Mesorhizobium septentrionale sp. nov. and *Mesorhizobium temperatum* sp. nov., isolated from *Astragalus adsurgens* growing in the northern regions of China

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Ninety-five rhizobial strains isolated from *Astragalus adsurgens* growing in the northern regions of China were classified into three main groups, candidate species I, II and III, based on a polyphasic approach. Comparative analysis of full-length 16S rRNA gene sequences of representative strains showed that candidate species I and II were *Mesorhizobium*, while candidate species III, which consisted of non-nodulating strains, was closely related to *Agrobacterium tumefaciens*. The phylogenetic relationships of the three candidate species and some related strains were also confirmed by the sequencing of *glnA* genes, which were used as an alternative chromosomal marker. The DNA–DNA relatedness was between 11·3 and 47·1 % among representative strains of candidate species I and II and the type strains of defined *Mesorhizobium* species. Candidate III had DNA relatedness of between 4·3 and 25·2 % with type strains of *Agrobacterium tumefaciens* and *Agrobacterium rubi*. Two novel species are proposed to accommodate candidate species I and II, *Mesorhizobium septentrionale* sp. nov. (type strain, SDW014^T = CCBAU 11014^T = HAMBI 2582^T) and *Mesorhizobium temperatum* sp. nov. (type strain, SDW018^T = CCBAU 11018^T = HAMBI 2583^T), respectively. At least two distinct *nodA* sequences were identified among the strains. The numerically dominant *nodA* sequence type was most similar to that from the *Mesorhizobium tianshanense* type strain and was identified in strains belonging to the two novel species as well as other, as yet, undefined genome types. Host range studies indicate that the different *nodA* sequences correlate with different host ranges. Further comparative studies with the defined *Agrobacterium* species are needed to clarify the taxonomic identity of candidate species III.

Published online ahead of print on 14 May 2004 as DOI 10.1099/ijs.0.02840-0.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences obtained in this work are AF508207, AF508208 and AF508209 for strains SDW014^T, SDW018^T and SDW052, respectively. Those for the partial *glnA* gene sequences determined in this study are AJ579875–AJ579885.

A phylogenetic tree is available as supplementary material in IJSEM Online.

INTRODUCTION

Plants within the family Leguminosae are agriculturally important and are planted worldwide for many purposes, including food and fodder production, as green manures, as soil coverage to reduce erosion, as herbal medicines and in gardening/landscaping. It has been shown that different plants in the same geographical region can share the same rhizobial group for nodulation; examples are *Mesorhizobium tianshanense* (Chen *et al.*, 1995), *Mesorhizobium plurifarium*

(de Lajudie *et al.*, 1998) and *Rhizobium hainanense* (Chen *et al.*, 1997). Conversely, the same plant species may associate with different rhizobial species; for example, soybean is nodulated by *Bradyrhizobium japonicum*, *Bradyrhizobium liaoningense* (Xu *et al.*, 1995), *Mesorhizobium tianshanense* (Chen *et al.*, 1995), *Sinorhizobium fredii* and *Sinorhizobium xinjiangense* (Chen *et al.*, 1988; Peng *et al.*, 2002) in different regions of China. However, the geographical ranges of host plants and microsymbionts are often restricted and rhizobial inocula are necessary in many cases when legumes are introduced into new areas (e.g. Sullivan *et al.*, 1996). Considering the specificity of the symbiosis between rhizobia and their host plants and the adaptation of these bacteria to their environments, characterization of local isolates is important to explore these microbial resources and optimize legume use.

The genus *Astragalus*, consisting of 1500–2000 species, is one of the largest genera in the family Leguminosae and many species form nitrogen-fixing symbioses in association with root-nodule bacteria (Allen & Allen, 1981). More than 70 species within this genus have been recorded in China and they have many economic uses; for example, in herbal medicine, as resources for honey production, as green manure (Li *et al.*, 2000; Nagasawa *et al.*, 2001; Shon *et al.*, 2002) and for bioremediation (Sriprang *et al.*, 2002). Different rhizobial groups have been identified among the nodule isolates from *Astragalus* species grown in China and other countries (Guo *et al.*, 1999; Laguerre *et al.*, 1997; Wang & Chen, 1996; Wdowiak & Malek, 2000). This lack of specificity is not universal: *Astragalus sinicus*, an important green manure in the southern regions of China, has been reported to nodulate almost exclusively with *Mesorhizobium huakuii* (Chen *et al.*, 1991; Zhang *et al.*, 2000). Among the *Astragalus* species used in China, *Astragalus adsurgens* has been planted over a vast area in desert and very dry regions to protect soils from wind erosion. In light of the important role of *Astragalus adsurgens* in improving the environment in the northern regions of China, we previously isolated rhizobia from nodules of this plant and characterized them using genetic methods, including rep-PCR fingerprinting, AFLP fingerprinting, and 16S rRNA and 23S rRNA PCR-RFLP (Gao *et al.*, 2001). This earlier work indicates that *Astragalus adsurgens* is nodulated by diverse rhizobial populations, including three dominant genomic groups (Gao *et al.*, 2001). We have further characterized these strains using phenotypic analysis, DNA–DNA hybridization and sequencing of 16S rRNA, glutamine synthetase (*glnA*) and the symbiosis-associated *nodA* gene to explore, and better understand, the diversity and systematic relationships of the rhizobia associated with *Astragalus adsurgens*. The results indicate that two of the three dominant genomic groups are novel species of *Mesorhizobium* and the third is a non-symbiotic group related to *Agrobacterium* species.

