ความหลากหลายของไรโซเบียมถั่วเหลืองใน 16 ตำบลของจังหวัดพิษณุโลก

นางสุจิตกัลยา มฤครัฐอินแปลง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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DIVERSITY OF SOYBEAN RHIZOBIA IN 16 SUBDISTRICTS OF PHITSANULOK PROVINCE

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Microbiology
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Field of Study	Microbiology
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สุจิตกัลยา มฤครัฐอินแปลง : ความหลากหลายของไรโซเบียมถั่วเหลืองใน 16 ตำบลของจังหวัดพิษณุโลก (DIVERSITY OF SOYBEAN RHIZOBIA IN 16 SUBDISTRICTS OF PHITSANULOK PROVINCE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รองศาสตราจารย์ ดร.กาญจนา ชาญสง่าเวช, 172 หน้า

ไรโซเบียมถั่วเหลืองเป็นแบคทีเรียย้อมติดสีแกรมลบที่ตรึงไนโตรเจนในปมรากถั่วเหลือง ปัจจุบันยังมีข้อมูลไม่มากด้านอนุกรมวิธานแบบพอลิฟาสิกของไรโซเบียมถั่วเหลืองในประเทศไทย วัตถุประสงค์ของงานวิจัยนี้เพื่อแยกและใช้อนุกรมวิธานแบบพอลิฟาสิกจำแนกชนิดสายพันธุ์ไรโซบียมถั่วเหลืองที่แยกจากดิน 16 ตำบลของจังหวัดพิษณุโลก โดยใช้ถั่วเหลืองจำนวน 5 พันธุ์เป็นกับดักล่อให้ไรโซเบียมถั่วเหลืองเข้าสร้างปม (Host trapping method) และนำปมมาแยกแบคทีเรียได้ทั้งหมดจำนวน 340 ไอโซเลต แบ่งออกเป็น 2 ประเภท คือประเภทเพิ่มจำนวนเร็วและประเภทเพิ่มจำนวนช้า ตามลักษณะการปรากฏของโคโลนีบนอาหารวุ้นสูตร YM ที่มีคองโกเรด 0.25 ไมโครกรัมต่อมิลลิลิตร ผลการทดลองพบว่ามี 138 ไอโซเลต และ 202 ไอโซเลต ที่จัดเป็นประเภทเพิ่มจำนวนเร็วและประเภทเพิ่มจำนวนช้า ตามลำดับ เปรียบเทียบความเหมือนของลายพิมพ์ดีเอ็นเอจาก RAPD-PCR พบว่าแบคทีเรียประเภทเพิ่มจำนวนช้า 202 ไอโซเลตประกอบด้วย 121 สายพันธุ์ การพิสูจน์ว่าแบคทีเรียที่แยกได้เป็นไรโซเบียมถั่วเหลืองหรือไม่โดยใช้ถั่วเหลือง 5 พันธุ์ พบว่าแบคทีเรียประเภทเพิ่มจำนวนเร็วทั้ง 138 ไอโซเลตไม่สร้างปมที่รากถั่วเหลือง ดังนั้น จึงไม่จัดว่าเป็นไรโซเบียมถั่วเหลืองประเภทเพิ่มจำนวนเร็ว ในทางตรงกันข้าม แบคทีเรียประเภทเพิ่มจำนวนช้าทั้ง 121 สายพันธุ์ สร้างปมที่รากถั่วเหลือง แสดงว่าเป็นไรโซเบียมถั่วเหลืองประเภทเพิ่มจำนวนช้า ปฏิกิริยาบรอมไทมัลบลูบนอาหารวุ้นบรอมไทมัลบลูแสดงให้เห็นว่ามีไรโซเบียมถั่วเหลือง 2 แบบ แบบที่ 1 ขับสารที่มีฤทธิ์เป็นด่างหลังจากบ่มที่ 30°C เป็นเวลา 5 วัน จากนั้นจะขับสารที่มีฤทธิ์เป็นกรดหลังจากบ่มต่อที่ 30°C เป็นเวลาอีก 5 วัน แบบที่ 2 ขับสารที่มีฤทธิ์เป็นด่างตลอดระยะเวลาการบ่มที่ 30°C เป็นเวลา 10 วัน ผลการจำแนกชนิดโดยใช้ลำดับนิวคลีโอไทด์ของ 16S rDNA ของไรโซเบียมถั่วเหลืองประเภทเพิ่มจำนวนช้า 20 สายพันธุ์ที่เลือกแบบสุ่ม พบว่า ไรโซเบียมถั่วเหลืองจำนวน 12 สายพันธุ์ (STB8, STB119, STB120, STB147, STB173, STB176, STB179, STB185, STB220, STB238, STB245 และ STB327) มีความสัมพันธ์ทางวิวัฒนาการใกล้ชิดกับ *B. elkanii* ไรโซเบียมถั่วเหลืองจำนวน 6 สายพันธุ์ (STB30, STB54, STB67, STB96, STB250 และ STB310) มีความสัมพันธ์ทางวิวัฒนาการใกล้ชิดกับ *Bradyrhizobium japonicum* ไรโซเบียมถั่วเหลืองจำนวน 2 สายพันธุ์ (STB169 และ STB264) มีความสัมพันธ์ทางวิวัฒนาการใกล้ชิดกับ *B. yuanmingense* และ ผลการทดลองพบว่าไม่สามารถใช้ลำดับนิวคลีโอไทด์ของยีน *nodY* และผลการใช้/ไม่ใช้แหล่งคาร์บอนและไนโตรเจนจำนวน 95 แหล่งในการจำแนกชนิดไรโซเบียมถั่วเหลือง นอกจากนี้ผลการสร้างเดนโดรแกรมจากลายพิมพ์ดีเอ็นเอของไรโซเบียมถั่วเหลืองจำนวน 121 สายพันธุ์ พบความหลากหลายด้านดีเอ็นเอ งานวิจัยนี้เป็นรายงานแรกที่พบ *B. yuanmingense* ในประเทศไทยและเป็นรายงานแรกที่พบว่าไรโซเบียมถั่วเหลืองในประเทศไทยประกอบด้วย natural variants

ภาควิชา จุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา จุลชีววิทยา ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2553

4973852323 : MAJOR MICROBIOLOGY

KEYWORDS : soybean rhizobia / polyphasic taxonomy / diversity / Phitsanulok province

SUJIDKANLAYA MARUEKARAJTINPLAENG : DIVERSITY OF SOYBEAN RHIZOBIA IN 16 SUBDISTRICTS OF PHITSANULOK PROVINCE. THESIS ADVISOR : ASSOC. PROF. KANJANA CHANSA-NGAVEJ, Ph.D., 172 pp.

Soybean rhizobia are Gram negative bacteria that fix nitrogen in root nodules of soybeans. At present, there is not much information on polyphasic taxonomy of soybean rhizobia in Thailand. The aim of the experiments is to isolate and characterize, by polyphasic taxonomy, soybean rhizobium strains in soils from 16 subdistricts in Phitsanulok province. Host trapping method was used to isolate bacteria from root nodules of 5 soybean cultivars grown in soils from the 16 subdistricts. A total of 340 isolates were purified and categorized into fast- and slow-growers based on their visible growth on yeast extract mannitol with 0.25 $\mu g.ml^{-1}$ Congo red agar plates. The results indicated there were 138 and 202 isolates of fast- and slow-growers, respectively. Identical RAPD-PCR fingerprints revealed the 202 slow-growing isolates consisted of 121 strains. Authentication tests employing 5 soybean cultivars showed all the 138 fast-growing isolates did not nodulate soybean roots. Thus, they were not fast-growing soybean rhizobia. On the contrary, all the 202 slow-growing isolates were found to nodulate soybean roots. Therefore, they were slow-growing soybean rhizobia. Bromthymol blue (BTB) reactions on BTB agar plates showed there were two types of slow-growing soybean rhizobia. Type 1 secreted alkali product(s) after 5-day incubation then secreted acidic product(s) upon prolonged incubation for another 5 days. Type 2 secreted only alkali product(s) after 10-day incubation. Results from nucleotide sequences of 16S rDNA of randomly-selected 20 slow-growing strains revealed 12 strains (STB8, STB119, STB120, STB147, STB173, STB176, STB179, STB185, STB220, STB238, STB245 and STB327) were related to *B. ellkanii*. 6 strains (STB30, STB54, STB67, STB96, STB250 and STB310) were closely related to *Bradyrhizobium japonicum*, and 2 strains (STB169 and STB264) were related to *B. yuanmingense*. The results indicated that *nodY* sequences and results on utilization/non-utilization of 95 carbon and nitrogen sources could not be used to identify soybean rhizobium strains. In addition, dendrograms constructed from DNA fingerprints of the 121 soybean rhizobium strains revealed genetic diversity. This research is the first report on the findings of *B. yuanmingenseas* as well as natural variants of soybean rhizobia in Thailand.

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Contents

Chapter

List of Tables

Page

List of Figures

Page

Page

CHAPTER I

INTRODUCTION

Soybean rhizobia are motile Gram negative, non-spore forming rods with width 0.5-0.9 μm and length 1.2-3.0 μm (Jordan, 1984). There are two categories of soybean rhizobia : Fast-growers and slow-growers. In 1992, Elkan and Bunn listed some of the properties of fast- and slow- growing soybean rhizobia as shown in Table 1.1

Table 1.1 Some properties of fast- and slow-growing soybean rhizobia (Elkan & Bunn, 1992)

Property	Soybean rhizobia	
	Fast-growers	Slow-growers
1. Doubling time	Less than 6 h	More than 6 h
2. Number and typeof flagella	2-6 peritrichous flagella	1 subpolar flagellum
3. Positions of *nif*H and *nif*DK which encode the Fe protein and the alpha and beta subunits of the Mo-Fe protein which make up the enzyme nitrogenase	On the same operon	On different operons
4. Positions of other *nif* and *fix* genes	On pSym plasmid	On chromosome

At present, there are two recognized strains of fast-growing soybean rhizobia, namely *Sinorhizobium fredii* and *S. xinjiangense* (Chen et al., 1988 ; Peng et al., 2002). In addition, there are 4 recognized strains of slow-growing soybean rhizobia which are *Bradyrhizobium elkanii, B. japonicum*, *B. liaoningense* and the relatively newly-discovered *B. yuanmingense* biovar that nodulates soybean (Appunu et al. 2008; Jordan, 1982; Kuykendall et al., 1992, Xu et al., 1995).

In People's Republic of China, most soybean rhizobia were found to be fast-growing soybean rhizobium *Sinorhizobium fredii* (Camacho et. al., 1992; Dowdle and

Bohlooh, 1985). In 2004 Chen et al. reported that continuous investigation and uses of new molecular biology techniques in People's Republic of China had led to the discovery of new species of soybean rhizobia in China such as *S. xinjiangense* (Chen et al., 1988) and *Bradyrhizobium liaoningense* (Xu et al., 1995).

In other leading soybean exporting countries such as Brazil, in 1975, the Brazilian government required that rhizobium strains used in the commercial production of inoculants for leguminous plants including soybeans had to be recommended by Brazilian public research institutes. Since 1985, the recommendation has been enforced by RELARE (Rede de LaboratÓrios para a Recomendação de Estirpes de *Rhizobium*), a network of laboratories for the identification of the most effective rhizobium strain for the production of inoculant for each leguminous species. However, the maintenance and distribution of rhizobium strains to the inoculant industry belong to a government agency, "SEMIA culture collection of Rhizobium", with the following URL http://wdcm.nig.ac.jp/CCINFO/CCINFO.xml?443 which distributes *Rhizobium* strains for free. In USA, the US Department of Agriculture (USDA) also distributes rhizobium strains for free.

In Thailand, the literature survey in Chapter II revealed that most of the published research conducted with soybean rhizobia since 1991 did not identify soybean rhizobia by polyphasic taxonomy which is an established concept widely used in bacterial identification (Vandamme et al., 1996). The aim of this research for dissertation is to isolate and characterize, by polyphasic taxonomy, soybean rhizobia from 16 subdistricts in Phitsanulok province. One reason Phitsanulok areas were chosen as soil collection sites for soybean rhizobia is because areas that used to be planted with soybeans are now being used to grow other crops which provide more financial return such as sugarcane and banana. According to Mr Weerachai Tangsaijai, the Bang Rakam district agricultural officer, soybean cultivar CM2 is only grown in 7 subdistricts of Bang Rakam district, Phitsanulok province, namely, Phan Sao, Plug Raed, Bang Rakam, Khui Muang, Bueng Kok. Nikhom Phattana, and Nong Kula. It is hoped that the results obtained from this research will contribute to the record of soybean rhizobium diversity in Thailand for use as a pool for the selection of soybean rhizobia for the production of soybean biofertilizers to increase soybean yields in order to increase

income for soybean growers and to encourage farmers to continue to cultivate soybean as a rotational crop to reduce the use of nitrogen chemical fertilizers, to protect the soil environments and to reduce trade deficit due to the import of 85% of soybean consumed in Thailand as reported in the website www.feeduser.com.

New findings reported in this dissertation include the first record of the presence of *Bradyrhizobium yuanmingense* in Thai soils, the finding of natural variants of slow-growing soybean rhizobia, and the finding that when grown on yeast extract mannitol agar containing the indicator dye Bromthymol blue, *Bradyrhizobium elkanii* strains and *B. yuanmingense* strain STB169 secreted alkali product(s) throughout the 10-day incubation period while *B. japonicum* and *B. yuanmingense* strain STB264 secreted alkali product(s) in the first 5-day incubation and secreted acidic product(s) in the last 5 days of incubation. This dissertation also presents for the first time sequences of *nodY* of 20 strains of slow-growing soybean rhizobia with phylogenetic trees constructed with *nodY* nucleotide sequences. In addition, some fast-growing bacteria which did not nodulate soybeans were obtained during the isolation of soybean rhizobia. These fast-growing bacteria are candidates for the possibility of developing inoculants containing PGPR (Plant Growth-Promoting Rhizobacteria) and for further research on their capacity to break down genistein which is a plant signal molecule in root nodulation. This additional research results will contribute to either the discovery of new formulations using either soybean rhizobial strain(s) alone or in combination with PGPR for the production of inoculants to improve soybean yields. Another potential discovery is the presence of genistein-degrading fast-growing bacteria in soybean rhizosphere which may lead to failure of soybean rhizobium inoculants due to biodegradation and subsequent disappearance of genistein, the signal molecule.

CHAPTER II

LITERATURE SURVEY

2.1 Previous research on soybean rhizobium diversity in Thailand

Soybeans are grown in the northern, upper central, and some parts of the north-eastern parts of Thailand. Figure 2.1 illustrates the percentages of soybean cultivation areas in different provinces of Thailand.

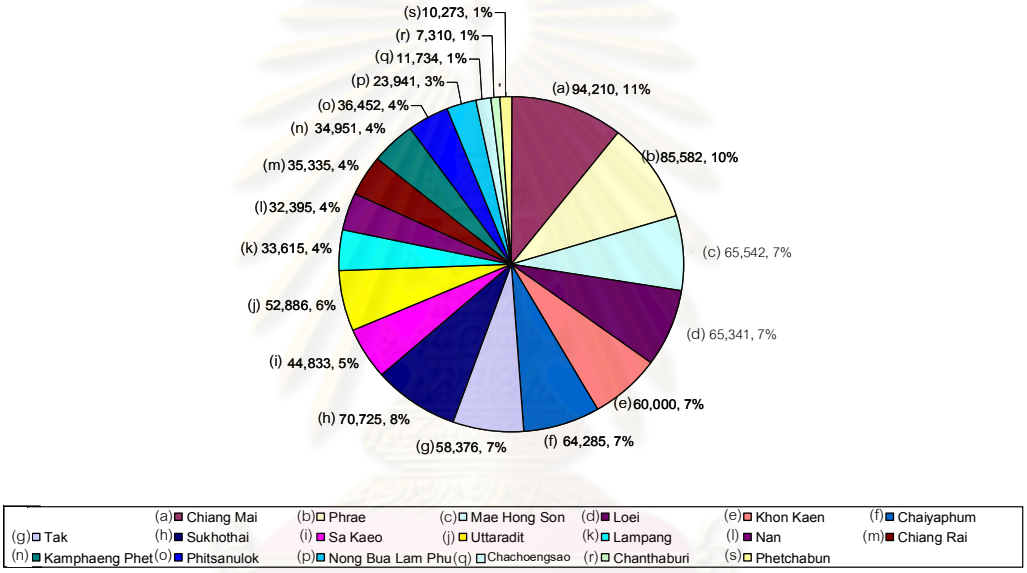


Figure 2.1 Percentages of soybean cultivation areas in different provinces of Thailand in the crop year 2008. Units in *rai* and percent. Data were obtained from the Department of Agriculture in 2009.

Figure 2.1 shows soybean is mostly cultivated in Chiangmai province in the northern part of Thailand covering the area of 94,210 *rai* or 11% of the total area for soybean cultivation. Although the area planted with soybeans in Phitsanulok is about 36,452 *rai* or 4% of the total soybean cultivation area, there is a need to isolate and characterize soybean rhizobia in Phitsanulok province because at present there is no record of soybean rhizobium diversity in the area.

In 1991 Thompson et al. isolated 1,536 bacterial isolates from root nodules of uninoculated soybeans and cowpeas grown in 2 meter long experimental rows in 25

sites in soybean-cultivation areas at Hang Dong, San Sai, Mae Rim, Mae Thaeng, Mae Sariang, Mae Hong Son, Khum Yuom, Mae La Noi, Sri Sachanalai, Sawankalok, Sri Samrong, Uttaradit, and Doi Tao. Seeds of local soybean cultivars Black Yodson, San Keuw, SJ2, SJ5, 7842, and the following soybean cultivars from US breeding programs were used: Peking, Improved Pelican, Hardee, Bossier, Bragg, and Forrest. All isolates were stained with conjugated fluorescent dye fluorescin isothiocyanate and polyclonal antisera from each of the following soybean rhizobium strains: THA7, USDA140, USDA24, USDA31, USDA110, USDA122, TAL379, and the following strains isolated by Thompson et al. (1991): 7DX9, 7DX36, 10DX21, 10EX51, and 1C3. All serologically-stained isolates were examined with UV fluorescence microscope. The results indicated most strains belonged to serogroups of local soybean rhizobium strains 1C3 and 7DX9. However, the researchers reported there were too many cross-reactions for the serological method to be used to differentiate different soybean rhizobium strains. Intrinsic antibiotic resistance (IAR) patterns were thus conducted on 116 isolates with 10 $\mu g.ml^{-1}$ of each of the following antibiotics: vancomycin, rifampicin, spectinomycin, and tetracycline, 40 $\mu g.ml^{-1}$ of each of the following antibiotics: streptomycin, kanamycin, erythromycin and 200 $\mu g.ml^{-1}$ of polymixin. Nearly all (93%-100%) of the isolates were found to be resistant to spectinomycin, vancomycin, tetracycline, and polymixin. Therefore, these four antibiotics were not useful in the differentiation of different strains. However, 83%, 69%, 68%, and 17% of the isolates were found to be resistant to kanamycin, streptomycin, rifampicin, and erythromycin, respectively. Therefore, qualitative differences in the resistance and susceptibility to these last four antibiotics were used to separate isolates into various groups for testing effectiveness in nodulation and in nitrogen fixation. Thompson et al. (1991) found that out of the 356 rhizobium isolates tested on 6 soybeans cv. Black Yodson. SJ5, Improved Pelican, Bossier, Peking and Hardee, only 5.6% of the isolates yielded less than 20 nodules per plant and that most of the isolates significantly increased ($p < 0.001$) nitrogen yield of plants over that obtained for the uninoculated control. The results indicated most of the soybean rhizobium isolates obtained by Thompson et al. (1991) from soybean-cultivation areas in the northern part of Thailand were effective in nodulation and in nitrogen fixation. However, Thompson et al. (1991) did not employ any method to group

identical soybean rhizobium isolates into the same strains. In addition, the authors did not identify any of the soybean isolates.

Most of the other previous research on the diversity of soybean rhizobia in Thailand was either incomplete or did not employ many strains of soybean rhizobia in the research. In 1997, Nuntagij et al. partially characterized soybean rhizobia isolated from soybean cultivation areas in Thailand. The authors did not report the sites of soybean cultivation areas where 5 strains were isolated. The 5 strains were tentatively identified as *Bradyrhizobium japonicum* strains THA2, THA5, THA6, THA7, and THA211 based on their inability to produce IAA (3-Indole Acetic Acid). In addition, 5 reference strains were used in the research: *Bradyrhizobium japonicum* strains TAL102, USDA136b, SM5, USDA76 and 61A101C. The 10 strains were characterized based on their IAA production, growth on YMA at pH 3.0-9.0, and growth at 17^0C, 28^0C, 37^0C, and 42^0C. Research was also conducted on the effects of pH 4.5 or 6.8 on activities of 19 enzymes using the APIZYM-kit (API system, France). Strains THA2, THA7, THA211 were found to be acid-tolerant at pH 3.0. The optimum range of pH for the 5 isolated strains were found to be pH 6.0-8.0. All the 5 strains except THA7 were found to grow at 42^0C. pH 4.5 and 6.8 were found to have different effects on the 19 enzymes of the 5 strains. An interesting result was the report on RAPD-PCR fingerprints of the 10 strains using each of primers 1-6 purchased from Pharmacia Biotech (Uppsala, Sweden). Diversity One Software (New York, USA) was used to construct a dendrogram from the similarity of the RAPD fingerprint profiles. The dendrogram revealed three clusters with the first cluster consisting of reference strains USDA136b, USDA76, 61A101C, and SM5 which were temperate strains. The Thai strain THA211 was found to belong to the same cluster as the temperate strains. The Thai strains THA2, THA5, and THA7 which were found to be acid-tolerant were found to cluster in the second cluster while the Thai strains THA6 and THA102 were found to belong to the third cluster. The researchers did not comprehensively identify all the 5 isolated strains obtained in their study. In addition, no information was given on the locations of soybean cultivation fields in Thailand where the 5 soybean rhizobium strains were isolated.

In 1998, Teaumroong and Boonkerd employed primers REP, ERIC, and RAPD to obtain PCR-DNA fingerprints of 18 strains of *Bradyrhizobium japonicum*. The

sequence of RAPD primer was 5'GGAAGTCGCC3'. Sequences of REP abd ERIC were shown in Figure 2.2.

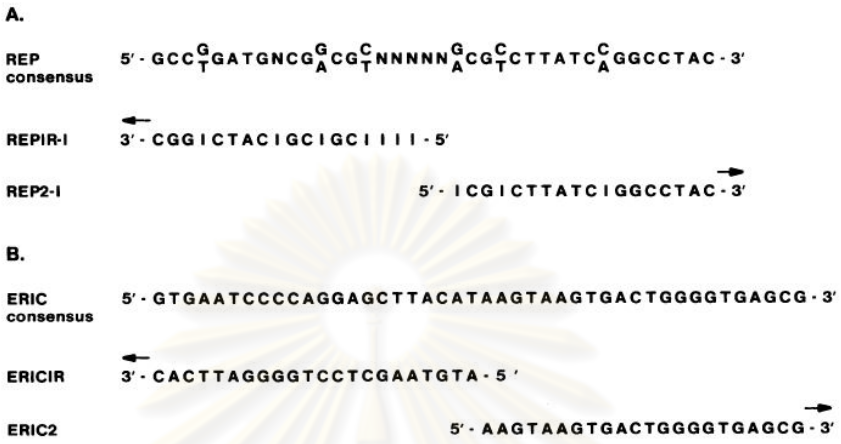


Figure 2.2 Nucleotide sequences of the REP and ERIC primers. (A) REP consensus sequence and nucleotide sequences of the two REP primers (REP1R-I and REP2-I), positioned relative to the REP consensus sequence. The I's denoted inosines. (B) ERIC consensus sequence and nucleotide sequences of the two ERIC primers (ERICIR and ERIC2), positioned relative to the ERIC consensus sequence. The arrows denoted the direction of *Taq* polymerase extension (de Bruijn, 1992).

Six of the 18 strains were *Bradyrhizobium japonicum* USDA strains (USDA8-0, USDA8-T, USDA35, USDA94, USDA117, and USDA136). Five of the strains were the same strains as used by Nuntagij (1997), namely, THA2, THA5, THA7, TAL102(USDA110), and TAL211. Therefore Teaumroong and Boonkerd (1998) reported on new 7 *Bradyrhizobium japonicum* strains. As with Nuntakij (1997), the authors did not provide details on where in Thailand the soybean nodules used for the isolation of the soybean rhizobia were obtained. The authors also did not elaborate on how the 7 strains were identified as *B. japonicum*. Combined banding patterns of the fingerprints obtained from using each of the set of primers (REP, ERIC, and RAPD) were used in the construction of a dendrogram with Primer Version 3.1B program. Figure 2.3 showed the dendrogram.

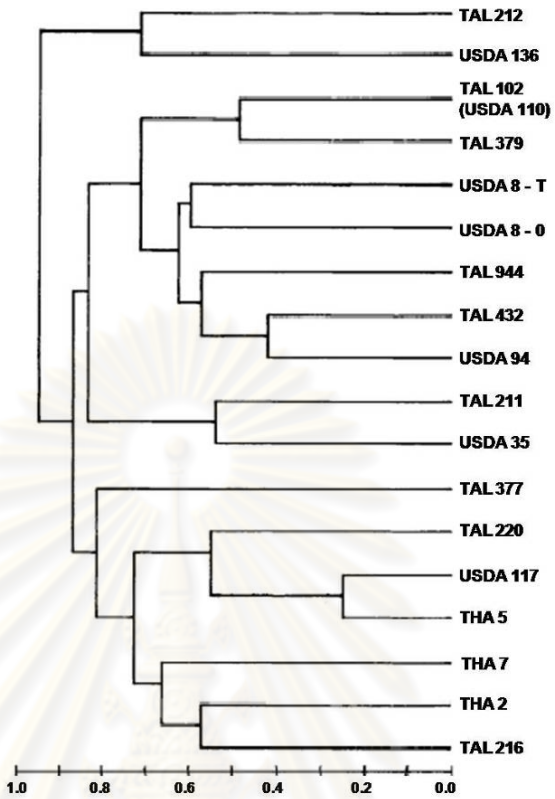


Figure 2.3 Dendrogram constructed by using combined banding patterns of the fingerprints obtained from using each of the set of primers (REP, ERIC, and RAPD) (Teaumroong and Boonkerd, 1998).

The dendrogram indicated 3 clusters at about 80% similarity. Cluster 1 consisted of TAL212 and USDA136. Cluster 2 consisted of two subgroups 2.1 and 2.2 with subgroup 2.1 containing three groups as follows:

Group 2.1.1 consisted of TAL102(USDA110),and TAL379

Group 2.1.2 consisted of USDA8-T and USDA8-0

Group 2.1.3 consisted of TAL944, TAL432, and USDA94

Subgroup 2.2 consisted of TAL211 and USDA35. Cluster 3 comprised two subgroups with TAL377 in subgroup 3.1 and there were two groups in subgroup 3.2. Group 3.2.1 contained TAL220, USDA117, and THA5. Group 3.2.2 contained THA7, THA2, and TAL216. Both Teaumroong and Boonkerd (1998) and Nuntagij et al. (1997) showed the same results that strain TAL211 belonged to a different cluster from strains THA5, THA7, and THA2.

In 1996 Yokoyama et al. obtained RFLP patterns of *nodDYABC* of 123 soybean rhizobia which consisted of 62 strains isolated in Thailand, 46 strains isolated in Japan, and 15 USDA strains. Dendrogram constructed from the RFLP patterns showed 4 clusters with all the Japanese and USDA strains belonged to Clusters 1 and 2 while Clusters 3 and 4 contained only the Thai isolates. In 1999 Ando and Yokoyama worked with 14 strains of Thai isolates of *Bradyrhizobium* sp. (*Glycine max*), 3 strains of Thai isolates of *Bradyrhizobium elkanii* , 8 USDA strains of *Bradyrhizobium japonicum* and 4 USDA strains of *Bradyrhizobium elkanii*. Genomic DNA of these 29 strains were digested with each to the following four restriction enzymes : *Bam*HI, *Hind*III, *Pst*I, and *Eco*RI. DNA fragments obtained were hybridized with 3.5 kb *Bgl*II fragment containing *nifDK*. 16 RFLP patterns obtained were used to construct a dendrogram with the Unweighted Pair Group Method with Arithmatic Averages (UPGMA) method using the PHYLIP software. The results were shown in Figure 2.4

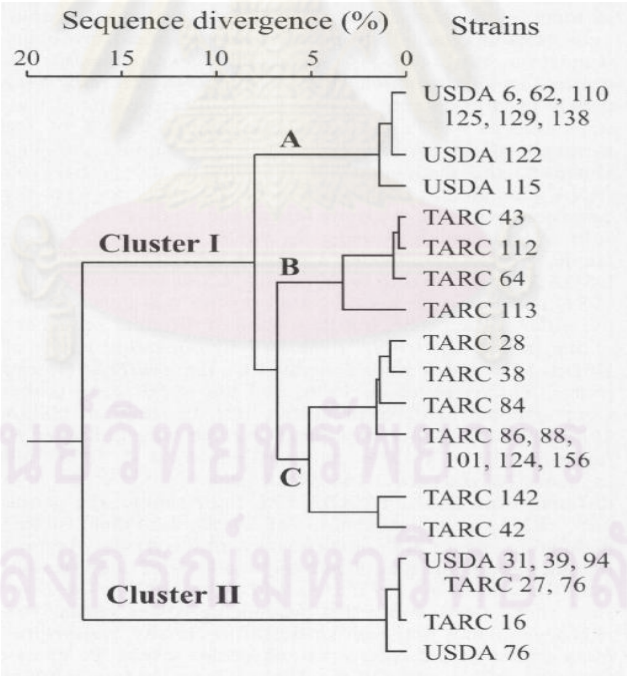


Figure 2.4 A dendrogram of 29 soybean rhizobia strains constructed with UPGMA method using the PHYLIP software (Ando and Yokoyama, 1999).

The results as shown in Figure 2.4 showed 2 clusters. Cluster 1 consisted of 3 groups (A, B, and C). Group A contained all the *B. japonicum* USDA strains 6, 62, 110,

115, 122, 125, 129, and 138. Group B contained 4 isolates from Thailand. Group C contained 10 isolates from Thailand. Partial 16S rDNA sequencing of the 246 bp at positions 44-337 showed 7 of the Thai isolates belonging to Groups B and C had identical sequences with the partial 16S rDNA sequences of *B. japonicum* USDA6, 115, and 129. Cluster 2 consisted of the 4 *B. elkanii* USDA strains 31, 39, 76, and 94 and three strains isolated in Thailand. The results as reported by Ando and Yokoyama (1999) indicated that the 14 strains of Thai isolates of *Bradyrhizobium* sp. (*Glycine max*) which were found to belong to Groups B and C formed a distinct group of slow-growing soybean rhizobia which were intermediate between the *B. japonicum* and *B. elkanii* USDA strains used in the study.

In 1999 Yokoyama et al. reported on phenotypic characterization by serological and intrinsic antibiotic resistance properties of 94 strains of soybean rhizobia isolated from Bangkok, Chiangmai, Nakorn Sawan, Saraburi, and Uttaradit. From the results obtained from the ELISA (Enzyme-Linked Immunosorbent Assay) using polyclonal antisera prepared against 15 USDA standard serotype strains of *B. japonicum* (USDA4, 6, 62, 110, 115, 122, 123, 124, 125, 127, and 129) and *B. elkanii* (USDA31, 46, 76, and 94), the 94 strains isolated in Thailand were characterized into 14 different cross-reaction groups corresponding to 12 of the 15 antisera used. The authors reported that the Thai strains showed a high degree of cross-reactivity. In addition, antibiotic resistance tests of the 94 Thai strains were carried out with the following antibiotics: 10 $\mu g.ml^{-1}$each of spectinomycin and streptomycin, 15 $\mu g.ml^{-1}$each of kanamycin and nalidixic acid, 25 $\mu g.ml^{-1}$ of rifampicin, and 50 $\mu g.ml^{-1}$each of neomycin and polymyxin. The Thai soybean rhizobia were found to be resistant to kanamycin, nalidixic acid, neomycin, and polymyxin. The results indicated that the Thai isolates were not closely related to the USDA strains of *B. japonicum* and *B. elkanii*.

Since the Ando and Yokoyama's and Yokoyama et al, 's studies in 1999 there have not been any published articles on the isolation and characterization of soybean rhizobia in Thailand. Research on soybean rhizobia in Thailand after the year 1999 concentrated on the determination of suitable cultivars of soybean as hosts for native rhizobia (Shutsrirung et al. 2002a,b,c). This dissertation is the first serious effort to isolate and characterize soybean rhizobia in Phitsanulok, Thailand , employing

polyphasic taxonomy which includes the characterization of several genetic and phenotypic properties including colony morphology, Bromthymol blue reaction, number and type of flagella, specific growth rates at different temperatures, ability/inability to utilize 95 carbon and nitrogen sources using the Biolog™ test kit, PCR-DNA Fingerprinting , and sequencing of 16S rDNA and *nodY*. Polyphasic taxonomy has been used in bacterial taxonomy since 1996 (Vandamme et a. 1996).

2.2 Soybean rhizobium genetics

Soybean rhizobium genetics is relatively well-documented both in terms of genetics of nodulation (Stacey, 1995) and of nitrogen fixation (Fischer, 1994). Loh and Stacey (2003) reported nodulation genes in *B. japonicum* included *nodD1, nodD2, nodYABC* and *nwsB*. *B. japonicum* responses to the gradient of flavonoids, genistein and daidzein, secreted by soybean roots while the fast- grower *S. fredii* responses to the gradient of soybean flavonoids daidzein and coumestrol in the initial step of soybean–rhizobial signal perception (Machado et al., 1998 ; Pueppke et al., 1998). The flavonoids enter the rhizobial periplasm to form complexes with NodD1. NodD1-flavonoid complexes induce expression of *nodD1* and *nod(Y)ABC* operon for the synthesis of Nod factors (Long, 1996). Banfalvi et al. (1988) reported that in *B. japonicum*, *nodY* is a 420-bp gene located within the 785-bp region between *nodD1* and *nodABC*. In *B. elkanii*, there is no *nodY* gene but, in its place, there is *nodK* gene which is 402 bp in size. *Bradyrhizobium* sp. (*Parasponia*) also contains a *nodK* gene in the same position (Scott 1986). The *nodY* and *nodK* genes share less than 30% sequence similarity. *B. japonicum* and *B. elkanii* strains were shown to be distinguishable on the basis of their hybridization to *nodY* and *nodK* genes.Therefore, one difference between *B. japonicum* and *B. elkanii* is the former contains *nodY* while the latter contains *nodK* in the corresponding place.

The complex between genistein and NodD1 formed in the periplasm acts as a transcriptional activator which binds to the promoter regions of *nodD1* and *nodYABC* which are known as *nodD1*box and *nodY*box respectively. Transcription and translation of *nodA, nodB,* and *nodC* lead to the synthesis of the first three enzymes in the synthesis of Nod factor which is essential for root hair deformation and nodulation process. NodC, N-acetylglucosaminyl transferase catalyses the joining of N-

acetylglucosaminyl units by ß-1,4 glycosidic linkages. NodB, N-deacetylase, catalyses the removal of an acetyl group from the N-acetylglucoaminyl group at the non-reducing end of the Nod factor. NodA, N-acyl transferase, catalyses the transfer of an acyl group (C18:1) to the N-glycosyl unit at the non-reducing end of the Nod factor. Figure 2.5 shows synthesis of nod factors which are produced by soybean rhizobia and whose function (s) on nodulation process is still unknown.

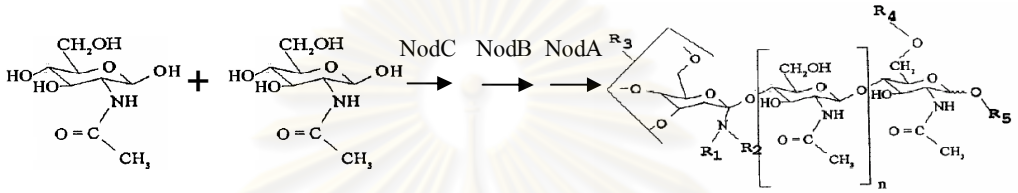
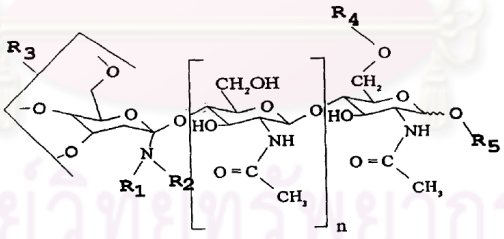


Figure 2.5 Synthesis of Nod factors catalysed by enzymes encoded by *nodC*, *nodB* and *nodA* (Stacey, 1995).

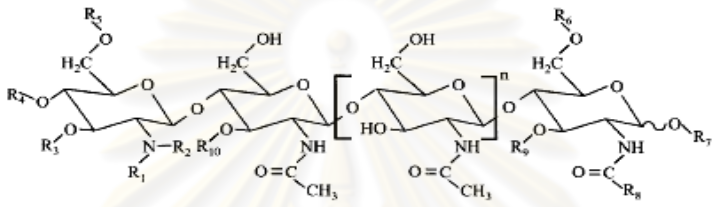
Chemical structures of nod factors of *B. japonicum* differ from those of *B. elkanii*. Nod factor of *Bradyrhizobium japonicum* consists of 5 N-acetylglucosaminyl units with side chains as indicated in Figure 2.5. The chemical structures of Nod factors of *B. japonicum* strains USDA 110 and USDA 135 and *B. elkanii* strain USDA 61 are shown in Figure 2.6.



STRAIN	R1	R2	R3	R4	R5	n
***B. japonicum* USDA110**						
NodBj-V(C18:1,MeFuc)	C18:1	H	H	2-O-MeFuc	H	3
***B. japonicum* USDA135**						
NodBj-V(C18:1, MeFuc)	C18:1	H	H	2-O-MeFuc	H	3
NodBj-V(Ac,C18:1,MeFuc)	C18:1	H	Ac	2-O-MeFuc	H	3
NodBj-V(C16:0,MeFuc)	C16:0	H	H	2-O-MeFuc	H	3
NodBj-V(Ac,C16:0,MeFuc)	C16:0	H	Ac	2-O-MeFuc	H	3
NodBj-V(C16:1, MeFuc)	C16:1	H	H	2-O-MeFuc	H	3
***B. elkanii* USDA61**						
NodBe-V(C18:1,MeFuc)	C18:1	H	H	2-O-MeFuc	H	3
NodBe-V(Ac,C18:1,MeFuc)	C18:1	H	Ac	2-O-MeFuc	H	3
NodBe-V(Cb,C18:1,NMe,MeFuc)	C18:1	Me	H	2-O-MeFuc	H	3
NodBe-V(Ac,Cb,C18:1,MeFuc)	C18:1	H	Ac,Cb	2-O-MeFuc	H	3
NodBe-fV(C18:1,MeFuc)	C18:1	H	H	2-O-MeFuc	H	2
NodBe-fV(Cb,C18:1,MeFuc)	C18:1	H	Cb	2-O-MeFuc	H	2
NodBe-fV(C18:1,Fuc,Gro)	C18:1	H	H	Fuc	Gro	2
NodBe-fV(C18:1,NMe,Fuc,Gro)	C18:1	Me	H	Fuc	Gro	2
NodBe-fV(Cb,C18:1,Fuc,Gro)	C18:1	H	Cb	Fuc	Gro	2
NodBe-fV(Cb,C18:1,NMe,Fuc,Gro)	C18:1	Me	Cb	Fuc	Gro	2

Figure 2.6 Summary of the various chito-oligosaccharides nodulation signals produced by *B. japonicum* strains USDA110 and USDA135 and *B. elkanii* strain USDA61. Abbreviations: AC, acetyl; Cb, carbamoyl; 2-0-MeFuc, 2-0-methylfucose; Fuc, fucose; Me, methyl; Gro, glycerol (Stacey et al. 1995).

