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Biodiversity of Bradyrhizobia Nodulating *Lupinus* spp.

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The genetic structure of *Bradyrhizobium* isolates recovered from three *Lupinus* species (*Lupinus campestris*, *Lupinus montanus*, and *Lupinus exaltatus*) grown in Mexico was examined. Among 41 *Bradyrhizobium* isolates, 18 electrophoretic types (ETs) were distinguished by multilocus enzyme electrophoresis of five metabolic enzymes. The mean genetic diversity, 0.64, indicated that there was great genetic diversity in the population sampled. Most isolates (63%) fell into two closely related clusters (clusters I and II) and were the types most frequently isolated from the root nodules of *L. montanus* and *L. campestris*. ET cluster III isolates were frequent nodule occupants of *L. exaltatus*. The isolates also were assigned to three main groups by using Curie point pyrolysis mass spectrometry. In general, the multilocus enzyme electrophoretic data and pyrolysis mass spectrometric data agreed. We determined the 16S rRNA sequences of representative *Lupinus* isolates and of *Bradyrhizobium japonicum* USDA 6^T and found that the lupine isolates were highly related to the *B. japonicum* type strain, although not all *B. japonicum* type strains (subcultures maintained in different bacterial collections) had identical small-subunit rRNA.

There are approximately 200 plant species in the genus *Lupinus*. The majority of them are indigenous to the Americas, but some species occur in the Old World (2). Plant species native to the Americas are more genetically diverse than the dominant European species (2). *Lupinus* spp. are valuable for soil improvement, as a source of protein, and as green manure. Sweet lupine is grown in Europe (especially Poland, Russia, and Spain), Australia, South Africa, and the United States. Although the genus *Lupinus* is not cultivated in Mexico, approximately 30 native species grow in the highlands (35).

As is the case with many leguminous plants, *Lupinus* species form nitrogen-fixing symbioses with soil bacteria collectively known as rhizobia (8). *Lupinus* spp. are nodulated by *Bradyrhizobium* strains, as well as by *Rhizobium* strains (23–26, 34, 37, 38). Microsymbionts from *Lupinus* spp. have been less well-characterized than microsymbionts from many other host legumes. *Bradyrhizobium* populations associated with *Lupinus* spp. in the Americas have been the subject of a single study (1). Ludwig et al. (30) reported the small-subunit (SSU) and large-subunit rRNA nucleotide sequences of one *Lupinus* strain (DSM 30140) that is related to the genus *Bradyrhizobium*. Analyses with additional lupine isolates have not been performed.

The taxonomy of the genera *Rhizobium* and *Bradyrhizobium* is being reexamined, and several new *Rhizobium* species have been proposed (31, 58). In contrast, there are only three validly described species of the genus *Bradyrhizobium*. *Bradyrhizobium japonicum* was the only species recognized in *Bergey's Manual of Systematic Bacteriology* (26). Since then, *Bradyrhizobium elkanii* (29) and *Bradyrhizobium liaoningense* (56) have been proposed to encompass soybean symbionts. *Bradyrhizobium* strains have been isolated from many different genera of plants (3–5, 12, 13, 32, 33, 45, 46, 49, 59), and these isolates appear to be diverse. It seems likely, therefore, that additional centers of taxonomic variation occur in the genus *Bradyrhizobium* and that these need to be highlighted and described. Analyses of the genetic diversity of bacterial populations, in addition to their taxonomic value, help unravel the genetic structure (clonality) of bacterial groups.

Rapid characterization methods are needed to determine the full extent of the biodiversity encompassed by the genus *Bradyrhizobium*. Molecular and chemical techniques, such as multilocus enzyme electrophoresis (MLEE) (40) and Curie point pyrolysis mass spectrometry (PyMS) (14), provide fast approaches for measuring the extent of species diversity and are very useful for primary grouping of strains.

