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Genetic Structure of Indigenous Non-nodulating and Nodulating Populations of *Bradyrhizobium* in Soils from Thailand

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Abstract

One hundred and thirty symbiotic and non-symbiotic strains of *Bradyrhizobium* were directly isolated from inoculated soybean and uninoculated legume-free virgin field soils in Thailand using a direct selection medium. About 47% and 58% of the isolates obtained from inoculated and uninoculated fields, respectively, were characterized as being non-symbiotic bradyrhizobia. Partial and nearly full-length sequence analyses of regions encoding 16S rRNA indicated that the non-symbionts were closely related to *Bradyrhizobium elkanii* (79–99% identity) and *Bradyrhizobium japonicum* (96–100% identity). Southern hybridization analyses showed that DNA from the non-symbiotic bradyrhizobia failed to hybridize to *nif* and *nod* gene probes. rep-PCR DNA fingerprint analyses, done using the BOXA1R primer, indicated the symbionts and non-symbionts could be separated into two distinct clusters. There was no relationship between the geographical origin of isolates and groups made based on serological reaction or their ability to nodulate soybean, cowpea, mungbean, or siratro. These results indicate that a relatively large percentage of non-nodulating bradyrhizobia are present in Thai soils.

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1. Introduction

Members of the genus *Bradyrhizobium* are slow-growing, gram-negative soil bacteria which invade and form nitrogen-fixing nodules on the roots of soybean, peanut, cowpea, and mungbean (Jordan and Allen, 1974). Within these root nodules bradyrhizobia fix atmospheric dinitrogen into ammonia, providing the nitrogen requirements of cultivated legumes (Hartmann and Amarger, 1991).

The assessment of the genetic diversity and genetic relationship among strains of natural populations of rhizobia and bradyrhizobia has provided valuable information about bacterial genotypes that are well adapted to certain environments (Niemann et al., 1997). However, there are several opposing viewpoints concerning the influence of host plants on the diversity of legume symbionts. While Martinez-Romero and Caballero-Mellado (1996) suggested that agriculture creates highly selective and homogenous environments that reduce bacterial diversity, Kennedy and Smith (1995) and Palmer and Young (2000) have argued that cultivation results in greater diversity.

Non-symbiotic rhizobia have been reported to represent a significant component of rhizobial populations in the environment (Laguerre et al., 1993; Rao et al., 1994; Segovia et al., 1991; Soberon-Chavez and Najera, 1989; Sullivan et al., 1995; Sullivan et al., 1996). While the origin of non-symbiotic rhizobia is unknown, they are either derived from relatively common saprophytic soil bacteria, or may be non-nodulating descendants of rhizobia that associated with native legumes that were once present at the site (Sullivan et al., 1996). Sullivan and Ronson (1998) reported the non-symbiotic rhizobia evolved into symbionts of *Lotus corniculatus* by acquiring a 500 kb genetic element. This symbiotic island was subsequently shown to contain genes essential for nodulation and nitrogen fixation (Sullivan et al., 2001). These studies suggest that non-symbiotic soil bacteria can become symbionts of legumes in nature via a single step process involving plasmid transfer and the relatively strong selection pressure exerted by a host plant.

While there have been several reports concerning the isolation and characterization of non-symbiotic rhizobia, there is little information about the occurrence of non-symbiotic bradyrhizobia and whether they represent a significant proportion of soil bacteria. This is in part due to the lack of an effective method to isolate bradyrhizobia from soil without the use of a trap host. Interestingly, although Saito et al. (1998) reported that slow-growing

and oligotrophic soil bacteria are phylogenetically related to *B. japonicum*, and failed to nodulate legume hosts and hybridize to *nod* and *nif* gene probes, these strains were incidentally isolated based on their oligotrophic characteristics. Tong and Sadowsky (1994) reported the development of a selective medium (BJSM) that allows for the direct isolation of bradyrhizobia from soils. This medium, and that of Gault and Schwinghamer (1993), facilitates the ecological study of bradyrhizobia in soils without bias introduced by the use of trap hosts.

In this report, we describe the use of BJSM to examine the prior influence of soybean inoculants on the genetic diversity of *Bradyrhizobium* directly isolated from field sites in Thailand. Remarkably, about 47% and 58% of the isolates obtained from inoculated and uninoculated fields, respectively, in Thailand were characterized as being non-symbiotic bradyrhizobia.

2. Materials and Methods

Soil samples

Soil samples were collected from heavily inoculated and uninoculated fields in Thailand. The soil samples from heavily-inoculated soybean fields were collected in the Khon Kaen (Northeastern) and Chiang Mai (Northern) Provinces, where *B. japonicum* inoculant strains USDA110, USDA122, and *Bradyrhizobium* sp. strains THA2, and THA5 were used for soybean cultivation almost every year for the last 20 years. Soil samples were also collected from a field in Chiang Mai province that was devoid of legumes, and had never received inoculation with any *Bradyrhizobium* strains.

Bacteria used, media and growth conditions

The *Bradyrhizobium* and *Rhizobium* strains used in this study are shown in Table 1. Cultures were routinely grown on arabinose-gluconate (AG) medium at 28°C (Sadowsky et al., 1987). Pure cultures were maintained on AG agar slants at 4°C and frozen in 50% glycerol at –80°C.

