THE CHARACTERIZATION AND MONITORING OF SELECTED RHIZOBIAL STRAINS ISOLATED FROM TREE LEGUMES

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การศึกษาลักษณะและติดตามเชื้อไรโซเบียมที่คัดเลือกได้จากไม้ยืนต้นตระกูลถั่ว

นายมนต์ชัย มนัสสิลา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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มนต์ชัย มนัสสิลา: การศึกษาลักษณะและติดตามเชื้อไรโซเบียมที่คัดเลือกได้จากไม้ยืนต้นตระกูลถั่ว (THE CHARACTERIZATION AND MONITORING OF SELECTED RHIZOBIAL STRAINS ISOLATED FROM TREE LEGUMES) อาจารย์ที่ปรึกษา: รศ. ดร. หนึ่ง เตียอำรุง, 70 หน้า, ISBN 974-533-312-3

การศึกษาลักษณะของเชื้อไรโซเบียมที่แยกและคัดเลือกได้จากได้จากปมต้นกระถินณรงค์ (*Acacia auriculiformis* Cunn.), กระถินเทพา (*A. mangium* Willd.), สาธร (*Millettia leucantha* Kurz.), ประดู่กิ่งอ่อน (*Pterocarpus indicus* Willd.) และ แดง (*Xylia xylocarpa* Taub.) ดำเนินการโดยทำการศึกษาทางสรีรวิทยา ได้แก่ การสร้างกรด-เบส, ความสามารถในการผลิต IAA, ความสามารถในการสร้างปมกับพืชตระกูลถั่วชนิดอื่น พบว่าไรโซเบียมทุกสายพันธุ์อยู่ในกลุ่มที่เจริญช้า ส่วนใหญ่ไม่มีการสร้าง IAA ยกเว้นสายพันธุ์ AA3, AA10, PT36 และ DX51 ที่สามารถสร้าง IAA ได้ และไม่มีการสร้างปมในถั่วเหลือง ยกเว้นสายพันธุ์ AA67, PT36 และ PT59 นอกจากนี้ยังทำการศึกษาโดยใช้เทคนิคทางพันธุศาสตร์ ได้แก่ การใช้เทคนิค rep-PCR, nodA PCR-RFPL และ การอ่านลำดับเบส ของ 16S rRNA ยีน เพื่อศึกษาความสัมพันธ์ในระดับพันธุกรรม จากผลการศึกษาเชื้อไรโซเบียมทั้งหมด 44 สายพันธุ์ พบว่าทุกสายพันธุ์มีความสัมพันธ์ใกล้ชิดกับ *B. elkanii* และ *Bradyrhizobium* spp. ยกเว้นสายพันธุ์ AA67 และ PT59 ที่มีความคล้ายคลึงกับ *B. japonicum* เป็นอย่างมากทั้งในด้านของสรีรวิทยาและพันธุกรรม ส่วนการติดตามเชื้อไรโซเบียมโดยการใช้ระบบ GUS-gene ในการติดตามพบว่าสายพันธุ์ที่ทำการคัดเลือกออกมา สามารถสร้างปมกับพืชเจ้าบ้านได้ดีกว่าเชื้อไรโซเบียมเจ้าถิ่น โดยมีความสามารถในการสร้างปมสูงถึง 63-100% โดยการทดลองปลูกในดินจากป่าในเขตอุทยานแห่งชาติทับลาน จังหวัดนครราชสีมา

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา.....

ปีการศึกษา 2546 ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

MONCHAI MANASSILA: THE CHARACTERIZATION AND MONITORING OF SELECTED RHIZOBIAL STRAINS ISOLATED FROM TREE LEGUMES THESIS ADVISOR: ASSOC. PROF. NEUNG TEAUMROONG, Dr. rer. nat, 70 pp. ISBN 974-533-312-3

Tree legume rhizobia in this study were isolated and selected from nodules of five legumes; *Acacia auriculiformis* Cunn., *A. mangium* Willd., *Millettia leucantha* Kurz., *Pterocarpus indicus* Willd. and *Xylia xylocarpa* Taub. Characterizations of these strains were conducted on the basis of physiological properties such as acid-base production, IAA production and cross inoculation with other legumes. Most of the strains were slow grower, not produce IAA except 4 strains (AA3, AA10, PT36 and DX51) and 3 strains (AA67, PT36 and PT59) were able to nodulate soybean. Genetic properties were also determined by rep-PCR and nodA PCR-RFLP technique. The 16S rRNA sequencing was used to identify and generated phylogenetic tree analysis. From 44 rhizobial strains the results showed that most selected strains form tree legumes were closely to with *B. elkanii* and *Bradyrhizobium* spp. except strains AA67 and PT59 which most likely belong to *B. japonicum.* To monitor the selected strains by using GUS marker system, the result indicated the selected strains has a higher competitive ability than indigenous strains. The nodule occupancy was in the range of 63-100% when tree legume were planted in soil sample from Tablan National park, Nakhonratchasima.

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Monchai Manassila

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LIST OF ABBREVIATIONS

bp	base pair
°C	degree celcius
dNTP	deoxynucleotide 5’ triphosphate
et al.	Et alia (and other)
g	gram
l	litre
µg	microgram
µl	microlitre
mg	milligram
ng	nanogram
min	minute
ml	millilitre
mM	millimole
pmol	picomol
ppm	parts per million
REP	repetitive extragenic palindromic
RFLP	Restriction Fragment Length Polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
rpm	revolution per minute
UV	ultraviolet
W/V	weigth per volume

CHAPTER I

INTRODUCTION

Thailand has approximately 15,000 species of plants, which account for 8% of the estimated total number of plant species found. The forest resource in Thailand has been in the critical situation since 1961. In the past time, area 53% of Thailand was covered with forest. But in 1989 only 28% of forest area were remained. To conserve the remaining forest, Thai government developed the National Forest Policy, which was passed a cabinet resolution on 3 December 1985. It was declared that 40% of the country should be kept under forest and divide as follows: A) 50% of the country area shall be kept as protection forest for nature conservation, reaction and environmental quality protection. B) 25% of the country shall be designated as production forest for production timber and other forest products. The 7th Social and Economic Plan (1993-1996) proposed that Thailand should maintain 40% of the total area, the same as mentioned in the Thailand National Forest Policy, but the protected forest should be increased from 15% to 25% while the production forest should be reduced from 25% to 15% of the total area of the country. In addition, the department of forestry has initiated the forestry restoration program for private sectors by promotion various kind of plants. Thirty-eight to fifty-five plant species as *Acacia* spp., *Rhizosphora* spp., *Cassia* spp., *Xylia* spp., *Pterocarcus* spp., *Millettia* spp., etc. are among the most favorable. The main plants are belonging to leguminous plant. Their special characteristics may be summarized as usually long lived and low maintenance, and

therefore enhance the sustainability of farming system. Moreover, they have a rapid growth because of nitrogen fixing ability. They can supply N-rich for cropping system nearby. They can stabilize sloping land against soil erosion because of their deep-root. They can provide a source of timber and firewood for either domestic or industrial use and a source of fruit and vegetables for human consumption and can be used in a multiple ways including contributing rich organic mulches to improve cropping land, providing fuel wood and timber (Gutteridge, 1994).

1.1 Legumes

Legumes have been used in agriculture since ancient times. Legume seeds or pulses were among the first source 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 contain 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 important as high quality forages for livestock both in cultivated pasture and in naturally occurring associations. All of plants used by human, only the grasses are more important than the legumes but it is the legumes that show the most promising for future exploitation and development. The legumes are third group of flowering plants comprising over 18,000 species in 650 genera, which are well distributed in most environments throughout the world. Taxonomists have divided the legumes into three families:

- Caesalpiniaceae contain about 2,800 species, most of which are trees of tropical Savannah's and forests of Africa, South America and Asia (Williams, 1983).

- **Mimosaceae** also contains about 2,800 species. These are predominantly small trees and shrubs of semiarid tropical regions of Africa, the America and Australia. *Acacia* species are the best known examples of this family.

- **Fabaceae** contains over 12,000 species, mainly herb and a small shrub distributed worldwide, and includes the well-known grain legumes such as beans and peas (Gutteridge, 1994).

1.2 Tree Legumes

Until recently, researchers have largely neglected tree legumes because their utilization and management fell between the disciplines of forestry and pasture agronomy. However, they are now receiving increased research attention because of their multipurpose value and some distinctive features, which set them apart from herbaceous legumes. Their special characteristics may be summarized as follows:

- Are usually long-lived and low maintenance, and therefore enhance the sustainability of farming system.

- Provide high quality forage for feeding of livestock.

- Stabilize sloping lands against erosion because of their deep-root habit.

- Supply N-rich mulch for cropping system.

- Can be used to colonize and rehabilitate adverse environment e.g. saline or arid location.

- Provide a source of timber and fire wood for either domestic or industrial use.

- Are used in farming systems as living fences, as shade tree for plantation crops, and as living trellises for climbing crops.

- Are a source of fruits and vegetable for human consumption.

Tree legumes can therefore be regarded as truly multipurpose tree for agriculture.

1.3 Biological Nitrogen Fixation (BNF)

Nitrogen is commonly the most limiting element in agricultural production and or the most of expensive to purchase as fertilizer. There is an abundant supply form air (78% air is nitrogen gas, amounting to about 8,000 pounds nitrogen in the air over every area at land). However, the nitrogen in air is a stable gas normally unaviable to plant. Biological Nitrogen Fixation (BNF) involves the enzymatic reduction of nitrogen gas to ammonia. The ammonia produced then can be incorporated by enzymatic for the growth and maintenance of the cell. BNF is unique to bacteria, animal and plants that fix N_2 must be do in associate with bacteria (Stacey, 1992). Many leguminous plants are able to utilize this atmospheric nitrogen through an association with rhizobia, bacteria that are hosted by the root system of certain nitrogen fixing plants. This self sufficiency, with many free the plant from the need amendment with manufacture N fertilizers, is achieved by symbiotic fixation of inert gas from atmospheric into NH_4^+ in the soil that is used in amino acid and protein synthesis. The overall of BNF can be summarized in the following equation (Hansen, 1994).

$$\mathbf{N_2 + 8H^+ + 8e^- + 16\ MgATP \xrightarrow{Nitrogenase} 2NH_3 + H_2 + 16\ MgADP + 16Pi}$$

The components of N_2-fixation process were nitrogenase enzyme and leghemoglobin, in nodules of legumes, nitrogenase synthesis normally follow very

shortly after bacteria are released from infection threads. The enzyme nitrogenase is comprised of two easily separable proteins designated the iron (Fe) protein component II and the molybdenum-iron (MoFe) protein or component I. The MoFe protein is a tetramer ($\alpha 2\beta 2$) of 220 kD Molecular mass. The MoFe protein contain 2 atoms of Mo and 24 to 32 atoms of Fe and S per molecule. The role of MoFe protein is to transfer electrons to N_2 and H^+. The Fe-protein is a homodimer. The Fe-protein has two Mg-ATP binding site and as ATP binds to these sites, the potential of electron at the (4Fe-4S) clster is reduced, allowing to Fe-protein to donate electrons to the MoFe protein (Vance, 1998). It is responsible for conversion (reduction) the atmospheric N to NH_4^+ and synthesized in the bacteroid cytosol. The assimilation of N_2 fixed in the bacteroids is exported through the inner and outer membrane of the microsymbiont and through the peribacteroid membrane into host plant cytosol. The nitrogenase enzyme complex is rapidly and irreversibly denatured by O_2. Thus the enzyme is functional only in low O_2 environments. The important contributor to solving the O_2 dilemma is leghemoglobin an O_2 binding protein found within nodule infected cells. This plant protein which is very similar to animal hemoglobin gives nodules their pink color. Leghemoglobinfaccilitates the diffusion of available O_2 though the plant cell cytoplasm to bacterial cells at concentrations which allow oxidative phosphorylation to occur without inactivation of nitrogenase activity (Vance, 1998).

1.4 Characteristics of the rhizobia

Rhizobia or root nodule bacteria are middle size, rod shaped cell, 0.5-0.9 μm in width and 1.2-3.0 μm in length. They do not from endospores, are gram-negative,

and are mobiled by a single polar flagellum or two to six peritrichous flagella. Uneven gram staining is frequently encountered with rhizobia, depending on the age of the culture. Cell from a young culture and nodule bacteroids usually show even gram stianing while older and longer cell give a banded appearance with unstained area. These unstained areas have been identified to be large granules of polymeric beta-hydroxybutyric acid (PHBA). The PHBA is refractile under phase contrast microscopy. Rhizobia are predominantly aerobic chemoorganotrophs and are relatively easy to culture. They grow well in the presence of O_2 and utilize relatively simple carbohydrates and amino compounds. With the exception of a few strains, they have not been fix N in the free-living from except under special conditions. Rhizobia can combine nitrogen gas from air to nitrogenous compound that plant can utilize as a direct nitrogen source. The enzyme nitrogenase is a complex of two enzymes, an Fe-containing protein and an Fe-Mo protein. It is responsible for the conversion (reduction) of atmospheric N into NH_4^+, and is synthesized in the cytosol of the bacteroids. The legume utilizes NH_4^+ to convert certain precursor metabolites such as α-ketoglutarate, phosphoenolpyruvate into amino acid, which, in turn, are synthesized into protein. The complex biochemical reaction whereby the inert atmospheric N is enzymatically reduced into a utilizable form for plant by the nitrogenase enzyme complex of the bacteroids is called biological nitrogen fixation. The symbiosis between leguminous plant and rhizobia is a complex interaction. This is a result from an effect between the legume and specific strain of rhizobium and legume are neither productive nor persistence without effective nodulation by appropriate rhizobia (Somasegaran and Hoben, 1994).

