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**Gustavo Saldaña · Virginia Martinez-Alcántara
José M. Vinardell · Ramón Bellogín · José E. Ruíz-Sainz
Pedro Alberto Balatti**

Genetic diversity of fast-growing rhizobia that nodulate soybean (*Glycine max* L. Merr)

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Abstract The fast-growing *Rhizobium* sp. strain NGR234, isolated from Papua New Guinea, and 13 strains of *Sinorhizobium fredii*, isolated from China and Vietnam, were fingerprinted by means of RAPD, REP, ERIC and ARDRA. ERIC, REP and RAPD markers revealed a considerable genetic diversity among fast-growing rhizobia. Chinese isolates showed higher levels of diversity than those strains isolated from Vietnam. ARDRA analysis revealed three different genotypes among fast-growing rhizobia that nodulate soybean, even though all belonged to a subcluster that included *Sinorhizobium saheli* and *Sinorhizobium meliloti*. Among *S. fredii* rhizobia, two strains, SMH13 and HH303, might be representatives of other species of nitrogen-fixing organisms. Although restriction analysis of the *nifD–nifK* intergenic DNA fragment confirmed the unique nature of *Rhizobium* sp. strain NGR234, several similarities between *Rhizobium* sp. strain NGR234 and *S. fredii* USDA257, the ARDRA analysis and the full sequence of the 16S rDNA confirmed that NGR234 is a *S. fredii* strain. In addition, ARDRA analysis and the full sequence of the 16S rDNA suggested that two strains of rhizobia might be representatives of other species of rhizobia.

Keywords *Sinorhizobium fredii* · Genotype · Genetic diversity · PCR · Symbiosis · Soybean

G. Saldaña · V. Martinez-Alcántara · P. A. Balatti (✉)
Instituto de Fisiología Vegetal,
Universidad Nacional de La Plata,
Cátedra de Microbiología Agrícola,
Facultad de Ciencias Agrarias y Forestales,
CC327 La Plata 1900 , Argentina
Tel.: +54-221-4233698, Fax: +54-221-4233698,
e-mail: pbalatti@isis.unlp.edu.ar

J. M. Vinardell · R. Bellogín · J. E. Ruíz-Sainz
Departamento de Microbiología, Facultad de Biología,
Universidad de Sevilla, Apdo 1095, 41080 Sevilla, Spain

Introduction

Soybean [*Glycine max* (L.) Merr], like other legumes, fixes atmospheric nitrogen in association with gram-negative soil bacteria of the genera *Bradyrhizobium* and *Sinorhizobium*. Members of the genus *Bradyrhizobium*, known for a long time as the sole symbiont of soybean (Fred et al. 1932), include slow-growing gram-negative bacteria with a long generation time (6–8 h) that alkalinize the media when grown in vitro and carry nodulation and nitrogen fixation genes on the chromosome (Jordan and Allen 1984).

Beginning in 1980, several researchers reported the isolation of fast-growing organisms (2–4 h generation time) that developed nitrogen-fixing nodules on soybean (Trinick 1980; Keyser et al. 1982; Dowdle and Bohlool 1985; Cleyet-Marel 1987). These new rhizobia initially were included in the genus *Rhizobium,* but are now known as the species *Sinorhizobium fredii* (Scholla and Elkan 1984; Chen et al. 1988; Young et al. 1988). Members of this genus acidify the media when grown in vitro and carry nodulation and nitrogen-fixation genes on symbiotic plasmids.

Fast-growing rhizobial isolates interact with soybean in a different manner. Trinick (1980) isolated the fast-growing *Rhizobium* sp. strain NGR234 from *Lablad purpureus*. This strain produces non-nitrogen-fixing nodules on soybean (Balatti et al. 1995). Keyser et al. (1982), Dowdle and Bohlool, (1985) and Cleyet-Marel (1987) isolated from the soils of China and Vietnam fast-growing organisms that nodulate soybean, some of them in a cultivar-specific manner. Based on physiological characteristics, some of the fast-growing strains were initially thought to belong to a transitional group. However, Rodriguez-Navarro et al. (1996) suggested that they are strains of *S. fredii* on the basis of the sequence of a hypervariable region of the 16S RNA, host specificity, lipopolysaccharide (LPS) profile, antibiotic resistance, salt tolerance, and restriction fragment length polymorphism (RFLP) analysis.

