APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1997, p. 4748–4758 Vol. 63, No. 12
0099-2240/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Genetic Diversity of Rhizobial Symbionts Isolated from Legume Species within the Genera *Astragalus*, *Oxytropis*, and *Onobrychis*

GISÈLE LAGUERRE,[1]* PETER VAN BERKUM,[2] NOËLLE AMARGER,[1] AND DANIELLE PRÉVOST[3]

Laboratoire de Microbiologie des Sols, Centre de Microbiologie du Sol et de L'Environnement, INRA, B.V. 1540, 21034 Dijon Cedex, France[1]; Soybean and Alfalfa Research Laboratory, USDA Agricultural Research Service, Beltsville, Maryland 20705[2]; and Centre de Recherches et de Développement sur les Sols et les Grandes Cultures, Agriculture et Agroalimentaire Canada, Sainte-Foy, Québec, Canada GIV 2J3[3]

Received 24 January 1997/Accepted 3 October 1997

The genetic diversity of 44 rhizobial isolates from *Astragalus*, *Oxytropis*, and *Onobrychis* spp. originating from different geographic locations was evaluated by mapped restriction site polymorphism (MRSP) analysis of 16S rRNA genes and by PCR DNA fingerprinting with repetitive sequences (REP-PCR). A comparison of tree topologies of reference strains constructed with data obtained by MRSP and by 16S rRNA gene sequence analyses showed that the topologies were in good agreement, indicating that the MSRP approach results in reasonable estimates of rhizobial phylogeny. The isolates were distributed into 14 distinct 16S rRNA gene types clustering into three major groups which corresponded with three of the genera within the legume symbionts. Most of the isolates were within the genus *Mesorhizobium*. Five were identified with different genomic species nodulating *Lotus* spp. and *Cicer arietinum*. Three *Astragalus* isolates were classified as *Bradyrhizobium*, one being similar to *Bradyrhizobium elkanii* and another being similar to *Bradyrhizobium japonicum*. Six of the isolates were related to species within the genus *Rhizobium*. Two were similar to *Rhizobium leguminosarum*, and the remainder were identified as *Rhizobium gallicum*. DNA fingerprinting by REP-PCR revealed a high level of diversity within single 16S ribosomal DNA types. The 44 isolates were distributed into 34 REP groups. Rhizobial classification at the genus and probably also the species levels was independent of geographic origin and host plant affinity.

The legume genus *Astragalus* is the largest in the plant family Fabaceae and is distributed among more than 100 subdivisions (1). This genus is taxonomically related to the genus *Oxytropis*, and both genera belong to the tribe Galegeae (36). *Astragalus* species are common in the northern half of the Northern Hemisphere, extending into the Arctic, and in the mountains of South America and Asia Minor. Species of *Oxytropis* are distributed throughout the north temperate, the subarctic, and the arctic regions. Emphases of earlier nodulation studies with species of *Astragalus* and/or *Oxytropis* were soil improvement and maintenance of reindeer herds in arctic regions. Also, these investigations were focused on species that could be used for forage, as a source of gum, or as indicators of selenium and uranium (1).

From about 1928 to 1960, communications which describe the characteristics of rhizobia of these legume species were inadequate for identification purposes. The rhizobia of *Astragalus* were divided into two cultural groups: one resembled the clover and pea rhizobia with rapid growth, and the other was similar to the slow-growing rhizobia. Isolates from *Oxytropis* were serologically similar to those from *Astragalus* (1).

More recently, rhizobia associated with *Astragalus alpinus*, *Oxytropis maydelliana*, and *Oxytropis arctobia*, three legume species indigenous to the Canadian high Arctic, were characterized for the selection of cold adaptation to improve nitrogen fixation at low temperatures (38). Independent of their plant host, 48 isolates of arctic rhizobia were divided into 11 groups by numerical analysis of phenotypic characteristics (38). All strains cross-nodulated these arctic legumes, and most of them were able to form nodules with the temperate species *Astragalus cicer*. No nodulation was observed on the remaining temperate legume species, *Coronilla varia*, *Lotus corniculatus*, *Medicago sativa*, and *Trifolium pratense*. These symbionts were acid producers similar to rhizobia belonging to the genera *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium*, the new designation of the genus of which [*Rhizobium*] *loti* is the type species (18); some grew slowly, which is a characteristic of the genus *Bradyrhizobium* but also of some rhizobia belonging to the genus *Mesorhizobium* (4, 40), or expressed nitrogenase activity in free-living culture as reported for some bradyrhizobia (21). Rhizobial isolates from *Astragalus* and from *Oxytropis* originating from Russia also nodulated *Astragalus cicer* and the temperate species *Oxytropis campanulata* (34). These isolates did not cluster with reference strains of the different genera of rhizobia as determined by numerical taxonomy. However, they were related to a cross-inoculation group which includes *Mesorhizobium loti* (33), which confirmed previous reports (7, 19, 20). Similarly, rhizobia originating from *Astragalus sinicus*, *Astragalus adsurgens*, and *Astragalus membranaceus* are phylogenetically related to *Mesorhizobium loti* based upon 16S rRNA gene sequences (35). Strains from *Astragalus sinicus*, an important winter-growing green manure in the People's Republic of China, were characterized as a new species, *Mesorhizobium huakuii* (5). It was proposed that *Mesorhizobium huakuii* was specific for *Astragalus sinicus* since estimates of DNA relatedness with strains from *Astragalus membranaceus*, *Astragalus adsurgens*, and *Astragalus aliginosus* were very low. Phyloge-

* Corresponding author. Mailing address: Laboratoire de Microbiologie des Sols, CMSE, INRA, 17, rue Sully, B.V. 1540, 21034 Dijon Cedex, France. Phone: 33 03 80 63 30 93. Fax: 33 03 80 63 32 24. E-mail: laguerre@dijon.inra.fr.

netically, *Mesorhizobium huakuii* and *Mesorhizobium loti* were shown to be closely related (53) and were classified in the same genus, *Mesorhizobium*, which also includes the chickpea-nodulating species, *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum* (18, 31, 32).

These reports would lead to the interpretation that rhizobia associated with *Astragalus* and *Oxytropis* species are closely related. However, the degree of their genetic diversity might be high because of the large number of species of *Astragalus* and *Oxytropis* and their diverse geographic origins. In the present study, we report the genetic relationships and genetic diversity among 39 isolates from several species of *Astragalus* and *Oxytropis* native to different geographic areas, mainly in North America. Five isolates from sainfoin (*Onobrychis viciifolia*, tribe Hedysareae, related to the tribe of Galegeae) were included because this legume species was effectively nodulated by rhizobia isolated from *Astragalus* and *Oxytropis* (37). The classification of rhizobia at the genus level is based largely on the phylogeny of the 16S rRNA genes, which is determined from a comparison of 16S ribosomal DNA (rDNA) nucleotide sequences (54). Since restriction fragment length polymorphism analysis of the PCR-amplified 16S rRNA genes is a rapid approach for estimating rhizobial phylogeny (24), we used mapped restriction site polymorphism (MRSP) as a classification method. PCR DNA fingerprinting with repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) sequences (REP-PCR) (8) was used to estimate genetic diversity at a higher level of resolution.

MATERIALS AND METHODS

Bacteria. The strains used in this study are listed in Table 1 and included 44 strains from *Astragalus*, *Oxytropis*, and *Onobrychis* spp. Additional reference or type strains representing species of *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, and *Agrobacterium* as well as some unclassified rhizobia from various host plants also were included.

Restriction pattern analysis and sequencing of 16S rDNA. For MRSP analysis, bacteria were grown on agar slopes of TY medium (3) at 28°C for 24 to 72 h depending on the species. Cells were suspended in sterile water, and the absorbance of the suspensions was adjusted to an optical density at 600 nm of 2. Cells were lysed with proteinase K as previously described (26). Nearly full-length 16S rDNA was amplified with the fD1 and rD1 primers (51) by mixing 5 μl of lysed cell suspensions with all PCR reagents as previously described (24). Subsamples of 8 μl of the PCR products were digested with each of eight restriction endonucleases (listed in Table 2) and the appropriate restriction buffer according to the recommendations of the manufacturer (Appligene, Illkirch, France). Analysis of digestion products by agarose gel electrophoresis was as previously described (24).