1.5 Taxonomy of rhizobium

Rhizobia are bacteria that form nitrogen-fixing on the roots or the shoots of legume. Classification schemes based on data derived from use of traditional physiological tests are separated into 2 groups by growth rate, I) Fast growing and II) Slow growing. Rhizobia are genetically diverse and physiologically heterogeneous group of bacteria that are nevertheless classified together by variety of their ability to nodulated members of leguminosae. The taxonomy of the rhizobia is in a state of flux at the moment, with a flood of proposals in a new name. This has been stimulated both by the isolation of symbionts from new source and by advances in molecular method, that have greatly increased the confidence with can assign strains to species and describe the relationship among species. Sequence comparisons of the small subunit from the ribosomal RNA (SSU rRNA) have become the standard method for assessing phylogenetic relationships among bacteria and such comparisons show the diversity of rhizobia very clearly. All rhizobia discovered so far are member of the alpha subdivision of the proteobacteria, but the branch that includes them all also carries other bacteria that are not root nodule symbionts (Young, 1996). Although these are useful attributes, the reliability of using the SSU rRNA gene alone as an approach for estimate phylogenies of bacteria has been brought into question, particularly for closely related taxa. To investigated the role of SSU rRNA similarity in species definition in comparison with DNA-DNA reassociation analyses. A similar correlation between SSU rRNA and DNA-DNA reassociation data from the member of *Rhizobiaceae* also is not linear. The conclusion for a parametric species definition the SSU rRNA nucleotide sequence data are most reliable to estimate relatedness

when these genes share more than 97% similarity (Berkum and Eardly1998). The current genera of Rhizobiaceae are summarized in Figure 1 and Table 1

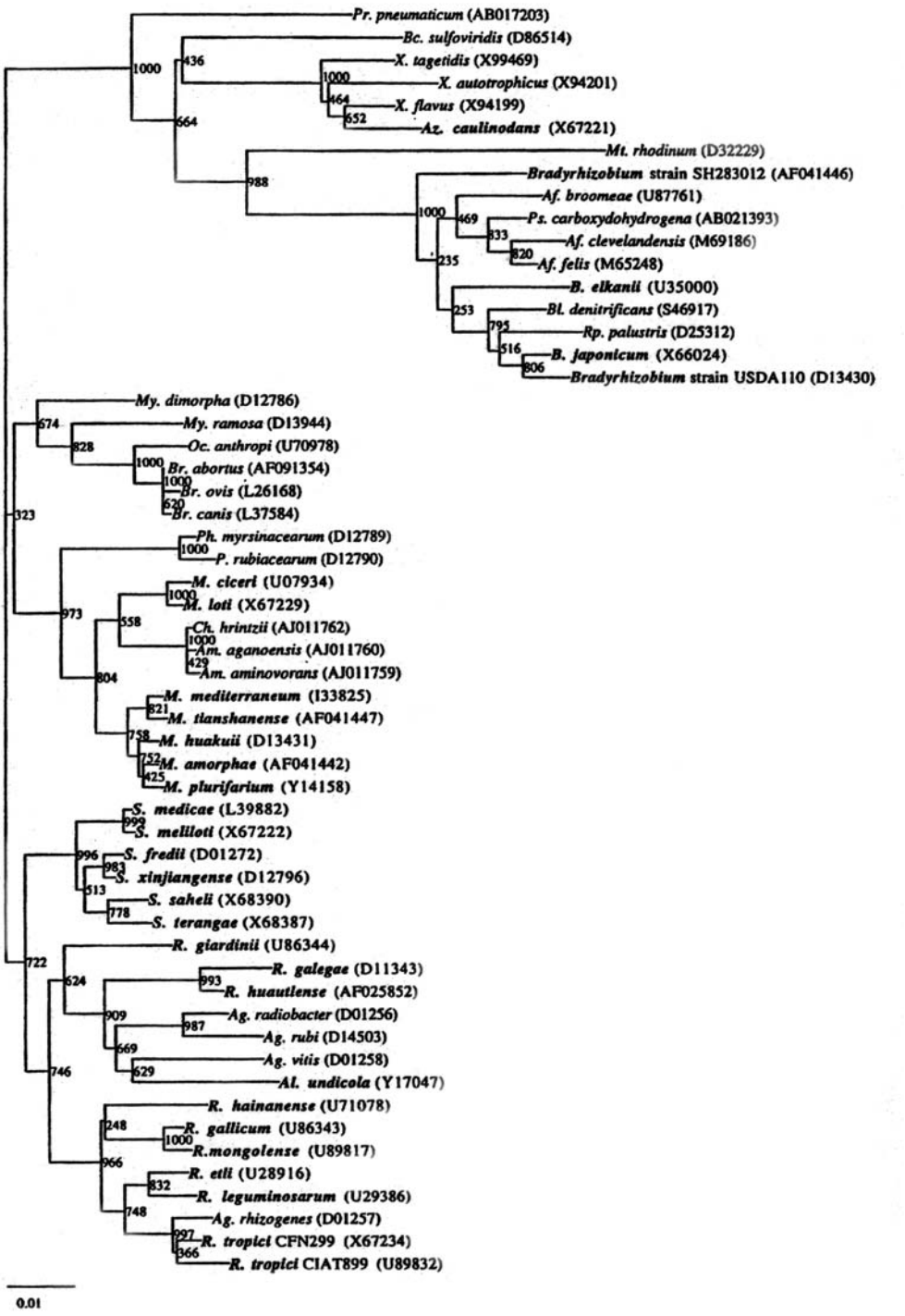

Figure 1. Phylogenetic relationship of the *Rhizobiaceae* within the α–subdiovition based upon aligned sequences of the mall subunit ribosomal RNA genes (Wang and Martinez-Romero, 2000)

Table 1. Current taxonomy of rhizobia (Martinez-Romero, 2000)

Genus	Species	Host
Allorhizobium	*A. undicola*	*Nepunia natans*
Azorhizobium	*A. caulinodans*	*Sesbania rostrata*
Bradyrhizobium	*B. elkanii*	*Glycine max*
	B. japonicum	*G. max*
	B. liaoningense	*G. max*
Mesorhizobium	*M. amorphae*	*Amorpha fructicosa*
	M. ciceri	*Cicer arietinum*
	M. huakuii	*Astragalus*
	M. loti	*Lotus*
	M. mediterraneum	*C. arietinum*
	M. plurifarium	*Acacia, Leucaena*
	M. tianshanse	*Glycyrrhiza, Sophora, Glycine* and others
Rhizobium	*R. etli*	*Phaseolus vulgaris, Mimosa affinis*
	R. galegae	*Galega*
	R. gallicum	*P. vulgaris*
	R. giardinii	*P. vulgaris*
	R. hainanense	*Stylosanthes, Centrocema, Desmodium, Tephrosia*
	R. huantlense	*S. herbacea*
	R. leguminosarum	*Vicia, Trifolium*
	R. mongolense	*Medicago ruthenica, P. vulgaris*
	R. tropici	*P. vulgaris, Leucaena*
Sinorhizobium	*S. arboris*	*A. senegal, Prosopis chilensis*
	S. fredii	*G. max*
	S. kostiense	*A. senegal, P. chilensis*
	S. medicae	*Medicago* spp
	S. meliloti	*M. sativa*
	S. saheli	*Sesbania*
	S. terangae	*Sesbania, Acacia*
	S. xinjiangense	*G. max*

The ability of certain rhizobia to infect and nodulated particular group of legume is important in the classification of rhizobia. Rhizobia are generally classified according to host-based system. The cross inoculation system has provided a useful classification system for rhizobial strains. But some major problem has historically been associated with its application. Cross inoculation group have provided a reasonably stable philosophical basis for the taxonomic scheme for grouping rhizobial strains, a variety of methods, including evaluation of DNA hybridization numerical taxonomic procedures, DNA base ratio, serology, intrinsic antibiotic resistant, genetic marker are being applied to a taxonomy in transition. These techniques as indicated above, have reinforced concepts of species designation derived by more classical method as well as detect occurrence of specific strains in soil and nodule (Tate, 1995). It is very difficult to boundary line between closely related species by conventional method. On the other hand, the comparison of nucleotide sequence is capable of revealing the phylogenetic relation among bacteria, and allows classification of the bacteria probable at genus or lower level (Oyaizu, et al., 1993). Chen et al., (1997) reported a new *Rhizobium* species on the basis of 16S rRNA gene sequence, DNA-DNA hybridization and phenotypic characterization. This new species belongs to the phylogenetic tree branch, which includes *R leguminosarum.* Nusawantara et al., (1999) studied in *M huakuii,* the phylogenetic approaches were used with Polymerase Chain Reaction and Restriction Fragment Length Polymorphism techniques (PCR-RFLP) analysis of full-length 16S rRNA gene, 16S rRNA gene analysis of the first 300 bp. sequence, bacteriophage typing and amplication of the genomic region by random primer. They found that *R. huakuii* bv. Rengei should be classified into subspecies of the new genus *Mesorhizobium* and renamed *M. huakuii* sub sp. Rengei.

1.6 Competition factors

Biofertilizer such as rhizobial or bradyrhizobial inocula have been achieved in Thailand with the leguminous plants. A major problem regards to the strains after used as inocula is the survival competition among indigenous soil bacteria. Rhizobial competition can be divided into several temporal stages: I) The free living stage, II) Rhizosphere colonization and early infection events, III) Nodule formation and N_2-fixation. The factors involved with the persistence and competition ability of rhizobia inocula are the strains, indigenous soil bacteria and cultivar. Thus, the efficiency in N_2-fixation and competition ability are the two key features needed for obtaining an appropriate inoculation and management. Nodulation competitiveness of the inoculum strains fail to infect target plants in natural systems. When inoculum is applied to soil that already contain indigenous rhizobia, the inoculant strain is almost unsuccessful. The reduction in percentage of nodule formed by the inoculant strain when the indigenous strain occours at level of only 10 rhizobia per gram soil. Eventhough, introduced strains are outnumbered by indigenous soil populations by as much as 250: 1, are not evenly distributed throughout the soil and are often not well adapted to general soil conditions. Therefore, inoculant rhizobia may assume remain only form 5-10 % of the nodule produced in the first year in field grown legumes (Sadowsky and Graham, 1998). Several attempts have been conducted to overcome this problem. The factors including environment and cell conditions are being improved the strains competitiveness on the basis of modern biotechnology approach.

1.6.1 Environmental factors

Environmental factors are known to influence the competitiveness of specific rhizobial inoculants include: soil water, nutrient limitation, soil acidity and soil temperature etc. Some of these factors might directly affect competitiveness, many most likely act by altering the persistence and survival of inoculated strains and only indirectly influence competitive interaction. Both establishment and activity of the legumes-Rhizobium symbiosis have been found to be extremely sensitive to drought stress. In dry condition, plant growth, root architecture and root exudates can be effected as well as low population level of rhizobia in soil. Therefore, symbiotic nitrogen fixation is highly sensitive to drought, which results in decreased N accumulation. Numerous studies have indicated that a nitrogenous signal (s), associated with N accumulation in the shoot and nodule, exists in legume plants so that N_2 fixation is inhibited early in soil drying (Serraj et al., 1999). In case of rhizobia, results from several studies have suggested that bradyrhizobia are more resistant to water stress than rhizobia. However, some gave the opposite results. This might caused from intrageneric strains diversity differences (Sadowsky and Graham, 1998).

Numerous nutrition in rhizosphere are influence the growth of rhizobia. N-limitation as the classic phenomenon being investigated particularly with bradyrhizobia association. Recently, Lopez-Garcia (2001) demonstrated that rhizobial N-starvation has positive influence on the symbiosis of *B. japonicum* with soybean plants, through parallel on the exopolysaccharide (EPS), capsular polysaccharide (CPS), *nod* gene induction and soybean lectin binding (SBL) stimulation of absorption, all of which resulted in increased nodulation efficiency and competitiveness. In addition, chemotactic ability and/or mobility confers a competitive advantage on rhizobia.

Interactions between chemoattractants and their receptors appear to affect the regulation motility in response source availability (Wei and Bauer, 1998).

Soil acidity effects on legume nodulation include reduced survival and growth of rhizobia in soil and seed, reduced attachment and root-hair infection and poor plant growth. Actually, acid pH has a much effect on the fast growing rhizobia than bradyrhizobia (Sadowsky and Graham, 1998). Introduction of rhizobial strains with acid tolerance property has been conducted in the central region of Argentina. This area was found become progessively more acidic over the last to decades. The inoculant is applied for alfalfa, however, the response to inoculation with specific rhizobia is strongly by native soil rhizobia that compete with the introduced strains (Broughton, et al., 2003).

