadas a plantas y su más certera identificación (Vinuesa *et al.*, 2005b). La diversidad de bacterias asociadas a plantas aún está poco explorada y seguramente tiene una función ecológica y agronómica muy importante. La utilización de *Rhizobium* como inoculante en la agricultura permite ahorros de millones de dólares (Mendes *et al*., 2004).

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VIII. Material suplementario.

8.3.1. Figure S1. Map of Mexico showing the location of field collection sites in Chiapas and Morelos.

8.3.2. Table S1 Phenotypic characteristics of *Sinorhizobium chiapanecum* strain ITTG S70^{T} and the reference related strains.

8.3.3. Table S2. Levels of total DNA-DNA relatedness as percent of hybridization of *Sinorhizobium chiapanecum* strain ITTG S70^{T} isolated of *A. angustissima* with the *S. mexicanum* and *S. terangae* strains.

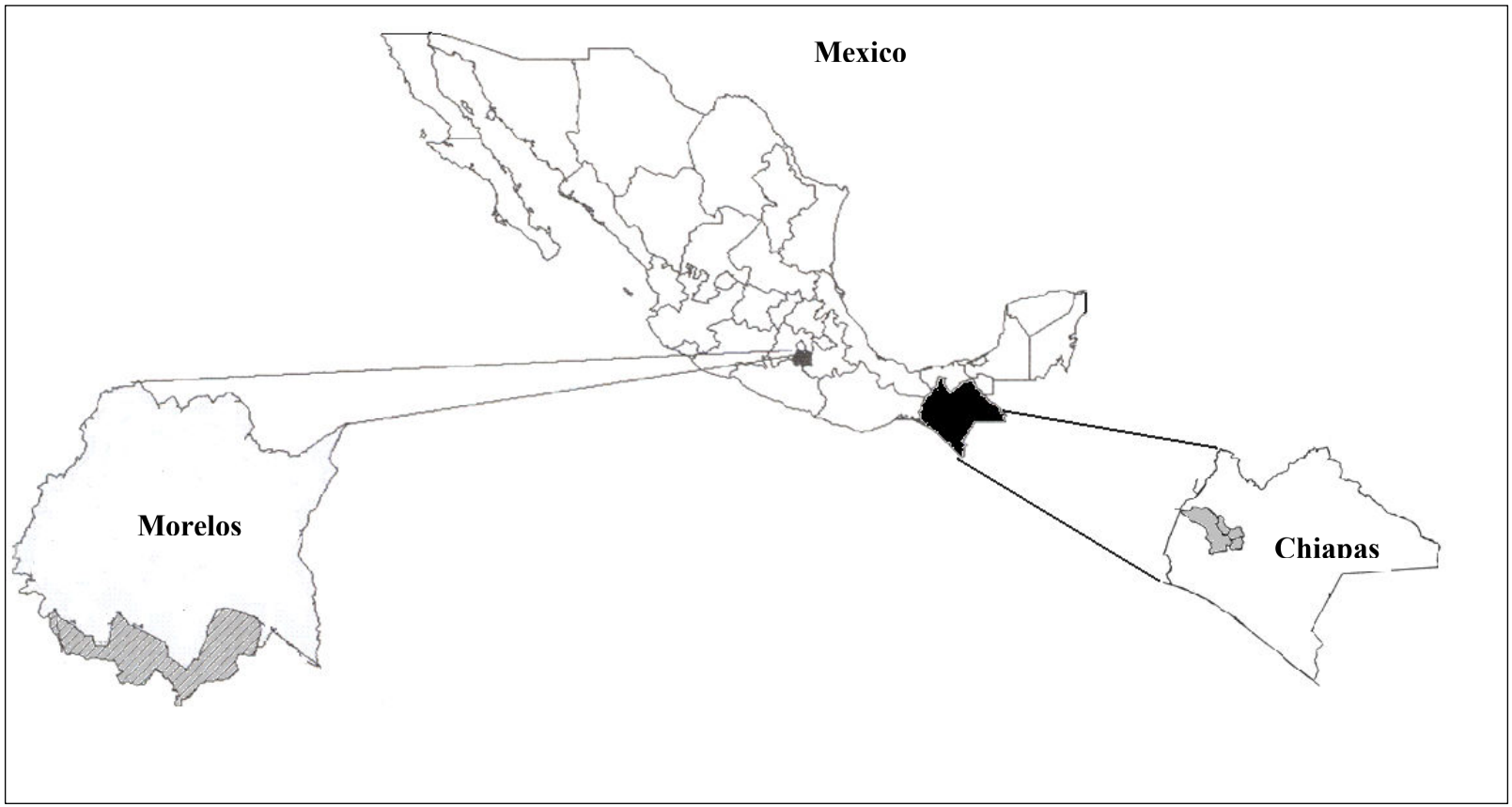

Figure S1. Map of Mexico showing the location of field collection sites in Chiapas and Morelos.

Table S1. Phenotypic characteristics of *Sinorhizobium chiapanecum* strain ITTG S70^{T} and the reference related strains.

Characteristic	Strains		
	S. chiapanecum ITTG S70^{T}	*S. mexicanum* ITTG R7^{T}	*S. terangae* ORS 1009^{T}
Growth in LB	-[a]	-	-
Tolerance to NaCl:			
0.5 %	+	+	+
1.0 %	+	+	+
2.0 %	+	-	-
3.0 %	-	-	-
Generation time (h) in YEM broth at 28^{o}C (pH=6.5)	2.33	2.05	2.03
Growth in PY at 37^{o}C (pH=6.5)	+	+	+
Growth in liquid PY pH 5 at 28^{o}C:	-	-	-
Acid production in YEM containing 25 µg L^{-1} (w/v) bromothymol blue as pH indicator	+	+	+
Tolerance to antibiotics (µg ml^{-1})			
Carbenicillin (20)	-	+	-
Ampicillin (10)	-	+	+
Choramphenicol (10)	-	-	-
Gentamicin (10)	-	-	-
Nalidix acid (120)	+	+	+
Neomycin (10)	-	-	-
Streptomycin (10)	-	-	-

a +, all strains positive ; -, all strains negative

Table S2. Levels of total DNA-DNA relatedness as percent of hybridization of *Sinorhizobium chiapanecum* strain ITTG S70^T isolated of *A. angustissima* with the *S. mexicanum* and *S. terangae* strains.

	DNA relatedness with:
	Sinorhizobium chiapanecum ITTG S70^T
Sinorhizobium chiapanecum	
ITTG S68	73.8
ITTG S70^T	100.0
ITTG S71	85.5
ITTG R11	70.0
Sinorhizobium mexicanum	
ITTG R7^T	32.5
ITTG R4	38.9
ITTG S4	37.6
ITTG S64	41.8
CFN ESH1	33.5
CFN ESH3	30.9
CFN ESH4	33.4
Sinorhizobium terangae	
ORS 19	55.5
ORS 604	56.6
ORS 1009^T	47.8
ORS 1073	53.0