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Phylogenetic and Genetic Relationships of *Mesorhizobium tianshanense* and Related Rhizobia

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The genetic and phylogenetic relationships for strains of *Mesorhizobium tianshanense* and its relatives were compared by an analysis of the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins, DNA-DNA hybridization, and full 16S rRNA gene sequencing. The strains of *M. tianshanense* formed a cluster which was distinct from those of other rhizobium species in the clustering analysis of SDS-PAGE. DNA-DNA relatedness between A-1BS (type strain of *M. tianshanense*) and the type or reference strains for *Mesorhizobium loti*, *M. huakuii*, *M. ciceri*, *M. mediterraneum*, and cluster U, an unnamed rhizobial group, ranged from 4.4 to 43.8%. The phylogenetic analysis based on the 16S rRNA gene sequences showed that *M. tianshanense* was closely related to the *Mesorhizobium* phylogenetic branch and could be distinguished from the other four species in this branch. These results further confirmed that these bacteria constitute a distinct rhizobial species.

The genetic approaches now widely applied to the taxonomy of root nodule bacteria have opened the possibility to infer their phylogenies and to correctly define the species and genera of these bacteria. The improved methods for identifying bacteria and a growing interest in characterization of new rhizobial isolates have brought about many changes in the taxonomy of rhizobia since 1984; a revised taxonomic system for these bacteria was proposed in "Bergey's Manual of Systematic Bacteriology" (16). Several reviews on the development of rhizobial taxonomy and phylogeny have been published recently (20, 33). The main developments include the descriptions of the genera *Azorhizobium* (8), *Sinorhizobium* (4, 6), and *Mesorhizobium* (14, 18), as well as many new species. Up to now, six distinct phylogenetic branches, *Azorhizobium*, *Bradyrhizobium*, the *Rhizobium-Agrobacterium rhizogenes* branch, *Mesorhizobium* (14), the *Rhizobium galegae-Agrobacterium* branch, and *Sinorhizobium*, have been identified, and all of them were located in the alpha subclass of *Proteobacteria* (6, 31–33). *Mesorhizobium* has been proposed recently by Jarvis et al. (14), and five species, *M. loti* (15), *M. huakuii* (3), *M. ciceri* (23), *M. mediterraneum* (22), and *M. tianshanense* (2) were included on the basis of the data from full sequences of 16S rRNA genes. A group of rhizobia named cluster U has been classified in this genus by 16S ribosomal DNA (rDNA) sequencing (6). Some isolates from nodules of *Amorpha fruticosa* also belong to it, as indicated by the PCR-based restriction fragment length polymorphism patterns of their 16S rDNA (unpublished data). *M. tianshanense* was described in our previous paper dealing with a group of rhizobia isolated from saline and arid soils in the Xinjiang region of China (2). Some of the strains in this species grow as slowly as *Bradyrhizobium* spp. Other strains grow faster than *Bradyrhizobium* but slower than *Rhizobium leguminosarum*. According to the data from the partial 16S rDNA sequence, this species belongs to the *M. loti-M. huakuii* branch (2). But its exact taxonomic and phylogenetic positions were controversial since the partial sequence of *M. tianshanense* A-1BS^T is identical to that of *M. ciceri* (18) and since the DNA-DNA relatedness between this species and some newly emerged species had not been determined. Also, the phylogenetic relationships based on the partial and the full sequences (31, 32, 34) may not be the same for some species, such as for *Rhizobium galegae*.

Currently, polyphasic taxonomy, including phenotypic and genotypic characterizations, is emphasized for classification of rhizobia (12). In order to confirm the taxonomic and phylogenetic positions of *M. tianshanense*, we performed an analysis of the full-length sequence of the 16S rRNA gene for A-1BS, the type strain of *M. tianshanense*. The DNA-DNA hybridization and whole-cell protein electrophoresis tests were also done for other *M. tianshanense* strains and some reference strains. The electrophoretic patterns of proteins have been widely used in bacterial classification, and different techniques have been developed (17, 21, 25). It has been proved that protein profiles can provide a level of discrimination similar to or slightly higher than that of DNA-DNA hybridization studies.

