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Study on Genetic Diversity and Phylogeny of Soybean Rhizobia in China

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Abstract

Fifty-two soybean rhizobia isolated from root nodules of nine soybean cultivars from soil samples collected from seven different sites in China were studied comparing with twenty three type strains of rhizobia by phenotypic characteristics, host specificity, 16S rDNA PCR-RFLP, 16S rDNA sequencing, REP-ERIC-PCR and DNA-DNA hybridization. Results of phenotypic characteristics indicated that twenty nine fast-growing strains could be distinguished with *Sinorhizobium xinjiangense* CCBAU110 and twenty three slow-growing strains with *Bradyrhizobium elkanii* USDA76, respectively. The similarities of some slow-growing strains corresponded to geographical sampling sites. All strains could be divided into two types by host specificity. Results of 16S rDNA PCR-RFLP, 16S rDNA sequence analysis, REP-ERIC-PCR and DNA-DNA hybridization revealed that twenty nine strains of fast-growing rhizobia were all clustered together with *Sinorhizobium fredii* USDA205 and *S. xinjiangense* CCBAU110. Similarly, twenty three strains of slow-growing rhizobia were highly related with *B. japonicum* and *B. liaoningense*.

Keywords: *Rhizobium*, 16S rDNA sequencing, REP-ERIC-PCR, DNA-DNA hybridization, genetic diversity

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1. Introduction

Soybean (*Glycine max* [L.] Merr.) is one of the main crops and plays an important role in the economy and life in China. Initially, *Bradyrhizobium* (*Rhizobium*) *japonicum*, which is a slow-growing, gram-negative bacterial species belonging to the family of Rhizobiaceae (Jordan, 1982) was originally described as the only nitrogen-fixing soybean root nodule symbiont. Hollis et al. (1981) reported three homology groups by using DNA-DNA hybridization among *B. japonicum* strains. Groups I and Ia corresponded to the species *B. japonicum*, whereas Group II was later given a new species name, *B. elkanii*, on the basis of genotypic and phenotypic results (Kuykendall et al., 1992). In fact, Date and Decker (1965) described 17 different serogroups of soybean bradyrhizobia. The variation in serological specificity may indicate that the *Bradyrhizobium* bacteria of soybean are genetically more diverse than just the two type species. Therefore, a comparative analysis made in further studies led to the proposal of *B. liaoningense*, a new group of extra-slow-growing species (Xu et al., 1995).

Keyser et al. (1982) described fast-growing rhizobia isolated from China, which also induced nodules that fixed atmospheric nitrogen on the roots of soybeans. Based on DNA hybridization and physiological differences, Scholla et al. (1984) proposed to designate this as a new species, *Rhizobium fredii*. However, Chen et al. (1988) found little relationship between *R. fredii* and the other fast-growing species.

This led to the proposal of a new genus, *Sinorhizobium*. A second species, *S. xinjiangens* was also proposed for fast-growing soybean-nodulating isolates isolated from Xinjiang region in China. Based on the results of partial 16S rDNA sequence, Jarvis et al. (1992) concluded that Chen's classification is not justified and that the fast-growing soybean-nodulating rhizobia should belong to *Rhizobium* and named *Rhizobium fredii*.

At the same time, the species status of *Sinorhizobium xinjiangense* was also questioned. According to the results of 16S rDNA sequences, Lajudie et al. (1994) proposed to restore the genus *Sinorhizobium* and the fast-growing soybean-nodulating rhizobia should be called *Sinorhizobium fredii* again. Currently, strains isolated from soybean may belong to one of the following five species of two genera: *Sinorhizobim fredii*, *S. xinjiangense*, *Bradyrhizobium japonicum*, *B. elkanii*, and *B. liaoningense*.

The genetic relationship, biodiversity and symbiotic effectiveness of 52 isolates from nine soybean cultivars in seven different geographic areas in China were studied. The results based on phenotypic characters, plasmid patterns, host specificity, 16S rDNA sequencing, REP-ERIC PCR fingerprinting, and DNA-DNA hybridization are reported in this paper.

2. Materials and Methods

Bacterial strains and soybean cultivars

Seeds of nine soybean cultivars were surface-sterilized with 1% (v/v) NaOCl for 3 min and rinsed 10 times with sterile distilled water. Germination was on moist paper towels at 28°C for 2–3 days. Plants were grown in Leonard jars containing sterile vermiculite moistened with nitrogen-free nutrition solution (Van Berkum, 1990). The surface-sterilized seeds were inoculated with 10% (w/v) soil samples collected from different sites in China. Plants were grown in a greenhouse for about 50 days, and all nodules were collected for *Rhizobium* isolation according to the procedure described by Vincent (1970) using yeast mannitol agar (YMA). The isolates were further purified by repeated plating and checked by microscopical examination. The effectiveness of the strains isolated was verified by plant pot infection tests with the original host plants. The isolates and reference strains used in this study are shown in Table 1.

Host specificity tests

Four commercial grown soybean cultivars (Aijiaozao, Houzimao, Heinong 33 and Williams) and *G. soja* were compared for host range. Purified isolates were used to inoculate soybean seedlings in sterile Leonard jars using the same procedure as for the *Rhizobium* isolation with the exception that soil samples were replaced with approximately 10^8 cell suspension cultures.

Table 1. Bacterial strains and their relevant characteristics.

Strain	Host	Geographic origin	Plasmid size[c] (MD)	16S rRNA RFLP[d]	
				Pattern	Genotype
HH2	Heinong 33	Huayuankou	480,235,130	AAAA	1
HH5	Heinong 33	Huayuankou	235,130	AAAA	1
HA1	Heinong 33	Anyang		BCBC	4
HA2	Heinong 33	Anyang		BCBC	4
HX1	Heinong 33	Yanglin		ADBC	5
HS1	Heinong 33	Weifang		BCBC	4
HS2	Heinong 33	Weifang		BCBC	4
AX5	Aijiaozao	Yanglin	270	AAAA	1
AA3	Aijiaozao	Anyang	450,250,200	AAAA	1
AA2	Aijiaozao	Anyang	450,250,200	ABAA	2

Table 1. Continued.