For sequencing, colonies of the bacteria were grown on the surface of modified arabinose-gluconate medium (46) and 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 these lysed cell suspensions were used in 120-μl PCR mixtures containing primers fD1 and rD1. PCR conditions for the amplification of the 16S rRNA genes were as described previously (48). The PCR products were purified by using QIAquick spin columns (Qiagen Inc., Chatsworth, Calif.) and were sequenced by using a model 370A DNA sequencer (Applied Biosystems Inc., Foster City, Calif.) as described by van Berkum et al. (48).

Analysis of MRSP and of sequence data. Each strain was assigned a composite 16S rDNA type defined by the combination of the restriction patterns obtained with the eight restriction endonucleases. The map locations of the restriction sites in the 16S rDNA genes were inferred from known gene sequences available in GenBank and checked by restriction fragment analysis of reference strains to correct possible errors in nucleotide sequences. Accession numbers of the sequences used are as follows: X67227 (*Rhizobium leguminosarum* LMG 8820), X67233 (*Rhizobium tropici* IIA CFN 299), X67234 (*Rhizobium tropici* IIB CIAT 899^T), U28916 (*Rhizobium etli* CFN 42^T), D12793 (*Rhizobium galegae* HAMBI 540^T), X67229 (*Mesorhizobium loti* NZP 2213^T), D12797 (*Mesorhizobium huakuii* CCBAU 2609^T), U07934 (*Mesorhizobium ciceri* UPM-Ca7^T), L38825 (*Mesorhizobium mediterraneum* UPM-Ca36^T), U86343 (*Rhizobium gallicum* R602sp^T), D12783 (*Sinorhizobium meliloti* USDA 1002^T), X67231 (*Sinorhizobium fredii* USDA 205^T), X68388 (*Sinorhizobium teranga* ORS 1009^T), X68390 (*Sinorhizobium saheli* ORS 609^T), D12781 (*Bradyrhizobium japonicum* USDA 6^T), Z35330 (*Bradyrhizobium japonicum* USDA 110), U35000 (*Bradyrhizobium elkanii* USDA 76^T), X67223 (*Agrobacterium tumefaciens* LMG 196), X67228 (*Agrobacterium rubi* LMG 156^T), X67225 (*Agrobacterium vitis* LMG 8750^T), X67221 (*Azorhizobium caulinodans* ORS 571^T).

For the sequence data, additional sequences of rhizobia, of closely related bacteria clustering with the *Bradyrhizobium* genus, and also of some other alpha *Proteobacteria* were used. The accession numbers were as follows: U69638 (*Bradyrhizobium japonicum* USDA 6^T), X87273 (*Bradyrhizobium* sp. [*Lupinus*] strain DSM 30140), X70401 (*Bradyrhizobium* sp. [*Acacia*] strain LMG 10689), X70403 (*Bradyrhizobium* sp. [*Acacia*] strain LMG 9966), X70404 (*Bradyrhizobium* sp. [*Enterolobium*] strain LMG 9980), X70405 (*Bradyrhizobium* sp. [*Lonchocarpus*] strain LMG 9514), U50164 (*Mesorhizobium loti* R8CS), U50165 (*Mesorhizobium loti* R88b), U50166 (*Mesorhizobium loti* ICMP 3153), M69186 (*Afipia clevelandensis*), M65248 (*Afipia felis*), M59060 (*Beijerinckia indica* ATCC 9039), S46917 (*Blastobacter denitrificans* LMC 8443^T), L26167 (*Brucella neotomae* ATCC 23459), D32226 (*Methylobacterium organophilum* JCM 2833), D12789 (*Mycoplana dimorpha* IAM 13154^T), L11663 (*Nitrobacter hamburgensis* X14^T), L11661 (*Nitrobacter winogradskyi* W^T), D12794 (*Ochrobactrum anthropi* IAM 14119^T), D25312 (*Rhodopseudomonas palustris* ATCC 17001^T), D30778 (*Rhodospirillum rubrum* ATCC 11170). Nucleotide sequences were aligned with the Clustal program (16) from the Bisance software (10).

Phylogeny from MRSP data was estimated by the parsimony method with the computer program PAUP (Phylogenetic Analysis Using Parsimony) (45). A dendrogram was also constructed by the neighbor-joining method (41) from a matrix of pairwise Euclidean distances squared-computed by using the NTSYS-pc analysis package (version 1.8; Exeter Software, Setauket, N.Y.).

Phylogeny from nucleotide sequence data was also estimated by the parsimony method with the computer program PAUP, and the robustness of the topologies was evaluated through 100 bootstrap replications (heuristic search). The Phylogenetic Inference Package (PHYLIP [15]) available in the Bisance software was used to compute a matrix of pairwise distances corrected for multiple base substitutions by the method of Kimura (22). A phylogenetic tree was constructed from the distance matrix by the neighbor-joining method, and a bootstrap confidence analysis (100 replications) was performed with the SEQBOOT and CONSENSE programs of PHYLIP.

REP-PCR fingerprinting. PCR DNA fingerprinting with the REP and ERIC primers (8, 50) and statistical cluster analysis were as previously described (49). Each of the gels was photographed with Kodak Xpan film to produce black and white negatives. These photographs were scanned into an IBM platform computer for scoring the presence of PCR products of specific molecular sizes in each of the lanes by using the computer program Pro-Score version 2.17 (DNA Proscan, Inc., Nashville, Tenn.). With this software, it is possible to score a 1 or a 0 for the presence or absence of a band at a specific molecular size in each of the lanes. All the bands in each of the lanes of all gels were scored to produce binary matrices of the images. The matrices were exported from Pro-Score as text files and were modified for analysis with NTSYS-pc. The similarities between lanes were estimated with simple matching coefficients, and phenograms (not shown) were produced by the Sahn clustering analysis program.

Nucleotide sequence accession number. The 16S rRNA gene sequence of the *Bradyrhizobium elkanii* type strain USDA 76 has been deposited in the GenBank database under accession no. U35000.

RESULTS

MRSP analysis of PCR-amplified 16S rRNA genes. A single DNA fragment of about 1,480 bp representing the 16S rRNA genes was amplified with each of the 44 isolates from *Astragalus* spp., *Oxytropis* spp., and *Onobrychis* spp. The restriction patterns, following digestion with eight restriction endonucleases, were compared to those of type or reference strains. A total of 98 restriction sites were identified, and these represented about 380 bp of the 16S rRNA genes since four-base cutting restriction enzymes were used. Of those 98 restriction sites, 69 sites were polymorphic (Table 2).

From the combined data, we identified 14 composite 16S rDNA types among the 44 isolates (Table 3) and a total of 36 types when the reference strains were included in the analysis (Tables 1 and 2). The 24, 15, and 5 rhizobial strains from different *Astragalus*, *Oxytropis*, and *Onobrychis* species were divided into 12, 6, and 2 16S rDNA types, respectively (Table 3). Among isolates originating from the same plant species, distinctly different 16S rDNA types were detected (Table 1). For example, the seven isolates from *Astragalus alpinus* belonged to five different types. None of the *Astragalus* isolates tested, including those from *Astragalus sinicus*, had the same 16S rDNA type as the reference strain for *Mesorhizobium huakuii*, which also originated from *Astragalus sinicus*. In con-

TABLE 1. Bacterial strains or 16S rDNA sequences used in this study and their distribution in 16S rDNA types