Isolation and initial characterization of Bradyrhizobium strains from Thailand soils

Bradyrhizobium isolates were extracted from soil samples by using a modification of the gelatin-ammonium phosphate method of Kingsley and Bohlool (1981). Ten-fold serial dilutions of soil suspensions were plated onto BJSM selective medium (Tong and Sadowsky, 1994).

Table 1. Strains used in this study and their sources

Strains	Source[1]
Bradyhizobium elkanii: SEMIA5019	1
USDA 31, USDA 94	2
Bradyhizobium japonicum: USDA 110, USDA 117	2
Sinorhizobium fredii: USDA193, USDA205	2
Rhizobium tropici: IFO15247	3
Rhizobium sp.: TAL1402	4
Bradyrhizobium sp. TAL211, TAL377	4
THA2, THA7, THA205, THA301	5
Indigenous symbiotic isolates[2] KKI1^{S}, KKI13^{MC}, KKI17-KKI18^{S}, KKI25-KKI26^{S}, KKI29-KKI35^{S}, KKI36^{MC}, KKI37-KKI39^{S}, KKI40^{MC}, CMI2^{MC}, CMI3-CMI4^{S}, CMI6^{MC}, CMI7^{S}, CMI9^{S}, CMI12-CMI13^{S}, CMI16^{S}, CMI18^{S}, CMI20^{S}, CMI21^{MC}, CMI26^{S}, CMI28^{S}, CMI30^{S}, CMI31-CMI34^{MC}, CMI35^{S}, CMI36^{MC}, CMI37-CMI38^{S}, CMI39-CMI40^{MC}, CMU2^{S}, CMU7^{S}, CMU8^{MC}, CMU11-CMU12^{MC}, CMU17^{S}, CMU25-CMU26^{S}, CMU35^{MC}, CMU38-CMU39^{S}, CMU40-CMU45^{MC}, CMU46-CMU47^{S}, CMU48^{MC}, CMU49-CMU50^{S}	This study
Indigenous non-symbiotic isolates KKI2-KKI12, KKI14-KKI16, KKI19-KKI24, KKI27, KKI28, CMI1, CMI5, CMI8, CMI10, CMI11, CMI14, CMI15, CMI17, CMI19, CMI22-CMI25, CMI27, CMI29, CMU1, CMU3-CMU6, CMU9, CMU10, CMU13-CMU16, CMU18-CMU24, CMU27-CMU34, CMU36, CMU37	This study

[1]Source: 1, People's Republic of China; 2, U.S. Department of Agriculture (USDA), Beltsville, MD; 3, Institute for Fermentation (IFO), Osaka, Japan; 4, NifTAL, Paia, Hawaii; 5, THA (Ministry of Agriculture and Cooperative), Thailand; [2]Ability to form nodules on: (S) soybean (*Glycine max* cv. SJ5), or (MC) mungbean (*Vigna radiata*) and cowpea (*Vigna unguiculata* cv. SUT1).

Pure cultures of each isolate were obtained by repeated streaking onto BJSM and AG media. The abbreviations, KKI, CMI and CMU, are used to refer to those isolates obtained from inoculated fields in Khon Kaen and Chiang Mai, and the uninoculated field in Chiang Mai, respectively. Pure cultures were examined for cell morphology, gram stain reaction, colony characteristics on AG medium containing congo red, the ability to alter the pH of media containing bromthymol blue, and the inability to grow on glucose-peptone and nutrient

agar media (Somasegaran and Hoben, 1994). The isolates were also evaluated for their ability to grow on nutrient agar (Difco, MI).

Plant nodulation tests

Plant nodulation tests were done using the MPN assay of Somasegaran and Hoben (1994), except that a gelatin-ammonium phosphate solution (Kingsley and Bohlool, 1981) was used as the extractant instead of water. Plants were set-up using a completely randomized experimental design. Soybean (*Glycine max* cv. SJ5, Thai local variety), mungbean (*Vigna radiata*) and cowpea (*Vigna unguiculata* cv. SUT1) were used as host plants. Siratro (*Macroptilium atropurureum*) was also used as a test host for those strains subjected to 16S rDNA sequence analysis. Plants for these studies were grown in modified Leonard jars as previously described (Sadowsky et al., 1987).

Sequence analysis of partial and nearly full-length 16S rDNAs

Partial and nearly full-length sequences of genes encoding 16S rRNA were obtained from 13 non-symbiotic isolates and used for phylogenetic analyses. Partial 16S rDNA was amplified using universal primers UN16S 926f (5'-AAACTYAAAKGAATTGACGG -3') and UN16S 1392r (5'-ACGGGCGGTGTGTRC -3') (Lane, 1991). PCR reaction mixtures contained buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.5 µM of each primer, 1.0 µl of genomic DNA and 0.5 U of Taq polymerase in a final reaction volume of 50 µl. PCR conditions were as follows: 1 cycle at 98°C for 5 min, 34 cycles consisting of 95°C for 30 s, 62°C for 30 s, 72°C for 1 min, and 1 cycle at 72°C for 5 min. PCR products were separated on a 1.0% agarose gel and purified using QIA Quick Gel Extraction Kit (Qiagen, Valencia, CA). The purified PCR products were sequenced by the University of Minnesota Advanced Genetic Analysis Center (AGAC).

