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*Full Length Research Paper*

# Characterization and monitoring of selected rhizobial strains isolated from tree legumes in Thailand

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**Tree legume *rhizobia* were isolated from five tree legumes; *Acacia auriculaformis Cunn.*, *A. mangium* Willd., *Milletia leucantha* Kurz., *Pterocarpus indicus* Willd., and *Xylia xylocarpa* Taub. grown in Thailand. Forty four highly effective rhizobial strains were selected on the basis of nitrogenase activity, number of nodules and plant biomass. The selected strains were characterized in both terms of physiology and genetics. Most of the strains are slow grower and able to nodulate cowpea rather than soybean. In addition, IAA production could be detected only from few strains. When almost complete 16S rRNA sequences were analysed, the results indicated that most of the selected strains most likely belong to *Bradyrhizobium elkanī* and *Bradyrhizobium* sp. except strains AA67 and PT59 which most likely belong to *B. japonicum*. The nodule occupancy of selected strains in forest soil condition was investigated by using GUS reporter gene. The nodule occupancy is in the range of 63 - 100%. This suggests the appropriate strains should be produced as inoculum for further application in reforestation programmes in Thailand.**

**Key words:** Tree legume rhizobia, *Acacia auriculaformis, Acacia mangium, Milletia leucantha, Pterocarpus indicus, Xylia xylocarpa.*

## INTRODUCTION

The forest resource in Thailand has been in a critical situation since 1961. In the past, 53% of Thailand was covered with forest but in 1989 only 28% remained forest areas. To conserve the remaining forest, the Department of Forestry has initiated the forestry restoration programme for private sectors by promotion of various kinds of plants. Thirty-eight to fifty-five plant species such as *Acacia* spp., *Rhizosphora* spp., *Cassia* spp., *Xylia* spp., *Pterocarcus* spp., *Milletia* spp., etc. are among the most favorable (http://www.forest.go.th/stat.htm). Most plants belong to the leguminous plant type. Members of the leguminosae, about 18,000 species, play an important ecological role with representatives in nearly every type of plant on earth. Most species are able to form nitrogen-fixing symbiosis with bacteria known as rhizobia. Galiana et al. (1994) demonstrated that *Acacia mangium* responds positively to inoculation with *Bradyrhizobium* strains under field conditions. *Acacia* species have become the major tree plantation species in South-Asia. In Indonesia and Malaysia, *A. manguim* is currently the most planted tropical tree species in the world since more than 600,000 ha of industrial plantations of *A. mangium* have already been established, while many forestation projects are planned for the coming years (Turnbull et al., 1998).

However, rhizobial symbiosis with some native tree legumes has not been elucidated. In this study, our aims are (i) to characterize the selected rhizobia strains from tree legumes such as *Acacia auriculiformis* Cunn, *A. mangium* Willd, *Millettia leucantha* Kurz, *Pterocarpus indicus* Willd and *Xylia xylocarpa* Taub in terms of some physiological features and genetic relationship, and (ii) to

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monitor the persistence of selected inocula in soil condition on the basis of reporter gene application.

## MATERIALS AND METHODS

### Bacterial strains and strains selection

The rhizobia type strains *Bradyrhizobium japonicum* USDA110 and *B. elkanii* USDA94 were used throughout this study. *A. auriculiformis* Cunn, *A. mangium* Willd, *M. leucantha* Kurz, *P. indicus* Willd, and *X. xylocarpa* Taub seeds were surface sterilized and the dormancy period broken by soaking in 98% sulfuric acid for 5 min. The excess acid was drained off and seeds were washed with sterilized water 4 - 5 times. Seeds were germinated in the Petri dish containing sterilized moist tissue paper and incubated at room temperature, in a dark place for 3 days. Germinated seeds were grown in pot culture containing soil samples collected from field grown tree legumes in Thailand. Rhizobial were isolated from their nodules. The high efficiency of $N_2$–fixation from various tree legume rhizobia strains were screened by the acetylene reduction activity (ARA) method (Somasegaran and Hoben, 1994). The selected rhizobia strains and related host plants were summarized in Table 1. *Escherichia coli* DH5α donor strains (harboring plasmid pBBR_*nif*HGUS) and *E. coli* HB101 helper strains (harboring plasmid pRK2013 containing *tra* gene), which are resistant to kanamycin, were grown in Luria-Bertani broth (LB) containing kanamycin (50 µg/ml) at 37°C overnight.

### Acid-alkaline production

Rhizobial strains were cultured on Yeast-Malt extract agar containing bromthymol blue (BTB) as the acid/base indicator and incubated at 28°C for 10 days. Colony form-ing was observed every day as well as the changing of color-medium. For an acid-producing strain, the medium color changed from green to yellow while alkaline production changed from green to blue (Somasegaran and Hoben, 1994).

### 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 (D-mannitol 10 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/l, pH 6.8) culture broth after incubation at 28°C for 10 days. The mixture was further incubated in the dark at 30°C for 30 min. Results were compared with a positive control of 1.0 g of IAA in distilled water and ethanol (1:1 for 1.0 ml) (**Nuntagij** et al., 1997).

