Phylogenetic analysis of the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* on the basis of 16S rRNA gene and internally transcribed spacer region sequences

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A total of 128 strains was isolated from more than 23 legume hosts in Korea. Phylogenetic relationships between these Korean isolates and reference strains of the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* were analysed using their 16S rRNA gene and internally transcribed spacer (ITS) region sequences. Among the *Bradyrhizobium* strains, dendrograms based on both the 16S rRNA gene and ITS region sequences produced two main groups. The ITS tree yielded at least two new clusters that were discernable from the seven previously delineated genospecies. Large discrepancies were revealed between phylogenetic dendrograms based on 16S rRNA gene and ITS region sequences for members of the genus *Rhizobium*, reflecting their taxonomic heterogeneity. The amalgamation of *Rhizobium* and former members of *Agrobacterium* was confirmed using the 16S rRNA tree. Phylogenetic analysis of ITS region sequences showed that the *Rhizobium giardinii* clade (group II) and the *Rhizobium radiobacter*/*Rhizobium rubi* clade (group III) could be tentatively recognized as groups that are separable from the core group (group I), which includes *Rhizobium leguminosarum*. Dendrograms based on the 16S rRNA gene and ITS region sequences of *Mesorhizobium* strains were highly conflicting due to the poor taxonomic resolution of the 16S rRNA gene sequences and the low confidence in the ITS dendrogram. Several Korean isolates within the genus *Mesorhizobium* are thought to represent novel taxa when considering their relatively low ITS region sequence similarities (<80%) to the reference strains.

Published online ahead of print on 10 September 2004 as DOI 10.1099/ijs.0.63097-0.

Abbreviation: ITS, internally transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and ITS sequences reported in this paper are AF345254–AF345271, AF345273–AF345289, AF508136–AF508141, AF508143–AF508146, AF508148, AF509909–AF509921, AF509923–AF509932, AF510346–AF510369, AF510371, AF510372, AF510374–AF510388, AF510578–AF510635, AF510881–AF510887, AF510890–AF510927, AF511490–AF511506, AF511543–AF511558, AF517932, AF517933, AF541973 and AF541974.

INTRODUCTION

Some genera within the α -*Proteobacteria* are associated with plants, inducing the formation of nitrogen-fixing nodules on the roots and stems of leguminous plants or causing gall disease and root hair disease. According to *Bergey's Manual of Systematic Bacteriology*, the rhizobia and agrobacteria were classified into four genera (*Rhizobium*, *Bradyrhizobium*, *Agrobacterium* and *Phyllobacterium*) within the family *Rhizobiaceae* (Jordan, 1984). Since then, rhizobial and agrobacterial taxa have been added to and revised at the genus

and species levels. The genus *Sinorhizobium* was proposed for a group of fast-growing, nodule-forming, soybean rhizobia, *Rhizobium fredii* (Chen *et al.*, 1988), and this was later confirmed by de Lajudie *et al.* (1994). Jarvis *et al.* (1997) created a new genus, *Mesorhizobium*, for *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum* and *Rhizobium tianshanense*. *Allorhizobium undicola* was the name given to nitrogen-fixing strains that nodulated *Neptunia natans* in Senegal (de Lajudie *et al.*, 1998). Also, agrobacteria were classified as *Agrobacterium tumefaciens*, *Agrobacterium radiobacter*, *Agrobacterium rhizogenes*, *Agrobacterium rubi* and *Agrobacterium vitis* on the basis of plasmid-borne phytopathological traits. Most recently, species belonging to the genera *Allorhizobium* and *Agrobacterium* have been reclassified in the genus *Rhizobium* (Young *et al.*, 2001). An overview of all members of these genera is available (<http://www.bacterio.cict.fr/>).

Rhizobium, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium* and the former *Agrobacterium* were traditionally classified on the basis of phenotypic characteristics such as nodulation, and pathogenic and physiological properties. However, nodulation and pathogenic properties have become less important in the taxonomic evaluation of these genera. Recently, high sequence variation in the internally transcribed spacer (ITS) region has been shown to be more informative for taxonomic evaluation of *Bradyrhizobium* strains (van Berkum & Fuhrmann, 2000; Willems *et al.*, 2001b).