Strain	Host plant	Geographic origin[d]	Source[a] or reference(s)	16S rDNA type[b]
Rhizobium sp. (*Astragalus*) strains	*Astragalus* spp.			
USDA 3357	*A. adsurgens*	North Dakota	USDA	27
AA1	*A. alpinus*	Alberta, Canada	AgCan	28
AA2	*A. alpinus*	Alberta, Canada	AgCan	20
N1	*A. alpinus*	NWT, Canada	AgCan	25
N31	*A. alpinus*	NWT, Canada	AgCan	20
N36	*A. alpinus*	NWT, Canada	AgCan	1
N39	*A. alpinus*	NWT, Canada	AgCan	21
USDA 3348	*A. alpinus*	North Dakota	USDA	1
USDA 3855	*A. americanus*	Alaska	USDA	25
USDA 3139	*A. canadiensis*	North Dakota	USDA	35
9B2	*A. cicer*	Alberta, Canada	Nitragin	26
9B5	*A. cicer*	Alberta, Canada	Nitragin	26
9B9	*A. cicer*	Alberta, Canada	Nitragin	26
USDA 3548	*A. eucosmos*	Alaska	USDA	20
USDA 3549	*A. eucosmos*	Alaska	USDA	19
USDA 3356	*A. gummifer*	Unknown	USDA	27
USDA 3142	*A. hypoglottis*	North Dakota	USDA	31
USDA 3143	*A. hypoglottis*	South Dakota	USDA	27
USDA 3152a	*A. mollissimus*	Oklahoma	USDA	34
USDA 3147	*A. onobrychis*	Maryland	USDA	27
USDA 3135	*A. sinicus*	Japan	USDA	28
USDA 3466	*A. sinicus*	China	USDA	28
USDA 3153	*Astragalus* sp.	Maryland	USDA	27
USDA 3358	*Astragalus* sp.	China	USDA	24
Rhizobium sp. (*Oxytropis*) strains	*Oxytropis* spp.			
N33	*O. arctobia*	NWT, Canada	AgCan	20
N38	*O. arctobia*	NWT, Canada	AgCan	25
USDA 4004	*O. arctica*	Alaska	USDA	20
USDA 4003	*O. deflexa*	Alaska	USDA	19
USDA 4006	*O. deflexa*	Alaska	USDA	20
USDA 4007	*O. deflexa*	Alaska	USDA	20
N13	*O. maydelliana*	NWT, Canada	AgCan	25
N20	*O. maydelliana*	NWT, Canada	AgCan	18
N40	*O. maydelliana*	NWT, Canada	AgCan	25
OMO1	*O. monticola*	Alberta, Canada	AgCan	20
OMO2	*O. monticola*	Alberta, Canada	AgCan	20
118H1	*O. riparia*	Unknown	Nitragin	6
USDA 3119	*O. riparia*	Washington, D.C.	USDA	24
USDA 3121	*O. riparia*	Washington, D.C.	USDA	20
OS1	*O. splendens*	Alberta, Canada	AgCan	20
Rhizobium sp. (*Onobrychis*) strains	*Onobrychis* spp.			
USDA 3736	*O. transcaucasia*	Unknown	USDA	6
116A15	*O. viciifolia*	Alberta, Canada	Nitragin	6
SM2	*O. viciifolia*	Alberta, Canada	AgCan	6
USDA 3172	*O. viciifolia*	Oregon	USDA	24
USDA 3173	*O. viciifolia*	Idaho	USDA	24
Reference strains				
Rhizobium spp. strains				
R. leguminosarum bv. viciae USDA 2370^T	*Pisum sativum*	USA	USDA	1
R. leguminosarum bv. trifolii USDA 2071	*Trifolium repens*	Unknown	USDA	1
R. leguminosarum bv. phaseoli H132	*Phaseolus vulgaris*	France	24	1
R. tropici IIB CIAT 899^T	*Phaseolus vulgaris*	Colombia	29	2
R. tropici IIA CFN 299	*Phaseolus vulgaris*	Brazil	29	3
R. etli CFN 42^T	*Phaseolus vulgaris*	Mexico	42	4
R. gallicum R602sp^T	*Phaseolus vulgaris*	France	2	6
R. giardinii H152^T	*Phaseolus vulgaris*	France	2	8
R. galegae HAMBI 540^T	*Galega orientalis*	Finland	27	9
Rhizobium sp. strain USDA 3497	*Leucaena leucocephala*	USA	USDA	7
Rhizobium sp. strain OR191	*Medicago sativa*	USA	14	5
Sinorhizobium spp. strains				
S. meliloti USDA 1002^T	*Medicago sativa*	USA	USDA	13
S. meliloti CC2013	*Medicago sativa*	Australia	13	13

Continued on following page

TABLE 1—*Continued*

Strain	Host plant	Geographic origin[d]	Source[a] or reference(s)	16S rDNA type[b]
S. medicae M1, M3, M75, M102, M161	*Medicago* spp.	Syria	13, 39	14
S. fredii USDA 205^T	*Glycine max*	China	USDA	15
S. saheli ORF 609^T	*Sesbania cannabina*	Senegal	9	16
S. teranga ORF 1007	*Acacia laeta*	Senegal	9	17
Mesorhizobium spp. strains				
M. loti NZP 2213^T	*Lotus tenuis*	New Zealand	17, 18	18
M. loti NZP 2037, cluster U4	*Lotus divaricatus*	New Zealand	9, 17, 18	28
M. loti NZP 2234	*Lotus corniculatus*	New Zealand	17, 18	28
M. loti MSDJ 865	*Lotus corniculatus*	France	18, 24	27
M. huakuii CCBAU 2609^T	*Astragalus sinicus*	China	5, 18	29
M. ciceri UPM-Ca7^T	*Cicer arietinum*	Spain	18, 32	19
M. mediterraneum UPM-Ca36^T	*Cicer arietinum*	Spain	18, 31	22[c]
Mesorhizobium sp. (*Cicer*) genomic species 4 strain IC-60	*Cicer arietinum*	India	18, 31	20
Mesorhizobium sp. strain USDA 3233	*Cicer arietinum*	USA	USDA	19
Mesorhizobium sp. strain MSDJ 2184	*Coronilla varia*	France	24	23
Bradyrhizobium spp. strains				
B. japonicum USDA 6^T	*Glycine max*	Japan	USDA	30
B. japonicum USDA 110	*Glycine max*	USA	USDA	31
B. elkanii USDA 76^T, 61, 94	*Glycine max*	USA	23	35
B. elkanii USDA 340	*Glycine max*	Japan	23	35
Bradyrhizobium sp. strain MSDJ 718	*Lupinus luteus*	France	24	32
Bradyrhizobium sp. strain VK7, VK4	*Lupinus* sp.	South Africa	PPRI	33
Azorhizobium caulinodans ORS 571^T	*Sesbania rostrata*	Senegal	11	36[c]
Agrobacterium spp. strains				
A. rhizogenes A4			L. Jouanin	2
A. tumefaciens C58			GMI	10
A. rubi^T				11[c]
A. vitis^T				12[c]

[a] Sources: USDA, *Rhizobium* culture collection, Beltsville Agricultural Research Center, Beltsville, Md.; AgCan, Agriculture and Agri-Food Canada, Sainte-Foy, Quebec, Canada; Nitragin, LiphaTech, Milwaukee, Wis.; PPRI, Plant Protection Research Institute, Pretoria, South Africa; GMI, INRA, Castanet-Tolozan, France; L. Jouanin, INRA, Biologie Cellulaire, Versailles, France.

[b] The 16S rDNA types are defined in Table 2.

[c] The 16S rDNA type was determined on the basis of the predicted restriction patterns from the known 16S rDNA sequence available in the GenBank database.

[d] NWT, Northwest Territories; USA, United States.

trast, the same 16S rDNA type was present among strains isolated from different plant species and genera. For example, 16S rDNA type 24 was identified among isolates from the three plant genera, *Astragalus*, *Oxytropis*, and *Onobrychis*. Thirty isolates shared 16S rDNA types with the reference strains of various rhizobial species (Table 3). However, eight, four, and two isolates from *Astragalus* spp., *Oxytropis* spp., and *Onobrychis viciifolia*, respectively, were divided into five rDNA types, which did not correspond with those identified with any of the type and reference strains.

The genetic relationships among the 36 rDNA types were estimated from the restriction site differences shown in Table 2 by using Dollo's parsimony analysis. One hundred equally parsimonious trees were obtained from an heuristic search. The topologies of all these trees were similar. An example is shown in Fig. 1A. A very similar tree was obtained by a phenetic approach with the neighbor-joining method (not shown). The cluster E represented the classification of the *Bradyrhizobium* species and was clearly separate from the four other main clusters with strains classified as *Rhizobium* (clusters A and B), *Sinorhizobium* (cluster C), and *Mesorhizobium* (cluster D) and from *Azorhizobium caulinodans*. Differences in restriction sites between pairs of genotypes ranged from 19 to 37 in number between the *Bradyrhizobium* and the other clusters. Cluster E included one isolate from *Astragalus*, USDA 3152a, in addition to two other isolates from *Astragalus* which had 16S rDNA types identical with that of *Bradyrhizobium japonicum* USDA 110 or that of the four *Bradyrhizobium elkanii* reference strains analyzed. Differences in restriction sites ranged from 0 to 10 in number among these three isolates from *Astragalus* and the *Bradyrhizobium* strains from soybean or from lupin. Within cluster E, the arithmetic mean of differences in restriction sites was 3.7. The 13 other isolates with 16S rDNA types differing from those of the reference strains were grouped in cluster D with the species of *Mesorhizobium* and other strains from *Cicer arietinum* and *Coronilla varia*. This cluster included most of the isolates (35 of 44). Differences in restriction sites between pairs of genotypes ranged from 0 to 11 in number (arithmetic mean of 3.8) within cluster D and from 12 to 34 between this cluster and the other clusters. Arithmetic means of differences in restriction sites within clusters A, B, and C were 4.4, 8.8, and 5.6, respectively.