Soil temperature is another variable that has important effects on nodulation of legume. As an example, soil bean evolved under sub-tropical condition and its optimal growth temperature is 25-30 °C. soil temperature below this range restrict nodulation and nodulation. Low temperature also decrease biosynthesis and secretion of genistein (flavonoid) from plant roots (Zhang and Smith, 1996). Therefore, applying flavonoid or related flavonoid to soybean growing at low soil temperature, might significantly improve nodulation and nitrogen fixation.

1.6.2 Biotic factors

Biotic factors influencing competition for nodulation include: exopolysaccharide, nod factor, some antibiotic production, hydrogen recycling system and some indigenous substance will be discussed.

Exopolysaccharide (EPS) is forming the outer layer of cell surface, contribute to cell protection against environmental influences, attachment to surfaces, nutrient gathering and antigenicity. Furthermore, the structural diversity enable EPS to

function as informational molecules is cell to cell communications. The ability to establish an effective symbiosis is severely in mutants of many rhizobia deficient in EPS production. Whereas mutant of *S. meliloti* SU47 over producing EPS I are able to infect alfalfa nodule. In some symbiotic systems addition of purified EPS restored the ability of EPS mutants to infect nodule (Becker and Puhler, 1998). Another gene found in *R. etli* which plays a critical role in both determination of cell surface characteristics and nodulation competitiveness is *ros*R has sequence similarity to a family of transcriptional represser involved in EPS production. When the *rosR.* mutants were constructed, changes in colony characteristics and competitive in *P. vulgaris* nodulation also observed (Bittinger and Handelsman, 1999). Eventhough, the regulation of EPS production is not clearly understand. Many different regulators were identified in rhizobia. The environmental factors influencing EPS production and mechanisms of regulation still need to be investigated.

In general, symbiotic interactions are controlled by signal exchange between the two partners. Plant secrete flavonoid, phenolic compounds that, in conjunction with the bacterial activator protein NodD, induce the expression of rhizobial nodulation *(nod, nol* and *noe)* genes. As a result, rhizobia produce Nod-factors. Few years ago, attempt to increases in Alfalfa nodulation, N_2-fixation and plant biomass by specific DNA amplification (SDA) in *S. meliloti* was performed. Recombinant plasmid contained *nod*D1*, nod*ABC and an essential *nif*N from the nod regulon of the symbiotic plasmid of *S. meliloti.* The results suggested that the copy number of symbiotic region was critical in determining the plant phenotype. In case of the strains with moderate increase in copy number, symbiotic properties were improved significantly. The inoculation of alfalfa with transconjugant strains resulted in an

enhancement of plant growth (Castillo, et al., 1999). Another gene which presumably involved in nodulation competitiveness on common bean with *R. etli* is *slp* (stomatin like protein). You, et al., 1998 found that *slp* gene may provide a growth or competition advantage to rhizobial strain in the infection thread. The mutant strain without *slp* may have a disadvantage in ion exchange and nutrient uptake and loss of capacity for competition for nodule invasion. In addition, bradyoxetin regulate *nod* gene activity, have antibiotic activity, inhibiting growth of competing bacteria in the rhizosphere. Besides these mode of action, bradyoxetin is also similar to that of munegeic acid, a known siderophore. Consistent with the fact that bradyoxetin regulate *nod* gene expression, a corresponding increase *nolA* expression and a concomitant decrease in *nod* gene expression was noted under iron-starved condition (Loh and Stacy, 2003).

Production of antirhizobacterial compounds by rhizobial inoculant strains could be a useful strategy in limiting nodulation by indigenous. One of antirhizobacterial compounds that had a potential for enhancing the nodulation competitiveness is trifolitoxin (TFX). TFX is ribosomally synthesized, posttranslationally modified peptide produced by *R. leguminosarum* bv. trifolii T24 (Breil, *et al.,* 1993). TFX inhibits member of a specific clade of the α subdivision of the division Proteobacleria that includes legume symbionts, plant pathogens, and animal pathogens. Under laboratory condition, the TFX production phenotype has been shown to significantly increase the nodulation competitiveness *of Rhizobium* and *Sinorhizobium* strains (Robleto, et al., 1997). Another antirhizobacterial compound known as bacteriocin is also interesting. *R. leguminosarum* strains have been shown to produce bacteriocin which have been well characterized. *R.*

leguminosarum strain 248 contains the symbiotic plasmid which includes determinants for medium bacteriocin production. Analysis of the deduced amino acid sequences revealed that similarly to hemolysin and leukotoxin. For the nodule competition experiments under controlled conditions showed that the bacteriocin can play a statistically significant role in competition against certain test strains (Oresnile, et al, 1999). A phytotoxin produced by certain strains of soybean bradyrhizobia so called rhizobitoxine is also play important role in nodulation competitiveness. Rhizobitoxine (2-amino-4-(2-amino-3-hydropropoxy)-*trans*-but-3-enoic acid) increases nodulation and competitiveness via inhibition of endogenous ethylene synthesis in host plants, because ethylene acts as a plant hormone that restrict, nodulation in many legumes. Recently, Okasaki, et al., (2003) demonstrated that rhizobitoxine production by *B. elkanii* USDA 94 gave this strain a nodulation competitiveness about 10 times greater than that of a non-rhizobitoxine producing mutant strain on Siratro plant. Rhizobitoxine enhancement of competitive nodulation occurred at the late stage in the time course of nodulation.

Some adequate nodulations caused from toxic substances released from seed and root exudates These can inhibit the growth of nodulating rhizobacteria. In case of mimosine, a free amino acid produced in large quantities in the leafs, seeds and other part of legume leucaena *(Leucaena leucocephala).* It is toxic not only animals grazing on leucaena foliage but also inhibit growth of rhizobia. However, attempts to provide the nodulation competition advantage, some rhizobial strain able to degrade mimosine was isolated. *Rhizobium* sp. strain 1145 can degrade mimosine under 2 major steps; in the first step mimosine is degraded to 3-hydroxy-4-pyridone (HP) which is then converted to pyruvate, formate and ammonia in the subsequent step. The

midD gene encodes an aminotransferase required for degrading mimosine into HP was found (Awaya, et.al., 2003). Therefore, this might be a possibility to use gene manipulation technique to provide the competition advantage for selected rhizobial strains.

Since symbiosis requires a respiratory chain that has a high affinity for O_2 and is efficiently coupled to ATP production because N_2-fixation is an energy-consuming process, requiring up to 20 ATP molecules to reduce just one molecule of N_2. The three-subunit terminal oxidase (*cbb*$_3$) is responsible for bacterial terminal oxidase production. Recently, attempt to overproduce *cbb*$_3$ terminal oxidase in *R. etli* can enhance symbiotic nitrogen fixation in *P.vulgaris* cv. negro jamapa (Soberon, et al., 1999). According to the nitrogen fixation to reduce N_2 to NH_3 by nitrogenase, H_2 is evolved during reduction reaction. Thus, H_2 production due to nitrogenase activity is a source of inefficient in *Rhizobium-legume* symbioses. Certain rhizobial strains synthesize a hydrogen uptake (Hup) system that recycles the evolved H_2. The ability to utilize H_2 is a desirable characterization for generating more productive and efficient rhizobial inoculants. However, this phenotype has been found only is a few *Rhizobium* and *Bradyrhizobium*. To extend this ability to other Hup$^-$ strains, Hup system from *R. leguminosarum* bv. viciae UPM791 were transferred by triparental maiting. The final results showed the increasing of hydrogenase activity (Brito, 2000). Furthermore, *R. leguminosarum* bv. viciae UPM791 induces hydrogenase activity in pea *(Pisum sativum* L.) bacteroids but not in free-living cells. The symbiotic induction of hydrogenase structural gene *(hup*SL*)* is mediated by *nif*A*,* the general regulator for nitrogen fixation process. In addition, bacteroids from pea plants

grown in low-nickel concentration induced higher level of hydrogenase activity and were able to recycle all hydrogen evolved by nodules (Brito, et al., 2002).

1.7 Strains Monitoring

To monitor rhizobia inocula after application into the field, the competitive ability of an inoculant strain is an important factor to determine the beneficially outcome of inoculant. Thus, the rhizobial behavior has been studied in soil. Bacterial competition studies require a specific method for marking the competing strain so that they can be easily identified. The one of common methods used were either serological marker (Anyango, 1998).

Serological reaction of rhizobia generally involve two major reactants "antigen and antibody" (Vincent, 1982). Antigen is any substance that provokes an immune response when introduced into the tissue of an animal or human. In work with rhizobia, rabbit are commonly used for immunization. Antigens of rhizobia can be categorized into somatic, flagellar and capsular depening on their derivation. Somatic antigen (O antigen) are closely related to the rhizobial cell wall. Some somatic antigens may be tightly bound to the call wall. They are not removed by washing of the cell. Therefore, these antigen are only detected when whole cell of rhizobia react with the antibody. Flagellar (H antigen) of the rhizobia are also antigenic. They are heat labile and are commonly detected. Finally, the Capsular (K antigen) are surface antigens and are found outside the sell (Somasegaran and Hoben, 1994).

Antibodies are protien complexes produced by the immune system. There are five different classes of antibodies or immunoglobulins (Igs) as IgA, IgD, IgE, IgG and IgM (Fig. 2).

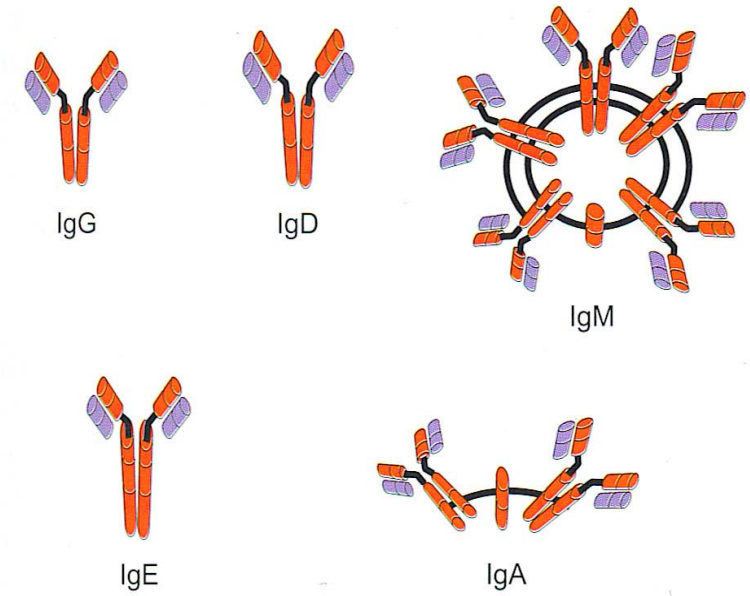

Figure 2. Schematic representation of the 5 classes of immunoglobulins

There are two major structural fragments or regions of Igs called the Fc and Fab regions (Fig. 3). The antigen binding fragment (Fabs) of the IgG immunoglobulin are the two regions at the top of molecule, as indicated, are the site of antibody-antigen interaction. The Fc region is the tail of antibody and is the fragment, that is recognized by the host (Dowd and Maier, 2000)

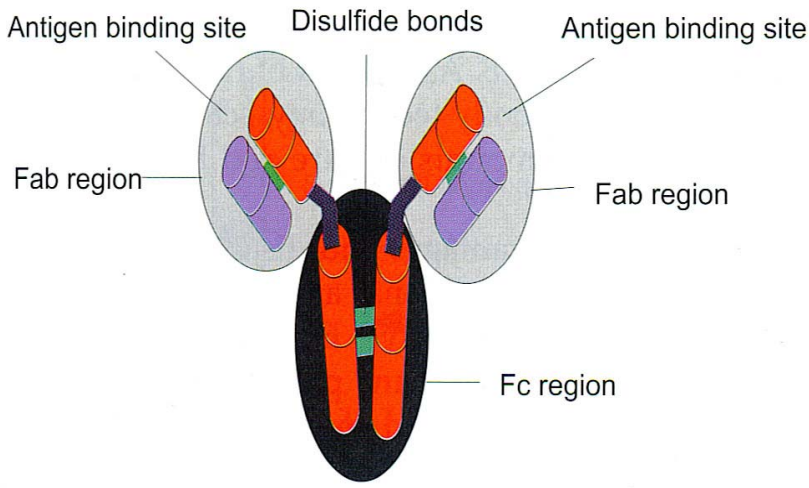

Figure 3. Schematic representation of an IgG antibody, showing the various regions associated with the antigen/antibody interaction

For rhizobial research, the most important of these are the IgG and IgM antibodies. The antigen which are detected by agglutination are those attached to cell and sufficiently close to the surface to be accessible to antibody. The surface somatic antigens of the rhizobia are the most strain specific. Flagellar antigens are generally more widely shared among strains, within a species or among closely related species (Vincent, 1982).

