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Rhizobium etli bv. mimosae, a novel biovar isolated from *Mimosa affinis*

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Fifty rhizobial isolates from root nodules of *Mimosa affinis*, a small leguminous plant native to Mexico, were identified as *Rhizobium etli* on the basis of the results of PCR–RFLP and RFLP analyses of small-subunit rRNA genes, multilocus enzyme electrophoresis and DNA–DNA homology. They are, however, a restricted group of lineages with low genetic diversity within the species. The isolates from *M. affinis* differed from the *R. etli* strains that originated from bean plants (*Phaseolus vulgaris*) in the size and replicator region of the symbiotic plasmid and in symbiotic-plasmid-borne traits such as *nifH* gene sequence and organization, melanin production and host specificity. A new biovar, bv. mimosae, is proposed within *R. etli* to encompass *Rhizobium* isolates obtained from *M. affinis*. The strains from common bean plants have been designated previously as *R. etli* bv. phaseoli. Strains of both *R. etli* biovars could nodulate *P. vulgaris*, but only those of bv. mimosae could form nitrogen-fixing nodules on *Leucaena leucocephala*.

Keywords: *Rhizobium etli*, *Mimosa affinis*, symbiotic plasmid, genetic diversity

INTRODUCTION

The Leguminosae is one of the largest families of plants. A remarkable characteristic of many species in this family is that they establish symbioses with nitrogen-fixing bacteria. In comparison with the large number of leguminous species, there is a very limited number of species within the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Azorhizobium* (for reviews see Martínez-Romero & Caballero-Mellado, 1996; Young & Haukka, 1996) and the newly proposed *Mesorhizobium* (Jarvis *et al.*, 1997) and *Allorhizobium* (de Lajudie *et al.*, 1998b) that form nodules on the roots and sometimes on the stems of legumes. Species such as *Rhizobium galegae* (Lindström, 1989), *Rhizobium huautlense* (Wang *et al.*, 1998), *Rhizobium mongolense* (van Berkum *et al.*, 1998), *Rhizobium hainanense* (Chen *et al.*, 1997), *Mesorhizobium huakuii* (Chen *et al.*, 1991), *Mesorhizobium ciceri* (Nour *et al.*, 1994), *Mesorhizobium mediterraneum* (Nour *et al.*, 1995), *Mesorhizobium tianshanense* (Chen *et al.*, 1995; Tan *et al.*, 1997) and *Azorhizobium caulinodans* (Dreyfus *et al.*, 1988) have been proposed from the study of novel isolates obtained from leguminous plants that had not been previously analysed.

The family Leguminosae is considered to be of tropical or subtropical origin (Raven & Polhill, 1981). Many tropical species are virtually unknown elsewhere. In a survey of legume-nodule bacteria based on small-subunit (SSU) rRNA gene sequences, most of the *Rhizobium* and *Bradyrhizobium* strains belonging to new varieties were isolated from the tropics and subtropics (Oyaizu *et al.*, 1993). The subfamily Mimosoideae seems to have branched earlier than the subfamily Papilionoideae in the evolution of the family Leguminosae (Doyle, 1995), and less is known about symbiotic bacteria from the Mimosoideae than from the Papilionoideae. *Leucaena*, *Acacia* and *Mimosa* are genera within the subfamily Mimosoideae. Root-nodule isolates from species within the genera *Leucaena* and *Acacia* have been found to be highly diverse (Dupuy *et al.*, 1994; Wang *et al.*, 1999b; de Lajudie *et al.*, 1994, 1998a). *Rhizobium* strains from *Mimosa caesalpiniaefolia* were isolated by Campelo & Dobereiner (1969). Extensive isolations were made by

Abbreviations: ET, electrophoretic type; MLEE, multilocus enzyme electrophoresis; SSU, small-subunit.

The GenBank accession number for the *nifH* gene sequence of isolate Mim2 is AF107621.

Table 1. Isolates and strains used in this study and their relevant characteristics

Isolate or strain	SSU rRNA PCR–RFLP pattern*	ET†	Plasmid profile (kbp)‡
Isolates from *M. affinis* in Huautla			
Mim2, Mim5, Mim6, Mim8, Mim10, Mim1-1, Mim1-3, Mim3-4, Mim3-6, Mim4-4, Mim4-5, Mim6-2, Mim6-3	DBEC	2	390, 450, **600**, 1000
Mim1-2	DBEC	2	390, 450, **600**, >1000
Mim1-4, Mim8-5, Mim10-2	DBEC	2	390, **600**, 1000
Mim7-5	DBEC	2	100, 390, 450, **600**, 1000
Mim2-3,Mim2-4, Mim2-5, Mim2-6, Mim6-4, Mim6-5, Mim7-2, Mim7-3, Mim9-3, Mim9-4, Mim10-3, Mim10-4	DBEC	2	ND
Mim7-4	DBEC	3	200, 350, 530, **630**, 800
Mim3-5, Mim3-7, Mim4-2, Mim4-3, Mim5-2, Mim5-3, Mim5-4, Mim5-5, Mim8-2	DBEC	4	390, 510, **600**, 1000
Mim8-3, Mim8-4, Mim9-2, Mim9-5, Mim10-1	DBEC	4	ND
Mim1, Mim7, Mim11, Mim12	DBEC	5	185, 300, 300, 510, **600**, 900
Mim9	DBEC	5	ND
Isolates from *M. affinis* in Cuernavaca soil			
MimC1	CBEC	ND	ND
MimC2	CBEC	ND	ND
Reference strains			
R. etli CFN42^T	DBEC	6	150, 175, 270, **390**, 510, 630
R. etli F8	ND	1	ND
R. etli Bra5	ND	2	ND
R. etli Viking1	ND	7	ND
R. tropici CFN299	EBFD	8	185, 225, **410**, >1000
R. tropici CIAT899^T	FBDB	ND	ND
R. leguminosarum USDA2370^T	DBDB	ND	ND
R. galegae HAMBI540^T	IBDF	ND	ND
R. gallicum R602sp^T	CBAD	ND	ND
R. giardinii H152^T	AAAI	ND	ND
Rhizobium sp. Lc37	CBEC	ND	ND
M. loti NZP2213^T	GGFF	ND	ND
M. huakuii CCBAU2609^T	FHGF	ND	ND
M. ciceri USDA3378^T	GGFF	ND	ND
M. mediterraneum USDA3392^T	HGGF	ND	ND
Sinorhizobium sp. Lc28	ADEA	ND	ND
S. meliloti USDA1002^T	ADAA	ND	ND
S. fredii USDA205^T	EDAA	ND	ND

* RFLP patterns of SSU rRNA gene PCR-fragments digested individually with restriction enzymes *Msp*I, *Hin*fI, *Hha*I and *Sau*3AI are indicated by four letters. Agarose gels (3 %) in 0·5 × TBE buffer were used for separation of the digests.

