

Presence of natural variants of *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum* and detection of *Bradyrhizobium yuanmingense* in Phitsanulok province, Thailand

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ABSTRACT: Soybean rhizobia are Gram negative bacteria that fix nitrogen in root nodules of soybeans. Selection of soybean rhizobia from present and previous soybean cultivation areas is one way to obtain efficient strains for inoculant production. At present, information on the diversity of soybean rhizobia in Thailand is scarce. The experiments aimed to isolate and characterize soybean rhizobium strains in soils from 16 subdistricts in Phitsanulok province, Thailand. Host trapping method was used to isolate bacteria from root nodules of 5 soybean cultivars grown in soils from the 16 subdistricts. Identical RAPD-PCR fingerprints revealed the 202 slow-growing isolates consisted of 121 strains. Authentication tests using 5 soybean cultivars showed all the 121 slow-growing strains had nodulated soybean roots. Four types of colony morphology on yeast extract mannitol containing congo red agar plates were obtained for all the isolated strains. Bromothymol blue (BTB) reactions on BTB agar plates revealed two types of slow-growing soybean rhizobia. Type 1 secreted alkali products after 5-day incubation, and acidic products upon prolonged incubation for another 5 days. Type 2 secreted only alkali products after 5- and 10-day incubation. Results from nucleotide sequences of 16S rDNA revealed 7, 6, and 2 strains of *Bradyrhizobium elkanii*, *B. japonicum*, and *B. yuanmingense*, respectively. The *B. elkanii* and *B. japonicum* strains were found to be natural variants with different RAPD-PCR fingerprints. This study is the first report on the findings of *B. yuanmingense* as well as natural variants of slow-growing soybean rhizobia in Thailand.

KEYWORDS: slow-growing soybean rhizobia, genetic diversity, 16S rDNA sequences, bromothymol blue reactions, RAPD-PCR fingerprints

INTRODUCTION

Soybean rhizobia are Gram negative, non-spore-forming bacteria that fix nitrogen in root nodules of soybeans. Efficient strains of soybean rhizobia have been selected for commercial production of soybean rhizobium biofertilizers world-wide^{1–3}. Presently, there are two recognized strains of fast-growing soybean rhizobia, namely *Sinorhizobium fredii* and *S. xinjiangense*^{4,5}. In addition, there are 4 recognized strains of slow-growing soybean rhizobia, i.e., *Bradyrhizobium elkanii*, *B. japonicum*, *B. liaoningense*, and the recently-discovered *B. yuanmingense* biovar that nodulates soybeans^{6–9}. At present, there is not much information on polyphasic taxonomy of soybean rhizobia in Thailand^{10–15}. Strains of soybean rhizobia in Thailand could be used as a pool to select

soybean rhizobia suitable for the production of biofertilizers. Increasing soybean yields would increase income for soybean growers and encourage farmers to continue cultivating soybeans as a rotational crop. Reducing nitrogen chemical fertilizer usage would reduce the extent of eutrophication and protect the soil environments for sustainable agriculture. The aim of this study is to isolate and characterize soybean rhizobia from 16 subdistricts in Phitsanulok province, one of the main soybean-cultivating areas in Thailand. Host-trapping method was employed to isolate 202 slow-growing bacterial isolates from soybean root nodules. Identical RAPD-PCR fingerprints using either RPO1 or CRL-7 as the primer revealed the 202 slow-growing isolates consisted of 121 strains. All the strains were found to nodulate soybean roots. Two types of bromothymol blue (BTB) reactions were

obtained for the isolated slow-growing soybean rhizobium strains. The BTB reaction, with the secretion of alkali products in the first 5-day incubation and the secretion of acidic products upon further incubation, has not been reported on slow-growing soybean rhizobia. Results from nucleotide sequences of 16S rDNA revealed 7, 6, and 2 strains of *B. elkanii*, *B. japonicum*, and *B. yuanmingense*, respectively. The *B. elkanii* and *B. japonicum* strains were found to be natural variants with different RAPD-PCR fingerprints.

MATERIALS AND METHODS

Soil collection sites

Soil samples were collected from 16 subdistricts of Phitsanulok province, Thailand (16° 49' 35" N, 100° 15' 37" E).

