Rhizobium ciceri sp. nov., Consisting of Strains That Nodulate Chickpeas (*Cicer arietinum* L.)

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The taxonomic status of 16 collection strains of chickpea (*Cicer arietinum* L.) rhizobia which were previously determined to belong to two groups (groups A and B) were compared with reference strains belonging to different genera and species of the family *Rhizobiaceae*. We used the following taxonomic, phylogenetic, and phenotypic characteristics and approaches to study these organisms: DNA homology, guanine-plus-cytosine content, restriction fragment length polymorphism of the amplified 16S-intergenic spacer rRNA gene, partial 16S rRNA sequencing, and auxanographic tests performed with 147 carbon sources. Similar groups of chickpea strains were identified by the different approaches. The chickpea strains were found to belong to the genus *Rhizobium* regardless of the phylogenetic group to which they belonged (group A or B). All strains fell into a tight cluster which included *Rhizobium loti* and *Rhizobium galegae*, and the group B strains were closely related to *R. loti*. An analysis of partial 16S ribosomal DNA sequences revealed identical nucleotide sequences for the slowly growing strains and fast-growing strains that were used as representatives of groups A and B, respectively, and these organisms fell into the *Rhizobium-Agrobacterium* lineage. When the sequences of these organisms were compared with the partial sequences of *Rhizobium huakuii* and *R. loti*, one- and two-nucleotide mismatches were observed, respectively, indicating that the chickpea rhizobia are closely related to these two species. The DNA-DNA hybridization data revealed that the chickpea rhizobia exhibited low levels of homology (less than 17%) to previously described *Rhizobium* and *Bradyrhizobium* species. Moreover, when we compared chickpea strains to *R. loti* and *R. huakuii*, the most closely related species as determined by the partial 16S rRNA sequence analysis, the homology values ranged from 21 to 52% and the ΔT_m values were greater than 5°C (ΔT_m is the difference between the denaturation temperatures of the heterologous and homologous duplexes). These results confirmed that the rhizobia that nodulate chickpeas cannot be assigned to a previously described species. Within the chickpea rhizobia, the DNA homology values obtained when members of groups A and B were compared were less than 38%, indicating that the group A and group B organisms belong to different species. Furthermore, these organisms can be distinguished from each other by the results of phenotypic tests. We propose that the group B chickpea rhizobia should be assigned to a new species, *Rhizobium ciceri*; UPM-Ca7 is the type strain of *R. ciceri*.

Currently, the bacteria which form nitrogen-fixing nodules on leguminous plants are divided into three genera, *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*. According to *Bergey's Manual of Systematic Bacteriology* (24), the genus *Rhizobium* contains three species, *Rhizobium meliloti*, *Rhizobium loti*, and *Rhizobium leguminosarum* (with three biovars, *R. leguminosarum* bv. *phaseoli*, *R. leguminosarum* bv. *trifolii*, and *R. leguminosarum* bv. *viciae*). Since the publication of *Bergey's Manual of Systematic Bacteriology*, the following five new species have been recognized: *Rhizobium galegae* (28), *Rhizobium fredii* (37), *Rhizobium huakuii* (7), *Rhizobium tropici* (29), and *Rhizobium etli* (38). Several other rhizobial groups have been described but have not been named yet (2, 47).

The rhizobia that nodulate chickpeas (*Cicer arietinum* L.) have been studied intermittently, and in all of the taxonomic studies of the *Rhizobiaceae* that have been performed either no strain or only a few strains belonging to this group were included. In *Bergey's Manual of Systematic Bacteriology* (24), the chickpea rhizobia are classified as members of either *R. loti*

or an undetermined *Bradyrhizobium* sp. group. The separation of these organisms into two different genera was based on the wide range of generation times observed with the strains, which led to classification of the fast and slow growers into the genera *Rhizobium* and *Bradyrhizobium*, respectively. In support of this separation, Chakrabarti et al. (6) determined levels of DNA-DNA hybridization for two slowly growing and two fast-growing chickpea strains and found very low levels of homology between members of the two groups.

Characterization of the fast-growing *Lotus* rhizobia was based on the results of an extensive study that included various strains that belonged to the *Rhizobiaceae* and were isolated from a wide range of host plants; these strains included three chickpea isolates (9). Relying on this report, Jarvis et al. (22) described the new species *R. loti* (with type strain NZP 2213), which was isolated from *Lotus corniculatus*. Two of the three chickpea strains tested exhibited 85 to 88% DNA homology with CC809a, an isolate that was obtained from *Lotus maroccanus* and was later assigned to *R. loti* (9, 22). However, it is important to note that this strain exhibited only 47% DNA homology with strain NZP 2213^T (T = type strain). According to Wayne et al. (41), strains that exhibit DNA reassociation values of less than 70% do not belong to the same species. Thus, the genomic group that includes CC809a and the two chickpea strains should not be assigned to *R. loti*. Furthermore, Chakrabarti et al. (6) determined levels of DNA-DNA hybrid-

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ization for four chickpea strains and NZP 2213^T and found very low homology values (8 to 32%) regardless of the generation times of the organisms.

On the other hand, extensive studies have demonstrated the uniqueness of the chickpea rhizobia. The results of host specificity experiments (16) clearly distinguished distinct cross-inoculation groups; 70 of the 71 chickpea isolates examined nodulated only the original host plant and did not nodulate the 88 species examined that belonged to the families Fabaceae and Mimosaceae. Similar conclusions were reached by Dadarwal (10), who studied the cross-infectivity of chickpea rhizobia by using cowpea group hosts. Immunochemical approaches have revealed that there are no serological cross-reactions between chickpea rhizobia and other *Rhizobium* species (10, 26). Cadahia et al. (5), using cultural characteristics and polymorphism of *nifHD* genes, did not find any criteria which defined groups within the chickpea rhizobia.

The numerous previously described contradictory conclusions about the characteristics and classification of the chickpea rhizobia were mainly due to the fact that few strains were tested or to the lack of approaches that permit workers to analyze genetic distances between genera. Few criteria exist for comparisons at the genus level; the most reproducible and universal criterion is the genetic distance between strains, as determined by 16S rRNA sequences. In the case of chickpea rhizobia, no such sequences have been available.

In order to reexamine the biodiversity of the chickpea rhizobia, we tested some of the collection strains used by Cadahia et al. (5); the strains used were chosen to represent various geographical origins (33). Sixteen strains were compared by using multilocus enzyme electrophoresis, restriction fragment length polymorphism (RFLP) of the 16S rRNA gene, and more than 150 phenotypic characteristics (33). The results of all of the approaches were very consistent, and our data resulted in the delineation of two phylogenetically distant groups (groups A and B) and correlated relatively well with strain generation times (slow and fast growers). Nevertheless, the two groups which we distinguished must be precisely identified by comparison with other taxa belonging to the *Rhizobiaceae*.

Therefore, in order to determine the taxonomic status of the two chickpea rhizobium groups, we analyzed the same 16 strains and compared them with representatives of previously described genera and species belonging to the *Rhizobiaceae*, including *R. loti* (in which some of the chickpea rhizobia have been classified previously). Consistent with the proposed minimal standards for the description of species (18, 41), the following taxonomic, phylogenetic, and phenotypic characteristics and approaches were used to study the chickpea rhizobia: DNA homology, guanine-plus-cytosine (G+C) content, RFLP of the amplified 16S-intergenic spacer (IGS) rRNA gene, partial 16S rRNA sequencing, and phenotypic characteristics, including auxanographic tests performed with 147 carbon sources.

MATERIALS AND METHODS

Strains and culture. The origins of the 16 chickpea collection strains and 13 reference strains belonging to the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* used in this study are shown in Table 1. The *R. huakuii* strain and 16S rRNA sequence became available at a time when most of our experiments had been terminated. We included this organism in the 16S rRNA sequence comparisons and DNA homology study.

