

Genomic Heterogeneity of Strains Nodulating Chickpeas (*Cicer arietinum* L.) and Description of *Rhizobium mediterraneum* sp. nov.

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The genetic diversity of chickpea strains was studied by using 30 isolates obtained from nodules on chickpeas growing in uninoculated fields over a wide geographic range. The following taxonomic approaches were used: DNA-DNA relatedness analysis, restriction fragment length polymorphism analysis of the amplified 16S ribosomal DNA (rDNA) intergenic spacer (IGS), and total 16S rRNA sequence analysis. The division of chickpea-infective strains into two major phylogenetic groups (groups A and B) that has been described previously was confirmed by the polymorphism of the 16S IGS rDNA. We identified a total of five genomic species, including the previously described species *Rhizobium ciceri*. All of the group B strains except one were homogeneous and belonged to a single genomic species corresponding to *R. ciceri*. Group A was heterogeneous, containing three genomic species and five strains that remained unclassified, and its members had very different PCR restriction fragment length polymorphism profiles. The complete 16S rRNA sequences of strains representing the two major groups, *R. ciceri* UPM-Ca7^T (T = type strain) and genomic species 2 strain UPM-Ca36^T, exhibited 19 mismatches. Both of these strains belonged to the *Rhizobium loti*-*Rhizobium huakuii* branch; *R. ciceri* UPM-Ca7^T was closely related to *R. loti*, and strain UPM-Ca36^T was clearly separated from *R. ciceri* and closely related to *R. huakuii*. Thus, genomic species 2 could be distinguished from *R. ciceri* by its 16S rRNA sequence, by DNA relatedness data, by the polymorphism of the 16S IGS rDNAs, and by previously described multilocus enzyme electrophoresis results and phenotypic characteristics. Therefore, we propose that strains belonging to genomic species 2 should be classified in a new species, *Rhizobium mediterraneum*, and that strain UPM-Ca36 should be the type strain.

Numerous studies to clarify the taxonomy and phylogeny of bacteria that are able to induce nodules on leguminous plants are now being carried out. Rapid changes in the classification of these organisms are occurring, and new genera and especially new species are being described. In 1984 in *Bergey's Manual of Systematic Bacteriology* (15) the following species in the genus *Rhizobium* were recognized: *Rhizobium leguminosarum*, *Rhizobium meliloti*, and *Rhizobium loti*. Since then, isolates obtained from new or partially studied host plants have led to the delineation of six new species, *Rhizobium galegae* (18), *Rhizobium fredii* (29), *Rhizobium huakuii* (4), *Rhizobium etli* (30), *Rhizobium tropici* (20), and *Rhizobium ciceri* (25). Recently, De Lajudie et al. (7) studied isolates obtained from different *Acacia* and *Sesbania* host plants and found that on the basis of 16S rRNA sequences most of the strains clustered on the *R. meliloti*-*R. fredii* branch. On the basis of polyphasic results, these authors described a distinct genus for this group, the genus *Sinorhizobium*. The genus *Sinorhizobium* includes *Sinorhizobium meliloti* and *Sinorhizobium fredii* (previously *R. meliloti* and *R. fredii*, respectively), as well as two new species, *Sinorhizobium teranga* and *Sinorhizobium saheli*.

Bacteria that are able to establish an effective nitrogen-fixing symbiosis with chickpeas have been studied intermittently, and no strains or only a few strains have been included in previous taxonomic studies of the family *Rhizobiaceae*, which has led to

contradictory conclusions. On the one hand, several authors concluded that the chickpea rhizobia form a unique group on the basis of the specificity of the host plant-rhizobium relationship (10), serological and antigenic characteristics (6, 17), cultural traits, and the polymorphism of the *nifHD* genes (3). On the other hand, Jarvis et al. (14) proposed that the chickpea strains should be classified as *R. loti* or *Bradyrhizobium* sp. (*Cicer arietinum* L.) strains, depending on their generation times, and this view prevailed until recently. These contradictory conclusions led us to reexamine the diversity of the chickpea rhizobia, and we used the following genomic and phenotypic approaches to do this: multilocus enzyme electrophoresis, an analysis of the polymorphism of the 16S intergenic spacer (IGS) ribosomal DNA (rDNA), an analysis of auxanographic and cultural characteristics, a 16S rRNA sequence analysis, and a DNA relatedness analysis (24, 25). Our conclusions were as follows: (i) all of the chickpea *Rhizobium* strains belong to the genus *Rhizobium* regardless of generation time; (ii) these organisms constitute a separate branch that is closely related to but can be clearly differentiated from *R. loti*; (iii) two phylogenetic lineages (groups A and B) are present; and (iv) the homogeneous group B strains belong to the recently described species *R. ciceri* (25). Groups A and B appeared to be genetically divergent, and preliminary DNA-DNA relatedness results obtained with two strains belonging to each group suggested that the group A strains do not belong to the species *R. ciceri* (25).

In order to examine the genetic diversity of chickpea strains at the species level, we determined DNA-DNA relatedness values since DNA-DNA relatedness is the major criterion used to delineate genospecies (11, 33). We also examined ribosomal

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TABLE 1. Bacterial strains examined

