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Diverse *Mesorhizobium plurifarium* populations native to Mexican soils

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Abstract Forty-six *Mesorhizobium* strains associated with the leguminous plants *Leucaena leucocephala* and *Sesbania herbacea* in an uncultivated Mexican field were characterized using a polyphasic approach. The strains were identified as *Mesorhizobium plurifarium* based upon the close relationships with the reference strains for this species in PCR-based restriction fragment length polymorphism analyses, sequencing of 16S rRNA genes, multilocus enzyme electrophoresis, and DNA-DNA hybridization. Although the strains isolated from both plants formed the same group in multilocus enzyme electrophoresis and cross-nodulations were observed in the laboratory, different electrophoretic types were obtained from the two plants grown in natural soils, indicating the existence of a preferable association between the plants and the rhizobia. The *M. plurifarium* strains from Mexico and the reference strains from Africa and Brazil formed different phenotypic clusters in a numerical taxonomy. The Mexican strains did not grow at 37 °C and were sensitive to salty-alkaline conditions, while the reference strains from Africa and Brazil grew at 42 °C and were more resistant to salty-alkaline conditions. These results demonstrate that both the plants and environmental factors affected the evolution of rhizobia and that the Mexican strains had adapted to the neutral soils and the cool climate where they were isolated.

Keywords *Mesorhizobium plurifarium* · *Leucaena* · *Sesbania* · Phylogeny · Diversity

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Introduction

Leucaena leucocephala and *Sesbania herbacea* are two leguminous species native to Mexico. *L. leucocephala* originated in Mexico and Guatemala and has been introduced to other continents. As a rapid-growing tropical tree, this plant has been used for many purposes: seeds as food in Mexico; trees as firewood or materials for paper, fiber, and furniture production; leaves as foliages, etc. Earlier reports indicated that this plant nodulated with fast-growing rhizobial groups (Sanginga et al. 1995) and inoculation was needed for nodulation in many areas (Peoples et al. 1995). Further research showed that *Rhizobium tropici* (Martínez-Romero et al. 1991), *Mesorhizobium plurifarium* (de Lajudie et al. 1998), and some unnamed groups (de Lajudie et al. 1998; Gao et al. 1994; Jarvis 1983) could nodulate this plant in South America and in Asia. Our previous study indicated that *L. leucocephala* nodulated with diverse rhizobial groups belonging to *Mesorhizobium, Rhizobium*, and *Sinorhizobium* in an uncultivated Mexican field (Wang et al. 1999b). Based on these results, it seemed that while *L. leucocephala* is not a selective plant for nodulation, the absence of rhizobia able to nodulate *L. leucocephala* is still possible in some regions where it has not been planted previously.

Sesbania herbacea is an annual wild plant growing naturally in waterlogged fields and, like other *Sesbania* species, it is a potential green manure in lowlands (Becker et al. 1990). It formed root nodules with *Rhizobium huautlense* (Wang et al. 1998) in flooded fields and with diverse rhizobial groups within the genera *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* in a dry field in Mexico (Wang et al. 1998; Wang and Martínez-Romero 2000). However, the rhizobia from the dry field failed to nodulate in flooded

soils and were outcompeted by *R. huautlense* in nodulation assays (Wang and Martínez-Romero 2000). Thus, it seems that the rhizobia from the dry field are not natural microsymbionts for *S. herbacea*.

It has been reported that *Leucaena* and *Sesbania* species belonged to the same cross-nodulation group (Trinick 1980). Among the different rhizobial groups nodulating *L. leucocephala* and *S. herbacea* in the Mexican field, *Mesorhizobium* strains have been isolated from both plants, and patterns identical to that of *M. plurifarium* were obtained by PCR-based restriction fragment length polymorphism (RFLP) of 16S rRNA genes (Wang et al. 1999b; Wang and Martínez-Romero, 2000). These *Mesorhizobium* strains occupied 38.1% and 52% of the nodules on *L. leucocephala* and on *S. herbacea,* respectively (Wang et al. 1999b; Wang and Martínez-Romero 2000). Although *M. plurifarium* was defined recently for genetically diverse strains from Africa and Brazil (de Lajudie et al. 1998), little is known about the distribution and diversity of this bacterium in other geographic regions. With the aim of verifying the relationships between the Mexican strains of *M. plurifarium* and those from Africa and Brazil, we characterized the *Mesorhizobium* strains from the plants *L. leucocephala* and *S. herbacea* grown in Mexico.

Materials and methods

Bacterial strains

All strains used in this study are listed in Table 1. The 46 *Mesorhizobium* strains from *L. leucocephala* and *S. herbacea* have identical patterns (rDNA type I) of PCR-base RFLP of 16S rRNA genes (Wang et al. 1998; 1999b; Wang and Martínez-Romero 2000). The 26 isolates from *S. herbacea* were obtained in 1997 (Wang et al. 1998) and 1998 (Wang and Martínez-Romero 2000) by growing *S. herbacea* plants in pots filled with natural soils from a well-drained field in Cuernavaca, Morelos, Mexico. The strains from *L. leucocephala* were obtained from the same field (Wang et al. 1999b). Characteristics of the soils were described previously (Wang and Martínez-Romero 2000). Routine methods and PY medium (peptone of casein 5 g, yeast extract 3 g, $CaCl_2$ 0.6 g, distilled water 1 l, and 18 g agar l^{-1} for solid medium) were used for growing and maintenance of the bacteria.