TABLE 1. Bacterial strains used

Strain ^a	Host plant	Source or reference
<i>Rhizobium</i> sp. group A		
IC-72M	<i>Cicer arietinum</i> L.	5
Ca-7	<i>Cicer arietinum</i> L.	5
IC-6	<i>Cicer arietinum</i> L.	5
IC-60	<i>Cicer arietinum</i> L.	5
H-45	<i>Cicer arietinum</i> L.	1
UPM-Ca142	<i>Cicer arietinum</i> L.	5
UPM-Ca36	<i>Cicer arietinum</i> L.	5
<i>Rhizobium</i> sp. group B		
UPM-Ca7 ^T	<i>Cicer arietinum</i> L.	5
UPM-Ca116	<i>Cicer arietinum</i> L.	5
IC-2091	<i>Cicer arietinum</i> L.	1
IC-2018	<i>Cicer arietinum</i> L.	1
3HOa10	<i>Cicer arietinum</i> L.	5
3HOa9	<i>Cicer arietinum</i> L.	5
522	<i>Cicer arietinum</i> L.	5
USAB67	<i>Cicer arietinum</i> L.	5
<i>Rhizobium</i> sp. group C strain 43-b	<i>Cicer arietinum</i> L.	5
<i>R. loti</i> NZP 2213 ^T	<i>Lotus tenuis</i>	9
<i>R. galegae</i>		
ATCC 43677 ^T	<i>Galega orientalis</i>	28
HAMBI 1141	<i>Galega officinalis</i>	28
<i>R. leguminosarum</i> bv. <i>viciae</i> JISP24	<i>Pisum sativum</i>	MSDJ ^b
<i>R. leguminosarum</i> bv. <i>trifolii</i> USDA 3Dim37	<i>Trifolium</i> spp.	MSDJ
<i>R. huakuii</i> CCBAU 2609 ^T	<i>Astragalus sinicus</i>	7
<i>R. meliloti</i> RCR 2011	<i>Medicago sativa</i>	29
<i>R. fredii</i> USDA 205 ^T	<i>Glycine max</i>	37
<i>R. tropici</i> CFN 299	<i>Phaseolus vulgaris</i>	29
<i>B. japonicum</i>		
USDA 110	<i>Glycine max</i>	46
USDA 135	<i>Glycine max</i>	MSDJ
<i>Bradyrhizobium</i> sp. strain LL113	<i>Lupinus luteus</i>	MSDJ
<i>Azorhizobium caulinodans</i>		
FY12	<i>Sesbania rostrata</i>	34
SG05	<i>Sesbania rostrata</i>	34

^a Groups A through C were described by Nour et al. (33). Strains were obtained from the Departamento de Microbiología, E.T.S. de Ingenieros Agrónomos, Universidad Politécnica, Madrid, Spain. USDA, United States Department of Agriculture, Beltsville, Md.; ATCC, American Type Culture Collection, Rockville, Md.; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland; NZP, Culture Collection of the Department for Scientific and Industrial Research, Palmerston North, New Zealand; CFN, Centro de Investigación sobre Fijación de Nitrogeno, Universidad Nacional Autónoma de México, Cuernavaca, México; FY and SG, Office de Recherche Scientifique et Technique Outre Mer, Dakar, Senegal; CCBAU, Culture Collection of Beijing Agricultural University, Beijing, People's Republic of China.

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Most strains were grown and maintained on yeast extract-mannitol medium (40); yeast extract-lactate medium (34) was used for the *Azorhizobium* strains. These media were used for all procedures and experiments except extraction of chickpea rhizobium DNA; for DNA extraction maltose was used as the carbohydrate instead of mannitol in order to limit extra slime formation.

DNA extraction. DNA was extracted by using the procedure of Brenner et al. (3), with slight modifications. To further purify chickpea rhizobium DNA, extra chloroform-isoamyl alcohol extraction steps were performed.

Determination of base composition. The average G+C contents of chickpea rhizobium DNAs were determined by high-performance liquid chromatography, as described previously (17).

DNA-DNA hybridization and thermal stability of duplexes. Total DNAs from *R. loti* NZP 2213^T, *R. galegae* ATCC 43677^T, *R. huakuii* CCBAU 2609^T, and chickpea rhizobium strains UPM-Ca142, 3HOa9, UPM-Ca7^T, and IC-60, were labelled in vitro by nick translation using tritium-labelled nucleotides (Amersham International, Amersham, England). DNA-DNA hybridization experiments were performed at the optimal temperature for DNA reassociation (70°C) by using the S1 nuclease-trichloroacetic acid procedure of Grimont et al. (20).

The thermal stability of reassociated DNA was estimated by determining the temperature (T_m) at which 50% of the double-stranded DNA became denatured and lysed by S1 nuclease, as described previously (8), with slight modifications (12). The temperature of the hybridization mixture was increased in 5°C steps from 70 to 100°C. The divergence between DNAs was determined by calculating ΔT_m (the difference between the T_m values of the heterologous and homologous duplexes).

PCR amplification and restriction enzyme analysis. Strains IC-6 and UPM-Ca7^T, which were representatives of the groups A and B (33), respectively, were compared with all of the reference strains except the *R. huakuii* strain. The oligonucleotides used to amplify the 16S-IGS region of the rRNA gene were 5'-GGAGAGTTAGATCTTGGCTCAG-3' (FGPS6-63) and 5'-CCGGGTTTCCCCATTCGG-3' (FGPL132'-38), which were derived from *rrn* sequences of *Frankia* sp. (32). DNA was amplified by using the protocol of Mullis and Faloona (30), with some modifications. PCRs 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 µM 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 *Taq*I DNA polymerase (Gibco-BRL, Cergy-Pontoise, France). Amplification was performed 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 finally 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.

Restriction enzyme analyses of PCR products were performed in a total volume of 20 µl by using 10 to 12 U of the following seven restriction endonucleases: *Alu*I, *Cfo*I, *Msp*I, *Nde*II, and *Sau*96.1 supplied by Boehringer Mannheim, Meylan, France, and *Hae*III and *Rsa*I supplied by Gibco-BRL. The reaction conditions used were those recommended by the manufacturers. The restricted fragments were separated by horizontal electrophoresis in TBE buffer by using a 3% (wt/vol) Nusieve agarose gel (FMC, Rockland, Maine) containing 1 µg of ethidium bromide per ml. The molecular weight standards used were 123-bp and 1-kb ladders (Gibco-BRL). The gels were electrophoresed at 2.3 V/cm for 3 h.

The distances that the restricted fragments migrated from the wells were measured, and the profiles were compared. Bands smaller than 90 bp were not taken into consideration, since they were not visualized reproducibly. The genetic relationship between two amplified DNAs was evaluated by using similarity coefficients (F). F values were calculated by using the following formula: $F = 2n_{xy}/n_x + n_y$, where n_{xy} is the number of bands shared by strains x and y with all restriction endo-

nucleases used and n_x and n_y are the total numbers of bands obtained for strains x and y, respectively. A genetic distance for each pair of strains was estimated by using the mathematical model defined by Nei and Li (31), and a dendrogram was constructed by using the unweighted pair group method with arithmetic average algorithm of Sneath and Sokal (39).

Nucleotide sequencing of 16S rRNA genes. The following four chickpea rhizobia strains that were representatives of the previously described phylogenetic groups (33) were tested: group A strains IC-72M and UPM-Ca36 and group B strains UPM-Ca7^T and IC-2018. DNA coding for the preliminary section of the 16S rRNA was amplified by using the conditions described above and oligonucleotides 5'-AGAGTTAGATC TTGGCTCAG-3' (FG1-157) and 5'-CCAGTGTGGCCG GTCGCCCTCTC-3' (FGPS305'-78), which were derived from *rrn* sequences of *Frankia* sp. (32). To check the efficiency of the amplification reactions, 5-µl portions of the PCR products were analyzed by electrophoresis on a 0.8% (wt/vol) agarose gel. The amplified products were purified with a GeneClean kit (Bio 101, La Jolla, Calif.) as specified by the manufacturer and then concentrated in 10 µl of sterilized ultrapure water. A 1-µl aliquot was electrophoresed on a 0.8% (wt/vol) agarose gel for a final check before sequencing.