Strain ^a	RFLP group ^b	Geographic origin	Reference(s)
<i>R. ciceri</i> strains			
UPM-Ca7 ^T (= ATCC 51585 ^T)	B ^c	Spain	3, 24, 25
3HOa10	B ^c	United States	3, 24, 25
IC-2091 (= LMG15046)	B ^c	India	3, 24, 25
UPM-Ca116	B ^c	Spain	3, 24, 25
522	B ^c	Russia	3, 24, 25
IC-2018	B ^c	Turkey	3, 24, 25
CP-76	B ^d	Morocco	This study
CP-77	B ^d	Morocco	This study
CP-92	B ^d	Syria	This study
<i>Rhizobium</i> sp. (<i>C. arietinum</i> L.) strains			
3HOa9	B ^c	United States	3, 24, 25
Ca-7	A ^c	India	3, 24, 25
IC-72M (= ATCC 51586)	A ^c	India	3, 24, 25
IC-60	A ^c	India	3, 24, 25
H-45	A ^c	India	3, 24, 25
IC-6	A ^c	India	3, 24, 25
UPM-Ca142	A ^c	Spain	3, 24, 25
UPM-Ca36 ^T	A ^c	Spain	3, 24, 25
CP-8	A ^d	Syria	This study
CP-12	A ^d	India	This study
CP-14	A ^d	India	This study
CP-18	A ^d	Lebanon	This study
CP-19	A ^d	Syria	This study
CP-24	A ^d	Syria	This study
CP-28	A ^d	Tunisia	This study
CP-47	A ^d	Morocco	This study
CP-54	A ^d	Morocco	This study
CP-78	A ^d	Italy	This study
USAB67	C ^c	India	3, 24, 25
CP-9	C ^d	Jordan	This study
CP-91	C ^d	Nepal	This study

^a ATCC, American Type Culture Collection, Rockville, Md.; LMG, Collection of Bacteria of the Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium. Strains whose designations begin with CP were obtained from D. Beck, International Center for Agricultural Research in Dry Areas, Aleppo, Syria.

^b RFLP groups obtained by amplification and digestion of 16S IGS rDNA.

^c Data from references 24 and 25.

^d Data from this study.

gene polymorphism, which classically has allowed workers to clarify phylogenetic relationships between species. The method which we used was a PCR restriction fragment length polymorphism (RFLP) analysis of the 16S IGS rDNA since this method allowed us to rapidly position a large number of strains phylogenetically and used the more divergent IGS, which increased the discriminative power of the analysis. Furthermore, the inability of partial 16S rRNA sequences to distinguish groups A and B, which were highly divergent as determined by polyphasic approaches (25), led us to determine whether these taxa belong to a unique phylum by using total 16S rRNA sequencing. In this study, we analyzed 30 chickpea strains, including 15 collection strains used in our previous studies (24, 25) and 15 isolates obtained from root nodules on chickpeas growing in uninoculated fields in diverse geographic regions in the Middle East and Far East.

MATERIALS AND METHODS

Strains and culture. The origins of the 30 strains which we studied are shown in Table 1. All of the strains whose designations begin with CP were obtained from the International Center for Agricultural Research in Dry Areas, Aleppo, Syria. These strains were isolated from root nodules on chickpeas growing in areas with no history of rhizobium inoculation. The other strains which we used are collection strains obtained from T. Ruiz-Argueso, Universidad Politécnica,

Madrid, Spain. The bacteria were maintained on yeast extract-mannitol agar slants (32) at 4°C, and repeated streaking was performed to check for purity. Cells were grown at 28°C in yeast extract-mannitol medium; however, in order to limit exopolysaccharide formation, maltose was used instead of mannitol.

DNA extraction. Previously described methods were used to extract (2) and purify (25) DNA.

DNA-DNA hybridization and thermal stability of duplexes. Native DNAs from *R. ciceri* UPM-Ca7^T (T = type strain), IC-2018, 3HOa9, Ca-7, IC-60, and CP-18 were labelled by nick translation (26) by using tritium-labelled nucleotides (Amersham International, Amersham, England). Levels of DNA relatedness were determined by using the S1 nuclease-trichloroacetic acid method (12) at the optimal temperature for DNA reassociation (70°C).

The thermal stability of each reassociated DNA was estimated by determining its denaturation temperature (the temperature at which 50% of the double-stranded DNA became denatured and hydrolyzable by S1 nuclease) as described previously (5), with slight modifications (9). The temperature of the mixture was increased from 70 to 95°C in 5°C increments. The levels of divergence between hybrid DNAs were inversely proportional to the thermal stabilities of the hybrids; this was expressed by calculating the ΔT_m , which was the difference between the denaturation temperatures of the heterologous and homologous duplexes.

Amplification and restriction enzyme analysis of 16S IGS rDNA. The oligonucleotides used as primers for amplification of the 16S IGS rDNA region by the PCR were 5'-GGAGAGTTAGATCTTGGCTCAG-3' (primer FGPS6-63; 5' end of the 16S rRNA gene) and 5'-CCGGGTTTCCCATTCGG-3' (primer FGPL132'-38; 5' end of the 23S rRNA gene) (23). Amplification reactions were carried out by using the protocol of Mullis and Faloona (21), with some modifications. PCR were performed in a final volume of 50 µl under a thin surface layer of paraffin oil to prevent evaporation. Each reaction mixture contained approximately 0.5 µg of genomic DNA, buffer (10 mM Tris HCl [pH 8.2], 1.5 mM MgCl₂, 50 mM KCl, 0.01% [wt/vol] gelatin), 20 µM dATP, 20 µM dTTP, 20 µM dGTP, 20 µM dCTP, each primer at a concentration of 0.1 µM, and 2.5 U of *TaqI* DNA polymerase (Gibco-Bio-Rad Laboratories, Cergy-Pontoise, France). Amplification reactions were carried out with a dry-block thermal cycler (Biometra Trio thermoblock) by using the following program: initial denaturation for 3 min at 95°C, 35 cycles consisting of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C, and final extension for 3 min at 72°C. To analyze the amplification products, 5-µl aliquots were electrophoresed on horizontal 0.8% (wt/vol) agarose gels in TBE buffer (89 mM Tris borate, 89 mM boric acid, 2 mM EDTA; pH 8) at 4 V/cm for 1 h. The gels were stained in an aqueous solution containing 0.4 mg of ethidium bromide per liter and were photographed under UV light (302 nm) by using Ilford type HP5 film. The sizes of amplified fragments were estimated by comparing them with a 1-kb ladder that was used as a molecular weight standard (Gibco-Bio-Rad Laboratories). The size of the IGS was then calculated by subtracting the size of the 16S rDNAs of members of the *Rhizobiaceae* (34) (approximately 1,500 bp) from the size of the 16S IGS rDNA amplified region.