† ETs were designated according to the combination of electrophoretic patterns of 10 metabolic enzymes in starch gels.

‡ Plasmid patterns were detected in Eckhardt gels (0·75 % agarose) and the sizes of the plasmids were estimated from their migration distances using the computer program SEQAID II version 3.5 (Rhoads & Roufa, 1989) and the plasmids of *R. etli* CFN42 (Romero *et al.*, 1991) and *R. tropici* CFN299 (Geniaux *et al.*, 1995; Martínez *et al.*, 1987) as molecular size standards. The symbiotic plasmids identified by *nif* gene probing are marked in bold.

ND, Not done.

Trinick (1980) from *Mimosa invisa* and *Mimosa pudica*. Some of these isolates were capable of nodulating and fixing nitrogen in *Leucaena*, and the reverse was also true. Four isolates from *M. invisa* and *M. pudica* nodules obtained from the Philippines were similar to *Rhizobium leguminosarum* and *Bradyrhizobium japonicum* and to a new lineage related to *R. galegae*, on the basis of comparison of 16S rRNA gene sequences (Oyaizu *et al.*, 1993).

In *Rhizobium* species, a significant proportion of the genome and its diversity is composed of plasmids, with the genetic information for nodulation and nitrogen fixation being located on the so-called symbiotic plasmids (reviewed by Martínez *et al.*, 1990). Specifically, a number of *nod* genes involved in the production of Nod factors (Dénarié *et al.*, 1996), which are key signal molecules in nodule formation (Relic *et al.*, 1994), and *nifHDK* genes that encode for nitrogenase are located on the symbiotic plasmids. Symbiotic plasmids may be transferred among *Rhizobium* species as well as to related genera, such as *Agrobacterium*, under laboratory conditions (Martínez *et al.*, 1987; Novikova & Safronova, 1992). Plasmid transfer in natural rhizobial populations has been assessed by comparing chromosomal and plasmid phylogenies (Amarger *et al.*, 1997; Haukka *et al.*, 1998; Souza & Eguiarte, 1997), and some earlier literature was reviewed by Martínez-Romero (1994) and Martínez-Romero & Caballero-Mellado (1996). Recently, the complete nucleotide sequence of a very-broad-host-range symbiotic plasmid showed that different genes in this plasmid may have different origins, suggesting a chimeric nature for symbiotic plasmids (Freiberg *et al.*, 1997).

Phaseolus vulgaris has been documented as a promiscuous host for rhizobia (Martínez *et al.*, 1985) and diverse bean-nodulating rhizobia have been recorded (van Berkum *et al.*, 1996; Eardly *et al.*, 1995; Geniaux *et al.*, 1993; Hernández-Lucas *et al.*, 1995; Laguerre *et al.*, 1993). Species in which strains have originated from bean plants include *Rhizobium etli* (Segovia *et al.*, 1993), *Rhizobium gallicum* (Amarger *et al.*, 1997), *Rhizobium giardinii* (Amarger *et al.*, 1997), *R. leguminosarum* and *Rhizobium tropici* (Martínez-Romero *et al.*, 1991). Strains within *R. mongolense* (van Berkum *et al.*, 1998) and various *Sinorhizobium* species (summarized by Hernández-Lucas *et al.*, 1995) were isolated from other host plants, but they also nodulated bean plants. In the reported phylogenetic trees based on the full SSU rRNA gene sequence (such as Amarger *et al.*, 1997; Wang *et al.*, 1998, 1999a), *R. etli* was closer to *R. leguminosarum* than to other species, and this was also the case when *nodD* gene sequences of *R. leguminosarum* bv. phaseoli and *R. etli* were compared (Laguerre *et al.*, 1996).

To describe more completely the diversity of *Rhizobium* from tropical areas, we isolated and analysed symbiotic bacteria from *Mimosa affinis*, a legume species native to Sierra de Huautla, a rainforest nature reserve in central Mexico. *M. affinis* is a small, annual plant that has never been domesticated and whose symbiotic bacteria have not been characterized. Our results showed that they corresponded to a limited group of lineages within *R. etli*, the dominant symbiotic bacterium for *Phaseolus vulgaris* (Segovia *et al.*, 1993; Caballero-Mellado & Martínez-Romero, 1999). Thus, we considered it of interest to pursue the molecular comparison of symbionts from *P. vulgaris* and *M. affinis*, with the aim of better understanding how *Rhizobium* lineages diverge and diversify.

METHODS

Nodule isolation and culture. Eleven *M. affinis* plants were collected from two populations 20 km apart in Sierra de Huautla, and one plant was grown in Cuernavaca soil. Nodules were surface-sterilized and were crushed on PY (3 g yeast extract, 5 g peptone and 0·7 g calcium chloride l^{-1}) plates. Bacterial isolates and reference strains (Table 1) were maintained in YM medium stabs (Vincent, 1970). Melanin production was detected in cultures grown on semi-solid PY medium (0·3 g agar l^{-1}) with added tyrosine (100 mg l^{-1}) and copper sulphate (20 mg l^{-1}).

Plant nodulation tests. Seeds of *Leucaena leucocephala* cv. Peruvian and *Phaseolus vulgaris* cv. Negro Xamapa were surface-sterilized (Martínez *et al.*, 1985) and pre-germinated seeds were placed in flasks with cotton or vermiculite with N-free plant nutrient solution (Fåhraeus, 1957). Plants were maintained in growth chambers at 28 °C with a photoperiod of 15 h. Nodulation of the plants was observed after 4 weeks of growth and the nitrogen-fixation ability of the nodules was assessed by comparison of the leaf colour of the inoculated plants with control plants that were not inoculated.

SSU rRNA gene typification. Primers fD1 and rD1 (Weisburg *et al.*, 1991) were used to synthesize SSU rRNA gene fragments by PCR with *Taq* polymerase. The PCR fragments were digested individually with restriction enzymes *Msp*I, *Sau*3AI, *Hin*fI and *Hha*I and the RFLP of the amplified SSU rRNA genes were visualized in agarose gels as described by Laguerre *et al.* (1994). Patterns were compared with type strains from the majority of described *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* species. The RFLP of the rRNA operons were determined by hybridizing Southern blots of *Eco*RI digests with a PCR product of the full 16S rRNA gene from *Bradyrhizobium japonicum* USDA6.