### Inoculation with other legumes

Seeds of soybean (*Glycine max*) and mungbean (*Vigna radiata*) were surface sterilized in 95% ethanol for 10 s before the addition of 3% sodium hypochlorite to immerse the seeds completely. After a 5 min drain-off of the sterilant, seeds were rinsed six times with sterilized water. The sterilized seeds were put on a plate containing sterilized moist tissue paper and kept in a dark place for 1 - 2 days. Seeds were germinated in the Petri dish containing sterilized moist tissue paper and incubated at room temperature in a dark place for 3 days. Germinated seeds were grown in Leonard jars containing sterilized sand then inoculated with 1 ml of rhizobial culture ($10^7$ - $10^8$ cells/ml/seed). The Leonard jars were put on a shelf light that set up 12 h in the light and 12 h in the dark. Nodule formation was observed 45 days after planting.

### Genomic DNA extraction

Rhizobial strains were cultured in HM medium (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/l, pH 6.8). After cultivation for 5 - 7 days on a rotary shaker at 200 rpm at 28°C, total genomic DNA from each bradyrhizobial isolate was prepared as described previously (Hartmann and Amarger, 1991).

### DNA primers and PCR conditions

DNA fingerprint was used to investigate the redundancy of the strains. Rep-PCR fingerprint was obtained by using BOXAIR primer (5′ CTACGGCAAGGCGACGCTGACG 3′) (Sadowsky et al., 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 in total volume of 50 µl. The PCR reaction condition was used as follows; 95°C for 2 min 1 cycle, 94°C for 30 s, 53°C for 1 min, 56°C for 8 min 35 cycles and finally 65°C for 16 min 1 cycle. Products from PCR were separated on 2% agarose gel, stained with ethidium bromide. NodA PCR was amplified by NodA-1 (5′ TGCRGTGGAARNTRNNCTGGGAAA 3′) and NodA-2 (5′GGNCCGTCRTRAAWGTCARGTA 3′) primer (Haukka, 1997). The PCR mixture contained 50 ng of DNA template, 12.5 pM of each primer, 2.5 mM of dNTP, 1X PCR buffer and 2.5 U Taq DNA polymerase 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 s, 49°C for 1 min, 72°C for 1 min 35 cycles and finally 72°C for 5 min 1 cycle. The partial fragment of 16S rDNA was amplified by a forward primer Fd-1 (5′ AGAGTTTGATCCTGGCTCAG 3′) and reverse primer Rp-2 (5′ ACGGCTACCTTGTTACGACTT 3′) (Nuswantara et al. 1999). Each PCR reaction contained 50 ng of DNA template, 12.5 pM of each primer, 2.5 mM of dNTP, 1X PCR buffer and 2.5 U Taq DNA polymerase 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 s, 50°C for 30 s, 74°C for 1 min 35 cycles and finally 74°C for 10 min 1 cycle. The full-length 16S rRNA gene was obtained in the next step by using the primer walking technique to amplify 16S rDNA PCR product between 480 bp to 500 bp on DNA template. The Flp primer (5'CAGCAGCCGCGGTAATACG 3') (this study) 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 polymerase 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 s, 65°C for 30 s and 74°C for 30 s 35 cycles and finally 74°C for 10 min 1 cycle.

### DNA Direct sequencing from PCR products

The direct sequencing of 16S rDNA gene was generated with a Perkin Elmer's ABI PRISM™ 377 DNA sequencer. For automated sequencing, the PCR products were purified using a gene clean II kit (Bio101 Lab) following the manufacturer's instructions. Sequencing reactions were done by using an 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 the subsequent DNA sequencing PCR, amplification and the subsequent DNA precipitation with ethanol were done following the manufacturer's instructions (Applied Biosystem, USA).

