CHAPTER III

MATERIALS AND METHODS

3.1 Soil collection sites

Soil samples from 16 subdistricts of Phitsanulok province were kindly collected on December 22, 2005, and February, 22, 2006, by Assistant Professor Dr. Wipa Homhaul, Department of Agricultural Sciences, Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Phitsanulok province, Thailand. Location of each subdistrict is shown in Figure 3.1. The samples were sent to the Agricultural Chemistry Research Group, Department of Agriculture, Ministry of Agriculture and Co-operatives, for analysis of organic matter, concentrations of available Phosphorus, available Potassium, Sodium, Calcium, Magnesium, Iron, Manganese, Zinc, Copper, Chorine, and Sulfur, as well as water holding capacity and moisture contents.

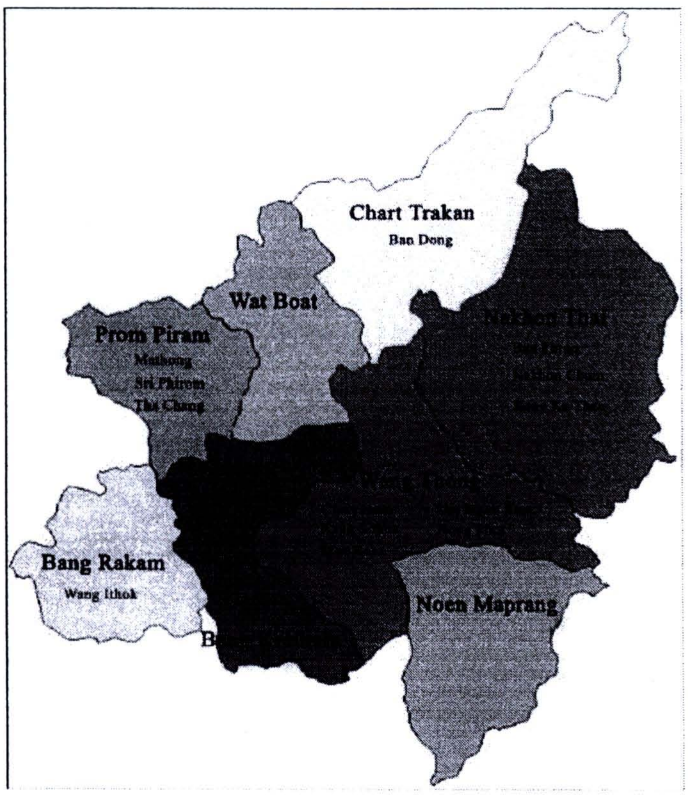

Figure 3.1 Map of Phitsanulok province which consists of 9 districts. Soil samples were collected from 16 subdistricts indicated by smaller letters.

3.2 Isolation of bacteria from root nodules

Bacteria were isolated from root nodules of 5 soybean cultivars (ST1, ST2, SJ5, CM2, and CM60) by the host trapping method as described by Somasegaran and Hoben (1994). A total of 80 earthernware pots with plates were autoclaved at 121^0 C for 20 min. After the pots cooled down, 4 kg of soil sample from each of the 16 subdistricts were placed in each pot. 10 seeds of each soybean cultivar were placed in each pot. The seeds were grown with nitrogen-free medium (Appendix A) for 28 days in a temperature-controlled greenhouse at 28^0–32^0C. All nodules from each pot were collected, surfaced sterilized briefly with 95% ethanol and submerged in 5% H_2O_2 for 5 minutes, rinsed 6 times with sterilized deionized water. Each nodule was aseptically cut into two halves. 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 $\mu g.ml^{-1}$. The composition of YM medium was as described by Somasegaran and Hoben (1994) and was given in Appendix A. YM plates were incubated at 25 ^{0} C for 1, 5, and 10 days. Different pinkish colonies were picked and streaked on YM agar plates containing Congo red at the final concentration 25 $\mu g.ml^{-1}$. Single colonies were streaked onto YM slants and kept at 4^0 C for short-term storage with subculturing every 3 months. Long-term storage was carried out by incubating each isolate in YM broth for 1-5 days, then 100 µl 20% glycerol was added into 100 µl of broth culture and stored at -80^0 C. Each isolate was provided with a code and soil collection sites as well as soybean cultivars used in the host trapping method were recorded.