Several molecular methods have been used to examine genetic relationships of closely related rhizobial strains. These include allozyme and RFLP analysis (Eardly et al. 1990; Demezas et al. 1991), pulsed-field gel electrophoresis-fingerprinting (Huber and Selenska-Pobell 1994), DNA–DNA hybridization analysis (Van Berkum et al. 1993); fatty acid analysis (Jarvis and Tigue 1994); DNA fingerprinting, polymerase chain reaction (PCR); and RFLP analysis (Kuykendall et al. 1992). Some of the fingerprinting strategies with PCR include random amplified polymorphic DNA (RAPD) (Nieman et al. 1997; Sikora et al. 1997; Vinuesa et al. 1998), enterobacter repetitive intergenic consensus (ERIC), and repetitive extragenic palindromic elements (REP) (De Bruijn 1992; Selenska Pobell 1994). These methods are useful tools for phylogenetic and diversity studies, each providing information on different parts of the genome.

Sequence analysis of the 16S RNA gene is a powerful method for assigning strains to species, provided the relevant species are represented in the 16S RNA sequence database (Fox et al. 1992). A somewhat simpler though still informative approach is amplified rDNA restriction analysis (ARDRA), which has been described as a useful tool to clarify the taxonomy of several organisms (Heyndrickx et al. 1996), although it has been found that bacteria may have multiple copies of the 16S RNA gene (Van Berkum 1998).

Fast-growing rhizobia isolated from geographically distinct locations have been only partially characterized. Sadowsky et al. (1983) and Stowers and Eaglesham (1984) described physiological and symbiotic characteristics of fast-growing rhizobia isolated from the soils of China. Dowdle and Bohlool (1985) and Cleyet-Marel (1987) and Buendía Claveria et al. (1989) also characterized those rhizobia isolated from the province of Hubei in Honghu County, China (HH serie) and Vietnam (SMH and SX series). Sadowsky et al. (1987) and Krishnan and Pueppke (1994) studied the antigenic relationship of fast-growing rhizobia.

Using REP, ERIC and RAPD PCR, we fingerprinted the genomes of 12 representative strains of fast-growing rhizobia, *S. fredii* isolated from each of three geographical distant places, and *Rhizobium* sp. strain NGR234. In addition, we also analyzed the 16S rDNA of fast-growing rhizobia and closely related bacteria initially by means of ARDRA and later by sequencing the full-length 16S rDNA. The objective of this work was: (a) to establish the genetic diversity among *Rhizobium* sp. strain NGR234 and fast-growing rhizobia that nodulate soybean; (b) to test whether diversity among rhizobial isolates at one site is comparable to that at another site; (c) to confirm that *Rhizobium* sp. strain NGR234 and other fast growing bacteria are *S. fredii* strains.

Materials and methods

Rhizobial strains

Sinorhizobium fredii strains were provided by Dr. Ruiz Sainz (Universidad de Sevilla, Spain) and Dr. Steven Pueppke (University of Missouri, USA). The strains were cultured in liquid yeast extract-mannitol broth (YEM) (Vincent 1970) in a rotary shaker at 30 °C and 150 rpm. Forty-eight-h-old cultures were supplemented with 7% glycerol, frozen in liquid nitrogen, and kept in the freezer at –70 °C as stock cultures. Strains were maintained in YEM agar slopes at 5 °C either in YEM or TY (Beringer 1974). The list of fast-growing rhizobia used in the experiments is presented in Table 1.

Genomic DNA

DNA was isolated as described by Meinhardt et al. (1993). Briefly, 25-ml 4-day-old cultures were centrifuged 10 min at 12,300×*g* at 5 °C. After discarding the supernatant, the pellet was resuspended

Table 1 Rhizobial strains from the genus *Sinorhizobium, Rhizobium*, *Mesorhizobium* and *Bradyrhizobium* that were used in this work

Strain	Place of origin	
Rhizobium spp. NGR234	Papua, New Guinea	Trinick (1980)
Sinorhizobium fredii		
USDA257	China	Keyser et al. (1982)
USDA205	China	Scholla and Elkan (1984
USDA196	China	Keyser et al. (1982)
USDA192	China	Keyser et al. (1982)
USDA191	China	Keyser et al. (1982)
HH103	China	Dowdle and Bohlool (1985)
HH102	China	Dowdle and Bohlool (1985)
HH303	China	Dowdle and Bohlool (1985)
HH003	China	Dowdle and Bohlool (1985)
SMX11	Vietnam	Cleyet-Marel (1987)
SMH12	Vietnam	Cleyet-Marel (1987)
SMH13	Vietnam	Cleyet-Marel (1987)
SMH15	Vietnam	Cleyet-Marel (1987)
Sinorhizobium saheli USDA4993	Senegal	De Lajudie et al. (1994)
Sinorhizobium meliloti USDA1002	USA	Dangeard (1926)
Mesorhizobium ciceri USDA3383	Spain	Nour et al. (1994)
Mesorhizobium loti USDA3471	New Zealand	Jarvis et al. (1982)
Bradyrhizobium japonicum E109	Argentina	INTA, Argentina

in lysis buffer consisting of TE (pH 8.0) with 10% sarkosyl (Sigma). The cell suspension was then treated with digested pronase (Sigma) for 1 h. DNA was treated three times with phenol-chloroform and once with chloroform. DNA was precipitated by adding isopropanol and 3 M sodium acetate to the remaining aqueous phase. After storage overnight at –20 °C, the DNA was pelleted in a microfuge (Fisher V235) and resuspended in 100 µl of sterilized distilled water.