A representative non-symbiotic isolate from each soil sample was used to generate nearly full-length 16S rDNA using primers 16Sa (5'-CGCTGGCGGCAGGCTTAACA -3') and 16Sb (5'- CCAGCCGCAGGTTCCCCT -3') (van Berkum and Fuhrmann, 2000). PCR was done as described above, except the reaction conditions used were as follows: 1 cycle at 98°C for 5 min, 35 cycles consisting of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and 1 cycle at 72°C for 3 min. PCR products were separated on 1.0% agarose gels and purified using a QIA Quick Gel Extraction Kit (Qiagen, Valencia, CA). Nearly full-length 16S rDNA was obtained by primer walking. Primers were designed by using Primer Designer 4.0 software (Scientific & Educational Software, Durham, NC). The purified products were sequenced as described above. Partial and nearly full-

length 16S rDNA sequences were aligned using Blast (http://www.ncbi.nlm.nih.gov) and Sequence Match ver. 2.7 of the RDP databases (http://www.cme.msu.edu/RDP/htm/analyses.htm/).

The following accessions were used for sequence comparisons: *Bradyrhizobium japonicum* accessions – AF363129, AF363130, AF363115, AF363123, AF363125, AF363124, AF363121, AF363113, AF363119, AF363133, AF239842, AF239843, AF239844, AF236086, AF234888, AF293377, AF293378, AF208504, AF208508; *Bradyrhizobium elkanii* accessions – AF363116, AF363122, AF239845, AF239846, AF234890, AF237422, AF293376, AF208512, AF208516, AF293379, AF293380, AF208518, AF293373, AF293374, AF208510; *Bradyrhizobium* spp. accessions – AF363139, AF363118, AF363145, AF363144, AF363149, X70401, X70402, X70403, X70404, X70405, Z94821, Z94805, AF178436, AF178435, AF321215, AF384135, AF384136, AF178437, AF178438; *Afipia* accessions – U87768, U87767, U87770, U87769, U87766; *Rhodopseudomonas* sp. accessions AF123085, AF095928; and *Rhodobacter palustris* accession – AY084709.

Serological studies

The serological reactions of indigenous bradyrhizobial isolates from Thailand were examined using antisera prepared against 15 standard rhizobial and bradyrhizobial strains (SEMIA 5019, IFO15247, TAL 211, TAL 377, TAL 1402, THA 2, THA 7, THA 205, THA 301, USDA 31, USDA 94, USDA 110, USDA 117, USDA 193, USDA 205). Three symbiotic and one non-symbiotic bradyrhizobial isolates (KKI1, KKI17, CMU8, and KKI8), which were directly obtained from soil samples, were also used to produce antisera. Antisera were produced in white, female, New Zealand rabbits, and immunological specificity was determined by agglutination reaction in test tubes at 52°C for 2 hr (Somasegaran and Hoben, 1994). Antigens from the indigenous and standard bradyrhizobia were prepared as described by Somasegaran and Hoben (1994).

rep-PCR fingerprint analysis

rep-PCR DNA fingerprinting analysis was also used to investigate the relatedness of the *Bradyrhizobium* strains (Judd et al., 1993). Total genomic DNA from each *Bradyrhizobium* isolate was prepared as described previously (Hartmann and Amarger, 1991). Genomic DNA concentrations were adjusted to 50 ng/µl and kept at –20°C. rep-PCR fingerprints were obtained by using the BOXA1R primer (5'- CTACGGCAAGGCGACGCTGAC -3') (Versalovic et al., 1998). PCR reactions were carried out essentially as described by Dombek et al. (2000), except that 2 µl of DNA solution was used as template instead of whole-

cell suspensions. The PCR reaction conditions used were as follows; 1 cycle at 95°C for 2 min, 30 cycles consisting of 94°C for 30 s, 92°C for 30 s, 50°C for 1 min, and 1 cycle at 65°C for 8 min. PCR products were electrophoresed at 4°C for 18 h at 70 V, and stained for 20 min with 0.5 μg/ml ethidium bromide. Gel images were captured and photographed using a FOTO/Analyst Archiver electronic documentation system (Fotodyne Inc., Hartland, WI), and rep-PCR DNA fingerprint analysis was done as described by Dombek et al. (2000) using Bionumerics Software (version 1.5; Applied Maths, Kortrijk, Belgium). A dendrogram was constructed based on the Pearson correlation method. The relatedness of isolates was assessed by multi-dimensional scaling analysis, using the similarity matrix generated by the Pearson correlation coefficient, with 0.5% optimization and ignoring uncertain bands.

Plasmid and probe isolation

Plasmid pRJ676 (Hennecke, 1981) and cosmid pR32 (Sadowsky et al., 1991) were isolated by the alkaline lysis method (Sambrook et al., 1989). pRJ676 was digested with *Hind*III to obtain a 9 kb fragment containing the *B. japonicum nifHDKE* nitrogenase structural genes (Hennecke, 1981). Cosmid pR32, containing the *B. japonicum* nodulation genes was digested with *Hind*III, to release five previously described subclones, pMJS9, pMJS12, pMJS18, pMJS22 and pMJS24 encoding *nolA, nodD1YABC, nodD2, nodSUIJ,* and *nodZ* (Sadowsky et al., 1987; Sadowsky et al., 1991). Probe DNA was radiolabeled with [^{32}P]dCTP by using the Rediprime II random prime labeling system (Amersham, U.K.) and purified by using QIA Quick Gel Extraction Kit (Qiagen, Valencia, CA).