METHODS

Bacterial strains. Strains used in this study are listed in Table 1. The 95 rhizobial strains from *Astragalus adsurgens* were obtained as part of an earlier study (Gao *et al.*, 2001). Some reference strains representing the defined species or unnamed groups in *Rhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Agrobacterium* and *Escherichia coli K-12* were included in different analyses. All the strains were kept in 20 % (v/v) glycerol at −20 °C and cultured in YMA medium (Vincent, 1970) at 28 °C.

Phenotypic characterization and numerical taxonomy. The 95 isolates from *Astragalus adsurgens* and 11 reference strains for related species were analysed using a total of 133 phenotypic characteristics as described previously (Gao *et al.*, 1994). The Ssm coefficient and UPGMA (Sneath & Sokal, 1973) were used for clustering analysis of phenotypic features.

Sequencing and phylogenetic analysis of 16S rRNA, *glnA* and *nodA* genes. To clarify the phylogenetic relationships, three strains, SDW014^T, SDW018^T and SDW052, representing clusters 2, 11 and 6 (Table 1) identified by numerical taxonomy were used for sequence analyses of the 16S rRNA gene. These three strains and some others were used in the sequence analyses of *glnA* and *nodA* genes to further check the phylogenetic relationships and the diversity. The full-length 16S rRNA genes were PCR-amplified and sequenced directly as described by Hurek *et al.* (1997). Internal gene fragments (495 bp) of glutamine synthetase I (*glnA*) were amplified and sequenced directly using the PCR primers and methods described by Turner & Young (2000). Internal *nodA* gene sequences (470–476 bp) were amplified and sequenced directly using the PCR primers and methods of Zhang *et al.* (2000). The newly acquired sequences were aligned with other rhizobial sequences in the databases for the 16S rRNA, *glnA* or *nodA* gene using CLUSTAL_X (Thompson *et al.*, 1997), which was also used to construct and bootstrap (1000 replicates) the corresponding phylogenetic trees. All trees were visualized using TreeView (Page, 1996).

DNA base composition and DNA–DNA hybridization. Total DNA was extracted from each strain using the method of Marmur (1961). The G+C content of DNA was measured using the thermal denaturation method of Marmur & Doty (1962) and *E. coli K-12* as standard. DNA homology was determined using the spectrophotometric method of De Ley *et al.* (1970).

Symbiotic properties. Strains SDW014^T and SDW018^T representing candidate species I and II (Table 1), respectively, were used for cross-nodulation tests with 11 leguminous plant species: *Pisum sativum*, *Phaseolus vulgaris*, *Vigna unguiculata*, *Glycine max*, *Leucaena leucocephala*, *Macroptilium atropurpureum*, *Galega officinalis*, *Astragalus sinicus*, *Medicago sativa*, *Trifolium repens* and *Lotus corniculatus*. Seed treatment and inoculation were performed using the standard method of Vincent (1970).

RESULTS AND DISCUSSION

A polyphasic approach has been suggested to accurately define taxonomic groups among rhizobia (de Lajudie *et al.*, 1998). Of the available methods, 16S rRNA gene sequencing is the most reliable for genus identification. Species identification is normally based upon both genetic or genomic comparison and phenotypic characterization. Genetic distance of 0·5 in multilocus enzyme electrophoresis (Martínez-Romero *et al.*, 1991; Wang *et al.*, 1999), 70 % DNA–DNA relatedness in DNA hybridization (Graham *et al.*, 1991; Wayne *et al.*, 1987) and 80 % similarity in numerical taxonomy (Chen *et al.*, 1991; Gao *et al.*, 1994) have been used or suggested as reference borders for species differentiation. These values are used in relevant analyses in this work.

Table 1. Strains used in this study

Rhizobial strains isolated from *Astragalus adsurgens*, reference strains and their grouping results in numerical taxonomy and in analyses of molecular biology. The groups of AFLP and rep-PCR and genotypes of 16S and 23S rRNA were defined in a previous work (Gao *et al.*, 2001).

Isolate or strain	Cluster in numerical taxonomy	Group of		Genotype of rRNA	
		AFLP	rep-PCR	16S	23S
Candidate species I (*Mesorhizobium septentrionale*)					
SDW012	2	7	10		
SDW014^T	2	7	10	3	3
SDW033, SDW034, SDW035, SDW036	2	11	2		
SDW073	2	11	2	3	3
SDW048	2	11	6	3	3
SDW020, SDW022, SDW028, SDW030, SDW065, SDW067	2	11	7		
SDW068	2	11	7	3	3
SDW043, SDW047	2	11	9		
SDW044	2	11	9	3	3
SDW032	2	11	13	3	3
SDW037	2	11	13		
SDW021, SDW025, SDW060, SDW061, SDW064	2	11	16		
SDW072	2	11	16	3	3
Candidate species II (*Mesorhizobium temperatum*)					
SDW015	1	7	3		
SDW016	1	7	3	5	5
SDW018^T	11	7	3	5	5
SDW029, SDW039, SDW050, SDW055	11	10	3		
SDW026, SDW038, SDW049	11	10	3	5	5
NM026	11	17	3	5	5
NM300	11	17	3		
Candidate species III (*Agrobacterium* sp.)					
SDW002, SDW003, SDW004, SDW005, SDW011, R084, SDW041, SDW051, SDW053, SDW054, SDW059, SDW063, SDW069, SDW070, SH394, SH395	6	14	31		
SDW007, SDW052	6	14	31	10	12
SDW019	6	15	31	10	12
***Mesorhizobium* spp.**					
SDW006, SDW008, SDW009	1	7	4		
SDW001	1	7	9		
SDW010	1	7	9	4	8
NM159	1	12	19	4	8
SH286	1	29	9	4	8
SDW013	2	7	15		
SDW046	2	1	11	4	7
SDW040	2	2	5	4	7
SDW27	2	28	21	4	7
SDW017	2	9	29	4	4
SDW023, SDW066	3	10	3		
SDW045	Single	31	14	7	9
SH138	4	5	24	5	5
SH152	4	7	6	3	3
SDW071	4	11	2		
SDW074	11	12	6	3	3
***Rhizobium* spp.**					
SDW027	5	7	10	9	11
SDW062	5	12	11	13	15
SX211a	5	21	33	8	10

Table 1. cont.

