Some Physiological and Molecular Biology Aspects of High Effiency N_2-Fixation Rhizobial Strains in Forage Legumes

Miss Kamonluck Teamtisong

A thesis Submitted in Partial Fulfillment of the Requirements
for the Degree in Master of Science in Biotechnology
Suranaree University of Technology
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ISBN 974-7359-66-9

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คุณสมบัติทางสรีรวิทยาและชีววิทยาอณูบางประการของไรโซเบียมที่มีประสิทธิภาพในการตรึงไนโตรเจนได้สูงในพืชอาหารสัตว์ตระกูล

นางสาวกมลลักษณ์ เทียมไชสง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2543

ISBN 974-7359-66-9

KAMONLUCK TEAMTISONG : SOME PHYSIOLOGICAL AND MOLECULAR BIOLOGY ASPECTS OF HIGH EFFIENCY N_2-FIXATION RHIZOBIAL STRAINS IN FORAGE LEGUMES
THESIS ADVISOR : ASSISTANT PROFESSOR NEUNG TEAUMROONG, Dr. rer. nat, 93 PP.
ISBN

Forage legumes rhizobia in this study were isolated from four tropical forage legumes; *Desmanthus virgatus, Stylosanthes hamata, Chamaecrista rotundifolia* (Wynn cassia) and *Centrosema pubescens*. The high efficiency N_2-fixation rhizobial strain were obtained from Department of Agriculture, Bangkok. Characterizations of these strains were conducted along with their physiological properties such as acid-base production, IAA production, intrinsic antibiotic resistance profiles and 19 different substrates utilization (APIZYM-test). Genetic relatedness was also determined. Using *nif* and *nod* probes for Southern blot hybridization analyses and RAPD, REP-PCR for distinguishing among the strains were employed. In addition, *nif*H and *nod*A PCR-RFLP and 16S rDNA sequences were used to investigate the phylogenic relationships. Characterization of effective *D. virgatus* rhizobia, it was found that most of them were belong to fast-grower group. By using antibiotic resistant it was clearly showen that most of them were susceptible to erythromycin. Moreover, among these strains non of them produced α-monosidase and α-fucosidase. In case of *D. virgatus* rhizobia, one of them seemed to be the *Rhizobium tropici* and the rest were closely related in intraspecies level. This was confirmed by doing cross nodulation test between rhizobial strains and host plants, *Phaseolus vulgaris,* prior to detecting with direct nodule PCR approach, *nif*H and *nod*A PCR-RFLP and 16S rDNA sequences. However, when distinguish the other host plant rhizobia isolated from *C. pubescens*, *S. hamata* and *Ch. rotundifolia* by using random primers and RFLP of nod-PCR products, the result suggested that there were great divergents in each plant host. For *C pubescens* rhizobia, DASA24008 and DASA24015, sequences were aligned with the *B. japonicum* 83% identities and 100% identities but DASA24016 were aligned with *Rhizobium spp*. 16S rRNA gene, respectively. In case of *S. hamata* rhizobia both of DASA25005 and 25015 were aligned with *B. japonicum*, 94% identities, on the other hand strain DASA25005 were aligned with *R. etli*, 98%. *Ch.*

rotundifolia rhizobia, DASA29007 were aligned with *B. japonicum*, 96% identities, but DASA29015 were aligned with *Pseudomonas reactans*, 98% identities.

สาขาวิชาเทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา.....
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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

กมลลักษณ์ เทียมไธสง : คุณสมบัติทางสรีรวิทยาและชีววิทยาอณูบางประการของไรโซเบียมที่มีประสิทธิภาพในการตรึงไนโตรเจนได้สูงในพืชอาหารสัตว์ตระกูลถั่ว
(SOME PHYSIOLOGICAL AND MOLECULAR BIOLOGY ASPECTS OF HIGH EFFIENCY N_2-FIXATION RHIZOBIAL STRAINS IN FORAGE LEGUMES) อาจารย์ที่ปรึกษา : ผศ. ดร. หนึ่ง เตียอำรุง, 93 หน้า, ISBN

พืชอาหารสัตว์ตระกูลถั่วเป็นแหล่งอาหารสัตว์ที่มีความสำคัญสำหรับการปศุสัตว์ เนื่องจากมีราคาถูกและมีปริมาณโปรตีนค่อนข้างสูง ดังนั้นในการศึกษาครั้งนี้จึงได้ศึกษาเชื้อไรโซเบียมที่มีประสิทธิภาพในการตรึงไนโตรเจนสูง ซึ่งได้รับการคัดเลือกจากกรมวิชาการเกษตรโดยพืชอาหารสัตว์ตระกูลถั่วสี่ชนิด ได้แก่ ถั่วไมยรา (*Desmanthus virgatus*),ถั่วฮามาตา (*Stylosanthes hamata*), ถั่วลาย (*Centrocema pubescens*) และถั่ววินแคสเซีย (*Chamaecrista rotundifolia*, Wynn Cassia) จากนั้นทำการศึกษาลักษณะของสายพันธุ์ที่คัดเลือกได้โดยใช้ทั้งเทคนิคทางสรีรวิทยาและทางพันธุศาสตร์โมเลกุล

เทคนิคทางสรีรวิทยาได้แก่ การผลิตกรดและเบส, ความสามารถในการผลิตกรดอินโดล (Indole Acetic Acid: IAA), ความต้านทานต่อสารปฏิชีวนะ และความสามารถในการผลิตเอนไซม์ 19 ชนิด ส่วนเทคนิคทางพันธุศาสตร์โมเลกุล ได้แก่ การตรวจสอบ *nif* gene และ *nod* gene ด้วยวิธีไฮบริไดเซชั่น, การใช้เทคนิคทาง PCR โดยใช้ REP และ RAPD เป็น primer, การใช้ *nif*H และ *nod*A เป็น primer สำหรับศึกษาโดยใช้เทคนิค PCR-RFLP และการใช้เทคนิคการหาลำดับเบสของ 16S rDNA

จากการศึกษาทั้งลักษณะทางสรีรวิทยาและการใช้เทคนิคทางพันธุศาสตร์โมเลกุล พบว่าไรโซเบียมที่แยกได้จาก *D. virgatus* ทั้งหมดจะเป็นกลุ่มที่เจริญเร็ว และสามารถผลิต IAA ได้ ส่วนที่แยกได้จากพืชอาหารสัตว์ตระกูลถั่วชนิดอื่นจะพบทั้งที่เจริญเร็วและเจริญช้า ส่วนผลการศึกษาการต้านทานต่อยาปฏิชีวนะพบว่า ส่วนมากจะมีความไวต่อ erythromycin นอกจากนี้ยังพบว่าเกือบทุกสายพันธุ์ไม่สามารถผลิตเอนไซม์ α-manosidase และ α-fucosidase โดยพบว่าไรโซเบียมที่แยกได้จาก *D. virgatus* บางสายพันธุ์จะมีความคล้ายคลึงกับ *R. tropici* มาก ซึ่งจากผลการปลูกเชื้อไขว้กับพืชอาศัย 2 ชนิดได้แก่ถั่วไมยรา และถั่วแดงหลวง (*Phaseolus vulgaris*) แสดงให้เห็นว่า DASA23047 คล้ายคลึงกับ *R. tropici* นอกจากนี้ผลของ *nif*H และ *nod*A PCR-RFLP แสดงให้เห็นอย่างชัดเจนว่า DASA23015, DASA23076, DASA23079 และ *R. tropici* มีความคล้ายคลึงกันมาก ซึ่งสามารถยืนยันจากผลของการหาลำดับเบสของ 16S rDNA

ส่วนไรโซเบียมที่แยกได้จากพืชอาหารสัตว์ชนิดอื่น พบว่า ในแต่ละชนิดจะมีความแตกต่างกันค่อนข้างมากในพืชอาศัยชนิดเดียวกัน อย่างไรก็ตามเมื่อคัดเลือกสายพันธุ์ที่มีประสิทธิภาพสูงในการตรึงไนโตรเจนในพืชแต่ละชนิดมาศึกษาลำดับเบสของ 16S rDNA พบว่า ไรโซเบียมที่แยกได้จากถั่วลาย สายพันธุ์ DASA24008 และ DASA24015 มีความคล้ายคลึงกับ *Bradyrhizobium japonicum* ถึง 83 และ 100% ตามลำดับ ส่วน DASA24016 มีความใกล้เคียงกับ *Rhizobium spp.* 93% ในกรณีของไรโซเบียมที่แยกได้จากถั่วฮามาตาพบว่าสายพันธุ์ DASA25005 และ DASA25015 มีความคล้ายคลึงกับ *B. japonicum* ถึง 94% ส่วนสายพันธุ์ DASA25008 มีความใกล้เคียงกับ *R. etli* ถึง 98% สำหรับไรโซเบียมที่แยกได้จากถั่ววินแคสเซีย จากผลการทดลองพบว่า DASA29015 นั้นไม่มีความคล้ายคลึงกับไรโซเบียมกลุ่มใดเลย แต่มีความใกล้เคียงกับ *Pseudomonas reactans* ถึง 98%

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ลายมือชื่อนักศึกษา.....
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ACKNOWLEDGEMENT

This work was carried out at the School of Biotechnology, Institute of Agriculture Technology, Suranaree University of Technology during the year 1997-2000. The following funding bodies are acknowledged for financial support: NRCT-JSPS under the Large Scale Cooperative Research Program in Biotechnology (JSPS-NRCT/DOST/LIPI/VCC) and Suranaree University of Technology.

I would like to express my deepest sense of gratitude to my advisor Dr. Neung Teaumroong for his encouragement, valuable suggestion, constructive criticism of the manuscript. He gave me full freedom in my work, while at the same time he was always most supportive, and my deep appreciation in also expressed to my co-advisor, Dr. Nantakorn Boonkerd and Dr. Achara Nuntakij.

Both Drs. Neung and Nantakorn showed great appreciation of my desire to see the world and my enthusiasm for travelling. With their support I gained numerous opportunities to participate in scientific meetings and visiting Tsukuba University. During these travels I was able to establish important contacts and make great friends.

Beside people making my life fun, I very much appreciate all those people who have made things work. My colleagues at Science and Technology Equipment Center (F3) and students in School of Biotechnology, especially Pee Pook, Pee Moo, Pooky, Nuch, Hi, Jeab and Of for their encouragement, patience and friendliness.

Finally, my warm thank are due to my family, my parents and my younger brother. Especially, I would like to mention my Momma, who always stand by my side in a figurative sense, while I was in good times and difficult times. I always keep her in my mind.

Kamonluck Teamtisong

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LIST OF ABBREVIATIONS

ADP	adenosine 5′- diphosphate
ARA	acetylene reduction assay
ATP	adenosine 5′- triphosphate
BNF	biological nitrogen fixation
bp	base pair
°C	degree celsius
cm	centimeter
cv	coefficient of variation
dATP	deoxiadenosine 5′- triphosphate
dCTP	deoxycytidine 5′- triphosphate
dGTP	deoxyguanosine 5′- triphosphate
DNA	deoxyribonucleic acid
DTTP	deoxythymine 5′- triphosphate
EDTA	ethylene diamine teraacetic acid
ERIC	enterobacterial repetitive intergenic consensus
e.g	for example
et al.	Et alia (and others)
g	gram
h	hour
IAA	indole acetic acid
Kda	kilodalton
L	litre
M	molar
μM	micromolar
μm	micrometer
μmol	micromole
μl	microlitre
μg	microgram
mg	milligram

min	minute
ml	millilitre
mM	millimole
PCA	Polymerase chain reaction
pmol	picomole
r	resistant
RAPD	random amplification polymerphism DNA
REP	repetitive extragenic polindromic
RFLP	Restriction fragment length polimorphism
RNA	ribonucleic acid
rDNA	ribosomal DNA
rRNA	ribosomal RNA
rpm	revolution per minute
s	susceptible
sm	susceptible with mutated colony
UV	ultraviolet
vol/vol	volume per volume

1. Introduction

The efficiency of animal production is depend upon the optimum utilization of the feed for growth, development, and reproduction. A wide variety of feeds and feedstuffs of different origin are used in animal production. Forage quality is the best defined as production per animal. One major role of forage is to provide feed for livestock, which convert fiber into human food such as milk and meat. There are about 6 to 10 major food crops, but hundreds of grasses, legumes, forbs, and shrubs contribute as forages. Forages differ in their inherent quality, potential, and there are seasonal variation in forage quality. The best estimate of the potential of a forage for animal production is the intake multiplied by it digestibility (Jorgensen and Howard, 1982). Legumes generally are higher in intake, digestibility, rate of passage, and potential for animal performance than grass (Jerome and Vivien, 1995). Many would maintain that the role of legume is of greater importance in agriculture and environmental protection. Therefore, they are higher protein content, palatability and digestibility relative to grasses. Thus, while the mean protein content of grass is just over 75 g kg^{-1}, that for tropical forage legume averages around 170 g kg^{-1}. Consequently, improved pasture with grass-legume mixture offer a viable means of enhancing animal productivity in the tropics. Interest in leguminous fodder tree has provided another dimension to this strategy since they are capable yielding high –protein forage during critical dry periods of the year (D'Mello and Devendra, 1995). Furthermore, theses biological resourse also provide feed for wild life, converses soil by reducing soil erosion, and protect water quality. These conservation measures contribute to the quality of the environment, the health of society, and recreational enjoyment. Most of all, forage legumes grow symbiotically with nitrogen bacteria that form nodules on the roots. These bacteria able to reduce atmospheric nitrogen (N_2) to forms that are available to the plant. Thus, legumes are valuable component in forage mixtures with grasses and in crop rotations with corn and other cereal crops to decrease dependence on fertilizer N. These may have economic benefits to the producer or rancher and environmental benefits to society (Barnes, R. F. et al. (eds). 1995).