Analysis of multilocus enzyme electrophoresis (MLEE)

The method described previously (Caballero-Mellado and Martínez-Romero 1994) was used for protein extraction. Aconitase (ACO), alanine dehydrogenase (ALD), esterase (EST), glucose-6-phosphate dehydrogenase (G6P), NADP-dependent glutamate dehydrogenase (GD2), hexokinase (HEX), isocitrate dehydrogenase (IDH), indophenol oxidase (IPO), malate dehydrogenase (MDH), and phosphoglucomutase (PGM) were analyzed by electrophoresis in starch gels and selective staining as described (Selander et al. 1986). Electrophoretic types (ETs) were designed based upon the combined electrophoretic patterns of all ten enzymes. The ten ETs among the strains *from L. leucocephala* were defined in our previous work (Wang et al. 1998) and were included in order to compare the genetic relationships between them and the isolates from *S. herbacea*. Cluster analysis was carried out using the neighbor-joining method (Nei and Li 1979), and statistic analysis of linkage disequilibrium among the tested populations was done using a Monte Carlo procedure (Souza et al. 1992). Genetic diversity of the populations is presented as the arithmetic average (H) of the genetic diversity at each enzyme locus as estimated using the formula $h=[1-\Sigma x_i^2]\ [n/(n-1)]$ (Selander et al. 1986).

DNA-DNA hybridization and determination of DNA base composition

DNA (3 µg) was extracted with a DNA/RNA extraction kit (Amersham), restricted with *Eco*RI, and then used to estimate DNA-DNA relatedness by the filter hybridization method (Wang et al. 1998). The reactions were hybridized at 65 °C and the membranes were washed under high-stringency conditions (twice with 2×SSC/0.1% SDS at room temperature for 10 min; once with 0.1×SSC/0.1% SDS at 65 °C for 15 min). DNA G+C mol% was determined by the spectrophotometric method (De Ley 1970) using *Escherichia coli* K12 DNA as standard.

Sequence analysis of 16S rRNA genes

The almost-complete 16S rRNA genes were amplified by a PCR procedure using universal primers 25f (5′-AAC TKA AGA G TT TGA TCC TGG CTC-3′) and 1492r (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) (Hurek et al. 1997). The PCR products were sequenced directly using six cy5-labeled primers (Hurek et al. 1997). The 16S rRNA gene sequences obtained in this work were aligned together with those of related bacterial species using the PILEUP program in the Wisconsin package (Genetic Computer Group 1995). A phylogenetic tree was constructed and bootstrapped using the programs in the CLUSTALW 1.7 package (Thompson et al. 1994) and was visualized with the TreeView program (Page 1996).

Phenotypic characterization and numerical taxonomy

Basal medium supplemented with 0.01% of NH_4NO_3 (Wang et al. 1998) was used to test the utilization of carbohydrates, organic acids, alcohols, and amino acids as sole carbon source. Liquid basal medium supplied with 1% (w/v) mannitol was used to test the utilization of amino acids as sole nitrogen source. Liquid basal medium without yeast extract was used to test the requirement of growth factors by adding 1% (w/v) mannitol and 0.01% NH_4NO_3, or 1% (w/v) casein peptone as carbon and nitrogen sources. Resistance to antibiotics, tolerance to salt (NaCl), pH range, and maximum temperature for growth were determined on PY plates. Acid or alkaline reaction in litmus milk (Sigma) was recorded after a 4-week incubation. Growth in Luria-Bertani medium was tested in broth. Semisolid YMA medium (Berger 1961) in tubes covered with mineral oil was used for fermentation testing. Acid/alkaline production was verified in YMA with 0.0025% bromol trimethyl blue as pH indicator. Generation time was estimated in PY broth using a spectrophotometric method (Yelton et al. 1983). Growth of the bacteria was observed after incubation for 5–7 days at 28 °C for all experiments, except that for the maximum temperature for growth. The data were used in clustering analysis using the Ssm coefficient and UPGMA method (Sneath and Sokal 1973).

Cellular plasmid contents and identification of symbiotic plasmids

The cellular plasmid profiles were visualized using the method of Eckhardt (1978) as modified by Hynes and McGregor (1990). Molecular sizes were estimated from migration of the plasmid bands using the plasmids of *Rhizobium etli* CFN42 as standard (Wang et al. 1999b). The plasmids were transferred onto nylon membrane and hybridized with a PCR-amplified *nifH* gene fragment (Wang et al. 1998) and a cloned *nodDAB* gene probe (Wang et al. 1999a) using the methods described previously (Wang et al. 1999a).

Table 1 Isolates, strains, and plasmids used in this research and some relevant features. *ET* Electrophoretic type, *MLEE* multilocus enzyme electrophoresis, – not analyzed, *NO* not observed