Southern hybridization analyses

Total genomic DNA from symbiotic and non-symbiotic isolates from each soil sample was isolated as previously described (Sadowsky et al., 1987; Sadowsky, 1994). Genomic DNA was digested with *Eco*RI or *Pst*I and restriction fragments were individually separated by horizontal electrophoresis on 0.8% agarose gels at 30 V for 24 h. Gels were stained and photographed as described above. DNAs on agarose gels were transferred to Nytran immobilization membranes (Schleicher & Schuell, Keene, NH) as described (Sambrook et al., 1989). Southern hybridizations were done using a Robbins Scientific hybridization incubator (Sunnyvale, CA) as described by the manufacturer, except for a few modifications; prehybridization were done at 65°C overnight in 20 ml of the prehybridization solution (0.25 M Na_2HPO_4, pH

7.4, 0.001 M EDTA, 1% BSA, 7% SDS), hybridizations were carried out at 65°C overnight, and membranes were finally washed in 0.2X SSC at 65°C.

Statistical analyses

BJSM plate and MPN plant assays were set-up in a completely randomized experimental design, with three replicates per treatment. The number of bradyrhizobial cells g^{-1} soil in each treatment were compared by using the Analysis of Variance subroutine of the IRRISTAT Program (Biometrics and Bioinformatics Unit, International Rice Research Institute, Manila, Philippines). Significant differences were assessed using Duncan's multiple ranges analysis with $\alpha = 0.05$.

3. Results and Discussion

Isolation of Bradyrhizobium from heavily- and un-inoculated Thai soils

The BJSM selective medium was used to directly isolate bradyrhizobia from Thai soils without trap host bias or enrichment. Ten-fold serial dilutions of each soil suspension were spread-plated onto BJSM medium and incubated at 28°C for 7 d. Single colonies were randomly selected and restreaked onto fresh AG medium in order to obtain pure cultures. While some contaminants were detected on BJSM, they were not selected and were discarded. Forty typical *Bradyrhizobium*-like colonies were subsequently collected from the heavily-inoculated soybean field soils in Khon Kaen (KKI) and Chiang Mai (CMI), and 50 colonies were collected from an uninoculated field in Chiang Mai (CMU) using the BJSM. All the isolated bacteria were rod-shaped, gram negative cells. The colonies were about 1 mm in size, opaque-white on AG medium, and pink on medium amended with congo red. The isolates produced an alkaline reaction on medium containing bromthymol blue, showed poor to no growth on peptone glucose agar, and failed to grow on nutrient agar. These characteristics are similar to those seen for the "typical" control bradyrhizobia used.

Comparison of enumeration methodologies

Bacterial counts in soils were determined at the time of sampling using two methodologies: plate counts on the BJSM selective medium and the most-probable-number (MPN) plant infection assay using soybean (*Glycine max* cv. SJ5; Thai local variety), mungbean (*Vigna radiata*) and siratro (*Macroptilium atropurpureum*) as hosts. The number of bacteria per gram soil estimated from total plate count on BJSM and MPN plant infection assay are shown in Table 2.

Table 2. Comparison of the number of bradyrhizobia in three Thai soils by plant infection MPN and BJSM assays

Soil source[1]	Number of bradyrhizobia g^{-1} as determined by:			
	BJSM[3]	MPN assay on host[2]		
		Soybean	Mungbean	Siratro
KKI	6.6×10^6 b[4]	1.2×10^6 Bb	4.4×10^4 Ab	2.2×10^6 Bb
CMI	2.2×10^6 ab	7.2×10^5 Bb	1.5×10^4 Aa	1.2×10^6 Bb
CMU	1.1×10^6 a	8.6×10^4 ABa	8.6×10^3 Aa	3.0×10^5 Ba

[1]Source: KKI, inoculated field in Kohn Kaen; CMI, inoculated field in Chiang Mai, CMU, uninoculated field in Chiang Mai. [2]Mean numbers (n = 4). [3]Values shown are means of triplicate analyses. [4]Means follows by the same lower case letter in a column, or upper case letter in a row, are not significantly different at $\alpha = 0.05$.

Results in Table 2 show that all the tested soils contained between 1.1–6.6 × 10^6 *Bradyrhizobium*-like cells g^{-1} soil, as estimated on BJSM. While cell numbers from soils at the two inoculated sites did not differ significantly (α = 0.05), the number of cells obtained from KKI differed significantly from that of the uninoculated soil, CMU. However there was no significant difference between bradyrhizobial counts at CMI and CMU, the two sampling sites in Chiang Mai.

In contrast, the number of soybean bradyrhizobia g^{-1} soil at the three sites, as estimated by the MPN plant infection assay, ranged from $8.6 \times 10^4 - 1.2 \times 10^6$. There was no significant difference ($\alpha = 0.05$) between the number of soybean bradyrhizobia in the KKI and CMI sites, both of which have previously been inoculated for soybean cultivation. However, there was a significant difference ($\alpha = 0.05$) in bradyrhizobial numbers between the inoculated and the uninoculated fields. The number of bradyrhizobia which nodulated mungbean from the KKI, CMI, and CMU sites ranged from $8.6 \times 10^3 - 4.4 \times 10^4$ g^{-1} soil, and there was a significant difference between the number of bradyrhizobia at the KKI site to those at the CMI and CMU sites. Similarly, there was variation in the MPN estimated number of siratro-nodulating bacteria number at the three sites, which ranged from $3.0 \times 10^5 - 2.2 \times 10^6$ g^{-1} soil. Mean numbers of siratro-nodulating bradyrhizobia obtained from both the KKI and CMI inoculated fields, did not differ significantly from each other ($\alpha = 0.05$), however they were significantly greater than those found in soils at the CMU site. Interestingly, when siratro was used as trap host plant, the number of bradyrhizobia g^{-1} soil estimated by MPN plant infection assay were about 1.8

to 3.5 times less than those obtained by plate count on the BJSM selective medium. Tong and Sadowsky (1994) previously reported that MPN counts for soybean nodulating bacteria were generally 0.5-fold greater than those obtained on BJSM. This variance either reflects a large proportion of non-nodulating bradyrhizobia in soils, or the presence of colonies on the selective medium that were not bradyrhizobia.