MLEE is a technique based on the polymorphism of a significant number of genes that encode housekeeping enzymes. This method detects different alleles of diagnostic genes by analyzing the electrophoretic mobilities of the enzymes which they encode. The different electromorphs of each enzyme reflect different alleles at the corresponding gene locus of the enzyme, and the electromorphic profiles for a number of enzymes are identified as multilocus phenotypes or electrophoretic types (ETs) which reflect chromosomal genotypes (40).

The speed, reproducibility, and universality of Curie point PyMS make it a particularly attractive method for determining the taxonomic integrity of bacterial groups classified by using other characterization procedures (17, 19). In general, good congruence has been found between classifications generated by using PyMS and more standard procedures; this has been true, for example, in comparative taxonomic studies of gordonae (18) and streptococci (54). It is particularly encouraging that PyMS and DNA-DNA pairing data give similar patterns of relatedness (16, 36).

The primary aim of the present investigation was to analyze

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TABLE 1. Sources of *Bradyrhizobium* isolates and assignment to electrophoretic groups

ET	Isolate(s)	Source[a]	Host plant
1	FN 24	Felipe Neri, Mexico	*Lupinus montanus*
1	CICS 42, CICS 48	Ex-hacienda Mayorazgo, Mexico	*L. montanus*
2	AME 75	Amecameca, Mexico	*L. campestris*
3	CICS 53	Ex-hacienda Mayorazgo, Mexico	*L. montanus*
4	FN 22	Felipe Neri, Mexico	*L. montanus*
5	FN 13	Felipe Neri, Mexico	*L. montanus*
6	HUI 59, HUI 63	Huitzilac, Mexico	*L. campestris*
7	CICS 43	Ex-hacienda Mayorazgo, Mexico	*L. montanus*
8	AME 103	Amecameca, Mexico	*L. campestris*
9	CICS 55, CICS 70	Ex-hacienda Mayorazgo, Mexico	*L. montanus*
9	HUI 58, HUI 68	Huitzilac, Mexico	*L. campestris*
9	AME 72	Amecameca, Mexico	*L. campestris*
9	USDA 3060, USDA 3061	USDA	*L. angustifolius*
10	AME 76	Amecameca, Mexico	*L. campestris*
11	FN 2	Felipe Neri, Mexico	*L. montanus*
11	HUI 57	Huitzilac, Mexico	*L. campestris*
11	Km 50-96, Km 50-97	Km-50, Mexico	*L. campestris*
12	HUI 67	Huitzilac, Mexico	*L. campestris*
12	Km 50-81	Km-50, Mexico	*L. campestris*
13	FN 40	Felipe Neri, Mexico	*L. montanus*
13	HUI 60, HUI 61, HUI 66	Huitzilac, Mexico	*L. exaltatus*
13	Km 50-82, Km 50-87	Km-50, Mexico	*L. campestris*
13	Km 50-89	Km-50, Mexico	*L. campestris*
14	FN 29	Felipe Neri, Mexico	*L. montanus*
15	USDA 3042	USDA	*L. albus*
16	FN 8	Felipe Neri, Mexico	*L. exaltatus*
16	Km 50-90	Km-50, Mexico	*L. campestris*
16	AME 102	Amecameca, Mexico	*L. exaltatus*
17	FN 1, FN 14	Felipe Neri, Mexico	*L. exaltatus*
18	FN 7	Felipe Neri, Mexico	*L. montanus*
18	CICS 52	Ex-hacienda Mayorazgo, Mexico	*L. exaltatus*
ND[b]	USDA 6	USDA	*Glycine max*
ND	USDA 110	USDA	*G. max*

[a] USDA, U.S. Department of Agriculture Culture Collection, Beltsville, Md.; Strains whose designations begin with CICS and AME were obtained from the state of Mexico, Mexico; strains whose designations begin with FN, Km 50, and HUI were obtained from the state of Morelos, Mexico.

[b] ND, not determined.

and characterize *Bradyrhizobium* strains isolated from native *Lupinus* species growing in undisturbed highlands in Mexico by using MLEE. Representatives of groups defined in the MLEE analyses were characterized by Curie point PyMS and by determining the sequence of the 16S rRNA genes.