Chemical structures of Nod factors of the fast- growing *S. fredii* differ from those of the slow- growers in the fatty acid side chain of the non– reducing end of Nod factors and the number of N- acetylglucosamine units as shown in Figure 2.7.



Rhizobial strain	Nod factor substitutions[a,b] R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}	n^c
B. elkanii USDA61	C18:1,C16:0	Me,H	Cb,Ac[f] H	Cb,Ac,H	Cb,Ac,H	2-*O*-MeFuc,Fuc	Gro,H	Me	H	H	1, 2
B. japonicum USDA110	C18:1[g]	H	H	H	H	2-*O*-MeFuc	H	Me	H	H	2
B. japonicum USDA135	C18:1,C16:0,C16:1[h]	H	H	H	Ac,H	2-*O*-MeFuc	H	Me	H	H	2
S. fredii HH103	C16:0,C16:1 C18:0,C18:1[i]	H	H	H	H	2-*O*-MeFuc,Fuc	H	Me	H	H	0, 1, 2
S. fredii USDA191[e]	C16:0,C16:1 C18:0,C18:1	H	H	H	H	2-*O*-MeFuc,Fuc	H	Me	H	H	0, 1, 2
S. fredii USDA257	C18:1	H	H	H	H	2-*O*-MeFuc,Fuc	H	Me	H	H	0, 1, 2

Figure 2.7 Nod factors structures and their specific substitutions in fast- and slow-growing soybean rhizobia (Gil- Serrano et al., 1997 ; Haeze and Holstera, 2002).

In addition, in 2002 Loh et al. discovered Bradyoxetin in the broth culture of stationary phase cells of *B. japonicum*. In the same year the authors constructed 4 mutants : *B. japonicum* JWS21 (*nwsB* Sm^rSp^r) ; *B. japonicum* JNWS24 (JNWS21 harboring pBGAlac1 with *nolA-lacZ* translational fusion); *B. japonicum* JNWS31 (JNWS21 harboring pZB32 with *nodY-lacZ* translation fusion); *B. japonicum* JNWS41 (JNWS21 harboring pPRJ1248 with *nodD2-lacZ* translational fusion). The mutants were used to demonstrate that at high cell density the expression of *nolA* and *nodD2* increased while that of *nodY* decreased and that *nwsB* was essential for the density-dependent full expression of *B. japonicum nod, nolA,* and *nodYABC*. NwsB was

postulated to sense the presence of Bradyoxetin at high cell density which led to the activation of *nolA* and *nodD2* which inhibited the expression of *nodYABC* leading to a decrease in Nod factor synthesis. The results implied that the number of *B. japonicum* cells in a rhizobial biofertilizer should be optimal for optimal expression of nodulation genes *nodYABC* for Nod factor synthesis.

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 ° 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 30^0C 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).

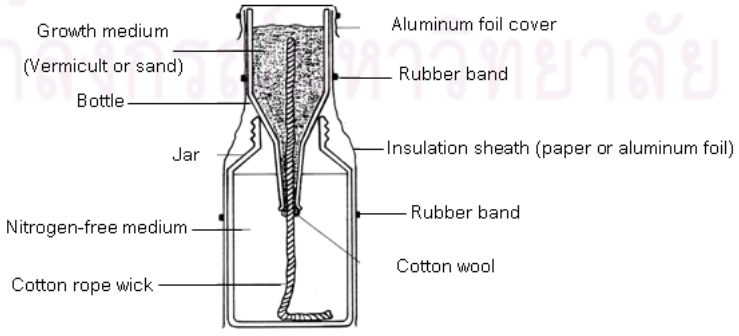


Figure 3.2 Diagram of a Leonard jar (Somasegaran and Hoben, 1994).

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 30^0C 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 25^0C, 30^0C, 37^0C, and 40^0C 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 μg.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 = $(OD_{590} - OD_{750}) x - (OD_{590} - OD_{750}) A_1 x1000$

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 *nodY*f and *nodY*r. Composition of PCR mixture was as follows : 10x PCR buffer 2 µl, 10mM dNTPs 2 µl, primer *nodY*f (10 pmol•µl $^{-1}$) and primer *nodY*r (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^{o}C 15 seconds, 50^{o}C 30 seconds and 72^{o}C 90 seconds(5 cycles), 95^{o}C 15 seconds, 60^{0} C 30 seconds, 72^{0} C 90 seconds (25 cycles) followed by 72^{o}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 :

*nodY*f : 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.

CHAPTER IV

RESULTS

4.1 Chemical and physical properties of soils from collection sites

Table 4.1 showed chemical and physical properties of soil samples from 16 subdistricts in Phitsanulok province where soil samples were kindly collected in 2006 A.D. by Assistant Professor Dr. Wipa Homhaul, Faculty of Agriculture, Natural Resources and Environment, Naresuan University. Most of the soil samples were acidic with average pHs ranging from 4.30 to 6.67. The organic matter contents were relatively low, ranging from 0.91% in Nong Phra subdistrict to 2.85% in Mathong subdistrict. Moisture contents and water holding capacity were found to be in the range of 0.50% in Ban Dong subdistrict to 12.74% in Sri Phirom subdistrict and 27.29% in Nong Pra subdistrict to 53.44 % in Mathong subdistrict, respectively. The concentrations of metals were as shown in Table 4.2. Na contents were found to range from 70 ppm in Ban Dong subdistrict to 186 ppm in Ban Phrao subdistrict. The results indicated a wide range of Ca contents from 124 ppm in Hua Ro subdistrict to 1948 ppm in Sri Phirom subdistrict. Mg contents were found to have a relatively wide range from 11 ppm in Nong Phra subdistrict to 361 ppm in Mathong subdistrict. A low Fe content of 25.7 was found in Ban Dong subdistrict while high Fe content of 319.2 ppm was found in Mathong subdistrict. Zn, Cu and S contents were low in all soil samples. The results showed soil sample from Ban Dong subdistrict was low in moisture, Na, Fe, and S contents while Mathong soil sample was found to have high water holding capacity and high Mg, Fe, and Zn contents. Sri Phirom soil sample was found to have high moisture, Ca, and Cu contents.

Table 4.1 Chemical and physical properties of soils collected in 2006 A.D. from the collection sites in 16 subdistricts in Phitsanulok province.

Subdistricts Soil properties	Average soil pH	Organic matter (%)	Available P (ppm)	Available K (ppm)	Water holding capacity (%)	Moisture content (%)
Ban Pa	4.51	1.11	8	90	34.41	1.11
Hua Ro	5.04	1.61	23	62	30.32	0.81
Wat Phrik	6.12	0.94	19	85	33.83	1.42
Wang Ithok	6.30	1.54	27	93	40.11	1.83
Mathong	4.98	2.85	38	115	53.44	2.56
Sri Phirom	4.57	1.98	28	62	41.10	12.74
Tha Chang	6.67	2.38	22	69	42.88	2.15
Ban Dong	5.12	1.11	5	49	27.91	0.50
Ban Phrao	4.68	2.64	32	83	43.56	2.99
Nakhon Chum	4.30	2.38	20	48	32.14	1.21
Nong Kathao	5.52	1.80	26	75	35.77	2.04
Chaiyanam	5.51	2.48	17	185	40.99	2.35
Kaeng Sopha	5.50	1.88	33	75	34.35	1.73
Mae Raka	4.60	2.18	7	105	40.56	1.73
Nong Phra	4.92	0.91	9	20	27.29	0.60
Tha Muen Ram	4.70	1.98	30	144	35.31	1.52

Table 4.2 Concentrations of metals in soil samples collected in 2006 A.D. from the collection sites in 16 subdistricts in Phitsanulok province (units in ppm).

Subdistricts Concentrations of metals	Na	Ca	Mg	Fe	Mn	Zn	Cu	Cl^-	S
Ban Pa	106	315	81	127.0	35.3	1.35	1.04	26.6	13
Hua Ro	82	124	48	147.7	46.1	0.73	0.35	53.3	2
Wat Phrik	96	1072	278	22.7	44.4	0.75	1.18	53.3	36
Wang Ithok	94	1164	255	85.5	61.3	1.23	2.04	26.6	16
Mathong	168	1490	361	319.2	118.0	2.83	4.16	53.3	44
Sri Phirom	151	1948	306	287.0	85.1	2.17	2.98	53.3	24
Tha Chang	104	131	122	77.8	74.5	1.80	2.75	53.3	11
Ban Dong	70	2156	132	25.7	30.9	0.39	0.38	26.6	1
Ban Phrao	186	224	43	237.5	141.8	2.39	1.65	80.0	20
Nakhon Chum	81	1240	156	199.3	36.6	2.75	0.65	53.3	3
Nong Ka Thao	157	1321	124	174.4	122.0	1.68	1.57	79.9	70
Chaiyanam	145	831	127	80.0	121.6	2.05	1.61	53.3	18
Kang Sopha	85	1130	131	83.8	123.2	2.20	1.39	79.9	2
Mae Raka	183	406	150	191.8	170.1	1.94	1.34	26.6	10
Nong Phra	82	342	11	57.1	26.7	0.52	0.39	26.6	1
Tha Muen Ram	132	104	83	227.8	115.0	1.78	1.23	53.3	21

4.2 Isolation and characterization of bacteria from root nodules of soybeans

Table 4.3 showed 340 bacterial isolates obtained by the host trapping method using 5 soybean cultivars. The isolates were categorized into fast- growers based on the visibility of colonies within 24 h growth on yeast extract mannitol agar (YMA) containing 25 $ug.ml^{-1}$ congo red. Those isolates with colonies visible on YMA with congo red plates after at least 5-day incubation were recoded as slow-growers. Appendix C showed colony morphology of all 340 isolates. Figure 4.1 showed colony morphology of representative isolates.

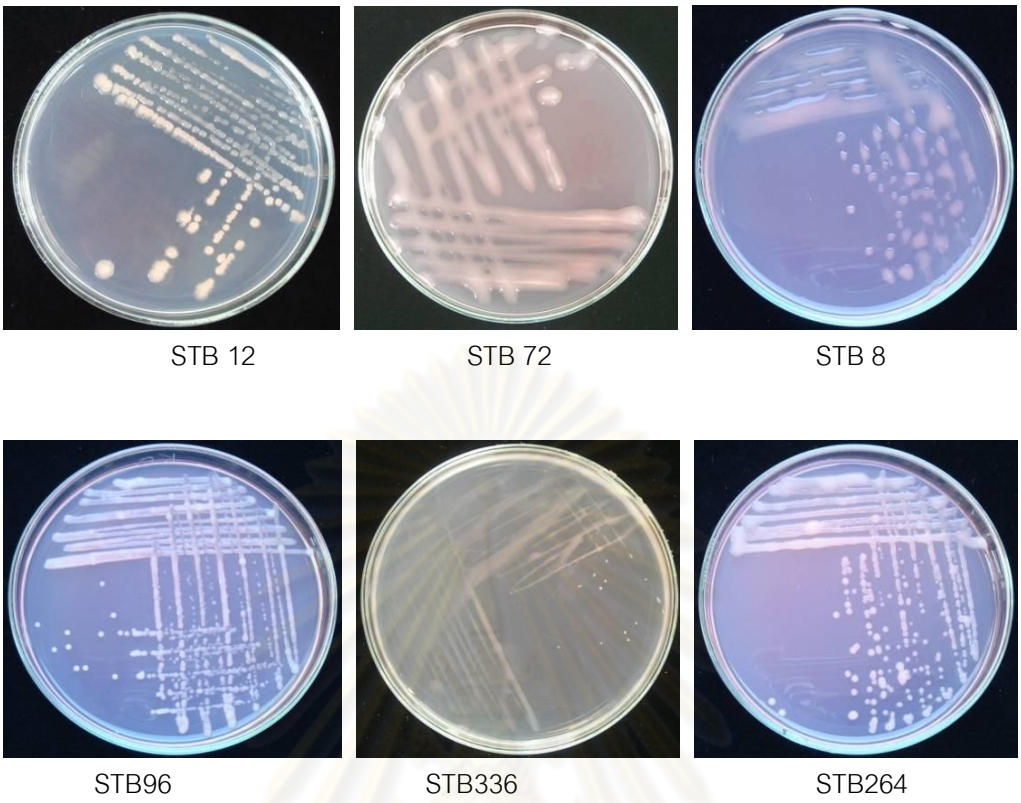


Figure 4.1 Colony morphology of some representative fast- (STB 12, STB 72) and slow-growing bacteria isolated from root nodules of soybeans (STB 8, STB 96, STB336, and STB 264).

The results as shown in Figure 4.1 showed there were two and four types of colonies for fast- and slow-growing bacteria isolated from root nodules of soybeans respectively. After 24 h growth on YMA plates, the first type of colonies of fast-growers as represented by STB12 was opaque and regular while the second type as represented by STB72 was relatively large, round (diameter up to 0.5 mm), and slimy. Colonies of the isolated slow-growing bacteria on YMA plates after 5-day incubation were small with up to 0.1 mm in diameter. The first type of colonies as represented by STB8 was irregular and slimy . The second type (STB96) was small, round and pearly while the third type was very small with diameter less than 0.1 mm (STB336) and the fourth type was round and shiny as represented by STB264 in Figure 4.1. Table 4.3 showed codes of bacteria isolated by the host trapping method from root nodules of 5 soybean cultivars. The Table indicated the nature of fast- and slow-growing.

Table 4.3 Bacteria isolated from root nodules of 5 soybean cultivars grown in soils from 16 subdistricts in Phitsanulok province.

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
Ban Pa	ST 1	STB 1	S
		STB 2	F
		STB 3	S
		STB 4	S
	ST 2	STB 5	S
		STB 6	S
	SJ 5	STB 7	F
		STB 8	S
		STB 9	S
		STB 10	S
	CM 2	STB 11	F
		STB 12	F
		STB13	F
	CM 60	STB 14	S
		STB 15	S
		STB 16	S
		STB 17	F
		STB 18	F
		STB 19	F
		STB 20	S
Hua Ro	ST 1	STB 21	S
		STB 22	S
		STB 23	F
	ST 2	STB 24	F
		STB 25	F
		STB 26	F

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
		STB 27	F
		STB 28	S
		STB 29	S
	SJ 5	STB 30	S
		STB 31	F
		STB 32	F
		STB 33	F
		STB 34	F
	CM 2	STB 35	F
		STB 36	F
		STB 37	F
		STB 38	S
		STB 39	F
		STB40	F
	CM 60	STB 41	S
		STB 42	F
		STB 43	F
		STB 44	F
Wat Phrik	ST 1	STB 45	F
		STB 46	S
		STB 47	F
		STB 48	F
		STB 49	F
	ST 2	STB 50	S
		STB 51	S
	ST 2	STB 52	S
		STB 53	S
		STB 54	S
	SJ 5	STB 55	F

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	SJ5	STB 56	F
		STB 57	S
		STB 58	S
		STB 59	S
	CM 2	STB 60	S
		STB 61	S
		STB 62	S
	CM 60	STB 63	S
		STB 64	S
		STB 65	F
		STB 66	F
Wang Ithok	ST 1	STB 67	S
		STB 68	S
		STB 69	S
		STB 70	S
		STB 71	S
	ST 2	STB 72	F
		STB 73	F
		STB 74	F
	SJ 5	STB 75	F
		STB 76	F
		STB 77	F
		STB 78	F
		STB 79	F
	CM 2	STB 80	F
		STB 81	F
		STB 82	F
		STB 83	F
		STB 84	F

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	CM2	STB 85	F
		STB 86	F
	CM 60	STB 87	F
		STB 88	F
		STB 89	S
		STB 90	F
Mathong	ST 1	STB 91	F
		STB 92	F
		STB 93	F
		STB 94	F
	ST 2	STB 95	S
		STB 96	S
		STB 97	F
		STB 98	S
		STB 99	S
	SJ 5	STB 100	S
		STB 101	F
		STB 102	F
		STB 103	F
		STB 104	S
		STB 105	S
	CM 2	STB 106	S
		STB 107	S
		STB 108	S
		STB 109	S
	CM 60	STB 110	F
		STB 111	F
		STB 112	F

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	ST 1	STB 113	S
		STB 114	S
		STB 115	S
	ST 2	STB 116	S
		STB 117	S
		STB 118	S
		STB 119	S
Sri Phirom	SJ 5	STB 120	S
		STB 121	F
		STB 122	F
		STB 123	F
	CM 2	STB 124	S
		STB 125	F
		STB 126	S
		STB 127	S
		STB 128	F
		STB 129	F
		STB 130	F
	CM 60	STB 131	S
		STB 132	S
		STB 133	S
		STB 134	S
Tha Chang	ST 1	STB 135	S
		STB 136	S
		STB 137	S
		STB 138	S
	ST 2	STB 139	S
		STB 140	S
		STB 141	S

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	ST 2	STB 142	S
		STB 143	F
	SJ 5	STB 144	F
		STB 145	F
		STB 146	F
	CM 2	STB 147	S
		STB 148	F
		STB 149	F
	CM 60	STB 150	S
		STB 151	S
		STB 152	F
		STB 153	F
Ban Dong	ST 1	STB 154	S
		STB 155	F
		STB 156	S
	ST 2	STB 157	S
		STB 158	S
		STB 159	F
		STB 160	F
		STB 161	F
		STB 162	S
		STB 163	S
		STB 164	F
	SJ 5	STB 165	F
		STB 166	F
		STB 167	F
	CM 2	STB 168	S
		STB 169	S
		STB 170	F

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	CM2	STB 171	S
		STB 172	F
		STB 173	S
		STB 174	F
		STB 175	F
		STB 176	S
		STB 177	S
	CM 60	STB 178	S
		STB 179	S
		STB 180	S
		STB 181	S
		STB 182	S
		STB 183	S
		STB 184	S
		STB 185	S
Ban Phrao	ST 1	STB 186	S
		STB 187	S
	ST 2	STB 188	S
		STB 189	S
		STB 190	S
		STB 191	F
		STB 192	F
		STB 195	F
		STB 196	F
	CM 2	STB 197	F
		STB 198	S
		STB 199	F
		STB 200	S
	CM 60	STB 201	S

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	CM 60	STB 202	S
		STB203	S
Nakhon Chum	ST 1	STB 204	S
		STB 205	F
		STB 206	S
		STB 207	F
		STB 208	F
	ST 2	STB 209	S
		STB 210	F
		STB 211	F
		STB 212	F
		STB 213	S
		STB 214	S
	CM 2	STB 219	F
		STB 220	S
	CM 60	STB 221	S
		STB 222	S
		STB 223	S
		STB 224	S
		STB 225	F
Nong Ka Thao	ST 1	STB 226	S
		STB 227	S
		STB 228	S
		STB 229	F
		STB 230	F
	ST 2	STB 231	S
		STB 232	S
		STB 233	S
		STB 234	S

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	CM 2	STB 241	S
		STB 242	S
		STB 243	S
		STB 244	F
		STB 245	S
	CM 60	STB 246	S
		STB 247	F
		STB 248	S
		STB 249	F
		STB 250	S
Chaiyanam	ST 1	STB 251	S
		STB 252	S
	ST 2	STB 253	S
		STB 254	S
	SJ 5	STB 324	S
		STB 325	S
		STB 326	S
	CM 2	STB 255	S
		STB 256	S
		STB 257	F
		STB 258	F
	CM 60	STB 259	S
		STB 260	S
		STB 261	S
Kang Sopha	ST 1	STB 262	F
		STB 263	F
		STB 264	S
		STB 265	S
	ST 2	STB 266	S

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	ST 2	STB 267	F
		STB 268	S
		STB 269	S
	SJ 5	STB 270	S
		STB 271	S
		STB 272	S
	CM 2	STB 273	S
		STB 274	S
		STB 275	S
	CM 60	STB 276	S
		STB 277	F
		STB 278	F
		STB 279	F
		STB 280	S
Mae Raka	ST 1	STB 281	S
		STB 282	S
		STB 283	F
	ST 2	STB 284	S
		STB 285	S
		STB 286	S
		STB 287	S
		STB 288	S
	SJ 5	STB 289	F
		STB 290	F
		STB 291	F
	CM 2	STB 327	S
		STB 328	S
		STB 329	S
	CM 60	STB 292	S

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	CM 60	STB 293	F
		STB 294	S
Nong Phra	ST 1	STB 295	S
		STB 296	S
		STB 297	F
		STB 298	S
		STB 299	S
	ST 2	STB 330	S
		STB 331	S
		STB 332	S
	SJ 5	STB 300	S
		STB 301	F
		STB 302	S
		STB 303	F
		STB 304	F
	CM 2	STB 333	S
		STB 334	S
		STB 335	S
	CM 60	STB 305	F
		STB 306	S
		STB 307	F
		STB 308	S
		STB 309	F
Tha Muen Ram	ST 1	STB 310	S
		STB 311	F
		STB 312	F
		STB 313	F
		STB 314	F
	ST 2	STB 315	S

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	SJ 5	STB 336	S
		STB 337	S
		STB 338	S
	CM 2	STB 339	S
		STB 340	S
	CM 60	STB 320	S
		STB 321	S
		STB 322	F
		STB 323	S

The results as shown in Table 4.3 indicated 138 and 202 isolates of fast- and slow-growing bacteria were obtained respectively. It was noted that in Wang Ithok subdistrict all bacteria isolated from root nodules of soybean cultivars ST2, SJ5, and CM2 were fast-growers. Only fast-growing bacteria were also isolated from root nodules of soybean cultivar SJ5 when soybeans were grown in soils from Tha Chang, Ban Dong, Ban Phrao, and Mae Raka subdistricts. Isolation of bacteria from root nodules of soybean cultivar CM2 grown in soil from Ban Pa also showed only fast-growing bacteria were obtained. Other soil-soybean cultivar combinations where only fast-growing bacteria were isolated were Mathong-ST1 and Mathong-CM60. Physical characteristics of soil samples from Wang Ithok and Tha Chang subdistricts, as shown in Tables 4.1 and 4.2, showed there were no distinct soil properties in these two subdistricts when compared with physical properties of soil samples collected from other subdistricts except that the two soil samples from Wang Ithok and Tha Chang were least acidic with pHs 6.30 and 6.67. It is not known if the relativey high soil pHs were conducive to more fast-growing bacteria. In terms of the soybean cultivars used as the host traps, in order to attract more fast-growing bacteria to the root nodules, the attractant flavonoid signal molecules secreted by roots of soybean cultivar SJ5 might be qualitatively and/or quantitatively different from those secreted by roots of other soybean cultivars. There

have been reports that different soybean cultivars secrete different kinds and different quantities of the attractant flavonoid molecules. Since RAPD-PCR fingerprinting of all the 340 isolates to determine which isolates were the same strains was time-consuming, it was decided that, instead of waiting for the results of the determination of which isolates belonged to the same strains, determination of bromthymol blue reactions and authentication of all the fast- and slow-growing isolates would be carried out for all isolates.

4.3 Bromthymol blue reactions

The results of Bromthymol blue (BTB) reactions of some representative fast- and slow-growing isolates were shown in Figure 4.2. The results showed that all the fast-growing isolates secreted acidic product(s) to turn the dye to yellow color. There were two types of BTB reactions for the slow-growing isolates with the unexpected results for the slow-growing isolates. Somasegaran and Hoben (1994) stated that fast- growing soybean rhizobia secreted acidic product(s) which turned the color of the indicator bromthymol blue into yellow while slow- growing soybean rhizobia secreted alkali product(s) which renderedd the bromthymol blue indicator blue color. The results obtained from this experiment indicated that, based on the bromthymol blue reactions, the slow-growing isolates could be divided into two groups with group 1 containing the isolates which secreted alkali product(s) throughout the 10-day incubation period while group 2 consisted of the slow-growing isolates which secreted alkali products after 5-day incubation and acidic product(s) was secreted after 10-day incubation. Figure 4.2 showed bromthymol blue reaction of some representative of fast- and slow-growing isolates.

STB 97 STB 8 STB 30

Figure 4.2 Bromthymol blue reactions of some representative of fast- (STB 97) and slow-growing isolates which secreted alkaline product(s) throughout the 10-day incubation period (STB8) and secreted alkali product(s) after 5-day incubation and acidic product(s) was secreted after 10-day incubation (STB 30).

4.4 Authentication test of bacterial isolates from root nodules of soybeans

Figures 4.3 and 4.4 showed representative results of authentication test of fast-growing (STB 23, STB 83, STB 87, STB 170, STB 191, STB 192, STB240) and slow-growing (STB 1, STB 5 STB 8, STB 272, STB310) bacterial isolates from root nodules of 5 soybean cultivars grown in soils from 16 subdistricts of Phitsanulok province.

ST1 ST2 SJ5 CM2 CM60

ST1 ST2 SJ5 CM2 CM60

ST1 ST2 SJ5 CM2 CM60

ST1 ST2 SJ5 CM2 CM60

ST1 ST2 SJ5 CM2 CM60

ST1 ST2 SJ5 CM2 CM60

Negative Control:

ST1 ST2 SJ5 CM2 CM60

Positive Control:

ST1 ST2 SJ5 CM2 CM60

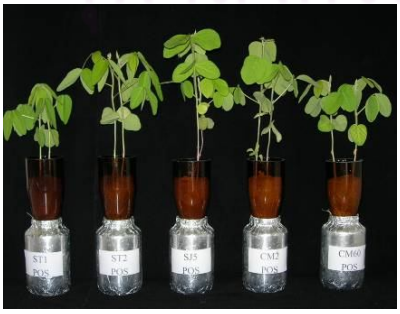
Figures 4.3 Representative authentication test results of fast-growing bacterial isolates (STB 23, STB 83, STB 87, STB 170, STB 191, STB 192, and STB240) grown in Leonard jars with two germinating seeds per jar. Five ml of each bacterial suspension in yeast extract mannnitol broth were added to each germinating seed of soybean cultivars ST1, ST2, SJ5, CM2, and CM60 in Leonard jars which were placed in a 28^0C- 32^0C temperature-controlled greenhouse for 28 days before the observation of roots. Negative and positive control soybean plants were found to contain no root nodules.

ST1 ST2 SJ5 CM2 CM60

ST1 ST2 SJ5 CM2 CM60

Negative Control:

ST1 ST2 SJ5 CM2 CM60



Positive Control:

ST1 ST2 SJ5 CM2 CM60

Figure 4.4 Representative authentication test results of slow-growing bacterial isolates (STB1, STB5, STB 8, STB 272, and STB 310) grown in Leonard jars with two germinating seeds per jar. Five ml of each bacterial suspension in yeast extract mannnitol broth were added to each germinating seed of soybean cultivars ST1, ST2, SJ5, CM2, and CM60 in Leonard jars which were placed in a 28^0C- 32^0C temperature-controlled greenhouse for 28 days before the observation of root nodules. Negative and positive control soybean plants were found to contain no root nodules.

Figures 4.3 and 4.4 showed the representative fast-growing bacterial isolates did not cause root nodule formation while the representative slow-growing isolates formed root nodules in all the 5 soybean cultivars used in the experiments. The rationale behind the use of 5 soybean cultivars in the authentication test, especially in the authentication test of fast-growing isolates, was because fast-growing soybean rhizobia had been shown to be relatively more soybean host-specific than slow-growing soybean rhizobia. For example, in 1998 Pueppke et al. reported that fast-growing soybean rhizobium, *Sinorhizobium fredii* strain USDA257 could nodulate soybean *Glycine max* cv. Peking but not cv. McCall while *Sinorhizobium fredii* strain USDA191 could nodulate both soybean cultivars. The researchers employed reverse-phase HPLC and electrospray mass spectrophotometry to determine types and quantities of flavonoids secreted by roots of germinating seeds of each soybean cultivar. The results indicated root exudates of both soybean cultivars contained the same flavonoids genistein, daidzein, and coumestrol with soybean cultivar Peking secreted relatively high concentration of daidzein (average 1371 pmol. seedling^{-1}). The authors suggested that different environments regarding different flavonoid levels in the rhizosphere of soybean cultivars McCall and Peking, and possibly the differential ability to uptake the flavonoid signal molecules could explain different host-specificity in soybean and *S. fredii* system. Therefore, in the authentication experiments, 5 soybean cultivars which had been recommended to soybean growers by the Department of Agricultural Extension, Ministry of Agriculture and Cooperatives, were used to take into account possible specific host requirements of fast-growing soybean rhizobia.

Authentication test results indicated the 138 fast-growing root nodule bacteria did not cause nodule formation in roots of the 5 soybean cultivars. They were found not to be fast-growing soybean rhizobia. On the other hand, all the 202 slow-growing isolates were found to cause nodule formation on roots of all the 5 soybean cultivars grown in Leonard jars in a 28^0 C - 32^0 C temperature-controlled greenhouse. The 202 bacterial root nodule isolates were authenticated to be slow-growing soybean rhizobia.

4.5 PCR-DNA fingerprints of 202 slow-growing soybean rhizobia

Figures 4.5-4.16 showed PCR-DNA fingerprints of 202 slow-growing soybean rhizobium isolates obtained from 16 subdistricts in Phitsanulok province. The results showed some isolates had identical fingerprints when either RPO1 or CRL-7 was used as the primer in obtaining the fingerprints. Isolates with identical DNA fingerprints were regarded as the same strains using the lowest code number of the isolates as each strain's code number. For example, isolates STB4 and STB30 had identical DNA fingerprints when either RPO1 or CRL-7 was used as the primer. Therefore the two isolates were regarded as the same strain STB4. Table 4.4 showed isolates with identical DNA fingerprints were grouped into the same strains with a total of 43 strains. Figure 4.17 showed 33 strains with different fingerprints from each other and from other isolates. Therefore the 33 strains constitute distinctive strains. All together, PCR-DNA fingerprints revealed a total of 121 slow-growing soybean rhizobium strains were obtained in this research.

RPO1 CRL-7

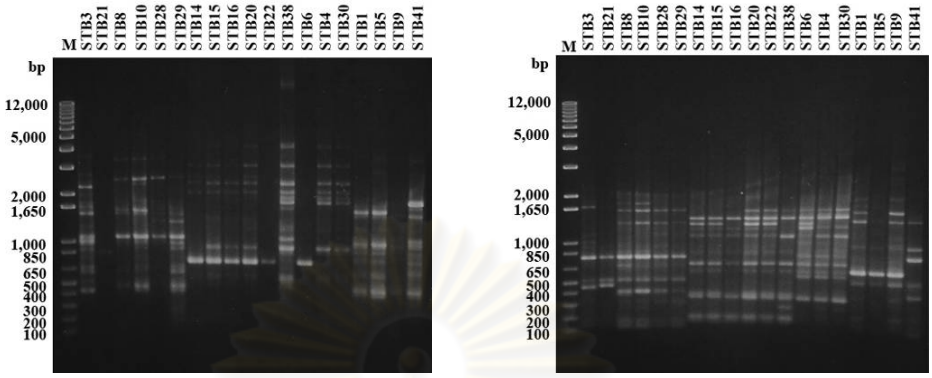


Figure 4.5 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Ban Pa and Hua Ro subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB1 = STB5 = STB9, STB3 = STB21, STB4 = STB30, STB8 = STB10 = STB28 = STB29, STB20 = STB22, STB14 = STB15.

RPO1 CRL-7

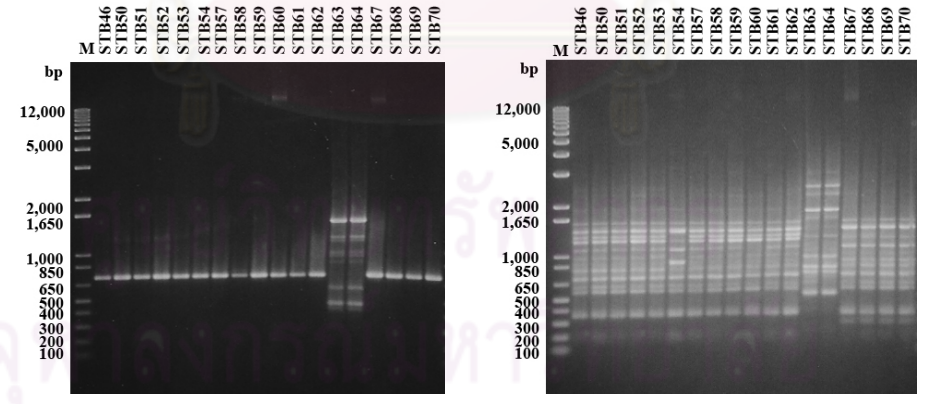


Figure 4.6 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Wat Phrik and Wang Ithok subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB46 = STB50 = STB51 = STB52 = STB53 = STB57 = STB58 = STB59 = STB60 = STB61 = STB62, STB63 = STB64, STB67 = STB68 = STB69 = STB70.

RPO1 CRL-7

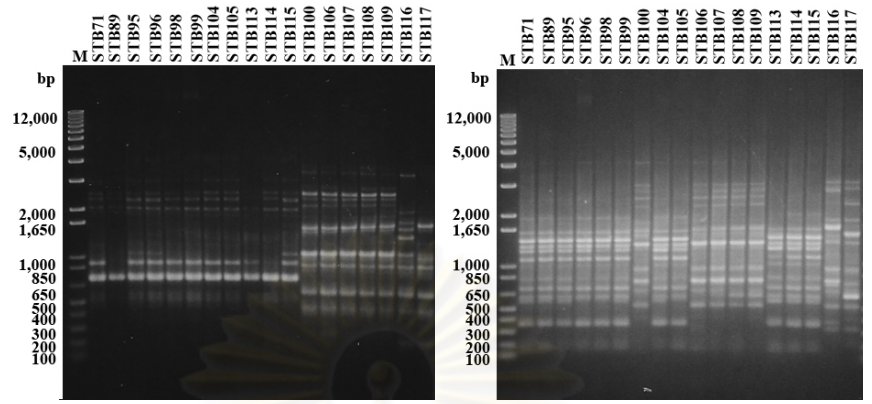


Figure 4.7 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Wang Ithok, Mathong and Sri Phirom subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB71= STB95 = STB96 = STB98 = STB99 = STB104 = STB105 = STB115, STB100 = STB106 = STB107 = STB108 = STB109.

RPO1 CRL-7

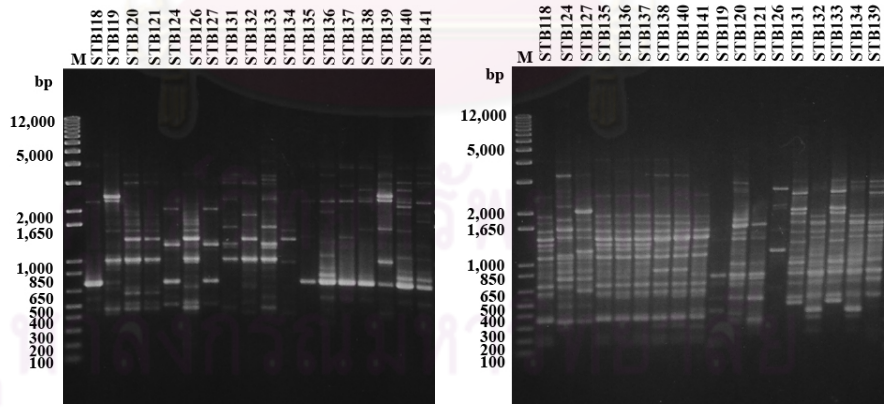


Figure 4.8 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Sri Phirom and Tha Chang subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB118 = STB135 = STB136 = STB137, STB131 = STB133, STB132 = STB134, STB138 = STB141.

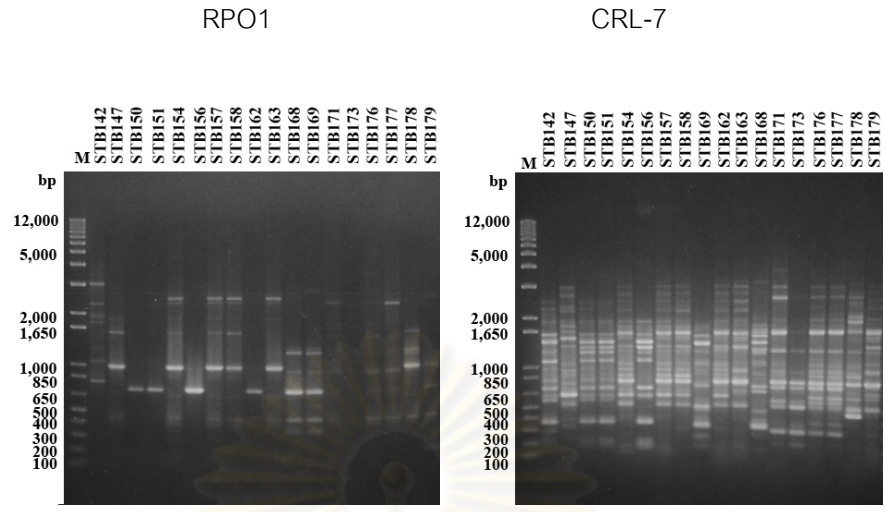


Figure 4.9 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Tha Chang and Ban Dong subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB150 = STB151 = STB156, STB154 = STB157 = STB158, STB171 = STB176 = STB177.

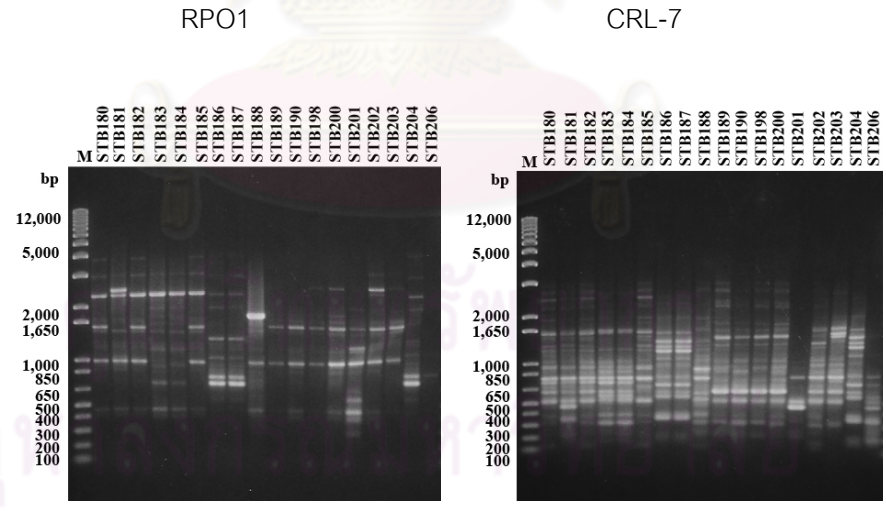


Figure 4.10 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Ban Dong, Ban Phrao and Nakhon Chum subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB180 = STB182, STB189 = STB190 = STB198 = STB200, STB183 = STB184, STB186 = STB187 = STB204.

RPO1 CRL-7

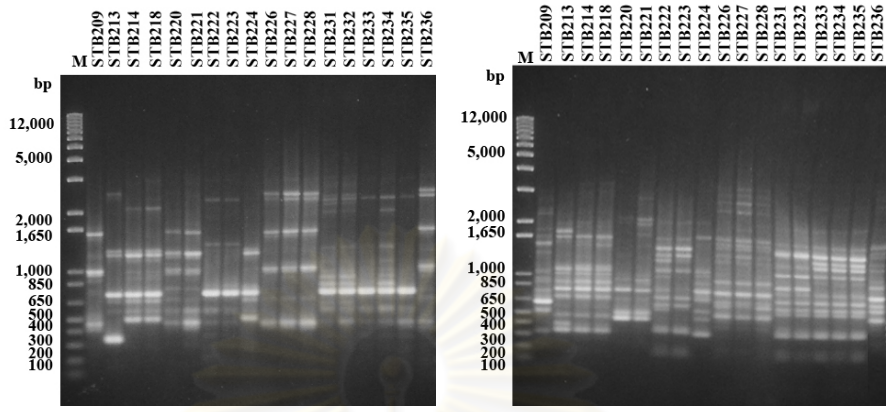


Figure 4.11 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Nakhon Chum and Nong Kathao subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB214 = STB218 , STB220 = STB221, STB222 = STB223, STB226 = STB227 = STB228, STB231 = STB232, STB233 = STB235.