MATERIALS AND METHODS

Bacterial strains. The tested strains are listed in Table 1. All strains of *M. tianshanense* were characterized in our previous research (2) and maintained in 20% (vol/vol) glycerol-water solution at −20°C. The bacteria were grown on yeast mannitol agar (28) medium at 28°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins. Bacterial strains were grown at 28°C for 2 days in flasks on tryptone-yeast extract medium, which contained (per liter) 5 g of tryptone (Oxoid), 3 g of yeast extract (Oxoid), 0.7 g of $CaCl_2 \cdot 2H_2O$ (pH 6.8–7.0). Then each culture was centrifuged at 12,000 × *g* for 3 min. The pellet was washed once in 10 mM Tris-HCl, pH 7.6, and the cells were suspended in 0.5 ml of 10 mM Tris-HCl, pH 7.6 (21). The concentration of protein was adjusted to achieve an optical density at 280 nm of 1.0 in an ultraviolet spectrophotometer. After ultrasonic disruption on ice for 30 s with a microtip probe at 40 W, 2× treatment buffer (0.5 g of SDS, 3 ml of glycerol, 1 ml of 2-mercaptoethanol, 4 mg of bromophenol blue, 2 ml of 1 M Tris-hydrochloride, and distilled water for a final volume of 10 ml at pH 6.8) was added. The samples were stored at −20°C for as long as 1 week before being analyzed by electrophoresis. The SDS-polyacrylamide gel (200 by 200 mm and 1 mm thick) and a shark's tooth comb were used for electrophoresis. The samples were incubated at 100°C for 2 min before the gel was run. Twenty-five samples per gel were subjected to discontinuous slab gel electrophoresis in an SDS-Tris-glycine buffer system, as described by Laemmli (17). The protein patterns were visualized by silver staining (25). The bands of protein for each strain were scanned with a Densitometer Extra-Scanner (LKB

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TABLE 1. List of bacterial strains examined

Strain[a]	Host	Origin[b]	Source[c]
M. loti NZP 2213	*Lotus coniculatus*	New Zealand	NZP
M. ciceri USDA 3378	*Cicer arietinum*		USDA
M. mediterraneum USDA 3392	*Cicer arietinum*		USDA
Cluster U USDA 4413	*Acacia senegal*	Senegal	USDA
S. meliloti USDA 1002	*Medicago sativa*	United States	USDA
R. leguminosarum USDA 2370		United States	USDA
R. tropici CIAT 899	*Phaseolus vulgaris*		CNPBS
R. galegae HAMBI 540	*Galega orientalis*	Finland	HAMBI
M. huakuii CCBAU 2609	*Astragalus sinicus*	Nanjing	CCBAU
S. fredii USDA 205	*Glycine soja*	Henan	USDA
B. japonicum USDA 6	*Glycine max*	United States	USDA
B. japonicum SEMIA 5601	*Glycine max*	Brazil	SEMIA
B. japonicum SEMIA 5079	*Glycine max*	Brazil	SEMIA
M. tianshanense strains			
A-1BS^T	*Glycyrrhiza pallidiflora*	Xinjiang	CCBAU
6	*Glycyrrhiza uralensis*	Xinjiang	CCBAU
032B	*Caragana plourensis*	Xinjiang	CCBAU
060A	*Halimodendron holodendron*	Xinjiang	CCBAU
016Bm	*Swainsonia salsula*	Xinjiang	CCBAU
91x10	*Halimodendron holodendron*	Xinjiang	CCBAU
005B	*Sophora alopecuroides*	Xinjiang	CCBAU
009B	*Glycine max*	Xinjiang	CCBAU
91x01	*Glycine max*	Xinjiang	CCBAU
91x05	*Sorphora alopecuroides*	Xinjiang	CCBAU
91x07	*Sorphora alopecuroides*	Xinjiang	CCBAU
91x72	*Sorphora alopecuroides*	Xinjiang	CCBAU
91x11	*Caragana plourensis*	Xinjiang	CCBAU
91x09	*Sophora alopecuroides*	Xinjiang	CCBAU
91x13	*Glycyrrhiza sp.*	Xinjiang	CCBAU