3.3 RAPD-PCR fingerprinting of bacteria isolated from root nodules

Each root nodule bacterial isolate which was stored in YM slants at 4^0 C was activated by streaking one loop on to 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, 30^0 C for 1-5 days. Cells were harvested by centrifugation at 8,000 rpm, 4^0 C, 10 min, washed once with 0.85% NaCl to get rid of extracellular polysaccharides before use in chromosomal DNA isolation and RAPD-PCR fingerprinting.

3.3.1 Chromosomal DNA isolation

Cells were broken by incubating in EDTA-lysozyme solution (2.5 $mg.ml^{-1}$) at 37^0 C for 1 h followed by freezing at -20^0 C for 5 min and thawing at 80^0 C for 5 min for 2 cycles. 250 μl DNAzol® (Molecular Research Center) were added to the solution with gentle mixing by inverting the eppendorf tubes for hydrolysis of total RNA. Broken cells were centrifuged at 10,000 rpm, 4^0 C, 10 min to get rid of cell debris. Supernatant was transferred to fresh eppendorf tubes. Chromosomal DNA was precipitated with ice-cold absolute ethanol at -80^0 C for 15 min after adjusting the solution to acidic condition with 300 μl 3M Sodium acetate. DNA precipitate obtained from centrifugation at 12,000 rpm, 4^0 C, 10 min was dissolved in 20 μl high quality distilled water overnight. Quantity of chromosomal DNA preparation was obtained by OD_{260} readings with OD_{260} of 1.0 equals 50 $\mu g.ml^{-1}$ double stranded DNA (Sambrook et al, 1989). Quality of chromosomal DNA preparation was obtained through 0.8% agarose gel electrophoresis by standard method (Sambrook et al., 1989). Good DNA preparation showed no smear in the agarose gel.

3.3.2 RAPD-PCR fingerprinting

RAPD-PCR fingerprinting was obtained by the PCR method using either RPO1 or CRL-7 as the primer. RPO1 primer (5'AATTTTCAAGCGTCGTGCCA3') anneals to the 20 bp conserved region (bases 5-25) of *nifHDK* promoter of *Rhizobium leguminosarum* biovar *trifolii* (Richardson et al., 1995; Schofield and Watson, 1985). CRL-7 is an arbitrarily GC rich primer (5'GCCCGCCGCC3') which has been used in RAPD-PCR fingerprinting (Welsh and McCleland, 1990; Williams et al., 1990). PCR mixture consisted of 2 μl 10x PCR buffer, 2.0 μl 10mM dNTPs, 0.2 μl 100 $pmole.\mu l^{-1}$ primer CRL-7 or RPO1, 0.2 μl *Taq* polymerase (5U. μl^{-1}), DNA 200 ng, distilled water to 20 μl. PCR program was as follows: 95 ^{0}C 15 seconds, 55^0 C 30 seconds, 72^0 C 90 seconds for 5 cycles, 95^0 C 15 seconds, 60^0 C 30 seconds, 72^0C 90 seconds for 25 cycles, followed by 72^0C 10 minutes. PCR products were separated on 1.25% agarose gel electrophoresis. Gels were stained with 10 $mg.ml^{-1}$ 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.

3.3.3 Assigning bacterial isolates with identical RAPD-PCR fingerprints to the same strains

RAPD-PCR fingerprints of all the isolated bacteria were compared. Isolates with identical fingerprints were assigned to the same strains.

3.4 Determination of fast- and slow-growing bacterial strains and determination of colony morphology

One loop of each isolated bacterial strain was streaked onto YM agar plate containing 25 $\mu g.ml^{-1}$ Congo red. The plates were incubated at $30^{0}C$ until visible colonies were obtained. If colonies were visible after 1- day incubation, the strain was determined to be a fast-grower. On the other hand, if colonies were visible after 5- day incubation, the strain was determined to be a slow-grower. All colonies were photographed for determination of types of colony morphology.

3.5 Authentication tests of fast- and slow-growing bacterial strains

All fast- and slow-growing bacterial strains were authenticated to determine if they were fast- or slow-growing soybean rhizobia by observing formation of nodules on soybean roots grown in Leonard jars as described by Somasegaran and Hoben (1994). Seeds of each of the 5 soybean cultivars (ST1, ST2, SJ5, CM2 and CM60) were surfaced sterilized as previously reported in section 3.2 and allowed to aseptically germinate on seedling agar (0.75% agar) at 30^{0} C in the dark for 48 h. Two germinating seeds, each with approximately 1.0 cm radicle, were placed in a Leonard jar (Figure 3.2).

