Characterization of soybean rhizobia at different levels using PCR based techniques *

ZHANG XUE-XIAN^{1,2} MARTIN MUILENBURG² TAK AN LIE² ZHOU JUN-CHU¹ LI FU-DI¹

1 Department of Microbiology, Huazhong Agricultural University, Wuhan 430070, China

2 Department of Microbiology, Wageningen Agricultural University, Wageningen, The Netherlands

Abstract Nineteen standard USDA strains representing three recognized soybean rhizobia, *Sinorhizobium fredii*, *Bradyrhizobium japonicum* and *B. elkanii*, were examined by restriction fragment length polymorphism (RFLP) analysis of 16S rRNA genes amplified by polymerase chain reaction (PCR). Four composite genotypes were obtained from the combined data of the RFLP analysis with three endonucleases. *S. fredii*, *B. japonicum*, *B. elkanii* II and IIa reference strains fell into these four genotypes respectively. No genotype was shared by two species. Therefore, 16S rDNA PCR-RFLP is a rapid tool for the identification of soybean rhizobia. According to 16S rDNA PCR-RFLP, 22 fast-growing and 19 slow-growing soybean rhizobia strains from China were identified to *S. fredii* and *B. japonicum*, respectively.

The reference strains and wild type isolates were also examined by 16S~23S intergenic spacer PCR-RFLP analysis. PCR results indicated that the length of the intergenic region between *S. fredii* and *Bradyrhizobium* sp. is different. All fast-growing *S. fredii* strains produced one larger band (2.1 kb), and all slow-growing *Bradyrhizobium* strains produced one smaller band (2.0 kb). Based on 16S~23S RFLP analysis, 22 *S. fredii* isolates can be further separated into two "genotypes" that originated from two different regions in China; 19 *B. japonicum* strains from Heilongjiang Province (northeast of China) can be separated into 11 genotypes. These wild type isolates were also characterized at strain level by REP (repetitive extragenic palindromic) and ERIC (enterobacterial repetitive intergenic consensus) PCR fingerprinting.

Key words soybean, rhizobia, genetic diversity, restriction fragment length polymorphism

Introduction

The soybean, *Glycine max* (L.) Merr., originated from China, and now is cultivated worldwide. It plays a major role in the world food system. Nowadays, three groups of symbionts of this plant are recognized. One is slow growing *Bradyrhizobium japonicum*, which was firstly found and has been examined extensively for 100 years; another is fast growing *Sinorhizobium fredii* which was discovered just more than 10 years ago in some Chinese soils. The third is *Bradyrhizobium elkanii*, which represents a specialized group of slow growing rhizobia, and was proposed recently by Kuykendall et al. (1992) based on genetic analysis. According to growth speed and/or some physiological characteristics, it is very easy to distinguish *S. fredii* from *Bradyrhizobium* strains, but it is not so easy to distinguish *B. elkanii* from *B. japonicum*. Therefore, an easy and rapid method is needed to identify slow growing soybean microsymbionts. Restriction fragment length polymorphism (RFLP) analysis of 16S rDNA gene amplified by polymerase chain reaction (PCR) was recently used successfully in the differentiation of *Rhizobium*, *Bradyrhizobium* and *Agrobacterium* species (Laguerre et al. 1994), but only one *B.*

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japonicum strain was analysed, and *B. elkanii* was not included in their study. In this paper, we investigated the value of the RFLP analysis of PCR-amplified 16S rRNA gene for identifying soybean rhizobia species, and the work was conducted with 19 reference strains and 41 wild type isolates from China.