Polymerase chain reaction

PCR was carried out with REP, ERIC and RAPD primers (DNAgency, Malvern, Penn., USA). ERIC and REP primers sequences were according to de Bruijn (1992). The sequences of the RAPD primers selected at random were OPA02 5′ tgccgagctg3′, H04 5′ggaagtcgcc′3; Oligo124 5′attgcgtccgag′3. All PCR reactions were carried out in a Thermolyne thermocycler in a final volume of 15 µl. Amplification reactions with REP and RAPD primers contained 30 ng of template DNA. The reaction buffer was made up of 1.5 mM $MgCl_2$, 50 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25 °C), 0.1% Triton X-100, 50 mg BSA ml^{-1}. The reaction mixture also contained 0.2 mM dNTPs, 1 U *Taq* DNA polymerase (Promega Biotech.) and 30 pmol primer.

ERIC amplifications were carried out under similar conditions except that the buffer contained 2.5 mM $MgCl_2$. The temperature cycling used for REP-PCR and ERIC-PCR was as described by De Bruijn (1992). PCR products were analyzed by horizontal electrophoresis in 2% agarose gels in Tris-Borate-EDTA buffer (1×).

ARDRA and 16S rDNA full sequence

Escherichia coli 8–27 forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer 1522–1541 5′-AAGGAGGTGATCCAGCCGCA-3′ were used to amplify nearly full-length 16S DNA genes (Field et al. 1997). PCR was done in a 100-µl reaction mixture containing 1× PCR buffer, 1.5 mM $MgCl_2$, 5% dimethylsulfoxide, 200 µM each nucleotide (Promega Biotech), 15 pmol of each primer, 1 U of *Taq* polymerase, and 50 ng of purified template DNA. The temperature profile was as follows: initial denaturation at 95 °C for 3 min 30 s; 35 cycles of denaturation at 94 °C for 1 min 10 s, annealing at 57 °C for 45 s, and extension at 72 °C for 2 min 10 s, and a final extension at 6 min 10 s. PCR products were precipitated by adding 2.5 vol of ethanol and 10% sodium acetate 3 M. The PCR products were resuspended in TE, pH 8.0. Aliquots of the PCR products were digested with *Alu*I, *Hin*fI, *Hae*III and *Rsa*I (Promega Biotech) as recommended by the manufacturer.

16S rDNA generated by PCR was sequenced. Ten to 20 ng of template DNA were amplified in a 100-µl reaction containing 0.2 mM dNTPs, 15 pmol of each primer, 2.5 U *Taq* polymerase (Amersham Pharmacia), 50 mmol KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0. The reactions were carried out in a Thermocycler PTC-100 (MJ Research) programmed as described above. DNA fragments obtained by PCR were purified by a PCR Clean Up Kit (Roche) and sequenced by the dideoxynucleotide method described by Sanger et al. (1977). Sequences were confirmed by sequencing them three times. Individual sequences were assembled by means of the GCG Sofware (University of Wisconsin) (Devereux et al. 1984) and aligned by means of BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

PCR of the intergenic sequence *nifD–nifK* DNA fragment

The *nifD–nifK* fragment was amplified with primer FGPD807 5′-CACTGCTACCGGTCGATGAA-3′, corresponding to the *nifD* sequence conserved among nitrogen-fixing bacteria (Jamann et al. 1993), and reverse FGPK492 5′-GATGACCTCGGCCAT-3′ (Laguerre et al. 1996). The 100-µl PCR reaction mixture contained 1× PCR buffer, 1.5 mM $MgCl_2$, 5% dimethylsulfoxide, 200 µM each nucleotide (Promega Biotech), 15 pmol each primer, 1 U *Taq* polymerase, and 50 ng purified template DNA. The temperature profile was identical to the profile used for the 16S rDNA amplification.

Data analysis

The sizes of the DNA bands were estimated by comparison with the 1-kb ladder marker (Promega Biotech). Bands were recorded in the binary form i.e. (1)=presence and (0)=absence and assembled in a data matrix table. The UPGMA algorithm was used for hierarchical cluster analysis. Pairwise comparisons were calculated using Simple Matching (Sm) coefficient. In addition, a dendrogram was built by using NTSYS-pc package (Rohlf 1990).

