International Journal of Systematic Bacteriology (1999), 49, 51–65 Printed in Great Britain

Diversity of rhizobia associated with *Amorpha fruticosa* isolated from Chinese soils and description of *Mesorhizobium amorphae* sp. nov.

E. T. Wang,[1,3] P. van Berkum,[2] X. H. Sui,[3] D. Beyene,[2] W. X. Chen[3] and E. Martínez-Romero[1]

Author for correspondence: E. T. Wang. Tel: +52 73 131697. Fax: +52 73 175581. e-mail: ewang@cifn.unam.mx

[1] Centro de Investigación sobre Fijación de Nitrógeno, UNAM, Apdo Postal 565-A, Cuernavaca, Morelos, Mexico

[2] Alfalfa and Soybean Research Laboratory, Agricultural Research Service, US Department of Agriculture, Beltsville, MD 20705, USA

[3] Department of Microbiology, College of Biology, China Agricultural University, Beijing 100094, People's Republic of China

Fifty-five Chinese isolates from nodules of *Amorpha fruticosa* were characterized and compared with the type strains of the species and genera of bacteria which form nitrogen-fixing symbioses with leguminous host plants. A polyphasic approach, which included RFLP of PCR-amplified 16S rRNA genes, multilocus enzyme electrophoresis (MLEE), DNA–DNA hybridization, 16S rRNA gene sequencing, electrophoretic plasmid profiles, cross-nodulation and a phenotypic study, was used in the comparative analysis. The isolates originated from several different sites in China and they varied in their phenotypic and genetic characteristics. The majority of the isolates had moderate to slow growth rates, produced acid on YMA and harboured a 930 kb symbiotic plasmid (pSym). Five different RFLP patterns were identified among the 16S rRNA genes of all the isolates. Isolates grouped by PCR-RFLP of the 16S rRNA genes were also separated into groups by variation in MLEE profiles and by DNA–DNA hybridization. A representative isolate from each of these DNA homology groups had a separate position in a phylogenetic tree as determined from sequencing analysis of the 16S rRNA genes. A new species, *Mesorhizobium amorphae*, is proposed for the majority of the isolates, which belonged to a moderately slow- to slow-growing, acid-producing group based upon their distinct phylogenetic position, their unique electrophoretic type, their low DNA homology with reference strains representing the species within the genus *Mesorhizobium* and their distinct phenotypic features. Strain ACCC 19665 was chosen as the type strain for *M. amorphae* sp. nov.

Keywords: *Mesorhizobium amorphae*, *Amorpha fruticosa*, polyphasic taxonomy, diversity, rhizobia

INTRODUCTION

Low-input sustainable agriculture depends upon the management of leguminous crops because these plants form nitrogen-fixing symbioses with five genera of bacteria belonging to the family *Rhizobiaceae* (Chen *et al.*, 1988; de Lajudie *et al.*, 1994; Dreyfus *et al.*, 1988; Jarvis *et al.*, 1997; Jordan, 1984). Because of their value in agriculture, many legume species are introduced and are cultivated outside their native range. False indigo, *Amorpha fruticosa*, is a leguminous shrub native to the South-Eastern and Mid-Western United States (Allen & Allen, 1981) and has been cultivated in Asia for more than half a century. *Amorpha fruticosa* is useful as a windbreak and as soil cover for erosion control, but also provides food for wildlife. The pods yield amorphin, which is toxic to aphids, cinch bugs and cucumber beetles, and a repellent against cattle flies. In China, the leaves are used as green manure and the seeds are a source of oil used in the manufacture of glycerol. Additional products from this plant may

Abbreviations: ET, electrophoretic type; I_A, index of association; MLEE, multilocus enzyme electrophoresis.

The GenBank accession numbers for the 16S rRNA gene sequences of isolates ACCC 19665^T, ACCC 19667, H15003, HL56 and SH283012, and of *M. tianshanense* A-1BS^T are AF041442–AF041447, respectively.

Table 1. Isolates, strains and their relevant characteristics

Isolate or strain*	16S rRNA RFLP†		ET‡	Plasmid size (kb)§	Geographic origin
	Group	Pattern			
Mesorhizobium amorphae					
ACCC 19665^T, ACCC 19676, B101, B104, B105, B106, B270, B271, B273, B274, B278, B280, B281, B283, B284, B286, B287, B292	1	AAAA	1	930	Beijing
ACCC 19674, ACCC 19675, B107	1	AAAA	1	930, 150	Beijing
B108	1	AAAA	1	930, 550	Beijing
ACCC 19662, ACCC 19663, B102, ACCC 19664, B103, B269, B272, B275, B279, B288	1	AAAA	2	930, 550	Beijing
SH190012	1	AAAA	3	930	Shaanxi
B109, B110	1	AAAA	4	930	Beijing
SH109	1	AAAA	4	930	Shaanxi
ACCC 19660, ACCC 19666, ACCC 19670, ACCC 19672, ACCC 19673	1	AAAA	5	930, 150	Beijing
B289, B291	1	AAAA	6	930	Beijing
***Mesorhizobium* sp. (*Amorpha*)**					
HL56	2	BABA	7	930, 550	Heilongjiang
B276, B277, B282, B285, B290	2	BABA	8	930	Beijing
N206	3	ABAA	9	930	Ningxia
SH15003	3	ABAA	10	930	Shaanxi
***Rhizobium* sp. (*Amorpha*)**					
ACCC 19667, ACCC 19677	4	DBDB	11	930, 610, 490, 440	Beijing
***Bradyrhizobium* sp. (*Amorpha*)**					
SH28301, SH283012	5	GCHE	12	NO	Shaanxi
Standard reference strains					
M. huakuii CCBAU 2609^T	3	ABAA	16		
M. tianshanense A-1BS^T	2	BABA	17		
M. ciceri UPM-Ca7^T	13	CABA	15		
M. loti NZP 2213^T	12	ABCA	13		
M. mediterraneum UPM-Ca36^T	12	ABCA	14		
M. plurifarium ORS 1037	17	AAKA	18		
R. etli CFN 42^T	9	DBEC		630, 510, 390, 270, 170, 150	
R. tropici A CFN 299	8	EBFD		> 1000, 410, 225, 185	
R. tropici B CIAT 899^T	7	FBDB			
R. leguminosarum USDA 2370^T	4	DBDB	19		
R. galegae HAMBI 540^T	6	GBDA			
A. caulinodans ORS 571^T	14	HBGC			
S. meliloti USDA 1002^T	18	FBIF			
S. fredii USDA 205^T	19	KBJF			
S. saheli ORS 609^T	11	LBIF			
S. terangae ORS 1009^T	10	LBIA			
B. japonicum USDA 6^T	15	MDLH			
B. japonicum USDA 110	15	MDLH			
B. elkanii USDA 76^T	16	NEMH	20		
Plasmid or cosmid					
pMR133	A 2·0 kb insert of the *nodDAB* of *R. tropici* CFN 299 cloned into pUC18 (unpublished)				
pEM15	pSup205 derivative with region *nifKDH* of *R. etli* CFN 42^T (Morett *et al.*, 1988)				

For footnotes see facing page.

have pharmaceutical properties (Konoshima *et al.*, 1993; Mitscher *et al.*, 1981).

The import of legumes from their native habitats and their cultivation as an introduced crop may significantly impact the microbiology within soils. The rhizobia specific for the introduced crop may be transmitted along with the host and subsequently may establish a symbiosis and populate the new soil environment. The introduction of these specific rhizobia may occur either by management through inoculation, or by seed-borne bacteria as described for common bean seeds (Pérez-Ramirez *et al.*, 1998). Occasionally, remnants of a rhizobial population which have lost symbiotic determinants may become recipients of genetic information from closely related inoculated strains, acquiring the ability for symbiosis with an introduced legume host as was reported for *Lotus corniculatus* in New Zealand (Sullivan *et al.*, 1995, 1996). The possibility also exists that exotic legumes are nodulated by rhizobia which are members of the local soil population. For instance, common beans normally form symbioses with *Rhizobium etli* in their native habitat (Piñero *et al.*, 1988; Segovia *et al.*, 1993), but in Europe they may be nodulated by the two newly proposed species *Rhizobium giardinii* and *Rhizobium gallicum* (Amarger *et al.*, 1997). The ability of an introduced legume species to form symbioses with local rhizobia can vary according to host promiscuity. In the case of common bean, promiscuity is evident from its ability to be nodulated by *Rhizobium leguminosarum* (Eardly *et al.*, 1995; Young, 1985), *Rhizobium tropici* (Martínez-Romero *et al.*, 1991) and *Rhizobium mongolense* (van Berkum *et al.*, 1998a), in addition to *R. gallicum* and *R. giardinii* (Amarger *et al.*, 1997).

The rhizobia associated with *Amorpha fruticosa* originating from soils of the legume's native habitat have not been studied and no molecular evolutionary characterization of these bacteria has been reported. In China, *Amorpha fruticosa* is nodulated without inoculation, but limited information is available about the rhizobia which form these symbioses. Six isolates originating from *Amorpha fruticosa* growing in China were clustered into two phenotypic groups in a numerical analysis which included isolates from other host legumes (unpublished data). Several isolates from Japan were related to *Mesorhizobium loti* based upon partial sequence analysis of their 16S rRNA genes (Oyaizu *et al.*, 1993). Because so little information is available about the rhizobia of *Amorpha fruticosa*, our objective was to characterize isolates from nodules of plants growing in Chinese soils using a polyphasic approach.