Restriction enzyme digestions of PCR products were performed in 20-µl (total volume) mixtures containing 7 µl of amplified DNA and 10 to 12 U of the following restriction endonucleases: *AluI*, *CfoI*, *MspI*, *NdeII*, and *Sau 96.1* (all obtained from Boehringer Mannheim, Meylan, France) and *HaeIII* and *RsaI* (both obtained from Gibco-Bio-Rad Laboratories). The reaction conditions used were those recommended by the manufacturers. The restricted fragments were separated by horizontal gel electrophoresis in TBE buffer by using a 3% (wt/vol) Nusieve (FMC, Rockland, Maine) agarose gel containing 1 µg of ethidium bromide per ml. The molecular weight standards used were 123-bp and 1-kb ladders (Gibco-Bio-Rad Laboratories). The gels were electrophoresed at 2.3 V/cm for 3 h.

The size of each restricted fragment was calculated, and the profiles of the strains were compared. Bands of fragments shorter than 90 bp were not taken into consideration, since they were not resolved reproducibly. Thus, the sizes of the PCR products estimated by adding the sizes of the restriction fragments were slightly smaller than the sizes estimated for the undigested fragments. The genetic relationship between two amplified DNAs was evaluated by using the similarity coefficient *F*, which was calculated as follows: $F = 2n_{xy}/n_x + n_y$, where n_{xy} was the number of comigrating fragments (considering all enzymes) and n_x and n_y were the total numbers of bands for strains *x* and *y*, respectively. The genetic distance for each pair of strains was determined by calculating the *D* value as described by Nei and Li (22), and a dendrogram was constructed by using the unweighted pair group with arithmetic average algorithm of Sneath and Sokal (31).

Nucleotide sequences of 16S rRNA genes and data analysis. The 16S rRNA genes were amplified by using the PCR conditions described above and primers FGPS5-255 (5'-TGGAAAGCTTGATCTGGCT-3') and FGPS1509'-153 (5'-AGGAGGGGATCCAGCCGCA-3'), which contain restriction sites for *HindIII* and *BamHI* (the underlined portions), respectively (1). The amplified products were purified with a GeneClean kit (Bio 101, La Jolla, Calif.) and were transformed into *Escherichia coli* DH5αF' (Bethesda Research Laboratories) as described by Maniatis et al. (19). Plasmid purification from strain DH5αF' was performed by alkaline lysis in which a QIAGEN Midi-prep kit (Diagen, Düsseldorf, Germany) was used. Seven primers (primers FGPS310-20 [5'-GAGACACGGCCAGACTCT-3'], FGPS485-292 [5'-CAGCAGCCGCGTAA-3'], FGPS747-293 [5'-AACAGGATTAGATAC-3'], FGPS1047-295 [5'-ATGTTGG

TABLE 2. Levels of DNA relatedness for 30 chickpea strains

Source of unlabelled DNA	RFLP group ^a	% Relatedness to ³ H-labelled DNA from:					
		UPM-Ca7 ^T	IC-2018	3HOa9	CP-18	Ca-7	IC-60
<i>R. ciceri</i> strains							
UPM-Ca7 ^T	B	100 ^b	ND ^c	ND	ND	ND	26
3HOa10	B	78	ND	37	ND	41	31
IC-2091	B	83	72	35	40	30	29
UPM-Ca116	B	68	ND	38	34	31	31
522	B	75	71	35	30	32 (12.0) ^d	30
IC-2018	B	ND	100	28	28	20	27
CP-77	B	65	89	32	28	28	30
CP-76	B	ND	70	36	31	ND	ND
CP-92	B	ND	102	30	29	23	27
Genomic species 1 strain 3HOa9	B	53	42	100	31	27	29
Genomic species 2 strains							
UPM-Ca142	A	48	38	31	81	53 (6.0)	43
UPM-Ca36 ^T	A	42	40	28	82	50 (6.5)	43
CP-18	A	ND	41	30	100	56 (6.5)	37
CP-12	A	ND	39	24	99	49	32
CP-24	A	35	32	24	100	48	35
CP-8	A	ND	32	23	99	52	36
CP-28	A	ND	34	21	100	45	34
Genomic species 3 strains							
Ca-7	A	36	32	26	63 (6.0)	100	ND
IC-72M	A	34	ND	ND	ND	104	ND
CP-14	A	ND	36	24	54 (6.5)	91	40
Genomic species 4 strains							
IC-60	A	36	30	24	46 (10.5)	37	100
H-45	A	38	ND	ND	ND	44	77
Unclassified strains							
IC-6	A	29	34	27	63 (6.5)	56 (5.5)	37
CP-47	A	ND	35	29	56	ND	38
CP-54	A	ND	34	17	55 (8.0)	53	36
CP-19	A	ND	37	24	ND	47	35
CP-78	A	ND	34	24	48	49	33
USAB67	C	29	27	23	33	23	36
CP-91	C	ND	23	20	20	19	17
CP-9	C	ND	33	22	29	25	26

^a Groups determined by analyzing RFLP of the amplified and digested 16S IGS rDNA region (24, 25; this study).

^b Level of DNA relatedness at 70°C.

^c ND, not determined.

^d The numbers in parentheses are ΔT_m values (in degrees Celsius).

GTAAAGTC-3'], FGPS1176'-112 [5'-GGGGCATGATCACTTGACGTC-3'], FGPS910'-270 [5'-AGCCTTGCGGCCGTACTCCC-3'], and FGPS505'-313 [5'-GTATTACCGCGGCTGCTG-3']], as well as primers T3 and T7, which were supplied by Pharmacia LKB, Uppsala, Sweden, were necessary to sequence both strands of the 16S rRNA molecule. The sequencing procedure was performed by using T7 sequencing kits (Pharmacia LKB) and was based on the dideoxy chain termination method described by Sanger et al. (28).

