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Systematic and Applied Microbiology 30 (2007) 280–290
SYSTEMATIC AND APPLIED MICROBIOLOGY
www.elsevier.de/syapm

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

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Received 31 October 2006

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.
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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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doi:10.1016/j.syapm.2006.12.002

[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^{TM} 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.

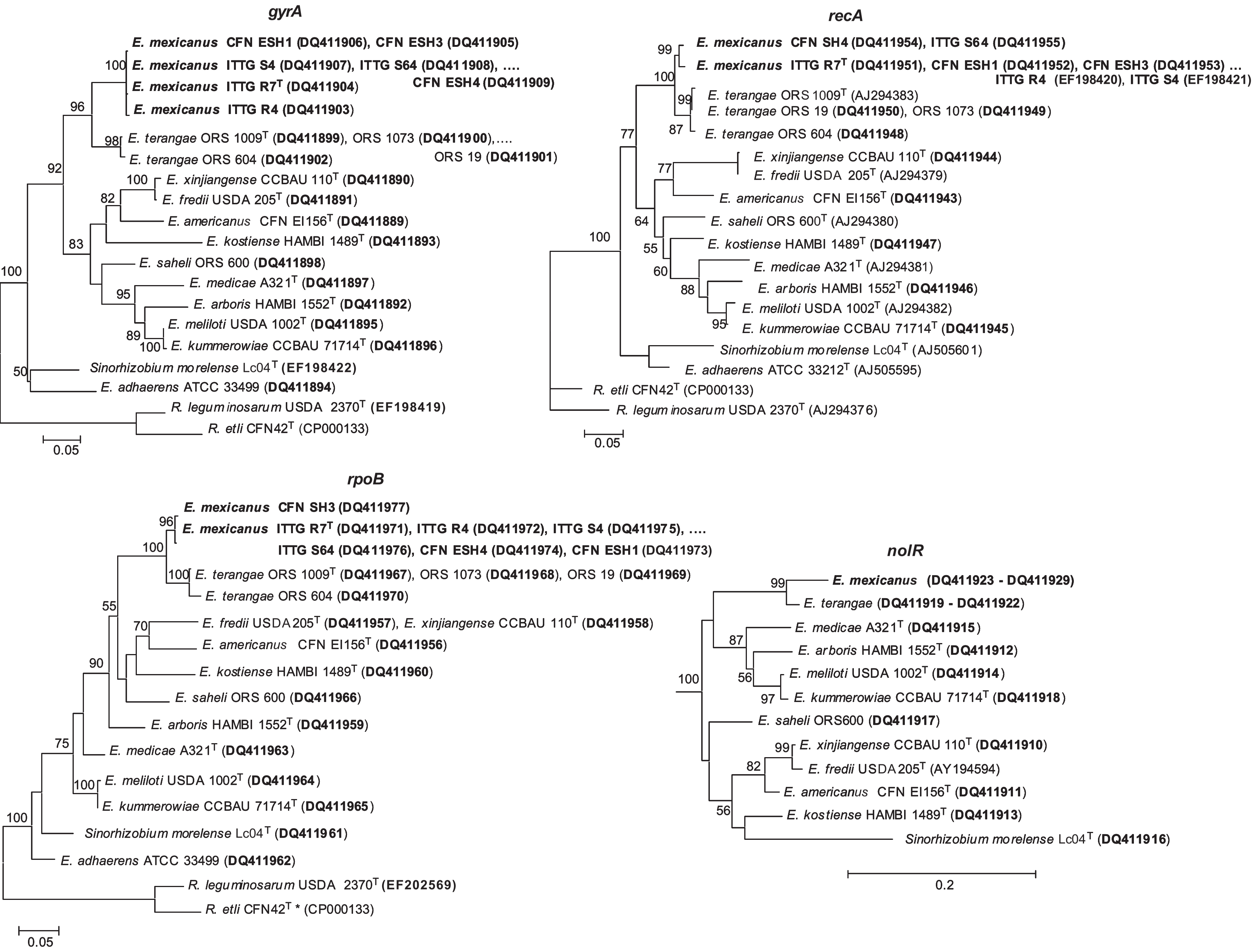
Multilocus enzyme electrophoresis (MLEE)