We compared the topologies of the phylogenetic trees constructed with the MRSP data and with aligned 16S rRNA gene nucleotide sequences. The 16S rRNA gene nucleotide sequence of *Bradyrhizobium elkanii* USDA 76^T was determined to permit such a comparison since this type strain was included in our MRSP analysis. The 16S rRNA gene nucleotide se-

TABLE 2. Data matrix of restriction sites[a] between 16S rDNA types of rhizobia and *Agrobacterium* strains[b] revealed by restriction analysis

16S rDNA type	*Alu*I 47*[c]	*Alu*I 47**[c]	*Alu*I 106	*Alu*I 122	*Alu*I 532	*Alu*I 741	*Alu*I 755	*Alu*I 788	*Alu*I 901	*Alu*I 921	*Alu*I 1190	*Alu*I 1374	*Cfo*I 42	*Cfo*I 47*[c]	*Cfo*I 320	*Cfo*I 492	*Cfo*I 952	*Cfo*I 1351	*Cfo*I 1356	*Dde*I 47*[c]	*Dde*I 108	*Dde*I 359	*Dde*I 558	*Dde*I 1034	*Dde*I 1062	*Dde*I 1173	*Hae*III −5[d]	*Hae*III 20	*Hae*III 172	*Hae*III 206	*Hae*III 357	*Hae*III 531	*Hae*III 649	*Hae*III 657
1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
2	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
3	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	0	0	0
4	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	1	0	0	0	0	1	1	0	0	0	1	1	1	0	0	0
5	0	0	0	0	1	0	0	0	0	0	1	1	1	0	1	1	0	0	0	0	0	0	0	1	1	0	0	0	1	1	1	0	0	0
6	0	0	0	0	1	0	0	0	0	0	1	1	1	0	1	1	0	0	1	0	0	0	0	1	1	0	0	0	1	1	1	0	0	0
7	0	0	0	0	1	0	0	0	0	0	1	1	1	0	1	1	0	0	1	0	0	0	0	1	1	0	0	0	1	1	1	0	0	0
8	0	0	0	0	1	0	0	0	1	0	1	1	1	0	1	1	0	0	1	0	1	1	0	1	1	0	0	0	0	1	1	0	0	0
9	0	0	0	1	1	0	0	0	0	1	1	1	1	0	1	1	0	0	1	0	0	1	0	1	1	0	0	0	0	1	0	0	0	0
10	0	0	1	0	1	0	0	0	1	0	1	1	0	0	1	1	0	0	1	0	0	1	0	1	1	0	0	0	0	1	1	0	0	0
11	0	0	1	0	1	0	0	0	1	0	1	1	0	0	1	1	0	0	1	0	0	1	0	1	1	0	0	0	0	1	1	0	0	0
12	0	0	1	0	1	0	0	0	1	0	1	1	1	0	1	1	0	0	1	0	0	1	0	1	1	0	0	0	0	1	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
14	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
15	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
16	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
17	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
18	0	0	0	1	0	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
19	0	0	0	1	0	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
20	0	0	0	1	0	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
21	0	0	0	1	0	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	1	0	1	0	0	1	1	0	0	0
22	0	0	0	1	0	1	0	1	0	0	1	0	1	0	1	1	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0
23	0	0	0	1	0	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
24	0	0	0	1	0	1	0	1	0	0	1	0	1	0	1	1	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
25	0	0	0	1	0	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
26	0	0	0	1	0	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
27	0	0	0	1	0	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
28	0	0	0	1	0	1	0	1	0	0	1	0	1	0	1	1	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
29	0	0	0	1	0	1	0	1	0	0	1	0	1	0	1	1	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
30	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	0	1	0	1	0	0	1
31	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	0	0	1	0	1	0	0	1
32	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	1	0	1	0	0	1
33	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	1	1	0	0	1	0	1	0	0	1
34	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	1	0	0	1	0	1	0	0	1
35	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	1	1	1	0	0	1	0	1	0	0	1
36	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	1	0	1	0

[a] The position of each restriction site is given according to the nucleotide numbering of the sequence of *Rhizobium leguminosarum* ATCC 14480 (GenBank accession no. X67227) after alignment of the 16S rDNA sequences of rhizobia and *Agrobacterium* strains available in GenBank. The fD1 primer is −20 bp upstream of position 1; the rD1 primer corresponds to positions 1437 and 1453. Only the restriction sites that are polymorphic are given. The following restriction sites are conserved among the sample of strains examined in this study: *Alu*I 187, *Alu*I 229, *Alu*I 373, *Alu*I 775, *Alu*I 982, *Cfo*I 772, *Cfo*I 887, *Cfo*I 1022, *Dde*I −4 (restriction site located on primer fD1), *Dde*I 235, *Dde*I 262, *Dde*I 671, *Dde*I 1209, *Hae*III 272, *Hae*III 843, *Hae*III 1121, *Hae*III 1300, *Hin*fI 1251, *Msp*I 418, *Msp*I 1074, *Msp*I 1296, *Nde*II −13 (restriction site localized on primer fD1), *Nde*II 220, *Nde*II 244, *Nde*II 327, *Nde*II 1268, *Nde*II 1436, *Rsa*I 805.

[b] The distribution of the strains among 16S rDNA types is given in Table 1.

[c] Restriction sites located within the 72-bp insertion present in *Rhizobium tropici* IIA strains (52) at position 47 according to the nucleotide numbering of the sequence of *Rhizobium leguminosarum* ATCC 14480. *Alu*I 47*, *Cfo*I 47*, *Dde*I 47*, and *Nde*II 47* generated restriction fragments of 89, 56, 106, and 100 bp, respectively. The restriction fragment generated by *Alu*I 47*-*Alu*I 47** was 50 bp.

[d] Restriction site located within a 6-bp insertion present in these strains upstream of position 1 according to the nucleotide numbering of the sequence of *Rhizobium leguminosarum* ATCC 14480 (47).

[e] The position of these restriction sites missing in the known sequences was estimated.

quences of *Afipia* and *Nitrobacter* species, *Rhodopseudomonas palustris*, *Blastobacter denitrificans*, *Bradyrhizobium* sp. (*Lupinus*) strain DSM 30140, and *Bradyrhizobium japonicum* were more similar (similarity values ranging from 99.6 to 97.3%) than the sequences between *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* (96.3% homology), even though the latter two are classified within the same genus and nodulate the same legumes. The 16S rRNA gene sequences of *Bradyrhizobium elkanii* and the bradyrhizobia isolated from several tropical leguminous trees (12) were very similar (99.4% homology). The topologies of the phylogenetic trees constructed from the sequencing data by the distance matrix method with the neighbor-joining algorithm (Fig. 1B) and by parsimony analysis (not shown) were very similar. Also, the topologies of trees constructed from the MRSP and the sequencing data corresponded well (Fig. 1A and B), from which we concluded that MRSP is a powerful preliminary approach for the determination of the putative phylogeny of newly isolated legume symbionts.