Isolate or strain[a]	ET[b]	Plasmid (kb)[c]	Reference
Mesorhizobium strains from Mexico (group I in both MLEE and numerical taxonomy)			
Ls7, Ls8[d], Ls26, Ls30, Ls43, Ls44, Ls57, Ls64	1	NO[a]	Wang et al. (1999b)
Le45	1	330	Wang et al. (1999b)
Ls20	2	NO	Wang et al. (1999b)
Ls23	3	540	Wang et al. (1999b)
Ls24	3	440	Wang et al. (1999b)
Ls25	4	440	Wang et al. (1999bb)
Ls70	4	NO	Wang et al. (1999b)
Ls29	5	330, 220	Wang et al. (1999b)
Ls38	6	400	Wang et al. (1999b)
Ls45	7	440	Wang et al. (1999b)
Ls50	8	NO	Wang et al. (1999b)
Ls63	9	480	Wang et al. (1999b)
Ls69	10	NO	Wang et al. (1999b)
Ls32	10	90	Wang et al. (1999b)
Sn2, Sn7, Sn10, Sn11, Sn16, Sn24, Sn25	11	NO	Wang et al. (1998)
Sn3	11	250	Wang et al. (1998)
Sn20	11	600	Wang et al. (1998)
Sn26	11	290	Wang et al. (1998)
Sn5	12	NO	Wang et al. (1998)
Sn6, Sn8	13	NO	Wang et al. (1998)
Sn23	13	600	Wang et al. (1998)
Sn9	14	NO	Wang et al. (1998)
Sn18	15	NO	Wang et al. (1998)
Sn19	16	NO	Wang et al. (1998)
Sn21	17	NO	Wang et al. (1998)
CS2	11	NO	Wang and Martinez-Romero 2000
CS1	18	NO	Wang and Martinez-Romero 2000
CS4	19	460	Wang and Martinez-Romero 2000
CS7	20	500	Wang and Martinez-Romero 2000
CS13[d]	20	NO	Wang and Martinez-Romero 2000
CS9	21	360	Wang and Martinez-Romero 2000
CS12	22	240	Wang and Martinez-Romero 2000
CSF1	23	600	Wang and Martinez-Romero 2000
Reference strains			
Mesorhizobium plurifarium LMG11892^T	24	230	de Lajudie et al. 1998
LMG11883	25	NO	de Lajudie et al. 1998
USDA4413 (ORS1037)	26	NO	de Lajudie et al. 1998
LMG10056	27	NO	de Lajudie et al. 1998
LMG9970	28	NO	de Lajudie et al. 1998
LMG10093	–	NO	de Lajudie et al. 1998
Mesorhizobium amorphae ACCC19665^T	35	930	Wang et al. 1999a
Mesorhizobium ciceri USDA3378^T	29	NO	Nour et al. 1994
Mesorhizobium huakuii USDA4779^T	32	–	Chen et al. 1991
Mesorhizobium loti NZP2213^T	34	–	Jarvis et al. 1982
Mesorhizobium mediterraneum USDA3392^T	31	–	Nour et al. 1995
Mesorhizobium tianshanense USDA3592^T	30		Chen et al. 1995
Mesorhizobium sp. SH0174	33	NO	Tan et al. 1999
Mesorhizobium sp. SH2672	–	NO	Tan et al. 1999
SH1701	–	320	Tan et al. 1999
SH18611	–	320	Tan et al. 1999
Rhizobium etli CFN42^T	Its plasmids (630, 510, 390, 270, 150 kb) were used as standard of molecular size (Segovia et al. 1994)		
Escherichia coli K12	Standard for estimation of DNA G+C mol% (Tm)		
Plasmid pMR133	A 2.0-kb *Eco*RI–*Pst*I fragment containing *nodDAB* from symbiotic plasmid of *R. tropici* CFN299 cloned into pUC18 (Wang et al. 1999a)		

[a]Strains with prefix "Ls" or "Le" were from *Leucaena leucocephala* (Wang et al. 1999b) and strains with prefix "Sn" or "CS" were from *Sesbania herbacea* (Wang et al. 1998; Wang and Martínez-Romero 2000)

[b]ETs were designated according to combined electrophoretic patterns of 10 metabolic enzymes

[c]Molecular sizes of plasmids were estimated from Eckhardt gel using the plasmids of *R. etli* CFN42 as standards (Wang et al. 1998)

[d]These two strains were not included in the numerical taxonomy

Cross-nodulation

Standard methods (Berger 1961) were used for the surface sterilization, germination, and inoculation of seeds. Strains were incubated overnight in 5 ml PY broth at 28 °C with agitation, and an aliquot of 100 µl was inoculated onto each of the seeds. Strains Sn2 and CS1 from *S. herbacea* were inoculated onto germinated seeds of *L. leucocephala*. Strain Ls38 from *L. leucocephala* and *M. plurifarium* LMG11892 from *Acacia senegal* (de Lajudie et al. 1998) were inoculated onto germinated seeds of *S. herbacea*. Representatives of the Mexican strains and *M. plurifarium* were also inoculated onto bean seeds. Surface-sterilized seeds of *L. leucocephala* and *S. herbacea* without inoculation of the rhizobia were included as controls. Plants were grown in 250-ml flasks filled with 7 g cotton and N-free plant nutrient solution (Fahraeus 1957) under natural sunlight. The roots of the plants were kept under aseptic conditions by sealing the flask with cotton throughout the growing period.

Results

MLEE analysis

In this work, 13 ETs were identified among the 26 isolates from *S. herbacea*. These differed from both the ten ETs obtained from the isolates of *L. leucocephala* and from the 12 ETs of reference strains for the defined species (Fig. 1).

