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Characterization of halotolerant rhizobia isolated from root nodules of *Canavalia rosea* from seaside areas

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Abstract

Twelve nodule isolates from *Canavalia rosea*, an indigenous leguminous halophyte growing in the seaside areas of southern Taiwan, were effective symbionts for the original host and able to grow at NaCl concentrations up to 3–3.5% (w/v). The taxonomy of these isolates was investigated using a polyphasic approach, including phenotypic characteristics, banding patterns of total proteins from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), genomic fingerprint patterns from random amplified polymorphic DNA (RAPD) analysis, pulsed-field gel electrophoresis (PFGE) analysis, amplified 16S rDNA restriction analysis (ARDRA), 16S rRNA gene sequencing, and *nifH* gene sequencing. Based on the SDS–PAGE, RAPD, PFGE and ARDRA results, the 12 isolates are highly diverse. The 16S rRNA and *nifH* gene sequences were determined for isolates with distinct ARDRA patterns and compared with other members of the rhizobial species. We propose these isolates should be classified into the genus *Sinorhizobium* and distinguished from the current species of this genus. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Symbiosis; *Canavalia rosea*; *Sinorhizobium*; 16S rRNA gene; *nifH* gene; Halotolerant

1. Introduction

The Leguminosae is one of the largest plant families. It is composed of about 750 genera containing 16 000–19 000 species distributed worldwide [1]. Rhizobia are soil bacteria capable of forming root or stem nodules on leguminous plants, where they undertake symbiotic fixation of atmospheric nitrogen. Based on current studies and the application of modern molecular taxonomic techniques, the rhizobial bacteria are divided into six genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* with approximately 30 species [2–4]. Phylogenetically, these bacteria belong to the α-Proteobacteria [3].

Soil salinization is one of the most serious forms of land degradation in the world. Salinated soil contains very little nitrogen and thus is not suitable for cultivation of most plants. An appropriate solution to this situation would be cultivation of plants that are able to fix nitrogen through symbiotic systems. Most plants, however, are sensitive to even low levels of salinity. In addition, the symbiotic rhizobia of legumes are also sensitive to salinity during both the free-living stage and the symbiotic process [5].

Some leguminous plants naturally growing on seashores are tolerant to the saline habitat. *Canavalia rosea* (Sw.) DC. (common name: baybean) is a perennial twining herb and is widely distributed in the open and sandy seaside area in southern Taiwan. *C. rosea* was found to form root nodules [6], and thus the symbiotic bacteria that induce root nodules on this plant must also be tolerant of highly saline habitats. The characteristics of the rhizobial symbionts of *C. rosea*, however, have not been studied. In this work we investigated 12 halotolerant isolates from root nodules of *C. rosea* in the seaside regions of southern Taiwan by characterizing their diversity. The relationships of these isolates with other rhizobial species are discussed.

2. Materials and methods

2.1. Isolation of rhizobia

The rhizobial isolates were isolated from three sandy seaside areas (Fangliao, Banana and South Bay) of south-

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ern Taiwan. Root nodules of *C. rosea* were located on the lateral roots and had a spherical shape (2–4 mm in diameter) with a pink color. Root nodules were immersed in 95% (v/v) ethanol for 10 s, sterilized in 0.1% (w/v) mercuric chloride for 5 min and then washed six times with sterile distilled water. Individual nodules were crushed with sterile glass rods and streaked onto yeast extract mannitol (YEM) agar [7] containing 0.0025% (w/v) Congo red. After an incubation of 3 days at 28°C, single colonies were selected and restreaked on YEM agar for purity.

2.2. *Plant inoculation test*

The ability of the isolated rhizobia to induce root nodules was confirmed by inoculating them onto the original host plant, *C. rosea*. Seeds of *C. rosea* were sterilized with concentrated sulfuric acid for 90 min, rinsed six times with sterile distilled water, and germinated on 1% water agar plates. After 72 h, three germinating seeds were transferred aseptically to each sterilized Leonard jar containing sterilized nitrogen-free solution [7]. For each rhizobial isolate, three plants were inoculated with 1 ml of bacterial suspension in nitrogen-free solution containing 10^8 bacterial cells. Plants were grown in a growth chamber at 28°C with an 18-h photoperiod for 6 weeks. These tests were done in duplicate and results were compared to plants which were not inoculated.

2.3. *Phenotypic characterization*

The colony morphology of isolates was examined on both YEM and tryptone-yeast extract (TY) [8] agar plates. After an incubation of 3 days at 28°C, individual colonies were characterized based on their size, color, shape, and capacity to produce exopolysaccharide gum.