METHODS

Isolates and strains. The isolates and reference strains used in this study are shown in Table 1. Fifty-five isolates were obtained from three different geographic regions in Northern China. Forty-eight isolates originated from the Western suburbs of Beijing. One isolate was from Heilongjiang Province, which is in North-Eastern China approximately 1000 km from Beijing, and six were from Ningxia and Shaanxi Provinces, which are in North-Western China, approximately 1000 km from Beijing and 2000 km from Heilongjiang Province. The ACCC isolates (from the Agricultural Center Culture Collection, Chinese Academy of Agriculture, Beijing, China) and isolates B101–B292 were obtained in 1985 and 1995, respectively. The remaining isolates were collected in 1993. The plants from which isolations were made were grown in soils poor in organic matter ($< 0{\cdot}1$ %) without the addition of fertilizer. Isolations were made according to the procedure described by Vincent (1970) using yeast mannitol agar (YMA). Cultures used for further study were purified from single colonies on YMA or MAG (van Berkum, 1990) agar plates after 7 d incubation at 28 °C.

Symbiotic performance. Seeds of *Amorpha fruticosa* were scarified with fine sandpaper and were surface-sterilized with 1% (v/v) NaOCl solution for 3 min. Germination was on moist paper towels or on 0·75% water-agar plates. Germinated seedlings were planted in Leonard jars (Leonard, 1943) containing sterile vermiculite moistened with nitrogen-free nutrition solution as described previously (van Berkum, 1990). Five millilitres of a 1- or 2-d-old MAG culture for the fast- or slow-growing isolates, respectively, was used to inoculate six seedlings in each jar. Nodulation was examined after 4 weeks growth in a growth room with day and night cycles of 16 and 8 h, and temperatures of 25 and 20 °C, respectively. The selected hosts recommended by Graham *et al.* (1991) and also *Astragalus sinicus*, *Cicer arietinum* and *Glycyrrhiza uralensis* were examined for nodulation by isolates ACCC 19665^T, ACCC 19667 and SH283012. Nodulation of *Amorpha fruticosa* by the type strains of described species was also investigated. The cross-

NO, No plasmid observed.

* ACCC, Agricultural Center Culture Collection, Chinese Academy of Agriculture, Beijing, China; CCBAU, Culture Collection of Beijing Agriculture University, Beijing, China; CFN, Centro de Investigación sobre Fijación de Nitrógeno, UNAM, Cuernavaca, México; CIAT, Centro Internacional de Agricultura Tropical, Cali, Columbia; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland; NZP, Division of Scientific and Industrial Research, Palmerston North, New Zealand; ORS, ORSTOM Collection, Institut Français de Recherche Scientifique pour le Développement en Coopération, Dakar, Senegal; USDA, Beltsville *Rhizobium* Culture Collection, Beltsville Agricultural Research Center, Beltsville, MD, USA.

† Four letters were arbitrarily assigned to represent specific fingerprint patterns obtained from PCR-RFLP analysis of 16S rRNA genes digested with restriction endonucleases *Msp*I, *Hin*fI, *Hha*I and *Sau*3AI, respectively. Different combinations of letters were then used to define RFLP groups.

‡ Electrophoretic type representing different combinations of nine enzyme patterns distinguishable with starch gel electrophoresis.

§ The approximate plasmid sizes were calculated from their mobilities in 0·7% agarose gel with plasmids of *R. etli* CFN 42^T as molecular size standard marker and *R. tropici* CFN 299 as reference. The standard deviation was 12 kb.

nodulation tests were done in sterilized plastic cups filled with vermiculite and the plants were grown in sunlight as described previously (Wang *et al.*, 1998).

PCR-RFLP analysis and determination of nucleotide sequences of the 16S rRNA genes. The PCR procedure described by van Berkum *et al.* (1996) was used for amplification of the 16S rRNA gene fragments (approximately 1500 bp) using primers fD1 (5′ AGAGTTTGATCCTGGCTCAG 3′) and rD1 (5′ AAGGAGGTGATCCAGCC 3′) (Weisburg *et al.*, 1991), which corresponded to positions 8–27 and 1524–1540, respectively, of the *Escherichia coli* 16S rRNA gene. The products in 10 µl subsamples of the reaction mixtures were digested with 5 U each of *Hha*I, *Hin*fI, *Msp*I or *Sau*3AI and the restriction fragments were separated by electrophoresis in 2% (w/v) agarose gels (Laguerre *et al.*, 1994). Similarities among the 16S rRNA gene sequences were estimated from the proportion of shared restriction fragments (Nei & Li, 1979) and a dendrogram was constructed from the resulting distance matrix using the unweighted pair group method with averages (UPGMA) (Sneath & Sokal, 1973). Template DNAs were prepared from 50 ml MAG-grown cells of isolates ACCC 19665^T, HL56, SH15003, ACCC 19667 and SH283012, and the type strain of *Mesorhizobium tianshanense*, A-1BS^T . These were purified by CsCl ultracentrifugation (Navarro *et al.*, 1993) and were used to generate the PCR products of the 16S rRNA genes for sequencing analysis. Full-length sequences of the 16S rRNA genes were determined with purified PCR products and an Applied Biosystems International 373 DNA Sequencer using a Dye Deoxy Terminator Cycle Sequencing Kit as described previously (van Berkum *et al.*, 1996).

Analysis of the sequence data. The sequences were aligned using the PILEUP program in the Wisconsin package of the Genetics Computer Group (Madison, WI, USA). Aligned sequences were analysed using the Molecular Evolutionary Genetics Analysis (MEGA) package version 1.01 (Kumar *et al.*, 1993) to produce a Jukes–Cantor distance matrix (Jukes & Cantor, 1969) and to construct an optimal unrooted tree using the neighbour-joining method (Saitou & Nei, 1987). This package was also used to generate bootstrap confidence values from 500 replications of each sequence and to derive the nucleotide sequence similarities of the 16S rRNA genes.

Multilocus enzyme electrophoresis (MLEE). Culture extracts for MLEE analysis were prepared from 40 ml PY (Noel *et al.*, 1984) broth cultures grown overnight at 28 °C and were stored according to the procedure of Caballero-Mellado & Martínez-Romero (1994). Starch gel electrophoresis and selective staining for the nine metabolic enzymes malic enzyme, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, NADP-independent glutamate dehydrogenase, indophenol oxidase, phosphoglucomutase, hexokinase, esterase and phosphoglucose isomerase were done according to the procedures described by Selander *et al.* (1986). Distinctive mobility variants (electromorphs) of each enzyme, numbered in order of decreasing anodal mobility, were equated with alleles at the corresponding structural gene locus. Allele profiles or electrophoretic types (ETs) were equated with multilocus genotypes. The genetic distance between pairs of ETs was estimated as the proportion of loci at which dissimilar alleles (mismatches) occurred. Clustering of ETs from a matrix of pairwise genetic distances was by the method described by Nei & Li (1979). Genetic diversity (h) at an enzyme locus was calculated as $h=[1-\Sigma x_i^2]\,[n/(n-1)]$, where x_i is the frequency of the ith allele at the locus and n is the number of ETs in the population. The extent of linkage disequilibrium (non-random combinations of alleles) among ETs was estimated by comparing observed and expected moments of allelic mismatch frequency distributions. An 'index of association' or I_A, originally described by Brown *et al.* (1980) was used to demonstrate a non-random association of alleles. Equations for the calculation of I_A and its associated error variance are described by Maynard Smith *et al.* (1993). A Monte Carlo procedure with 10000 iterations as recommended by Souza *et al.* (1992) was used to estimate the extent of linkage disequilibrium.

Determination of DNA base composition and DNA–DNA hybridization. Cultures were grown in 5·0 ml PY broth at 28 °C until late-exponential phase and were centrifuged at 12000 *g* for DNA preparation using a DNA/RNA isolation kit from USB as described by Chirgwin *et al.* (1979). These samples were used to estimate DNA G+C content (T_m) by the method of De Ley (1970) and to estimate DNA homologies by using the filter method as described by van Berkum *et al.* (1996).

Plasmid content and identification of the symbiotic plasmid. The plasmid contents of each of the isolates and each of the type strains for each species within the genus *Mesorhizobium* were visualized using a modified Eckhardt procedure (Eckhardt, 1978; Hynes & McGregor, 1990). Plasmid mobilities determined in 0·7% agarose gels were used for estimating the approximate molecular sizes of plasmids with the computer program Seqaid II version 3.5 (Rhoads & Roufa, 1989). The plasmids of *R. etli* CFN 42^T (Romero *et al.*, 1991) and of *R. tropici* CFN 299 (Geniaux *et al.*, 1995; Martínez *et al.*, 1987) were used as reference molecular size markers. Symbiotic plasmids were identified by Southern hybridization analysis using as probes a 2·0 kb *Eco*RI–*Pst*I fragment containing *nodDAB* of *R. tropici* CFN 299 cloned in pUC18 (unpublished) and a 5·1 kb *Bam*HI restriction fragment containing *nifHDK* of *R. etli* CFN 42^T (Morett *et al.*, 1988) as described previously (Wang *et al.*, 1998). The type strain of *R. etli*, CFN 42, and the type A strain of *R. tropici*, CFN 299, were used as positive controls.

Determination of reiteration of *nifH*. Reiteration of *nifH* was determined by Southern hybridization analysis as described above for plasmid hybridization. A 600 bp *Sal*I fragment carrying *nifH* of *R. etli* (Morett *et al.*, 1988) as probe and total genomic *Eco*RI or *Bam*HI digests were used. The type A strain of *R. tropici*, CFN 299, was used as a reference.