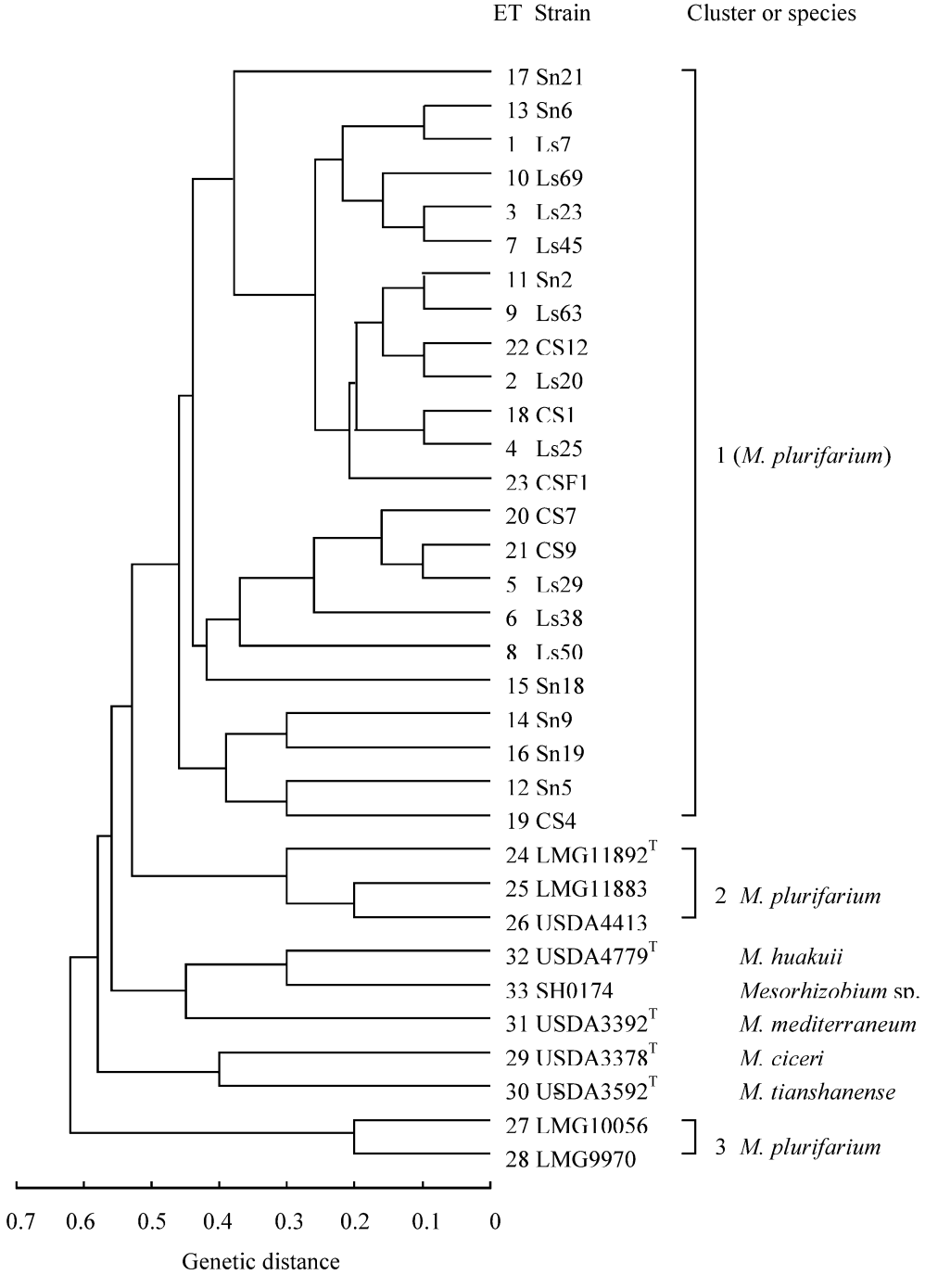

Fig. 1 Dendrogram showing genetic relationships among the Mexican strains from *Leucaena leucocephala* and *Sesbania herbacea*. This dendrogram was produced by cluster analysis using the neighbor-joining method (Nei and Li 1979) based upon MLEE data of 10 metabolic enzymes

Table 2 Genetic diversity of the *Mesorhizobium* strains from *L. leucocephala* and *S. herbacea*

Enzyme locus[a]	23 ETs from Mexican soils[b]		28 ETs including *M. plurifarium*[b]	
	Alleles	Diversity (*h*)[c]	Alleles	Diversity (*h*)[c]
IDH	5	0.524	6	0.556
MDH	1	0	3	0.331
PGM	2	0.165	2	0.199
GD2	3	0.517	3	0.449
ACO	2	0.087	3	0.204
HEX	4	0.591	4	0.620
G6P	4	0.588	4	0.597
IPO	3	0.609	3	0.660
ALD	2	0.087	2	0.072
EST	5	0.509	5	0.633
Average	3.1	0.374	3.7	0.432
Vo/Ve	1.1194[d]		1.1317[d]	

[a]See Materials and methods for the abbreviations
[b]The 23 ETs refer to the strains from Mexican soils and the 28 ETs include the 23 ETs of the Mexican strains and the 5 ETs of the reference strains for *M. plurifarium*
[c]Diversity was calculated using the formula $h=[1-\Sigma x_i^2][n/(n-1)]$ (Selander et al. 1985)
[d]Not significantly different from 1.0, indicating a linkage equilibrium as described previously (Souza et al. 1992)

Except for the enzyme MDH, polymorphism with two to five electrophoretic alleles was found for all ten enzymes analyzed in the Mexican strains (Table 2). The genetic diversity (*H*) was 0.374 when the 23 ETs from Mexico were considered and was slightly higher (0.432) when the five reference strains for *M. plurifarium* were considered together with the 23 ETs from Mexican soils (Table 2). These values of diversity were lower than those reported for other *Rhizobium* species, 0.660 in the case of a *R. leguminosarum* bv. viceae population and 0.487 in the case of a *R. etli* population (Martínez-Romero and Caballero-Mellado 1996). In a cluster analysis, all 23 ETs from Mexico were clustered together at the genetic distance of 0.48 (Fig. 1). Isolates from both host plants were intermingled in this cluster, which was further linked to three reference strains of *M. plurifarium* from *Acacia senegal* grown in Senegal (cluster II) at a genetic distance of 0.52. Another two strains of *M. plurifarium* from *Chamaecrista ensiformiiis* and *Leucaena diversifolia* grown in Brazil formed cluster III, which had a genetic distance 0.62 with the strains in clusters I and II. Linkage equilibrium was detected between the 23 ETs from Mexico and the five ETs of the *M. plurifarium* reference strains. Linkage disequilibrium was observed when all the reference strains for other defined *Mesorhizobium* species were included.

DNA-DNA hybridization and DNA nucleotide composition

The degree of DNA relatedness between CS7 and CS13, two isolates of ET20,was 100%. Based upon this finding and previous data (Wang et al. 1998), we considered that strains of the same ET belonged to the same genomic group; thus, hybridizations between different ETs were carried out. Using 70% DNA relatedness as the specific border as suggested (Brenner et al. 2001; Graham et al. 1991; Wayne et al. 1987), genomic groups were defined as shown in Table 3. Eight ETs represented by strains LS7 and Sn2 had DNA relatedness 87–100% and were classified as genomic group I. Seven other ETs represented by Ls38 formed genomic group II, in which the DNA-DNA relatedness was 69.3–87.9%. The remaining eight ETs could not be included in any genomic group due to their low degree of DNA-DNA relatedness (30–60%) with the reference strains Sn2, Ls38, and Ls29. The DNA-DNA relatedness between the reference strain Sn2 and *M. plurifarium* LMG9970 and LMG10056 was about 88%. The DNA relatedness between the other reference strains for *M. plurifarium* and strains Ls38 and Ls29 varied from 18 to 61%. The DNA relatedness between strains Sn2, Ls38, and Ls29 and the type strains for other *Mesorhizobium* species was 8.5–44.7% (Table 3). The DNA G+C mol% (T_m) of the representative strains Sn2, LS7, Ls29, and Ls38 was 63.2, 62.8, 63.2, and 63.5, respectively; these values were within the range of *Mesorhizobium* (59–65%) (Table 4).