Isolation of bacteria from root nodules

Bacteria were isolated from root nodules of 5 local soybeans (*Glycine max* (L.) Merrill) cultivars ST1, ST2, SJ5, CM2, and CM60, by the host trapping method as described¹⁶. A small-looped needle was used to collect pink root tissue to spread onto yeast extract mannitol (YM) agar plates containing congo red at the final concentration of 25 µg/ml. The composition of YM medium was mannitol 10 g, K₂HPO₄ 0.5 g, MgSO₄ · 7 H₂O 0.2 g, NaCl 0.1 g, yeast extract 0.5 g, deionized water 1 l, pH 6.8. YM plates were incubated at 25 °C for 5 and 10 days. Different pinkish colonies were picked and streaked on YM agar plates containing congo red at the final concentration 25 µg/ml. Single colonies were streaked onto YM slants and kept at 4 °C for short-term storage with subculturing every 4 months. Long-term storage was by storing culture broth in 10% glycerol at –80 °C.

Chromosomal DNA isolation

Each root nodule bacterial isolate stored in YM slants at 4 °C was activated by streaking one loop onto YM agar plate. The plate was incubated for 1–5 days until visible colonies were observed. One loop of culture was inoculated into YM broth in a 250 ml flask and incubated in a temperature-controlled shaker at 200 rpm, 30 °C for 5 days. Cells were harvested by centrifugation at 8000g, 4 °C, 10 min, washed once with 0.85% NaCl to remove extracellular polysaccharides before chromosomal DNA isolation and RAPD-PCR fingerprinting.

Cells were broken by incubating in EDTA-lysozyme solution (2.5 mg/ml) at 37 °C for 1 h followed by freezing at –20 °C for 5 min and thawing at 80 °C for 5 min for 2 cycles. 250 µl DNAzol

(Molecular Research Centre) were added to the solution with gentle mixing by inverting the eppendorf tubes for hydrolysis of total RNA. Broken cells were centrifuged at 12 000g, 4 °C, for 10 min to get rid of cell debris. Supernatant was transferred to new eppendorf tubes. Chromosomal DNA was precipitated with ice-cold absolute ethanol at –80 °C for 15 min after adjusting the solution to acidic condition with 300 µl 3 M sodium acetate. DNA precipitate obtained from centrifugation at 17 500g, 4 °C, 10 min was dissolved in 20 µl high quality distilled water overnight. Quantity and quality of chromosomal DNA preparation were obtained by OD₂₆₀, OD₂₈₀ and 0.8% agarose gel electrophoresis by standard methods¹⁷.

RAPD-PCR fingerprinting

PCR mixture consisted of 2 µl 10× PCR buffer, 2.0 µl 10 mM dNTPs, 0.2 µl 100 pmol/µl primer RPO1 or CRL-7, 0.2 µl *Taq* polymerase (5 U/µl), DNA 200 ng, distilled water to 20 µl. Reported sequences of primers^{18,19} RPO1 and CRL-7 were as follows: RPO1, 5'-AATTTTCAAGCGTCGTGCCA-3'; CRL-7, 5'-GCCCCGCCG-3'. PCR program was as follows: 95 °C 15 s, 55 °C 30 s, 72 °C 90 s for 5 cycles, 95 °C 15 s, 60 °C 30 s, 72 °C 90 s for 25 cycles, followed by 72 °C 10 min. PCR products were separated on 1.25% agarose gel electrophoresis. Gels were stained with 10 mg/ml ethidium bromide for 10 min, destained in distilled water for 30–45 min before taking a Polaroid photo (FUJI Film FP-3000B) with BIO-RAD UV transilluminator equipped with a polaroid camera set-up. Isolates with identical RAPD-PCR fingerprints were assigned to the same strains.

Authentication tests of slow-growing bacterial strains

All the 121 slow-growing bacterial strains were authenticated to determine if they were slow-growing soybean rhizobia by observing formation of nodules on soybean roots grown in Leonard jars as described¹⁶ using seeds of each of the 5 soybean cultivars (*G. max* cv. ST1, ST2, SJ5, CM2, and CM60).

