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Characterization of bacteria isolated from wild legumes in the north-western regions of China

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Nodule isolates from 11 species of wild legumes in north-western China were characterized by numerical taxonomy, PCR-based 16S rRNA gene RFLP and sequence analyses, DNA–DNA hybridization, restriction patterns of *nodDAB* and *nifH* genes, and symbiotic properties. Based on the results of numerical taxonomy, most of the 35 new isolates were grouped into five clusters (clusters 7, 9, 12, 14 and 15). Clusters 7 and 12 were identified as *Mesorhizobium amorphae* and *Agrobacterium tumefaciens*, respectively, based on their high DNA homologies with the reference strains for these species, their 16S rRNA gene analysis and their phenotypic features. Results of 16S rDNA PCR-RFLP analysis showed that cluster 9 belonged to *Rhizobium*. Clusters 14 and 15 were identified as *Mesorhizobium* based on their moderately slow-growing, acid-producing characters and the high similarity of their 16S rDNA PCR-RFLP patterns to those of *Mesorhizobium* species. These two clusters were genomic species distinct from all described species based on analysis of DNA relatedness within this genus. The isolates in cluster 12 (*Agrobacterium tumefaciens*) failed to nodulate their original host and other selected hosts and they did not hybridize to *nif* or *nod* gene probes. The possibility of opportunistic nodulation of these isolates is discussed. Identical restriction patterns were obtained in the *nif* or *nod* gene hybridization studies from the three isolates within cluster 15, which were isolated from the same host species. The isolates from different host plants in each of clusters 9 and 14 produced different *nodDAB* RFLP patterns, but similar *nifH* RFLP patterns appeared (one band for each isolate). Different patterns were observed among different clusters from both the *nod* and *nif* gene hybridization studies. Cross-nodulation was recorded among the isolates and the host plants in the same cluster and promiscuous properties were found among some of the hosts tested.

Keywords: wild leguminous plants, diversity of rhizobia, polyphasic taxonomy

INTRODUCTION

Microbial biodiversity is considered to be one of the most valuable resources for mankind. Rhizobia are useful bacterial resources because of their symbiotic nitrogen-fixing ability. They form nodules on roots or stems of many leguminous plants and reduce N_2 to ammonia which can be used by the host plants. With the continual investigation of rhizobial resources and the application of modern molecular biological techniques, six genera with about 30 species have been described, including *Allorhizobium* (de Lajudie *et al.*, 1998a), *Azorhizobium* (Dreyfus *et al.*, 1988), *Bradyrhizobium* (Jordan, 1984; Kuykendall *et al.*, 1992; Xu *et al.*, 1995), *Mesorhizobium* (Jarvis *et al.*, 1982, 1997; Chen *et al.*, 1991, 1997; Nour *et al.*, 1994, 1995; de Lajudie *et al.*, 1998a; Wang *et al.*, 1999a), *Rhizobium* (Amarger *et al.*, 1997; van Berkum *et al.*, 1998; Chen *et al.*, 1997; Jordan, 1984; Lindström, 1989; Martínez-Romero *et al.*, 1991; Segovia *et al.*, 1993; Wang *et al.*, 1998) and *Sinorhizobium* (Chen *et al.*, 1988; de Lajudie *et al.*, 1994; Rome *et al.*, 1996). Phylogenetically, these bacteria belong to the α-*Proteobacteria* (Stackebrandt *et al.*, 1988; Sawada *et al.*, 1993; Willems & Collins,

Table 1. Isolates, reference strains and plasmids used in this research

Strain/isolate	Host plant	Geographic origin	Source or donor†
Cluster 7 (*Mesorhizobium amorphae*)			
SH10901, SH15003, SH190012	*Amorpha fruticosa*	Shaanxi*	CCBAU
SH2773	*Sophora viciifolia*	Gansu*	CCBAU
Cluster 9			
SH2462, SH246012	*Coronilla varia*	Gansu*	CCBAU
SH17113, SH1718	*Coronilla varia*	Shaanxi*	CCBAU
SH22623	*Gueldenstaedtia multiflora*	Gansu*	CCBAU
SH1456	*Amphicarpaea trisperma*	Shaanxi*	CCBAU
SH27121, SH28931, SH0975	*Amphicarpaea trisperma*	Gansu*	CCBAU
Cluster 12			
SH21321	*Caragana pruinosa*	Ningxia*	CCBAU
SH215214	*Caragana microphylla*	Ningxia*	CCBAU
SH19312, SH19352	*Glycyrrhiza pallidiflora*	Ningxia*	CCBAU
SH1124	*Sophora viciifolia*	Shaanxi*	CCBAU
Cluster 14			
SH27422, SH2671, SH2672, SH2851, SH28512	*Gueldenstaedtia multiflora*	Gansu*	CCBAU
SH13020, SH0232	*Gueldenstaedtia multiflora*	Shaanxi*	CCBAU
SH283, SH2830	*Amorpha fruticosa*	Gansu*	CCBAU
SH18611	*Caragana intermedia*	Shaanxi*	CCBAU
SH1701, SH1707	*Glycyrrhiza uralensis*	Shaanxi*	CCBAU
SH0211	*Sophora viciifolia*	Shaanxi*	CCBAU
Cluster 15			
SH0172, SH1081, SH2751	*Glycyrrhiza multiflora*	Gansu*	CCBAU
Single strain SH226022	*Gueldenstaedtia verna*	Gansu*	CCBAU
Agrobacterium rhizogenes IAM 13570^T			IAM
Agrobacterium radiobacter IAM 12048^T			IAM
Agrobacterium tumefaciens IAM 13129^T			IAM
Agrobacterium rubi HAMBI 1187^T			HAMBI
Agrobacterium vitis IAM 14140^T			IAM
Azorhizobium caulinodans ORS 571^T	*Sesbania rostrata*	Senegal	ORS
Bradyrhizobium elkanii USDA 76^T	*Glycine max*	USA	USDA
Bradyrhizobium japonicum B15	*Glycine max*	Liaoning*	CCBAU
Bradyrhizobium japonicum USDA 6^T	*Glycine max*	Japan	USDA
Bradyrhizobium japonicum USDA 110	*Glycine max*		USDA
Mesorhizobium amorphae ACCC 19665^T	*Amorpha fruticosa*	Beijing*	CCBAU
Mesorhizobium sp. HL56	*Amorpha fruticosa*	Heilongjiang*	CCBAU
Mesorhizobium ciceri USDA 3378^T (UPM-Ca7^T)	*Cicer arietinum*	Spain	USDA
Mesorhizobium huakuii A106, PL-52	*Astragalus sinicus*	Hubei*	CCBAU
Mesorhizobium huakuii CCBAU 2609^T	*Astragalus sinicus*	Nanjing*	CCBAU
Mesorhizobium loti NZP 2227, NZP 2234	*Lotus* sp.		NZP
Mesorhizobium loti NZP 2213^T	*Lotus corniculatus*	New Zealand	NZP
Mesorhizobium mediterraneum USDA 3392^T (UPM-Ca36^T)	*Cicer arietinum*	Spain	USDA
Mesorhizobium plurifarium LMG 11892^T (ORS 1032^T)	*Acacia senegal*	Senegal	LMG
Mesorhizobium plurifarium USDA 4413 (ORS 1037)	*Acacia senegal*	Senegal	USDA
Mesorhizobium tianshanense 6, A-1BS^T	*Glycyrrhiza uralensis*	Xinjiang*	CCBAU
Rhizobium etli CFN 42^T	*Phaseolus vulgaris*	Mexico	CFN
Rhizobium galegae HAMBI 1185	*Galega* sp.	UK	HAMBI
Rhizobium galegae HAMBI 503	*Galega officinalis*	USA	HAMBI
Rhizobium galegae HAMBI 540^T	*Galega orientalis*	Finland	HAMBI
Rhizobium galegae 59A2		USA	USDA
Rhizobium giardinii H152^T	*Phaseolus vulgaris*	France	USDA