To determine the ability of the bacteria to produce acids, each isolate was grown on duplicate YEM agar plates containing 0.025% (w/v) bromothymol blue. After an incubation of 72 h at 28°C, the pH change was scored on the basis of the color change of the medium. The ability of the rhizobial isolates to grow in basic or acidic media was tested by streaking them on YEM agar plates whose pH was adjusted to 4.0, 5.0, 6.0, 9.0, 10.0, 11.0 or 12.0 with HCl or NaOH. The ability of the isolates to grow at different concentrations of salt was tested by streaking isolates on TY agar plates containing 1, 2, 3, 3.5, or 4% (w/v) NaCl. Tolerance to high temperatures was tested by plating on TY agar and incubating at 38, 40, 42 and 45°C. All plates were incubated for 72 h and scored for viability based on colony formation.

For carbon substrate assimilation tests, Biolog GNII microtiter test plates (Biolog) were used. Early exponential-phase cultures were used as inocula for the test plates (150 μl well^{-1}). Plates were incubated at 28°C and examined after 24 and 48 h to allow for the development of a purple color indicative of substrate oxidation.

2.4. *Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of total cellular proteins*

The isolates were cultured on YEM medium for 72 h at 28°C. Total bacterial cellular protein extracts were prepared and analyzed by 12% SDS–PAGE. The patterns of SDS–PAGE assays were analyzed by Pearson product–moment correlation coefficients [9] using the 1D Advanced and Dendro software package (Advanced American Biotech Imaging, Inc.).

2.5. *Random amplified polymorphic DNA (RAPD) analysis*

The rhizobial isolates were grown on YEM agar at 28°C for 3 days. Two to three colonies of each isolate were harvested and washed twice with sterile distilled water. The cell pellets were suspended in 100 μl of sterile distilled water, boiled for 10 min, and centrifuged briefly. RAPD fingerprints were performed using the primer OPAB-11 (5′-GTGCGCAATG-3′) from the Operon RAPD 10-mer kit (Operon Technologies, Inc.). PCR was carried out in a 25 μl reaction containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 100 μM each of dATP, dTTP, dGTP and dCTP, 5 pmol of primer OPAB-11, 2.5 μl of supernatant of boiled bacterial cells, and 0.5 units of *Taq* DNA polymerase (Perkin-Elmer). The PCR was performed for 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C in a thermal cycler (GeneAmp PCR system 2400, Perkin-Elmer) and the PCR products were analyzed on 1.4% agarose gels in 0.5× TBE buffer (45 mM Tris–borate, 1 mM EDTA).

2.6. *Pulsed-field gel electrophoresis (PFGE) analysis*

Total DNA plugs were performed as described by Corich et al. [10]. The plugs were incubated overnight with lysis buffer (6 mM Tris–HCl pH7.6, 1 M NaCl, 10 mM EDTA, 0.5% (w/v) Brij, 0.2% (w/v) sodium deoxycholate, 1% (w/v) sodium lauroyl sarcosine, 1 mg ml^{-1} lysozyme, 10 μg ml^{-1} RNase) at 37°C with gentle shaking. The lysis buffer was then replaced with ESP buffer (0.5 mM EDTA, 1% (w/v) sodium lauroyl sarcosine, 100 μg ml^{-1} proteinase K) and incubated at 50°C for 48 h. The buffer was changed twice. The plugs were then washed with TE buffer (10 mM Tris–HCl pH7.6, 1 mM EDTA) and digested with *Xba*I (Boehringer Mannheim). DNA fragments were then subjected to electrophoresis on a 1.2% Seakem GTG agarose (FMC) gel in 0.5×TBE buffer at 200 V with a pulse time increased from 5 to 35 s for 24 h at 14°C using Pharmacia-LKB 2015 equipment.

2.7. *Amplified rDNA restriction analysis (ARDRA)*

Primers fD1 and rD1 [11] were used for amplification of rhizobial 16S rRNA genes by PCR. These primers correspond to nucleotide positions 8–27 and 1524–1540 of the *Escherichia coli* 16S rRNA gene, and can be used for amplifying nearly full-length 16S rRNA genes. A 50 μl PCR cocktail containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 100 μM each of dATP, dTTP, dGTP and dCTP, 15 pmol of each primer, 2.5 μl of supernatant of boiled bacterial cells, and 1.0 unit *Taq* DNA polymerase (Perkin-Elmer) was subjected to 35 cycles using a GeneAmp PCR system 2400 thermocycler (Perkin-Elmer). The amplification cycles included one cycle of 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C; and a final extension for 5 min at 72°C. The PCR products were checked by electrophoresis in 1.0% agarose. The remaining PCR products were purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim). 15 μl each of purified PCR products was digested with *Hin*fI, *Msp*I and *Nde*II (Boehringer Mannheim) and resolved in 3% Metaphore agarose (FMC) gels of 15 cm long at 6.7 V cm^{-1} for 4 h.