[a] Genus names not identified in the text are spelled out in the footnote to Table 2.
[b] Origins listed without a country are all in China.
[c] NZP, Division of Scientific and Industrial Research, Palmerston North, New Zealand; USDA, Beltsville *Rhizobium* Culture Collection, Beltsville Agricultural Research Center, Beltsville, Md.; CNPBS, Centro National de Pesquisa em Biologia do solo, Iseropedica 23851, Rio de Janeiro, Brazil; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland; CCBAU, Culture Collection of Beijing Agricultural University, Beijing, China; SEMIA, Equipe de Microbiologia do Solo, Instituto de Pesquisas Agronomicas (IPAGRO)-Secretaria de Agricultura, Porto Alegre, Rs, Brazil.

Co.), and a clustering analysis was performed by using the Sj coefficient and the unweighted-average linkage method (26).

DNA-DNA hybridization. The DNAs were extracted by the standard method (19). The total DNAs were digested with *Eco*RI restriction endonuclease and were subjected to electrophoresis in 1% agarose gels (11). For each lane, 3 μg of DNA was used. A photograph of the gel was taken after staining with ethidium bromide, and then the DNAs were Southern blotted onto nitrocellulose (Hybond-N+; Amersham). The protocols specified by the manufacturer were used for Southern blotting, fixation by UV cross-linking, and hybridization at 65°C. The random primer labeling kit (Rediprime; Amersham Life Science) and [^{32}P]GTP were used for labeling the total DNA probe from *M. tianshanense* A-1BS^T by following the instructions of the manufacturer. After exposure of an X ray film, the nitrocellulose with DNAs was cut into strips according to the lanes on the film. Then the strips were placed in 19-ml scintillation vials, and liquid scintillation counting was performed. The DNA-DNA homology was expressed as the percentage calculated by dividing the count for each lane by the count for the homology lane and multiplying by 100.

Amplification and cloning of 16S rRNA gene. The total DNAs were used as a template for PCR. A large fragment of the 16S rRNA gene was amplified in a 100-μl reaction mixture by using universal forward primer P1 (5′-CGgatccAG AGTTTGATCCTGGTCAGAACGAACGCT-3′, corresponding to positions 8 to 37 in *Escherichia coli* 16S rDNA [1]) and universal reverse primer P6 (5′-C GggatccTACGGCTACCTTGTTACGACTTCACCCC-3′, positions 1479 to 1506) (31, 32). The lowercase letters in the primer indicate the restriction site of *Bam*HI. The protocol described by Chun and Goodfellow (5) and a PCR kit purchased from Promega Co. (Madison, Wis.) were used. Amplified 16S rDNA was purified from 0.8% low-melting-point agarose gel by using the method of Wieslander (30). Purified rDNA and plasmid pUC18 vector were cut with *Bam*HI and ligated at 16°C for 16 h. Ligated plasmids were transformed into *E. coli* DH5α, and transformants were selected on the basis of the results of the blue-white screening procedure (24).

DNA sequencing. Plasmids containing 16S rDNA were extracted and purified according to the methods of Tiesman and Rizzino (27). Purified plasmids were sequenced by using the fmol DNA sequencing system (Promega Co.) according to the manufacturer's instructions. Six primers for sequencing were selected based on the work of Yanagi and Yamasato (32) and Willems and Collins (31). They were P1 and P6 and an additional four primers, P2 through P5, with, respectively, the following sequences (*E. coli* numbering system): 5′-GCTAGT TGGTGGGGTAA-3′ (positions 247 to 263), 5′-CGTGCCAGCAGCCGCGGT -3′ (positions 514 to 531), 5′-TAGATACCCTGGTAGTCC-3′ (positions 799 to 806), and 5′-CCGCAACGAGCGCAACCC-3′ (positions 1097 to 1115).