Table 1 *(cont.)*

Strain/isolate	Host plant	Geographic origin	Source or donor†
Rhizobium hainanense 112	*Centrosema pubescens*	Hainan*	CCBAU
Rhizobium hainanense I66^T	*Desmodium smuatum*	Hainan*	CCBAU
Rhizobium hainanense H14	*Desmodium heterophyllum*	Hainan*	CCBAU
Rhizobium huautlense S02^T	*Sesbania herbacea*	Mexico	CFN
Rhizobium gallicum USDA 2914^T (R602sp^T)	*Phaseolus vulgaris*	France	USDA
Rhizobium leguminosarum 162X68	*Trifolium* sp.	USA	USDA
Rhizobium leguminosarum USDA 2370^T		USA	USDA
Rhizobium mongolense USDA 1844^T	*Medicago luthenica*	Inner Mongolia*	USDA
Rhizobium tropici type A CFN 299	*Phaseolus vulgaris*	Mexico	CFN
Rhizobium tropici type A C-05-I	*Phaseolus vulgaris*	Brazil	CFN
Rhizobium tropici type B BR853	*Leucaena leucocephala*	Brazil	CFN
Rhizobium tropici type B CIAT 899^T	*Phaseolus vulgaris*	Columbia	CFN
Sinorhizobium fredii 2048	*Glycine soja*	Liaoning*	CCBAU
Sinorhizobium fredii USDA 194, USDA 205^T	*Glycine soja*	Henan*	USDA
Sinorhizobium meliloti USDA 1002^T		USA	USDA
Sinorhizobium meliloti 102F28	*Medicago sativa*		
Sinorhizobium meliloti H1	*Melilotus albus*	Heilongjiang*	CCBAU
Sinorhizobium terangae LMG 7834^T (ORS 1009^T)	*Acacia laeta*	Senegal	LGM
Sinorhizobium xinjiangense CCBAU 110^T	*Glycine max*	Xinjiang*	CCBAU
Sinorhizobium xinjiangense CCBAU 108, Rx22	*Glycine max*	Xinjiang*	CCBAU
K12	Standard for DNA G+C estimation		
HD5α	Host for plasmid		
Phage and plasmid M13	Bacteriophage vector for clone		
pMR133	A 2·0 kb *Eco*RI-*Pst*I insert with *nodDAB* of *R. tropici* CFN 299 cloned into pUC18 (M. Rosenblueth, unpublished)		

^{T}, type strain.

* Province or city of China.

† CCBAU, Culture Collection of Beijing Agricultural University, Beijing, China; ACCC, Agricultural Center of Culture Collection, Chinese Academy of Agriculture, Beijing, China; CFN, Centro de Investigación sobre Fijación de Nitrógeno, UNAM, Cuernevaca, Mexico; CIAT, Centro Internacional de Agricultura Tropical, Cali, Columbia; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland; IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; LMG, Collection of Bacteria of the Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium; NZP, Culture Collection of the Department for Scientific and Industrial Research, Biochemistry Division, Palmerston North, New Zealand; ORS, ORSTOM Collection, Institut Français de Recherche Scientifique pour le Développement en Coopération, Dakar, Senegal; USDA, *Rhizobium* Culture Collection, Beltsville Agricultural Research Center, USDA, Beltsville, MD, USA.

1993; Yanagi & Yamasato, 1993; Young, 1994). The genera *Azorhizobium* and *Bradyrhizobium* are phylogenetically divergent from the other rhizobia. The remaining genera, *Allorhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*, are closely related to each other and some of them are intermingled with plant pathogenic bacteria belonging to the genus *Agrobacterium*. The need for further taxonomic revision of the rhizobia–agrobacteria group is evident (de Lajudie *et al.*, 1994; Lindström *et al.*, 1995; Sawada *et al.*, 1993; Young & Haukka, 1996).

Since the 1980s, the research group of W. X. Chen has been investigating rhizobial resources in China. Our studies have mainly focused on the reservation of rhizobial biodiversity and characterization of the isolates for potential use in reforestation or sustainable agriculture. A lot of rhizobial isolates have been characterized by a polyphasic approach and some species, *Mesorhizobium huakuii* (Chen *et al.*, 1991), *Mesorhizobium tianshanense* (Chen *et al.*, 1995), *Mesorhizobium amorphae* (Wang *et al.*, 1999a), *Rhizobium hainanense* (Chen *et al.*, 1997) and *Sinorhizobium xinjiangense* (Chen *et al.*, 1988), have been described based on experimental results. A great diversity of rhizobia populations has been found in Chinese soil.

The north-western regions of China, including the provinces of Qinghai, Shaanxi, Gansu and Autonomous Regions of Xinjiang and Ningxia, are temperate regions with arid and semi-arid climates and soils that are poor in organic matter. In this vast area, some herbaceous and woody legumes grow well. Most of these legumes are indigenous to these regions and

form root nodules. These legumes play important roles in the environment as foliage for wild animals, as wind breaks to control soil erosion or as resources for production of Chinese medicines. Our previous studies have shown that rhizobia in that area have some unique features, such as increased tolerance to NaCl and alkaline conditions (Chen *et al.*, 1988). Several species have been described for isolates from that region (Chen *et al.*, 1988, 1995; Wang *et al.*, 1999a).

The aims of this research were to characterize rhizobial isolates from the north-western regions of China by both phenotypic and genetic methods and to clarify the diversity and taxonomic positions of the local rhizobial populations.

METHODS

Isolation and inoculation tests. The root nodules were obtained from the fields of Shaanxi and Gansu Province and Autonomous Region of Ningxia in the summer of 1992. Rhizobia were isolated from fresh nodules by the standard method on YMA medium (Vincent, 1970). Single colonies were picked up and checked for purity by repeated streaking and by microscopic examination. All strains and isolates were incubated at 28 °C and maintained on YMA slants at 4 °C except where specifically indicated, or in 20% (v/v) glycerol at −20 °C. All the isolates were tested for nodulation ability on the original hosts as described previously (Chen *et al.*, 1991; Vincent, 1970). Thirty-five new isolates collected from north-western China and some reference strains for the described species used in this study are listed in Table 1.