REP-PCR DNA fingerprinting. The diversity of isolates within each of the 16S rDNA types was determined by REP- and ERIC-PCR DNA fingerprinting. The reproducibility of

TABLE 2—*Continued*

*Hae*III 920 or 1040[c]	*Hae*III 950	*Hae*III 1378	*Hae*III 1421	*Hin*fI 82	*Hin*fI 277	*Hin*fI 473	*Hin*fI 569	*Hin*fI 920 or 800[c]	*Hin*fI 933	*Msp*I 109	*Msp*I 129	*Msp*I 382	*Msp*I 404	*Msp*I 527	*Msp*I 540	*Msp*I 573	*Msp*I 659	*Msp*I 914	*Msp*I 920	*Msp*I 926	*Msp*I 951	*Msp*I 1375	*Nde*II 47[*c]	*Nde*II 166	*Nde*II 502	*Nde*II 562	*Nde*II 914	*Nde*II 930	*Nde*II 949	*Nde*II 1181	*Rsa*I 87	*Rsa*I 401	*Rsa*I 663	*Rsa*I 1306
0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	1
0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1
0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	1
0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	1	1	1	0	0	0	0	1	0	0	1
0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	1	1	1	0	0	0	0	1	0	0	1
0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	1
1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	1
0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	1
0	0	0	0	1	0	0	0	0	0	1	0	1	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1
0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
0	1	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
0	0	0	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1
0	0	0	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	1
0	0	0	0	1	0	0	1	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	1
0	0	0	0	1	0	0	1	0	0	0	0	1	1	1	1	0	1	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1
0	0	0	0	1	0	0	1	0	0	0	0	1	1	1	1	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
0	0	0	0	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
0	0	0	0	1	0	0	1	0	1	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
1	0	0	0	1	0	0	1	0	1	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1
0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1
0	0	0	0	1	1	0	1	0	1	1	0	0	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
0	0	0	0	1	1	0	1	0	0	1	0	0	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
0	0	0	0	1	1	0	1	0	1	1	0	0	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
0	0	1	1	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	1	1	0	1
0	0	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	1	1	0	1
0	0	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	1	1	0	1
0	0	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	1	1	0	1
0	0	1	1	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	1	1	0	1	0	1	0	0	0	0	1	1	0	1
0	0	1	1	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	1	1	0	1	0	1	0	0	0	0	1	1	0	1
0	0	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0

the REP-PCRs was checked for some strains by comparison of the banding patterns obtained independently in two different laboratories. The major bands were reproducible, and the strains were recognizable by the banding patterns obtained in the two laboratories. Both REP- and ERIC-PCR analyses yielded similar levels of resolution. From the data, we were able to differentiate most isolates. Several classified within the same 16S rDNA types produced very similar fingerprints and were grouped together (Fig. 2). Thirty-four groups of REP- plus ERIC-PCR fingerprints (REP groups) were recorded among the 44 isolates (Table 3). Based upon data from the REP-PCR analysis, we observed that within most of the 16S rDNA types the isolates were genetically heterogeneous. Within 16S rDNA type 20, which was the largest group, none of the 11 isolates appeared to be closely related, although some were isolated from the same plant species and originated from the same geographic region (e.g., Fig. 2B and E, strains USDA 4006 and USDA 4007, lanes D and B; strains OMO1 and OMO2, lanes N and O). In contrast, isolates from different plant species (in rDNA types 25 and 27) or genera (in rDNA types 6 and 24) and/or from different geographic locations had similar electrophoretic patterns. For example, within 16S rDNA type 6, the REP and ERIC electrophoretic patterns of strain 116A15 from *Onobrychis* (Fig. 2C and F, lane B) were similar to those of strain 118H1 from *Oxytropis* (Fig. 2B and E, lane G); within 16S rDNA type 24, the patterns produced with strain USDA 3358 isolated from *Astragalus* in China (Fig. 2A and D, lane M) were similar to those of strain USDA 3119 isolated from *Oxytropis* in the United States (Fig. 2B and E, lane E).

DISCUSSION

The usefulness of PCR-restriction fragment length polymorphism analysis of rhizobial 16S rDNA to obtain preliminary estimates of phylogenetic classification for identification purposes has been demonstrated previously (24). We have improved the method by constructing a database of mapped restriction sites in the 16S rRNA genes of bacteria in the family *Rhizobiaceae*. Restriction site analysis is more reliable because, in pairwise comparisons, it eliminates the problem of scoring restriction fragments of identical molecular size but corresponding to different gene regions. It also facilitates the detection of double or triple bands of similar length in a single

TABLE 3. Diversity of REP groups within the 16S rDNA types and according to the host plant of origin

16S rDNA type[a]	No. of strains	Distribution of REP groups[b] among:		
		Astragalus spp.	*Oxytropis* spp.	*Onobrychis* spp.
1 (*R. leguminosarum*)	2	a, b		
6 (*R. gallicum*)	4		c	c, d, e
18 (*M. loti* NZP2213^T)	1		f	
19 (*M. ciceri*)	2	g	h	
20 (*Mesorhizobium* sp. [*Cicer*] strain IC60)	11	i, j, k	l, m, n, o, p, q, r, s	
21	1	t		
24	4	u1	u2	v, v
25	5	w, x	y1, y2, z	
26	3	α1, α2, α3		
27 (*M. loti* MSDJ865)	5	β, β, χ1, χ1, χ2		
28 (*M. loti* NZP2234)	3	δ, ε1, ε2		
31 (*B. japonicum* USDA 110)	1	ϕ		
34	1	γ		
35 (*B. elkanii*)	1	η		
No. of 16S rDNA types		12	6	2

[a] 16S rDNA types are defined in Table 2. Some of these genotypes included type (T) and/or reference strains of the species indicated in parentheses (see also Table 1).
[b] The classification of each strain into REP groups is given in Fig. 2.

strain. Besides the greater definition, the restriction maps produced in this study enable classification of newly isolated rhizobia by scoring restriction site polymorphism without having any longer to include reference strains in the experiment.

Twenty species of rhizobia belonging to five genera have been described elsewhere (2, 6, 18, 54). Reference strains belonging to 17 of these recognized species were analyzed in this study, and all these species were differentiated with the MRSP analysis. An exception was *Rhizobium tropici* IIB and *Agrobacterium rhizogenes*, which could not be differentiated by restriction site analysis of the 16S rRNA genes as was reported previously (24). The isolates from the three legume hosts examined in our study were classified within three of the genera, *Rhizobium Mesorhizobium*, and *Bradyrhizobium*.

Although only a few isolates from each plant species were sampled, two to five distinct 16S rDNA types were identified among the symbionts of 10 of the 12 plant species. Several of the 16S rDNA types were shared by isolates which had originated from different host legumes. We made similar observations with the REP-PCR analysis but at a finer level of classification. This method is highly discriminating, which permitted identification of genetic diversity at the intraspecies level (8, 25, 46). From the REP-PCR data, we identified only three examples in which rhizobia from a single plant species appeared to be closely related (the two strains from *Astragalus sinicus*, two strains from *Onobrychis viciifolia*, and three strains from *Astragalus cicer*). These results agree with previous classifications by serology (1), by numerical taxonomy (34, 38), and by cross-infection experiments in which rhizobia from *Astragalus*, *Oxytropis*, and *Onobrychis* spp. were grouped independently of their plant origin (37, 38).

We also concluded from our data that rhizobial classification was independent of origin since isolates with different 16S rDNA types were from the same geographic regions. For example, the four *Astragalus* isolates from North Dakota had four distinct 16S rDNA types and were classified within three rhizobial genera. Similarly, the 16 isolates originating from the Arctic regions (Alaska and Northwest Territories of Canada) were distributed into six 16S rDNA types. Some of these 16S rDNA types also included isolates which had originated from temperate climatic regions. However, we identified from the REP-PCR analysis that genetically similar rhizobia may be present in soils of different continents.

Most of the isolates (35 of 44) were classified within the genus *Mesorhizobium*. This is consistent with the reports indicating that *Astragalus* and *Oxytropis* rhizobia are related to *Mesorhizobium loti* based on partial sequencing of 16S rRNA genes (35), phage sensitivity determinations (33), and cross-inoculation experiments (33, 34). Our classification of the *Coronilla* isolate within this genus was consistent with the report that isolates of *Coronilla varia* nodulated *Astragalus cicer* (34). Most, but not all, temperate-zone isolates of *Astragalus* and *Oxytropis* nodulate *Lotus* species (33, 34). However, the eight arctic strains that we grouped in this genus do not nodulate *Lotus corniculatus* or *Coronilla varia* (38). This difference in host range between the arctic and temperate-region rhizobia of *Astragalus* and *Oxytropis* may indicate variability in genetic determinants for nodulation and/or variation in susceptibility of host legumes for nodulation by these rhizobia.