MATERIALS AND METHODS

***Bradyrhizobium* strains.** Thirty-eight new *Bradyrhizobium* strains isolated from nodules of *Lupinus* spp. (Table 1) were maintained in YM (50) broth containing 25% (vol/vol) glycerol at −80°C. The previously described representatives of the genus *Bradyrhizobium*, which are also shown in Table 1, were collection strains from the Beltsville Agricultural Research Center.

MLEE. The test strains were grown for 48 h in 30 ml of PY medium, and cells were harvested by centrifugation at 10,000 × *g* for 10 min at −4°C. The pelleted cells were suspended in 0.3 ml of 10 mM $MgSO_4 \cdot 7H_2O$ containing 300 μg of lysozyme. The cell suspensions were incubated for 10 min at room temperature and disrupted by repeated freezing (−70°C for 15 min) and thawing. The disrupted cell preparations were stored at −70°C.

The procedures used for separating proteins by starch gel electrophoresis and selective staining of enzymes were the procedures described by Selander et al. (40, 41). Five metabolic enzyme activities were examined. Xanthine dehydrogenase, glucose-6-phosphate dehydrogenase, and phosphoglucomutase were assayed in Tris-citrate (pH 5.3), whereas alcohol dehydrogenase and isocitrate dehydrogenase were assayed in Tris-citrate (pH 8.0). The electrophoretic mobility of each of these enzymes was determined three times for each strain.

Each distinct electromorphic profile was referred to as an ET. The genetic diversity (h) of each enzyme locus was calculated as follows: $h = (1 - \Sigma x_i^2)\,[n/(n-1)]$, where x_i is the frequency of the ith allele at the locus and n is the number of ETs in the population assayed. The mean genetic diversity per locus is the arithmetic mean of the h values for the five loci (40). The genetic distance between each pair of ETs was estimated by determining the proportion of loci at which dissimilar alleles occurred. Clustering by the average linkage method was determined from a matrix of pairwise genetic distances (42).

PyMS. Thirteen *Bradyrhizobium* isolates (strains CICS 42, CICS 43, CICS 52, CICS 70, FN 13, FN 22, FN 29, Km 50-81, Km 50-89, Km 50-90, and Km 50-96 and reference strains USDA 3042 and USDA 3061) were analyzed. Glycerol stock cultures were used to inoculate sterile nitrocellulose membrane filters placed over YM agar plates. The inoculated plates were incubated at 30°C for 48 h. Single colonies were used to smear ferronickel alloy foils (Horizon Instruments, Ltd., Heathfield, East Sussex, United Kingdom) with a sterilized plastic loop. The inoculated foils were inserted into pyrolysis tubes (Horizon Instruments), and the samples were heated at 80°C for 5 min. Each culture was analyzed in triplicate.

The samples were processed in a single batch with a Horizon Instruments model RAPyD 400× pyrolysis mass spectrometer. Curie point pyrolysis was performed at 530°C for 2.4 s with a temperature rise time of 0.6 s. The inlet heater was set at 160°C, and the quadrupole mass spectrometer was set at scanning intervals of 0.35 s. Integrated ion counts for each sample at unit mass intervals from 51 to 400 were recorded, together with total ion counts and the sample pyrolysis sequence, without background removal.

PyMS data analysis. Variation between spectra due to inoculum size was normalized by iterative renormalization (20), and individual masses were ranked according to their characteristicity values (9) prior to a principal-component analysis. Principal components accounting for less than 0.1% of the total variance were discarded. Canonical variate analysis was then performed to generate sample groups on the basis of the retained principal components by taking into account the sets of triplicates (53). The resultant data were presented in a two-dimensional ordination diagram.

PCR amplification and sequence analysis of the SSU rRNA gene. Colonies of the test strains grown on the surface of MAG medium (47) were placed in 200-μl portions of 0.1% Tween 20 (polyoxythylene sorbitan monolaurate), and the cells were lysed by incubating the suspensions at 95°C for 10 min. Samples (4 μl) of the lysed cell suspensions were used in 120-μl PCR mixtures containing primers fD1 and rD1 (51). The PCR conditions used for amplification of the SSU rRNA genes have been described previously (48). The PCR products, purified by using QIAquick spin columns (Qiagen, Inc., Chatsworth, Calif.), were sequenced by using a model 370A DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.) as described previously (48).