In this study, rhizobial strains were isolated from root nodules of several legume hosts in Korea. Their ITS regions and 16S rRNA genes were sequenced and compared with those of reference strains. Phylogenetic analysis of these sequences was performed to compare the dendrograms generated from these two types of sequences and to evaluate the use of the ITS region as a taxonomic marker.

METHODS

Bacterial strains and media. The bacterial strains used in this study are listed in Table 1. All strains were grown on yeast extract-mannitol (YM) agar (Vincent, 1970) or nutrient broth (0.5% peptone, 0.5% meat extract, pH 7.0) at 30 °C. Strains were preserved using two methods: deep-freezing with 15% glycerol and freeze-drying with 15% skimmed milk.

PCR amplification of the 16S rRNA gene and ITS region.

The 16S rRNA gene was amplified using universal primers fD1 and rP2 (Weisburg *et al.*, 1991). The ITS region was amplified using primers R16-1 (5'-CTTGTACACACCGCCCGTCA-3') and R23-3R (5'-GGTACTTAGATGTTTCAGTTC-3'), which were redesigned from the primers of Nakagawa *et al.* (1994). Primers R16-1 and R23-3R were located at positions 1389–1408 of the 16S rRNA gene and at 189–209 of the 23S rRNA gene sequence of *Escherichia coli*, respectively (GenBank accession no. J01695) (Brosius *et al.*, 1978). Each PCR mixture contained the following: 20 pmol each primer, 200 µM dNTPs (Promega), 2.5 U *Taq* DNA polymerase (Promega), approximately 50 ng genomic DNA and *Taq* polymerase buffer in a final reaction volume of 50 µl. The DNA thermal cycler (Perkin-Elmer) used for PCR amplification was programmed as follows: an

Table 1. Bacterial strains isolated from legume hosts in Korea

For each strain, the generic position was determined on the basis of 16S rRNA gene and ITS sequence analysis.

Isolate(s)	Genus	Legume plant from which strain(s) were isolated
Aei-1	<i>Bradyrhizobium</i>	<i>Aeschynomene indica</i>
Aei-2	<i>Mesorhizobium</i>	<i>Aeschynomene indica</i>
Aei-3–Aei-6	<i>Rhizobium</i>	<i>Aeschynomene indica</i>
Amf-1–Amf-5	<i>Mesorhizobium</i>	<i>Amorpha fruticosa</i>
Crj-1, Crj-2	<i>Bradyrhizobium</i>	<i>Crotalaria juncia</i>
Crj-3, Crj-4	<i>Rhizobium</i>	<i>Crotalaria juncia</i>
Glm-1–Glm-8	<i>Bradyrhizobium</i>	<i>Glycine max</i>
Glm-9–Glm-15	<i>Rhizobium</i>	<i>Glycine max</i>
Gls-1	<i>Bradyrhizobium</i>	<i>Glycine soja</i>
Gls-2–Gls-5	<i>Rhizobium</i>	<i>Glycine soja</i>
Kusp-1	<i>Rhizobium</i>	<i>Kummerowia stipulacea</i>
Kus-1–Kus-6	<i>Bradyrhizobium</i>	<i>Kummerowia striata</i>
Kus-7, Kus-8	<i>Rhizobium</i>	<i>Kummerowia striata</i>
Leb-1–Leb-4	<i>Mesorhizobium</i>	<i>Lepedeza bicolor</i>
Leb-5–Leb-14	<i>Bradyrhizobium</i>	<i>Lepedeza bicolor</i>
Leb-15	<i>Rhizobium</i>	<i>Lepedeza bicolor</i>
Loc-1	<i>Mesorhizobium</i>	<i>Lotus corniculatus</i>
Mes-1	<i>Bradyrhizobium</i>	<i>Medicago sativa</i>
Mes-2	<i>Sinorhizobium</i>	<i>Medicago sativa</i>
Pha-1–Pha-10	<i>Bradyrhizobium</i>	<i>Phaseolus angularis</i>
Phr-1–Phr-7	<i>Bradyrhizobium</i>	<i>Phaseolus radiatus</i>
Phr-8, Phr-9	<i>Rhizobium</i>	<i>Phaseolus radiatus</i>
Pis-1	<i>Rhizobium</i>	<i>Pisum sativum</i>
Put-1	<i>Bradyrhizobium</i>	<i>Pueraria thunbergiana</i>
Put-2, Put-3	<i>Mesorhizobium</i>	<i>Pueraria thunbergiana</i>
Rop-1–Rop-4	<i>Mesorhizobium</i>	<i>Robinia pseudoacacia</i>
Trp-1–Trp-6	<i>Rhizobium</i>	<i>Trifolium pratense</i>
Trr-1–Trr-9	<i>Rhizobium</i>	<i>Trifolium repens</i>
Vif-1	<i>Rhizobium</i>	<i>Vicia faba</i>
Viv-1	<i>Rhizobium</i>	<i>Vicia villosa</i>
Vga-1	<i>Bradyrhizobium</i>	<i>Vigna angularis</i>
Vgn-1–Vgn-3	<i>Bradyrhizobium</i>	<i>Vigna nakashimae</i>
Vgn-4, Vgn-5	<i>Mesorhizobium</i>	<i>Vigna nakashimae</i>
Vgs-1–Vgs-4,	<i>Bradyrhizobium</i>	<i>Vigna sinensis</i>
Vgs-6–Vgs-9		
Vgs-5, Vgs-10,	<i>Rhizobium</i>	<i>Vigna sinensis</i>
Vgs-11		
Wif-1	<i>Mesorhizobium</i>	<i>Wisteria floribunda</i>
NK-1, NK-2	<i>Mesorhizobium</i>	Not known
NK-3	<i>Bradyrhizobium</i>	Not known
NK-4	<i>Rhizobium</i>	Not known