Phenotypic characterization and numerical taxonomy. The generation time was determined spectrophotometrically (Yelton *et al.*, 1983) or by plate counting (Somasegaran & Hoben, 1994). For the spectrophotometric method, the isolates were grown in YM or PY broth (Noel *et al.*, 1984), since some isolates grew poorly in YM broth. Isolates were grown in YM broth (Vincent, 1970) for up to 7 d and the numbers of cells were counted each day. A_{600} was determined with a Beckman DU 650 spectrophotometer every 4 h for the fast-growing rhizobia and every 8 h for the slow- or moderately slow-growing rhizobia.

The 148 phenotypic features described previously (Gao *et al.*, 1994) and the following features were used for characterization of the isolates and strains: L-arabitol, fructose, glycogen, inulin, DL-malate, D-mannose, melibiose, sodium pyruvate, sodium acetate, sodium citrate, sodium laurate, sodium oxalate, D-sorbitol, starch, tartrate, trehalose, D-turanose, urea, vanillic acid, glycine, L-proline, L-phenylalanine and L-tyrosine as sole carbon sources; DL-citrulline, DL-cystine, L-cystine, hypoxanthine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-serine, D- and L-threonine, L-tyrosine, urea and D-valine as sole nitrogen sources (at a final concentration of 0·1%); resistance to antibiotics (concentrations given in μg ml^{-1}) chloramphenicol (100, 150 and 300), chlortetracycline (100 and 150), erythromycin (50, 100, 150 and 200), kanamycin sulfate (200, 300 and 400), lincomycin (50, 100, 200 and 350), neomycin (50 and 100), penicillin GK (200 and 350), qianglimycin (50 and 75), streptomycin (50), tetracycline (75); and resistance to dye (0·1%), Bismarck brown and erythrosin B.

The unweighted pair group method with arithmetic means (UPGMA; Sneath & Sokal, 1973) was used for clustering analysis of phenotypic features.

PCR-based 16S rDNA RFLP and partial sequencing. Primers fD1 and rD1 (Weisburg *et al.*, 1991), which correspond to *Escherichia coli* 16S rRNA gene positions 8–27 and 1524–1540, respectively, and procedures described previously (van Berkum *et al.*, 1996) were used for PCR amplification of 16S rRNA genes (approximately 1·5 kb) from reference strains or representative isolates. Restriction endonucleases *Msp*I, *Hin*fI, *Hha*I and *Sau*3AI (Amersham), as recommended by Laguerre *et al.* (1994), were used separately to digest a liquor of 10–15 μl PCR products. The restriction patterns were analysed by electrophoresis in 3% (w/v) agarose gels (Wang *et al.*, 1998).

For clustering analysis, RFLP patterns of the 16S rDNAs were converted to a two-dimensional binary matrix. Sequence divergence between the 16S rDNA sequences of each strain pair were estimated from the proportion of shared restriction fragments. A dendrogram was constructed from the distance matrix by the method of Nei & Li (1979).

For sequence analysis, DNA extracted by the method of Marmur (1961) was used as a template to amplify a 260 bp fragment of 16S rRNA gene using primers Y1 and Y2 and the procedure described by Young *et al.* (1991). The PCR fragment was cloned into phage M13 and sequenced as described previously (Chen *et al.*, 1995). The sequences obtained in this work (GenBank accession nos AF068252 for SH22623 and AF068253 for SH19312) were compared with those of related species obtained from the GenBank database. All the sequences were aligned using the PILEUP program in the Wisconsin package (Genetics Computer Group, 1994) and a phylogenetic tree was constructed using the CLUSTAL W package (Thompson *et al.*, 1994). This package was also used for bootstrap analysis from 1000 replications of each sequence.

Estimation of DNA base compositions and DNA–DNA hybridization. DNAs extracted according to the method of Marmur (1961) were used for estimating the G+C content (mol%) by the thermal denaturation method (T_m) (De Ley, 1970; Marmur & Doty, 1962) and for determination of DNA homology by the spectrophotometric method (De Ley *et al.*, 1970). DNA from *E. coli* K-12 was used as the standard for estimation of G+C content. The DNA homologies among the novel groups and described species were also estimated by filter hybridization as described by van Berkum *et al.* (1998). The DNA–RNA isolation kit, rediprimers, Hybond-N+ positively charged nylon membrane and Rapid-hyb buffer from Amersham were used for DNA extraction, labelling probes and hybridization under stringent conditions, as specified by the manufacturer.

Restriction patterns of *nodDAB* and *nifH* genes. Total DNA extracted with the DNA–RNA isolation kit was digested with *Eco*RI or *Bam*HI and separated by electrophoresis in 0·6% agarose gels. DNA–DNA hybridization was carried out as described above for the filter hybridization. A 2·0 kb *Eco*RI/*Pst*I fragment containing *nodDAB* of *Rhizobium tropici* CFN 299 (Wang *et al.*, 1998) and a 500 bp PCR fragment of *nifH* from *Sinorhizobium meliloti* using primers nifH-1 and nifH-2 (Eardly *et al.*, 1992) were used as probes. Hybridization was performed under stringent conditions at 65 °C. The patterns were recorded by autoradiography with Kodak X-ray film.

Symbiotic properties. The cross-nodulation was examined among the isolates and the host species within each cluster. The species *Phaseolus vulgaris* and *Leucaena leucocephala* were also used for the test since their promiscuous properties are well-documented (Martínez-Romero & Caballero-

Mallado, 1996; Wang *et al.*, 1999b). Seed treatments and inoculation were performed by using the methods of Vincent (1970) with some modifications (Chen *et al.*, 1991). The effectiveness of the nodules for nitrogen-fixation was estimated from the pink colour of the nodules and the dark green colour of leaves compared to control plants (without inoculation).

RESULTS

Isolation and nodulation tests

In this study, root nodules were collected from 11 leguminous species within five genera (Table 1). The species *Amorpha fruticosa*, *Sophora viciifolia* and *Caragana* spp. are shrubs. *Glycyrrhiza* spp., important resources of Chinese medicine, are semi-shrubby plants. *Coronilla varia*, *Gueldenstaedtia* spp. and *Amphicarpaea trisperma* are perennial herbaceous plants. Among these legume species, *Amorpha fruticosa* was introduced from America as a wind break plant and now it grows wildly in many places in China. The other species are native to the region. Nodulation ability has not been recorded for *Gueldenstaedtia multiflora* (Allen & Allen, 1981). Fifteen of the 35 isolates were fast growers which formed single colonies with diameters of 2–3 mm within 3 d on YMA; 20 isolates were slow- or moderately slow-growing rhizobia forming single colonies less than 1 mm in diameter in 5–7 d incubation. For most of the isolates, nodulation and nitrogen-fixation abilities were confirmed by the formation of pink nodules on their original hosts and the dark green leaves of nodulated plants compared with the control plants (without inoculation) except for five isolates (SH19312, SH19352, SH1124, SH215214 and SH21321), which could not nodulate the original host plants. Since these five isolates were originally from root nodules, they were also included in further studies.