RPO1 CRL-7

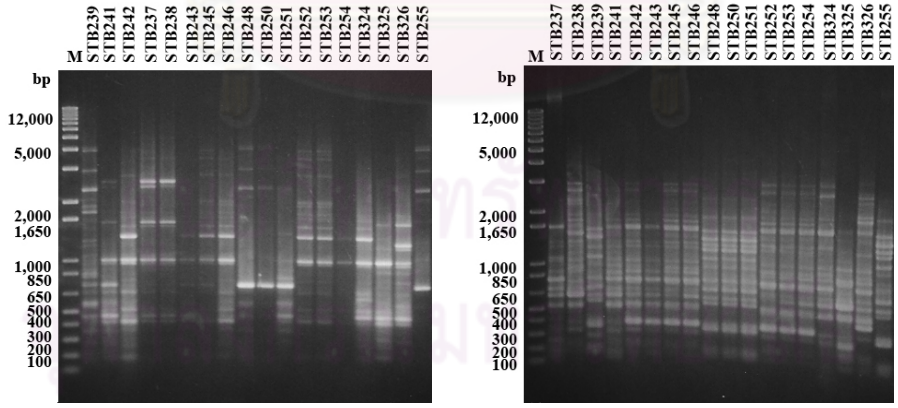


Figure 4.12 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Nong Kathao and Chaiyanam subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB248 = STB251, STB252 = STB253, STB242 = STB245 = STB246, STB252 = STB253 = STB 324.

RPO1 CRL-7

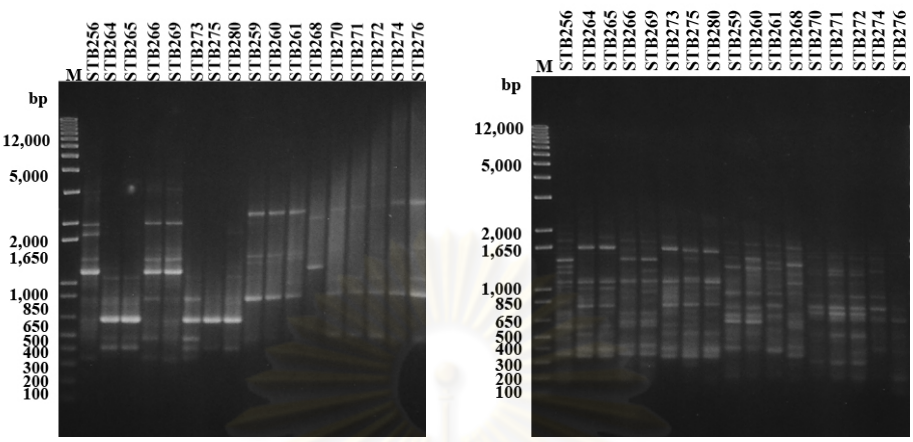


Figure 4.13 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Chaiyanam and Kang Sopha subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB259 = STB260, STB264 = STB265 = STB280, STB266 = STB269, STB271 = STB272.

RPO1 CRL-7

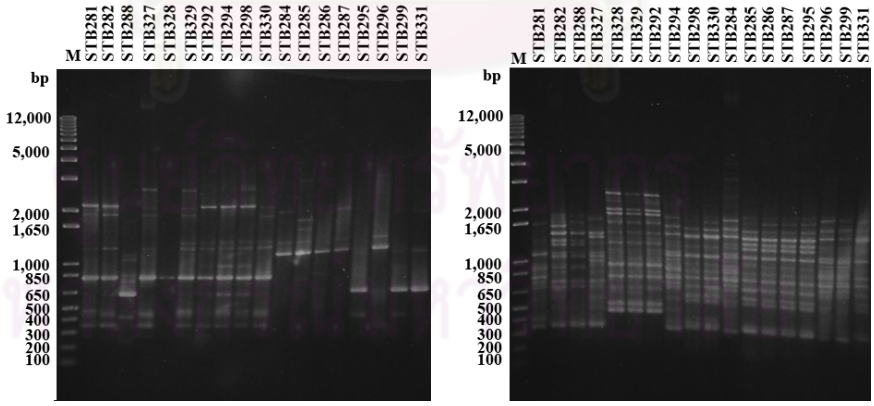


Figure 4.14 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Kang Sopha and Nong Phra subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB299 = STB331, STB286 = STB287.

RPO1 CRL-7

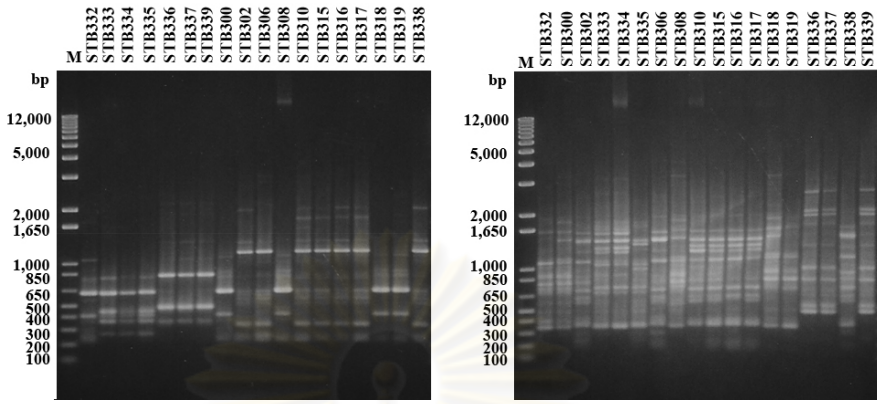


Figure 4.15 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Nong Phra and Tha Muen Ram subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB300 = STB308, STB302 = STB306, STB310 = STB315 = STB316 = STB317 = STB338, STB333 = STB334, STB336 = STB337 = STB339.

RPO1 CRL-7

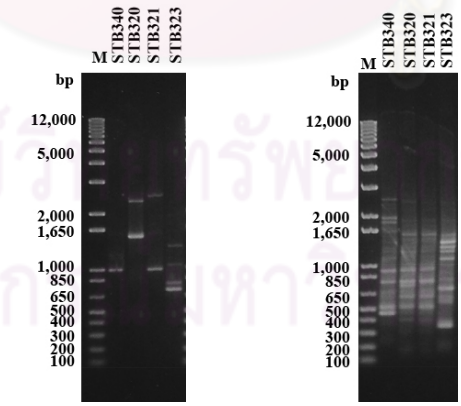
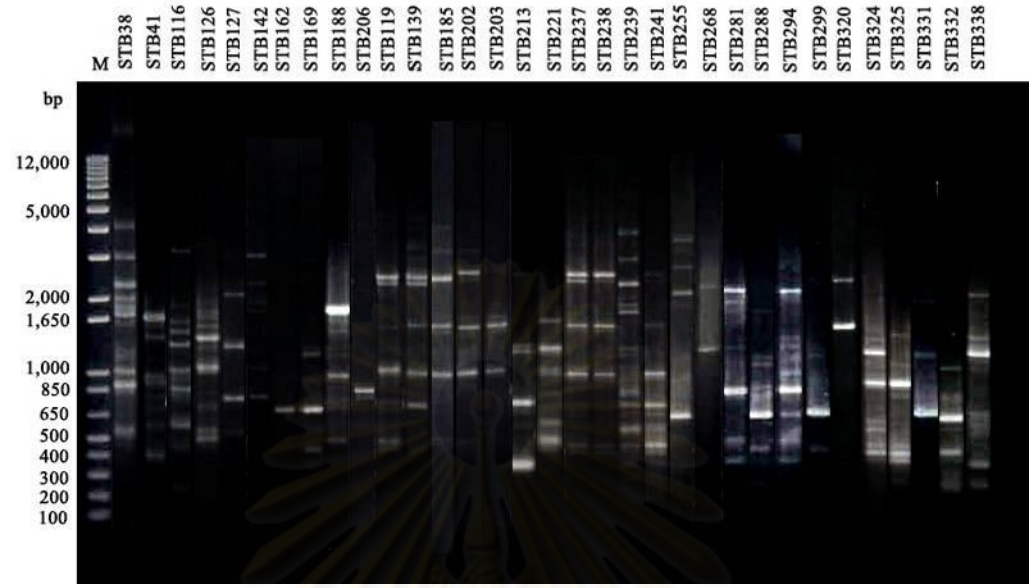


Figure 4.16 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Tha Muen Ram subdistrict, Phitsanulok province.

RPO1



CRL-7

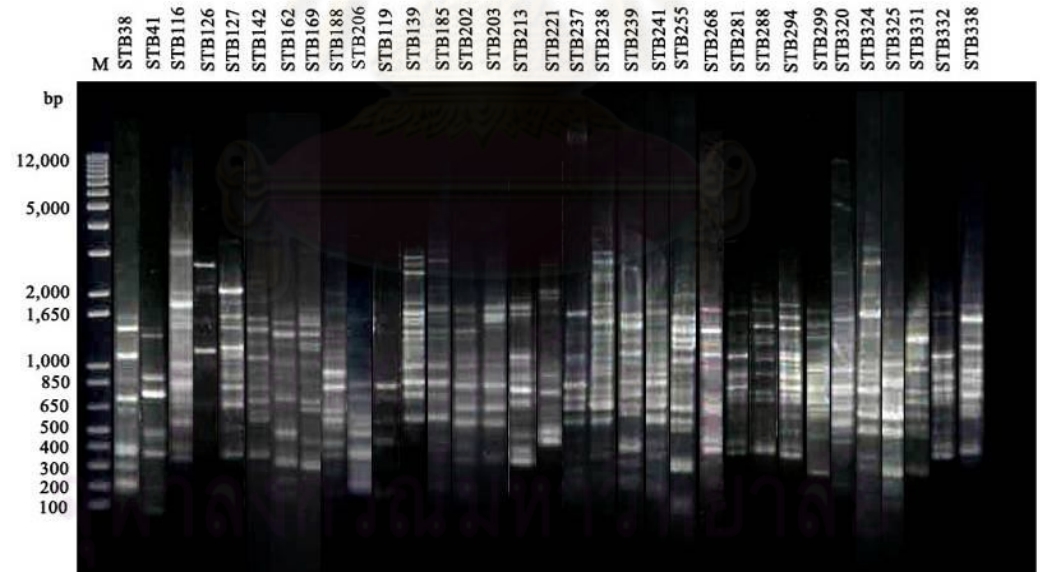


Figure 4.17 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from several subdistricts of Phitsanulok province which have distinct individual fingerprints.

Table 4.4 Slow-growing soybean rhizobium isolates with identical RAPD-PCR fingerprints were grouped into the same strains.

Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
STB1	STB1	Ban Pa	ST 1
	STB5	Ban Pa	ST 2
	STB9	Ban Pa	SJ 5
STB3	STB3	Ban Pa	ST1
	STB21	Hua Ro	ST 1
STB4	STB4	Ban Pa	ST1
	STB30	Hua Ro	SJ 5
STB6	STB6	Ban Pa	ST2
STB8	STB8	Ban Pa	SJ 5
	STB10	Ban Pa	SJ 5
STB14	STB14	Ban Pa	CM60
	STB15	Ban Pa	CM60
STB16	STB16	Ban Pa	CM60
STB20	STB20	Ban Pa	CM60
	STB22	Hua Ro	ST1
STB28	STB28	Hua Ro	ST2
	STB29	Hua Ro	ST 2
STB38	STB38	Hua Ro	CM2
STB41	STB41	Hua Ro	CM2
STB46	STB46	Wat Phrik	ST1
	STB50	Wat Phrik	ST2
	STB51	Wat Phrik	ST2
	STB52	Wat Phrik	ST2
	STB53	Wat Phrik	ST2
	STB57	Wat Phrik	SJ5

Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
	STB58	Wat Phrik	SJ5
	STB59	Wat Phrik	SJ5
	STB60	Wat Phrik	CM2
	STB61	Wat Phrik	CM2
	STB62	Wat Phrik	CM2
STB54	STB54	Wat Phrik	ST2
STB63	STB63	Wat Phrik	CM2
	STB64	Wat Phrik	CM2
STB67	STB67	Wang Ithok	ST1
	STB68	Wang Ithok	ST1
	STB69	Wang Ithok	ST1
	STB70	Wang Ithok	ST1
STB71	STB71	Wang Ithok	ST2
	STB95	Mathong	SJ5
	STB96	Mathong	CM2
	STB98	Mathong	SJ 5
	STB99	Mathong	ST2
	STB104	Mathong	CM2
	STB105	Mathong	CM2
	STB115	Sri Phirom	ST1
STB89	STB89	Wang Ithok	ST2
STB100	STB100	Mathong	SJ 5
	STB106	Mathong	CM2
	STB107	Mathong	CM2
	STB108	Mathong	CM60
	STB109	Mathong	SJ5
STB113	STB113	Sri Phirom	CM60

Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
STB114	STB114	Sri Phirom	CM2
STB116	STB116	Mathong	ST2
STB117	STB117	Sri Phirom	ST2
STB118	STB118	Sri Phirom	SJ5
	STB135	Tha Chang	SJ5
	STB136	Tha Chang	CM60
	STB137	Tha Chang	CM2
STB119	STB119	Mathong	ST2
STB120	STB120	Sri Phirom	SJ5
STB121	STB121	Sri Phirom	SJ5
STB124	STB124	Sri Phirom	CM2
STB126	STB126	Sri Phirom	CM2
STB127	STB127	Sri Phirom	CM2
STB131	STB131	Sri Phirom	ST2
	STB133	Sri Phirom	ST2
STB132	STB132	Sri Phirom	CM60
	STB134	Sri Phirom	CM60
STB138	STB138	Tha Chang	SJ5
	STB141	Tha Chang	CM2
STB139	STB139	Tha Chang	ST2
STB140	STB140	Tha Chang	CM2
STB142	STB142	Tha Chang	ST2
STB147	STB147	Tha Chang	CM2
STB150	STB150	Tha Chang	CM60
	STB151	Tha Chang	CM60
	STB156	Ban Dong	ST1
STB154	STB154	Ban Dong	CM2

Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
	STB157	Ban Dong	CM2
	STB158	Ban Dong	ST2
STB162	STB162	Ban Dong	ST2
STB163	STB163	Ban Dong	ST1
STB168	STB168	Ban Dong	CM2
STB169	STB169	Ban Dong	CM2
STB171	STB171	Ban Dong	CM2
	STB176	Ban Dong	CM2
	STB177	Ban Dong	CM2
STB173	STB173	Ban Dong	CM2
STB178	STB178	Ban Dong	CM60
STB179	STB179	Ban Dong	CM60
STB180	STB180	Ban Dong	CM60
	STB182	Ban Dong	CM60
STB181	STB181	Ban Dong	CM60
STB183	STB183	Ban Dong	CM60
	STB184	Ban Dong	CM60
STB185	STB185	Ban Dong	CM60
STB186	STB186	Ban Phrao	ST1
	STB187	Ban Phrao	ST1
	STB204	Nakhon Chum	ST1
STB188	STB188	Ban Dong	ST2
STB189	STB189	Ban Phrao	ST 2
	STB190	Ban Phrao	ST 2
	STB198	Ban Phrao	CM2
	STB200	Ban Phrao	CM2
STB201	STB201	Ban Phrao	CM60

Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
STB202	STB202	Ban Dong	CM60
STB203	STB203	Ban Dong	CM60
STB206	STB206	Nakhon Chum	ST2
STB209	STB209	Nakhon Chum	ST 2
STB213	STB213	Nakhon Chum	ST2
STB214	STB214	Nakhon Chum	ST2
	STB218	Nakhon Chum	SJ5
STB220	STB220	Nakhon Chum	CM2
	STB221	Na Khon Chum	CM2
STB222	STB222	Na Khon Chum	ST1
	STB223	Na Korn Chum	ST2
STB224	STB224	Nakhon Chum	CM60
STB226	STB226	Nong Kathao	ST1
	STB227	Nong Kathao	ST1
	STB228	Nong Kathao	ST1
STB231	STB231	Nong Kathao	ST2
	STB232	Nong Kathao	ST2
STB233	STB233	Nong Kathao	SJ5
	STB235	Nong Kathao	ST2
STB234	STB234	Nong Kathao	SJ5
STB236	STB236	Nong Kathao	SJ5
STB237	STB237	Nong Kathao	SJ5
STB238	STB238	Nong Kathao	SJ5
STB241	STB241	Nong Kathao	CM2
STB242	STB242	Nong Kathao	CM2
	STB245	Nong Kathao	ST1
	STB246	Nong Kathao	ST1

Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
STB243	STB243	Nong Kathao	CM2
STB248	STB248	Nong Kathao	CM2
	STB251	Chaiyanam	CM2
STB250	STB250	Ban Dong	CM2
STB252	STB252	Chaiyanam	CM60
	STB253	Chaiyanam	ST2
	STB324	Chaiyanam	SJ5
STB254	STB254	Chaiyanam	ST2
STB255	STB255	Chaiyanam	CM2
STB256	STB256	Chaiyanam	CM2
STB259	STB259	Chaiyanam	CM60
	STB260	Chaiyanam	CM60
STB264	STB264	Kang Sopha	ST1
	STB265	Kang Sopha	ST1
	STB280	Kang Sopha	CM60
STB261	STB261	Chaiyanam	CM60
STB266	STB266	Kang Sopha	ST2
	STB269	Kang Sopha	ST2
STB268	STB268	Kang Sopha	ST2
STB270	STB270	Kang Sopha	SJ5
STB271	STB271	Kang Sopha	SJ5
	STB272	Kang Sopha	SJ5
STB273	STB273	Kang Sopha	CM2
STB274	STB274	Kang Sopha	CM2
STB275	STB275	Kang Sopha	CM2
STB276	STB276	Kang Sopha	CM60
STB281	STB281	Mae Raka	ST1

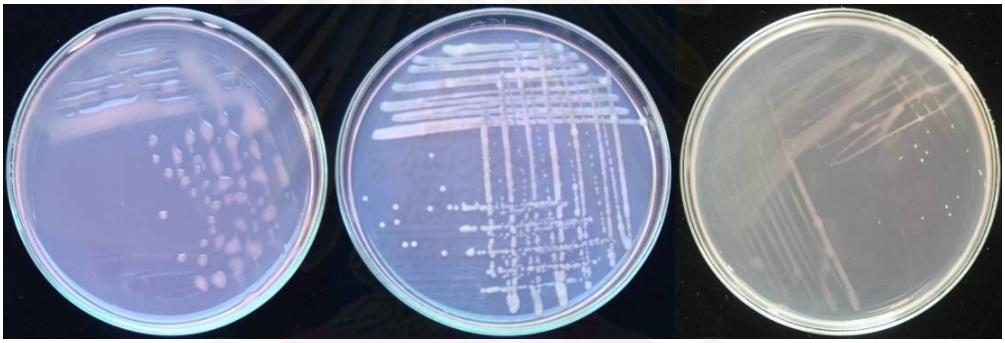
Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
STB282	STB282	Mae Raka	ST2
STB284	STB284	Mae Raka	CM2
STB285	STB285	Mae Raka	ST2
STB286	STB286	Mae Raka	ST2
	STB287	Mae Raka	ST2
STB288	STB288	Mae Raka	ST2
STB292	STB292	Mae Raka	CM2
STB294	STB294	Mae Raka	CM60
STB295	STB295	Nong Phra	SJ5
STB296	STB296	Nong Phra	CM2
STB298	STB298	Nong Phra	ST1
STB299	STB299	Nong Phra	ST1
	STB331	Nong Phra	ST2
STB300	STB300	Nong Phra	SJ5
	STB308	Nong Phra	CM60
STB302	STB302	Nong Phra	SJ5
	STB306	Nong Phra	CM60
STB310	STB310	Ta Muen Ram	ST1
	STB315	Ta Muen Ram	ST2
	STB316	Ta Muen Ram	ST2
	STB317	Ta Muen Ram	ST2
	STB338	Ta Muen Ram	SJ5
STB318	STB318	Ta Muen Ram	ST2
STB319	STB319	Ta Muen Ram	ST2
STB320	STB320	Tha Muen Ram	CM60
STB321	STB321	Ban Dong	ST1
STB323	STB323	Ta Muen Ram	CM60

Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
STB325	STB325	Chaiyanam	SJ5
STB326	STB326	Chaiyanam	SJ5
STB327	STB327	Mae Raka	CM2
STB328	STB328	Mae Raka	ST1
STB329	STB329	Mae Raka	ST1
STB330	STB330	Nong Phra	ST2
STB332	STB332	Nong Phra	ST2
STB333	STB333	Nong Phra	CM2
	STB334	Nong Phra	CM2
STB336	STB336	Kang Sopha	CM60
	STB337	Ta Muen Ram	SJ5
	STB339	Ta Muen Ram	SJ5
STB335	STB335	Nong Phra	CM2
STB340	STB340	Tha Muen Ram	CM2

4.6 Polyphasic characterization of slow-growing soybean rhizobia

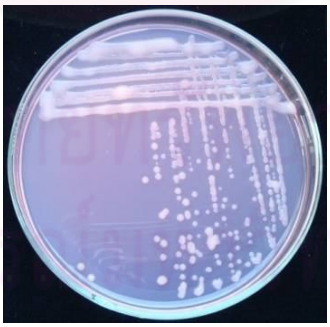
4.6.1 Colony morphology

Figure 4.18 showed representative colony morphology of 4 slow-growing soybean rhizobium strains. The results indicated Type I colonies were irregular and slimy after 10- day incubation on YMA with congo red medium. Type II colonies were round and pearly. Type III colonies were round and shiny with very small colonies (diameter less than 0.01 mm) while Type IV colonies were round and slimy. All the slow-growing soybean rhizobia were found not to absorb congo red.



STB 8 STB96 STB336

Type I colony Type II colony Type III colony



STB264

Type IV colony

Figure 4.18 Colony morphology of representative slow-growing soybean rhizobia isolated from soils from 16 subdistricts in Phitsanulok province. Type I colonies

appeared irregular and slimy. Type II colonies appeared round and pearly. Type III colonies were round and shiny with very small colonies (diameter less than 0.01 mm). Type IV colonies were round and slimy.

4.6.2 Bromthymol blue reactions

Table 4.5 showed colony morphology and Bromthymol blue (BTB) reactions of the 121 slow-growing soybean rhizobium strains. The results of BTB reactions showed most of the soybean rhizobia with Type I colonies secreted alkali product(s) throughout the 10-day incubation period except STB1, STB117, STB209, STB274, STB319, STB328, STB330, and STB335. Soybean rhizobia with Type II colonies secreted alkali product(s) after 5-day incubation and secreted acidic product(s) after 10-day incubation except STB285, STB286, and STB295. All soybean rhizobia of Type III colony with the smallest colonies which were less than 0.01 mm in diameter (STB28, STB41, STB116, STB139, STB162, STB163, STB188, STB321, STB326, STB336, and STB340) except STB168 were found to secrete alkali product(s) throughout the 10-day incubation period. Type IV colonies (STB264 and STB302) were found to secrete alkali product(s) after 5-day incubation and secrete acidic product(s) after 10-day incubation.

Table 4.5 Colony morphology and BTB reactions of 121 strains of slow-growing soybean rhizobia isolated from soils from 16 subdistricts of Phitsanulok province. (+ = H^+ secretion; - = OH^- secretion).

Strain no.	Code	Subdistrict	Type of colony	Bromthymol blue reactions after incubation for	
				5 days	10 days
1	STB1	Ban Pa	I	-	+
2	STB3	Ban Pa	I	-	-
3	STB4	Ban Pa	II	-	+
4	STB6	Ban Pa	II	-	+
5	STB8	Ban Pa	I	-	-
6	STB14	Ban Pa	II	-	+

7	STB16	Ban Pa	II	-	+
8	STB20	Ban Pa	II	-	+
9	STB28	Hua Ro	III	-	-
10	STB38	Hua Ro	II	-	+
11	STB41	Hua Ro	III	-	-
12	STB46	Wat Phrik	II	-	+
13	STB54	Wat Phrik	II	-	+
14	STB63	Wat Phrik	I	-	-
15	STB67	Wang Ithok	II	-	+
16	STB71	Wang Ithok	II	-	+
17	STB89	Wang Ithok	II	-	+
18	STB100	Mathong	I	-	-
19	STB113	Sri Phirom	II	-	+
20	STB114	Sri Phirom	II	-	+
21	STB116	Sri Phirom	III	-	-
22	STB117	Sri Phirom	I	-	+
23	STB118	Sri Phirom	II	-	+
24	STB119	Sri Phirom	I	-	-
25	STB120	Sri Phirom	I	-	-
26	STB121	Sri Phirom	I	-	-
27	STB124	Sri Phirom	II	-	+
28	STB126	Sri Phirom	I	-	-
29	STB127	Sri Phirom	II	-	+
30	STB131	Sri Phirom	I	-	-
31	STB132	Sri Phirom	I	-	-
32	STB138	Tha Chang	II	-	+
33	STB139	Tha Chang	III	-	-
34	STB140	Tha Chang	II	-	+
35	STB142	Tha Chang	II	-	+
36	STB147	Tha Chang	I	-	-

37	STB150	Tha Chang	II	-	+
38	STB154	Ban Dong	I	-	-
39	STB162	Ban Dong	III	-	-
40	STB163	Ban Dong	III	-	-
41	STB168	Ban Dong	III	-	+
42	STB169	Ban Dong	I	-	-
43	STB171	Ban Dong	I	-	-
44	STB173	Ban Dong	I	-	-
45	STB178	Ban Dong	I	-	-
46	STB179	Ban Dong	I	-	-
47	STB180	Ban Dong	I	-	-
48	STB181	Ban Dong	I	-	-
49	STB183	Ban Dong	I	-	-
50	STB185	Ban Dong	I	-	-
51	STB186	Ban Phrao	I	-	-
52	STB188	Ban Phrao	III	-	-
53	STB189	Ban Phrao	I	-	-
54	STB201	Ban Phrao	I	-	-
55	STB202	Ban Phrao	I	-	-
56	STB203	Ban Phrao	I	-	-
57	STB206	Nakhon Chum	II	-	+
58	STB209	Nakhon Chum	I	-	+
59	STB213	Nakhon Chum	II	-	+
60	STB214	Nakhon Chum	II	-	+
61	STB220	Nakhon Chum	I	-	-
62	STB222	Nakhon Chum	II	-	+
63	STB224	Nakhon Chum	II	-	+
64	STB226	Nong Kathao	I	-	-
65	STB231	Nong Kathao	II	-	+
66	STB233	Nong Kathao	II	-	+

67	STB234	Nong Kathao	II	-	+
68	STB236	Nong Kathao	I	-	-
69	STB237	Nong Kathao	I	-	-
70	STB238	Nong Kathao	I	-	-
71	STB241	Nong Kathao	I	-	-
72	STB242	Nong Kathao	I	-	-
73	STB243	Nong Kathao	I	-	-
74	STB248	Nong Kathao	II	-	+
75	STB250	Nong Kathao	II	-	+
76	STB252	Chaiyanam	I	-	-
77	STB254	Chaiyanam	I	-	-
78	STB255	Chaiyanam	II	-	+
79	STB256	Chaiyanam	II	-	+
80	STB259	Chaiyanam	I	-	-
81	STB259	Chaiyanam	I	-	-
82	STB264	Kaeng Sopha	IV	-	+
83	STB266	Kaeng Sopha	II	-	+
84	STB268	Kaeng Sopha	I	-	-
85	STB270	Kaeng Sopha	I	-	-
86	STB271	Kang Sopha	I	-	-
87	STB273	Kang Sopha	II	-	+
88	STB274	Kang Sopha	I	-	+
89	STB275	Kang Sopha	II	-	+
90	STB276	Kang Sopha	I	-	-
91	STB281	Mae Raka	I	-	-
92	STB282	Mae Raka	I	-	-
93	STB284	Mae Raka	II	-	+
94	STB285	Mae Raka	II	-	-
95	STB286	Mae Raka	II	-	-
96	STB288	Mae Raka	II	-	+

97	STB292	Mae Raka	I	-	-
98	STB294	Mae Raka	I	-	-
99	STB295	Nong Phra	II	-	-
100	STB296	Nong Phra	II	-	+
101	STB298	Nong Phra	I	-	-
102	STB299	Nong Phra	II	-	+
103	STB300	Nong Phra	II	-	+
104	STB302	Nong Phra	IV	-	+
105	STB310	Tha Muen Ram	II	-	+
106	STB318	Tha Muen Ram	II	-	+
107	STB319	Ta Muen Ram	I	-	+
108	STB320	Tha Muen Ram	I	-	-
109	STB321	Tha Muen Ram	III	-	-
110	STB323	Ta Muen Ram	II	-	+
111	STB325	Chaiyanam	I	-	-
112	STB326	Chaiyanam	III	-	-
113	STB327	Mae Raka	I	-	-
114	STB328	Mae Raka	I	-	+
115	STB329	Mae Raka	I	-	-
116	STB330	Nong Phra	I	-	+
117	STB332	Nong Phra	I	-	-
118	STB333	Nong Phra	I	-	-
119	STB335	Nong Phra	I	-	+
120	STB336	Tha Muen Ram	III	-	-
121	STB340	Tha Muen Ram	III	-	-

The observed ability of *B. elkanii* strain NA7, *B. japonicum* strain S76, *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 to secrete either acidic or alkali product(s) in response to surrounding pHs were shown in Tables 4.6- 4.13.

Table 4.6 Responses of *B. elkanii* strain NA7 and *B. japonicum* strain S76 grown in yeast extract mannitol broth (YMB) with and without 30 mM NEDA buffer at the initial pH of 4.0, 30^0C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM NEDA			pH in YMB without 30mM NEDA		
		1	2	average	1	2	average
B.elkanii NA7	1	3.94	3.94	3.94	5.57	5.52	5.55
	2	3.98	3.98	3.98	5.74	5.72	5.73
	3	3.98	3.98	3.98	5.32	5.34	5.33
	4	3.98	3.98	3.98	4.50	4.63	4.57
	5	3.99	4.00	4.00	4.31	4.27	4.29
B. japonicum	1	3.94	3.94	3.94	5.55	5.61	5.58
S76	2	3.99	3.98	3.99	5.91	5.90	5.91
	3	3.98	3.98	3.98	5.82	5.83	5.83
	4	3.98	3.98	3.98	5.60	5.95	5.78
	5	3.99	3.99	3.99	5.52	4.70	5.11

The results as shown in Table 4.6 indicated that in the presence of 30 mM NEDA buffer, pH of YMB medium was maintained close to the initial pH of 4.0. However, when there was no buffer in the medium, cells of *B. elkanii* strain NA7 and *B. japonicum* strain S76 were found to secrete alkali product(s) to increase pHs of the medium to a less acidic range.

Table 4.7 Responses of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 grown in yeast extract mannitol broth (YMB) with and without 30 mM NEDA buffer at the initial pH of 4.0, 30^0C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM NEDA			pH in YMB without 30mM NEDA		
		1	2	average	1	2	average
B. liaoningense	1	3.98	3.98	3.98	4.80	5.40	5.10
SK3	2	3.98	3.99	3.99	5.39	5.19	5.29
	3	4.00	4.00	4.00	5.43	5.52	5.48
	4	4.00	3.99	4.00	5.67	5.49	5.58
	5	3.99	3.99	3.99	4.62	5.55	5.09
B. yuanmingense	1	3.98	3.98	3.98	4.54	4.61	4.58
STB264	2	3.98	3.98	3.98	4.47	4.60	4.54
	3	3.99	3.99	3.99	5.36	5.06	5.21
	4	3.99	3.99	3.99	5.66	4.61	5.14
	5	3.99	3.99	3.99	5.40	5.55	5.48

The results as shown in Table 4.7 indicated that in the presence of 30 mM NEDA buffer, pH of YMB medium was maintained close to the initial pH of 4.0. However, when there was no buffer in the medium, cells of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 were found to secrete alkali product(s) to increase pHs of the medium to a less acidic range.

Table 4.8 Responses of *B. elkanii* strain NA7 and *B. japonicum* strain S76 grown in yeast extract mannitol broth (YMB) with and without 30 mM NEDA buffer at the initial pH of 5.0, 30^0C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM NEDA			pH in YMB without 30mM NEDA		
		1	2	average	1	2	average
B.elkanii NA7	1	4.96	4.96	4.96	6.29	6.30	6.30
	2	4.99	4.99	4.99	6.32	6.31	6.32
	3	5.00	4.99	5.00	6.11	6.12	6.12
	4	5.01	5.02	5.02	5.82	5.75	5.79
	5	5.02	5.03	5.03	5.52	5.50	5.51
B. japonicum	1	4.97	4.98	4.98	6.25	6.25	6.25
S76	2	5.00	4.99	5.00	6.40	6.38	6.39
	3	5.00	5.00	5.00	6.34	6.35	6.35
	4	5.01	5.02	5.02	6.37	6.28	6.33
	5	5.01	5.02	5.02	6.16	6.20	6.18

The results as shown in Table 4.8 indicated that in the presence of 30 mM NEDA buffer, pH of YMB medium was maintained close to the initial pH of 5.0. However, when there was no buffer in the medium, cells of *B. elkanii* strain NA7 and *B. japonicum* strain S76 were found to secrete alkali product(s) to increase pHs of the medium to a less acidic range.

Table 4.9 Responses of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 grown in yeast extract mannitol broth (YMB) with and without 30 mM NEDA buffer at the initial pH of 5.0, 30^0C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM NEDA			pH in YMB without 30mM NEDA		
		1	2	average	1	2	average
B. liaoningense	1	5.03	5.03	5.03	6.37	6.43	6.40
SK3	2	5.04	5.03	5.04	6.33	6.26	6.30
	3	5.03	5.04	5.04	6.21	6.21	6.21
	4	5.03	5.02	5.03	6.27	6.04	6.16
	5	5.02	5.03	5.03	5.98	5.97	5.98
B. yuanmingense	1	5.01	5.02	5.02	6.27	6.25	6.26
STB264	2	5.03	5.02	5.03	6.37	6.35	6.36
	3	5.03	5.03	5.03	6.27	6.21	6.24
	4	5.03	5.03	5.03	6.20	6.12	6.16
	5	5.02	5.02	5.02	6.25	6.13	6.19

The results as shown in Table 4.9 indicated that in the presence of 30 mM NEDA buffer, pH of YMB medium was maintained close to the initial pH of 5.0. However, when there was no buffer in the medium, cells of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 were found to secrete alkali product(s) to increase pHs of the medium to a less acidic range.

Table 4.10 Responses of *B. elkanii* strain NA7 and *B. japonicum* strain S76 grown in yeast extract mannitol broth (YMB) with and without 30 mM MES buffer at the initial pH of 6.0, 30^0C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM MES			pH in YMB without 30mM MES		
		1	2	average	1	2	average
B.elkanii NA7	1	6.03	6.03	6.03	6.51	6.46	6.49
	2	6.07	6.07	6.07	6.62	6.61	6.62
	3	6.06	6.05	6.06	6.49	6.53	6.51
	4	6.05	6.05	6.05	6.29	6.47	6.38
	5	6.05	6.04	6.05	6.26	6.29	6.28
B. japonicum	1	6.04	6.04	6.04	6.47	6.47	6.47
S76	2	6.06	6.06	6.06	6.60	6.55	6.58
	3	6.06	6.06	6.06	6.59	6.57	6.58
	4	6.06	6.06	6.06	6.43	6.56	6.50
	5	6.05	6.06	6.06	6.54	6.52	6.53

The results as shown in Table 4.10 indicated that in the presence of 30 mM MES buffer, pH of YMB medium was maintained close to the initial pH of 6.0. However, when there was no buffer in the medium, cells of *B. elkanii* strain NA7 and *B. japonicum* strain S76 were found to secrete alkali product(s) to increase pHs of the medium to a less acidic range.

Table 4.11 Responses of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 grown in yeast extract mannitol broth (YMB) with and without 30 mM MES buffer at the initial pH of 6.0, 30^{0}C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM MES			pH in YMB without 30mM MES		
		1	2	average	1	2	average
B. liaoningense	1	6.08	6.08	6.08	6.70	6.68	6.69
SK3	2	6.09	6.09	6.09	6.61	6.61	6.61
	3	6.08	6.08	6.08	6.57	6.54	6.56
	4	6.09	6.08	6.09	6.62	6.66	6.64
	5	6.06	6.07	6.07	6.65	6.65	6.65
B. yuanmingense	1	6.08	6.08	6.08	6.69	6.67	6.68
STB264	2	6.09	6.09	6.09	6.64	6.61	6.63
	3	6.08	6.09	6.09	6.62	6.55	6.59
	4	6.07	6.08	6.08	6.52	6.52	6.52
	5	6.05	6.07	6.06	6.60	6.60	6.60

The results as shown in Table 4.11 indicated that in the presence of 30 mM MES buffer, pH of YMB medium was maintained close to the initial pH of 6.0. However, when there was no buffer in the medium, cells of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 were found to secrete alkali product(s) to increase pHs of the medium to a less acidic range.

Table 4.12 Responses of *B. elkanii* strain NA7 and *B. japonicum* strain S76 grown in yeast extract mannitol broth (YMB) with and without 30 mM HEPES buffer at the initial pH of 7.0, 30^0C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM HEPES			pH in YMB without 30mM HEPES		
		1	2	average	1	2	average
B.elkanii NA7	1	7.08	7.08	7.08^{abcde}	7.12	7.13	7.13^{ab}
	2	7.04	7.05	7.05^{cdefg}	7.14	7.14	7.14^{a}
	3	7.01	7.03	7.02^{efg}	7.11	7.10	7.11^{abc}
	4	7.01	7.01	7.01^{fg}	7.13	7.00	7.07^{bcdef}
	5	7.01	7.01	7.01^{fg}	7.12	7.11	7.12^{ab}
B. japonicum	1	7.09	7.09	7.09^{abcd}	7.12	7.12	7.12^{ab}
S76	2	7.08	7.09	7.09^{abcde}	7.11	7.13	7.12^{ab}
	3	7.01	7.03	7.02^{fef}	7.11	7.01	7.06^{bcdef}
	4	7.02	7.02	7.02^{efg}	7.00	7.05	7.03^{defg}
	5	7.02	7.04	7.03^{defg}	6.98	7.00	6.99^{g}

The results as shown in Table 4.12 indicated that in the presence of 30 mM HEPES buffer, pH of YMB medium was not maintained close to the initial pH of 7.0. However, when there was no buffer in the medium, cells of *B. elkanii* strain NA7 and *B. japonicum* strain S76 were found to secrete alkali product(s) to increase pHs of the medium to a slightly alkali to neutral range. The same letter superscripts indicated there were no significant differences at $p < 0.05$ as determined by the Duncan's Multiple Range Test.

