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Genetic diversity of rhizobia from *Leucaena leucocephala* nodules in Mexican soils

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Abstract

***Leucaena* species are leguminous plants native to Mexico. Using two *L. leucocephala* cultivars grown in different soils, we obtained 150 isolates from the nodules. Twelve rDNA types were identified which clustered into groups corresponding to *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* by restriction fragment length polymorphism (RFLP) of amplified 16S rRNA genes. Types 2, 4, 5, 6, 10, 11, and 12 were distinct from all the defined species. Others had patterns indistinguishable from some recognized species. Most of the isolates corresponded to *Sinorhizobium*. Forty-one electrophoretic types (ETs) were identified among the isolates based on the different combinations of electrophoretic patterns of 13 metabolic enzymes. ETs were clustered into groups in general agreement with the rDNA types. Diverse plasmid patterns were obtained among the isolates, but common plasmids were observed among most isolates within rDNA types 5, 10, and 11. The symbiotic plasmids were identified among most of the isolates, except for the *Mesorhizobium* isolates. The affinities of host cultivars for different rhizobial groups and the impact of soil cultivation on the soil populations of rhizobia were analysed from the estimation of isolation frequencies and diversity. The results showed differences in rhizobial populations in cultivated and uncultivated soils and also differences in rhizobia trapped by *L. leucocephala* cv. Cunningham or Peruvian.**

Keywords: 16S rDNA fingerprinting, diversity, *L. leucocephala*, MLEE, plasmid patterns, *Rhizobium*

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Introduction

Leucaena leucocephala, with varieties from shrubs to trees, is a fast-growing legume plant belonging to the subfamily Mimosoideae. This species is native to North America and has been used for reforestation both in the Americas and other areas (Allen & Allen 1981; Martínez-Romero *et al*. 1997). The leaves of the plant are high in protein (C/N ratio 9.80) and are used as forage (Sanginga *et al*. 1995). It forms nodules with *Rhizobium* spp. and obtains 59–100% of its nitrogen from the nitrogen-fixing symbiosis. The level of N_2 fixation in this plant is similar to or higher than that of crop legumes such as soybean and peanut (200–300 kg N/ha/crop), and most of the fixed nitrogen can be returned to the soil in the leaf litter (Peoples *et al*. 1995a). A survey by NifTAL of 305 soil samples from 17 countries indicated that 51% of the samples had no microsymbionts associated with *L. leucocephala* and the others varied from 1 to more than 1000 cells/gram of soil (Peoples *et al*. 1995b). Another report showed that *L. leucocephala* had a significant response to inoculation in more than 38% of the cases (Peoples *et al*. 1995b). However, the inoculation responses varied according to the inoculant strains, geographical areas, and host cultivars (Peoples *et al*. 1995b; Sanginga *et al*. 1995). *L. leucocephala* was considered to have a narrow symbiotic range because it was nodulated only by fast-growing strains within *Rhizobium* (Sanginga *et al*. 1995). It forms a cross-nodulation group with species in the genera *Mimosa, Acacia, Sesbania, Vigna, Lablab, Phaseolus* and some others (Trinick 1980). Rhizobia associating with this plant have been isolated from South America, Asia, Africa, and Australia. Some of these isolates have been classified as belonging to the species *Rhizobium tropici* (Martínez-Romero *et al*. 1991) and *Mesorhizobium plurifarium* (De Lajudie *et al*. 1998), while others remain unnamed (Trinick 1980; Jarvis 1983; Martínez *et al*. 1985; Wedlock & Jarvis 1986; Zhang *et al*. 1991; Oyaizu *et al*. 1993; De Lajudie *et al*. 1994; Gao *et al*. 1994;

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Kuykendall *et al*. 1996). Strains originally isolated from other host species can also nodulate *L. leucocephala,* for example *R. gallicum and R. giardinii* isolated from common bean (Amarger *et al*. 1997), *R. huautlense* from *Sesbania herbacea* (Wang *et al*. 1998), and *R. etli* bv. mimosae from *Mimosa affinis* (unpublished data).

L. leucocephala is cultivated in Mexico (Martínez-Romero *et al*. 1997), as well as in Hawaii and the Phillipines. The diversity of rhizobial populations from *L. leucocephala* growing in Mexico, where the plant is native, has not been described.

L. leucocephala was suggested as a selective host when defining the host range of new rhizobial species (Graham *et al*. 1991). It was also a host to distinguish *R. tropici* from *R. etli* as it was not nodulated by the latter (Martínez-Romero *et al*. 1991; Segovia *et al*. 1993). However, one *R. etli* strain was found to nodulate *L. leucocephala* cv. Cunningham (Hernández-Lucas *et al*. 1995). Consequently, the aims of this research were to determine if *R. etli* strains were natural symbionts of *L. leucocephala* and to describe the diversity of rhizobia nodulating *L. leucocephala* in Mexican soils. To achieve this, we isolated and characterized 150 isolates trapped by *L. leucocephala* cv. Cunningham and cv. Peruvian grown in soils planted previously with bean which we presumed to be rich in *R. etli* strains, and in soils not previously used for cultivation.

Materials and methods

Plant trapping tests and isolation of nodules

Seeds of *Leucaena leucocephala* cv. Cunningham and cv. Peruvian were surface sterilized in concentrated H_2SO_4 for 20 min and germinated in water-agar (0.7%) at 28 °C (Vincent 1970). They were then grown in pots filled with soils from Cuernavaca, Morelos, Mexico from which common bean plants had been harvested and the *Rhizobium etli* populations from bean nodules were analysed (Caballero-Mellado & Martínez-Romero 1998). Uncultivated soils from the same site were also used for the Peruvian cultivar. This location has not been used for cropping for the last 15 years. More than 10 species of wild legume plants, such as *Trifolium* sp., *Medicago* sp., *Mimosa* spp., *Aeschynomene* spp., *Crotalaria* spp., *Dalea* spp., *Desmodium* spp., *Macroptilium atropurpureum*, and *L. esculenta* grow in this area. In each treatment, 50 seeds were placed in five pots and grown in an open field. The nodules were collected and isolated after 2 months (from May to July 1996) using standard methods (Vincent 1970) and PY medium (5 g of peptone of casein, 3 g of yeast extract, 0.6 g of $CaCl_2$, 1 L of water). Isolates were purified by streaking twice on PY plates and single colonies were picked for further study. Cultures were grown at 28 °C and maintained in 20% glycerol at – 20 °C. The nodulation ability of each isolate was confirmed by inoculating the original host (cv. Peruvian) as described previously (Wang *et al*. 1998). The isolates and reference strains used are listed in Table 1.

Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis of 16S rRNA genes

The methods of van Berkum *et al*. (1996) were used for DNA extraction and PCR amplification of almost full length 16S rRNA genes (rDNA) from the isolates using primers fD1 and rD1 (Weisburg *et al*. 1991) corresponding to positions 8–27 and 1524–1540 of *Escherichia coli* 16S rDNA. Subsamples (10–15 μL) of the PCR-amplified 16S rDNAs were individually digested with the restriction nucleases *Msp*I, *Hin*fI, *Hha*I and *Sau*3AI, as described previously (Wang *et al*. 1998). RFLPs of the 16S rDNAs were analysed by horizontal electrophoresis in 3% (w/v) agarose gels (Wang *et al*. 1998) and the fragments were numbered from the gels. The new isolates were grouped according to their RFLP patterns into rDNA types and compared with those of the type strains of related species. Similarity of each strain pair was calculated using the formula: $Sj = 2a/(2a + b + c)$ (Sneath & Sokal 1973), where a is the number of fragments shared by both strains; b and c are the numbers of dissimilar fragments occurring in each one. A dendrogram was constructed by cluster analysis of RFLP data using the UPGMA method of Nei & Li (1979). The isolation frequency of each rDNA type was calculated for different host cultivars and different soils. To determine if the frequencies of isolates from various rDNA types were significantly different between two host cultivars and two soil types, contingency table tests were performed. Data were grouped according to Cochran's criteria to satisfy the conditions required for the test (Cochran 1954).

Multilocus enzyme electrophoresis (MLEE)

The reported methods were used for growing bacteria in 40 mL PY broth and for protein extraction (Caballero-Mellado & Martínez-Romero 1994). Starch gel electrophoresis and selective staining of the enzymes were performed as described by Selander *et al*. (1986). The following 13 metabolic enzymes were analysed: aconitase (ACO), adenylate kinase (ADK), alanine dehydrogenase (ALD), esterase (EST), glucose-6-phosphate dehydrogenase (G6P), hexokinase (HEX), indophenol oxidase (IPO), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), malic enzyme (ME), phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), and xanthine dehydrogenase (XHD). Different bands, or alleles, at the corresponding structural gene locus of each enzyme were numbered by order of migration distance.

Table 1 Isolates, strains, and their relevant characteristics

ET*	Isolate or strain†	16S rDNA RFLP pattern‡	Plasmid§	
			Size (kb)	pSym
	rDNA type 1	FFFF		
1	Ls16		540	
	Ls32		90	
	Ls69		NO	
2	Ls50		NO	
3	Ls38		400	
4	Ls63		ND	
5	Ls23		540	
	Ls24		440	
6	Ls25		440	
	Ls70		NO	
7	Ls45		440	
8	Ls7,Ls8,Ls26,Ls30,Ls43,Ls44, Ls57,Ls64		NO	
	Le45		330	
9	Ls20		NO	
10	Ls29		330,220	
	rDNA type 2	EAAA		
11	Ls22		530,210	
	rDNA type 3	DDAB		
16	Lc09,**Lc14,Lc15**,Lc35,Lc36		670	670
17	Le33		NO	
18	Le48		> 1000, 670, 440	670
19	Ls41		570, 440	570
	rDNA type 4	EDEA		
20	Le109		670, 270	670
28	Ls37		ND	
	rDNA type 5	ADEA		
21	Le16		NO	
22	Le10		>1000, 630, 440	630
	Le14,Le15		630	
23	Le40		630,260	
	Le43		630, 300	
	Le49		630, 60	
	Le50		630	
24	Le25,Le26		630, 400	
25	Le20		>1000, 800, 730, 300	730
26	Lc28,Lc05, Le35, Le38, Ls10, Ls37, Ls46, Ls48		ND	
	Lc01,Lc02,Le41,Ls49,Ls53		>1000¶, 630, < 5	630
	Lc03,Le18		>1000¶, 630, 260	
	Lc06,Le2,Le23,Le27,Le28,Le106		>1000¶, 630, 50	630
	Le5,Le11,Le24,**Le29**,Le38		>1000¶, 630, 60	630
	Lc17,Le12,Le31,Le34		>1000¶, 630, 440, 190	630
	Le1,Le13,Le46		>1000¶, 630	630
	Le7,Le22,Le30,Le54,Le57,Le58, Le111,Ls1,Ls11,Ls54,Ls58		NO	
	Le8,Le9,Le44		>1000¶, 630, 260, 50	630
	Le17		>1000, 630, 400, 260	630
	Le42		630, 150, 100	
	Le47		800, 630	
	Le51,Le60		630, < 5	
	Le55,Le114		630, 190	
	Le56		630, 150	
	Le59		630, 260, < 5	

Table 1 *Continued*

			Plasmid§	
ET*	Isolate or strain†	16S rDNA RFLP pattern‡	Size (kb)	pSym
27	Lc24,Lc42,Lc36a,Le19		630, 50	
	Lc56		NO	
29	Le3		>1000, 630, 320	630
30	Le53		NO	
31	Le36		NO	
	Le37		>1000, 630	630
32	Ls3,Ls6		630, 260	
	rDNA type 6	DDEF		
33	Le101,Le102,Ls33		NO	
	Ls9		490	490
34	Le39		770, 450	770
	rDNA type 7	DDAF		
35	Ls14		>1000, 570, 350, 80	670
	rDNA type 8	CBAD		
12	Lc52		>1000	
	rDNA type 9	ACBH		
13	Ls31		>1000	
14	Le52		770, 630	630
15	Ls27		NO	
	rDNA type 10	CBEC		
36	Lc51		850, 440	
37	Lc37		850, 440, 70	440
	Ls42,Ls52		850, 440	
38	Lc41		850, 570	570
	Lc50		NO	
	Lc54,Le6,Ls4,Ls13		850, 440	
	rDNA type 11	AAAA		
39	Lc04,Lc18,Lc19,Lc21,Lc29, Lc44, Lc56a		>1000¶, 670, 540	
40	Lc57		670, 70	670
	rDNA type 12	AAAB		
41	Ls55		>1000, 310, 270	
	Ls18		740	
Unclassified strains				
38	Cli80, Leu2, Dal4	CBEC	960,440	440
Reference strains				
Agrobacterium rhizogenes IAM13570^T		JCBH		
Ag. rubi IAM13569^T		IBAF		
Ag. tumefaciens IAM13129^T		IBAF		
Ag. vitis IAM14140^T		IBAG		
Bradyrhizobium elkanii USDA76^T		LLDJ		
B. japonicum USDA6^T, USDA110		KKCJ		
Bradyrhizobium sp. SH283012		MMHJ		
Mesorhizobium amorphae ACCC19665^T		FHFF		
M. ciceri UPM-Ca7^T		GGFF		
M. huakuii CCBAU2609^T		FHGF		
M. loti NZP2213^T		GGFF		
M. mediterraneum UPM-Ca36^T		HGGF		
M. tianshanense A-1BS^T		HIFF		
M. plurifarium ORS1037		FFFF		
Rhizobium etli CFN42^T		CJAC	630, 510, 390, 270, 150	
R. galegae HAMBI540^T		ICAF		
R. gallicum R602sp^T		CBAD		
	FL27	CBAD	>1000, 550, 380	