Multilocus enzyme electrophoresis (MLEE). Cell extracts from the new isolates and from reference strains were prepared from 40 ml liquid cultures in PY medium. The following metabolic enzymes were assayed: isocitrate, malate, alanine, threonine and glucose-6-phosphate dehydrogenases, hexokinase, phosphoglucomutase, indophenol oxidase and esterase. Enzymic activities were revealed with the standard procedures described by Selander *et al.* (1986). The mean genetic diversity for a locus was calculated as $h = [1 - \Sigma x_i^2][n/(n-1)]$, where x_i is the frequency of the i-th allele at the locus and n is the number of electrophoretic types (ETs) in the sample. Mean diversity (H) is the arithmetic mean of h over all loci assayed. A cluster analysis was performed using the neighbour-joining method (Nei & Li,

Table 2. Plasmids used in this study

Plasmid	Description
pEM15	pSUP205 derivative with *nifKDH* of *R. etli* CFN42 (Morett *et al.*, 1988)
pH3	pSUP202 derivative containing the replicator region (*RepA*, *RepB*, *RepC* genes and intergenetic sequences) of p42d in a 5·6 kbp *Hin*dIII fragment (Ramírez-Romero *et al.*, 1997)
pKRE-1	Cosmid (M. A. Cevallos, unpublished); the same fragment as outlined for pH3 was cloned in pRK7813, a wide-host-range vector (Stanley *et al.*, 1987)
pAGS10	3·6 kbp *Eco*RI–*Bam*HI fragment containing the entire *lpsβ*1 and *lpsβ*2 region of CFN42 plasmid b cloned in pRK7813, Tc^r (García-de los Santos & Brom, 1997)
pAGS4	718 bp internal fragment (*Xho*I–*Bam*HI) of *lpsβ*2 from pAGS10 cloned in pBluescript II SK(−), Cb^r (García-de los Santos & Brom, 1997)
pUC19	Cloning vector (Sambrook *et al.*, 1989)
pRK2013	Helper plasmid for conjugation, Km^r (Ditta *et al.*, 1980)

1979) based on the genetic distance data and a dendrogram was constructed. Linkage disequilibrium was calculated as described previously (Souza *et al.*, 1992).

Plasmid analysis. The cellular plasmid contents of some isolates were visualized in 0·75 % agarose gels by a modified Eckhardt technique (Hynes & McGregor, 1990). Plasmids of *R. etli* CFN42 (Romero *et al.*, 1991) and *R. tropici* CFN299 (Geniaux *et al.*, 1995; Martínez *et al.*, 1987) were used as molecular size standards and as positive controls for hybridization analyses. The electrophoretic plasmid patterns were hybridized as described previously (Wang *et al.*, 1998) to the following probes labelled with [^{32}P]dCTP (Amersham): a 600 bp internal *Sal*I fragment of *nifH* from pEM15 (Morett *et al.*, 1988), a 5·6 kbp *Hin*dIII fragment from pH3 containing the replicator region of CFN42 plasmid d (Ramírez-Romero *et al.*, 1997), a PCR-amplified internal lipopolysaccharide gene (*lpsβ*1) fragment (250 bp) from pAGS10 (García-de los Santos & Brom, 1997) and pAGS4, in which a 0·718 kbp internal fragment of *lpsβ*2 was cloned (García-de los Santos & Brom, 1997) (Table 2).

DNA fingerprinting and hybridization. DNA was isolated by standard methods (Sambrook *et al.*, 1989) from representative isolates and reference strains. DNA fingerprinting patterns were visualized after electrophoretic separation in 1·0 % agarose gels of fragments digested with the restriction enzymes *Bam*HI or *Eco*RI. λ DNA digested with *Bst*EII was used as the molecular size marker for estimation of fragment sizes in hybridization tests. The DNA restriction products were transferred to Hybond-N$^+$ membranes (Amersham) and hybridized under stringent conditions at 65 °C. The probes used for plasmid hybridization were also used for this experiment. Additional probes to evaluate DNA–DNA homology were total DNA digested with *Eco*RI from either CFN42 or Mim2 used in filter DNA hybridization, as described previously (Wang *et al.*, 1998).

***nifH* gene sequence.** PCR products were obtained from a representative isolate, Mim2, using *Pwo* DNA polymerase (Boehringer) and primers nifH-1 and nifH-2, corresponding to nucleotides 256 and 856 of the *Sinorhizobium meliloti nifH* gene (Eardly *et al.*, 1992). The PCR products were checked by electrophoresis in 1 % agarose and were purified from the gel using a Nucleotrap extraction kit for nucleic acids. The PCR fragments were cloned in the *Sma*I site of pUC19 vector (Sambrook *et al.*, 1989) by using the PCR cloning kit (blunt-end) from Boehringer as specified by the manufacturer and the resulting clones were extracted using the High Pure plasmid isolation kit (Boehringer) and sequenced using an AutoRead sequencing kit on an ALF DNA sequencer (Pharmacia). The sequence obtained from Mim2 was compared with other sequences in the database. Sequences were aligned using the PILEUP program in the Wisconsin package version 8.1 (Genetics Computer Group, 1995). The neighbour-joining method and bootstrap analysis (confidence values estimated from 500 replications of each sequence) in the CLUSTAL W package (Thompson *et al.*, 1994) were used to produce a phylogenetic tree. The following *nifH* sequences from GenBank were used: *Mesorhizobium* sp. DWO366 (Z95226), *R. etli* CFN42 (M15941), *R. etli* Olivia 4 (M55227), *R. gallicum* FL27 (M55226), *R. leguminosarum* bv. trifolii (K00490), *Rhizobium* sp. BR6001 (Z95230), *R. tropici* CIAT899 (M55225), *Sinorhizobium fredii* USDA191 (Z95229), *S. meliloti* CC169 (M55231), *Sinorhizobium saheli* ORS609 (Z95221) and *Sinorhizobium terangae* ORS1009 (Z95218).

Matings. Transconjugants of Mim1, a representative isolate from *M. affinis*, were obtained by transferring Tn*5-mob*-labelled plasmids (a–f) from CFN42 derivatives (Brom *et al.*, 1992) in triparental matings using plasmid pRK2013 as a helper. Transconjugants were selected by their resistance to kanamycin (50 μg ml^{-1}). Nodulation tests were performed for the transconjugants containing two symbiotic plasmids from both the donor (CFN42) and the receptor (Mim1) on *P. vulgaris* and *L. leucocephala* plants. pKRE-1 (M. A. Cevallos, unpublished), a cosmid containing the replicator region of the symbiotic plasmid, plasmid d, of CFN42, was introduced into isolates Mim1, Mim7-4 and Mim7-5 and transconjugants were selected by their resistance to tetracycline (3 μg ml^{-1}).

RESULTS

Isolation and phenotypic characteristics

One hundred and forty isolates were obtained from the nodules on 11 *M. affinis* plants collected from Huautla and two isolates were obtained from one plant grown in soil from Cuernavaca. All the isolates from Huautla had the same colony morphology and growth rate on PY medium. All of them formed gummy and pearly colonies >2 mm in diameter after 3 d incubation on PY plates. Colonies of the two isolates from Cuernavaca soil were not gummy. Fifty isolates from Huautla were chosen randomly for further characterization.