Strain	Host	Geographic origin	Plasmid size[c] (MD)	16S rRNA RFLP[d] Pattern	Genotype
AA1	Aijiaozao	Anyang		ADBC	5
AB3	Aijiaozao	Renqiu	230	ABAA	2
AB4	Aijiaozao	Renqiu	230	AAAA	1
BH7	Bragg	Huayuankou	235,130,70	AAAA	1
BB1	Bragg	Renqiu	230	AAAA	1
BS14	Bragg	Weifang	250,100	AAAA	1
BS3	Bragg	Weifang		BCBC	4
BJ4	Bragg	Jiamusi		BCBC	4
BA1	Bragg	Anyang		ADBC	5
BA3	Bragg	Anyang		ADBC	5
BX4	Bragg	Yanglin		ADBC	5
WH5	Williams	Huayuankou	480,235,130	AAAA	1
WH10	Williams	Huayuankou	480,235,130	AAAA	1
WO	Williams	HongHu	480,235,130	AAAA	1
WX5	Williams	Yanglin		ADBC	5
WX1	Williams	Yanglin		ADBC	5
MH1	Houzimao	Huanyuankou	320,235	AAAA	1
MA2	Houzimao	Anyang	230	ABAA	2
MB2	Houzimao	Renqiu	230	AAAA	1
MS7	Houzimao	Weifang		BCBC	4
MS13	Houzimao	Weifang		BCBC	4
MS1	Houzimao	Weifang	250	ABAA	2
LS7	Longmu 1	Weifang	250,100	AAAA	1
LB1	Longmu 1	Renqiu	230	AAAA	1
LS1	Longmu 1	Weifang		BCBC	4
LA2	Longmu 1	Anyang		ADBC	5
LA1	Longmu 1	Anyang		ADBC	5
JH1	Jindou 19	Huayuankou	250,145	ABAB	3
JH2	Jindou 19	Huayuankou	250,70	ABAA	2
JB2	Jindou 19	Renqiu	230	AAAA	1
JX2	Jindou 19	Yanglin	270,130	ABAA	2
JX4	Jindou 19	Yanglin	270,130	AAAA	1
JA1	Jindou 19	Anyang	230	AAAA	1
JA4	Jindou 19	Anyang	520,250,200,70	ABAA	2
JO1	Jindou 19	Honghu	250,70	AAAA	1
DH1	Taixinghei	Huayuankou	480,235,130,70	AAAA	1
DS6	Taixinghei	Weifang	470,230	AAAA	1
DX2	Taixinghei	Yanglin		BCBC	4
DX3	Taixinghei	Yanglin		BCBC	4
DS8	Taixinghei	Weifang		BCBC	4
KS2	Kobbe	Weifang		BCBC	4
KX1	Kobbe	Yanglin		ADBC	5

Table 1. Continued.

Strain	Host	Geographic origin	Plasmid size[c] (MD)	16S rRNA RFLP[d] Pattern	Genotype
B. japonium					
USDA6^T (=ATCC10324)	*Glycine max*	CAU[b]		BCBC	4
USDA110	*Glycine max*	Own lab		BCBC	4
B. elkanii USDA76^T	*Glycine max*	CAU		ICBG	25
Sinorhizobium					
S. fredii USDA205^T	*Glycine max*	CAU		AAAA	1
S. meliloti USDA1002^T		CAU		AEAA	7
S. arboris HAMBI1552^T		CAU		AEEA	8
S. xinjiangensis CCBAU110^T		CAU		AGCA	6
Mesorhizobium					
M. amorphae ACCC19665^T		CAU		CDCD	9
M. plurifarium MG11892^T		CAU		EGCD	10
M. huakuii CCBAU2609^T		CAU		CGCA	11
M. ciceri USDA3378^T		CAU		DCCD	12
M. tianshanense CCBAU3306^T		CAU		DHCD	13
M. mediteraneum USDA3392^T		CAU		AHCE	14
Rhizobium					
R. mongolense USDA1844^T		CAU		FFCA	15
R. giardinii USDA2914^T(H152^T)		CAU		FGCA	16
R. huantiense SO2^T		CAU		FCBA	20
R. galegae HAMBI540^T		CAU		GFBA	21
R. leguminosarum USDA2370^T		CAU		GEBA	22
R. etli CFN42^T		CAU		HFFF	23
R. hainnanense CCBAU57015		CAU		GEBA	25
A. tumefaciense IAM13129^T		CAU		HICA	17
A. vivis IAM14140^T		CAU		GECA	18
A. undicola LMG11875^T		CAU		HJDA	19

[a]ACCC, Agricultural Center Culture Collection, Chinese Academy of Agriculture, Beijing, China; CCBAU, Culture Collection of Beijing Agriculture University, Beijing, China; CFN, Centro de Investigation sobre Fijacion de Nitrogeno, UNAM, Cuernavaca, Mexico; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland; USDA, Beltsville *Rhizobium* Culture Collection, Beltsville, Agricultural Research Center, Beltsville, MD, USA; LMG, Collection of *Bacterium* of the Laboratorium voor, Microbiology, University of Ghent, Ghent, Belgium; T, Type strain. [b]CAU, China Agricultural University. [c]The approximate plasmid sizes were calculated from their mobility in 0.7% agarose gel with plasmids of *S. fredii* USDA205T as molecular size marker as reference (Broughton et al., 1984). [d]Four letters arbitrarily assigned to represent specific fingerprint patterns obtained from PCR–RFLP analysis of 16S rRNA genes digested with endonucleases *Hinf*I, *Msp*I, *Sau*3AI, *Hha*I, respectively. Different combinations of letters were then used to define RFLP genotypes.

DNA isolation

Total DNA was isolated from 2 × 1.5 ml liquid cultures using the procedure described by Ausubel et al. (1987). The DNA concentration was determined by comparison with known concentrations of λDNA in agarose gel electrophoresis.