Table 1 *Continued*

ET*	Isolate or strain†	16S rDNA RFLP pattern‡	Plasmid§ Size (kb)	Plasmid§ pSym
	R. giardinii H152^T	AAAI		
	R. hainanense I66^T, I12	ACBH		
	R. huautlense S02^T	ICAF		
	R. leguminosarum USDA2370^T	CCAH		
	R. mongolense USDA1844^T	CBAD		
	R. tropici CIAT899^T	ACBH		
	CFN299	BEBC	>1000, 410, 225, 185	
	Sinorhizobium fredii USDA205^T	EDAA		
	S.meliloti USDA1002^T	ADAA		
	1021	ND	1700, 1340	
	S. medicae A321^T	ADAA		
	S. saheli ORS609^T	DDAB		
	S. terangae ORS1009^T	DDAF		

*Electrophoretic type determined by multilocus enzyme electrophoresis.
Ls, isolates from *Leucaena leucocephala* cv. Peruvian grown in uncultivated soils; Le, isolates from cv. Peruvian in cultivated soils; Lc, isolates from cv. Cunningham in cultivated soils; T, type strain. Isolates in bold were used for pSym identification.
‡ 16S rDNA types were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) of 16S rDNA. The four letters represent different electrophoretic patterns digested by *Msp*I, *Hin*fI, *Hha*I, or *Sau*3AI, respectively.
§The plasmid profiles were determined in Eckhardt gels (0.75%). The molecular sizes were estimated from their migration using plasmids of *R. etli* CFN42 and of *S. meliloti* 1021 as standards. Symbiotic plasmids (pSym) were identified by hybridization with *nifH* (*S. meliloti* USDA1002) and *nodDAB* (*R. tropici* CFN299).
¶ Not for all the isolates.
ND, not done; NO, not observed.

Electrophoretic types (ETs) were assigned based on the combined electrophoretic patterns of all 13 enzymes. Genetic diversity at an enzyme locus was calculated as described by Selander *et al*. (1986) using the formula $h = [1 - \sum x_i^2][n/(n-1)]$. The mean diversity ($H$) is the arithmetic average of the h values. The proportion of loci with dissimilar alleles was estimated as the genetic distance between pairs of ETs. The UPGMA method of Nei & Li (1979) was used for cluster analysis of ETs based on a matrix of pairwise genetic distances. Linkage disequilibrium was performed to estimate the degree of clonality or genetic recombination among the populations studied. The ratio of the variance in mismatch observed (V_O) to the expected (V_E) was iterated 1000 or 10 000 times in different populations using a Monte Carlo procedure (Souza *et al*. 1992).

Electrophoretic estimation of cellular plasmid content

The Eckhardt (1978) method as modified by Hynes & McGregor (1990) was used to reveal the plasmids. Isolates were grown in 5 mL of PY broth overnight for fast-growing bacteria or 24 h for slow- or moderately slow-growing rhizobia. The plasmids of *R. etli* CFN42 (Romero *et al*. 1991) and *Sinorhizobium meliloti* 1021 (Honeycutt *et al*. 1993) were used as references for estimating the molecular sizes of the plasmids.

Hybridization of plasmids with 16S rDNA and symbiotic genes

The Southern hybridization procedure was used. The Hybond™-N+ nucleic acid transfer membrane, *redi*prime, and the rapid hybridization solution from Amersham were used as specified by the manufacturer. The probes were PCR products of 16S genes from *Bradyrhizobium japonicum* USDA6, a 2.0-kb *Eco*RI-*Pst*I fragment containing *nodDAB* genes from *R. tropici* CFN299 (Wang *et al*. 1998) and a 580-bp PCR-amplified fragment of *nifH* (Eardly *et al*. 1992) from *S. meliloti* USDA1002.

RFLP analysis of symbiotic gene loci

Total DNAs were extracted using a DNA/RNA extraction kit (Amersham) and were digested with *Eco*RI or *Bam*HI. Three micrograms of digested DNAs from each isolate was separated by electrophoresis in 0.6% agarose gels and hybridized with symbiotic genes as described above for plasmid hybridization. These experiments were performed in duplicate with DNA samples extracted independently.

Results

PCR–RFLP of 16S rDNA

Twelve 16S rDNA types were distinguished among the 150 isolates from *Leucaena leucocephala* (Table 1). A dendrogram (Fig. 1) was constructed based on the RFLP patterns. In Fig. 1, the species within the genera *Bradyrhizobium, Sinorhizobium*, and *Mesorhizobium* were clustered into groups I, II, and IV, respectively. Group III contained species of *Agrobacterium* and *Rhizobium* and could be further divided into subgroups representing either *Agrobacterium* or *Rhizobium* strains. The exception was *A. rhizogenes* which clustered in the subgroup containing *R. leguminosarum* (Fig. 1). With the restriction enzymes used, indistinguishable patterns were obtained among closely related species such as *A. tumefaciens* and *A. rubi; R. hainanense*, *R. tropici* type B and *A. rhizogenes*; *R. gallicum* and *R. mongolense*; *R. galegae* and *R. huautlense*; *M. loti* and *M. ciceri*; and *S. meliloti* and *S. medicae* (Table 1 and Fig. 1).

The new isolates fell into three genera: *Mesorhizobium*, *Rhizobium* and *Sinorhizobium.* rDNA type 1 had identical RFLP patterns with *M. plurifarium*. rDNA types 2–7 were clustered in the genus *Sinorhizobium*. rDNA types 2, 4, and 5 showed close relationships with *S. fredii* and *S. meliloti*. rDNA types 3, 6, and 7 formed a subgroup with *S. terangae* and *S. saheli*. Among the group of *Rhizobium* isolates, five rDNA types were clustered into different subgroups and no one belonged to the subgroup containing *Agrobacterium tumefaciens*. rDNA types 8 and 10 formed a subgroup with *R. mongolense, R. gallicum*, and *R. etli*. rDNA type 9 shared the same position with *R. tropici* CIAT899, *R. hainanense*, and *A. rhizogenes*. rDNA types 11 and 12 formed a small cluster with *R. giardinii* (Amarger *et al*. 1997) within the subgroup containing *R. leguminosarum*. Three unclassified strains, Cli80, Leu2 (CFN234) (Hernández-Lucas *et al*. 1995), and Dal4 (CFN265), from *Clitoria ternatea*, *Leucaena* sp. and *Dalea leporina*, respectively, were identified as *Rhizobium* rDNA type 10 together with some new isolates. This result was in agreement with rDNA sequencing data (Hernandez-Lucas *et al*. 1995).