Analysis of sequence data. The sequences were aligned by using the PILEUP program in the Wisconsin package of the Genetics Computer Group (Madison, Wis.). Nucleotide differences were determined for *B. japonicum* IAM 12608^T (33) (accession no. D11345), LMG 6138^T (4) (accession no. X66024), and USDA 6^T, *B. japonicum* USDA 110 (accession no. D13430 and Z35330), *B. elkanii* USDA 76 (accession no. U35000), and lupine isolates Km 50-90, CICS 70, and FN 13. The aligned sequences were analyzed by using the SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE programs of the software package PHYLIP, version 3.5c (10). Trees were constructed by using DRAWGRAM. *Azorhizobium caulinodans* LMG 6465 (accession no. X67221) was used as the outgroup species, and only *B. japonicum* USDA 6^T was included. The other sequences used were the sequences of *Blastobacter denitrificans* LMC 8443^T (accession no. S46917), *Rhodopseudomonas palustris* ATCC 17001 (accession no. D25312), *Afipia clevelandensis* ATCC 35685 (accession no. M69186), *Afipia felis* (accession no. M65248), and *Bradyrhizobium* sp. strain BR 6011 (accession no. X70401) from *Lonchocarpus costatus*; strains BR 29 (accession no. X70402) and BR 3621 (accession no. X70403) from *Acacia mangium*; strain BR 4406 (accession no. X70404) from *Enterolobium ellipticum*; and strain ORS 133 (accession no. X70405) from *Acacia albida* (4).

Nucleotide sequence accession numbers. The SSU rRNA gene sequences of strains FN 13, Km 50-90, and USDA 6^T have been deposited in the GenBank database under accession no. U69636, U69637, and U69638, respectively.

RESULTS

Genetic diversity among *Bradyrhizobium* isolates. The metabolic enzymes assayed were polymorphic, and the number of alleles per locus ranged from four to six (Table 2). With *Bradyrhizobium* extracts it was difficult to reveal the whole set of metabolic enzymes usually used in MLEE analysis with legume symbionts. This problem has been noted before in MLEE analysis of *Bradyrhizobium* spp. (1). For this reason we report results obtained with only five enzymes instead of the customary 10 to 12 enzymes. Nevertheless, 18 distinctive ETs were

TABLE 2. Genetic diversity at five enzyme loci among ETs of *Bradyrhizobium* strains isolated from *Lupinus* spp.

Enzyme[a]	pH of Tris-citrate buffer	No. of alleles	Genetic diversity
IDH	8	6	0.594
ADH	8	5	0.641
G6P	6.3	6	0.684
XHD	6.3	4	0.604
PGM	6.3	6	0.690
Avg		5.4	0.643

[a] IDH, isocitrate dehydrogenase; ADH, alcohol dehydrogenase; G6P, glucose-6-phosphate dehydrogenase; XHD, xanthine dehydrogenase; PGM, phosphoglucomutase.

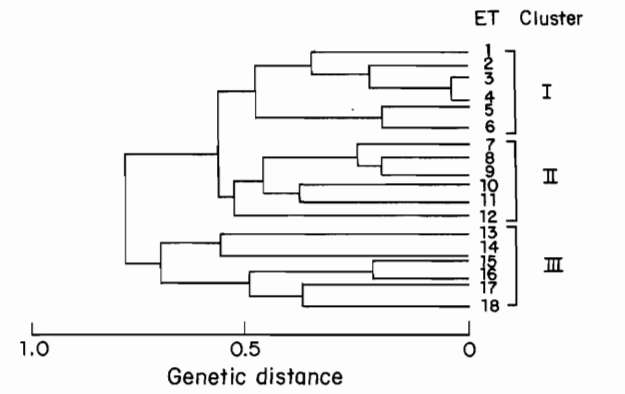

FIG. 1. Genetic relatedness among the 18 ETs of *Bradyrhizobium* strains isolated from *Lupinus* spp. determined by MLEE.