2.8. 16S rRNA gene sequencing

For sequence analysis, purified PCR products of 16S rRNA genes were cloned by using pGEM-T Easy Vectors System (Promega). Sequence analysis was performed using an Applied Biosystems 377 DNA Sequencer and the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit. Using the PILEUP program of the Wisconsin Genetics Computer Group (GCG) package aligned the determined sequences, together with reference sequences obtained from the GenBank database. All the sequences used were almost full length and were derived from the type strain wherever possible. The neighbor-joining method and bootstrap analysis (confidence values estimated from 1000 replications of each sequence) in the CLUSTAL w 1.7 program [12] were used to produce a phylogenetic tree. The tree was drawn using the TREEVIEW program [13]. Sequence identities were calculated using the BioEdit program [14].

2.9. nifH gene sequence

The *nifH* gene was amplified using primers nifH-1 and nifH-2 [15], which amplify a 601-bp fragment between positions 256 and 856 in the *Sinorhizobium meliloti* sequence. The PCR products were purified from the gel using JETQUICK Gel Extraction Spin Kit (Genomed). The cloning, sequencing and analysis procedure described were the same as above.

3. Results

3.1. Phenotypic characteristics of rhizobial isolates

A total of 12 isolates (Table 1) were recovered from root nodules of *C. rosea* plants growing on three sandy seaside areas around the southern part of Taiwan. All isolates were authenticated by their ability to nodulate the original host plant. These rhizobial isolates lacked the ability to absorb Congo red, and produced acid during their growth leading to a color change on YEM medium containing bromothymol blue. The colony morphology of these isolates on both YEM and TY agar plates was 2–7 mm with copious mucoid slime or extensive areas of confluent watery slime after an incubation of 3 days at 28°C.

All isolates grew at 38°C, and five of them (C1, C3, C10, C11, and C12) grew at 42°C. All isolates grew at salt concentrations as high as 3%, and three of them (C2, C10, and C11) could also grow at 3.5%, but not at salt concentrations higher than 4%. All isolates grew at acidic conditions of pH 5.0–6.0 and at basic conditions of up to pH 9.0. Three of them (C10, C11, and C12) even grew at a basic condition as high as pH 11.0.

Among the 95 carbon sources tested, all of the 12 *C. rosea* isolates could utilize 50 of them as sole carbon

Table 1
Rhizobial isolates of *C. rosea* and their relevant characteristics

Isolate	SDS–PAGE protein profile	RAPD genotype	PFGE genotype	ARDRA pattern[a]	Accession number for 16S rRNA gene sequence	Accession number for *nifH* gene sequence
C1	I	A	A	AAA	–[b]	–
C2	I	B	B	AAA	AF227754	AF230186
C3	I	C	A	AAA	–	–
C4	II	D	C	BBB	AF227753	AF230187
C5	III	E	D	ACB	AF227755	AF230188
C6	II	D	E	BBB	–	–
C7	III	E	D	ACB	–	–
C8	III	D	F	ACB	–	–
C9	IV	D	G	ADB	AF227756	AF230189
C10	V	F	H	AAA	–	–
C11	V	F	H	AAA	–	–
C12	V	G	I	AAA	–	–

[a] Three letters indicate ARDRA patterns digested individually with restriction enzymes *Hin*fI, *Msp*I and *Ned*II.

[b] –, not determined.