Sequences were aligned and compared by using the Clustal program (13). Matrix pairwise comparisons were corrected for multiple base substitutions by the method of Kimura (16), and a phylogenetic tree was constructed by the neighbor-joining method (27). Gap-containing positions were omitted from the computations. A bootstrap confidence analysis was performed by using 1,000 replications to determine the reliability of the tree topology obtained (8).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of strains UPM-Ca7^T and UPM-Ca36^T have been deposited in the GenBank data library under accession numbers U07934 and L38825, respectively. The accession numbers of the nucleotide sequences of the 16S rRNAs of the organisms belonging to the *Rhizobiaceae* and other related bacteria used for comparison are as follows: *R. loti* group B strain LMG4284, X67230; *R. loti* group A strain LMG6125^T, X67229; isolate ORS1001, obtained from *Acacia senegal*, X68389; *R. huakuii*, D12797; *Phyllobacterium myrsinacearum*, D12789; *S. meliloti* LMG6133, X67222; *S. fredii* LMG6217, X67231; *R. galegae* LMG6214, X67226; *Agrobacterium tumefaciens* LMG196, X67223; *R. tropici* IFO15247, D11344; *R. leguminosarum* group A strain LMG9518, X67233; *R. tropici* group IIB strain LMG9517, X67234; *Rhizobium* sp. strain LMG9509, X67232; *Agrobacterium rhizogenes* LMG152, X67224; *Bradyrhizobium japonicum*, X66024; and *Azorhizobium caulinodans* LMG6465, X67221.

RESULTS

DNA-DNA hybridization experiments. The levels of DNA relatedness of 30 strains and the ΔT_m values of 10 strains with six reference strains are shown in Table 2. Labelled DNAs from *R. ciceri* UPM-Ca7^T and IC-2018 exhibited DNA relatedness values of 65 to 100% with seven other group B strains, and together these strains constituted a tight genomic group which corresponded to *R. ciceri* since it contained the type strain of the species (strain UPM-Ca7). Strain 3HOa9 is the only group B strain which did not belong to *R. ciceri*; this strain exhibited 53% relatedness with the type strain of *R. ciceri* and less than 38% relatedness with eight other *R. ciceri* strains. Twelve group A strains fell into three genomic species. Genomic species 2 contained seven strains which exhibited 81 to 100% DNA relatedness with strain CP-18 and 46 to 63% DNA relatedness (ΔT_m , 6.0 to 10.5°C) with the other group A strains. This genomic species exhibited less than 40% DNA relatedness with group B and C strains. Genomic species 3 comprised three strains that exhibited more than 91% DNA relatedness with strain Ca-7 and 37 to 56% DNA relatedness (ΔT_m , >6.0°C) with the other group A strains. These organisms exhibited low levels of DNA relatedness (20 to 41%) with

TABLE 3. 16S IGS rDNA restriction patterns of 24 chickpea strains

Strain	Restriction enzyme pattern ^a							16S IGS rDNA type
	<i>RsaI</i>	<i>AluI</i>	<i>CfoI</i>	<i>HaeIII</i>	<i>MspI</i>	<i>NdeII</i>	<i>Sau 96.1</i>	
Group A strains								
CP-18	R1	A1	C1	H1	M1	N1	S1	I
CP-12	R1	A1	C1	H1	M1	N1	S1	I
CP-24	R1	A1	C1	H1	M1	N1	S1	I
CP-8	R1	A1	C1	H1	M1	N1	S1	I
CP-28	R1	A1	C1	H1	M1	N1	S1	I
H-45	R2	A2	C2	H2	M2	N2	S2	II
UPM-Ca142	R3	A3	C3	H3	M3	N3	S3	III
CP-54	R4	A4	C4	H4	M4	N4	S4	IV
IC-6	R4	A5	C5	H5	M5	N5	S5	V
CP-19	R1	A6	C6	H6	M6	N6	S6	VI
IC-72M	R5	A7	C7	H7	M7	N7	S7	VII
Ca-7	R5	A7	C7	H7	M7	N7	S7	VII
CP-14	R5	A7	C7	H7	M7	N7	S7	VII
CP-47	R1	A8	C1	H8	M4	N8	S8	VIII
CP-78	R6	A9	C8	H9	M8	N9	S9	IX
Group B strains								
CP-76	R1	A10	C9	H10	M9	N10	S10	X
UPM-Ca7 ^T	R1	A10	C10	H10	M9	N10	S10	XI
CP-92	R1	A10	C10	H11	M10	N10	S11	XII
CP-77	R1	A10	C10	H11	M10	N10	S12	XIII
522	R7	A11	C11	H12	M11	N11	S8	XIV
3HOa9	R1	A12	C12	H13	M12	N12	S13	XV
Group C strains								
CP-9	R8	A13	C13	H2	M13	N13	S14	XVI
CP-91	R8	A13	C13	H2	M13	N13	S14	XVI
USAB67	R1	A14	C14	H14	M14	N14	S15	XVII

^a The numbers of electromorphs obtained with restriction enzymes *RsaI*, *AluI*, *CfoI*, *HaeIII*, *MspI*, *NdeII*, and *Sau 96.1* were 8, 14, 14, 14, 14, 14, and 15, respectively; the numbers of bands larger than 90 bp per profile obtained with these enzymes were 4 to 5, 6 to 9, 6 to 10, 8 to 12, 8 to 10, 4 to 6, and 6 to 8, respectively.

group B strains. Genomic species 4 comprised two strains, IC-60 and H-45, which exhibited 77% DNA relatedness with each other and less than 43% DNA relatedness with the remaining strains.

A total of eight strains belonging to groups A and C did not fall into any of the five genomic groups described above since they exhibited less than 63% DNA relatedness with all reference strains. These unclassified strains were not studied further by this method; however, they may represent one or more additional genomic species.

PCR amplification and RFLP of 16S IGS rDNA. A total of 24 strains were included in the RFLP study; 9 collection strains were used to represent the two major PCR RFLP-delineated clusters (24), and there were 15 new chickpea isolates. Gel electrophoresis of undigested PCR products revealed that all strains produced a single 2,500-bp band. The estimated length of the IGS between the 16S and 23S rDNAs found by subtracting the average length of the 16S rDNAs of members of the *Rhizobiaceae* (34) (1,500 bp) from the 2,500 bp of 16S IGS rDNA was 1,000 bp.