Phenotypic characterization. The phenotypic characters of 55 isolates from *Amorpha fruticosa* and the type strains of the described species were examined by recording growth after incubation times of 3 or 7 d for fast- and moderately slow-growing cultures, respectively. Reference strains used in phenotypic characterization were *R. leguminosarum* USDA 2370^T, *Sinorhizobium fredii* USDA 205^T, *M. loti* NZP 2213^T, *M. tianshanense* A-1BS^T, *Bradyrhizobium japonicum* USDA 6^T and USDA 110, and *Bradyrhizobium elkanii* USDA 76^T. Resistance to the presence of agar diffusion discs containing 10 µg ampicillin, 10 U bacitracin, 75 µg cefoperazone, 30 µg cefuroxime, 5 µg ciprofloxacin, 30 µg novobiocin, 10 U penicillin G, 100 U polymyxin B, 10 µg streptomycin and 30 µg tetracycline (Difco) was determined by growth on MAG as reported previously (van Berkum *et al.*, 1998a). The method and agar basal medium described by Ladha & So (1994) were used to investigate utilization of sole carbon substrates for growth. Filter-sterilized acetate,

L-arabinose, alanine, citrate, D-fructose, fumarate, D-glucose, L-leucine, L-lysine, *meso*-inositol, DL-malate, malonate, maltose, L-ornithine, D-raffinose, saccharic acid, sorbose, sucrose and xylose were added to the sterile basal medium at a final concentration of 0·2 %. Basal medium supplemented with 0·2 % mannitol or without addition of carbon substrate was used as the positive and negative control, respectively. The same basal medium was used to test the use of amino acids as the sole nitrogen sources for growth. Filter-sterilized arginine, glycine, lysine, methionine, phenylalanine, threonine, tryptophan and tyrosine were added to the sterilized basal medium at a final concentration of 10 mM. Basal medium containing 0·01 % $(NH_4)_2SO_4$ or without the addition of a nitrogen substrate was used as the positive and negative control, respectively. The appearance of single colonies was scored as positive.

Tolerance to NaCl at concentrations of 1·0, 1·5, 2·0, 2·5 and 3·0 % (w/v) was determined by growth on supplemented YMA. Ability to grow at 37 and 40 °C, and the ability to grow at pH 5·0, 9·0 or 9·5 were also determined on YMA. Acid/alkali production in litmus milk was recorded after 28 d incubation in Difco litmus milk medium. Ability to grow in LB (nutrition) broth (Yanagi & Yamasato, 1993) was determined with 5 ml medium. Production of acid or alkali was examined on YMA supplemented with 0·0025 % bromothymol blue as pH indicator (van Berkum, 1990). Growth rates of isolates ACCC 19660, ACCC 19663, ACCC 19665^T, B101, B289, SH109, SH19012, HL56, B276, N206, SH15003, ACCC 19667, ACCC 19677, SH28301 and SH283012, which represented each of the five 16S rRNA gene patterns, were determined in YM broth by following the increase in culture optical density at 600 nm (Yelton *et al.*, 1983) with a Beckman DU 650 spectrophotometer every 4 h for the faster growing cultures and every 12 h for more slowly growing cultures. Cell shape and size were determined by Gram-staining and microscopy.

RESULTS

Isolation and nodulation

Most of the 55 isolates grew moderately slowly to slowly, produced acid and were effective for symbiotic nitrogen fixation on the original trap host. *Amorpha fruticosa* formed determinate nodules with diameters of 1–4 mm on plants which were 4 weeks old. The isolates ACCC 19667 and ACCC 19677 differed from the other 53 by exhibiting faster growth since they formed colonies larger than 2 mm in diameter after 3 d incubation, and by forming nodules on *Amorpha fruticosa* which were ineffective for nitrogen fixation. Isolates HL56, SH283012 and SH28301 also differed in symbiotic response since they were only moderately effective for nitrogen fixation with *Amorpha fruticosa*.

Nodulation of *Amorpha fruticosa* was also observed in response to inoculation with *R. tropici* strain CFN 299. However, *R. leguminosarum* USDA 2370^T, *R. tropici* CIAT 899^T, *R. etli* CFN 42^T, *Rhizobium galegae* HAMBI 540^T, *Sinorhizobium meliloti* USDA 1002^T, *S. fredii* USDA 205^T, *Sinorhizobium saheli* ORS 609^T, *Sinorhizobium terangae* ORS 1009^T, *M. loti* NZP 2213^T, *Mesorhizobium huakuii* CCBAU 2609^T, *Mesorhizobium ciceri* UPM-Ca7^T, *Mesorhizobium mediterraneum* UPM-Ca36^T, *M. tianshanense* A-1BS^T , *Mesorhizobium plurifarium* ORS 1037, *B. japonicum* USDA 6^T, *B. elkanii* USDA 76^T and *Azorhizobium caulinodans* ORS 571^T did not nodulate *Amorpha fruticosa*. Isolate ACCC 19665^T, representing the main group (group 1) of the isolates, failed to nodulate *Astragalus sinicus*, *Cicer arietinum*, *Galega officinalis*, *Glycine max*, *Glycyrrhiza uralensis*, *Leucaena leucocephala*, *Lotus corniculatus*, *Macroptilium atropurpureum*, *Medicago sativa*, *Phaseolus vulgaris*, *Pisum sativum*, *Trifolium albus* and *Vigna unguiculata*. Also, strain ACCC 19667 did not nodulate *Phaseolus vulgaris* and *Pisium sativum*, whilst SH283012 did not nodulate *Glycine max*.

RFLP of 16S rRNA genes

Five distinct RFLP patterns with the PCR-amplified 16S rRNA genes were identified among the isolates from *Amorpha fruticosa* and were used to define groups 1–5 (Table 1). The majority of the isolates (43) had identical fingerprint patterns (pattern AAAA). These isolates originated from Beijing and Shaanxi Province. The second-most prevalant PCR-RFLP pattern (pattern BABA) was observed in six isolates which originated from Beijing and Heilongjiang Province. Each of the other three PCR-RFLP patterns (ABAA, DBDB and GCHE) were observed in analyses with the remaining six isolates. The five PCR-RFLP patterns observed among the isolates from *Amorpha fruticosa* were compared to those obtained with strains representing the bacterial species and genera forming symbioses with leguminous host plants. The first and the last patterns (AAAA and GCHE) were distinct from those observed with the reference strains (Table 1). From clustering analysis of the PCR-RFLP data we concluded that the 16S rRNA genes of the isolates within these two groups were characteristic of the genera *Mesorhizobium* and *Bradyrhizobium* (Fig. 1). The remaining three patterns were characteristic of the type strains for *M. tianshanense*, *M. huakuii* or *R. leguminosarum*. The described species formed clusters according to their estimated phylogeny based on the 16S rRNA gene nucleotide sequences (Amarger *et al.*, 1997; de Lajudie *et al.*, 1994; van Berkum *et al.*, 1998a, Wang *et al.*, 1998).

MLEE analysis

The 55 isolates were analysed for variation in electrophoretic mobility of nine enzyme loci. A total of 12 distinctive multilocus ETs were identified (Table 1). The largest number of isolates in any single ET was twenty-two, which is represented by strain ACCC 19665^T (Tables 1 and 2). Across all 55 isolates, the enzymes were polymorphic with two to eight alleles per locus (Table 2). However, across the majority of the isolates (43 isolates from ET 1–ET 6), only three enzymes were polymorphic (Table 2). Across all the isolates, the *h* values per enzyme locus ranged from

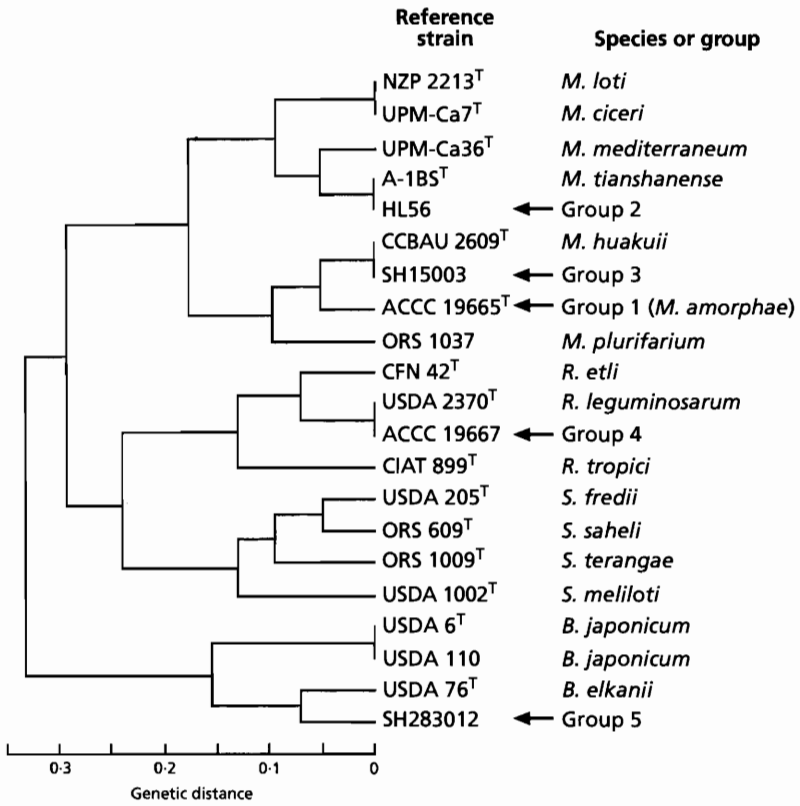

Fig. 1. Dendrogram showing the preliminary phylogenetic positions of isolates from *Amorpha fruticosa* based on the RFLP patterns of PCR-amplified 16S genes. Clustering analysis was performed using the method of Nei & Li (1979) from the genetic distance estimated from the fragments shared by each strain pair. The five groups, represented by isolates marked with arrows, consist of a total of 55 isolates from *Amorpha fruticosa* growing in Chinese soils, divided according to RFLP patterns. Isolates within each group shared the same RFLP patterns when digested with *Msp*I, *Hin*fI, *Hha*I or *Sau*3AI.