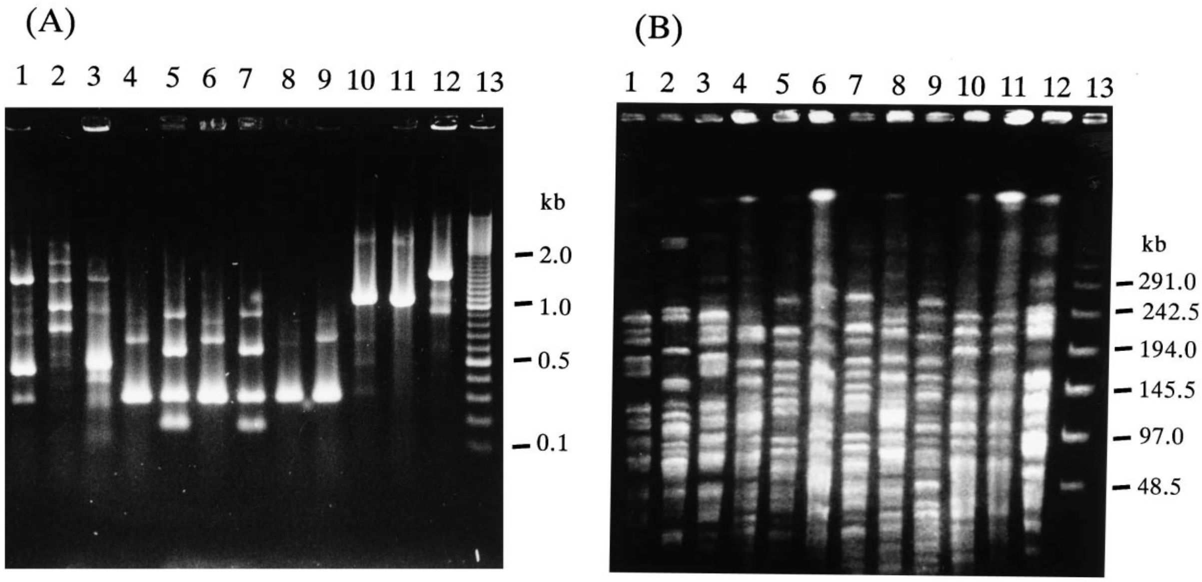

Fig. 1. A: RAPD analysis of the *C. rosea* isolates. Lane 1, isolate C1; lane 2, isolate C2; lane 3, isolate C3; lane 4, isolate C4; lane 5, isolate C5; lane 6, isolate C6; lane 7, isolate C7; lane 8, isolate C8; lane 9, isolate C9; lane 10, isolate C10; lane 11, isolate C11; lane 12, isolate C12; lane 13, 100-bp DNA ladder marker. B: PFGE analysis of the *C. rosea* isolates. The genomic DNA was digested with *Xba*I before PFGE analysis. Lane 1, isolate C1; lane 2, isolate C2; lane 3, isolate C3; lane 4, isolate C4; lane 5, isolate C5; lane 6, isolate C6; lane 7, isolate C7; lane 8, isolate C8; lane 9, isolate C9; lane 10, isolate C10; lane 11, isolate C11; lane 12, isolate C12; lane 13, λ concatemer marker.

source as follows: *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, *m*-inositol, α-D-lactose, maltose, D-mannitol, D-mannose, D-melibiose, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, methyl pyruvate, mono-methyl succinate, *cis*-aconitic acid, formic acid, β-hydroxybutyric acid, D,L-lactic acid, malonic acid, quinic acid, alaninamide, L-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, L-leucine, L-ornithine, L-proline, L-serine, L-threonine, urocanic acid, D,L-camitine, glycerol, glucose 1-phosphate and glucose 6-phosphate. Thirty-three carbon sources were utilized to varying extents, including α-cyclodextrin, dextrin, glycogen, Tween 40, Tween 80, *N*-acetyl-D-galactosamine, lactulose, β-methyl D-glucoside, acetic acid, citric acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketobutyric acid, propionic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-aspartic acid, hydroxy-L-proline, L-phenylalanine, γ-aminobutyric acid, inosine, uridine, thymidine, 2-aminoethanol and 2,3-butanediol. Fourteen carbon sources were not utilized by any isolate, including *i*-erythritol, ρ-hydroxy phenylacetic acid, itaconic acid, α-ketoglutaric acid, α-ketovaleric