Table 4.13 Responses of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 grown in yeast extract mannitol broth (YMB) with and without 30 mM HEPES buffer at the initial pH of 7.0, 30^0C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM HEPES			pH in YMB without 30mM HEPES		
		1	2	average	1	2	average
B. liaoningense	1	7.11	7.12	7.12	7.60	7.59	7.60
SK3	2	7.17	7.16	7.17	7.52	7.47	7.50
	3	7.12	7.13	7.13	7.63	7.58	7.61
	4	7.12	7.10	7.11	7.64	7.63	7.64
	5	7.11	7.13	7.12	7.67	7.70	7.69
B. yuanmingense	1	7.11	7.12	7.12	7.60	7.56	7.58
STB264	2	7.14	7.15	7.15	7.62	7.63	7.63
	3	7.10	7.12	7.11	7.59	7.51	7.55
	4	7.12	7.11	7.12	7.58	7.64	7.61
	5	7.12	7.14	7.13	7.69	7.68	7.69

The results as shown in Table 4.13 indicated that in the presence of 30 mM HEPES buffer, pH of YMB medium was maintained close to the initial pH of 7.0. However, when there was no buffer in the medium, cells of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 were found to secrete alkali product(s) to increase pHs of the medium to a more alkali range.

Table 4.14 Responses of *B. elkanii* strain NA7 and *B. japonicum* strain S76 grown in yeast extract mannitol broth (YMB) with and without 30 mM HEPES buffer at the initial pH of 8.0, 30^0C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM HEPES			pH in YMB without 30mM HEPES		
		1	2	average	1	2	average
B.elkanii NA7	1	8.03	8.04	8.04^{def}	8.05	8.07	8.06^{bcde}
	2	8.09	8.07	8.08^{abc}	7.99	8.00	8.00^{g}
	3	8.10	8.10	8.10^{a}	8.01	8.01	8.01^{fg}
	4	8.10	8.10	8.10^{a}	8.03	8.02	8.03^{efg}
	5	8.09	8.09	8.09^{ab}	8.09	8.11	8.10^{a}
B. japonicum	1	8.03	8.04	8.04^{def}	8.01	8.03	8.02^{fg}
S76	2	8.06	8.06	8.06^{bcde}	7.80	7.87	7.84^{j}
	3	8.03	8.06	8.05^{cdef}	7.90	7.92	7.91^{i}
	4	8.06	8.06	8.06^{bcde}	7.90	7.91	7.91^{i}
	5	8.06	8.07	8.07^{abcd}	7.93	7.97	7.95^{h}

The results as shown in Table 4.14 indicated that in the presence of 30 mM HEPES buffer, pH of YMB medium was not maintained close to the initial pH of 8.0. However, the results showed that when there was no buffer in the medium, cells of *B. elkanii* strain NA7 and *B. japonicum* strain S76 were found to secrete acidic product(s) to decrease pHs of YMB medium to a lesser alkali range. The same letter superscripts indicated there were no significant differences at $p < 0.05$ as determined by the Duncan's Multiple Range Test.

Table 4.15 Responses of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 grown in yeast extract mannitol broth (YMB) with and without 30 mM HEPES buffer at the initial pH of 8.0, 30^0C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM HEPES			pH in YMB without 30mM HEPES		
		1	2	average	1	2	average
B. liaoningense	1	8.01	7.99	8.00[a]	7.74	7.74	7.74[e]
SK3	2	8.02	8.00	8.01[a]	7.83	7.76	7.80[cd]
	3	8.01	7.99	8.00[a]	7.87	7.83	7.85[bcd]
	4	7.99	7.99	7.99[a]	7.83	7.88	7.86[bc]
	5	8.02	8.02	8.02[a]	7.77	7.92	7.85[bcd]
B. yuanmingense	1	8.01	8.01	8.01[a]	7.73	7.75	7.74[e]
STB264	2	8.01	8.01	8.01[a]	7.87	7.87	7.87[b]
	3	8.00	7.98	7.99[a]	7.79	7.77	7.78[cd]
	4	7.98	7.98	7.98[a]	7.87	7.86	7.87[bc]
	5	8.01	8.00	8.01[a]	7.86	7.94	7.90[b]

The results as shown in Table 4.15 indicated that in the presence of 30 mM HEPES buffer, pH of YMB medium was maintained close to the initial pH of 8.0. However, when there was no buffer in the medium, cells of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 were found to secrete acidic product(s) to lower pHs of the medium to a lesser alkali range. The same letter superscripts indicated there were no significant differences at $p < 0.05$ as determined by the Duncan's Multiple Range Test.

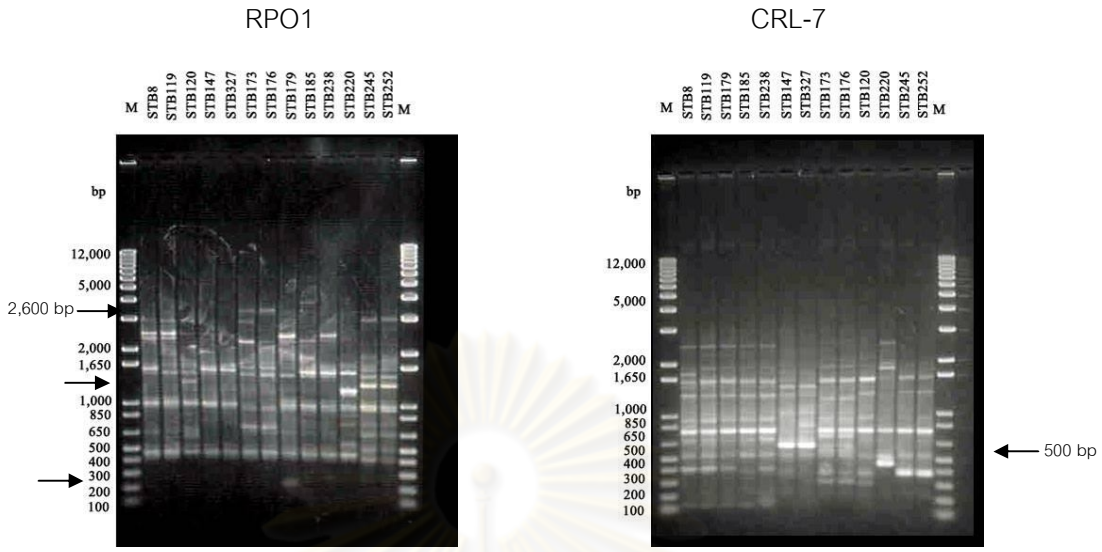
The results on the maintenance of pHs when each of the three buffers was used were satisfactory in all experiments except when 30mM HEPES was used to maintain the medium pH for *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 at pH7.0 where the pHs were slightly above 7.0 as shown in Table 4.13 and when the buffer was used to maintain the medium pH for *B. elkanii* strain NA7 and *B. japonicum* strain S76 at

the initial pH of 8.0 as shown in Table 4.14 where the buffered medium was slightly above pH 8.0.

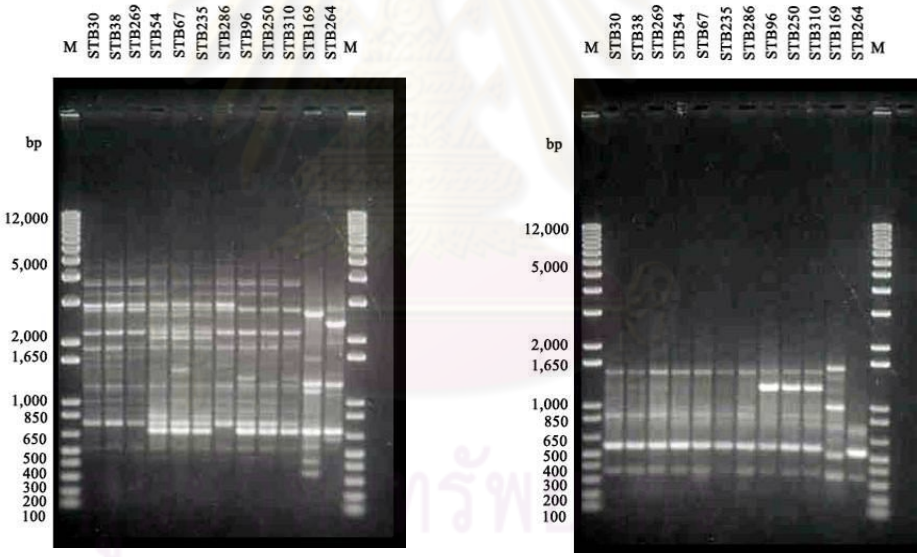
The three buffers were chosen for their effective buffering capacity as reported by Good et al. (1966). The responses of *B. elkanii* strain NA7, *B. japonicum* strain S76, *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 to changes in pHs of YMB medium when no buffer was used as shown in Tables 4.6 – 4.15 showed clearly that when pHs of the medium were in the acidic range, the slow-growing soybean rhizobium cells secreted alkali product(s) to increase the pH values to a less acidic range. On the other hand, when the medium pHs were in the neutral range, the cells were found to secrete alkali product(s) to increase the medium pHs to a slightly alkali range. However, when the medium pHs were 8.0, the cells were found to secrete acidic products to lower pHs of the medium to a lesser alkali range. Thus, the results obtained showed an ability of the slow-growing soybean rhizobia to secrete either acidic or alkali products to change pHs of the surrounding medium.

4.6.3 RAPD-PCR fingerprints of 25 randomly-selected STB slow-growing soybean rhizobium isolates

Since there was a high cost involved in the sequencing service (5,400 baht per one sequence of 16S rDNA and 1,200 baht per one sequence of *nodY*), in the use of the Transmission Electron Microscope to determine the number and type of flagella by negative staining (approximately 5,000 baht until a reportable picture of a strain was obtained), and a high cost of the Biolog™ test kit (1,200 baht per one GN2 MicroPlate and inoculation fluid, and approximately 3 plates were used to obtain 3 replicates of results per strain), it was not possible to carry out polyphasic taxonomy for all the of 121 slow-growing soybean rhizobium strains. Therefore, 25 slow-growing STB isolates were randomly selected for further use in polyphasic taxonomy. In order to find out if the randomly-selected 25 STB strains were different strains, RAPD-PCR fingerprints of the 25 randomly-selected isolates were obtained as shown in Figures 4.19 (a,b).



(a)



(b)

Figure 4.19 PCR-DNA fingerprints using either RPO1 or CRL-7 as the primer of (a) 13 randomly-selected isolates and (b) 12 randomly-selected isolates. DNA fingerprints indicated the following isolates were the same strains : STB245 = STB 252 (results obtained from Figure 4.19a); STB30 = STB38 = STB269 = STB286; and STB54 = STB235 (results obtained from Figure 4.19b).

DNA fingerprints as shown in Figure 4.19 (a,b) indicated that, in fact, the 25 randomly-selected isolates consisted of 20 strains because some isolates were found to have identical DNA fingerprints so they were the same strains . Thus, the 20 strains chosen for further polyphasic taxonomy tests including 16S rDNA and *nodY* isolation and sequencing were: STB8, STB119, STB120, STB147, STB173, STB176, STB179, STB185, STB220, STB238, STB245, and STB327 (results obtained from Figure 4.19a); and STB30, STB54, STB67, STB96, STB250, STB310, STB169, STB264, (results obtained from Figure 4.19b). The observed RAPD-PCR DNA fingerprints with CRL-7 as the primer as shown in Figure 4.19 indicated less variations in the DNA fingerprint banding patterns. Further analysis of the DNA banding patterns in Figure 4.19a revealed the presence of natural variants which had near identical fingerprints except for differences either in the presence or the absence of some DNA bands. For example, RPO1 fingerprints in Figure 4.19a showed that only strains STB8, STB119, STB173 and STB185 had the 2,400 bp bands while only STB8, STB119, STB179, and STB238 had the 2,600 bp DNA bands ; only the STB strains 120 and 245 had the 1,600 bands; only STB173, STB176 and STB245 had the 700 bp DNA bands while only STB179 had the 250 bp band. Similarly, the CRL-7 fingerprints as shown in Figure 4.19a showed only the STB147 and STB327 strains had the 500 bp bands but did not have the 700 bp bands. Figure 4.19b showed that all the 6 STB strains 30, 54, 67, 96, 250, and 310 were natural variants . RPO1 fingerprints showed only STB96 and STB310 had 1,450 bp bands. In addition, only strains STB 54, 67, 96, 250, and 310 had the 700 bp bands. The CRL-7 fingerprints as shown in Figure 4.19b showed STB96, 250, and 310 had an extra 1,260 bp band.

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

4.6.4.1 Identification by 16S rDNA sequences

Table 4.16 showed identification of the selected 20 strains of slow-growing soybean rhizobia by using the Blast program to compare the obtained 16S rDNA sequences with those sequences deposited in the GenBank database. The Blast program indicated the following identification of the 20 STB strains:

STB8 (determined length 1452 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium elkanii* strain S127 . All the compared sequences had the following homology: Identities = 1451/1454 (99%), gaps = 3/1454.

STB30 (determined length 1453 bp) could be related to *Bradyrhizobium japonicum* strain HMS-02 with identities = 1451/1455 (99%), gaps = 4/1455.

STB54 (determined length 1451 bp) could be related to *Bradyrhizobium japonicum* strain HMS-02 with identities = 1446/1454 (99%), gaps = 4/1454.

STB67(determined length 1455 bp) could be related to *Bradyrhizobium japonicum* strain HMS-02 with identities = 1446/1454 (99%), gaps = 4/1454.

STB96 (determined length 1451 bp) could be related to *Bradyrhizobium japonicum* strain HMS-02 with identities = 1451/1453 (99%), gaps = 2/1415.

STB119 (determined length 1451 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium elkanii* strain S127 or *Bradyrhizobium elkanii* strain GZ1. All the compared sequences had the following homology: Identities = 1451/1452 (99%), gaps = 0/1452.

STB120 (determined length 1451 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 with identities = 1451/1451 (100%), Gaps = 0/1451 or *Bradyrhizobium elkanii* strain SEMIA 5002 with identities = 1450/1452 (99%), gaps = 2/1452.

STB147 (determined length 1451 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium elkanii* strain S127or *Bradyrhizobium elkanii* strain GZ1. All the compared sequences had the following homology: Identities = 1448/1454 (99%), gaps = 5/1454.

STB169 (determined length 1449 bp) could be related to *Bradyrhizobium* sp. GX5 or *Bradyrhizobium liaoningense* strain LYG2 with identities = 1449/1454 (99%), Gaps =

4/1454 or *Bradyrhizobium yuanmingense* strain TTC4 with identities = 1447/1452 (99%), gaps = 4/1452.

STB173 (determined length 1452 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium elkanii* strain S 127 or *Bradyrhizobium elkanii* strain GZ1. All the compared sequences had the following homology: Identities = 1450/1454 (99%), gaps = 3/1454.

STB176 (determined length 1452 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 with identities = 1449/1455 (99%), gaps = 5/1455 or *Bradyrhizobium elkanii* strain SEMIA 5002 with identities = 1449/1455 (99%), gaps = 5/1455.

STB179 (determined length 1451 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium elkanii* strain S127 or *Bradyrhizobium elkanii* strain GZ1. All the compared sequences had the following homology: Identities = 1451/1451 (100%).

STB185 (determined length 1452 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 with identities = 1450/1455 (99%), gaps = 3/1455.

STB220 (determined length 1451 bp) could be related *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium elkanii* strain S 127 or *Bradyrhizobium elkanii* strain GZ1 with identities = 1448/1451 (99%) and there was no gap.

STB238 (determined length 1451 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium*

elkanii strain S127 or *Bradyrhizobium elkanii* strain GZ1 with identities = 1450/1454 (99%), gaps = 4/1454.

STB245 (determined length 1450 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium elkanii* strain S 127 or *Bradyrhizobium elkanii* strain GZ1 with identities = 1449/1454 (99%), gaps = 5/1454.

STB250 (determined length 1451 bp) could be related to *Bradyrhizobium japonicum* strain HMS-02 with identities = 1449/1453 (99%), gaps = 2/1453.

STB264 (determined length 1450 bp) could be related to *Bradyrhizobium* sp. GX5 or *Bradyrhizobium yuanmingense* strain TTC4 with identities = 1444/1453 (99%), gaps = 5/1453.

STB310 (determined length 1449 bp) could be related to *Bradyrhizobium japonicum* strain HMS-02 with identities = 1449/1452 (99%), gaps = 2/1452.

STB327(determined length 1454 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 with identities = 1451/1455 (99%), gaps = 3/1455 or *Bradyrhizobium elkanii* strain SEMIA 5002 with identities = 1450/1455 (99%), gaps = 5/1455.

Table 4.16 showed identification of the 20 slow-growing soybean rhizobium STB strains based on homology of 16S rDNA sequences as obtained by the Blast program of the National Center for Biotechnology Information (NCBI).

Table 4.16 Summary of identification of 20 slow-growing soybean rhizobium STB strains based on 16S rDNA sequences.

Strain	Size of 16S rDNA (bp)	Percent homology with sequences in GenBank	Identification
STB8	1452	1451/1454 (99%) with 3 gaps	*Bradyrhizobium elkanii*
STB30	1453	1451/1455 (99%) with 4 gaps	*B. japonicum*
STB54	1451	1446/1454 (99%) with 4 gaps	*B. japonicum*
STB67	1455	1449/1451 (99%) with no gap	*B. japonicum*
STB96	1451	1451/1453 (99%) with 2 gaps	*B. japonicum*

STB119	1451	1451/1452 (99%) with no gap	*B. elkanii*
STB120	1451	1450/1452 (99%) with 2 gaps	*B. elkanii*
STB147	1451	1448/1454 (99%) with 5 gaps	*B. elkanii*
STB169	1449	1449/1454 (99%) with 4 gaps 1447/1452 (99%) with 4 gaps	*B. liaoningense* *B. yuanmingense*
STB173	1452	1450/1454 (99%) with 3 gaps	*B. elkanii*
STB176	1452	1449/1455 with 5 gaps	*B. elkanii*
STB179	1451	1451/1451(100%) with no gap	*B. elkanii*
STB185	1452	1450/1455 (99%) with 3 gaps	*B. elkanii*
STB220	1451	1448/1451 (99%) with no gap	*B. elkanii*
STB238	1451	1450/1454 (99%) with 4 gaps	*B. elkanii*
STB245	1450	1449/1454 (99%) with 5 gaps	*B. elkanii*
STB250	1451	1449/1453 (99%) with gaps	*B. japonicum*
STB264	1450	1444/1453 (99%) with 5 gaps	*B. yuanmingense*
STB310	1449	1449/1452 (99%) with 2 gaps	*B. japonicum*
STB327	1454	1450/1455 (99%) with 5 gaps	*B. elkanii*

Table 4.16 indicated that the 20 STB strains consisted of 12 *B. elkanii* strains (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).

Figures 4.20 (a-c) showed dendrograms constructed with sequences of 16S rDNA of the slow-growing soybean rhizobium STB strains.

(a)

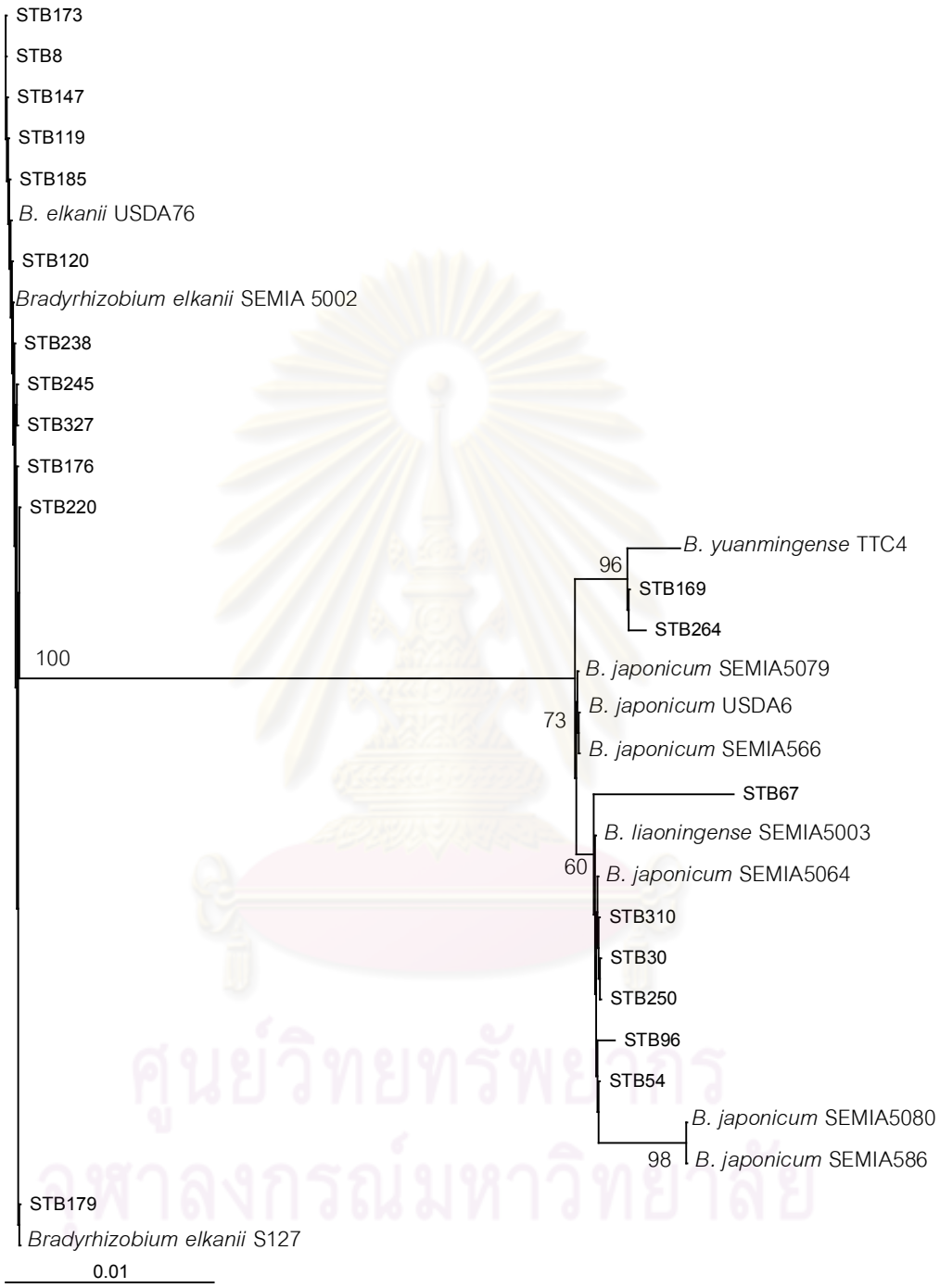


Figure 4.20a Dendrogram obtained from 16S rDNA sequences of 20 slow-growing soybean rhizobium STB strains as well as some reference strains. The Maximum Likelihood Method was used in the dendrogram construction.

(b)

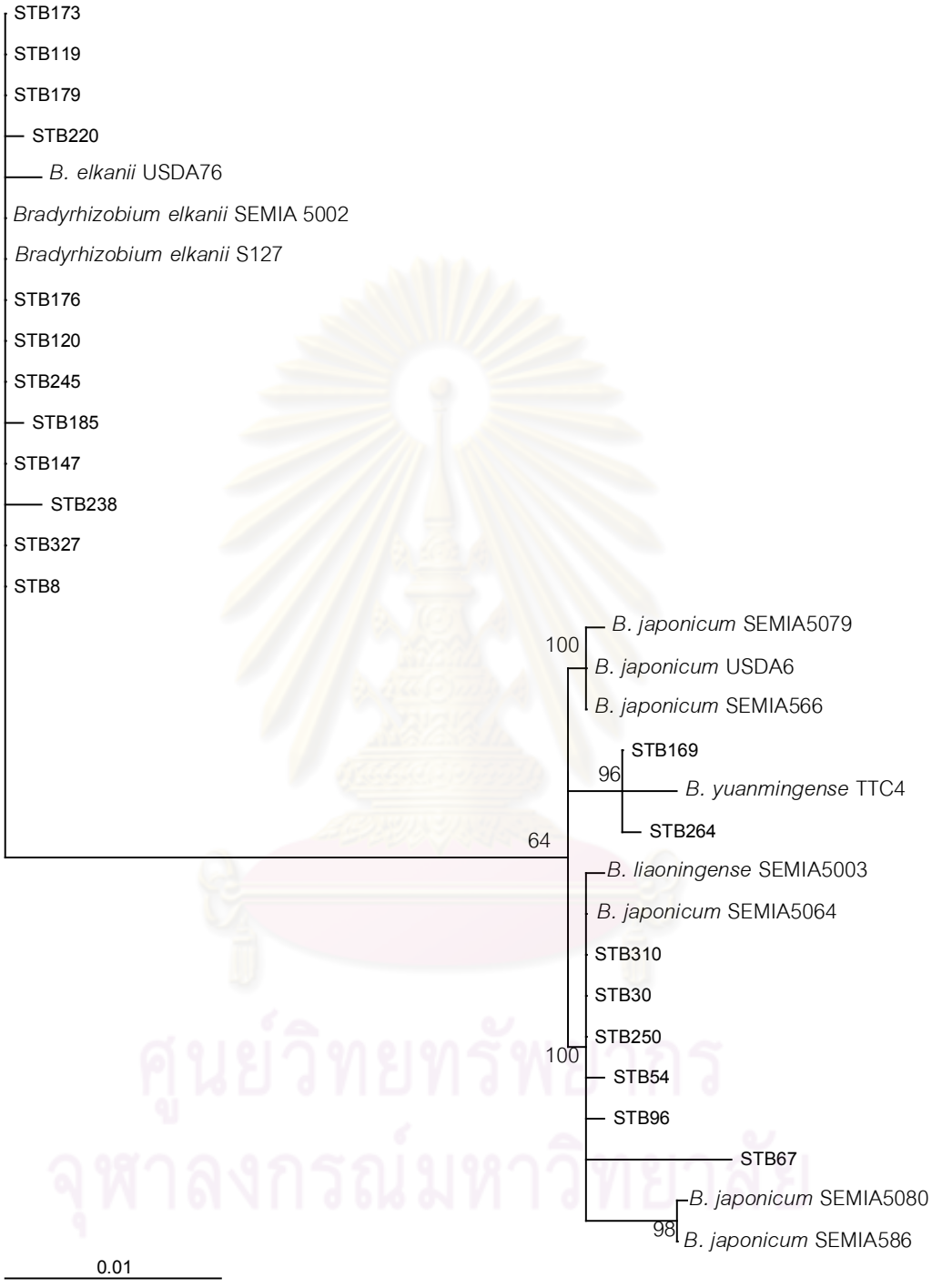


Figure 4.20b Dendrogram obtained from 16S rDNA sequences of 20 slow-growing soybean rhizobium STB strains as well as some reference strains. The Maximum Parsimony Method was used in the dendrogram construction.

(c)

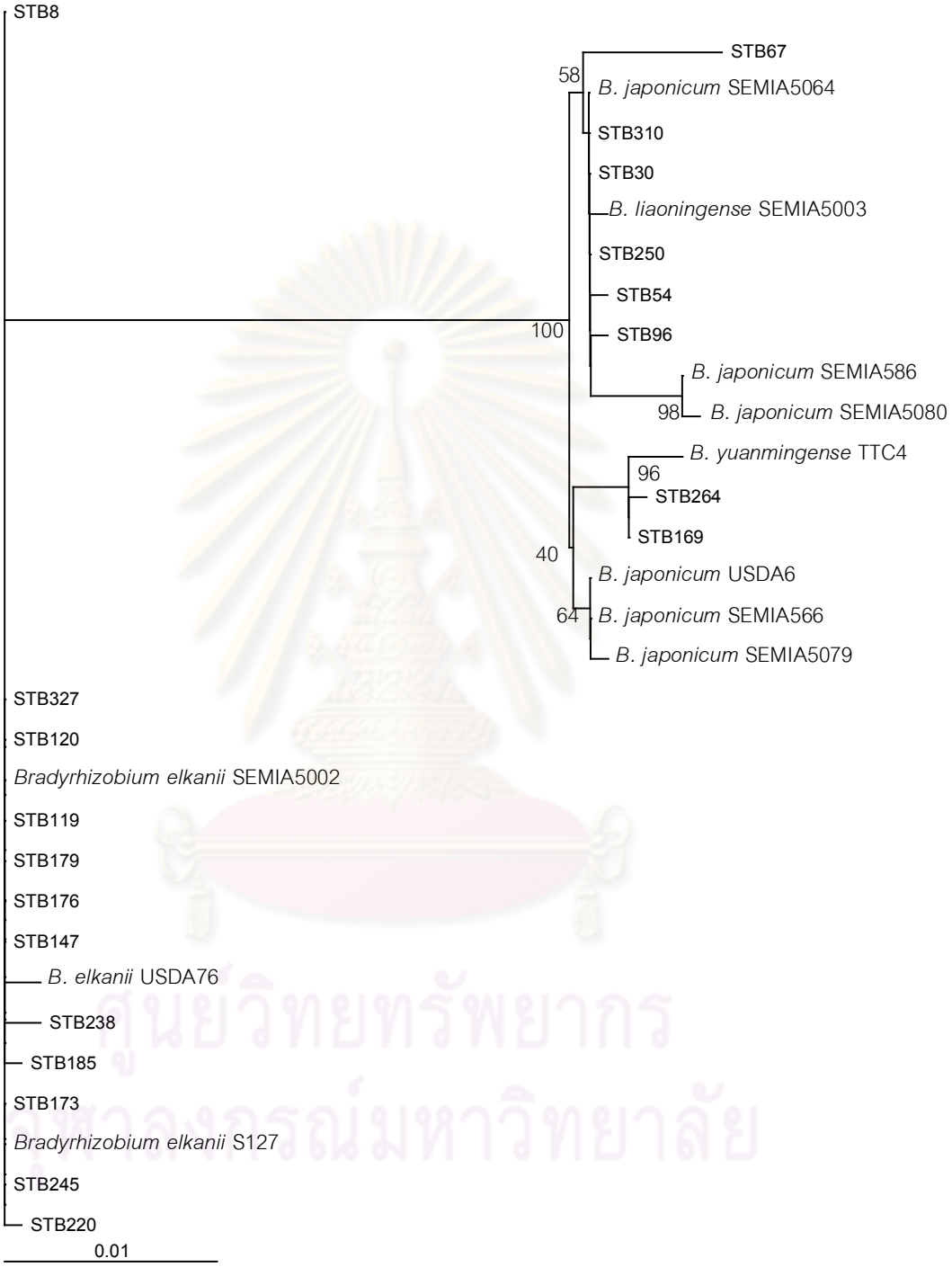


Figure 4.20c Dendrogram obtained from 16S rDNA sequences of 20 slow-growing soybean STB strains as well as some reference strains. The Neighbor-Joining Method was used in the dendrogram construction.

Figures 4.20 (a-c) showed dendrograms obtained from 16S rDNA sequences of 20 slow-growing STB strains as well as some reference strains when the Maximum Likelihood method, the Maximum Parsimony method, and the Neighbor-Joining method were used in the dendrogram construction, respectively. The results showed minor discrepancies when the three methods were used in the dendrogram construction. All the three dendrograms showed the 20 STB strains and the reference strains were grouped into 5 clusters with a minor discrepancy in Cluster I where the Maximum Likelihood dendrogram grouped the following 11 STB strains: STB173, STB8, STB147, STB119, STB185, STB120, STB238, STB245, STB327, STB176, and STB220 in the same cluster as the reference strains *B. elkanii* SEMIA 5002 and USDA 76 with STB179 grouped with *B. elkanii* S127 which had been reported as a natural variant of *B. elkanii* SEMIA 566 (Barcellos et al., 2007). However, the Maximum Parsimony method grouped STB179 into the same cluster as the 11 STB strains to yield a total of 12 STB strains of *B. elkanii* (Figure 4.20b) and the Neighbor-Joining method separated STB8 from the other STB *B. elkanii* strains (Figure 4.20c). The discrepancies observed in the three 16S rDNA dendrograms probably reflected the presence of natural variants in the *B. elkanii* STB strains.

All the three methods used in the dendrogram construction from 16S rDNA sequences revealed Cluster II consisted of the following 6 STB strains: STB310, STB30, STB250, STB96, and STB54 which were grouped with *B. japonicum* SEMIA5064/*B. liaoningense* SEMIA5003 with STB67 occupying a separate branch. The finding that *B. japonicum* SEMIA5064 and *B. liaoningense* SEMIA5003 were grouped in the same cluster probably reflected a close phylogenetic relationship between the two slow-growing soybean rhizobia species. Cluster III revealed STB169 and STB264 were closely related to *B. yuanmingense* TTC4. In addition, all the three dendrograms showed Cluster IV consisted of the reference strains *B. japonicum* SEMIA5079, USDA6, and SEMIA566. Cluster V consisted of the reference strains *B. japonicum* SEMIA5080 and SEMIA586.

4.6.4.2 Identification of slow-growing soybean rhizobia by using *nodY* sequences

Table 4.17 showed identification of the 20 slow-growing soybean rhizobium STB strains by using the Blast program to compare the obtained *nodY* sequences with those

sequences in the GenBank database. The Blast program indicated the following results for *nodY* of STB strains:

STB8 (determined length 363 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 359/361 (99%), gap = 1/361 or *Bradyrhizobium elkanii* USDA94 *nodK* with Identities = 359/361 (99%), gap = 1/361.

STB30 (determined length 363 bp) could be *Bradyrhizobium* sp. TARC112 *nodY* with identities = 341/342 (99%) with 1 gap.

STB54 (determined length 364 bp) could be *Bradyrhizobium* sp. TARC 112 *nodY* with identities = 362/364 (99%), gaps = 1/364.

STB67(determined length 360 bp) could be *Bradyrhizobium* sp. TARC 112 *nodY* with identities = 350/353 (99%), gaps = 3/353.

STB96 (determined length 383 bp) could be *Bradyrhizobium* sp. TARC 112 *nodY* with identities = 353/357 (98%), gaps = 2/357.

STB119 (determined length 354 bp) could be *Bradyrhizobium elkanii* strain USDA 76 *nodY* or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 325/329 with no gap.

STB120 (determined length 360 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 347/352 (98%), gaps = 2/352 or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 347/352 (98%), gaps = 2.

STB147 (determined length 351 bp) could be *Bradyrhizobium elkanii* strain USDA 76 *nodY* with identities = 342/346 (98%), gaps = 4.

STB169 (determined length 362 bp) could be *Bradyrhizobium elkanii* USDA 31 *nodY* with identities = 329/367 (89%), gaps = 9/367.

STB173 (determined length 361 bp) could be *Bradyrhizobium elkanii* strain USDA 76 *nodY* or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 349/352, gaps = 3.

STB176 (determined length 357 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 354/361 (98%), gaps = 3/361 or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 354/361 (98%), gaps = 3/361.

STB179 (determined length 359 bp) could be *Bradyrhizobium elkanii* strain USDA 76 *nodY* or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 341/345 (98%), gap = 1.

STB185 (determined length 360 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 358/361 (99%), gaps = 2/361 or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 358/361 (99%), gaps = 2/361.

STB220 (determined length 359 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 329/345 (95%), gaps = 3/345 or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 334/352 (94%), gaps = 3/352.

STB238 (determined length 359 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 341/353 (96%), gaps = 3/353 or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 341/353 (96%), gaps = 3/353.

STB245 (determined length 358 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 328/330 (99%), gaps = 1/330 (0%) or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 326/327 (99%), gaps = 1/327.

STB250 (determined length 363 bp) could be *Bradyrhizobium* sp. TARC 112 *nodY* with identities = 363/363 (100%),with no gap.

STB264 (determined length 363 bp) could be *Bradyrhizobium elkanii* USDA 31 *nodY* with identities = 363/364 (99%), gap = 1/364.

STB310 (determined length 360 bp) could be *Bradyrhizobium* sp. TARC 112 *nodY* with identities = 357/365 (97%), gaps = 6/365.

STB327(determined length 347 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 344/347 (99%), gaps = 1/347 or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 344/347 (99%), gaps = 1/347.

Table 4.17 showed identification of the 20 slow-growing soybean rhizobium STB strains based on homology of *nodY* sequences as obtained by the Blast program of the National Center for Biotechnology Information (NCBI).

Table 4.17 Identification of 20 slow-growing soybean rhizobium STB strains based on sequences of *nodY.*

Strains	Size of *nodY* (bp)	Percent homology with sequences in GenBank	Identification
STB8	363	359/361 (99%) with 1gap	*B.elkanii* strains USDA76, USDA94
STB30	363	341/342 (99%) with 1 gap	*Bradyrhizobium* sp. TARC112
STB54	364	362/364 (99%) with 1 gap	*Bradyrhizobium* sp. TARC112
STB67	360	350/353(99%) with 3 gaps	*Bradyrhizobium* sp. TARC112
STB96	363	353/357(98%) with 2 gaps	*Bradyrhizobium* sp. TARC112
STB119	354	325/329(98%) with no gap	*B. elkanii* strains USDA76, USDA94
STB120	360	347/352(98%) with 2 gaps	*B. elkanii* strains USDA76, USDA94
STB147	351	359/361(99%) with 1 gap	*B. elkanii* strains USDA76, USDA94
STB169	362	329/367(89%) with 9 gaps	*B. elkanii* strain USDA 31
STB173	361	349/356(98%) with 6 gaps	*B. elkanii* strains USDA76, USDA94
STB176	357	354/361(98%) with 3 gaps	strains USDA76, USDA94
STB179	359	341/345 (98%) with 1 gap	*B. elkanii* strains USDA76, USDA94
STB185	360	358/361(99%) with 2 gaps	strains USDA76, USDA94
STB220	359	329/345(95%) with 3 gaps	*B. elkanii* strains USDA76, USDA94
STB238	359	341/353(96%) with 3 gaps	*B. elkanii* strains USDA76, USDA94
STB245	358	328/330 (99%) with 1 gap	*B. elkanii* strains USDA76, USDA94
STB250	363	361/363(99%) with no gap	*Bradyrhizobium* sp. TARC112
STB264	363	363/364(99%) with 1 gap	*B. elkanii* strain USDA31
STB310	360	357/365 (97%) with 6 gap	*Bradyrhizobium* sp. TARC112
STB327	347	344/347 (99%) with 1 gap	*B. elkanii* strains USDA76, USDA94

It is interesting to note that when homology of *nodY* sequences of the 20 slow-growing soybean rhizobium STB strains and those of the strains deposited in the GenBank database were used to identify the 20 STB strains, 12 of the STB strains were found to be related to *B. elkanii* strains USDA76, USDA94, 6 STB strains were found to be related to *Bradyrhizobium* sp. TARC112, and two strains (STB 169 and STB 264) were found to be related to *B. elkanii* strain USDA31.