MLEE analysis

Forty-one ETs were identified among the 150 isolates (Table 1). The cluster diagram based on the genetic distances between the isolates is shown in Fig. 2. The isolates within each rDNA type were grouped together by alloenzyme analysis and most of the rDNA types were separated by around 0.4 genetic distance. Ten ETs identified among isolates in rDNA type 1 (FFFF) formed a group at 0.43 genetic distance and linked to the others at 0.9. Twenty-one ETs were distinguished within six *Sinorhizobium* rDNA types which formed four clusters or branches. Only one isolate, Ls22, corresponding to rDNA type 2 was an ET distant from other ETs of *Sinorhizobium* and *Rhizobium*. Four ETs of rDNA type 3 were a distinct cluster. Thirteen ETs in types 4 and 5 clustered together and formed the largest group among our isolates. Three ETs within rDNA types 6 and 7 formed another cluster. The 10 ETs representing isolates among the genus *Rhizobium* formed five groups or branches corresponding to the five rDNA types. rDNA types 8, 9, and 10 were linked to each other at 0.6 distance and then linked to *Sinorhizobium* groups. rDNA types 11 and 12 (related to *R. giardinii*) were linked together above 0.7 and formed a deep branch with a distance of 0.84 from other *Rhizobium* and *Sinorhizobium* rDNA types.

The genetic diversity in each enzyme locus and the linkage disequilibrium estimation were performed hierarchically for all isolates, for each general group, and for each rDNA type (or MLEE group) (Tables 2 and 3). When different rDNA types were considered together, all of the 13 enzymes analysed were polymorphic. Mean alleles for all isolates, for *Sinorhizobium,* and for *Rhizobium* rDNA types were 5.77, 3.31, and 3.15, respectively. The mean genetic diversities (H) were 0.683, 0.400, and 0.579, respectively, for the same three populations. These populations are also in linkage disequilibrium because their V_O/V_E values are significantly different from 1, which means they have clonal genetic structure or no genetic exchange among the different rDNA types (Table 2). The genetic diversity was rather limited when each rDNA type or MLEE group was analysed separately (Tables 2 and 3). The alleles ranged from one to four and the H values ranged from 0.180 to 0.308 for different rDNA types or MLEE groups. Linkage equilibrium was observed within all these subpopulations, indicating genetic recombination among bacteria within each rDNA type or MLEE group. However, the sample sizes were small (two to 13 ETs in each) and the confidence of these V_O/V_E values might be questionable.

Electrophoretic plasmid profiles

Diverse plasmid profiles were observed among the isolates. The patterns and the molecular sizes of the plasmids are shown in Table 1 and examples of these profiles are shown in Fig. 3. Isolates within a rDNA type had common plasmids in some cases. For example, most isolates of rDNA type 5 harboured a 630 kb plasmid (Fig. 3b). A 850 kb plasmid was characteristic of rDNA type 10 and a 670 kb plasmid was revealed among all eight isolates of rDNA type 11 (Table 1). No common plasmids were observed among the *Mesorhizobium* (rDNA type 1) isolates. We were unable to detect plasmids in some of the isolates (Table 1). Seventeen different plasmid profiles were identified among the 69 isolates belonging to rDNA type 5 (ADEA) (Table 1, Fig. 3b). The number of bands

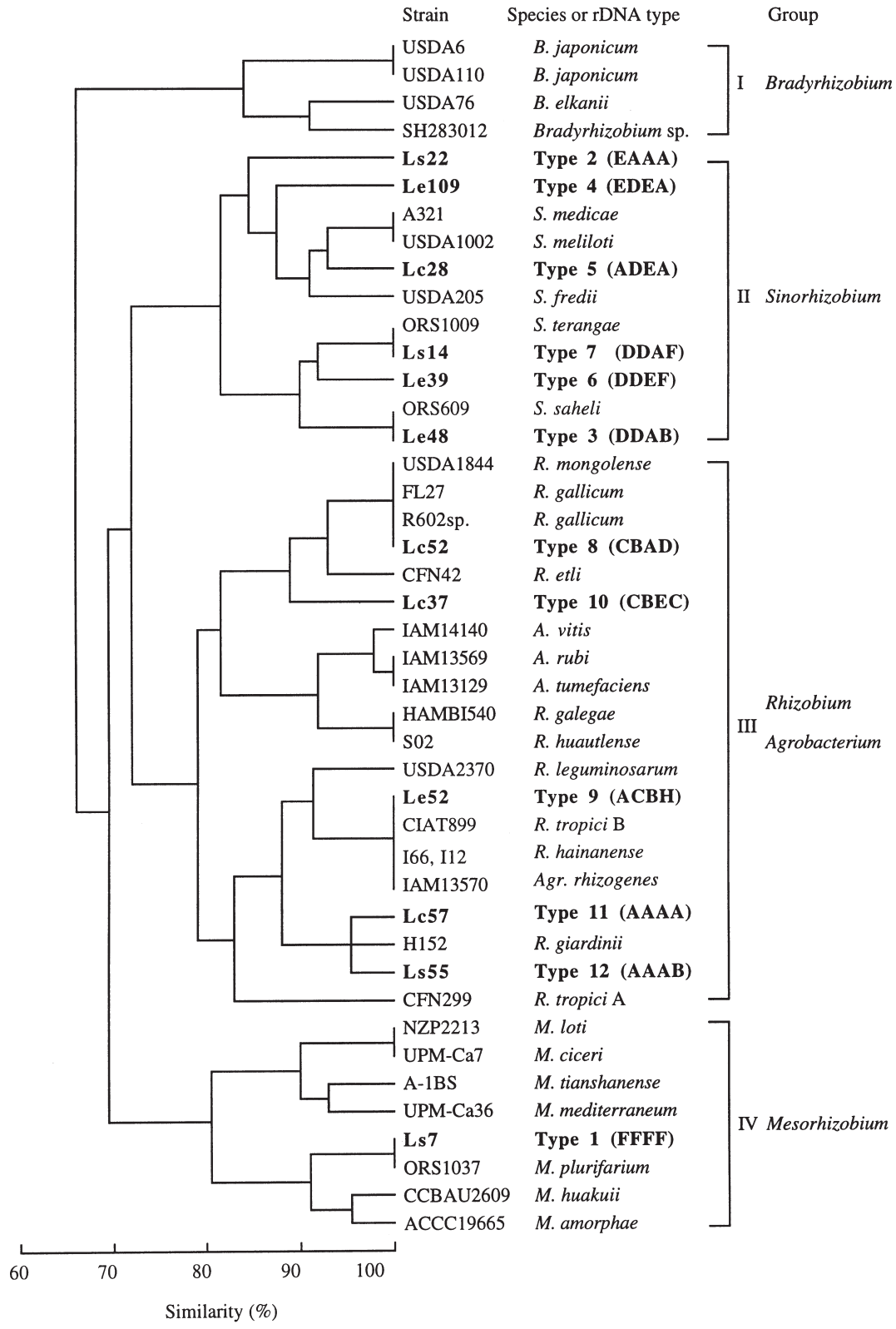

Fig. 1 Dendrogram based on the restriction fragment length polymorphism (RFLP) patterns of polymerase chain reaction (PCR)-amplified 16S rDNAs showing the phylogenetic relationships of the new isolates from *Leucaena leucocephala*. The 12 rDNA types were obtained from 150 isolates. Similarities were estimated from the proportion of shared fragments by each isolate pair. Cluster analysis was performed using the method of Nei & Li (1979).