The restriction patterns obtained for the strains are shown in Table 3. Each restriction pattern was designated by a letter representing the enzyme, followed by a number representing the profile type. The seven enzymes tested generated 4 to 12 fragments per profile and a total of 178 different bands. Depending on the enzyme used, 8 to 15 electromorphs were obtained; *RsaI* was the least discriminatory enzyme. The 24 strains analyzed produced 17 different combinations of 16S IGS rDNA electromorphs, which were designated I to XVII (Table 3). None of the enzymes was able to distinguish among strains Ca-7, IC-72M, and CP-14, between strains CP-9 and CP-91, or among strains CP-8, CP-18, CP-12, CP-24, and CP-

28. The similarity coefficients for and genetic distances between pairs of strains are shown in Table 4. The values for similarity coefficients and genetic distances ranged from 59.2 to 100% and from 0 to 4.5 substitutions per site, respectively. An unweighted pair group with arithmetic average dendrogram was constructed from the distance matrix data (Fig. 1). A major division at 0.038 separated the strains into two clusters corresponding to the two major groups obtained previously (groups A and B) (24). Three strains formed an outgroup (group C) that diverged at 0.04.

16S rRNA gene sequencing. The phylogenetic relationship between group A and B strains was estimated by determining the total 16S rRNA gene sequences of two strains, *R. ciceri* UPM-Ca7^T (group B) and a representative group A strain, strain UPM-Ca36^T, which belongs to genomic species 2. The 16S rRNA sequences of the following organisms belonging to the *Rhizobiaceae* and other related bacteria were used for comparison: *R. loti* group B strain LMG4284; *R. loti* group A strain LMG6125^T; an isolate obtained from *Acacia senegal*, strain ORS1001; *R. huakuii*; *P. myrsinacearum*; *S. meliloti* LMG6133; *S. fredii* LMG6217; *R. galegae* LMG6214; *Agrobacterium tumefaciens* LMG196; *R. tropici* IFO15247; *R. leguminosarum* group A strain LMG9518; *R. tropici* group IIB strain LMG9517; *Rhizobium* sp. strain LMG9509; *Agrobacterium rhizogenes* LMG152; *B. japonicum*; and *Azorhizobium caulinodans* LMG6465.

The phylogenetic tree obtained and the bootstrap confidence limits for the tree are shown in Fig. 2. The similarity values and observed numbers of nucleotide differences for members of the branch comprising *R. ciceri*, *R. loti*, *Rhizobium* sp. strain UPM-Ca36^T (genomic species 2), strain ORS 1001, *R. huakuii*, and *P. myrsinacearum* are shown in Table 5. As

TABLE 4. Levels of similarity and genetic distances for chickpea strains based on the results of a PCR RFLP analysis of 16S IGS rDNA

Strain	% Similarity or genetic distance (%) ^a																
	CP-28	UPM-Ca142	CP-54	CP-47	IC-6	H-45	Ca-7	CP-78	3HOa9	CP-92	CP-77	CP-76	UPM-Ca7 ^T	522	CP-19	CP-9	USAB67
CP-28 ^b		3.4	3.4	2.5	3.2	2.9	3.3	3.6	3.6	3.8	3.7	4.0	4.0	4.5	3.1	4.1	4.2
UPM-Ca142	67.3		2.3	3.2	2.9	3.0	3.5	3.2	3.8	3.7	3.6	3.7	3.7	3.9	2.7	4.4	3.9
CP-54	67.3	76.3		2.2	2.6	3.3	3.5	3.0	3.2	3.7	3.6	3.7	3.7	4.1	3.0	4.3	4.4
CP-47	74.2	68.8	77.1		2.8	2.7	2.1	2.6	3.4	3.3	3.2	3.6	3.6	3.2	2.4	4.0	3.8
IC-6	68.7	71.6	73.5	72.3		3.4	3.0	3.0	3.3	4.1	4.0	3.8	3.8	3.4	2.8	3.9	3.4
H-45	71.4	70.2	68.0	73.1	67.4		3.2	3.5	3.8	3.7	3.6	3.7	3.7	4.1	3.0	3.0	3.6
Ca-7 ^c	68.0	66.7	66.7	77.9	70.1	68.8		3.1	4.2	3.9	3.8	4.1	4.1	3.8	3.1	4.3	4.3
CP-78	66.0	68.8	70.7	73.7	70.1	66.7	69.4		3.7	3.9	3.8	3.9	3.9	3.8	2.9	4.5	4.3
3HOa9	66.0	64.6	68.6	67.3	68.0	64.6	61.4	65.3		3.3	3.2	3.1	3.1	4.3	3.1	3.9	3.5
CP-92	64.6	65.3	65.3	68.1	62.5	65.3	63.9	63.9	68.0		0.3	1.3	1.1	2.6	3.3	4.1	4.3
CP-77	65.3	66.0	66.0	68.8	63.2	66.0	64.6	64.6	68.7	96.8		1.0	0.8	2.5	3.0	4.1	4.1
CP-76	62.6	65.3	65.3	66.0	64.6	65.3	61.9	63.9	70.0	85.4	88.4		0.2	2.9	3.0	4.5	4.3
UPM-Ca7 ^T	62.6	65.3	65.3	66.0	64.6	65.3	61.9	63.9	70.0	87.5	90.5	97.9		2.9	3.0	4.1	4.3
522	59.4	63.9	62.0	68.8	67.3	61.9	64.6	64.6	60.8	73.5	74.2	71.4	71.4		3.8	4.3	4.1
CP-19	70.0	72.9	70.7	75.8	72.2	70.8	69.4	71.4	69.3	68.0	70.8	70.1	70.1	64.6		3.7	4.0
CP-9 ^d	62.0	60.4	60.6	63.2	63.9	70.8	61.2	59.2	63.4	61.9	62.5	59.8	61.9	60.6	65.3		3.2
USAB67	61.4	63.9	60.0	64.6	67.3	66.0	60.6	60.6	66.7	61.2	61.9	61.2	61.2	62.0	62.6	68.7	

^a Levels of similarity and genetic distances are shown on the lower left and on the upper right, respectively. Genetic distance values were derived from pairwise levels of similarity between restriction fragments corrected by using the method of Nei and Li (22).

