The diversity of root nodule bacteria associated with endemic legume species in Southeast Asia and its application for sustainable agriculture

（東南アジア産マメ科植物と共生する根粒細菌の多様性と
持続型農業への応用に関する研究）

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Abstract

The recent exploration of tropical wild legume species (tress and shrubs) has evidenced as unexpected diversity of rhizobia symbionts, resulting in the description of numerous rhizobial new species. However, there is a general lack of information on the genetic and functional diversity of rhizobia nodulating endemic legumes especially, in Southeast Asia, the unique geological history in the world. These areas containing high concentrations of endemic legume species. The diversity of *Bradyrhizobium* strains isolated from *Aeschynomene americana* L. (weed in rice field) in Thailand was emphasized and used as model. The results from phenotypic characteristics and multilocous sequence analysis of 16S rRNA gene and 3 housekeeping genes (*dnaK*, *recA*, and *glnB*), indicated that the isolated strains were non-photosynthetic bacteria and assigned to the genus *Bradyrhizobium*, in which *B. yuanmingense* was the dominant species. Some of the other species including *B. japonicum*, *B. liaoningense*, and *B. canariense* were minor species. These isolated strains were divided into 2 groups, *nod*-containing and divergent *nod*-containing strains, based on Southern blot hybridization and PCR amplification of *nodABC* genes. Surprisingly, the *A. americana* isolated strains could nodulate *Aeschynomene* that belong to different cross inoculation (CI) groups including *A. afraspera* and *A. indica*. This is the first discovery of bradyrhizobia (non-photosynthetic and *nod*-containing strain) originating from CI-group 1 nodulating roots of *A. indica* (CI-group 3).

Moreover, one of these strains, *Bradyrhizobium* sp. DOA9 showed a broad host range, being able to colonize and efficiently nodulate the roots of most plants tested of the dalbergioid, millettioid, and robinoid tribes. In addition, DOA9 carried two replicons, a chromosome (cDOA9) and a single megaplasmid (pDOA9) larger than 352 kb.

Interestingly, genes for nodulation (*nodA*, *B*, *C*) and nitrogen fixation (*nifH*) were localized on the megaplasmid. These results suggested that *Bradyrhizobium* sp. DOA9 has the unusual combination of a broad host range, bacteroid differentiation, and symbiosis-mediating replicons.

To broaden the scope of the study, non-leguminous symbioses were also investigated. Rice is the most important food croup in Asia. The biological nitrogen fixation (BNF) from rice root-associated bacteria has a great potential to improve the sustainable rice production. Even though attempts have been made to use endophytic diazotrophic bacteria as biofertilizers for rice cultivation, their community structure and complexity in rice tissues is not well understood. The population of viable endophytic diazotrophic bacteria in cultivated rice was investigated under different soil conditions. Some isolates were closely related to *Enterobacter dissolvens*, *Brevundimonas aurantiaca*, *Pantoea agglomerans*, *Pseudomonas* spp., and Enterobacteriaceae. Nested PCR-DGGE analysis with *nifH* primer demonstrated less diazotrophic bacterial diversity in the roots of rice cultivated in paddy soil amended with nitrogen fertilizer than in unfertilized and previously uncultivated soil. Plant tissue type was found to dictate the endophytic diazotrophic community structure rather than the type of soil or fertilizer amendment.

On the other hand, Plant colonization by bradyrhizobia is found not only in leguminous plants but also in non-leguminous species such as rice. The rice endophytic bradyrhizobia were isolated on the basis of oligotrophic property, selective medium and nodulation on siratro. Six bradyrhizobial strains were obtained exclusively from rice with crop rotational system, not from rice monoculture system. The isolates were separated into photosynthetic bradyrhizobia (PB) and non-photosynthetic bradyrhizobia (Non-PB).

Interestingly, Thai bradyrhizobial strains promoted rice growth of Thai rice cultivar rather than the Japanese strains.

Furthermore, rhizobial inoculants have been used as an environmental friendly source of nitrogen fertilizer for several decades. However, there are a number of factors that impede the nodulation on legumes. The coinoculation with bradyrhizobia and PGPR leads to an increased number of the most active nodules and plant yield as well as a greater nitrogen fixation. Selected 12 PGPR performed a significant capability of promoting nodulation and N_2-fixing efficiency of soybean with both of the commercial bradyrhizobial strains, USDA110 and THA6. Moreover, isolates S141 and S222, which are closely related to *Bacillus subtilis* and *Staphylococcus* sp., were selected for co-inoculation with USDA110 and THA6. The effect of co-inoculation experiment under field condition could increase the seed yields. Therefore, the efficiency to enhance soybean N_2-fixation by co-inoculation of S141 and S222 with *Bradyrhizobium* strategy could be developed for supreme soybean inoculants.

Most of all, *Rhizobium* is a biofertilizer for leguminous crops. To formulate this form of fertilizer, the suitable sterilization processes of carrier are important. In this study, carriers could be efficiently sterilized by irradiation at 10-20 kGy, or by autoclaving with tyndallization approach. The number of strain tested, *Bradyrhizobium* sp. PRC008 was in the range of 10^8-10^9 cfu/g in both irradiated and autoclaved peat after 6 months storage. These results indicated that carrier material had an important influence on inoculant quality, while sterilization processes using gamma irradiation and autoclaving with tyndallization approach could be used for efficient rhizobial inoculant production with peat based carrier.

Abbreviations

AG agar	arabinose-gluconate agar
ANFICOs	anaerobic N2-fixing consortia
ANOVA	analysis of variance
ARA	acetylene reduction activity
BNF	biological nitrogen fixation
$CaCO_3$	calcium carbonate
CFU	colony-forming unit
CI	cross-inoculation
C^o	degree Celsius
DAI	days after inoculation
DAPI	4', 6-diamidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DIC microscope	differential interference contrast microscope
DIG	digoxigenin
DOA	department of agriculture
GUS	beta-glucuronidase
HM agar	mueller hinton Agar
IAA	Indole-3-acetic acid
IRLC	inverted repeat lacking clade
kGy	kilogray
MLSA	multilocus sequence analysis
MPN	most-probable number
N-fertilizer	nitrogen fertilizer

N:P:K	nitrogen:phosphorus:potassium
N_2-fixing	nitrogen fixing
NCBI	National Center for Biotechnology Information
Nif	nitrogense
Nod	nodulation
Non-PB	non-photosynthetic bradyrhizobia
PB	photosynthetic bradyrhizobia
PCA	plate count agar
PCR-DGGE	polymerase chain reaction denaturing gradient gel electrophoresis
PDA	potato dextrose agar
PGPR	Plant Growth Promoting Rhizobacteria
PI	propidium iodide
SDS	sodium dodecyl sulfate
SUT	Suranaree university of technology
TAE buffer	tris-acetate-EDTA buffer
YEM	yeast extract-mannitol

ORIGINAL PAPERS

This thesis is based on the following articles, referred to in the text by their Roman numerals.

I Prakamhang, J., K. Minamisawa, **K. Teamtisong**, N. Boonkerd and N. Teaumroong. 2009. The communities of endophytic diazotrophic bacteria in cultivated rice (*Oryza sativa* L.). Appl. Soil Ecol. 42:141-149. (Prakamhang *et al.*, 2009).

II Rujirek Noisangiam, **Kamonluck Teamtisong**, Panlada Tittabutr, Nantakorn Boonkerd, Uchiumi Toshiki, Kiwamu Minamisawa, and Neung Teaumroong. 2012. Genetic Diversity, Symbiotic Evolution, and Proposed Infection Process of *Bradyrhizobium* Strains Isolated from Root Nodules of *Aeschynomene americana* L. in Thailand. Appl. Environ. Microbiol. 78:6236-6250. (Noisangiam *et al.*, 2012).

III Panlada Tittabutr, **Kamonluck Teamthisong**, Bancha Buranabanyat, Neung Teaumroong, Nantakorn Boonkerd. 2012. Gamma Irradiation and Autoclave Sterilization Peat and Compost as the Carrier for Rhizobial Inoculant Production. Journal of Agricultural Science. 4 (12): 59-67. (Tittabutr *et al.*, 2012).

IV Kamonluck Teamtisong, Pongpan Songwattana, Rujirek Noisa-ngiam, Pongdet Piromyou, Nantakorn Boonkerd, Panlada Tittabutr, Kiwamu Minamisawa, Achara Nantakit, Shin Okazaki, Mikiko Abe, Toshiki Uchiumi, Neung Teaumroong. 2014. Divergent *nod*-containing *Bradyrhizobium* sp. DOA9 with a megaplasmid and its host range. Microbes and Environments. 29 (4): 370-376. (Teamtisong *et al.*, 2014).

V Janpen Prakamhang, **Kamonluck Teamtisong**, Panlada Tittabutr, Nantakorn Boonkerd, Toshiki Uchiumi, Mikiko Abe, Neung Teaumroong. 2015. Proposed some interactions at molecular level of PGPR coinoculated with *Bradyrhizobium diazoefficiens* USDA110 and *B. japonicum* THA6 on soybean symbiosis and its potential o field application. Applied Soil Ecology. 85:38-49. (Prakamhang *et al.*, 2015)

VI Pongdet Piromyou, Teerana Greetatorn, **Kamonluck Teamtisong**, Takashi Okubo Ryo Shinoda, Achara Nuntakij, Panlada Tittabutr, Nantakorn Boonkerd, Kiwamu Minamisawa, Neung Teaumroong. 2014. Preference of endophytic bradyrhizobia in different rice cultivars and the implication of rice endophyte evolution. (submitted to AEM)

AIM OF THIS STUDY

The recent exploration of tropical wild legume species (tress and shrubs) has evidenced as unexpected diversity of rhizobia symbionts, resulting in the description of numerous rhizobial new species. However, there is a general lack of information on the genetic and functional diversity of rhizobia nodulating endemic legumes especially, in Southeast Asia, the unique geological history in the world. These areas containing high concentrations of endemic legume species. Thus, the aim of this thesis became to explore the diversity of bradyrhizobial strains isolated from root nodules of endemic legume. The diversity of *Bradyrhizobium* strains isolated from *A. americana* L. (weed in rice field) in Thailand was emphasized and used as model. In addition, symbiotic genes and the replicons for symbiosis genes were analyzed. To broaden the scope of the study, non-leguminous symbioses were also investigated such as endophytic bradyrhizobia in rice. Finally, the application of isolated strains for inoculant production was also considered.

