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Identification of strains of *Mesorhizobium huakuii*, root nodule bacteria of *Astragalus sinicus*, by the polymerase chain reaction

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Strains of *Mesorhizobium huakuii*, root nodule bacteria of *Astragalus sinicus*, were identified from DNA polymorphism amplified by the polymerase chain reaction (PCR) using some known or random primers. Five strains of *M. huakuii* isolated in Japan and China were examined using SPH (a random primer), nifHDK (a part of the sequence of *nif* gene) and ERIC (both ends of an repetitive intergenic consensus DNA sequence of enteric bacteria). DNA bands amplified with primers showed distinctly different band patterns between groups of strains isolated in Japan and those isolated in China. Although it was sometimes difficult to identify strains belonging to the same group with only one primer, strains of a group could be identified by comparison of the results obtained with different PCR primers. Strains of *M. huakuii* isolated randomly from a rice field soil were examined by PCR, and several types of strains were found to survive at the same site. This identification method using PCR was also useful to investigate the infection rate of inoculated strains in a pot cultivation experiment.

Key words : *Mesorhizobium huakuii*, *Astragalus sinicus*, PCR, ERIC, nifHDK

Introduction

The taxonomy of root nodule bacteria of Chinese milk vetch (*Astragalus sinicus* L.) remained unclear until it was classified as *Rhizobium huakuii* by Chen *et al.* in 1991³⁾. It was reclassified as *Mesorhizobium huakuii* according to its physiological and phylogenetic properties to distinguish it from other rhizobial bacteria. Chinese milk vetch is a leguminous plant, being cultivated in the temperate area of Far East Asia especially in China, and it has not been paid attention by agricultural researchers of the western world except in a few cases⁶⁾. The role of Chinese milk vetch as green manure in rice fields, however, has become lower in China with the increase of oil crops such as oil seed rape as cash crops²⁾. In Japan also, Chinese milk vetch was widely grown as a winter crop on rice fields as green manure, but the cultivation area of Chinese milk vetch has rapidly decreased after World War II with the spread of chemical fertilizers. However, the role of Chinese milk vetch is now being reassessed since the soil fertility of arable land in Japan is thought to be continuously lower and the establishment of a sustainable agriculture system is required.

The root nodule bacteria of *A. sinicus* are considered to survive as indigenous heterotrophic inhabitants of Japanese soils, because Chinese milk vetch has been grown on

rice fields since the 17th century. Therefore the nodules of *A. sinicus* are usually formed by indigenous root nodule bacteria. In such cases, the effect of the inoculation of *M. huakuii* must be evaluated by checking if the root nodules are formed by the inoculant strains or other native ones. It is rather difficult to identify the strains of root nodule bacteria by such conventional methods as intrinsic antibiotic resistance, serological reaction, enzyme linked immunosorbent assays (ELISA) or restriction fragment length polymorphisms (RFLP) which require complicated procedures. Recently, the polymerase chain reaction (PCR) using random primers has become one of the most effective methods to identify bacterial strains and it has been used for rhizobial bacteria. However, PCR has not been applied to *M. huakuii*. In this paper, I report the utility and detailed conditions of PCR to identify strains of *M. huakuii*. This method was also applied to estimate the infection rate of inoculated strains on the root of *A. sinicus* in a pot experiment.

Methods and Materials

Strains of M. huakuii

The strains used in the experiment were the five strains listed in Table 1. Three of them were isolated in Japan and the other two in China. The strain 103T is the

Table 1. Strains used in the experiments

Strains	Origin
103T (NAU)	Type stain of <i>M. huakuii</i> (Nanjing Agricultural University, China).
13005 (ACCC)	Strain used in China (Agricultural Culture Collection, China)
B3	Isolate by Murooka, Hiroshima Univ., Japan.
901 (IAC)	Isolate by Tokachi Federation of Agr. Coop., Japan
912 (IAC)	Isolate in this research, Ishikawa Agr. Coll., Japan

Table 2. Primers used for PCR

Symbol	Base sequence	Remark	Reference
SPH 1	5'-GACGACGACCGACGAC-3'	Random primer	6)
nifHDK	5'-GGTTATCGAAATCAGCAGCCACAGCGC-3'	Part of <i>nif</i> gene	9)
ERIC 1R	5'-ATGTAAGCTCCTGGGGATTAC-3'	Intergenic consensus repetitive sequence of enteric bacteria	4)
ERIC 2	5'-AAGTAAGTGACTGGGGTGAGCG-3'		

type strain of *M. huakuii*.