**Table 1.** The selected rhizobia strains and host plants.

| Host plants | Plant dry weight (g/plant) | Nodule No | Nodule fresh weight (g/plant) | μM $C_2H_4$/plant/h |
|---|---|---|---|---|
| ***A. auriculiformis*** | | | | |
| control | 0.47$^{b}$ | 0 | 0 | 0.235$^{b}$ |
| AA1 | 1.33$^{a}$ | 22$^{ab}$ | 0.091$^{a}$ | 0.825$^{ab}$ |
| AA3 | 1.50$^{a}$ | 26$^{ab}$ | 0.099$^{a}$ | 0.601a$^{b}$ |
| AA4 | 1.32$^{a}$ | 37$^{ab}$ | 0.203$^{a}$ | 1.370$^{a}$ |
| AA5 | 1.26$^{a}$ | 29$^{ab}$ | 0.166$^{a}$ | 1.421$^{a}$ |
| AA10 | 0.89$^{ab}$ | 30$^{ab}$ | 0.121$^{a}$ | 0.861$^{ab}$ |
| AA43 | 1.27$^{a}$ | 16$^{b}$ | 0.198$^{a}$ | 0.650$^{ab}$ |
| AA67 | 1.13$^{a}$ | 40$^{ab}$ | 0.202$^{a}$ | 1.255$^{a}$ |
| AA75 | 1.20$^{ab}$ | 43$^{a}$ | 0.253$^{a}$ | 1.422$^{a}$ |
| AA91 | 1.05$^{ab}$ | 39$^{ab}$ | 0.174$^{a}$ | 0.960$^{ab}$ |
| ***A. mangium*** | | | | |
| control | 0.58$^{b}$ | 0 | 0 | 0.333$^{c}$ |
| AM13 | 1.82$^{ab}$ | 62$^{a}$ | 0.469$^{a}$ | 2.896$^{a}$ |
| AM16 | 2.40$^{a}$ | 36$^{ab}$ | 0.378$^{ab}$ | 1.479$^{abc}$ |
| AM30 | 1.23$^{ab}$ | 22$^{b}$ | 0.243$^{abc}$ | 1.442$^{abc}$ |
| AM32 | 1.60$^{ab}$ | 29$^{ab}$ | 0.165$^{bc}$ | 1.192$^{abc}$ |
| AM37 | 1.22$^{ab}$ | 24$^{b}$ | 0.114$^{c}$ | 1.401$^{abc}$ |
| AM42 | 1.55$^{ab}$ | 50$^{ab}$ | 0.240$^{abc}$ | 1.421$^{abc}$ |
| AM45 | 1.84$^{ab}$ | 44$^{ab}$ | 0.337$^{abc}$ | 1.587$^{abc}$ |
| AM47 | 1.80$^{ab}$ | 37$^{ab}$ | 0.243$^{abc}$ | 1.686$^{abc}$ |
| AM49 | 2.85$^{a}$ | 24$^{b}$ | 0.256$^{abc}$ | 2.195$^{ab}$ |
| ***M. leucantha*** | | | | |
| control | 1.22$^{b}$ | 0 | 0 | 0.373$^{b}$ |
| ML37 | 2.89$^{a}$ | 36$^{ab}$ | 0.479$^{a}$ | 4.712$^{a}$ |
| ML49 | 2.69$^{a}$ | 37$^{ab}$ | 0.316$^{a}$ | 3.871$^{a}$ |
| ML70 | 2.43$^{a}$ | 18$^{ab}$ | 0.212$^{a}$ | 2.750$^{ab}$ |
| ML96 | 2.46$^{a}$ | 31$^{ab}$ | 0.326$^{a}$ | 2.986$^{ab}$ |
| Ml100 | 2.24$^{ab}$ | 47$^{a}$ | 0.436$^{a}$ | 4.890$^{a}$ |
| ML116 | 2.43$^{a}$ | 37$^{ab}$ | 0.295$^{a}$ | 2.857$^{ab}$ |
| ML118 | 2.63$^{a}$ | 21$^{ab}$ | 0.307$^{a}$ | 2.897$^{ab}$ |
| ML119 | 2.53$^{a}$ | 12$^{b}$ | 0.208$^{a}$ | 2.273$^{ab}$ |
| ***P. indicus*** | | | | |
| **control** | 0.61$^{de}$ | 0 | 0 | 0.369$^{d}$ |
| PT26 | 0.90$^{b-e}$ | 33$^{ab}$ | 0.178$^{ab}$ | 1.270$^{bcd}$ |
| PT27 | 1.24$^{ab}$ | 50$^{a}$ | 0.236$^{a}$ | 2.234$^{ab}$ |
| PT30 | 1.10$^{abc}$ | 18$^{bc}$ | 0.206$^{ab}$ | 2.379$^{a}$ |
| PT31 | 1.50$^{a}$ | 25$^{bc}$ | 0.258$^{a}$ | 2.562$^{a}$ |
| PT32 | 1.04$^{bcd}$ | 10$^{c}$ | 0.064$^{c}$ | 1.179$^{cd}$ |
| PT36 | 1.28$^{ab}$ | 24$^{bc}$ | 0.208$^{ab}$ | 2.378$^{a}$ |
| PT41 | 1.05$^{bcd}$ | 22$^{bc}$ | 0.180$^{ab}$ | 2.225$^{ab}$ |
| PT53 | 0.73$^{cde}$ | 17$^{bc}$ | 0.102$^{bc}$ | 1.348$^{bc}$ |
| PT59 | 0.92$^{b-e}$ | 18$^{bc}$ | 0.122$^{bc}$ | 1.868$^{abc}$ |
| PT70 | 0.57$^{e}$ | 14$^{bc}$ | 0.212$^{ab}$ | 1.664$^{abc}$ |
| ***X. xylocarpa*** | | | | |
| **control** | 0.60$^{b}$ | 0 | 0 | 0.232$^{b}$ |
| DX1 | 1.28$^{a}$ | 11$^{bc}$ | 0.075$^{abc}$ | 1.041$^{ab}$ |
| DX11 | 1.11$^{ab}$ | 22$^{a}$ | 0.126$^{a}$ | 1.068$^{a}$ |
| DX16 | 1.13$^{ab}$ | 5$^{cde}$ | 0.041$^{abc}$ | 0.929$^{ab}$ |