16S rDNA PCR-RFLP and DNA sequence analysis

Primers fD1 and rD1 (Weisburg et al., 1991) were used to amplify the 16S rRNA gene according to the PCR procedure described by van Berkum et al. (1996). The products in 15 µl samples of the reaction mixture were digested with 5 µ restriction enzyme *Msp*I, *Sau*3AI, *Hinf*I or *Hha*I individually, and the results visualized by electrophoresis in 2% (w/v) agarose gels (Laguerre et al., 1994). Restriction fragments were compared with those of the type species of *Sinorhizobium*, *Rhizobium* and *Bradyrhizobium* species, and a dendrogram was constructed from resulting distance matrix using the unweighted pair group method with averages (UPGMA) (Sneath et al., 1973). Based on the RFLP results of the 16S rRNA genes from 52 soybean rhizobia tested, three distinct strains were chosen for completed sequencing. Purified rDNA's were cloned by using a pMD18-T vector kit (TaKaRa Biotechnology, Co., Ltd., Dalian) according to the manufacturer's instructions. Purified plasmids were sequenced with four primers, M13R, M13F, W1F and W2F corresponding to the following sequences: 5'-CGCCAGGGTTTTCCCAGTCACGAC-3',
5'-AGCGGATAACAATTTCACACAGG-3',
5'-GGACTTAACCCAACATCTCA-3' and
5'-GTGCCAGCAGCCGCGGTAAT-3'.

The generated rDNA sequences and sequences of reference strains from GenBank were aligned using CLUSTAL W program. Aligned sequences were analyzed using the Molecular Evolutionary Genetics Analysis (MEGA) package version 1.01 (Kumar et al., 1993) to generate an optional unrooted tree using the neighbour-joining method (Saitou et al., 1987).

The accession numbers of 16S rDNA sequences used in the phylogenetic analysis were: AY148432 (BA1), AY148433 (AB3), AY148434 (JH1), AF250354 (*S. xinjiangensis* CCBAU110), X67231 (*S. fredii* LMG 6217), X68387 (*S. terangae*), X68390 (*S. saheli* LMG 7837), Z78204 (*Sinorhizobium* sp.), U28916 (*R. etli* CFN 42), D13431 (*Mesorhizobium huakuii*), D14503 (*Agrobacterium rubi*), Z30542 (*A. larrymoorei*), X94200 (*Azorhizobium caulinodans*), U35000 (*Bradyrhizobium elkanii*), X70402 (*Bradyrhizobium* sp. LMG 9520), D13429 (*B. japonicum* strain USDA94), M69186 (*Afipia clevelandensis*), M65248 (*A. felis*), S46917 (*Blastobacter denitrificans*), Z35330 (*B. japonicum* strain USDA110), D12781 (*B. japonicum* strain USDA6), U69638 (*B. japonicum*), and X86065 (*B. liaoningense*).

Plasmid profiles

The plasmids isolated from strains were tested by using a modified Eckhardt procedure (Eckhardt, 1978; Hynes et al., 1970) and recorded using an image recording apparatus. Plasmid mobility was determined by electrophoresis in 0.7% agarose gels. Plasmids of *S. fredii* USDA205 were used for estimating relative molecular sizes.

Phenotypic characterization and numerical taxonomy

The phenotypic characters of 52 isolates and 5 type-strains of USDA205, CCBAU110, USDA110, USDA6 and USDA76 were examined. The growth ability was recorded after 3 days of incubation for fast-growing strains or after 7–10 days incubation for slow-growing strains, respectively. The following features were analyzed: (i) Utilization of mannitol, L-arabinose, D-glucose, sucrose, dulcitol, maltose, lactose, D-fructose, sodium succinate, D-sorbitol, dextrin and D-galactose as the sole carbon source; (ii) Utilization of L-tryptophan, L-glutamine, L-arginine, L-asparagine, L-histidine, L-lysine and L-valine as nitrogen sources; (iii) Tolerance to streptomycin (10, 30 μg/ml), kanamycin (1, 5, 10 μg/ml), spectinomycin (5, 10 μg/ml), tetracycline (1, 5 μg/ml), gentamicin (25, 50 μg/ml), nalidixic acid (25, 50 μg/ml), and rifamycin (10, 20 μg/ml) added to YMA medium; (iv) Growth on YMA medium containing 1.0, 1.5, 2.0, 3.0 and 4.0% NaCl (w/v); (v) Growth on YMA medium at pH 5.0, 6.0, 9.0 and 10.0; (vi) Absorption of Congo-red (1/1000) on YMA medium; (vii) Production of melanin on TY medium (Maria et al., 1999); (viii) Growth at 25°C, 33°C, 35°C and 37°C on YMA; (ix) Growth curve was determined in YM broth by detecting the optical density at 600 nm, which was used to determine the generation times of strains tested. To test utilization of carbon compounds, the different carbon sources were added at a final concentration of 1 g/l to a carbon-free basic medium (White, 1972). Growth on nitrogen compounds was tested at a concentration of 0.5 g/l added to nitrogen-free basic medium. All of the nutrients were filter-sterilized before adding to the basic medium. Growth ability on different media was tested in petri dishes by inoculation of approximately 10^5 bacteria from a freshly prepared bacterial suspension. Plates were incubated at 28°C, and the growth ability was recorded after 3 or 7–10 days.

All results were coded as 1 for positive and 0 for negative. Similarity values were calculated by using a simple matching coefficient (Sokal and Michener, 1958). A dendrogram was constructed from the similarity matrix by the unweighted pair group method with arithmetic means (UPGMA) (Sneath and Sokal, 1973) by using the NTSYS-PC analysis package (version 1.8, Exeter Software, Setauket, NY).

REP-ERIC PCR genomic fingerprintings

Genomic DNA was isolated and amplified by using primers of enterobacterial repetitive intergenic consensus (ERIC) or repetitive extragenic palindromic (REP) (De Bruijn, 1992; Versalovic et al., 1991) and a PTC-100 thermocycler from MJ Research Inc. The fragments were visualized after electrophoresis in a 1.5% agarose gel. Reproducibility of REP-ERIC PCR was tested according to Vinuesa's procedure (Vinuesa et al., 1998). Clustering analysis of PCR results was performed using NTSYS-PC program, with the UPGMA and the simple matching coefficients.

DNA base composition and DNA-DNA hybridization

Strains were grown in Roux flasks on TY medium until late-exponential phase, and high-molecular mass DNA was prepared following Marmur's method (Marmur, 1961). The average G+C mol% of DNA were measured by thermal denaturation method (De Ley, 1970) and calculated by using the equation of Marmur and Doty (Marmur et al., 1962) as modified by De Ley (1991). DNA-DNA hybridization was performed with the initial renaturation rate method (De Ley, 1970).