In rhizobial serology, both cultured cell and nodule antigens used for strain identification. Three forms of serological reactions have been widely used in the study of rhizobia as agglutination, immunofluorescence and Enzyme-Linked Immunosorbant Assay (ELISA) (Vincent, 1982). For agglutination, the process in which the antigen are linked together by their corresponding antibodies. The agglutination reaction depend on a firm structural relationship between an exposed bacterial antigen and antibody. Rhizobial antibodies can be conjugated to these fluorescing chemical dye. The chemical dye commonly used for labeling the specific antibody is fluorescien isothiocyanate (FITC). This technique called immunofluorescence or fluorescent antibody (FA) technique (Somasegaran and Hoben, 1994). ELISA is one of the several immunoassay used in detecting antigen and antibodies. ELISA has also been used in studies of rhizobial serological diveristy, the competitive ability of certain strain and in quality control of inoculants (Nambiar, et. al., 1984). For quantification of specific microorganism in soil by FA technique, Schmidt (1974) devised a method of concentrating cells released from soil particles onto a membrane filter and reacting them with the antisera directly on the filter surface. By this method, *Rhizobium japonicum* present in soil at cell concentration above 10^4/g soil were enumerated in the present of a mixed soil population. In case of

Thai soybean cultivars interact with different *B. japonicum* strains and they were investigated for nodule occupy. The nodule competition assay was conducted by using a fluorescent antibody technique to detect rhizobial strains nodules (Payakapong, et al., 2004). These methods have their limitations. In all cases there may be substantial background activity. Bacterial strains with cross-reacting antigenic determinants are common and many in soil bacteria (Anyango, 1998).

A technique, which base on molecular biology and provides significant advantage is marker gene. The marker gene techniques has been extensively used in several laboratories, it is able to facilitate better sensitivity and simplicity of assay as well as possible to use in the field. The commonly used reporter gene such as β-galactosidase (*lac*Z), luciferase (*lux*AB), green fluorescent protein (*gfp*) have been widely accomplished. In this recently year, the development of bioluminescent reporter for detection of 2,4-D degradation in aqueous sample and demonstrate its use in slurries contain (Hay et al., 2000). Normander (1999) reported on the single cell distribution viability and activity of *gfp*-tagged *Psudomonas fluorescens* in the rhizosphere of barley planted and agricultural soil. Luciferase marker gene (*lux*AB) tagged *S. meliloti* strains was studies under the field condition which deficient in indigenous *S. meliloti* (Schwieger et al., 2000). A marker gene used as reporter gene and widely applied to ecology of bacteria that interact with plants such as rhizobial strain is β-glucuronidase (*gus*A) gene (Teaumroong, 1998).

In this study, the *gusA* gene encoding the enzyme β-glucuronidase (GUS) is inserted into the genome of recipient strains. Marked bacteria can then be detected by virtue of this GUS activity using the GUS substrate X-glcA (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) which forms a blue color on cleavage by GUS. This detection

may take place on agar media, in which case blue colonies are formed, or in nodules, with nodules occupied by the marked strain turning blue while nodules marked by other, competing strains do not change color (Anyango, 1998).

1.8 Objectives

The aims of thesis are to characterize the effective tree legume rhizobial strains performing high N_2-fixing capacity in *Acacia auriculiformis* Cunn., *A. mangium* Willd., *Millettia leucantha* Kurz., *Pterocarpus indicus* Willd. and *Xylia xylocarpa* Taub. in term of some physiological features and genetic relationship. In addition, to monitor the persistence of selected inocula in soil condition will also be elucidated on the basis of reporter gene application.

CHAPTER II

MATERIALS AND METHODS

2.1 Rhizobial Strains

The rhizobial type strains *Bradyrhizobium japonicum* USDA110, *B. elkanii* USDA94 were used throughout this study. Rhizobial strains from five tree legumes (*Acacia auriculiformis* Cunn., *A. mangium* Willd., *Milletia leucantha* Kurz., *Pterocarpus indicus* Willd., and *Xylia xylocarpa* Taub.) were isolated from their nodules collected from field grown tree legumes in Thailand. Some of them were obtained from Department of Agriculture, Bangkok. Isolation, purification and authentication were carried out according to the methods described by Somasegaran and Hoben (1994). The high efficiency of N_2–fixation from various tree legumes rhizobial strains were screened by Dr. Achara Nuntagij, Department of Agriculture, Bangkok. The selected rhizobial strains and related host plants were summarized in Table 2.

Table 2. The selected rhizobial strains and host plants.

Host plants	Selected strains
Acacia auriculiformis Cunn.(Aa)	Aa1, 3, 4, 5, 10, 43, 67, 75, 91
Acacia mangium Willd. (Am)	Am13, 16, 30, 32, 37, 42, 45, 47, 49
Millettia leucantha Kurz. (Ml)	Ml37, 49, 70 96, 100, 116, 118, 119
Pterocarpus indicus Willd. (Pt)	Pt26, 27, 30, 31, 32, 36, 41, 53, 59, 70
Xylia xylocarpa Taub. (Dx)	Dx1, 11, 16, 23, 24, 38, 51, 52

2.2 Strain Characterization

2.2.1 Physiological characteristics

2.2.1.1 Acid-Alkaline Production

Rhizobial strains were cultured on Yeast-Malt extract 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 color-medium. For acid-production strain, the medium color was changed from green to yellow while alkaline-production from green to blue.

2.2.1.2 Indole Acetic Acid (IAA) Production

IAA production was determined by adding 2 ml of 0.01 M $FeCl_3$ in 35% $HClO_4$ into 1 ml of Tris-TMRT culture broth after incubated at 28°C for 10 days. The mixture was incubated in the dark at 30°C for 30 minutes. Results were compared with positive control of 1.0 g of IAA in distilled water and ethanol (1:1 for 1.0 ml) (Nuntagij et al., 1997)

2.2.1.3 Inoculation with other legumes

To study the ability of nodule formation action in various tree legume host plants, other host plants including soybean (*Glycine max*) and mungbean (*Vigna radiata*) were also conducted. Seed plants were surface sterilized in 95% ethanol for 10 sec before added 3% sodium hypochlorite to immerse the seed completely. After 5 min drain of the sterilant, seeds were rinsed six times with steriled water. The sterilized seeds were put on plate containing wet tissue and kept in the dark place for 1-2 days. Germinated seeds were grown in leonard jar containing sterilized sand then inoculated with 1 ml of rhizobial culture (10^7-10^8 cell/ml/seed). The leonard jars were

put on the shelf light that set up 12 h in light and 12 h in dark. Nodule formation was observed after 45 days planting.

2.2.2 Genotypic characteristics

2.2.2.1 Genomic DNA extraction

Prior to isolate the genomic DNA, rhizobial strains were cultured in HM medium. After cultivation for 5-7 days on rotary shaker for 200 rpm, at 28°C, bacterial cells were pelleted before resuspened in 0.5 ml TEN 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. Bacterial pellets were resuspended in 200 µl of 20% sucrose in TEN buffer, then 100 µl of 10% SDS were add prior to incubate with 20 µl of 2.0 mg/ml lysozyme for 30 min at 37°C. Then, 75 µl of 5 M NaCl, was added and the mixtures were extracted twice with saturated phenol and followed with phenol:chloroform:isoamylalcohol (25:24:1). The upper phase was taken. DNA was precipitated with two volumes of cold 99% ethanol and 50 µl of 3M sodium acetate. DNA pellets were dried under vacuum before dissolved in TE buffer and treated with 10 mg/ml of RNaseA at 55°C for 15 min.

2.2.2.2 DNA primers and PCR conditions

The DNA primers used for PCR in this study were summarized in Table 3.

Table 3. Summary of DNA primer sequences uesd in this study of DNA.

Primer	Sequence	Ref.
BoxAIR	(5′-CTACGGCAAGGCGACGCTGACG 3′)	Sadowsky, 1996
Fd-1	(5′-AGAGTTTGATCCTGGCTCAG- 3′)	Nuswantara, 1999

Table 3. (continue)

Primer	Sequence	Ref.
Rp-2	(5′-ACGGCTACCTTGTTACGACTT 3′)	Nuswantara, 1999
NodA-1	(5′-TGCRGTGGAARNTRNNCTGGGAAA-3′)	Haukka, 1997
NodA-2	(5′-GGNCCGTCRTRAAWGTCARGTA-3′)	Haukka, 1997
Flp	(5’ CCAGCAGCCGCGGTAATACG 3’)	This study

Rep-PCR DNA fingerprint was used to investigate the redundant of the strains. Rep-PCR fingerprint were obtained by using BoxAIR primer (Sadowsky, 1996). The PCR reaction contained 50 ng of DNA template, 50 pmol of primer, 2.5 mM of dNTP, 1X PCR buffer, and 2.5 U Taq DNA polymerase (Promega, USA) in total volume of 50 µl. Each PCR was performed with GeneAmpPCR system 9600 (Perkin Elmer, USA). The PCR reaction condition was used as follows; 95°C for 2 min 1 cycle, 94°C for 30 sec, 53°C for 1 min, 56°C for 8 min 35 cycle and final 65°C for 16 min 1 cycle. Products from PCR were separated on 2% agarose gel, stained with ethidium bromide and viewed under UV light in gel documentation (UVP, UK).

NodA PCR was amplified by NodA-1 and NodA-2 primer. The PCR mixture contained 50 ng of DNA template, 12.5 pmol of each primer, 2.5 mM of dNTP, 1X PCR buffer and 2.5 U Taq DNA polymeraes (Promega, USA) in total volume of 50 µl. The PCR reaction condition was used as follows; 93 °C for 2 min 1 cycle, 94°C for 45 sec, 49°C for 1 min, 72°C for 1 min 35 cycle and final 72°C for 5 min 1 cycle.

The partial fragment of 16S rDNA was amplified by a forward primer Fd-1 and reverse primer Rp-2 (Nuswantara, 1999). Each PCR reaction contained 50 ng of DNA template, 12.5 pmol of each primer, 2.5 mM of dNTP, 1X PCR buffer and 2.5 U

Taq DNA polymeraes (Promega, USA) in total volume of 50 µl. The PCR reaction condition was used as follows; 94°C for 3 min 1 cycle, 94°C for 30 sec, 50°C for 30 sec, 74°C for 1 min 35 cycle and final 74°C for 10 min 1 cycle. The full-length 16S rRNA gene was obtained in the next step by using primer walking technique to amplify 16S rDNA PCR product between 540 bp to 560 bp on DNA template. The Flp primer was used in this step for DNA sequencing. The PCR reaction contained 50 ng of DNA template, 12.5 pmol of Flp and Rp-2 primers and 2.5 mM of dNTP, 1X PCR buffer, 2.5 U Taq DNA polymeraes (Promega, USA) in total volume of 50 µl. The PCR reaction condition was used follows; 94°C for 3 min 1 cycle, 94°C for 30 sec, 65°C for 30 sec and 74°C for 30 sec 35 cycle and final 74°C for 10 min 1 cycle.

2.2.2.3 Dendogram analysis

The unweighted pair group method using arithmetic mean average (UPGMA) implemented in Phylogenetic Inference Package (Ntsys ver. 2.1) was used to construct the a dendogram from the respective genetic distance.

2.2.2.4 Direct sequencing from PCR products

The direct sequences of 16S rDNA gene was generated with Perkin Elmer's ABI PRISM™ 377 DNA sequencer. For automated sequencing, the PCR products were purified using gene clean II kit (Bio101 Lab) following the manufacturer's instructions. Sequencing reactions were done by using ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq® DNA Polymerase (Applied Biosystem, USA). 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 (Applibio system, USA).

2.2.2.5 DNA sequence analysis

Generally only one strand of the 16S rDNA fragments was sequenced with the automated sequencer. Each PCR product was sequenced in both directions and the sequences were assembled and checked with the autoassembler 1.4 program (Perkin Elmer) and transferred directly to a sequence analysis program. In this study, to search for homologous sequence in the data bank, Gene Bank (American), BlastN 2.0.13 was employed.

2.2.2.6 Phylogenetic tree analysis

ClustalX ver 1.81 program (Boostrap value = 1000) was used to construct the phylogenetic tree from nucleotide sequence of 16S rDNA PCR product.

2.3 Mornitoring of rhizobia inocula

2.3.1 Recombinant plasmid

Plasmid pBBR *nif*HGUS was obtained from Prof. Y. Murooka (Department of Engineering, Osaka University). The plasmid was depicted in Fig. 4. This 7,600 bp plasmid encodes kanamycin resistance and a *gus* translational fusion of the *nif*H gene derived from *Mesorhizobium huakii*.