The dendrogram based on the 16S rDNA full sequence was produced by the neighbor-joining method in ClustalW version 1.81, and the results were ploted by means of NJPlot WIN95.

Results

Analysis of the fingerprint patterns obtained with ERIC, REP and RAPD PCR amplification revealed a considerable genetic diversity among fast-growing rhizobial strains.

In Fig. 1 fingerprints of fast-growing rhizobia generated with ERIC (Fig. 1a), REP (Fig. 1b) and RAPD (Fig. 1c) primers are presented. The results were analyzed by UPGMA and by principal coordinate analysis (PCOORD) (Rohlf 1990). Both methods generated similar results; therefore, only those of the cluster analysis are presented. The reproducibility of each PCR genomic fingerprinting protocol was confirmed by repeating the amplifications at least three times. Cluster analysis based on the Dice coefficient yielded a significantly high cophenetic correlation coefficient (CCC) suggesting that the level of distortion between the similarity matrix and cluster analysis was low. The CCC for the cluster analysis was 0.92 for REP and ERIC and 0.86 for RAPD.

Amplification of genomic DNA with ERIC primers generated five to eight DNA fragments for each strain tested and the sizes of the DNA bands ranged from 0.1 to 2.0 kb (Fig. 1a).The amplification with REP primers generated a more complex amplification pattern than ERIC PCR (Fig. 1b). Each strain generated 7–14 bands that ranged in size from 0.1 to 2.8 kb. The fingerprints generated with three RAPD primers (OPA02, H04, Oligo124) were used for cluster analysis, and the relationship established among the isolates was similar to those found based on REP PCR fingerprints. In Fig. 1c, a representative PCR reaction with primer Oligo124 is presented.

Since the analysis of the fingerprints obtained with REP, ERIC and RAPD generated very similar dendrograms, the amplification pattern obtained with each protocol was analyzed in a unique matrix. This analysis resulted in the dendrogram presented in Fig. 2. The CCC obtained with the Dice coefficient was higher than 0.90. The dendrogram did not differ significantly from those generated by any of the PCR fingerprinting methods alone. Strain USDA257 was clustered separately from other fast-growing rhizobia, showing a similarity index of 62%. The cluster that included the rest of the strains showed three main groups. One cluster included only strain NGR234. Cluster 2 grouped all the strains isolated from the soils of

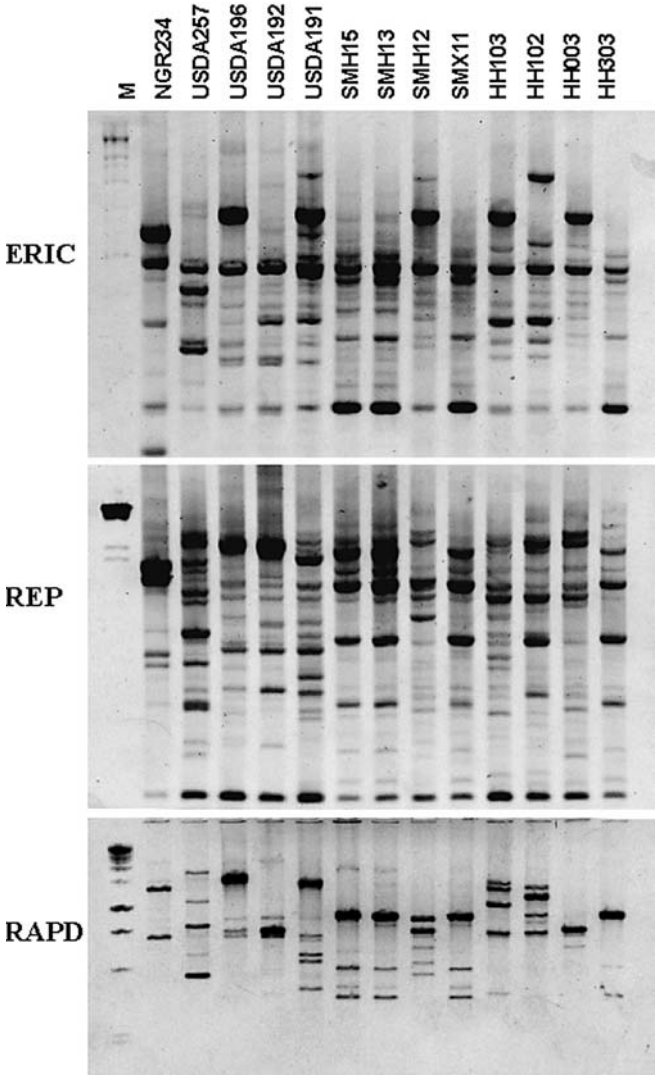

Fig. 1 ERIC, REP and RAPD fingerprints of *Rhizobium* sp NGR234 and *Sinorhizobium fredii* strains isolated from three different geographical areas. Fingerprints are representative of three replicates using the same genomic DNA as template source. RAPD analysis was done with primer 02. USDA strains were isolated from East central China; HH strains were isolated from Central China; SMX and SMH strains were isolated from nodulated soybean plants growing in Vietnam