1. Leguminous plants

Legumes have been used in agriculture since the ancient times. Legumes seed or plues were among the first sources of human food and their domestication and cultivation in many areas occurred at the same time as that of the major cereals. Nutritionally they are 2-3 times richer in protein than cereal grains and many also containing oil. Leguminous mulches have

always been used as a source of nutrient rich organic matter and nitrogen for crops. In more recent times legumes have become improtant as high quality forage for livestock both in cultivated pastures and in naturally occuring associations (Gutteridge and Shelton, 1994).

The legumes are the third largest group of flowering plants comprising over 18,000 species in 650 genera which are well distributed in most environments throughout the word. Taxonomists have divied the legumes into three families (Gutteridge and Shelton, 1994);

1.1 The Caesalpiniaceae contains about 2,800 species, most of which are trees tropical savanahs and forests of Africa, South America and Asia (Williams, 1983)

1.2 Mimosaceae also contains about 2,800 species, there are predominantly small trees and shrubs of semi arid tropical regions of Africa, the Americas and Australia. *Acacia* species are the best know examples of the family.

1.3 Fabaceae contains over 12,000 species, mainly herbs and small herbs distributed worldwide, and includes the well-known grain legumes such as beans and peas.

Utilization of legumes in farming systems

Legumes have a signification role in many farming systems of the tropics and subtropics through their contribution to

- Enhanced nutritive value of the animal diet.
- Biological nitrogen (N) fixation. The legume-rhizobial symbiosis converts atmospheric N to forms of N which can be taken up by plants and cycled within the plant-animal-soil system in way which increase its productivity.
- Landscape stability soil erosion and runoff are reduced by leguminouse cover in plantation, by contour-planted trees, shrubes and hedge rows on sloping land and by the use of sod – forming legumes such as *Arachis pintoi* in perennial pastures (Humphreys, 1995).

2. Forage Legumes

The main role of the forage legume is to improve fodder quality as pasture legumes are rich in N and provide an extra source of protein for the grazing animals, particularly during the dry season when the grasses are poorly nutrition. At the same time the legume can contribute to the overall N economy of the pasture through N_2- fixation, giving rise to an increased N content of the associated grasses. Research in the use of tropical legumes in association with permanent grass pasture began after world war II, but intensive work on a wide scale began in the early 1960s,. Presently, in the most tropical countries, researchers appreciate

the potential value of tropical legumes in providing nitrogen (N), improving forage quality, and increasing carrying capacity compared with grasses alone without N fertilization. Native or naturalized tropical forage legumes in many instances have been contributed to the grazing system without noticed by the cattle producer. Cutting experiments with tropical legume grass associations have shown that as much as 300-400 kg. N ha^{-1} yr^{-1} can be fixed by some tropical legumes. For the benefits of a legume to cattle production are exemplified by a long-term study from Carimaqua in the Colombia Llanos (Lascono and Estrada, 1989). *Brachiaria decumbens* cv. Basilisk was planted on a low pH oxisol with or without strips of the twining legume *Pueraria phaseoloides* CIAT 9900 and the fertilized with P, N, Mg and S. This study showed positive effects of the legume on cattle production. Both through its ingestion by the animals and its influence on the growth and quality of the companion grass. On the other hand, the benefits of forage legumes in mixed farming systems using ruminants and crops are shown further by study (Gibson, 1987) in northeast Thailand near Khon Kaen on a light upland soil of the Korat series (oxic paleustults) which was formerly under open dipterocarp forest. The results showed that the beneficial influence of the grazed legume was strongly evident in improved crop yields if soil nutrient constraints to legume growth and N fixation were overcame.

Therefore, legumes are needed in perenial pastures used for ruminant production but are assuming a grater role in mixed farming practice where the legume contributes to sustaining crop yields and to protecting the environment. This occurs in the context of ;

- tree crops grown with pasture
- annual crops rotated with pastures in a ley system
- shrub legumes partially feed to livestock and grown with annual crops, and
- annual crops inter cropped with annual legumes (Humphreys, 1994).
-

3. Characteristics of tropical forage legumes

The primary concern is that the legume should give a large yield of good quality fodder, rich in protein, but it is equally important that the legume should be able to establish and persist in the sward. Thus the legume must not only be well adopted to the environment in a given region, but must be compatible with the dominant grasses in the pasture under glazing (Griller and Wilson, 1993). Selected legumes must be able to yield well and persist with minimal fertilizer additions, or preferably without their use (Toledo, 1985). In Thailand many legumes have been

presented, but legumes which used for forage belong to four species; *Desmanthus virgatus* (L.) Willd. (or *Mimosa virgata* L.), *stylosanthes hamata*, *Chamaecrista rotundifolia* (wynn cassia) and *Centrosema pubescens*. These groups of legume have been considers that most promising since they contained high amount of crude protein productivity in the range of 0.2-0.3 t/ha and some of them such as *Desmanthus spp*. and *C. pubescens* were drought tolerant. Thus, rendering this study focused on the group of these plants.

3.1 *Desmanthus virgatus* (l.) Willd.

The species displays a range of morphology and habit from event shrubs 2-3 m tall to prostrate herbaceous types less than 50 cm in high. Originally from Central and South America *D. virgatus* is now naturalized in many countries, including those in Southeast Asia and the Pacific (Allen and Allen. 1981). Leaves contain 24-30% crude protein with an is vitro dry matter digestibility of *D. virgatus* was 58% while that for leucaena was 65%. High yield from dense stands of *D. virgatus* have been obtained from a number of regions.

3.2 *Centrosema spp.*

Several of the 35 species of *Centrosema* have been proved to be very useful pasture legumes. The genus has a wide distribution in tropical and subtropical America and *C. pubescens* was the first species to be widely used as pasture improvement due to its productive growyh, good herbage quality and ability to combine with grasses (Clements et al., 1983). *C. pascuorum* is a herbaceous, twing annual native to tropical South and Central America, mainly in semiarid regions or those with long dry seasons. It is utilized in the Northern Territory of Australia as a glazing legume where there is a short but reliable wet season (Oram, 1990b).

3.3 Stylosanthes spp.

The genus *Stylosanthe*s, which contains about 30 species that occur throughout tropics, has been on important source of plants for pasture improvement across a wide range of climates. *S. hamata* and *S. scabra* are species which are well adopted to drier regions. Legume has been very successful in Northern Australia as a pasture legumes. It has also been used in Thailand, India and several West Africa countries (Giller, K. E. and Wilson, K. J., 1991).

3.4 Chamaecrista rotundifolia

This annual or short-lived perennial semievect to prostrate herb is native from Mexico and Caribbesn region extending into Brasil and Urwguay. It is commercialized in Australia, where it is successful in seasonally dry are as with 700-1,400 mm of rainfall. Leaves

are bifoliolate with subrotund to oborate leaflets 0.5-1.0 cm long. Yellow flowers are produced in the late summer and early fall at higher latitudes (Kretschmer, A. E. JR. and Piman, W. D., 1995)

4. Biological nitrogen fixation symbiosis

All plants use soil N in the form of nitrate (NO^-_3) or ammonium (NH^+_3) to meet their nutritional needs for growth and reproduction. Because mechanical harvest or grazing of forages by animals removes N in the plant dry matter of fields or range, soil amendment with manufactured fertilizers is often necessary to sustain forage or animal productivity at a profitable level (Darrella and Gray, 1995). Many forage, pasture, or rang systems require supplementation with purchased N fertilizers, with influences profitability and may affect environmental quality. Dinitrogen (N_2) fixation by legumes provides an opportunity for forage plants to obtain N form the earth's atmosphere rather than commercial fertilizer. This may have economic benefits to the producer or rancher and environmental benefits to society.

4.1 Symbiosis : A Process for nitrogen self-sufficiency

Several forage, pasture, and rangeland species have the genetic capacity for N self-sufficiency ; legumes such as alfalfa, clovers, medics, trefoils, vetches, and lespedezas are examples. This self –sufficiency, with may free the plants from the need for amendment with manufactured N fertilizers, is achieved by symbiotic fixation of inert gaseous N_2 from the atmosphere into NH^+_4 as indicated in the equation belowed. Ammonium produced from symbiosis is identical to the NH^+_4 in the soil that is used in amino acid and protein synthesis (Barnes, R. F. et al., 1994).

$$N_2 + 8\,\text{ferridoxin}^- + 8H^+ + 16\,MgATP^- + 18\,H_2O \rightarrow 2\,NH_4 + 2\,OH^- + 8\,\text{ferridoxin} + 16\,MgADP^- + 16\,H_2PO_4^- + H_2$$

4.2 How nodules form ?

Root infection by rhizobia is multi-step process that is initiatled by preinfection event in the rhizosphere. Rhizobia respond by positive chemotaxis to plant root exudates and move toward localized sites on the legume roots (Barbour et al., 1994; Caetano-Anolles, et al., 1992; Cooper et al., 1994; Gaworzewska et al., 1982; Gulash et al., 1984). Both *Bradyrhizobium* and *Rhizobium spp*. are attacted by amino acids, dicarboxylic acids present in the exudates, and very low concentrations of excreted components, such as flavonoids, that may not

have high nutritional value. (Aguilar et al., 1988; Armitage et al., 1988; Caetano- Anolles et al., 1988; Kape et al., 1991; Peters et al., 1990).

Initially it was speculated that attachment of *Rhizobium* and *Bradyrhizobium spp.* to legume roots was involved in host specificity. Specific adherence of compatible rhizobia was proposed to be mediated by specific binding of particular polysaccharide structures present on the bacterial cell surface to host plant lectins (Bohlool et al., 1974; Hamblin et al., 1973). At the surface of the root, bacteria alter the growth of the epidermal hairs on the root, such that they grow deformed, even curled (Yao and Vincent, 1969 ; Dazzo and Gerdiol, 1984). As this happens the cell of the root cortex, under the epidermis, begin dividing (Libbenga and Harkes, 1973 ; Newcomd, 1981). Bacteria trapped in a curled root hair, or between a hair and another cell, proliferate and begin to infect the outer plant cells, as they do, the invaded plant cell is stimulated to produce a cell wall sheath, "infection thread" (Callaham and Torry, 1981). As cell divisions in the plant root establish the body of the nodule, infection threads ramify and penetrate individual target cells within the nodule. Bacteria are released into the plant cytoplasm itself, enveloped in plant plasma membrane (Robertson *et al*., 1978). The bacteria and plant cells differentiate and begin symbiotic nitrogen fixation and metabolite exchange (Sutton *et al*., 1981 ; Verma and Long, 1983).

5 Rhizobium and plants interaction in molecular level

Rhizobium genetic has been greatly advanced by transpose on mutagenesis, recombinant cloning, and plasmid transfer experiments,(Kondorsi and Johnston, 1981 ; Denarie *et al*., 1981 ; Long, 1984). The fast growing rhizobium species typically have large plasmids, one or more of which carry symbiotic genes and are designated *pSym*. This vary form *R. leguminosarum* plasmids of about 200-300 kilobases (Kb) to the large "megaplasmids" (1200-1500 Kb) *of R. meliloti*. In some other symbionts, such as *Bradyrhizobium*, symbiotic genes are appearently not located on plasmids. Genetic analysis of plants also have been used successfully to define some host symbiotic loci (Rolfe and Gresshoff, 1988). .

5.1 Rhizobial genes controlling infection, nodulation and host range

The rhizobium genes essential for infection and nodule formation can be divided into two classes. First class includes several set of genes involved in the formation of the bacterial cell surface, such as genes determining the synthesis of exopolysaccharide (*exo* genes),

lipopolysaccharide (*lps* genes), capsular polysaccharides or K^- antigenes, and -1,2-glucans (*ndr* genes). A possible role of *exo* and *lps* genes in the determination of host specificity has been suggested, but not clear genetic evidence has yet been given that *Rhizobium* surface components are major determinants of host range specificity.