Symbiotic properties of Bradyrhizobium-like cells isolated on BJSM

The isolates obtained from the three soils on BJSM medium were evaluated for their ability to form root nodules on soybean, cowpea, and mungbean. Results of nodulation studies in Table 3 show that non-symbiotic bradyrhizobial-like cells were recovered from inoculated and uninoculated field sites, and constituted 55%, 40%, and 58% of total bradyrhizobial populations for the inoculated KKI and CMI, and CMU sites, respectively. These results indicate that non-symbiotic bradyrhizobia can remain in soils in the presence or absence of a legume host. This is consistent with results obtained by Segovia et al. (1991) who found that non-symbiotic rhizobia were abundant in the rhizosphere, and Sullivan et al. (1996) who found that non-symbiotic rhizobia persist in soil in the absence of a legume.

Table 3. Numbers of symbiotic and nonsymbiotic isolates obtained from three Thai soils

Soil source[1]	Total isolates	Isolates nodulating: Soybean	Mungbean or Cowpea	Nonsymbiotic isolates
KKI	40	15 (37.5)[2]	3 (7.5)	22 (55.0)
CMI	40	15 (37.5)	9 (22.5)	16 (40.0)
CMU	50	11 (22.0)	10 (20.0)	29 (58.0)
Total	130	41 (31.5)	22 (16.9)	67 (51.5)

[1]Soil: KKI and CMI, inoculated fields in Khon Kaen and Chiang Mai, respectively. CMU, uninoculated field in Chiang Mai. [2]Numbers in parentheses refer to percentage in each category.

Several studies have reported that non-symbiotic rhizobia were more numerous than symbiotic rhizobia in rhizosphere and soil samples (Laguerre et al., 1993; Segovia et al., 1991; Soberon-Chavez and Najera, 1989). Sullivan et

al. (1996) suggested that symbiotic properties in some bacteria are genetically unstable, raising the possibility that non-symbiotic rhizobia are a significant component of rhizobial populations in the soil. They hypothesized that the isolates that were originally non-symbiotic bacteria could acquire symbiotic genes from inoculant strains. Genetic transfer of symbiotic genes from inoculants to non-symbiotic bacteria in soil would thus contribute to the rapid diversification of populations of legume symbionts in soils when inoculated legumes are cultivated (Rao et al., 1994; Sullivan et al., 1995).

While the origin of non-symbiotic rhizobia is unknown, there are at least two possible explanations for their presence in field sites. It is possible that non-symbiotic rhizobia are relatively common saprophytic bacteria in soil. Alternatively, the non-symbiotic rhizobia may be descendants of rhizobia from native legumes once present at the site that have lost their capacity to nodulate and fix nitrogen (Sullivan et al., 1996). Presumably, this also holds true for the bradyrhizobia.

Sequence analysis of genes encoding 16S rRNA

In order to ascertain whether the non-symbiotic isolates belong to the genus *Bradyrhizobium*, the partial 16S rDNA gene sequences of 13 non-symbiotic isolates were determined using primer pair 926f and 1392r (Lane, 1991). PCR results indicated that each isolate had a single PCR product of about 500 bp (data not shown). All the partial 16S rRNA sequences were very similar to those of *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, or *Bradyrhizobium liaoningensis*. Of the 13 non-symbiotic isolates examined, four (KKI14, CMU16, CMU28, and CMU29) had 16S rDNA sequences showing 79–99% similarity to *B. elkanii* strains USDA94, USDA31, USDA130, USDA90, USDA23, USDA121, USDA101, PRY52, PRY49, SEMIA 5019, SEMIA587, and to several *Bradyrhizobium* sp. strain including LMG10689, LMG9966, LMG9520, LMG9980. Consequently, these isolates were assigned to the genus *Bradyrhizobium*. The 16S rDNA sequence of KKI28 was identical to *B. japonicum* strains USDA62, USDA4, USDA38, USDA135, USDA129, USDA127, USDA126, USDA125, USDA124, USDA123, USDA122 and *Bradyrhizobium liaoningense* strain 2281. The remaining 8 isolates (KKI16, KKI20, KKI21, CMI1, CMI11, CMI17, CMI23, CMU3) had 16S rDNA sequences with 96–99% identity to several bacteria including *B. japonicum*, *Rhodopseudomonas*, and *Afipia*. These non-symbiotic isolates were also very similar to *B. japonicum* strains MSDJ5569, MSDJ5568, MSDJ5563, USDA59, USDA32, PRY42, PRY40, PRY1, SEMIA5079, and SEMIA566. The nucleotide sequences of genes encoding for 16S rRNA in these strains have been deposited in GenBank under accession numbers AF509893-AF509908.