initial extensive denaturation step at 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min; and a final extension step at 72 °C for 10 min.

Cloning and sequencing. PCR products were electrophoresed on 1.5% agarose gel. In the ITS region, one or two bands from each of the strains were observed. Each band was eluted and purified with a QIAquick gel extraction kit (Qiagene). Purified DNAs were ligated into pGEM-T easy vector (Promega) and ligated plasmids were then

used to transform *Escherichia coli* DH5 α F' cells (Sambrook *et al.*, 1989). Nucleotide sequences were determined with an Applied Biosystems 377 sequencer. Sequencing of the ITS region was conducted with three primers, R16-1, R23-3R and ILEF (5'-GTAG-CTCAGTTGGTTAGAGC-3'). ILEF was designed from a conserved region (tRNA^{Ile} coding region) within the ITS region.

Phylogenetic analysis. Nucleotide sequences were aligned using the program MEGALIGN (DNASTAR). An evolutionary distance matrix was generated as described by Jukes & Cantor (1969). An evolutionary tree for the datasets was inferred by the neighbour-joining method of Saitou & Nei (1987) using the neighbour-joining program of MEGA2 (<http://www.megasoftware.net>). The stability of relationships was assessed by performing bootstrap analysis of the neighbour-joining data based on 1000 resamplings.

RESULTS AND DISCUSSION

16S rRNA gene and ITS region sequence determination

A total of 128 bacterial strains was isolated from more than 23 legume hosts in Korea (Table 1). Phylogenetic analysis was carried out using 16S rRNA gene sequences of about 1450 nt from all strains used in this study, corresponding to nt 50–1501 of the *Escherichia coli* 16S rRNA gene sequence (Brosius *et al.*, 1978). Most of the strains produced one band in PCR amplification of the ITS region. However, six strains (*Mesorhizobium mediterraneum* USDA 3392^T, *Rhizobium gallicum* R602sp^T, *Rhizobium rubi* DSM 6772^T, *Sinorhizobium kostiense* LMG 19227^T, Leb-2 and Glm-15) produced two bands. The sequence similarities between two clones of the same strain ranged from 77 % (two clones of *S. kostiense* LMG 19227^T) to 98 % (two clones of *R. gallicum* R602sp^T) and all these sequences were included in the phylogenetic analysis. Phylogenetic analysis of these ITS clones revealed that each pair of ITS clones for these strains, except for Glm-15, whose two ITS clones were positioned in separate clades, were closely related (Figs 1 and 3).