The 50 selected *Rhizobium* isolates from Huautla and reference strains for *R. etli* (Table 1) were resistant to

nalidixic acid (20 μg ml^{-1}) and did not grow in LB. These isolates had duplication times of 3 h in PY liquid medium, similar to *R. etli* strains. No melanin production was obtained from the isolates even after 1 month incubation in soft agar containing tyrosine and copper, while CFN42 readily produced the brown pigment.

Nodulation tests

All 50 isolates from *M. affinis* obtained in Huautla were capable of nodulating *P. vulgaris* cultivar Negro Xamapa, with nodules appearing around 5–6 d after inoculation. The mean number of nodules per plant ranged from 30, in the case of inoculation with isolate Mim5, to 60, in the case of inoculation with isolate Mim1, after 17 d. The nodules were pink and the leaves of the nodulated plants were dark-green, as were the positive controls inoculated with *R. etli* CFN42, while the control non-inoculated plants were yellow-green. Isolates Mim1, Mim2, Mim3-7, Mim7 and Mim7-4 formed nodules on *L. leucocephala* and nodulated plants were green, while non-inoculated plants or those inoculated with *R. etli* strains CFN42 and F8 did not have nodules and were yellow.

Ribosomal types

The ribosomal types were defined on the basis of the PCR–RFLP and RFLP patterns of SSU rRNA genes. RFLP patterns of PCR-synthesized SSU rRNA genes were identical in *R. etli* strain CFN42 and in all 50 isolates from Huautla, and differed from those of *R. tropici* CIAT899 and CFN299, *S. meliloti* USDA1002, *R. galegae* HAMBI540, *Mesorhizobium loti* NZP2213, *R. leguminosarum* USDA2370, *M. huakuii* CCBAU-2609, *M. ciceri* USDA3378, *M. mediterraneum* USDA3392, *S. fredii* USDA205, *R. gallicum* FL27 and *R. giardinii* H152 (Table 1). The two strains isolated from *M. affinis* sown in Cuernavaca soil had patterns identical to each other and to one of our previously isolated strains from *Leucaena* (Lc37), which corresponded to a novel lineage within the genus *Rhizobium* (Wang *et al.*, 1999b) (Table 1). Additional characteristics of these two isolates from Cuernavaca will be reported elsewhere and only the characterization of the isolates from Huautla is presented here. Hybridization patterns (RFLPs) of *Eco*RI-restricted DNAs to the 16S rRNA gene were identical in *R. etli* CFN42 and in the isolates from *M. affinis* obtained in Huautla (not shown).

Electrophoretic types

The 50 isolates from *M. affinis* in Huautla were grouped into four ETs (ET 2–5) (Table 1) based on the analysis of ten enzymes. ETs 3, 4 and 5 were composed only of isolates from *M. affinis*, corresponding to one, 14 and five isolates in each. ET 2 contained 30 isolates and a reference strain, Bra5. The other reference strains

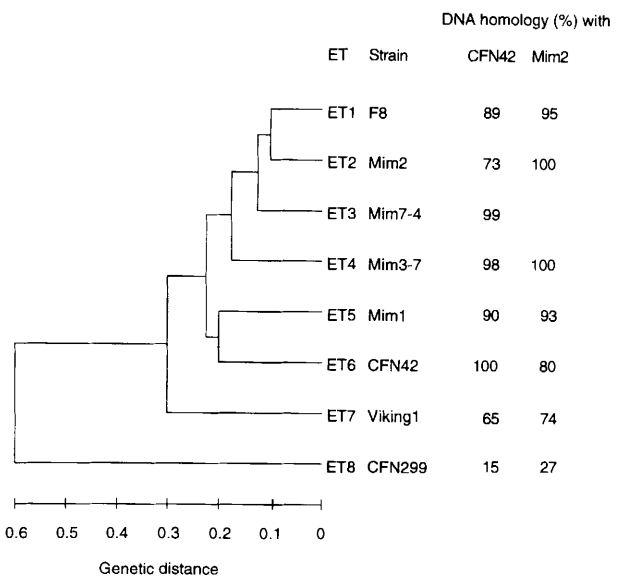

Fig. 1. Genetic relatedness of isolates from *M. affinis*, *R. etli* reference strains and *R. tropici* CFN299 defined by analyses of MLEE and DNA–DNA homology. The dendrogram was constructed from the MLEE data for 10 metabolic enzymes using the neighbour-joining method (Nei & Li, 1979) based on the genetic distance (Selander *et al.*, 1986) of each ET pair. ET 2 contains 30 isolates and strain Bra5; ET 3 contained isolate Mim7-4 only; ET 4 represents 14 isolates; ET 5 consists of five isolates. DNA–DNA homology was estimated by the filter-hybridization method (Wang *et al.*, 1998).

for *R. etli*, CFN42, F8 and Viking1, and *R. tropici* CFN299 formed distinct ETs. The isolates from *M. affinis* and the reference strains for *R. etli* were intermingled in a close cluster (genetic distance less than 0·3) that was distant from *R. tropici* strain CFN299 (genetic distance 0·6) (Fig. 1). We obtained a mean of 12·5 isolates per ET among the isolates from *M. affinis*, which is larger than the mean of 1·2 obtained with isolates from *P. vulgaris* (Pérez-Ramírez *et al.*, 1998). The mean genetic diversity (H) for ETs from *Mimosa* was 0·2. ETs of the isolates and reference strains for *R. etli* were in linkage equilibrium, with the V_o/V_e value (0·951) close to 1, meaning that the microsymbionts of *M. affinis* and *P. vulgaris* share a pool of chromosomal genes with no barriers for chromosomal recombination.