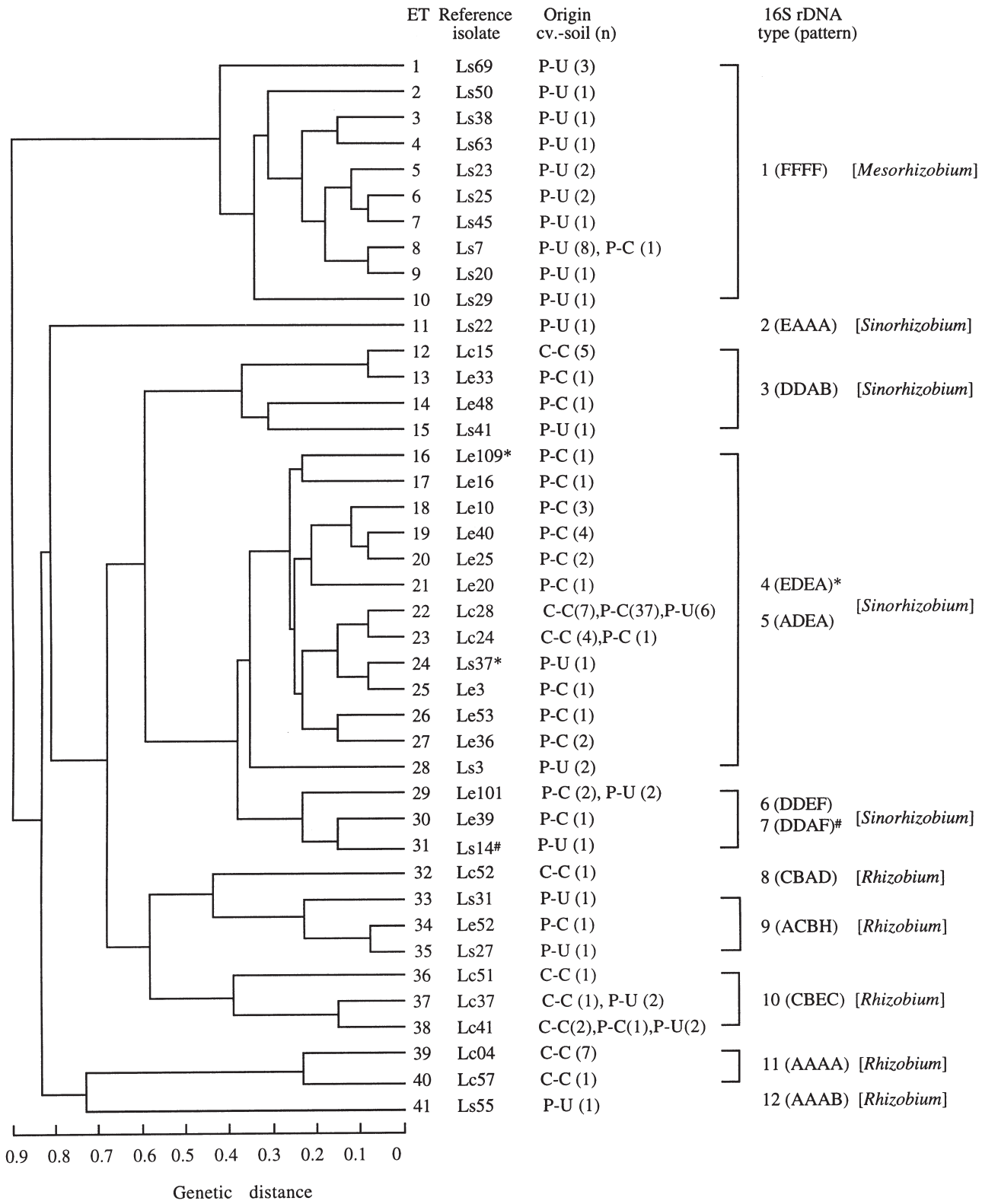

Fig. 2 Genetic relationships among the rhizobial isolates from *Leucaena leucocephala* determined by multilocus enzyme electrophoresis (MLEE) analysis of 13 metabolic enzymes. The number of isolates (*n*) for each electrophoretic type (ET) is shown in parentheses. P-U, cv. Peruvian grown in uncultivated soils; P-C, cv. Peruvian grown in cultivated soils; C-C, cv. Cunningham grown in cultivated soils. For 16S rDNA type see Fig. 1.

Table 2 Genetic diversity at 13 enzyme loci among rhizobia from *Leucaena leucocephala* (based on the generic groups)*

Enzyme locus	Total (41 ETs)		*Mesorhizobium* (10 ETs)		*Sinorhizobium* (21 ETs)		*Rhizobium* (10 ETs)	
	Allele	*h*	Allele	*h*	Allele	*h*	Allele	*h*
MDH	4	0.654	1	0	2	0.096	3	0.378
IDH	7	0.786	3	0.600	4	0.648	5	0.756
G6P	9	0.727	4	0.533	2	0.096	3	0.511
ME	4	0.590	1	0	2	0.096	2	0.500
ADK	7	0.808	3	0.622	3	0.338	4	0.644
PGI	8	0.814	1	0	5	0.606	3	0.600
EST	8	0.837	3	0.689	4	0.587	3	0.689
XDH	4	0.655	2	0.356	3	0.462	3	0.511
IPO	5	0.308	3	0.622	3	0.186	2	0.356
HEX	8	0.684	2	0.200	5	0.596	5	0.822
ALD	6	0.732	1	0	4	0.612	3	0.622
ACO	3	0.622	NO	NO	3	0.308	2	0.533
PGM	3	0.668	1	0	3	0.567	3	0.600
Mean	5.77	0.683	2.08	0.302	3.31	0.400	3.15	0.579
V_O/V_E	4.12†		1.26		2.78 †		2.64†	

*See Fig. 2 and Table 1 for the electrophoretic types (ETs).
†Significantly different from 1 which indicates linkage disequilibrium or clonal genetic structure.
ACO, aconitase; ADK, adenylate kinase; ALD, alanine dehydrogenase; EST, esterase; G6P, glucose-6-phosphate dehydrogenase; HEX, hexokinase; IPO, indophenol oxidase; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; PGM, phosphoglucomutase; PGI, phosphoglucose isomerase; XHD, xanthine dehydrogenase . NO, no activities were observed.