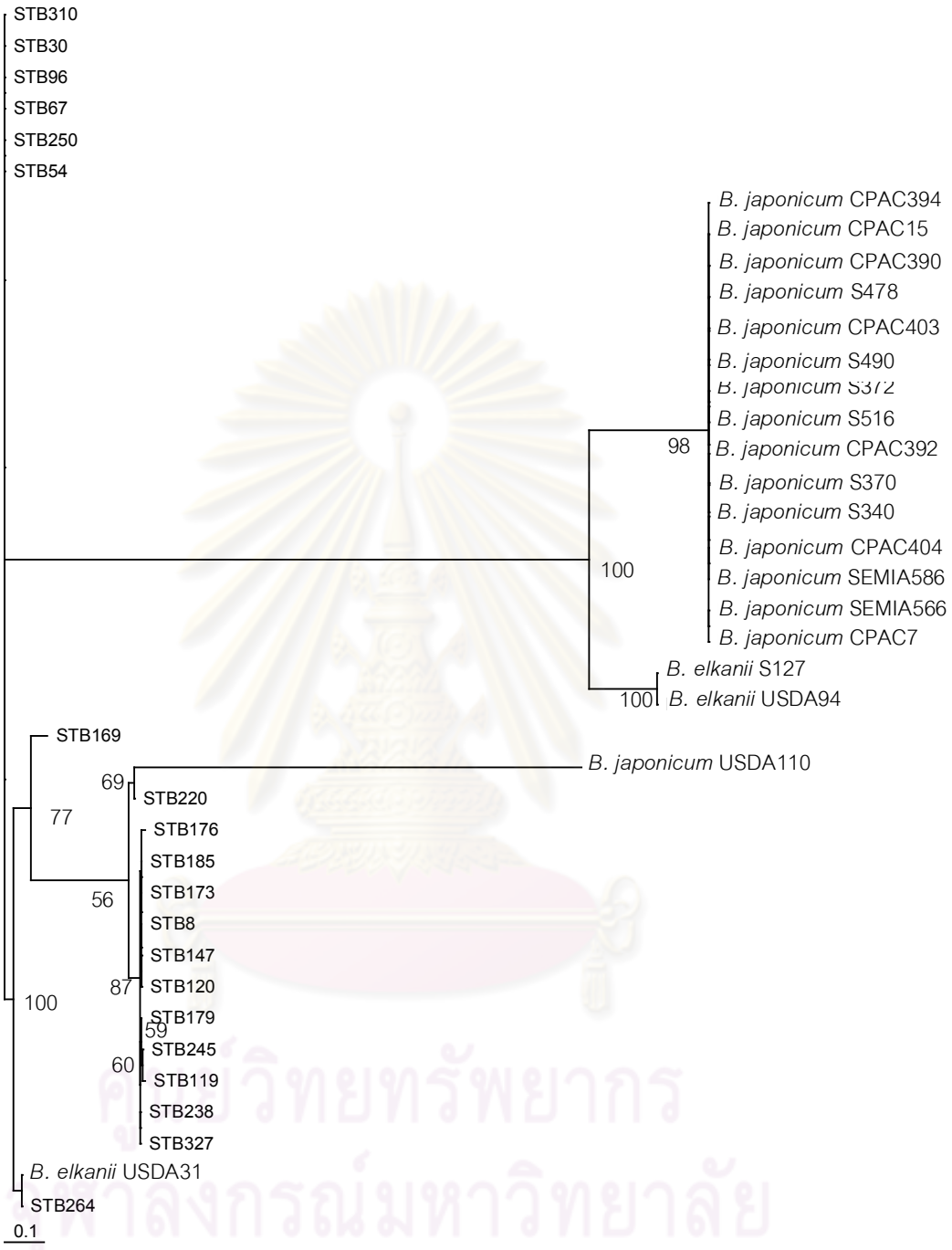


Figure 4.21a Dendrogram obtained from *nodY* sequences of 20 selected slow-growing strains as well as some reference strains. The Maximum Likelihood Method was used in the dendrogram construction.

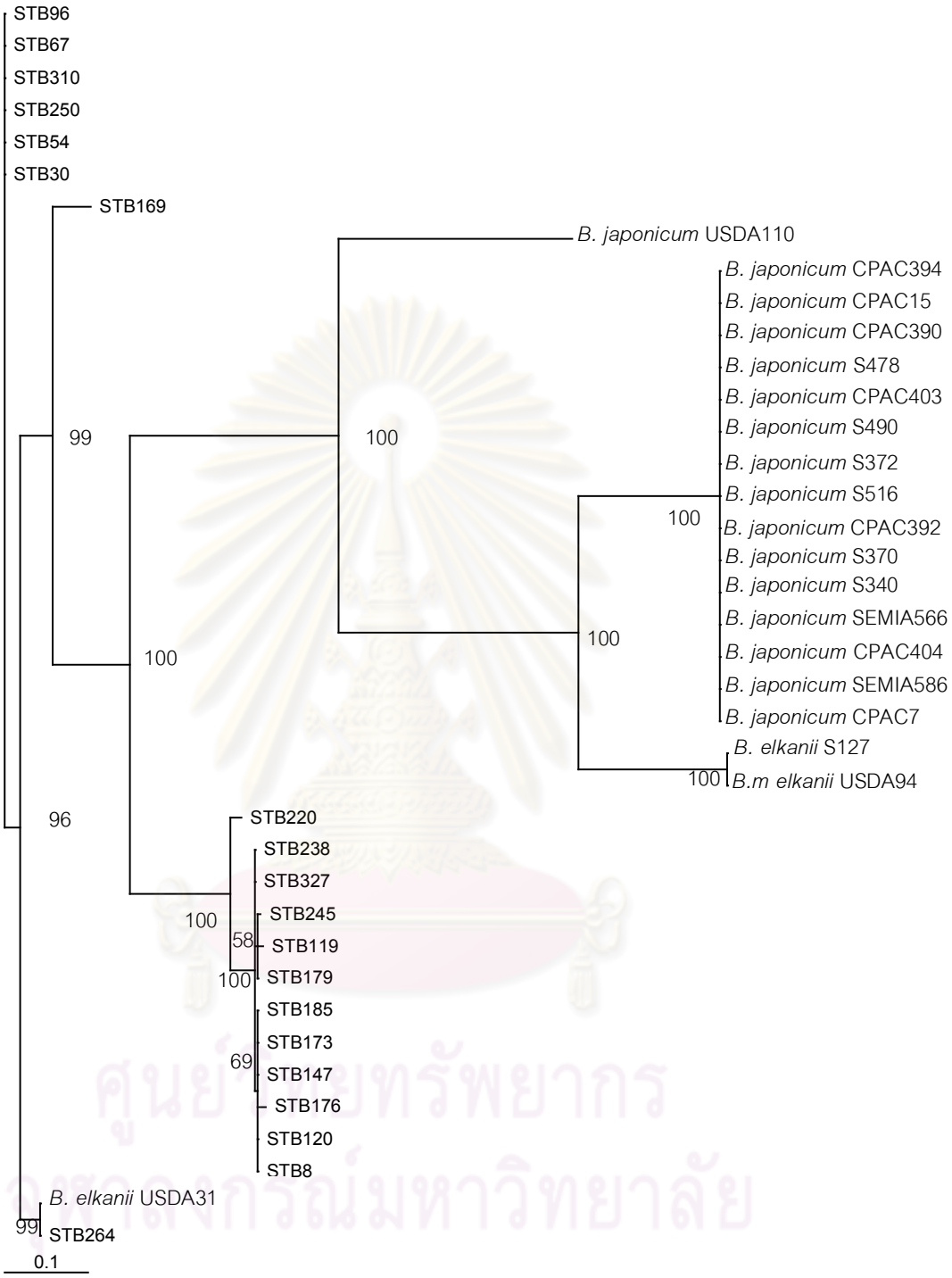


Figure 4.21b Dendrogram obtained from *nodY* sequences of 20 selected slow-growing strains as well as some reference strains. The Maximum Parsimony Method was used in the dendrogram construction.

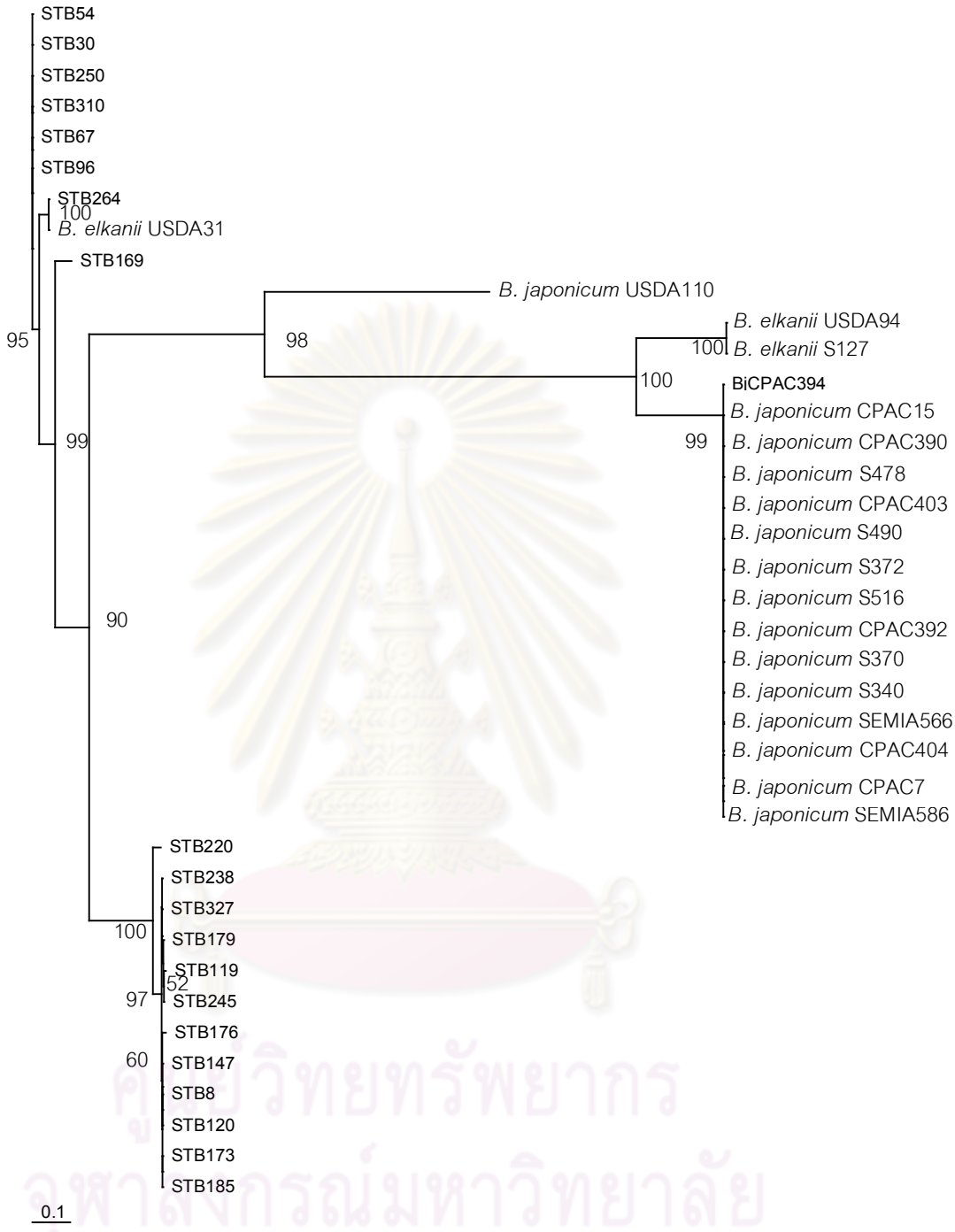


Figure 4.21c Dendrogram obtained from *nodY* sequences of 20 selected slow-growing strains as well as some reference strains. The Neighbor-Joining Method was used in the dendrogram construction.

Identification of slow-growing soybean rhizobia by using *nodY* sequences

The dendrograms constructed with *nodY* sequences of 20 STB strains and 19 reference strains as shown in Figures 4.21 (a-c) indicated a minor discrepancy when the Maximum Likelihood, Maximum Parsimony, and Neighbor-Joining methods were used in the dendrogram construction. The dendrogram constructed with the Maximum Likelihood method showed strain STB 220 as closely related to *B. japonicum* strain USDA 110 while the other methods of dendrogram construction showed the strain as closely related to all the *B. elkanii* STB strains. Apart from the discrepancy, all the three dendrograms showed the 39 strains were grouped into 5 clusters. Cluster 1 consisted of 11 or 12 *B. elkanii* STB strains (depending on whether strain STB220 was regareded as closely related to *B. elkanii* or not): STB8, 119, 120, 147, 173, 176, 179, 185, 220, 238, 245, and 327). Cluster 2 consisted of the 6 *B. japonicum* STB strains: STB 30, 54, 67, 96, 250, and 310. Cluster 3 consisted of one *B. yuanmingense* strain STB 169 which was found to be related to *B. japonicum/B. elkanii* strains S127 and USDA94 series. Cluster 4 showed *B. yuanmingense* strain STB264 as related to *B.elkanii* strain USDA 31. Cluster 5 contained the rest of the 18 reference strains. The results showed the same phylogenetic relationships as obtained with the 16S rDNA dendrograms except for the fact that STB strains 169 and 264 were not shown to be closely related to *B. yuanmingense* because there were no *nodY* sequences of *B. yuanmingense* deposited in the GenBank database for downloading.

Since 16S rDNA sequences are relatively longer (1500 bp) than *nodY* sequences (approximately 350 bp) and there are not many sequences of *nodY* deposited in the Genbank database, the identification and phylogenetic relationship determination of slow-growing soybean rhizobia were more reliable when 16S rDNA sequences were used in the identification and phylogenetic relationship determination. Therefore, the 20 slow-growing soybean rhizobium strains were identified as 12 *B. elkanii* strains, 6 *B. japonicum* strains, and 2 *B. yuanmingense* strains. This research provided the first record of the presence of *B. yuanmingense* in Thailand. Appendix D showed 16S rDNA and *nodY* sequences of 20 slow-growing soybean rhizobium STB strains. Figure 4.22 showed the distribution of the 20 STB strains according to the isolation sites of 16 subdistricts in Phitsanulok province.

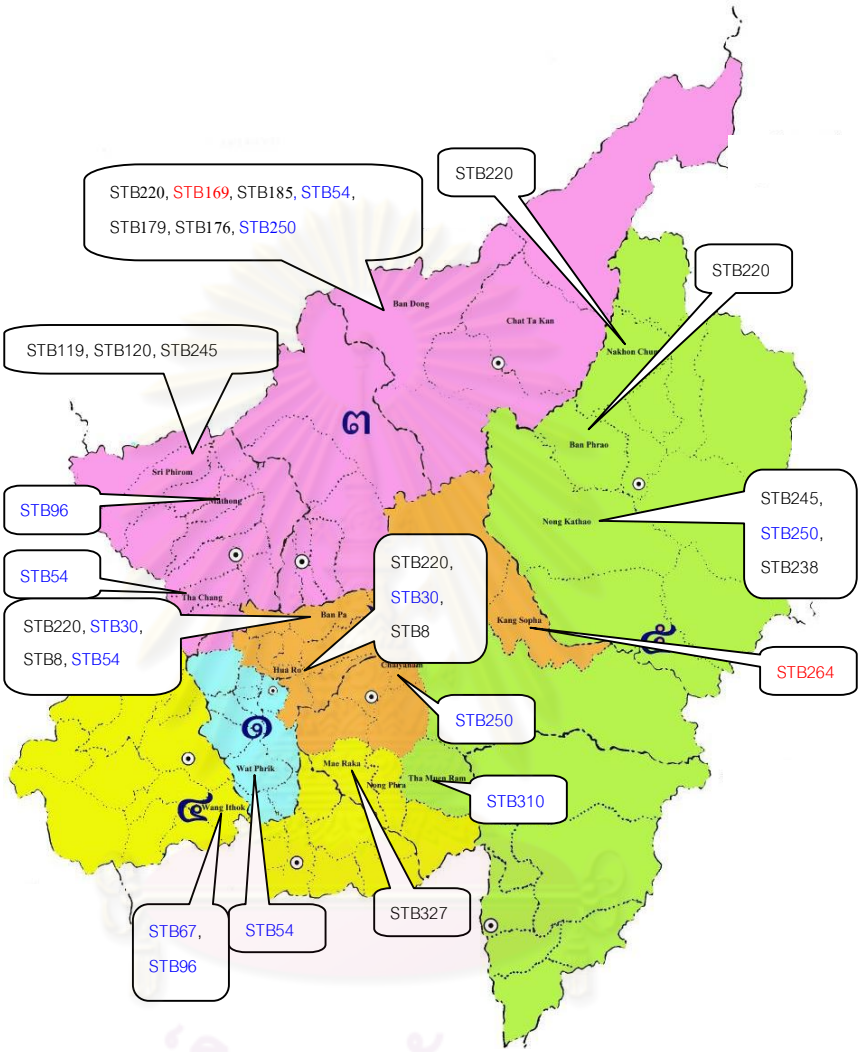
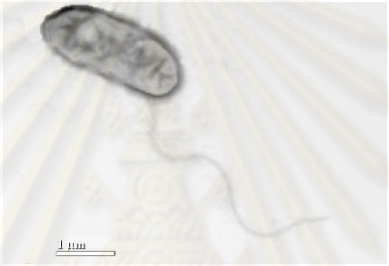


Figure 4.22 Distribution of 12 *Bradyrhizobium elkanii* STB strains (STB8, STB119, STB120, STB147, STB173, STB176, STB179, STB185, STB220, STB238, STB245, and STB327), 6 *Bradyrhizobium japonicum* STB strains (STB30, STB54, STB67, STB96, STB250 and STB310), and 2 *Bradyrhizobium yuanmingense* (STB169 and STB264) in 16 subdistricts in Phitsanulok province, Thailand.

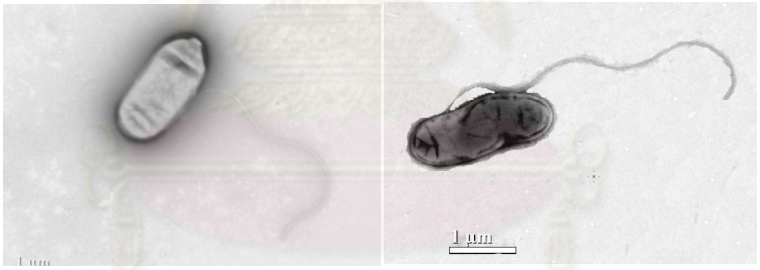
The distribution of the 20 slow-growing soybean rhizobium STB strains as shown in Figure 4.22 showed the presence of *B. elkanii* in almost all of the 16 subdistricts.

4.6.5 Number and type of flagella as determined by negative staining

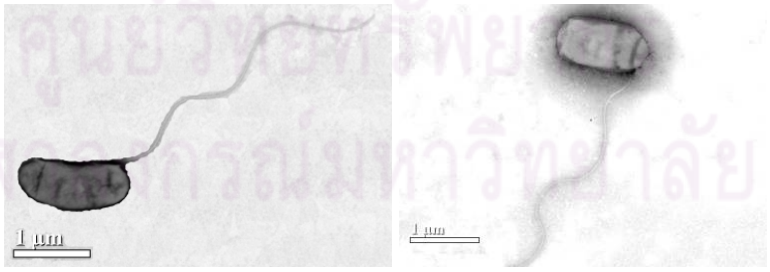
Five strains of slow-growing soybean rhizobia were selected for negative staining of flagella. The results in Figure 4.23 showed each of the 5 selected strains had one sub-polar flagellum as expected. Elkan and Bunn (1992) reported slow-growing soybean rhizobia had one sub-polar flagellum. The length of flagella may play a role in the competitive ability of soybean rhizobia in the root nodulation process (Vlassak and Vanderleyden, 1997). However, other factors including soybean cultivars also play a role in the competitive ability of soybean rhizobia in the root nodulation process (Payakapong et al., 2004).



B. japonicum STB 30



B. japonicum STB96 *B. elkanii* STB120



B. elkanii STB220 *B. yuanmingense* STB264

Figure 4.23 Transmission electron micrographs of 5 slow-growing soybean rhizobium STB strains.

4.6.6 Determination of growth at different temperatures

Figures 4.24 showed growth at different temperatures in terms of CFU/ml of 5 selected strains of isolated slow-growing soybean rhizobia. Table 4.18 showed the calculated specific growth rates.

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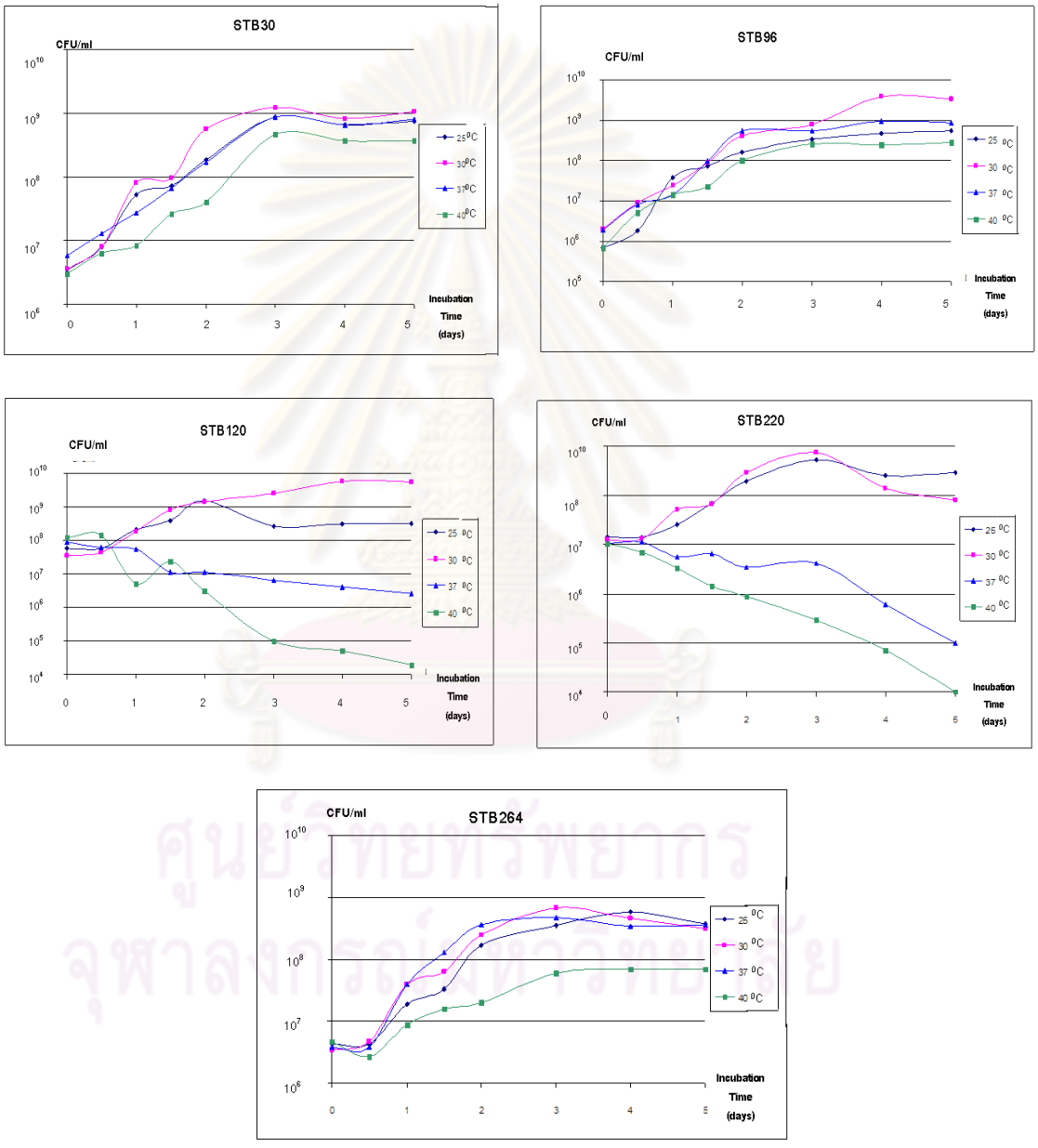

Figures 4.24 Growth at different temperatures in terms of CFU/ml of 5 selected strains of isolated slow-growing soybean rhizo

Table 4.18 Specific growth rates of 5 slow-growing STB strains.

Strains	Specific growth rates at different temperatures (days^{-1})			
	25^{0}C	30^{0}C	37^{0}C	40^{0}C
B. japonicum STB 30	1.99	2.54	1.69	1.26
B. japonicum STB 96	2.07	1.74	1.89	1.99
B. elkanii STB 120	1.53	1.13	Cells died	Cells died
B. elkanii STB 220	1.21	1.36	Cells died	Cells died
B.yuanmingense STB264	1.83	2.14	2.30	0.74

The growth and specific growth rates of the 5 selected STB strains as shown in Figure 4.24 and Table 4.18 indicated the *B. japonicum* and the *B.yuanmingense* strains grew better at the relatively low temperatures at 25^{0}C and 30^{0}C. The *B. elkanii* strains were found to be heat-sensitive. The results showed different intrinsic abilities of the slow-growing soybean rhizobia to grow at different temperatures.

4.6.7 Ability/Inability to utilize different kinds of carbon and nitrogen sources

Table 4.19 showed results on the ability/inability of the 20 slow-growing soybean rhizobium STB strains and three reference strains of slow-growing soybean rhizobia to use different kinds of carbon and nitrogen sources as determined by the Biolog^{TM} test kit.

Table 4.19 Ability/Inability to utilize 95 carbon and nitrogen sources as determined by the Biolog^{TM} test kit of the 20 slow-growing soybean rhizobium STB strains and three reference strains. + indicates the strains could use the carbon and nitrogen sources. – indicates the strains could not use the carbon and nitrogen sources. Numbers indicate the total numbers out of the 20 test strains that could use the carbon and nitrogen sources.

Carbon/Nitrogen sources	*B. elkanii* NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	Numbers of strains
α-Cyclodextrin	-	-	-	4
Dextrin	+	+	+	15

Glycogen	-	-	+	1
Tween 40	+	+	+	20
Tween 80	+	+	+	20
N-Acetyl-D-Galactosamine	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-
Adonitol	+	-	-	2
L-Arabinose	+	+	+	20
D-Arabitol	+	+	-	18
D-Cellobiose	-	+	-	2
i-Erythritol	-	-	-	-
D-Fructose	+	+	-	16
L-Fucose	+	-	-	10
D-Galactose	+	+	+	20
Gentiobiose	-	-	-	-
α-D-Glucose	+	+	+	20
m-Inositol	-	-	-	-
α-D-Lactose	-	-	-	-
Lactulose	-	-	-	-
Maltose	-	-	-	-
D-Mannitol	+	+	+	20
D-Mannose	+	+	+	20
D-Melibiose	-	-	-	-
β-Methyl-D-Glucoside	+	-	-	-
D-Psicose	+	-	-	9
D-Raffinose	-	-	-	-
L-Rhamnose	-	+	-	8
D-Sorbitol	+	-	-	9
Sucrose	-	-	-	-
D-Trehalose	-	-	-	-
Turanose	-	-	-	-

Xylitol	-	-	-	-
Pyruvic Acid Methyl Ester	+	+	+	20
Succinic Acid Mono-Methyl-Ester	+	+	+	20
Acetic Acid	+	+	+	20
Cis-Aconitic Acid	-	-	-	-
Citric Acid	+	-	-	9
Formic Acid	+	+	-	15
D-Galactonic Acid Lactone	+	+	-	15
D-Galacturonic Acid	+	-	-	11
D-Gluconic Acid	+	+	+	20
D-Glucosaminic Acid	+	-	-	11
D-Glucuronic Acid	+	-	-	2
α-Hydroxybutyric Acid	-	+	+	8
β-Hydroxybutyric Acid	+	+	+	20
γ-Hydroxybutyric Acid	+	+	+	20
p-Hydroxy Phenylacetic Acid	+	-	+	3
Itaconic acid	-	+	+	8
α-Keto Butyric Acid	-	+	+	5
α-Keto Glutaric Acid	+	+	+	15
α-Keto Valeric Acid	-	+	+	11
D,L-Lactic Acid	+	+	+	20
Malonic Acid	+	-	+	3
Propionic Acid	+	+	+	20
Quinic Acid	+	-	-	2
D-Saccharic Acid	+	+	+	18
Sebacic Acid	+	-	+	14
Succinic Acid	+	+	+	20
Bromosuccinic Acid	+	+	+	20
Succinamic Acid	+	+	+	20
Glucuronamide	+	-	-	7

L-Alaninamide	+	+	-	14
D-Alanine	+	-	-	11
L-Alanine	+	-	+	5
L-Alanyl-glycine	+	+	-	3
L-Asparagine	+	-	-	8
L-Aspartic Acid	+	-	+	11
L-Glutamic Acid	+	-	-	12
Glycyl-L-Aspartic Acid	+	-	-	3
Glycyl-L-Glutamic Acid	-	-	+	5
L-Histidine	-	-	-	-
Hydroxy-L-Proline	-	-	-	-
L-Leucine	+	+	+	20
L-Ornithine	-	-	+	1
L-Phenylalanine	+	+	+	20
L-Proline	+	+	-	13
L-Pyroglutamic Acid	+	+	+	18
D-Serine	+	-	-	10
L-Serine	+	-	-	2
L-Threonine	-	+	-	5
D,L-Carnitine	-	-	-	-
γ-Amino Butyric Acid	-	+	-	5
Urocanic Acid	-	-	-	-
Inosine	-	-	-	-
Uridine	-	-	-	-
Thymidine	-	-	-	-
Phenyethyl-amine	-	-	-	-
Putrescine	-	-	-	-
2-Aminoethanol	-	-	-	-
2,3-Butanediol	+	-	-	1
Glycerol	+	+	+	18

D,L-α-Glycerol Phosphate	+	-	-	3
α-D-Glucose-1-Phosphate	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-

The results indicated all the 20 slow-growing soybean rhizobium STB strains could utilize Tween 40, Tween 80, L-Arabinose , D-Galactose, α-D-Glucose, D-Mannitol, D-Mannose , Pyruvic Acid Methyl Ester, Succinic Acid Mono-Methyl-Ester, Acetic Acid, D-Gluconic Acid, β-Hydroxybutyric Acid , γ-Hydroxybutyric Acid, D,L-Lactic Acid, Propionic Acid, Succinic Acid , Bromosuccinic Acid , Succinamic Acid , L-Leucine , and L-Phenylalanine. The 20 slow-growing soybean rhizobium STB strains could not utilize many carbon and nitrogen sources as shown in Table 4.19. The results indicated that there were variations in the ability to use different kinds of carbon and nitrogen sources even within the strains that were identified as belonging to the same genus and species. The results confirmed the existence of genetic variations as observed in the different RAPD-PCR fingerprints of strains belonging to the same genera and species. Appendix E showed a summary of representative results of ability/inability to use 95 carbon/nitrogen sources by three reference strains and by *B. elkanii* strain STB327, *B. japonicum* strain STB310 and *B. yuanmingense* strain STB264.

4.7 Genetic diversity of 121 slow-growing soybean rhizobium STB strains

Figure 4.25 (a,b) showed dendrograms constructed from DNA fingerprints of 121 slow-growing soybean rhizobium STB strains when either RPO1 or CRL-7 was used as the primer. The dendrograms showed two main groups of slow-growing soybean rhizobia as follows:

Group 1 consisted of 2 subgroups : Subgroups 1.1 and 1.2.

Subgroup 1.1 contained three clusters :

Cluster 1.1.1 consisted of 2 subclusters, the first subcluster 1.1.1.1 consisted of 11 STB strains (STB209, STB220, STB226 , STB236 , STB231 , STB234 , STB233 , STB222 , STB214 , STB224 ,and STB213) which were found to be closely related to *B. elkanii* STB220. The second subcluster 1.1.1.2 consisted of 12 strains (STB171 , STB173 , STB179 , STB150 , STB162 , STB142 , STB168 , STB169 , STB147 , STB178 , STB154 , and STB163) which were found to be closely related to *B. elkanii*

STB147, *B. elkanii* STB173, *B. elkanii* STB176, and *B. elkanii* STB179/ *B. yuanmingense* STB169.

Cluster 1.1.2 consisted of 2 subclusters: subcluster 1.1.2.1 consisted of 7 strains (STB116, STB117, STB100, STB113, STB96, STB114, and STB89) which were found to be closely related to *B. japonicum* STB96. Subcluster 1.1.2.2 contains 16 strains (STB295, STB299, STB288, STB328, STB281, STB282, STB292, STB327, STB328, STB330, STB294, STB298, STB284, STB286, STB285, and STB296) which were found to be closely related to *B. elkanii* STB327.

Cluster 1.1.3 consisted of 12 strains (STB261, STB270, STB268, STB271, STB274, STB276, STB259, STB256, STB266, STB264, STB273, and STB275) which were found to be closely related to *B. yuanmingense* STB264.

Subgroup 1.2 consisted of 2 clusters:

Cluster 1.2.1 consisted of 2 subclusters. The first subcluster 1.2.1.1 consisted of 8 strains (STB250, STB255, STB248, STB243, STB254, STB237, STB238, and STB252) which were found to be closely related to *B. elkanii* STB238/*B. japonicum* STB250. The second subcluster 1.2.1.2 consisted of 4 strains (STB325 , STB326 , STB245 , and STB241) which were found to be closely related to *B. elkanii* STB245. The third subcluster 1.2.1.3 consisted of 9 strains (STB333, STB335, STB332, STB318, STB319, STB300, STB336, STB302, and STB310) which were found to be closely related to *B. japonicum* STB310.

Cluster 1.2.2 consisted of 4 strains (STB46, STB56, STB67, and STB63) which were found to be closely related to *B. japonicum* STB54 and *B. japonicum* STB67.

Group 2 consisted of 2 subgroups: subgroup 2.1 and subgroup 2.2. Subgroup 2.1 was divided into three clusters:

Cluster 2.1.1 consisted of 12 strains (STB321, STB340, STB131, STB120, STB121, STB126, STB132, STB28, STB8, STB3, STB1 and STB201) which were found to be closely related to *B. elkanii* STB8 and *B. elkanii* STB120.

Cluster 2.1.2 consisted of 10 strains (STB180, STB320, STB189, STB203, STB202, STB183, STB185, STB119, STB181 and STB139) which were found to be closely related to *B. elkanii* STB185 and *B. elkanii* STB119.

Cluster 2.1.3 consisted of 4 strains (STB188, STB41, STB38, and STB4) which were found to be closely related to *B. japonicum* STB30.

Subgroup 2.2. contained 12 strains whose identities could not be obtained from the experimental results because no previously-identified soybean rhizobia were grouped in the same subgroup 2.2.

Dendrograms obtained from RPO1 and CRL-7 DNA fingerprints showed identical clustering of the 121 slow-growing soybean rhizobia strains used in this research. The strains were divided into two groups, namely, Groups 1 and 2. Group 1 was divided into two subgroups. Subgroup 1.1 contained 3 clusters. Cluster 1.1.1 contained two subclusters 1.1.1.1 and 1.1.1.2. Cluster 1.1.2 contained two subclusters 1.1.2.1 and 1.1.2.2. Cluster 1.1.3 formed one cluster. Subgroup 1.2 contained two clusters. Cluster 1.2.1 contained three subclusters 1.2.1.1, 1.2.1.2, and 1.2.1.3. Cluster 1.2.2 formed one cluster. Group 2 contained two subgroups. Subgroup 2.1 contained three clusters 2.1.1, 2.1.2, and 2.1.3. Subgroup 2.2 contained one cluster. The clustering was summarized in Figure 4.25(a). The results as shown in Figure 4.25(b) showed 73 strains of slow-growing soybean rhizobia constituted seven types of strains which were found to be closely related to *B. elkanii*. The first type consisted of 11 strains in subcluster 1.1.1.1 which were found to be closely related to *B. elkanii* STB220. The second type contained 12 strains in subcluster 1.1.1.2 which were found to be closely related to *B. elkanii* STB147, *B. elkanii* STB173, *B. elkanii* STB176, *B. elkanii* STB179/ *B. yuanmingense* STB169. The third type of slow-growing soybean rhizobia contained 16 strains in subcluster 1.1.2.2 which were found to be closely related to *B. elkanii* STB327. The fourth type of slow-growing soybean rhizobia consisted of 8 strains in subcluster 1.2.1.1 which were found to be closely related to *B. elkanii* STB328 / *B. japonicum* STB250. The fifth type of slow-growing soybean rhizobia consisted of 4 strains in subcluster 1.2.1.2 which were found to be closely related to *B. elkanii* STB245. The sixth type of slow-growing soybean rhizobia consisted of 12 strains in cluster 2.1.1 which were found to be closely related to *B. elkanii* STB8 and *B. elkanii* STB120. The seventh type of slow-growing soybean rhizobia consisted of 10 strains in cluster 2.1.2

which were found to be closely related to *B. elkanii* STB185 and *B. elkanii* STB119. In addition, 24 strains of isolated slow-growing soybean rhizobia in subclusters 1.1.2.1, 1.2.1.3 and in clusers 1.2.2, and 2.1.3 were found to be closely related to *B. japonicum* STB96, *B. japonicum* STB310, and *B. japonicum* STB54, *B. japonicum* STB67, and *B. japonicum* STB30 repectively. Moreover, 12 strains of slow-growing soybean rhizobia in cluster 1.1.3 were found to be closely related to *B. yuanmingense* STB264. With the twelve strains of unidentified slow-growing soybean rhizobia, the total number of slow-growing soybean rhizobia was 121 strains consisting of 73 *B. elkanii* strains, 24 *B. japonicum* strains, 12 *B. yuanmingense* strains and 12 unidentified strains.

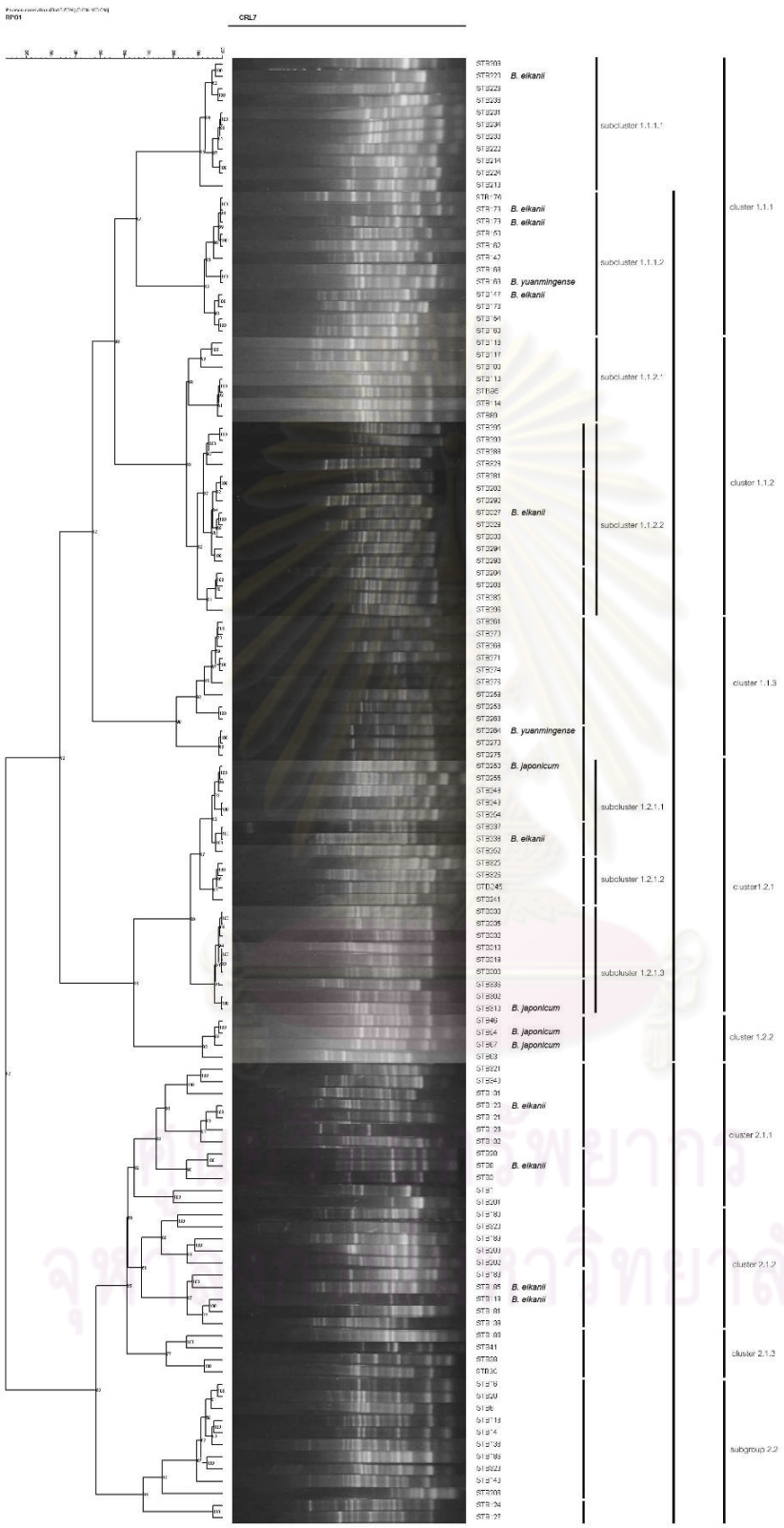

Figure 4.25 (a) Dendrogram constructed from DNA fingerprints using CRL-7 as the primer. Detail is given in text.