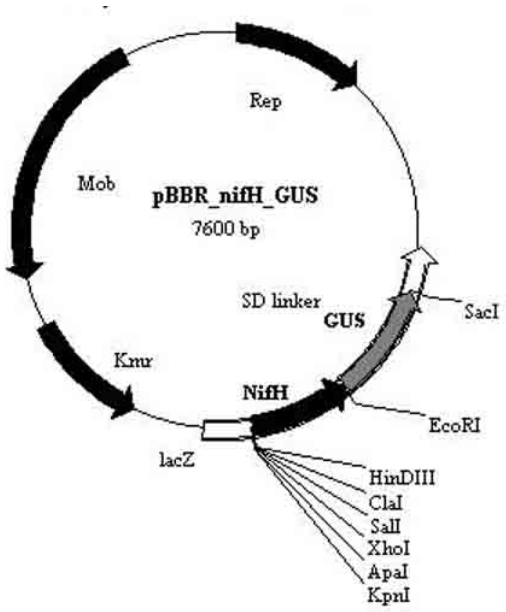

Figure 4. Diagram of recombinant plasmid pBBRI*nif*HGUS containing GUS gene, *nif*Hpromoter, Mob gene and Km^r gene

2.3.2 Bactarial growth condition

E. coli DH5α donor strains (harboring plasmid pBBR_*nif*HGUS) and HB101 helper strains (haboring plasmid pRK2013 containing *tra* gene) which resistant to kanamycin were grown in Luria-Bertani broth (LB) containing kanamycin (50 μg/ml) at 37°C for overnight.

2.3.3 Triparental mating

Each of 1 ml of donor, helper and recipient (selected rhizobial strain) mixture was pelleted by centrifugation at 3000 rpm for 2 min. Cells mixture was washed twice with HM broth and resuspened in 0.5 ml in the same medium. Cell suspension from donor, helper and recipient were mixed in 1:1:5 ratio, respectively and 100 μl of cell suspension was applied onto a membrane filter placed on HM plate. The filters were then incubated at 28°C for 3 days. The cell slurry was scraped and transferred into a

microcentrifuge tube before 1 ml HM broth was added. The cell slurry was mixed with HM and then 100 μl of cell suspension was plated on HM agar containing 50 μg/ml of kanamycin, 50 μg/ml X-gluc and tetracyclin 25 μg/ml. After incubation at 28°C for 7-10 days, blue forming colonies were selected as transconjugants.

2.3.4 Gus staining

For the detection of GUS-marked rhizobia in plant nodule, the nodule was cut in a half. The nodule was immersed in a microtiter plate containing the GUS assay solution (40 μl X-Gluc 20mg/ml in N, N-Dimethylformamide, SDS 20 mg, Methanol 2 ml, 1M sodium phosphate buffer 0.2 ml and distilled water 7.76 ml), in vacuum for 120 min before incubated for overnight at 28°C.

2.4 Seed preparation

Acacia auriculiformis Cunn., *A. mangium* Willd., *Milletia leucantha* Kurz., *Pterocarpus indicus* Willd., and *Xylia xylocarpa* Taub. seeds were surface sterilized and break dormancy period before to soaking into 98% sulfuric acid for 5 min. The excess acid was drained off and seeds were washed with sterilized water for 4-5 times. Seeds were germinated in the petri dish containing moist tissue paper and incubated at room temperature, in dark place for 3 days.

2.5 Soil characterization

Soil sample was collected from the area in Tablan National park, Nakhonratchasima. For soil characterization was conducted under analytical standard method by Department of soil Science, Faculty of Agriculture, Kasetsart University.

The soil had the following characteristics: 79% sand, 18% silt and 3% clay. The total organic matter was 0.3%, the pH was 5.4. The soil consist of phosphorus 32 ppm, potassium 30 ppm, calcium 240 ppm and magnesium 70 ppm.

2.6 Culture media

Composition per liter of each medium was as following ;

2.6.1 Yeast manitol medium (YM)

The medium containing	: D-manitol	10.0	g
	: Yeast extract	0.4	g
	: NaCl	1.0	g
	: $MgSO_4 \cdot 7H_2O$	0.2	g
	: H_2O	1000	ml
	pH 6.8		

2.6.2 HM medium

The medium containing	: Sodium Glutamate	1.0	g
	: Na_2HPO_4	0.125	g
	: $NaSO_4$	0.25	g
	: NH_4Cl	0.32	g
	: $MgSO_4 \cdot 7H_2O$	1.8	g
	: $FeCl_3$	0.004	g
	: $CaCl_2 \cdot 2H_2O$	0.013	g
	: HEPES	1.3	g
	: MES	1.1	g
	: Yeast extract	1.0	g

: L-arabinose 1.0 g

: H_2O 1000 ml

pH 6.8

2.6.3 N-free medium

The medium containing : $CaCl_2$ 0.1 g

: KH_2PO_4 0.1 g

: $MgSO_4 \cdot 7H_2O$ 0.12 g

: $Na_2HPO_4 \cdot 2H_2O$ 0.15 g

: Ferric citrate 0.005 g

: trace element stock solution 1 ml

: H_2O 1000 ml

pH 6.8-7.0

Trace element stock solution : $MnSO_4 \cdot 4H_2O$ 2.03 g

: $CuSO_4 \cdot 5H_2O$ 0.08 g

: $ZnSO_4 \cdot 7H_2O$ 0.22 g

: H_3BO_3 2.86 g

: $Na_2MoO_4 \cdot 2H_2O$ 0.14 g

: H_2O 1000 ml

2.6.4 Tris-TMRT medium

The medium containing : D-manitol 10.0 g

: Yeast extract 0.2 g

: $CaCl_2 \cdot 2H_2O$ 0.2 g

: $MgSO_4 \cdot 7H_2O$ 0.25 g

: Tris-base 1.21 g

: L-Tryptophane 0.061 g

: H_2O 1000 ml

pH 6.8

2.6.5 Luria-Bertani (LB) medium

: Bacto peptone 10.0 g

: Yeast extract 5.0 g

: NaCl 5.0 g

: H_2O 1000 ml

pH 7.0

2.7 Chemical

All chemicals used were laboratory grade, or otherwise specified.

2.7.1 Bromthymol Blue (BTB) : Stock solution 0.5 g/100 ml ethanol
Add 5 ml stock/l of YMA
Final concentration of BTB ; 25 ppm.

2.7.2 Congo Red (CR) : Stock solution 0.25 g/100 ml distilled water
Add 10 ml stock/l of YMA
Final concentration of CR ; 25 ppm.

2.7.3 0.01 M $FeCl_3$ in 35% $HClO_4$ for IAA production test

2.7.4 Kanamycin sulphate : Stock 50 mg/ml (in DW)

Final concentration = 50 μg/ml

2.7.5 X-gluc : Stock 50 mg/ml (in DMSO)

Final concentration = 50 μg/ml

2.7.6 Regent for PCR reaction

: 10 X buffer product from promega (USA)

: dNTP mixture product from promega (USA)

: $MgCl_2$ product from promega (USA)

: Taq polymerase product from promega (USA)

: Primer product from Bioservice unit

(Bangkok, Thailand)

2.7.7 Reagent for gel electrophonic

: 10X TBE buffer

: Ethidium bromide for gel staining

: Agarose gel

2.8 Other equipments

- UV transilluminator : UVP (Ultra Violet Product), UK.
- Hot air oven : Memmert, Germany.
- Incubator : Shellab, USA.
- Electrophoresis set : Biorad, Subcell GT, USA.
- Gel documentation : UVP, UK.
- Refrigerator 4 °C : Sanyo, Thailand.
- Freezer -20 °C : Heto, HLLF 370, Denmark.

- Deep freezer -70 °C : Heto, Ultra Freeze, Denmark.
- Laminar flow : Holten, Larminarair, Denmark.
- Shaker and shaker water bath : Heto, Maxishake, Denmark.
- Water bath : Heto, Denmark.
- Microwave : Turbora, Chaina
- Centrifuge : Hereaus, Labofuge 400R
- Thermal cycler : Perkin Elmer, GeneApPCR System 9600, USA.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Physiological Characteristics

3.1.1 Determination of acid-alkaline production

Acid and alkaline production in Yeast extract-Manitol (YM) medium has been used as a tool to indicate the general character of rhizobia. From selected 44 effective rhizobial strains, the acid-alkaline production characteristics were determined. Most of them produced alkaline and changed the medium color from green to blue color. The result indicated that every strains were slow-grower group (Table 4). The colony forming size was varied in the range of diameter 1 to 3 mm. Slow growing rhizobia can produce alkaline while fast growing rhizobia produce acid (Young, 1996). Thus, most of effective tree legume rhizobia in this study most likely belong to genus *Bradyrhizobium.*

3.1.2 Determination of IAA production

In this study only 4 out of 44 rhizobial strains (*A. auriculiformis* AA3 and AA10, *X. xylocarpa* DX51 and *P. indicus* PT36) could produced Indole Acetic Acid (IAA) (Table 4). Therefore these isolates might rather closely related to *B. elkanii* than *B. japonicum* when determined along with single IAA production characteristic. Indole-3-acetic acid (IAA), was a phytohormone that prometes cell growth and elongation and influences rooting. IAA production was visually determined by the development of red color in the Tris-YMRT broth medium (Minamisawa and Fukai,

1991). In the previous report IAA-attenuated mutants from *B. elkanii* USDA31 significantly reduce the nodule number on soybean root. It has been suggested that bacterial IAA production is related to nodulation and host response (Fukuhara et al., 1994). Minamisawa et al., (1997) reported that selected 44 diverse isolates from the nodule of *Glycine max* cultivars and *Macroptilium artropurpureum* (sirato) based on IAA production, hydrogenase phenotype, *nif* restriction fragment length polymorphism and RS fingerprinting. The selected isolates included 19 *B. japonicum* isolates which contain 10 hydrogen uptake positive isolates and 24 *B.elkanii* isolates separate by IAA production and 30 different RS fingerprints to maintain the genetic diversity. Thus, IAA production could be one of the criteria for classification of *Bradyrhizobium* because *B. elkanii* could produced and secrete IAA while *B. japonicum* does not produced IAA (Minamisawa et al., 1996).

3.1.3 Cross inoculation test with other legumes

The ability of certain rhizobia to infect and nodulated particular group of legume is important in the classification of rhiobia. The cross inoculation system has provided a reasonably stable phylosophical basis for taxonomic scheme for grouping rhizobia strain. The result indicated that selected rhizobial strains could nodulated soybean for 3 isolates which were, 1 isolate from *A. auriculifoemis* (AA67) and 2 isolates from *P. indicus* (PT36 and PT59). The results were correlated with IAA production result because these isolates did not produce IAA except PT36 could produce IAA. These isolates were assumed to be *B. japonicum* group. As for IAA production, *B. elkanii* can produce and excrete IAA in culture media, whereas *B. japonicum* can not produce (Minamisawa *et al*., 1996). Therefore, strain PT36 was assumed to be *B. elkanii* when compared by IAA production and inoculation test.

Twenty rhizobial isolates from tree legume could nodulated mungbean. Four isolates from *A. auriculifoemis* (AA1, 3, 4 and 67), 4 isolates from *A. mangium* (AM16, 42, 45 and 47), 4 isolates *M. leucantha* (ML49, 70, 96 and 100), 4 isolates from *P. indicus* (PT26, 27, 36 and 59) and 4 isolates from *X. xylocarpa* (DX1, 11, 38 and 51) the results show in Table 4. The cross inoculation result showed that the phenotypic of soybean nodulated rhizobial (AA67 and PT59) most likely belong to *B. japonicum* and mungbean nodulated rhizobial were most likely *B. elkanii*. For the rest of strains might be assumed as *Bradyrhizobium* spp.

Table 4. Summary of tree legume rhizobia in each plants and some of their characteristics

Host plants	Characteristics						
	Growth rate		IAA production		Other legumes nodulation		BoxAIR patterns
	Fast	Slow	+	-	*Glycine max*	*Vigna radiata*	
A. auriculiformis							
AA1		✔		✔		✔	1
AA3		✔	✔			✔	1
AA4		✔		✔		✔	2
AA5		✔		✔			2
AA10		✔	✔				3
AA43		✔		✔			4
AA67		✔		✔	✔	✔	5
AA75		✔		✔			5
AA91		✔		✔			6
A. mangium							
AM13		✔		✔			7
AM16		✔		✔		✔	8
AM30		✔		✔			9
AM32		✔		✔			10
AM37		✔		✔			11
AM42		✔		✔		✔	12

Table 4. (continue)

Host plants	Characteristics						
	Growth rate		IAA production		Other legumes nodulation		BoxAIR patterns
	Fast	Slow	+	-	*Glycine max*	*Vigna radiata*	
AM45		✔		✔		✔	13
AM47		✔		✔		✔	14
AM49		✔		✔			15
Millettia leucantha							
ML37		✔		✔			16
ML49		✔		✔		✔	17
ML70		✔		✔		✔	18
ML96		✔		✔		✔	19
Ml100						✔	
ML116		✔		✔			20
ML118		✔		✔			20
ML119		✔		✔		✔	21
Pterocarpus indicus							
PT26		✔		✔		✔	22
PT27		✔		✔		✔	22
PT30		✔		✔			23
PT31		✔		✔			23
PT32		✔		✔			23
PT36		✔	✔		✔	✔	24
PT41		✔		✔			25
PT53		✔		✔			26
PT59		✔		✔	✔	✔	27
PT70		✔		✔		✔	27
Xylia xylocarpa							
DX1		✔		✔		✔	28
DX11		✔		✔		✔	28
DX16		✔		✔			28
DX23		✔		✔			28
DX24		✔		✔			29
DX38		✔		✔		✔	30
DX51		✔	✔			✔	31
DX52		✔		✔			32