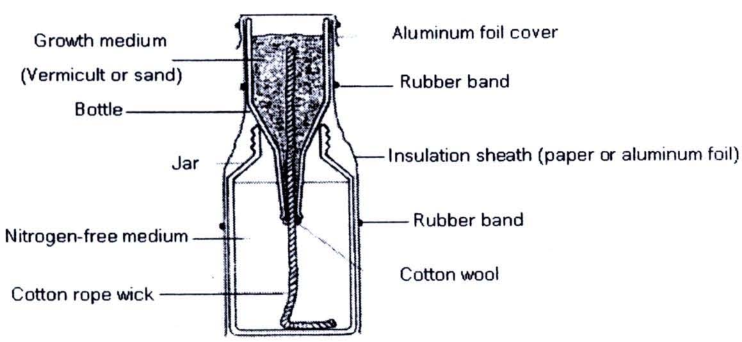

Figure 3.2 Diagram of a Leonard jar (Somasegaran and Hoben, 1994).

Negative or positive control soybean plants were grown in Leonard jars with no inoculation but with N-free medium or N-free medium containing 0.05% KNO_3 as plant growth solutions respectively. Soybeans were grown in a temperature-controlled greenhouse (28^0C-32^0 C) for 28 days with nitrogen-free medium (Appendix A). Roots were cut and nodules observed. Soybean rhizobia produced root nodules while other bacterial isolates did not produce root nodules.

3.6 Polyphasic taxonomy of 20 selected strains of slow-growing soybean rhizobia

3.6.1 Colony morphology

For polyphasic taxonomy, 20 strains of slow-growing soybean rhizobia were randomly chosen based on differences in their DNA fingerprints. Colony morphology was obtained by the method as described in section 3.4 .

3.6.2 Bromthymol blue reactions

Bromthymol blue reactions were obtained by streaking cells onto YMA with the indicator dye Bromthymol blue at 25 $\mu g.ml^{-1}$ final concentration. The plates were incubated at 30^0C for 5 days and 10 days with observation for color of the indicator dye on the plates at the end of the 5- and 10-day incubation periods. According to Somasegaran and Hoben (1994), fast-growing rhizobia changed color of the indicator dye to yellow while slow-growing soybean rhizobia turned the indicator dye to blue. Additional experiments for the determination of responses to changes in pHs of the medium were performed with each of the following strains : *Bradyrhizobium elkanii* strain NA7 (Chanthapetch, 2009), *Bradyrhizobium japonicum* strain S76 (Chansa-ngavej et al., 2008), *Bradyrhizobium liaoningense* (Suptaweewut and Chansa-ngavej, 2010), and *Bradyrhizobium yuanmingense* strain SYB264 (this study). Each strain was grown in YMB medium with or without 30mM buffer. The type of buffers used depended on the buffering capacity as shown in Table 3.1. For example, the buffer used to maintain pH of the medium at pH 4.0 or 5.0 was NEDA. Cells grown for 5 days in an incubator shaker at 200 rpm, 30^0C, for 5 days were harvested and pH of the supernatant measured with a pH meter (Beckman). Duncan's multiple range test was carried out with SPSS program version 15.0 for Windows.

Table 3.1 Buffering capacity of buffers (Sigma)

pH	Buffers	Useful pH Range
4.0 5.0	NEDA (cis-5-Norbornene-endo-2,3-dicarboxylic anhydride)	4.0 – 5.4
6.0	MES (2-(N- Morpholino)ethanesulfonic acid hydrate)	5.5 - 6.7
7.0 8.0	HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid)	6.8 - 8.2

3.6.3 Negative staining of flagella

Cells kept in YMA agar slants were activated by streaking onto YMA with Congo red plates, incubated at 30^{0}C for 5 days before restreaking on fresh YMA with Congo red plates and incubated for two more days. It was found that cells needed to be reactivated twice as described above in order to get healthy–looking cells for the negative staining of flagella. One loop of high quality distilled water was added onto one colony on the plate. A copper grid was touched on the cell suspension and left to partially air-dry for 3 minutes before adding 6 μl of 1% phosphotungstic acid to the grid, left for 1 min then dried the grid completely with the rugged edge of a torn filter paper. The grid was stored overnight in a clean petri dish before observing for flagella with a Transmission Electron Microscope (JOEL model JEM-2100) at the Scientific and Technological Equipment Center, Chulalongkorn University.