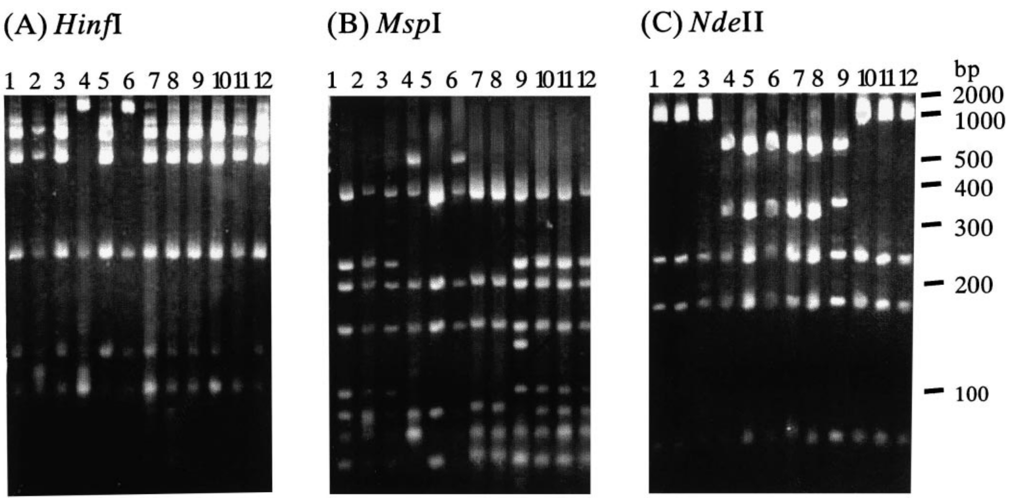

Fig. 2. Restriction patterns of PCR products of 16S rRNA genes digested with *Hin*fI (A), *Msp*I (B), or *Nde*II (C). Lane 1, isolate C1; lane 2, isolate C2; lane 3, isolate C3; lane 4, isolate C4; lane 5, isolate C5; lane 6, isolate C6; lane 7, isolate C7; lane 8, isolate C8; lane 9, isolate C9; lane 10, isolate C10; lane 11, isolate C11; lane 12, isolate C12. Molecular mass standards (in bp) are indicated on the right.

acid, D-saccharic acid, sebacic acid, D-alanine, L-pyroglutamic acid, D-serine, phenylethylamine, putrescine and D,L-α-glycerol phosphate.

3.2. *SDS–PAGE of total bacterial proteins*

The total proteins of the 12 isolates were analyzed by SDS–PAGE. From protein electropherograms of the isolates analyzed by Pearson product–moment correlation coefficients (data not shown), the 12 isolates could be classified into five patterns (Table 1).

3.3. *RAPD and PFGE fingerprinting*

To determine the genomic diversity of the 12 *C. rosea* isolates, RAPD and PFGE analyses were performed. Seven RAPD fingerprinting patterns were observed among the 12 *C. rosea* isolates (Fig. 1A). Results of PFGE (Fig. 1B) showed that the 12 isolates could be allocated to nine unique restriction patterns. Both RAPD and PFGE results are also summarized in Table 1.

3.4. *ARDRA analysis and 16S rRNA gene sequencing*

Four distinct RFLP patterns with the amplified 16S rRNA genes were identified among 12 isolates (Fig. 2 and Table 1). The 16S rRNA gene sequences of single isolates each representing the four RFLP patterns were determined. The 16S rRNA sequences were compared with those of other rhizobial species available from the GenBank database. Isolates C2, C4, C5 and C9 were found to be closely related but distinct members of the *Sinorhizobium* lineage (Fig. 3). The levels of sequence identity are shown in Table 2.

3.5. *nifH gene sequencing*

Based on the ARDRA and 16S rRNA gene sequencing results, isolates C2, C4, C5 and C9 were chosen for sequencing of their *nifH* genes. A dendrogram showing *nifH* sequence relatedness among our isolates and those from related rhizobial species obtained from the GenBank is presented in Fig. 4.

4. Discussion

Twelve new isolates from naturally occurring root nodules of *C. rosea* plants growing in seaside areas of southern Taiwan were characterized as fast-growing and acid-producing, and produced copious exopolysaccharide slime on YEM medium. These isolates utilize a wide range of carbohydrates and salts of organic acids as carbon sources which are also diagnostic for fast-growing root nodule bacteria [22].

It is known that salt stress significantly reduces nitrogen fixation in legumes. Hashem et al. [16] have proposed that salt stress may decrease the efficiency of the rhizobium–legume symbiosis by reducing plant growth and photosynthesis, and hence, nitrogen demand, by decreasing survival and proliferation of rhizobia in the soil and rhizosphere, or by inhibiting very early symbiotic events such as chemotaxis and root hair colonization, thus directly interfering with root nodule function. To date, only some rhizobial strains have been shown to grow under high salt conditions. *S. meliloti* is one of the most halotolerant rhizobia, and several strains have been reported to grow at high salt concentrations ($>3\%$) [17,18]. Of the other halotolerant rhizobium strains which have been isolated, most originate from tropical leguminous trees [17,19,20]. In our study, the 12 rhizobia isolated from *C. rosea* grew in 3% (w/v) NaCl and three isolates even grew at 3.5% NaCl, a salinity equivalent to that of seawater.

The ARDRA analysis showed that the 12 *C. rosea* isolates could be classified into four RFLP patterns of the 16S rRNA genes (Fig. 2 and Table 1). The sequence sim-