3. Results

Strain identification

Fifty-two rhizobial isolates (Table 1) were isolated and purified from root nodules of nine soybean cultivars. Twenty-nine of them grew fast and produced acid on YMA. Twenty-three strains grew slowly and produced alkali on YMA.

Nodulation and host specificity

The fifty-two soybean isolates could all induce nodules on their original host, resulting in a very efficient nitrogen-fixing symbiosis. Strains isolated from soybean cultivar Williams, Heinong 33 and *G. soja* were found to be effective on soybean cultivar Aijiaozao and Houzimao, but the strains isolated from Aijiaozao, Houzimao and Jindou 19 were ineffective on Williams, Heinong 33 and *G. soja*.

Numerical analysis of phenotypic data

The results of phenotypic characteristics of fifty-two isolates were

summarized in Table 2. Compared with type strains USDA205, CCBAU110, USDA110, USDA6 and USDA76, all strains tested could grow at 25–33°C and at pH 6.0–8.0, but no growth occurred at 37°C. The generation time was 2.4–3.9 h for fast-growing strains and 6.9–8.3 h for slow-growing rhizobia. The results of seven antibiotics resistance tests showed that all strains were resistant to nalidixic acid (25 μg/ml) and susceptible to kanamycin (10 μg/ml) and rifamycin (20 μg/ml). Mannitol and L-arabinose were good growth substrates for all strains tested.

Table 2. Phenotypic characteristics of strains tested.

Characteristics	% of positive strains[a]		Characteristics	% of positive strains[a]	
	Fast-growers	Slow-growers		Fast-growers	Slow-growers
pH 5.0	20–30	90–99	L-lysine	70–90	95–100
pH 10.0	90–95	0	L-arginine	70–90	95–100
35°C	30–70	5–10	L-tryptophan	70-90	95–100
NaCl (1%)	100	0	Tolerance[b]		
Congo red absorption	90–95	90–95	Str (10)	90–95	70–90
Production of melanin	95–100	0	Str (30)	70–90	5–30
Utilization of carbon source			Km (1)	95–100	95–100
Mannitol	100	100	Km (5)	70–90	50–70
L-arabinose	100	100	Km (10)	0	0
Dulcitol	20–30	0	Spe (5)	10–30	0
D-sorbitol	20–70	90–99	Spe (10)	0	0
D-fructose	90–99	70–90	Tc (1)	30–50	90–95
D-galactose	90–99	70–90	Tc (5)	0	30–70
Maltose	90–99	70–90	Gen (25)	50–70	90–95
Lactose	90–99	5–10	Gen (50)	10–30	50–70
Sucrose	95–100	50–70	Nx (25)	100	100
Utilization of nitrogen source			Nx (50)	70–90	50–70
L-histidine	3–10	95–100	Rif (10)	40–60	40–60
L-valine	3–10	95–100	Rif (20)	0	0
L-glutamine	3–10	95–100			
L-asparagine	3–10	5–10			

[a]Strains which can grow, absorb Congo red or produce melanin are positive. [b]Antibiotic concentration in μg ml^{-1}.

The numerical analysis of phenotypic data from above tests were used in this analysis. The resulting dendrogram is presented in Fig. 1. At a similarity coefficient of 42%, two groups could be distinguished and corresponded to

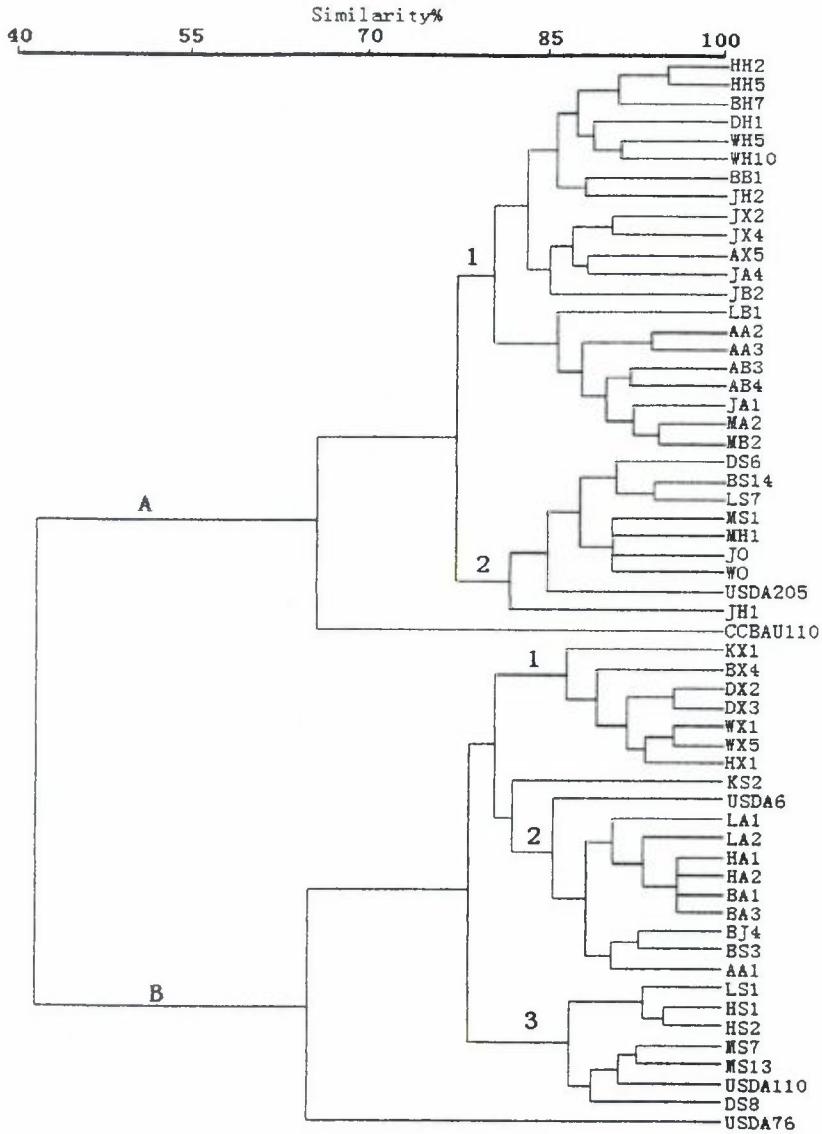

Figure 1. Dendrogram of isolates, type strain *S. fredii*, *S. xinjiangense*, *B. japonicum* and *B. elkanii* based on phenotypic characteristics using UPGMA algorithm. Characteristics of growth, absorbing Congo red, tolerance to antibiotics, or producing melanin were positive and were coded as 1, otherwise, they were negative and coded 0. Similarity values were calculated by using a simple matching coefficient. A dendrogram was constructed from the similarity matrix by the unweighted pair group method with arithmetic means (UPGMA) by using the NTSYS-PC analysis package. All strains could be distinguished into two groups at a level of similarity of 42% which corresponded to *Sinorhizobium* (group A) and *Bradyrhizobium* (group B), respectively.