An important factor to the study on biodiversity of the rhizobia population is to have an efficient method to characterize individual strains. Methods that give information about genetic relationships at molecular level are particularly useful. Recently, a molecular method, which is based on differences in the relative position and occurrence of repetitive DNA sequence in the bacterial genome, has been developed. These sequences are divided into two classes: class I consists of repetitive extragenic palindromic (REP) elements, and class II consists of enterobacterial repetitive intergenic consensus (ERIC). After determination of the DNA sequences of these repeated elements, RFLP/ERIC-specific oligonucleotide primers were constructed to probe the genomes of a large variety of bacteria (Versalovic et al. 1991). Using PCR techniques in combination with REP or ERIC primers, the DNA sequences between the repeated elements can be amplified and very characteristic patterns of PCR products can be obtained when separated on agarose gels. These REP/ERIC-PCR fingerprinting techniques have been used successfully for characterization of *Rhizobium meliloti* isolates (de Bruijn 1992) and closely related *Bradyrhizobium* strains (Judd et al. 1993).

The intergenic region of the 16S and 23S rRNA genes is not well conserved, it exhibits a large degree of sequence, length and frequency variation. Therefore, the PCR-RFLP patterns of the intergenic spacers (IGS) between 16S and 23S rDNAs have been more useful in the characterization of closely related bacterial strains, comparing with 16S rDNA PCR-RFLP (Navarro et al. 1992, Jensen et al. 1993).

In this study, 41 wild type soybean rhizobia isolates from China have been characterized at strain level using REP/ERIC-PCR fingerprinting techniques. The possibility of 16S~23S IGS PCR-RFLP in the diversity study of soybean rhizobia (at subspecies level) was also investigated.

Materials and methods

Bacteria strains

Soybean rhizobia reference strains *S. fredii*: USDA 191, 205, 217, 257; *B. japonicum*: USDA 6, 38, 115, 122, 123, 127, 129, 136, 142, 143, 2110; *B. elkanii* II: USDA 46, 86, 130, and *B. elkanii* IIa: USDA94 were from the culture collection of the U. S. Department of Agriculture. All the wild type isolates from different areas of China were kept in our laboratories.

Sample preparation for direct amplification of DNA from cultured cells

Fast growing rhizobia strains were grown in YEM liquid medium at 30°C to a concentration of approximately 10^8 cells per ml. Then 5 ml culture and 0.5 ml sterile nanopure water (SNW) were mixed, and pelleted by centrifugation. The pellets were washed two times with SNW. Finally cell suspensions were diluted with SNW to approximately 10^4 to 10^5 cell per amplification reaction. For slow growing strains, fresh cultures were used alternatively.

PCR amplification

A pair of primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3') were chosen for PCR amplification of 16S rDNA. They are derived from conserved regions of 16S rRNA genes, and capable of amplifying nearly full-length 16S rDNA.

Primer pHr (5'-TGCGGCTGGATCACCTCCTT-3') and p23SR01 (5'-GGCTGCTTCTAAGC-CAAC-3') were selected from amplification of 16S~23S intergenic spacers (Fig. 1). For each reaction, approximately $10^4 \sim 10^5$ rhizobial cells were used. The optimized reaction was carried out in a 100 μ L volume containing a reaction buffer (10 mmol/L Tris-HCl; 50 mmol/L KCl; 2.5 mmol/L $MgCl_2$; 5 μ L W_1 -detergent and 0.001% gelatin), 0.22 mmol/L deoxynucleotide triphosphates (dNTPs), 100 ng each of primers, and 1.25 units of *Taq* DNA polymerase (Life Technologies). The samples were overlaid with two drops of mineral oil (Sigma). The following program was used: 93°C for 3 min (initial denaturation); 35 cycles at 93°C for 1 min (denaturation), 48°C for 2 min (annealing) and 72°C for 3 min (extension); 72°C for 7 min (final extension) and a final soak at 4°C. The amplification was performed with a DNA thermal cycler (Perkin-Elmer Cetus). The PCR products were digested with three enzymes (*Msp*I, *Hae*III, *Cfo*I) from Life Technologies.

Fig. 1 Schematic representation of the ribosomal RNA operon showing approximate localization of priming binding sites for PCR amplification.