Isolate or strain	Cluster in numerical taxonomy	Group of		Genotype of rRNA	
		AFLP	rep-PCR	16S	23S
SX211b	5	21	33		
NM179	5	22	36	21	21
SDW058	5	25	30	12	14
SX251a	6	14	38	24	12
NM349	6	16	26	18	12
SDW042	6	16	26		
SDW056	6	23	20	19	20
SDW057	6	23	20		
SDW16	6	7	34	11	13
N211	6	27	7		
SDW024	Single	18	16	22	16
NM170	Single	18	22	17	19
Rhizobium leguminosarum					
NM115	8	3	32	14	16
NM277, NM366	8	3	32		
Reference strains					
Rhizobium sp. (*Astragalus*) CA8593	9	6	1	9	11
Rhizobium sp. (*Astragalus*) CA8561	9	6	1		
Rhizobium sp. (*Astragalus*) X59	9	7	1	20	11
Rhizobium sp. (*Astragalus*) JL84	7	27	34	11	13
Rhizobium sp. (*Astragalus*) SX042	7	27	7	11	13
Rhizobium leguminosarum USDA 2370^T	8	26	12	14	16
Rhizobium leguminosarum 127K17	8	19	8		
Sinorhizobium meliloti USDA 1002^T	12	20	22	17	19
Sinorhizobium meliloti 102F28	12	20	22		
Mesorhizobium huakuii CCBAU 2609^T	10	7	35	23	22
Mesorhizobium huakuii A106	10				

Phenotypic characterization and numerical taxonomy

The results of numerical taxonomy are summarized in Fig. 1 and Table 1. Most of the strains tested are grouped into 12 clusters at a similarity level of 83 %. The others are single strains. Among these, the clusters 8, 10 and 12 correspond to *Rhizobium leguminosarum*, *Mesorhizobium huakuii* and *Sinorhizobium meliloti*, respectively. Clusters 7 and 9 are composed of the reference strains from two unnamed rhizobial groups isolated from *Astragalus* species (Wang & Chen, 1996). Clusters 1 to 6 and 11 consist only of strains from *Astragalus adsurgens*, of which, clusters 2, 6 and 11 are the largest. The 31 strains in cluster 2 and the 11 strains in cluster 11 form single colonies of less than 1 mm in diameter within 7–10 days and produce acid on YMA medium. Cluster 6 contains 26 strains that form single colonies of 2–3 mm in diameter within 3–5 days and produce acid on YMA medium.

It seems that the clusters defined by similarity values of more than 83 % in numerical taxonomy are more conservative than are the groups defined by rep-PCR fingerprinting, AFLP fingerprinting, and 16S rRNA and 23S rRNA PCR-RFLP (Gao *et al.*, 2001) and are largely in good agreement with the rRNA RFLP. All the main clusters based on numerical taxonomy contain strains corresponding to several groups of AFLP or rep-PCR, and to more than one genotype of rRNA. For instance, cluster 2 includes *Mesorhizobium* strains belonging to AFLP groups 7, 11, 1, 2, 28 and 9; rep-PCR groups 10, 15, 2, 6, 7, 9, 13, 16, 11, 5, 21 and 29; and three genotypes of rRNA. Strains in AFLP group 7 (Gao *et al.*, 2001) are found in numerical taxonomy clusters 1, 2, 4, 5, 6, 9, 10 and 11. These results suggest that AFLP and rep-PCR analyses are more valuable for revealing genetic diversity than for estimating taxonomic relationships. In order to clarify further the genetic relationships among strains within the larger numerical taxonomy groups, the full-length 16S rRNA and partial *glnA* gene were sequenced for some strains.

16S rRNA and *glnA* gene sequencing

Full-length (~1500 bp) 16S rRNA genes were sequenced for strains SDW014^T, SDW018^T and SDW052, chosen to represent the three main numerical clusters (2, 11 and 6) (Table 1). The phylogenetic relationships of these sequences