Preparation of DNA for PCR

The bacteria to be tested were cultured in TY liquid medium (Bactotryptone (Difco) 5 g, yeast extract (Difco) 3 g, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ 1.3 g, distilled water 1 L, pH 7.0) at 26 °C for about two days until the logarithmic growth stage. The cultured bacterial body was collected by centrifugation ($10,000 \times g$, 10 min), and washed with physiological saline solution. The collected bacterial body was suspended with an adequate amount of sterilized water and dispensed into 1.5 mL microtubes and centrifuged again. To the precipitant was added 200 μL of sterilized water, and the suspension was stocked at -20°C . The suspension was melted just before use, and 10 μL of Proteinase K solution (1 mg mL^{-1}) and 50 μL of BL buffer solution (Tris 40 mM, Tween-20 1%, Nonidet P-40 0.5%, EDTA $\cdot 2\text{ Na}$ 1 mM, pH 8.0) were added and incubated at 60 °C for 20 min to dissolve the bacterial body. The reaction was stopped by heating at 95 °C for 10 min and the supernatant was collected by centrifugation and used as crude DNA solution. The optical density at 260 nm of the crude DNA solution was measured and the concentration of DNA was adjusted to $150\text{ }\mu\text{g }\mu\text{L}^{-1}$ for PCR.

PCR

The reaction solution contained 10 μL of $10\times$ buffer (Takara Bio), 1.5 mM MgCl_2 , 0.2 mM dNTP, 1 μM Primer(s), 2.5 units of Taq DNA polymerase (Takara Bio) and 5 μL of the diluted crude DNA solution (750 μg as template DNA), the total volume of which was adjusted to 100 μL . The primers used in PCR were as listed in Table 2. SPH 1 was a random single primer and the oth-

ers were directed primers that are a part of a known sequence (nifHDK) or of a consensus sequence (ERIC). The reaction mixture was taken into a 1.5 mL microtube, overlaid with 100 μL of mineral oil and set in a thermal cycler (Astec PC-700). The temperature condition of PCR with SPH 1 and nifHDK was as follows: pre-run at 94 °C for 1 min, 30 cycles of denaturing at 94 °C for 1 min, annealing at 50 °C for 1 min, polymerization at 72 °C for 1.5 min, and post-run at 65 °C for 2 min. The temperature condition of PCR with ERIC primers was as follows: pre-run at 95 °C for 5 min, 30 cycles of denature at 94 °C for min, annealing at 52 °C for 1 min, polymerization at 65 °C for 8 min, and post-run at 65 °C for 16 min. The PCR products were separated from mineral oil by addition of 100 μL of chloroform isoamyl alcohol mixture (24 : 1 v/v) and electrophoresed with 2% agarose (Amplisize agarose, Bio-Rad) in $1\times$ TBE (pH 8.2) under a 100 V for about 45 min. As DNA size markers, a 100 base-pair ladder and Kilo base-pair ladder (Pharmacia) were electrophoresed at the same time. The agarose gel was stained with ethidium bromide solution ($2\text{ }\mu\text{g L}^{-1}$) and DNA fragments were detected under UV light of 260 nm.

Inoculation experiment with the strains of root nodule bacteria

Air-dried soil (gray lowland soil, sandy clay loam) was mixed with river sand at the ratio of 1 : 1, and 2.5 kg of the mixture per Wagner pot of 1/5,000 a was used for the pot cultivation experiment. Nodule bacteria shown in Table 1 were cultured in TY liquid medium at 26 °C for about two days and the suspension of bacteria ($3\sim 9\times 10^8$ CFU mL^{-1} , 2 mL per pot) was inoculated to the seedlings of *A. sinicus* (cv. Gifu-hinode-wase), which were sown at

the density of nine per pot. Calcium superphosphate and potassium chloride were supplied as basal fertilizers at the rate of 0.5 g per pot as P_2O_5 and K_2O , respectively. *A. sinicus* was grown in a greenhouse at about 20°C for 2 months. The cultivation experiment was carried out with three replicates. The growth and nitrogen content of *A. sinicus* were measured after harvesting, and the root nodules were counted and randomly sampled at the rate of ten nodules per treatment. The isolated bacteria from colonies formed on yeast extract mannitol agar medium (YM) were subjected to PCR and examined for nodule formation by inoculated strains or other indigenous strains of *M. huakuii*.