DNA–DNA homology and fingerprinting

In agreement with the MLEE results, we found close similarity of isolates from *M. affinis* and *R. etli* reference strains from *P. vulgaris* on the basis of DNA–DNA homology (Fig. 1). When total DNA from *R. etli* CFN42 was used as a probe in three independent assays, the homology was 73–99% with isolates Mim2, Mim7-4, Mim3-7 and Mim1, representing ETs 2, 3, 4 and 5, respectively; homology was lower with Viking1 (65%) and *R. tropici* CFN299

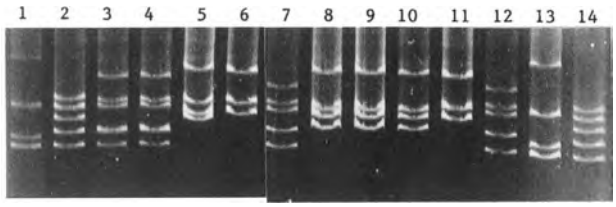

Fig. 2. Examples of plasmid profiles of isolates from *M. affinis* obtained in Huautla (lanes 3–12). Lanes: 3, Mim1 (185, 300, 300, 510, 600, 900 kbp); 4, Mim7 (sizes as lane 3); 5, Mim1-3 (390, 450, 600, 1000); 6, Mim1-4 (390, 600, 1000); 7, Mim7-4 (200, 350, 530, 630, 800); 8, Mim5-4 (390, 510, 600, 1000); 9, Mim4-3 (as lane 8); 10, Mim4-2 (as lane 8); 11, Mim8-5 (as lane 6); and 12, Mim7-4 (as lane 7). Molecular sizes were estimated from migration distances using the computer program SEQAID II version 3.5 (Rhoads & Roufa, 1989) and the plasmids of *R. tropici* CFN299 (lanes 1 and 13) (185, 225, 410, >1000 kbp) (Geniaux *et al.*, 1995; Martínez *et al.*, 1987) and *R. etli* CFN42 (lanes 2 and 14) (150, 270, 390, 510, 630 kbp) (Romero *et al.*, 1991) as molecular size markers.

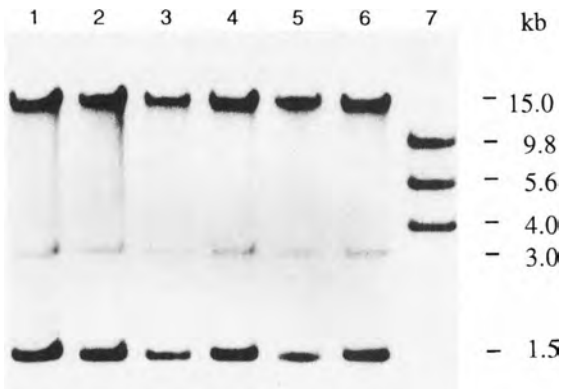

Fig. 3. Example of autoradiogram of *nifH* gene hybridization patterns obtained in *Bam*HI digests of DNAs from the isolates and a reference strain. Lanes: 1, Mim3-7; 2, Mim1-1; 3, Mim7-5; 4, Mim5-3; 5, Mim10-4; 6, Mim9-2; 7, *R. etli* CFN42 (Martínez *et al.*, 1985). Isolates Mim1, Mim5, Mim2-5, Mim5-5, Mim7-2, Mim7, Mim12, Mim1-3, Mim3-6, Mim4-2, Mim6-4, Mim8-3, Mim9-5 and Mim10-1 gave patterns identical to those of Mim3-7. The molecular mass marker was λ DNA digested with *Bst*EII and the sizes of the hybridizing bands were estimated using the computer program SEQAID II version 3.5 (Rhoads & Roufa, 1989).

(15%). When DNA from isolate Mim2 was used as a probe (two independent assays), homology with *R. etli* strains CFN42 and F8 was 80 and 95%.

Identical DNA fingerprints were obtained from the restriction of total DNA with *Eco*RI among some isolates within an ET, and these patterns were similar among related ETs (not shown), predominantly in the lower parts of the gels, as observed previously for related ETs of *Acetobacter diazotrophicus* (Caballero-Mellado & Martínez-Romero, 1994).

Plasmid profiles and symbiotic plasmid characterization

All isolates from *M. affinis* tested had plasmids (Table 1) ranging from 100 kbp to larger than 1000 kbp. Different plasmid patterns were observed (Fig. 2). Megaplasmids ($\geqslant$1000 kbp) were observed in all the isolates tested from ETs 2 and 4. Plasmid profiles seemed to be conserved in genetically related isolates. Nine isolates within ET 4 shared the same plasmid pattern, and the four isolates in ET 5 had another identical pattern. Although four different plasmid patterns (with three to five plasmids in each) were obtained in 17 ET 2 isolates, two common bands (390 and 600 kbp) were observed in all of them. The 600 kbp plasmid was common to all the isolates tested within ETs 2, 4 and 5, but was not observed in Mim7-4, the only isolate in ET 3.

The symbiotic plasmids, identified by hybridization of Eckhardt gels to *R. etli nif* genes, were 600 kbp in most of the isolates from *M. affinis* tested (Mim1-1, Mim1-3, Mim1-4, Mim8-5, Mim4-2, Mim4-3, Mim5-4, Mim1 and Mim7, representing ETs 2, 4 and 5) and slightly larger (630 kbp) in Mim7-4, the sole isolate corresponding to ET 3. Multiple copies of the *nifH* gene were observed in the isolates and in *R. etli* reference strains (Fig. 3). The *nifH* gene organization was the same in all isolates from *M. affinis* tested representing ETs 2, 4 and 5, which had symbiotic plasmids with the same molecular size, and different from that found in *R. etli* strains from *P. vulgaris* (Fig. 3). Two strongly hybridizing bands of around 15 and 1·5 kbp and one faint band (3·0 kbp) were obtained from the isolates (Fig. 3).

The replicator region of the symbiotic plasmid, pSym, from *R. etli* strain CFN42 (Ramírez-Romero *et al.*, 1997) did not hybridize to any plasmid in isolates Mim1, Mim5-5, Mim7-4 or Mim7-5, representing the four ETs. Furthermore, the introduction of plasmid pKRE-1 carrying this replicator did not promote any plasmid loss in Mim7-4 (ET 3) or Mim7-5 (ET 2), but a 300 kbp plasmid was lost upon introduction of pKRE-1 in Mim1 (ET 5).

Two clones each containing a PCR-amplified fragment from Mim2 *nifH* gene were sequenced. Over the nucleotides compared, both clones had identical sequences. The sequence identities among the *nifH* gene obtained from isolate Mim2 and those from related species obtained from GenBank are presented in Table 3. A dendrogram showing *nifH* sequence relatedness is shown in Fig. 4. The Mim2 *nifH* gene is closely related to the *R. etli* CFN42 *nifH* gene (97·3% identity in nucleotide sequences, 98·9% identity in amino acid sequences). *R. gallicum* bv. gallicum, *R. etli* CFN42, *R. etli* Olivia 4 and the isolate Mim2 formed a close cluster that was supported by high bootstrap values (Fig. 4). Their sequence identities were greater than 95·7%, while they had identities to other species of 83·5–90·5%.

Table 3. Sequence identities (%) of internal *nifH* gene fragments (558 bp) among *R. etli* bv. mimosae Mim2 and related strains

Sequences were aligned and compared using SEQED and GAP programs in the Wisconsin package.