There were nine distinct 16S rDNA types among the 35 strains within the genus *Mesorhizobium*, and five of these types included reference strains of rhizobia which nodulate *Lotus* spp. or *Cicer arietinum*. The phylogenetic positions of these isolates were independent of host plant origin and were intertwined with those of the reference strains of *Mesorhizobium loti*, *Mesorhizobium ciceri*, *Mesorhizobium mediterraneum*, *Mesorhizobium* genomic species 4 (*Cicer arietinum*), *Mesorhizobium* sp. (*Coronilla*), and *Mesorhizobium huakuii*. Two isolates of *Astragalus sinicus* had the same 16S rDNA type as one of the reference strains of *Mesorhizobium loti* (NZP 2234) and were differentiated from *Mesorhizobium huakuii*. Our report and those of others (31, 43) describing the lack of a relationship between rhizobial 16S rDNA type within *Mesorhizobium* and host plant affinity complicate the rhizobial phylogenetic schemes. On the basis of DNA-DNA hybridizations, rhizobia classified as *Mesorhizobium loti* represented several different genomic species because they had less than 50% DNA homology and had 16S rRNA genes with more than two nucleotide mismatches (43). Although MRSP is less sensitive than sequencing analysis for estimating phylogeny, five to seven differences in restriction sites were identified between the 16S rRNA genes of two reference strains of *Mesorhizobium loti* analyzed in our study and the type strain, which may indicate that these strains also represent distinctly different genomic species. This would be consistent with DNA-DNA hybridization data indicating that the type strain, NZP 2213, and the

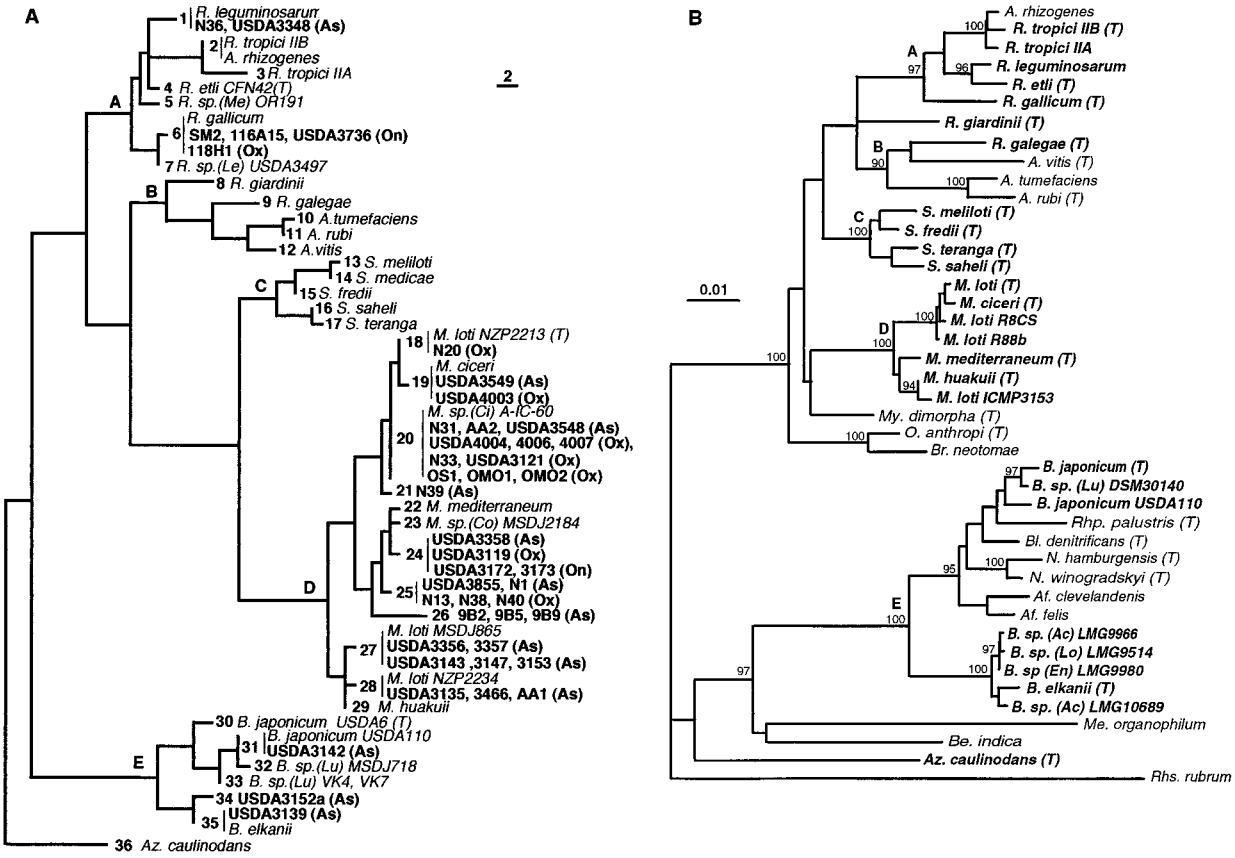

FIG. 1. Phylogenetic trees showing the relationships between rhizobia and several related taxa from the alpha subgroup of the *Proteobacteria* on the basis of 16S rRNA gene analyses. (A) One of the 100 most parsimonious trees obtained from the MRSP data by the parsimony method; the tree length was 183 steps. (B) Tree obtained from the nucleotide sequence data by the neighbor-joining method; significant bootstrap probability values are indicated at the branching points (only values greater than 90% are shown). The horizontal branches are drawn proportionally to the number of restriction site changes (A) or nucleotide substitutions per site (B). *R*., *Rhizobium*; *A*., *Agrobacterium*; *S*., *Sinorhizobium*; *M*., *Mesorhizobium*; *My*., *Mycoplana*; *O*., *Ochrobactrum*; *Br*., *Brucella*; *B*., *Bradyrhizobium*; *Rhp*., *Rhodopseudomonas*; *Bl*., *Blastobacter*; *N*., *Nitrobacter*; *Af*., *Afipia*; *Me*., *Methylobacterium*; *Be*., *Beijerinckia*; *Az*., *Azorhizobium*; *Rhs*., *Rhodospirillum*; As, *Astragalus*; Me, *Medicago*; On, *Onobrychis*; Ox, *Oxytropis*; Le, *Leucaena*; Ci, *Cicer*; Co, *Coronilla*; Lu, *Lupinus*; Ac, *Acacia*; Lo, *Lonchocarpus*; En, *Enterolobium*; (T), type strain.

reference strain NZP 2234 included in our study were only 60% related (7). The lateral transfer of symbiotic genes from introduced rhizobia to closely related nonsymbiotic soil bacteria was one of the proposed mechanisms by which this diversity arose (43, 44).

Three of the *Astragalus* isolates were classified as *Bradyrhizobium*, and their phylogenetic positions were intermixed with the bradyrhizobia of soybean and lupin. We conclude from the 16S rRNA gene sequences that the soybean bradyrhizobia are phylogenetically divergent, which is consistent with their separation into two distinct species (23). The phylogenetic distances of *Bradyrhizobium elkanii* and two strains of *Bradyrhizobium japonicum* were greater than those among *Rhodopseudomonas palustris*, *Blastobacter denitrificans*, the *Afipia* and the *Nitrobacter* species, and *Bradyrhizobium japonicum*. The 16S rRNA gene nucleotide sequences among strains of *Bradyrhizobium elkanii* probably are very similar because no polymorphism was detected among the four strains of *Bradyrhizobium elkanii* examined by MRSP analysis. In contrast, five differences in restriction sites were observed between the two strains of *Bradyrhizobium japonicum* included in this analysis. Therefore, *Bradyrhizobium elkanii* may be phylogenetically more homogeneous than *Bradyrhizobium japonicum*. The reported nucleotide sequence of the lupin type strain DSM 30140 (USDA 3051, ATCC 10319) has indicated a phylogenetic relationship within *Bradyrhizobium* close to that of *Bradyrhizobium japonicum* (28), but a relationship with the other closely related genera was not presented. Similarly, we conclude from our work that at least some other lupin strains are closely related to *Bradyrhizobium japonicum*. Both from MRSP and from the sequencing data, we identified two subgroups within the *Bradyrhizobium* cluster, one including *Bradyrhizobium japonicum* and several lupin strains and the other including *Bradyrhizobium elkanii* and bradyrhizobia of several tropical leguminous trees (12, 30). The three bradyrhizobia isolated from *Astragalus* spp. were distributed between these two subgroups.

The six isolates within the genus *Rhizobium* also were phylogenetically heterogeneous. They represented two 16S rDNA types which differed by seven restriction sites. Two isolates had the same 16S rDNA type as *Rhizobium leguminosarum*, and the others were identified with *Rhizobium gallicum*, a species recently proposed which was isolated from *Phaseolus vulgaris* growing in French soil (2).