Analysis of sequence data. The generated rDNA sequence and those of reference strains (obtained from the EMBL, GenBank, and DDBJ data Libraries) were aligned pairwise, and the similarities were calculated and converted to a distance matrix with the Jukes-Cantor coefficient in the DNADIST program (PHYLIP version 3.572c) (10). Deletions and insertions of more than one base length were counted as one change. A dendrogram was produced by the neighbor-joining method of the NEIGHBOR program. Finally, an unrooted tree was generated with the DRAWTREE program (PHYLIP version 3.572c).

Nucleotide sequence accession number. The 16S rDNA sequence determined in this study has been deposited in the GenBank under the accession number U71079.

RESULTS AND DISCUSSION

SDS-PAGE of whole-cell protein. In this research, 15 strains of *M. tianshanense* and 10 type or reference strains for rhizobial species were analyzed numerically, and also a computer-processed image (BAU software, unpublished) was produced (Fig. 1).

The dendrogram in Fig. 1 showed that all *M. tianshanense* strains formed a cluster at the similarity level of 84%, while the representative strains of other rhizobial species were distinguished from *M. tianshanense* and from each other. The relationships among the strains tested in this study were similar to those from DNA-DNA hybridization (2). SDS-PAGE analysis of protein provided further evidence that *M. tianshanense* strains form a unique genetic group.

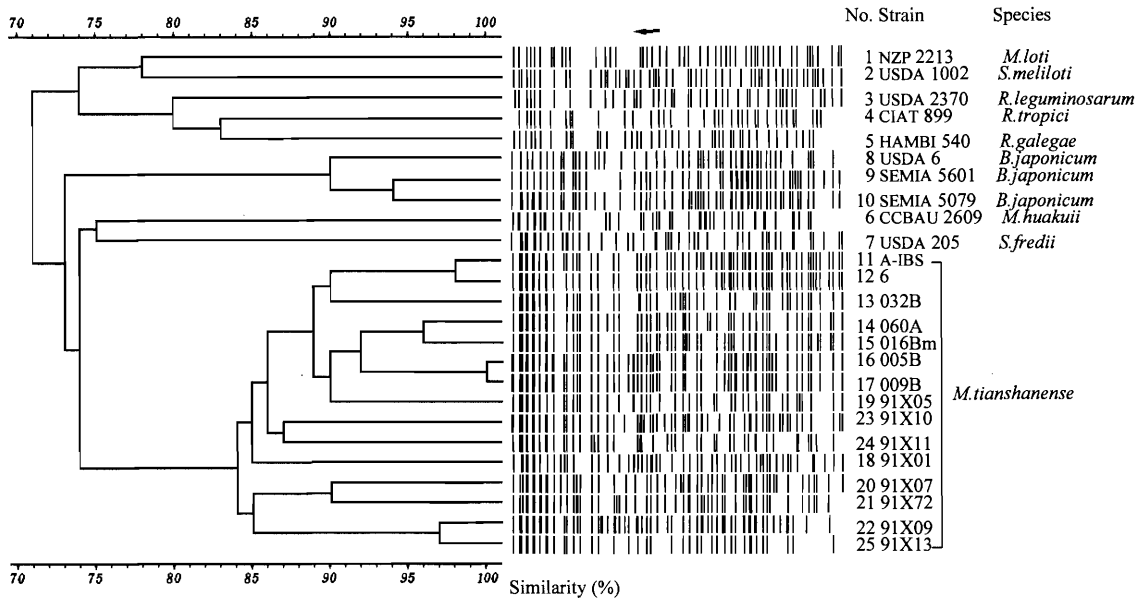

FIG. 1. Dendrogram showing the relationships among the SDS-PAGE whole-cell protein patterns of *M. tianshanense* and reference strains of *Rhizobium*, *Bradyrhizobium*, and *Sinorhizobium*. Computer-processed images are also shown. Genus names not introduced in the text are spelled out in the footnote to Table 2.