The second class consists of the nodulation (*nod* or *nol*) genes. Some of the *nod* genes appear to be interchangable for nodulation function between different species and biovars and are therefore designated as common *nod* genes, on the other hand, some *nod* genes are involved in the nodulation of particular host and are hence called host-specific *nod* (*hsn*) genes (Kondorosi et al., 1984). In most *Rhizobium* species studied to date, the *nod* genes reside on large symbiotic plasmids (*pSym*) that also carry the *nif* and *fix* nitrogen-fixation genes. In *R. loti* and *Bradyrhizobium* and *Azorhizobium spp*., the symbiosis-related genes are localized on the chromosome (Appelbaum et al., 1988; Chua et al., 1985; Goethals et al., 1989). Most *Rhizobium nod* genes are not expressed in cultured cell but are included in the presence of the plant. This induction is cased mostly by flavonoids secreted by the plant and also requires the participation of the transcriptional-activation protein NodD. During the last few years, it has become clear that a major function of the *nod* genes is to ensure signal exchange between the two symbiotic partners (fig. 1). In the first step, flavonoids exsceted by the plant induce, inconjunetion with the NodD protein, the transcription of bacterial *nod* genes (Fisher et al., 1992; Schlaman et al., 1992). In the second step, the bacterium, by means of the structural *nod* genes produce lipooligosaccharide signals (Nod factors) (Denarie et al., 1993; Denarie et al., 1992; Spaink, 1992) that induce various root responses (Spaink et al., 1992; Truchet et al., 1991).

Fig. 1 Schematic representation of the interaction between *Rhizobium* specises and legume roots. Plant-secreted flavonoids, in conjugation with bacterial NodD protein, regilate the transcription of bactrial *nod* genes, here exemplified by *nod*ABCDEFL genes of *R. leguminosarum*. The bacterial *nod* gene products are involved in the synthesis of Nod factors. These Nod factors are able to induce the critical steps leading to nodule formation: (a) root hair deformation; (b) cortical cell divition; (c) at least some steps of the infection process; and (d) increased flavonoid production (Franssen, et al., 1992).

6 Rhizobial *nif* and *fix* genes

Genes for nitrogen fixation in rhizobium are generally divided into two groups : those with homologes in free-living nitrogen fixation systems, such as *Klebsiella*, are referred to as *nif* genes ; those that are shown to be required for symbiotic nitrogen fixation, but whose function is not known to be anologous to a free-living function, are referred to as *fix* genes. Both nif and fix gene mutants are able to case nodule development, but the nodules do not fix nitrogen (Nod+ Fix-). The *nif* and *fix* loci in three system are shown in figure 2. (Sharon , 1989).

Fig. 2 Maps showing *nif* and *fix* genes in three organisms with different linkage relationships.
(Top) *R. meliloti*. (middle) *R. leguminosarum* biovar trifolii and leguminosarum (composite). (Bottom) *B. japonicum*. *FixN* in *R. meliloti* is probably a muliltigene region (David et al., 1988).

7 Advantages of BNF in farming systems

The availability of soil N exerts the most significant control of plant yield in the humid and sub humid tropics, and primary goal of farmers is to maintain a level of soil organic matter whose continual breakdown will remove their contain. The legume-rhizobial symbiosis provides farmers with inexpensive source of N whose production is environmentally "clean" ; it dose not involve the consumption of fossil fuel, as occurs in the production of fertilizer N, which contributes to global warming and exacerbates the foreign exchange balance of tropical countries lacking oil sources.

The level of N fixation from effectively nodulated legumes depends upon the growth rate of the legume and soil conditions; usually ; 15-40 kg N is fixed for each 1000 DM of shoots grown. This may be sufficient for some high level intensive dairy production enterprise, but insufficient in many farming systems (Humphreys, 1995).

In term of biological nitrogen fixation (BNF), pasture legumes are among the more efficient member of the Leguminoseae. Several pasture species can fix nitrogen in excess of their own needs and BNF rates can be high (Gibson, 1977). This is a result of an effective symbiosis between the legume and specific strains of *Rhizobium* and pasture legumes are neither productive nor persistent without effective nodulation by appropriate rhizobia. Therefore, one of the impediments to greater nitrogen fixation efficiency is the inability to apply superior rhizobia as an inoculant so that they will form majority of the nodules on the legume plants. BNF has appeared as a breeding and selection concern only recently and awaits exploitation (Smith and Knight, 1984

; Mytton et al., 1988). To obtain the necessary strains of *Rhizobium* for inoculations, an evaluation and characterization process concurrent with host plant selections becomes mandatory.

8 Taxonomy of rhizobiua

The taxonomy of the rhizobia is in the state of flux at the monent, with a flood of proposals for new names. This has been stimulated both by the isolation of symbionts from new sources and by the advences in molecular methods,notably DNA sequencing and DNA-DNA hybridization, that have greatly increased the confidence with which we can assign strains to species and describe the relationships among species(Young, 1996). It has been suggested that a bacterial species could simply be defined as a group of strains sharing 70% or greater DNA-DNA relatedness, measured under specified conditions (Stackebrandt and Goebel ,1994; Vandamme et al., 1996). This degree of relatedness has been found to correlate with the 16S rRNA sequence similarity of 97% or greater. In addition, the formed species should be supported by phenotypic characteristics which can be used in strain identification (Graham et al., 1991). Nowaday, if a new species is to be defined use of the so-called 'polyphasic taxonomy' is recommended. This approach is an attempt to integrate different kinds of information (phenotypic, genotypic and phylogenetic) on a microorganism of interest into a consensus type of classification system (Graham et al., 1991; de Lajudie et al. 1994; Vandamme et al., 1996). Many of the recent changes in bacterial classification have been prompted by a desire to bring taxonomy into line with our increasing knowledge of evolutionary relationships, that is phylogeny. Sequences of the small subunit ribosomal RNA (SSU or 16S rRNA), supported by the less extensive data for other genes, now form the main basis for ideas on bacterial phylogeny. The SSU data support the well established subdivision of rhizobia into tree genera: *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*. These all line within the alpha subdivision of the *Proteobacteria*, but on quite distinct brancher, each of which also includes many bacterial species that are not rhizobia. Fig. 3. Shows a phylogeny based on SSU sequences of almost all *Rhizobium* species and a selection of related non-rhizobia. Table 1 gives a list of the species names in current use and references to the papers in which they were published.

Fig. 3 Phylogenetic tree of rhizobia some related bacteria in alpha subdivition of the Proteobacteria. The tree is constructed by the Neighbor-Joining method (Saitou and Nei, 1987) from SSU rRNA sequences. All the sequences used are almost full length and are derived from the type strain wherever possible. "*Mesorhizobium*" *tianshanens*e, " *M* " , *mediterraneum* and *Bradyrhizobium liaonigngense* were omitted as full sequences were not available (Young, 1996).

Table 1 Currently described species of rhizobia.

Species	Representative strains	Reference
Rhizobium		Jordan 1984
Rhizobium leguminosarum Bv. Viciae, trifolii and phaseoli	ATCC 10004^T	Jordan 1984
Rhizobium tropici type B	CIAT 899^T	Martinez-Romero et al. 1991
Type A	CFN 299	Martinez-Romero et al.1991
Rhizobium etli	CFN 42^T, CE-3	Segovia et al. 1993
Rhizobium hainanense	CCBAU 57015^T	Chen et al. 1997
Sinorhizobium		de Lajudie et al. 1994
Sinorhizobium meliloti	ATCC 9930^T	Jordan 1984
Sinorhizobium fredii	USDA 205^T	Scholla and Elkan 1984
Sinorhizobium teranga	ORS 1009^T	de Lajudie et al. 1994
Sinorhizobium saheli	ORS 609^T	de Lajudie et al. 1994
Sinorhizobium medicae	A 321^T, CC169	Rome et al. 1996
Mesorizhobium		Javis et al. 1997
Mesorizhobium loti	NZP 2213^T	Javis et al. 1997
Mesorizhobium huakii	CCBAU 2609^T	Chen et al. 1995
Mesorizhobium ciceri	UPM-Ca7^T	Nour et al. 1995
Mesorizhobium mediterraneum	UPM-Ca36^T	Nour et al. 1995
Mesorizhobium tianshanense	CCBAU 3306^T	Chen et al. 1995
(Rhizobium)		
(Rhizobium) galegae	HAMBI 540^T	Linstrom 1989
Bradyrhizobium		Jordan 1982
Bradyrhizobium	ATCC 10324^T	Jordan 1984
Bradyrhizobium	USDA 76^T	Kuyendall et al. 1992
Bradyrhizobium	2281^T	Xu et al. 1995
Azorhizobium		Dreyfus et al. 1988
Azorhizobium caulinodans	ORS 571^T	Dreyfus et al. 1988

9. Genotypic diversity and phylogeny of rhizobium

Before the development of molecular methods, bacteria used to be classified according to their phenotypic features. Nowadays, however, the taxonomy and phylogeny of bacteria is based increasingly on genotypic characteristics, that is on the genetic constitution of the organism. the bacterial genome can divided into two parts : chromosomal DNA and plasmid DNA. It has become apparent that the phylogenies of these two components do not necessarity agree. This is especially interesting, because in fast-growing rhizobia the symbiotic genes

commonly reside in plasmids, which can transfer between strains. Thus instead of being a permanent association, the chromosomal background-plasmid combinations can vary. However, also symbiotic genes locates in the chromosome have been shown to be transferable. Nevertheless, sequence comparison of symbiotic genes from different strains and species is possible without a knowledge of their genomic location.

Table 2 examples of techniques applied for studying the chromosomal diversity of rhizobia.

Method	References
Multilocus enzyme electrophoresis (MLEE)	Pinero et al. 1988; Eardly et al. 1990; Souza et al. 1994
Fatty acid methyl ester (FAME)	So et al. 1994; Jarvis et al. 1996
Whole-cell protein analysis	Moreira et al. 1993; de Lajudie et al. 1994
Pulsed-field gel electrophoresis (PFGE)	Corich et al. 1991; Sobral et al. 1991
DNA-DNA hybridization	Rinaudo et al. 1991; Laguerre et al. 1993
DNA restriction fragment length Polymorphism (RFLP)	Young and Wexler 1988; Kailalainen and Linstrom 1989; Urtz and Elkan 1996
16S rRNA gene sequencing	Martinez-Romero et al. 1991; young et al. 1991; Willems and Collins 1993; Yanaji and Yamasato 1993
Restriction fragment length polymorphism of PCR-amplified DNA fragments (PCR-RFLP)	Laguerre et al. 1994; Paffetti et al. 1996 Nick et al. 1994
PCR of repetitive DNA sequences (rep-PCR Using REP, ERIC, BOX primers)	de Bruiln 1992; Nick and Linstrom 1994 Versalovic et al. 1994
Ribosomal DNA spacer (IGS) polimorphism	Nour et al. 1995; Laguerre et al. 1996; Gurtler and Stanisich 1996
PCR with random or arbitary primers(RAPD, AP-PCR)	Harrison et al. 1992; Richardson et al. 1995; Selenska-Pobell et al. 1996
Insertion sequence elements (IS) typing	Hartmann and Amarger 1991; Kosier et al1993; Rice et al. 1994, Minamizawa et al., 1992

9.1 Chromosomal diversity

Summary of some techniques have been used for studying the chromosomal diversity of rhizobia was shown in table 2. The first three methods give the phenotypic information, whereas the rest give genotypic information derived from the DNA (Vandamme *et al*., 1996) Restriction fragment length polymorphism (RFLP) analysis of Southern blotted genomic DNA with various chromosomes and symbiotic plasmid probe has been used to type strains in different species of Rhizobium. Distinist symbiotic gene hybridization patters could be found in identical chromosomal types, and correlation were not always found between chromosomal any symbiotic plasmid types, suggesting interstain transfer and recombination of symbiotic sequence in the course of evolution. Therefore, for a proper characterization of Rhizobium strains, chromosomal and symbiotic gene markers should be concomitantly used. Although the techniques based in PCR amplification of DNA have rapidly gained great popularity in the studies of bacterial diversity. Recently various methods based on the PCR have been proposed to characterize *Rhizobium* strains and to examine genetic relationships in Rhizobium groups. Direct sequencing of genes coding for 165 rRNA (16S rDNA) amplified by PCR and RFLP analysis of these PCR-amplified sequences (PCR-RFEP analysis) have been used to establish genetic relationships and to characterize *Rhozobium* strains at the species and higher levels. DNA sequence analysis of 16S rRNA regions has revealed a much greter diversity than previously recognized (Martinez-Romero et al., 1996, Oyaizu e al., 1993 and Young et al., 1991), leading to important revisions in the taxonomy and systematics of this group of bacteria and in the description of new genera and species (Lindstrom et al., 1998; Martinez et al., 1996; Young and Haukka, 1996). Moreover, amplified rDNA restriction analysis (ARDRA), a technique that is suitable to group strains at the genus or species level of taxanomic resolution (Heyndrickx et al., 1996). Other PCR methods base on direct analysis by gel electrophoresis of multiple DNA fragments amplified stimultaneously have been proposed to fringerprint Rhizobium strains. Dooley et al. proposed this approach for identification and phylogenetic grouping of Rhizobium isolates. DNA fringerprints can also be generated by using pairs of primers derived from repetitive extragenetic palindromic (REP) In addition, another short primers with arbitrary nucleotide sequences, RAPD (random amplified polymorphic DNA) has also been in this study sine it simply detect polymorphisms which allow rapid identification and isolation of chromosome-specific DNA fragment.