Primers REP1R-I and REP2-I for REP-PCR, ERIC1R and ERIC2 for ERIC-PCR were described by Judd et al. in 1993. All the primers used in this study were synthesized by Pharmacia Biotech, Woerden, The Netherlands. The REP/ERIC-PCR reaction was carried out in a 25 μ L volume. DNA was amplified by mixing template (approximately 10^4 to 10^5 cells with the polymerase reaction buffer (10 mmol/L Tris-HCl, 50 mmol/L KCl; 3.5 mmol/L $MgCl_2$ and 0.001% gelatin), 0.6 mmol/L dNTPs, 50 pmols of two opposing primers, and 1.25 units of *Taq* DNA polymerase. Temperature profile for REP-PCR: 95°C for 7 min; 35 cycles at 90°C for 1 min, 40°C for 1 min, 65°C for 8 min; 65°C for 16 min, and final soak at 4°C. Temperature profile for ERIC-PCR: 95°C for 7 min; 35 cycles at 94°C for 1 min, 52°C for 1 min, 65°C for 8 min; 65°C for 16 min, and a final soak at 4°C. Amplified DNA was examined by horizontal electrophoresis in 1.5% agarose gel with 8~10 μ L aliquots of PCR products.

Results and discussion

Characterization of soybean rhizobia at species level using 16S rDNA PCR-RFLP analysis

DNAs of all 19 reference strains and 41 wild type isolates tested were amplified with primers pA and pH. All strains produced a single band of about 1500 bp (data not shown). This size corresponded to the expected length of the 16S rRNA genes of bacteria.

Three restriction enzymes (*Hae*III, *Msp*I and *Cfo*I) were chosen to digest the PCR products. Three distinct restriction patterns were detected with each enzyme (Fig. 2). The different patterns obtained for each enzyme and each strain are listed in Table 1. Four different combinations of patterns representing four different 16S rDNA genotypes were detected in 19 reference strains. Four *S. fredii* reference strains were classified into genotype 1; 11 *B. japonicum* reference strains were classified into genotype 3; three *B. elkanii* II strains and one *B. elkanii* IIa strain fell into genotype 4 and 5, respectively. None of these genotypes were shared by two

species. Therefore, 16S rDNA PCR-RFLP can be used to identify soybean microsymbionts.

For the unclassified wild type isolates from Chinese soil, all slow growing strains fell into genotype 3, suggesting that they should be *B. japonicum*; all fast growing strains but one were classified into genotype 1.

Characterization of soybean rhizobia at subspecies level using 16S~23S IGS PCR-RFLP

A pair of primers (pHr and p23SRO1) were chosen for amplification of 16S~23S intergenic spacers of soybean rhizobia wild type isolates from China. Results indicated that all fast growing *S. fredii* strains produced one larger band (2.1 kb), and all slow growing *Bradyrhizobium* strains produced one smaller band (2.0 kb). Same results were obtained with all the reference strains tested. Therefore, the length of the intergenic region between *S. fredii* and *Bradyrhizobium* sp. is different (Fig. 3).

Fig. 2 Restriction patterns of PCR amplified fragments of 16S rRNA genes digested with *Hae*III, *Cfo*I and *Msp*I. The lanes assignment are given in Table 1.

Table 1 16S rDNA genotypes and restriction patterns revealed by RFLP analysis of PCR-amplified 16S rRNA genes

Rhizobia	Restriction patterns with			Genotype
	<i>Hae</i> III	<i>Msp</i> I	<i>Cfo</i> I	
<i>S. fredii</i>				
USDA 205,257,217,191	a	a	a	1
<i>B. japonicum</i>				
USDA 6,2110,115,129	b	c	b	3
USDA 127,122,38,142				
USDA 123,136,143				
<i>B. elkanii</i> II				
USDA 86,130,46	b	c	c	4
<i>B. elkanii</i> IIa				
USDA94	c	c	c	5
Unclassified strains				
Fast growing				
1,2,3,6,7,8,9,10,11				
77,78,79,107,116,117	a	a	a	1
118,119,120,121,122				
76	a	b	a	2
Slow growing				
88,89,91,92,82,84,87	b	c	b	3
93,73,69,67,124,123				