DNA-DNA hybridization. The DNA-DNA hybridization is an important criterion for definition of bacterial species (29), and different methods have been recommended for determining these values among rhizobia (12). Usually, the DNA-DNA relatedness among distinct species should be lower than 70%, and the conservation in the ribosomal gene sequences should be lower than 95% between distinct genera, among other criteria (20). In our previous research, the spectrophotometer method described by De Ley et al. (7) was used for measuring the DNA-DNA hybridizations among the strains of *M. tianshanense* and between A-1BS, the type strain of *M. tianshanense*, and type strains for other described rhizobial species (2). The *M. tianshanense* strains formed a homologous-DNA group at the species level (DNA-DNA relatedness higher than 70%). The DNA-DNA relatedness between A-1BS^T and other type strains was lower than 70%. But the relatedness between A-1BS^T and *M. huakuii* CCBAU 2609 was 63.5%, which was near the value of 70%, one criterion used to define a species (12, 29). It seemed that there was no big difference between *M. tianshanense* and *M. huakuii*. As a result, the validity of *M. tianshanense* was questioned (18). In this study, Southern blotting methods (11) were used for estimating DNA-DNA hybridizations between A-1BS^T and type strains for related species. The DNA-DNA relatedness between A-1BS^T and type or reference strains for *M. loti*, *M. huakuii*, *M. ciceri*, *M. mediterraneum*, and cluster U was 4.4, 13.9, 18.2, 43.8, and 35.6%, respectively. And the DNA-DNA relatedness between A-1BS^T and *M. huakuii* CCBAU 2609 (12%) was confirmed by M. Gillis (11a). According to these data, *M. tianshanense* strains form a distinct homologous-DNA group.

Nucleotide sequence analysis. The 16S rRNA gene sequence of strain A-1BS^T was compared with the partial sequence of the 16S rRNA gene of the same strain, as determined in the previous research (2). The alignment of the variable fragments between primers Y1 and Y2 (34) obtained in previous partial sequencing is identical with that obtained in this research. The variations among the sequences of A-1BS^T and those of 27 other species in the family *Rhizobiaceae* and related bacteria were calculated. The similarity values are shown in Table 2.

On the basis of the K_{nuc} values, an unrooted phylogenetic tree (Fig. 2) was generated by the DRAWTREE program. The relationships among the described species are similar to those described in references 6 and 31 to 33. The *Bradyrhizobium* species formed one branch; *Azorhizobium* occupied the second branch; the species of *Sinorhizobium* constituted the third branch; the fourth branch included *Rhizobium* species and *Agrobacterium rhizogenes*; *R. galegae* and *Agrobacterium* species formed the fifth branch; and the *Mesorhizobium* branch is the sixth one. *M. tianshanense*, represented by A-1BS^T, fell in the *Mesorhizobium* branch. The similarities between *M. tianshanense* and *M. loti*, *M. huakuii*, *M. ciceri*, *M. mediterraneum* and cluster U are 97.4, 97.9, 96.4, 96.4, and 97.4%, respectively. Based on these data, it is clear that *M. tianshanense* is a member of the *Mesorhizobium* branch and is distinct from all of the related species. In this study, it was found that 3.6% of the base pairs of the full sequences of 16S rDNAs for *M. tianshanense* and *M. ciceri* are different (Table 2), although the two species have identical sequences for a 260-bp fragment between primers Y1 and Y2 (14, 18). A similar situation exists for *R. galegae* and *Sinorhizobium fredii-Sinorhizobium meliloti* (31, 32, 34). Within a 260-bp fragment, *R. galegae* is only 1.2% (3 bp) different from *M. loti* (31). But the difference is 6.3% (about 90 bp) when the full-length sequences are compared (32, 34). Eardley and Biever (9) reported that cluster analysis of polymorphic nucleotide sequence positions in full and partial segments of the 16S rRNA genes of *R. galegae*, *M. loti*, and *Agrobacterium tumefaciens* revealed marked disagreement in phylogenetic tree topology depending on the portions of the genes included in the analysis. In another case, phylogenetically related rhizobial strains, such as *Sinorhizobium* strains, were found to have a large number of similar-but-distinct 16S rDNA sequences (13). Also, some rhizobial strains, such as the type strain for *Sinorhizobium saheli*, have two different sequences for 16S genes (13). In this case, the importance of DNA-DNA hybridization experiments in drawing the species boundaries was emphasized by Haukka et al. (13). Up to now, five species have been distinguished in the *Mesorhizobium* branch.