The specific aims of this study were

1. To investigate the diversity of bradyrhizobial strains isolated from root nodules of endemic legume; *Aeschynomene americana* L. in Thailand by using phenotypic characterization and phylogenetic analyses of the 16S rRNA gene and a combination of the 3 housekeeping genes (*dnaK, glnB*, and *recA*) **(II).**
2. To determine symbiotic genes based on Southern blot hybridization and PCR amplification of symbiotic genes. In addition, the replicons and localization for symbiosis genes were analyzed (**IV** and **II**).
3. To investigate the non-leguminous symbiosis; Endophytic bradyrhizobia (**I, VI)**.
4. To apply the knowledge for the non-leguminous species such as rice and inoculant production for sustainable agriculture (**V, III**)

Chapter 1: General Introduction

1-1 Diversity of rhizobia nodulating endemic legumes in Southeast Asia

Rhizobia are soil bacteria having capability to form specialized nodules on the roots of legumes and fix atmospheric nitrogen in exchange for carbon from the host plant. The Fabaceae (Leguminosae) family consists of over 19,000 species under three subfamilies, the Papilionoideae, the Mimosoideae and the polyphyletic Caesalpinioideae (Lewis *et al.*, 2005). Of these, only 22% of Caesalpinioideae species have been found to be nodulated by rhizobia, versus 96% in the Mimosoideae and 96% in the Papilionoideae (Sprent, 2009). There have been intensive efforts to determine the full range of legumes that can be nodulated, especially in the tropical region, where legume diversity is very high (Sprent, 2009; Doyle, 2011). The recent exploration of tropical wild legume species (tress and shrubs) has been evidenced as unexpected diversity of symbionts, resulting in the description of numerous new species of rhizobia. For examples, Yao *et al.* (2002) reported the novel bradyrhizobium, *Bradyrhizobium yuanmingense* sp. Nov, isolated from the nodule of *Lespedeza* in China (Yao *et al.*, 2002). Whereas the majority of legumes form symbioses with members of genus *Rhizobium* which relatives in class α-proteobacteria. Moreover, nodulation of *Mimosa* by β-proteobacteria was also found, for example, *Cupriavidus taiwanensis* is generally isolated from nodules on the pan-tropical weeds *M. pudica* and *M. diplotricha* in Taiwan (Chen *et al.*, 2001; Chen *et al.*, 2003), India (Verma *et al.*, 2004) and China (Liu *et al.*, 2011). In addition, *Burkholderia* spp. such as *B. mimosarum* and *B. phymatum* are also often isolated from these two Mimosa species as well as from other species as *M. pigra*, which is a hugely aggressive invader in South East Asia (Chen *et al.*, 2005; Liu *et al.*, 2011) and Australia (Parker *et al.*, 2007), and is rated in the top 10 world's worst weeds (Lowe *et al.*, 2000). With further regard to

β-rhizobia from southeast Asia, it is interesting to note that Trinick and Galbraith (1980) isolated many strains of so-called *Rhizobium* from invasive *Mimosa* spp. in Papua New Guinea in the early 1960s, and many of these isolates were later identified as *Burkholderia* and *Cupriavidus* spp. (Elliott *et al.*, 2007; Elliott *et al.*, 2009). In addition, the well known non-legume *Parasponia andersonii* was originated from Indonesia, Malaysia and Papua New Guinea is nodulated by *Sinorhizobium* sp. NGR 234 (Pueppke and Broughton, 1999).

1-2 Endemic legumes in Thailand: *Aeschynomene americana*

In Thailand, rhizobial symbioses with some native tree legumes such as *Acacia auriculiformis* Cunn, *A. mangium* Willd, *Millettia leucantha* Kurz, *Pterocarpus indicus* Willd and *Xylia xylocarpa* Tau were elucidated. The results of almost completed 16S rRNA sequences indicated that the most of the selected strains most likely belong to *Bradyrhizobium elkanii* and *Bradyrhizobium* sp. (Manassila *et al.*, 2007). In addition, the partial sequences of the 16S rDNA of the bacteria nodulating medicinal legume *Indigofera tinctoria* strains revealed that the microsymbionts of the plant species were related to members of five distinct genera: *Rhizobium, Sinorhizobium*, *Bradyrhizobium*, *Cupriavidus* and *Pseudoalteromonas* (Leelahawonge et al., 2010*)*.

The legume in genus *Aeschynomene* comprises about 175 to 180 species, distributed throughout the tropical and subtropical regions of the world (Queiroz and Cardoso, 2008). Half of them were described from the New World, mainly South and Central America, and the other half have been found across the tropical regions of Africa, Southeast Asia, Australia and the Pacific Islands (Rudd, 1955; Verdcourt, 1971). Half of the species were hydrophytes growing in marshes, temporary or permanent ponds, rice

fields, waterlogged meadows, and along streams and riverbanks. The remaining species were more xeric and were found in savannas or dry forests (Chaintreuil *et al.*, 2013).

Taxonomically, *Aeschynomene* belongs to the dalbergioid clade, which is in subfamily Papilionoideae of the Fabaceae (Lavin *et al.*, 2001). Dalbergioid legumes, a large, mostly pantropical, group of papilionoids characterized by the presence of the Aeschynomeneae type of root nodule (Lavin *et al.*, 2001; Sprent and Gardens, 2001). The infection process in *Aeschynomene* species is rather primitive, as it occurs via "crack entry" by intercellular infection of epidermal fissures generated by the emergence of lateral roots (Alazard and Duhoux, 1990; Bonaldi *et al.*, 2011). Infection threads are never formed, and infection cells are not interspersed with uninfected ones (Sprent, 2007). In Thailand, *A. americana* has been used for forage legume and green manures (Santipracha *et al.*, 1987; Prachunkanchana *et al.*, 1993). Actually, *A. americana* is the leguminous weed grown in rice field for only four months a year (during July until December). Our previous study indicated that the bacterial strains from its nodules were assigned to the genus *Bradyrhizobium.* Interestingly, the bradyrhizobial strains nodulating *A. americana* have never been detected in the paddy soil. Thus, the bradyrhizobial strains nodulating *A. americana* can survive during without genuine host and reform nodules in the following year. They may proliferate their cells in rice tissues even though *A. americana* cannot grow well each year during December until July the following year (tentatively eight months). Therefore, the isolated strain from this plant should be very intriguing to investigate for further understand their genetic and functional diversity.

1-3 Genotypic diversity and phylogeny of *Aeschynomene* symbionts

Aeschynomene species establish a symbiotic relationship with bacteria of the genus *Bradyrhizobium.* Three cross-inoculation (CI) groups among *Aeschynomene*

species have been described according to their symbiotic bacteria (Lavin *et al.*, 2001; Miché *et al.*, 2010). Members of CI group 1, such as *A. americana* and *A. elaphroxylon*, are nodulated only on their roots, by non-photosynthetic bradyrhizobia. Members of CI group 2, such as *A. afraspera* and *A. nilotica*, are nodulated on their roots and stems, by both non-photosynthetic and photosynthetic bradyrhizobia. CI group 3 species, such as *A. indica* and *A. sensitiva*, are nodulated on their roots and stems by photosynthetic bradyrhizobia. CI group 3 species are nodulated by *Bradyrhizobium* sp. strains BTAi1 and ORS278 in a Nod factor–independent manner (Miché *et al.*, 2010). Chaintreuil *et al.* (2013) conducted a molecular phylogenetic analysis of 38 different *Aeschynomene* species. The phylogeny was reconstructed with both the chloroplast DNA *trn*L intron and the nuclear ribosomal DNA ITS/5.8S region. From the maximum likelihood analysis, four groups of *Aeschynomene* were demonstrated. All *Aeschynomene* species using a Nod-independent symbiotic process clustered in the same clade (Chaintreuil *et al.*, 2013).

1-4 Non-leguminous symbiosis; Endophytic bradyrhizobia

Nitrogen-fixing bacteria that contribute to plant growth stimulation or to disease prevention and suppression are referred to as plant growth-promoting rhizobacteria (PGPR). In non-legume agrosystems, agriculturally important grasses such as sugar cane, wheat, sorghum, and maize contain numerous diazotrophic bacteria, such as *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp., and *Azospirillum* spp.

The plant infection and colonization by bradyrhizobia are not only found in leguminous plants but also in non-leguminous species such as rice (Chaintreuil *et al.*, 2000; Okubo *et al.*, 2013). Rice is the most important food crop in Asia. The high-yielding rice production requires huge amounts of nitrogen fertilizers. The biological nitrogen fixation (BNF) from rice root-associated bacteria has a great potential to

improve the sustainable rice production. Bradyrhizobial *A. indica* symbionts are reported to be nitrogen-fixing rice endophytes in many studies (Chaintreuil *et al.*, 2000; Mano and Morisaki, 2008; Kaga *et al.*, 2009). Nitrogen-fixing activity was detected in African wild rice plants (*Oryza breviligulata*) inoculated with *Bradyrhizobium* sp. ORS278, and the inoculated rice showed a very high growth promotion effect, giving 20% increases in both shoot and grain yields compared with a control (Chaintreuil *et al.*, 2000).