Sequencing of 16S rDNA

16S rDNA of each of randomly-selected 20 strains was isolated by PCR using standard methods and primers 27f (5'-GAGTTTGATCCTGGCTCAG-3') and 1492r (5'-ACGGCTACCTTGTTACGACTT-3')²⁰. Each PCR product of approximately 1500 bp was sent to the BioDesign Co. Ltd., Thailand Science Park, for sequencing with 9 primers²⁰. The BIOEDIT program (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) was used to obtain sequences of sense strands

of 16S rDNAs. Sequences of the 16S rDNA were deposited at Genbank with the accession numbers HQ533228–HQ533247. Identities of the 20 randomly-selected slow-growing soybean rhizobia were obtained by comparing sequences of 16S rDNA with available sequences deposited at GenBank (<http://www.ncbi.nlm.nih.gov/>) using the BLAST program.

Colony morphology and bromothymol blue reactions

Colony morphology and bromothymol blue reactions were obtained by streaking cells onto agar plates containing YM medium with either congo red or bromothymol blue at 25 µg/ml final concentration. For bromothymol blue reactions, the plates were incubated at 30 °C for 5 days and 10 days with observation for colour of the indicator dye on the plates at the end of the 5- and 10-day incubation periods. According to Somasegaran and Hoben¹⁶, fast-growing rhizobia changed colour of the indicator dye to yellow while slow-growing rhizobia turned the indicator dye to blue.

RESULTS

16S rDNA sequencing

Table 1 shows BLAST results of the 20 slow-growing soybean rhizobium STB strains based on homology of 16S rDNA sequences. The results indicate that the 20 STB isolates consisted of 12 *B. elkanii* isolates (STB8, STB119, STB120, STB147, STB173, STB176, STB179, STB185, STB220, STB238, STB245, and STB327); 6 *B. japonicum* strains (STB30, STB54, STB67, STB96, STB250, and STB310); one *B. yuanmingense* strain (STB264), and one *B. liaoningense/yuanmingense* strain (STB169). Fig. 1 shows that identical RAPD-PCR fingerprints when CRL-7 was used as the primer indicated that isolates STB119, STB179, STB185, and STB238 were the same strain, isolates STB147 and STB327 were the same strain, isolates STB173 and STB176 were the same strain, isolates STB245 and STB252 were the same strain. The fingerprint data in conjunction with 16S rDNA sequences indicated strains STB8, STB119, STB120, STB147, STB173, STB220, and STB245 were 7 natural variants of *Bradyrhizobium elkanii*.

Fig. 2 shows RAPD-PCR fingerprints of *Bradyrhizobium japonicum* isolates when either RPO1 or CRL-7 was used as the primer. Identical fingerprints suggest that isolates STB30, STB38, STB269, and STB286 were the same strain, isolates STB54 and STB235 were the same strain. The fingerprints data in conjunction with 16S rDNA

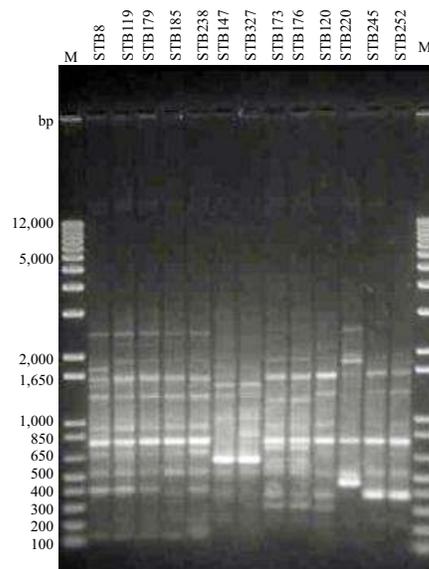


Fig. 1 RAPD-PCR fingerprints of *Bradyrhizobium elkanii* isolates when CRL-7 was used as the primer. Identical RAPD-PCR fingerprints showed isolates STB119, STB179, STB185, and STB238 were the same strain, isolates STB147 and STB327 were the same strain, isolates STB173 and STB176 were the same strain, isolates STB245 and STB252 were the same strain. The fingerprints data in conjunction with 16S rDNA sequences indicated strains STB8, STB119, STB120, STB147, STB173, STB220, and STB245 were 7 natural variants of *B. elkanii*.

sequences identify the strains STB30, STB54, STB67, STB96, STB250, and STB310 were 6 natural variants of *B. japonicum*.