3.2 Genotypic characteristics

3.2.1 BOXAIR Fingerprints

BOXAIR primer was used to determined the strains redundant. Enlight to 8-16 bands were generated by amplification of rhizobia genomic DNA. The DNA samples were generated 32 different patterns from 44 isolates (Fig 5). The results patterns of DNA fragment were compared and selected one from the same pattern for further study. For the phynotypic patterns were different from genotypic patterns. One from this genotypic patterns were selected and focused on the basis of genetic information. The relative position of the repeated sequence in the genome of a particular bacterial isolate appear to be conserved in closely related strains and are distinct in diverse species and genera (Sadowsky et. al., 1996)

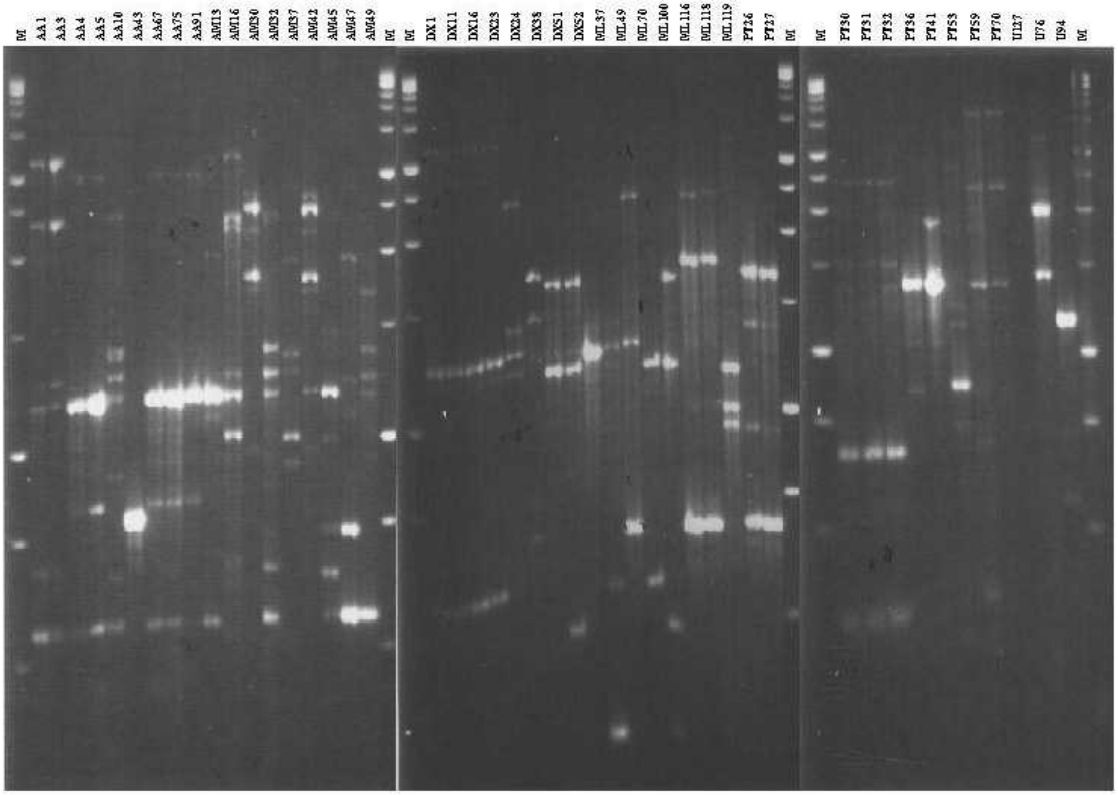

Figure 5. Example of BoxAIR fingerprint patterns from tree legume rhizobial genomic DNA

3.2.2 NodA analysis

The nodA PCR fragment about 700-1,500bp were generated by using NodA primer. The 3 restriction enzymes *Hha*I, *Hinf*I and *Ras*I were used for digestion. The restriction patterns were summarized in Table 5. The nodulation gene (*nod* gene) is a key role in the initiation of nodulate to legume, *nod*ABC are responsible for genes result in the synthesis of the chitin backbone and mutation in these gene the loss of production of Nod signals (Bradley Day et al., 1997). The *nod*A gene, which is present in a single copy in all rhizobia, and whose product, an nod factor acyl transferase, interacts with two substrates, an acyl chain donor and a substituted chito oligomeric acceptor (Debelle, 2001).

Table 5. Restriction pattern of tree legume rhizobia and type strains generated by RFLP analysis of PCR amplified *nodA* gene

Strains	**Restriction pattern of *nodA* gene digested with**		
	***Hha*I**	***Hinf*I**	***Rsa*I**
M. huakii	A	A	A
M. ciceri	B	B	B
R. leguminosarum	C	C	C
R. trifolii	D	D	D
R. tropici	E	E	E
S. meliloti	F	F	F
B. elkanii	G	G	G
B. japonicum	H	H	H
AA4	I	I	I
AA10	J	J	J
AA43	K	K	K
AM13	L	K	L
AM16	J	L	M
AM30	M	K	K

Table 5. (Continue)

Strains	Restriction pattern of *nodA* gene digested with		
	***Hha*I**	***Hinf*I**	***Rsa*I**
AM32	N	M	N
AM37	K	K	K
AM47	J	K	K
AM49	J	K	K
DX1	J	K	K
DX24	O	N	O
DX38	P	O	P
DX51	Q	K	Q
ML37	R	P	R
ML49	S	Q	S
ML70	K	K	K
ML96	T	K	T
ML116	T	K	T
ML118	U	R	U
PT27	T	S	V
PT30	V	T	W
PT36	W	T	W
PT53	X	K	W
PT59	Y	U	X

From combined data of PCR digested product, the dendrogram was generated by Ntsys program ver 2.1. The result was showed in Fig 6. The results showed that nodA PCR-RFLP can be separated into two main clusters and two strains (AA43 and AM37) as out group. The dendrogram of *nod*A PCR-product analysis showed that rhizobial strains in each group of tree legume were scatered in every clusters. The results indicated that no relationship between nodA patterns and host plants specific. However, some strains shared the same *nod*A PCR-RFLP pattern as strains AM13, 45, 47, and ML96. This might imply that they were most likely belong to the same species (see also in Table 6).

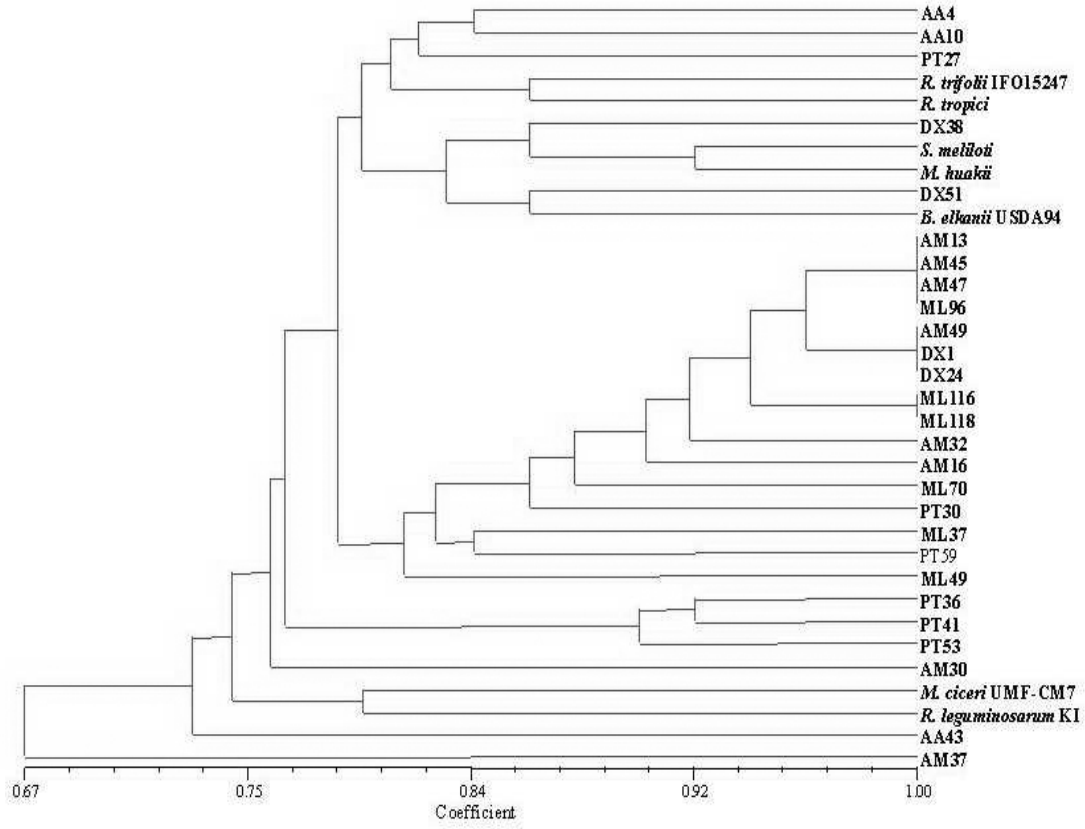

Figure 6. Dendrogram analysis of tree legume rhizobia on the basis of nodA PCR-RFLP (combination of the restriction enzymes *Hha*I, *Hinf*I, *and Rsa*I

However, symbiotic gene as *nod* could not indicated the relationships among even closer genera. For example, *nod*A of *B. elkanii* USDA94 was clearly separated from *nod*A of *B. japonicum* USDA110 and the same case as *M. huakii* and *M. ciceri*. Therefore, this supports that symbiotic genes may have evolutionary histories different from those of 16S rRNA genes. In rhizobia, symbiotic genes be consisted of those genes involved in nitrogen fixation (*nif, fix)* and nodulation (*nod)*. These elements may be located in potentailly transfer genomic elements in all species of *Rhizobium* and *Sinorhizobium,* and in *Mesorhizobium amorphae* and *M. huakii,* or are transferable chromosomal fragments in *M. loti* and *B. japonicum.* These discoveries were also somewhat similar with our results obtained from Thai soil. One hundred and thirty symbiotic and non-symbiotic strains of *Bradyrhizobium* were directly isolated from inoculated soybean and uninoculated legume-free virgin field soil in Thailand using a direct selection medium. About 47% and 58% of the isolates obtained from inoculated and uninoculated field, respectively, were characterized as being non-symbiotic bradyrhizobia. Partial and near full-length sequence analyses of regions encoding 16S rRNA indicated that the non-symbiosis were closely related to *B. elkanii* (79-99%) and *B. japonicum* (98-100%) identity (Pongsilp, et al., 2002). In addition, comparative sequencing has revealed that the symbiotic genes have different evolutionary histories from 16S rRNA genes (Wang, 2000).

Haukka et. al.(1998), reported that three phylogenetic groups of *nod*A and *nif*H in *Sinorhizobium.* With in the large group of African sinorhizobia, similar symbiotic gene types were found in different backgrounds, indicating the lateral transfer of symbiotic genes across species boundaries. Type strains for *S. terangae*

and *S. saheli* have a close relationship in phylogenies of 16S r RNA and *nif*H. But the type strains for *S. saheli* has a totally different position in the phylogram of *nod*A.

Nodulation system by rhizobia is strictly restricted to the family Leguminoceae. The nodulation system was not found in family Rosaceae which closest family to the Leguminoceae (Oyaizu et al., 1993). Galiana et al., (1994) showed that only a restricted range of *Bradyrhizobium* strains were able to produce effective nodules on *A. mangium* and *A. mangium* was a specific host. Ferro et al., (2000) reported in *Acacia* species could be classified in three groups according to their ability to be nodulated in the field by fast-growing rhizobia of the *Rhizobium, Sinorhizobiun* and *Mesorhizobium* (*A. senegal, A. raddiana* and *A. cyanophylla*) by *Bradyrhizobium* (*A. albida, A. mangium* and *A. auriculiformis*) or both type of rhizobia (*A. seyal*). In addition, *A. mangium* is often spontaneously nodulated in its native area and introduction zones by indigenous nitrogen fixing bacteria mainly of the genus *Bradyrhizobium* (Prin et at., 2003).

Hence, in *M. loti* a symbiotic cluster can be transferred to other strains and integrated into a phenylalanine-specific tRNA, this island structure is well conserved on a similar chromosome. Therefore, it is generally accepted that symbiosis genes in rhizobia have evolved by horizontal gene transfer and genomic rearrangements thereafter. Therefore it may also be possible in tree legume. Thus, from the results obtained from this investigation, particularly no relationships was clearly found between host plant and *nod*A support the horizontal gene transfer phenomena.