Our results demonstrate that the three genera of legumes, *Astragalus*, *Oxytropis*, and *Onobrychis*, native to arctic and temperate climatic zones, have widely divergent symbionts at the genus level. The polymorphism detected in their 16S rRNA genes with patterns in some cases matching those of different

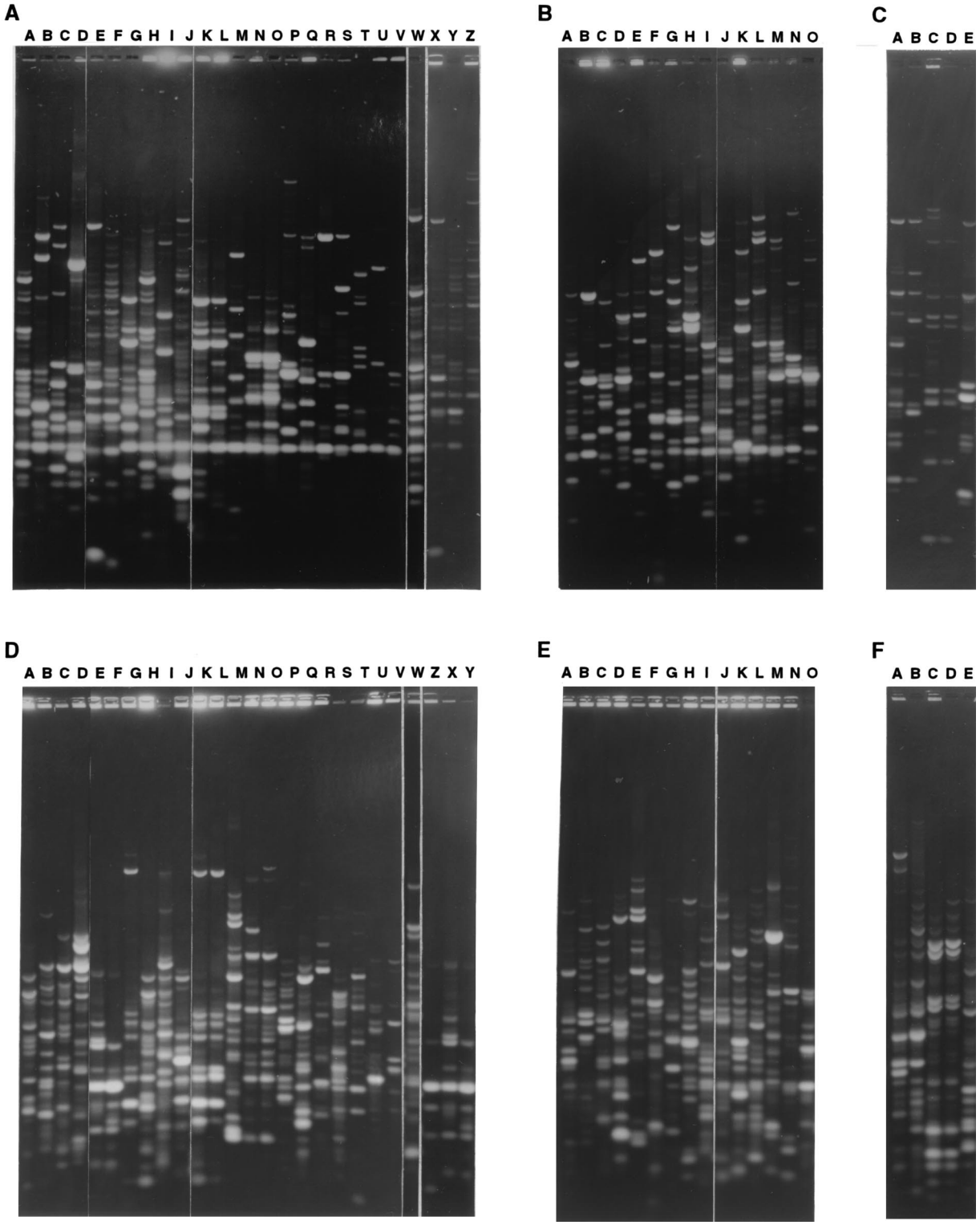

FIG. 2. Electrophoretic patterns generated by REP (A to C)- and ERIC (D to F)-PCR among *Astragalus*, *Oxytropis*, and *Onobrychis* rhizobial strains. (A and D) Lanes A through Z, *Astragalus* strains USDA 3357 (β), USDA 3855 (w), USDA 3348 (a), USDA 3139 (η), 9B2 (α1), 9B9 (α2), USDA 3356 (χ1), USDA 3143 (β), USDA 3142 (ϕ), USDA 3152a (γ), USDA 3147 (χ1), USDA 3153 (χ2), USDA 3358 (u1), USDA 3135 (ε1), USDA 3466 (ε2), USDA 3548 (k), USDA 3549 (g), N1 (x), N31 (i), N36 (b), AA1 (δ), AA2 (j), N39 (t), 9B2 (α1), 9B9 (α2), and 9B5 (α3), respectively. (B and E) Lanes A through O, *Oxytropis* strains USDA 4004 (l), USDA 4007 (n), USDA 4003 (h), USDA 4006 (o), USDA 3119 (u2), USDA 3121 (p), 118H1 (c), N33 (m), N38 (y1), N13 (z), N20 (f), N40 (y2), OS1 (q), OMO1 (r), and OMO2 (s), respectively. (C and F) lanes A through E, *Onobrychis* strains SM2 (d), 116A15 (c), USDA 3172 (v), USDA 3173 (v), and USDA 3736 (e), respectively. The result of the clustering into REP groups from the combined data of REP plus ERIC PCRs is indicated within parentheses for each strain; closely related fingerprints (similarity values greater than 80% according to the statistical clustering analysis [not shown]) were assigned the same letter.

type strains may be an indication that these rhizobial symbionts are diverse at the species level as well.

ACKNOWLEDGMENTS

We thank F. Revoy and K. Lee Nash for technical assistance and Desta Beyene for providing quality control of the cultures.

This work was partly funded by le Fond pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR), Action concertée de soutien à la coopération scientifique internationale, Gouvernement du Québec, Canada.