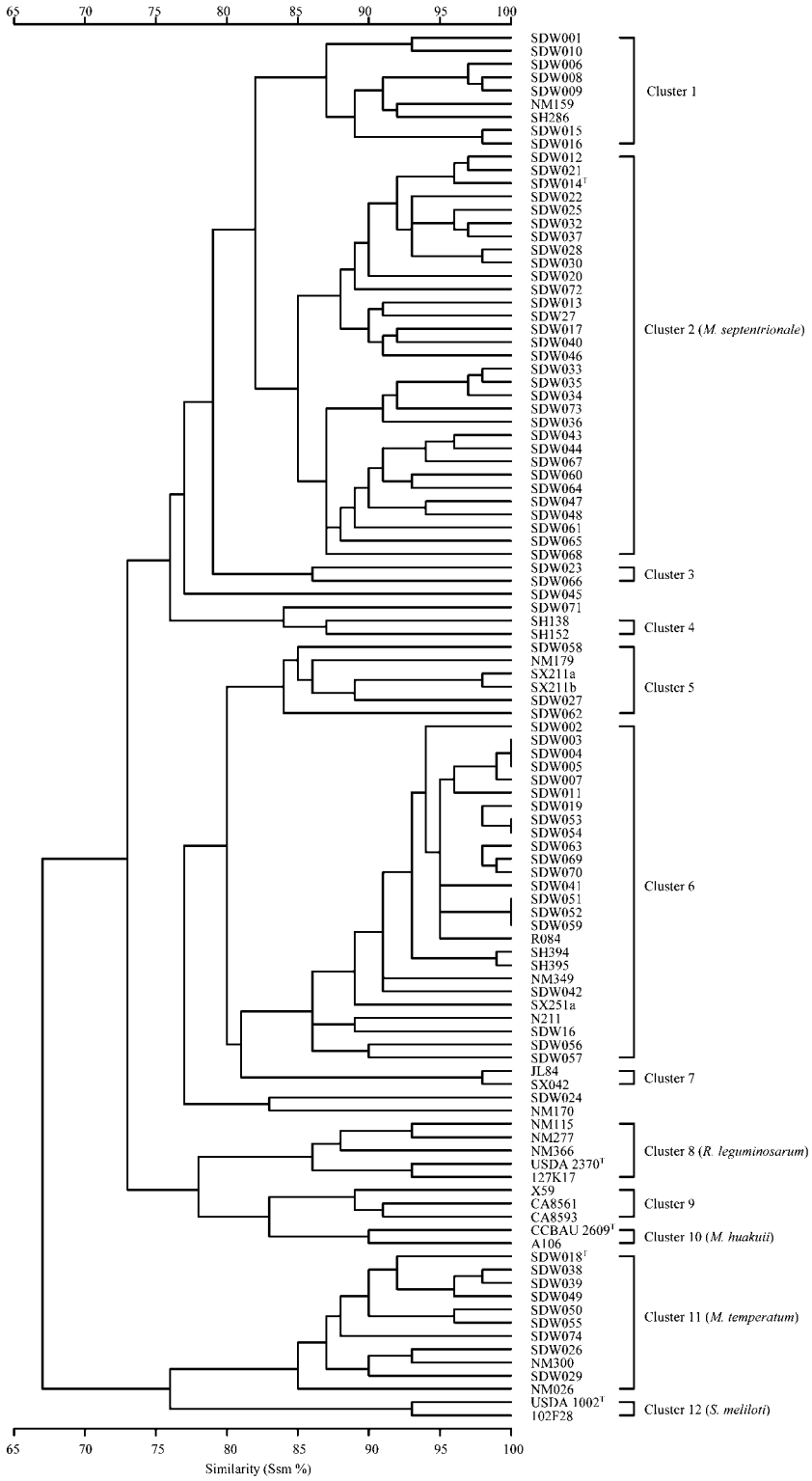

Fig. 1. Dendrogram showing the phenotypic similarities among the rhizobial isolates from *Astragalus adsurgens* in China. Clustering analysis was performed using the UPGMA method (Sneath & Sokal, 1973).

(Fig. 2) are in good agreement with those estimated from the 900 bp partial 16S rRNA gene sequences determined previously (Gao *et al.*, 2001), which clearly showed that strains SDW014^T and SDW018^T are related to *Mesorhizobium* species. Strain SDW014^T shares 99 % sequence identity with *Mesorhizobium amorphae*, *Mesorhizobium huakuii* and *Mesorhizobium plurifarium*, which form a subgroup within the genus *Mesorhizobium*. Strain SDW018^T is closely related to *Mesorhizobium mediterraneum* (99 % sequence identity). Strain SDW052 is related to *Agrobacterium tumefaciens* and *Agrobacterium rubi*, with 99 and 97 % sequence identity, respectively.

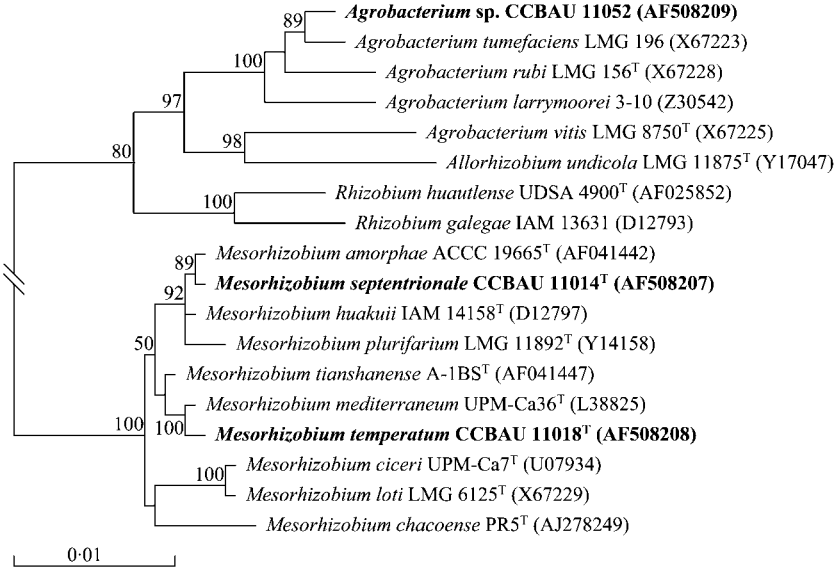

Fig. 2. Simplified phylogenetic tree (complete tree is available in IJSEM Online) showing the phylogenetic relationships among *Mesorhizobium septentrionale*, *Mesorhizobium temperatum* and the *Agrobacterium* group identified in this work and some related bacteria in the *α-Proteobacteria*. The tree was constructed from full-length sequences of the 16S rRNA genes using the neighbour-joining method in the CLUSTAL_X package (Thompson *et al.*, 1997). Bootstrap values (1000 replicates) greater than 60 % are shown on the appropriate nodes. Bar, 0·01 % nucleotide divergence.