Sinorhizobium and *Bradyrhizobium*, respectively. Within the group A, two subgroups could be distinguished at a similarity level of 78%. Twenty-one strains from Renqiu of Hubei Province, Yanglin of Shanxi Province, Huayuankou and Anyang of Henan Province constituted subgroup 1. Subgroup 2 consisted of eight strains from Huayuankou of Henan Province, Weifang of Shandong Province and Honghu of Hubei Province. It was clear that the twenty-nine fast-growing strains analyzed showed higher similarity with USDA205 than to CCBAU110, its similarity was 78% and 65%, respectively. Within group B, we could distinguish twenty-three rhizobia into three subgroups at a similarity level of 80%. Seven strains in subgroup 1 and six strains in subgroup 3 were isolated from Yanglin or Weifang, respectively. The subgroup 2 contained 10 isolates from Weifang, Anyang and only one strain, BJ4, isolated from Jiamusi of Heilongjiang Province. The type strain USDA6 and USDA110 belonged to subgroup 2 and 3, respectively, but USDA76 branched far from all of slow-growing strains tested.

Plasmid profiles

Fourteen different plasmid profiles were observed among twenty-nine fast-growing rhizobia. The plasmid number observed ranged from 1 to 4 (Table 1, Fig. 2). The majority of them had two or three plasmids. But no plasmid was observed in any of the slow-growing rhizobia examined. In spite of little differences in phenotypic character analysis, isolates HH5, DH1, BH7, JH1, JH2 and MH1 from Huayuankou of Heinan Province shown six plasmid profiles types, while only a 230 MD plasmid was observed with strains isolated from Renqiu of Hebei Province. Plasmid profiles of strains JH2, JH1, JA4 and JX2 originated from host soybean Jindou 19 are different. Although different plasmid profiles were observed among rhizobia from different soil samples, Strains MA2 and LB1 had the same plasmid profiles. Our results indicated that plasmid profiles were mainly correlated with the host plant and the geographical origin. It is also possible that the plasmids may have been inherited through genetic exchange. Rhizobia might be transmitted with seeds to different sites when the new host plant was introduced (Perez-Ramirez et al., 1998) and different soils may harbor the same rhizobia able to nodulate different soybean hosts.

PCR-RFLP analysis of the 16S rRNA genes

A 1.5kb DNA fragment was obtained by PCR amplification from all isolates and twenty-three type strains tested. Five distinct RFLP patterns were identified among the isolates (Table 1). Comparing with twenty-three type

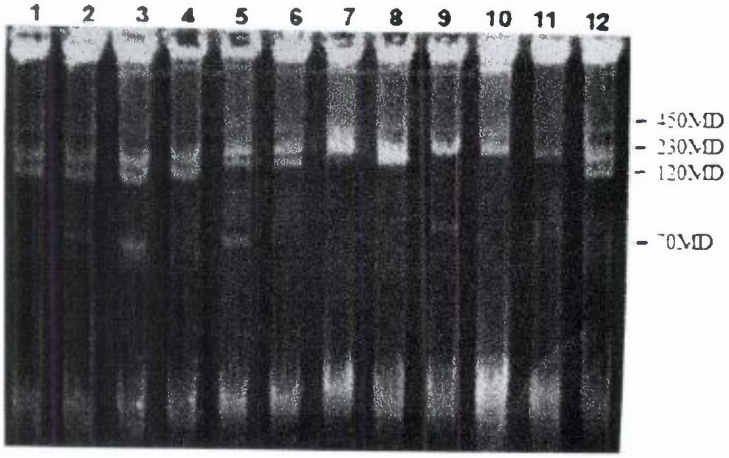

Figure 2. Plasmid electrophoretic patterns of represent fast-growing rhizobia. Lanes: 1, HH5; 2, DH1; 3, BH7; 4, JH1; 5, JA4; 6, AA2; 7, MS1; 8, DS6; 9, LS7; 10, MH1; 11, MA2; 12,USDA205. The approximate plasmid sizes were calculated from their mobility in 0.7% agarose gel with plasmids (70, 120, 230, 450 MD) of *S. fredii* USDA205^T as molecular size marker (Broughton et al., 1984).

strains, patterns 2, 3 and 5 (ABAA, ABAB and ADBC) were distinct from those observed with the type strains. However, the patterns 1 and 4 (AAAA and BCBC) were identical with USDA205 and USDA6, respectively. The results of clustering analysis demonstrated that there were two groups at the 52% similarity level within fifty-two strains tested (Fig. 3). Representative strains AB3, JH1 and BA1 showing difference with USDA205 and USDA6 were chosen for completed sequence analysis. Comparing with data of type strains, a phylogenic tree was constructed (Fig. 4). Strains AB3 and JH1 were clustered with USDA205 and CCBAU110, and strain BA1 clustered with *B. japonicum* and *B. liaoningense*. It was difficult to place isolates AB3 and JH1 into *S. fredii* or *S. xinjiangense*, because their 16S rRNA gene nucleotide sequences were very similar (more than 99.6%). Meanwhile, isolate BA1 showed only 2 basepair differences to that of *B. liaoningense*. Therefore DNA-DNA hybridization and other methods are required to study biodiversity within these closely related strains.