**Table 1.** Contd.

| | | | | |
|---|---|---|---|---|
| DX23 | 1.19$^{ab}$ | 15$^{b}$ | 0.126$^{a}$ | 0.727$^{ab}$ |
| DX24 | 1.26$^{a}$ | 8$^{cd}$ | 0.066$^{abc}$ | 0.646$^{ab}$ |
| DX38 | 1.13$^{ab}$ | 5$^{cde}$ | 0.120$^{ab}$ | 0.958$^{ab}$ |
| DX51 | 1.43$^{a}$ | 2$^{de}$ | 0.011$^{bc}$ | 0.441$^{ab}$ |
| DX52 | 0.89$^{ab}$ | 6$^{cde}$ | 0.076$^{abc}$ | 0.836$^{ab}$ |

*Same letters are not significantly different at LSD P<0.05 level.

#### DNA sequences and analyses

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 auto assembler 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. ClustalX ver 1.81 program (Boostrap value = 1,000) was used to construct the sequences alignment from nucleotide sequence of 16S rDNA PCR product. The unweighted pair group method using arithmetic mean average (UPGMA) implemented in Phylogenetic Inference package (Ntsys ver. 2.1) was used to construct the dendogram from the respective genetic distance.

#### Triparental mating and GUS activity

Each of 1 ml of donor, helper and recipient (selected rhizobia strain) cell mixture was pelleted by centrifugation at 3,000 rpm for 2 min. Cell mixture was washed twice with HM broth and resuspended in 0.5 ml with the same medium. Cell suspensions from donor, helper and recipient were mixed together in a 1:1:5 ratio, respectively, and 100 µl of cell suspension was spread onto a membrane filter placed on HM plate. The filters were then incubated at 28°C for 3 days. The cell slurry was collected and transferred into a microcentrifuge tube before 1 ml HM broth was added. The cell slurry was mixed with HM broth and then 100 µl of cell suspension was plated on HM agar containing 50 µg/ml of kanamycin and 50 µg/ml X-gluc. After incubation at 28°C for 7-10 days, blue forming colonies were selected as transconjugants. Seeds were inoculated with 1 ml of each transconjugants. The experiment was replicated three times. For the detection of GUS-marked rhizobia in a plant nodule, the nodule was cut in half. The nodule was immersed in a microtiter plate containing the GUS assay solution (40 µl X-Gluc 20 mg/ml in N, N-Dimethyl-formamide, 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 being incubated overnight at 28°C.

#### Soil characterization

A soil sample was collected from an area in Tablan National Park, Nakhonratchasima province. Soil chara-cterrization was conducted under the analytical standard method by the Department of Soil Science, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. The soil had the following characteristics: 79% sand, 18% silt and 3% clay. The total organic matter was 0.3%, and the pH was 5.4. The soil minerals consist of phosphorus 32 ppm, potassium 30 ppm, calcium 240 ppm and magnesium 70 ppm.

## RESULTS

### Some physiological characteristics

The rhizobial strains were isolated from five tree legumes nodules. One hundred and eighteens rhizobial strains were obtained from *A. auriculiformis* Cunn., 180 strains from *A. mangium* Willd, 126 strains from *M. leucantha* Kurz, 177 strains from *P. indicus* Willd, and 126 strains from *X. xylocarpa* Taub. The most effective strains were selected on the basis of plant dry weight, nodule number, nodule dried weight and ARA activity (Table 1). The 44 effective rhizobial strains were selected and the acid-alkaline production characteristics were firstly determined. Most of them produced alkaline and changed the medium colour from green to blue. The result indicated that all strains were in the slow-grower group (Table 2). The colony forming size varied in the range of diameter between 1 to 3 mm. In this study, only 4 out of 44 rhizobial strains (*A. auriculiformis* strains AA3 and AA10, *X. xylocarpa* strain DX51 and *P. indicus* strain PT36) could produce Indole Acetic Acid (IAA) (Table 2). Twenty rhizobial isolates from tree legumes could nodulate mungbean. There were 4 isolates from *A. auriculifoemis* (AA1, 3, 4 and 67), 4 isolates from *A. mangium* (AM16, 42, 45 and 47), 4 isolates from *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). Only three rhizobial strains could nodulate soybean, which was an isolate from *A. auriculiformis* (AA67) and 2 isolates from *P. indicus* (PT36 and PT59) (Table 2).