TABLE 2. 16S rDNA similarity values for rhizobia, agrobacteria, and related bacteria

Bacterium (no. and name)	% Similarity to bacterial species no.:																												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1. *A. rhizogenes*																													
2. *A. rubi*	93.8																												
3. *A. tumefaciens*	94.6	98.6																											
4. *A. vitis*	94.4	94.8	95.4																										
5. *A. caulinodans*	89.8	89.9	89.5	89.3																									
6. *S. fredii*	95.7	94.6	95.3	94.5	89.9																								
7. *R. galegae*	95.1	94.9	95.2	96.2	89.7	94.1																							
8. *R. leguminosarum*	98.1	93.4	94.2	93.9	90.1	95.0	95.9																						
9. *M. loti*	93.7	93.6	93.1	93.3	89.7	95.1	93.7	93.3																					
10. *S. meliloti*	96.1	94.3	94.9	94.1	89.9	98.8	94.1	95.2	95.6																				
11. *R. tropici A*	99.4	93.8	94.6	93.9	89.6	95.6	94.8	97.8	93.4	95.9																			
12. *R. tropici B*	99.3	93.6	94.5	94.1	89.9	95.6	94.9	98.0	93.5	95.6	99.1																		
13. R. sp. I66	96.5	93.3	94.1	93.9	89.8	95.2	95.4	96.9	92.6	95.3	96.4	96.3																	
14. Cluster U	93.8	93.9	93.5	93.7	90.2	95.8	94.0	93.3	98.0	95.4	93.5	93.5	92.7																
15. *M. tianshanense*	93.7	94.9	94.4	93.9	89.7	95.5	94.6	93.1	97.4	95.4	93.4	93.5	92.7	97.4															
16. *S. saheli*	94.9	94.3	94.7	94.1	89.4	98.1	93.7	94.4	95.1	97.3	94.7	94.5	94.4	95.7	95.3														
17. *S. teranga*	94.6	94.1	94.3	94.1	90.0	97.9	93.7	94.1	94.3	97.4	94.5	94.1	94.0	94.7	94.5	97.8													
18. *B. japonicum*	87.9	87.1	87.2	86.5	88.5	88.6	87.9	88.1	88.6	88.6	87.9	88.0	88.4	87.9	88.0	88.7	87.9												