identified among the 41 *Bradyrhizobium* isolates (Table 3). The *h* values per enzyme locus ranged from 0.59 to 0.69, and the overall mean genetic diversity for the five enzyme loci was 0.640 (Table 2). A total of 10 of the 18 ETs were represented by more than one isolate, and in some cases *Bradyrhizobium* strains from the same site gave identical multilocus genotypes (ET 6 was represented by two isolates from Huitzilac, Mexico). In other cases, the same ETs were isolated from different locations; for example, ET 9 was found in isolates from both the state of Morelos and the state of Mexico.

Genetic relatedness of ETs. The different ETs were clustered into three major divisions, designated clusters I, II, and III (Fig. 1). Cluster I, which encompassed isolates from ETs 1 to 6, had a genetic distance of 0.50. Similarly, cluster II was comprised of isolates assigned to ETs 7 to 12. Cluster III showed the greatest level of heterogeneity; it included isolates in ETs 13 to 18. This cluster also contained USDA 3042. Clusters I and II joined at a genetic distance of 0.56, and cluster III diverged at a genetic distance of 0.73.

Geographic variation. The mean genetic diversity per locus for the eight ETs from the state of Mexico was 0.70. The corresponding value for the nine ETs from the state of Morelos was 0.57. The total diversity (H_T) for the 18 ETs was 0.650, and the mean diversity within the two geographic regions (H_S) was 0.63. Consequently, the interregional component of diversity, $F_{ST} = (H_T - H_S)/H_T$, was 0.030, which indicated only a mild degree of regional differentiation.

PyMS characterization of *Bradyrhizobium* isolates. Excellent agreement was found among the results of the triplicate analyses of each strain. The 13 test strains were assigned to three groups, as shown on the ordination diagram (Fig. 2). A total of 10 of the 13 strains fell into PyMS groups that corresponded to the three ET clusters. Strains FN 13 and CICS 42 were assigned to group 1; these strains belonged to cluster I in the MLEE analysis. The second group contained three strains, CICS 70, Km 50-96, and reference strain USDA 3061 (cluster II ETs). Eight strains with ETs that corresponded to the three different MLEE clusters were found in PyMS group 3. Isolates FN 29, USDA 3042, Km 50-90, Km 50-89, and CICS 52 had MLEE cluster III ETs; strains CICS 43 and Km 50-81 belonged to cluster II; and FN 22 had a cluster I ET.

Nucleotide sequences of the SSU rRNA genes. Double-stranded PCR products of the SSU rRNA genes from three

TABLE 3. Allele profiles at five enzyme loci of the 18 ETs of *Bradyrhizobium* strains isolated from *Lupinus* spp.

ET	IDH allele[a]	ADH allele	G6P allele	XHD allele	PGM allele
1	7	8	7	4	6
2	6	7	2	6	4
3	5	5	5	5	4
4	4	6	5	4	4
5	4	4	8	6	4
6	7	2	8	6	2
7	8	7	8	6	8
8	2	5	2	4	4
9	2	2	2	2	2
10	2	2	2	6	8
11	2	2	4	4	4
12	2	2	4	4	2
13	2	2	4	2	4
14	4	4	7	5	5
15	4	4	4	6	8
16	4	6	4	2	2
17	4	4	4	2	2
18	4	4	2	2	4

[a] IDH, isocitrate dehydrogenase; ADH, alcohol dehydrogenase; G6P, glucose-6-phosphate dehydrogenase; XHD, xanthine dehydrogenase; PGM, phosphoglucomutase.