Colony morphology and bromothymol blue reactions

Colonies of all the 7 *B. elkanii* strains were irregular and slimy after 10-day incubation on YMA with congo red medium. Colonies of all the 6 *B. japonicum* strains were round, pearly, and about 1 mm in diameter. Colony morphology of *B. yuanmingense* strain STB169 was irregular and slimy, while that of *B. yuanmingense* strain STB264 was round, slimy, and about 1 mm in diameter. All the isolated slow-growing soybean rhizobia did not absorb congo red. Bromothymol blue (BTB) reactions on BTB agar plates revealed two types of reactions. In the first type of reaction, cells were found to secrete alkali product(s) after 5-day incubation then secreted acidic product(s) upon prolonged incubation for another 5 days. Cells with the second type of BTB reaction were found to secrete only alkali product(s) after 5- and 10-day

Table 1 Summary of BLAST results of 20 slow-growing soybean rhizobium STB isolates based on 16S rDNA sequences.

Isolate	Size of 16S rDNA (bp)	Homology with sequences in GenBank	BLAST result*
STB8	1451	1451/1451 (100%) with no gap	<i>B. elkanii</i> strain SEMIA 6096 (9)
STB30	1451	1450/1452 (99%) with 2 gaps	<i>B. japonicum</i> strain HMS-02
STB54	1452	1450/1452 (99%) with 1 gap	<i>B. japonicum</i> strain HMS-02
STB67	1451	1449/1451 (99%) with no gap	<i>B. japonicum</i> strain HMS-02
STB96	1450	1450/1451 (99%) with 1 gap	<i>B. japonicum</i> strain HMS-02
STB119	1451	1450/1451 (99%) with no gap	<i>B. elkanii</i> strain SEMIA 6096 (9)
STB120	1451	1451/1451 (100%) with no gap	<i>B. elkanii</i> strain SEMIA 6096 (9)
STB147	1450	1450/1451 (99%) with 1 gap	<i>B. elkanii</i> strain SEMIA 6096 (9)
STB169	1449	1449/1451 (99%) with 2 gaps	<i>B. yuanmingense</i> strain TTC4 (9)
STB173	1452	1450/1452 (99%) with 1 gap	<i>B. elkanii</i> strain SEMIA 6096 (9)
STB176	1452	1449/1454 with 5 gaps	<i>B. elkanii</i> strain SEMIA 6096 (9)
STB179	1451	1451/1451(100%) with no gap	<i>B. elkanii</i> strain SEMIA 6096 (9)
STB185	1451	1450/1451 (99%) with no gap	<i>B. elkanii</i> strain SEMIA 6096 (9)
STB220	1451	1450/1451 (99%) with no gap	<i>B. elkanii</i> strain SEMIA 6096 (9)
STB238	1451	1450/1452 (99%) with 2 gaps	<i>B. elkanii</i> strain SEMIA 6096 (9)
STB245	1451	1451/1451 (100%) with no gap	<i>B. elkanii</i> strain SEMIA 6096 (9)
STB250	1451	1450/1451 (99%) with no gap	<i>B. japonicum</i> strain HMS-02
STB264	1451	1449/1452 (99%) with 2 gaps	<i>B. yuanmingense</i> strain TTC4 (9)
STB310	1449	1448/1451 (99%) with 2 gaps	<i>B. japonicum</i> strain HMS-02
STB327	1451	1450/1451 (99%) with no gap	<i>B. elkanii</i> strain SEMIA 6096 (9)

* Brackets contain number of BLAST matches that are identical to the sequence.

incubation. All the *B. japonicum* and *B. yuanmingense* strains were found to secrete alkali followed by acidic products, while all the *B. elkanii* strains were found to secrete alkali product(s) throughout the 10-day incubation.

DISCUSSION

Colony morphology and bromothymol blue reactions in slow-growing soybean rhizobia