Numerical taxonomy

Two hundred and twelve features were analysed to determine phenotypic characters and group the isolates. Sixteen clusters could be distinguished at a similarity level of 78–84% (Fig. 1) using the strains of described species as references. One isolate, SH226022, from *Gueldenstaedtia verna* and a strain of *Rhizobium tropici* type A C-05-I could not be included in any cluster.

Clusters 1–6, 8, 10, 11, 13 and 16 consisted of strains from described species corresponding to *Rhizobium leguminosarum*, *Sinorhizobium fredii*, *Sinorhizobium meliloti*, *Sinorhizobium xinjiangense*, *Mesorhizobium huakuii*, *Mesorhizobium loti*, *Rhizobium galegae*, *Rhizobium tropici* type B, *Rhizobium hainanense*, *Mesorhizobium tianshanense* and *Bradyrhizobium japonicum*, respectively. Cluster 7 was composed of an isolate (SH2773) from *Sophora viciifolia* and three isolates from *Amorpha fruticosa*, two of which had been assigned to *Mesorhizobium amorphae* (Wang *et al.*, 1999a). Clusters 9, 12, 14 and 15 consisted only of new isolates.

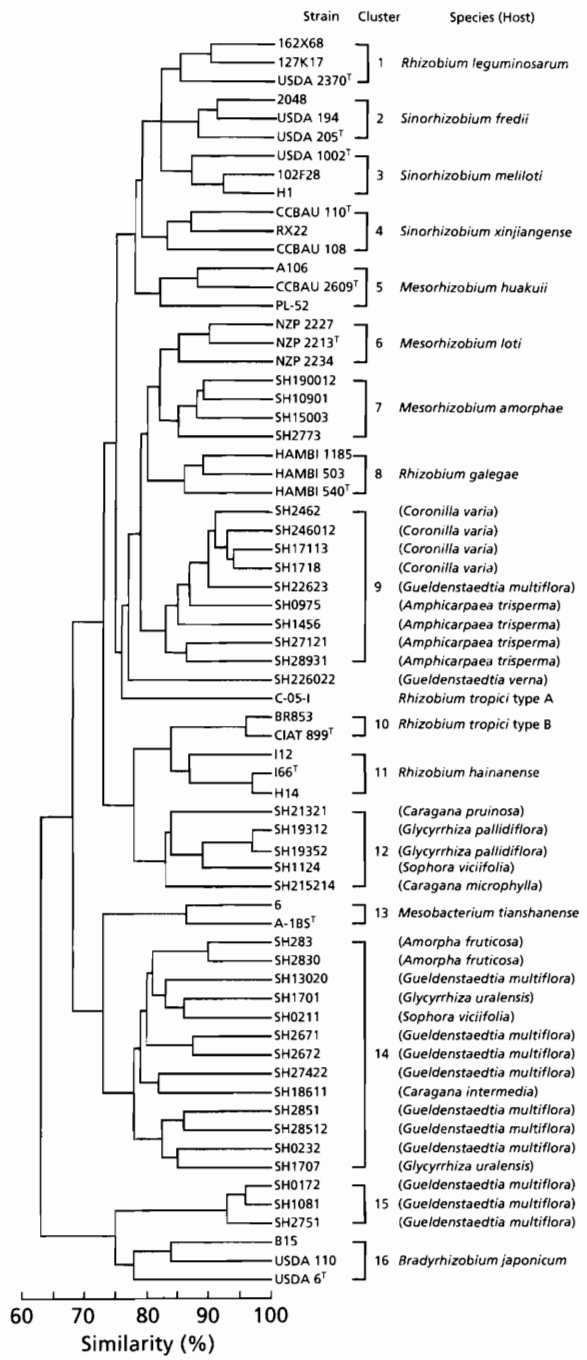

Fig. 1. Dendrogram showing the phenotypic similarities among the isolates and strains. The UPGMA method was used for the cluster analysis (Sneath & Sokal, 1973).

Cluster 9 consisted of nine isolates from *Coronilla varia*, *Gueldenstaedtia multiflora* and *Amphicarpaea trisperma*. These isolates produced acid on YMA and had a generation time of 2·8–3·2 h in PY broth as determined spectrophotometrically (3·2 h for SH22623, 3·0 h for SH1456 and 2·8 h for SH1718). Their colonies looked like those of *Rhizobium legum-*

Table 2. Distinctive features of the new clusters and related species defined in numerical taxonomy

Strains: 1, *Rhizobium leguminosarum* ($n = 3$); 2, *Rhizobium galegae* ($n = 2$); 3, cluster 9 ($n = 9$); 4, *Mesorhizobium tianshanense* ($n = 2$); 5, *Mesorhizobium loti* ($n = 3$); 6, cluster 7 ($n = 4$); 7, cluster 14 ($n = 13$); 8, cluster 15 ($n = 3$); 9, cluster 12 ($n = 5$). The values given in the table are the percentage of positive strains; +, all strains positive; −, all strains negative. All the strains in the clusters or species listed in this table could not use ferulaic acid, glycogen, starch, vanillic acid, threonine, L-phenylalanine, D-tryptophan or L-tryptophan as sole carbon source. No strains could use D-tryptophan or D-valine as sole nitrogen source. All strains were sensitive to the following antibiotics (μg ml^{-1}): aureomycin (50 and 100), chloramphenicol (300), doxycycline (50 and 100), gentamicin (50 and 100), neomycin (100), terramycin (100 and 300), tetracycline (5–300). No strains could grow in medium with Bromocresol purple (0·1 %), Methylene red (0·1 %) or NaCl (3·5 %). No peptonization was observed in Litmus milk.

Characteristic	1	2	3	4	5	6	7	8	9
Utilization as sole carbon source:									
Amygdalin	+	50	−	−	−	−	−	−	−
Calcium malonate	+	+	−	−	−	−	−	+	−
Dextrin	−	−	−	−	−	−	−	+	−
Dulcitol	+	+	−	−	−	25	14	+	80
D-Fucose	−	−	−	−	−	−	−	67	−
Inositol	+	+	+	−	+	75	50	67	+
Inulin	−	−	−	−	−	−	−	67	−
D-Melezitose monohydrate	−	−	−	−	−	25	−	+	20
D-Ribose	+	−	−	−	−	−	−	−	20
Salicin	+	+	−	−	−	50	−	67	40
Sodium acetate	+	+	−	−	−	−	−	+	−
Sodium oxalate	−	−	−	−	−	−	−	+	−
D-Sorbitol	+	+	+	+	+	+	−	+	+
Xylose	+	−	+	+	−	25	−	−	40
L-Alanine	+	+	+	−	−	−	−	−	−
L-Glutamic acid	+	+	77	−	−	50	36	−	60
L-Histidine	+	+	67	−	−	+	−	−	+
Utilization as sole nitrogen source:									
L-Cystine	+	+	+	+	+	+	14	+	+
Glycine	−	−	−	50	+	75	14	+	80
D-Methionine	−	−	−	−	33	−	−	+	20
L-Methionine	+	−	−	+	+	+	50	+	+
D-Serine	−	−	23	−	−	−	21	+	80
L-Tyrosine	+	−	67	+	+	+	21	+	+
Urea	+	+	+	+	67	50	50	+	80
Antibiotic resistance (μg ml^{-1}):									
Bacitracin (300)	+	+	+	+	+	+	36	−	+
Chloramphenicol (100)	33	−	−	+	−	+	33	−	+
Erythromycin (300)	−	+	−	−	−	−	36	−	+
Penicillin G (50)	33	−	44	+	+	+	+	−	+
Polymixin E (100)	33	50	33	−	33	−	−	−	+
Streptomycin (50)	33	−	33	50	+	−	+	−	+
Chemical tolerance (%):									
Giemsa's stain (0·1)	67	50	−	+	67	+	+	33	+
Erythrosin B (0·1)	+	+	+	−	67	+	86	−	+
NaCl (2·5)	−	−	−	−	33	−	71	−	80
Growth at 40 °C	67	50	67	50	33	−	71	−	+
Enzyme activity									
Catalase	+	+	+	−	+	50	50	33	+
Oxidase	+	−	−	−	−	25	43	−	+
Litmus milk alkali production	+	+	+	−	33	25	−	67	20
Litmus milk reduction	−	−	−	+	−	50	−	−	40