Table 2. Genetic diversity (*h* values) at nine enzyme loci among isolates from *Amorpha fruticosa*

Enzyme locus*	Group 1 (6 ETs)†		*Mesorhizobium* (10 ETs)		All isolates (12 ETs)	
	No. of alleles	*h* value	No. of alleles	*h* value	No. of alleles	*h* value
EST	3	0·733	6	0·889	8	0·909
IPO	1	0	2	0·356	4	0·394
ME	1	0	2	0·467	2	0·530
G6P	1	0	2	0·200	4	0·455
IDH	2	0·600	3	0·733	5	0·927
GD2	1	0	3	0·600	5	0·728
HEX	1	0	3	0·511	5	0·669
PGM	1	0	3	0·356	4	0·394
PGI	3	0·600	4	0·800	5	0·833
Mean	**1·6**	**0·214**	**3·1**	**0·546**	**4·7**	**0·649**
I_A value	−0·06±0·28		2·44±0·25		3·04±0·20	

*EST, esterase; IPO, indophenol oxidase; ME, NADP-malate dehydrogenase; G6P, glucose-6-phosphate dehydrogenase; IDH, isocitrate dehydrogenase; GD2, NADP-dependent glutamate dehydrogenase; HEX, hexokinase; PGM, phosphoglucomutase; PGI, phosphoglucose isomerase.

†Groups defined on the basis of PCR-RFLP analysis of 16S rRNA genes (see Table 1).

0·39 to 0·93. When the most divergent isolates were omitted from the analysis (isolates from ET 11 and ET 12), the *h* values ranged from 0·20 to 0·89, whilst across isolates belonging to ET 1–ET 6, the *h* values ranged from 0 to 0·73 (Table 2). The overall mean diversity per locus (*h*) of all isolates was 0·65. A dendrogram illustrating the relative genetic distance among the twelve ETs is shown in Fig. 2. The isolates within each of the clusters derived from the MLEE data had the same 16S rRNA gene PCR-RFLP fingerprint patterns. Exceptions were isolates N206 and SH15003, which were separated in clustering analysis of the MLEE data even though they had generated identical PCR-RFLP patterns in the analysis of the 16S rRNA genes.

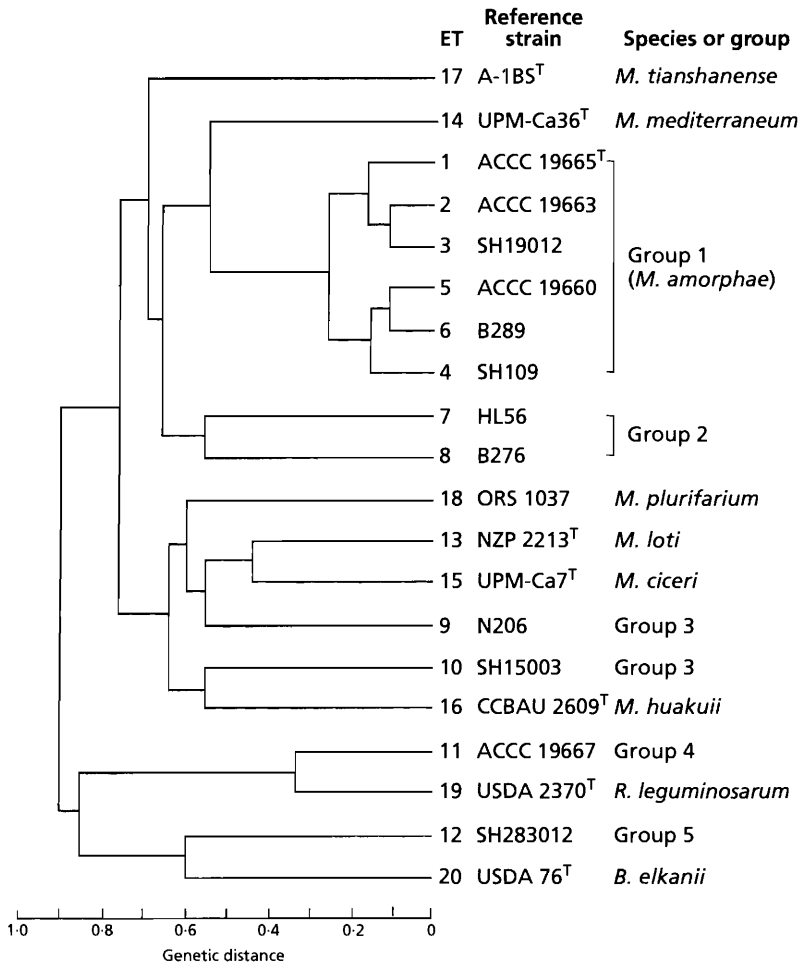

Fig. 2. Genetic relationships derived from MLEE studies on isolates obtained from *Amorpha fruticosa* and type strains representing species of *Mesorhizobium*, *R. leguminosarum* and *B. elkanii*. A total of 55 isolates from *Amorpha fruticosa* were represented by the 12 ETs observed. Groups were defined by RFLP patterns of PCR-amplified 16S rRNA genes.

Forty-nine of the isolates were in two distinct groups represented by eight ETs. The remaining six isolates (four ETs) were either associated with the type strains for *Mesorhizobium* (ET 9 and 10) or clustered with *R. leguminosarum* (ET 11) or with *B. elkanii* (ET 12).

The observed variance of the ET mismatch distribution for the first ten ETs, which represented all *Mesorhizobium* isolates, was significantly larger than expected ($I_A = 2{\cdot}44 \pm 0{\cdot}25$), implying a clonal population structure among the chromosomal lineages (Table 2). Further subdivision of the isolates into the first six ETs (group 1) resulted in an I_A value ($-0{\cdot}06 \pm 0{\cdot}28$) that did not significantly differ from zero.

Determination of DNA G+C content and DNA hybridization

In Table 3 are listed the isolates which we chose to represent our collection for the purpose of determining DNA homology and DNA G+C contents. The DNA G+C values among these isolates varied from 61 to 64 mol%, which is within the range expected for rhizobia. DNA homologies between isolates within the same PCR-RFLP group ranged from 64 to 100% (Table 3). Across isolates with different RFLP patterns, the DNA homologies ranged from 10 to 50%. With the exception of ACCC 19667, the DNA homologies between the isolates and the reference strains for the species ranged from 7 to 37% (Table 3). In the case of isolate ACCC 19667, the DNA homology with the type strain for *R. leguminosarum* (USDA 2370) was 70%.

Phylogeny of the isolates

Phylogeny was reconstructed from 16S rRNA gene sequences of single isolates each representing the five different patterns identified in the PCR-RFLP analysis. The majority of the isolates, represented by ACCC 19665^T, SH15003 and HL56, were grouped with species in the genus *Mesorhizobium* (Fig. 3). The isolates ACCC 19665^T and SH15003 clustered with type strains for *M. huakuii*, *M. mediterraneum* and *M. tianshanense*, whilst isolate HL56 was more closely related to *M. loti* and *M. ciceri*.

We also determined the 16S rRNA gene sequence of the type strain for *M. tianshanense* because the full-length sequence reported by Tan *et al.* (1997) and the partial sequence reported by Chen *et al.* (1995) over the same 260 bp region differed by six nucleotides. The

Table 3. DNA–DNA homologies among the reference strains for isolates from *Amorpha fruticosa* and type strains for described species

The membranes were hybridized overnight at 65 °C and washed twice in 2 × SSC at room temperature for 10 min and in 1 × SSC at 65 °C for 15 min. Values are means ± standard error.

Isolate or strain*	DNA homology with reference strain (%)				
	ACCC 19665^T	HL56	SH15003	ACCC 19667	SH283012
Group 1 (*M. amorphae*)					
ACCC 19665^T	100	44 ± 9	35 ± 0		
B101	80 ± 16				
ACCC 19663	85 ± 3				
SH19012	75 ± 0				
SH109	76 ± 8				
ACCC 19660	86 ± 0				
B289	95 ± 7				
Group 2					
B276		67 ± 2			
HL56	45 ± 3	100	50 ± 2		
Group 3					
N206	40 ± 1	37 ± 8	64 ± 0		
SH15003	42 ± 5	40 ± 1	100		
Group 4 (*Rhizobium* sp.)					
ACCC 19667	10 ± 2			100	
ACCC 19677				100	
Group 5 (*Bradyrhizobium* sp.)					
SH283012	19 ± 5	18 ± 1	10 ± 1		100
SH28301			8 ± 0		95
M. loti NZP 2213^T	35 ± 0	37 ± 0	32 ± 0		13 ± 3
M. mediterraneum UPM-Ca36^T	29 ± 1	31 ± 7	22 ± 3		
M. ciceri UPM-Ca7^T	30 ± 1	25 ± 2	17 ± 1		
M. huakuii CCBAU 2609^T	23 ± 4	33 ± 5	26 ± 1		
M. plurifarium ORS 1037	33 ± 0	25 ± 5	21 ± 3		
M. tianshanense A-1BS^T	24 ± 3	20 ± 4	22 ± 1		
S. meliloti USDA 1002^T	7 ± 1				
S. fredii USDA 205^T	7 ± 1				11 ± 1
S. saheli ORS 609^T	10 ± 1				
S. terangae ORS 1009^T	11 ± 0				
R. leguminosarum USDA 2370^T	7 ± 0			70 ± 2	7
R. tropici B CIAT 899^T	17 ± 1	37 ± 5			17 ± 0
R. tropici A CFN 299	14 ± 0			26 ± 0	
R. etli CFN 42^T	11 ± 1			34 ± 5	
R. galegae HAMBI 540^T	17 ± 0				
B. elkanii USDA 76^T	7				32 ± 0
B. japonicum USDA 6^T	7 ± 2				19 ± 0
A. caulinodans ORS 571^T	8 ± 1				