Results and Discussion

DNA fragments were amplified with any primers used in this experiment as shown in Fig. 1. Electrophoresed DNA band patterns were similar in each group of Chinese strains and Japanese strains, and it was easy to determine to which group isolated strains belonged. Within a group, identification with only one primer such

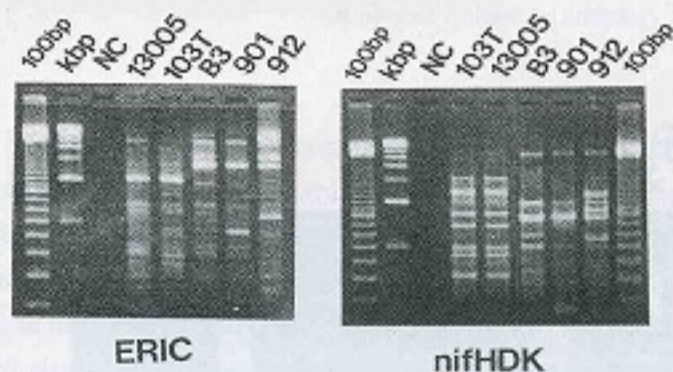


Fig. 1. DNA band patterns of tested strains of *Mesorhizobium huakuii* by PCR.

Left, ERIC primers; Right, nifHDK primer.

M 100, 100 base-pair ladder DNA size marker; Mk, kilo base-pair ladder DNA size marker; NC, negative control.



Fig. 2. DNA band patterns of strains isolated from a field soil by PCR with nifHDK primer.

M 100, 100 base-pair ladder DNA size marker; Mk, kilo base-pair ladder DNA size marker; NC, negative control.

as nifHDK was sometimes difficult, but the strains could be identified by comparison of the results obtained with different primers. This PCR method is considered to be useful to investigate the infection rate of inoculated nodule bacteria in *A. sinicus*.

The nodule bacteria isolated from nodules which were randomly sampled from *A. sinicus* grown in one rice field showed various DNA polymorphisms by PCR. This suggests that various types of indigenous strains of *M. huakuii* inhabit even in the soil of the same site.

The inoculation experiment was carried out with Wagner pots of 1/5,000 a to confirm the effect of strains of *M. huakuii* as inoculants in a condition where indigenous nodule bacteria survived. As shown in Table 3, the growth and nitrogen content were significantly stimulated by the inoculation of 13005, B3 and 901 except 103T. The investigation of isolates from nodules of *A. sinicus* by PCR (Figs. 3 and 4) suggested that a high percentage of the nodules were infected with the inoculants except 103T. As summarized in Table 4, the infection rate with 103T, 13005, B3 and 901 was 0%, 100%, 63% and 88%, respectively. Although 103T is the type strain of *M. huakuii*, it seemed to lack a plasmid containing *nod* gene which is related to the nodulation of leguminous plants¹³. The ability of 103T to form nodules in *A. sinicus* was not confirmed also in this experiment. Growth and total nitrogen content of the plants inoculated with the strains except 103T were significantly higher than the control without inoculation, but there was no significant difference between the three inoculants of 13005, B3 and 901.

There was no significant difference in the number of big nodules (>3 mm in diameter) between the treatments, but the total number of nodules was significantly increased by the inoculation treatments except 103T. It is estimated that inoculation treatments caused the increase of small nodules in *A. sinicus*.