To more precisely determine the phylogenetic positions of the non-symbiotic isolates, nearly full-length 16S rDNA sequences were obtained for three non-symbiotic isolates representing each soil sample (KKI14, CMI23, and CMU16). The rDNA from these isolates were amplified using primer pair 16Sa and 16Sb (van Berkum and Fuhrmann, 2000), resulting in a characteristic single band of about 1,500 bp in size (data not shown). Sequence analyses of nearly full-length rDNA indicated that matches to database entries were in general agreement with those obtained using partial 16S rRNA analysis. Sequence alignments done using BLAST indicated that two of the isolates (KKI14 and CMU16) had 16S rDNA sequences with 96–99% similarity to many strains of *B. elkanii* and *B. lupini* subgroups. Similar results were obtained using matching algorithms (Sequence Match) on the RDP server. Sequence analysis of nearly full-length rDNA from CMI23 using Blast and Sequence Match indicated that this isolate had two regions of homology (94–99%) to *B. japonicum*, *Bradyrhizobium* sp., *Afipia* genosp., and *Rhodopseudomonas* sp. strains. Taken together, these results indicate that the non-symbiotic isolates obtained in this study are phylogenetically related to *Bradyrhizobium* sp. strains, and as such appear to bonafide non-symbiotic bradyrhizobia.

Generally speaking, our results are in agreement with Young (1996) and Willems et al. (2001) who demonstrated that the phylogenetic cluster formed by all bradyrhizobia includes a number of bacteria that are not rhizobia, including *Rhodopseudomonas*, *Nitrobacter*, and *Afipia*. Interestingly, Saito et al. (1998) found that full 16S rRNA gene sequences of four isolates of slow-growing, oligotrophic, bacteria were closely related to *B. japonicum* (similarity values of 98.1–98.8%), although none of the strains showed hybridization to *nod* gene probes of *B. japonicum*, and only one strain exhibited hybridization to *nifDK*- and *hupSL*- specific DNA. These results further suggest that there are many metabolically diverse soil bacteria that are closely related to *B. japonicum*, although they do not have symbiotic functionality.

Serological identity of bradyrhizobial isolates

A total of 130 isolates indigenous to soils at the three sites were examined for their serological relatedness to each other by using antisera produced against the somatic antigens of 19 *Rhizobium* and *Bradyrhizobium* strains. Among the 19 antisera tested, 11 were prepared against SEMIA, IFO, TAL, and USDA strains, which originated in temperate regions, and the eight remaining antisera were prepared against THA strains, and indigenous tropical Thai isolates. Results of this analysis indicated that 77 of 130 isolates (59.2%) demonstrated serological reaction (by agglutination) to the tested antisera, most cross-reacting with more than one antiserum. However, 53 isolates,

representing 40.8% of the total tested, failed to show agglutination cross-reaction with any of tested antisera, and none of the isolates reacted with antisera against SEMIA5019, IFO15247, THA2, and USDA205. Fifty-six of the serologically-reactive isolates, representing 43.1% of the total number, reacted with antiserum prepared against THA205. The remaining serologically-reactive isolates were distributed into each of 19 somatic serogroups, consisting of 1 to 11 isolates (Table 4). There was no apparent relationship between symbiotic ability and soil of isolation, and serological reaction, both symbiotic and non-symbiotic isolates reacted belonged to the same serogroups and came from the same or different soils (data not shown). However, there was an apparent relationship between serological reaction and nodulation ability. For example, the 22 isolates in groups 1,2,3,4,13,14,16, and 19 all failed to nodulate any of the three tested legume hosts.

Table 4. Agglutination reactions of indigenous bradyrhizobial isolates with 19 antisera against *Rhizobium* and *Bradyrhizobium* strains

Group	Cross-reaction with antisera	Sym. group[1]	No. of isolates[2]
1	TAL211, TAL 1402, THA205	N	5 (3.9)
2	TAL211, THA205, USDA193	N	4 (3.1)
3	TAL211, THA205, KKI1, KKI17	N	3 (2.3)
4	TAL211, THA205, KKI17	N	4 (3.1)
5	TAL377, THA205	S	6 (4.6)
6	THA7, THA205, THA301, USDA31, USDA117	S	7 (5.4)
7	THA7, THA205, THA301, USDA110	M	3 (2.3)
8	THA7,THA205, KKI8	S	1 (0.8)
9	THA7, THA301, USDA110	M	2 (1.5)
10	CMU8	N, M	11 (8.5)
11	THA 205	N, S	8 (6.2)
12	THA205, USDA 31, KKI1, KKI17	S	6 (4.6)
13	THA205, USDA 94	N	1 (0.8)
14	THA205, KK18	N	2 (1.5)
15	THA205, CMU8	S	6 (4.6)
16	USDA 31	N	1 (0.8)
17	USDA 94	M	1 (0.8)
18	USDA 31, KKI1, KKI17	S	4 (3.1)
19	USDA 110, KKI8	N	2 (1.5)

[1]S = Symbiotic isolates nodulating soybean, M = symbiotic isolates nodulating mungbean and cowpea, and N = nonsymbiotic isolates for soybean, mungbean, siratro, and cowpea.
[2]Values represent number of isolates reacting with antisera in each group with % of total number in parentheses.