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),….
ORS 19 (DQ411901)
E. terangae ORS 604 (DQ411902)
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)
100
96
98
92
100
82
83
95
89
100
50
100
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)
99
100
99
87
77
77
64
55
60
88
95
100
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)
96
100
100
55
70
90
75
100
100
0.05
nolR
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 USDA 205T (AY194594)
E. americanus CFN EI156T (DQ411911)
E. kostiense HAMBI 1489T (DQ411913)
Sinorhizobium morelense Lc04T (DQ411916)
99
87
56
97
100
99
82
56
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 $10\,\mu g\,ml^{-1}$ of chloramphenicol, gentamicin, neomycin and streptomycin; $120\,\mu g\,ml^{-1}$ of nalidixic acid, and $20\,\mu g\,ml^{-1}$ 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

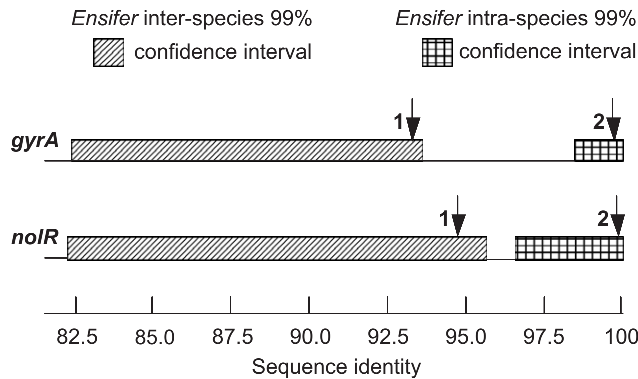


Fig. 2. 99% confidence intervals of identity means within and between *Ensifer* species. Arrows indicate the average identities value: (1) new species isolates compared to *Ensifer terangae* and (2) new species isolates compared among themselves. The confidence intervals and identities average values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

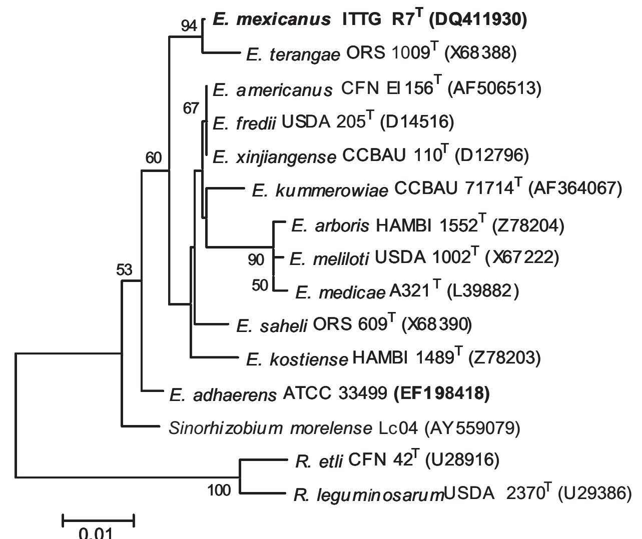


Fig. 3. Neighbor-joining phylogenetic tree of almost complete *rrs* sequences (1417 nt, from position 28 to 1444 of the *E. meliloti* 1021 *rrs* gene). Only bootstrap values $\geqslant 50\%$ are shown. Type strains are indicated by superscript T. *E. mexicanus* is shown in bold. The accession numbers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