### Genotypic characteristics

BOXAIR primer was used to determine the redundant strains (data not shown). PCR product 8 - 16 bands were generated by amplification of rhizobia genomic DNA. The DNA samples generated 32 different patterns from 44 isolates (Table 2). The results of DNA fragments were compared and only one from the same pattern was selected for further study. The *nod*A PCR fragments, in size about 700 - 1,500 bp, were generated by using nod A primer. The results showed that *nod*A PCR-RFLP can be separated into two main clusters and two strains (AA43 and AM37) were as an out group. The dendrogram of *nod*A PCR- product analysis (Figure 1) showed that rhizobial strains in each group of tree legumes were scattered in all clusters. The results indicated that there was no relationship between *nod*A patterns and the host plants specificity. However, some different plant host strains shared the same nodA PCR-RFLP pattern as strains AM13, 45, 47 (from *A. mangium*) and ML96 (from *M. leucantha*).

To classify the selected rhizobial strains in the genus

**Table 2.** Summary of tree legume rhizobia in each plant 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 |
| AM45 | | ✔ | | ✔ | | ✔ | 13 |
| AM47 | | ✔ | | ✔ | | ✔ | 14 |
| AM49 | | ✔ | | ✔ | | | 15 |
| ***M. leucantha*** | | | | | | | |
| ML37 | | ✔ | | ✔ | | | 16 |
| ML49 | | ✔ | | ✔ | | ✔ | 17 |
| ML70 | | ✔ | | ✔ | | ✔ | 18 |
| ML96 | | ✔ | | ✔ | | ✔ | 19 |
| Ml100 | | ✔ | | ✔ | | ✔ | 20 |
| ML116 | | ✔ | | ✔ | | | 21 |
| ML118 | | ✔ | | ✔ | | | 21 |
| ML119 | | ✔ | | ✔ | | ✔ | 22 |
| ***P. indicus*** | | | | | | | |
| PT26 | | ✔ | | ✔ | | ✔ | 23 |
| PT27 | | ✔ | | ✔ | | ✔ | 23 |
| PT30 | | ✔ | | ✔ | | | 24 |
| PT31 | | ✔ | | ✔ | | | 24 |
| PT32 | | ✔ | | ✔ | | | 24 |
| PT36 | | ✔ | ✔ | | ✔ | ✔ | 25 |
| PT41 | | ✔ | | ✔ | | | 26 |
| PT53 | | ✔ | | ✔ | | | 27 |
| PT59 | | ✔ | | ✔ | ✔ | ✔ | 28 |
| PT70 | | ✔ | | ✔ | | ✔ | 28 |
| ***X. xylocarpa*** | | | | | | | |
| DX1 | | ✔ | | ✔ | | ✔ | 29 |
| DX11 | | ✔ | | ✔ | | ✔ | 29 |
| DX16 | | ✔ | | ✔ | | | 29 |
| DX23 | | ✔ | | ✔ | | | 29 |
| DX24 | | ✔ | | ✔ | | | 30 |
| DX38 | | ✔ | | ✔ | | | 31 |
| DX51 | | ✔ | | | | ✔ | 32 |
| DX52 | | ✔ | | ✔ | | ✔ | 33 |