Sequencing analysis of 16S rRNA

The nucleotide sequences of the 16S rRNA genes from strains Ls29, Ls38 ad Sn2 have been deposited in GenBank under the accession numbers AF516881, AF516882, and AF516883, respectively. Strains Sn2, Ls38, and Ls29, representatives of genomic groups I, II, and the variegated isolates of Mexican *Mesorhizobium* respectively, were grouped into the *Mesorhizobium* cluster and were most related to *M. plurifarium* in the phylogenetic tree (Fig. 2). The sequence identity between them and the *Mesorhizobium* species ranged from 97 to 99%.

Phenotypic characterization and numerical taxonomy

In this study, 96 unit features were analyzed, 60 of which were variable among the 67 strains, including reference strains for the defined species. In the clustering analysis (Fig. 3), the Mexican strains formed a single cluster (cluster I) at a similarity level of 82%, while the reference strains for the *Mesorhizobium* species formed separate clusters similar to those reported previously (de Lajudie et al. 1998; Tan et al. 1999). Five *M. plurifarium* strains formed a cluster at a similarity of 87% that was further linked to rDNA type I strains CS3, Ls8, and another *M. plurifarium* strain LMG9970. These results indicated that the Mexican strains from both plants had similar phenotypic features, but with minor differences among them. Some of the distinctive features are summarized in Table 4, and some important features of the Mexican strains are presented here.

Cells of the Mexican strains are 1.2–4 μm in length and 0.5–1.5 μm in width, depending on the strains. The gener-

Table 3 DNA-DNA relatedness among the *Mesorhizobium* strains from Mexico estimated by filter-hybridization under stringent conditions at 65 °C (presented as mean±standard error from duplicate hybridization). *T* Type strain

Strain	DNA relatedness (% ±SD) with strain			
	Ls7	Sn2	Ls38	Ls29
DNA group I				
Ls7 (ET1)	100	87.6±12.4		
Ls63 (ET9)	100±0			
Sn2 (ET11)	100±0	100		
Sn19 (ET16)	91.6±1.5			
Sn21 (ET17)		97.8±2.2		
CS1 (ET18)		88.1±11.9		
CS4 (ET19)		84.6±15.4		
CSF1 (ET23)		100±0		
DNA group II				
Ls23 (ET3)	30.6±0.4		69.3±4.1	
Ls25 (ET4)	47.2±0.2		87.9±6.7	
Ls38 (ET6)	31.4±1.6	41.2±3.0	100	
Ls45 (ET7)	44.9±1.9		82.7±0.7	
Ls69 (ET10)	27.4±3.0		73.5±2.0	
Sn6 (ET13)	33.0±6.6		76.1±13.4	
CS12 (ET22)	55.0±8.2		79.3±3.1	
Other Mexican strains				
Ls20 (ET2)	45.5±0.2		46.5±9.6	30.3±5.6
Ls29 (ET5)	29.4±5.0	23.0±1.7	27.4±7.4	100
Ls50 (ET8)	15.6±2.5	42.1±8.3	26.2±6.2	45.6±0.7
Sn5 (ET12)	17.4±0.2		18.4±0.3	58.6±2.6
Sn9 (ET14)	15.4±0.4		25.7±2.0	46.6±4.1
Sn18 (ET15)	14.8±0.8		23.5±3.5	60.8±1.2
CS7 (ET20)		26.9±4.5	29.0±4.8	34.0±5.7
CS9 (ET21)	12.1±1.0		21.8±3.6	42.4±4.3
Reference strains				
M. plurifarium LMG11892^T	29.6±1.8	26.8±1.6	18.0±2.6	12.6±1.2
LMG11883		61.0±4.2	29.1±4.3	21.5±1.9
LMG11893		56.9		
LMG9970		88.4±7.4		
LMG10056		87.2±1.0		
M. amorphae ACCC19665^T			25.4±3.8	15.3±1.4
M. tianshanense USDA3592^T	12.0±1.1	30.3	16.6±2.4	13.3±1.2
M. loti NZP2213^T	31.4±2.2		27.2±4.0	16.4±1.5
M. ciceri UDSA3378^T	44.7±3.9		14.2±2.1	9.0±0.8
M. mediterraneum USDA3392^T	23.6±0.6		13.8±2.0	11.8±1.0
M. huakuii USDA4779^T	36.8±4.3		18.0±2.6	8.5±0.8

ation times of strains Ls7, Ls29, Ls38, Ls50, Ls63, Sn2, Sn21, CS1, CS4, and CSF1 are 4, 5, 7, 4.3, 4.3, 6.5, 4, 5.3, 8 and 4 h, respectively, similar to those of other mesorhizobia, as indicated in Table 4. All of the Mexican strains can use D-galactose, fumarate, malate, pyruvate, L-phenylalanine, and L-cystidine as sole carbon source, and L-phenylalanine and L-cystidine as sole nitrogen source but they cannot use methanol or gluconate as carbon source. All strains are sensitive to 300 µg kanamycin ml^{-1} , 5 µg tetracycline ml^{-1} , and 50 µg carbenicillin ml^{-1} , but resistant to 100 µg erythromycin ml^{-1}. No bacterial growth was obtained in LB or in PY medium supplied with 1.5% NaCl. The pH range for growth is from 4.5 to 8.0 and varies from strain to strain. None of these strains grows at 37 °C.