Table 3 Genetic diversity at 13 enzyme loci among rhizobia from *Leucaena leucocephala* (based on rDNA types*)

Enzyme locus	Type 3 (4 ETs)		Types 4 + 5 (13 ETs)		Types 6 + 7 (3 ETs)		Type 9 (3 ETs)		Type 10 (3 ETs)		Type 11 (2 ETs)	
	Allele	*h*	Allele	*h*	Allele	*h*	Allele	*h*	Allele	*h*	Alles	*h*
MDH	1	0	1	0	1	0	1	0	2	0.668	1	0
IDH	1	0	4	0.603	2	0.668	3	1.000	1	0	2	1.000
G6P	1	0	1	0	1	0	2	0.668	1	0	2	1.000
ME	1	0	1	0	1	0	1	0	1	0	1	0
ADK	2	0.500	1	0	1	0	1	0	2	0.668	1	0
PGI	2	0.666	2	0.154	2	0.668	1	0	1	0	1	0
EST	2	0.666	3	0.590	1	0	1	0	2	0.668	1	0
XDH	1	0	2	0.154	1	0	1	0	2	0.668	1	0
IPO	1	0	2	0.154	1	0	1	0	1	0	2	1.000
HEX	3	0.833	1	0	2	0.668	1	0	2	0.668	1	0
ALD	1	0	4	0.871	2	0.668	1	0	2	0.668	1	0
ACO	1	0	1	0	1	0	2	0.668	1	0	1	0
PGM	3	0.833	2	0.545	2	0.668	1	0	1	0	2	1.000
Mean	1.54	0.269	2.00	0.236	1.38	0.257	1.31	0.180	1.46	0.308	1.31	0.308
V_O/V_E	1.66†		1.13†		0.38†		3.00†		2.25†		ND	

*See Fig. 2 and Table 1 for the rDNA types.
†Not significantly different from 1 implying genetic recombination.
ACO, aconitase; ADK, adenylate kinase; ALD, alanine dehydrogenase; EST, esterase; G6P, glucose-6-phosphate dehydrogenase; HEX, hexokinase; IPO, indophenol oxidase; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; PGM, phosphoglucomutase; PGI, phosphoglucose isomerase; XHD, xanthine dehydrogenase . ND, not done; ET, electrophoretic type.

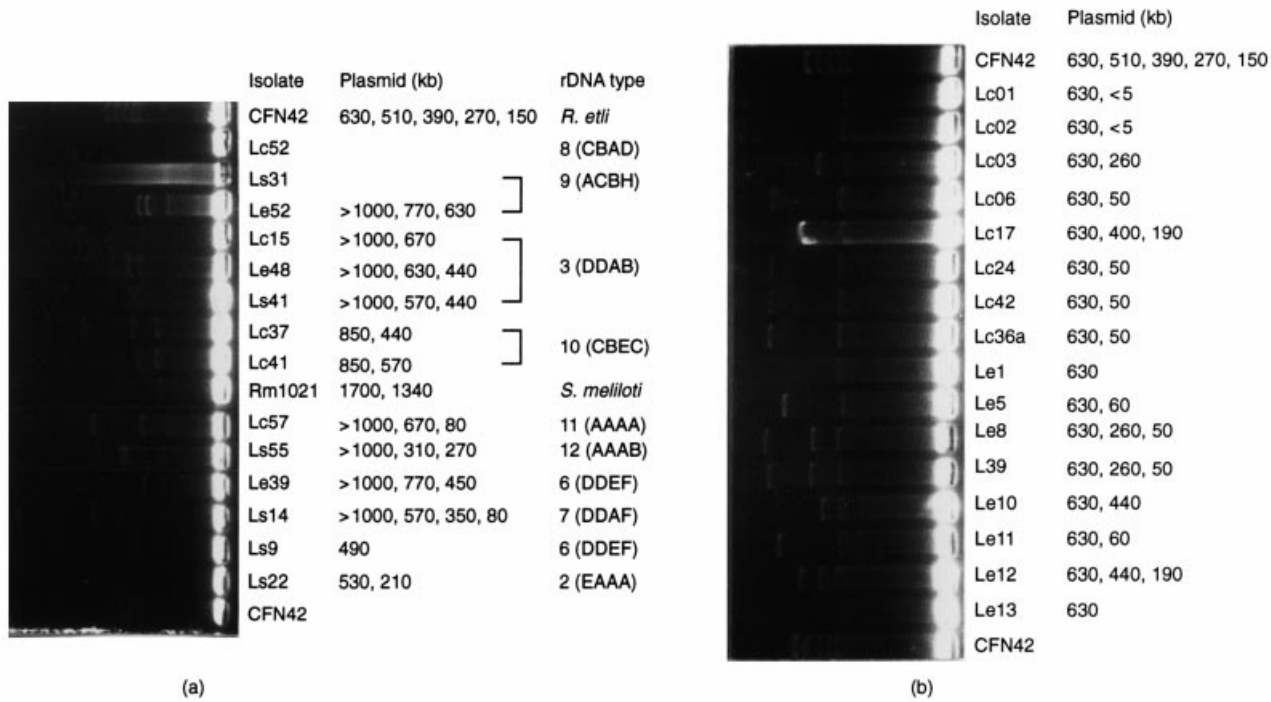

Fig. 3 Examples of electrophoretic plasmid profiles of the isolates in different rDNA types (a) and within rDNA type 5 (b). The patterns were obtained by electrophoresis for 9 h at 80 V in 0.75% Eckhardt gels.

varied from none to three and the molecular sizes ranged from < 5 to > 1000 kb. The megaplasmids (> 1000 kb) are seen as very faint bands in Fig. 3(b) but were more clearly observed in other gels (not shown). Even within the same ET, ET 26, there were 15 different profiles (from none to three bands).