REP-ERIC PCR

The fifty-two isolates were further analyzed for REP-ERIC PCR finger-printing and more heterogeneous groups were identified within each 16S rDNA

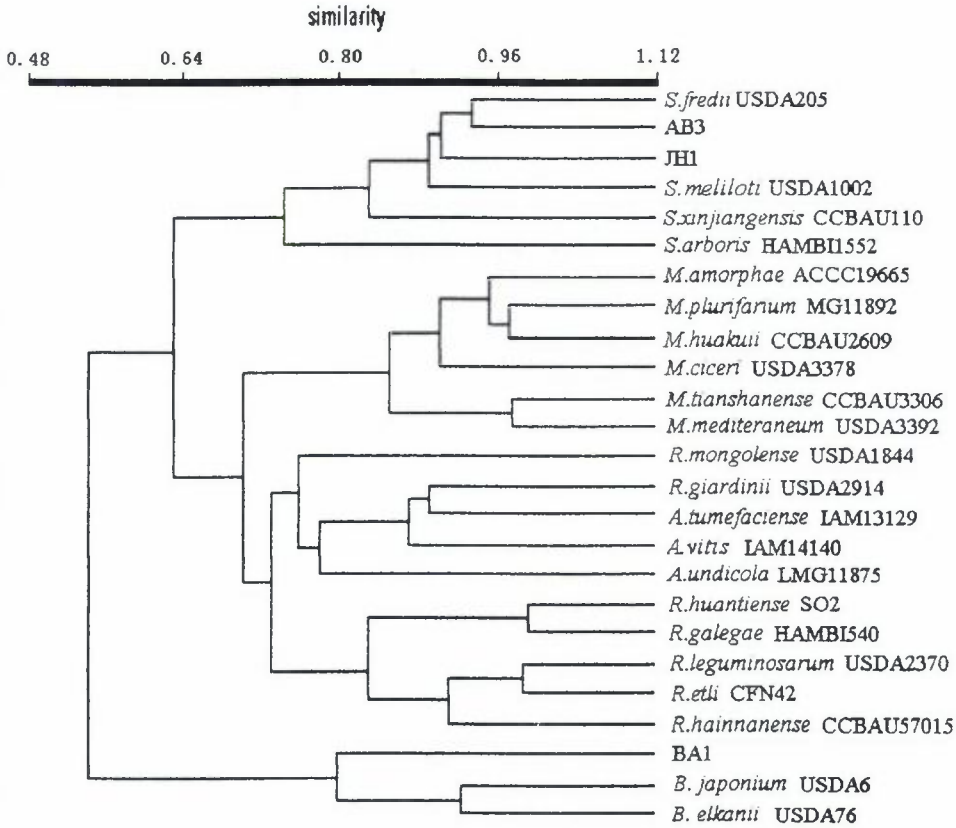

Figure 3. Dendrogram showing the preliminary phylogenetic positions based on RFLP patterns of PCR-amplified 16S rRNA gene. 16S rDNA restriction fragments were compared with those of the type species of *Sinorhizobium, Rhizobium* and *Bradyrhizobium* species. The dendrogram was constructed using the same method as for Fig. 1. Clustering analysis was performed using UPGMA method from fragments shared by each strain pair.

genotypes of the isolates. Clustering analysis based on results of REP-ERIC PCR revealed two groups at 28% similarity level corresponding to *Sinorhizobium* and *Bradyrhizobium* (Fig. 5). Group A were further divided into five subgroups including USDA205 at 62% similarity level. Strain CCBAU110 was clustered into a separate group at a level of 45%. Group B was also further divided into three subgroups at 64% similarity level. Strain USDA76 was linked to these subgroups at a level of 52%. Subgroup 1 including ten isolates and USDA6 could be further separated into two clusters. The isolates of cluster a were all isolated from Weifang (Shandong Province). Similarly, cluster 3 could also be separated into two clusters: isolates of cluster a were all isolated from Anyang (Henan

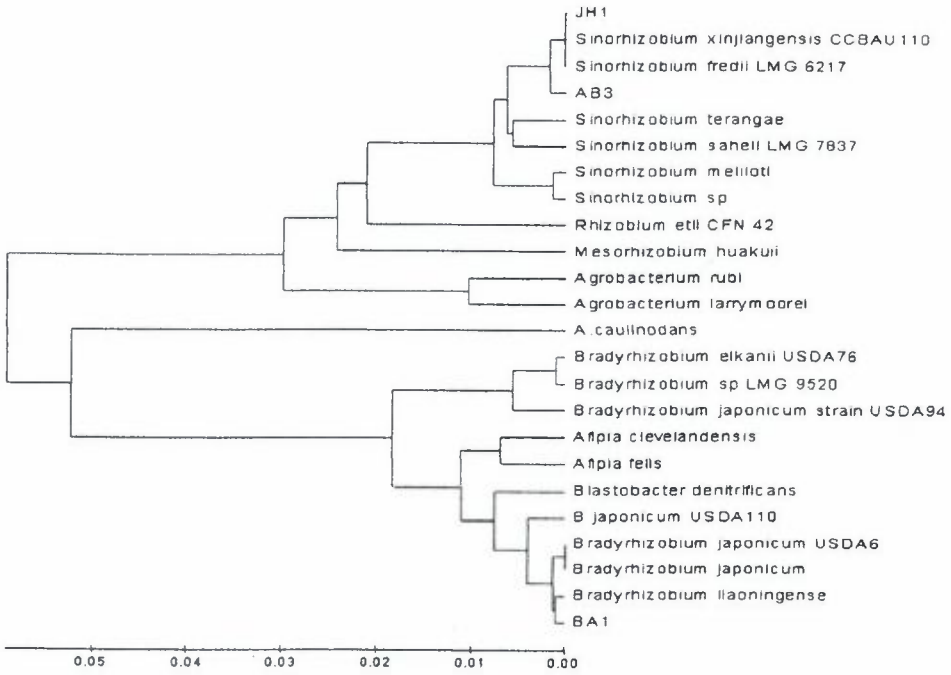

Figure 4. Phylogenetic tree of the α-subclass proteobacteria obtained by using complete 16S rRNA gene sequences. The length of the aligned sequence was 1,380 bases. The sequences were aligned using clustal W program and neighbour-joining method were used.

Province) and cluster b from Yanglin (Shanxi Province). Cluster 2 includes only isolates BJ4, LS1, DS8, and *B. japonicum* USDA110. According to the above results, the similarity of isolates appeared to be more closely related with geographical origins (e.g., subgroup 1 of group A and subgroup 3 of group B) than host plant differences. The existence of distinct subgroups and clusters within group A and B at lower similarity level (<75%) showed the high level genetic divergence among the Chinese soybean rhizobia.