All of the identified *B. elkanii* strains secreted alkali product(s) throughout the 10-day incubation period while *B. japonicum* and *B. yuanmingense* strains secreted alkali product(s) in the first 5 days of incubation and secreted acidic product(s) in the last 5 days of incubation. According to Somasegaran and Hoben¹⁶, the indicator dye bromothymol blue is green in YMA with pH 6.8. All slow-growing soybean rhizobia have been reported so far to turn the colour of bromothymol blue to blue due to the secretion of alkali product(s) while all fast-growing soybean rhizobia have been reported to turn the indicator dye to yellow due to the secretion of acidic products. However, this study, demonstrates for the first time that slow-growing soybean rhizobia *B. japonicum* and *B. yuanmingense* exhibit two types of bromothymol blue reactions depending on the length of the incubation time. The strains were found to secrete alkali product(s) during the first 5 days of incubation as expected for slow-

growing soybean rhizobia. In addition, they were found to secrete acidic product(s) during the last 5 days of incubation. The results could be interpreted as *B. japonicum* and *B. yuanmingense* might be able to adjust pH of the surroundings as follows: when the pH of the surroundings are in the alkali range, the two slow-growing species secrete acidic product(s) to adjust the surrounding pHs to the acidic range and vice versa.

Predominance of slow-growing soybean rhizobia in 16 subdistricts of Phitsanulok province and the prevalence of natural variants

The authentication test results on isolated bacteria from root nodules obtained in the experiments indicated that only slow-growing soybean rhizobia were obtained. One reason for the predominance of slow-growing soybean rhizobia in Phitsanulok province, Thailand, is the acidity of the soil samples which were in the range of 4.5–6.5. Soybean rhizobia on the Okinawa Islands in Japan are mainly fast-growing soybean rhizobia due to the alkalinity of the soils²¹.

This study is the first report on the record of *B. yuanmingense* in Thailand. Previously there were reports on the isolation and characterization of *B. yuanmingense* strains that nodulated mungbean and soybean^{9,22} and *B. yuanmingense* strain that did not nodulate soybean but nodulated legume species of

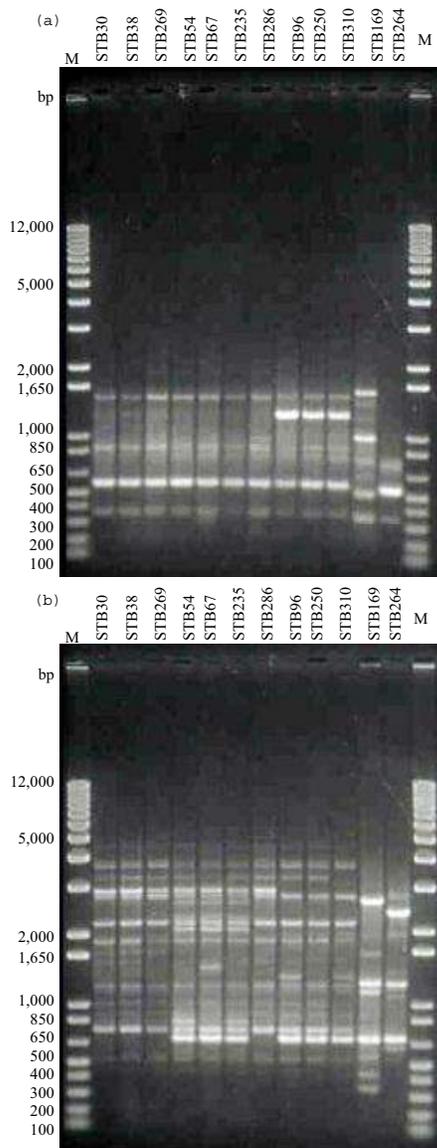


Fig. 2 RAPD-PCR fingerprints of *Bradyrhizobium japonicum* isolates when (a) RPO1 or (b) CRL-7 was used as the primer. Identical RAPD-PCR fingerprints showed isolates STB30, STB38, STB269, and STB286 were the same strain, isolates STB54 and STB235 were the same strain. The fingerprints data in conjunction with 16S rDNA sequences indicated strains STB30, STB54, STB67, STB96, STB250, and STB310 were 6 natural variants of *B. japonicum*.

the genus *Lespedeza*²³. In this study, two *B. yuanmingense* strains STB169 and STB264 were isolated. In addition, this study reveals natural variants of slow-growing soybean rhizobia for the first time in Thailand. The evidence was RAPD-PCR fingerprints when either RPO1 or CRL-7 was used as the primer

(Fig. 1 and Fig. 2). The results showed the 7 STB *B. elkanii* strains (STB8, STB119, STB120, STB147, STB173, STB220, and STB245) and the 6 *B. japonicum* STB strains (STB30, STB54, STB67, STB96, STB250, and STB310) had different DNA fingerprints. Previously, natural variants in slow-growing soybean rhizobia were reported from Brazil, where natural variants of *B. japonicum* SEMIA 566 strain used in Brazilian commercial inoculants from 1966–1978 were found²⁴. All the natural variants reported in Brazil might have arisen from genetic adaptations to the soil environments in Brazil and possibly by lateral gene transfer^{25, 26}.