10. Objective

To characterize the effective rhizobial strains for forage legumes in terms of physiology and genetic relatedness.

2. Materials and Methods

1. Rhizobial Strains

Strains of rhizobia from four forage legumes were isolated from their nodules collected from field grown forage legumes in Thailand at the following proviences; Nakhon Ratchasima, Chonburi, Lopburi, Ratchaburi, Lampang, Khon Kean, Srisaket and Chai Nat. Some of them were obtained from Department of Agricluture and NifTAL project (University of Hawaii). Isolation, purification and authentication were carried out according to the methods described by Somasegaran and Hoben (1994). The rhizobial reference strains; *Mesorhizobium ciceri* UMF-CM7, *R. trifolii* IFO15247, *R. leguminosarum* Kl, *R. etli* CFN42, *M. huakii* and *Bradyrhizobium japonicum* USDA110, *Sinorhizobium meliloti* were kindly provided from Prof. Y. Muruooka, Dapartment of Bittechnology, Osaka University. The high efficiency N_2-fixation rhizobial strains from various forage legumes were obtained from Dr. Achara Nuntagij, Department of Agriculture, Bangkok (as summarized in table 3).

Table 3 High efficiency N_2-fixation rhizobial strains from various forange legumes.

Host plants	Rhizobial strains
1. *Desmanthus virgatus*	DASA23008, DASA23010, DASA23011, DASA23015*, DASA23017, DASA23024, DASA23027, DASA23028, DASA23029, DASA23022, DASA23043, DASA23044, DASA23045, DASA23046, DASA23047, DASA23048, DASA23052, DASA23053, DASA23056, DASA23059, DASA23068, DASA23076*, DASA23079*, DASA23088
2. *Centrosema pubescens*	DASA24002, DASA24005, DASA24006, DASA24007, DASA24008*, DASA24009, DASA24012, DASA24015*, DASA24016*, DASA24019
3. *Stylosanthes hamata*	DASA25001, DASA25005*, DASA25008*, DASA25015*, DASA25016, DASA25018, DASA25019, DASA25020, DASA25021, DASA25023
4. *Chamaecrista rutundifolia*	DASA29004, DASA29007*, DASA29010*, DASA29011, DASA26012, DASA29012, DASA29013, DASA29014, DASA29015*, DASA29017

* The higher eficiency N_2-fixation rhizobial strains in each host plants.

2. Strain Characterization

2.1 Physiological property

A. Acid-Alkaline Production

Rhizobial strains were streaked on Yeast-Malt extract Agar (YMA) (Somasegaran, P, and Hoben, H. J., 1994). This medium contained 5 g/l manitol, 0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4.7H_2O$, 0.1 g/l NaCl, 0.5 g/l Yeast extract, distilled water 1 l and 1.5% agar. Bromthymol blue (BTB) was added as the acid/base indicator and incubated at 28°C for 10 days. colony forming was observed everyday as well as the changing of colour-medium. For acid-production strain, the medium colour was changed from green to yellow while alkaline-production from green to blue.

B. IAA (Indole Acetic Acid) Production

IAA production was determined by adding 2 ml of 0.01 M $FeCl_3$ in 35% $KclO_4$ in to 1 ml of Tris-TMRT culture broth (10.0 g/l D-manitol, 0.2 g/l Yeast-extract, 0.2 g/l $CaCl_2.2H_2O$, 0.25 g/l $MgSO_4.7H_2O$, 1.21 g/l Tris-base and 0.061 g/l L-tryptophan, pH 6.8) after incubated at 28°C for 10 days. The mixture were incubated in the dark at 30°C for 30 minutes. Results were compared with positive control of 1.0 g IAA in distilled water and ethanol (1:1 for 1.0 ml) (Nuntagij, et al., 1997).

C. Antibiotic resistance profiles

Plates containing an antibiotic, as well as a nonselective control plate, were incubated with 10 µl of inoculum. Resistance to a particular concentration of antibiotic was defined as the ability of a strain to form colonies at that concentration. The antibiotics and the concentration used in this study are listed in Table 4. Al preparations were filter sterilized by using 0.45 µm membrane filters. antibiotics were added to molten agar after sterilization and cooling to 50°C. (Teaumroong, et al., 1995).

D. APIZYM test

The APIZYM-kit (API system 5, La Balme le, Gro Hes, Mantalieu-Vercieu, France) against 19 kinds of substrates was used and enzyme activity of the strains were qualitatively assayed from colour development along with recommendation in the manuscript.

Table 4 Type and concentration of antibiotic used in the study.

Antibiotic	Concentration (µg/ml)	Solvent
Carbenicillin (Car)	500	Distilled water
Choloramphenicol (Chl)	500	Distilled water
Erythromycin (Ery)	250	50% ethanol
Nalidixic acid (Nal)	50	0.35 N NaOH
Streptomycin (Str)	100	Distilled water
Tetracycline (Tet)	100	50% ethanol
Trimethoprim (Tmp)	50	70% ethanol

2.2 Genotypic characteristics

A. Genomic DNA extraction

Prior to isolate the genomic DNA from rhizobial strains, reduction of exopolysaccharide was employed by culturing the strains in HM medium (Na_2HPO_4 0.125 g, Na_2SO_4 0.25 g, NH_4Cl 0.32 g, $MgSO_4.7H_2O$ 0.18 g, $FeCl_3$ 0.004 g, $CaCl_2.2H_2O$ 0.013 g, HEPES 1.3 g, MES 1.1 g, yeast extract 0.25 g, L-Arabinose 1.0 g per distilled water 1 l with final pH 6.8). After cultivation for 3-5 days on shaking condition for 200 rpm, at 30^{o}C, bacterial cells were pelleted in 0.1% lauroylsarcosine in TE buffer [0.1 M NaCl, 10 mM Tris-Cl (pH 8), 1 mM EDTA (pH 8)] and centrifugation at 13,000 rpm for 2 min. Cells were resuspended in 60 µl of 25% sucrose in TE fuffer, 700 µl of TE buffer and 80 µl of 1.0% lauroylsarcosine prior to incubation with 20 µl of 2.0 mg/ml lysozyme for 30 min at 37^{o}C. After that, add 75 µl of 5 M NaCl, the mixtures were extracted twice with saturated phenol and once with chloroform: isoamylalcohol (24:1). The aqueous phase was precipitated with two volumes of cold 99% ethanol. DNA pellets were dried under vacuum before dissolved in TE buffer and stored at 4^{o}C.

B. DNA primers and PCR conditions

The primers used for PCR in this study are summarized in Table 5. The use of primers Y1 and Y2 for amplification and sequencing of a short fragment of 16S rDNA was described Young et al. (1991). Primers *nod*A-1 and *nod*A-*2* was described by Haukka et al. (1997), and they amplify most of *nod*A (starting from base 14) and the beginning of *nod*B. Primers *nif*H-1 and *nif*H-2 amplifying *nif*H were described by Eardly et al. (1992). Table 6 lists the components used for the master mix. The polymerase, buffer and $MgCl_2$ were purchased from GibcoBRL, USA. Fifty ng of DNA template was used per reaction and PCR condition for each primer showed in Table 7.

Table 5 Primer sequences and positions of reference species used in this study.

Prime	Sequence*	Position in ref. Species or reference
Y1	5'-TGGCTCAGAACGAACGCTGGCGGC-3'	20-43 *E. coli*
Y2	5'-CCCACTGCTGCCTCCCGTAGGAGT-3'	361-338 *E. coli*
*nof*A-1	5'-TGCRGTGGAARNTRNNCTGGGAAA-3'	14-37 *S. meliloti*
*nod*A-2	5'-GGNCCGTCRTCRAAWGTCARGTA-3'	65-43 *S. meliloti* (nod B)
*nif*H-1	5'-AAGTGCGTGGAGTCCGGTFF-3'	256-275 *Smeliloti*
*nif*H-2	5'-GTTCGGCAAGCATCTGCTCG-3'	856-837 *S. meliloti*
REPIR-I	5'-NNNNCGNCGNCATCNGGG-3'	Judd, A. K. et al., 1993
REP2-I	5'-NCGNCTTATCCNGGCCTAC-3'	Judd, A. K. et al., 1993
RAPD	5'-GGAAGTCGCC-3'	Judd, A. K. et al., 1993

* Symbols used : A; Adenine, G; Guanine, C; Cytocine, T; Thymine, R; Purine (A or G), W; 'weak' (A or T) and N; Unknown (A, C, G or T).

Table 6 Components of the PCR reactions.

Components	Final conc./50µl	Stock conc.	Volumes
Buffer	1X	10X	5 µl
$MgCl_2$	1.5 mM	25 mM	3 µl
DNTP mixture	0.2 mM each	2.5 mM	4 µl
Forward primer*	12.5 pmol (125 nM)	5 pmol/µl	2.5 µl
Reverse primer*	12.5 pmol (125 nM)	5 pmol/µl	2.5 µl
Taq-polymerase	1 unit	varies	0.2 µl
PCR grade water	-	-	22.8 µl

* The given primer concentrations were used for Y1/Y2 and *nif*H primers. For *nod*A primers were used at a final concentration of 20 pmol (400 nM).

Table 7 The PCR conditions for the different primers.

Primer	Denaturation	Annealing	Primer extension	No. of cycles
Y1	93°C, 2 min	-	-	1
Y2	93°C, 45 sec	62°C, 45 sec	72°C, 2 min	34
			72°C, 5 min	1
*nod*A-1	93°C, 2 min	-	-	1
*nod*A-2	93°C, 45 sec	49°C, 1 min	72°C, 1 min	34
			72°C, 5 min	1
*nif*H-1	94°C, 30 sec	-	-	1
*nif*H-2	94°C, 30 sec	55°C, 30 sec	72°C, 1 min	30
			72°C, 5 min	1
RERIR-I	95°C, 6 min	-	-	1
RER2-I	94°C, 1 min	40°C, 1 min	65°C, 8 min	30
			65°C, 16 min	1
RAPD	94°C, 1 min	36°C, 1 min	72°C, 2 min	45

C. Souther Blot Hybridization

To determine nitrogene fixing gene localization, plasmid pRj 676 was isolated from *E. coli* by alkaline lysis. The pRj 677 was digested with the restriction enzyme *Hind* III to obtain a 9 kb *Hind* III fragment containing the *nif*HDKE gene from *Bradyrhizobium japonicum* (Hennecke, H., 1981). On the other hand, to determine nod gene localization, plasmid pRjUT 10 was isolated from *E. coli*DH1. The pRjUT 10 was also digested with the restriction enzyme *Hind* III to obtain a 3.9 kb *hind* III fragment containing the *nod* DYABC gene. Each fragment was tagging with Dig-11-dUTP and used as probe according to manuscript. Genomic DNA isolated from each rhizobial strain was digested with ghe restriction enzyme *EcoR* I. DNA hybridization was carried out at 55^{o}C for 24 hr. The hybridized DNA was detected on the nylon-membrane with anti-digoxigenin-alkaline phosphatase conjugate and a colour reaction specific for alkaline phosphatase (Boehringer Manheim, Germany).

D. Direct PCR amplification from root nodule

DNA templates were prepared from nodules after surface sterilization and directly amplified the bacteroid genomes in nodule by modification method of Aguilar et al, 1998. Template DNA from nodule rhizobia was prepared as follows: a single nodule was surface steriled with hydrogen peroxide and rinsed with sterile water. The nodule was crushed in a plastic microfuge tube by using a glass rod and 300 µl of sterile water was added after crushing and vortexed. After a short pulse of centrifugation at 4000 rpm for 10s in an Eppendorf Centrifuge model 5415C to remove debris, 30 µl of the supernatant was transferred to another tube and the rhizobia ware pelleted by centrifugation, suspended in 50 µl of sterile distilled water and incubated in boiling water bath for 5 min. Subsampled of 5 to 10 µl were used for the PCR assay.