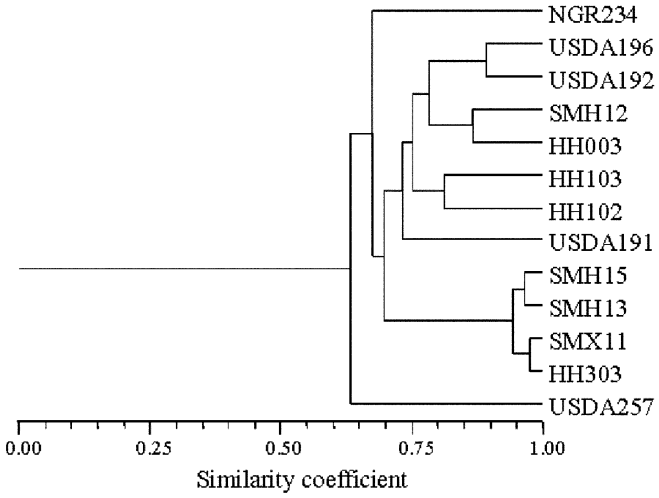

Fig. 2 Cluster analysis of fast-growing rhizobia isolated from different geographical areas. The cluster was built based on the matrix generated with DNA fragments amplified by ERIC, REP and RAPD PCR. The coefficient used was Simple Matching (Sm) and the CCC value was 0.95

Table 2 The 16S rRNA restriction pattern of fast-growing rhizobia and closely related species obtain with four restriction enzymes

	Restriction enzyme			
	*Hin*fI	*Hae*III	*Rsa*I	*Alu*I
Rhizobium sp. strain NGR234	HiB	HaA	RB	AI
Sinorhizobium fredii				
USDA205(type strain)	HiB	HaA	RB	AI
USDA191	HiB	HaA	RB	AI
USDA192	HiB	HaA	RB	AI
USDA196	HiB	HaA	RB	AI
USDA257	HiB	HaA	RB	AI
HH003	HiB	HaA	RB	AI
HH102	HiB	HaA	RB	AI
HH103	HiB	HaA	RB	AI
HH303	HiD	HaA	RB	AII
SMX11	HiD	HaA	RB	AII
SMH12	HiD	HaA	RB	AII
SMH13	HiC	HaA	RB	AIII
SMH15	HiD	HaA	RB	AII
Sinorhizobium saheli	HiB	HaA	RB	AI
Sinorhizobium meliloti	HiB	HaA	RB	AI
Mesorhizoprhizobium ciceri	HiA	HaA	RB	AIV
Mesorhizobium loti	HiE	HaA	RB	AIV
Bradyrhizobium japonicum	HiB	HaB	RA	AV

China and strain SMH12. Cluster 3 included highly related fast-growing rhizobia as suggested by the high similarity index of 92%. This cluster comprised strains isolated from Vietnam as well as strain HH303.

Full-length DNA 16S rDNA of 13 *S. fredii* strains and *Rhizobium* sp. strain NGR234 were amplified by PCR and the products digested with *Hin*fI, *Rsa*I, *Alu*I or *Hae*III. These enzymes generated three different restriction patterns (Table 2). The sizes of the fragments were estimated by comparing the restriction analysis with type strains of the species in the USDA culture collection (USDA) and by summing up the restriction fragments to make up the full-length 16S rDNA.

Because we were concerned about the identity of fast-growing rhizobia, 16S rDNA restriction analyses of fast-growing rhizobia were compared with those of closely related species, such as *Sinorhizobium saheli, Sinorhizobium meliloti, Mesorhizobium ciceri, Mesorhizobium loti* and *Bradyrhizobium japonicum* (Table 1). A 1,400-bp fragment of the 16S rDNA was amplified by PCR and restricted with endonuclease *Hin*fI, *Rsa*I, *Alu*I or *Hae*III. Five different restriction patterns were identified (Table 2) Restriction pattern type I included *Rhizobium* sp. strain NGR234, *S. fredii* strains USDA205, USDA191, USDA192,