Table 2
16S rRNA gene sequence identity levels between sinorhizobia

Strain	Percentage identity/No. of nucleotide differences[a]											
	1	2	3	4	5	6	7	8	9	10	11	12
1. Isolate C2		98.6	98.4	98.2	97.8	98.7	98.3	97.8	97.6	99.1	99.1	98.5
2. Isolate C4	21		98.6	97.4	97.9	98.9	98.2	98.1	97.6	98.2	98.5	98.7
3. Isolate C5	23	20		98.0	98.6	99.7	98.7	98.6	98.4	98.8	98.2	99.5
4. Isolate C9	27	39	30		98.8	98.3	98.0	98.6	98.4	98.6	98.0	98.1
5. *S. arboris* HAMBI 1552^{T}	27	30	19	17		98.8	98.4	99.5	99.1	98.2	97.6	98.6
6. *S. fredii* USDA 205^{T}	19	17	4	25	16		99.0	98.9	98.6	99.1	98.4	99.8
7. *S. kostiense* HAMBI 1489^{T}	25	26	18	30	21	15		98.2	98.0	98.5	98.4	98.9
8. *S. medicae* A 321^{T}	32	28	20	21	8	16	27		99.3	98.0	97.6	98.7
9. *S. meliloti* IAM 12611^{T}	31	31	19	18	8	14	25	3		97.9	97.2	98.8
10. *S. saheli* ORS 609^{T}	12	27	16	20	25	13	22	29	25		98.8	98.9
11. *S. terangae* ORS 1009^{T}	12	20	26	28	35	24	23	36	37	17		98.2
12. *S. xinjangensis* IAM 14142^{T}	19	16	5	25	19	3	16	19	15	13	24	

[a] The values in the upper right part of the table are percentage identities and those in the lower left part of the table are numbers of nucleotide differences observed. The strains used in the comparison are indicated in the left-hand column.