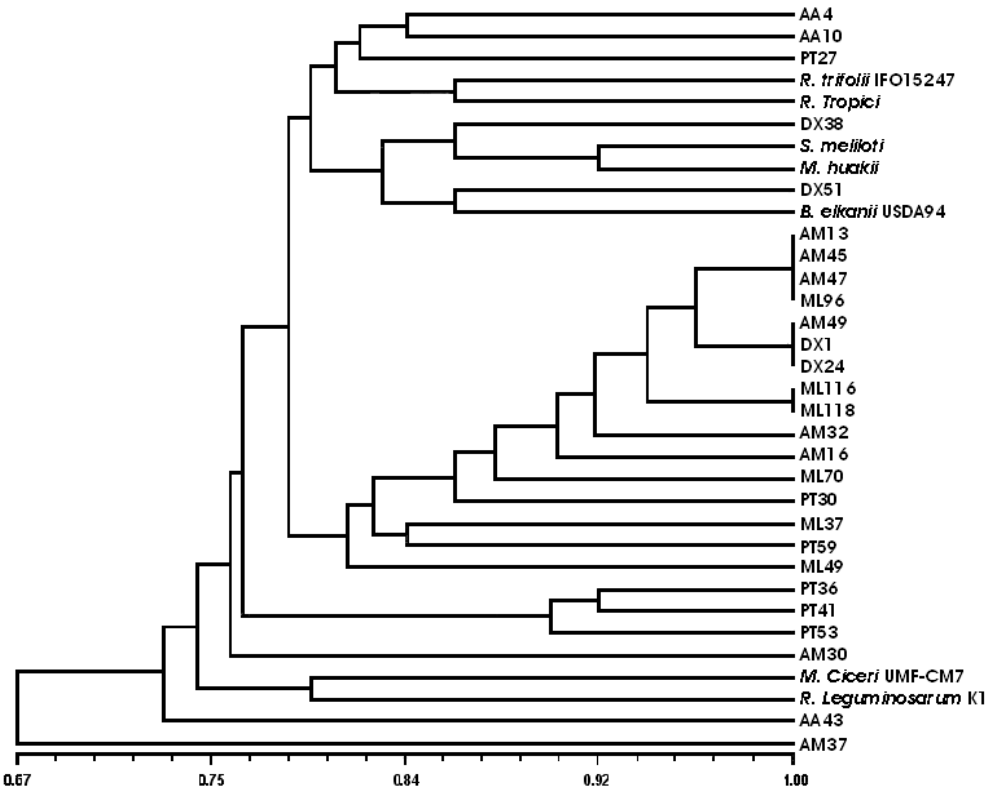


**Figure 1.** Dendrogram analysis of tree legume rhizobia on the basis of nodA PCR-RFLP (combination of the restriction enzymes *Hha* I, *Hinf* I, and *Rsa* I).

level, the sequence analyses of 16S rDNA were conducted. The genomic DNA from 29 rhizobia was randomly selected and used as a DNA template for each 16S rDNA analysis. The results of PCR amplification generated the single fragment about 1,500 bp in size. For classification of rhizobia in the genus level, the nucleotide sequences of the 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 a clustalX program. A nucleotide sequence between 480 - 500 bp was selected for the new primer and used in the nucleotide sequence. Approximately 621 - 835 bp of nucleotide sequence was obtained from the Flp primer. Two sequences from each isolate were combined by the Gene Application program. Thus, new sequences from each isolate have a length between 1,065 - 1,317 bp. The nearly full length sequences of 16S rRNA were compared to other 16S rRNA genes from the database.

The results of similarity, strains homology, and identified genus on the basis of phenotypic characteristics were compared in Table 3. For *A. auriculiformis*, rhizobial strains AA1, 10, 43, and 67 should belong to *B. japonicum* (97 - 99% homology), but strain AA4 belongs to *B. elkanii* (98% homology) when considered along with 16S rRNA sequences results, whilst strain AA67 showed both the same phenotypic and DNA sequences as *B. japonicum.* However, rhizobial strains AA1 and AA4 showed the same phenotypic characteristics as *B. elkanii.* The rhizobial strains AA10 and AA43 were assumed to be *Bradyrhizobium* sp. when determined from phenotypic characteristics. In *A. mangium* rhizobial strains AM13, 16, 32 and 45 were *B. elkanii,* while strains AM37 and 49 were *B. japonicum* and AM42 was *Bradyrhizobium* sp. when determined from the 16S rDNA sequences. The phenotypic characteristics showed that AM13, 32, 37 and 49 should belong to *Bradyrhizobium* sp., while strains AM16, 42 and 45 belong to *B. elkanii. X. xylocarpa* rhizobial strains DX24 and 51 were closely related to *B. elkanii,* while strain DX38 was *B. japonicum* when compared from 16S rDNA sequences. However, phenotypic data indicated that strains DX38 and 51 should be *B. elkanii* and DX24 was *Bradyrhizobium* sp. Rhizobial strains ML37, 96, 100 and 119 isolated from *M. leucantha* were closely related to *B. elkanii,* when analysed from 16S rDNA sequences, while strains ML49 and ML116 were *Bradyrhizobium* sp. and ML70 was *B. japonicum.* Phenotypic characteristics indicated that most strains most likely belong to *B. elkanii*, and only ML37 and 49 strains were *Bradyrhizobuim* sp. For *P. indicus* rhizobia strains PT30, 36 and 59 belong to *B. japonicum* and PT27 was *Bradyrhizobium* sp. from genotypic data, whilst from phenotypic data, strains PT27 and 36 were

**Table 3.** 16S rDNA sequences results compared with strains from data base and identified strains from phenotypic characteristics.

| Strains | Compared with strains from data base | % Homology | Accession No. | Identifidation from phenotypepic |
|---|---|---|---|---|
| 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 gene, | 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, | 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, | 98% | AY507968 | *Bradyrhizobium sp.* |
| AM37 | *Bradyrhizobium japonicum* strain USDA 124 16S ribosomal RNA gene, | 98% | AY507969 | *Bradyrhizobium sp.* |
| AM42 | *Bradyrhizobium* sp. 16S 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 gene, | 97% | AY507971 | *Bradyrhizobium sp.* |
| DX24 | *Bradyrhizobium elkanii* strain USDA 121 16S ribosomal RNA gene, | 95% | AY507973 | *Bradyrhizobium sp.* |
| 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.* |
| Strains | Compared with strains from data base | %Homology | Accession No. | Identifidation from phenotypic |
| 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* |

**Table 4.** The results of nodule occupancy of wild types and transconjugants rhizobia.

| Strains | Non-Innoculation | | Wild type | | Marked | | % Nodule Occupancy |
|---|---|---|---|---|---|---|---|
| | Soil (X) | Sand | Soil (X) | Sand (X) | Soil (X) | Sand (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 |

closely related to *B. elkanii,* PT30 was *Bradyrhizobium* sp. and PT59 was *B. japonicum.*