Sequencing was performed by using the dideoxy chain termination method of Sanger et al. (36), with the slight modifications described by Winship (43). The sequences of both strands of each amplified DNA were determined. The sequences were aligned and compared by using the Clustal V program (21). The 16S rRNA sequences of the following organisms belonging to the *Rhizobiaceae* and related bacteria found in the GenBank Data Library were used for comparison (accession numbers are in parentheses): *R. loti* NZP 2213^T (X63825), *R. galegae* ATCC 43677^T (X63823), *Rhizobium* sp. (*Phaseolus*) strain H152 (L19662), *Rhizobium* sp. (*Phaseolus*) strain FL27 (M55234), *Rhizobium* sp. (*Leucaena*) strain TAL 1145 (X63824), *R. tropici* CIAT899^T (M55233), *R. tropici* IIA strain CO-5 (X67234), *R. etli* Olivia 4 (M55235), *Rhizobium* sp. (*Medicago*) strain Or191 (M55236), *R. leguminosarum* bv. phaseoli ATCC 8002 (M55494), *R. meliloti* ATCC 9930^T (M55241), *R. meliloti* CC169 (M55242), *R. fredii* USDA 205^T (M74163), *Rhizobium* sp. (*Medicago*) strain Or191 (M55236), *R. leguminosarum* bv. phaseoli ATCC 8002 (M55494), *R. meliloti* ATCC 9930^T (M55241), *R. meliloti* CC169 (M55242), *R. fredii* USDA 205^T (M74163), *Rhizobium* sp. (*Medicago*) strain Or191 (M55236), *R. leguminosarum* bv. phaseoli ATCC 8002 (M55494), *R. meliloti* ATCC 9930^T (M55241), *R. meliloti* CC169 (M55242), *R. fredii* USDA 205^T (M74163), *Rhizobium* sp. (*Medicago*) strain Or191 (M55236), *R. leguminosarum* bv. phaseoli ATCC 8002 (M55494), *R. meliloti* ATCC 9930^T (M55241), *R. meliloti* CC169 (M55242), *R. fredii* USDA 205^T (M74163), *Rhizobium* sp. 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FIG. 1. RFLP analysis of amplified 16S-IGS rRNA genes from chickpea rhizobia and other members of the *Rhizobiaceae*, using restriction enzyme *Sau*96.1. *B.*, *Bradyrhizobium*; *A.* *Azorhizobium*; *R.* *Rhizobium*; *R. leg. bv. viciae*, *R. leguminosarum* bv. *viciae* JISP24; *R. leg. bv. trifolii*, *R. leguminosarum* bv. *trifolii* USDA 3Dim37; *B. sp. (lupin)* LL113, *Bradyrhizobium sp. (Lupinus)* strain LL113.

Grottes, Vercieu, France) that included 49 carbohydrates (API 50CH), 49 organic acids (LRA 50AO), and 49 amino acids (LRA 50AA) were used. The inoculation procedures were performed as recommended by the manufacturer, and the galleries were incubated at 28°C. Growth (as shown by turbidity compared with a control) was recorded every 24 h for 5 consecutive days. The genetic distances between strains were estimated by using the Jaccard similarity coefficient (39). In this procedure the sum of effective matches was divided by the total possible number of matches for each pairwise calculation. Clustering based on a matrix of pairwise genetic distances was performed by using the unweighted pair group method with arithmetic average algorithm of Sneath and Sokal (39).

RESULTS

PCR-RFLP analysis of 16S-IGS rDNA. In a previous study two genetic groups, groups A and B, were distinguished on the basis of the polymorphism of rRNA genes (33). In this study, two representative strains (one belonging to each group) were compared with reference strains belonging to known genera and species of the *Rhizobiaceae*. The DNAs of chickpea rhizobium strains UPM-Ca7^T and IC-6 and reference strains were amplified with the 16S-IGS rRNA primers designated FGPS6-63 (5' end of the 16S subunit) and FGPL132'-38 (5' end of the 23S subunit). An electrophoretic analysis of uncut PCR products revealed a single band having the same intensity for all strains. The sizes of the fragments represented by the bands were approximately 2,500 bp for the chickpea rhizobium, *R. galegae*, *R. loti*, and *Azorhizobium* strains; 2,800 bp for the *R. tropici*, *R. meliloti*, *R. leguminosarum* bv. *trifolii*, *R. leguminosarum* bv. *viciae*, and *R. fredii* strains; 1,800 bp for *B. japonicum* USDA 110; 2,000 bp for *Bradyrhizobium sp. (Lupinus)*; and 2,200 bp for *B. japonicum* USDA 135.

The seven restriction enzymes which we used produced polymorphic patterns for the strains studied. *Rsa*I was the least

discriminating enzyme since nine different profiles were found among the 15 strains tested. *Cfo*I, *Nde*II, and *Msp*I produced a unique pattern for each strain. Restriction enzymes *Alu*I, *Sau*96.1 (Fig. 1), and *Hae*III produced 14 profiles; the profiles of *R. loti* NZP 2213^T and UPM-Ca7^T were the same with these enzymes. The similarity coefficient values ranged from 0.236 to

FIG. 2. Genetic distances, based on the results of an RFLP analysis of the amplified 16S-IGS rRNA region, between chickpea rhizobia and other members of the *Rhizobiaceae*. For abbreviations and the strains used, see the legend to Fig. 1. Groups A and B were described by Nour et al. (33).

TABLE 2. Utilization of sole carbon sources by chickpea rhizobia, *R. loti*, and *R. galegae*

Carbon sources	<i>Rhizobium</i> sp. (<i>Cicer</i>) group A	<i>Rhizobium</i> sp. (<i>Cicer</i>) group B	<i>R. loti</i> NZP 2213 ^T	<i>R. galegae</i> ATCC 43677 ^T
Glycerol, D-arabinose, L-arabinose, ribose, D-xylose, D-glucose, D-fructose, D-mannose, rhamnose, inositol, mannitol, sorbitol, <i>N</i> -acetylglucosamine, maltose, sucrose, trehalose, D-turanose, L-fucose, D-arabitol, succinate, fumarate, D-malate, L-malate, pyruvate, L-histidine, D-tryptophan, L-tryptophan, L-glutamate, DL-kynurenine, L-proline, betaine, DL-4-aminobutyrate, 2-aminobenzoate, 3-aminobenzoate, 4-aminobenzoate, glucosamine	+	+	+	+
Galactose, DL-lactate, L-tyrosine	+	+	+	—
D-Lyxose, DL-glycerate, L-aspartate	—	+	+	+
L-Ornithine, β-alanine	—	+	+	—
L-Xylose, L-alanine, L-leucine	—	+	—	—
Amygdalin, D-raffinose, gluconate, 5-ketogluconate, glutarate, DL-3-OH-butyrate, D-tartrate, mesotartrate, <i>m</i> -OH-benzoate, tryptamine	—	—	—	+
L-Phenylalanine, L-lysine	—	—	+	—

0.884 (data not shown). On the basis of the genetic distances obtained, we constructed a dendrogram (Fig. 2) which revealed the following two main groups: a *Rhizobium*-*Azorhizobium* group and a group consisting of *Bradyrhizobium* strains. These two groups were separated by a genetic distance of approximately 0.12. *R. loti*, *R. galegae*, and the chickpea rhizobium strains formed a cluster which branched off from the remaining rhizobium and azorhizobium strains at a genetic distance of 0.08. The chickpea rhizobia grouped with *R. loti* and were separated from the two *R. galegae* strains at a genetic distance of about 0.065. Strain UPM-Ca7^T, a group B strain, was separated from *R. loti* NZP 2213^T at a genetic distance of 0.01.

Phenotypic tests. The RFLP analysis of rRNA genes grouped the chickpea rhizobia close to *R. loti* and *R. galegae*. Thus, phenotypic tests were performed with the type strains of these two species and representative strains belonging to chickpea rhizobium groups A and B. The results of tests to determine assimilation of sole carbon sources are shown in Table 2. The following 61 compounds were not assimilated by any of the strains: erythritol, β-methylxyloside, α-methyl-D-mannoside, α-methyl-D-glucoside, salicin, inulin, melezitose, amidon, glycogen, acetate, propionate, butyrate, isobutyrate, *N*-valerate, isovalerate, *N*-caproate, heptanoate, caprylate, pelargonate, caprate, oxalate, malonate, maleate, adipate, pimelate, suberate, azelate, L-tartrate, levulinate, citraconate, itaconate, mesaconate, aconitate, phenylacetate, benzoate, *o*-hydroxybenzoate, *p*-hydroxybenzoate, D-mandelate, L-mandelate, phthalate, isophthalate, terephthalate, glycine, D-alanine, L-isoleucine, L-norleucine, DL-norvaline, DL-2-aminobutyrate, L-serine, L-cysteine, L-methionine, L-citrulline, creatine, urea, acetamide, ethylamine, butylamine, amylamine, benzylamine, diaminobutane, and spermine. It is interesting that when we considered the amino acids utilized by at least one strain, *R. galegae* and the group A strains assimilated about 50% of these amino acids, whereas *R. loti* and the group B strains assimilated 77 and 70%, respectively. In this respect, the chickpea rhizobium group A strains resemble *R. galegae*, whereas the group B strains resemble *R. loti*. When we took all 147 characteristics into account, the dendrogram obtained (Fig. 3) showed that *R. loti* NZP 2213^T joins the group B chickpea rhizobium strains at a value of approximately 0.23, and the group A chickpea rhizobium strains join at a distance of 0.32. The type strain of *R. galegae* joins at a distance of 0.41.