3.6.4 Growth rates at different temperatures

One loop of activated cells of each of 5 randomly selected strains was inoculated into 50 ml YMB in a 250-ml Erhenmeyer flask. Cells were incubated at 25^{0}C, 30^{0}C, 37^{0}C, and 40^{0}C for 5 days. Samples were taken daily for serial dilutions and plating on YMA with Congo red at the final concentration of 25 μg.ml^{-1} . Specific growth rates were determined from growth curves and the standard formula: $N_t = N_0 e^{\mu t}$

when N_t = colony forming unit (CFU.ml^{-1}) at time t

N_0 = initial CFU.ml^{-1}

μ = specific growth rate (day^{-1})

T = incubation time (days)

3.6.5 Ability / inability to utilize carbon and nitrogen sources

Ability / inability of each of the 20 randomly-selected soybean rhizobium strains to utilize carbon and nitrogen sources was determined using the Biolog™ test kit. The Biolog processing machine at The Center for Agricultural Biotechnology, Kasetsart University, Kamphangsaen Campus, Nakorn Pathom Province was used for data processing. Cells were streaked on either YMA with 25 $\mu g.ml^{-1}$ Congo red or, in the case of and pearly colonies, TY medium, incubated at 30^0 C for 5 days before suspending in an inoculation fluid which was supplied by the manufacturer (Biolog, USA) until the transmission were 52% as measured by the spectrophotometer supplied by the manufacturer of the Biolog™ instrument. 150 μl of the cell suspension were filled into each of the 96- well Biolog™ GN2 MicroPlates for Gram negative bacteria, incubated at 30^0 C for 24 h before measurements of dual wavelength optical density at 590 nm and 750 nm. Figure 3.3 showed layout of the 96 wells on the Biolog™ MicroPlate for determination of ability / inability to utilize 95 carbon / nitrogen sources by Gram negative bacteria (GN2 Microplate, Biolog™).

A1 water	A2 α-cyclodextrin	A3 dextrin	A4 glycogen	A5 tween 40	A6 tween 80	A7 N-acetyl-D-galactosamine	A8 N-acetyl-D-glucosamine	A9 adonitol	A10 L-arabinose	A11 D-arabitol	A12 cellobiose
B1 i-erythritol	B2 D-fructose	B3 L-fucose	B4 D-galactose	B5 gentiobiose	B6 α-D-glucose	B7 m-inositol	B8 α-D-lactose	B9 lactulose	B10 maltose	B11 D-mannitol	B12 D-mannose
C1 D-melibiose	C2 β-methyl D-glucoside	C3 D-psicose	C4 D-raffinose	C5 L-rhamnose	C6 D-sorbitol	C7 sucrose	C8 D-trehalose	C9 turanose	C10 xylitol	C11 methyl-pyruvate	C12 mono-methyl-succinate
D1 acetic acid	D2 cis-aconitic acid	D3 citric acid	D4 formic acid	D5 D-galactonic acid lactone	D6 D-galacturonic acid	D7 D-gluconic acid	D8 D-glucosaminic acid	D9 D-glucuronic acid	D10 α-hydroxybutyric acid	D11 β-hydroxybutyric acid	D12 γ-hydroxybutyric acid
E1 p-hydroxy phenylacetic acid	E2 itaconic acid	E3 α-keto butyric acid	E4 α-keto glutaric acid	E5 α-keto valeric acid	E6 D,L-lactic acid	E7 malonic acid	E8 propionic acid	E9 quinic acid	E10 D-saccharic acid	E11 sebacic acid	E12 succinic acid
F1 bromo succinic acid	F2 succinamic acid	F3 glucuronamide	F4 alaninamide	F5 D-alanine	F6 L-alanine	F7 L-alanyl-glycine	F8 L-asparagine	F9 L-aspartic acid	F10 L-glutamic acid	F11 glycyl-L-aspartic acid	F12 glycyl-L-glutamic acid
G1 L-histidine	G2 hydroxy L-proline	G3 L-leucine	G4 L-ornithine	G5 L-phenylalanine	G6 L-proline	G7 L-pyroglutamic acid	G8 D-serine	G9 L-serine	G10 L-threonine	G11 D,L-carnitine	G12 γ-amino butyric acid
H1 urocanic acid	H2 inosine	H3 uridine	H4 thymidine	H5 phenyl ethylamine	H6 putrescine	H7 2-amino ethanol	H8 2,3-butanediol	H9 glycerol	H10 D,L-α-glycerol phosphate	H11 glucose-1-phosphate	H12 glucose-6-phosphate