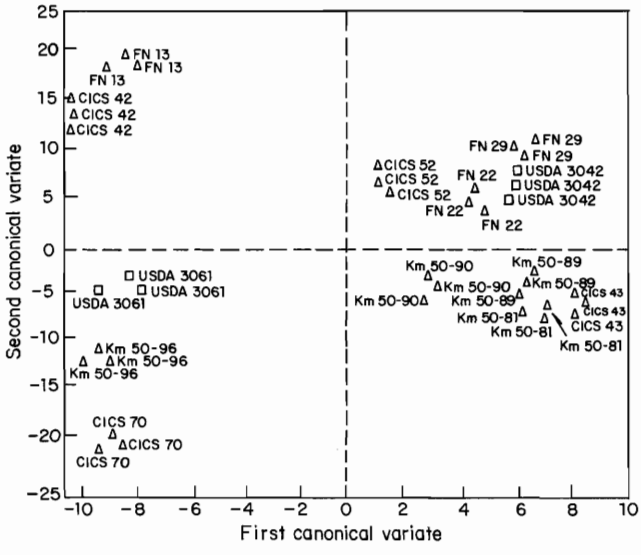

FIG. 2. Principal-component–canonical variate analysis ordination diagram of PyMS data for the *Bradyrhizobium* strains. The first two axes accounted for 70% of the total variation found among all of the strains. Symbols: △, representative isolates; □, reference strains.

TABLE 4. Aligned sequences of SSU rRNA genes

Strain(s)	Nucleotide at position[a]:																				No. of differences from consensus sequence
	48	50	104	149	336	372	489	510	586	646	678	860	861	1045	1261	1264	1271	1272	1428	1472	1487	
IAM 12608^T	C	T	G	T	A	A	G	G	C	A	G	C	G	G	T	G	C	A	C	C	C	10
USDA 6^T	T	A	G	C	A	A	G	C	G	G	G	G	C	G	T	T	C	G	T	C	C	1
LMG 6138^T	T	A	G	C	A	A	G	C	G	G	G	G	C	G	T	T	C	G	T	C	C	1
Km 50-90, CICS 70	T	A	G	C	A	A	G	C	G	A	G	G	C	A	T	T	T	G	T	C	C	2
FN 13	T	A	G	C	A	A	G	C	G	A	G	G	C	A	C	T	C	A	T	T	C	4
USDA 110[d]	C	T	G	T	A	A	G	G	C	A	G	C	G	G	T	G	C	A	C	C	C	10[b]
USDA 76	T	A	G	C	A	A	G	C	G	A	G	G	C	A	T	T	C	G	T	C	C	1[c]
Consensus	T	A	G	C	A	A	G	C	G	A	G	G	C	G	T	T	C	G	T	C	C	

[a] *E. coli* 16S rRNA gene numbering (accession no. J01859). Only positions at which there are differences in the *Bradyrhizobium* sequences are shown.
[b] Five additional nucleotide differences are not shown.
[c] Thirty-eight additional nucleotide differences are not shown.
[d] Accession no. D13430 has 13 nucleotide differences with USDA 110 sequence, accession no. Z35330 (not shown).

isolates representing the three ET clusters (strains FN 13, CICS 70, and Km 50-90, from ET clusters I, II, and III, respectively) and from *B. japonicum* USDA 6^T were generated for the sequence analysis. USDA 6 is the *B. japonicum* type strain; it was introduced to the U.S. Department of Agriculture collection in 1929 from Japan and was provided to the American Type Culture Collection in 1948, where it became ATCC 10324. Subcultures of the type strain exist in many collections, and other designations of the type strain are 3I1b6, SEMIA 5052, RCR 3425, IITA 2116, ACCC 15032, NZP 5549, IAM 12608, and LMG 6138. SSU rRNA gene sequences have been reported for LMG 6138^T and IAM 12608^T. Nevertheless, we chose to sequence USDA 6^T because it has been kept in the Beltsville collection since 1929. The SSU rRNA sequences of *B. japonicum* USDA 6^T and LMG 6138^T were identical and different from that of IAM 12608^T (Tables 4 and 5). The sequences of LMG 6138^T and USDA 6^T were the sequences most similar to the consensus sequence derived from considering *B. japonicum* USDA 6^T, LMG 6138^T, IAM 12608^T, USDA 110, Km 50-90, and FN 13 and *B. elkanii* USDA 76 SSU rRNA gene sequences (Table 4). IAM 12608^T should be reexamined by other criteria to define the reasons for the discrepancy with the other type strains.