DNA sequencing. The fragment sequenced was located between nucleotide positions 44 and 337 in the *Escherichia coli* 16S rRNA sequence. This region, which has been used exten-

sively for phylogenetic and taxonomic studies of members of the *Rhizobiaceae* (11, 27, 44, 46), is long and variable enough to permit reliable comparisons. The corresponding amplified DNA fragments obtained for strains IC-72M and UPM-Ca36 (group A) and for strains UPM-Ca7^T and IC-2018 (group B) were 260 bp long. The four strains were identical at all 260 nucleotide positions determined (Fig. 4). When we compared these sequences with the *R. loti* and *R. huakuii* sequences, there were two and one nucleotide mismatches, respectively. The similarity coefficients for the chickpea rhizobia ranged from 0.772 with *Rhodospirillum rubrum* to 0.996 with *R. huakuii* (Table 3). When the sequence was compared with the *R. loti* and *R. galegae* sequences, the similarity coefficients were 0.992 and 0.972, respectively. The phylogenetic tree obtained by using the partial 16S rRNA sequences is shown in Fig. 5. All of the chickpea rhizobium strains belonging to groups A and B belonged to the *Rhizobium*-*Agrobacterium* lineage, and none belonged to the *Bradyrhizobium*-*Rhodopseudomonas* lineage. The chickpea rhizobium strains formed a tight cluster with *R. huakuii*, *R. loti*, and *R. galegae*.

DNA analysis. The G+C contents of the DNAs of representative chickpea rhizobium strains were as follows: strain 3HOa9, 64 mol%; and strains UPM-Ca7^T and IC-2091, 63 mol%.

FIG. 3. Distances, based on sole carbon source assimilation data, between chickpea rhizobia, *R. loti*, and *R. galegae*. *R.*, *Rhizobium*. Groups A and B have been described previously (33).