Figure 3.3 Layout of carbon and nitrogen sources in the 96 wells of a 96-well Biolog™ GN2 MicroPlate for the determination of ability / inability to utilize 95 carbon and nitrogen sources by Gram negative bacteria (Biolog™ manual, 2001).

According to the Biolog™ manual (2001), each dual wavelength optical density reading is determined by the processing unit of the Biolog™ instrument according to the following equation :

Optical density at dual wavelength = $(OD_{590} - OD_{750})\ x - (OD_{590} - OD_{750})\ A_1 \times 1000$

Where x = any well

A_1 = control well with no carbon/nitrogen sources

Since database in the Biolog™ instrument at the Center for Agricultural Biotechnology, Kasetsart University, Nakorn Pathom Province , Kamphangsaen Campus does not contain data of ability / inability to utilize the 95 carbon / nitrogen sources by soybean rhizobia, it was not possible to identify soybean rhizobia using the Biolog™ database. Therefore, results were recorded as the ability/inability to use the 95 carbon / nitrogen compounds. The following type strains : *B. elkanii* NBRC 14791, *B. japonicum* 14783, and *B. liaoningense* NBRC 100396 which were purchased from NITE Biological Resource Center (NBRC) (NITE = National Institute of Technology and Evaluation), Japan, were also used in the Biolog tests.

Interpretation of the Biolog data was arbitrarily set as follows:

+ = Dual wavelength (DWL) of test wells > DWL of control well (A_1) plus 25% < 0.25

++ = DWL of test wells > 0.25 < 0.50

+++ = DWL of test wells > 0.50

3.6.6 Sequencing of 16S rDNA

16S rDNA of each of the randomly-selected 20 strains was isolated by PCR using primers 27f and 1492r. Composition of PCR mixture was as follows : 10x PCR buffer 2 µl, 10mM dNTPs 2 µl, primer 27f (10 pmol•µl^{-1}) and primer 1492r (10 pmol•µl^{-1}) 0.25 µl each for a total of 0.5 µl, DNA 200 ng, *Taq* polymerase(5 units•µl^{-1}) 0.2 µl, distilled water to 20 µl. PCR program was as follows: 95°C 30 minutes, 48°C 1 minute, 72°C 2 minutes (30 cycles) followed by 48°C 1 minute, 72°C 5 minutes (1 cycle). Sequences of the primers 27f and 1492r were as described by Dorsch and Stackebrandt (1992) : 27f (9-27)* : 5'GAGTTTGATCCTGGCTCAG3', 1492r (1492-1512)* : 5'ACGGCTACCTTG TTACGACCT3'

* = Positions of nucleotides on consensus sequence of 16S rDNA of *E. coli*

Each PCR mixture containing a product of approximately 1,500 bp was sent to the Genome Institute, Thailand Science Park (until January 2009), and to the BioDesign Company, Thailand Science Park (from February 2009 because the Genome Institute discontinued the sequencing service). The following 9 primers were used in the sequencing :

27 f (9-27)* : 5'GAGTTTGATCCTGGCTCAG3'

1492r (1492-1512)* : 5' ACGGCTACCTTGTTACGACCT3'

343r (343-357)* : 5'CTGCTGCCTCCCGTA3'

519r (519-536)* : 5'GTATTACCGCGGCTGCTC3'

787r (787-803)* : 5'CTACCAGGGTATCTAAT3'

907r (907-926)* : 5'CCGTCAATTCATTTGAGTTT3'

1100r (1100-1115)* : 5'AGGGTTGCGCTCGTTG3'

1385r (1385-1401)* : 5'CGGTGTGTACAAGGCCC3'

1241f (1224-1241)* : 5'TACACACGTGCTACAATG3'

* Positions of nucleotides on consensus sequence of 16S rDNA of *E. coli*

The BioEdit program which is a free software on the Internet at http://www.mbio.ncsu.edu/BioEdit/bioedit.html was used to obtain sequences of sense strands of 16S rDNAs.