Multilocus sequencing analysis in the identification and determination of phylogenetic relationships in natural variants of slow-growing soybean rhizobia

The average size of isolated 16S rDNAs of the 20 slow-growing STB strains around 1450 bp were in the same range previously reported^{27, 28}. However, sequences of only one gene, 16S rDNA, cannot be used to resolve differences amongst natural variants of the 7 *B. elkanii* and 6 *B. japonicum* STB strains observed in PCR-DNA fingerprints (Fig. 1 and Fig. 2). Therefore, there is a trend towards identification and phylogenetic relationship determination by multilocus sequence analysis²⁹, which has been used to identify slow-growing soybean rhizobia³⁰.

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Note: Nucleotide sequence data reported are available in the GenBank database under the accession numbers: HQ533228–HQ533247.

REFERENCES

- Bala A, Karanja N, Murwira M, Lwimbi L, Abaidoo R, Giller K (2011) Production and use of rhizobial inoculants in Africa. www.N2Africa.org, 21 pp.
- Aguilar OM, López MV, Riccillo PM (2001) The diversity of rhizobia nodulating beans in Northwest Argentina as a source of more efficient inoculant strains. *J Biotechnol* **91**, 181–8.
- Goday LP, Vasconcelos ATR, Chueire LMO, Souza RC, Nicolás MF, Barcellos FG, Hungria M (2008) Genomic panorama of *Bradyrhizobium japonicum* CPAC15, a commercial inoculant strain largely established in Brazilian soils and belonging to the same serogroup as USDA 123. *Soil Biol Biochem* **40**, 2743–53.
- Chen WX, Yan GH, Li JL (1988) Numerical taxonomic study of fast-growing soybean rhizobia and a proposal