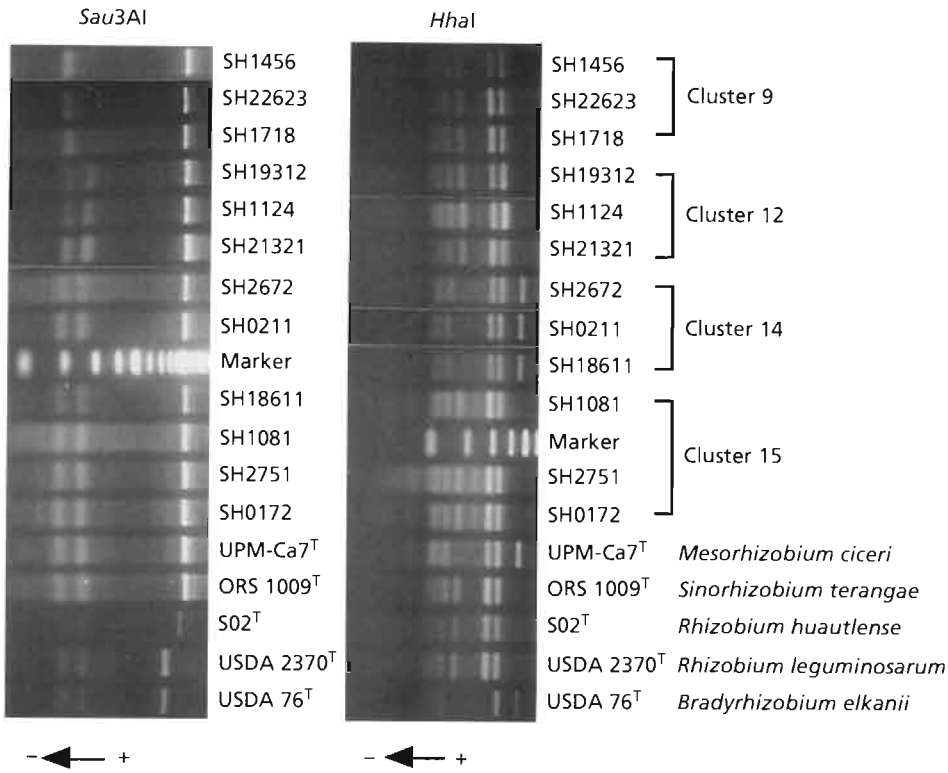

Fig. 2. Examples of RFLP of PCR-amplified 16S rRNA genes separated by electrophoresis in 3% (w/v) agarose gels. The marker is DNA molecular mass marker XIV (100 bp ladder) from Boehringer Mannheim; the smallest band of the marker is 100 bp.

inosarum and they produced a lot of polysaccharides on YMA medium. Most of the isolates in this cluster did not grow in LB (Yanagi & Yamasato, 1993), with the exception of SH27121 and SH28931 which did grow in LB medium.

Cluster 12 contained five isolates which failed to nodulate their original hosts *Caragana pruinosa*, *Caragana microphylla*, *Glycyrrhiza pallidiflora* and *Sophora viciifolia*. They were fast-growing, acid-producing bacteria with generation time of 2 h (SH19312, SH1124 and SH21321) in YM broth as determined spectrophotometrically. Their colonies were transparent with a white centre. All grew in LB medium.

Cluster 14 comprised 13 moderately slow-growing isolates from *Amorpha fruticosa*, *Gueldenstaedtia multiflora*, *Glycyrrhiza uralensis*, *Sophora viciifolia* and *Caragana intermedia*. They produced acid on YMA medium and formed 1-mm-diameter transparent colonies after 7 d incubation. Their generation time was 5·6–6·5 h, estimated by the plate-counting method. The generation time of isolate SH2672 (6·0 h), which is representative of this cluster, was also determined by the spectrophotometric method. No growth in LB was observed from these isolates.

Cluster 15 consisted of three slow- or moderately slow-growing isolates from *Gueldenstaedtia multiflora*. The generation time of these isolates was 8·0–8·3 h, estimated spectrophotometrically in PY broth. They formed colonies of less than 1 mm in diameter on YMA plates within 7 d. No apparent acid or alkali production was observed on YMA after 7 d. Acid production occurred only after 2 weeks. They did not grow in LB. All three isolates produced water-soluble brown pigments on PY agar medium after 7 d.

Phenotypic characteristics

Of the 212 features tested, all the isolates and reference strains were unable to grow in media containing sodium formate, sodium salicylate, D-glutamic acid, L-methionine, β-alanine or L-threonine as sole carbon source; however, they were all able to grow on mannitol as sole carbon source. All of them could use L-alanine, L-glutamic acid and L-proline as sole nitrogen source. All tested isolates and strains were sensitive to doxycycline, gentamicin and neomycin (all at 300 μg ml^{-1}), and to 0·1% bromothymol blue and gentian violet, but resistant to bacitracin (5, 50 and 100 μg ml^{-1}) and erythromycin (5 and 50 μg ml^{-1}). Growth of all isolates was inhibited at pH 3·0 and by 5·0% (w/v) NaCl on YMA, but all grew at pH 5·0.

The distinctive phenotypic features of nine related clusters, including *Rhizobium leguminosarum*, *Rhizobium galegae*, *Mesorhizobium loti*, *Mesorhizobium tianshanense*, clusters 7, 9, 12, 14 and 15, are presented in Table 2.