Plasmid hybridizations and RFLP of symbiotic gene loci

No hybridization to a 16S rDNA probe was observed with the plasmids or megaplasmids of the isolates analysed (not shown). This is similar to other megaplasmids in *Rhizobium* (Geniaux *et al*. 1995), but not in *Brucella* (Michaux *et al*. 1993; Humas-Bilak *et al*. 1998). Symbiotic plasmids were identified in some isolates by hybridization to the *nifH* and *nodDAB* genes (Table 1). In general, the common plasmids in each rDNA type corresponded to the pSym, but some exceptions were observed. Among the representative isolates analysed (Fig. 4), no or very weak hybridization signals with the *nifH* or *nodDAB* genes were observed from Ls22 (rDNA type 2), Lc52 (rDNA type 8) and Ls55 (rDNA type 12). The others showed only one *nifH* hybridization band in both the *Eco*RI and *Bam*HI digestions (Fig. 4a, b). These results may indicate that they have only one *nifH* gene copy. However, there were no two isolates sharing the same *nifH* RFLP patterns in both *Eco*RI and *Bam*HI digestions which might imply the existence of diverse symbiotic plasmids among our isolates although they were from the same host and the same site. Different patterns with multiple bands were observed upon hybridization with the *nodDAB* genes among the isolates tested (not shown). These bands may be derived from internal cuts within the target fragments or may correspond to reiterated copies of *nod* genes. The *nif* and *nod* gene hybridization patterns were reproducible even for the faint bands in the *nodDAB* hybridizations.

The isolation frequencies of rDNA types and ETs

The isolation frequencies of the different rDNA types were estimated comparing different treatments (Table 4). Statistically significant differences ($P < 0.001$) were obtained when two cultivars and when two soil types were tested. The affinities of the two cultivars for the different groups were estimated from the isolation frequency of each rDNA type from cultivated soils. Five rDNA types were identified among the 31 isolates from cv. Cunningham (Lc isolates) and seven types were identified among 64 isolates from cv. Peruvian (Le isolates). Three rDNA types were shared by both of the plant cultivars with different frequencies. rDNA types 8 and 11 were isolated only from cv. Cunningham, while rDNA types 1, 4, 6, and 9 were isolated only from cv. Peruvian. The cv. Peruvian showed high affinity with the isolates of rDNA type 5 which represented 85.94%, while each of the other

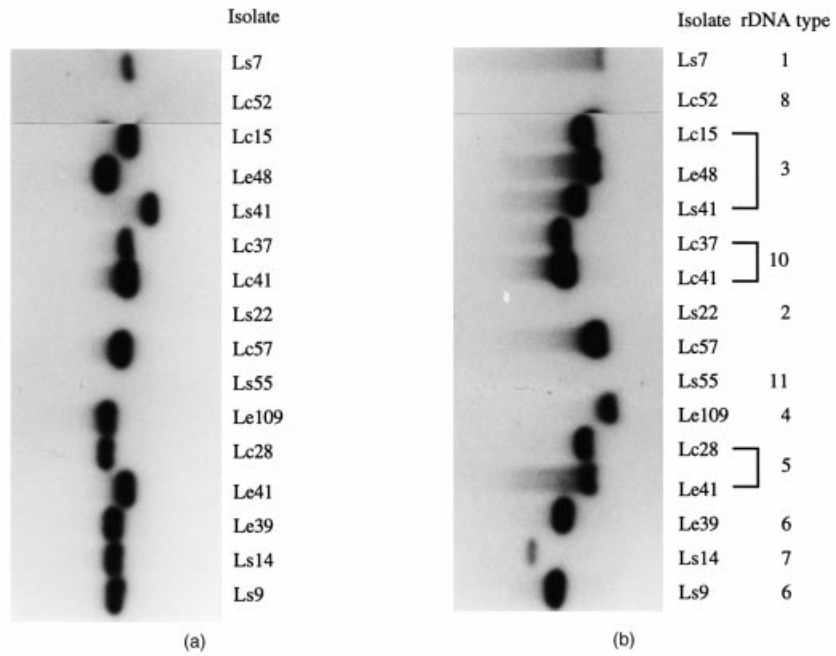

Fig. 4 Southern hybridization patterns of the *nifH* gene. The total DNAs from the isolates were digested with *Eco*RI (a) or *Bam*HI (b) and separated by electrophoresis in 0.6% agarose gels. The probe was a 580 bp polymerase chain reaction (PCR) fragment of *nifH* from *Sinorhizobium meliloti* USDA1002.

six rDNA types was less than 5%. The frequencies of the five rDNA types among isolates from cv. Cunningham varied from 3.23% in the case of rDNA type 8 to 35.48% in the case of rDNA type 5, indicating moderate affinity of this cultivar for different rhizobia. The main difference between these two cultivars was for type 11, which consisted of 25.81% of the isolates from cv. Cunningham but none from cv. Peruvian. The different frequencies of the rDNA types between these two cultivars demonstrated that they had different affinities for rhizobia from different genera and species. The isolates of rDNA type 5 (*Sinorhizobium*) were encountered in the highest numbers in both cultivars.

The effects of soil cultivation on *Rhizobium* populations were analysed by comparison of the number of rDNA

Table 4 Isolation frequencies of different 16S rDNA types from two varieties of *Leucaena leucocephala* and from cultivated and uncultivated soils*

rDNA type (pattern)	Cunningham-C		Peruvian-C		Peruvian-U	
	Number	Frequency	Number	Frequency	Number	Frequency
1 (FFFF)	0	0	1	1.56	16	38.10†
2 (EAAA)	0	0	0	0	1	2.38
3 (DDAB)	5	16.13	2	3.13	1	2.38
4 (EDEA)	0	0	1	1.56	1	2.38
5 (ADEA)	11	35.48	55	85.94	12	28.57
6 (DDEF)	0	0	3	4.69	2	4.76
7 (DDAF)	0	0	0	0	1	2.38
8 (CBAD)	1	3.23	0	0	0	0
9 (ACBH)	0	0	1	1.56	2	4.76
10 (CBEC)	6	19.35	1	1.56	4	9.52
11 (AAAA)	8	25.81	0	0	0	0
12 (AAAB)	0	0	0	0	2	4.76
Total	31	100.00	64	100.00	42	99.99

*Significant differences ($P < 0.001$) of the frequencies were observed by X^2 tests when two cultivars or two soil types were compared.
†Six *Mesorhizobium* isolates listed in Table 1 (Ls53 to Ls70) were not included in this estimation. They were chosen according to their growth rate to increase the number of isolates in this group.
C, cultivated soils; U, uncultivated soils.

types and their frequencies in the populations trapped by cv. Peruvian from cultivated and uncultivated soils (Table 4). Seven rDNA types were isolated from both soils, while four others (with one or two isolates in each) were isolated only from the uncultivated soils. The main differences in the frequencies occurred in rDNA types 1 (*Mesorhizobium*) and 5 (*Sinorhizobium*). Among the isolates from uncultivated soils, the frequency of rDNA type 1 isolates was 38.10%, while that of rDNA type 5 was 28.57%, very different from those in cultivated soils (1.56 and 85.94%, respectively).

The correspondence of ETs to host cultivars and soil types is presented in Fig. 2. The differences between host varieties and soil types were also found in this analysis. Among the 41 ETs, only four were isolated from both cultivars of the host plant, and four were common to both the cultivated and uncultivated soils.