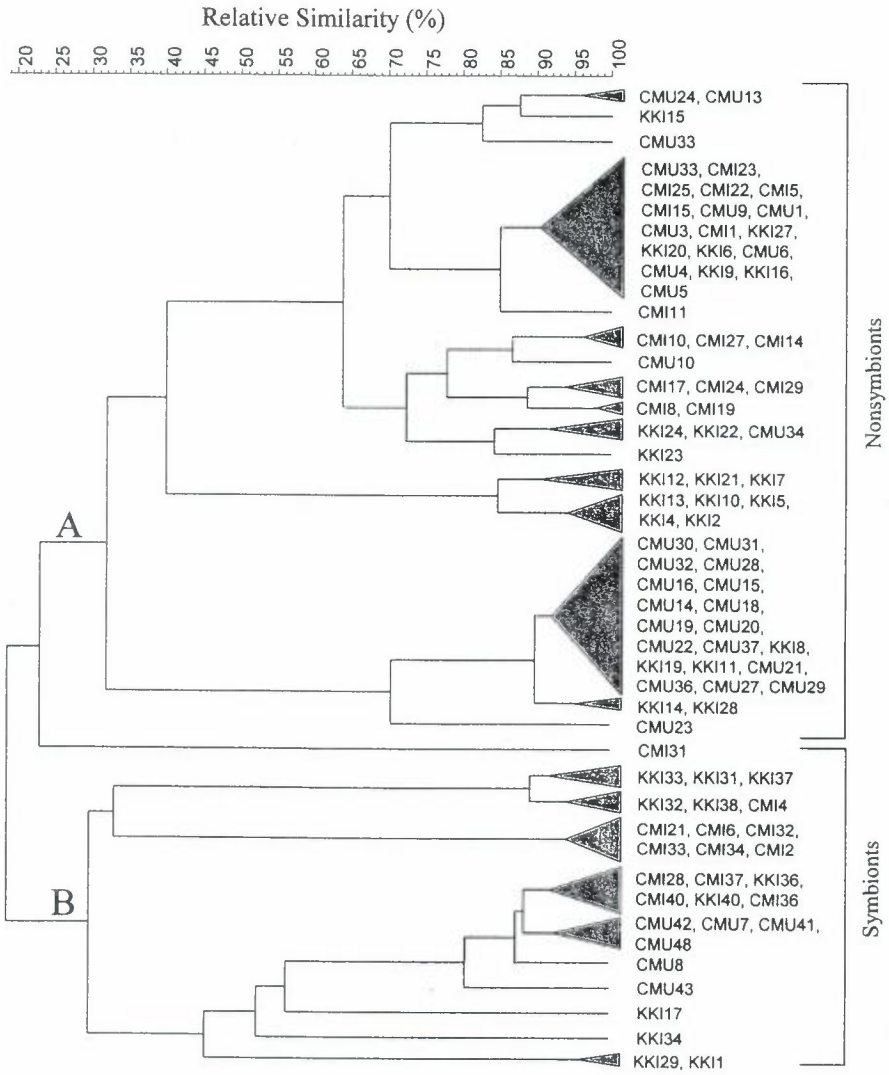

Figure 1. Genetic relatedness of nonsymbiotic and symbiotic bradyrhizobia recovered from Thai soils. Relatedness assessed by using rep-PCR DNA fingerprinting with BOXA1R primers. The dendrogram was created by using Pearson's correlation method and with position tolerance optimization set to 0.5%.

Generally speaking, the number of isolates reacting with antisera from temperate region strains was less than those reacting against antisera prepared

from Thai local strains. Similar results were reported by Thompson et al. (1991) who examined the serological properties of 1,500 root-nodule bacteria isolated from five different hosts in northern Thailand. In addition, Yokoyama et al. (1996) reported that 24 of 62 Thai strains reacted strongly with antiserum prepared against USDA31, by enzyme-linked immunosorbent assay and the agglutination test. In our studies, 18 of 130 Thai isolates also reacted with antiserum against USDA31. Consequently, either the USDA bradyrhizobial strains share antigens in common with those in Thai soils, or they are direct descendants of the Thai bradyrhizobia.

BOXA1R PCR fingerprints

Ninety-four, indigenous, Thai bradyrhizobial strains were examined for similarities by using rep-PCR DNA fingerprinting and BOXA1R primers. Individual lanes generally contained from 15 to 30 PCR products, ranging from approximately 300 to 4,500 bp in size (data not shown). To determine the relatedness of isolates, a dendrogram based on BOX-derived fingerprint data was constructed by using Pearson correlations. Multi-dimensional scaling analysis (MDS) was done to examine the fidelity of the groups.

The resulting dendrogram (Fig. 1) was found to reflect the overall diversity and symbiotic characteristics of the indigenous bradyrhizobia analyzed in this study, Cluster A was comprised of the 65 non-symbiotic isolates and 1 symbiotic isolate, and Cluster B contained 28 of 29 symbiotic isolates. These two main clusters diverged at an average estimated similarity value of 20%, reflecting deep divergence and possibly separate evolutionary lineages. The rep-PCR analysis revealed clearly distinct patterns for the symbiotic and non-symbiotic isolates, regardless of their site of origin, and this was reflected in MDS analyses (Fig. 2).

This result indicated that there was significant genetic diversity among bradyrhizobia that were isolated directly from soil samples. However, some of the isolates were closely related to each other. Other studies examining the genetic structure of rhizobia from different geographical origins have reported similar results. For example, similar strains of *Rhizobium leguminosarum* are found on native legumes in western North America and in Europe (Strain et al., 1995).