- that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *Int J Syst Evol Microbiol* **38**, 392–7.
5. Peng GX, Tan ZY, Wang ET, Reinhold-Hurek B, Chan WF, Chen WX (2002) Identification of isolates from soybean nodules in Xinjiang region as *Sinorhizobium xinjiangense* and genetic differentiation of *S. xinjiangense* from *Sinorhizobium fredii*. *Int J Syst Evol Microbiol* **52**, 457–62.
 6. Jordan DC (1982) Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow growing, root nodule bacteria from leguminous plants. *Int J Syst Evol Microbiol* **32**, 136–9.
 7. Kuykendall LD, Saxena B, Devine TE, Udell SE (1992) Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. *Can J Microbiol* **38**, 501–5.
 8. Xu LM, Ge C, Cui Z, Li J, Fan H (1995) *Bradyrhizobium liaoningense* sp. nov. isolated from the root nodules of soybeans. *Int J Syst Evol Microbiol* **45**, 706–11.
 9. Appunu C, N' Zoue A, Laguerre G (2008) Genetic diversity of native bradyrhizobia isolated from soybeans (*Glycine max* L.) in different agricultural-ecological-climatic regions of India. *Appl Environ Microbiol* **74**, 5991–6.
 10. Thompson JA, Bhromsiri A, Shutsrirung A, Lillakan S (1991) Native root-nodule bacteria of traditional soybean growing areas of northern Thailand. *Plant Soil* **135**, 53–65.
 11. Yokoyama T, Ando S, Murakami T, Imai H (1996) Genetic variability of the common *nod* gene in soybean bradyrhizobia isolated in Thailand and Japan. *Can J Microbiol* **42**, 1209–18.
 12. Nuntagij A, Abe M, Uchiumi T, Seki Y, Boonkerd N, Higashi S (1997) Characterization of *Bradyrhizobium* strains isolated from soybean cultivation in Thailand. *J Gen Appl Microbiol* **43**, 183–7.
 13. Teamroong N, Boonkerd N (1998) Detection of *Bradyrhizobium* spp. and *B. japonicum* in Thailand by primer-based technology and direct DNA extraction. *Plant Soil* **204**, 127–34.
 14. Ando S, Yokoyama T (1999) Phylogenetic analyses of *Bradyrhizobium* strains nodulating soybean (*Glycine max*) in Thailand with reference to the USDA strains of *Bradyrhizobium*. *Can J Microbiol* **45**, 639–45.
 15. Yokoyama T, Ando S, Tsuchiya K (1999) Serological properties and intrinsic antibiotic resistance of soybean bradyrhizobia isolated from Thailand. *Soil Sci Plant Nutr* **45**, 505–15.
 16. Somasegaran P, Hoben HJ (1994) *Handbook for Rhizobia*. Methods in Legume-Rhizobium Technology. New York: Springer-Verlag, pp 240–58.
 17. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, Book 1, Cold Spring Harbor Laboratory Press, New York, p A3.
 18. Richardson AE, Viccars LA, Watson JM, Gibson AH (1995) Differentiation of *Rhizobium* strains using the polymerase chain reaction with random and directed primers. *Soil Biol Biochem* **27**, 515–24.
 19. Mathis JN, McMillin DE (1996) Detection of genetic variation in *Bradyrhizobium japonicum* USDA 110 variants using DNA fingerprints generated with GC rich arbitrary PCR primers. *Plant Soil* **186**, 81–5.
 20. Dorsch M, Stackebrandt E (1992) Some modifications in the procedure of direct sequencing of PCR amplified 16S rDNA. *J Microbiol Meth* **16**, 271–9.
 21. Suzuki K, Oguro H, Yamakawa T, Yamamoto A, Akao S, Saeki Y (2008) Diversity and distribution of indigenous soybean-nodulating rhizobia in the Okinawa islands, Japan. *Soil Sci Plant Nutr* **54**, 237–46.
 22. Appunu C, N' Zoue A, Moulin L, Depret G, Laguerre G (2009) *Vigna mungo*, *V. radiata* and *V. unguiculata* plants sampled in different agronomical-ecological-climatic regions of India are nodulated by *Bradyrhizobium yuanmingense*. *Syst Appl Microbiol* **32**, 460–70.
 23. Yao ZY, Kan FL, Wang ET, Wei GH, Chen WX (2002) Characterization of rhizobia that nodulate legume species of the genus *Lespedeza* and description of *Bradyrhizobium yuanmingense* sp. nov. *Int J Syst Evol Microbiol* **52**, 2219–30.
 24. Barcellos FG, Menna P, Batista JSS, Hungria M (2007) Evidence of horizontal transfer of symbiotic genes from a *Bradyrhizobium japonicum* inoculant strain to indigenous diazotrophs *Sinorhizobium (Ensifer) fredii* and *Bradyrhizobium elkanii* in a Brazilian savannah soil. *Appl Environ Microbiol* **73**, 2635–43.
 25. Wright BE (2004) Stress-directed adaptive mutations and evolution. *Mol Microbiol* **52**, 643–50.
 26. Boucher Y, Douady CJ, Papke RT, Walsh DA, Boudreau MER, Nesbo CL, Case RJ, Doolittle WF (2003) Lateral gene transfer and the origins of prokaryotic groups. *Annu Rev Genet* **37**, 283–328.
 27. Binde DR, Menna P, Bangel EV, Barcellos FG, Hungria M (2009) rep-PCR fingerprinting and taxonomy based on the sequencing of the 16S rRNA gene of 54 elite commercial rhizobial strains. *Appl Microbiol Biotechnol* **83**, 897–908.
 28. Menna P, Hungria M, Barcellos FG, Bangel EV, Hess PN, Martínez-Romero E (2006) Molecular phylogeny based on the 16S rRNA gene of elite rhizobial strains used in Brazilian commercial inoculants. *Syst Appl Microbiol* **29**, 315–32.
 29. Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, Stackebrandt E, Van de Peer Y, Vandamme P, Thompson FL, Swings J (2005) Re-evaluating prokaryotic species. *Nat Rev Microbiol* **3**, 733–9.
 30. Vinuesa P, Rojas-Jiménez K, Contreras-Moreira B, Mahna SK, Prasad BN, Moe H, Selvaraju SB, Thierfelder H, Werner D (2008) Multilocus sequence analysis for assessment of the biogeography and evolutionary genetics of four *Bradyrhizobium* species that nodulate soybeans on the Asiatic continent. *Appl Environ Microbiol* **74**, 6987–96.