3.2.3 Full length 16S r DNA sequence

To classify the selected rhizobial strains at genus level, the sequence analyses of 16S rDNA were conducted. The genomic DNA from 29 rhizobia was randomly selected and used as DNA template for each 16S rDNA analysis. The results of PCR amplification were generated the single fragment in size about 1,500 bp. For classification of rhizobia at the genus level, the nucleotide sequences of PCR fragment were selected. The first sequence in each sample was obtained between 650-750 bp, then the full length sequence was done by using the primer walking technique. Full-length primer (Flp) was designed by nucleotide alignment in clustalX program (Fig 7.).

```
              481                                                    540
Am30_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAGCG
Am39_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCG
U76_             CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Pt27_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Am37_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Pt59_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Pt53_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Am13_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Pt28_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Pt36_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
U94_             CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Aa1_             CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Aa4_             CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Aa67_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Am32_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Aa10_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Am16_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Aa43_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Dx38_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Pt56_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Pt31_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Pt32_            CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGG
Am45_            CCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG
Ml49_            CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCG
                 ********************
```

Figure 7. Sequence alignment of 16S rRNA gene (position from 480-540) from tree legumes rhizobia (*similar sequence from sequence alignment).

Nucleotide sequence between 480-500 bp were selected for new primer and used in nucleotide sequence. Approximately 621-835 bp of nucleotide sequence was obtained from Flp primer. Two sequences from isolate were combined with Gene Application program (http://genome.cs.mtu.edu/). Thus, new sequences from each isolate have the length between 1065-1317 bp. The nearly full length sequences of 16S rRNA were compared to other 16S rRNA from data base. The results of % similarity, strains homology, and identified genus on the basis of phenotypic characteristics were compared in Table 6.

Table 6. Sequence results compared with strains from data based and identified strains from phenotypic characteristics

Strains	Compared with strains from data based	%Homology	Accession No.	Identifidation from phenotypic
AA1	*Bradyrhizobium japonicum* strain DASA01059 16S ribosomal RNA gene	99%	AY519470	*B. elkanii*
AA4	*Bradyrhizobium elkanii* strain USDA 121 16S ribosomal RNA gene,	98%	AY507964	*B. elkanii*
AA10	*Bradyrhizobium japonicum* strain DASA02007 16S ribosomal RNA gene	98%	AY507965	*Bradyrhizobium sp.*
AA43	*Bradyrhizobium japonicum* gene for 16S rRNA, partial sequence,	98%	AY519471	*Bradyrhizobium* sp
AA67	*Bradyrhizobium japonicum* strain USDA 110 16S ribosomal RNA gene	97%	AY519472	*B. japonicum*
AM13	*Bradyrhizobium elkanii* 16S ribosomal RNA gene, partial sequence;	98%	AY507966	*Bradyrhizobium* sp
AM16	*Bradyrhizobium elkanii* strain USDA 101 16S ribosomal RNA gene,	98%	AY507967	*B. elkanii*
AM32	*Bradyrhizobium elkanii* strain USDA 23 16S ribosomal RNA gene, partial	98%	AY507968	*Bradyrhizobium* sp.
AM37	*Bradyrhizobium japonicum* strain USDA 124 16S ribosomal RNA gene,	98%	AY507969	*Bradyrhizobium* sp.
AM42	*Bradyrhizobium* sp16S ribosomal RNA gene,	97%	AY507970	*B. elkanii*
AM45	*Bradyrhizobium elkanii* 16S ribosomal RNA gene	98%	AY519473	*B. elkanii*
AM49	*Bradyrhizobium japonicum* gene for 16S rRNA, partial sequence,	97%	AY507971	*Bradyrhizobium* sp.
Dx24	*Bradyrhizobium elkanii* strain USDA 121 16S ribosomal RNA gene,	95%	AY507973	*Bradyrhizobium* sp.

Table 6. (continue)

Strains	Compared with strains from data based	%Homology	Accession No.	Identifidation from phenotypic
Dx38	*Bradyrhizobium japonicum* strain USDA 124 16S ribosomal RNA gene	97%	AY507974	*B. elkanii*
Dx51	*Bradyrhizobium elkanii strain* USDA 23 16S ribosomal RNA gene,	97%	AY507975	*B. elkanii*
ML37	*Bradyrhizobium elkanii* 16S ribosomal RNA gene	98%	AY507976	*Bradyrhizobium* sp.
ML49	*Bradyrhizobium* sp. LMG9250 16S ribosomal RNA gene	98%	AY507977	*B. elkanii*
ML70	*Bradyrhizobium japonicum* strain DASA02007 16S ribosomal RNA gene	98%	AY507978	*B. elkanii*
ML96	*Bradyrhizobium elkanii* 16S ribosomal RNA gene	98%	AY507979	*B. elkanii*
ML100	*Bradyrhizobium elkanii* 16S ribosomal RNA gene	99%	AY507980	*B.elkanii*
ML116	*Bradyrhizobium* sp. strain Da3-1 16S ribosomal	97%	AY507981	*Bradyrhizobium* sp.
ML119	*Bradyrhizobium elkanii* strain USDA 121 16S ribosomal RNA gene,	97%	AY507982	*B. elkanii*
Pt27	*Bradyrhizobium* sp. AMKT 2020 16S ribosomal RNA gene	98%	AY507983	*B. elkanii*
Pt30	*Bradyrhizobium japonicum* strain THA7 16S ribosomal RNA gene	99%	AY507984	*Bradyrhizobium* sp.
Pt36	*Bradyrhizobium japonicum* strain DASA03066 16S ribosomal RNA gene	97%	AY507985	*B. elkanii*
Pt59	*Bradyrhizobium japonicum* strain USDA 124 16S ribosomal RNA gene,	98%	AY507987	*B. japonicum*

***** strains AM30, 47, DX1, ML118, PT 41 and PT53 did not sequence

This table showed the strains identification compared with phenotypic and genotypic characteristics. For *A. auriculiformis*, strains AA1, 10, 43, and 67 were belong to *B. japonicum* from genotypic charcteristics but in phenotypic characteristic AA1 was *B. elkanii* and AA10 and 43 was *Bradyrhizobium* sp. Strain AA4 showed the same result in both genotypic and phenotypic characteristics. In *A. mangium* strains AM13, 16, 32 and 45 were *B. elkanii,* while strains AM37 and 45 were *B. japonicum* and AM42 was *Bradyrhizobium* sp. from genotypic data. The phenotypic characteristics showed that AM13, 32, 37 and 49 belong to *Bradyrhizobium* sp.,

AM16, 42 and 45 belong to *B. elkanii.* Strains DX24 and 51 were *B. elkanii,* whilst DX38 was *B. japonicum* when compared from data base. The phenotypic of DX38 and 51 indicated that they were *B. elkanii* and DX24 was *Bradyrhizobium* sp. Strains ML37, 96, 100 and 119 isolated from *M. leucantha* were *B. elkanii,* ML49 and ML116 were *Bradyrhizobium* sp. and ML70 was *B. japonicum* when identified from 16S rDNA sequences. From phenotypic characteristics most of strains were *B. elkanii* only ML37 and 49 strains were *Bradyrhizobuim* sp. For *P. indicus* rhizobial strains PT30, 36 and 59 were *B. japonicum* and PT27 was *Bradyrhizobium* sp. from genotypic data whilst from phenotypic data strains PT27 and 36 were to *B. elkanii,* PT30 was *Bradyrhizobium* sp. and PT59 was *B. japonicum.* The results suggested that determination by both phenotypic and genotypic characteristics are very important for classification particularly for unknown rhizobial strains.

3.2.4 Phylogenetic tree analysis

The phylogenetic tree was constructed from 16S rDNA sequence by using ClustalX program. The phylogenetic tree of the 16S rDNA sequence was showed in Fig 8. 16s rRNA data were compared with other rhizobial reference sequence: *B. japonicum* USDA110, *B. elkanii* USDA94. While *M. huakii* IFO15243, *M. tianshanse* USDA3592,*R. leguminosarum* IAM12609, *S. fredii* ATCC35423, *S. meliloti* IAM12611 and *A. caulinodans* ORS571 were used as the out group. Phylogenetic analysis of the isolates revealed that PT30 and ML 49 were separated from other isolates. The other isolates were separated into 3 clusters. Cluster I is the out group of the type strains contained many genera of rhizobia, such as *M. huakii* IFO15243, *M. tianshanse* USDA3592, *R. leguminosarum* IAM12609, *S. fredii* ATCC35423, *S. meliloti* IAM12611 and *A. caulinodans* ORS571. Cluster II, the main cluster, which

had 3 subgroups. Subgroup I contained the isolates represent to *B. japonicum* and *B. elkanii.* The isolate PT36 was in this cluster. From the previous results, these rhizobial strains could nodulate soybean, mungbean and produce IAA. The results indicated that PT36 isolate was closely related with *B. elkanii* rather than *B. jajonicum*. Strain PT 59 was member in this cluster, this isolate was closely related with *B. jajonicum* rather than *B. elkanii*. On the other hand, subgroup II and subgroup III are containing many isolates. In this group only 1 isolates was found that able to produce IAA (AA67). Although some isolates could nodulated mungbean but this group were not related to *B. japonicum* and *B. elkanii.* Eventhough Oyaizu, et al., (1993), reported *P. indicus* was nodulated by *Bradyrhizobium* sp. However the phylogenetic tree indicated that rhizobial strains from *P. indicus* were closely related with both of *B. japonicum* and *B. elkanii*. For *X. xylocarpa* rhizobial strains were distributed in subgroup II as the same in *M. leucactha* rhizobial strain except ML49 were separated as out-group. de Lajudie, et al., (1998) studied *Acacia* species in Senegal, rhizobial strains from *Acacia* belong to *S. terangae* and new species *M. plurifarium*. The results are contrast from this study, since only found *Acacia* species only belong to *Bradyrhizobium* sp in this study.

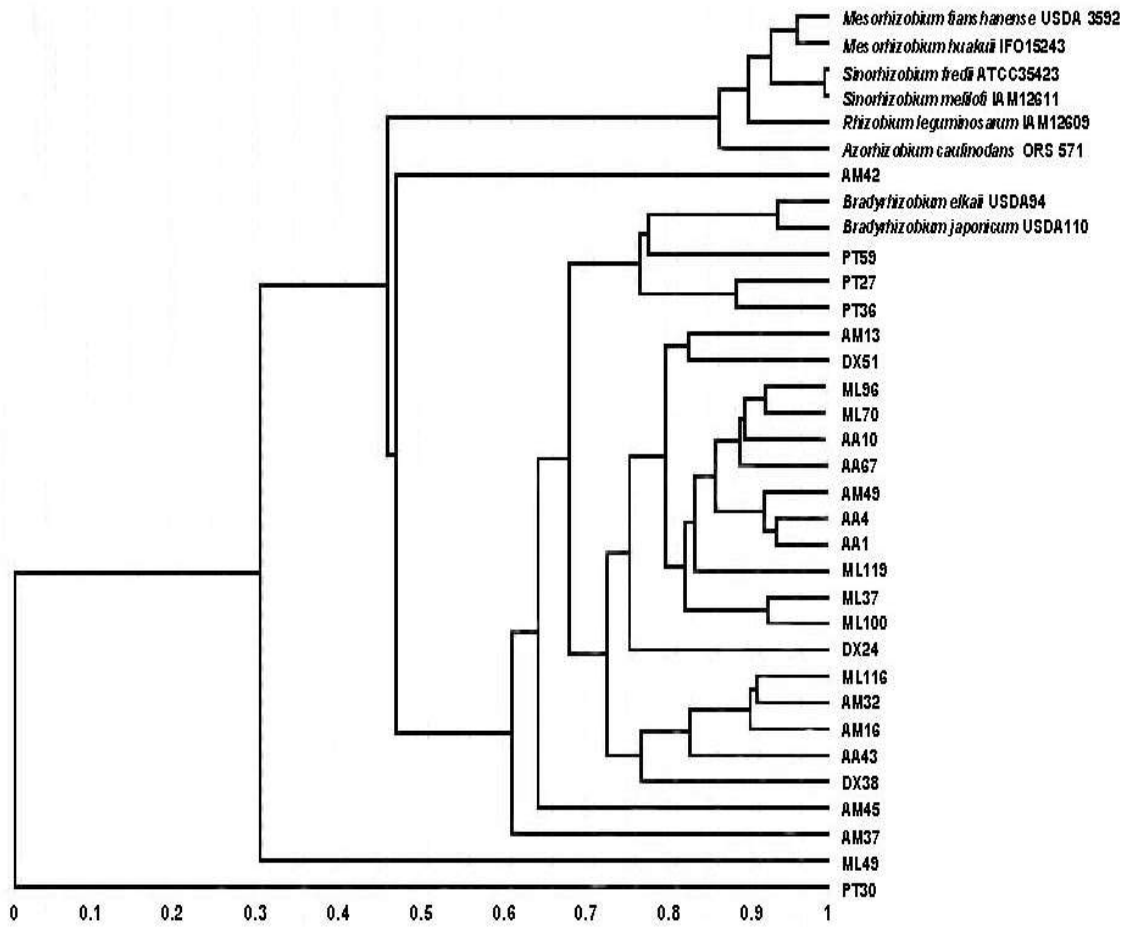

Figure 8. Phylogenetic tree derived from 16S rDNA sequence from tree legume

From those effective rhizobial strains isolated from tree legumes, the results indicated that most of them were most likely belong to *B. japonicum, B. elkanii* and *Bradyrhizobium* sp. The reletionships between host plants and 16S rDNA sequence were not found. The phylogenetic tree showed that rhizobial strains in each tree legume were spread all clusters indicated the nonspecific 16S rDNA and host plants. Initially, *Bradyrhizobium* (*Rhizobium*) *japonicum* was only recognized species which nodulated soybean. Later *B. japonicum* was proposed to the separate species, *B. elkanii* for one of the soybean nodulating group (Kuykendall, et al., 1992). In

addition, recently the third species, *B. liaoningense* was proposed for a group of extra slow growing *Glycine* isolated (Xu, et al., 1995). *B. liaoningense* was also genotypically highly related to *B. japonicum* but phenotypically distinct, whereas *B. elkanii* is more distantly, related to there two species (Willems, et al., 2001). However, when the results of cross nodulation were determined the plant host nodulation characteristics did not show phylogenetical relationships. Ramsubhag, et al., (2002) found that slow growing pigeon pea isolates were phylogenetically related to *B. elkanii*. However, all of those strains could not be classified as *B. elkanii* since they did not all have the necessary phenotypic traits including the ability to nodulated soybean.