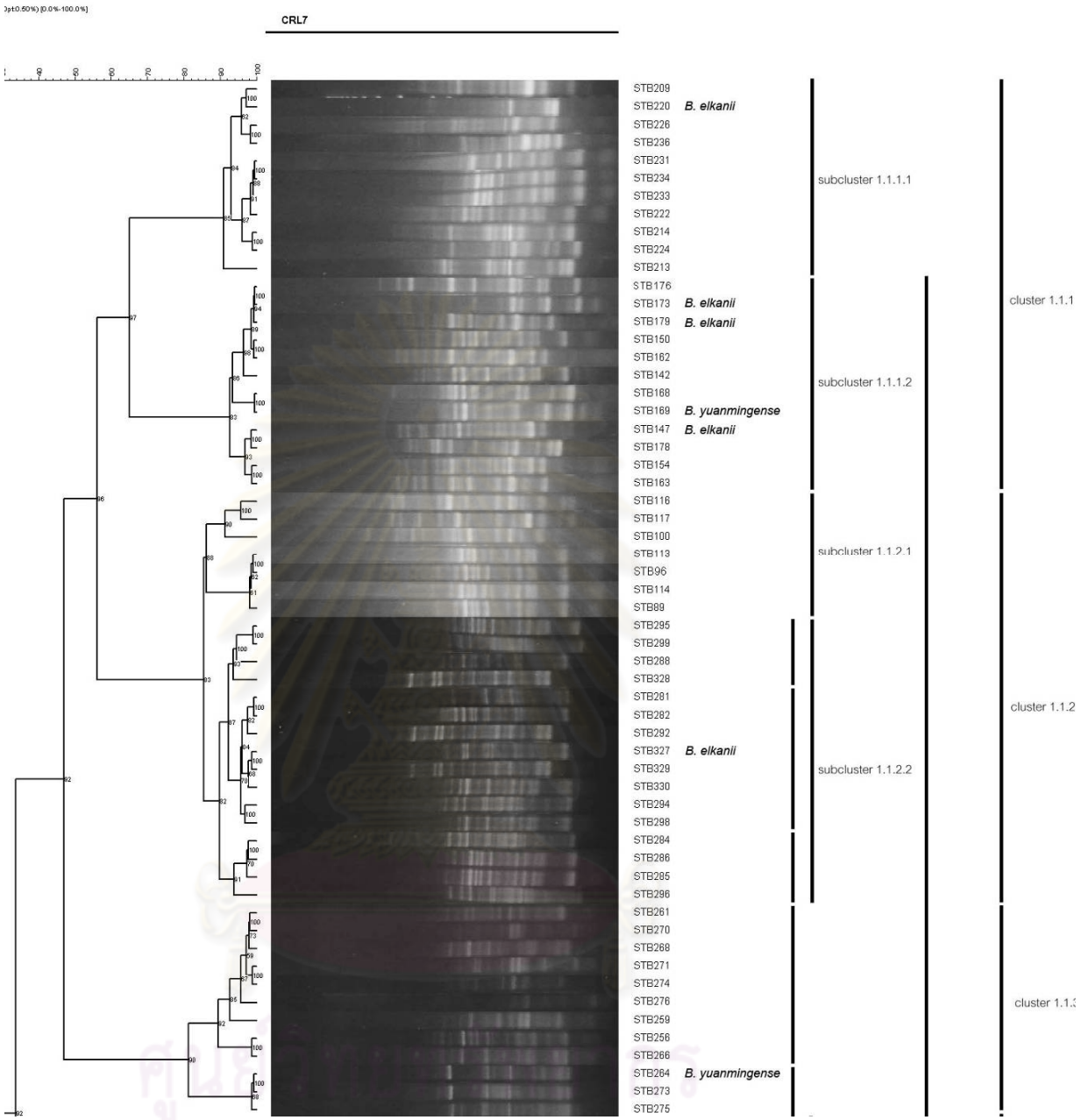

Figure 4.25 (a) Dendrogram constructed from DNA fingerprints using CRL-7 as the primer. Detail is given in text.

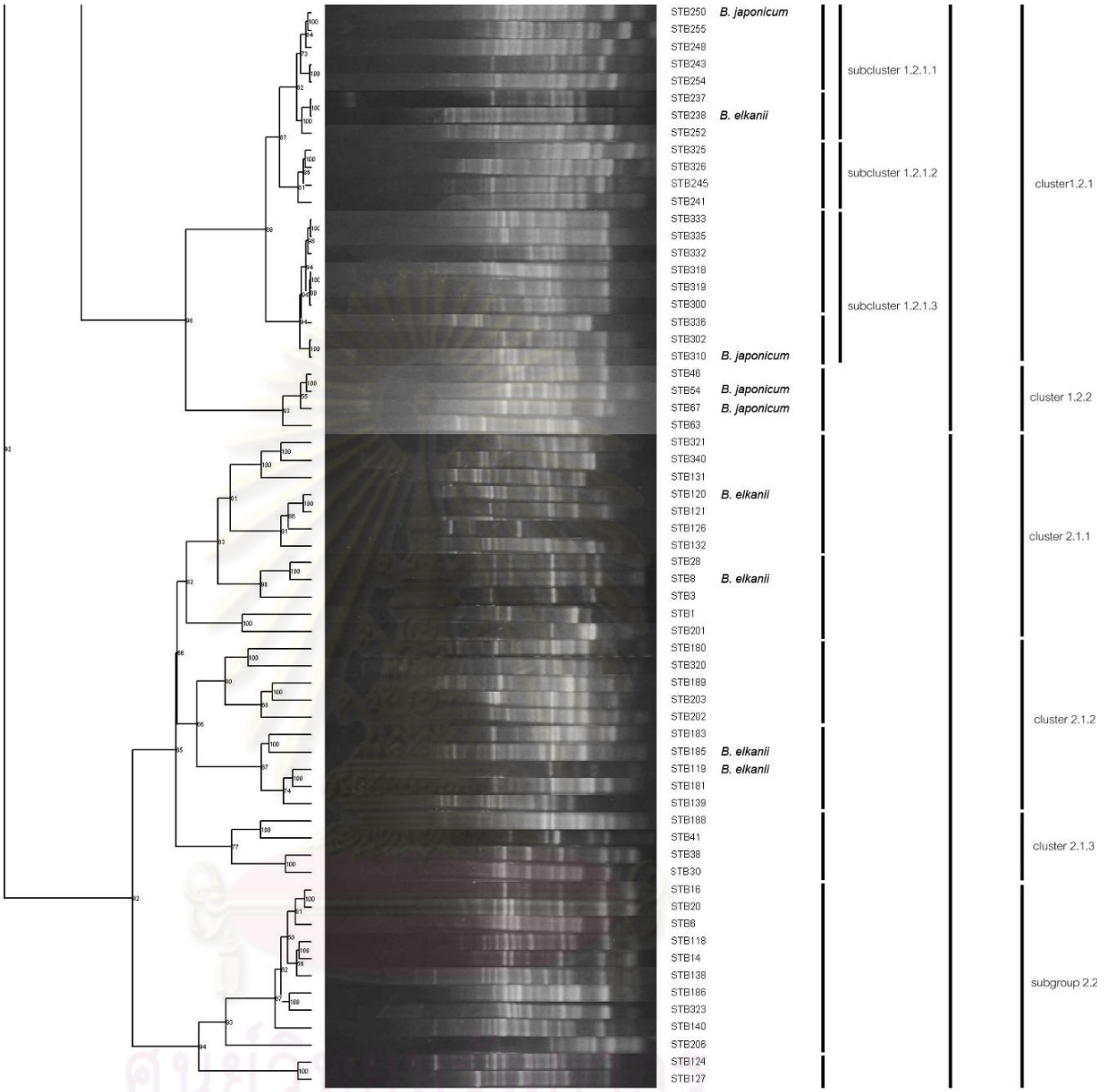

Figure 4.25 (a) Dendrogram constructed from DNA fingerprints using CRL-7 as the primer. Detail is given in text.

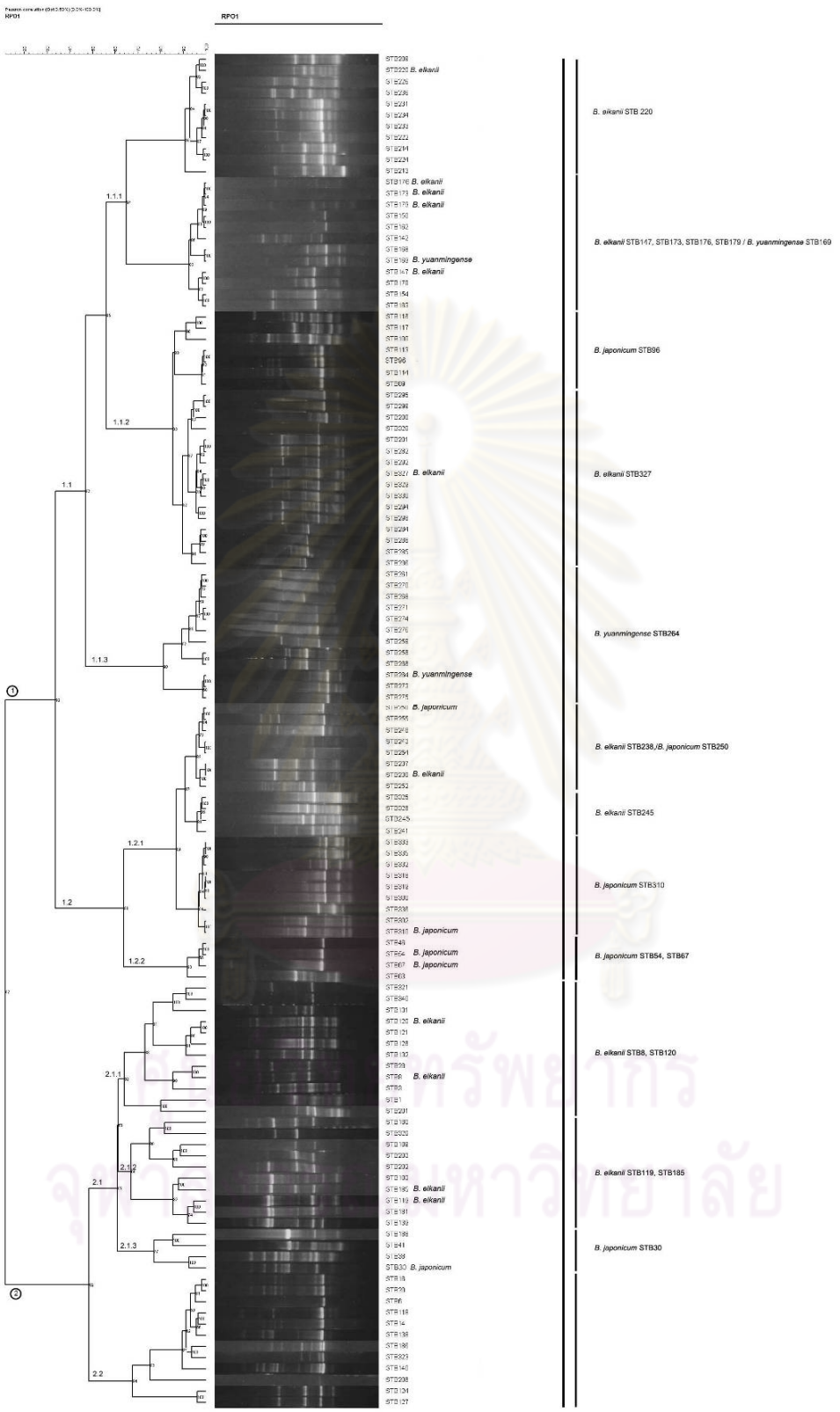

Figure 4.25 (b) Dendrogram constructed from DNA fingerprints using RPO1 as the primer. Detail is given in text.

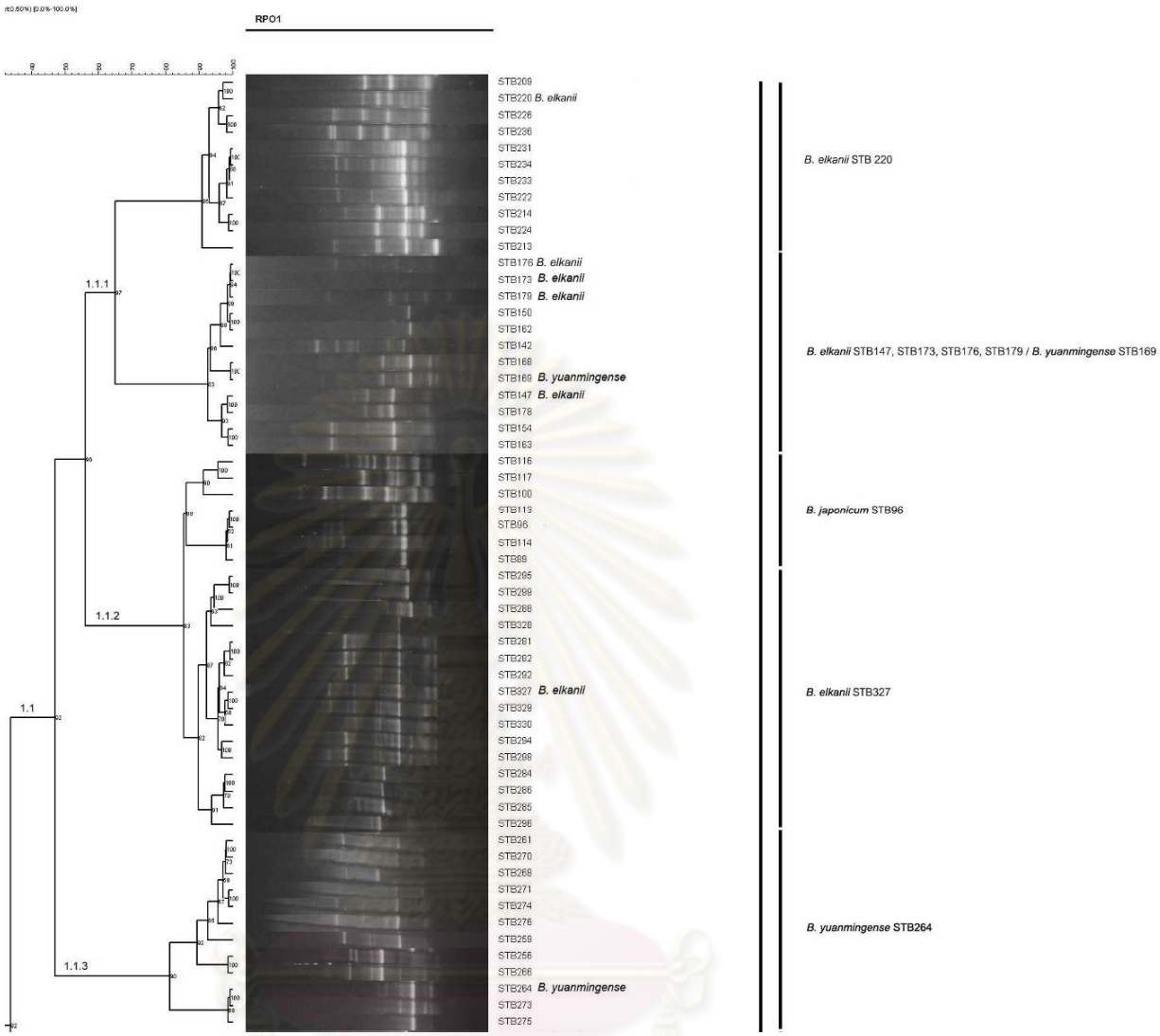

Figure 4.25 (b) Dendrogram constructed from DNA fingerprints using RPO1 as the primer. Detail is given in text.

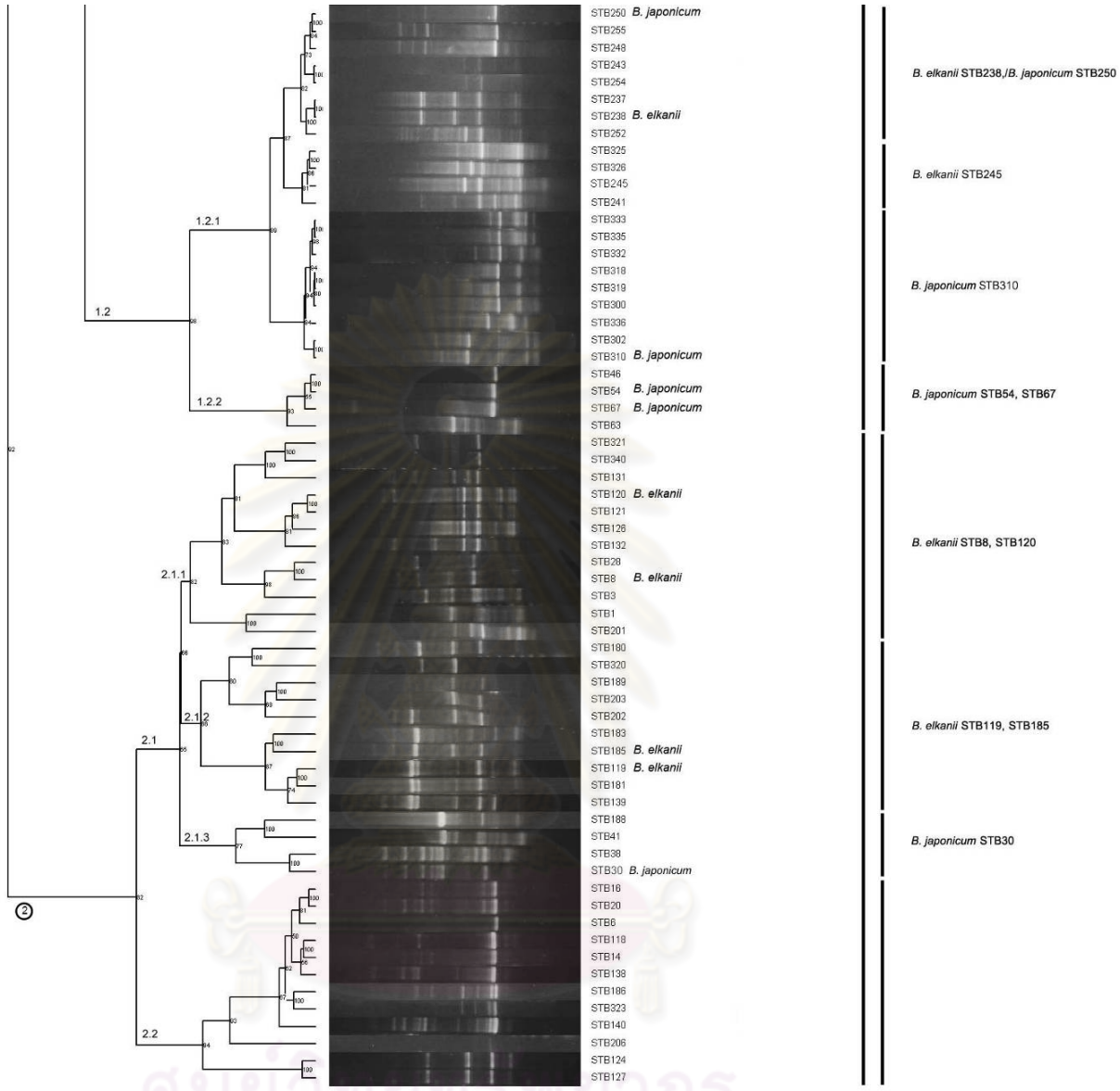

Figure 4.25 (b) Dendrogram constructed from DNA fingerprints using RPO1 as the primer. Detail is given in text.

CHAPTER V

DISCUSSION

5.1 Colony morphology and Bromthymol blue reactions in slow-growing soybean rhizobia

The results obtained for colony morphology, Bromthymol blue reactions and the identification of slow-growing soybean rhizobia as shown in Figures 4.1, 4.2 and Tables 4.5 and 4.16 showed that in most cases, it is possible to predict the type of Bromthymol blue reactions from the colony morphology. Almost all of the identified *Bradyrhizobium elkanii* strains and *B. yuanmingense* strain STB169 with Type I colony morphology (irregular and slimy colonies) secreted alkali product(s) throughout the 10-day incubation period while the slow-growing *B. japonicum* with Type II colony and *B. yuanmingense* strain STB264 secreted alkali product(s) in the first 5-day incubation and secreted acidic product(s) in the last 5-day of incubation. According to Somasegaran and Hoben (1994), the indicator dye Bromthymol blue is green in YMA with pH 6.8. Fast-growing soybean rhizobia secrete acidic product(s), therefore, Bromthymol blue is changed to yellow color. Slow-growing soybean rhizobia turn the color of Bromthymol blue to blue due to the secretion of alkali product(s). Other researchers also reported that fast-growing soybean rhizobia showed an acid Bromthymol blue reaction while slow-growing soybean rhizobia showed an alkali Bromthymol blue reaction (Alberton et al., 2006; Chen et al., 2002; Chen et al., 2004; Hungria et al., 2001). However, in this research , it is demonstrated for the first time that two types of Bromthymol blue reactions were found in slow-growing soybean rhizobia as described above. The experimental results showed that during growth on YMA with Bromthymol blue at the initial pH of 6.8, strains of *B. elkanii* and *B. liaoningense* as well as *B. yuanmingense* strain STB169 secreted alkali product(s) which turned the medium blue throughout the 10-day incubation time while strains of *B. japonicum* and *B. yuanmingense* strain STB264 were found to secrete acidic product(s) during the first 5-day incubation and secrete acidic product(s) during the last 5-day incubation. The results could be

interpreted as *B. elkanii*, *B. liaoningense* as well as *B. yuanmingense* strain STB169 could survive under alkali condition with no need for an adaptation to changes of pHs in the surroundings while *B. japonicum* and *B. yuanmingense* strain STB264 preferred an acidic condition. Therefore, when pHs of the surroundings were in the alkali range, *B. japonicum* secreted acidic product(s) to adjust the surrounding pHs to the acidic range.

The findings that slow-growing soybean rhizobia could secrete either acidic or alkali products to change pHs of the surroundings were confirmed by the experimental results on responses of *B. elkanii* strain NA7, *B. japonicum* strain S76, *B. liaoningense* strain SK3, and *B. yuanmingense* strain STB264 to the pHs of the medium with and without buffer as shown in Tables 4.6 to 4.15. The results showed that in the absence of buffer, when the initial pHs of the medium were acidic (pH 4.0, 5.0, and 6.0), the four slow-growing soybean rhizobium strains secreted alkali product(s) to turn the values of pHs of the supernatant to more than those of the initial pHs. On the other hand, when initial pHs of the medium were 7.0 or 8.0, cells of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 were found to secrete acidic product(s) to turn pHs of the supernatant to the values of pHs in an acidic range. In terms of an adaptation for growth and survival in soils with different pHs, it would be advantageous for slow-growing soybean rhizobia to be able to change pHs of the surrounding soils to the optimum pH for growth and survival by secreting either acidic or alkali products, depending on pHs of the surroundings.

5.2 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 was the acidity of the soil samples which were in the range of 4.5-6.5. Suzuki et al. (2008) reported that soybean rhizobia on the Okinawa Islands in Japan were mainly fast-growing soybean rhizobia due to the alkalinity of the soils. In addition, Han et al. (2008) also reported the presence of fast-growing soybean rhizobia as well as the slow-growing *B. liaoningense* in saline alkali soils in Xinjiang, People's Republic of China. The first report on the isolation of fast-growing soybean rhizobium, *Rhizobium fredii* was carried out by Keyser

et al. in 1982. Since then other researchers have isolated fast-growing soybean rhizobia from Hubei province in mainland China (Camacho et al., 2002; Dowdle and Bohlool, 1985; Stephen and Bohlool, 1985), and in Brazil (Hungria et al., 2001).

This dissertation is the first report on the record of *B. yuanmingense* in Thailand. Previously there were two published papers on the isolation and characterization of *B. yuanmingense* strain that nodulated soybean (Appunu et al., 2009) and *B. yuanmingense* strain that did not nodulate soybean but nodulated legume species of the genus *Lespedeza* (Yao et al., 2002). In this dissertation, two strains of *B. yuanmingense*, namely, strains STB169 and STB264 were isolated. The two strains were found to have different colony morphology and BTB reactions and shown in the Appendices C and D. Future taxonomic work on all the 76 slow-growing soybean rhizobium STB strains will be carried out by Multilocus Sequencing Analysis (MLSA) (Gevers et. al., 2005). In addition, this dissertation presented two lines of evidence for the detection of natural variants of slow-growing soybean rhizobia for the first time in Thailand. The first evidence was 16S rDNA dendrograms (Figures 4.20, a-c) grouped the 12 STB strains (STB8, STB119, STB120, STB147, STB173, STB176, STB179, STB185, STB220, STB245, STB283, and STB327) and the 6 STB strains (STB30, STB54, STB67, STB96, STB250, and STB310) into the same species of *B. elkanii* and *B. japonicum*, respectively. The second line of evidence showed the STB strains which were grouped into the same species had different DNA fingerprints as shown in Figure 4.19a for the 12 STB strains of *B. elkanii* and the 6 STB strains of *B. japonicum* (Figure 4.19b). Figure 4.19 showed the above-mentioned STB strains 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 to 1978 were found (Barcellos et al., 2007). In fact, all the reference strains used in the construction of *nodY* dendrograms were quoted from the paper by Barcellos et al. (2007) with the expectation that some of the Thai natural variants might have close phylogenetic relationship with the Brazilian natural variants. However, the *nodY* dendrograms showed all the Brazilian natural variants were found in the same cluster. It was not surprising to find the Brazilian natural

variants in the same cluster because they were all arose from genetic adaptations to the soil environments in Brazil and by possibly by lateral gene transfer (Boucher et al.,2003; Wright, 2004).

5.3 Multilocus Sequencing Analysis (MLSA) in the identification and determination of phylogenetic relationship in natural variants of slow-growing soybean rhizobia STB strains

The average size of isolated 16S rDNAs of the 20 slow-growing STB strains around 1450 bp were in the same range as those reported by Binde et al. (2009) and Menna et al. (2006). The dendrograms constructed with sequences of *nodY* of the 20 slow-growing soybean rhizobium STB strains yielded less satisfactory results because the two *B. yuanmingense* strains STB169 and STB264 could not be identified by *nodY* sequences. The results agreed with other researchers who reported the limitations of using homology to sequences deposited in GenBank database for the identification purpose. If less numbers of sequences of genes of interest are deposited in the GenBank database, the chance of being able to identify a particular species is reduced. In addition, sequences of only one gene, 16S rDNA, cannot be used to resolve differences amongst natural variants of the 12 *B. elkanii* and 6 *B. japonicum* STB strains observed in PCR-DNA fingerprints (Figure 4.19). Moreover, phenotypic differences in the ability to use/not use 95 carbon and nitrogen sources determined by the Biolog™ tests of the 20 STB strains could not be used to resolve differences in the 12 and 6 natural variants of *B. elkanii* and *B. japonicum*. Therefore, there is a trend towards identification and phylogenetic relationship determination by the Multilocas Sequence Analysis (MLSA) which was first introduced by Gevers et al. (2005) .In 2008 Vinuesa et al. employed Multilocus Sequence Analysis to identify soybean rhizobia.

5.4 Further research on the collection of soybean rhizobia obtained

The genetic diversity of the 121 strains of slow-growing soybean rhizobia as shown in Figures 4.25 (a,b) is an example to indicate that Thailand has a vast collection of soybean rhizobium strains which could be tested for their suitability for use in the production of inoculants for field trials. In 2009, Chansa-ngavej applied for a Thai patent on the selection method for soybean rhizobia that could be used to produce soybean

rhizobium biofertilizers which could be kept at room temperature. The method required the selection of strains that grew well at 25^0C and 30^0C which were used to represent soil temperatures in the northern, upper central, and some parts of the north-eastern part of Thailand where soybeans are grown. The selected strains should not increase in numbers when grown at 37^0C and 40^0C which were chosen as representation of room temperatures where soybean rhizobium biofertilizers are kept during storage and transportation. The results of growth curves obtained when cells of the 5 selected strains of slow-growing soybean rhizobia were grown at 25^0C, 30^0C, 37^0C, and 40^0C as shown in Figure 4.24 showed the strains STB30, STB96, STB120, STB220, and STB264 did not meet the criteria set up for the selection of soybean rhizobia that could be used in the production and field testings of soybean rhizobium biofertilizers that could be kept at room temperature. The rationale of maintaining no growth when grown at 37^0C and 40^0C is to keep the numbers of cells constant at the original minimum10^8 CFU/ml as stipulated in the Royal Gazette for the standard quality of biofertilizers. If the number of rhizobium cells in the biofertilizers is more than 10^8 CFU/ml, there may be inhibition of nodulation gene expression by the quorum sensing mechanism (Loh et al., 2001, 2002a, b, 2003, Sharma et al., 2003).

The remaining 116 strains of slow-growing rhizobia obtained in this dissertation could be further used in the determination of growth at different temperatures to find desirable strains for the production of inoculants for field trials. Other desirable properties of soybean rhizobium strains that need to be determine for the selection for use in the production of biofertilizers include competitive ability to outcompete indigenous soybean rhizobia in root nodulation, nitrogen fixation ability and survival in the fields as well as formulations (Vlassak and Vanderleyden, 1997). The results on slow-growing soybean rhizobia in 16 subdistricts in Phitsanulok province presented in this dissertation are thus a contribution to the study of soybean rhizobium diversity which has a far-reaching effect on the development of soybean rhizobium biofertilizers which would increase income of soybean growers and would contribute to the preservation of the soil environments for sustainable agriculture in some parts of Thailand. However, a lot more research in terms of basic and applied sciences in soybean rhizobium

technology still needs to be carried out in Thailand before we reach the same advanced stage of research and applications in soybean rhizobium technology as found in developed countries where the whole genome of *B. japonicum* has been sequenced (Kaneko et al., 2002) and approximately 51% of the whole genome of *B. japonicum* CPAC 15 have been sequenced (Goday et al., 2008). Finally, a lot of efforts are still needed in the popularization of the use of soybean rhizobium biofertilizers in Thailand (Chanaseni and Kongngoen, 1992) when compared with the use of soybean rhizobium inoculants in other countries (Abaidoo et al., 2007; Aguilar al., 2001; Brutti et al., 1998; Chen et al, 2000, 2004; Hungria et al.,2001; de Jensen et al, 2004; Judd et al, 1993; Minamisawa et al, 1999, Thomas- Oates et al., 2003 and Yanni, 2004).

5.5 Significance of discovering fast-growing bacteria in soybean rhizosphere

Soybean roots secrete flavonoids such as genistein which creates a gradient along which soybean rhizobia move towards the roots to form nodules (Kosslak et al, 1987). Other bacterial populations in soybean rhizosphere have been known to break down these signal flavonoid molecules. The presence of various bacterial populations capable of breaking down the flavonoid molecules could contribute to reduced extent of nodulation of soybeans resulting in lower soybean yields. In this research, isolation and identification by 16S rDNA sequences were obtained for several fast-growing, acid-secreting bacteria belonging to *Agrobacterium tumefaciens* (STB170, Ban Dong) and *Rhizobium tropici* (STB23, Hua Ro and STB97, Mathong). The collection of fast-growing bacteria from the rhizosphere of soybeans provide sets of soybean rhizobia and bacteria in the soybean rhizosphere which form base-line data for further research on dynamics of bacterial populations in soybean rhizosphere as well as study on the impact of rhizospheric bacterial populations on soybean root nodule formation (Taboran and Chansa-ngavej, 2009). In 2010, Udomchotphruet and Chansa-ngavej reported on reversed-phase HPLC conditions which could be used to determine concentrations of genistein. The aim of the research is to detect if fast-growing bacteria isolated from rhizosphere of soybeans could *in vitro* break down genistien, the signal molecule for soybean root nodulation.

CHAPTER VI

CONCLUSION

Polyphasic taxonomy was employed in the Identification of slow-growing soybean rhizobia obtained from the host trapping method using 5 soybean cultivars grown in soils from 16 subdistricts in Phitsanulok province, Thailand. A total of 340 isolates were purified and categorized into fast- and slow-growers based on their visible growth on yeast extract mannitol with 0.25 μg.ml^{-1} Congo red agar plates. Identical RAPD-PCR fingerprints revealed the 202 slow-growing isolates consisted of 121 strains. Authentication tests showed all the 138 fast-growers did not nodulate soybean roots. Thus, they were not fast-growing soybean rhizobia. On the contrary, all the 121 slow-growing strains were found to nodulate soybean roots. Therefore, they were slow-growing soybean rhizobia. Comparisons of 16S rDNA sequences of 20 slow-growing strains with corresponding sequences deposited with GenBank revealed the slow-growing soybean rhizobia consisted of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii and B. yuanmingense*. This research is the first report on the presence of *B. yuanmingense* in Thailand.

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APPENDICES

APPENDIX A

BACTERIAL GROWTH MEDIA AND PLANT NUTRIENT SOLUTIONS

Preparation of all bacterial growth media and plant nutrient solutions are as described by Somasegaran and Hoben (1994) unless otherwise stated.

Yeast Extract Mannitol Broth (YMB)

Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	0.5 g
Deionized water	1.0 g

pH of medium was adjusted to 6.8 with 0.1 N NaOH. The medium was autoclaved at 121°C for 15 min.

Yeast Extract Mannitol Agar (YMA)

YMB	1 liter
Agar	15 g

Agar was added to 1 liter of YMB. The solution was shaken to suspend the agar then autoclaved at 121°C for 15 min. After autoclaving, the medium was shaken to ensure even mixing of melted agar with medium before pouring onto petri dishes and left to solidify.

YMA with Congo Red

Congo Red stock solution: 250 mg of Congo Red dissolved in 100 ml of deionized water. 10 ml of Congo Red stock solution were added to 1 liter of YMA. The final Congo Red concentration was 25 $\mu g.ml^{-1}$. The medium was autoclaved at 121°C or 15 min.

YMA with Bromthymol Blue (BTB)

Bromthymol Blue stock solution: 0.5 g of Bromthymol Blue were dissolved in 100 ml of ethanol. 5 ml of Bromthymol Blue stock solution were added to 1 liter of YMA. The final Bromthymol Blue concentration was 25 $\mu g.ml^{-1}$. The medium was autoclaved at 121°C for 15 min.

N-free Nutrient Solution

Stock Solutions	Chemicals	g/liter
1	$CaCl_2.2H_2O$	294.1
2	KH_2PO_4	136.1
3	$FeC_6H_5O_7.3H_20$	6.7
	$MgSO_4.7H_2O$	123.3
	K_2SO_4	87.0
	$MnSO_4.H_2O$	0.338
4	H_3BO_3	0.247
	$ZnSO_4.7H_2O$	0.288
	$CuSO_4.5H_2O$	0.100
	$CoSO_4.7H_2O$	0.056
	$Na_2MoO_2.7H_2O$	0.048

Warm water was used to prepare stock solutions to get the ferric-citrate into solution. Ten liters of full-strength plant culture solution were prepared as follows:

- To 5 liters of water, add 5 ml of each stock solution and mix,
- Adjust pH to 6.8 with 1 N HCl,
- Dilute to 10 liters by adding water,

Tryptone-Yeast extract (TY) Medium

Tryptone	5.0 g
Yeast extract	3.0 g
$CaCl_2 \cdot H_2O$	0.87 g
Deionized water	1000 ml

pH of medium was adjusted to 6.8 with 0.1 N NaOH. The medium was autoclaved at 121°C for 15 min.

APPENDIX B

CHEMICALS AND SOLUTIONS

1. Solutions for DNA extraction

Saline-EDTA solution

15 mM NaCl, 10 mM EDTA, pH 8.0

0.9 g NaCl, 0.29 g EDTA

were added to distilled water. The final volume was made to 100 ml. 0.1 N NaOH was used to adjust pH to 8.0 before autoclaving at 121°C for 15 min.

DNAzol

DNAzol solution (Molecular Research Lab, MRL) was used according to the manufacturer's instruction.