Similarly, Laguerre et al. (1997) evaluated the genetic diversity of 44 rhizobial isolates from different host plants and different geographic locations, and reported that rhizobial classification at the genus, and probably also the species, levels was independent of geographic origin and host plant affinity.

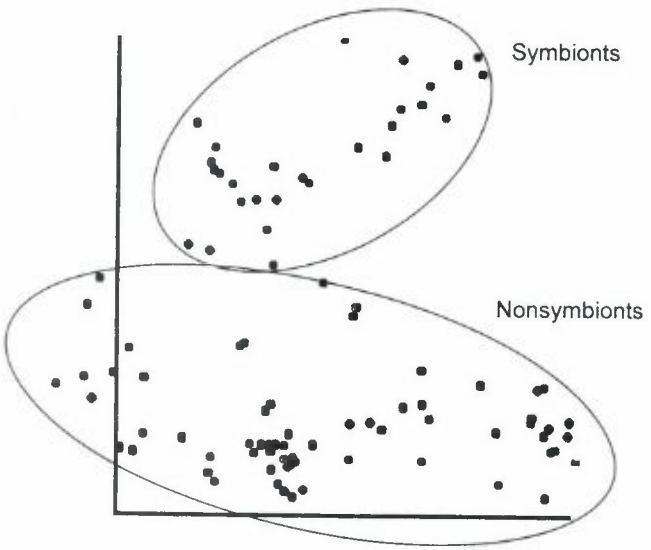

Figure 2. Multi-dimensional scaling (MDS) analysis of rep-PCR DNA fingerprints from nonsymbiotic and symbiotic bradyrhizobia. Analysis was done with the similarity matrix generated using Pearson's correlation coefficients, with 0.5% optimization, and ignoring uncertain bands. Symbols: o Nonsymbionts, o Symbionts. For purposes of clarity, only two of the three dimension axes are presented.

Lafay and Burdon (1998) also found that there are great similarities between rhizobial communities in southeastern Australia and at the distant and climatically different sites in Queensland, and del Papa et al. (1999) demonstrated the existence of similar populations of alfalfa-nodulating rhizobia in geographically distant regions that have soil acidity as a common feature.

Data obtained from our serological studies reported here are consistent with the rep-PCR fingerprint patterns. The isolates which exhibited high similarity by rep-PCR DNA fingerprinting were grouped into identical serogroups. Nevertheless, the results indicated that some isolates belonging to the same serogroup formed distinct clusters based on PCR fingerprint patterns.

Hybridization to symbiotic probes

Twenty-four, indigenous, Thai bradyrhizobial isolates, including symbiotic and non-symbiotic strains obtained from the three soil samples, were examined

for the presence of nitrogen fixation and nodulation genes by Southern hybridization to *nif* and *nod*-specific gene probes. The *nod* gene probe, derived from restriction fragments of cosmid pR32, was chosen for these studies because it allows for the simultaneous probing of 12 nodulation genes. Results of these studies revealed that while all of the symbiotic isolates exhibited *nif* and *nod* gene-specific hybridization signals, the non-symbiotic isolates failed to hybridize to *nif* and *nod* gene probe. The symbiotic isolates yielded from 1 to 13 *nif*-specific hybridization signals, with approximate sizes ranging from 1.2 to 16.5 kb, and possessed 1 to 8 *nod*-specific fragments, with estimated size of 0.9 to 18.8 kb (data not shown). These results indicate that the failure of the non-symbiotic bradyrhizobia to nodulate or fix nitrogen with the three legume test hosts is due to a lack of symbiotic genes.

4. Conclusions

In our studies, genotypic and phenotypic criteria were used to evaluate the diversity of *Bradyrhizobium* strains that were directly isolated from soils using a selective medium. Among the 130 isolates obtained, 67 were non-symbiotic on three test hosts (soybean, siratro, mungbean, and cowpea) that are normally nodulated by *Bradyrhizobium* strains. This is a remarkable number and points to the fact that we are only just now learning about diversity among soil bacterial populations, some of which could previously only be isolated using specific trap host plants. Moreover, since the isolates analyzed were obtained directly from soils without a legume trap host, this study provides information about genetic diversity of bradyrhizobial populations, in which the non-nodulating bradyrhizobia are a significant component.

Based on 16S rRNA sequence analyses, most of the non-symbionts that were obtained from the inoculated field in Khon Kaen and all of the non-symbionts obtained from the inoculated field in Chiang Mai were classified as *B. japonicum* and most of the non-symbionts obtained from the uninoculated field in Chiang Mai were classified as *B. elkanii*. These results clearly show that non-symbiotic bradyrhizobia remain in soil both in the presence and absence of a legume plant. The effects of land management practices on bacterial diversity are not known. While it has been suggested that agriculture creates highly selective and homogenous environments that reduce bacterial diversity (Martinez-Romero and Caballero-Mellado, 1996), it has also been shown that inoculation of soybean and red clover with superior strains, over a period of several years, may help to displace native strains (Rao, 1999). Conversely, it has also been argued that cultivation results in more diverse populations (Palmer and Young, 2000), and a greater diversity of substrate utilization (Kennedy and Smith, 1995). In our studies, while inoculation clearly influenced

the diversity of isolates recovered from the field sites using BJSM, the results are not clear-cut. Taken together these results indicate that further studies are needed to examine the evolution of nodulating and non-nodulating root nodule bacteria that lack plasmid-encoded symbiotic genes and the influence of agronomic practices on these disparate populations.

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