Recent studies have used other core metabolism genes to investigate the reliability of 16S rRNA gene-based species definitions among rhizobia (Turner & Young, 2000; Gaunt *et al.*, 2001). In this study, PCR amplification and sequencing of the partial *glnA* gene was undertaken. The 11 obtained sequences have been deposited in the GenBank/EMBL/DDBJ databases under accession numbers AJ579875–AJ579885, which are from the representatives of clusters 2 (SDW014^T, SDW017, SDW037 and SDW040), 11 (SDW018^T and NM026) and 6 (SDW052 and NM349) identified by numerical taxonomy and also for representatives of two minor clusters, 1 (SDW010) and 5 (SDW058 and SX211a). The relationships inferred from these sequences are in agreement with the earlier 16S rRNA gene analysis (a supplementary phylogenetic tree is available in IJSEM Online). The two cluster 11 strains, SDW018^T and NM026, have identical sequences that are most closely related to *Mesorhizobium mediterraneum*. The *glnA* sequencing results also support the clustering of strains in cluster 6 and their affiliation with *Agrobacterium* species. The cluster 2 sequences are more divergent but are all *Mesorhizobium*-like. Strains SDW014^T and SDW037 group together and are not closely related to any other described type strain for which a sequence is available. The two other cluster 2 strains, SDW040 and SDW017, are not closely affiliated with strain SDW014^T or SDW037, or with any of the previously described *Mesorhizobium* type strain sequences. Of the strains examined from the minor numerical taxonomy clusters, SDW010 (cluster 1) is closely related to *Mesorhizobium tianshanense*, and SDW058 and SX211a (cluster 5) are probably *Rhizobium* spp. that are unrelated to any described type strain. All these findings corroborate the earlier partial 16S rRNA gene data of Gao *et al.* (2001).

Based upon the numerical taxonomy and the sequencing data of this work and those of rep-PCR fingerprinting, AFLP fingerprinting, 16S rRNA and 23S rRNA PCR-RFLP from an earlier study (Gao *et al.*, 2001), we defined three candidate species (I, II and III) among the strains from *Astragalus adsurgens* (Table 1). Twenty-seven of the 31 strains in cluster 2 (Fig. 1) were defined as candidate species I. Twenty-four of the 27 strains in candidate species I were in AFLP group 11 and three strains were in AFLP group 7 but they shared the same rRNA genotype. This indicates that the strains in candidate species I are highly related both in phenotypic and in genotypic characteristics. The rep-PCR results (Gao *et al.*, 2001), which clustered these 27 strains into eight groups, indicate that there is genetic diversity within the candidate species I strains. Four strains in cluster 2, SDW040, SDW046, SDW017 and SDW27, were not included in candidate species I because they had different AFLP patterns and 16S rRNA and 23S rRNA genotypes, and did not group with SDW014^T in either partial 16S (Gao *et al.*, 2001) or *glnA* phylogenies.

Candidate species II consisted of 10 of the 11 isolates within cluster 11 and 2 isolates in cluster 1 (Fig. 1), which have the same rRNA genotype. They belonged to a single rep-PCR group and three AFLP groups (Gao *et al.*, 2001). One strain, SDW074, of cluster 11 was not included in candidate species II because its 16S rRNA and 23S rRNA genotypes (Table 1) are identical to the strains of candidate species I. This strain will require more detailed analysis to determine its true taxonomic position.

Candidate species III is composed of 19 of the 26 isolates in numerical taxonomy cluster 6 (Fig. 1). Seven strains, SDW16, SX251a, NM349, SDW042, SDW056, SDW057 and N211, were not included in candidate species III mainly due to their different rRNA genotypes. All 19 strains in candidate species III belong to rep-PCR group 31 and AFLP groups 14 and 15 (Gao *et al.*, 2001), suggesting that they are highly related both phenotypically and genetically.

Table 2. DNA–DNA relatedness among strains representing candidate species I, II and III and the type strains of some recognized species in the genera *Mesorhizobium* and *Agrobacterium*

Candidate species I, *Mesorhizobium septentrionale* (SDW014^T); candidate species II, *Mesorhizobium temperatum* (SDW018^T); candidate species III, *Agrobacterium* sp. (SDW052).

Type or reference strain	DNA relatedness (%) with strain:		
	SDW014^T	SDW018^T	SDW052
Mesorhizobium amorphae ACCC 19665^T	13·3	24·1	
Mesorhizobium chacoense LMG 19008^T	25·5	28·3	
Mesorhizobium plurifarium LMG 11892^T	25·5	11·3	
Mesorhizobium huakuii CCBAU 2609^T	32·9	46·2	
Mesorhizobium ciceri USDA 3383^T	16·8	32·9	
Mesorhizobium loti NZP 2213^T	31·1	29·9	
Mesorhizobium mediterraneum USDA 3392^T	28·9	47·1	
Mesorhizobium tianshanense A-1BS^T	23·7	45·8	
Agrobacterium tumefaciens IAM 13129^T			25·2
Agrobacterium rubi IAM 13569^T			4·3
SDW014^T	100	32·1	
SDW012	87·6		
SDW034	81·5		
SDW018^T		100	
SDW016		92·9	
SDW017	0		
SDW046	0		

Comparative analyses of representative 16S rRNA and partial *glnA* gene sequences indicate that these strains are *Agrobacterium* spp. most closely related to *Agrobacterium tumefaciens* (Fig. 2). The failure of these strains to nodulate *Astragalus adsurgens* indicates either that they have lost their symbiotic genes rapidly during the isolation procedure or that they were never symbiotic (Gao *et al.*, 2001). The identification of non-symbiotic *Agrobacterium* strains from *Astragalus adsurgens* nodules showed again that non-symbiotic strains may occupy some nodules. Similar observations have been reported by Tan *et al.* (1999) and by de Lajudie *et al.* (1999). Further work is needed to clarify the genetic relationships between the candidate species III strains and pathogenic species within the genus *Agrobacterium*.