2. Electrophoresis Buffer

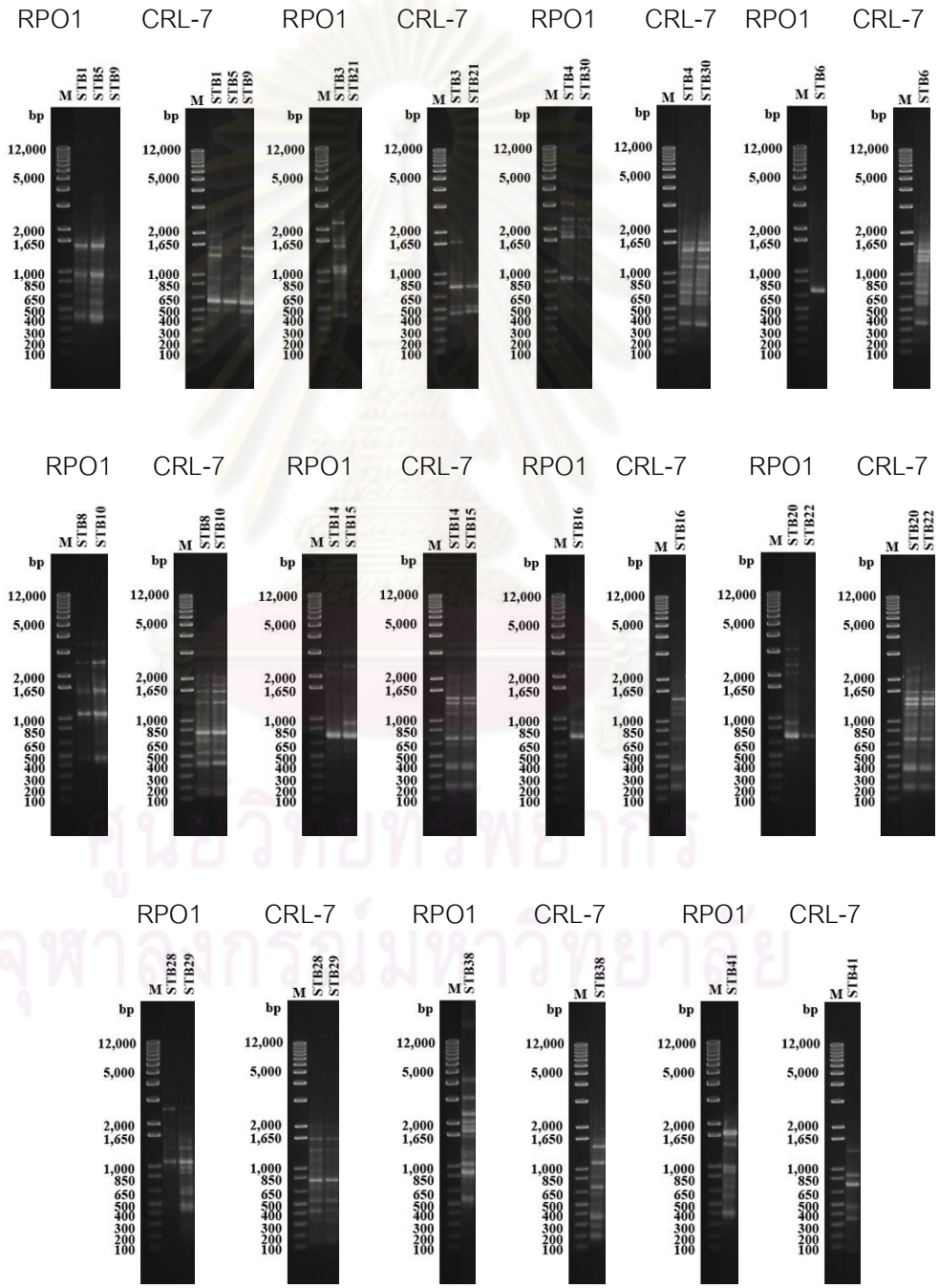
50X Tris Acetate Buffer (TAE buffer)

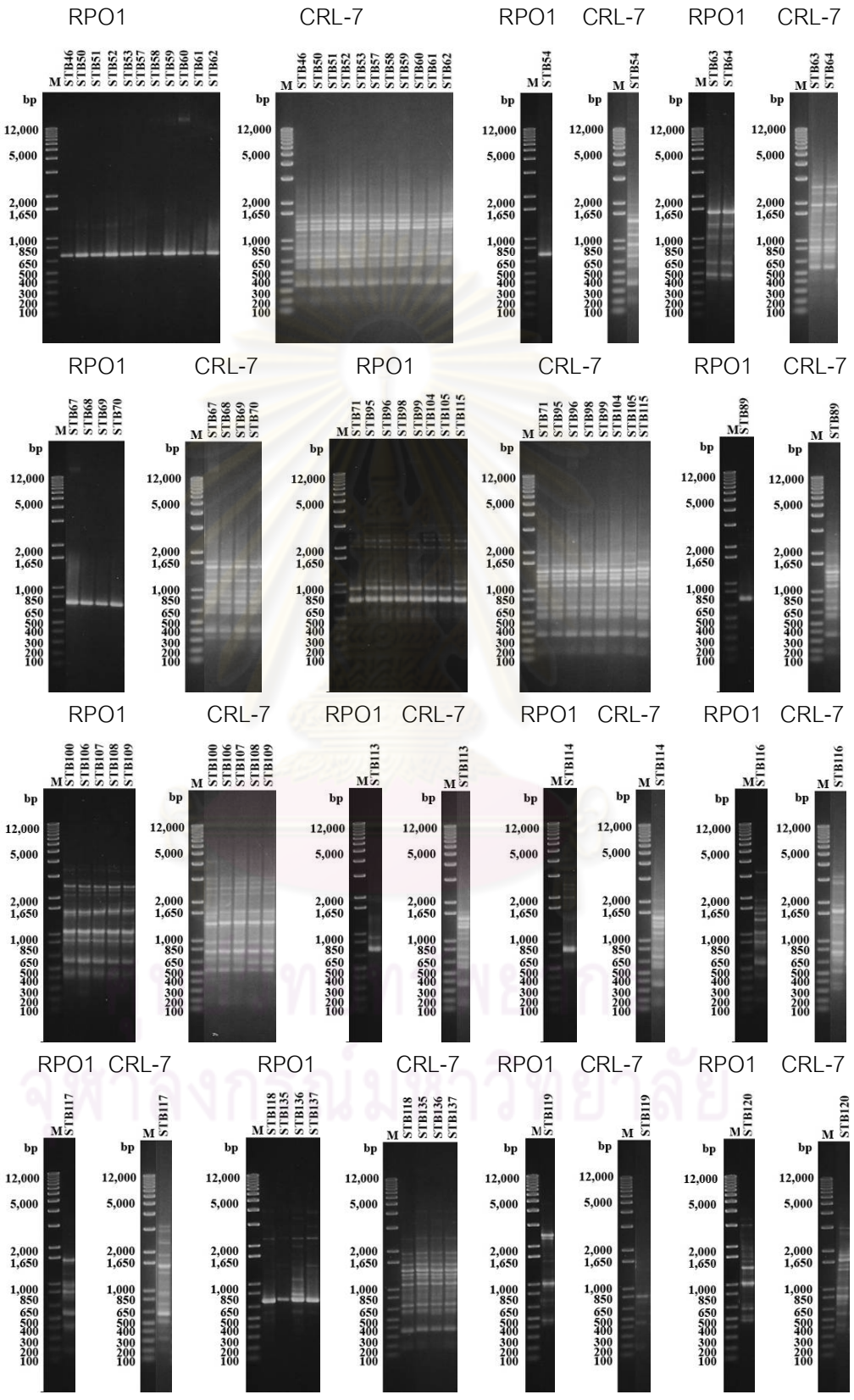
Tris base	242	g.
glacial acetic acid	57.1	ml
0.5 M EDTA pH 8.0	100	ml

were added to double distilled water. 6 N HCl was used to adjust pH to 8.0. The final volume was added to 1000 ml.

APPENDIX C

RAPD-PCR FINGERPRINTS OF 202 SLOW-GROWING SOYBEAN RHIZOBIUM ISOLATES GROUPED AS THE SAME STRAINS

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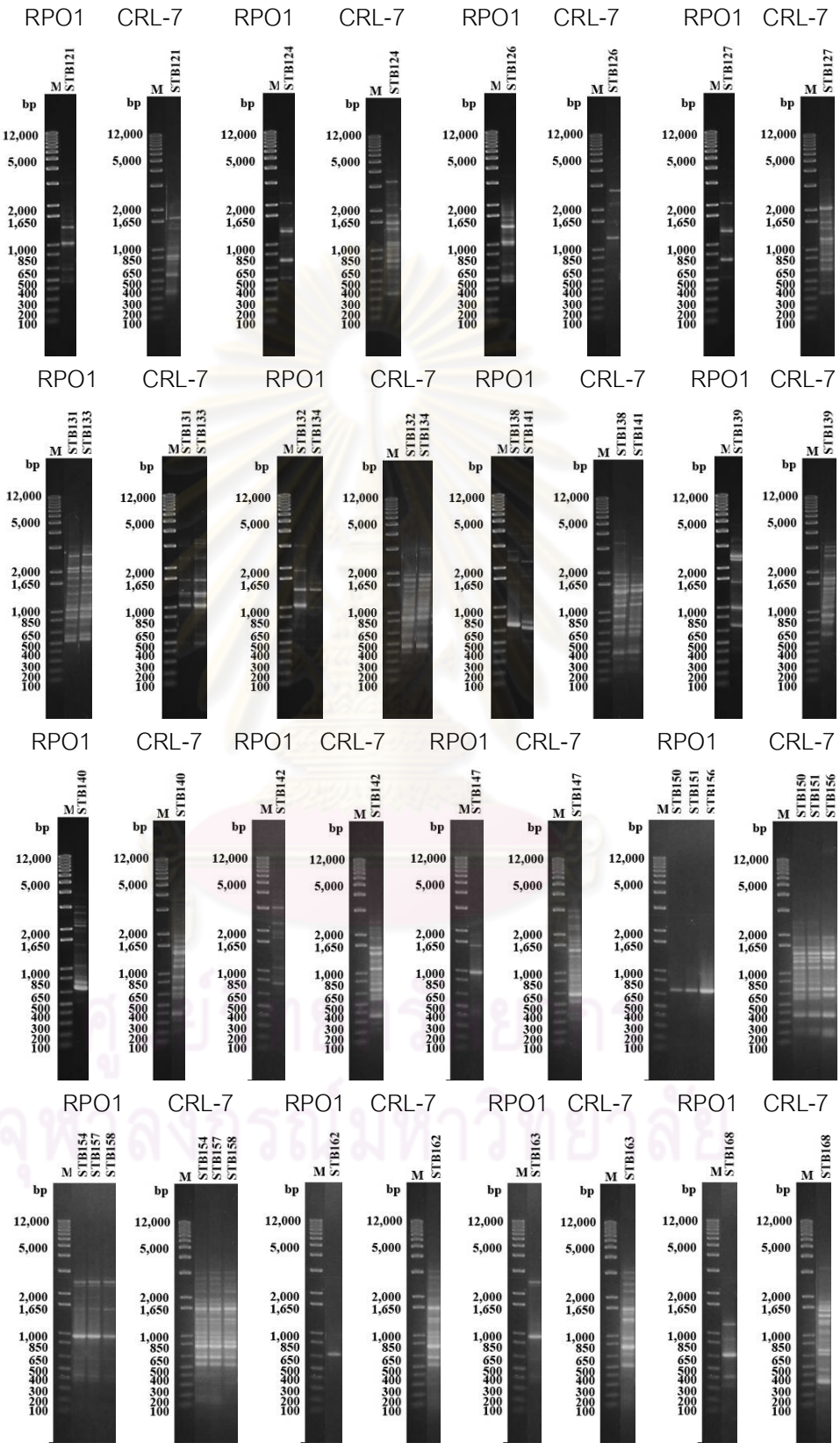
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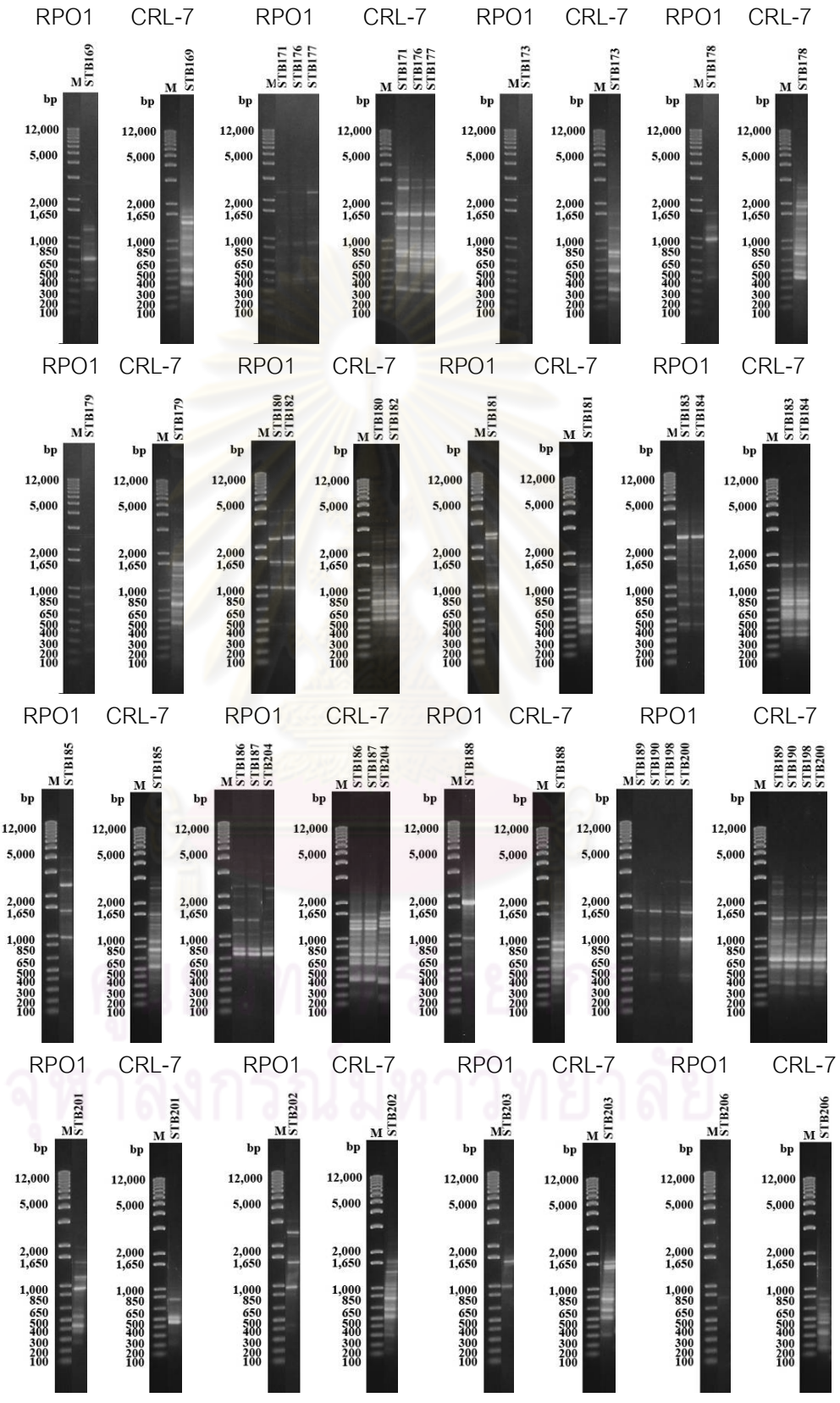
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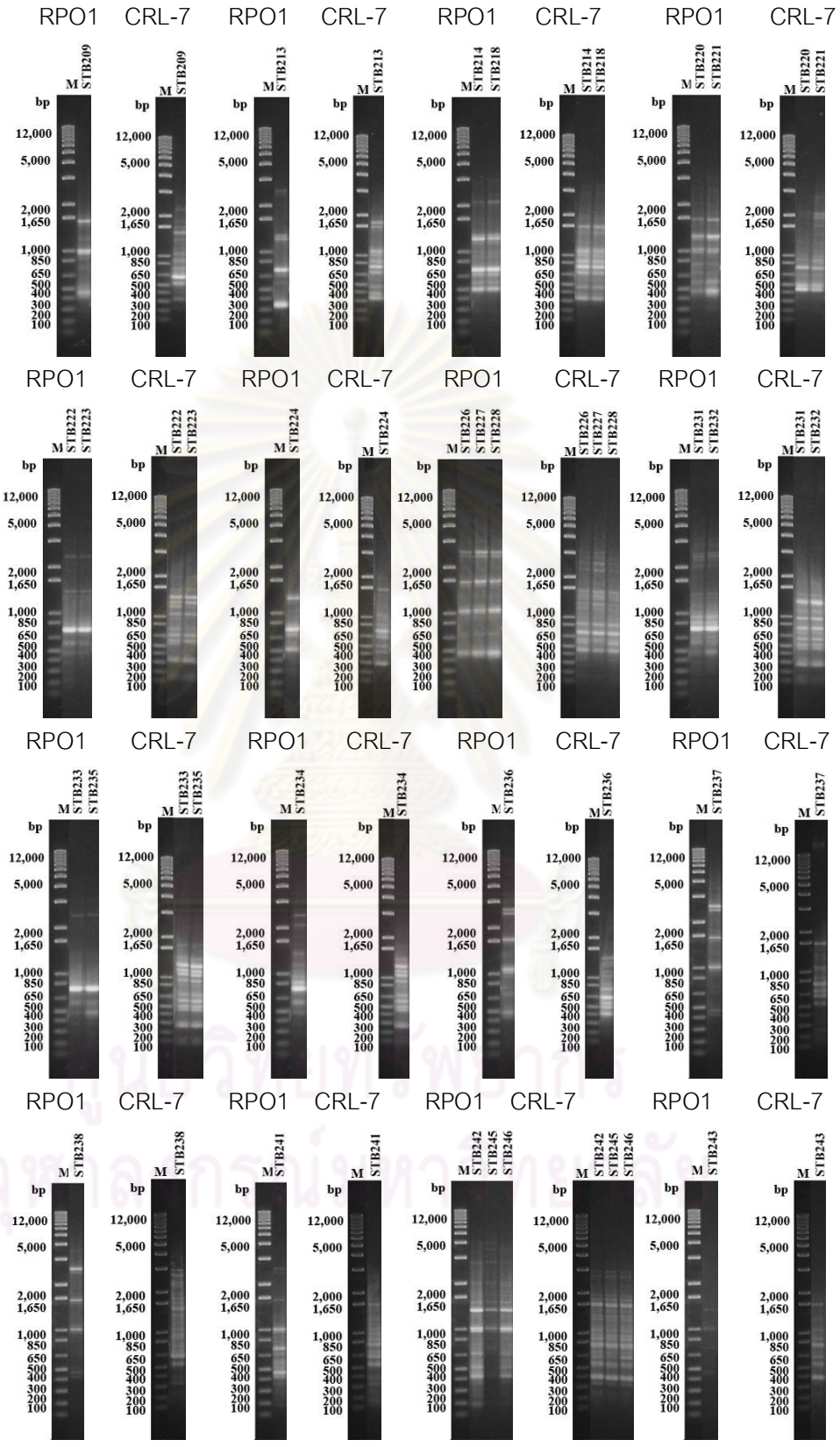
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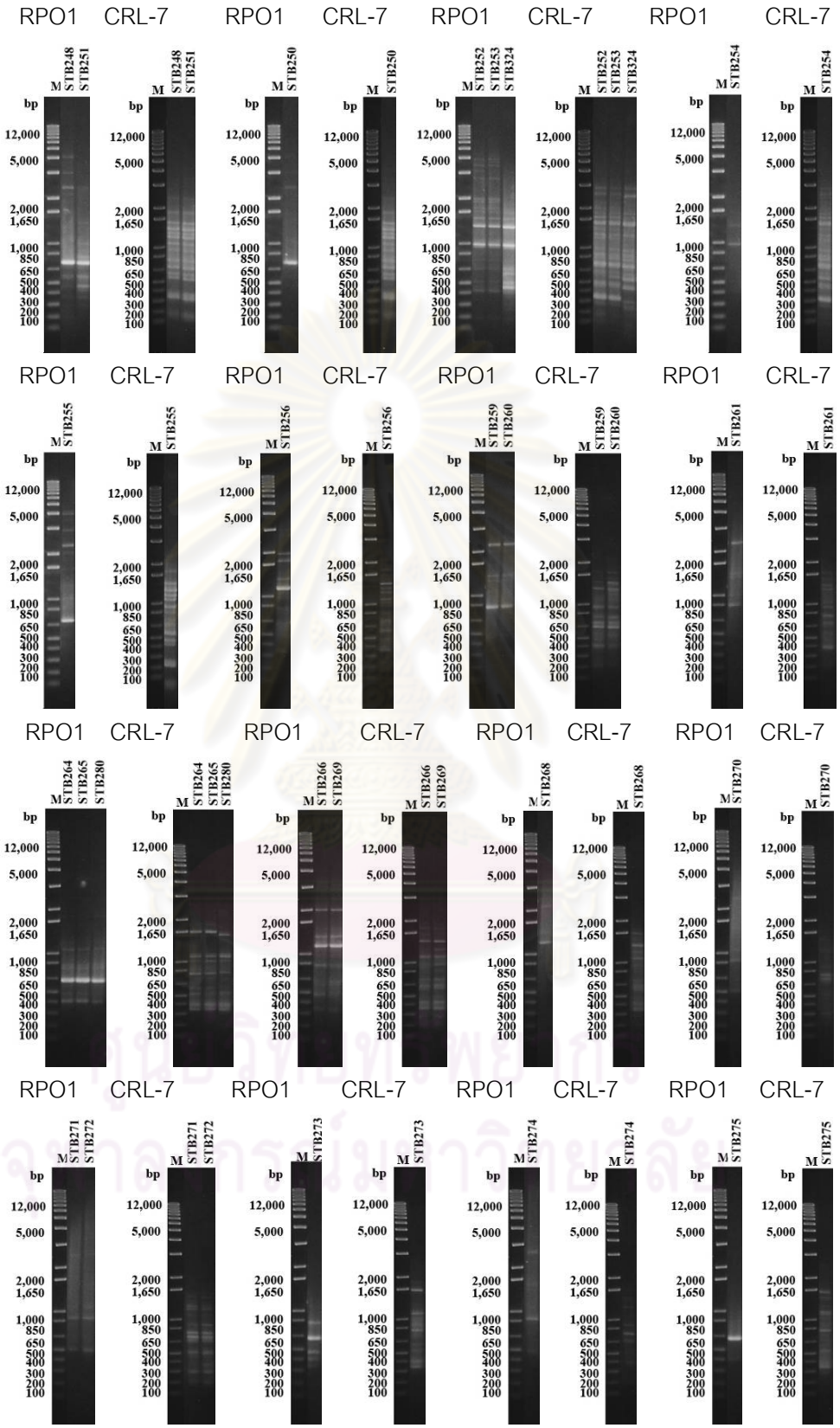
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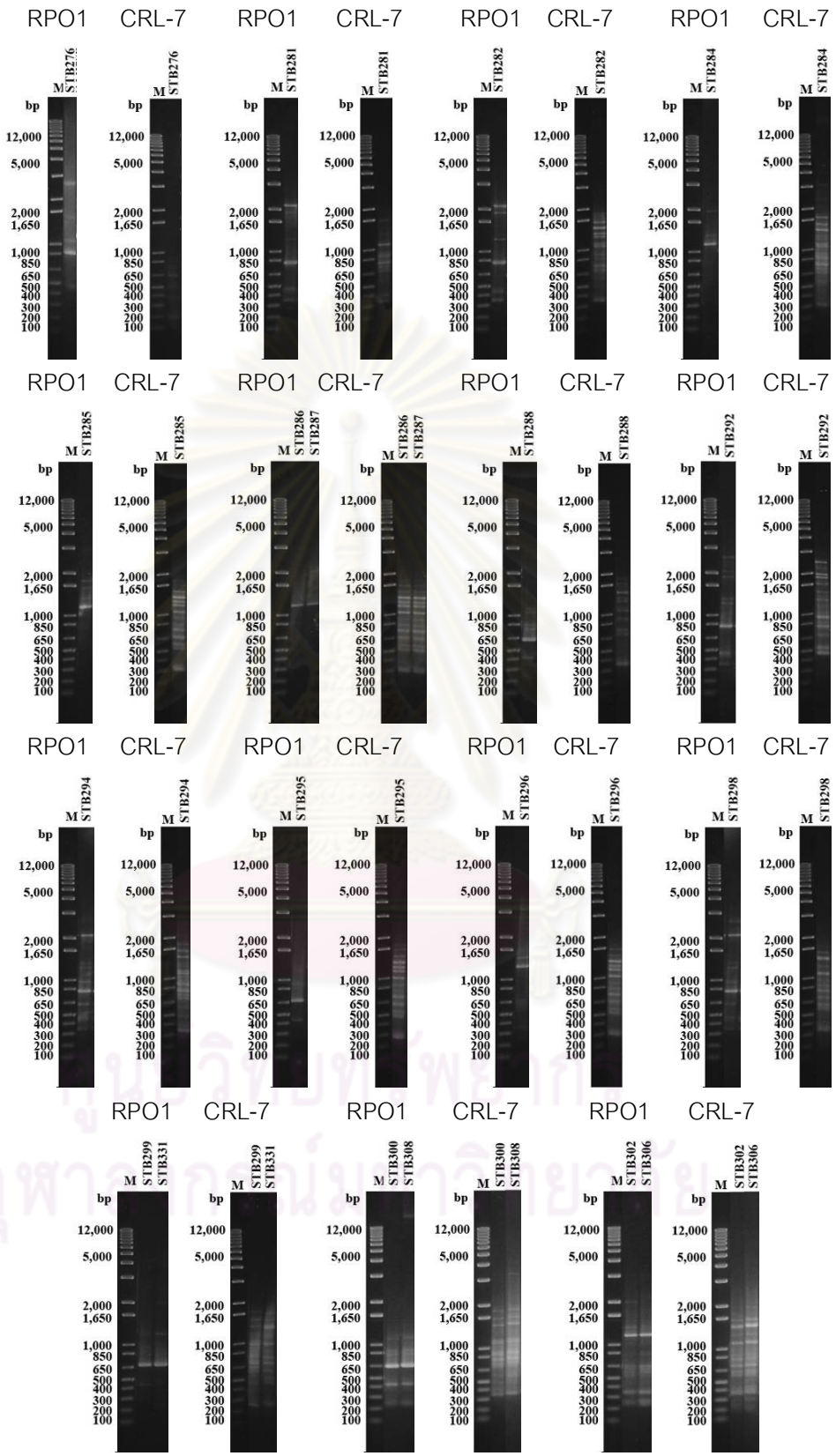
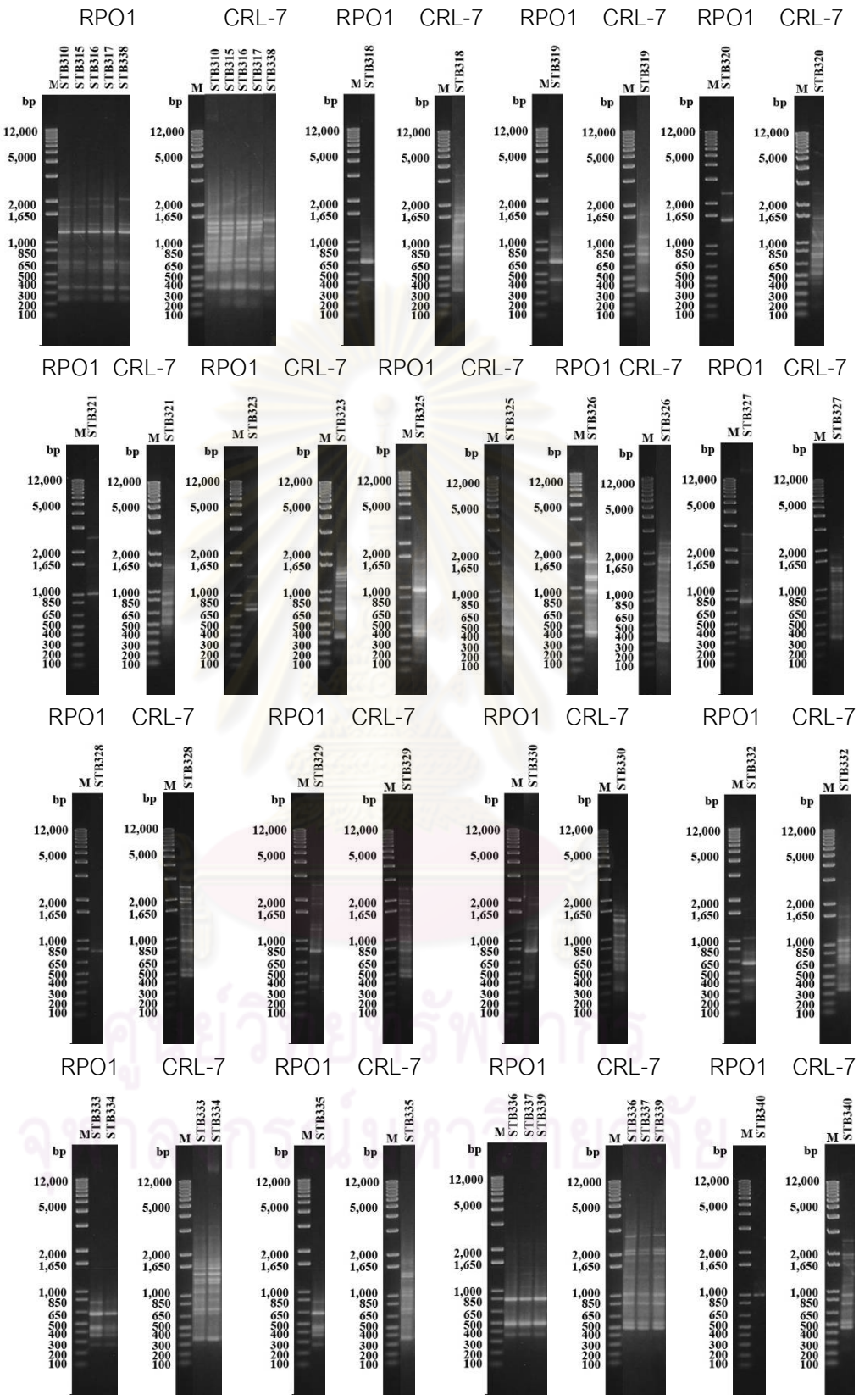

RPO1
CRL-7
M
STB276
STB281
STB282
STB284
STB285
STB286
STB287
STB288
STB292
STB294
STB295
STB296
STB298
STB299
STB331
STB300
STB308
STB302
STB306
bp
12,000
5,000
2,000
1,650
1,000
850
650
500
400
300
200
100


RPO1
CRL-7
M
STB310
STB315
STB316
STB317
STB338
STB318
STB319
STB320
STB321
STB323
STB325
STB326
STB327
STB328
STB329
STB330
STB332
STB333
STB334
STB335
STB336
STB337
STB339
STB340
bp
12,000
5,000
2,000
1,650
1,000
850
650
500
400
300
200
100

APPENDIX D

16S rDNA AND *nodY* SEQUENCES

Figure G.1 16S rDNA sequence of *B. elkanii* STB8 with sequences of primers in boxes.

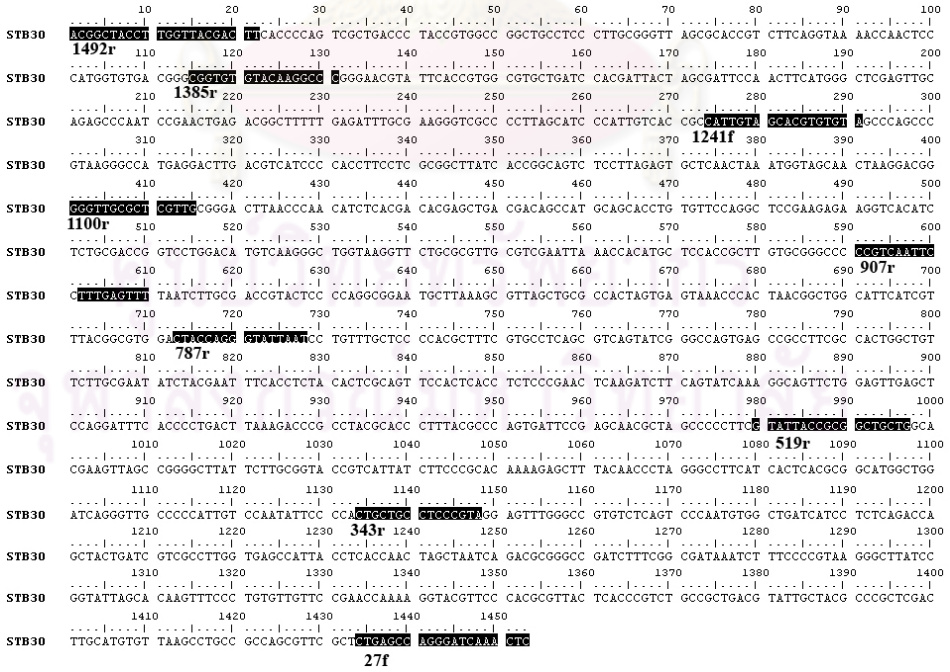


Figure G.2 16S rDNA sequence of *B. japonicum* STB30. Primer sequences in boxes.

Figure G.3 16S rDNA sequence of *B. japonicum* STB54. Sequences of primers are in boxes.

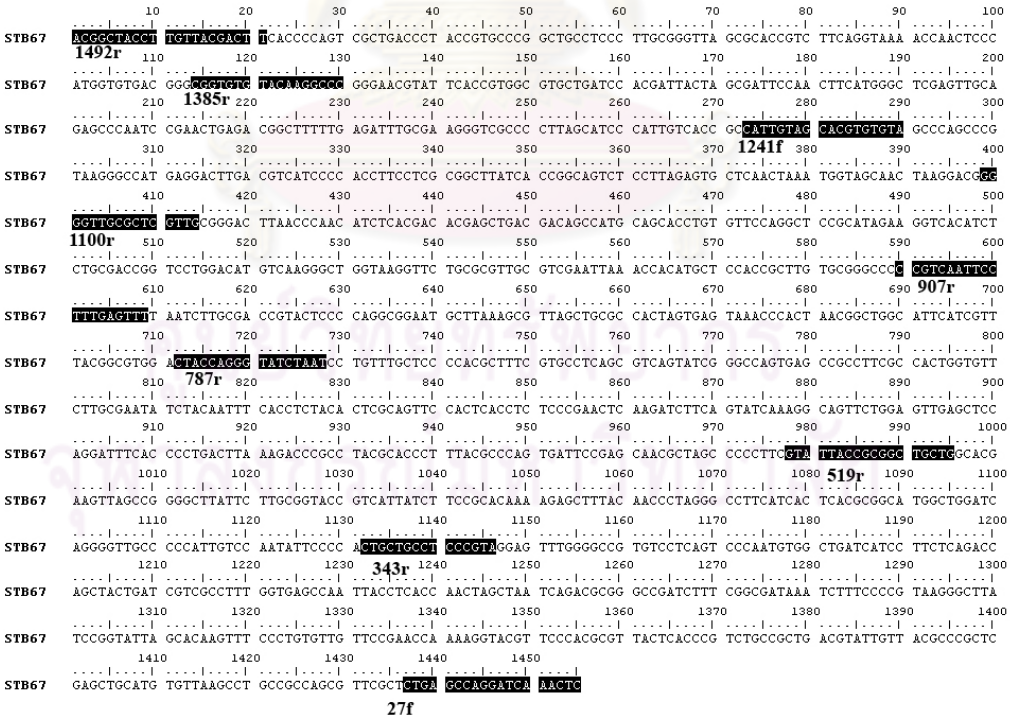


Figure G.4 16S rDNA sequence of *B. japonicum* STB67. Sequences of primers are in boxes.

Figure G.5 16S rDNA sequence of *B. japonicum* STB96. Sequences of primers are in boxes.

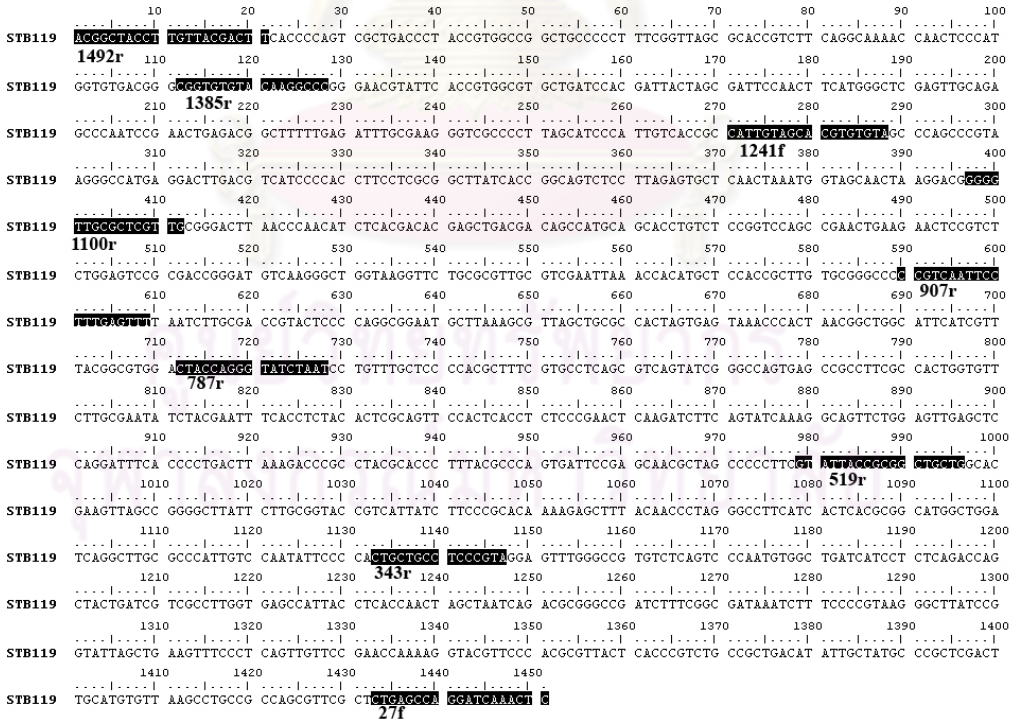


Figure G.6 16S rDNA sequence of *B. elkanii* STB119 with sequences of primers in boxes.

Figure G.7 16S rDNA sequence of *B. elkanii* STB120 with sequences of primers in boxes.

Figure G.8 16S rDNA sequence of *B.elkanii* STB147 with sequences of primers in boxes.

Figure G.9 16S rDNA sequence of *B. yuanmingense* STB169 with with sequences of primers in boxes.

Figure G.10 16S rDNA sequence of *B. elkanii* STB173 with sequences of primers in boxes.

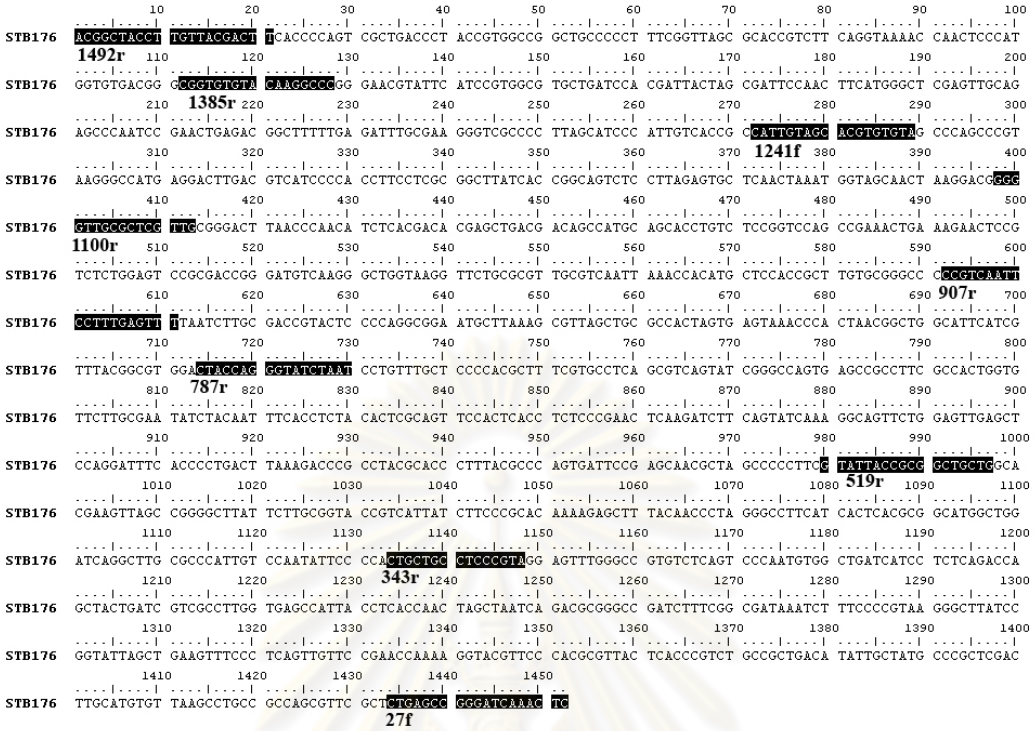


Figure G.11 16S rDNA sequence of *B. elkanii* STB176 with sequences of primers in boxes.

Figure G.12 16S rDNA sequence of *B. elkanii* STB179 with sequences of primers in boxes.

Figure G.13 Sequence of 16S rDNA of *B. elkanii* STB185 with sequences of primers in boxes.

Figure G.14 Sequence of 16S rDNA of *B. elkanii* STB220 with sequences of primers in boxes.

Figure G.15 Sequence of 16S rDNA of *B. elkanii* STB238 with sequences of primers in boxes.