19. *M. ciceri*	93.2	93.0	92.6	92.9	89.1	94.4	93.2	92.8	98.2	94.3	92.9	93.0	92.2	97.6	96.4	94.2	93.3	88.2											
20. *M. mediterraneum*	93.2	93.4	92.9	93.3	90.1	94.1	93.6	92.8	96.7	93.8	92.9	93.1	91.7	97.5	96.4	93.9	94.6	87.9	97.9										
21. *B. denitrificans*	89.1	88.2	88.2	87.5	88.1	89.4	88.6	88.7	88.4	88.6	88.7	88.9	88.5	88.5	88.7	89.6	88.7	97.4	88.4	88.6									
22. *M. dimorpha*	94.4	94.7	94.5	94.4	90.9	96.9	94.1	94.0	95.9	96.1	94.1	94.1	93.8	97.0	96.4	96.7	95.6	88.6	95.1	95.0	89.9								
23. *M. bullata*	93.9	93.5	93.3	92.5	91.2	95.6	92.7	93.8	93.9	95.0	94.2	94.1	93.6	94.7	94.2	94.9	94.9	89.1	92.8	92.9	85.8	96.1							
24. *P. myrsinacearum*	94.1	93.1	93.1	92.8	90.6	96.0	92.6	93.6	96.7	95.6	93.7	93.9	92.9	96.7	95.1	95.6	94.9	88.4	95.8	95.1	88.5	96.1	94.7						
25. *P. rubiacearum*	93.8	93.0	92.9	92.6	90.4	95.9	92.4	93.3	96.6	95.5	93.4	93.6	92.6	96.5	95.0	95.7	94.7	88.2	95.6	95.0	88.4	95.9	94.6	99.9					
26. *M. huakuii*	93.9	94.1	93.6	93.9	90.4	95.9	94.1	93.5	98.2	95.5	93.6	93.7	92.9	99.6	97.9	95.9	94.8	88.6	97.4	97.5	89.3	97.1	94.9	96.6	96.5				
27. *S. xinjiangensis*	95.8	94.6	95.2	94.6	89.9	99.8	94.2	95.1	95.2	98.9	95.7	95.4	95.3	96.0	95.3	98.3	98.1	88.9	94.6	94.4	89.6	97.1	95.8	96.2	96.1	96.1			
28. *O. anthropi*	87.9	88.0	88.1	88.3	88.4	87.9	89.2	88.1	87.3	87.1	88.0	88.1	88.4	86.2	87.4	87.3	87.4	87.1	87.1	87.0	87.4	88.1	88.4	87.7	87.4	87.1	87.9		
29. *B. elkanii*	87.6	87.6	87.6	87.0	88.6	89.1	87.9	87.4	88.9	88.6	87.9	87.9	87.1	88.6	89.0	89.4	90.1	95.5	88.0	89.6	95.3	89.5	90.1	88.7	88.6	89.4	89.3	87.1	
30. *R. etli*	96.4	92.9	93.7	93.1	89.4	95.3	93.9	96.6	93.7	95.4	96.2	96.1	96.0	93.9	93.7	95.1	94.6	89.0	92.9	92.9	89.4	94.8	94.9	93.6	93.5	94.0	95.5	87.9	88.8