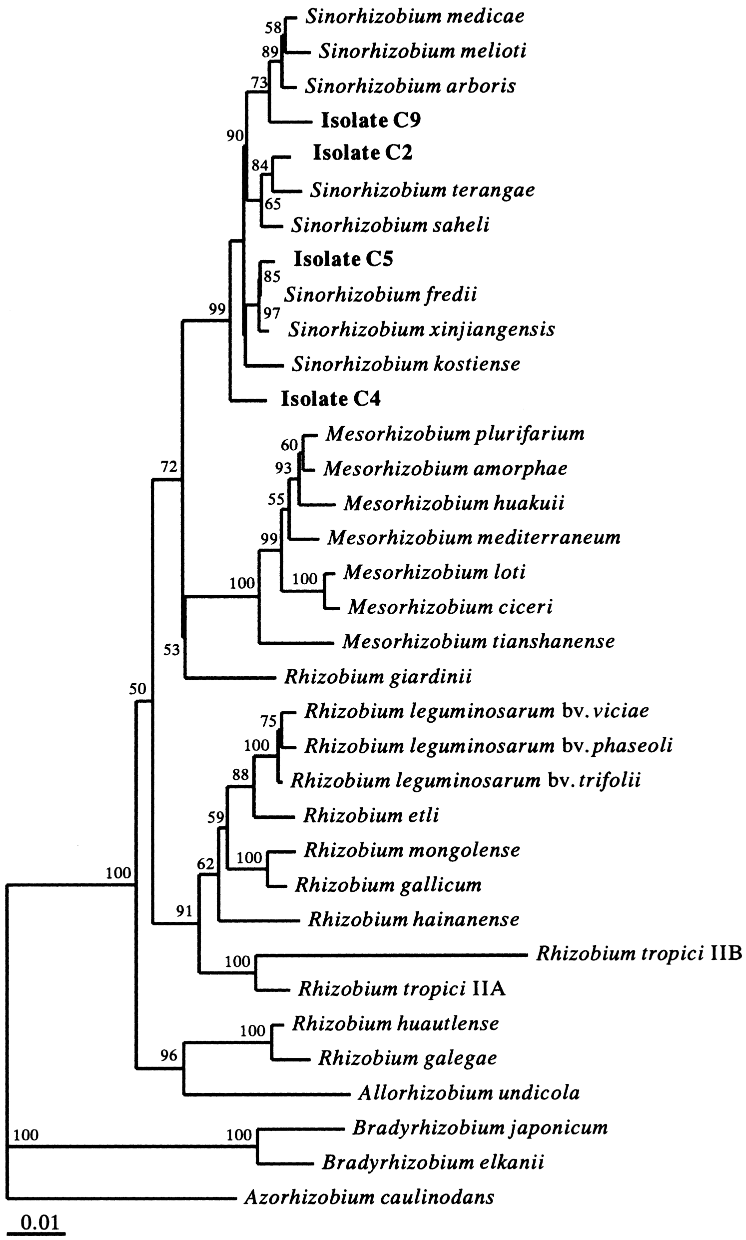

Fig. 3. Phylogenetic tree based on the rhizobial 16S rRNA gene sequences and constructed using the neighbor-joining method. Only bootstrap values greater than 50% are shown. Bar, 0.01 substitution per site. The sequences are derived from the type strains wherever possible. The following strains were used (the numbers in parentheses are accession numbers): *Allorhizobium undicola* LMG 11875 (Y17047), *Azorhizobium caulinodans* LMG 6465 (X67221), *Bradyrhizobium japonicum* USDA 6 (U69638), *B. elkanii* USDA 76 (U35000), *Mesorhizobium amorphae* ACCC 19665 (AF041442), *M. ciceri* UPM-Ca7 (U07934), *M. huakuii* IAM 14158 (D12797), *M. loti* LMG 6125 (X67229), *M. mediterraneum* UPM-Ca36 (L38825), *M. plurifarium* LMG 11892 (Y14158), *M. tianshanense* A-1BS (U71079), *Rhizobium etli* USDA 9032 (U28916), *R. galegae* LMG 6214 (X67226), *R. gallicum* R602sp (U86343), *R. giardinii* H 152 (U86344), *R. hainanensis* I 66 (U71078), *R. huautlense* SO 2 (AF025852), *R. leguminosarum* bv. viciae USDA 2370 (U29386), *R. leguminosarum* bv. phaseoli USDA 2671 (U29388), *R. leguminosarum* bv. trifolii LMG 8820 (X67227), *R. mongolense* USDA 1844 (U89817), *R. tropici* IIA CFN 299 (X67233), *R. tropici* IIB CIAT 899 (X67234), *Sinorhizobium arboris* HAMBI 1552 (Z78204), *S. fredii* USDA 205 (X67231), *S. kostiense* HAMBI 1489 (Z78203), *S. medicae* A 321 (L39882), *S. meliloti* IAM 12611 (D12783), *S. saheli* ORS 609 (X68390), *S. terangae* ORS 1009 (X68388) and *S. xinjiangensis* IAM 14142 (D12796).

ilarities of their 16S rRNA genes ranged from 97.4 to 98.8% between the four representative isolates (Table 2). Analysis of a phylogenetic tree (Fig. 4) revealed the relationships between our isolates and Sinorhizobia, as well as their relationships to other genera of rhizobia. The results showed that our isolates are closely related to species of *Sinorhizobium* and more distantly to species from the other genera of rhizobia. We therefore propose that the 12 isolates should be assigned to the genus *Sinorhizobium*. The results also indicate that these isolates diverge from each other in the different species of *Sinorhizobium*. The species *S. fredii*, *S. meliloti*, *S. xinjiangensis*, *S. saheli*, *S. terangae*,