Cellular plasmid contents and identification of symbiotic plasmid

In this work, no common plasmids were found between the Mexican strains and the *M. plurifarium* strains (Table 1). No hybridization with the *nifH* or *nodDAB* probe*s* was observed on plasmids, indicating that these bacteria have their symbiotic genes on chromosome.

Cross-nodulation

Sn2 and CS1, representing the strains from *S. herbacea*, formed root nodules on all nine seedlings of *L. leucocephala*. Two of the six *S. herbacea* seedlings were nodulated by strain Ls38 (from *L. leucocephala*). The nodules on both

Table 4 Distinctive features of the *Mesorhizobium* strains from Mexico and the defined *Mesorhizobium* species. *ND* Not reported or not done; *NO* not observed, *d* varied

Characteristic	Mexican strains	*M. plurifarium*	*M. amorphae*	*M. chacoense*[a]	*M. ciceri*	*M. huakuii*	*M. loti*	*M. mediterraneum*	*M. tianshanense*
Maximum temperature for growth (°C)	35.5	42	35.5	37	40	37–39	39	40	ND
Maximum NaCl conc. for growth (%, w/v)	≤1.0	2.0	≤1.0	ND	2.0	1.0	2.0	2.0	1.0
pH range for growth	4.5–8.0	4.5–9.0	5.0–8.5	ND	5.0–10	5.0–9.5	4.0, <10.0	5.0, <10.0	5.0–8.5
DNA G+C mol% (T_m)	63	63–64	64	62	63–64	59–64	59–64	63–65	59–63
Growth in LB medium	–	+	–	ND	–	–	–	–	–
Colony size[b] (mm, days of incubation)	0.5–2.0, 3 d	0.5–2.0, 2–3 d	1.0, 7 d	1.0–3.0, 7 d	2.0–4.0, 3–5 d	2.0–4.0, 5–6 d	1.0, 7 d	>2.0, 4–5 d	1.0–2.0, 5–7 d
Generation time (h)	4–7	6–13	6–13	10–24	ND	4–6	ND	ND	5–15
16S rDNA PCR-RFLP patterns[c]	FFFF	FFFF	FHFF	ND	GGFF	FHGF	GGFF	HGGF	HIFF
Symbiotic plasmid	NO	NO	930 kpb	NO	NO	117–251 MD[d]	NO	NO	NO
Natural host (genus or species)	*Leucaena leucocephala*	*Acacia*, *Leucaena*, *Prosopis*	*Amorpha fruticosa*	*Prosopis*	*Cicer*	*Astragalus sinicus*	*Lotus*	*Cicer*	*Glycine max*, *Glycyrrhiza*, *Sophora*

[a]Data from Velázquez et al. (2002)
[b]On YMA for *M. chacoense* and on PY for rest species
[c]Four letters were designed for each strain to present the RFLP patterns correcting the digestion of *Msp*I, *Hinf*I, *Hha*I and *Msp*I. Different letters indicate different patterns
[d]Data from Guo et al. (1999)

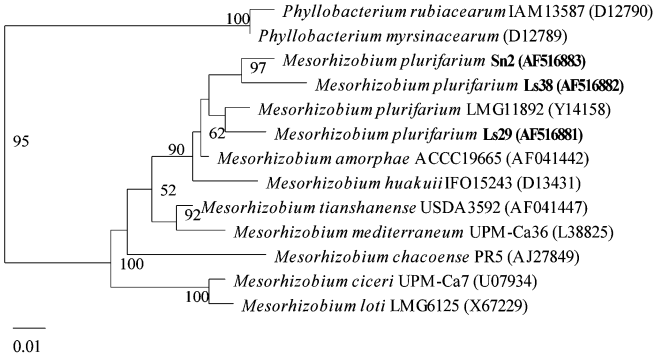

Fig. 2 Simplified phylogenetic tree of 16S rRNA genes showing the relationships among the tested *Mesorhizobium* strains and related species. The GenBank accession numbers are given in *parentheses*. Bootstrap values greater than 50% are presented at the branch points. *Scale bar* 0.1% nucleotide substitutions

plants were of normal size and pink inside, indicating that effective symbiosis had been established. *M. plurifarium* LMG11892^T did not nodulate *S. herbacea*. On the bean plants, no nodules were formed with *M. plurifarium* LMG11883 while a number of nodules were formed with strains Sn2, Ls7, Ls38, Ls29, *M. plurifarium* LMG11892^T, and LMG9970. However, the nodulated plants grew as poorly as the non-inoculated controls. The nodule sizes varied from 1 to 4 mm in diameter. Some of them were pink but most were white or green, indicating that they were ineffective. The re-isolation results of five to ten nodules from each inoculation confirmed that the nodules were formed by *Mesorhizobium* strains.

Discussion

In this work, the relationships among the Mexican strains of *Mesorhizobium* obtained from the two host plants *L. leucocephala* and *S. herbacea* were investigated using a polyphasic approach (Gillis et al. 2001) that included genetic, phylogenetic, and phenotypic characterizations. The Mexican strains from both plants had identical PCR-based RFLP patterns, as reported previously (Wang and Martínez-Romero 2000), and high sequence similarities (around 99%) of 16S rRNA genes according to sequence analysis (Fig. 2). The strains formed a single cluster in MLEE analysis (Fig. 1) at a genetic distance of 0.48. The linkage equilibrium detected among these strains (Table 2) indicated that they were from the same gene pool (Selander et al. 1986). In numerical taxonomy, most of the strains formed a single cluster (Fig. 3), with a similarity level of 80%. Although different genomic groups were defined among the Mexican strains in the DNA-DNA hybridization (Table 3), the existence of mediate DNA-DNA relatedness from 40 to 61% among the genomic groups indicated that they were a continuum population. According to polyphasic taxonomy (Gillis et al. 2001), taxonomic conclusions should be made based upon a comprehensive analysis of all the valuable data. Thus, DNA-DNA relatedness is not a determinative value for the definition of species. Also, it has been indicated that 70% DNA relatedness cannot be used as a universal criterion for bacterial speciation (Gillis et al. 2001; Ward 1998). With a comprehensive consideration of all the grouping results obtained in this work along with previous PCR-RFLP analysis (Wang et al. 1999b; Wang and Martínez-Romero 2000), we conclude that the Mexican strains from both plants form a single species.