[a] Bacterial species are as follows: *A. rubi*, *Agrobacterium rubi*; *A. vitis*, *Agrobacterium vitis*; *A. caulinodans*, *Azorhizobium caulinodans*; *S. redii*, *Sinorhizobium redii*; *R. tropici*, *Rhizobium tropici*; R. sp. I66, *Rhizobium* sp. strain I66; *S. teranga*, *Sinorhizobium teranga*; *B. japonicum*, *Bradyrhizobium japonicum*; *B. denitrificans*, *Bradyrhizobium denitrificans*; *M. dimorpha*, *Mycoplana dimorpha*; *M. bullata*, *Mycoplana bullata*; *P. myrsinacearum*, *Phyllobacterium myrsinacearum*; *P. rubiacearum*, *Phyllobacterium rubiacearum*; *S. xinjiangensis*, *Sinorhizobium xinjiangensis*; *O. anthropi*, *Ochrobactum anthropi*; *B. elkanii*, *Bradyrhizobium elkanii*; *R. etli*, *Rhizobium etli*.

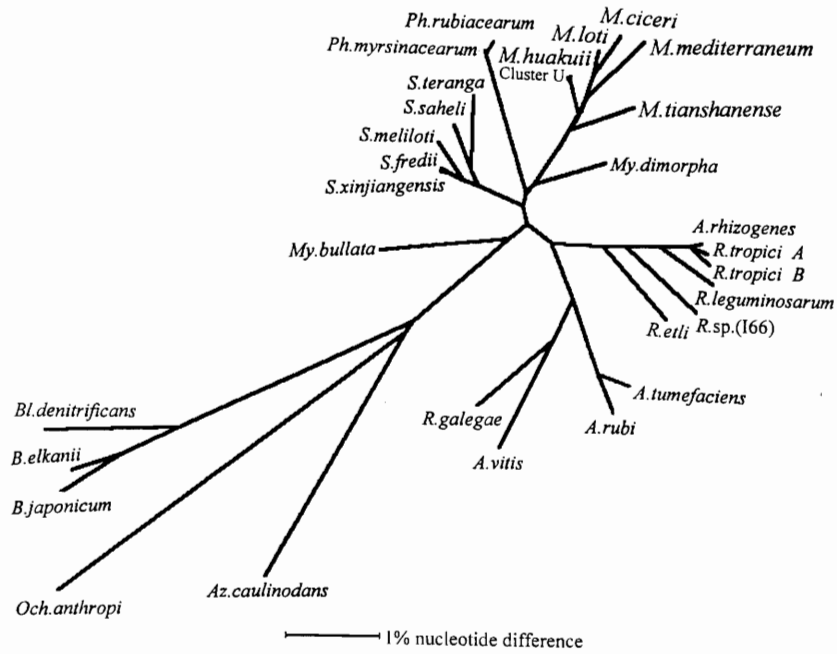

FIG. 2. Unrooted tree obtained by majority rule and strict consensus tree program PHYLIP, version 3.572c. Abbreviations: R, *Rhizobium*; M, *Mesorhizobium*; Ph, *Phyllobacterium*; My, *Mycoplana*; S, *Sinorhizobium*; Och, *Ochrobactrum*; A, *Agrobacterium*; Az, *Azorhizobium*; B, *Bradyrhizobium*; Bl, *Blastobacter*. GenBank accession numbers: *A. rubi* LMG 156, X67228; *A. tumefaciens* LMG 196, X67223; *A. rhizogenes* LMG 152, X67224; *A. vitis* LMG 8750, X67225; *S. fredii* LMG 6217, X67231; *S. meliloti* LMG 6133, X67222; *S. xinjiangensis* IAM 14142, D12796; *S. saheli* LMG 7837, X68390; *S. teranga* LMG 6463, X68387; *R. galegae* LMG 6215, X67226; *R. leguminosarum* LMG 8820, X67227; *M. loti* LMG 6125, X67229; *R. tropici* B LMG 9518, X67234; *R. tropici* A LMG 9517, X67233; *R. etli* ATCC 14483, U47303; *M. huakuii* IAM 14158, D12791; *M. ciceri* UPM-Ca7, U07934; *M. mediterraneum* UPM-Ca36, L38825; *B. japonicum* LMG 6138, X66024; *P. myrsinacearum* IAM 13584, D12789; *P. rubiacearum* IAM 13587, D12790; *M. dimorpha* IAM 13154, D12786; R. sp. I66, U71078; *M. bullata* IAM 13153, D12785; *O. anthropi* IAM 14119, D12794; *A. caulinodans* LMG 6465, X67221; *B. elkanii* ATCC 49852, U35000; *B. denitrificans* LMG8443, X66025; Cluster U LMG 7836, X68389. In the above strain designations, the following abbreviations are used: LMG, Culture Collection Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium; IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; ATCC, American Type Culture Collection, Rockville, Md. Genus names are spelled out either in the text or in the footnote to Table 2.

The results in this paper and those in our previous paper (2) clearly proved that *M. tianshanense* is a distinct species in the *Mesorhizobium* branch.

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