1-5 Applications

Bradyrhizobium plays a special role in the nitrogen cycle of agroecosystems by infecting the roots of soybean (*Glycine max*) and forming dinitrogen-fixing nodules. In this way, significant amounts of nitrogen are fixed and transferred to the plant, reducing the need for nitrogen fertilizer. However, there are a number of factors, which impede the nodulation on soybean root. Furthermore, the inoculation dose is an important factor in agricultural application of microbial inoculants. In addition, enhancement of legume nitrogen fixation by coinoculation of rhizobia with some plant growth promoting rhizobacteria (PGPR) is an alternative approach to improve the nitrogen availability in sustainable agricultural production systems. Some PGPR strains are capable of promoting growth of leguminous plants, nodulation, and nitrogen fixation when coinoculated with rhizobia. Examples of these are *Azospirillum* (Aung *et al.*, 2013), *Azotobacter* (Wu *et al.*, 2012), *Bacillus* (Atieno *et al.*, 2012), *Serratia* (Zahir *et al.*, 2011), and *Streptomyces* (Tokala *et al.*, 2002).

Using rhizobial inoculant is a clean technology for sustainable agriculture. Rhizobial inoculants have been used as an environmental friendly source of nitrogen fertilizer for several decades to reduce putting chemical nitrogen fertilizer into the soil, as well as the cost of legume production. However, the quality of inoculant is varied

according to the physicochemical and biological properties of material as well as the sterilization method applied to carrier (Khavazi *et al.*, 2007; Swelim *et al.*, 2010). Since contaminant microorganism is the main problem that affects the quality and shelf-life of rhizobial inoculant, sterilized carrier is necessary to be accomplished prior injection of the pure culture of rhizobia into carrier.

Chapter 2: The diversity of bradyrhizobium strains isolated from root nodules of endemic legume; *Aeschynomene americana* L. in Thailand

2-1 Introduction

Aeschynomene establishes symbiotic relationship with bacteria belonging to the genus *Bradyrhizobium* and the nitrogen-fixing nodules are formed on roots and/or stems (Molouba *et al.*, 1999; Nzoué *et al.*, 2009). Three cross-inoculation (CI) groups among *Aeschynomene* species have been described according to their symbiotic bacteria (Miché *et al.*, 2010). CI-group 1, such as *A. americana* and *A. elaphroxylon*, are nodulated only on their roots by non-photosynthetic bradyrhizobia. CI-group 2, such as *A. afraspera* and *A. nilotica*, and CI-group 3, such as *A. indica* and *A. sensitiva*, are nodulated on their roots and stems. CI-group 2 is nodulated by both non-photosynthetic and photosynthetic bradyrhizobia which are also able to nodulate CI-group 3 (Miché *et al.*, 2010). However, CI-group 3 is only nodulated by photosynthetic bradyrhizobia, such as *Bradyrhizobium* sp. BTAi1 and ORS278.

Phylogenetic analyses indicated that the bacterial symbionts associated to CI-group 3 *Aeschynomene* belonged to the genus *Bradyrhizobium* but form a separate sub-branch distinct from the non-photosynthetic species, *B. japonicum* and *B. elkanii* (Molouba *et al.*, 1999). Multilocus sequence analyses (MLSA) of a number of housekeeping genes were used to study the evolutionary relationships and delineate species in the genus *Bradyrhizobium* (Vinuesa *et al.*, 2005; Nzoué *et al.*, 2009; Rivas *et al.*, 2009). Three housekeeping genes, *dnaK*, *glnB* and *recA* produced a well-revolved phylogeny of photosynthetic bradyrhizobia. These genes have been suggested to use for MLSA analyses in *Bradyrhizobium* genus. However, only little information of the non-photosynthetic bradyrhizobia isolated from *Aeschynomene* in CI-group 1 was obtained

(Molouba *et al.*, 1999; Nzoué *et al.*, 2009). Therefore, in this study, phylogenetic analyses of 16S rRNA gene and combination of 3 housekeeping genes, *dnaK*, *glnB* and *recA*, were used to elucidate the taxonomic relationships of the *A. americana.* In addition, symbiotic genes based on southern blot hybridization and PCR amplification of symbiotic genes were determinated.

2-2 Materials and Methods

2-2-1 Soil samples, collection sites, and Bradyrhizobium isolation and screening

Bradyrhizobial strains were isolated from nodules of *A. americana*, which is a local Thai variety confirmed by *matK* sequence. The *A. americana* was grown in soil collected from 11 rice field areas, where *A. americana* is found, in Thailand. These areas are located in different regions in the north and northeast of Thailand, including Phitsanulok (17° 31’ 4.77N, 100° 19’ 3.92E and 17° 31’ 31.82N, 100° 19’ 11.11E), Uttaradit (17° 18’ 7.97N, 100° 13’ 53.33E, 17° 47’ 33.99N, 100° 6’ 58.96E, 17° 47’ 48.81N, 100° 6’ 57.12E, and 17° 41’ 22.21N, 100° 8’ 34.04E), Chiangmai (19° 3’ 44.04N, 98° 56’ 26.53E and 19° 4’ 17.28N, 98° 56’ 13.93E), Lampang (17° 31’ 13.50N, 99° 11’ 6.34E), Lopburi (17° 22’ 8.99N, 99° 48’ 2.13E), and Nakhon Ratchasima (14° 22’ 29.58N, 101° 43’ 30.47E). After 45 days of growing, nodules were harvested, surface-sterilized by immersion in 70% ethanol for 30 s and then in 3% hydrogen peroxide for 3 min, and washed five times with sterilized water. Each nodule was crushed and then streaked and purified on yeast extract mannitol (YEM) agar (Somasegaran and Hoben, 1994) plates. These isolated strains were reinoculated onto the host plant under sterilized condition to verify their nodulation ability. The isolated strains showing different BOXAIR1-genomic patterns were selected for further study. BOXAIR1 fingerprints were obtained by using BOXA1R primer (5’-CTACGGCAAGGCGACGCTGAC-3’)

(Versalovic *et al.*, 1994).

2-2-2 Bacterial strains, plasmids, and growth conditions

In total, 40 isolated strains used in this study are listed in Table 1. Bradyrhizobial strains were cultured in HM medium (Cole and Elkan, 1973) for further analyses.

2-2-3 Phenotypic characterization

Colony color and morphology were observed during cultivation on agar medium. The production of photosynthetic pigment from the bradyrhizobial strains was observed on the HM agar medium. In order to detect bacterial chlorophyll, bacterial strains were grown aerobically at 28°C for 7 days under a 12/12 h light–dark cycle. Cell pellets were extracted in the dark with cold acetone–methanol (7:2 [v/v]) for 30 min (Lorquin *et al.*, 1997). Absorbance of the supernatant was observed at a wavelength range from 350 to 800 nm. The nitrogen-fixing activity of the bacterial cultures was examined by acetylene reduction assay as described previously (Prakamhang *et al.*, 2009). The reaction was carried out in a 21-ml test tube containing 7 ml of bacterial culture in LG medium (Turner and Gibson, 1980) supplemented with arabinose and 0.005% yeast extract, and incubated at 28°C for 5 days.

2-2-4 Plant test

Bradyrhizobial strains were grown for 5 days in HM broth. All plants were grown in a growth room with controlled environmental conditions of 25°C on a 12/12 h light/dark cycle. Seeds of peanut (*Arachis hypogaea*), mungbean (*Vigna radiata*), soybean (*Glycine max* cv. SJ5), siratro (*Macroptilium atropurpureum*), and *Sesbania rostrata* were sterilized as described (Somasegaran and Hoben, 1994). Seeds of *A. americana* (a local Thai variety), *A. afraspera* (provided by Prof. Eric Giraud), and *A. indica* (ecotype Tottori, Japan) were sterilized by incubation in concentrated sulfuric acid for 25 min. The seeds were washed with sterilized water, and then soaked in sterilized

water overnight at ambient temperature. All seeds were germinated on 0.8% water agar. Cross-nodulation experiments were performed in plastic pouches (Somasegaran and Hoben, 1994). Symbiotic abilities of bradyrhizobial strains were determined in Leonard jars containing sterilized vermiculite and inoculated with 1 ml of bacterial culture, equivalent to 10^7 cells. N-free Hoagland's solution (Hotter and Scott, 1991) was added to each jar as required. After 6 weeks, plants were harvested and the entire plant was used to analyze for nitrogenase activity by measurement of acetylene reducing activity (ARA) (Somasegaran and Hoben, 1994). After ARA assay, nodules were detached from the roots and the number of the nodule was scored. The root, shoot, and nodules dry weights were determined after drying at 70°C for 72 h.

2-2-5 DNA extraction, PCR amplification, and sequencecing (16S rRNA genes, house keeping genes, nodulation genes)

Bradyrhizobial genomic DNA was prepared as described previously (Manassila *et al.*, 2007). Primers used are listed in Table 2. The thermal cycler was programmed as follows: an initial denaturation at 95ºC for 5 min; 35 cycles of denaturation at 94ºC for 1 min, annealing at appropriated temperature (see Table 2) for 45 s, and extension at 72ºC for 1 min; and a final extension at 72ºC for 10 min. Gene fragments were amplified using the Go Taq® Flexi DNA polymerase Kit (Promega, Germany). The PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Germany) and sequenced using the same primers as for the PCR. DNA sequencing was carried out by Macrogen, Inc. (Seoul, Korea).