Three enzymes (*Hae*III, *Cfo*I & *Msp*I) were used for restriction analysis. From 7 to 11 distinct restriction patterns were detected with each enzyme (Fig. 4). Sixteen different combinations of patterns represent 16 different 16S~23S intergenic region genotypes (Table 2). Ten genotypes were found within 18 *B. japonicum* strains from Heilongjiang Province (Northeast of China). Five genotypes were detected in 16 *S. fredii* strains, and the genotypes are correspondent to their geographic origin. All the tested *S. fredii* strains but one from Jilin Province (Northeast of China) were classified into genotype 1. Meanwhile, the *S. fredii* strains from Hubei Province (Centre of China) fell into genotype 4.

Characterization of Chinese soybean rhizobia at strain level using REP/ERIC-PCR fingerprinting

Based on REP/ERIC profiles, many differences were found among the fast growing and slow growing strains tested. The results of fast growing strains were listed in Table 3, and part of ERIC-PCR fingerprinting patterns were shown in Fig. 5. Computer analysis await being done to estimate their genetic relationships.

Fig. 3 Electrophoresis pattern of the PCR products of 16S~23S intergenic spacer. Lanes 1~5: slow growing strains; lanes 6~10: fast growing strains; Lanes M: λ DNA *Eco*RI/*Hind*III

Fig. 4 Restriction patterns of PCR-amplified fragments of 16S~23S intergenic region digested with *Hae*III and *Msp*I. The lane assignments (lanes a to h) are given in Table 2. Lane M, 100 bp DNA ladder

Table 2 16S~23S intergenic spacer genotypes and restriction patterns revealed by RFLP analysis of PCR-amplified 16S~23S intergenic region

Strains	Restriction patterns of			Genotypes	Geographic origin
	<i>Hae</i> III	<i>Msp</i> I	<i>Cfo</i> I		
Fast growing					
1,2,3,6,7,8,10	a	a	a	1	Jilin
9	b	a	h	2	Jilin
USDA257	c	b	b	3	Shanghai
114,117,119	d	c	c	4	Hubei
120,121,122					
76	g	h	i	12	Liaolin
Slow growing					
87,93	e	d	f	5	Heilongjiang
88	e	e	e	6	Heilongjiang
USDA6	f	e	e	7	?
67,73,83,92	e	f	g	8	Heilongjiang
69,81,82	e	d	f	9	Heilongjiang
66	f	g	d	10	Heilongjiang
71	f	g	f	11	?
91,124	e	f	j	13	Heilongjiang
84,123	e	d	d	14	Heilongjiang
72	f	g	f	15	Heilongjiang
89	e	e	k	16	Heilongjiang

Table 3 REP/ERIC fingerprinting genotypes of soybean rhizobia strains from Chinese soil

Strains	REP-PCR pattern	ERIC-PCR pattern	Genotype	Geographic origin
Fast growing				
1,2	R1	E1	1	Jilin
3,6,10	R1	E2	2	Jilin
7,8	R1	E3	3	Jilin
9	R2	E4	4	Jilin
11	R1	E5	5	Jilin
114	R3	E7	6	Jilin
116	R4	E1	7	Hubei
117	R5	E7	8	Hubei
118	R6	E1	9	Hubei
119	R8	E6	11	Hubei
120	R7	E7	10	Hubei
121	R9	E6	12	Hubei
122	R10	E7	13	Hubei
USDA257	R11	E8	14	Shanghai
Slow growing				
84	R24	E9	15	Heilongjiang
87	R26	E10	16	Heilongjiang
88	R23	E11	17	Heilongjiang
89	R22	E12	18	Heilongjiang

Table 3 (continued)