	43	53	63	73	83	93
1 <i>R. sp. (Cicer)</i> UPM-Ca7	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA	CGGGTGAGTA
2 <i>R. loti</i> NZP 2213	AGGCTTAACA	CATGCAAGTC	GAGCG--CCT	CGCAAGAGG-	-AGCGGCAGA	CGGGTGAGTA
3 <i>R. galegae</i> ATCC 43677	AGGCTTAACA	CATGCAAGTC	GAGCG--CCT	CGCAAGAGG-	-AGCGGCAGA	CGGGTGAGTA
4 <i>R. huakuii</i> CCBau 2609	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA	CGGGTGAGTA
5 <i>R. sp. (Phaseolus)</i> H152	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA	CGGGTGAGTA
6 <i>R. sp. (Leucaena)</i> TAL 1145	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA	CGGGTGAGTA
7 <i>R. tropici</i> CIAT 899	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA	CGGGTGAGTA
8 <i>R. tropici</i> C-05	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CTCGAGGGG-	-AGCGGCAGA	CGGGTGAGTA
9 <i>R. sp. (Phaseolus)</i> FL27	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA	CGGGTGAGTA
10 <i>R. etli</i> Olivia 4	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA	CGGGTGAGTA
11 <i>R. sp. (Medicago)</i> Or191	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA	CGGGTGAGTA
12 <i>R. leg. bv. phas.</i> 8002	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA	CGGGTGAGTA
13 <i>R. meliloti</i> ATCC 9930	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA	CGGGTGAGTA
14 <i>R. meliloti</i> CC169	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA	CGGGTGAGTA
15 <i>R. fredii</i> USDA 205	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA	CGGGTGAGTA
16 <i>A. tumefaciens</i> LMG 196	AGGCTTAACA	CATGCAAGTC	GAACG--CCC	CGCAAGGGG-	-AGTGGCAGA	CGGGTGAGTA
17 <i>A. rhizogenes</i> LMG 152	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA	CGGGTGAGTA
18 <i>A. rubi</i> LMG 156	AGGCTTAACA	CATGCAAGTC	GAACG--CTC	CGCAAGGAG-	-AGTGGCAGA	CGGGTGAGTA
19 <i>A. vitis</i> LMG 8750	AGGCTTAACA	CATGCAAGTC	GAGCG--CCT	CGCAAGAGG-	-AGCGGCAGA	CGGGTGAGTA
20 <i>B. japonicum</i> USDA 110	AGGCTTAACA	CATGCAAGTC	GAGCGGGCGT	AGCAATACGT	CAGGTGCAGA	CGGGTGAGTA
21 <i>Az. cauli.</i> ORS 571	AGGCTTAACA	CATGCAAGTC	GAACGGGCC	-TTCCG--GG-T	CAGTGGCAGA	CGGGTGAGTA
22 <i>Ro. quintana</i>	AGGCTTAACA	CATGCAAGTC	GAGCGCACCT	TTTTAGAGT	GAGCGGCAAA	CGGGTGAGTA
23 <i>Rp. palustris</i> 216	AGGCTTAACA	CATGCAAGTC	GAACGGGCGT	AGCAATACGT	CAGTGGCAGA	CGGGTGAGTA
24 <i>Rb. sphaeroides</i> ATCC 17023	AGGCTTAACA	CATGCAAGTC	GAGCGC-CGT	AGCAATACGG	-AGCGGCAGA	CGGGTGAGTA
25 <i>Rs. rubrum</i> ATCC 11170	AGGCTTAACA	CATGCAAGTC	GAACGCATCC	CTTCG--GGAT	GAGTGGCGCA	CGGGTGAGTA
	103	113	123	143	153	163
1 <i>R. sp. (Cicer)</i> UPM-Ca7	ACGCGTGGGA	ATCTACCCAT	CTCTACGGAA	CAACTCCGGG	AAACTGGAGC	TAATACCGTA
2 <i>R. loti</i> NZP 2213	ACGCGTGGGA	ATCTACCCAT	CTCTACGGAA	CAACTCCGGG	AAACTGGAGC	TAATACCGTA
3 <i>R. galegae</i> ATCC 43677	ACGCGTGGGA	ATCTACCCAT	CCCTACGGAA	CAACTCCGGG	AAACTGGAGC	TAATACCGTA
4 <i>R. huakuii</i> CCBau 2609	ACGCGTGGGA	ATCTACCCAT	CTCTACGGAA	CAACTCCGGG	AAACTGGAGC	TAATACCGTA
5 <i>R. sp. (Phaseolus)</i> H152	ACGCGTGGGA	ATCTACCCAT	CTCTACGGAA	TAACTCAGGG	AAACTTGTGC	TAATACCGTA
6 <i>R. sp. (Leucaena)</i> TAL 1145	ACGCGTGGGA	ATCTACCCAT	CTCTACGGAA	TAACTCAGGG	AAACTTGTGC	TAATACCGTA
7 <i>R. tropici</i> CIAT 899	ACGCGTGGGA	ATCTACCCAT	TGCTACGGAA	TAACTCAGGG	AAACTTGTGC	TAATACCGTA
8 <i>R. tropici</i> C-05	ACGCGTGGGA	ATCTACCCAT	TTCTACGGAA	TAACTCAGGG	AAACTTGTGC	TAATACCGTA
9 <i>R. sp. (Phaseolus)</i> FL27	ACGCGTGGGA	ACGTACCCCT	TACTACGGAA	TAACTCAGGG	AAACTTGTGC	TAATACCGTA
10 <i>R. etli</i> Olivia 4	ACGCGTGGGA	ACGTACCCCT	TACTACGGAA	TAACTCAGGG	AAACTTGTGC	TAATACCGTA
11 <i>R. sp. (Medicago)</i> Or191	ACGCGTGGGA	ACGTACCCCT	TACTACGGAA	TAACTCAGGG	AAACTTGTGC	TAATACCGTA
12 <i>R. leg. bv. phas.</i> 8002	ACGCGTGGGA	ATCTACCCCT	GACTACGGAA	TAACTCAGGG	AAACTTGTGC	TAATACCGTA
13 <i>R. meliloti</i> ATCC 9930	ACGCGTGGGA	ATCTACCCCT	TTCTACGGAA	TAACTCAGGG	AAACTTGTGC	TAATACCGTA
14 <i>R. meliloti</i> CC169	ACGCGTGGGA	ATCTACCCCT	TTCTACGGAA	TAACTCAGGG	AAACTTGTGC	TAATACCGTA
15 <i>R. fredii</i> USDA 205	ACGCGTGGGA	ATCTACCCCT	TTCTACGGAA	TAACTCAGGG	AAACTTGTGC	TAATACCGTA
16 <i>A. tumefaciens</i> LMG 196	ACGCGTGGGA	ATCTACCCCT	CCCTGCGGAA	TAGCTCCGGG	AAACTGGAAT	TAATACCGCA
17 <i>A. rhizogenes</i> LMG 152	ACGCGTGGGA	ATCTACCCCT	TTCTACGGAA	TAACTCAGGG	AAACTTGTGC	TAATACCGTA
18 <i>A. rubi</i> LMG 156	ACGCGTGGGA	ATCTACCCCT	CCCTGCGGAA	TAGCTCTGGG	AAACTTGTGC	TAATACCGCA
19 <i>A. vitis</i> LMG 8750	ACGCGTGGGA	ATCTACCCCT	CCCTACGGAA	TAGCTCCGGG	AAACTGGAAT	TAATACCGTA
20 <i>B. japonicum</i> USDA 110	ACGCGTGGGA	ACGTACCCCT	TGGTTCCGAA	CAACACAGGG	AAACTTGTGC	TAATACCGGA
21 <i>Az. caulinodans</i> ORS 571	ACGCGTGGGA	ATCTACCCCT	CAGTTCCGAA	TAACCCAGGG	AAACTTGTGC	TAATACCGGA
22 <i>Ro. quintana</i>	ACGCGTGGGA	ATCTACCCCT	CTCTACGGAA	TAACACAGAG	AAATTTGTGC	TAATACCGTA
23 <i>Rp. palustris</i> 216	ACGCGTGGGA	ACGTACCCCT	TGGTTCCGAA	CAACACAGGG	AAACTTGTGC	TAATACCGGA
24 <i>Rb. sphaeroides</i> ATCC 17023	ACGCGTGGGA	ATGTGCCCAA	AGGTACGGAA	CAACCAAGGG	AAACTTTGGC	TAATACCGTA
25 <i>Rs. rubrum</i> ATCC 11170	ACGCGTGGGA	ACGTACCCCT	GAGTCCGGAA	TAATCTTTGG	AAACGAGGAC	TAATACCGCA
	173	183	193	203	213	223
1 <i>R. sp. (Cicer)</i> UPM-Ca7	TACGTCC--TT	CGGGGAGAAAG	ATTTATCGGA	GATGGATGAG	CCCGCGTTGG	ATTAGCTAGT
2 <i>R. loti</i> NZP 2213	TACGTCC--TT	CGGGGAGAAAG	ATTTATCGGA	GATGGATGAG	CCCGCGTTGG	ATTAGCTAGT
3 <i>R. galegae</i> ATCC 43677	TACGTCC--TT	CGGGGAGAAAG	ATTTATCGGG	GATGGATGAG	CCCGCGTTGG	ATTAGCTAGT
4 <i>R. huakuii</i> CCBau 2609	TACGTCC--TT	CGGGGAGAAAG	ATTTATCGGA	GATGGATGAG	CCCGCGTTGG	ATTAGCTAGT
5 <i>R. sp. (Phaseolus)</i> H152	TACGTCC--TT	CGGGGAGAAAG	ATTTATCGGA	GATGGATGAG	CCCGCGTTGG	ATTAGCTAGT
6 <i>R. sp. (Leucaena)</i> TAL 1145	TGTGTCC--TT	CGGGGAGAAAG	ATTTATCGGG	GAAGGATGAG	CCCGCGTTGG	ATTAGCTAGT
7 <i>R. tropici</i> CIAT 899	TGTGTCC--TT	CGGGGAGAAAG	ATTTATCGGG	AAGAGATGAG	CCCGCGTTGG	ATTAGCTAGT
8 <i>R. tropici</i> C-05	TGTGTCC--TT	CGGGGAGAAAG	ATTTATCGGG	AAAGGATGAG	CCCGCGTTGG	ATTAGCTAGT
9 <i>R. sp. (Phaseolus)</i> FL27	TGTGTCC--TT	CGGGGAGAAAG	ATTTATCGGT	AAGGATCGG	CCCGCGTTGG	ATTAGCTAGT
10 <i>R. etli</i> Olivia 4	TGTGTCC--TT	CGGGGAGAAAG	ATTTATCGGT	AAGGATCGG	CCCGCGTTGG	ATTAGCTAGT
11 <i>R. sp. (Medicago)</i> Or191	TGTGTCC--TT	CGGGGAGAAAG	ATTTATCGGT	AAGGATCGG	CCCGCGTTGG	ATTAGCTAGT
12 <i>R. leg. bv. phas.</i> 8002	TGTGTCC--TT	CGGGGAGAAAG	ATTTATCGGT	CAAGGATGAG	CCCGCGTTGG	ATTAGCTAGT
13 <i>R. meliloti</i> ATCC 9930	TGAGCCC--TT	CGGGGAGAAAG	ATTTATCGGG	AAAGGATGAG	CCCGCGTTGG	ATTAGCTAGT
14 <i>R. meliloti</i> CC169	TAAGCCC--TT	CGGGGAGAAAG	ATTTATCGGG	AAAGGATGAG	CCCGCGTTGG	ATTAGCTAGT
15 <i>R. fredii</i> USDA 205	TGAGCCC--TT	CGGGGAGAAAG	ATTTATCGGG	AAAGGATGAG	CCCGCGTTGG	ATTAGCTAGT
16 <i>A. tumefaciens</i> LMG 196	TACGCCC--TA	CGGGGAGAAAG	ATTTATCGGG	GTATGATGAG	CCCGCGTTGG	ATTAGCTAGT
17 <i>A. rhizogenes</i> LMG 152	TGTGTCC--TT	CGGGGAGAAAG	ATTTATCGGG	AAAGGATGAG	CCCGCGTTGG	ATTAGCTAGT
18 <i>A. rubi</i> LMG 156	TACGCCC--TA	CGGGGAGAAAG	ATTTATCGGG	GATGGATGAG	CCCGCGTTGG	ATTAGCTAGT
19 <i>A. vitis</i> LMG 8750	TACGCCC--TT	CGGGGAGAAAG	ATTTATCGGG	GTATGATGAG	CCCGCGTTGG	ATTAGCTAGT
20 <i>B. japonicum</i> USDA 110	TAAGCCC--TT	ACGGGGAGAAAG	ATTTATCGCC	GAAAGATCGG	CCCGCGTTGG	ATTAGCTAGT
21 <i>Az. caulinodans</i> ORS 571	TACGTCC--GA	AAGGAGAAAG	ATTGATCGCT	GAAGGATCGG	CCCGCGTTGG	ATTAGCTAGT
22 <i>Ro. quintana</i>	TACGTCCCTC	TGGGAGAAAG	ATTTATCGGA	GGTGGATGAG	CCCGCGTTGG	ATTAGCTAGT
23 <i>Rp. palustris</i> 216	TAAGCCC--TT	ACGGGGAGAAAG	ATTTATCGCC	GAAAGATCGG	CCCGCGTTGG	ATTAGCTAGT
24 <i>Rb. sphaeroides</i> ATCC 17023	TGTGTCC--GA	GAGGGAGAAAG	ATTTATTGCC	TTTGGAGCAG	CCCGCGTTGG	ATTAGCTAGT
25 <i>Rs. rubrum</i> ATCC 11170	TACGCCC--TT	AGGGGAGAAAG	ATTTATCGCT	CCAAGATCGG	CCCGCGTTGG	ATTAGCTAGT