2-2-6 Southern blot hybridization of nodulation genes

The nodulation gene probes were obtained through a PCR amplification using genomic DNA of bradyrhizobial strains as a template. These bradyrhizobial strains included *B. japonicum* USDA110, *Bradyrhizobium* sp. ORS285, the isolated strain

SUTN6-2 (99% identity to *B. yuanmingense* based on 16S rRNA gene sequence, JN578798), and the isolated strain SUTN7-2 (99% dentity to *B. canariense* based on 16S rRNA gene sequence, JN578800). The strains SUTN6-2 and SUTN7-2 were used as representatives of *B. yuanmingense* and *B. canariense*, respectively. DNA fragments of *nodA*, *nodB*, and *nodC* of *B. japonicum* USDA110 were amplified from the primer pairs nodA28/nodA627, nodB26/nodB625, and nodCF/nodCI (Table 2), respectively. DNA fragments of *nodA*, *nodB*, and *nodC* of the isolated strains SUTN6-2 and SUTN7-2 were amplified from the primer pairs nodAYF46/nodAYR595, nodB26/nodB625, and nodCF/nodCI (Table 2), respectively. Each *nod* gene probe was verified by DNA sequencing. The *nodA* probe obtained from *B. japonicum* USDA110 (600 bp) shared 100% to that of *B. japonicum* USDA110, from the strain SUTN6-2 (550 bp) shared 95% identity to that of *B. yuanmingense*, and from the strain SUTN7-2 shared 93-95% identity to that of various strains of *Bradyrhizobium* sp. The *nodB* probe obtained from *B. japonicum* USDA110 (600 bp) shared 100% to that of *B. japonicum* USDA110, from the strain SUTN6-2 (530 bp) shared 95% identity to that of *B. yuanmingense,* and from the strain SUTN7-2 (530 bp) shared 87% identity to that of *B. yuanmingense*. The *nodC* probe (1,000 bp) obtained from *B. japonicum* USDA110 shared 100% identity to that of *B. japonicum* USDA110, from the strain SUTN6-2 shared 93% identity to that of *B. yuanmingense*, and from the strain SUTN7-2 shared 96% identity to that of *B. canariense*. DNA probes were labeled overnight by random priming and hybridized using DIG High Prime DNA Labeling and Detection Starter Kit I (Roche). Bradyrhizobial genomic DNAs were digested with the *Eco*RI and separated in a 1% agarose gel. Southern blotting was carried out by capillary transfer to a Hybond-N+ nylon membrane (Amersham, Cardiff, UK), as described previously (Sambrook *et al.*, 1989). The membranes were hybridized at 44°C for 18 h. Sequentially, membranes were twice washed in 2X SSC-0.1% SDS at

25°C for 5 min and in 0.5X SSC-0.1% SDS at 65°C for 15 min. For low stringent condition, the membranes were hybridized at 40°C for 18 h, and then twice washed in 2X SSC-0.1% SDS at 25°C for 5 min and in 0.5X SSC-0.1% SDS at 60°C for 15 min.

2-2-7 Phylogenetic analysis

DNA sequences of gene fragments generated in this study were subjected to the algorithm BLASTN to identify the most similar sequences available in the database. DNA sequences of each gene from relative strains in the family *Bradyrhizobiaceae*, of other rhizobia and of outgroups were obtained from the GenBank database. Multiple alignments were performed with MUSCLE from phylogeny.fr (Dereeper *et al.*, 2008). Unaligned regions and gaps were excluded from the analyses. In total, 1351, 677, 458, and 587, nucleotide positions were used for the phylogenetic analyses of 16S rDNA, *dnaK*, *glnB*, and *recA*, respectively. Phylogenetic trees were reconstructed by the maximum likelihood using the PhyML (Guindon and Gascuel, 2003) and confidence levels were estimated for 1000 replicates. In comparison, phylogenetic trees were also reconstructed by the distance neighbor-joining method (Saitou and Nei, 1987) using the MEGA 4.1 package (Kumar *et al.*, 2008).

2-2-8 Statistical analysis

The data were subjected to analysis of variance (ANOVA) using the program SPSS version 11.5. Duncan's multiple range tests was used to identify differences between means at $p \leq 0.05$.

2-3 Results

2-3-1 Bacterial isolation and phenotypic characteristics

After nodule isolation and re-nodulation of *A. americana* a total of 246 bacterial strains were obtained. Identical BOXAIRI profiles were found with the isolated strains from different geographic origins. Thus, the 40 isolated strains showing different BOXAIR1 fingerprints (data not shown) were chosen for further study.

Most of the strains formed 1.5-2 mm diameter colonies within 4-5 days of incubation, when grown on HM medium. Photosynthetic pigment was neither observed from growing on plate culture nor from extracting of broth culture with acetone–methanol although, the peaks of bacterial chlorophyll (780 nm) and carotenoids (460-490 nm) of photosynthetic *Bradyrhizobium* sp. BTAi1 and ORS278 (positive control) could be detected.

2-3-2 Phylogenetic analysis of 16S rRNA gene

The 40 *A. americana* representative strains were selected for determination of the sequences of 16S rRNA gene. Sequences from several reference strains were added, which consisted of different *Bradyrhizobium* species as well as *Rhodopseudomonas palustris*. *Mesorhizobium loti* MAFF303099 was chosen as an outgroup strain to root the tree. On the basis of 16S rRNA gene sequence similarity, there are 2 major phylogenetic lineages including a lineage 1 of *B. elkanii* and a lineage 2 where other species of *Bradyrhizobium* and photosynthetic bradyrhizobia were included, with a strong bootstrap support (98%) (Fig.1). All of the isolated strains belonged to the lineage 2 including various *Bradyrhizobium* species; *B. yuanmingense, B. japonicum*, *B. liaoningense*, *B. canariense*, and also *R. palustris*. In the lineage 2, photosynthetic bradyrhizobial strains formed a separated branch with a strong bootstrap support (98%), supporting that the isolated strains were non-photosynthetic *Bradyrhizobium*.

2-3-3 Cross nodulation test

Nodulation test on the original host *A. americana* indicated that most of the isolated strains could produce up to 100 nodules per plant after 45 days of inoculation. As well as nodulating their original host, all isolated strains could efficiently nodulate *A. hypogaea*, *V. radiata*, and *M. atropurpureum,* but they failed to nodulate *S. rosrtata* and *G. max* (Table 3). *B. japonicum* USDA110 had ability to form root nodules on all tested plants, except on *A. americana* and *S. rostrata*.

2-3-4 Presence of nodulation genes

Southern blots of *Eco*RI-digested DNA derived from the isolated strains were hybridized to probes of *nodA*, *nodB*, and *nodC* from different bradyrhizobial strains. The Southern blot hybridization results are shown in Table 3 (see Fig. S1 in the supplemental material). Based on Southern blot hybridization results, the isolated strains were divided into 2 groups, *nod*-containing strains and divergent *nod*-containing strains. From 40 strains, only 18 strains, showing signals of *nodA*, *nodB*, and *nodC* after hybridization to the probes obtained from *B. japonicum* USDA110, were called "*nod*-containing strains". On the other hand, the remaining 22 strains failed to hybridize all *nod*-gene probes from *B. japonicum* USDA110 under stringent conditions, except the strains SUTN1-8, SUTN1-12, SUTN2-1, SUTN2-3, and SUTN4-3 that showed signals of some *nod* genes. However, under low stringent conditions, the remaining 22 strains showed weak signals of *nodC* after hybridization to probes obtained from *B. japonicum* USDA110, and of *nodA* and *nodB* after hybridization to probes obtained from the other bradyrhizobial strains (SUTN6-2 and SUTN7-2). Therefore, these strains were called "divergent *nod*-containing strains".

2-3-5 Phylogenetic analyses of *dnaK*, *recA*, and *glnB*

The phylogenetic trees based on sequences of *dnaK*, *recA*, and *glnB* were

constructed, in order to determine the taxonomic position of the isolated strains. Taxonomic positions of the isolated strains in the combination tree (Fig. 2) were almost similar with their taxonomic positions in the 16S rRNA gene tree (Fig. 1). In the combination tree, the isolated strains were clustered with *B. yuanmingense* and *B. japonicum* and divided from the group of *B. elkanii* and CI-group 1 strains (ORS301 and ORS304) with 98% bootstrap support. Further, the non-photosynthetic bradyrhizobia isolated from CI-group 1 were clearly distinguished from the bradyrhizobia isolated from CI-group 2 and 3 with 99% bootstrap support. In addition, in *B. yuanmingense* cluster, the number of divergent *nod*-containing strains formed a separate sub-cluster distinct from the majority of *nod*-containing strains, except the strains SUTN6-2, SUTN8-1, and SUTN8-2. In *B. yuanmingense* cluster, the combination of *dnaK+recA+glnB* sequence analyses indicated that the *nod*-containing strains shared 98-99% sequence identities to that of *B. yuanmingense* but the divergent *nod*-containing strains shared only 96-97% sequence identities to that of *B. yuanmingense*.