Lupine isolates Km 50-90 and CICS 70 had identical SSU rRNA nucleotide sequences, and these sequences differed from the sequence obtained for lupine isolate FN 13 by four nucleotides. Reconstructing the phylogeny with the aligned SSU rRNA sequences which we obtained and sequences obtained from GenBank showed that the three lupine isolates were closely related to *B. japonicum* and to *Blastobacter denitrificans* (Fig. 3).

DISCUSSION

The genetic diversity of bradyrhizobia isolated from nodules collected from three different *Lupinus* species growing in diverse geographical areas in Mexico was similar to the genetic diversity reported for isolates from four hosts, including two *Lupinus* species, *Lupinus albus* and *Lupinus angustifolius*, in the United States (1). Our data may indicate that there are host plant preferences for different bacterial chromosomal types. Isolates from the nodules of *Lupinus montanus* and *Lupinus campestris* dominated the first two ET clusters. The third cluster contained isolates from *Lupinus exaltatus*. Isolates from the same geographical region had the same ETs, although in some cases isolates with the same ET originated from different geographical locations.

Since our MLEE analysis was based on results from relatively few enzymes, we considered it necessary to support the MLEE grouping by an alternative method, such as PyMS. The reason why we were unable to reveal several enzymes in the MLEE analysis is unknown but may be related to the previously reported problems in obtaining sharp protein bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels with *Bradyrhizobium* strains (4).

PyMS provides a simple and reproducible method for distinguishing a large number of closely related strains (14, 15, 17). Previously, it has been used to discriminate between *B. japonicum* strains in order to evaluate the fate of inoculants (27), and it has been used for characterization of *Rhizobium meliloti* (16). This method is very sensitive to the growth conditions of cultures, as is fatty acid methyl ester analysis of bacteria (22). In the present study we found that the MLEE groups were supported by the PyMS data. A total of 10 of the 13 strains studied by PyMS fell into groups that corresponded

TABLE 5. Number of nucleotide differences in 1,441 bp of the SSU rRNA gene

Strain	No. of nucleotide differences				
	Km 50-90 and CICS 70	FN 13	USDA 110[a]	*B. elkanii* USDA 76	IAM 12608^T
B. japonicum USDA 6^T and LMG 6138^T	3	5	9	40	11
Bradyrhizobium sp. (*Lupinus*) strain Km 50-90		4	8	39	
Bradyrhizobium sp. (*Lupinus*) strain FN 13			10	40	
B. japonicum USDA 110[a]				42	

[a] Accession no. Z35330

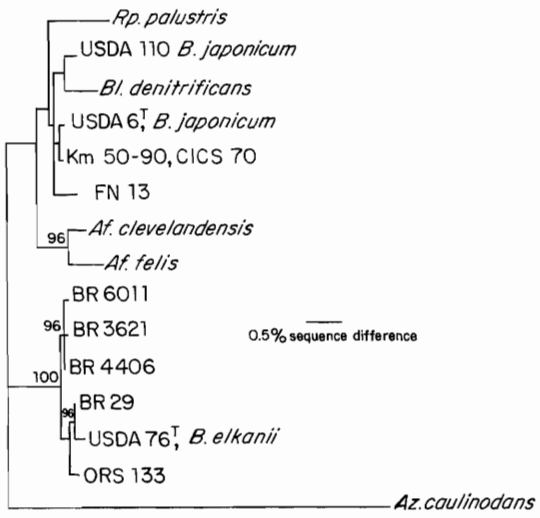

FIG. 3. Optimal unrooted phylogenetic tree obtained by using the unweighted pair group arithmetic average method tree selection criteria when Jukes-Cantor distances were estimated from SSU rRNA gene sequences. The tree shows the relationships of the lupine isolates with the genus *Bradyrhizobium* and several other genera of the α *Proteobacteria* whose sequences were obtained from the database under the accession numbers indicated in Materials and Methods; USDA 110 accession no. Z35330 was used. A majority rule consensus tree was derived based on 1,000 bootstrap replicates. The levels of support in the data for the presence of nodes at or above 95% are indicated. Abbreviations: *Rp.*, *Rhodopseudomonas*; *B.*, *Bradyrhizobium*; *Bl.*, *Blastobacter*; *Af.*, *Afipia*; *Az.*, *Azorhizobium*.