* Groups defined on the basis of PCR-RFLP analysis of 16S rRNA genes (see Table 1).

sequence we report for *M. tianshanense* in the same 250 bp region was 1 bp different to that reported by Chen *et al.* (1995). We used PCR products directly as templates for sequencing, whilst Tan *et al.* (1997) used a cloned PCR fragment. This difference in methodology may explain the disparity of the results since *Mesorhizobium* harbours two 16S rRNA genes (van Berkum *et al.*, 1998b) and they may show sequence heterogeneity (Cilia *et al.*, 1996; Wang *et al.*, 1997). Since the 16S rRNA gene nucleotide sequence determined by Tan *et al.* (1997) and by us differ, reconstructions of the phylogeny also differ. In our case, the estimated similarities of the 16S rRNA genes were 99·6 and 99·1 % between *M. tianshanense* and *M. mediterraneum*, and between *M. tianshanense* and *M. huakuii*, respectively.

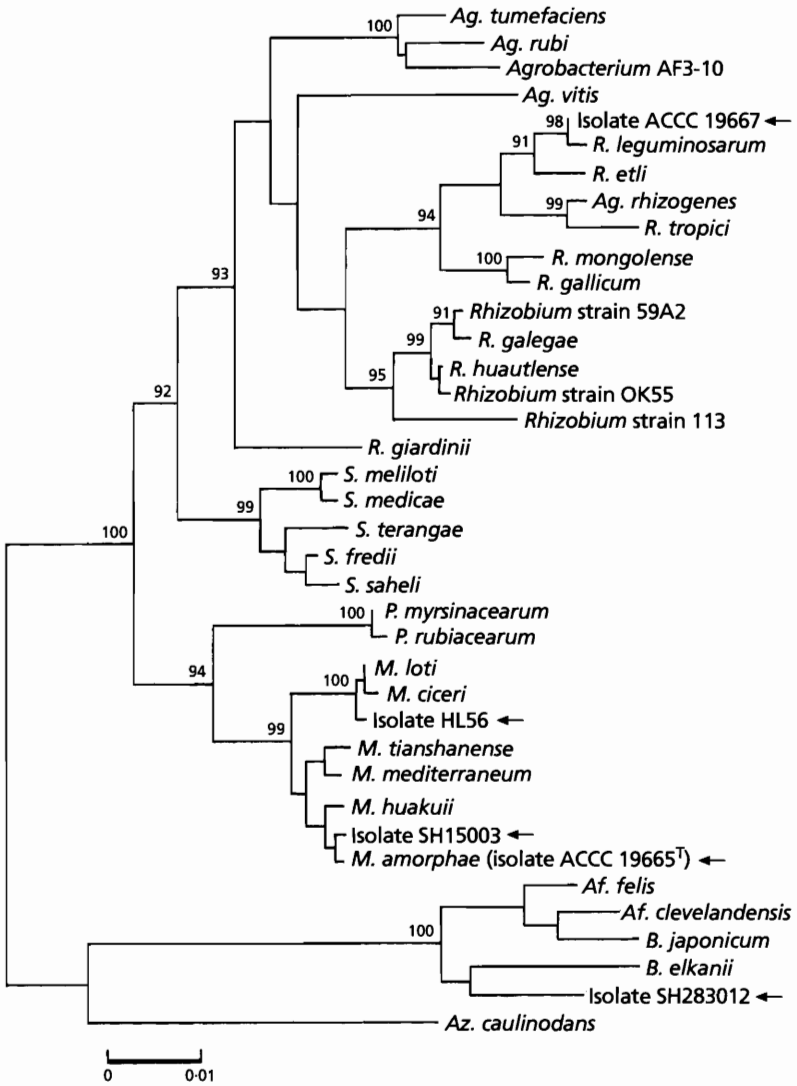

Fig. 3. Phylogenetic relationships of *Mesorhizobium amorphae* and several other isolates originating from *Amorpha fruticosa* within the α-*Proteobacteria* based upon aligned sequences of the 16S rRNA genes. Jukes–Cantor distances and the neighbour-joining method were used to construct an optimal unrooted tree. Five hundred trees were generated in a bootstrap analysis to derive a majority rule consensus tree. The levels of support for the presence of nodes above a value of 90% are indicated in the tree. Representative sequences in the tree which were obtained from GenBank and used in the phylogenetic analysis were: *Agrobacterium rubi* (D14503), *Agrobacterium vitis* (D14502), *Agrobacterium rhizogenes* NCPPB2991 (D14501), *Agrobacterium tumefaciens* NCPPB2437 (D14500), *Agrobacterium* sp. strain AF3-10 (Z30542), *Afipia clevelandensis* (M69186), *Afipia felis* (M65248), *Azorhizobium caulinodans* (X94200), *B. elkanii* (U35000), *B. japonicum* (U69638), *M. ciceri* (U07934), *M. huakuii* (D13431), *M. loti* (X67229), *M. mediterraneum* (L38825), *Phyllobacterium myrsinacearum* (D12789), *Phyllobacterium rubiacearum* (D12790), *R. etli* (U28916), *R. galegae* (X67226), *R. galegae* strain 59A2 (AF025853), *R. gallicum* (U86343), *R. giardinii* (U86344), *R. huautlense* (AF025852), *R. leguminosarum* bv. *viciae* (U29386), *R. mongolense* (U89817), *R. tropici* (U89832), *Rhizobium* sp. strain OK 55 (D14510), *Rhizobium* sp. strain 113 (D14512), *S. fredii* (X67231), *Sinorhizobium medicae* (L39882), *S. meliloti* (X67222), *S. saheli* (X68390) and *S. terangae* (X68387). The sequences were aligned using the PILEUP program in the Wisconsin package of the Genetics Computer Group (Madison, WI, USA) and the aligned sequences were analysed using the Molecular Evolutionary Genetics Analysis (MEGA) package version 1.01 (Kumar *et al.*, 1993). The scale bar shows 1% nucleotide substitutions.

From the 16S rRNA gene sequence data we concluded that the isolate ACCC 19667 was a member of the genus *Rhizobium*. Its 16S rRNA nucleotide sequence and that of the type strain for *R. leguminosarum* were 99·8% similar. In our current reconstruction *R. galegae* and *Rhizobium huautlense* had common ancestry with *R. leguminosarum* as we previously reported (Wang *et al.*, 1998). The fifth isolate (SH283012) and the type strain for *B. elkanii* were distantly related since their 16S rRNA gene nucleotide sequences were only 97·3% similar.

Plasmid contents and symbiotic plasmid identification

Five different plasmid profiles were observed among the isolates from *Amorpha fruticosa*. The number of plasmids observed ranged from none in the case of isolates SH28301 and SH283012 to four in the case of ACCC 19667 and ACCC 19677 (Table 1, Fig. 4). The majority of the isolates haboured one or two plasmids. With the exception of isolates SH28301 and SH283012, each of the isolates harboured a 930 kb plasmid. In isolates ACCC 19665^T, B104, SH190012, ACCC 19660, ACCC 19666, HL56, B276, SH15003 and N206, which represented groups within *Mesorhizobium*, the 930 kb plasmid appeared to be the symbiotic plasmid (pSym) since it hybridized with the cloned *nodDAB* genes of *R. tropici* CFN 299 and the cloned *nifDHK* genes of *R. etli* CFN 42^T. None of the plasmids of isolate ACCC 19667 hybridized with these two clones.

Because we observed a pSym in isolates from *Amorpha fruticosa* which were species within *Mesorhizobium*, we also examined reference strains within this genus for the presence of plasmid-borne symbiotic genes. Although we observed from one to three plasmids in NZP 2213^T (*M. loti*), CCBAU 2609^T (*M. huakuii*), UPM-Ca36^T (*M. mediterraneum*) and A-1BS^T (*M. tianshanense*), none hybridized with the *nod* and *nif*

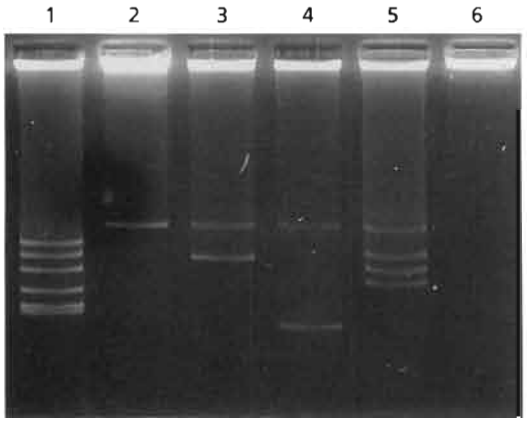

Fig. 4. Plasmid electrophoretic patterns obtained with *R. etli* CFN 42^T and isolates from *Amorpha fruticosa*. Lanes: 1, CFN 42^T (630, 510, 390, 270, 170 and 150 kb); 2, ACCC 19665^T (930 kb); 3, ACCC 19663 (930 and 550 kb); 4, ACCC 19660 (930 and 150 kb); 5, ACCC 19667 (930, 610, 490 and 440 kb); 6, SH283012 (no plasmids). The plasmids were visualized by using a modified Eckhardt procedure (Hynes & McGregor, 1990) and their mobilities were determined in 0·7% agarose gels. The approximate molecular sizes were estimated by using plasmids of CFN 42^T as molecular size references.

genes tested. In the case of UPM-Ca7^T (*M. ciceri*) and ORS 1037 (*M. plurifarium*) no plasmids were observed.