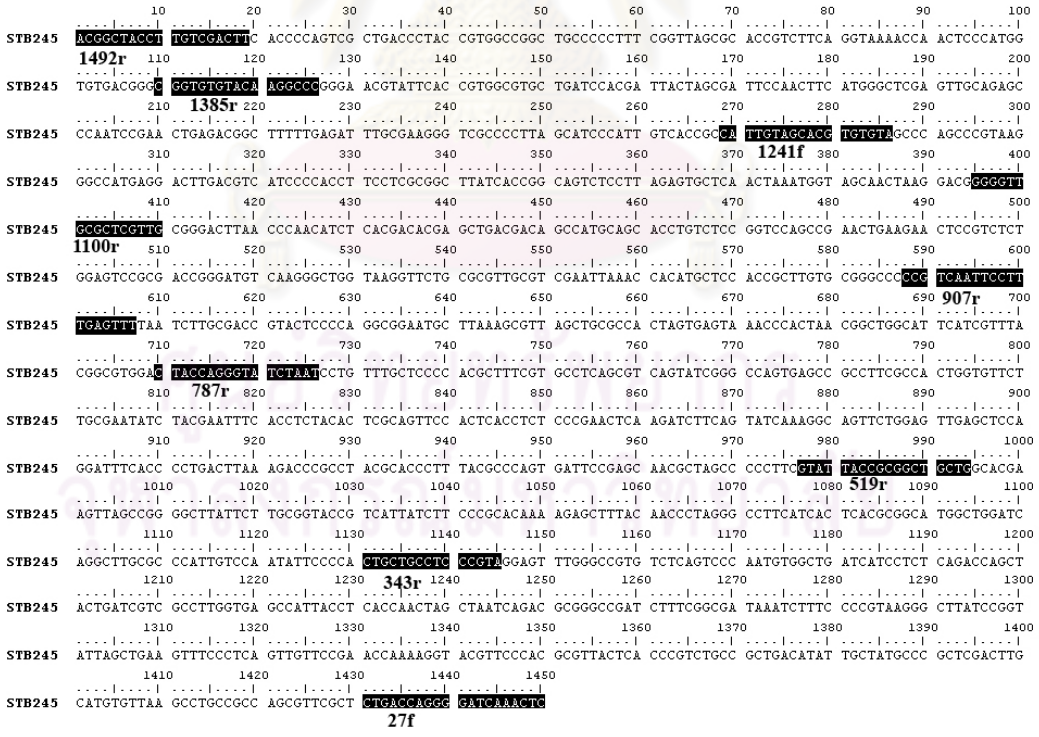


Figure G.16 Sequence of 16S rDNA of *B. japonicum* STB245 with sequences of primers in boxes.

Figure G.17 Sequence of 16S rDNA of *B. yuanmingense* STB264 with sequences of primers in boxes.

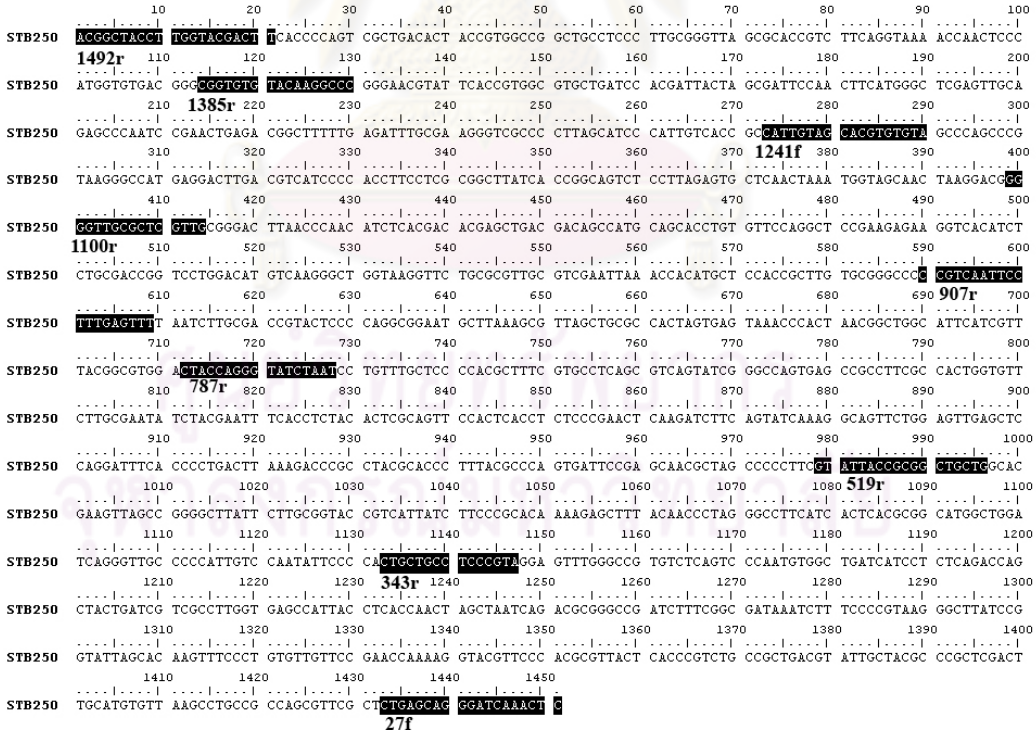


Figure G.18 Sequence of 16S rDNA of *B. japonicum* STB250 with sequences of primers in boxes.

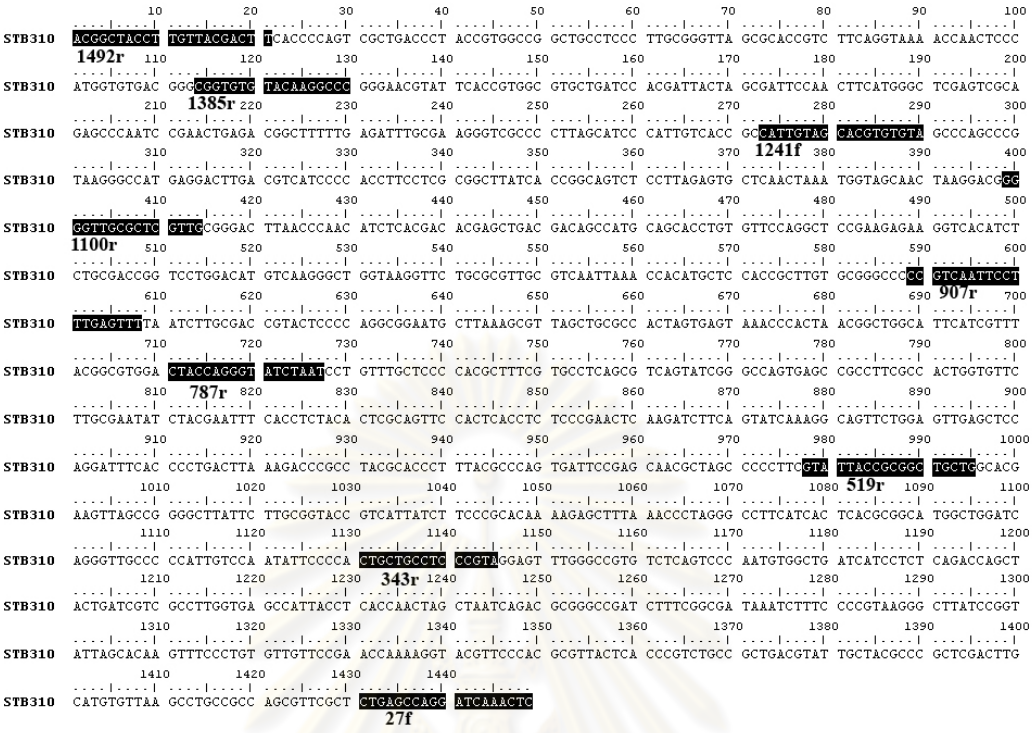


Figure G.19 Sequence of 16S rDNA of *B. japonicum* STB310 with sequences of primers in boxes.

Figure G.20 Sequence of 16S rDNA of *B. elkanii* STB327 with sequences of primers in boxes.

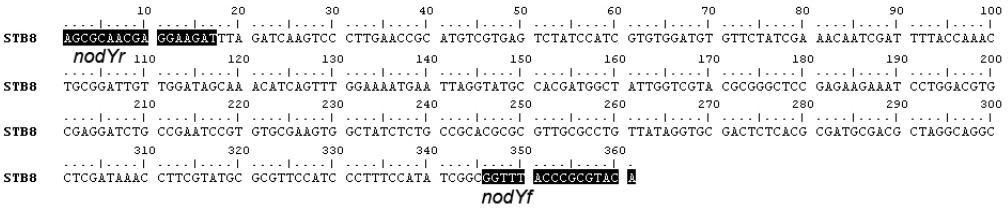


Figure G.21 Sequence of *nodY* of *B. elkanii* STB8 with sequences of primers in boxes.

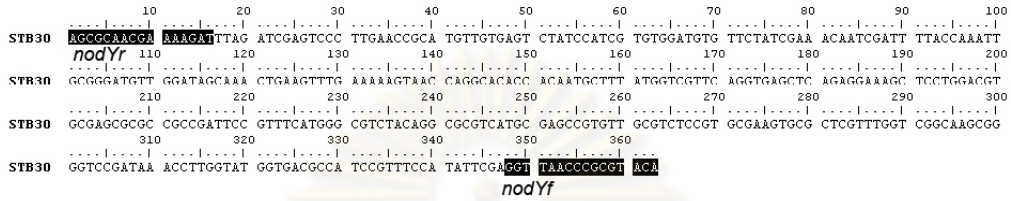


Figure G.22 Sequence of *nodY* of *B. japonicum* STB30 with sequences of primers in boxes.

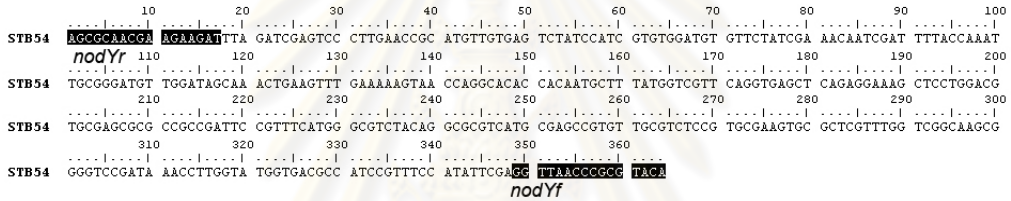


Figure G.23 Sequence of *nodY* of *B. japonicum* STB54 with sequences of primers in boxes.

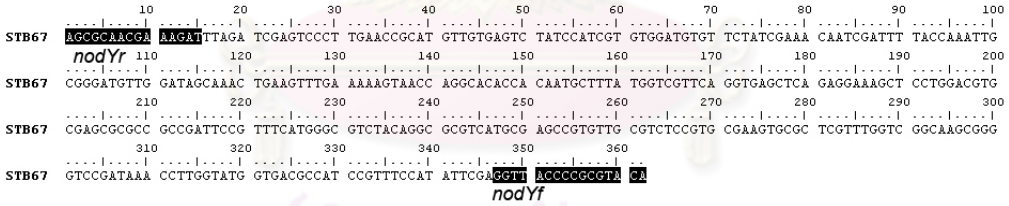


Figure G.24 Sequence of *nodY* of *B. japonicum* STB67 with sequences of primers in boxes.

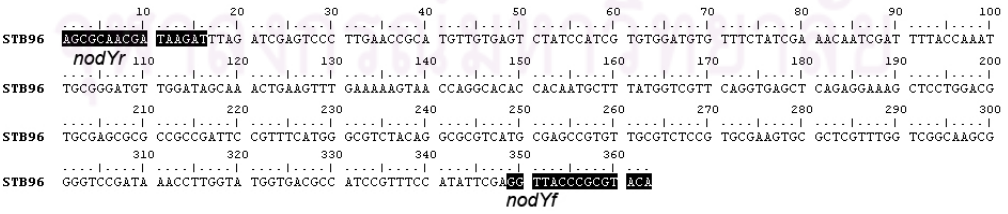


Figure G.25 Sequence of *nodY* of *B. japonicum* STB96 with sequences of primers in boxes.

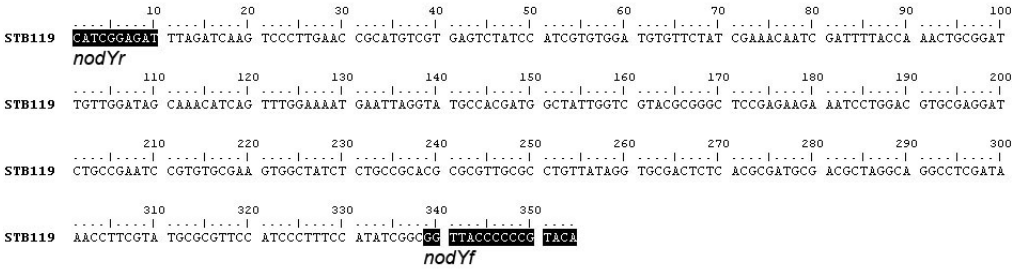


Figure G.26 Sequence of *nodY* of *B. elkanii* STB119 with sequences of primers in boxes.

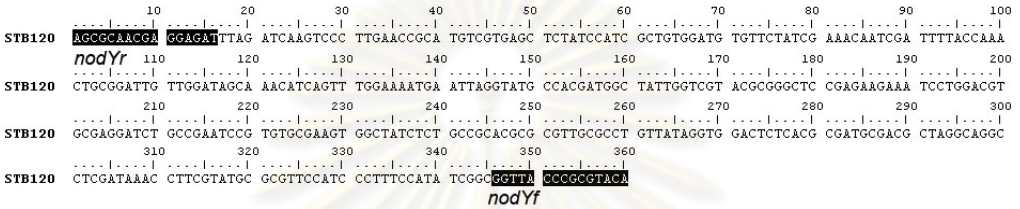


Figure G.27 Sequence of *nodY* of *B. elkanii* STB120 with sequences of primers in boxes.

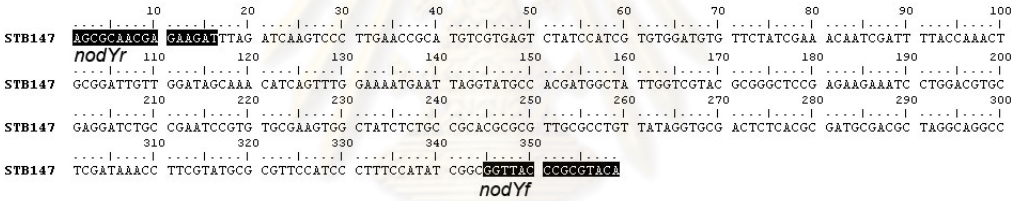


Figure G.28 Sequence of *nodY* of *B. elkanii* STB147 with sequences of primers in boxes.

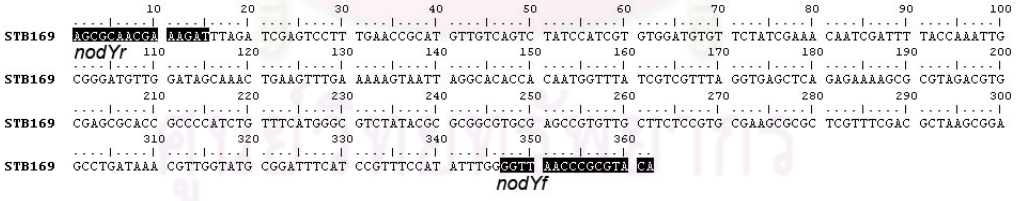


Figure G.29 Sequence of *nodY* of *B. yuanmingense* STB169 with sequences of primers in boxes.

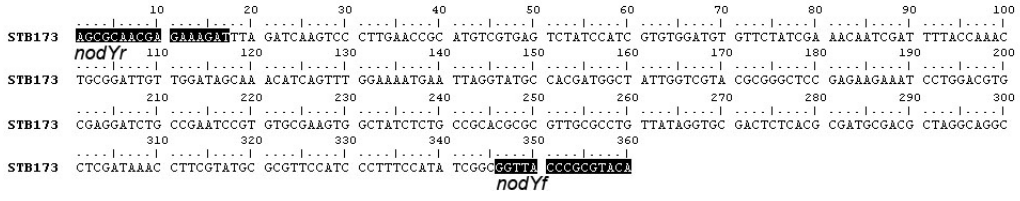


Figure G.30 Sequence of *nodY* of *B. elkanii* STB173 with sequences of primers in boxes.

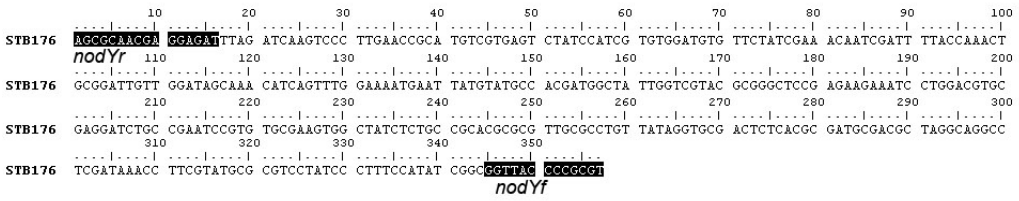


Figure G.31 Sequence of *nodY* of *B. elkanii* STB176 with sequences of primers in boxes.

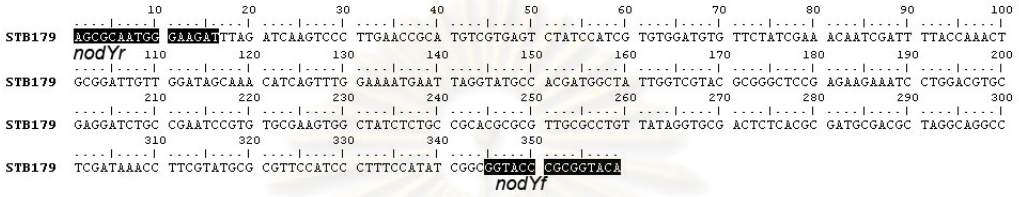


Figure G.32 Sequence of *nodY* of *B. elkanii* STB179 with sequences of primers in boxes.

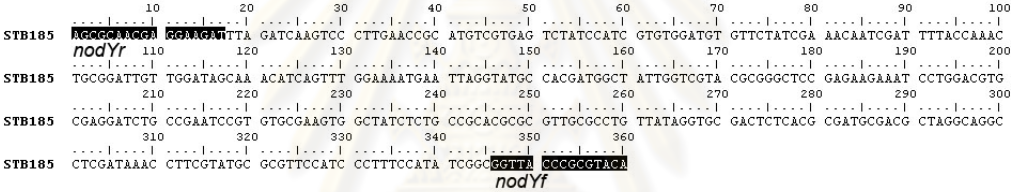


Figure G.33 Sequence of *nodY* of *B. elkanii* STB185 with sequences of primers in boxes.

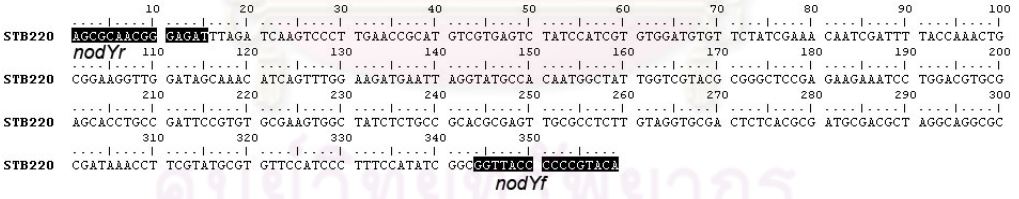


Figure G.34 Sequence of *nodY* of *B. elkanii* STB220 with sequences of primers in boxes.

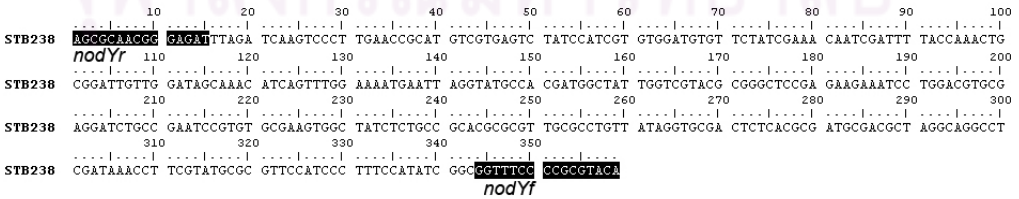


Figure G.35 Sequence of *nodY* of *B. elkanii* STB238 with sequences of primers in boxes.

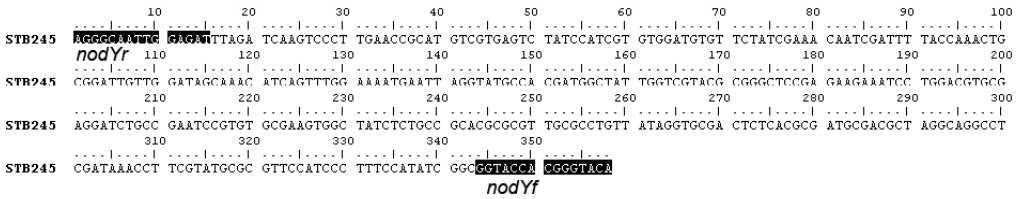


Figure G.36 Sequence of *nodY* of *B. elkanii* STB245 with sequences of primers in boxes.

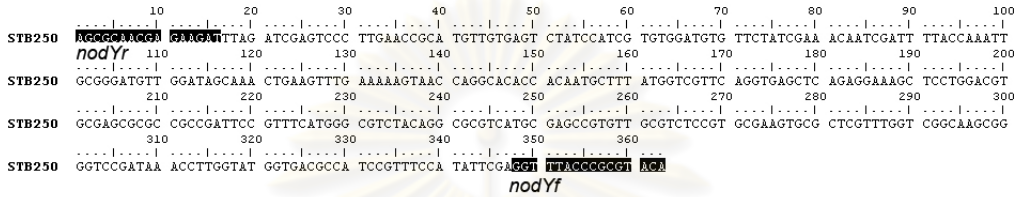


Figure G.37 Sequence of *nodY* of *B. japonicum* STB250 with sequences of primers in boxes.

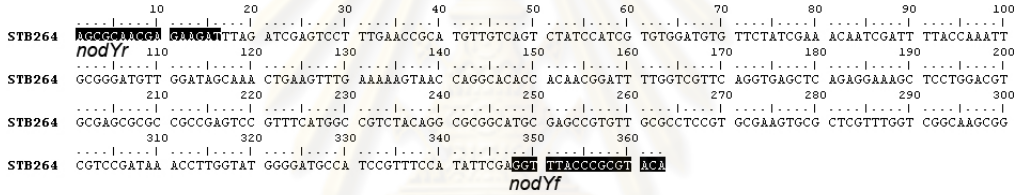


Figure G.38 Sequence of *nodY* of *B. yuanmingense* STB264 with sequences of primers in boxes.

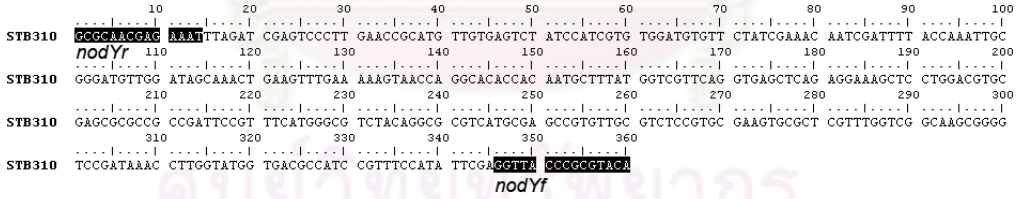


Figure G.39 Sequence of *nodY* of *B. japonicum* STB310 with sequences of primers in boxes.

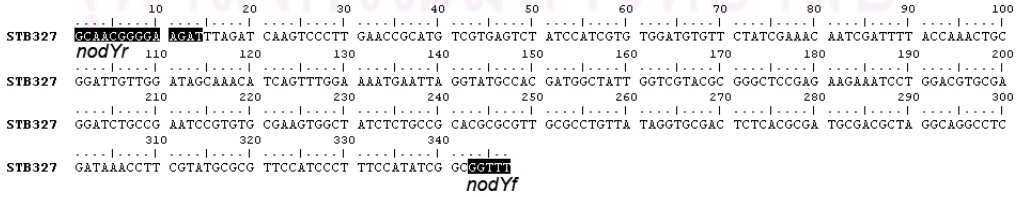


Figure G.40 Sequence of *nodY* of *B. elkanii* STB327 with sequences of primers in boxes.

APPENDIX E

SUMMARY OF ABILITY/INABILITY OF THREE REFERENCE STRAINS AND THREE REPRESENTATIVE STB STRAINS TO USE 95 CARBON AND NITROGEN SOURCES

Table G.1 Determination of ability/inability to utilize 95 carbon/nitrogen sources by reference strains and by *Bradyrhizobium elkanii* strain STB 327.

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Summary of results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	STB327 Grown on TY			Summary of results from 3 determinations
				1st	2nd	3rd	
□-Cyclodextrin	-	-	-	-	-	-	-
Dextrin	+	+	+	-	+	+	+
Glycogen	-	-	+	-	-	+	-
Tween 40	++	++	++	++	++	++	++
Tween 80	++	++	++	++	++	++	++
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-
Adonitol	+	-	-	-	+	+	+
L-Arabinose	++	++	++	+	+	+	+
D-Arabitol	+	++	-	+	+	+	+
D-Cellobiose	-	+	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-
D-Fructose	+	+	-	+	+	+	+
L-Fucose	+	-	-	+	+	+	+
D-Galactose	+	+	+	+	+	+	+
Gentiobiose	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Summary of results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	STB327 Grown on TY			Summary of results from 3 determinations
				1st	2nd	3rd	
□-D-Glucose	++	++	++	+	+	+	+
m-Inositol	-	-	-	-	-	-	-
□-D-Lactose	-	-	-	-	-	-	-
Lactulose	-	-	-	-	+	++	+
Maltose	-	-	-	-	-	-	-
D-Mannitol	++	++	++	+	+	+	+
D-Mannose	++	++	++	-	-	-	-
D-Melibiose	-	-	-	-	-	-	-
□-Methyl-D-Glucoside	+	-	-	-	-	-	-
D-Psicose	+	-	-	+	+	+	+
D-Raffinose	-	-	-	-	-	-	-
L-Rhamnose	-	+	-	-	-	+	-
D-Sorbitol	+	-	-	-	+	+	+
Sucrose	-	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	+	-
Turanose	-	-	-	-	+	-	-
Xylitol				-	+	-	-
Pyruvic Acid Methyl Ester	+++	+++	+++	+	++	++	++
Succinic Acid Mono-Methyl-Ester	+++	+++	+++	+	+	++	+
Acetic Acid	+++	+++	+++	+	+	+	+
Cis-Aconitic Acid	-	-	-	+	+	-	+
Citric Acid	+	-	-	+	+	+	+
Formic Acid	+	+	-	+	++	++	++
D-Galactonic Acid Lactone	++	++	-	+	+	++	+
D-Galacturonic Acid	+	-	-	-	+	+	+
D-Gluconic Acid	++	++	++	+	+	+	+

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Summary of results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	STB327 Grown on TY			Summary of results from 3 determinations
				1st	2nd	3rd	
D-Glucosaminic Acid	+	-	-	+	+	+	+
D-Glucuronic Acid	+	-	-	-	+	+	+
□-Hydroxybutyric Acid	-	++	++	-	-	-	-
□-Hydroxybutyric Acid	+++	+++	+++	++	++	++	++
□-Hydroxybutyric Acid	+++	+++	+++	+	+	++	+
p-Hydroxy Phenylacetic Acid	+	-	+	-	+	+	+
Itaconic acid	-	++	++	-	-	+	-
□-Keto Butyric Acid	-	++	+	-	-	-	-
□-Keto Glutaric Acid	+	++	+	+	+	+	+
□-Keto Valeric Acid	-	++	+	-	-	-	-
D,L-Lactic Acid	+++	+++	+++	+	++	++	++
Malonic Acid	+	-	+	-	+	+	+
Propionic Acid	++	++	++	+	+	++	+
Quinic Acid	+	-	-	-	+	++	+
D-Saccharic Acid	+++	+++	+	++	++	++	++
Sebacic Acid	+	-	+	+	++	++	++
Succinic Acid	+++	+++	+++	+	+	+	+
Bromosuccinic Acid	++	++	++	+	+	+	+
Succinamic Acid	+++	+++	+++	++	++	++	++
Glucuronamide	+	-	-	+	-	+	+
L-Alaninamide	+	++	-	+	+	+	+
D-Alanine	+	-	-	+	+	+	+
L-Alanine	+	-	+	-	+	+	+
L-Alanyl-glycine	+	+	-	-	+	+	+
L-Asparagine	+	-	-	-	+	+	+

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Summary of results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	STB327 Grown on TY			Summary of results from 3 determinations
				1st	2nd	3rd	
L-Aspartic Acid	+	-	+	-	++	++	++
L-Glutamic Acid	+	-	-	+	++	++	++
Glycyl-L-Aspartic Acid	+	-	-	-	+	+	+
Glycyl-L-Glutamic Acid	-	-	+	-	+	-	-
L-Histidine	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	+	++	++	+	+	+	+
L-Ornithine	-	-	+	-	-	-	-
L-Phenylalanine	+	++	+	-	+	+	+
L-Proline	+	++	-	-	+	+	+
L-Pyroglutamic Acid	++	++	++	+	+	+	+
D-Serine	+	-	-	+	+	+	+
L-Serine	+	-	-	-	+	+	+
L-Threonine	-	+	-	-	-	-	-
D,L-Carnitine	-	-	-	-	+	-	-
□-Amino Butyric Acid	-	+	-	-	-	+	-
Urocanic Acid	-	-	-	+	-	-	-
Inosine	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-
Phenyethyl-amine	-	-	-	-	-	+	-
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	+	-	-	-	+	+	+
Glycerol	+	++	+	+	+	+	+
D,L-□-Glycerol Phosphate	+	-	-	-	+	+	+
□-D-Glucose-1-	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Summary of results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	STB327 Grown on TY			Summary of results from 3 determinations
				1st	2nd	3rd	
Phosphate							
D-Glucose-6-Phosphate	-	-	-	-	+	-	-

Table G.2 Determination of ability/inability to utilize 95 carbon/nitrogen sources by reference strains and by *Bradyrhizobium japonicum* strain STB 310.

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Summary of results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	STB310 Grown on TY			Summary of results from 3 determinations
				1st	2nd	3rd	
□-Cyclodextrin	-	-	-	+	+	-	+
Dextrin	+	+	+	+	+	+	+
Glycogen	-	-	+	-	-	-	-
Tween 40	++	++	++	++	++	++	++
Tween 80	++	++	++	++	+++	+++	+++
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-
Adonitol	+	-	-	-	-	-	-
L-Arabinose	++	++	++	++	++	++	++
D-Arabitol	+	++	-	++	+	++	++
D-Cellobiose	-	+	-	++	+	-	+
i-Erythritol	-	-	-	-	-	-	-
D-Fructose	+	+	-	+	+	++	+

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Summary of results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	STB310 Grown on TY			Summary of results from 3 determinations
				1st	2nd	3rd	
L-Fucose	+	-	-	-	-	-	-
D-Galactose	+	+	+	+	+	-	+
Gentiobiose	-	-	-	-	-	-	-
□-D-Glucose	++	++	++	++	++	++	++
m-Inositol	-	-	-	-	-	-	-
□-D-Lactose	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-
D-Mannitol	++	++	++	++	+	++	++
D-Mannose	++	++	++	++	++	++	++
D-Melibiose	-	-	-	-	-	-	-
□-Methyl-D-Glucoside	+	-	-	-	-	-	-
D-Psicose	+	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-
L-Rhamnose	-	+	-	+	+	-	+
D-Sorbitol	+	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	-
Turanose	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+++	+++	+++	+++	+++	+++	+++
Succinic Acid Mono-Methyl-Ester	+++	+++	+++	+++	+++	+++	+++
Acetic Acid	+++	+++	+++	+++	+++	+++	+++
Cis-Aconitic Acid	-	-	-	-	-	-	-
Citric Acid	+	-	-	-	-	-	-
Formic Acid	+	+	-	+	+	++	+

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Summary of results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	STB310 Grown on TY			Summary of results from 3 determinations
				1st	2nd	3rd	
D-Galactonic Acid Lactone	++	++	-	+	++	++	++
D-Galacturonic Acid	+	-	-	-	+	-	-
D-Gluconic Acid	++	++	++	++	++	++	++
D-Glucosaminic Acid	+	-	-	-	-	-	-
D-Glucuronic Acid	+	-	-	-	-	-	-
□-Hydroxybutyric Acid	-	++	++	++	++	+++	++
□-Hydroxybutyric Acid	+++	+++	+++	+++	+++	+++	+++
□-Hydroxybutyric Acid	+++	+++	+++	+++	+++	+++	+++
p-Hydroxy Phenylacetic Acid	+	-	+	+	+	-	+
Itaconic acid	-	++	++	++	++	++	++
□-Keto Butyric Acid	-	++	+	++	++	++	++
□-Keto Glutaric Acid	+	++	+	++	++	++	++
□-Keto Valeric Acid	-	++	+	++	++	++	++
D,L-Lactic Acid	+++	+++	+++	+++	+++	+++	+++
Malonic Acid	+	-	+	-	-	-	-
Propionic Acid	++	++	++	++	++	++	++
Quinic Acid	+	-	-	-	-	-	-
D-Saccharic Acid	+++	+++	+	+++	+++	+++	+++
Sebacic Acid	+	-	+	+	-	-	-
Succinic Acid	+++	+++	+++	+++	+++	+++	+++
Bromosuccinic Acid	++	++	++	++	++	++	++
Succinamic Acid	+++	+++	+++	+++	+++	+++	+++
Glucuronamide	+	-	-	-	-	-	-
L-Alaninamide	+	++	-	++	++	++	++

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Summary of results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	STB310 Grown on TY			Summary of results from 3 determinations
				1st	2nd	3rd	
D-Alanine	+	-	-	-	-	-	-
L-Alanine	+	-	+	-	-	-	-
L-Alanyl-glycine	+	+	-	+	+	-	+
L-Asparagine	+	-	-	-	-	-	-
L-Aspartic Acid	+	-	+	-	-	++	-
L-Glutamic Acid	+	-	-	+	-	-	-
Glycyl-L-Aspartic Acid	+	-	-	-	+	-	-
Glycyl-L-Glutamic Acid	-	-	+	-	-	-	-
L-Histidine	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	+	++	++	++	++	++	++
L-Ornithine	-	-	+	-	-	-	-
L-Phenylalanine	+	++	+	++	++	+++	++
L-Proline	+	++	-	++	++	-	++
L-Pyroglutamic Acid	++	++	++	++	++	+++	++
D-Serine	+	-	-	-	-	-	-
L-Serine	+	-	-	-	-	-	-
L-Threonine	-	+	-	+	+	++	+
D,L-Carnitine	-	-	-	-	-	-	-
□-Amino Butyric Acid	-	+	-	+	+		+
Urocanic Acid	-	-	-	+	-	-	-
Inosine	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-
Phenyethyl-amine	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	+	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Summary of results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	STB310 Grown on TY			Summary of results from 3 determinations
				1st	2nd	3rd	
Glycerol	+	++	+	++	++	+++	++
D,L-□-Glycerol Phosphate	+	-	-	-	+	-	-
□-D-Glucose-1-Phosphate	-	-	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	-	-

Table G.3 Determination of ability/inability to utilize 95 carbon/nitrogen sources by reference strains and by *Bradyrhizobium yuanmingense* strain STB 264.

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Summary of results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	STB264 Grown on TY			Summary of results from 3 determinations
				1st	2nd	3rd	
□-Cyclodextrin	-	-	-	+	+	-	+
Dextrin	+	+	+	+	+	+	+
Glycogen	-	-	+	-	-	-	-
Tween 40	++	++	++	+	+	++	+
Tween 80	++	++	++	+	++	++	++
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-
Adonitol	+	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Summary of results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	STB264 Grown on TY			Summary of results from 3 determinations
				1st	2nd	3rd	
L-Arabinose	++	++	++	++	++	+++	++
D-Arabitol	+	++	-	+	+	+	+
D-Cellobiose	-	+	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-
D-Fructose	+	+	-	+	+	+	+
L-Fucose	+	-	-	+	-	+	+
D-Galactose	+	+	+	+	+	+	+
Gentiobiose	-	-	-	-	-	-	-
□-D-Glucose	++	++	++	+	+	+	+
m-Inositol	-	-	-	-	-	-	-
□-D-Lactose	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-
D-Mannitol	++	++	++	+	+	+	+
D-Mannose	++	++	++	+	+	+	+
D-Melibiose	-	-	-	-	-	-	-
□-Methyl-D-Glucoside	+	-	-	-	-	-	-
D-Psicose	+	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-
L-Rhamnose	-	+	-	+	-	+	+
D-Sorbitol	+	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	-
Turanose	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+++	+++	+++	+	++	++	++
Succinic Acid Mono-	+++	+++	+++	++	++	++	++

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Summary of results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	STB264 Grown on TY			Summary of results from 3 determinations
				1st	2nd	3rd	
Methyl-Ester							
Acetic Acid	+++	+++	+++	+	+	++	+
Cis-Aconitic Acid	-	-	-	-	-	-	-
Citric Acid	+	-	-	-	-	-	-
Formic Acid	+	+	-	+	+	++	+
D-Galactonic Acid Lactone	++	++	-	+	-	+	+
D-Galacturonic Acid	+	-	-	-	-	-	-
D-Gluconic Acid	++	++	++	+	+	+	+
D-Glucosaminic Acid	+	-	-	-	-	-	-
D-Glucuronic Acid	+	-	-	-	-	-	-
□-Hydroxybutyric Acid	-	++	++	+	+	+	+
□-Hydroxybutyric Acid	+++	+++	+++	++	+++	+++	+++
□-Hydroxybutyric Acid	+++	+++	+++	++	++	++	++
p-Hydroxy Phenylacetic Acid	+	-	+	+	-	+	+
Itaconic acid	-	++	++	+	-	+	+
□-Keto Butyric Acid	-	++	-	+	-	+	+
□-Keto Glutaric Acid	-	++	-	+	-	-	-
□-Keto Valeric Acid	-	++	-	+	+	+	+
D,L-Lactic Acid	+++	+++	+++	++	++	++	++
Malonic Acid	-	-	-	-	-	-	-
Propionic Acid	++	++	++	+	+	+	+
Quinic Acid	+	-	-	-	-	-	-
D-Saccharic Acid	+++	+++	+	+	-	-	-
Sebacic Acid	+	-	+	+	-	+	+

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Summary of results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	STB264 Grown on TY			Summary of results from 3 determinations
				1st	2nd	3rd	
Succinic Acid	+++	+++	+++	++	++	++	++
Bromosuccinic Acid	++	++	++	++	++	++	++
Succinamic Acid	+++	+++	+++	++	++	++	++
Glucuronamide	+	-	-	+	-	-	-
L-Alaninamide	+	++	-	-	-	-	-
D-Alanine	+	-	-	+	-	-	-
L-Alanine	+	-	+	-	-	-	-
L-Alanyl-glycine	+	+	-	-	-	-	-
L-Asparagine	+	-	-	-	-	-	-
L-Aspartic Acid	+	+	+	-	-	-	-
L-Glutamic Acid	+	-	-	+	-	+	+
Glycyl-L-Aspartic Acid	+	-	-	+	-	-	-
Glycyl-L-Glutamic Acid	-	-	+	+	-	-	-
L-Histidine	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	+	++	++	+	-	+	+
L-Ornithine	-	-	+	-	-	-	-
L-Phenylalanine	+	++	+	+	+	+	+
L-Proline	+	++	-	+	+	-	+
L-Pyroglutamic Acid	++	++	++	++	++	++	++
D-Serine	+	-	-	-	-	-	-
L-Serine	+	-	-	-	-	-	-
L-Threonine	-	+	-	+	+	+	+
D,L-Carnitine	-	-	-	-	-	-	-
□-Amino Butyric Acid	-	+	-	+	-	-	-
Urocanic Acid	-	-	-	-	-	-	-
Inosine	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Summary of results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	STB264 Grown on TY			Summary of results from 3 determinations
				1st	2nd	3rd	
Thymidine	-	-	-	-	-	-	-
Phenyethyl-amine	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	+	-	-	-	-	-	-
Glycerol	+	++	+	+	+	+	+
D,L-□-Glycerol Phosphate	+	-	-	-	-	-	-
□-D-Glucose-1-Phosphate	-	-	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	-	-

BIOGRAPHY

Mrs Sujidkanlaya Maruekarajtinplaeng was born on May 9, 1970. She obtained a Bachelor of Science Degree in Microbiology from Khon Kaen University, Thailand, in 1993, and a Master of Science Degree in Industrial Microbiology from Chulalongkorn University in 1999. She has been a faculty member at the Faculty of Science and Technology, Phranakhon Sri Ayutthaya University since 2006.

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