G+C mol% of DNA and DNA-DNA hybridization

Eight representative isolates and three type strains were chosen and the results are listed in Table 3. The G+C mol% of five fast-growing isolates varied from 60.2 to 62.7 mol%, and for three slow growers from 60.8 to 63.5 mol%. The high degree of DNA hybridization (73–90%) between five fast-growing rhizobia and USDA205 are summarized in Table 4. Low DNA hybridization

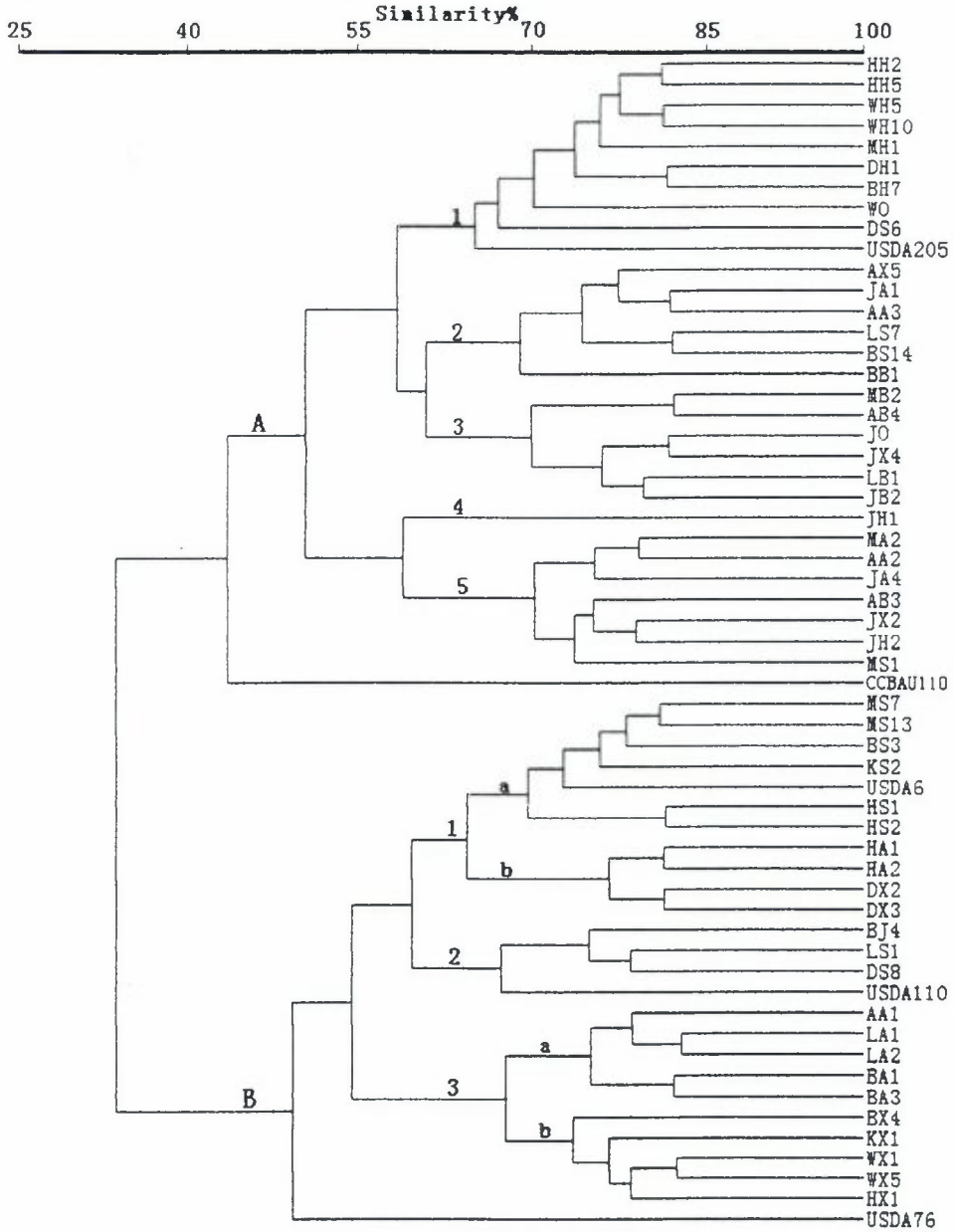

Figure 5. Dendrogram of isolates and type strain *S. fredii*, *S. xinjiangense*, *B. japonicum* and *B. elkanii* based on results of REP-ERIC PCR using UPGMA algorithm. The tree which was constructed using UPGMA method.

Table 3. Tm and G+Cmol% of representative strains tested.

Strains	Tm (°C)	G+Cmol%	Strains	Tm (°C)	G+Cmol%
HH2	76.3	60.2	MS7	77.8	63.2
AA3	76.9	61.3	LS1	76.6	60.8
JB2	77.2	62.0	BA1	77.9	63.5
JH1	77.3	62.2	USDA110	76.3	60.2
AB3	77.5	62.7	USDA6	78.1	63.8
USDA205	77.3	62.3	USDA76	78.3	64.2
CCBAU110	78.5	64.7			

Table 4. DNA homology between representative strains.

	DNA homology of strains (%)			DNA homology of strains (%)		
Strains	USDA205	CCBAU110	Strains	USDA110	USDA6	USDA76
HH2	82	54	MS7	75	93	21
AA3	90	57	LS1	92	76	28
JB2	85	43	BA1	72	75	33
JH1	76	55				
AB3	73	27				

with CCBAU110 was observed (less than 57%). Similarly, DNA homologies between slow-growing isolates and USDA6 or USDA110 were from 72–93%, while only less than 33% homology was observed with USDA76. Hybridization between slow-growing isolates and *B. liaoningense* was not performed since its type strain was not available.