^b Strains CP-12, CP-24, CP-18, and CP-8 had the same levels of similarity and genetic distances as CP-28.

^c Strains IC-72M and CP-14 had the same levels of similarity and genetic distances as Ca-7.

^d Strain CP-91 had the same levels of similarity and genetic distances as CP-9.

previously described on the basis of partial 16S rRNA sequences (25), the *R. ciceri* type strain clustered on the *R. loti* branch. Total sequence comparisons performed with *R. ciceri* gave similarity coefficients of 0.998 (three nucleotide differences) and 0.999 (two nucleotide differences) with *R. loti* groups A and B, respectively. This group formed a tight cluster that was corroborated by a bootstrap confidence value of 100. Chickpea strain UPM-Ca36^T grouped with *R. huakuii* and strain ORS1001 cluster U and exhibited similarity coefficients of 0.992 (11 nucleotide differences) and 0.987 (17 nucleotide differences) with these two taxa. Thus, chickpea strains *R. ciceri* UPM-Ca7^T and UPM-Ca36^T fell into different clusters. The similarity coefficient for these two strains was 0.986, corresponding to 19 base differences. All of the strains mentioned above formed part of a larger group (bootstrap confidence value, 98) which was separated from other *Rhizobium* species (*R. leguminosarum*, *R. galegae*, *R. tropici*) by strains belonging to other genera of the *Rhizobiaceae*, such as the genera *Sinorhizobium*, *Agrobacterium*, and *Phyllobacterium* (Fig. 2), as well as by organisms belonging to other families not represented in this study, such as *Rochalimea*, *Mycoplana*, and *Brucella* strains (7).

DISCUSSION

In a study of the diversity of rhizobial isolates that nodulate chickpeas (*C. arietinum* L.), we recently determined that there are two phylogenetic lineages (groups A and B) by using a polyphasic approach (24, 25). We showed that these two groups of strains belong to the same genus, the genus *Rhizobium*, but are distinct from all previously described species. Preliminary DNA homology results obtained with four strains showed that the two group B strains did not belong to the same genomic species as the group A strains. The group B strains clustered close to *R. loti*, but could be clearly separated from this species on the basis of their partial 16S rRNA sequences,

the results of a PCR RFLP analysis of the 16S IGS rDNA, their phenotypic characteristics, and DNA homology values; thus, these strains were assigned to a new species, *R. ciceri*. The group A strains were more found to be more heterogeneous and remained unnamed. In this study we examined 30 chickpea strains isolated from very diverse geographic areas representing the different regions where chickpeas are grown. The results of a PCR RFLP analysis of the 16S IGS rDNA, which previously correlated well with the results obtained with other approaches (24, 25), were used to group the new strains. Because of the heterogeneity observed previously, and the fact that some strains could not be classified as *R. ciceri* strains, we questioned how many new species the chickpea strains belong to, performed DNA-DNA hybridization experiments, and determined ΔT_m values. Moreover, despite the high levels of divergence between members of groups A and B that we found, these organisms had identical partial 16S rRNA sequences (25). Thus, in order to determine the phylogenetic position of these organisms, their total 16S rRNA sequences were determined.

The clusters obtained when we used the PCR RFLP method with 30 strains confirmed that the chickpea isolates should be divided into two major phylogenetic groups, groups A and B (24, 25). These groups diverged at a genetic distance of approximately 0.038 substitution per site. The DNA relatedness values were consistent with these results since the group A strains exhibited higher average DNA relatedness values with each other than with group B strains. The level of genetic diversity observed within the groups varied, depending on the group considered. The group B strains appeared to be very homogeneous, but much greater heterogeneity was observed among the group A strains when DNA-DNA relatedness and PCR RFLP methods were used. Most of the group B strains belonged to the same genomic species, *R. ciceri*; the only exception was group B strain 3HOa9, which exhibited low levels of DNA relatedness with *R. ciceri* strains and was excluded