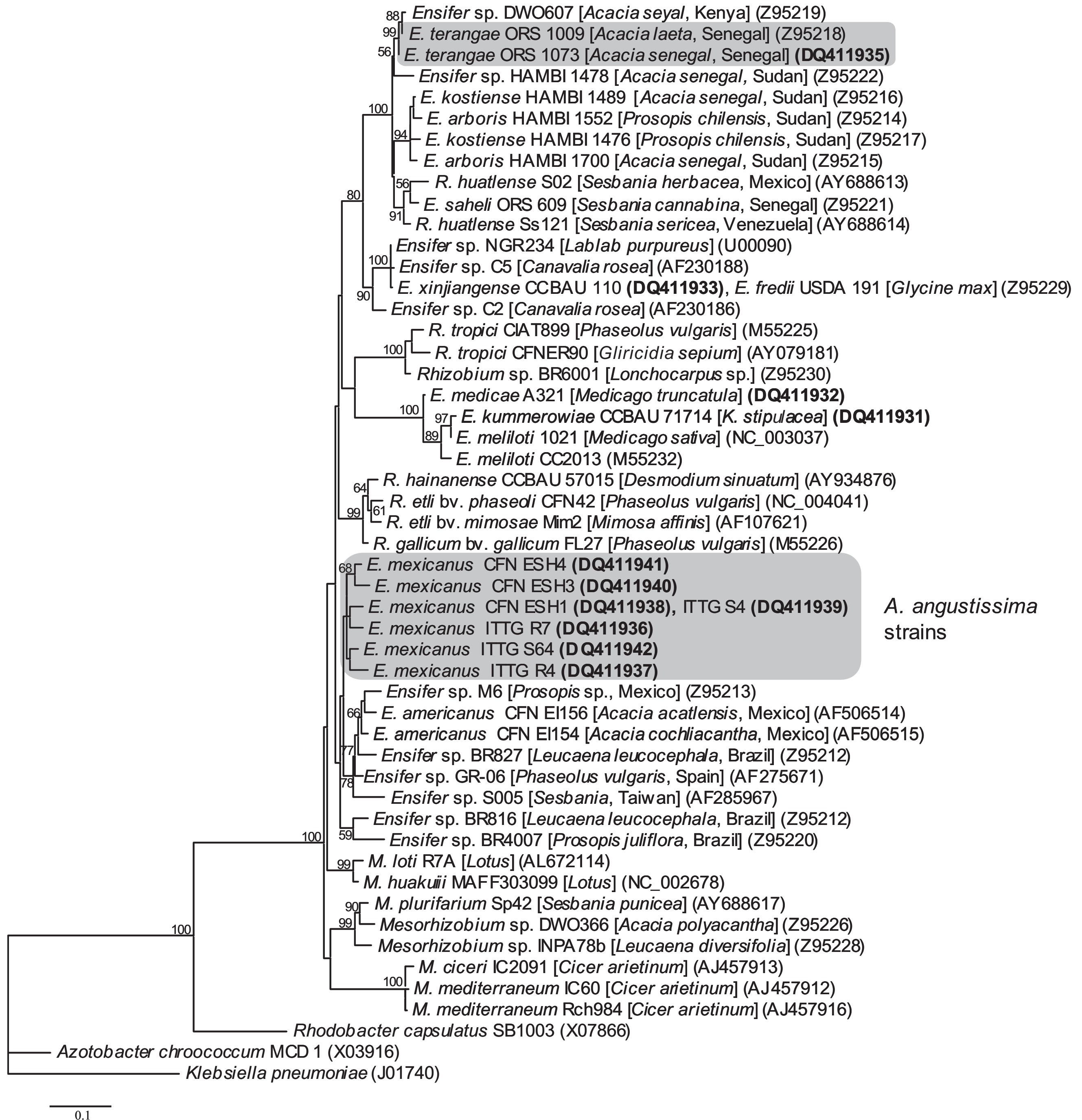


Fig. 4. Maximum likelihood phylogenetic tree of partial *nifH* gene sequences from the *Acacia angustissima* strains and representative rhizobia. Alignment length includes 475 nt from positions 313 to 787 of the corresponding gene from *E. meliloti* 1021. Only bootstrap values ⩾50% are shown. The isolation 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.

Acknowledgments

We thank Aryana Chávez for field work at the Sumidero Canyon and Tuxtla Gutiérrez in Chiapas, Edith Ponce and Ivonne Toledo for technical assistance and soil sample collection at the Sierra de Huautla, Morelos. We also thank W.X. Chen, P. de Lajudie, E.T. Wang and K. Lindström for providing bacterial strains, and P. Vinuesa for his advice in the selection of the best evolutionary models for the phylogenetic analyses. We thank Michael Dunn for reading this manuscript. Financial support was from PAPIT-DGAPA Grant IN 201106.

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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