	233	243	253	263	273	283
1 <i>R. sp. (Cicer)</i> UPM-Ca7	TGGTGGGGTA	ATGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
2 <i>R. loti</i> NZP 2213	TGGTGGGGTA	ATGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
3 <i>R. galegae</i> ATCC 43677	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
4 <i>R. huakuii</i> CCBAU 2609	TGGTGGGGTA	ATGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
5 <i>R. sp. (Phaseolus)</i> H152	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
6 <i>R. sp. (Leucaena)</i> TAL 1145	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
7 <i>R. tropici</i> CIAT 899	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
8 <i>R. tropici</i> C-05	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
9 <i>R. sp. (Phaseolus)</i> FL27	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
10 <i>R. etli</i> Olivia 4	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
11 <i>R. sp. (Medicago)</i> Or191	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
12 <i>R. leg. bv. phas.</i> 8002	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
13 <i>R. meliloti</i> ATCC 9930	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
14 <i>R. meliloti</i> CC169	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
15 <i>R. fredii</i> USDA 205	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
16 <i>A. tumefaciens</i> LMG 196	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
17 <i>A. rhizogenes</i> LMG 152	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
18 <i>A. rubi</i> LMG 156	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
19 <i>A. vitis</i> LMG 8750	TGGTGGGGTA	AAGGCCTACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
20 <i>B. japonicum</i> USDA 110	TGGTAGGGTA	ACGGCCTACC	AAGGCGACGA	TCAGTAGCTG	GTCTGAGAGG	ATGATCAGCC
21 <i>Az. caulinodans</i> ORS 571	TGGTAGGGTA	ATGGCTCACC	AAGGCGACGA	TCAGTAGCTG	GTCTGAGAGG	ATGATCAGCC
22 <i>Ro. quintana</i>	TGGTAGGGTA	AGGGCTCACC	AAGGCGACGA	TCCATAGCTG	GTCTGAGAGG	ATGATCAGCC
23 <i>Rp. palustris</i> 216	TGGTAGGGTA	ATGGCTCACC	AAGGCGACGA	TCAGTAGCTG	GTCTGAGAGG	ATGATCAGCC
24 <i>Rb. sphaeroides</i> ATCC 17023	TGGTGGGGTA	ACGGCCACC	AAGGCGACGA	TCAGTAGCTG	GTCTGAGAGG	ATGATCAGCC
25 <i>Rs. rubrum</i> ATCC 11170	TGGCGGGTA	ATGGCCACC	AAGGCGACGA	TCGGTAGCTG	GTCTGAGAGG	ATGGCAGCCA
	293*	303				
1 <i>R. sp. (Cicer)</i> UPM-Ca7	ACATTGGGAC	TGAGACACGG				
2 <i>R. loti</i> NZP 2213	ACATTGGGAC	TGAGACACGG				
3 <i>R. galegae</i> ATCC 43677	ACATTGGGAC	TGAGACACGG				
4 <i>R. huakuii</i> CCBAU 2609	ACACTGGGAC	TGAGACACGG				
5 <i>R. sp. (Phaseolus)</i> H152	ACATTGGGAC	TGAGACACGG				
6 <i>R. sp. (Leucaena)</i> TAL 1145	ACATTGGGAC	TGAGACACGG				
7 <i>R. tropici</i> CIAT 899	ACATTGGGAC	TGAGACACGG				
8 <i>R. tropici</i> C-05	ACATTGGGAC	TGAGACACGG				
9 <i>R. sp. (Phaseolus)</i> FL27	ACATTGGGAC	TGAGACACGG				
10 <i>R. etli</i> Olivia 4	ACATTGGGAC	TGAGACACGG				
11 <i>R. sp. (Medicago)</i> Or191	ACATTGGGAC	TGAGACACGG				
12 <i>R. leg. bv. phas.</i> 8002	ACATTGGGAC	TGAGACACGG				
13 <i>R. meliloti</i> ATCC 9930	ACATTGGGAC	TGAGACACGG				
14 <i>R. meliloti</i> CC169	ACATTGGGAC	TGAGACACGG				
15 <i>R. fredii</i> USDA 205	ACATTGGGAC	TGAGACACGG				
16 <i>A. tumefaciens</i> LMG 196	ACATTGGGAC	TGAGACACGG				
17 <i>A. rhizogenes</i> LMG 152	ACATTGGGAC	TGAGACACGG				
18 <i>A. rubi</i> LMG 156	ACATTGGGAC	TGAGACACGG				
19 <i>A. vitis</i> LMG 8750	ACATTGGGAC	TGAGACACGG				
20 <i>B. japonicum</i> USDA 110	ACATTGGGAC	TGAGACACGG				
21 <i>Az. caulinodans</i> ORS 571	ACATTGGGAC	TGAGACACGG				
22 <i>Ro. quintana</i>	ACACTGGGAC	TGAGACACGG				
23 <i>Rp. palustris</i> 216	ACATTGGGAC	TGAGACACGG				
24 <i>Rb. sphaeroides</i> ATCC 17023	ACATTGGGAC	TGAGACACGG				
25 <i>Rs. rubrum</i> ATCC 11170	CACCTGGGAC	TGAGACACGG				

FIG. 4. Alignment of chickpea rhizobium partial 16S rRNA gene sequences with sequences of other members of the *Rhizobiaceae* and related bacteria. *R.*, *Rhizobium*; *R. leg. bv. phas.*, *R. leguminosarum* bv. phaseoli; *A.*, *Agrobacterium*; *B.*, *Bradyrhizobium*; *Az.*, *Azorhizobium*; *Ro.*, *Rochalimaea*; *Rp.*, *Rhodopseudomonas*; *Rb.*, *Rhodobacter*; *Rs.*, *Rhodospirillum*. *, nucleotide mismatches between *Rhizobium* sp. (*Cicer*), *A. loti*, *R. galegae*, and *R. huakuii*.

On the basis of the results of the partial 16S rRNA sequence analysis, which placed the chickpea rhizobia close to *R. loti*, *R. huakuii*, and *R. galegae*, DNA relatedness values were therefore determined by using *R. loti* NZP 2213^T, *R. galegae* ATCC 43677^T, and *R. huakuii* CCBAU 2609^T as the reference strains. The results are shown in Table 4. *R. loti* gave low homology values (21 to 45%) and ΔT_m values ranging from 5.5 to 11.5°C with all of the chickpea rhizobia. Likewise, *R. galegae* exhibited low levels of homology (0.4 to 8%) with both the chickpea rhizobia and *R. loti*. The homology values for *R. huakuii* and the chickpea rhizobia were 18 to 52%. Moreover, the levels of DNA homology between three chickpea rhizobia (UPM-Ca142, IC-60, and 3HOa9) and other species, including *R. leguminosarum* bv. viciae, *R. leguminosarum* bv. trifolii, *R. leguminosarum* bv. phaseoli, *R. meliloti*, *R. fredii*, *B. japonicum*,

and *Bradyrhizobium* sp. (*Lupinus*) were very low, ranging from 1 to 17% (data not shown). The levels of DNA homology between group A and B strains were tested by using two strains belonging to each group (group B strains UPM-Ca7^T and IC-2091 and group A strains IC-60 and H-45) (Table 5). Strain UPM-Ca7^T exhibited a high level of homology (83%) with the other group B strain, IC-2091, and low levels of homology (36 to 38%) with the group A strains. Similarly, IC-60 exhibited 77 and 26 to 29% homology with strain H-45 (group A) and the group B strains, respectively.

DISCUSSION

Previously, bacteria that are able to establish effective symbiotic relationships with chickpeas were classified as *R. loti*