DNA base composition and DNA–DNA hybridization

To date, eight species have been described within genus *Mesorhizobium* (de Lajudie *et al.*, 1998; Jarvis *et al.*, 1997; Velázquez *et al.*, 2001). All these species share more than 97 % sequence similarity among their 16S rRNA genes (de Lajudie *et al.*, 1998) and their definition relies mainly on the genetic and phenotypic grouping results, including DNA–DNA hybridization. Strains SDW014^T, SDW018^T and SDW052 representing the candidate species I, II and III, respectively, were chosen for determination of DNA base composition and for DNA–DNA hybridization with the type strains of *Mesorhizobium* and *Agrobacterium* species. The DNA G+C content of SDW014^T and SDW018^T was 59·4 and 65·1 %, respectively. The results of DNA–DNA hybridization are shown in Table 2. DNA–DNA relatedness values of greater than 80 % are detected among the strains within each of the candidate species I and II. Among SDW014^T, SDW018^T and the type strains of *Mesorhizobium* species, the DNA–DNA relatedness ranges from 11·3 to 47·1 %. Strain SDW052 has DNA–DNA relatedness of 25·2 and 4·3 % with *Agrobacterium tumefaciens* and *Agrobacterium rubi*, respectively. These values clearly indicate that candidate species I and II are genomic species distinct from each other and from the type strains for defined *Mesorhizobium* species. The very low DNA–DNA relatedness between SDW014^T and SDW017 and SDW046 supports the exclusion of the latter two and related strains from candidate species I (Table 1).

As well as the different genotypes of rRNA, the utilization of fructose, glucose, inositol, malate, succinate, sucrose and turanose as carbon source can differentiate candidate species I from candidate species II. These two candidate species also can be differentiated from other *Mesorhizobium* species by their geographical origins and the natural host origins. Based upon the proposed standards for describing novel species (Graham *et al.*, 1991; Wayne *et al.*, 1987) and the results presented here and in previous work (Gao *et al.*, 2001), we believe that candidate species I and II represent two novel species within the genus *Mesorhizobium*. Since all the strains are from the temperate region in northern China, we propose the names *Mesorhizobium septentrionale* sp. nov. and *Mesorhizobium temperatum* sp. nov. for candidate species I and II, respectively.

Symbiotic properties and *nodA* gene sequencing

Host range is an important feature for the root- and/or stem-nodule bacteria and cross-nodulation with the selected hosts is required for the description of novel rhizobial species (Graham *et al.*, 1991). The cross-nodulation results showed that strain SDW014^T could nodulate *Phaseolus vulgaris*, *Glycine max*, *Leucaena leucocephala*, *Macroptilium atropurpureum* and *Lotus corniculatus*, but not *Pisum sativum*, *Vigna unguiculata*, *Galega officinalis*, *Astragalus sinicus*, *Medicago sativa* or *Trifolium repens*. Strain SDW018^T could nodulate *Phaseolus vulgaris*, *Vigna unguiculata*, *Glycine max*, *Leucaena leucocephala*, *Medicago sativa* and *Lotus corniculatus*, but not *Pisum sativum*, *Macroptilium atropurpureum*, *Galega officinalis*, *Astragalus sinicus* or *Trifolium repens*. These results indicated that the type strains for the two novel species have different nodulation spectrums. This difference may be explained by the sequencing results of the nodulation genes.

Different rhizobial species have been isolated and subsequently recognized from *Astragalus* hosts, including *Astragalus adsurgens*, as mentioned in the Introduction. This conclusion has been substantiated in the present study, since these rhizobia also have diverse phenotypic characteristics by numerical taxonomy. However, diversity of the symbiotic gene, *nodA*, superficially appears to be markedly less than measures of chromosomal diversity. Most of the *nodA* sequences identified are most similar to that described for the type strain of *Mesorhizobium tianshanense* even though the novel isolates belong to different species and even to different genera (Fig. 3); for example, *nodA* of the proposed type strain of *Mesorhizobium temperatum*, SDW018^T, differs from that of *Mesorhizobium tianshanense* at one synonymous site. Similarly, the *nodA* sequences of SDW037 (*Mesorhizobium septentrionale*), SDW062 (*Mesorhizobium temperatum*) and SX211a (unknown *Rhizobium* sp.) are also closely related to the *Mesorhizobium tianshanense* sequence (Fig. 3). These results might suggest that *Astragalus adsurgens* is symbiotically fastidious (has restricted requirements of the Nod factor signals for nodulation) but that the symbiotype (defined by symbiotic gene composition) functions in a range of rhizobial genetic backgrounds. The *nodA* sequence from SDW014^T is considerably divergent relative to the other *nodA* sequences obtained from *Astragalus adsurgens* (Fig. 3) and is unrelated to any other described *nodA* sequence in the databases. Whilst the host ranges of SDW014^T and SDW018^T differ, *nodA* is only one component that contributes to the host range limits of each symbiotype. It is therefore necessary to investigate more of the symbiotic genes from *Astragalus adsurgens* strains to better understand the ecological and evolutionary relationships of these particular rhizobial symbionts. *Astragalus adsurgens* has been successfully planted in different regions in China as soil cover and foliage. The ability of the dominant symbiotype, associated with *Astragalus adsurgens*, to function in diverse chromosomal backgrounds and to nodulate a range of legumes may help to explain why the plant has been grown successfully over such a wide area.