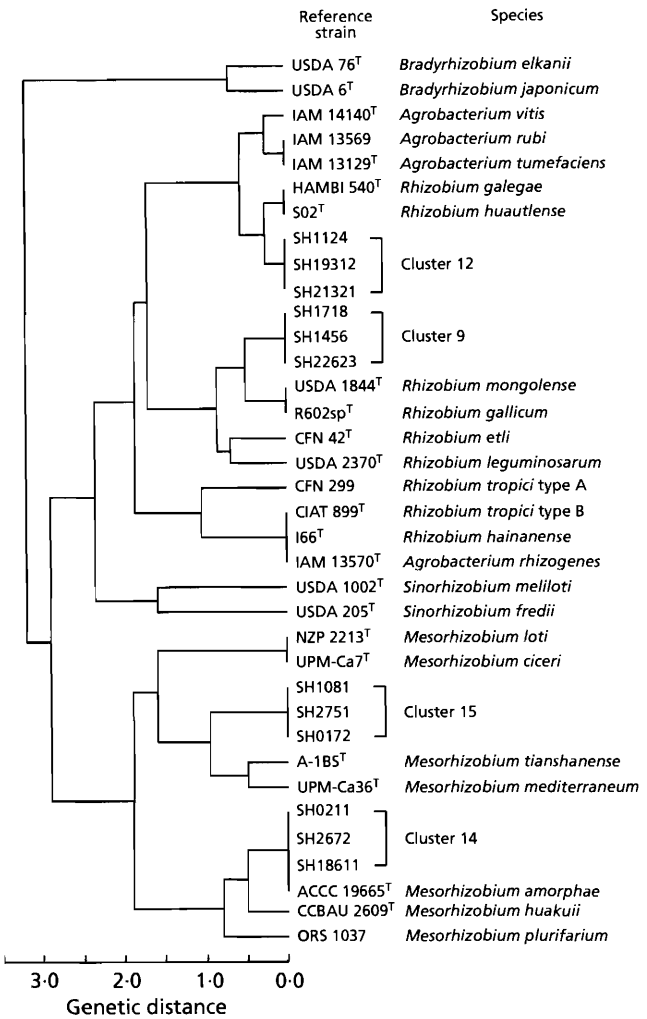

Fig. 3. Dendrogram showing the preliminary phylogenies of the new groups obtained from PCR-RFLP data of 16S rRNA genes. Genetic distances between each strain pair were estimated from the proportion of shared bands in the restriction patterns of *Msp*I, *Hin*fI, *Hha*I and *Sau*3AI, separated by electrophoresis in 3% (w/v) agarose gels and were used for cluster analysis using the Nei & Li (1979) method.

16S rDNA PCR-RFLP and sequence analysis

PCR-RFLP analysis was performed for representative isolates of the new clusters and for the reference strains of described species to estimate the validity of the grouping results in numerical taxonomy and the phylogenetic relationships of the new clusters. Some PCR-RFLP patterns of the isolates are shown as examples in Fig. 2. The representative isolates within each cluster showed the same patterns, but different patterns were observed among different clusters. Clusters 9, 12 and 15 had distinct patterns from all the described species and cluster 14 shared the same patterns as *Mesorhizobium amorphae* ACCC 19665^T (not shown). The phylogenetic relationships of these new clusters, estimated from RFLP results, are shown in Fig. 3. As reported previously (Laguerre *et al.*, 1994; Wang *et al.*, 1999b), the relationships among the described species (in Fig. 3) were similar to those based on analysis of 16S rRNA gene sequence (such as Amarger *et al.*, 1997; van Berkum *et al.*, 1998; Sawada *et al.*, 1993; Willems & Collins, 1993; Yanagi & Yamasato *et al.*, 1993). Cluster 9 was closely related to *Rhizobium gallicum* and *Rhizobium mongolense*. Cluster 12 was closely linked to *Rhizobium huautlense* and *Rhizobium galegae*. Cluster 14 had a close relationship with *Mesorhizobium amorphae* and cluster 15 was closely related to *Mesorhizobium tianshanense* and *Mesorhizobium mediterraneum*.

The phylogenetic relationships estimated from 16S rRNA gene PCR-RFLP data were also confirmed by the comparison of partial sequence data of this gene. The partial sequences for isolates SH22623 (cluster 9) and SH19312 (cluster 12) were determined from two clones for each and identical sequences were obtained from the two cloned fragments of each isolate. The partial sequence similarities between the representative strains, SH22623 and SH19312, and the known species were calculated. The results showed that SH22623 has only 1 base difference from both of the type strains of *Rhizobium gallicum* and *Rhizobium mongolense* (corresponding to 99·7% similarity). The sequence of SH19312 was closely related to that of *Agrobacterium tumefaciens* (99·0% similarity corresponding to 3 base differences).

DNA base composition and DNA–DNA hybridization

These experiments were designed according to the clustering results in numerical taxonomy and the phylogenetic relationships defined in 16S rRNA gene analyses. The results of DNA G+C content estimation and DNA hybridization are shown in Table 3. The DNA G+C content for all isolates was 59–63 mol%, which is in the range for *Rhizobium* (Jordan, 1984). High DNA homologies were obtained with both the hybridization methods from the isolates within cluster 9 (72·3–88·1%; mean of 78·4%), cluster 12 (72·4–100·0%; mean of 84·5%), cluster 14 (72·0–92·5%; mean of 77·3%) and cluster 15 (72·8–100%), indicating that they were genomic species.

The DNA homologies between the strains within cluster 7 and the type strain of *Mesorhizobium amorphae*, ACCC 19665^T, were 77·5–86·2%, which indicates that the cluster 7 isolates belong to *Mesorhizobium amorphae* (Wang *et al.*, 1999a).

Low DNA homologies (4·7–27·5%) were obtained between isolate SH22623, the representative of cluster 9, and most reference strains for the fast-growing rhizobia species and other clusters, which indicates that cluster 9 is a genomic group.

DNA homologies between the representative isolate of cluster 12 (SH19312) and the type strains of rhizobial species, including *Agrobacterium rhizogenes* which is closely related to *Rhizobium tropici* in phylogeny, were 4·4–28·0% (Table 3). High homologies were observed

between SH19312 and the reference strains of *Agrobacterium radiobacter* IAM 12048^T (72·6%) and *Agrobacterium tumefaciens* IAM 13129^T (86·2%). These results indicated that cluster 12 was a group belonging to *Agrobacterium tumefaciens* genomic species. It has been reported that *Agrobacterium tumefaciens* and *Agrobacterium radiobacter* are a single species based on their high DNA homology and the specific name *Agrobacterium tumefaciens* has remained after a controversial discussion (Bouzar, 1994; Sawada *et al.*, 1993).

The DNA homologies among the representative strains SH2672 for cluster 14 and strain SH0172 for cluster 15 and the reference strains for all the *Mesorhizobium* species and some species in other genera were lower than 34·2%, indicating that these two clusters were unique genomic species.