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

Prior to investigating the nodulation ability of selected rhizobia strains, the nodulation by indigenous rhizobia in forest soil was investigated with all tree legumes. For *A. auricuriformis* and *A. mangium* approximately 5 - 6 nodules/plant were found, for *X. xylocarpa*, as few nodules as 2 - 3 nodules/plant were observed. In *M. leucantha* were found approximately 4 - 5 nodules/plant and as high as 13 - 14 nodules were found in *P. indicus* (Table 4). When each of the selected rhizobia strains were inoculated into plants under sterilized soil conditions, it was found that the ability to nodulate was in the range of 6 - 7 nodules/plant. Rhizobial strain AA10 could form nodules in *A. mangium*, approximately 6 - 7 nodules/plant. Strain PT36 could form approximately 10 nodules/plant in *P. indicus*, while strains AM16, DX11 and ML100 could form as high as 16 - 17 nodules/plant in *A. mangium*, *X. xylocarpa* and *M. leucantha,* respectively. However when the selected strains were introduced into plants with forest the soil sample, the results showed that the number of nodules in each plant was reduced except in *P. indicus*. But when compared with the number of nodules formed by native rhizobia in soil, it seemed that introduced strains had an effect upon nodulation occupancy. For example *A. mangium, X. xylocarpa, M. leucantha* and *P. indicus* showed a higher number of nodules/plant (1 - 5 times), while this effect was not found in *A. auriculiformis.*

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 4. The GUS-staining in each plant was as depicted in Figure 2. Each plant was inoculated with the marked strains. Strain AA10 produced 71% of nodule occupancy, AM16 had 87%, DX11 had 63%, ML100 had 72% and PT36 had 100%. The numbers of nodules formed by marked strains were lower than those of wild type strains AA10, AM16 and PT36.

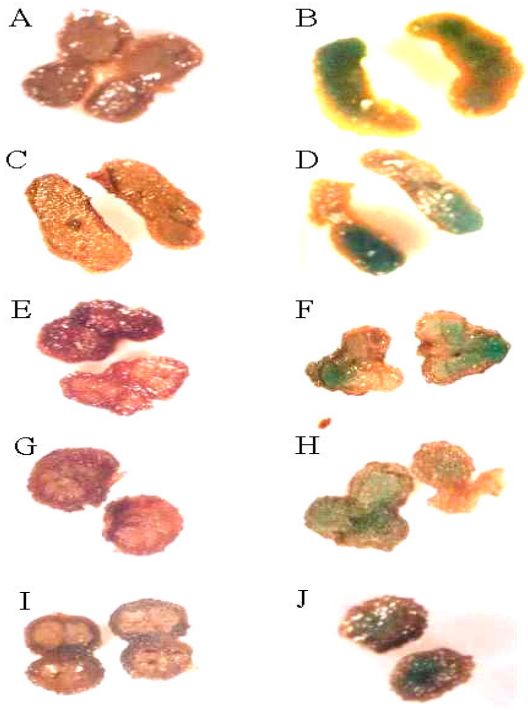


**Figure 2.** GUS activity in root nodule infected by transconjugant and compared with wild type. A, *A. auriculiformis* AA10 wild type; B, *A. auriculiformis* AA10 transconjugant; C, *A. manguim* AM16 wild type; D, *A. manguim* AM16 transconjugant; E, *X. xylocarpar* DX11 wild type; F, *X. xylocarpar* DX11 transconjugant; G, *M. leucantha* ML100 wild type;H, *M. leucantha* ML100 transcon-jugant; I, *P. indicus* PT36 wild type; and J, *P. indicus* PT36 transconjugant.

## DISCUSSION

Acid and alkaline production in Yeast extract-Manitol (YM) medium has been used as a tool to indicate the general character of rhizobia. Slow growing rhizobia can produce alkaline while fast growing rhizobia produces acid (Young, 1996). Most of the effective tree legumes rhizobia in this study therefore most likely belong to genus *Bradyrhizobium.* Indole-3-acetic acid (IAA) was a phytohormone that promotes cell growth and elongation

and influences rooting. IAA production was visually determined by the development of a red colour in the Tris-YMRT broth medium (Minamisawa and Fukai, 1991). IAA production could be one of the criteria for *Bradyrhizobium* classification because *B. elkanii* could produce and secrete IAA while *B. japonicum* does not produce IAA (Minamisawa et al., 1996). Therefore, these isolates might be rather closely related to *B. elkanii* than that of *B. japonicum*.

The cross inoculation results showed that the phenotype of soybean nodulating rhizobia (AA67 from *A. auriculaformis* and PT59 from *P. indicus*) most likely belongs to *B. japonicum* and mungbean nodulated rhizobia were most likely *B. elkanii*, when the results were compared from Genbank. The rest of the strains might be assumed to be *Bradyrhizobium* sp. The ability of certain rhizobia to infect and nodulate a particular group of legumes is important in the classification of rhizobia. The cross inoculation system has provided a reasonably stable philosophical basis for a taxonomic scheme for grouping rhizobial strains. The results were correlated with IAA production results because these isolates did not produce IAA, except that strain PT36 could produce IAA. These isolates were assumed to be the *B. japonicum* group. Therefore, strain PT36 was assumed to be *B. elkanii* when compared with the IAA production and inoculation test.