2-4 Discussion

Bradyrhizobia have been described in the nodules of *Aeschynomene* (So *et al.*, 1994) and most studies have focused on photosynthetic bradyrhizobial strains nodulating on *Aeschynomene* that belong to the CI-group 2 and CI-group 3 (Miché *et al.*, 2010). Extensive studies have been done for photosynthetic *Bradyrhizobium* sp. strains BTAi1 and ORS278, which nodulate *A. indica* and *A. sensitiva* belonging to CI-group 3. These strains lack canonical classical nodulation genes, so they are called "*nod*-independent strains" (Miché *et al.*, 2010). In this study, we focused on *A. americana*, which is a species belonging to CI group 1. We found that all of the strains isolated from *A. americana* belonged to the genus *Bradyrhizobium*, based on their molecular

characterization. *B. yuanmingense* was found to be the dominant species and some of the other species including *B. japonicum*, *B. liaoningense*, and *B. canariense* were the minor species. The bradyrhizobial strains isolated from *A. americana* were non-photosynthetic bacteria, thus they were distinguished from the photosynthetic strains nodulating the other *Aeschynomene* species belonging to the CI-group 2 and CI-group 3 (Miché *et al.*, 2010). Morover, all the strains isolated from *A. americana* were able to nodulate *M. atropurpureum*, *A. hypogaea*, and *V. radiata,* and some strains were able to nodulate *A. afraspera* (e.g. DOA9 and SUTN9-2) and *A. indica* (e.g. SUTN9-2). On the other hand, the photosynthetic strains BTAi1 and ORS278 were more specific with a narrow host-range; they were not able to nodulate *A. americana* (Molouba *et al.*, 1999). These results encourage the occurrence of non-specific and specific bradyrhizobia among *Aeschynomene* symbionts with the photosynthetic strains being highly specific, and the *A. americana* are commonly nodulated by *Bradyrhizobium* spp. of the cowpea-miscellaneous group (van Berkum *et al.*, 1995).

From the results of the canonical nodulation genes (*nodA*, *nodB*, and *nodC*), the isolated strains were separated into 2 groups, which were *nod*-containing strains and divergent *nod*-containing strains. The strains in both groups comprised various nodulation gene patterns based on a size of each hybridized signal (Fig. S1). This result indicated the genomic variation of these strains. Taxonomic positions of the divergent *nod*-containing strains in the housekeeping gene trees were congruent with their positions in the 16S rRNA gene tree, pointing out that the divergent *nod*-containing strains were closely related to *B. yuanmingense.*

Moreover, we found that the *A. americana* strains (SUTN9-2) could nodulate all CI-group representatives of *Aeschynomene* but the ORS285 could nodulate only the CI-group 2 and 3. This might be elucidated that the *nod* genes of the *A. americana* strains

broadened their host range. It was supported by the similar sequences of *nodA* and *nodB* with non-photosynthetic bradyrhizobial species, separating from the photosynthetic strains belonging to the CI-group 2. Alternatively, it was possible that the *A. americana* strains use another simple mode for invasion of the *Aeschynomene* species. For example, the invasion might involve direct invasion at the site of emerging lateral roots, and other signals (such as cytokinin) instead of the Nod-factor might play a role in triggering infection and nodule formation (Alazard and Duhoux, 1990; Boiero *et al.*, 2007; Giraud *et al.*, 2007). We found that the divergent *nod*-containing strain DOA9 could infect both *A. afraspera* and *A. indica* but could only establish nodule only of *A. afraspera.* Possibly, the divergence of the divergent *nod* genes might impair the nodulation ability to *A. indica*. Altogether from these results, gene loss and lateral transfer of nodulation genes might explain this finding. Moreover, the role of nodulation genes of the *A. americana* strains in the infection and nodulation of *Aeschynomene* species is still unclear. Therefore, it would be interesting to determine the role of the divergent *nod* gene and Nod-factor produced from the divergent *nod* gene products in nodulation ability.

This is the first description of the diversity of the non-photosynthetic bradyrhizobial strains nodulating the CI-group 1. Further more, we found that the canonical *nodABC* of the *nod*-containing strains differed from those of the other bradyrhizobia, and those of the divergent the *nod*-containing strains were more diverse. We hypothesized that each *nod* gene might diverge from various origins.

Chapter 3: Unusual symbiotic genes; Divergent *nod*-containing *Bradyrhizobium* sp. DOA9 with a megaplasmid and its host range

3-1 Introduction

A. americana belongs to CI group 1 are nodulated only on their roots, by non-photosynthetic bradyrhizobia. From the chapter 2, however, *A. indica* (CI group 3) was found to be nodulated by a non-photosynthetic strain isolated from *A. americana* (CI group 1). This strain could nodulate several CI groups of *Aeschynomene*, as well as peanut and mung bean (Noisangiam *et al.*, 2012).

Bradyrhizobium sp. DOA9 (Alphaproteobacteria) was isolated from root nodules of *A. americana* in Thailand. This strain was assigned to *B. yuanmingense* from phenotypic characteristics and multilocus sequences analysis of the 16S rRNA gene and house keeping genes (*dnaK*, *recA*, and *glnB*). DOA9 was called a divergent *nod*-containing strain base on Southern blot hybridization with *nod* gene (*nodA, B, C*) probes. In addition, on the basis of *nifH* sequence similarity, DOA9 was placed in a cluster of non-photosynthetic bradyrhizobial strains that able to fix nitrogen in the free-living form. Moreover, it can nodulate a wide range of legumes, including *M. atropurpureum*, *A. hypogaea*, *V. radiata*, and *A. afraspera* (Noisangiam *et al.*, 2012). As little was known about this strain, we investigated root colonization, infection, and nodulation efficiency of DOA9 in several species in the legume subfamilies Papilionoideae and Mimosoideae, as well as rice. We also investigated bacteroids and nodule development. Because symbionts with a broad host range generally carry multiple replicons in their genome (Flores *et al.*, 1998; Schuldes *et al.*, 2012; Weidner *et al.*, 2012), we examined the replicon structure and the localization and copy number of symbiosis genes.

3-2 Materials and Methods

3-2-1 Bacterial strains and growth conditions

Bradyrhizobium sp. DOA9, *B. japonicum* USDA110, and *Mesorhizobium loti* MAFF303099 were cultured at 28 °C in HM medium supplemented with L-arabinose (Cole and Elkan, 1973). GUS-tagged DOA9 was cultured in HM supplemented with streptomycin (200 μg/mL) (Noisangiam *et al.*, 2012).

3-2-2 Plant growth and inoculation

Seeds of peanut (*Arachis hypogaea*), mung bean (*Vigna radiata*), soybean (*Glycine max* cv. SJ5), siratro (*Macroptilium atropurpureum*), *Sesbania rostrata*, lupin (*Lupinus polyphyllus*), *Desmodium* sp., alfalfa (*Medicago sativa*), *Medicago truncatula*, and *Acacia mangium* seeds were sterilized as described previously (Somasegaran and Hoben, 1994). *Aeschynomene americana* (a local Thai variety), *A. afraspera*, *A. evenia* (provided by Eric Giraud), and *A. indica* (ecotype Tottori, Japan; ecotype Tomeshi, Japan; and a local Thai variety) were sterilized by incubation in concentrated sulfuric acid for 30 min. Seeds of *Indigofera tinctoria*, *Lotus japonicus*, *Stylosanthes hamata*, *Crotalaria juncea*, *Lespedeza* sp., *Leucaena leucocephala*, *Mimosa pudica*, *Neptunia natans*, and *Samanea saman* were sterilized by incubation in concentrated sulfuric acid for 10 min. Seeds of rice (*Oryza sativa* ssp. *indica*) were sterilized as described previously (Miché and Balandreau, 2001). All seeds were washed with sterilized water and then soaked in sterilized water overnight at ambient temperature. All seeds were germinated on sterilized 0.8% (w/v) water agar for 1 to 2 days at 28°C in the dark. The germinated seeds were transferred onto Hoagland's agar (Hotter and Scott, 1991). The *Bradyrhizobium* sp. DOA9 was washed with 0.85% NaCl, and the optical density at 600 nm (OD_{600}) was adjusted to 1 with sterilized water, corresponded to approximately 10^9 cells/mL. Then each seedling was inoculated with 100 μL of bacterial culture. All plants

were grown at 25°C under a 16h light/8h dark cycle at a light intensity of 639 μE m^{-2} s^{-1} for 7 to 14 days (Yuttavanichakul *et al.*, 2012). These samples were used for microscopic observations. Symbiotic abilities of DOA9 was determined in Leonard's jars containing sterilized vermiculite and inoculated with 1 mL of bacterial culture, as mention above. N-free Hoagland's solution was added to each jar as required. After 35 days, plants were harvested and the plant was used for analysis of nitrogenase activity by Acetylene Reduction Assay and the number of the nodule was scored. The plant dry weights were determined after drying at 70°C for 72 h.

3-2-3 Microscopic observation of root colonization and nodulation

Root colonization and nodulation were revealed by GUS staining. Samples inoculated with GUS-tagged DOA9 were immersed in GUS assay solution (40 mL 20 mg/mL X-Gluc in *N, N*-dimethyl-formamide, 20 mg SDS, 2 mL methanol, 0.2 mL 1 M sodium phosphate buffer, and 7.76 mL distilled water), in vacuum for 120 min and left in this solution for 12 h at 28°C. GUS staining was observed under a light microscope.

3-2-4 Differential interference contrast and fluorescence microscope observation of isolated bacteroids

Nodules were mashed in bacteroid extraction buffer (125 mM KCl, 50 mM Sodium succinate, 50 mM TES buffer, pH 7.0, with 1% (w/v) BSA) (McRae *et al.*, 1989). To remove the plant cell debris, the suspension was centrifuged at 100× *g* at 4°C for 10 min. To precipitate the bacteroids, the supernatant was centrifuged at 3000× *g* at 4°C for 10 min. The precipitate was observed by Differential Interference Contrast (DIC) microscopy. For fluorescence microscopy, the bacteroid fraction was first stained with 4′, 6-diamidino-2-phenylindole (DAPI; 50 μg/mL) and propidium iodide (PI; 2 μg/mL).