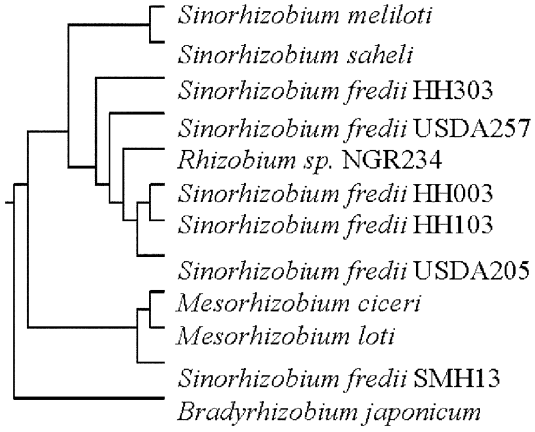

Fig. 3 Dendrogram generated based on the full 16S rDNA sequence by using the neighhbor-joining method of the ClustalW, version 1.81, software. Results were ploted using NJPlotWin95. The Genbank accesion numbers for the 16S rDNA sequences of *Rhizobium* sp. strain NGR234, *S. fredii* strains HH003, HH103, HH303, SMH13, USDA205 and USDA 257 are AY260147, AY260144, AY260145, AY260146, AY260148, AY260149 and AY260150, respectively

Table 3 *nifD–nifK* restriction patterns of fast-growing rhizobia isolated from the soils of China, Vietnam and Papua, New Guinea, revealed by RFLP analysis of PCR-amplified *nidD-nidK*. The combination of the restrictions of the enzymes is represented in the restriction pattern column. *A*, *B* and *C* indicate different restrictions patterns obtained with *Hae*III. *D*, *E* and *F* stand for different restriction patterns obtained with *Rsa*I

Rhizobia strains	DNA size (bp)	*Hae*III	*Rsa*I	Restriction pattern[a]
NGR234	850	A	D	I
USDA257	1200	B	E	II
USDA196	1200	B	E	II
USDA191	1200	B	E	II
SMH15	1200	B	E	II
SMH12	1200	B	E	II
SMH11	1200	B	E	II
HH103	1200	B	E	II
HH102	1200	B	E	II
SMH13	1200	C	F	III
HH003	1200	C	F	III

[a]The nifD–nifK restriction pattern is the result of combining the results obtained with two restriction enzymes

USDA196, USDA257, HH003, HH102, HH103, *Sinorhizobium saheli*, and *Sinorhizobium meliloti*. Restriction type II included 4 *S. fredii* strains SMX11, SMH12, SMH15 and HH303. Restriction type III corresponded to fast-growing strain SMH13. Restriction type IV included *Mesorhizobium loti* and *Mesorhizobium ciceri* while restriction type V included only *Bradyrhizobium japonicum*.

The full-length 16S rDNAs of *Rhizobium* sp. strain NGR234, *S. fredii* type strain USDA205, and *Sinorhizobium* strains HH003, HH103, HH303, USDA257 and SMH13 were sequenced and the sequences compared with those of *Sinorhizobium saheli, Sinorhizobium meliloti, Mesorhizobium ciceri, Mesorhizobium* loti and *Bradyrhizobium japonicum*. The sequence similarity found between the 16S rDNA genes of *S. fredii* type strain USDA257 and fast-growing rhizobia strain NGR234, SMH13, HH303, USDA257, HH003 and HH103 was 99.78%, 95.71%, 99.22%, 99.92% and 100%, respectively. Based on these sequences, a dendrogram was generated (Fig. 3) and it grouped the strains in two main clusters. One included all *Sinorhizobium* species and *Mesorhizobium* species, while the other cluster included only *Bradyrhizobium japonicum*. Furthermore, *S. fredii*, *Sinorhizobium saheli* and *Sinorhizobium meliloti* were all clustered together and apart from *Mesorhizobium ciceri*, *Mesorhizobium loti* and *S. fredii* strain SMH13 (Fig. 3)

In addition to the 16S rDNA, the *nifD–nifH* intergenic sequences of *S. fredii* strains and *Rhizobium* sp. strain NGR234 were amplified, yielding a 1,200-bp fragment and an 850-bp fragment, respectively (Table 3). The 1,200-bp fragment was digested with restriction endonucleases; the *S. fredii* strains generated only two different restriction patterns. One included only *S. fredii* strains HH003 and SMX13. The other restriction pattern included the remaining *S. fredii* strains. The 850-bp *nifD–nifH* intergenic sequence of strain NGR234, as expected, had a different restriction pattern (Table 3).

Discussion

Although more complex DNA fingerprints should generate more confident results, the DNA typing methods such as ERIC, REP or RAPD, used alone or in combination, generated reliable and similar clusters with high cophenetic correlation coefficient (CCC) (Fig. 1 and Fig. 2). A high CCC suggests a better fitness between the similarity matrix and the phenogram.