TABLE 3. Similarity coefficients obtained by using partial 16S rRNA sequences

Organism	Similarity coefficient with:															
	<i>Rhizobium</i> sp. (Cicer) strain UPM-Ca7 ^a	<i>R. loti</i> NZP 2213 ^T	<i>R. galegae</i> ATCC 43677 ^T	<i>R. huakuii</i> CCBAU 2609 ^T	<i>Rhizobium</i> sp. (Phaseolus) strain HI52	<i>Rhizobium</i> sp. (Phaseolus) strain TAL 1145	<i>R. tropici</i> CIAT899 ^T	<i>R. tropici</i> CO-5	<i>Rhizobium</i> sp. (Phaseolus) strain FL27	<i>R. etli</i> Olivia 4	<i>Rhizobium</i> sp. (Medicago) strain Or191	<i>R. leguminosarum</i> bv. phaseoli ATCC 8002	<i>R. meliloti</i> ATCC 9930 ^T	<i>R. fredii</i> USDA 205 ^T	<i>Agrobacterium tumefaciens</i> LMG 196	<i>Agrobacterium rhizogenes</i> LMG 152
<i>Rhizobium</i> sp. (Cicer) strain UPM-Ca7 ^a	1.000															
<i>R. loti</i> NZP 2213 ^T	0.992	1.000														
<i>R. galegae</i> ATCC 43677 ^T	0.972	0.980	1.000													
<i>R. huakuii</i> CCBAU 2609 ^T	0.996	0.988	0.968	1.000												
<i>Rhizobium</i> sp. (Phaseolus) strain HI52	0.972	0.964	0.968	0.968	1.000											
<i>Rhizobium</i> sp. (Leucaena) strain TAL 1145	0.952	0.944	0.940	0.948	0.964	1.000										
<i>R. tropici</i> CIAT899 ^T	0.937	0.929	0.925	0.933	0.948	0.976	1.000									
<i>R. tropici</i> CO-5	0.940	0.933	0.929	0.937	0.952	0.988	0.972	1.000								
<i>Rhizobium</i> sp. (Phaseolus) strain FL27	0.921	0.913	0.925	0.917	0.948	0.960	0.960	0.956	1.000							
<i>R. etli</i> Olivia 4	0.917	0.909	0.921	0.913	0.944	0.960	0.952	0.956	0.992	1.000						
<i>Rhizobium</i> sp. (Medicago) strain Or191	0.909	0.901	0.913	0.905	0.937	0.952	0.944	0.948	0.984	0.992	1.000					
<i>R. leguminosarum</i> bv. phaseoli ATCC 8002	0.944	0.937	0.933	0.940	0.956	0.984	0.972	0.976	0.964	0.964	0.956	1.000				
<i>R. meliloti</i> ATCC 9930 ^T	0.940	0.933	0.944	0.937	0.968	0.984	0.968	0.980	0.968	0.968	0.960	0.972	1.000			
<i>R. meliloti</i> CC169	0.944	0.937	0.948	0.940	0.972	0.980	0.964	0.976	0.964	0.964	0.956	0.968	0.996	1.000		
<i>R. fredii</i> USDA 205 ^T	0.921	0.913	0.933	0.917	0.925	0.913	0.901	0.901	0.893	0.893	0.885	0.905	0.917	0.921	0.917	1.000
<i>Agrobacterium tumefaciens</i> LMG 196	0.948	0.940	0.937	0.944	0.960	0.996	0.980	0.992	0.964	0.964	0.956	0.984	0.988	0.984	0.988	0.909
<i>Agrobacterium rhizogenes</i> LMG 152	0.929	0.921	0.940	0.925	0.937	0.909	0.893	0.897	0.893	0.889	0.881	0.901	0.913	0.917	0.913	0.964
<i>Agrobacterium rubi</i> LMG 156 ^T	0.933	0.940	0.960	0.929	0.937	0.925	0.913	0.913	0.905	0.905	0.897	0.917	0.929	0.933	0.929	0.968
<i>Agrobacterium vitis</i> LMG 8750 ^T	0.865	0.873	0.881	0.861	0.881	0.881	0.889	0.869	0.897	0.901	0.893	0.873	0.889	0.893	0.889	0.845
<i>B. japonicum</i> USDA 110	0.857	0.849	0.837	0.853	0.857	0.861	0.845	0.861	0.869	0.873	0.865	0.865	0.849	0.853	0.849	0.841
<i>Azorhizobium caulinodans</i> ORS 571 ^T	0.917	0.917	0.901	0.921	0.925	0.909	0.893	0.909	0.877	0.881	0.873	0.901	0.897	0.901	0.897	0.857
<i>Rochalmia quiniana</i>	0.853	0.861	0.865	0.849	0.865	0.873	0.853	0.881	0.885	0.877	0.857	0.873	0.877	0.873	0.845	0.861
<i>Rhodopseudomonas palustris</i> 216	0.853	0.861	0.869	0.849	0.865	0.857	0.857	0.849	0.865	0.865	0.857	0.857	0.861	0.857	0.861	0.821
<i>Rhodobacter sphaeroides</i> ATCC 17023	0.774	0.766	0.770	0.778	0.782	0.774	0.778	0.778	0.782	0.802	0.813	0.790	0.782	0.786	0.782	0.802
<i>Rhodospirillum rubrum</i> ATCC 11170																

^a The sequence of *Rhizobium* sp. (Cicer) strain UPM-Ca7^T is identical to the sequence of *Rhizobium* sp. (Cicer) strains IC-2018, IC-72M, and UPM-Ca36 (this study).

FIG. 5. Dendrogram derived from the aligned sequences of 16S rDNA fragments obtained from members of the *Rhizobiaceae* and related bacteria. We obtained identical sequences for the following four *Rhizobium* sp. (*Cicer*) strains: group A strains IC-72M and UPM-Ca36 and group B strains UPM-Ca7^T and IC-2018. *R.*, *Rhizobium*; *R. leg. bv. phaseoli*, *R. leguminosarum* bv. *phaseoli*; *A.*, *Agrobacterium*; *Ro.*, *Rochalimaea*; *Rb.*, *Rhodobacter*; *Rp.*, *Rhodopseudomonas*; *Rs.*, *Rhodospirillum*; *B.*, *Bradyrhizobium*; *Az.*, *Azorhizobium*. The unmarked strains of species in the dendrogram are the following: *R. huakuii* CCBAU 2609^T, *R. loti* NZP 2213^T, *R. galegae* ATCC 43677^T, *A. vitis* LMG 8750^T, *A. rubi* LMG 156^T, *A. tumefaciens* LMG 196, *R. leguminosarum* bv. *phaseoli* ATCC 8002, *A. rhizogenes* LMG 152, *Rhizobium* sp. (*Leucaena*) strain TAL 1145, *R. fredii* USDA 205^T, *Rhizobium* sp. (*Medicago*) strain Or191, *R. etli* Olivia 4, *Rhodobacter sphaeroides* ATCC 17023, *Rhodopseudomonas palustris* 216, *B. japonicum* USDA 110, *Azorhizobium caulinodans* ORS 571^T, and *Rhodospirillum rubrum* ATCC 11170.

(fast growers) or *Bradyrhizobium* sp. (slow growers) (9, 23). However, several authors have concluded that these organisms form a unique group on the basis of the specificity of the host plant-rhizobium relationship (16), serological and antigenic characteristics (10, 26), cultural traits, and the polymorphism of the *nifHD* genes (5). Recently, we reexamined the diversity among chickpea rhizobium strains, and our results, which were based on multilocus enzyme electrophoresis data, the polymorphism of the 16S-IGS rRNA genes, and phenotypic characteristics, led us to delineate two phylogenetic groups (33). Because of the relatively high levels of phenotypic and genotypic divergence that correlated with the variable generation times of these organisms, we questioned the taxonomic status of these two groups and particularly whether they belong to the same or different genera. Thus, in this study, in order to clarify the taxonomic position of these bacteria, we compared chickpea rhizobium strains with reference strains of genera and species belonging to the *Rhizobiaceae* by analyzing data from the following approaches: DNA-DNA hybridization, polymorphism of the 16S-IGS rRNA genes, partial 16S rRNA sequences, and phenotypic characteristics.

Amplification of the 16S-IGS rRNA gene generated identical 2,500-bp bands for all of the chickpea rhizobia. The deduced IGS fragment appeared to be relatively long (approx-

TABLE 4. Relative levels of homology between labelled *R. loti*, *R. huakuii*, or *R. galegae* DNA and unlabelled DNAs from *Rhizobium* sp. (*Cicer arietinum* L.) strains

Strain	% Reassociation with ³ H-labelled DNA from:		
	<i>R. loti</i> NZP 2213 ^T	<i>R. galegae</i> ATCC 43677 ^T	<i>R. huakuii</i> CCBAU 2609 ^T
<i>R. loti</i> NZP 2213 ^T	100 ^a	3	ND ^b
<i>R. galegae</i> ATCC 43677 ^T	4	100	ND
<i>R. huakuii</i> CCBAU 2609 ^T	ND	ND	100
<i>Rhizobium</i> sp. (<i>Cicer</i>)			
IC-60	21	8	29
H-45	30	5	28
IC-6	36 (10) ^c	5	34
UPM-Ca142	25 (10.5)	5	35
UPM-Ca36	31 (11.5)	7	29
Ca-7	23	7	27
IC-72M	22	7	33
UPM-Ca7 ^T	44 (7.5)	3	19
3HOa10	32	4	47
IC-2091	40 (6.5)	5	41
UPM-Ca116	45 (5.5)	4	49
3HOa9	36 (8.0)	5	52
522	41 (7.0)	6	42
IC-2018	34	1	35
USAB67	26 (11.0)	5	33
43-b	24	5	29

^a Level of relatedness at 70°C.