	1	2	3	4	5	6	7	8	9	10	11	12
1. *R. etli* Mim2	100											
2. *R. etli* CFN42	97·3	100										
3. *R. etli* Olivia 4	97·3	100·0	100									
4. *R. gallicum* FL27	96·1	95·7	95·7	100								
5. *R. tropici* CIAT899	90·2	89·4	89·4	90·4	100							
6. *Rhizobium* sp. Br6001	90·5	89·8	89·8	90·6	97·0	100						
7. *R. leguminosarum*	84·1	83·5	83·5	83·1	82·3	82·6	100					
8. *S. meliloti*	86·6	86·6	86·6	87·4	86·6	87·0	83·3	100				
9. *S. terangae* ORS1009	89·6	88·7	88·7	90·2	88·8	88·9	83·7	88·0	100			
10. *S. saheli* ORS609	88·7	88·3	88·3	88·4	87·2	88·2	83·7	87·8	94.3	100		
11. *S. fredii* USDA191	90·3	90·0	90·0	91·7	89·2	88·9	83·9	88·4	91·4	90·7	100	
12. *Mesorhizobium* sp. DWO366	89·8	89·6	89·6	90·4	89·0	90·0	82·2	87·2	88·9	88·9	89·8	100

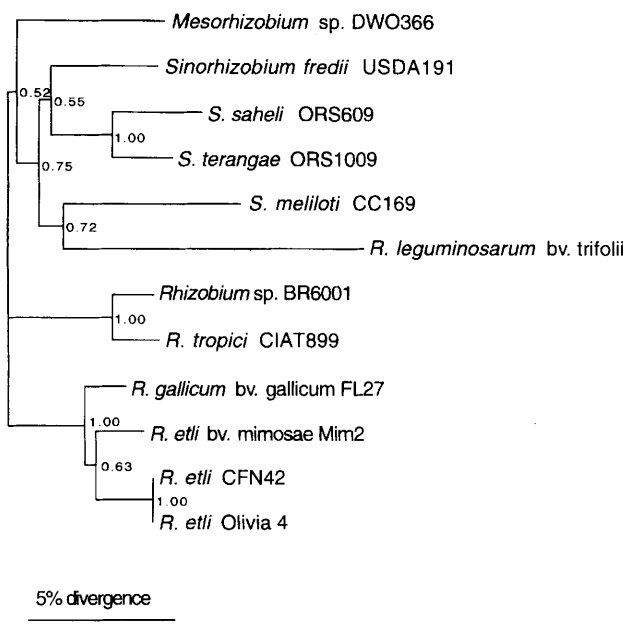

Fig. 4. Phylogram showing relatedness of partial *nifH* gene sequences of isolate Mim2, a representative of the 50 isolates from *M. affinis* obtained in Huautla, and reference strains. Sequences were aligned using the PILEUP program in the GCG package. The neighbour-joining method in the CLUSTAL W package (Thompson *et al.*, 1994) was used for the analysis of sequence data. Bootstrap confidence values were estimated from 500 replications of each sequence.

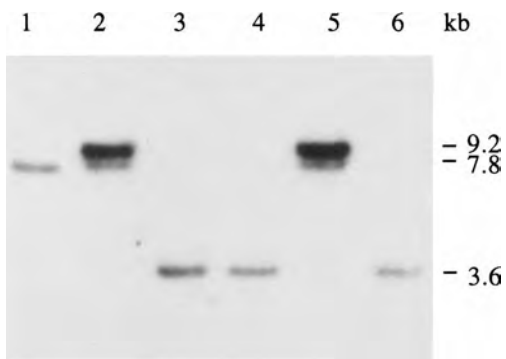

Fig. 5. Hybridization patterns of *lpsβ*1 gene (250 bp PCR fragment of pAGS10) from *R. etli* CFN42 to *Eco*RI digests of DNAs from new isolates and *R. etli* CFN42. Lanes: 1, CFN42; 2, Mim1; 3, Mim1-4; 4, Mim4-3; 5, Mim7; 6, Mim7-5. λ DNA digested with *Hin*dIII was used as the molecular size marker to estimate the sizes of the hybridizing bands.

General plasmid comparison

By hybridization with plasmid patterns, genes homologous to *lpsβ* were found either on a plasmid similar in size (185 kbp) to plasmid b of *R. etli* CFN42 (175 kbp), in isolates Mim1 and Mim7 (ET 5), or on a megaplasmid (1000 kbp), in Mim1-4, Mim7-5 (ET 2) and Mim4-3 (ET 4) (not shown). Two restriction patterns (1 and 2) were observed in the hybridization of *Eco*RI-digested total DNA from isolates Mim1-4, Mim7-5 (ET 2), Mim4-3 (ET 4), Mim1 and Mim7 (ET 5), representing different plasmid patterns, to *R. etli* CFN42 *lpsβ* genes (Fig. 5). Hybridization pattern 1 had two hybridizing bands (9·2 and 7·8 kbp) and was found in the isolates of ET 5 from *M. affinis* that have homology to *lpsβ*1 on a 185 kbp plasmid. Only one hybridizing band (3·6 kbp), which we designated pattern 2, was obtained from the isolates of ET 2 and ET 4. These isolates were found to have the *lpsβ* genes located on megaplasmids (1000 kbp). Identical hybridization results were obtained with the two *lpsβ* gene probes (*lpsβ*1 and 2), which demonstrated that the two *lpsβ* genes were in one gene cluster in the isolates, as for those in CFN42.

Conjugal transfer of plasmids

We transferred each of the six plasmids (a–f) from strain CFN42 (Brom *et al.*, 1992) to the isolate Mim1. Ten transconjugants (colonies) from each plasmid transfer were analysed. Plasmids a, b, c, d and e (150, 175, 270, 390 and 510 kbp, respectively) could be transferred easily to Mim1 receptors. We found that plasmids a and b were maintained in Mim1 transconjugants. It seems that CFN42 plasmids c and e substitute for the similarly sized plasmids (300 and 510 kbp, respectively) in isolate Mim1, since Tn*5* was maintained but the plasmid profiles were not changed and the hybridizations to Tn*5-mob* were obtained in the 270 and 510 kbp plasmids in the transconjugants. The CFN42 symbiotic plasmid, plasmid d, was maintained in Mim1 in all 10 transconjugants and caused the curing of a 300 kbp plasmid, the same one that was lost upon the introduction of pKRE-1 (carrying the pSym replicator region) but perhaps different from that which was lost with plasmid c transfer, according to their relative positions in Eckhardt gels. Transconjugants bearing symbiotic plasmids from both CFN42 and Mim1 were tested for nodulation in *Leucaena* and bean and they formed similar numbers of nodules as the original strains. Fifteen isolates from the nodules formed by Mim1 transconjugants with two symbiotic plasmids were kanamycin resistant (marker for the *R. etli* CFN42 symbiotic plasmid) and Eckhardt plasmid profiles showed that both symbiotic plasmids were maintained in these nodule isolates. Plasmid f was not maintained in Mim1, in comparison with transconjugants containing any of the other plasmids. Several kanamycin-resistant colonies obtained after the conjugal transfer of CFN42 plasmid f (630 kbp) did not have a plasmid corresponding to this size and Tn*5-mob* was shown by hybridization to be located on the megaplasmid in Mim1 recipients.