E. Restriction digestion of PCR products

The *nod*A PCR products of three effective rhozobial strains from each plant host were digested with the endonucleases *Rsa* I, *Hnif* I and *Hha* I. The reaction mixtures (15µl) contained 5-12 µl of PCR product, 5 U of enzyme and 1.5 µl of buffer specific for the enzyme (Promega, U.S.A.). The restriction products were electrophoresed on 2.5% agarose gel at 80V for about 1 hr. Ethidium bromide-stained gels were photographed by using a UVP gel documentation system and the band patterns were scored manually. followed as *nod*A, the *nif*H PCR products

were digested with the restriction enzyme *Taq* I, *Msp* I, *Alu* I and *Hae* III. The restriction products were electrophoresed in 10% acrylamide gel at 80 V for about 1.5 hr.

F. Phylogenetic tree analysis

A phylogenetic tree was constructed from the respective genetic distances by the unweighted pair group method using arithmetic average (UPGMA) implemented in Phylogenetic Inference Package (PHYLIP ver. 3.572).

G. Direct sequencing from PCR products

The partial 16S sequences were generated with Perkin Elmer's ABI PRISM™377 DNA sequencer. for automated sequencing, the PCR products were purified using Gene clean II kit (Qaigen) following the manufacturer's instructions. Purified products were run in a 1% agarose gel to estimate the amount of DNA. Sequencing reactions were done using ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq DAN Polymerase, FS (Perkin Elmer). An estimated amount of 100 ng of DNA was used for each reaction together with 1.6 pmol of primer, 4 µl of ready reaction mix and double distilled water to make up a 10 µl final volume. The same primers were used as for previous PCR amplifications. Cycle-Sequencing PCR amplification and following DNA precipitation with ethanol were done following the manufacturer's instructions.

H. Sequence analysis

Generally only one strand of the 16S fragments was sequenced with the automated sequencer each PCR product was sequenced in both directions and the sequences were assembled and cheched with the autoassembler 1.4 program. In this study, to search for homologous sequence in the data bank, Gene Bank (American), BLASTN 2.0.13 was used.

I. Nucleotide sequence accession numbers

The sequence for strains DASA23079, DASA24008, DASA24015, DASA24016, DASA25005, DASA25015, DASA29007 and DASA29015 were analyzed in the GenBank sequence database under accession no. AF260269.1/AF260296, AF239848.2/AF2309848, Y15341.1/PLY15341, AF260297.1/AF260297, AF159436.1/AF159436, U28939.1/REU28939, AF260297.1/AF260297, AF239848.2/AF239848 and AF255337.1/AF255337 respectively.

3. Results and Discussion

1. Physiological characteristics

1.1 Determination of acid-alkaline and IAA production

1.1.1 *D. virgatus* rhizobia

From 24 effective rhizobial strains investigated in this experiment, the results (Table 8) showed that most of them were fast-grower group and able to produced IAA. For the colony forming size were varied among the strains in the range of diameter 0.3-0.5 mm. This group of rhizobium was assumed to belong in genus *Rhizobium* rather than other genera (Young, 1996).

1.1.2 *C. pubescens* rhizobia

From 10 effective rhizobial strains investigated in this experiment, only 3 strains were found that belong to fast-grower (DASA 24006, 24008 and 24019) and only 2 out of them able to produce IAA (DASA 24006 and 24008). The rest of the strains were most likely be slow-grower and only one strain (DASA 24005) could produce IAA (Table 9). The colony forming size found that very small particulary in slow-grower group.

1.1.3 *S. hamata* rhizobia

From 10 effective rhizobial strains investigated in this experiment, only 3 strains were found that belong to slow-grower group and none of them able to produce IAA (DASA 25001, 25005 and 25008). While the rest were fast grown but 5 of them (DASA 25016, 25018, 25020, 25021 and 25023) able to produce IAA (Table. 10). The small colony forming were also found in the slow-grower group.

1.1.4 *Ch. rotundifolia* rhizobia

From 10 effective rhizobial strains investigated in this experiment, only 6 out of them were slow-grower (DASA 29011, 29012, 29014, 29015,29016 and 29017) while 3 strains were slow-grower and only one strain (DASA 29010) unable to produce neither acid nor alkali under this condition. Two fast-grower strains (DASA 29014 and 29015) and 2 slow-grower strains (DASA 29004 and 29007) were found that unable to produce IAA (Table 11).

For *C. pubescens*, *S. hamata* and *Ch. rotundifolia* rhizobia the results show that, rhizobial strains from these hosts could produced both acid and alkaline, but most of them could produced acid except in *C. pubescens* rhizobial strains. Compare rhizobial strains which contained ability to differently produced acid and base suggested that most of rhizibia which can produce acid also could produce IAA than alkaline producing rhizobia. Acid and alkaline production with Yeast extract-manitol (YEM) as the culture-medium has been employed as a general taxonomic character for rhizobia, slow-growing rhizobia produce alkali, while fast growing rhizobia produce acid (Stowers and Elkan, 1984). Norris (1965) has postulated that slow growing, alkali producing rhizobia have coevolved with legumes of tropical origin. Tropical soils are typically acid and hence the ability to produce alkaline would provide a selective advantage. However culture media carbon source and nitrogene source strongly influenced whether acid or alkali was produced (Stowers and Elkan, 1984). For IAA production, rhizobium can produce IAA as well as a number of IAA precusors asymbiotically. Soybean bradyrhizobia are comprised of two highly divergent species;B. *japonicum* and B. *elkanii* (Minamisawa et al., 1996). Although these two species differ in various phenotypic and genotypic traits, IAA production could be a criterion for the classification of soybean bradyrhizobia; *B. elkanii* strains exclusively synthesis and secrete IAA in the culture, while *B. japonicum* does not produce IAA (Minamisawa and Fukai, 1991) All strains in the is studied can produce IAA. It has been suggested that bacterial IAA production by *B. elkanii* is related to nodulation and host response.

In the previous paper (Fukuhara et al., 1994), IAA attenuated mutants from *B. elkanii* USDA 31 significantly reduced the nodule number on soybean roots. Yuhashi et al. (1995) suggested that *B. elkanii* – specific induction of outer cortical swelling (OCS) on soybean roots was probably due to the bacterial IAA production.

Table 8 Production of acid, alkali and IAA by *Desmanthus virgatus* rhizobial strains.

Rhizobial strain	Time to visible colony size (day)	Reaction (in YMA + BTB)	Colony characteristics	Colony dimeter (mm)	IAA production
DASA23008	1-2	Acid	Wet, translucent, convex, slime	3.0	+ + + +
DASA23010	1-2	Acid	Wet, translucent, convex, slime	3.0	+ + + +
DASA23011	1-2	Acid	Wet, translucent, convex, slime	0.5	+ + +
DASA23015	1-2	Acid	Wet, translucent, convex, slime	1.0	+ + +
DASA23017	1-2	Acid	Wet, off white, convex, slime	1.5	+ + +
DASA23024	1-2	Acid	Wet, off white, convex, slime	3.0	+ + + +
DASA23027	1-2	Acid	Wet, off white, convex, slime	1.5	+ +
DASA23028	1-2	Acid	Wet, off white, convex, slime	1.0	+ +
DASA23029	1-2	Acid	Wet, off white, convex, slime	0.5	+ + +
DASA23032	1-2	Acid	Wet, off white, convex, slime	1.0	+ +
DASA23043	1-2	Acid	Wet, off white, convex, slime	1.0	+ + +
DASA23044	1-2	Acid	Wet, translucent, convex, slime	1.0	+ + +
DASA23045	1-2	Acid	Wet, off white, convex, slime	1.0	+ + +
DASA23046	1-2	Acid	Wet, translucent, convex, slime	1.0	+ + +
DASA23047	1-2	Acid	Wet, translucent, convex, slime	1.0	+ + +
DASA23048	1-2	Acid	Wet, off white, convex, slime	1.0	+
DASA23052	1-2	Acid	Wet, off white, convex, slime	0.5	+
DASA23053	1-2	Acid	Wet, off white, convex, slime	1.0	+ +
DASA23056	1-2	Acid	Wet, off white, convex, slime	2.0	+ +
DASA23059	1-2	Acid	Wet, off white, convex, slime	2.0	+ + +
DASA23068	1-2	Acid	Wet, off white, convex, slime	1.5	+ +
DASA23076	1-2	Acid	Wet, off white, convex, slime	1.5	+ + +
DASA23079	1-2	Acid	Wet, off white, convex, slime	1.0	+ + +
DASA23088	1-2	Acid	Wet, off white, convex, slime	0.5	+
	-	-	-	-	-

Table 9 Production of acid, alkali and IAA of *Centrosema pubescens* rhizobial strains

Rhizobial strain	Time to visible colony size (days)	Reaction (in YMA+BTB)	Colony characteristics	Colony dimeter (mm)	IAA production
DASA 24002	3	Base	wet, white, translucent, slime	0.5-.1	-
DASA 24005	3	Base	wet, white, translucent, slime	0.5	+ + +
DASA 24006	3	Acid	wet, off white, translucent, slime	0.3	+ + +
DASA 24007	2	Base	wet, white, translucent, slime	1.0	-
DASA 24008	2	Acid	wet, white, translucent, slime	0.3	+
DASA 24009	3	Base	wet, white, translucent, slime	<0.5	-
DASA 24012	4	Base	wet, white, translucent, slime	<0.5	-
DASA 24015	3	Base	wet, white, translucent, slime	<0.5	-
DASA 24016	3	Base	wet, white, translucent, slime	<0.5	-
DASA 24019	2	Acid	wet, off white, translucent, slime	1.5-2.0	-

Table 10 Production of acid, alkali and IAA of *Stylosanthes hamata* rhizobial strains

Rhizobial strain	Time to visible colony size (days)	Reaction (in YMA+BTB)	Colony characteristics	Colony dimeter (mm)	IAA production
DASA 25001	4	Base	wet, white, translucent, slime	0.5	-
DASA 25005	3	Base	wet, white, translucent, slime	0.5	-
DASA 25008	5	Base	wet, white, translucent, slime	0.5-1.0	-
DASA 25015	3	Acid	wet, off white, translucent, slime	0.3	-
DASA 25016	2	Acid	wet, off white, translucent, slime	0.35	+ + +
DASA 25018	2	Acid	wet, off white, translucent, slime	0.2	+
DASA 25019*	2	Acid	wet, off white, translucent, slime	0.3	-
DASA 25020*	2	Acid	wet, off white, translucent, slime	0.35	+
DASA 25021	2	Acid	wet, off white, translucent, slime	0.4	+ + +
DASA 25023*	2	Acid	wet, off white, translucent, slime	0.35	+ +

Table 11 Production of acid, alkali and IAA of *Chamaecrista rotundifolia* rhizobial strains

Rhizobial strain	Time to visible colony size (days)	Reaction (in YMA+BTB)	Colony characteristics	Colony dimeter (mm)	IAA production
DASA 29004	3	Base	wet, white, translucent, slime	=<0.5	-
DASA 29007	5	Base	wet, white, translucent, slime	0.1	-
DASA 29010	3	neutral	wet, white, translucent, slime	=<0.5	+
DASA 29011	2	Acid	wet, off white, translucent, slime	0.1-0.2	+
DASA 29012	2	Acid	wet, off white, translucent, slime	0.3	+
DASA 29013	3	Base	wet, white, translucent, slime	=<0.5	+ + +
DASA 29014	2	Acid	wet, off white, translucent, slime	0.5-1.0	-
DASA 29015	2	Acid	wet, off white, translucent, slime	0.2	-
DASA 29016*	2	Acid	wet, off white, translucent, slime	0.2	+ + +
DASA 29017	2	Acid	wet, off white, translucent, slime	0.3	+ + +

1.2. Intrinsic antibiotic resistant profiles of forage legumes rhizobia

To determine the pattern of intrinsic antibiotic resistant, various kinds of antibiotics were employed. The results indicated that most of the strains in this study were sensitive to erythromycin. For the group of *D. virgatus* rhizobia, the antibiotic resistant profile were found that somewhat similar pattern performed within the group such as most of the strain were resisted to carbenicillin, nalidixic acid and trimothoprim. On the other hand, most of them also suscepted to both erythromycin and tetracycline. This might roughtly indicated the closely related among the group of *D. virgatus* rhizobia. Moreover, these profiles also were almost the same as reference strain *R. tropici*.

In case of *C. pubescens* rhizobia, most of them were found that resisted to trimethoprim and susceptible with erythromycin. Other profiles in other types of antibiotics were rather heterogenous than *D. virgatus* rhizobia.

For *S. hamata* rhizobia, most of the strains were suscepted to chloramphenicol and erythromycin. Among the all slow grower strain in this group, susceptibility to tetracyclin were found and for the all fast-grower, susceptibility to kanamycin were also observed.