The taxonomic position of the Mexican strains was clarified by comparing these strains with the reference strains for related species. The 16S rRNA gene phylogeny (Fig. 2) confirmed that the Mexican strains belonged to *Mesorhizobium* and were most related to *M. plurifarium*, as observed in the PCR-RFLP of the 16S rRNA genes (Wang et al. 1999b; Wang and Martínez-Romero 2000). Although they grouped into different clusters in MLEE analysis (Fig. 2), the genetic distance of 0.52 and the linkage equilibrium detected among the Mexican strains and some *M. purifarium* reference strains indicated that the strains were closely related genetically and that they were from the same gene pool. In the DNA-DNA hybridization, the relatedness between Mexican strains and the *M. plurifarium* reference strains was slightly to greatly higher than that with other *Mesorhizobium* species (Table 3). Although the Mexican strains and the *M. plurifarium* reference strains divided into two clusters in numerical taxonomy, two Mexican strains, CS3 and Ls8, were rather similar to the *M. plurifarium* reference strains (Fig. 3). Furthermore, the three distinctive features among the Mexican strains and the *M. plurifarium* reference strains were mainly those related to their geographic origins: growth at 37 °C and resistance to pH 9.0 and 2.0% (w/v) NaCl (Table 4). Thus, the Mexican strains and the *M. plurifarium* reference strains might be considered as different ecological types within the same species.

Based upon the comparative analyses between the Mexican strains and the reference strains for the defined *Mesorhizobium* species, and using a polyphasic taxonomy approach, the Mexican strains were identified as *M. plurifarium*. In reaching this conclusion, it was also considered that *M. plurifarium* included diverse groups in both the protein analysis and the DNA-DNA hybridizations (de Lajudie et al. 1998). This identification allowed *M. plurifarium* species to be easily differentiated from other *Mesorhizobium* species based on PCR-based RFLP analysis of 16S rRNA genes digested with *Msp*I, *Hinf*I, *Hha*I, and *Sau*3AI, as well as some other distinctive features (Table 4). It was also shown that the *M. plurifarium* strains have no symbiotic plasmids and harbor symbiotic genes in the chromosome. This characteristic could differentiate *M. plurifarium* from the symbiotic plasmid-harboring species *M. huakuii* (Guo et al. 1999) and *M. amorphae* (Wang et al. 1999a) (Table 4).

In this work, some discrepancies were observed among the grouping results obtained using different approaches, such MLEE (Fig. 1), numerical taxonomy of phenotypic features (Fig. 2), and DNA-DNA hybridization (Table 3). A similar situation was reported in other work (de Lajudie et al. 1998; Yan et al. 2000), thus demonstrating the ne-