Description of *Mesorhizobium septentrionale* sp. nov.

Mesorhizobium septentrionale (sep.ten.tri.o.na′le. L. neut. adj. *septentrionale* northern, implying that the strains were isolated from the northern parts of China).

Gram-negative, aerobic, non-spore-forming rods. Colonies on YMA medium are circular, convex, translucent and have a diameter of 1 mm within 7–10 days at 28 °C. Generation times are 6–9 h in PY broth (Wang *et al.*, 1999). Can use fructose, glucose, inositol, malate, maltose, D-mannose, melibiose, sodium succinate, D-sorbitol, sucrose, trehalose and turanose as sole carbon sources, and use hypoxanthine, L-isoleucine, L-phenylalanine and D-threonine as sole nitrogen sources. All strains have been deposited in the Culture Collection of China Agricultural University (CCBAU), China and in the Culture Collection of Helsinki University (HAMBI), Finland.

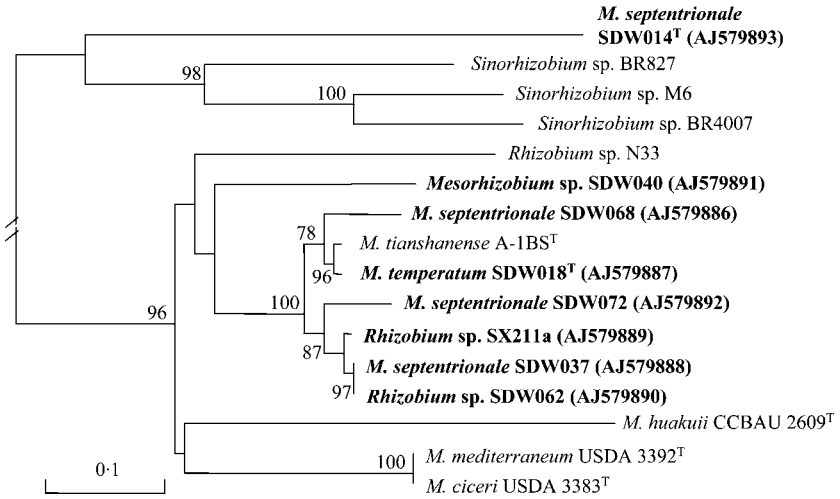

Fig. 3. Simplified neighbour-joining phylogeny estimated from partial *nodA* gene sequences using the K2P evolutionary model in CLUSTAL_X. Bootstrap values (1000 replicates) greater than 60 % are shown on the appropriate nodes. All sequences, other than those shown in bold, were taken from Zhang *et al.* (2000).

The type strain is SDW014^T (=CCBAU 11014^T=HAMBI 2582^T); it has the characteristics described for the species. The G+C content of the genomic DNA of the type strain is 59·4 %.

Description of *Mesorhizobium temperatum* sp. nov.

Mesorhizobium temperatum (tem.pe.ra′tum. L. neut. adj. *temperatum* temperate, implying that the strains were isolated from temperate zones).

Gram-negative, aerobic, non-spore-forming rods. Colonies on YMA medium are circular, convex, translucent and have a diameter of 1 mm within 7–10 days at 28 °C. The generation times are 5–9 h in PY broth (Wang *et al.*, 1999). Cannot use fructose, glucose, inositol, malate, maltose, D-mannose, melibiose, sodium succinate, D-sorbitol, sucrose, trehalose or turanose as sole carbon sources, but can use hypoxanthine and D-threonine as sole nitrogen sources. All the strains have been deposited in the Culture Collection of China Agricultural University (CCBAU), China and in the Culture Collection of Helsinki University (HAMBI), Finland.

The type strain is SDW018^T (=CCBAU 11018^T=HAMBI 2583^T); it has the characteristics described for the species. The G+C content of the genomic DNA of the type strain is 65·1 %.

ACKNOWLEDGEMENTS

This work was supported financially by EU-INCO contract no. IC18-CT96-0103 and by the Natural Science Foundation of China project no. 2001CB108905. We are grateful to H. G. Trüper for suggesting the names of the two novel species and to Mr Q. Chen for his technical support.

REFERENCES

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

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**, 392–397.

Chen, W. X., Li, G. S., Qi, Y. L., Wang, E. T., Yuan, H. L. & Li, J. L. (1991). *Rhizobium huakuii* sp. nov. isolated from the root nodules of *Astragalus sinicus*. *Int J Syst Bacteriol* **41**, 275–280.

Chen, W. X., Wang, E. T., Wang, S. Y., Li, Y. B., Chen, X. Q. & Li, Y. (1995). Characteristics 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., Tan, Z.-Y., Gao, J.-L., Li, Y. & Wang, E.-T. (1997). *Rhizobium hainanense* sp. nov., isolated from tropical legumes. *Int J Syst Bacteriol* **47**, 870–873.

