ความหลากหลายทางพันธุกรรมของไรโซเบียมถั่วเหลืองที่ตำบลหนองกุลา จังหวัดพิษณุโลก

Genetic Diversity of Soybean Rhizobia atNong Kula Subdistrict, PhitsanulokProvince

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ABSTRACT

Soybean rhizobia are bacteria in soybean root nodulesable to convert atmospheric nitrogen toammonia for the plant to assimilate for growth.At present,there has been an annual decline in soybean cultivation areas and Thailand imports approximately 85% of local soybean consumption resulting in a trade deficit and in an opportunity loss of sustainable maintenance of soil quality. The aims of this research were to isolate and identify soybean rhizobia from Nong Kula subdistrict, Phitsanulok province. Experimental methods consisted of growing soybeans, cultivar Chiangmai2 from seeds in a 15 x 24sq. m. plot in Nong Kula subdistrict, Phitsanulok province, collecting root nodules for the isolation of bacteria, DNA fingerprinting of root nodule bacterial isolates by PCR reaction, construction of a dendrogram from DNA fingerprints, and determination of soybean yield.48 strains of slow-growing root nodule bacteria ofdifferent DNA fingerprints which were closely related to *Bradyrhizobiumelkanii, B. japonicum,* and *B. yuanmingense* were obtained. These strains will be furtherselected forthe production of biofertilizers.

KeyWords: genetic diversity, soybean rhizobium, Nong Kula subdistrict, Phitsanulok province

บทคัดย่อ

ไรโซเบียมถั่วเหลืองเป็นแบคทีเรียในปมรากถั่วเหลืองซึ่งเปลี่ยนไนโตรเจนจากอากาศ เป็นแอมโมเนียให้ถั่วเหลืองใช้ในการเจริญ ในปัจจุบันพื้นที่เพาะปลูกถั่วเหลืองในประเทศไทยลดลงทุกปีและประเทศไทยนำเข้าถั่วเหลืองประมาณ 85% ของถั่วเหลืองที่บริโภคในประเทศ ทำให้ขาดดุลการค้าและขาดการบำรุงดินอย่างยั่งยืน วัตถุประสงค์ของงานวิจัยเพื่อแยกและจำแนกชนิดไรโซเบียมถั่วเหลืองจาก ต.หนองกุลา จ. พิษณุโลก วิธีทดลองประกอบด้วย การปลูกเมล็ดถั่วเหลืองพันธุ์เชียงใหม่ 2 ในแปลงทดลองขนาด 15 x 24ตารางเมตรใน ต.หนองกุลา จ. พิษณุโลก การเก็บรากติดปมของถั่วเหลืองการแยกแบคทีเรียประเภทเพิ่มจำนวนช้าจากปมรากถั่วเหลืองและหาลายพิมพ์ดีเอ็นเอโดยใช้ปฏิกิริยาพีซีอาร์การสร้างต้นไม้วิวัฒนาการจากลายพิมพ์ดีเอ็นเอและการหาผลผลิตถั่วเหลืองในแปลงทดลอง ผลการทดลองได้แบคทีเรียประเภทเพิ่มจำนวนช้าที่มีความหลากหลายด้านลายพิมพ์ดีเอ็นเอ จำนวน 48 สายพันธุ์ที่มีความใกล้ชิดทางวิวัฒนาการกับ *Bradyrhizobiumelkanii, B. japonicum,* และ *B. yuanmingense*จะใช้สายพันธุ์ไรโซเบียมถั่วเหลืองในการคัดเลือกสายพันธุ์เพื่อใช้ผลิตปุ๋ยชีวภาพเพื่อเพิ่มผลผลิตถั่วเหลือง เป็นการลดการขาดดุลการค้า และเพิ่มพื้นที่เพาะปลูกที่มีการบำรุงคุณภาพดินอย่างยั่งยืน

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INTRODUCTION

Soybean rhizobia are bacteria in soybean root nodules which are able be convert atmospheric nitrogen toammonia for soybeans' use for growth.At present,Thai farmers prefer not to grow soybeans in rotation with rice because the retail price for soybean seeds per *rai* is low (Table 1).

Table 1 Averageyields and retail prices per *rai*of some economic crops in Thailand in 2010 /2011.

Economic crops	Average yields (kg/*rai**) *One *rai* is 1,600 sq.m.	Average retail prices per kg. (baht)	Average retail price per *rai*(baht)
Rice	530	30.00	15,900.00
Corn	800	8.13	6,504.00
Soybeans	250	16.18	4,045.00

Source: การผลิตสินค้าเกษตรที่สำคัญ (http://www.oae.go.th/ewt_news.php?nid=9704).