For *Ch. rotundifolia* rhizobia, almost of the strain were resisted to both nalidixic acid and trimethoprim except only one strain DASA 29004. The results of intrinsic antibiotic resistant profiles obtained from all effective forage legumes rhizobia were summerized in Table 12.

Antibiotic sensitivity of rhizobial strains has been used extensively both for general characterization and for identification of marked strains (Beynon and Josey, 1980; Rupela et al., 1982; Eaglesham, 1987; Mueller et al., 1988; Date and Hurse, 1997, Batzli et al., 1992). In this study the rhizobial isolates varied in the number and type of antibiotics and host plants. The results of rhizobia isolates from different host plants indicated that rather similarity in each groups. Fast-growing isolates were generally more sensitive than slow-growing types. Reference fast-growing strains were on average more sensitive to the antibiotic levels tested compared to the slow growing isolates. This observation agreed with results of other studies that showed slow-growing rhizobia to be less senitive to antibiotics (Elkan, 1992). Mueller et al. (1988) observed that sensitivity of rhizobia may be low for those antibiotics that may be routinely produced by other soil organisms.

Table 12 Rhizobial strains used in this study and their intrinsic antibiotic profiles

Strain	Antibiotic resistance

	Car	Chl	Ery	Nal	Tmp	Tet	Kan	Str
M. ciceri	S	S	S	Sm	r	S	S	S
R. trifolii	S	S	S	Sm	r	S	S	S
S. meliloti	S	S	S	r	r	S	S	S
R. nhaesioli	S	S	S	r	r	S	S	S
R. tronici	r	S	S	r	r	S	S	Sm
R. leauminosarum	S	S	S	r	r	S	S	S
R. etli	S	S	S	r	Sm	S	S	r
M. huakii	S	S	S	r	Sm	S	S	Sm
Bradyrhizobium iaponicum	S	r	S	r	r	r	S	r
DASA 23008	S	S	S	Sm	r	S	Sm	r
DASA 23010	S	Sm	S	Sm	r	S	Sm	r
DASA 23011	r	Sm	S	r	r	S	Sm	r
DASA 23015	r	Sm	S	r	r	S	Sm	r
DASA 23017	r	Sm	S	r	r	S	Sm	r
DASA 23024	S	S	S	r	r	S	Sm	r
DASA 23027	r	Sm	S	r	r	S	Sm	r
DASA 23028	r	Sm	S	r	r	S	S	r
DASA 23029	r	Sm	S	r	r	S	S	r
DASA 23032	r	Sm	S	r	r	S	S	r
DASA 23043	r	Sm	S	r	r	S	S	r
DASA 23044	r	Sm	S	r	r	S	S	r
DASA 23045	r	Sm	S	r	r	S	S	r
DASA 23046	r	Sm	S	r	r	S	S	r
DASA 23047	r	S	S	r	r	S	S	r
DASA 23048	r	S	S	r	r	S	S	S
DASA 23052	r	Sm	S	r	r	S	S	S
DASA 23053	r	S	Sm	r	r	S	S	S
DASA 23056	r	Sm	Sm	r	r	S	S	S
DASA 23059	r	Sm	Sm	r	r	S	S	Sm
DASA 23068	r	Sm	Sm	r	r	S	S	S
DASA 23076	r	Sm	S	r	r	S	S	S
DASA 23079	r	Sm	S	r	r	S	S	r
DASA 23088	r	S	S	r	Sm	S	S	Sm
DASA 24002	r	r	S	r	r	S	S	S
DASA 24005	r	r	S	Sm	r	S	S	S
DASA 24006	S	r	S	r	r	r	r	S
DASA 24007	Sm	r	S	Sm	Sm	Sm	r	S
DASA 24008	Sm	Sm	S	r	r	S	S	Sm
DASA 24009	Sm	r	S	Sm	r	S	S	S
DASA 24012	S	S	S	Sm	r	S	Sm	S
DASA 24015	r	r	S	Sm	r	r	S	r
DASA 24016	S	S	S	S	r	S	S	r
DASA 24019	S	S	S	r	r	S	r	r
DASA 25001	r	S	S	S	r	S	S	r
DASA 25005	Sm	S	S	r	S	S	S	r
DASA 25008	Sm	S	S	r	Sm	S	r	S
DASA 25015	Sm	S	S	r	r	S	S	S
DASA 25016	r	S	S	r	r	r	Sm	S
DASA 25018	r	S	S	Sm	Sm	r	S	r
DASA 25019	S	S	S	S	r	r	S	S
DASA 25020	Sm	S	S	r	S	S	S	S
DASA 25021	S	S	S	S	r	r	S	S
DASA 25023	S	S	S	r	r	S	S	S
DASA 29004	S	S	S	S	S	S	S	S
DASA 29007	r	r	S	r	r	r	r	S
DASA 29010	r	r	S	r	r	r	r	Sm
DASA 29011	r	r	S	r	r	r	S	Sm
DASA 29012	r	Sm	S	r	r	r	S	S
DASA 29013	Sm	r	S	r	r	r	S	r
DASA 29014	r	r	S	r	r	r	S	Sm
DASA 29015	r	S	S	r	r	S	S	S
DASA 29016	S	S	S	r	r	S	r	r
DASA 29017	r	r	S	r	r	S	S	r

DASA 23_ _ _ ; *D. virgatus* rhizobial strains, DASA 24_ _ _ ; *C. pubescens* rhizobial strains, DASA 25_ _ _ ; *S. hamata* rhizobial strains and DASA 29_ _ _ ; *Ch. Rotundifolia* strains. r = resistant, S = susceptible and Sm = susceptible with mutated colony

1.3. APIXYM-test analyses

To detect the profile of enzymes activity generated by APIXYM-test, it was found that, enzymes which most of them could produce in high quantity were leucine arylamidase, phosphatase alkaline, phosphatase acid, valine arylamidase and α-glucosidase. On the other hand, the enzymes which most strains could neither produce nor produce in low quantity were esterase, lipase, chymotrypsin, α-galactosidase, glucuronidase, monosidase and fucosidase.

For *D. virgatus* rhizobial strains which most strains could produce in high quantity were phosphatase alkaline, leucine arylamidase, valine arylamidase, phosphatase acid, β -galactosidase, α -glucosidase and N-acetyl-glucosaminidase. On the other hand, *D. virgatus* rhizobial strains exhibited several carbohydrate degradation activity such as galactosidase, β-galactosidase, β-glucosidase, monosidase and fucosidase. However, this was differed from other bradyrhizobia which unable to produced these enzymes (Natagij, et al., 1997)

In case of *C. pubescens* rhizobial strains, the enzyme which most strains could produce in high quality were phosphatase alkaline, leucine arylamidase, phosphatase acid except strains DASA 24012, 24015 and 24016 which found that, all strains could produce alkali. Enzymes which most strains could not produce or produce in low quantity were esterase, lipase, chymotrypsin, α -galactosidase, β-galactosidase, glucuronidase, β-glucosidase, N-acetyl-glucosaminidase, monosidase and fucosidase.

For *Ch. rotundifolia* rhizobial strains, the enzymes which most strains could produce in high quality were leucine arylamide and phosphatase acid except strain DASA 25001. The enzymes which most strains could not produce or produce in low quality were esterase, lipase, chymotripsin, α-galactosidase, glucuronidase, N-acetyl-glucosaminidase, monosidase and fucosidase.

Ability to utilize as sole carbon sources large numbers of compounds is an important trait in numerical taxonomy (De Lajudie et al., 1994). Fast-but not slow-growing rhizobia are reported to be able to utilize as sole carbon sources certain mono-, di- and trisaccharides (Elkan, 1992).

2. Genotypic characteristics of forage legume rhizobia

Assessment of rhizobial genotypic diversity relevant to ecologically oriented studies requires a higher level of taxonomic resolution than can be achieved by 16S rDNA sequencing (Fox et al., 1992). Isolates of the same species, even of the same serotype, can significantly differ in their N_2-fixing efficiencies and in their abilities to occupy nodules in competition with other closely related strains (Streit et al., 1992; Triplett et al., 1992). PCR-based genomic fingerprints generated with short arbitrary primers (randomly amplified polymorphic DNA-PCR [RAPD-PCR]) (Welsh and Mcclelland, 1990) or primers binding to inter spersed repetitive sequences (repetitive extragenic palindromic PCR [rep-PCR]) (Versalovic et al., 1994) give the highest level of taxonomic resolution currently achievable by PCR methods (de Bruijn, 1992; Laquerre et al., 1996; Van Rossum et al., 1995). The high degree of reproducibility of the rep-PCR approach has recently been discussed (Tyler et al., 1997).

Genotypic characteristics in this study, used various methods such as DNA-DNA hybridization, REP-PCR, RAPD-PCR, PCR-RFLP and 16S rDNA sequencing for classify each forage legume rhizobium.

2.1 *D. virgatus* rhizobia

Twenty four *D. virgatus* rhizobia were examined from hybridization patterns using the restriction enzyme *Hind III* to digest the target DNAs and using common *nod*DYABC from *B. japonicum* as DNA probe.

The results of hybridization patterns were separated into four different groups (fig. 4). The fifteen strains, possessed the 6.0 kb and 3.0 kb fragment. Interestingly, this group showed the pattern similar to *R. tropici*. Another five strains, shared the 10.0 kb and 6.0 kb. For three strains, had the 6.0 kb fragment, while the remaining one strains had the 3.0 kb and 1.7 kb. The common *nod*DYABC genes that used as a DNA probe in this study are structurally and functually conserved among all rhizobia and required for both root hair curling and infection (Kandrosi et al., 1984; Lerouge et al., 1990; Roche et al., 1991; Truchet et al., 1991, Sanjuan et al., 1992). From this study demonstrated that the common *nod* genes of the *D. virgatus* rather nearly related among in the group of *R. tropici*.

Total genomic DNAs from 24 *D. virgatus* rhizobia were used as DNA template for PCR analysis using both of the REP and RAPD primers. PCR products were separated on 1.5% agarose

Fig. 4 Signal generated by *nod* DYABC probe hybridsed with *Hind III* restricted total DNA of *D. vergatus* rhizobia .The patterns were, from left to right, pattern I; 6.0 kb and 3.0kb, II; 10.0 kb and 6.0 kb, III; 6.0 kb and VI; 3.0 kb and 1.7 kb

gels and stained with ethidium bromide. The result of PCR-RAPD patterns was depicted in Fig 5. On averages, the PCR products generated fingerprint of 2 to 7 fragments of variable length, ranging from 250 bp to 1.5 kbp. The PCR products generated from RAPD primer, could be distinguished the strains into 6 different groups.

For using REP as primer, yielded multiple DNA products (Fig 6), the size of which ranged from approximately 300 bp to 3.0 kbp. The strains can be differentiated into 7 different groups. However, with REP and ERIC primers it is possible to get a fingerprint consisting of several fragments for each strain used, whereas performing a PCR with random primers the fingerprints contain fewer fragment, if any de Bruijn (1992) and Versalovic et al. (1992) presented that the REP and ERIC PCR is a useful tool for taxonomically studies.

To compare the relationship of PCR products and hybridization signal patterns showed some similarly related among the strains (Table 22). Interestingly, strain DASA 23047 shared the same DNA fingerprinting as *R. tropici* which assumed that group of effective rhizobial in *D. virgatus* probably shared the same cluster as *R. tropici*.

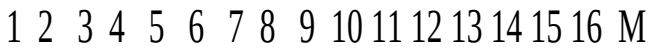

Fig.5 Some RAPD-PCR products from *D. virgatus* rhizobial strains,lane 1*; R. leguminosarum*, 2; *R. trifolii*, 3; *R. phosioli*, 4*; S. meliloti*, 5; *M. huakii*, 6; *M. ciceri*, 7; *R.etli*, 8; *R. tropici*, 9; DASA23047, 10; DASA23056, 11; dasa23010, 12; DASA23008, 13; DASA23052, 14; DASA23024, 15; DASA23079, 16; DASA23076 and M; 1 kb ladder marker.

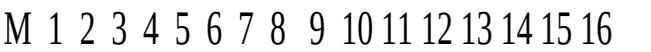

Fig. 6 Some REP-PCR products from *D. virgatus* rhizobial strains, M; 1 kb ladder marker, 1; DASA23010, 2; DASA23011, 3; DASA23015,4; DASA23017, 5; DASA23024, 6; DASA23027,

7; DASA23028, 8; DASA23029, 9; DASA23032, 10; DASA23045, 11; DASA23008, 12; DASA23046, 13; DASA23048, 14; DASA23047 and 15; DASA23044.

Table 15 Comparision of Eco *RI* RFLP, REP and RAPD patterns among effective *D. virgathus* rhizobial strains.