Hybridization of *nifH* gene loci

Only one *nifH* hybridization band was observed in both the *Eco*RI and *Bam*HI digests of the DNAs of the isolates ACCC 19665^T, HL56 and SH15003 (Fig. 5). The molecular sizes of the *Eco*RI or *Bam*HI restriction fragments hybridizing with *nifH* were the same across these three isolates. Weak to no hybridization signals were obtained with the *nifH* probe in analyses with the DNAs of isolates ACCC 19667 and SH283012 (Fig. 5).

Phenotypic characterization

The phenotypic characters of all the 55 isolates from *Amorpha fruticosa* are summarized in Table 4. Based upon phenotype, it was difficult to distinguish between group 1, represented by isolate ACCC 19665^T, and group 2, represented by HL56. Besides the presence of a pSym, the isolates within the three *Mesorhizobium* groups were distinguishable from the type strains for the species within *Mesorhizobium* by a combination of several phenotypic characters. These distinctive features are shown for the main group (group 1) of isolates from *Amorpha fruticosa* in Table 5.

DISCUSSION

Although *Amorpha fruticosa* was introduced into China approximately 50 years ago and was not inoculated, we observed that this legume host growing in Chinese soils was nodulated. The rhizobia which nodulated *Amorpha fruticosa* growing in Chinese soils were usually effective for nitrogen fixation since we isolated only 2 out of a total of 55 which were ineffective. Since *Amorpha fruticosa* is not native to China we are uncertain about the origin of the rhizobia which we isolated. It is possible that initially the rhizobia were seed-borne and were transmitted to China when the host was introduced, as has been suggested for the transmission of *R. etli* on seeds of

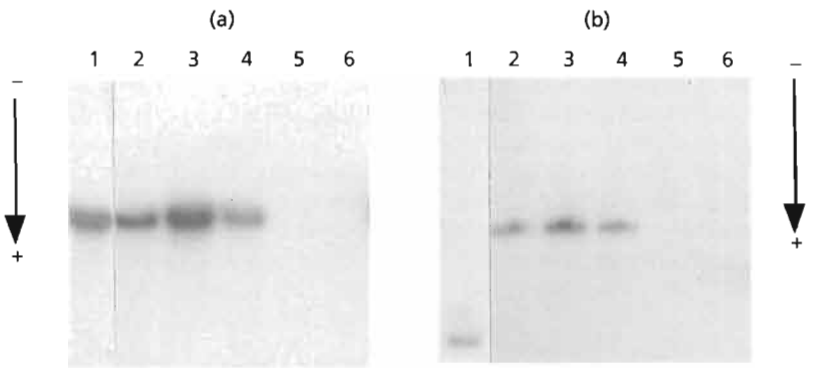

Fig. 5. Autoradiograms of Southern blots of total digested DNAs from *R. etli* CFN 299 and isolates from *Amorpha fruticosa* hybridized to a 600 bp *nifH* fragment from *R. etli* CFN 42^T (Morett *et al.*, 1988). (a) DNAs digested with *Eco*RI. Lanes: 1, CFN 299 (8·1 kb); 2, ACCC 19665^T (7·8 kb); 3, HL56 (7·8 kb); 4, SH15003 (7·8 kb); 5, ACCC 19667 (no band); 6: SH283012 (no band). (b) DNAs digested with *Bam*HI. Lanes: 1, CFN 299 (4·8 kb); 2, ACCC 19665^T (8·3 kb); 3, HL56 (8·3 kb); 4, SH15003 (8·3 kb); 5, ACCC 19667 (no band); 6, SH283012 (no band). The molecular sizes of the fragments were estimated from their migration in 0·7% agarose gel and λ DNA digested with *Bst*EII was used as molecular size markers. Arrows show the electrophoretic direction.

Table 4. Phenotypic characters and DNA G+C content (T_m) of isolates from *Amorpha fruticosa*

All the 55 isolates used L-arabinose, fumarate, rhamnose and L-xylose as sole carbon source. All grew at pH 5·0 and produced alkali in litmus milk. None grew with malonate, sorbose or lysine as sole carbon source. None grew at pH 9·0 or at 37 °C on YMA (Vincent, 1970). Values are the number of strains which showed growth, resistance or positive reactions.

Character	Group*				
	1 (n = 43)	2 (n = 6)	3 (n = 2)	4 (n = 2)	5 (n = 2)
Utilization as sole carbon source:					
Acetate	9	5	2	2	0
Citrate	0	0	2	0	2
D-Fructose, D-glucose, *meso*-inositol, sucrose, L-ornithine	43	6	2	2	0
Malate	43	5	2	0	2
Maltose	42	6	2	2	1
D-Raffinose	43	5	1	2	0
Saccharic acid	1	0	0	1	1
β-Alanine	43	6	2	0	0
Utilization as sole nitrogen source:					
Arginine	1	0	0	1	1
Glycine	0	0	0	0	1
Lysine	15	2	1	2	1
Methionine	0	1	1	2	0
Phenylalanine	4	6	2	2	1
Threonine	29	6	1	2	0
Tryptophan	15	4	2	0	2
Tyrosine	43	6	2	2	0
Resistance to antimicrobials:					
Bacitracin (10 U)	3	0	1	0	0
Cefoperazone (75 μg)	43	6	2	2	0
Cefuroxime (30 μg)	41	6	0	2	0
Novobiocin (30 μg)	35	2	2	1	0
Polymyxin B (100 U)	8	4	0	1	0
Ampicillin (10 μg)	41	6	1	2	0
Ciprofloxacin (5 μg)	25	6	2	2	0
Penicillin G (10 U)	31	6	0	0	0
Streptomycin (10 μg)	30	1	1	0	0
Tetracycline (30 μg)	42	6	2	2	0
Others:					
Tolerance to 1% (w/v) NaCl	13	1	1	0	1
Tolerance to 1·5% (w/v) NaCl	0	0	0	0	1
Growth in LB medium	0	1	1	0	0
Production of acid on YMA	43	6	2	2	0
Production of alkali on YMA	0	0	0	0	2
Generation time (h)	6–13	9–12	4–5	4	14–15
DNA G+C content (mol%) (T_m)	64	64	61–63	63	64

* Groups defined on the basis of PCR-RFLP analysis of 16S rRNA genes (see Table 1). n is the number of strains in the group.

Table 5. Characters distinguishing *M. amorphae* from other *Mesorhizobium* species

The distinguishing features were chosen according to Jarvis *et al.* (1997). Species: 1, *M. amorphae*; 2, *M. ciceri*; 3, *M. huakuii*; 4, *M. loti*; 5, *M. mediterraneum*; 6, *M. tianshanense*. +, > 95 strains showed positive reaction (growth); −, > 95% strains showed negative reaction (no growth); d, more than 5% but less than 95% strains showed positive reaction. ND, Not done; NO, not observed.

Character	1	2	3	4	5	6
Maximum temperature for growth (°C)	< 37	40	37–39	39	40	ND
Maximum NaCl concn for growth (%, w/v)	⩽ 1·0	2·0	ND	2·0	2·0	1·0
pH growth range	5·0, <9·0	5·0–10·0	5·0–9·5	4·0, <10·0	5·0, <10·0	ND
DNA G+C content (mol%)	64	63–64	59–64	59–64	63–64	59–63
Diameter of colonies:						
Incubation (d)	7	3–5	5–6	7	4–5	5–7
Size (mm)	⩽ 1·0	2·0–4·0	2·0–4·0	1·0	> 2·0	1·0–2·0
Symbiotic plasmid size (kb)	930	NO	NO	NO	NO	NO
Substrates used as sole carbon sources:						
Fumarate, D/L-malonate	+	+	+	+	+	−
meso-Inositol	+	+	−	+	+	−
D-Raffinose	+	−	+	d	−	d
L-Lysine	−	−	+	+	d	d
L-Ornithine	d	+	+	−	−	d
β-Alanine	+	+	d	−	−	+
L-Xylose	+	+	+	−	−	+

Phaseolus vulgaris (Pérez-Ramirez *et al.*, 1998). Alternatively, Chinese soils may harbour rhizobia which are able to nodulate *Amorpha fruticosa*, but which more usually form symbioses with native legumes. There is some precedence for the opportunistic nodulation of introduced legume species by indigenous rhizobia (Martínez-Romero & Caballero-Mellado, 1996). A comparative analysis of our Chinese isolates and those from the Americas may provide more insight into their origin.

The isolates from *Amorpha fruticosa* were examined using a polyphasic approach, which revealed genetic, phylogenetic and phenotypic characters of bacterial diversity. Generally, the results from each of the different approaches we used agreed. The 55 isolates were divided into five groups or genomic species, which were associated with three genera. The isolates were variable in their symbiotic effectiveness even if they belonged to the same genus. Phenotypes of growth rate and acid or alkali production on YMA distinguished isolates within the different genera. Variation among isolates within the genus level was demonstrated with results of MLEE, growth in the presence of 1·5% NaCl and differences in utilization of carbon and nitrogen sources.