As a result, there has been adecline in soybean cultivation areas in Thailand (Table 2). In addition, Thailand has imported approximately 85% of local soybean consumption(Table 3) resulting in a trade deficit and an opportunity loss of sustainable maintenance of soil quality.

Table 2 Soybean cultivation areas and average yields in both rainy and dry seasons in Thailand.

Year	Cultivation areas (x 1000*rai)*	Average yields (kg./*rai*)
2000	1,396	232
2001	1,154	236
2002	1,130	238
2003	961	246
2004	945	238
2005	929	250
2006	886	250
2007	831	253

Source: Office of Agricultural Economics, Ministry of Agriculture and Cooperatives. 2009. "Statistics of Agriculture in Thailand in 2007"(http://www.oae.go.th/statistic/yearbook50/section2/sec2table26.pdf)

Table 3 Quantities of soybeans imported and the local produce of Thailand from 2007 to 2010.

Year	Quantities of soybeans imported (million tons)	Import value (million baht)	Quantities of locally-grown soybeans (million tons)
2007	1.54	19,456	0.21
2008	1.72	32,225	0.20
2009	1.53	23,812	0.19
2010	1.82	25,795,	0.19

Source: สารสนเทศส่งเสริมการเกษตร (http://www.agriinfo.doae.go.th/).

There are two categories of soybean rhizobia: fast- and slow-growers (Elkan and Bunn, 1992). The research work on the isolation and characterization by 16S rDNA sequences of soybean rhizobia isolated from Nan and Phitsanulok provinces showedthat only slow-growing soybean rhizobia,*Bradyrhizobiumelkanii, B. japonicum, B. liaoningense* and *B. yuanmingense*were present in Thailand (Chansa-ngavej, 2011; Maruekarajtinplaeng, 2010). The research on genetic diversity of soybean rhizobia and the development of soybean rhizobium biofertilizers in Thailand is not as extensiveas inthe countries exporting soybean such as USA, Brazil, and Argentina (http://www.rizobacter.com.ar/risoja.html,http://www.americasbestinoculant.com/,http://www.beckerunderwood.com/en/inoculants, Appunu *et al*., 2008; Giongo *et al*., 2008; Menna *et al*., 2006). Therefore, to encourage Thai farmers to grow soybeans in rotation with rice,corn or sugar cane to increase their income and to reduce chemical fertilizer usage for sustainable maintenance of soil quality,more in-depth research on soybean rhizobium diversity, strain selection and the production and field tests of soybean rhizobium biofertilizersshould be carried out. Soybean rhizobium biofertilizers are produced by mixing selected strain(s) of soybean rhizobium with peat at 10^8 CFU/g biofertilizer (Somasegaran and Hoben, 1994). At present, the Soil Microorganisms Group, Department of Agriculture, in Bangkok, is the sole producer and distributor of soybean rhizobium inoculant in Thailand. Thus, it is inconvenient for farmers growing soybeans in the north, the upper central, and some parts in the northeastof Thailand to purchase soybean rhizobium biofertilizer. Therefore, the use of the biofertilizer in Thailand is not widespread. The aims of this research were to isolate and identify soybean rhizobia from Nong Kula subdistrict, Phitsanulok province. These rhizobium strainswill be further selected for the production of soybean rhizobium biofertilizers.

MATERIALS AND METHODS

1.Planting soybean seeds at Nong Kula subdistrict

A 15 x 24 m^2 experimental plot containing four 7.5 x6.0 m^2 subplots as described by Somasegaran and Hoben (1994) was set up in August 2009 at Nong Kula subdistrict in Phitsanulokprovince. Soybeanscultivar Chiangmai2 were grown from seeds in four rows in each randomly-selected 2 x 7.5 m^2 in each subplot.Soybean root nodules were collected on day 28after planting as described by Somasegaran and Hoben (1994). Yield was determined on day 95 after planting.

2. Isolation of bacteria from soybean root nodules

Root nodules were surfaced-sterilized by 5% H_2O_2 and rinsed to remove trace H_2O_2 with sterile deionized water as described bySomasegaran and Hoben (1994).Thebacteria within the sterile nodule extract were isolated by plating method using yeast extract mannitol (YM) agar medium with 25 µg. ml^{-1}congo red. Pure isolates weremaintained in YM agar slants at 4 ^{o}C for short-term storage and in 10% glycerol for long-term storage. Each isolate was cultivated in YM broth at 30^{o}C, 200 rpm,

for 4 days for RAPD-PCR fingerprinting. The composition of YM medium was described by Somasegaran and Hoben (1994) and as follows: (g/l), mannitol 10.0; K_2HPO_4 0.5; $MgSO_4$ 0.2; NaCl 0.1; yeast extract 0.5; deionized water 1 liter.