Hybridization of *nodDAB* and *nifH* genes

The *nod* and *nif* genes in rhizobia are responsible for host specificity and symbiotic nitrogen fixation and the reiteration of these genes are also characters for the species (Amarger *et al.*, 1997; Martínez *et al.*, 1987). The hybridization patterns of *Eco*RI-digested total DNA to *nifH* and *nodDAB* genes are shown in Fig. 4. Three isolates for cluster 12, as well as an *Agrobacterium* strain did not show hybridization signals with either of the probes. The representative isolates from different hosts in clusters 9 or 14 had different *Eco*RI (Fig. 4) and *Bam*HI restriction patterns of *nodDAB* with 2–4 bands (not shown). The three isolates of cluster 15 were from the same host species and they had the same *Eco*RI or *Bam*HI restriction patterns of *nod* genes (2 bands). The patterns obtained from *Mesorhizobium amorphae* ACCC 19665^T were different from those of clusters 14 and 15. Only one *nifH* hybridization band was observed from the isolates and strains analysed by *Eco*RI or *Bam*HI digestion. The sizes of the bands were the same in *Eco*RI digestion among isolates in each clusters, but different in *Bam*HI digestion (not shown).

Symbiotic properties

Host range is a requested event for the description or identification of the rhizobial species (Graham *et al.*, 1991). The isolates of cluster 9 (SH22623, SH2462, SH1718, SH1456 and SH27121) could share their host plants. All of them nodulated *Coronilla varia*, *Gueldenstaedtia multiflora* and *Amphicarpaea trisperma*, as well as *Phaseolus vulgaris*, but not *Leucaena leucocephala*. Strain SH22623 also nodulated *Glycyrrhiza pallidiflora*. The nodules of *Phaseolus vulgaris* formed by these isolates decayed very rapidly since most of the mature nodules were white or green. The isolates of cluster 12 (SH19312, SH1124, SH215214 and SH21321) did not nodulate any of *Caragana pruinosa*, *Caragana microphylla*, *Glycyrrhiza pallidiflora*, *Sophora viciifolia*, *Phaseolus vulgaris* or *Leucaena leucocephala*.

DISCUSSION

In this research, we characterized 35 nodule isolates from 11 temperate legume species in north-western China with a polyphasic approach including numerical taxonomy, 16S rDNA PCR-RFLP and sequence analysis, DNA–DNA hybridization, restriction patterns of *nodDAB* and *nifH* genes, and symbiotic properties. The grouping results from different methods generally correlated with each other. The new isolates were grouped into five clusters, 7, 9, 12, 14 and 15. They were also genomic species, as defined by the high DNA homologies among the isolates within each cluster. The taxonomic positions of these clusters were estimated from the 16S rRNA gene comparison by PCR-based RFLP and by the DNA–DNA hybridization to the reference strains for described species.

In current bacterial taxonomy, a polyphasic approach (Vandamme *et al.*, 1996) has been emphasized (Graham *et al.*, 1991) and used (Amarger *et al.*, 1997; de Lajudie *et al.*, 1994, 1998a, 1998b) for the description of both generic and specific taxa since no single method can produce perfect classification. In our previous studies, the species clusters were generally defined around the similarity of 80% in numerical taxonomy and they were always supported by DNA homology data (Chen *et al.*, 1991, 1995; Gao *et al.*, 1994; Wang & Chen, 1996). In our research, clusters 13, 14, 15 and 16 were separated at the similarity level of 78%; they grew slowly or moderately slowly. Clusters 1–12 were separated at the similarity level of 84%; they were fast-growing rhizobia or agrobacteria. The meaning of this definition has been supported by further characterization, including PCR-RFLP of 16S rRNA genes and DNA hybridization. The different similarity levels used to define the clusters in numerical taxonomy were reported for cluster analyses of auxanographic characteristics, protein patterns and repetitive extragenic palindrome PCR patterns (Amarger *et al.*, 1997; de Lajudie *et al.*, 1994, 1998a, 1998b).

The five isolates in cluster 12 were identified as *Agrobacterium tumefaciens* based upon their high DNA homologies and other results as well. Their taxonomic position was also confirmed in our previous study (Sui *et al.*, 1998). In a characterization of *Agrobacterium* isolates from China, two representatives for cluster 12 (SH19312 and SH19352) had 91% similarity to *Agrobacterium tumefaciens* strain C58 and more than 83% similarity to 13 other isolates in this species in terms of numerical taxonomy (Sui *et al.*, 1998). The DNA homology of SH19312 to the type strains *Agrobacterium tumefaciens* IAM 13129^T and *Agrobacterium radiobacter* IAM 12048^T was 81·5± 0·1%, estimated by the spectrophotometric method. These five strains were isolated from root nodules of the shrub or semi-shrubby species *Caragana* spp., *Glycyrrhiza pallidiflora* and *Sophora viciifolia*. However, their nodulation abilities were not confirmed by either the nodulation tests or symbiotic gene hy-

Table 3. DNA homologies (%) among the isolates and defined species

Species or cluster	DNA G+C (mol%; T_m)	Cluster 7 (SH190012)	Cluster 9 (SH22623)	Cluster 12 (SH19312)	Cluster 14 (SH2672)	Cluster 15 (SH0172)
Cluster 7 (*Mesorhizobium amorphae*)						
SH190012	62	100*	24·5*	28·0*	21·2*	20·2*
SH109	60	77·5*				
SH1503	62	78·2*	17·5±0·1	22·8±7·2		
SH2773	61	86·2*				
Cluster 9 (*Rhizobium* sp.)						
SH22623	62	24·5*	100*	4·7±0·6	4·4±0·1	3·2±1·1
SH1456	62		82·4±0·8			
SH1718	60		83·8±13·0			
SH2462	62		88·1*			
SH246012	62		72·5*			
SH17113	59		76·0*			
SH0975	60		81·4*			
SH27121	59		74·2*			
SH28931	61		72·3*			
Cluster 12 (*Rhizobium* sp.)						
SH19312	60	28·0*	4·7±0·6	100*	15·1*	18·6*
SH1124	62			100*		
SH21321	63			81·3±1·2		
SH19352	60			81·1*		
SH215214	62			72·4*		
Cluster 14 (*Mesorhizobium* sp.)						
SH2672	60	21·2*	4·4±1·0	15·1*	100*	14·9±3·1
SH0211	62				80·0±2·0	
SH18611	63				79·6±1·5	
SH13020	60				84·8*	
SH1701	63				78·3*	
SH2671	61				87·0*	
SH27422	61				72·0*	
SH2851	61				72·1*	
SH28512	60				72·0*	
SH0232	60				92·5*	
SH283	60				76·6*	
SH2830	61				79·3*	
SH1707	61				72·7*	
Cluster 15 (*Mesorhizobium* sp.)						
SH0172	ND	20·2*	3·2±1·1	18·6*	14·9±3·1	100*
SH2751	ND					72·8±1·0
SH1081	ND					100*
Agrobacterium caulinodans ORS 571				18·8±3·6		
Agrobacterium rhizogenes IAM 13570^T			5·7±0·1	9·8±1·0		
Agrobacterium radiobacter IAM 12048^T				72·6±2·6		
Agrobacterium tumefaciens IAM 13129^T			5·0±0·8	86·2±9·4		
Agrobacterium rubi IAM 13569^T			7·9±0·3	35·4*	4·8±0·6	
Agrobacterium vitis HAMBI 1817			5·0±0	11·8±0·8		
Bradyrhizobium elkanii USDA 76^T					2·9±0·4	4·5±0·7
Bradyrhizobium japonicum USDA 6^T				4·3±0·7	7·2±0·1	8·5±2·0
Mesorhizobium loti NZP 2213^T				6·8±1·1	13·4±0·8	34·2±3·6
Mesorhizobium mediterraneum UPM-Ca36^T					19·4±3·8	40·0±0·0
Mesorhizobium tianshanense A-1BS^T					16·8±1·0	17·6±2·8
Mesorhizobium ciceri UPM-Ca7^T					23·9±0·3	31·6±1·0
Mesorhizobium huakuii CCBAU 2609^T					15·2±4·4	9·8±0·6
Mesorhizobium amorphae ACCC 19665^T		85·2±3·2			21·2±4·2	13·6±3·5