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.

de Lajudie, P., Willems, A., Nick, G. & 8 other authors (1999). *Agrobacterium* bv. 1 strains isolated from tropical legumes. *Syst Appl Microbiol* **22**, 119–132.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). Quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

Gao, J. L., Sun, J. G., Li, Y., Wang, E. T. & Chen, W. X. (1994). Numerical taxonomy and DNA relatedness of tropical rhizobia isolated from Hainan Province, China. *Int J Syst Bacteriol* **44**, 151–158.

Gao, J. L., Terefework, Z. D., Chen, W. X. & Lindström, K. (2001). Genetic diversity of rhizobia isolated from *Astragalus adsurgens* growing in different geographical regions of China. *J Biotechnol* **91**, 155–168.

Gaunt, M. W., Turner, S. L., Rigottier-Gois, L., Lloyd-Macgilp, S. A. & Young, J. P. W. (2001). Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. *Int J Syst Evol Microbiol* **51**, 2037–2048.

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.

Guo, X. W., Zhang, X. X., Zhang, Z. M. & Li, F. D. (1999). Characterization of *Astragalus sinicus* rhizobia by restriction fragment length polymorphism analysis of chromosomal and nodulation genes regions. *Curr Microbiol* **39**, 358–364.

Hurek, T., Wagner, B. & Reinhold-Hurek, B. (1997). Identification of N_2-fixing plant- and fungus-associated *Azoarcus* species by PCR-based genomic fingerprints. *Appl Environ Microbiol* **63**, 4331–4339.

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

Laguerre, G., van Berkum, P., Amarger, N. & Prevost, D. (1997). Genetic diversity of rhizobial symbionts isolated from legume species within genera *Astragalus*, *Oxytropis*, and *Onobrychis*. *Appl Environ Microbiol* **63**, 4748–4758.

Li, J., Yu, L., Li, N. & Wang, H. (2000). *Astragalus mongholicus* and *Angelica sinensis* compound alleviates nephritic hyperlipidemia in rats. *Chin Med J* **113**, 310–314.

Marmur, J. (1961). A procedure for the isolation of DNA from microorganisms. *J Mol Biol* **3**, 208–218.

Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.

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.

Nagasawa, H., Watanabe, K., Yoshida, M. & Inatomi, H. (2001). Effects of gold banded lily (*Lilium auratum* Lindl) or Chinese milk vetch (*Astragalus sinicus* L) on spontaneous mammary tumourigensis in SHN mice. *Anticancer Res* **21**, 2323–2328.

Page, R. D. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.

Peng, G. X., Tan, Z. Y., Wang, E. T., Reinhold-Hurek, B., Chen, W. F. & Chen, W. X. (2002). Identification of isolates from soybean nodules in Xinjiang Region as *Sinorhizobium xinjiangense* and genetic

differentiation of *S. xinjiangense* from *Sinorhizobium fredii*. *Int J Syst Evol Microbiol* **52**, 457–462.

Shon, Y. H., Kim, J. H. & Nam, K. S. (2002). Effects of *Astragali radix* extract on lipopolysaccharide-induced inflammation in human amnion. *Biol Pharm Bull* **25**, 77–80.

Sneath, P. H. A. & Sokal, R. R. (1973). *Numerical Taxonomy. The Principles and Practices of Numerical Classification*. San Francisco: W. H. Freeman.

Sriprang, R., Hayashi, M., Yamashita, M., Ono, H., Saeki, K. & Murooka, Y. (2002). A novel bioremediation system for heavy metals using the symbiosis between leguminous plant and genetically engineered rhizobia. *J Biotechnol* **99**, 279–293.

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., Wang, E. T., Peng, G. X., Zhu, M. E., Martínez-Romero, E. & Chen, W. X. (1999). Characterization of bacteria isolated from wild legumes in the north-western regions of China. *Int J Syst Bacteriol* **49**, 1457–1469.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Turner, S. L. & Young, J. P. W. (2000). The glutamine synthetases of rhizobia: phylogenetics and evolutionary implications. *Mol Biol Evol* **17**, 309–319.

Velázquez, E., Igual, J. M., Willems, A. & 9 other authors (2001). *Mesorhizobium chacoense* sp. nov., a novel species that nodulates *Prosopis alba* in the Chaco Arido region (Argentina). *Int J Syst Evol Microbiol* **51**, 1011–1021.

Vincent, J. M. (1970). *A Manual for the Practical Study of Root Nodule Bacteria. IBP Handbook no.15*. Oxford: Blackwell Scientific.

Wang, S. Y. & Chen, W. X. (1996). Numerical taxonomy and DNA relatedness of rhizobia isolated from *Astragalus* spp. In *Diversity and Taxonomy of Rhizobia*, pp. 79–84. Edited by F. D. Li *et al.* Beijing: China Agricultural Scientech Press.

Wang, E. T., van Berkum, P., Sui, X. H., Beyene, D., Chen, W. X. & Martínez-Romero, E. (1999). Diversity of rhizobia associated with *Amorpha fruticosa* isolated from Chinese soils and description of *Mesorhizobium amorphae* sp. nov. *Int J Syst Bacteriol* **49**, 51–65.

Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Wdowiak, S. & Malek, W. (2000). Numerical analysis of *Astragalus cicer* microsymbionts. *Curr Microbiol* **41**, 142–148.

Xu, L. M., Ge, C., Cui, Z., Li, J. & Fan, H. (1995). *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. *Int J Syst Bacteriol* **45**, 706–711.

Zhang, X. X., Turner, S. L., Gao, X. W., Yang, H. J., Debelle, F., Yang, G. P., Denarie, J., Young, J. P. W. & Li, F. D. (2000). The common nodulation genes of *Astragalus sinicus* rhizobia are conserved despite chromosomal diversity. *Appl Environ Microbiol* **66**, 2988–2995.