3. DNA fingerprinting of bacterial isolates from Nong Kula subdistrict

One loopful of each root nodule bacterial isolate was inoculated into 50 ml of YM in a 250 ml Erlenmeyer flask. Cells grown at 30°C, 200 rpm for 4 days were harvested by centrifugation at 8000 rpm, 4°C, 5 min, washed once with 0.85% NaCl to get rid of polysaccharides. Cells were broken by incubation for 1 h with lysozyme in 100 µl saline-EDTA (2.5 $mg \cdot ml^{-1}$), 400 µl TE buffer, 20 µl 10%SDS followed by freezing and thawing at –20°C, 5 min and 80°C, 5 min, twice. RNA was hydrolyzed by adding 250 µl of DNAzol™ (Molecular Research Center). DNA was precipitated with 30 µl 3M sodium acetate and ice-cold 500 µl absolute ethanol and with incubation at –80°Cfor 15 min.,washed with 70% ethanol, air dried, and redissolved in sterilized distilled water. The quantity and qualityof chromosomal DNA preparations were determined by OD_{260}, OD_{260}/OD_{280} and 1.25% agarose gel electrophoresis (Sambrook *et al*., 1989).DNA fingerprint of each root nodule bacterial isolate was obtained by RAPD-PCR using CRL-7 (5'GCCCGCCGCC3', Mathis and McMillin, 1996) as the primer. PCR mixture consisted of 2.0 µl 10x PCR buffer, 2.0 µl 10mM dNTPs , 0.2 µl primer CRL-7 (100 $pmole.\mu l^{-1}$), 0.2 µl *Taq* polymerase (5 $U.\mu l^{-1}$), DNA 200 ng, and sterilized distilled water to 20 µl. PCR program was as follows: 95°C15 seconds, 55°C30 seconds, 72°C90 seconds for 5 cycles, 95°C15 seconds, 60°C 30 seconds, 72°C90 seconds for 25 cycles, followed by 72°C10 minutes. PCR products were separated by 1.25% agarose gel electrophoresis (Sambrook *et al*., 1989), stained in 0.5 µg/ml Ethidium bromide and photographed under UV light on Bio-rad UV transilluminator equipped with Polaroid camera using FUJI 3000 B Polaroid film.

4. Dendrogram construction from DNA fingerprints and grouping of the isolates

Root nodule bacterial isolates with identical fingerprints were assigned to the same strains. The dendrogram of DNA fingerprints was constructed with DNA Fingerprinting II Informatix software version 3.0 provided by the Bio-Rad Laboratories (Thailand) Co., Ltd. using the UPGMA algorithm. Fingerprints of *B.japonicum*strainsSTB30, STB54,STB67, STB96, STB250, and STB310,*B.elkanii*strains STB8, STB119, STB120, STB147, STB173, STB176, STB179, STB185, STB220, STB238, STB245, and STB327, and *B.yuanmingense*strains STB169 and STB264 which were isolated and characterized by Maruekarajtinplaeng (2010) were also used in the dendrogram construction.

RESULTS AND DISCUSSION

The average soybean yield in the experimental plot at Nong Kula subdistrict, Phitsanulok province was 139 kg/*rai*which was comparatively lower thanthe average soybean yields shown in Table 2. Figure 1 showeda dendrogram constructed with DNA fingerprints. It indicated that the 48 strains of root nodule bacteria isolated from Nong Kula subdistrict,

Phitsanulok province, were clearly separated into severalsubclusters. Strains in the second subcluster from the top of the dendrogram (NKL09243, NKL09668, and NKL09826) were closely related to *B. japonicum*STB310. Strains in the next subcluster (NKL09226,NKL09266,and NKL09204) were closely related *to B.elkanii*STB119 and STB120,whilestrain NKL09256 was closely related to *B.yuanmingense* STB169. In addition, strains NKL091044, NKL09117, and NKL09277 were closely related to *B.japonicum* STB96, STB67, and STB30, respectively.Moreover, strains NKL09663 and NKL09653 were in the same subcluster as *B. elkanii* STB327. Strains NKL09210 and NKL09269 were in the same subcluster as *B. elkanii* STB179. Strains NKL091052 and NKL091048 were closely related to*B. elkanii* STB220 and STB238 respectively. Finally, strains NKL09119, NKL09679, and NKL09281 were in the same subcluster as *B.yuanmingense* STB264. These results indicated that soybean rhizobia belonging to the same species had different DNA fingerprints.