Moreover, particular in *Acacia,* the high diversity of rhizobeaceae was found in term of nodule formation. *A. senegal* and *A. tortilis* in Senegal were found that colud be nodulated by *S. terangae* and *M. plurifarium* (de Lajudie, et al., 1998). Whereas, *A. albida* also in Senegal was found that mainly nodulated by *B. japonicum, B. elkanii* and *Bradyrhizobium* sp. (Dupuy, et al., 1994). On the other hand, fast growing group as *R. leguminosarum* was also able to form nodule in both *A. confusa* and *A. farnesiana,* in geograhpic origin of Japan and Philipines, respectively (Oyaizu, et al., 1993). This phenomenon due to signal molecules call nod factor. The compounds from all strains involved with *Acacia* tribe were found to be similar i.e., O-carbamoylated and substituted by on of ten sulfated metyl fucose (Ferro, et al., 2000). Therefore, a comprehensive and polyphasic study of soybean or host plants of interests using many strains is requried for defining more reliably the species limits of bradyrhizobia originating from nodules.

3.3 The nodulation ability of GUS-marked strains relative to the parental strain

Prior to investigate the nodulation ability of selected rhizobial strains, the nodulation of indigenous rhizobia in forest soil was investigated with every tree legumes. For *A. auricuriformis* and *A. mangium* approximate 5-6 nodules/plant was found, *X. xylocarpa* a few nodule as 2-3 nodules/plants was found. In *M. leucantha* an approximate 4-5 nodules/plants and higher nodules as 13-14 nodules were found in *P. indicus* (Table7). This implied that native rhizobia from Tablan National Park soil prefer *P. indicus* rather than other tree legumes. When each of selected rhizobial strains were inoculated into plants under sterilized condition, it was found that the ability of nodulation were in the range of 6-7 nodules/plant. Rhizobial strain AA10 could form nodule in *A. mangium* approximate 6-7 nodules/plant. Strain PT36 could form nodule approximate 10 nodules/plant in *P. indicus* while strain AM16, DX11 and ML100 could form nodule in higher number as 16-17 nodules/plant in *A. mangium*, *X. xylocarpa* and *M. leucantha,* respectively. However when introduced the selected strains into plant under forest soil condition, the results showed that the number of nodule in each plant was reduced except in *P. indicus*. But when compared with number of nodule formation by native rhizobia in soil, it seemed that introduced strains had the effect upon nodulation occupancy. For example in *A. mangium, X. xylocarpa, M. leucantha* and *P. indicus* showed the higher in number of nodules/plant for 1-5 time. While this effect was not found in *A. auriculiformis*. This once again was confirmed by introduction of marked strains.

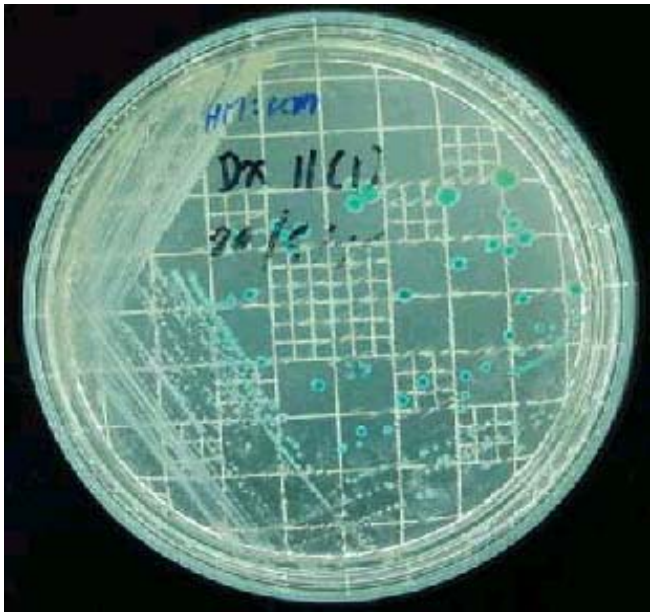

Figure 9. Blue colony forming with *gusA* activity

The results of nodulation ability were analysed on the basis of GUS stained nodules.The nodule occupancy data from the preliminary experiment in soil with GUS-marked transconjugants was summarized in Table 7. The GUS activity in each plants were showed in Fig 9. Each plant was inoculated with the marked strains. Strains AA10 performed 71% of nodules occupancy as AM16 87% DX11 63% ML100 72% and PT36 100%. The number of nodule formation by marked strains were lower than wild type in AA10, AM16 and PT36. This might caused by the marked strain interfere the nodulation ability. The *E. coli gusA* gene encoding β-glucuronidase is a very convenient and suitable marker for these purpose, GUS activity has not been reported in either plants or rhizobia (Wilson et al., 1995). Ayango et al*.,* (1998), used the transposon Tn5 as the delivery vehicle for the GUS marker gene, the transconjugants had to be screened for their ability to nodulate beans as efficiently as the parent strains. This is because the Tn5 transposon could cause insertion mutations, which may affect genes required for the symbiosis. In case no

significant differences observed between the transconjugant and their parental strains. Okazaki et al., (2003), reported *B. elkanii* USDA94 and mutant MA 941 were mixed in the inoculum at a 1:1 ratio, both strains formed similar number of nodule. This indicated that *gusA* insertion did not affect the nodulation competitiveness of parent strain USDA94. Denton et al., (2003) found that rhizobial strains RW185D and RW855C mark with *gusA* did not from their parental strains in nodulation competitiveness and effectiveness and were therefore used to test competitiveness of the field isolates against the commercial strain TA1. From the previous data show that *gusA* are suitable for genetic marker and selection of suitable inoculant.

Table 7. The results of nodule occupancy of wild type and transconjugants

Strains	Non-Innoculation		Wild type		Marked		% Nodule Occupancy
	Soil ($\overline{X}$)	Sand	Soil ($\overline{X}$)	Sand ($\overline{X}$)	Soil ($\overline{X}$)	Sand ($\overline{X}$)	Soil
Aa10	5.5 ± 2.12	-	4.0 ± 1.41	6.5 ± 2.12	4.0 ± 1.41	3.5 ± 0.71	71
Am16	5.3 ± 2.31	-	12.3 ± 2.52	17.3 ± 2.08	4.0 ± 1.41	3.5 ± 0.71	87
DX11	2.5 ± 0.71	-	11.7 ± 1.53	17.0 ± 7.55	11.0 ± 1.41	9.0 ± 1.41	63
Ml100	4.5 ± 0.71	-	11.7 ± 2.08	16.7 ± 1.53	11.0 ± 1.41	8.0 ± 1.41	72
Pt36	13.7 ± 1.53	-	17.7 ± 2.52	10.7 ± 1.53	8.0 ± 0.0	10.0 ± 7.07	100

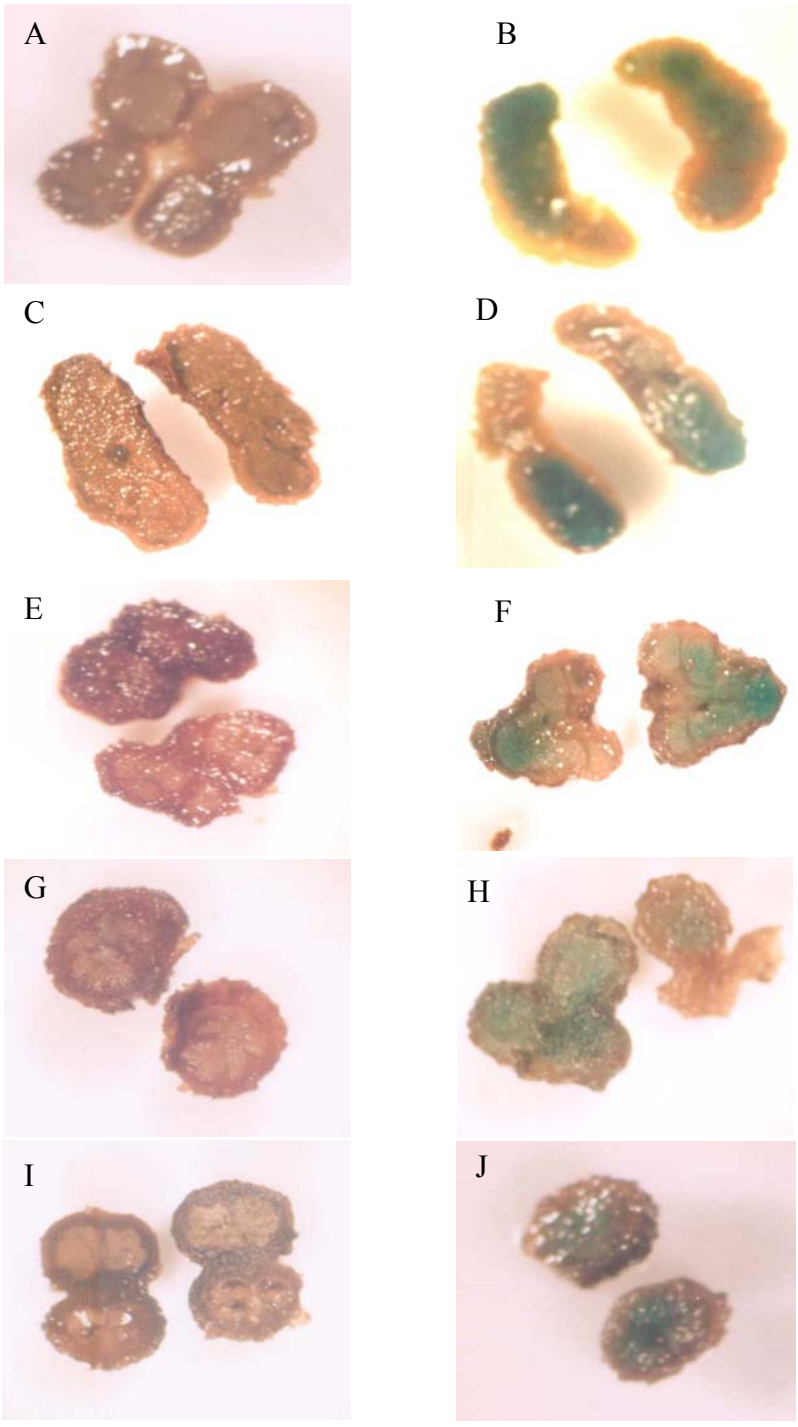

Figure 10. GUS activity in root nodule infected by transconjugant and compared with wild type.

A: *A. auriculiformis* AA10 wild type B: *A. auriculiformis* AA10 conjugant,
C: *A. manguim* AM16 wild type D: *A. manguim* AM16 conjugant
E: *X. xylocarpar* DX11 wild type F: *X. xylocarpar* DX11 conjugants
G: *M. leucantha* ML100 wild type H: *M. leucantha* ML100 conjugant
I: *P. indicus* PT36 wild type J: *P. indicus* PT36 conjugant

CHAPTER IV

CONCLUSION

The objectives of this study aim to charaterize and monitor the selected tree legume rhizobial strains. The rhizobial strains were isolated and selected from *Acacia auriculiformis* Cunn., *A. mangium* Willd., *Millettia leucantha* Kurz., *Pterocarpus indicus* willd. And *Xylia xylocarpa* Taub. Characterizations of selected rhizobial strains were carried out in both aspects of physiology and genetics. To characterization, different methods such as acid-alkaline production, IAA production and cross inoculation were used for study physiological characteristics. To determined the genetic characteristics BoxAIR-PCR, nodA-PCR, sequencing 16S rRNA gene and phylogenetic tree analysis were used to distinguish rhizobial strains.

The results from this study indicated that the phenotypic and genotypic characteristics from tree legumes rhizobia were closely related with *B. elkanii* and *Bradyrhizobium* spp. However strains PT36 and PT59 from *P. indicus* most likely belong to *B. japonicum*. The symbiotic gene as *nod* gene could not indicated the relationship among strains or even closer genera. To monitor the selected strains on the basis of marker gene, the results suggested that *gusA* marked strains performed higher nodule occupy than that of indigenous strains in soil sample from Tab-Lan national park. This implied that the selected strains perform better competition ability than indigenous strains. This enable to further study soil and other environmental factors which might affect the competition performance of different rhizobial strains.

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