3-2-5 DNA extraction and megaplasmid detection

Bradyrhizobium sp. DOA9 total DNA was prepared as described previously (Manassila *et al.*, 2007). Megaplasmids were isolated by electrophoresis (Thomas *et al.*, 1994; Argandona *et al.*, 2003) with the modifications described here. Bacteria were cultured on HM broth medium (Somasegaran and Hoben, 1994) with 0.05% (w/v) L-arabinose, 0.05% (w/v) yeast extract for *B. japonicum* USDA110 and *M. loti* MAFF303099, and no L-arabinose for *Bradyrhizobium* strain DOA9, to reduce polysaccharide production. USDA110 and DOA9 were cultured at 30°C for 3 to 5 days on a rotary shaker at 200 rpm until the late-log phase was reached. Then 1% (v/v) of these pre-cultures were inoculated into new tubes containing HM broth medium. The cultures were incubated for 3 days until the exponential growth phase (the mid-log phase) was reached. MAFF303099 was incubated for 2 days and inoculated into a fresh tube containing HM medium and then cultured under the same conditions for 24–36 h. After that, the cell pellets were harvested by centrifugation at 3000×*g* for 10 min. The cells were resuspended in 0.85% (w/v) NaCl to OD_{600}=1. The cells were harvested from 1 mL of cell suspension and washed with M9 salts (Sambrook *et al.*, 1989) containing 0.5 M NaCl, and then with 1 mL 0.1% (w/v) Sarcosyl. The supernatant was removed immediately and the sediment was resuspended in 50 μL of lysis buffer (1 mg/mL lysozyme, 1 mg/mL RNase A [first dissolved in 0.4 M sodium acetate, pH 4.0, and then boiled for 10 min (Plazinski *et al.*, 1985)], 0.1% (w/v) bromophenol blue in Tris-borate buffer (pH 8.2, 89 mM Tris base, 12.5 mM disodium EDTA, and 8.9 mM boric acid) (Eckhardt, 1978), and 40% (v/v) glycerol).

Before sample loading, electrophoresis was performed in 0.7% agarose gel, which leveled off with 0.5× TBE buffer (4°C) until it touched the gel. The wells were then filled with 50 μL Sodium Dodecyl Sulfate (SDS; 10% w/v) mixed with xylene cyanol

(1mg/mL). The current was run for 10–15 min at 100 V with reversed polarity until the SDS was 1 cm above the wells. After that, 50 µL of sample mixed with lysis solution was directly loaded and left for 15 min before 15 µL of Protinase K (5 mg/mL) in 40% (w/v) glycerol was overlaid. The wells were sealed with melted agarose gel, and 0.5× TBE buffer was then added to cover the gel. After 1 h, electrophoresis was carried out in a cold chamber at 4°C. The current was run at 10 mA for 14 h and at 50 mA for a further 10 h. The DNA in the gel was stained for 30 min in ethidium bromide (0.5 µg/mL) and washed with distilled water before being viewed under UV light.

3-2-6 Southern blot hybridization

The megaplasmid and chromosomal DNAs separated in the gel were used for Southern blot hybridization of nodulation (*nod*) and nitrogen fixation (*nifH*) genes as described (Noisangiam *et al.*, 2012). In brief, probes for *nodA* (550 bp), *nodB* (530 bp), and *nodC* (1 kb) were obtained through PCR amplification using genomic DNA of *B. yuanmingense* SUTN6-2, *B. canariense* SUTN7-2, and *B. japonicum* USDA110, respectively. DNA fragments of the respective strains were amplified with the primer pairs nodAYF46/nodAYR595 (*nodA*), nodB26/nodB625 (*nodB*), and nodC195/nodCI (*nodC*) (Noisangiam *et al.*, 2012). The probe for *nifH* was derived from *B. yuanmingense* using nifHF/nifHI primer pairs (Laguerre *et al.*, 2001). DNA probes were labeled overnight at 37°C by random priming, and hybridized with the Digoxigenin (DIG) High Prime DNA Labeling and Detection Starter Kit I (Roche, Switzerland). The DNA was capillary-transferred to a Hybond-N^+ nylon membrane (Amersham, Cardiff, UK) as described previously (Sambrook *et al.*, 1989). Low-stringency conditions were used for hybridization: membranes were hybridized at 40°C (*nodA* and *nodC* genes) or 42°C (*nodB* and *nifH*) for 18 h and then washed twice in 2× SSC + 0.1% (w/v) SDS at 25°C for 15 min and in 0.5× SSC + 0.1% (w/v) SDS at 62°C for 15 min

DOA9 genomic DNA was digested with *Eco*RI, *Eco*RV, *Hind*III, or *Not*I for evaluating the copy number of *nodB, nodC* and *nifH*; with *Eco*RI, *Hind*III, *Bgl*II, or *Not*I for *nodA*. Fragments were separated in 1% (w/v) agarose gels before hybridization as described above.

3-3 Results

3-3-1 Nodulation of subfamily Papilionoideae

We tested 19 species and 3 variety ecotypes of *A. indica* (Table 4). DOA9 was able to nodulate the roots of 15 species. Seven of the tested species were effective in term of nitrogen fixing efficiency, as indicated by significantly increase in plant dry weight when inoculated with DOA9 (Stowers and Elkan, 1980) (Table 4 and Table S2 in the supplemental material). The ineffective symbiotic was found in DOA9 with *S. hamata*, *M. atropurpureum*, *Lepedeza* sp., *L. japonicus*, *L. leucocephala*, and *S. saman*. This strain indicated the colonization at the lateral root (Fig. 3 A-D), and also on the root surface (Fig. 3 E). In addition, the nodulations were related with the colonization sites (Fig. 3 F-I). All of nodules were determinate (Fig. 3 F-J). Thin section of nodules in this group showed achynomenoid types (Fig. 3 K-O) (Sprent, 2007). DOA9 nodulated both genistoids but induced effective nodules only on *C. juncea*. Among the dalbergioids, DOA9 induced effective nodules on *A. americana*, *A. afraspera*, and *A. hypogaea*. Nodules in this group are clearly of the aeschynomenoid type (Fig. 3 K-L), which are formed via the crack infection pathway (Fig. 3 F and G) (Sprent, 2007). DOA9 induced nodules on all millettioids tested except *G. max*. Among the two robinioids, it nodulated only *L. japonicus*, but the nodules were ineffective (see Fig. S2 and Table S2 in the supplemental material). However, DOA9 could not nodulate *S. rostrata*. Among the

Inverted Repeat–Lacking Clade (IRLC), it nodulated only *M. sativa*, but the nodules were ineffective.

3-3-2 Nodulation of subfamily Mimosoideae

We tested 4 species of subfamily Mimosoideae; *Mimosa pudica, Leucaena leucocephala*, *Neptunia natans* and *Samanea saman* (Table 4). DOA9 nodulated *L. leucocephala* and *S. saman*, but nodules were not effective.

3-3-3 Root colonization and infection of rice

DOA9 colonized the roots and infected the tissues of rice. The expression of GUS is indicated by blue staining (Fig. 4). At 1 day after inoculation, histochemical staining of β-galactosidase activity revealed strong colonization of the root cap (Fig. 4A). At 5 days, this strain colonized the entire root (Fig. 4B) and intercellular cell (Fig. 4C and D) as showed in blue. However, We did not measure ARA in rice and the efficiency could not be decided based on the plant growth. It should be further analyzed by Kjeldahl method for total nitrogen content compared between inoculated and un-inoculated rice.

3-3-4 Microscopic observation of isolated bacteroids

Bacteroids isolated from various species of Papilionoideae were indistinguishable from the free-living form (Figs. 5, and 6). The shape and size of bacteroids in this group were not different from free living (Fig. 5 A-E). However, bacteroids from *A. hypogaea* were spherical (Figs. 5F, 6B, 6F, 6J). The bacteroids isolated from the nodules of four of five Papilionoideae species were not swollen and were the same size (2–4 μm) as the free-living form (Fig. 5A–E). Furthermore, the PI stained only the bacteroids from *A. hypogaea* (Fig. 6J).

3-3-5 Megaplasmid detection and hybridization

We successfully extracted both chromosomal and megaplasmid DNAs. The megaplasmid size was estimated from the plasmid profile of *M. loti* MAFF303099, which

contains megaplasmids of 208 and 352 kb (Kaneko *et al.*, 2000) (Fig. 7A lane 1).

3-3-6 Copy number

By using the probe for *nodA*, at least two fragments were detected (Fig. 8A). This suggests that *nodA* on the megaplasmid is single or two copies. For *nodB* and *nodC*, single fragment was detected on each lanes suggesting that these two genes are single copy on the megaplasmid (Fig. 8B, and C). At least two fragments were detectable probed by *nifH*. The result supports that two *nifH* genes are separately located both on the megaplasmid and on the chromosome (Figs. 7E, and 8D).

3-4 Discussion

DOA9 was able to nodulated the roots of plant tested, which form via the crack infection pathway (Sprent, 2007). This route of infection bypasses some of the complex processes involved in infection via root hairs, which depends on the Nod-factor structure (Goormachtig *et al.*, 2004). However, DOA9 could not induce nodules on *G. ma*x, even though soybean-nodulating bacteria are distributed among three genera (Chen *et al.*, 1988; Chen *et al.*, 1995). Rhizobia so far known to nodulate millettioids are all classical rhizobia, mainly slow-growing ("brady-") type (Sprent, 2007). Interestingly, this strain could nodulate the model legume *L. japonicus*. The *L. japonicus* might have permitted intercellular infection of the cortex (Madsen *et al.*, 2010). However, DOA9 could not nodulate *S. rostrata*, even though *Sesbania* accepts infection via the crack entry mode (Goormachtig *et al.*, 2004). Among the Inverted Repeat–Lacking Clade (IRLC), DOA9 nodulated only *M. sativa*, but the nodules were ineffective. These tribes of the Papilionoideae are generally nodulated with fast-growing α-rhizobia (Alphaproteobacteria) and usually show a high degree of specificity between symbiotic partners (Sprent, 2007).