PCR amplification using REP primers generated the more complex fingerprint and, together with ERIC PCR, the more confident one since the CCC was 0.92. The dendrogram built with RAPD-generated fingerprints was similar to those obtained with ERIC or REP, but RAPD-based analysis had a lower CCC value. The analysis of all DNA fragments amplified by REP, ERIC, and RAPD together generated a matrix that resembled those constructed from the fingerprint patterns generated by only one PCR method (Fig. 2). Considering the number of bands used, the CCC value was, as expected, higher than when calculated based either on ERIC, REP, or RAPD alone. Therefore, our discussion will be based on the dendrogram built by combining REP-ERIC-RAPD data (Fig. 2).

The levels of diversity of rhizobia have been found to be unrelated to the geographical sites of isolation. Geniaux et al. (1993) and Wong et al. (1994) analyzed diversity among isolates of *Bradyrhizobium japonicum* and rhizobia, respectively and found that strains isolated from distant places showed similar levels of diversity compared to rhizobia isolated from close places. Vietnamese strains

and Chinese strain HH303 had the highest levels of similarity (about 95%). Among Vietnamese strains, only strain SMH12 always clustered with strains isolated from China, although it shares with other Vietnamese strains, e.g. strains SMX11, SMH13, and SMH15, the same soybean host range and LPS profile. Furthermore, strains SMH12 and SMX11 were shown to have identical plasmid profiles, to use the same carbon sources, and to be resistant to the same antibiotics (Rodriguez-Navarro et al. 1996). The small size of the geographical area in which strains were collected might be an explanation for the higher level of similarity between Vietnamese strains.

Fast-growing rhizobial strains from China, USDA257, USDA196, USDA192, USDA191 HH003, HH102, HH103, and strain SMH12 from Vietnam were clustered together. The level of diversity found in this group was approximately 25%, much higher than that found among Vietnamese strains. However, some of these strains, e.g. USDA191, HH type strains and strain SMH12, share an unrestricted nodulation ability on soybean. All of them utilized the same carbon sources when cultured in vitro and showed the same antibiotic resistance. However, they differ in their LPS profile and their ability to synthesize melanin (Buendía-Claveria et al,.1989; Cleyet-Marel, 1987; Rodriguez-Navarro et al. 1996). Therefore, among fast-growing rhizobia the level of diversity varied.

The ability of fast-growing rhizobia to nodulate and fix nitrogen on soybean cultivars was unrelated to the geographical place of isolation. Vietnamese strains SMH15, SMH13, SMX11, and strain HH303 clustered together with a similarity coefficient of 95% and nodulated and fixed nitrogen on all cultivars tested. Chinese strains, including the HH and USDA isolates, were clustered together and included both strains that are cultivar-specific or that are fully compatible with soybean. *Rhizobium* sp. strain NGR234, which is unable to form nitrogen-fixing nodules on soybean, and USDA257, which nodulates soybean in a cultivar-specific manner, formed two other clusters. The ability of these organisms to nodulate soybean might have been a character lost during adaptation of some of these rhizobia to different environments. Pueppke and Broughton (1999) recently demonstrated that strains NGR234 and USDA257 have the broadest host range known; thus, it is possible that these strains perpetuate themselves in the environment by nodulating alternative legumes. Under these circumstances, rhizobium genes that are indispensable for soybean nodulation might have been lost after generations of no interaction with soybeans. Balatti et al. (1995) previously stated that strain NGR234 might lack one or more genes necessary to form Fix$^+$ nodules on *Glycine max*.

Strains NGR234 and USDA257 are phylogenetically closely related, and most probably considerable parts of their genomes are identical. These strains have a number of similarities even though they were isolated from nodules developed on different legumes, growing in widely separated geographical locations (Trinick 1980; Keyser et al. 1982). Both strains have an extremely broad host range (Pueppke and Broughton, 1999); carry *nod* and *nif* genes on large sym(biosis) plasmids (Broughton 1984); have two *nodD* alleles, *nodD1* on a 3-kb *Eco*RI fragment and *nodD2* on a 6.0-kb *Eco*RI fragment (Kondorosi et al. 1986; Nayudu and Rolfe 1987; Krishnan and Pueppke 1991); and have common *nodABC* genes unlinked to *nodD1* (Bachem et al. 1985; Krishnan and Pueppke 1991). The *nodD1* alleles of the two strains are very similar and diverge by less than 10% (Young et al. 1988), the *nodABC* genes are 99% identical (Relic et al. 1994), and the *nolXWBTUV* loci are very similar (Balatti et al. 1995). However, we found that the genome fingerprints of these two strains are as similar as those of other *S. fredii* are from each other. Interestingly, strain USDA257 has a lower level of similarity than strain NGR234 has with other *S. fredii* strains. The dendrograms built based on the fingerprints of strains NGR234 and USDA257 showed that these two strains are 62% similar, based on the analysis of the REP-ERIC and RAPD fingerprints (Fig. 2). The level of diversity found between strains NGR234 and USDA257 suggest that they have diverse genomes that might be responsible for the differences in their ability not only to synthesize Nod factors (Price et al. 1992; Bec-Ferté et al. 1993) but also to carry the *nol*R allele on *Eco*RI DNA fragments of different sizes (Kiss et al. 1998), and to establish a symbiosis with legumes. Strain NGR234 has a broader host range than strain USDA257 (Pueppke and Broughton 1999), but cannot induce nitrogen-fixing nodules on soybean cultivars like other *S. fredii*, such as strain USDA257 (Balatti and Pueppke 1992; Balatti et al. 1995; Videira et al. 2001).