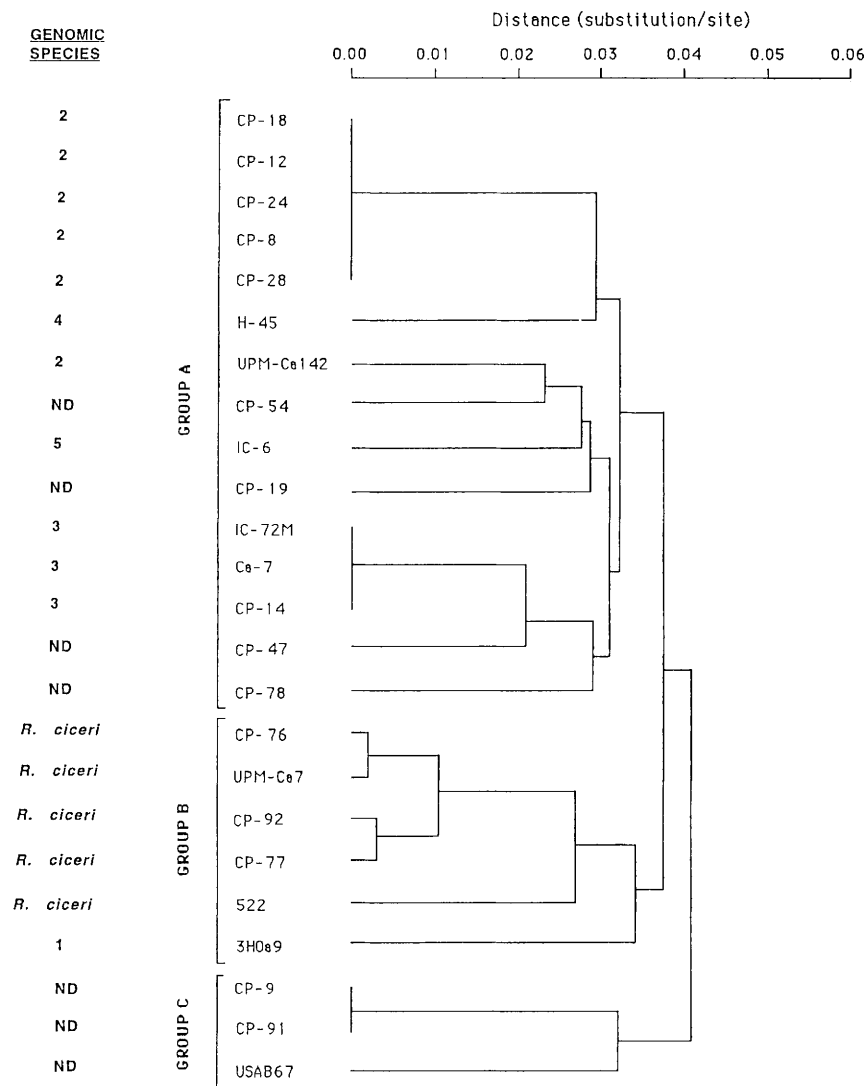


FIG. 1. Genetic distances between chickpea rhizobia based on the results of an RFLP analysis of the amplified 16S IGS rDNA region. ND, not determined.

from this species when the PCR RFLP method was used. This strain may represent a separate group B genomic species. The group A strains were grouped into three genomic species, and five strains remained unclassified. This heterogeneity is reflected by the divergent branches on the PCR RFLP dendrogram (Fig. 1). Our overall results revealed that there was a good correlation between DNA homology data, which led to the delineation of genomic species, and polymorphism of the 16S IGS rDNAs. For example, genomic species 3 strains IC-72M, Ca-7, and CP-14 formed a tight 16S IGS rDNA cluster among the group A strains. Similarly, all of the genomic species 2 strains except strain UPM-Ca142 grouped tightly (Fig. 1). We concluded that strains CP-9 and CP-91, which produced identical PCR RFLP profiles with all seven restriction enzymes tested, probably belong to the same genomic species. Thus, the PCR RFLP method used in this study not only is a good method for fingerprinting strains but also can be used to screen large numbers of strains and classify them into their probable genomic species.

Some of the genomic species which we identified have a wide geographic distribution, and others appear to be confined to

one geographic area. *R. ciceri* and genomic species 2 are found in the Mediterranean area, North Africa, North America, the Indian subcontinent, and Russia, while genomic species 3 and 4 were obtained from the Indian subcontinent. However, genomic species 3 and 4 included only a few strains, and thus it is not possible to confirm that they are limited to the Indian subcontinent.

Because of the great heterogeneity of the strains that nodulate chickpeas (there are at least five genomic species among the 30 isolates which we studied, and group A and B strains exhibit high levels of divergence), we reconsidered our previous conclusion that group A and B strains comprise a unique phylum based on identical partial 16S rRNA gene sequences. The partial sequence that includes the first 250 nucleotides from the 5' part of the 16S rRNA gene is commonly considered to represent the most heterogeneous region in the 16S rRNA gene, but in certain cases this assumption leads to erroneous phylogenetic positions. For example, the partial sequences of *R. galegae* strains placed this species on the *R. loti-R. huakuii* branch (25), far from its position on the *Agrobacterium vitis-Agrobacterium tumefaciens-Agrobacterium rubi* branch