Although, in general, the high sequence variation of the ITS region allows discrimination between closely related strains, the presence of multiple types of ITS region makes interpretation of phylogenetic relationships difficult (Chun *et al.*, 1999; Gürtler & Stanisich, 1996). This study showed that most rhizobial strains contained one type of ITS region sequence, suggesting that this region may be useful as a marker for phylogenetic analysis. For phylogenetic analysis of ITS region sequences, the full-length ITS region with about 10 nt of the 3'-terminal region of the 16S rRNA gene and about 40 nt of the 5'-terminal region of the 23S rRNA gene was used. All ITS region sequences analysed in this study varied from 751 to 1236 nt and contained two deduced tRNA genes, tRNA^{Ile} and tRNA^{Ala}. The ILEF primer for internal sequencing of the ITS regions was designed from the conserved tRNA^{Ile} region. Variable sequences within the ITS region were interspersed throughout the entire region, except for the two tRNA sequences. The ITS regions for all strains revealed high sequence variation (24–100 %). To simplify the tree topology, strains

with high sequence similarities, i.e. >99.8 % (16S rRNA gene) and >99.7 % (ITS region), were grouped into boxes at the same position in our phylogenetic trees (Figs 1, 2 and 3).

Phylogenetic analysis of the *Bradyrhizobium* group

For 16S rRNA gene sequence analysis of the *Bradyrhizobium* group, a total of 73 strains, including four type strains, 60 Korean isolates and nine strains of van Berkum & Fuhrmann (2000), was used. The ITS region sequence analysis was conducted with a total of 79 strains, including three type strains, 60 Korean isolates, nine strains of van Berkum & Fuhrmann (2000) and seven strains of Willems *et al.* (2001b). The 16S rRNA gene sequence similarities among the strains were more than 93.3 % and the ITS region sequence similarities were more than 44.4 %. Both trees produced two main groups: group I, including *Bradyrhizobium elkanii* strains, and group II, including *Bradyrhizobium japonicum* and *Bradyrhizobium liaoningense* strains (Fig. 1a, b). According to the 16S rRNA tree, group I was divided into two subgroups (groups Ia and Ib) (Fig. 1a). Group Ia consisted of *B. elkanii* strains, including *B. elkanii* USDA 76^T, and 11 Korean strains. Group Ib included only Korean strains (39 strains) isolated from different legume hosts. The phylogenetic positions of ten Korean isolates and strains of *B. japonicum*, *B. liaoningense* and *Bradyrhizobium yuanmingense*, which were clustered into group II, were not clear due to low bootstrap values (Fig. 1a). Based on phylogenetic analysis of the ITS region sequences, three groups with high bootstrap values were identified (Fig. 1b). Within group I, group Ia included 41 strains, all of which, except for LMG 8293, were isolated from Korean legume hosts. Among them, three strains (LMG 8293, Mes-1 and Vga-1) clustered independently. Group Ib included four *B. elkanii* strains, LMG 10676 and nine Korean isolates. Within group II, group IIa was composed of four *B. japonicum* strains (including LMG 6138^T), *B. liaoningense* LMG 18230^T and four Korean isolates, and group IIb included four *B. japonicum* strains, LMG 11950, LMG 10697 and six Korean isolates. Group III was composed of two strains (LMG 15404-c4 and LMG 10300). The members of subclusters (groups Ia and Ib) within group I of the 16S rRNA tree were firmly reflected in those of subclusters (groups Ia and Ib) within group I of the ITS tree, except for a new branch of LMG 8293, Vga-1 and Mes-1 that was not recognized in the 16S rRNA tree (Fig. 1a, b). Two branches (groups IIa and IIb) of group II, which were supported by relatively high bootstrap values, were determined in the ITS tree; this branching pattern was not evident in the 16S rRNA tree.

van Berkum & Fuhrmann (2000) conducted a phylogenetic study using the ITS region sequences of *Bradyrhizobium* strains isolated from soybean. Willems *et al.* (2001b) studied extensively the genus *Bradyrhizobium* on the basis of 16S rRNA gene and ITS sequences, amplified fragment length polymorphisms and DNA–DNA hybridization.

Fig. 1. Phylogenetic trees based on the 16S rRNA gene (a) and ITS region sequences (b) for the genus *Bradyrhizobium* and related strains. The branching pattern was produced by the neighbour-joining method. Numbers at nodes indicate levels of bootstrap support based on neighbour-joining analysis of 1000 resampled datasets. Bootstrap values below 40 % are not shown. Genospecies are noted according to Willems *et al.* (2001b).