DISCUSSION

On the basis of current taxonomy for root-nodule bacteria (Graham *et al.*, 1991), greater than 70% DNA–DNA homology, as well as distinctive phenotypic characters, is one of the main criteria for defining species, although in some cases strains sharing lower homology (40–60%) have been found within a single species, as in the cases of *R. tropici* types A and B (Martínez-Romero *et al.*, 1991) and *Mesorhizobium plurifarium* (de Lajudie *et al.*, 1998a). The SSU rRNA gene sequence identity is used mainly as a criterion to estimate the generic position of bacteria, and strains within some species, such as *M. loti*, can have divergent SSU rRNA genes (de Lajudie *et al.*, 1998a). In this study, identical PCR–RFLP patterns of SSU rRNA genes were found among the 50 isolates from *M. affinis* in Huautla and the type strain of *R. etli* in individual digestions with four restriction enzymes. This result indicated a close phylogenetic relationship between the isolates and the type strain of *R. etli*, since around 99% sequence identities were obtained between the SSU rRNA genes that shared identical PCR–RFLP patterns digested with the same four enzymes in our previous reports (Wang *et al.*, 1998, 1999a). This relationship between RFLP and sequence data was confirmed by a computer-simulated RFLP analysis of SSU rRNA genes (Moyer *et al.*, 1996). To describe the specific status of the isolates, we characterized them further by genetic and phenotypic approaches. We concluded that the new isolates from Huautla were members of the species *R. etli*, on the basis of their close relationships and linkage equilibrium in MLEE analysis, high DNA–DNA homology (73–99%), similar DNA fingerprints and several common phenotypic features (growth rate, colony morphology and resistance to nalidixic acid) to the reference strain of the species. Multiple *nifH* genes are a common characteristic of *R. etli* and *R. leguminosarum* (Martínez *et al.*, 1985; Quinto *et al.*, 1982; Segovia *et al.*, 1993; Sessitsch *et al.*, 1997) and they were also found in the isolates from *M. affinis* (Fig. 3). We did not include strain F16, the single *R. etli* strain reported to nodulate both bean and *Leucaena* plants (Hernández-Lucas *et al.*, 1995), since further analysis of the strain used in that study showed us that it did not correspond to *R. etli* (data not shown).

The limited genetic diversity (H = 0·2) of the isolates from *M. affinis* revealed by MLEE analysis might be related to the small sampling size (11 plants) and the restricted sampling site. However, far larger genetic diversity was observed among *R. etli* bv. phaseoli strains (H = 0·6) obtained from a more restricted geographical area (Caballero-Mellado & Martínez-Romero, 1999). The large diversity of *R. etli* bv. phaseoli strains is striking (Piñero *et al.*, 1988) and the species limits of *R. etli* have been difficult to define (Eardly *et al.*, 1995). This may result from the population of *R. etli* harbouring the bv. phaseoli plasmid enlarging and diversifying more than that of bv. mimosae.

Generally, an MLEE group at a genetic distance of less than 0·5 shares more than 70% DNA homology (Caballero-Mellado & Martínez-Romero, 1999; Martínez-Romero *et al.*, 1991; Segovia *et al.*, 1993; Wang *et al.*, 1999a). This is also the case in this study (Fig. 1). The DNA homology data were also generally related to the genetic distances obtained when the ETs within an MLEE group were compared (Fig. 1). Mim2 had homology of 95% with F8, 100% with Mim3-7, 93% with Mim1, 80% with CFN42 and 74% with Viking1 in DNA–DNA hybridization. The genetic distances between Mim2 and these five isolates or strains increased gradually from 0·1 for F8 to 0·3 for Viking1 in MLEE analysis. CFN42 had very similar genetic distances (0·20–0·23) to the isolates in ETs 1–5 in MLEE analysis and the DNA homology values were also quite similar (73–99%).

The metabolic enzymes tested in this study were found to be chromosomally encoded in *R. etli* strain CFN42, since strains cured of each of the plasmids presented

bands identical to the wild-type strain (S. Brom & J. Caballero-Mellado, personal communication). We observed conserved plasmid patterns in isolates within some ETs, such as ET 4 and ET 5. Although a common plasmid (the symbiotic plasmid) was found, different plasmid patterns were obtained among different ETs and among the 17 isolates within ET 2, the ET with the largest number of isolates, showing that plasmids constitute the most diverse components in these populations.

Even though extensive screenings of Mesoamerican legumes have been performed, we had previously isolated *R. etli* mainly from *Phaseolus* bean nodules and not from any wild legumes (Martínez *et al.*, 1985; Wang *et al.*, 1998, 1999b; Barrera *et al.*, 1997). It is remarkable that the new *R. etli* host plant is a member of the subfamily Mimosoideae that has not been cultivated and comes from an undisturbed area. Wild *Phaseolus* species have also been reported to occur in Huautla (O. Dorado, personal communication); thus, both hosts share a geographical distribution area, which may have facilitated their sharing of symbionts. We show here that, although very similar at the chromosomal level, isolates from *P. vulgaris* and *M. affinis* have differences attributable to diverging plasmids, especially the symbiotic plasmids.

The best characterized of all plasmids in *Rhizobium* are the symbiotic plasmids and, consequently, our comparison of isolates from *Phaseolus* and *Mimosa* is most complete for these plasmids. Differential nodulation specificity was found among isolates from *M. affinis* and *P. vulgaris*. Isolates from both *P. vulgaris* and *M. affinis* could nodulate *P. vulgaris*, but only the isolates from *M. affinis* could nodulate *L. leucocephala*. Different sizes of symbiotic plasmids were recorded among the isolates or strains from these two host plants (Table 1). The hybridization patterns for *nifH*, which had been located on the symbiotic plasmids, observed for the isolates from *M. affinis* (Fig. 3) were not similar to any of the *nifH* patterns reported in strains from *P. vulgaris* (Martínez *et al.*, 1985; Pérez-Ramírez *et al.*, 1998; Sessitsch *et al.*, 1997), indicating a different genetic organization of the symbiotic plasmid of the isolates from *M. affinis* from that of strains from *P. vulgaris*. No homology to the replicator sequence CFN42 pSym was observed by hybridization and by mating tests on the plasmids in the isolates from *M. affinis*. Melanin production, a typical feature of most *R. etli* strains from *P. vulgaris* (Martínez-Romero, 1996; Michiels *et al.*, 1994), is symbiotic-plasmid-encoded and none of the isolates from *M. affinis* produced it. We concluded that the symbiotic plasmids were different in the isolates from *P. vulgaris* and *M. affinis*.