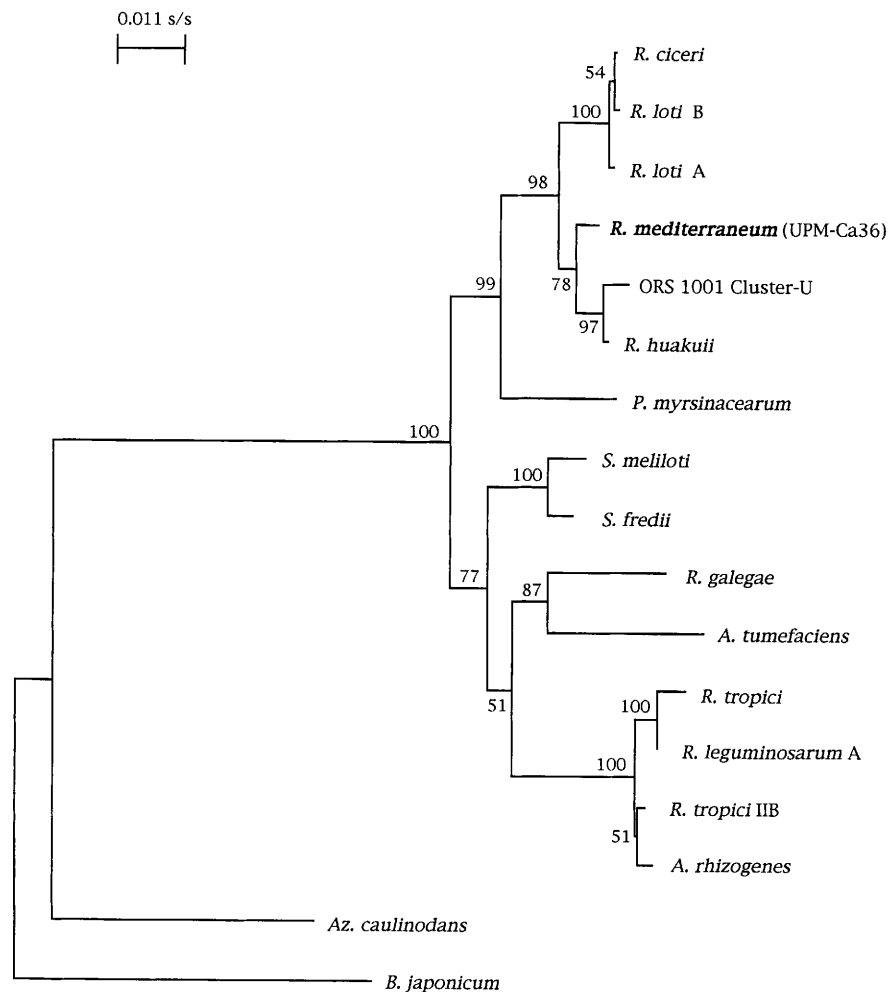


FIG. 2. Phylogenetic tree for the alpha subclass of the *Proteobacteria*, obtained by using complete 16S rRNA gene sequences and the neighbor-joining method. Significant bootstrap probability values are indicated at the nodes. Abbreviations: s/s, substitution per site, *R.*, *Rhizobium*; *P.*, *Phyllobacterium*; *S.*, *Sinorhizobium*; *A.*, *Agrobacterium*; *B.*, *Bradyrhizobium*; *Az.*, *Azorhizobium*.

determined by a total 16S rRNA sequence analysis (7). Our results showed that the position of *R. ciceri* on the 16S rRNA phylogenetic tree did not change and that this species clustered tightly with *R. loti*. Thus, in this case, the partial sequences accurately predicted the phylogenetic position of the strains, since of the three nucleotide differences in the total 16S rRNAs of *R. ciceri* and *R. loti* group A, two were observed in the partial sequences of the first one-fifth of the gene. In contrast, we were not able to predict levels of phylogenetic divergence for *R. huakuii* and *R. ciceri* on the basis of partial sequences, since 1 mismatch was observed in the first 250 nucleotides and there were 24 mismatches over the whole length of the gene. Strain UPM-Ca36^T, which represents genomic species 2, belongs to the *R. huakuii* branch and was well separated from the other reference strains, particularly *R. ciceri* UPM-Ca7^T (19 nucleotide differences), even though the partial sequences of the two strains were identical. Thus, on the basis of 16S rRNA gene sequence data, *R. ciceri* and genomic species 2 were phylogenetically distinct, constituting two distinct lineages of the *R. loti*-*R. huakuii* subgroup. Consistent with these results, genomic species 2 exhibited low levels of DNA homology with *R. ciceri* and other members of the *Rhizobiaceae* (<17%) (25).

On the basis of the cumulative genotypic results obtained in this study (DNA relatedness values, the results of the analysis of the polymorphism of the 16S IGS rDNA, and total 16S rRNA sequence data), as well as the previous phenotypic multilocus enzyme electrophoresis results and cultural and physiological characteristics (24, 25) which strongly separated groups A and B, we suggest that genomic species 2 is a species that is distinct from *R. ciceri*. Therefore, we propose that genomic species 2 strains isolated from *C. arietinum* L. should be assigned to a new species, *Rhizobium mediterraneum* (med.i.ter. ra'ne.um. L. adj. *mediterraneum*, of the Mediterranean, one of the primary centers of origin from which chickpea plants were disseminated throughout the world).

Genomic species 3 and 4 belong to group A together with *R. mediterraneum*, and even though they can be clearly distinguished from this species on the basis of DNA homology data and the polymorphism of the rDNA, they have not yet been differentiated by phenotypic features or 16S rRNA sequence data. Moreover, few strains belong to these two genomic species, and thus we propose that more data will be required before they are named. Similarly, the status of the three strains belonging to group C which differ from both group A and B strains needs to be clarified.

TABLE 5. Similarity coefficients and observed numbers of nucleotide differences based on whole 16S rRNA gene sequences

Taxon	Similarity coefficient or observed no. of differences ^a						
	<i>R. ciceri</i>	<i>R. loti</i> group A	<i>R. loti</i> group B	<i>Rhizobium</i> sp. strain UPM-Ca36 ^T	ORS1001 cluster U	<i>R. huakuii</i>	<i>P. myrsinacearum</i>
<i>R. ciceri</i>		3	2	19	28	24	46
<i>R. loti</i> group A	0.998		3	21	27	23	45
<i>R. loti</i> group B	0.999	0.998		20	30	24	46
<i>Rhizobium</i> sp. strain UPM-Ca36 ^T	0.986	0.985	0.985		17	11	50
ORS1001 cluster U	0.979	0.980	0.978	0.987		6	47
<i>R. huakuii</i>	0.982	0.983	0.982	0.992	0.996		48
<i>P. myrsinacearum</i>	0.966	0.967	0.966	0.963	0.965	0.964	

^a The similarity coefficients and the observed numbers of nucleotide differences are shown on the lower left and on the upper right, respectively.

Description of *Rhizobium mediterraneum* sp. nov. Gram-negative, aerobic, non-spore-forming rods. Colonies on yeast extract-mannitol agar are convex, opaque, and 2 mm in diameter within 4 to 5 days when cultures are incubated at 28°C. *R. mediterraneum* can be differentiated from other species belonging to the *Rhizobiaceae* by the following characteristics: total 16S rRNA sequence, DNA-DNA relatedness data, multilocus enzyme electrophoresis data (24), and polymorphism of the 16S IGS rDNA. Two representative strains of the species, UPM-Ca36^T and UPM-Ca142, which have been studied previously by performing different phenotypic tests and an isozyme polymorphism analysis (24), clearly group together and are separated from the other chickpea isolates. These two strains have the following characteristics in common with the other group A strains (24): the maximum temperature for growth is 40°C, they are able to grow on minimal media, they tolerate 2% (wt/vol) NaCl, and they grow at pH 9.5. The characteristics that differentiate UPM-Ca36^T and UPM-Ca142 from *R. ciceri* are their inability to grow at pH 10 and 5, their susceptibility to carbenicillin, and their resistance to chloramphenicol, nalidixic acid, and trimethoprim-sulfamethoxazole. Strain UPM-Ca36, which was isolated from nodulated chickpeas grown in Spain, is the type strain. This strain has the characteristics described above for *R. mediterraneum* sp. nov. Strain UPM-Ca36^T has been deposited in the American Type Culture Collection.

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