Strains	REP-PCR pattern	ERIC-PCR pattern	Genotype	Geographic origin
91	R17	E13	19	Heilongjiang
92	R18	E13	20	Heilongjiang
93	R19	E14	21	Heilongjiang
123	R20	E15	22	Heilongjiang
124	R21	E16	23	Heilongjiang
77	R25	E17	24	Heilongjiang
66	R14	E18	25	Heilongjiang
67	R13	E13	26	Heilongjiang
69	R12	E14	27	Heilongjiang
71	R13	E19	28	Heilongjiang
72	R14	E20	29	Heilongjiang
73	R15	E21	30	Heilongjiang
81	R23	E14	31	Heilongjiang
83	R17	E22	32	Heilongjiang
129	R27	E23	33	Heilongjiang
USDA6	R16	E24	34	

In general, nineteen slow growing indigenous isolates from Heilongjiang Province shared one 16S rDNA RFLP genotype, but they could be separated into 10 genotypes according to 16S ~ 23S IGS PCR-RFLP analysis; furthermore, their REP/ERIC patterns were completely different from each other. Similar results were obtained with fast growing isolates. So we can conclude that 16S rDNA PCR-RFLP, 16S ~ 23S IGS PCR-RFLP, and REP/ERIC-PCR fingerprinting can be used to characterize soybean rhizobia at species, sub-species, and strain level, respectively. These methods will be used to characterize more indigenous soybean rhizobia from Chinese soil in our laboratories.

China is the soybean gene center. According to the coevolution theory (Lie, 1984), more and more variations among Chinese soybean rhizobia population should be found in the future.

Fig. 5 ERIC-PCR fingerprinting profiles of some soybean rhizobia isolates from Chinese soil. Lanes a to j are slow growing strains 66, 67, 69, 71, 72, 73, 81, 83, 129, and type strain USDA6 respectively; lanes indicated with M are λ DNA *Eco*RI/*Hind*III.

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采用 PCR-RFLP 技术在不同水平上鉴定大豆根瘤菌

张学贤^{1,2} M.木论贝格² 李德安¹ 周俊初¹ 李卓隼¹

1(华中农业大学微生物系, 武汉 430070)

2(Department of Microbiology, Wageningen Agricultural University, Wageningen, The Netherlands)

摘要 采用 16S rRNA 基因 PCR 扩增与限制性酶切片段多态性分析(RFLP)技术对选自弗氏中华根瘤菌(*S. fredii*)、大豆慢生根瘤菌(*B. japonicum*)和埃氏慢生根瘤菌(*B. elkanii*)的 19 株代表菌进行了比较分析,根据用 3 种限制性内切酶的 RFLP 分析结果,可将供试菌株分为 *S. fredii*, *B. japonicum*, *B. elkanii* II 和 *B. elkanii* IIa 等 4 种基因型。各类菌株之间没有交叉,因此本研究采用的 PCR-RFLP 技术不失为一种快速鉴别大豆根瘤菌的新方法。采用本技术已将分离自中国的 22 株快生菌和 19 株慢生菌分别鉴定为 *S. fredii* 和 *B. japonicum*。

对供试参比菌株和野生型菌株进行的 16S~23S 基因间隔 DNA(IGS)的 PCR-RFLP 分析结果表明:*S. fredii* 和 *B. japonicum* 菌株的 IGS 长度不同,所有供试 *S. fredii* 菌株的 IGS 为 2.1 kb,而供试 *B. japonicum* 菌株则为 2.0 kb。依据 RFLP 的差异,可将来自中国两个不同地区的 *S. fredii* 株区分为 2 个基因型,而来自中国东北黑龙江地区的 19 株 *B. japonicum* 菌株则可分为 11 个基因型。对上述野生型菌株还进行了 REP-PCR 和 ERIC-PCR 分析并确定其具有菌株水平的特异性。

关键词 大豆,根瘤菌,遗传多样性,限制性酶切片段长度多态性