The soil used in this experiment was estimated to have a history of growing *A. sinicus*. However, as it was used in an air-dried condition, the density of indigenous *M. huakuii* was rather low, less than 10^3 CFU g^{-1} and this might have caused the high inoculation effect in this experiment. It may be necessary to confirm the effect of inoculation in fresh soil. The inoculation of some strains of *M. huakuii* was quite effective for Chinese milk vetch in soils which had no history of growing Chinese milk vetch in such countries as U. S. A. and Nepal^{1,8)}. However, in China where Chinese milk vetch had been grown for a long period, the effect of inoculation was sometimes not so clear²⁾ and it has not been confirmed whether nodules of Chinese milk vetch were really formed by inoculated strains of *M. huakuii*.

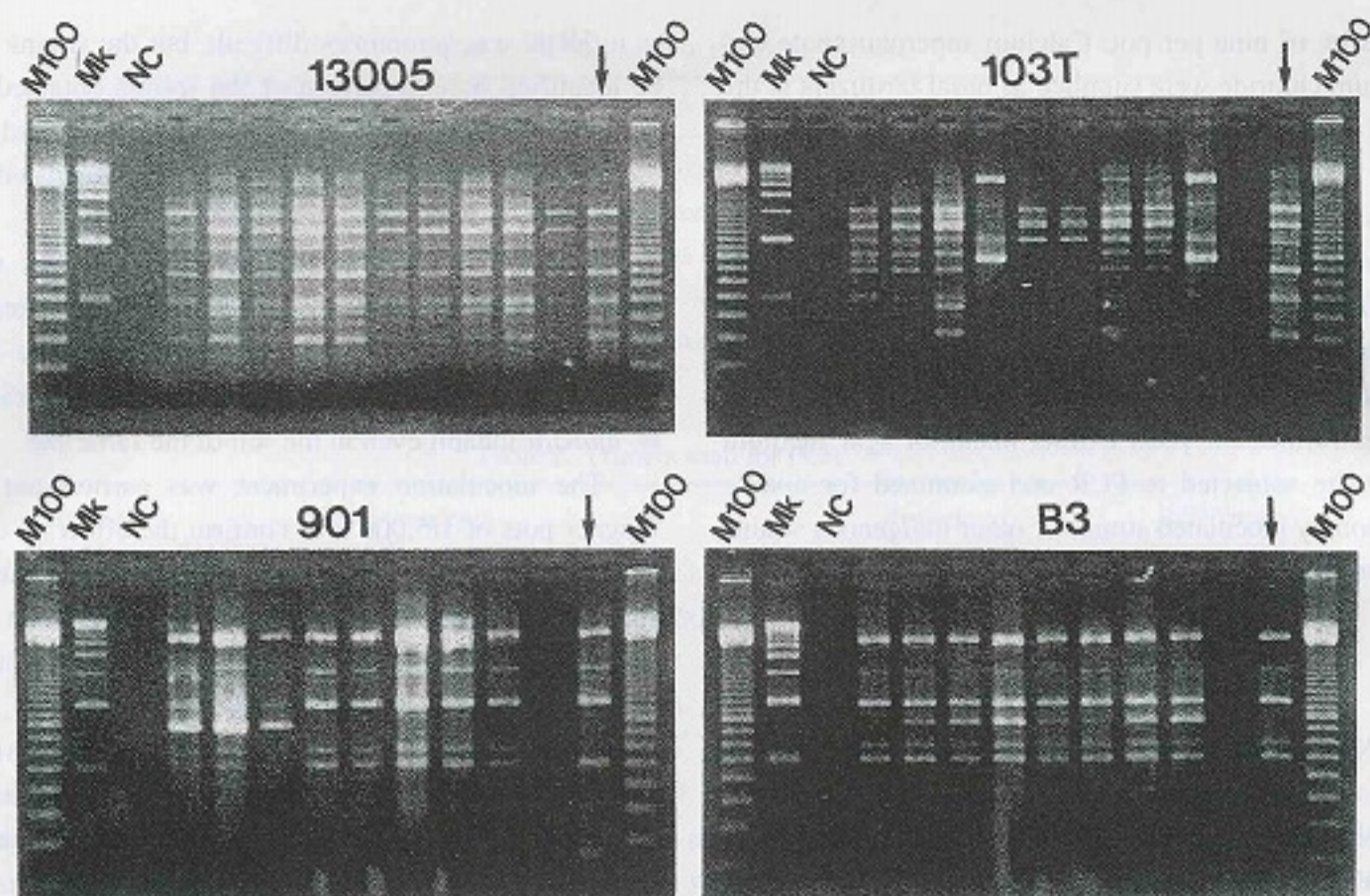


Fig. 3. DNA band patterns of isolates in the pot experiment obtained by PCR with nifHDK primer. M 100, 100 base-pair ladder DNA size marker ; Mk, kilo base-pair ladder DNA size marker ; NC, negative control. The symbol of each plate indicates the treatment, and arrows indicate the inoculated strains (13005, B3, 901, 103T).