Strain	Eco RI RFLP	REP	RAPD
DASA 23015	E 1	RE 1	RA 1
DASA 23043	E 1	RE 1	RA 1
DASA 23044	E 1	RE 1	RA 1
DASA 23048	E 1	RE 1	RA 1
DASA 23056	E 1	RE 1	RA 1
DASA 23059	E 1	RE 1	RA 1
DASA 23088	E 1	RE 1	RA 1
DASA 23047	E 1	RE 1	RA 5
R. tropici	E 1	RE 1	RA 5
DASA 23011	E 1	RE 2	RA 1
DASA 23027	E 1	RE 2	RA 1
DASA 23046	E 1	RE 2	RA 1
DASA 23053	E 1	RE 4	RA 1
DASA 23076	E 1	RE 5	RA 2
DASA 23079	E 1	RE 5	RA 2
DASA 23052	E 1	RE 7	RA 6
DASA 23028	E 2	RE 1	RA 1
DASa 23029	E 2	RE 1	RA 1
DASA 23032	E 2	RE 2	RA 1
DASA 23068	E 2	RE 2	RA 1
DASA 23045	E 2	RE 4	RA 1
DASA 23008	E 3	RE 3	RA 3
DASA 23010	E 3	RE 3	RA 3
DASA 23024	E 3	RE 6	RA 4
DASA 23017	E 4	RE 2	RA 1

E1 : hybridization signals in size 6 and 3 kb., E2 : hybridization signal in size 10 and 6 kb., E3 : hybridization in size 6 kb and E4 : uncleared signals.

Morever, these rhizobial strains were similar to type strain of *R. tropici* and some strain such as DASA 23047 was most likely belong to *R. tropici* according to DNA analyses results such as REP in combination with RAPD-PCR patterns which were generated in dendrogram (Fig.7). To confirm this phenomenon, strain DASA 23047 was inoculated onto kidney bean (*Phaseolus vulgaris*) which was the plant host of *R. tropici*, at the same time *R. tropici* was also inoculated seeds of *D. virgatus*. Both of two plants nodulation were observed and again confirmed by directly amplified genomic DNA from bacteroids in nodules by using REP as primer (Fig. 8). The results suggested that strain DASA 23047 was most likely belong or closely related to *R. tropici*. However, from some studies of host range analysis of rhizobia that nodulate soybeans and *Phaseolus* beans, they revealed that wide host range of some rhizobia species; *Sinorhizobium fredii* strains isolated from *Glycine max* and *G. soja* have nodulated *P. vulgaris* as well as 60 other legume species (Keyser et al., 1982). In addition, *B. japonicum* can induces nodules on a wide range of leguminous plants (Jordan, 1984).

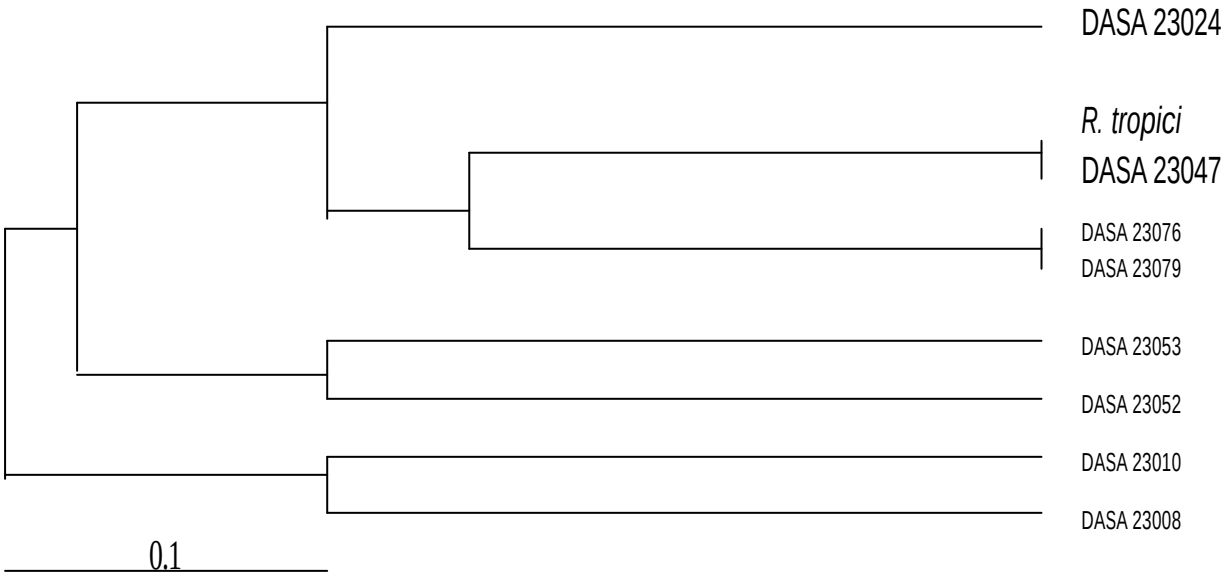

Fig.7 Dendogram showing intra-specific variation of *D. virgatus* rhizobia and *R. tropici* obtained by combination of REP and RAPD analysis.

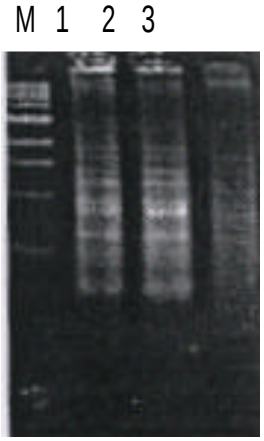

Fig.8 REP-PCR products directly generated from root nodules., Lane 1 ; PCR products from *R. tropici*, Lane 2 ; PCR products from nodule of kidney bean (*Phaseolus vulgaris*) inoculated with DASA 23047 and Lane 3 ; PCR products from a nodule of *D. virgatus* inoculated with *R. tropici* .

Diversity in rhizobia nodulating tropical legumes has been revealed by many studies, and these bacteria belong to several bacterial genera and species. Many slow growing strains isolated from tropical legumes belong to the genus *Bradyrhizobium* (Gao et al., 1994 and Jordan, 1984). A unique group of strains inducing stem and root nodules on *Sesbania rostrata*, a tropical legume of Africa, constitute the genus *Azorhizobium* (Dreyfus et al., 1988). *R. tropici* and *R. etli* were proposed for the rhizobia isolated form the common bean (*P. vulgaris*) and *Leucaena leucocephala* in the tropical region.

To determine more detail in genetic relatedness, three out of ten effective rhizobial strains were chosen. From *D. virgatus* were DASA23015, 23076 and 23079, *C. pubescens* were DASA24002, 24005 and 24016, *S. hamata* were DASA25005, 25008 and 25015, *Ch. Rotundifolia* were DASA29007, 19010 and 29015. Restriction digestion of PCR products and sequencing of *D. virgatus* rhizobial strains were conducted. The results of RFLP analysis of PCR-amplified *nod*A digested with the restriction endonuclease *Hha I*, *Hinf I* and *Rsa I* were indicated

in Fig 9. The results distinct that all of them share the same pattern exceped SUT2 not consistant with other strains. Phylogenetic tree constructed from *nod*A-RFLP digested with enzyme *Hha I*

(Fig 10), *Hinf I* (Fig 11), *Rsa I* (Fig 12) and combination of three enzymes were demonstrated in Fig 13.

On the other hand, the results of RFLP analysis of PCR amplified from *nif*H region digested with the restriction endonucleases *Alu I*, *Msp I*, *Hae III*, and *Taq I* were showed in Fig. 14 (lane 16,17,and 18), Fig. 15 (lane 10), Fig. 16 (lane 10,11 and 12) and Fig. 17 (lane 10 and 19), respectively. The results clearly indicated that all of three strains, DASA 23015, 23076 and 23039 have same similar patterns . Previous study revealed that most of them share another characteristic such as IAA production, Acid-base production, intrinsic antibiotic resistance profiles, RAPD and REP-PCR patterns. As would be expected, these isolates consistent with the reference strains, *R. tropici*, but SUT2 distinct from other strains. Phylogenetic tree constructed from *nif*H-RFLP digested with enzymes *Alu I* (Fig. 18), *Msp I* (Fig. 19), *Hae III* (Fig. 20), *Taq I* (Fig. 21) and combination of four enzymes (Fig. 22), strongly suggested that rhizobia strains isolated from *D. virgatus* were resemble to *R. tropici*. The phylogenetic trees based on the *nod*A gene of the *D. virgatus* rhizobia also reveal closely related to *R. tropici* (Fig 13). However, some correlations between *nod* and *nif* gene have also been supported by Urtz and Elkan (1996), who used the RFLP technique to study the diversity among *Bradyrhizobia* that nodulate peanuts. In case of SUT2, the NodA tree result is not consistant with any reference strains used in this study, but the NifH tree result showed that it more related with *R. leguminosarum* than other reference strains used in this study.

Phylogenetic studies have been conducted on different nitrogen-fixation genes, e.g. *nif*D (Ueda et al., 1995b), but *nif*H has been the one most commonly used. *Nif*H is often part of *nif*HDK operon, where *nif*D and *nif*K code for the α- and β- subunits of component I in nitrogenase, respectively, and *nif*H codes for two identical subunits of component II, nitrogenase reductase. The nitrogenase enzyme complex, comprising the components I and II, is reponsible for the actual dinitrogen fixation. *Nif*HDK genes are highly conserved, *nif*H might be even the most conserved translated gene in bacteria (Ruvkan and Ausubel, 1980; Norel and Elmerich 1987). At a higher taxonomic level *nif*H and 16S rRNA phylogenies are similar, and, for example,

the *nif*H genes of species in the alpha subdivision of the *Proteobacteria* are quite distinct from the *nif*H genes of species in the gamma subdivision (Young, 1992). Therefore, in this study, one from three isolates were selected for 16S rDNA sequencing. Comparative sequence of about 300-bp fragment of the 16S rDNA genes of isolate (DASA23079) and *R. tropici* are presented in Fig. 23. The results shown distinct that, strains DASA 23015, 23076, 23079 are *R. tropici*, with 99% similarity. Furthermore, Young (1993) demonstrated that for most rhizobium species, phylogenetic positions based on partial 16S rRNA gene sequences data were very similar to phyolgenetic position based on complete sequence data.

Martinez-romero et al.,(1991) presented a new species, *R. tropici*. They were distinguished from other species at the molecular level by the results of whole-DNA hybridization tests, their multilocus enzymes electrophoresis profiles, and their ribosomal gene sequence. *R. tropici* contains two subgroups that correspond to type IIA and type IIB strains. In this study, *R. tropici* reveal the same type IIB strains, refer to IAA and antibiotic studied. For type IIB strains, they are resistant to chloramphenicol, carbenicillin, spectinomycin rifampin and the metals Ni, Pb, Co, Cu, Ag and Cr (Martinez et al., 1991), but type IIA strains are susceptible to both the antibiotics and metals. Moreover, type IIB strains can produce IAA, while type IIA unable to produce (Chen et al., 1997).

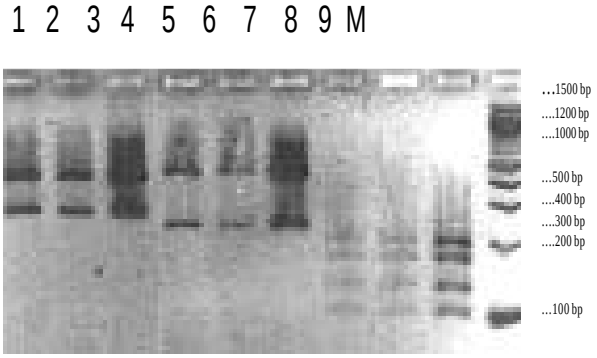

Fig. 9 Different restriction patterns of PCR-amplified *nod*A generated by *Rsa I* (lane 1-3), *Hinf I* (lane 4-6) and *Hha I* (lane 7-9). The samples were from left to right, 1; DASA23015, 2; DASA23076, 3; DASA23079, 4; DASA23015, 5; DASA23076, 6; DASA23079, 7; DASA23015, 8; DASA23076 and 9; DASA23079.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 1