REFERENCES

1. **Allen, O. N., and E. K. Allen.** 1981. The *Leguminosae*. University of Wisconsin Press, Madison, Wis.
2. **Amarger, N., V. Macheret, and G. Laguerre.** 1997. *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. Int. J. Syst. Bacteriol. **47:**996–1006.
3. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84:**188–198.
4. **Chen, W., E. Wang, S. Wang, Y. Li, X. Chen, and Y. Li.** 1995. Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xianjiang, People's Republic of China. Int. J. Syst. Bacteriol. **45:**153–159.
5. **Chen, W. X., G. S. Li, Y. L. Qi, E. T. Wang, H. L. Yuan, and J. L. Li.** 1991. *Rhizobium huakuii* sp. nov. isolated from the root nodules of *Astragalus sinicus*. Int. J. Syst. Bacteriol. **41:**275–280.
6. **Chen, W. X., Z. Y. Tan, J. L. Gao, Y. Li, and E. T. Wang.** 1997. *Rhizobium hainanense* sp. nov., isolated from tropical legumes. Int. J. Syst. Bacteriol. **47:**870–873.
7. **Crow, V. L., B. D. W. Jarvis, and R. M. Greenwood.** 1981. Deoxyribonucleic acid homologies among acid-producing strains of *Rhizobium*. Int. J. Syst. Bacteriol. **31:**152–172.
8. **de Bruijn, F. J.** 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. **58:**2180–2187.
9. **De Lajudie, P., A. Willems, B. Pot, D. Dewettinck, G. Maestrojuan, M. Neyra, M. D. Collins, B. Dreyfus, K. Kersters, and M. Gillis.** 1994. Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. Int. J. Syst. Bacteriol. **44:**715–733.
10. **Dessen, P., C. Fondrat, C. Valencien, and C. Mugnier.** 1990. Bisance: a French service for access to biomolecular sequence databases. CABIOS **6:**355–356.
11. **Dreyfus, B., J. L. Garcia, and M. Gillis.** 1988. Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. Int. J. Syst. Bacteriol. **38:**89–98.
12. **Dupuy, N., A. Willems, B. Pot, D. Dewettinck, I. Vandenbruaene, G. Maestrojuan, B. Dreyfus, K. Kersters, M. D. Collins, and M. Gillis.** 1994. Phenotypic and genotypic characterization of bradyrhizobia nodulating the leguminous tree *Acacia albida*. Int. J. Syst. Bacteriol. **44:**461–473.
13. **Eardly, B. D., L. A. Materon, N. H. Smith, D. A. Johnson, M. D. Rumbaugh, and R. K. Selander.** 1990. Genetic structure of natural populations of the nitrogen-fixing bacterium *Rhizobium meliloti*. Appl. Environ. Microbiol. **56:**187–194.
14. **Eardly, B. D., J. P. W. Young, and R. K. Selander.** 1992. Phylogenetic position of *Rhizobium* sp. strain Or 191, a symbiont of both *Medicago sativa* and *Phaseolus vulgaris*, based on partial sequences of the 16S rRNA and *nifH* genes. Appl. Environ. Microbiol. **58:**1809–1815.
15. **Felsenstein, J.** 1989. PHYLIP—phylogeny inference package (version 3.2). Cladistics **5:**164–166.
16. **Higgins, D. G., and P. M. Sharp.** 1988. Clustal: a package for performing multiple alignment on a microcomputer. Gene **73:**237–244.
17. **Jarvis, B. D. W., C. E. Pankhurst, and J. J. Patel.** 1982. *Rhizobium loti*, a new species of legume root nodule bacteria. Int. J. Syst. Bacteriol. **32:**378–380.
18. **Jarvis, B. D. W., P. van Berkum, W. X. Chen, S. M. Nour, M. P. Fernandez, J. C. Cleyet-Marel, and M. Gillis.** 1997. Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. Int. J. Syst. Bacteriol. **47:**895–898.
19. **Jensen, H. L.** 1967. Mutual host plant relationships in two groups of legume root nodule bacteria (*Rhizobium* spp.). Arch. Microbiol. **59:**174–179.
20. **Jensen, H. L., and A. L. Hansen.** 1968. Observations on host plant relations in root nodule bacteria of the *Lotus-Antythyllis* and *Lupinus-Ornithopus* groups. Acta Agric. Scand. **18:**135–142.
21. **Jordan, D. C.** 1982. Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing root nodule bacteria from leguminous plants. Int. J. Syst. Bacteriol. **32:**136–139.
22. **Kimura, M.** 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16:**111–120.
23. **Kuykendall, L. D., B. Saxena, T. E. Devine, and S. E. Udell.** 1992. Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. Can. J. Microbiol. **38:**501–505.
24. **Laguerre, G., M. R. Allard, F. Revoy, and N. Amarger.** 1994. Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. Appl. Environ. Microbiol. **60:**56–63.
25. **Laguerre, G., P. Mavingui, M. R. Allard, M. P. Charnay, P. Louvrier, S. I. Mazurier, L. Rigottier-Gois, and N. Amarger.** 1996. Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. Appl. Environ. Microbiol. **62:**2029–2036.
26. **Lemanceau, P., T. Corberand, L. Gardan, X. Latour, G. Laguerre, J. M. Boeufgras, and C. Alabouvette.** 1995. Effect of two plant species, flax (*Linum usitatissinum* L.) and tomato (*Lycopersicon esculentum* Mill.), on the diversity of soilborne populations of fluorescent pseudomonads. Appl. Environ. Microbiol. **61:**1004–1012.
27. **Lindström, K.** 1989. *Rhizobium galegae*, a new species of legume root nodule bacteria. Int. J. Syst. Bacteriol. **39:**365–367.
28. **Ludwig, W., R. Rossello-Mora, R. Aznar, S. Klugbauer, S. Springs, K. Reetz, C. Beimfohr, E. Brockmann, G. Kirchhof, S. Dorn, M. Bachleitner, N. Klugbauer, N. Springer, D. Lane, R. Nietupsky, M. Weizenegger, and K.-H. Schleifer.** 1995. Comparative sequence analysis of 23S rRNA from proteobacteria. Syst. Appl. Microbiol. **18:**164–188.
29. **Martinez-Romero, E., L. Segovia, F. M. Mercante, A. A. Franco, P. Graham, and M. A. Pardo.** 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. **41:**417–426.
30. **Moreira, F. M. S., M. Gillis, B. Pot, K. Kersters, and A. A. Franco.** 1993. Characterization of rhizobia isolated from different divergence groups of tropical Leguminosae by comparative polyacrylamide gel electrophoresis of their total proteins. Syst. Appl. Microbiol. **16:**135–146.
31. **Nour, S. M., J. C. Cleyet-Marel, P. Normand, and M. P. Fernandez.** 1995. Genotypic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. Int. J. Syst. Bacteriol. **45:**640–648.
32. **Nour, S. M., M. P. Fernandez, P. Normand, and J. C. Cleyet-Marel.** 1994. *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). Int. J. Syst. Bacteriol. **44:**511–522.
33. **Novikova, N. I., E. A. Pavlova, and E. V. Limeshchenko.** 1993. Phage sensitivity and host range of *Rhizobium* strains isolated from temperate legumes. Plant Soil **151:**45–53.
34. **Novikova, N. I., E. A. Pavlova, N. I. Vorobjev, and E. V. Limeshchenko.** 1994. Numerical taxonomy of *Rhizobium* strains from legumes of the temperate zone. Int. J. Syst. Bacteriol. **44:**734–742.
35. **Oyaizu, H., N. Naruhashi, and T. Gamou.** 1992. Molecular methods of analysing bacterial diversity: the case of rhizobia. Biodivers. Conserv. **1:**237–249.
36. **Polhill, R. M., and P. H. Raven (ed.).** 1981. Advances in legume systematics, part 1. Royal Botanic Gardens, Kew, England.
37. **Prévost, D., L. M. Bordeleau, and H. Antoun.** 1987. Symbiotic effectiveness of indigenous arctic rhizobia on a temperate forage legume: sainfoin (*Onobrychis viciifolia*). Plant Soil **104:**63–67.
38. **Prévost, D., L. M. Bordeleau, S. Caudry-Reznick, H. M. Schulman, and H. Antoun.** 1987. Characteristics of rhizobia isolated from three legumes indigenous to the Canadian high arctic: *Astragalus alpinus*, *Oxytropis maydelliana* and *Oxytropis arctobia*. Plant Soil **98:**313–324.
39. **Rome, S., M. P. Fernandez, B. Brunel, P. Normand, and J. C. Cleyet-Marel.** 1996. *Sinorhizobium medicae* sp. nov., isolated from annual *Medicago* spp. Int. J. Syst. Bacteriol. **46:**972–980.
40. **Ruiz-Argüeso, T., E. Cadahia, and A. Leyva.** 1988. Physiology and genetics of *Rhizobium* nodulating chickpeas (*Cicer arietinum* L.), p. 109–119. *In* D. P. Beck and L. A. Materon (ed.), Nitrogen fixation by legumes in Mediterranean agriculture. International Center for Agricultural Research in the Dry Areas, Aleppo, Syria.
41. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
42. **Segovia, L., J. P. W. Young, and E. Martinez-Romero.** 1993. Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I strains as *Rhizobium etli* sp. nov. Int. J. Syst. Bacteriol. **43:**374–377.
43. **Sullivan, J. T., B. D. Eardly, P. van Berkum, and C. W. Ronson.** 1996. Four unnamed species of nonsymbiotic rhizobia isolated from the rhizosphere of *Lotus corniculatus*. Appl. Environ. Microbiol. **62:**2818–2825.
44. **Sullivan, J. T., H. N. Patrick, W. L. Lowther, D. B. Scott, and C. W. Ronson.** 1995. Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. Proc. Natl. Acad. Sci. USA **92:**8985–8989.
45. **Swofford, D. L.** 1993. PAUP: phylogenetic analysis using parsimony, version 3-1.1. Illinois Natural History Survey, Champaign, Ill.
46. **van Berkum, P.** 1990. Evidence for a third uptake hydrogenase phenotype among the soybean bradyrhizobia. Appl. Environ. Microbiol. **56:**3835–3841.
47. **van Berkum, P.** Unpublished data.

48. **van Berkum, P., D. Beyene, and B. D. Eardly.** 1996. Phylogenetic relationships among *Rhizobium* species nodulating the common bean (*Phaseolus vulgaris* L.). Int. J. Syst. Bacteriol. **46:**240–244.
49. **van Berkum, P., R. B. Navarro, and A. A. T. Vargas.** 1994. Classification of the uptake hydrogenase-positive (Hup^+) bean rhizobia as *Rhizobium tropici*. Appl. Environ. Microbiol. **60:**554–561.
50. **Versalovic, J., T. Koeuth, and J. R. Lupski.** 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19:**6823–6831.
51. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.
52. **Willems, A., and D. Collins.** 1993. Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequence. Int. J. Syst. Bacteriol. **43:** 305–313.
53. **Yanagi, M., and K. Yamasato.** 1993. Phylogenetic analysis of the family *Rhizobiaceae* and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. FEMS Microbiol. Lett. **107:**115–120.
54. **Young, J. P. W., and K. E. Haukka.** 1996. Diversity and phylogeny of rhizobia. New Phytol. **133:**87–94.