Using 16S rDNA sequences for the identification of slow-growing soybean rhizobia, Maruekarajtinplaeng (2010)earlier reported that the *B.elkanii*type strains STB8, STB119, STB120, STB173, STB176, STB179, STB185, and STB238 and the *B.japonicum*type strains STB30, STB54, STB67, STB96, STB250, and STB310 had different RAPD-PCR fingerprints when the arbitrarily GC rich CRL-7 was used as the primer. Slow-growing soybean rhizobia might include more than the 4 species presently recognized worldwide as *Bradyrhizobiumelkanii (*Kuykendall *et al*., 1992*), B. japonicum (*Jordan, 1982*), B. liaoningense* (Xu *et al*., 1995),and *B. yuanmingense* (Appunu *et al*., 2008). Hence, further research should concentrate on identification and determination of phylogenetic relationships among soybean rhizobia isolated from Nong Kula subdistrict,Phitsanulok province. This will shade more light on the genetic diversity and strain identification of these organisms and will make contribution to soybean rhizobia taxonomy.

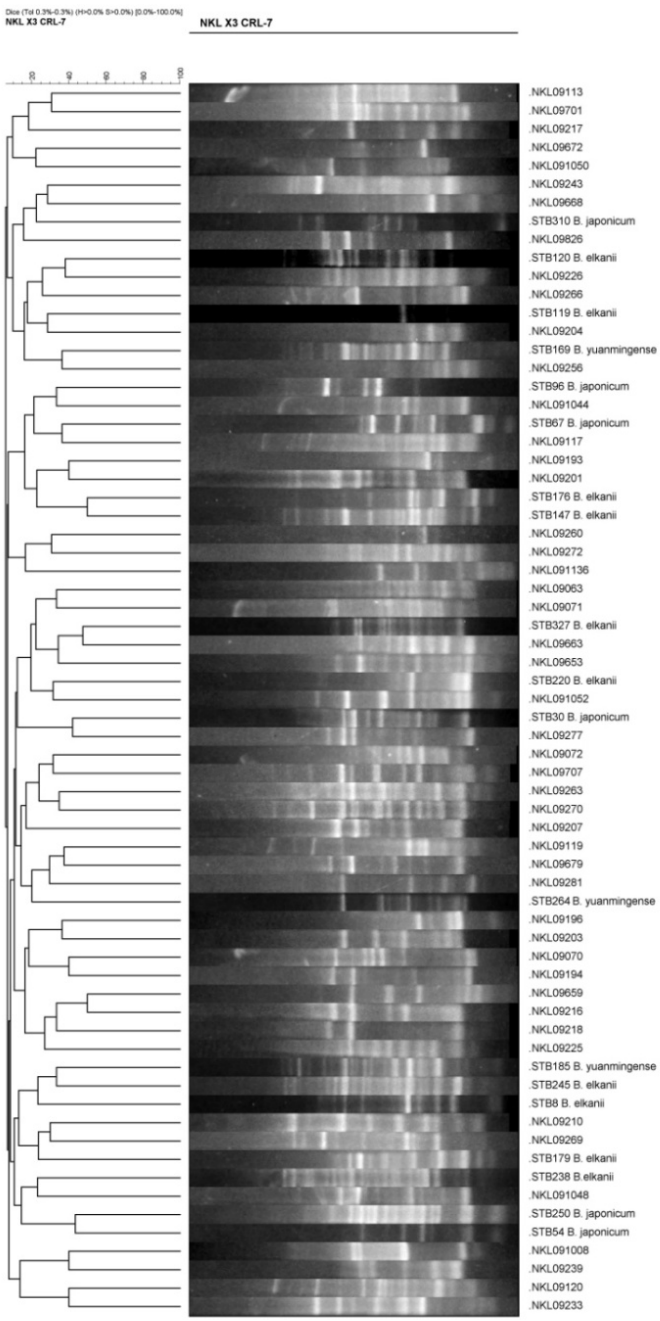

Figure 1 Adendrogramof relationships among 48 isolates of soybean root nodule bacteria and 19 type strains of *Bradyrhizobium* based onDNA Fingerprinting II Informatix software version 3.0.