4. Discussion

A polyphasic approach was used to study the taxonomic relationships of 52 soybean isolates obtained from China. Generally, the results obtained by using different methods agreed with each other. The 52 isolates tested were divided into two main groups corresponding to *Sinorhizobium* and *Bradyrhizobium*. The isolates were variable in their host specificity even if they belonged to the same group or subgroup. Strains isolated from Aijiaozao, Houzimao and Jindou 19 were ineffective on soybean cultivar Williams and Heinong 33. Keyser and Cregan (1987) also reported that *B. japonicum* serogroup 123 isolates only can

nodulate on North American soybean cultivar Clark. But the *Bradyrhizobium* spp. (TGx) isolates were found to be ineffective on the North American soybeans (Abaidoo et al., 2000). Some *S. fredii* strains formed effective nodules on the Asian soybean cultivar Peking, but they could only form ineffective nodules on North American commercial cultivar McCall (Keyser et al., 1982).

The effectiveness of 16S rDNA PCR-RFLP analysis used for preliminary estimation of phylogenetic classification has been demonstrated previously (Laguerre et al., 1994). The PCR-RFLP analysis of our study separated *Sinorhizobium* and *Bradyrhizobium* genera at 50% similarity level. Sequencing of the 16S rRNA gene is commonly used for the reconstruction of bacterial phylogenies (Maidak et al., 1994). Stackebrandt and Goebel (1994) reported that the difference was above species level when the similarity of 16S rDNA sequence was less than 97%, however the DNA homology values varied from 10 to 100%, while the similarity of the 16S rDNA sequence was more than 97%. Lateral gene transfer is known to occur at this level, while 16S rRNA genes show relatively little divergence (Sullivan et al., 1996; Haukka et al., 1996). Evidence was also obtained by van Berkum et al. (2003) that phylogenies based on 16s rDNA sequences were significantly different from those of the ITS region and 23S rDNA sequences. Considering the limitation of 16S rDNA sequence, they suggested that analysis of a wider variety of loci and comparative analytical methods should be used in taxonomic decisions. Other authors (Barrera et al., 1997; Urtz and Elkan, 1996; Willems et al., 2001) also suggested that, in addition to the 16S rDNA sequence analysis, other methods showing higher resolution power or DNA-DNA hybridization are needed to study relationship. According to the phylogenic tree only by 16S rDNA sequence analysis in our study, it is difficult to define the position of the isolates AB3, JH1, and AB1. Thus more data from other genetic information were needed to estimate their relationships among the species studied.

The results of REP-ERIC PCR analysis are highly discriminating for the identification of genetic diversity at the intraspecies level (De Bruijn, 1992; Laguerre et al., 1996; Van Berkum, 1990) and show high level genetic diversities among soybean rhizobia tested, because none of the isolates shared a similar REP-ERIC PCR fingerprint. The results of REP-ERIC PCR were consistent when compared to those obtained by phenotypic characterization. Strains isolated from the same soybean cultivars and the geographic regions were clustered together, except for a few which showed lower similarity (e.g., isolates HH2 and HH5, isolates WH5 and WH10). As for geographic regions, slow-growing rhizobia were closely shown in two dendrograms (e.g., isolates WX1, WX5, HX1, KX1 and BX4 were all isolated from Yanglin of Shanxi province and isolates AA1, LA1, LA2, BA1 and BA3 originated all from Anyang of Henan province).

However, in further analysis of the twenty-nine fast-growing strains, the results of the phenotypic characterization were inconsistent with those of 16S rDNA PCR-RFLP and REP-ERIC PCR, because the isolates JH1, WO and MS1, which belong to subgroup 2 of *Sinorhizobium*, were classified into different 16S rDNA genotypes and REP-ERIC PCR subgroups. Conversely, strains clustered into an identical subgroup of 16S rDNA and REP-ERIC PCR-RFLP were divided into different subgroups by phenotypic characterization (e.g., MS1 and JH2). The same 16S rDNA genotype can be also separated into different subgroups or clusters in the REP-ERIC PCR dendrogram. Thus the REP-ERIC PCR analysis is a suitable method to screen diversity in the phylogenetic groups defined by 16S rDNA PCR-RFLP analysis.

Because species are usually defined on the basis of DNA homology values (Van Berkum et al., 1998), DNA hybridization may be used to further study relationships in different REP-ERIC PCR subgroups. From our hybridization data, it is clear that there are different DNA hybridization levels among five fast-growing strains and USDA205 (73–90%), and also among three slow-growing strains, USDA6 and USDA110 (72–93%). A higher homology value (93%) between strains MS7 and USDA110 was obtained than between strains MS7 and USDA6 (75%), while the homology value (92%) between strains LS1 and USDA110 was higher than between LS1 and USDA6 (76%). Furthermore, it is remarkable, that strain MS7 and USDA6, LS1 and USDA110 were clustered together while they are in different subgroups in REP-ERIC PCR dendrogram. The diversities in classification of the strains USDA110 and USDA6 are also reported by other authors based on DNA homology (Hollis et al., 1981), on fatty acid methylester analyses (De Bruijn, 1992), on serology (Date and Decker, 1965) and on 16S rRNA gene and ITS region sequences (Van Berkum and Fuhrman, 2000).

It has been shown that a higher resolution could be obtained by using multi-combined typing methods. Here different typing methods were used and reliable and similar clusters were generated. Shortcomings of the phenotypically based methods have led to the development of many DNA-based techniques that minimize problems with reproducibility (Olive and Bean, 1999). RFLP typing give an ideal method for the differentiation among the members of well-defined species. This places strains in easily recognizable groups. PCR amplification using REP-ERIC primers which generated the more complex fingerprint confirmed REP-ERIC PCR as the most powerful typing method. But it has been argued whether the reproducibility of REP-ERIC-PCR depended on the experimental conditions. So it is very important to optimize experimental conditions and standard parameters such as templet concentration and primers, annealing temperature, type of *Taq* DNA polymerase, $MgCl_2$ concentration and the thermocycler used (Tyler et al., 1997). According to our results, REP-ERIC was the most discriminatory. The combination of other

methods showed a tendency to group according to their geographical origin.

Based on phenotypic characteristics, host specificity, 16S rDNA PCR-RFLP, 16S rDNA sequencing, REP-ERIC-PCR fingerprints and DNA-DNA hybridization we conclude that twenty nine strains of fast-growing rhizobia were all clustered together with *S. fredii* USDA205 and *S. xinjiangensis* CCBAU110, twenty three strains of slow-growing rhizobia were highly related with *B. japonicum* and *B. liaoningensis*.

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