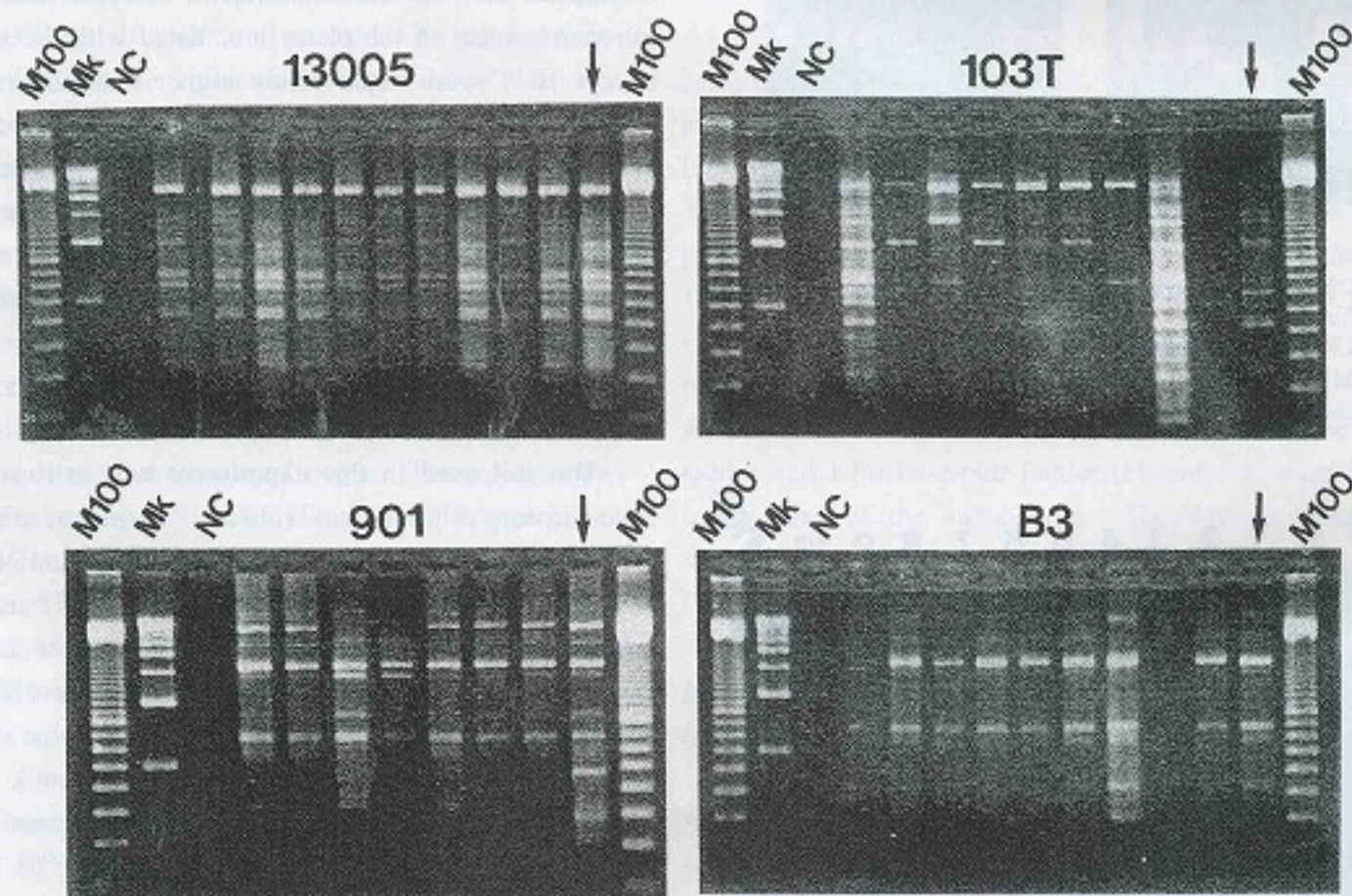


Fig. 4. DNA band patterns of isolates in the pot experiment obtained by PCR with ERIC primers. M 100, 100 base-pair ladder DNA size marker ; Mk, kilo base-pair ladder DNA size marker ; NC, negative control. The symbol of each plate indicates the treatment, and arrows indicate the inoculated strains (13005, B3, 901, 103T).

Table 3. Inoculation test by pot cultivation

Strains	Shoot growth	N content	Root nodules per plant		Infection rate
	D.W. (g) per pot	mg per pot	big (>5 mm)	total	infected / total (%)
Control	3.31 ± 0.18 a*	107 ± 1 a	2.9	8 a	—
103T	3.82 ± 0.13 a	153 ± 8 ab	6.0	18 a	0/8 (0)
13005	5.29 ± 0.70 b	244 ± 30 bc	1.2	100 b	10/10 (100)
901	6.59 ± 0.30 b	300 ± 17 c	3.5	146 c	5/8 (63)
B3	6.20 ± 0.77 b	276 ± 27 c	4.5	103 bc	7/8 (88)

* Same letters indicate no significant difference by Duncan's multiple range test at $p=0.05$ ($n=3$).

Table 4. Infection rates of inoculated strains in the pot experiment

Inoculated strain	Primer						Infection rate infected / total (%)
	nifHDK			ERIC			
	+	±	-	+	±	-	
103T	0*	7	2	0	0	8	0/8 (0)
13005	10	0	0	10	0	0	10/10 (100)
B3	3	5	0	7	1	0	5/8 (63)
901	5	3	0	5	2	0	7/8 (88)

+, same as the inoculated strain

±, almost the same but partly different from the inoculated strain

-, different from the inoculated strain

* number of isolates (= nodules)