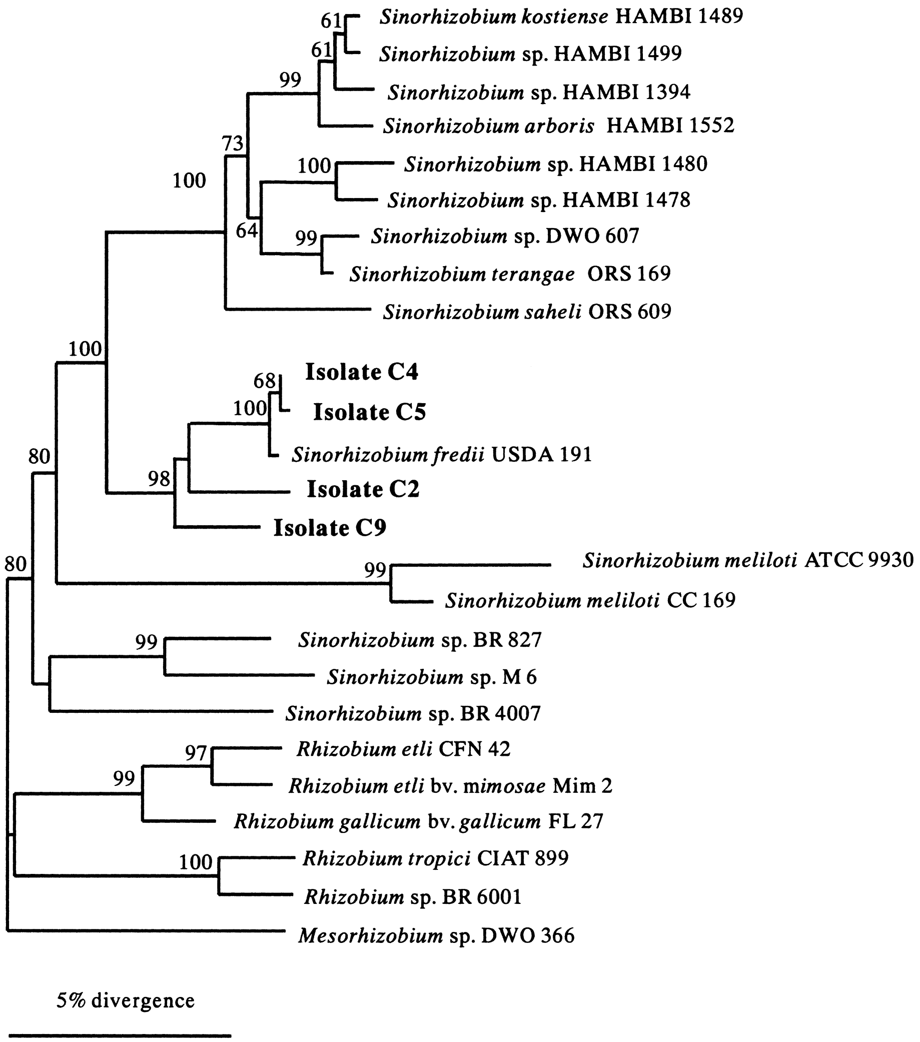

Fig. 4. Phylogenetic tree based on the partial *nifH* gene sequences and constructed using the neighbor-joining method. Only bootstrap values greater than 50% are shown. Sequences of the following strains were used (the numbers in parentheses are accession numbers): *M.* sp. DWO 366 (Z95226), *R. etli* CFN 42 (M15941), *R. etli* bv. mimosae Mim 2 (AF107621), *R. gallicum* FL 27 (M55226), *R. tropici* CIAT 899 (M55225), *R.* sp. BR 6001 (Z95230), *S. arboris* HAMBI 1552 (Z95214), *S. fredii* USDA 191 (Z95229), *S. kostiense* HAMBI 1489 (Z95216), *S. meliloti* ATCC 9930 (M55229), *S. meliloti* CC 169 (M55231), *S. saheli* ORS 609 (Z95221), *S. terangae* ORS 1009 (Z5218), *S.* sp. BR 4007 (Z95220), *S.* sp. BR 827 (Z95212), *S.* sp. DWO 607 (Z95219), *S.* sp. HAMBI1394 (Z95211), *S.* sp. HAMBI 1478 (Z95222), *S.* sp. HAMBI 1489 (Z95223), *S.* sp. HAMBI 1499 (Z95224) and *S.* sp. M 6 (Z95213).

S. medicae, *S. arboris* and *S. kostiense* have been described within the genus *Sinorhizobium* [4,21–23]. The closest similarity of isolate C2 was to *S. saheli* ORS 609^T and *S. terangae* ORS 1009^T, which have the closest 16S rRNA genes (99.1% sequence homology with both). The sequence identities of the 16S rRNA genes from the isolates C4 and C5 to *S. fredii* USDA 205^T are 98.9 and 99.7%, respectively. The 16S rRNA gene of isolate C9 is more closely related to *S. arboris* HAMBI 1552^T (98.8% sequence homology) than to that of other sinorhizobial species. The strains of *S. saheli* and *S. terangae* were isolated from different *Sesbania* and *Acacia* species of tropical leguminous trees in Senegal [22], that of *S. fredii* was identified from soybean nodules in China [21], and *S. arboris* was isolated from *Acacia senegal* and *Prosopis chilensis* in Sudan and Kenya [4]. Based on the phylogenetic result, isolates C2, C4 and C9 are closely related to these species but still distinguished from them. According to Graham et al. [24], assignment of rhizobium strains to new species should be based on the results of total DNA relatedness studies. The relationships between the *C. rosea* isolates, soybean isolates and leguminous tree isolates should be analyzed by using DNA–DNA hybridization in future work.

The *nifH* gene-based phylogram (Fig. 4) we obtained is similar to that reported previously [25]. The analysis revealed the close relationship between our isolates and the other sinorhizobia and their distance from the other genera of rhizobia. From sequence similarity of *nifH* genes, our isolates are much more related to each other, and to *S. fredii* USDA 191, than to other sinorhizobial species and strains. In general the phylogeny of *nifH* genes closely resembles that of 16S rRNA genes, suggesting the *nifH* genes probably share a common evolutionary history [26,27]. However, Haukka et al. [25] have reported that the *nifH* genes in *Sinorhizobium* isolates from leguminous trees are divided into two distinct phylogenetic groups based on their geographic origin, Africa or Latin America. They also reported similar *nifH* gene types were found in different chromosomal backgrounds, and suggested that lateral gene transfer has occurred across species boundaries [25]. The phylogenetic tree of *nifH* genes indicates that our isolates have a close relationship to *S. fredii* which originates from China. In the geographic view, Taiwan is near to mainland China. The geographic effect of sinorhizobia from Taiwan and China should be checked in a future survey. From our results, the *C. rosea* isolates are diverse in their 16S rRNA genes, and their genomes are also very diverse based on the RAPD and PFGE data. In general, on the basis of our data we support the hypothesis that intensive lateral gene transfer may play a role in generating new types of rhizobia.

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