Fig. 2. Phylogenetic tree based on the 16S rRNA gene for the genera *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* and related strains. The branching pattern was produced by the neighbour-joining method. Numbers at nodes indicate levels of bootstrap support based on neighbour-joining analysis of 1000 resampled datasets. Bootstrap values below 40% are not shown.

Using DNA–DNA hybridization data and ITS region sequence grouping of the *Bradyrhizobium* strains from several host plants, mainly *Glycine max*, *Aeschynomene* species and *Faidherbia albida*, seven genospecies were delineated (Willems *et al.*, 2001b). A subsequent DNA–DNA hybridization study revealed four more genospecies (Willems *et al.*, 2001c). Our ITS region sequence analysis, including representative strains of van Berkum & Fuhrmann (2000) and Willems *et al.* (2001b), yielded more than two new clusters discernable from the seven groups of Willems *et al.* (2001b) (Fig. 1b). One distinct cluster consisted of only Korean isolates from various host plants within group Ia. The other cluster was composed of three strains (LMG 8293, Vga-1 and Mes-1) within group Ia. Although, according to Willems *et al.* (2001b), LMG 8293 was supposed to be related to genospecies II, which was represented by *B. elkanii* LMG 6134^T, our results showed that this strain formed an independent cluster with Vga-1 and Mes-1. Furthermore, the ITS region sequences of two strains (LMG 15404-c4 and LMG 10300), which were classified as genospecies VI of Willems *et al.* (2001a), formed a distinct cluster separable from known species and the Korean isolates. The close relationship between the type strains of *B. japonicum* and *B. liaoningense* was confirmed by high sequence similarities (16S rRNA gene sequence similarity of 99.8% and ITS region sequence similarity of 88.2%) and a high DNA–DNA hybridization value (56%) (Willems *et al.*, 2001a, b).

Phylogenetic analysis of the *Rhizobium* group

For 16S rRNA gene sequence analysis, the type strains of 18 *Rhizobium* species and 45 Korean isolates were included. For the ITS region sequence analysis, 45 Korean isolates and 15 reference strains were used. Among the strains analysed, the 16S rRNA gene sequence similarities were shown to be above 89.4% and the ITS region sequence similarities were more than 31.4%. The 16S rRNA gene sequences of members of the genus *Rhizobium* (group I) formed one large cluster separable from the *Mesorhizobium* (group III) and *Sinorhizobium* (group II) groups (Fig. 2). Each of these main groups comprised subclusters that were supported by high bootstrap values. Within group I, four subgroups (groups Ia–Id) were produced. Thirty-two Korean isolates formed one large cluster with several *Rhizobium* species, including *Rhizobium leguminosarum* USDA 2370^T and *Rhizobium etli* CFN 42^T. Gls-3 clustered in group Ib with *Rhizobium galegae* ATCC 43677^T, *Rhizobium huautilense* SO2^T and *Rhizobium loessense* CCBAU 7190B^T. Group Ic comprised two Korean isolates (Kusp-1 and Aei-5) and *Rhizobium giardinii* H152^T. Ten Korean isolates were grouped into group Id with *Rhizobium radiobacter* NCPPB 2437^T, *Rhizobium rubi* IFO 13261^T, *Rhizobium vitis* NCPPB 3554^T and *Rhizobium undicola* LMG 11875^T.

The dendrogram generated from the ITS region sequences of these *Rhizobium* strains and Korean isolates revealed tree topologies comparable to those of the 16S rRNA dendrogram and produced three groups (I, II and III), which were

supported by high bootstrap values (Fig. 3a). Group I formed a large complex, including all the strains of groups Ia and Ib of the 16S rRNA tree and three strains (*R. undicola* LMG 11875^T, *R. vitis* LMG 8750^T and Kus-7) that were classified in group Id of the 16S rRNA tree. However, the phylogenetic relationships among the strains of group I on the basis of the ITS tree could not be clarified due to low bootstrap values. Group II of the ITS tree was composed of *R. giardinii* H152^T, Aei-5 and Kusp-1, which were found in group Ic of the 16S rRNA tree. The members of group III of the ITS tree were identical to those of group Id of the 16S rRNA tree, except for *R. undicola* LMG 11875^T, *R. vitis* LMG 8750^T and Kus-7 (Figs 2 and 3a).