Table 3 *(cont.)*

Species or cluster	DNA G+C (mol%; T_m)	Cluster 7 (SH190012)	Cluster 9 (SH22623)	Cluster 12 (SH19312)	Cluster 14 (SH2672)	Cluster 15 (SH0172)
Mesorhizobium sp. HL56					22·7±5·0	21·6±7·2
Mesorhizobium plurifarium ORS 1032^T					9·6±0·3	11·2±3·0
Rhizobium leguminosarum USDA 2370^T			9·8±1·2	9·4±1·6		
Rhizobium etli CFN 42^T			17·6±2·2	12·8±2·7		
Rhizobium galegae HAMBI 540^T			11·8±0·6	7·2±1·2	10·3±0·5	15·2±0·5
Rhizobium galegae 59A2				4·4±1·1		
Rhizobium giardinii H152^T			7·2±0·0	5·2±1·6	6·9±1·7	
Rhizobium hainanense I66^T			7·7±0·1		7·5±0·7	
Rhizobium huautlense S02^T			9·4±0·0	9·7±2·4		
Rhizobium mongolense USDA 1844^T			27·5±0·9	8·2±0·7		
Rhizobium tropici CIAT 899^T				12·2±1·5	2·9±0·1	
Sinorhizobium fredii USDA 205^T					7·7±0·3	
Sinorhizobium meliloti USDA 1002^T			18·1±0·1	6·3±1·0	7.0±0·0	7·0±2·6

ND, Not done; ^{T}, type strain.

*Values were obtained by spectrophotometric methods; these are without variation. Other values (mean value of the homologies ± variation estimated from at least two hybridizations) were obtained from filter hybridization experiments performed under highly stringent conditions.

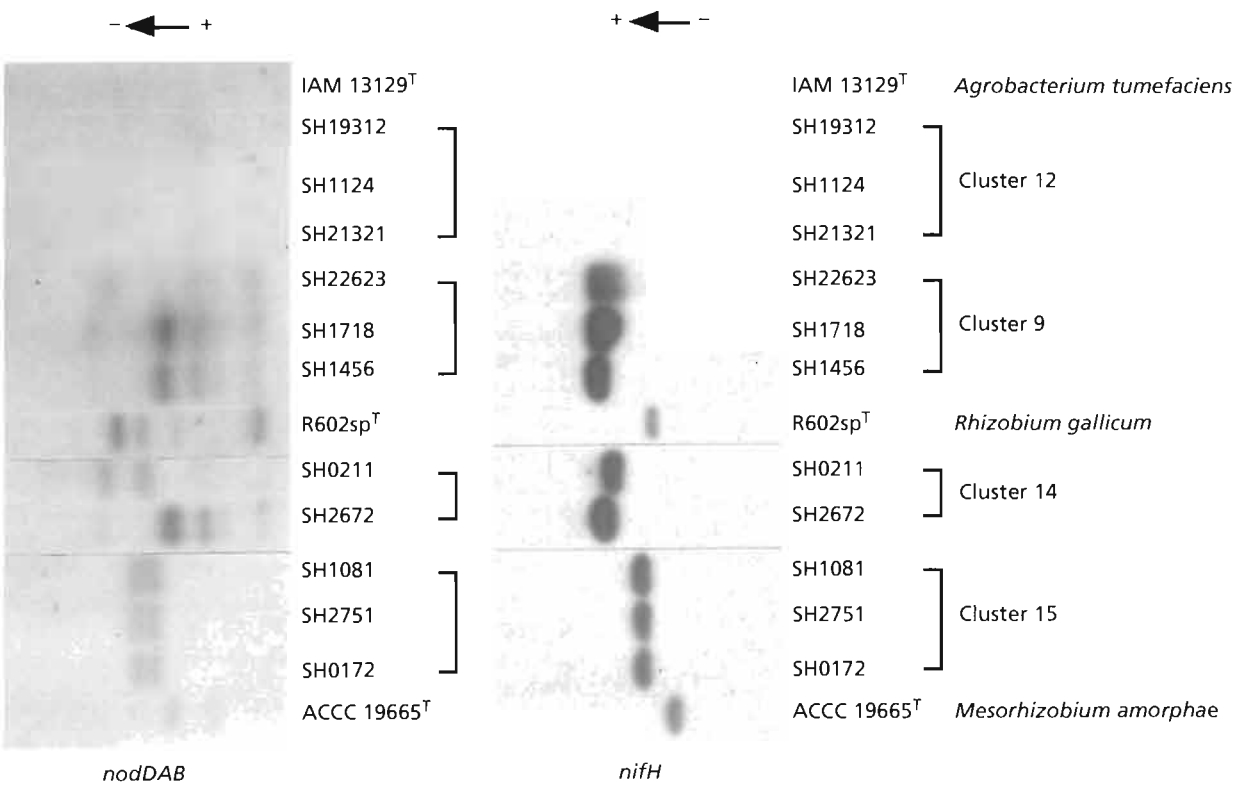

Fig. 4. Hybridization patterns of total DNA digested with *Eco*RI to *nifH* from *Sinorhizobium meliloti* USDA 1002^T or *nodDAB* from *Rhizobium tropici* CFN 299. DNA fragments were separated by electrophoresis in 0·7% agarose gels. Hybridization was performed under highly stringent conditions at 65 °C.

bridization. We estimated that they might form or only enter the nodules by chance in nature since Imshenetskii *et al.* (1976) reported an *Agrobacterium tumefaciens* strain that could form ineffective root nodules in 50% of inoculated lucerne plants.

The genus *Mesorhizobium* recently was separated from the genus *Rhizobium* based on the similarity of 16S rRNA gene sequences, slow or moderately slow growth rates and acid production on YMA (Jarvis *et al.*, 1997). The species within this genus have very

similar 16S rRNA gene sequences (>97·6 % similarity) and the strains within a single species, such as *Mesorhizobium loti*, may have 16S rRNA genes as divergent as those among different species (de Lajudie *et al.*, 1998b). Clusters 14 and 15 were unique genomic species within the genus *Mesorhizobium* based upon their slow or moderately slow growth rate, acid production on YMA and their high similarities of 16S rRNA gene PCR-RFLP patterns with those of *Mesorhizobium* species. Further studies for these two clusters will be done to clarify their specific positions.

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