In a subfamily Mimosoideae, DOA9 nodulated *L. leucocephala* and *S. saman*, but nodules were not effective. This limited result might have been due to host range specificity, as species of the tribe Mimoseae may be nodulated by β-rhizobia (Betaproteobacteria) (Barrett and Parker, 2006; Elliott *et al.*, 2007).

Bradyrhizobium sp. DOA9 is able to nodulate several species of legumes (Table 4) (Noisangiam *et al.*, 2012). In addition, *A. americana* plants are commonly nodulated by *Bradyrhizobium* spp. of the cowpea miscellaneous group (van Berkum *et al.*, 1995). These facts highlight the broad host range character of these bacteria. DOA9 also nodulated many of the dalbergioids, millettioids, and robinoids. As DOA9 contains divergent *nod*-genes (Noisangiam *et al.*, 2012), these genes might facilitate the broad host range nodulation ability. Moreover, the ability of DOA9 to invade roots via the cracks through which lateral roots emerge (Noisangiam *et al.*, 2012) might allow it to infect a wide variety of legumes; we found no infection threads in any sample. Overall, then, DOA9 shows a broad host range.

DOA9 colonized the roots and infected the tissues of rice. From previous study photosynthetic *Bradyrhizobium* strains induce N_2-fixing nodules on stems and roots of the genus *Aeschynomene* (Molouba *et al.*, 1999), and also form a natural endophytic association with the wild rice species *Oryza breviligulata* (Chaintreuil *et al.*, 2000), which grows in association with several aquatic legumes. However, we have found no reports of non-photosynthetic bradyrhizobial strains in endophytic association with rice. So it is interesting that the non-photosynthetic DOA9 could colonize and infect rice. In Thailand, the semi-aquatic *A. americana* frequently grows in association with rice. It will be interesting to study the genes in DOA9 that are involved in rice infection, which might also play a role in the early interaction between *A. americana* and DOA9. Such a study

might reveal a common determinant of the symbiotic interaction between rhizobia and ancestor plants before the symbiotic relationship evolved.

Bacteroids isolated from various species of Papilionoideae were indistinguishable from the free-living form. The shape and size of bacteroids in this group were not different from free living. However, bacteroids from *A. hypogaea* were spherical. It is widely accepted that the size and shape of N_2-fixing bacteroids vary widely and are controlled by the legume host rather than by the rhizobial genotype (Oke and Long, 1999).

Furthermore, the PI stained only the bacteroids from *A. hypogaea*. In addition, when the bacteroids fraction prepared from the nodules of *A. hypogaea* was plated on an agar media, no colony appeared. Although further quantitative analyses will be required, this result suggests that the bacteroids in the nodules of *A. hypogaea* lost productivity. These characteristics, the absence of PI staining, and the ability to form colonies on agar plates place DOA9 in the group that continues to reproduce after leaving the nodules (Mergaert *et al.*, 2006). However, the bacteroids isolated from *A. hypogaea* were spherical and non-reproductive after leaving the nodules. As bacteroids morphology (which is determined by the host legume) is linked to reproductive viability (Mergaert *et al.*, 2006), the host species can have implications for rhizobial evolution (Oono *et al.*, 2010).

DOA9 revealed one megaplasmid, which was larger than 352 kb. Broad-host-range symbionts generally carry multiple replicons on their genome. *Sinorhizobium* sp. NGR234, which has an extremely broad host range, has three replicons: a symbiosis plasmid, a megaplasmid, and a chromosome (Flores *et al.*, 1998); *S. fredii* USDA257 has a chromosome and a plasmid (Schuldes *et al.*, 2012); *S. fredii* HH103 has one chromosome and five plasmids (Weidner *et al.*, 2012); DOA9 has a chromosome (cDOA9) and a megaplasmid (pDOA9) larger than 352 kb. Many natural isolates of

Rhizobium spp. carry a variety of large plasmids (MacLean *et al.*, 2007). Genes for a few functions have been found on these large plasmids, particularly including *nod* and *nif* genes of fast-growing rhizobia. In contrast, the published genomes of *Bradyrhizobium* spp. are composed of a chromosome only, except that *Bradyrhizobium* sp. BTAi1 also harbors a plasmid of 228 kb (Cytryn *et al.*, 2008). To date, there have been no published reports of symbiosis genes localized on plasmids of *Bradyrhizobium* (MacLean *et al.*, 2007). Remarkably, symbiotic islands of *B. japonicum* are located on chromosome, while symbiosis genes of *Sinorhizobium* sp. are located on plasmids (Galibert *et al.*, 2001). This characteristic may contribute to the broad host range of these rhizobia. It will be interesting to find out that symbiosis genes on plasmid have extent host range.

DOA9 has at least two copies of *nifH*, one on the megaplasmid and the other on the chromosome. Photosynthetic *Bradyrhizobium* strains ORS278 and BTAi1 have two copies of *nifH* (Nzoué *et al.*, 2009), and *B. japonicum* USDA110 has one copy (Kaneko *et al.*, 2002), in all cases on the chromosome. We have already sequenced *nodA* of DOA9 (Accession No. DF 820426.1), which was used as a probe for Southern blot analysis in this study, and the fragment contains no restriction sites of *Eco*RI, *Hin*dIII, *Bgl*II, or *Not*I. Therefore, the hybridization data probed by *nodA* suggests that DOA9 has at least two copies of *nodA* on the megaplasmid (Fig. 8). An increase in copy number of symbiotic region has been indicated to promote symbiotic properties such as nodule number, nitrogenase activity and plant growth. The inoculation of alfalfa with *S. meliloti* with a moderate increase in copy number of symbiotic region resulted in enhancement of plant growth (Castillo *et al.*, 1999).

Therefore, this is the first report of symbiosis genes of *Bradyrhizobium* sp. located on a megaplasmid. Detailed investigation of both replicons of DOA9 would be useful. In addition, studies of the host range of rhizobia have been focused on *Sinorhizobium* sp.

strain NGR234, *S. fredii* USDA257, and *S. fredii* HH103. The genome sequences of these three strains share a high degree of synteny (Schmeisser *et al.*, 2009; Schuldes *et al.*, 2012; Weidner *et al.*, 2012), then the host range of rhizobia might be correlated with the number of specialized protein secretion systems they carry (Schmeisser *et al.*, 2009). Therefore, it would be interesting to determine and compare the protein secretion systems of DOA9.

Since many of photosynthetic bradyrhizobial (PB) strains were found to be naturally endophytic with rice (Chaintreuil *et al.*, 2000), the general conclusion was that rice probably evolved approximately 120 million years before legumes (Allen, 2002; Lavin *et al.*, 2005). Thus, PB strains might also be the ancestor of non-photosynthetic bradyrhizobia (van Berkum *et al.*, 1995). In the present study, DOA9 shows characteristics of broad host range in legume and rice infection. Therefore, DOA9 might be at the interval of evolution toward the Nod factor-independent symbiotic system but later this strain might loss the photosynthetic activity. Moreover, DOA9 is a divergent Nod-containing strain rendering incomplete Nod factors production. This may also support the idea that DOA9 would associate with host plants under Nod-independent manner since and during its evolution. To understand clearly the evolution of this group, whole genome analysis, symbiotic genes comparison and disruption as well as protein secretion system and lipopolysaccharide structure should be required.

Chapter 4: Non-leguminous symbiosis; The communities of endophytic diazotropic bacteria in cultivated rice (*Oryza sativa* L)

4-1 Introduction

In non-legume agrosystems, agriculturally important grasses such as sugar cane, wheat, sorghum, and maize contain numerous diazotrophic bacteria, such as *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp., and *Azospirillum* spp. Among graminaceous plants, the capacity to support associated nitrogen fixation is varied. For example, sugarcane which has been known to support BNF was traditionally studied without large amounts of N-fertilizer application (Lima *et al.*, 1987). Endophytic diazotrophic bacteria do not usually cause disease symptoms in the plants with which they are associated. They are capable of invading inner tissues including xylem vessels, and of systemic spreading (James and Olivares, 1998). The emphasis in these studies has been on endophytic bacteria belonging to several groups, especially on searching for diazotrophs that are able to contribute to the nitrogen requirements of gramineous plants. However, their functions for the plants are still disputed. Cultivated rice (*Oryza sativa* L.) is the most important staple crop, and nitrogen is the most important input required for rice production. In order to make rice cultivation sustainable and less dependent on chemical nitrogen fertilizer, there is a need to use diazotrophic bacteria that can make biologically fixed nitrogen available for the growth of rice plants. From previous study, the isolation of presumptive endophytic diazotroph bacteria from rice has been reported. Most studies have been concerned with the role of inoculated endophytic diazotrophic strains such as *Pantoea* sp. and *Ochrobactrum* sp. in deep-water rice (Verma *et al.*, 2004), *Herbaspirillum* sp. B501 in wild rice (You *et al.*, 2005), and *Pantoea agglomerans* YS19 (Feng *et al.*, 2006) and *Azoarcus* sp. strain BH72 (Reinhold-Hurek *et al.*, 2006) in rice

generally. However, prior to introducing any selected strains, the behavior of native species inside rice tissue should be clearly demonstrated. While the search for natural association and endophytic interaction of diazotrophs with rice is considered very promising, it is currently extremely difficult to isolate the organisms, because they are varied, and so far virtually uncharacterized. Also, the microbial community in rice is inherently complex, and assessments performed with such a complex population do not always reveal its specific components. Furthermore, the community structure of the bacterial population, both culturable and unculturable strains, inside the rice should be considered especially in relation to the actual rice field soil. A study published by Minamisawa *et al.* (2004) reported the existence of anaerobic N_2-fixing consortia (ANFICOs) consisting of N_2-fixing clostridia and diverse non-diazotrophic bacteria in many gramineous plants. Their work indicates that clostridia should be candidates for diazotrophic endophytes in grasses, and also demonstrates a new principle in environmental microbiology, which is that consortium of bacteria, rather than monocultures, may be responsible for a particular activity within a very complex environment.