Discussion

PCR–RFLP of 16S rRNA genes is a useful method for grouping new isolates and for estimating their phylogenetic positions (Laguerre *et al*. 1994; Wang *et al*. 1998, 1999). In this study, highly diverse rhizobial populations from *Leucaena leucocephala* nodules were revealed by the fingerprints of 16S rRNA genes. The 12 rDNA types identified among the new isolates were characterized as belonging to the genera *Mesorhizobium, Rhizobium*, and *Sinorhizobium* based on the similarities of their patterns with those of defined species. Some of them were indistinguishable from the defined species, but others, including types 2, 4, 5, 6, 10, 11, and 12 were distinct and corresponded to defined groups by MLEE analysis, and may represent some new species. The linkage disequilibrium among different rDNA types might be further evidence for the existence of different species among our isolates. The main microsymbionts of *L. leucocephala* in Mexican soils may be different from those in other places. In previous reports, some strains associated with *Leucaena* spp. were identified as members of different groups, such as *R. tropici* for strains from South America (Martínez-Romero *et al*. 1991), *Mesorhizobium plurifarium* (cluster U), *Bradyrhizobium* sp., and *Rhizobium* spp. for isolates from Senegal and Brazil (De Lajudie *et al*. 1994, 1998) and to an independent branch related to *R. tropici* (strain TAL1145) from Australia (George *et al*. 1993). In contrast, the majority of our isolates were *Sinorhizobium* with distinctive rDNA RFLP patterns (rDNA type 5). Another main group, the rDNA type 1 isolates, had the same RFLP patterns as *M. plurifarium* (Table 1 and Fig. 1) and as in this species did not seem to have the symbiotic genes borne on plasmids (Wang *et al*. 1999). The isolates corresponding to rDNA types 11 and 12 clustered with the most divergent species in the genus *Rhizobium*, *R. giardinii* (Amarger *et al*. 1997), in the dendrogram of PCR–RFLP of 16S rDNA (Fig. 1) and they formed branches very distant from others in MLEE analysis. These results might imply that these isolates were phylogenetically close to *R. giardinii*. Sanginga *et al*. (1995) concluded, mainly based on the fast-growing appearance of their nodule isolates, that *L. leucocephala* was nodulated by a restricted group of *Rhizobium*. Our data indicate that *L. leucocephala* is a very promiscuous host and may be nodulated by bacteria from at least three phylogenetically related genera.

L. leucocephala was considered as a host to distinguish *R. tropici* strains from *R. etli* (Segovia *et al*. 1993), but one *R. etli* strain was found to nodulate this plant (cv. Cunningham) (Hernández-Lucas *et al*. 1995). We tested 20 additional *R. etli* strains from our collection and none were able to nodulate *Leucaena* (unpublished data). To obtain an even larger sample of *R. etli* strains with which to clarify its host range, we decided to grow *Leucaena* plants in soils previously cultivated with beans and containing native and diverse populations of *R. etli*. From this soil we were unable to recover any *R. etli* strain with the two *Leucaena* cultivars used. However, *Leucaena* and bean plants may share some other rhizobia in the soil tested. A distinct cluster of isolates from beans grown in the same site was recognized by MLEE which linked to *R. etli* strains at a genetic distance of 0.85 (Caballero-Mellado & Martínez-Romero 1999). The relationship between this novel cluster and our isolates is unclear. Recent results from our laboratory (unpublished) showed that some rhizobial isolates from *Mimosa affinis* growing elsewhere in Mexico were *R. etli* bv. mimosae. These isolates can nodulate both *P. vulgaris* and *L. leucocephala*. So, *L. leucocephala* may no longer be considered as a host to distinguish *R. etli* from *R. tropici*, but a host to differentiate bv. mimosae from bv. etli within the species *R. etli*.

The legume–*Rhizobium* symbiosis has attracted much interest, especially in regard to the mechanisms involved in its specificity. Here, we report that the different *L. leucocephala* cultivars select rhizobial isolates from different rDNA types with different affinities (Table 4). This phenomenon is in agreement with previous reports that different *L. leucocephala* cultivars had distinct responses to different inoculant strains (Sanginga *et al*. 1995) and that the strains tested varied in their symbiotic competence (Kuykendall *et al*. 1996).

The diversity analysis performed showed that there was less isolate dominance in *L. leucocephala* cv. Cunningham. This cultivar was selected for its low mimosine content, making it less toxic as cattle forage. Mimosine has been shown to be a carbon and nitrogen source for some rhizobia from *Leucaena* (Soedario *et al*. 1994) and the strains that can use this compound may have a competitive advantage for nodulation (Soedario & Borthakur 1998). We have found differential utilization of

mimosine by the distinct groups described (unpublished data) and we will further explore it.

The effects of soil conditions on the selection of rhizobial populations have also been reported. We showed previously that *Sesbania herbacea* trapped *Mesorhizobium* sp. and *Rhizobium* spp. in Cuernavaca soils, but only *R. huautlense* strains in flooded areas in Huautla (Wang *et al*. 1998). In this research, more diverse populations were isolated from *Leucaena* plants grown in the uncultivated soils than from bean-cultivated soils. These effects may be attributable to the plant flora growing in the soils because there were no fertilizer additions when the common bean plants were grown before the *Leucaena* plants. We may conclude that the cultivation of common bean plants altered the soil rhizobial populations (the numerical predominance of members) even of those types that are not naturally its symbionts and that this effect is reflected in the populations encountered in *Leucaena* nodules.

Most *R. huautlense* strains have 900 and 400 kb plasmids (Wang *et al*. 1998) and most *Mesorhizobium amorphae* strains harbour a 930 kb symbiotic plasmid (Wang *et al*. 1999). Similar results were also reported for *R. leguminosarum* (Wernegreen *et al*. 1997). From the characterization of plasmid contents presented here, it seems clear that some plasmids always follow the rDNA types in some cases, and most of the isolates within a rDNA type, such as types 5, 10, and 11, harboured common plasmids, mainly pSym. However, variability among the symbiotic plasmids with similar molecular sizes was also observed in the restriction patterns with the *nifH* and *nodDAB* probes, showing that the most variable components in these populations are within plasmids.

Acknowledgements

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The group is working on biodiversity and taxonomy of nitrogen-fixing bacteria, including rhizobia isolates from native legumes in tropical areas and other endophytic nitrogen-fixing bacteria from maize, sugarcane, and banana plants. Ribotyping, alloenzyme polymorphism, DNA–DNA homology and some other genetic analyses are being used to describe the diversity of these bacteria and to estimate the effects of agricultural practices on them. These data, supported by sequence analysis of 16S rRNA genes, have led to the identification of new bacterial groups.