adequately to the three ET clusters. Strain Km 50-81, which fell into PyMS group 3 (clustering mainly with MLEE cluster III strains), belongs to MLEE cluster II, but it may be considered an outlier of this MLEE cluster; consequently, its recovery in a different PyMS group is not surprising. Strains CICS 43 and FN 22 were grouped differently by PyMS and MLEE. This disparity could be explained by the fact that the two methods measure different cell characteristics (15).

Based on MLEE and PyMS data, we chose strains to represent the different clusters to define the position of Mexican *Bradyrhizobium* spp. (*Lupinus*) in the general scheme of *Rhizobiaceae* phylogenies. Currently, the accepted approach for inferring bacterial phylogeny is to compare variations in ribosomal gene sequences. In the rhizobia, SSU rRNA gene sequences are commonly used to identify affinity at the genus level (6, 21, 52, 55, 57). While extensive use has been made of partial SSU rRNA sequences in the classification of rhizobia, this practice may be inadequate for reconstruction of phylogenetic relationships, as this minimally would require full-length sequence comparisons. It has been shown, for example, that reconstructions of the phylogenetic relationships of *Rhizobium galegae* and *Rhizobium etli* differ when partial or full-length SSU rRNA gene sequences are compared (39, 48). This inconsistency may be attributed to the highly conserved nature of the SSU rRNA sequences coupled with recombination events between divergent genes (7). While three ribosomal operons have been described in the genus *Rhizobium*, a single operon exists in the genus *Bradyrhizobium* (28). In the present investigation almost complete SSU rRNA sequences were compared, and the phylogenetic tree that we reconstructed is in perfect agreement with the relationships that were reported for *Bradyrhizobium* sp. strains BR 29, BR 6011, BR 3621, and BR 4406 (4). We found that these isolates are related to *B. elkanii* USDA 76.

We concluded from the SSU rRNA gene sequences that the Mexican lupine isolates are phylogenetically closely related to *B. japonicum*. Mexican soils do not harbor any native *B. japonicum* isolates which nodulate soybean (unpublished data). Our results are consistent with the observation of Ludwig et al. (30), who concluded that lupine strain DSM 30140 belonged to the genus *Bradyrhizobium* on the basis of an analysis of SSU and large-subunit rRNA genes. However, complementary DNA-DNA relatedness studies performed with a few strains (namely, Km 50-90, FN 13, and CICS 70) and *B. japonicum* USDA 6^T and USDA 110 revealed limited DNA homology (37% or less) (data not shown). This low level of DNA homology is not without precedent. It is worth mentioning that a report on DNA homology between *B. japonicum* ATCC 10324^T and *Bradyrhizobium* sp. (*Lupinus*) strain ATCC 10319 showed a very high homology value (99%), but a subsequent article reported that the American Type Culture Collection recognized that the *Lupinus* strain provided was contaminated with *B. japonicum* and a new *Lupinus*-nodulating sample exhibited only 35% homology with ATCC 10324^T (38). The similarity of the SSU rRNA gene sequences of the lupine isolates and the strains of *B. japonicum* is not inconsistent with this level of DNA homology because different bacterial species (with low levels of DNA homology) may possess SSU rRNA genes with identical or very similar nucleotide sequences (11, 43, 44). Therefore, a comprehensive analysis of DNA-DNA relatedness that includes lupine strains from diverse geographical origins and different *B. japonicum* strains will be required to define the taxonomic status of *Bradyrhizobium* spp. (*Lupinus*).

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