CONCLUSION

Soybean cultivar Chiangmai 2 grown in an experimental plot at Nong Kula subdistrict inPhitsanulok province yielded 139 kg/rai. A total of 133 bacterial isolates were obtained from root nodules of the soybean in the experimental plot. Identical DNA fingerprints were used to group bacterial isolates into the same strains with the resultant of 48 strains. A dendrogram of DNA fingerprints of the 48 strains as well as of 19 identified slow-growing soybean rhizobium strains revealed the presence of *B. elkanii, B. japonicum,* and *B. yuanmingense*among the isolated soybean rhizobia. The results showed that the same species of slow-growing soybean rhizobia had

different DNA fingerprints.Therefore,multilocus sequence analysis (MLSA) is being employed to shade more light on the genetic diversity of slow-growing soybean rhizobia which serve as a pool for strain selection for the production of biofertilizers to increase soybean productivity to reduce trade deficit and increase cultivation areas for sustainable soil quality maintenance.

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References

Appunu, C., A. N' Zoue and G. Laguerre. 2008. Genetic diversity of native bradyrhizobia isolated from soybeans (*Glycine max* L.) in different agricultural-ecological-climatic regions of India. Appl. Environ. Microbiol. 74(19): 5991-5996.

Chansa-ngavej, K. 2011.Selection and field trials of soybean rhizobium biofertilizers with DNA fingerprints and can be kept at room temperature. Paper presented at the 17th International Congress on Nitrogen Fixation. Fremantle, Australia. November 27-December 1,2011. 9 pp.

Chansa-ngavej.K., K.P.Ly and W. Chongfuengprinya. 2009. Research and development for commercial production of soybean rhizobium biofertilizers with DNA fingerprints and can be kept at room temperature. Proceedings of Thailand Research Symposium 2009. p. 12-22.

Elkan, G.H. and C.R. Bunn. 1992. The Rhizobia. In Balows, A., Truper, H.G., Dworkin, M., Harder, W., Schleifer, K-H (eds). The Prokaryotes.2nd Edition.Chapter 107. New York : Springer Verlag.

Giongo, A., A.Ambrosini, L.K.Vargas,J.R.J. Freire, M.H.Bodanese-Zanettiniand L.M.P. Passaglia. 2008. Evaluation of genetic diversity ofbradyrhizobia strains nodulating soybean [*Glycine max* (L.) Merrill] isolated from South Brazilian fields. Appl. Soil Ecol. 38: 261–269.

Jordan, D.C. 1982. Transfer of *Rhizoiumjaponicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow growing, root nodule bacteria from leguminous plants. Int. J. Syst. Bacteriol.32 : 136-139.

Kuykendall,L.D.,B.Saxena, T.E. Devine andS.E.Udell. 1992. Genetic diversity in *Bradyrhizobiumjaponicum*Jordan 1982 and a proposal for *Bradyrhizobiumelkanii*sp. nov.Can. J. Microbiol.38 : 501-505.

Maruekarajtinplaeng, S. 2010. Diversity of soybean rhizobia in 16 subdistricts of Phitsanulok province.Ph.D. thesis.Microbiology program.Chulalongkorn University.176 pp.

Mathis, J.N. and D.E. McMillin. 1996. Detection of genetic variation in

Bradyrhizobiumjaponicum USDA 110 variants using DNA fingerprints generated with GC rich arbitrary PCR primers. Plant and Soil.186 : 81-85.

Menna, P., M.Hungria, F.G.Barcellos, E.V. Banger, P.N. Hess and E. Martinez-Romeo. 2006. Molecular phylogeny based on the 16S rRNA gene of elite rhizobial strains used in Brazilian commercial inoculants. Syst. Appl. Microbiol. 29: 315-332.

Peng, G.X., Z.Y.Tan, E.T.Wang, B. Reinhold-Hurek, W.F.Chanand W.X. Chen. 2002. Identification of isolates from soybean nodules in Xinjiang region as *Sinorhizobiumxinjiangense* and genetic differentiation of *S. xinjiangense* from*Sinorhizobiumfredii*. Int. J. Syst. Evol. Mirobiol.52 : 457-462.

Sambrook, J., E.F. Fritsch and T. Maniatis.1989. Molecular cloning : A Laboratory Manual, 2nd Edition. New York : Cold Spring Harbor Laboratory Press. Book 1. p. A3.

Somasegaran, P. and H.Hoben. 1994. Handbook for Rhizobia ; Methods in legume-*Rhizobium* Technology. p. 340, 370-1, New York : Springer Verlag.

Xu, L.M., C.Ge, Z. Cui, J.Li and H. Fan. 1995.*Bradyrhizobiumliaoningense* sp. nov.isolated from the root nodules of soybeans. Int. J. Syst. Bacteriol. 45 (4) : 706-711.

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