We concluded from our investigation that the majority of the rhizobia originating from nodules of *Amorpha fruticosa* growing in Chinese soils are species within the genus *Mesorhizobium*. The 16S rRNA genes of the isolates in groups 1–3 were most similar to those of the type strains for the species within *Mesorhizobium* (Jarvis *et al.*, 1997). Phenotypically, these isolates also had the characteristics of *Mesorhizobium* since they had moderate to slow growth rates and produced acid on YMA (Jarvis *et al.*, 1997). Also, we established that the fast-growing, acid-producing, ineffective isolates were species of *Rhizobium*, and were most probably *R. leguminosarum* on the basis of the relatively high DNA homology (70%, Table 3) between isolate ACCC 19667 and the type strain of the species, USDA 2370. Even though this isolate was identified as *R. leguminosarum*, it does not possess the symbiotic determinants to nodulate *Pisium sativum* and *Phaseolus vulgaris*, hosts more commonly associated with this rhizobial species. The ineffective response on *Amorpha fruticosa* may not necessarily be because of their genus affiliation since *R. tropici* CFN 299 was effective with this host. Finally, we concluded that the remaining two isolates (group 5) were species of *Bradyrhizobium* based upon results from the 16S rRNA analysis and growth rate experiments. However, these isolates were distinct from *B. japonicum* and *B. elkanii* because of low DNA homology (Table 3).

Sequences of the 16S rRNA genes are most commonly used in the reconstruction of bacterial phylogenies (Maidak *et al.*, 1994). In our current reconstruction of the phylogeny of the isolates from *Amorpha fruticosa*, the position of *R. galegae* would indicate common ancestry with *R. leguminosarum* rather than with

Agrobacterium vitis. We first reported this new relationship between *R. galegae*, *R. leguminosarum* and *Agrobacterium vitis* when we proposed *R. huautlense* as a new species (Wang *et al.*, 1998). Most previously published phylogenies have indicated a commmon ancestry between *R. galegae* and *Agrobacterium vitis* (Amarger *et al.*, 1997; de Lajudie *et al.*, 1994; Tan *et al.*, 1997; van Berkum *et al.*, 1998a; Willems & Collins, 1993; Yanagi & Yamasato, 1993; Young & Haukka, 1996), which has resulted in a debate regarding whether to change the genus affiliation of *R. galegae* from *Rhizobium* to *Agrobacterium* (de Lajudie *et al.*, 1994; Young & Haukka, 1996). The difference in the phylogenetic relationships in our reconstruction probably result from the addition of sequences similar to that of *R. galegae* (Wang *et al.*, 1998) or perhaps from the different algorithms used to reconstruct phylogenetic trees. However, low bootstrap confidence values at the nodes separating *R. galegae* and other species of *Rhizobium* and *Agrobacterium* (Wang *et al.*, 1998) indicate that further changes may occur when additional related sequences are added. Therefore, we suggest the more cautious approach of retaining *R. galegae* within the genus *Rhizobium* and waiting for more evidence before reassignment of its genus affiliation.

The species within the genera *Rhizobium* and *Sinorhizobium* carry their determinants for symbiosis on plasmids (Martínez-Romero *et al.*, 1990). Species within the genera *Bradyrhizobium* and *Mesorhizobium* have been reported to carry their genes for symbiosis on the chromosome and a pSym is absent (Jarvis *et al.*, 1997; Martínez-Romero *et al.*, 1990). Here, we describe a common symbiotic plasmid (930 kb) as well as some intrinsic plasmids (150 or 550 kb) among the isolates within the genus *Mesorhizobium*. The presence of a pSym within *Mesorhizobium* is an unusual characteristic. However, there may be a precedence for the existence of a pSym in some species of the genus because Xu & Murooka (1995) indicated that the genes required for symbiosis in some *M. huakuii* strains were located on a large plasmid. However, we were unable to confirm this for the type strain of this species in our analysis.

All the isolates within *Mesorhizobium* had symbiotic plasmids of similar molecular size and had similar *nifH* gene hybridization patterns. From this evidence we concluded that these isolates shared the same pSym, indicating that the symbiotic genes may have been inherited through genetic exchange. However, the symbiotic genes of the isolates belonging to the other two genera were probably different since there were low or no hybridization signals with both the *nodDAB* and *nifH* probes we used.

None of the 16S rRNA gene sequences of the isolates within *Mesorhizobium* and those of the reference strains for species within *Mesorhizobium* were identical. However, this difference alone is insufficient evidence for their separate speciation (Stackebrandt & Goebel, 1994). Therefore, we used several additional approaches to determine whether our isolates belonged to any of the recognized species within the genus *Mesorhizobium*. Based upon the polyphasic approach of our investigation we conclude that the three groups represented by isolates ACCC 19665^T, HL56 and SH15003 are different from the described species of this genus. However, differences among the three groups were less distinct and, therefore, we hesitate to separate them into three different species. A possibility would be to classify them as different types within a single species, as for *R. tropici* (Martínez-Romero *et al.*, 1991) and *M. plurifarium* (de Lajudie *et al.*, 1998). However, for the moment, we prefer to propose only the most distinct group (group 1) as a new species within the genus *Mesorhizobium*, *Mesorhizobium amorphae*.

Description of *Mesorhizobium amorphae* sp. nov.

Mesorhizobium amorphae (a.mor'phae. N.L. n. *Amorpha* a systematic genus name of legume plants; N.L. gen. n. *amorphae* of *Amorpha*, the legume genus with which the species forms nitrogen-fixing symbioses).

Gram-negative, aerobic, non-spore-forming rods with cell size of 0·47–1·68 × 0·41–0·65 μm, which produce acid on YMA and alkali in litmus milk. Colonies on solid media are circular, translucent and with a diameter of 1 mm after 7 d growth at 28 °C. The generation times are from 6 to 13 h in YM broth. The maximum temperature for growth is below 37 °C. Growth on YMA is inhibited by 1·5% (w/v) NaCl and by a pH higher than 9·0. The species is resistant to cefoperazone (75 μg) and tetracycline (30 μg), uses L-arabinose, D-fructose, fumarate, D-glucose, *meso*-inositol, rhamnose, sucrose, L-xylose, β-alanine and ornithine as sole carbon sources, and tyrosine, NH_4Cl and NH_4SO_4 as sole nitrogen sources. The genes for symbiosis reside on a 930 kb plasmid. There is only one *nifH* gene copy. No evidence was obtained that the reference isolate, ACCC 19665^T, formed symbioses with any other host plant except for *Amorpha fruticosa*. The type strain is ACCC 19665^T and it has the characteristics described for the species.

ACKNOWLEDGEMENTS

This work was partly supported by the Natural Science Foundation of China, by USDA-FAS-OICD-RSED, USA, and by DGAPA IN202097 from UNAM, Mexico. We thank Dr D. Romero, Dr J. Caballero-Mellado and M. A. Rogel-Hernández for help and K. Lee Nash for technical support.

REFERENCES

Allen, O. N. & Allen, E. K. (1981). *The Leguminosae: a Source Book of Characteristics, Uses, and Nodulation.* Madison, WI: University of Wisconsin Press.

Amarger, N., Marcheret, V. & Laguerre, G. (1997). *Rhizobium*

gallicum sp. nov. and *Rhizobium giardinii* sp. nov. from *Phaseolus vulgaris* nodules. *Int J Syst Bacteriol* **47**, 996–1006.

van Berkum, P. (1990). Evidence for a third uptake hydrogenase phenotype among the soybean bradyrhizobia. *Appl Environ Microbiol* **56**, 3835–3841.

van Berkum, P., Beyene, D. & Eardly, B. D. (1996). Phylogenetic relationships among *Rhizobium* species nodulating the common bean (*Phaseolus vulgaris* L.). *Int J Syst Bacteriol* **46**, 240–244.

van Berkum, P., Beyene, D., Bao, G., Campbell, T. A. & Eardly, B. D. (1998a). *Rhizobium mongolense* sp. nov., is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.) Ledebour]. *Int J Syst Bacteriol* **48**, 13–22.

van Berkum, P., Ruihua, F., Campbell, T. A. & Eardly, B. D. (1998b). Some issues of relevance in the taxonomy of rhizobia. In *Highlights on Nitrogen Fixation Research*. Edited by E. Martínez & G. Hernández. New York: Plenum (in press).

Brown, A. H. D., Feldman, M. W. & Nevo, E. (1980). Multilocus structure of natural populations of *Hordeum spontaneum*. *Genetics* **96**, 523–536.

Caballero-Mellado, J. & Martínez-Romero, E. (1994). Limited genetic diversity in the endophytic sugarcane bacterium *Acetobacter diazotrophicus*. *Appl Environ Microbiol* **60**, 1532–1537.

Chen, W. X., Wang, E. T, Wang, S. Y., Li, Y. B., Chen, X. Q. & Li, Y. (1995). Characterization of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People's Republic of China. *Int J Syst Bacteriol* **45**, 153–159.

Chen, W. X., Yan, G. H. & Li, J. L. (1988). Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *Int J Syst Bacteriol* **38**, 392–398.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–5299.

Cilia, V., Lafay, B. & Christen, R. (1996). Sequence heterogeneities among 16S ribosomal RNA sequences and their effect on phylogenetic analyses at the species level. *Mol Biol Evol* **13**, 451–461.

De Ley, J. (1970). Re-examination of the association between melting point, buoyant density, and chemical base composition of DNA. *J Bacteriol* **101**, 738–754.

Dreyfus, B., Garcia, J. L. & Gillis, M. (1988). Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. *Int J Syst Bacteriol* **38**, 89–98.