^b ND, not determined.

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

imately 1,000 bp), like the IGS fragments of other *Rhizobium* species (length, approximately 1,000 to 1,300 bp). In contrast, the IGSs of *Bradyrhizobium* strains were found to be 300 to 700 bp long, values which are comparable to those obtained for the IGSs of other bacteria (200 to 600 bp) (4, 15, 32). These results suggest that the chickpea rhizobium strains are more closely related to the genus *Rhizobium* than to the genus *Bradyrhizobium*.

The analyses of rRNA polymorphism and 16S rRNA sequences led to the same conclusions; i.e., the chickpea rhizobium strains were grouped with the *Rhizobium* species and far from the *Bradyrhizobium* cluster. The position of the chickpea rhizobium strains among the different *Rhizobium* species was the same whatever approach was used. All of the strains fell into a tight cluster which included *R. loti* and *R. galegae*. The 16S rRNA sequence analysis showed that this cluster includes the *R. huakuii* strain. The genetic distances obtained by rRNA polymorphism analysis or phenotypic analysis placed the group B strains close to *R. loti*. Thus, even though the slowly growing chickpea rhizobium strains in group A were found to be less closely related to *R. loti*, they were shown to belong to the

TABLE 5. Levels of DNA relatedness between strains belonging to chickpea rhizobium groups A and B

Strain	% Reassociation with ³ H-labelled DNA from:	
	Group A strain IC-60	Group B strain UPM-Ca7 ^T
IC-60	100 ^a	36
H-45	77	38
UPM-Ca7 ^T	26	100
IC-2091	29	83

^a Level of relatedness at 70°C.

genus *Rhizobium*, demonstrating that the strain generation times which led to their classification as *Bradyrhizobium* strains are clearly a misleading criterion for classifying chickpea rhizobium isolates.

The four chickpea rhizobium strains that were used as representatives of groups A and B had identical partial 16S rRNA sequences. Since the region which we sequenced is considered to be the most variable in 16S rDNA (13), these results suggest that groups A and B form a unique phylogenetic lineage. When the chickpea rhizobium sequence was compared with the *R. huakuii* and *R. loti* sequences, we found one and two different nucleotides, respectively (Fig. 4), resulting in similarity coefficients of 0.996 and 0.992, respectively (Table 3). Because of these high similarity coefficients, separation of the chickpea rhizobia from *R. loti* and *R. huakuii* must be discussed. Similar values were found for the same 16S rRNA region with other members of the *Rhizobiaceae*. For example, a similarity coefficient of 0.996 was found for *R. meliloti* CC169 and *R. fredii* (Table 3). For these two species, sequencing of 97% of the 16S rDNAs revealed eight or nine nucleotide mismatches. Other workers have also reported very high similarity coefficient values for different species. For instance, strains of *Bacillus globisporus* and *Bacillus psychrophilus* exhibited 99.8% sequence identity (14), and *R. tropici* IIA and IIB exhibited 99.6 and 99.4% similarity with *Agrobacterium rhizogenes*, respectively (42). It is now well accepted that although 16S rRNA sequences can be used to establish relationships between genera and well-resolved species, very recently diverged species may not be recognizable perhaps because of molecular clock differences in the various taxa considered (14).

According to Wayne et al. (41) and Grimont (19), assignment of bacterial strains to species should be based on the results of total DNA relatedness studies. Strains that exhibit less than 70% reassociation and more than 5°C divergence do not belong to the same genomic species. The levels of DNA homology between the 16 chickpea rhizobia tested and *R. loti*, *R. huakuii*, and *R. galegae* were less than 52%, clearly demonstrating that neither of our two groups can be assigned to any of these three species. In particular, *R. loti*, which has been proposed as the species to which the fast-growing chickpea rhizobium strains belong, exhibited low levels of DNA homology and ΔT_m values greater than 5°C.

Phenotypic characteristics that distinguish between the chickpea rhizobium groups (groups A and B) and *R. loti* are shown in Table 6. *R. loti* strains assimilate L-phenylalanine and L-lysine, whereas members of groups A and B do not. *R. loti* cannot grow at 40°C and does not tolerate 2% (wt/vol) NaCl (24), while the majority of chickpea rhizobia do grow at 40°C and tolerate 2% (wt/vol) NaCl. Most *R. loti* strains can grow at pH 4 (24); however, no growth is observed with chickpea rhizobia at this pH. In contrast, *R. loti* cannot grow at pH 10, while numerous chickpea rhizobia (all group B strains) can.

In summary, all of the chickpea rhizobium strains tested belong to the genus *Rhizobium*, regardless of their generation times. They are clearly separated from *R. loti* and other *Rhizobium* species on the basis of data from DNA relatedness, 16S rRNA sequence, polymorphism of the 16S-IGS rRNA gene, and phenotypic characteristics. Within the chickpea rhizobia, the two previously described groups, groups A and B (33), belong to different lineages as determined by using various phenotypic and genotypic approaches, including low DNA-DNA homology values. On the basis of this evidence, we believe that the chickpea rhizobia belong in at least two different species. We propose that the group B chickpea rhizobia, which are more closely related to *R. loti*, should be assigned to a new species, *Rhizobium ciceri*. Presently, exten-

TABLE 6. Differentiating characteristics of *R. ciceri*, *Rhizobium* sp. (*Cicer arietinum* L.) group A, and *R. loti*

Characteristic	<i>R. ciceri</i>	<i>Rhizobium</i> sp. (<i>Cicer</i>) group A	<i>R. loti</i> NZP 2213 ^T
Utilization of the following sole carbon sources:			
L-Phenylalanine, L-lysine	—	—	+
D-Lyxose, DL-glycerate, L-aspartate	+	—	+
L-Ornithine, β -alanine	+	—	—
L-xylose, L-alanine, L-leucine	+	—	—
Growth at 39–40°C	+ ^a	+	—
Growth in the presence of 2% NaCl	+ ^a	+	—
Growth at pH 4	—	—	+ ^b
Growth at pH 5	+	—	+
Growth at pH 10	+	— ^c	—
Resistance to chloramphenicol	+	—	ND ^d
G+C content (mol %)	63–64	63–64	59–64

^a A total of 75% of the strains tested are positive.

^b Between 11 and 89% of the strains tested are positive (24).

^c Five of the seven strains tested are negative.

^d ND, not determined.

sive DNA relatedness studies consisting of a large number of strains reveal a great heterogeneity among the group A strains; thus, more results will be necessary before naming new species for the members of this group.

Description of *Rhizobium ciceri* sp. nov. *Rhizobium ciceri* (ci' ce. ri. N. L. gen. n. *ciceri*, of *Cicer*). Gram-negative, aerobic, non-spore-forming rods. Colonies on yeast extract-mannitol agar are circular, opaque, and 2 to 4 mm in diameter within 3 to 5 days at 28°C. The maximum temperature for growth is 40°C for most strains. These bacteria are able to grow on minimal media, can generally tolerate 2% (wt/vol) NaCl, and grow at pH values of 5.0 to 10.0. They are nalidixic acid, carbenicillin, chloramphenicol, and trimethoprim-sulfamethoxazole resistant and are able to assimilate up to 73 compounds as sole carbon sources (33). The DNA G+C content ranges from 63 to 64 mol%. These bacteria are distinguished from other *Rhizobium* and *Bradyrhizobium* spp. and particularly from their closest relative, *R. loti*, on the basis of phenotypic and genomic characteristics.

Furthermore, *R. ciceri* can be differentiated from group A chickpea rhizobium strains on the basis of the results of multilocus enzyme electrophoresis and polymorphisms of amplified 16S-IGS rRNA genes (33). Group A strains cannot grow at pH 5, generally cannot grow at pH 10, and are highly susceptible to chloramphenicol. *R. ciceri* assimilates 70% of the amino acids which we tested, whereas the group A strains utilize 50%. The following carbon sources are assimilated by *R. ciceri* and not by group A strains: D-lyxose, DL-glycerate, L-aspartate, L-ornithine, β -alanine, L-xylose, L-alanine, and L-leucine.

Strain UPM-Ca7, which was isolated from nodulated chickpeas grown in Spain, is the type strain. It has the characteristics described above for *R. ciceri* sp. nov. with respect to morphology, physiology, G+C content, and genotype.

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