The taxonomy of the genus *Rhizobium* remains controversial and, recently, Young *et al.* (2001) suggested that the former genus *Agrobacterium* be combined with *Rhizobium* based mainly on phylogenetic analysis of the 16S rRNA gene sequences without prejudice to phenotypic characters such as pathogenicity. In the phylogenetic trees based on 16S rRNA gene and ITS sequences, the *Rhizobium* species, including the Korean isolates, could be divided into several groups. Three major groups (I, II and III) in the ITS tree were supported by high bootstrap values and could be tentatively recognized as individual branches partially supported by the 16S rRNA tree (Fig. 2 and 3a). It could thus be suggested that the former *Rhizobium* and *Agrobacterium* be reclassified into more than two genera. However, such reclassification also might not be fully supported because of the presence of several peripheral groups and the discrepancy between the 16S rRNA and ITS trees.

Phylogenetic analysis of the *Mesorhizobium* and *Sinorhizobium* groups

For *Mesorhizobium* and related strains, the 16S rRNA tree was constructed for eight type strains and 22 related Korean strains and the ITS tree for 22 Korean isolates and seven type strains. Whereas the sequence similarities of the 16S rRNA gene were above 97.2%, the ITS region sequence similarities were above 49.2%. The 16S rRNA dendrogram grouped all *Mesorhizobium* and related strains into one cluster, group III (Fig. 2). Within group III, Wif-1 formed group IIIa with *Mesorhizobium ciceri* UPM-Ca7^T and *Mesorhizobium loti* LMG 6125^T. Twenty-one Korean isolates clustered with *Mesorhizobium plurifarium* LMG 11892^T, *Mesorhizobium amorphae* ACCC 19665^T and *Mesorhizobium huakuii* IFO 15243^T, forming group IIIb. The ITS tree of the *Mesorhizobium* and related strains resolved them into clearer branches than the 16S rRNA gene tree, but showed large disparities with the 16S rRNA tree (Fig. 3b). Due to the low ITS region sequence similarities (less than 80%) to reference strains, most of the Korean isolates, except for Amf-5, Put-3 and Leb-4, could not be placed into any known species, suggesting the presence of several novel taxonomic units. Characteristically, the high degree of relatedness (92.8%) of the ITS region sequences of *Mesorhizobium ciceri* LMG 14989^T and *Mesorhizobium loti* LMG 6125^T was consistent with the results of 16S rRNA

gene sequence analysis (Tan *et al.*, 1997) and high DNA–DNA hybridization (44 %) between these strains (Nour *et al.*, 1994).

For phylogenetic analysis of *Sinorhizobium* and related strains, the 16S rRNA gene sequences for nine type strains and one Korean isolate were used. For the ITS region sequences, seven type strains, one reference strain and one Korean isolate were used. The similarities of the 16S rRNA gene and ITS region sequences were over 97·1 and 45·7 %, respectively. According to the 16S rRNA dendrogram, one Korean isolate, Mes-2, was closely related to *Sinorhizobium meliloti* LMG 6133^T (Fig. 2). Based on ITS region sequences, the *Sinorhizobium* strains were divided into two clear clades with relatively high bootstrap values. Mes-2 formed one clade with *S. meliloti* LMG 6133^T, *Sinorhizobium medicae* LMG 18864, *Sinorhizobium fredii* LMG 6217^T and *Sinorhizobium xinjiangense* LMG 17930^T. Another clade included *Sinorhizobium arboris* LMG 14919^T and two clones of *S. kostiensis* LMG 19227^T (data not shown). The close similarity between *S. fredii* LMG 6217^T and *S. xinjiangense* LMG 17930^T, previously noted on the basis of 16S rRNA gene sequence similarity of 99·8 % (Tan *et al.*, 1997), was also confirmed by the ITS region sequence similarity (94·9 %).

Phylogenetic analysis of the Korean legume isolates on the basis of 16S rRNA gene and ITS region sequences showed their remarkable heterogeneity. To clarify the exact taxonomic positions of these isolates, more taxonomic methods, including other genetic methods and DNA–DNA hybridization, will be needed.

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