The PCR method with adequate primers is thought to be useful to identify the strains of *M. huakuii*, but there were some cases in which accurate identification was difficult because of low reproducibility of DNA band pattern. It was necessary to perform PCR amplification with standard strains under the same conditions. To increase the reliability of the PCR method, we must consider the culture conditions, refining procedure of DNA templates, temperature set of PCR and so on.

As it is known that *M. huakuii* easily lose plasmids during culture^{1,13}, the change of plasmid composition during the culture or in the soil may be one of the factors causing the variation of DNA band patterns.

Acknowledgements

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PCR 法によるレンゲ根粒菌の菌株識別

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幾つかの既知あるいはランダムプライマーを用いて DNA の一部を PCR 増幅し、そのバンドパターンをもとにレンゲ根粒菌の菌株を識別することを試みた。PCR 反応には国内外のレンゲ根粒菌 5 菌株を供試し、SPH 1 (ランダムプライマー)、nifHDK (窒素固定遺伝子 *nif* の一部)、および ERIC 1R と ERIC 2 (腸内細菌の遺伝子間反復配列の両端) のプライマーセットを用いた。いずれのプライマーでも PCR によって DNA の断片が増幅され、中国産と日本産のレンゲ根粒菌はそれぞれ類似のバンドパターンを示し、両グループの識別は容易であった。各グループ内の菌株間の識別はひとつのプライマーあるいはプライマーセットだけでは困難な場合もあったが、各プライマーによる結果を組み合わせると識別が可能であり、接種根粒菌の感染率の調査等に利用が可能であると考えられる。水田転換畑の同一圃場に栽培されたレンゲの根粒からランダムに分離したレンゲ根粒菌は多様なバンドパターンを示し、同一圃場でも数種類のレンゲ根粒菌が存在することが示唆された。ポット栽培による根粒菌接種栽培試験において形成された根粒から分離された根粒菌について PCR 法によって菌株を調べたところ、一部の接種菌株を除き、接種菌株による根粒形成が確認された。

キーワード : *Mesorhizobium huakuii*、レンゲ、PCR、ERIC、nifHDK