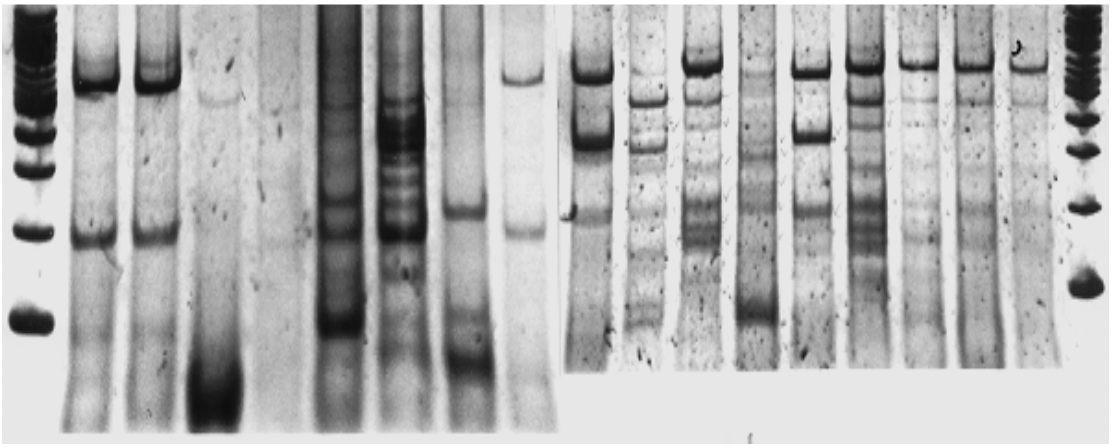

Fig. 14 Different restriction patterns of PCR-amplifield *nif*H digested by *Alu I.* The samples were, from left to right, 1:100 bp marker, 2:DASA24015, 3: DASA24016, 4; DASA25005, 5; DASA25008, 6; DASA25015 7; DASA29007, 8; DASA29010, 9; SUT2, 10; *S. meliloti*, 11; *R. phasioli*, 12: *R. tropici*, 13; *R. leguminosarum*, 14; *M. huakii*, 15; *R. tropici*, 16; DASA 23015, 17; DASA 23076 and 18; DASA 23079.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

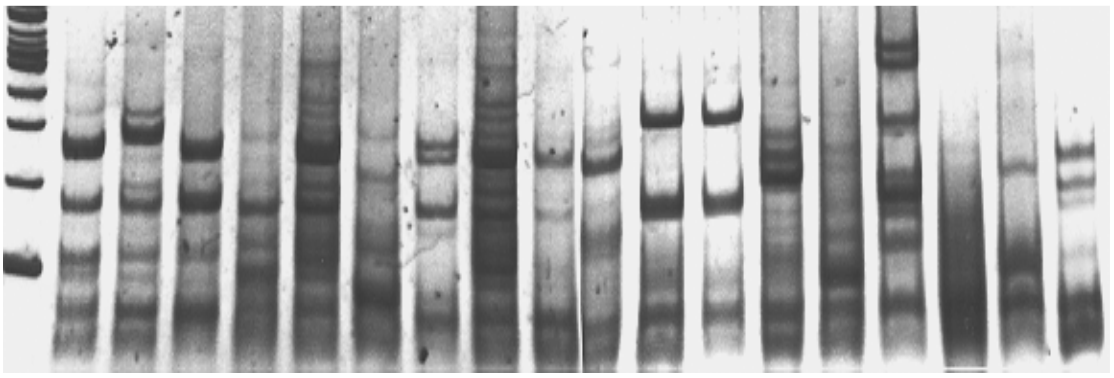

Fig. 15 Different restriction patterns of PCR-amplifield *nif*H generated by *Msp* I. The samples were, from left to right, 1:100 bp marker, 2; *M. ciceri*, 3; *R. tropici*, 4; *S. meliloti*, 5; *R. phasioli*, 6; *R. tropici*, 7; *R. leguminosarum*, 8; *M. huakii*, 9; DASA23076, 10; DASA24008, 11; DASA24015, 12; DASA24016, 13; DASA25008, 14; DASA25015, 15; DASA25016, 16; DASA29007, 17; DASA29010, 18; DASA29015 and 19; SUT2.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

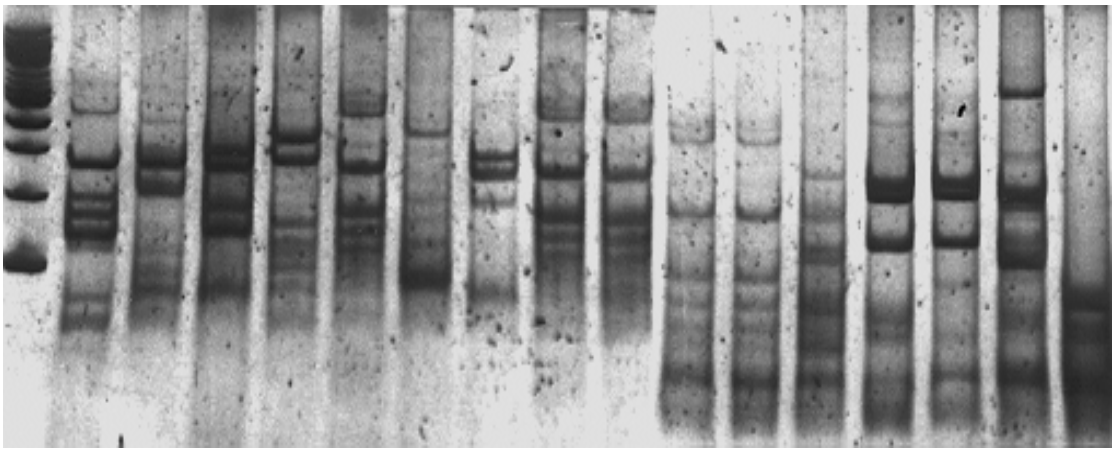

Fig. 16 Different restriction patterns of PCR-amplifield *nif*H digested by *Hae III*. The samples were, from left to right, 1:100 bp marker, 2; *M. ciceri*, 3; *R. tropici*, 4; *S. meliloti*, 5; *R. phasioli*, 6; *R. tropici*, 7; *R. leguminosarum*, 8; *M. huakii*, 9; DASA23015, 10; DASA23076, 11; *R. tropici* 12; DASA23079, 13; DASA24008, 14; DASA24015, 15; DASA24016, 16; DASA29007, and 17; DASA29010.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

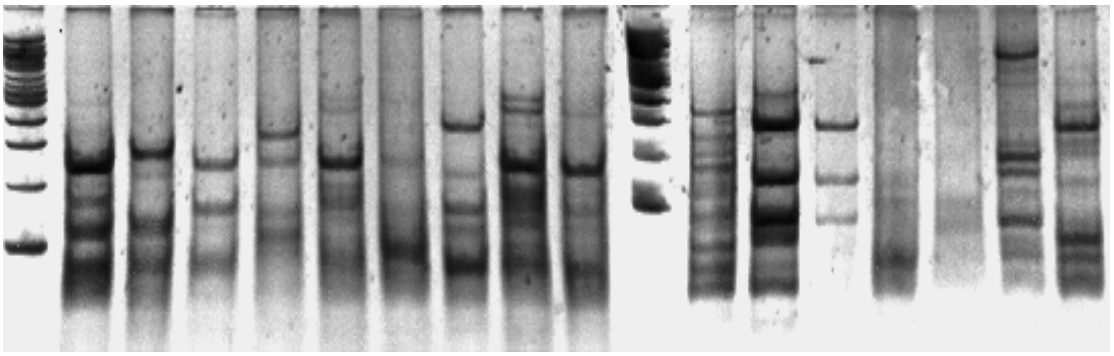

Fig. 17 Different restriction patterns of PCR-amplifield *nif*H generated by *Taq* I. The samples were, from left to right, 1and 11:100 bp marker, 2; *M. ciceri*, 3; *R. trifolii*, 4; *S. meliloti*, 5; *R. phasioli*, 6; *R. tropici*, 7; *R. leguminosarum*, 8; *M. huakii*, 9; DASA23015, 10; DASA23076, 12; DASA24008, 13; DASA24015, 14; DASA24016, 15; DASA25008, 16; DASA25015, 17; DASA29007, and 18; SUT2.

Conclusion

Since forage legumes are one of the plant, which have the potential to applied as high quality roughage in Thailand. Thus to explore and establish the management system using biological nitrogen fixation (BNF) instead of chemical fertilizers utilization were elucidated. The four tropical forage legumes; *Desmanthus virgatus*, *Stylosanthes hamata*, *Chamaecrista rotundifolia* (wynn cassia) and *Centrosema pubescens* were choosen in this study. The effective rhizobial strains for each host plant were isolated from various soil samples from agricultural areas in Thailand by using them as trapping host plant. The effective rhizobial strains were selected from plant nodules and determined the efficiency on the basis of high plant biomass productivity and N_2-fixing efficiency. These rhizobial strains were characterized in both means of physiology and genetics. In case of effective *D. virgatus* rhizobial strains, we found that most of the strains were fast grower, able to produce indole actic acid (IAA). Moreover, the genotypic characterization indicated that among these strains shared the genetic similarity with *Rhizobium tropici* rather than other genera in family Rhizobiaceae which has never been reported before. For other effective host plant rhizobial strains, both group of fast and slow grower were found and even when analyzed with *nif*H and *nod*A PCR-RFLP analysis and random primer using PCR technique, it was confirmed that great divergent of rhizobial strains able to establish in these host plants. However, the results from partial 16S rDNA sequence indicated that most of them closely relate to *Bradyrhizobium* spp. especially, *B. japonicum*. For *C. pascuorum* rhizobia, DASA24008 and DASA24015, sequences were aligned with the *B. japonicum* 83% identities and 100% identities but DASA24016 were aligned with *Rhizobium spp*., respectively. In case of *S. hamata* rhizobia both of DASA25005 and 25015 were aligned with *B. japonicum*, 94% identities, on the other hand ,DASA25005 were aligned with *R. etli*, 98% identities. And *Ch. rotundifolia* rhizobia, DASA29007 were aligned with *B. japonicum*, 96% identities, but DASA29015 were aligned with *Pseudomonas reactans*, 98% identities.

From this point of view, not only compensation of BNF application were promising but also regard with environment friendly programme management should be more extended to farmer level. In the context of sustainable agriculture, the use of forage legume should be considered as a biological strategy directed at sustainability. This research able to indicate profitable production without damage to environment. The suggestion in further step should be

more emphasized in regions and agricultural extension services to help poor farmers who have little afford on their area.

The data obtained from this study only able to elucidate the diversification in forage legume rhizobia but also important for quality control system in production level. Since the inoculant production in commercial scale must be considered the quality on terms of strains contamination and variation. Thus, these physiological and genetical data will provide fruitful information in further production.

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APPENDIX

Table 17 Isolated *D. virgatus, C. pubescens, S. hamata* and *Ch. rotundifolia* rhizobial strains from various sources.

Plant host	Strains	Place of isolation/source
Desmanthus virgatus	DASA23015	Sikhiu, Hakhon Ratchasima
	DASA23043	Niftal USA. TAL 997
	DASA23044	Chonburi
	DASA23048	Ratchaburi
	DASA23056	Maung, District Khon Kaen
	DASA23059	Maung, District Khon Kaen
	DASA23088	CB3126
	DASA23047	Lopburi
	DASA23011	Sikhiu, Nakhon Ratchasima
	DASA23027	Niftal USA. TAL 123
	DASA23046	Lopburi
	DASA23053	Jong, Lampang
	DASA23076	Department of Agriculture
	DASA23079	Department of Agriculture
	DASA23052	Jong, Lampang
	DASA23028	Niftal USA. TAL 137
	DASA23029	Niftal USA. TAL 137
	DASA23032	Niftal USA. TAL 209
	DASA23068	Department of Agriculture
	DASA23045	Chonburi
	DASA23008	Sikhiu, Hakhon Ratchasima
	DASA23010	Sikhiu, Hakhon Ratchasima
	DASA23024	Maung, District Khon Kaen
	DASA23017	Sikhiu, Hakhon Ratchasima

Table 17 (continued)

Plant host	Strains	Place of isolation/source
Centrosema pubescens	DASA24002	Niftal USA. TAL 652
	DASA24005	Niftal USA. TAL 655
	DASA24006	Chai Nat
	DASA24007	Chai Nat
	DASA24008	Chai Nat
	DASA24009	Niftal USA. TAL 984
	DASA24012	Nakhon Ratchasima
	DASA24015	Nakhon Ratchasima
	DASA24016	Pak Chong, Nakhon Ratchasima
	DASA24019	Pak Chong, Nakhon Ratchasima
Stylosanthes hamata	DASA25001	Niftal USA. TAL 85
	DASA25005	Niftal USA. TAL 794
	DASA25008	Niftal USA. TAL 1024
	DASA25015	Austraria Inoculum
	DASA25016	Department of Agriculture
	DASA25018	Department of Agriculture
	DASA25019	Department of Agriculture
	DASA25020	Department of Agriculture
	DASA25021	Department of Agriculture
	DASA25023	Department of Agriculture
Chamaecrista rotundifolia	DASA29004	Maung, District Chai Nat
	DASA29007	Maung, District Chai Nat
	DASA29010	Maung, District Chai Nat
	DASA29011	Maung, District Chai Nat
	DASA29012	Department of Agriculture
	DASA29013	Department of Agriculture
	DASA29014	Department of Agriculture
	DASA29015	Department of Agriculture
	DASA29016	Department of Agriculture
	DASA29016	Department of Agriculture

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