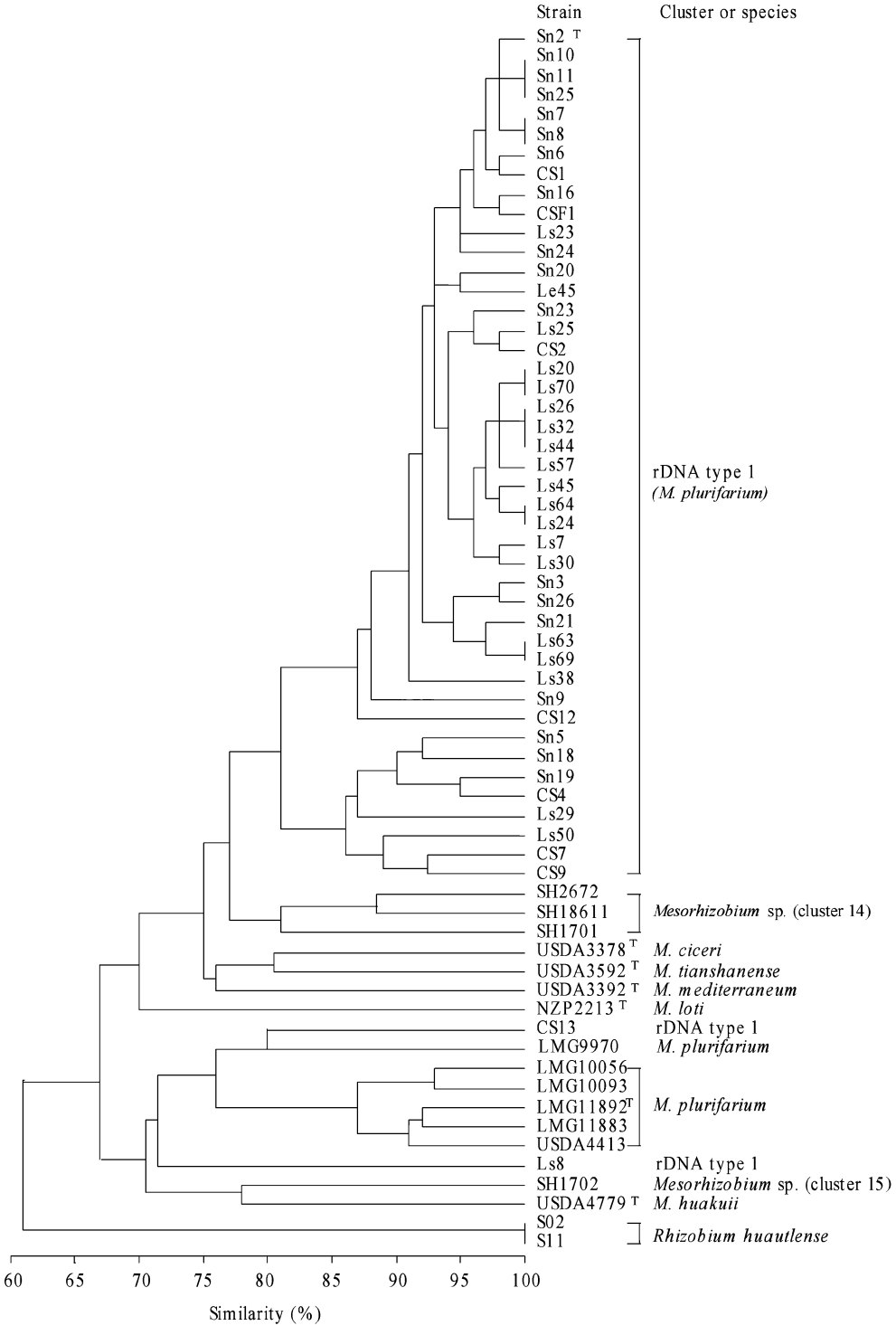

Fig. 3 Dendrogram showing the phenotypic similarities among the Mexican mesorhizobia and the reference strains for the defined *Mesorhizobium* species. The Ssm coefficient and UPGMA method (Sneath and Sokal 1973) were used for the cluster analysis

cessity of a polyphasic approach in bacterial taxonomy. Also, based upon our results, it is clear that the type strain of *M. plurifarium* does not represent all strains in this species, and reference strains representing diverse subgroups within a species should be used in taxonomic studies.

It seems that the *M. plurifarium* strains were native to Mexico based upon the fact that Mexico was the original center of *L. leucocephala* and there was no history of rhizobial inoculation in the field where the strains were obtained. Since the original center of a legume plant is also considered to be the center for the divergent evolution of rhizobia associated with that plant (Martínez-Romero and Caballero-Mellado 1996), *M. plurifarium* might also have originated in Mexico in association with *L. leucocephala*.

Although the Mexican strains were identified as *M. plurifarium*, diversity was detected among them. As shown in Table 3, the DNA-DNA relatedness varied from 12.1 to 100% and some mediate values were obtained. These data

might be evidence that the Mexican strains were native to where they were isolated because it has been reported that the long-established naturalized populations of various rhizobial species are highly diverse (Piñero et al. 1988; Young 1985; Young et al. 1987). The existence of genetically diverse groups may demonstrate that within the local *M. plurifarium* populations divergent evolution was taking place. This could have proceeded in two ways: (1) accumulation of gene mutations and lateral gene transfer (Dröge et al. 1999). In the laboratory, lateral gene transfer has been evidenced by transconjugation, as was the case for a symbiotic plasmid in fast-growing rhizobia (Rogel et al. 2001) and by comparative sequencing of related genes in the field, as revealed in *Mesorhizobium* (Sullivan et al. 1995) and in some other rhizobia (Young and Wexler 1988). The Mexican strains we characterized might be an example of divergent evolution by the accumulation of mutations, but the frequent gene exchange within the populations, as revealed by the linkage equilibrium in MLEE analysis, maintained them in the same gene pool or the same species. Our results also offer evidence that bacterial evolution was a continuum, as mentioned previously (Brenner et al. 2001; de Lajudie et al. 1998).

Due to their symbiotic feature, the evolution of rhizobia is associated with that of their host plants and their habitats. Both the plant genotype and environmental factors could affect the population structure and evolution of certain rhizobial species. The Mexican strains used in this work were isolated from an uncultivated field in Cuernavaca, which has an altitude of 1,100 m above sea level and a climate with almost constant temperature, similar to that of spring in temperate regions. The field has wet brown-sandy loam soils, pH 6.9, and is well-drained (Wang and Martínez-Romero 2000). Under these conditions, long-established rhizobia might have developed some features related to their habit, such as their lack of resistance to high temperature and their sensitivity to salty-alkaline conditions (Table 4). MLEE analysis also reflected the effects of environmental factors on genetic evolution since the three MLEE clusters of *M. plurifarium* strains were grouped exactly according to their geographic origins (Fig. 2).

The effects of plant genotype on the population structure and distribution of the rhizobial strains may be estimated from their affinities with different rhizobial types. The fact that different ETs were isolated from the two plant species under natural conditions might indicate the existence of different affinities between the plants and the rhizobial ETs (Table 1, Fig. 1). By contrast, cross-nodulations were obtained among the rhizobia from both plants under laboratory conditions. These results confirmed that *L. leucocephala* and *S. herbacea* belong to the same cross-nodulation group, as has been reported (Trinick 1980), but the host plants prefer to nodulate with some ETs rather than with others in the same species or in mixed populations. Also, the *M. plurifarium* strains formed nodules on common bean plants in laboratory but no *Mesorhizobium* strain was isolated from bean plants grown in the field where the strains were obtained (Caballero-Mellado and Martínez-Romero 1999). Considering these results and the fact that nodulation of mesorhizobia on *S. herbacea* was completely eliminated in the presence of *R. huautlense* (Wang and Martínez-Romero 2000), we conclud that the mesorhizobia were not natural microsymbionts for *Phaseolus vulgaris* and *S. herbacea*. These results remind us that the natural association among rhizobia and their host plants is more important than nodulation under controlled conditions.

In conclusion, we report the existence of *M. plurifarium* strains in Mexican soils as native microsymbionts for *L. leucocephala*. In addition, the strains may form nodules on *P. vulgaris* and *S. herbacea* under artificial conditions. These strains formed genetically diverse populations in the soil and may have developed some features allowing them to adapt to their native habitat.

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