3.6.7 Sequencing of *nodY*

nodY of each of the randomly-selected 20 strains was isolated by PCR using primers *nodYf* and *nodYr*. Composition of PCR mixture was as follows : 10x PCR buffer 2 μl, 10mM dNTPs 2 μl, primer *nodYf* (10 pmol•μl $^{-1}$) and primer *nodYr* (10 pmol•μl $^{-1}$) 2.5 μl each, DNA 200 ng, *Taq* polymerase (5 units•μl $^{-1}$) 0.2 μl, distilled water to 20 μl. PCR program was as follows: 95°C 15 seconds, 50°C 30 seconds and 72°C 90 seconds(5 cycles), 95°C 15 seconds, 60° C 30 seconds, 72° C 90 seconds (25 cycles) followed by 72°C 10 minutes. Each PCR mixture containing a PCR product of approximately 340 bp was sent to the Genome Institute, Thailand Science Park (until January 2009) and to the BioDesign Company, Thailand Science Park, from February 2009 due to the discontinuation of sequencing service by the Genome Institute. The following 2 primers which were designed by Emampaiwong (2006) were sent for use in the sequencing :

nodYf : 5'TGTACGCGGGTAAACC3'

*nodY*r : 5'AGCGCAACGAGAAGAT3'

3.6.8 Identification of slow-growing soybean rhizobia using sequences of 16S rDNA and *nodY*

Identities of the 20 randomly-selected slow-growing soybean rhizobia were obtained by comparing sequences of either 16S rDNA or *nodY* obtained in sections 3.6.6 and 3.6.7 with available sequences deposited at GenBank database at the Internet website http://www.ncbi.nlm.nih.gov/ using the Blast program.

3.6.9 Construction of dendrograms from 16S rDNA and *nodY* sequences

Dendrograms were constructed with either 16S rDNA or *nodY* sequences obtained in sections 3.6.6 and 3.6.7 as well as sequences of the following slow-growing soybean rhizobium strains obtained from GenBank database of the National Center for Biotechnology Information (USA) at the following website: http://www.ncbi.org. Brackets contain the accession numbers of the sequences, superscript T indicates the type strains: 16S rDNA : *Bradyrhizobium elkanii* strain USDA76^T (U35000), SEMIA 5002; *Bradyrhizobium japonicum* strains USDA6^T (U69638), SEMIA 566 (AF236086), SEMIA 586 (AF236087),S127 (DQ485704), SEMIA 5064 (FJ390925), SEMIA 5079 (FJ390956), SEMIA 5080 (AF234889) ; *B. liaoningense* SEMIA 5003 (FJ390906) ; *B. yuanmingense* TTC4 (FJ540937).

nodY sequences were obtained from the following *nodY-nodA* sequences: *Bradyrhizobium elkanii* strain USDA94 (U04609), *Bradyrhizobium japonicum* strains USDA110 (AF322013), SEMIA 586 (DQ485698), SEMIA 566 (DQ485700), CPAC 15 (DQ485694), CPAC 7 (DQ485696), S127 (DQ485703), S340 (DQ485705), S370 (DQ485706), S372 (DQ485707), S478 (DQ485708), S490 (DQ485709), S516 (DQ485710), CPAC 390 (DQ485711), CPAC 392 (DQ485716), CPAC 394 (DQ485713), CPAC 402 (DQ485717), CPAC 403 (DQ485719), CPAC 404 (DQ485720).

PHYLIP which is a free software in the Internet at http://evolution.gs.washington.edu.phylip.html. was used in the construction of the dendrograms. The software contains three methods for dendrogram construction, namely, the Maximum Likelihood method, the Maximum Parsimony method and the Neighbor- Joining method. All the three available methods were used in the construction of dendrograms. Bootstrap numbers were obtained with the BOOTSEQ program in the PHYLIP software using 100 replications.

3.6.10 Construction of dendrograms from DNA fingerprints of 121 slow-growing soybean rhizobia

Dendrograms of DNA fingerprints of 121 slow-growing strains of soybean rhizobia when either RPO1 or CRL-7 was used as the primer were constructed with DNA Fingerprinting II Informatix software version 3.0 provided by the Bio-Rad Laboratories (Thailand) Co Ltd. using the UPGMA algorithm.