The nodulation gene (*nod* gene) has a key role in the initiation of legume nodulation. *nod*ABC are responsible for gene results in the synthesis of the chitin backbone, and mutation in these genes for the loss of production of *nod* signals (Bradley et al., 1997). The *nod*A gene is present as a single copy in all rhizobia, and its product, a nod factor acyl transferase, interacts with two substrates, an acyl chain donor and a substituted chito-oligomeric acceptor. However, a symbiotic gene such as *nod* could not indicate 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 was the case with *Mesorhizobium huakii* and *M. ciceri* (Debelle et al., 2001). Therefore, this supports that symbiotic genes may have evolutionary histories different from those of 16S rRNA genes. In addition, comparative sequencing has revealed that symbiotic genes do have different evolutionary histories from 16S rRNA genes (Wang and Martinez-Romero, 2000). Galiana et al. (1994) demonstrated that only a restricted range of *Bradyrhizobium* strains was able to produce effective nodules on *A. mangium*, and *A. mangium* was a specific host. Ferro et al. (2000), reported that *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 types 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 al., 2003). Thus, from the results obtained from this investigation particularly, no relation-ship was clearly found between host plant and *nod*A which supports the horizontal gene transfer phenomena.

The relationships between host plants and the rhizobial 16S rDNA sequence in this study were also not found. The phylogenetic tree showed that rhizobial strains in each tree legume were spread to all clusters and indicated the non-specific relationship between the 16S rDNA sequence and host plants. Initially, *B.* (*Rhizobium*) *japonicum* was the 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* isolates (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 these 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 nodulate soybean. Moreover, particular in *Acacia,* the high diversity of rhizobeaceae was found in terms of nodule formation. *A. senegal* and *A. tortilis* in Senegal were found that could be nodulated by *S. terangae* and *M. plurifarium* (de Lajuide et al., 1998), whereas *A. albida*, also in Senegal, was found to be mainly nodulated by *B. japonicum, B. elkanii* and *Bradyrhizobium* sp. (Dupuy et al., 1994). This phenome-non is due to sig-nal molecules called the nod factor. The compounds from all strains involved with the *Acacia* tribe were found to be similar i.e., O-carbamoylated and substituted by an often sulfated methyl fucose (Ferro et al., 2000). Nearly full-length 16S rRNA sequences and partial 23S rRNA sequences confirmed that two isolates from *D. retusa* were highly similar or identical to *Bradyrhizobium* strains isolated from the legumes *Erythrina* and *Clitoria* (Papilio-noideae tribe Phaseoleae) in Panama. The rRNA sequences for five isolates from *L. atropurpureus, P. pinnatum* and *A. inermis* were not closely related to any currently known strains from Central America or elsewhere, but had affinities to the reference strains *B. japonicum* USDA 110 (three isolates) or to *B. elkanii* USDA 76 (two isolates) (Parker, 2004). Rasolomampianina et al. (2005) isolated and characterized 68 bacterial strains from eight species of *Dalbergia* endemic to Madagascar. The majority of the isolates (48) belonged to *Bradyrhizobium*, which is a common symbiont of most tropical legumes. Wang et al. (2006) characterized

71 isolates from root nodules of *Acacia* spp., *Albizia* spp. and *Leucaena leucocephala* grown in the subtropical zones of China. Based upon the cones-nsus of the grouping results of 16S rDNA PCR-RFLP, SDS–PAGE of whole-cell proteins, numerical taxonomy of phenotypic characters and 16S rRNA gene seque-ncing, a total of 14 putative species or lineages belonging to *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* were defined. All of the results showed that the isolates have a high diversity at both the species level and genetic level. They confirmed that the legume species within the genera *Acacia*, *Albizia* and *L. leucocephala* may harbor some common rhizobial species, but they also have different preferences of the microsymbionts. Therefore, a comprehensive and polyphasic study of host plants of interest including geographic origins using many strains is required for defining more reliably the species limits of bradyrhizobium originating from nodules.

Tablan National Park is a destroyed forest containing low organic matter soil. Five tree legumes were selected for a preliminary test in the soil sample and rhizobia strains were applied. The selected rhizobial strains showed a high potential for producing an inoculum. The nodule occupancy of transconjugants was found to be between 63 - 100%. *P. indicus* inoculated with strain PT36 in the soil sample was found to have the only nodule occupancy from a transconjugant. This implied that the inoculum strain for *P. indicus* has a nodulating ability higher than native rhizobial from Tablan National Park. This should enable a further study of soil and other environmental factors which might affect the competitive performance of different rhizobia strains. This technique can be developped for quality control programmes in inoculant production and application.

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