All *R. etli* bv. phaseoli strains have symbiotic plasmids with homology to the CFN42 pSym replicator region (Ramírez-Romero *et al.*, 1997), which was not the case in the isolates in *R. etli* bv. mimosae. In nature, strains could exist with both symbiotic plasmids found in the isolates from *P. vulgaris* and *M. affinis*, since these symbiotic plasmids belong to different compatibility groups (see Results) and have different replicator regions. Under laboratory conditions, Mim1 transconjugants carrying an additional symbiotic plasmid from CFN42 were stable, even after passage through bean nodules. Nevertheless, strains with two symbiotic plasmids may be less competitive for nodule formation and, as such, are not normally recovered from nodules.

The *nifH* gene-based phylogram we obtained is similar to that reported previously (Haukka *et al.*, 1998). Two different *R. etli* bv. phaseoli strains, CFN42 and Olivia 4, were found to have identical *nifH* sequences (accession numbers M15941 and M55227, respectively). The similarity of the *nifH* genes among bv. phaseoli and bv. mimosae plasmids suggests that they evolved from a common ancestor. It is remarkable that rhizobia that can nodulate *Leucaena* as well as bean, such as *R. etli* bv. mimosae (described here) and *R. gallicum* bv. gallicum FL27 (Eardly *et al.*, 1992), have *nifH* genes sharing the same ancestry as those of *R. etli* bv. *phaseoli* (Fig. 4). It seems that *R. etli* bv. phaseoli lost the capacity to nodulate *Leucaena* and became more specialized for bean. The evolution of *R. etli* bv. phaseoli from a generalist to a specialist might have been driven by the extensive cultivation of bean. Similarly, it seems that the *R. fredii* symbiotic plasmid (such as that from USDA257) evolved from a more promiscuous plasmid, as both NGR234 and USDA257 symbiotic plasmids are very similar (Krishnan *et al.*, 1992; X. Perret and W. J. Broughton, personal communication). Interestingly, USDA257, like *R. etli* bv. phaseoli, no longer nodulates *Leucaena*, and the genetic differences from NGR234 involved in this change of specificity have been analysed (Krishnan *et al.*, 1992). It has been assumed that coevolution has occurred in the *Rhizobium*–legume interaction, mainly related to symbiotic-plasmid-borne genes (Ueda *et al.*, 1995). Our data support this hypothesis, since both *R. etli* bv. mimosae and bv. phaseoli share the same gene pool, as revealed by MLEE analysis and DNA–DNA hybridization, but the symbiotic plasmids are different.

In *R. etli* bv. phaseoli, plasmids other than the pSym play a role in symbiosis (Brom *et al.*, 1992), for example CFN42 plasmid f has reiterated copies of *fixNOQP*, *fixGHI* and *fixK* and an unusual *fixL* gene, and bacteria cured of this plasmid are less efficient in nitrogen fixation (Girard *et al.*, 1998). Plasmids such as plasmids c, e and f from CFN42 seem to be conserved in isolates from both *Mimosa* and *Phaseolus*. The high DNA–DNA homology encountered among the isolates from *Mimosa* and *Phaseolus* is also indicative of chromosomal as well as of plasmid homology, as discussed previously for the interpretation of DNA–DNA homology values in rhizobia (Martínez-Romero, 1994). It was thus of interest to test the *lpsβ* genes as markers for plasmid b of *R. etli* CFN42. CFN42 plasmid b has also been assigned a role in the symbiosis with bean (Brom *et al.*, 1992) and is highly conserved among different *R. etli* and *R. legum-*

inosarum strains (García-de los Santos & Brom, 1997). The *lpsβ* genes are also conserved in isolates from *Mimosa*, although on a different replicon in some of them.

Scarce nodulation was obtained on *M. affinis* roots when plants were grown in Cuernavaca soil, and the two isolates were not *R. etli*, although *M. affinis* is also native to Cuernavaca (O. Dorado, personal communication). We found a similar situation with *R. huautlense*, a bacterium nodulating *Sesbania herbacea*, which was found only in Sierra de Huautla and not in Cuernavaca soils (Wang *et al.*, 1998). This probably indicates that Huautla, perhaps because of its geographical isolation, is a unique source of bacterial and plant diversity.

Tropical forests have a large diversity of plant species and also highly diverse microbial populations. Tropical plants may be hosts for new *Rhizobium* species, but this is not always the case. It was surprising that strains isolated from taxonomically distant host plants in previously unexplored environments corresponded to strains already described, largely from agricultural systems (Moreira *et al.*, 1998). Similarly, we did not recover a new rhizobial lineage from *M. affinis*, but rather a well-known species differing in its type of symbiotic plasmid and apparently also in other plasmids.

Common *Rhizobium* genetic backgrounds may harbour symbiotic plasmids with different specificities. The biovar designation in *Rhizobium* species is meant to refer to the symbiotic plasmids within a single type of chromosome, for example bv. viciae, bv. trifolii and bv. phaseoli within *R. leguminosarum* (Jordan, 1984) and bv. sesbaniae and bv. acaciae within *S. terangae* (Boivin *et al.*, 1997). When the species *R. etli* was described (Segovia *et al.*, 1993), the existence of biovars, bv. phaseoli and bv. viciae, within this species was discussed. Due to the small number of bv. viciae isolates analysed, the Subcommittee on the taxonomy of *Rhizobium* and *Agrobacterium* approved the species but deferred assignment of *R. etli* biovars for the time being (Martínez-Romero & Jarvis, 1993).

On the basis of our results, we propose a novel biovar within the species *R. etli*, bv. mimosae, for the isolates from *M. affinis* obtained in Huautla. The nodulation on *L. leucocephala* and non-production of melanin could be phenotypic features to distinguish the new biovar from the previously reported biovar, bv. phaseoli (Segovia *et al.*, 1993). The sizes, replicator regions and *nifH* gene organizations of the symbiotic plasmids and *nifH* gene sequences illustrate the genetic differences between these two biovars. Furthermore, there seems to exist a 'null' biovar of rhizobium lacking symbiotic plasmids. Non-symbiotic *R. etli* strains were recovered from the bean rhizosphere more frequently than symbiotic ones (Segovia *et al.*, 1991). The natural occurrence of these rhizobia, lacking plasmids but genetically intermingled with symbiotic bacteria, suggests that the former may be receptors in plasmid transfers at a frequency to prevent the non-symbiotic lineage from diverging from the symbiotic ones, a somewhat similar situation to that of genetic lateral transfer occurring in *M. loti* (Sullivan *et al.*, 1995).

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