ARDRA analysis revealed five different restriction patterns of the 16S rDNA among fast-growing *Rhizobia* (Table 2). This was surprising because the partial sequence of the 260-bp hypervariable region of the 16S rDNA of *S. fredii* strains USDA257, SMH12, SMX11, and HH103 was found to be identical (Balatti, unpublished results; Rodriguez-Navarro et al. 1996). Vinuesa et al (1998) found that the 260-bp partial sequence of the 16S rDNA was in full agreement with the results obtained by ARDRA.

ARDRA analysis and the sequence similarity between the full sequence of the 16S rDNA of *S. fredii* type strain USDA205 and *Rhizobium* sp.strain NGR234 indicated that NGR234 is a *S. fredii* strain (Table 2 and Fig. 3). Furthermore, *S. fredii* strains HH003 and HH103 are more closely related to type strain USDA205 than *Rhizobium* sp. strain NGR234 and USDA257 are. These results should be interpreted cautiously since rhizobia have been found to have multiple copies of the 16S RNA gene (Van Berkum, 1998). However, amplification of the *nifD–nifH* intergenic DNA region generated a fragment of 1,200 bp irrespective of the *S. fredii* template DNA used, but of only 850 bp when the genomic template DNA was isolated from strain NGR234 (Table 3). Restriction of the intergenic *nifD–nifK* fragment suggested that the sequence of NGR234 is not similar to that of other *S. fredii* strains. Laguerrre et al (1996) amplified the *nifD–nifH* intergenic region and the majority of the *Rhizobium* strains they tested generated a 1,250-bp fragment; only *Bradyrhizobium japonicum* generated a 700-bp fragment.

Restriction analysis and sequence similarity between the 16S rDNA genes also showed that HH303 diverged from *S. fredii* type strain USDA205. Rehus et al. (1998, 1999) found that the LPS of strain HH303 belonged to an untyped serogroup while the rest of *S.fredii* produced typed LPS.

Another fast-growing isolate, strain SMH13, presented a restriction pattern and a full 16S rDNA sequence different from those of the rest of the fast-growing *S. fredii* strains. This strain was less related to *S. fredii* than to *Mesorhizobium loti* and *Mesorhizobium ciceri*. The sequence similarity of the 16S rDNA sequence of strain SMH13 with type strain USDA205 was only 95.71%. Restriction analysis of the *nifD–nifH* intergenic region also showed that in strain SMH13 this region is different from that of the rest of the *S. fredii* isolates and the sequence is identical to the sequence of *S. fredii* strain HH003 (Table 3). In addition, we were unable to amplify from SMH13 template DNA a 260-bp fragment (Videira et al. 2002) that allowed us to identify fast-growing rhizobia that nodulate soybean specifically (Balatti unpublished results).

Based on REP, ERIC and RAPD fingerprints we conclude that diversity among *S. fredii* strains is considerable. The level of diversity among Chinese isolates is higher than among the Vietnamese strains analyzed. Three different 16S rDNA restriction patterns were identified among *S. fredii* fast-growing rhizobia although these isolates formed part of a cluster that included other fast-growing rhizobia, such as *Sinorhizobium meliloti* and *Sinorhizobium saheli*. Based on these preliminary results, we hypothesize that some of the strains isolated from China and Vietnam, such as HH303 and SMH13, may not be representatives of *S. fredii*, but of other species of rhizobia that nodulate soybean. Other more informative analyses, e.g., DNA–DNA hybridization, are needed to confirm this hypothesis.

Although restriction analysis of the *nifD–nifK* intergenic DNA fragment confirmed the relatively unique nature of *Rhizobium* sp. strain NGR234, the similarities between this strain and *S. fredii* strain USDA257 as well as the full sequence of the 16S rDNA confirmed that strain NGR234 is a *S. fredii* strain.

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