Eardly, B. D., Wang, F. S., Whittam, T. S. & Selander, R. K. (1995). Species limits in *Rhizobium* populations that nodulate the common bean (*Phaseolus vulgaris*). *Appl Environ Microbiol* **58**, 1809–1815.

Eckhardt, T. (1978). A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. *Plasmid* **1**, 584–588.

Geniaux, E., Flores, M., Palacios, R. & Martínez, E. (1995). Presence of megaplasmids in *Rhizobium tropici* and further evidence of differences between the two *R. tropici* subtypes. *Int J Syst Bacteriol* **45**, 392–394.

Graham, P. H., Sadowsky, M. J., Keyser, H. H. & 8 other authors (1991). Proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria. *Int J Syst Bacteriol* **41**, 582–587.

Hynes, M. F. & McGregor, N. F. (1990). Two plasmids other than the nodulation plasmid are necessary for formation of nitrogen-fixing nodules by *Rhizobium leguminosarum*. *Mol Microbiol* **4**, 567–574.

Jarvis, B. D. W., van Berkum, P., Chen, W. X., Nour, S. M., Fernandes, M. P., Cleyet-Marel, J. C. & Gillis, M. (1997). Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum*, and *Rhizobium tianshanense* to a new genus: *Mesorhizobium*. *Int J Syst Bacteriol* **47**, 895–898.

Jordan, D. C. (1984). Family III. *Rhizobiaceae*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 234–242. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Konoshima, T., Terada, H., Kokumai, M., Kozuka, M., Tokuda, H., Estes, J. R., Li, L., Wang, H. K. & Lee, K. H. (1993). Studies on inhibitors of skin tumour promotion. XII. Rotenoids from *Amorpha fruticosa*. *J Nat Prod* (*Lloydia*) **56**, 843–848.

Kumar, S., Tamura, K. & Nei, M. (1993). MEGA: Molecular Evolutionary Genetics Analysis, version 1.01. The Pennsylvania State University, University Park, PA 16802, USA.

Ladha, J. K. & So, R. B. (1994). Numerical taxonomy of photosynthetic rhizobia nodulating *Aeschynomene* species. *Int J Syst Bacteriol* **44**, 62–73.

Laguerre, G., Allard, M., Revoy, F. & Amarger, N. (1994). Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. *Appl Environ Microbiol* **61**, 56–63.

de Lajudie, P., Willems, A., Pot, B. & 7 other authors (1994). Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. *Int J Syst Bacteriol* **44**, 715–733.

de Lajudie, P., Willems, A., Nick, G. & 9 other authors (1998). Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarium* sp. nov. *Int J Syst Bacteriol* **48**, 369–382.

Leonard, L. T. (1943). A simple assembly for use in the testing for cultures of rhizobia. *J Bacteriol* **45**, 523–527.

Maidak, B. L., Larsen, N., McCaughey, M. J., Overbeek, R., Olsen, G. J., Fogel, K., Blandy, J. & Woese, C. R. (1994). The ribosomal database project. *Nucleic Acids Res* **22**, 3485–3487.

Martínez, E., Palacios, R. & Sánchez, F. (1987). Nitrogen-fixing nodules induced by *Agrobacterium tumefaciens* harboring *Rhizobium phaseoli* plasmids. *J Bacteriol* **169**, 2828–2834.

Martínez, E., Romero, D. & Palacios, R. (1990). The *Rhizobium* genome. *Crit Rev Plant Sci* **9**, 59–93.

Martínez-Romero, E. & Caballero-Mellado, J. (1996). *Rhizobium* phylogenies and bacterial genetic diversity. *Crit Rev Plant Sci* **15**, 113–140.

Martínez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A., Graham, P. & Pardo, M. A. (1991). *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int J Syst Bacteriol* **41**, 417–426.

Maynard Smith, J., Smith, N. H., O'Rourke, M. & Spratt, B. G. (1993). How clonal are bacteria? *Proc Natl Acad Sci USA* **90**, 4384–4388.

Mitscher, L. A., Park, Y. H., Al-Shamma, A., Hudson, P. B. & Haas, T. (1981). Amorfrutin A and B, bibenzyl antimicrobial agents from *Amorpha fruticosa*. *Phytochemistry* **20**, 781–785.

Morett, E., Moreno, S. & Espin, G. (1988). Transcription analysis of the three *nifH* genes of *Rhizobium phaseoli* with gene fusion. *Mol Gen Genet* **213**, 499–504.

Navarro, R. B., Vargas, A. A. T., Schroder, E. C. & van Berkum, P. (1993). Uptake hydrogenase (Hup) in common bean (*Phaseolus vulgaris*) symbioses. *Appl Environ Microbiol* **60**, 4162–4165.

Nei, M. & Li, W. H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci USA* **76**, 5269–5273.

Noel, K. D., Sánchez, F., Fernandez, F., Leemans, J. & Cevallos, A. (1984). *Rhizobium phaseoli* symbiotic mutants with transposon Tn5 insertions. *J Bacteriol* **158**, 148–155.

Oyaizu, H., Matsumoto, S., Minamisawa, K. & Gamou, T. (1993). Distribution of rhizobia in leguminous plants surveyed by phylogenetic identification. *J Gen Appl Microbiol* **39**, 339–354.

Pérez-Ramirez, N. O., Rogel-Hernández, M. A., Wang, E. T. & Martínez-Romero, E. (1998). Seeds of *Phaseolus vulgaris* bean carry *Rhizobium etli*. *FEMS Microbiol Ecol* **26**, 289–296.

Piñero, D., Martínez, E. & Selander, R. K. (1988). Genetic diversity and relationships among isolates from *Rhizobium leguminosarum* biovar *phaseoli*. *Appl Environ Microbiol* **54**, 2825–2832.

Rhoads, D. D. & Roufa, D. J. (1989). Seqaid II (tm) version 3.5. Molecular Genetics Laboratory, Kansas State University, KS, USA.

Romero, D., Brom, S., Martínez-Salazar, J., de Lourdes Girard, M., Palacios, R. & Davila, G. (1991). Amplification and deletion of a *nod-nif* region in the symbiotic plasmid of *Rhizobium phaseoli*. *J Bacteriol* **173**, 2435–2441.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Segovia, L., Young, J. P. W. & Martínez-Romero, E. (1993). Reclassification of American *Rhizobium leguminosarum* biovar *phaseoli* type I strains as *Rhizobium etli* sp. nov. *Int J Syst Bacteriol* **43**, 374–377.

Selander, R. K., Caugant, D. A., Ochman, H., Musser, J. M., Gilmour, M. N. & Whittam, T. S. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl Environ Microbiol* **51**, 873–884.

Sneath, P. H. A. & Sokal, R. R. (1973). *Numerical Taxonomy*. San Francisco: Freeman.

Souza, V., Nguyen, T. T., Hudson, R. R., Piñero, D. & Lenski, R. E. (1992). Hierarchical analysis of linkage disequilibrium in *Rhizobium* populations: evidence for sex? *Proc Natl Acad Sci USA* **89**, 8389–8393.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Sullivan, J. T., Patrick, H. N., Lowther, W. L., Barry Scott, D. & Ronson, C. W. (1995). Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. *Proc Natl Acad Sci USA* **92**, 8985–8989.

Sullivan, J. T., Eardly, B. D., van Berkum, P. & Ronson, C. W. (1996). Four unnamed species of nonsymbiotic rhizobia isolated from the rhizosphere of *Lotus corniculatus*. *Appl Environ Microbiol* **62**, 2818–2825.

Tan, Z. Y., Xu, X. D., Wang, E. T., Gao, J. L., Martínez-Romero, E. & Chen, W. X. (1997). Phylogenetic and genetic relationships of *Mesorhizobium tianshanense* and related rhizobia. *Int J Syst Bacteriol* **47**, 874–879.

Vincent, J. M. (1970). *A Manual for the Practical Study of Root-nodule Bacteria*. IBP handbook 15. Oxford: Blackwell Scientific Publications.

Wang, Y., Zhang, Z. & Ramanan, N. (1997). The actinomycete *Thermobispora bispora* contains two distinct types of transcriptionally active 16S rRNA genes. *J Bacteriol* **179**, 3270–3276.

Wang, E. T., van Berkum, P., Beyene, D., Sui, X. H., Dorado, O., Chen, W. X. & Martínez-Romero, E. (1998). *Rhizobium huautlense* sp. nov., a symbiont of *Sesbania herbacea* that has a close phylogenetic relationship with *Rhizobium galegae*. *Int J Syst Bacteriol* **48**, 687–699.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.

Willems, A. & Collins, D. (1993). Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequence. *Int J Syst Bacteriol* **43**, 305–313.

Xu, Y. & Murooka, Y. (1995). A large plasmid isolated from *Rhizobium huakuii* bv Renge that includes genes for both nodulation of *Astragalus sinicus* cv. Japan and nitrogen-fixation. *J Ferment Bioeng* **80**, 276–279.

Yanagi, M. & Yamasato, K. (1993). Phylogenetic analysis of the family *Rhizobiaceae* and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. *FEMS Microbiol Lett* **107**, 115–120.

Yelton, M. M., Yang, S. S., Edie, S. A. & Lim, S. T. (1983). Characterization of an effective salt-tolerant, fast-growing strain of *Rhizobium japonicum*. *J Gen Microbiol* **129**, 1537–1547.

Young, J. P. W. (1985). *Rhizobium* population genetics: enzyme polymorphism in isolates from peas, clover, beans and lucerne grown in the same site. *J Gen Microbiol* **131**, 2389–2408.

Young, J. P. W. & Haukka, K. E. (1996). Diversity and phylogeney of rhizobia. *New Phytol* **133**, 87–94.