The objective of this study was to determine the diversity of endophytic diazotroph on the basis of a culturable-based approach. The community structure under different soil conditions was also studied by cultivation-independent methods.

4-2 Materials and Methods

4-2-1 Rice and soil preparation

The cultivated Jasmine rice (*Oryza sativa* cultivar KDML105) was obtained from the Rice Research Center, Department of Agriculture, Thailand. The rice seeds were surface sterilized with 70% ethanol for 1 min and shaken in 10% (w/v) NaOCl solution

for 30 min. Seeds were then washed three times with sterilized distilled water with shaking (15 min each). Surface sterilized seeds were gnotobiotically germinated on sterilized wet tissue paper. After 7 days, rice seedlings were transferred into cement pots (120 cm in diameter, 60 cm high) containing 3 types of soil in three replicate pots per type of soil: paddy soil mixture, paddy soil mixed with chemical fertilizer, and uncultivated forest soil. The paddy soil mixture was obtained from 7 different rice fields in Nakhon Ratchasima, Thailand. Soil samples were analyzed by the Department of Soil Science, Faculty of Agriculture, Kasetsart University, Thailand. The soil characteristics are presented in Table 5. In the paddy soil mixture with chemical fertilizer treatment, plants were fertilized according to local custom i.e. seven days after transplantation, 100 g pot^{-1} of N:P:K (16:20:0) were added, and after 15 and 50 days, 50 g pot^{-1} urea [N:P:K (46:0:0)] were applied. The plants were grown under outdoor conditions in and were watered twice a week. The plants were studied at 3 growth stages (seedling, vegetative, and reproductive). Three rice plants were sampled randomly from each pot (27 plants in total), and tissue samples of roots, stems and leaves were examined.

4-2-2 Isolation of culturable endophytic diazotrophic bacteria

For isolation and maintenance of endophytic diazotrophic bacteria, a modified version of RMR medium was prepared essentially as described by Elbeltagy *et al.* (2001), except that the rice shoot extract was not supplemented. Freshly collected plants from all treatments were carefully washed with tap water and separated into leaf, stem, and root parts. They were surface sterilized following the previous method (Miyamoto *et al.*, 2004). The surface-sterilized plant materials were mechanically macerated with 0.8% saline solution and quartz sand and then decimally diluted in 0.8% saline solution. The dilutions were used to spread on N-free RMR medium for bacterial enumeration and seeded to N-free RMR semisolid medium for determinations of the N_2-fixing bacteria.

The N-free RMR medium was aerobically grown for 3 days at 30 ^{o}C. The tubes containing N-free semisolid medium were monitored for growth in the form of subsurface pellicles for 5 d after inoculation. Growth-positive tubes were tested for nitrogen fixation ability on the basis of the acetylene reduction assay (ARA).

4-2-3 Physiological properties

4-2-3-1 Acetylene reduction assay (ARA)

The nitrogen-fixing activity of the bacterial cultures was examined by acetylene reduction assay in a 21-ml test tube containing 7 ml of RMR semisolid culture. Acetylene gas was injected into the head atmosphere of the test tubes at a final concentration of 5% (v/v) and incubated for 24 h at 30 ^{o}C (Elbeltagy *et al.*, 2001). The ethylene concentration was assayed on a gas chromatograph equipped with a flame ionization detector and PE-Alumina column, 50 m x 0.32 mm x 0.25 μm (PerkinElmer, USA).

4-2-3-2 IAA production assay

Production of indole acetic acid was colorimetrically determined by mixing 5 ml Salkowski reagent (0.01 M $FeCl_2$ in $HClO_4$) with 1 ml culture supernatant followed by monitoring the color changes (Costacurta *et al.*, 1998). Pure indole-3-acetic acid (Sigma, USA) was used as standard.

4-2-4 Analysis of community structure of rice endophytic bacteria

4-2-4-1 Extraction of endophytic bacterial DNA from rice tissue

Total DNA was extracted using a modified potassium acetate method (Dellaporta *et al.*, 1983). The sterilized rice samples were homogenized in liquid N_2 and transferred into sterilized 1.5-ml tube containing 720 μl pre-heated extraction buffer (100 mMTris HCl, 50 mM EDTA, 500 mM NaCl and 1.25% (w/v) SDS). The samples were mixed and then incubated at 65 ^{o}C for 15 min. Proteins were precipitated by adding 225 μl 5 M potassium acetate and incubated on ice for 20 min before decanting supernatant into a

new tube. The DNA was precipitated by adding 2/3 volume cold isopropanol, and any remaining inhibitors were removed by reprecipitating the DNA with 300 µl 70% cold ethanol. The DNA was resuspended in 50 µl TE buffer and stored at -20 ^{o}C.

4-2-4-2 PCR amplification of 16S rRNA gene and *nifH* gene fragment

Amplification of 16S rRNA gene was performed using universal primers PRBA338F and PRUN518R (Ovreas et al., 1997). The GC-clamps (5'-GCGCCGCCGCGCGCGGCGGG CGGGGCGGGGGCACGGGGGG-3') were added to the 5'end of PRBA338F. The *nif*H primers used in this study were originally developed by (Poly *et al.*, 2001) and (Roesch *et al.*, 2006). The first PCR was performed with the forward primer PolF and the reverse primer PolR. The amplification product was 317 bp. The second PCR was performed with the forward primer nifHFor containing the GC clamp (5'-CGCCCGCCGCGCCCCGCGCCGT CCCGCCGCCCCCGCCCGACCCGCC TGATCCTGCACGCCAAGG-3') and the reverse primer nifHRev. The presence of PCR products was determined on 1% agarose gels stained with ethidium bromide. PCR products were stored at -20 ^{o}C before DGGE analysis.

4-2-4-3 Analysis of PCR products by denaturing gradient gel electrophoresis (DGGE)

All reagents were prepared as described in the Bio Rad D Gen Instruction Manual and Applications Guide (Bio Rad, Hercules, CA). DGGE gels were run using a BIO RAD DCode^{TM} Universal Mutation Detection System (Bio Rad, Hercules, CA). Three hundred µl of the PCR product were pooled, precipitated and resuspended in 30 µl of buffer. Gels for DGGE were 10% polyacrylamide gel (40%-acrylamide and N,N-methylenebisacrylamide solution (37.5:1, v/v), 40% (v/v) formamide, 7 M urea and 1X TAE) containing a linear gradient of the denaturant concentration ranging from 40% to 60% with 1 mm thick. The denaturing gradient gel was run for 12 h at 60 ^{o}C and 120 V.

After completion of electrophoresis, the gels were stained in an ethidium bromide solution (0.5 mg/ml) and documented on Gel documentation and analysis (Ultra Violet Product, USA).

4-3 Results

4-3-1 Enumeration of rice endophytic diazotrophic bacteria

The size of the total viable endophyte population detected was in the range of 10^3–10^6 CFU g^{-1} of rice tissue (fresh weight) in surface sterilized leaves, stems, and roots of rice (Table 6). The endophytic bacterial population was significantly affected by the type of soil, the growth stage of rice, the rice part, and by the interaction of these three factors (Table 6). Irrespective of the growth stage of rice and type of soil, the majority of endophytic bacteria were recovered from roots (2×10^5–3×10^6 CFU g^{-1} fresh weight) as compared to other parts of rice plants. While the endophytic bacterial population density in stem samples was 4×10^3–$\times 10^5$ CFU g^{-1} fresh weight, the lowest population density was present in leaf tissue with 1×10^3–2.7×10^4 CFU g^{-1} fresh weight.

4-3-2 Nitrogen fixing ability of rice endophytic strains

The nitrogen fixing ability of bacterial isolates grown in tubes containing RMR semisolid medium was assessed on the basis of acetylene reduction assay (ARA). Only 75 isolates with an ARA activity above 10 nmol C_2H_4 h^{-1} $tube^{-1}$ were selected for further study. During purification of single isolates from each consortium, ARA from both the consortium and single isolate were determined. The root consortium showed nitrogenase activity in the range of 40–4000, the stem consortium 180–5800 and the leaf consortium 300–900 nmol C_2H_4 h^{-1} $tube^{-1}$. Among the 75 isolates, the isolate VNS3-1 which was isolated from stems of seedlings growing in unfertilized soil exhibited the highest

nitrogenase activity (5840.07 nmol C_2H_4 h^{-1} $tube^{-1}$) (Table 7). The lowest ARA activity occurred in the leaf consortium.

Some chosen isolates were identified at the species level based on the full sequence analysis of 16S rRNA gene (Table 7). The results showed that they are closely related to *Enterobacter dissolvens*, *Brevundimonas aurantiaca*, *Pantoea agglomerans, Pseudomonas* spp., and *Enterobacteriaceae bacterium*.

4-3-3 Analysis of community structure of rice endophytic bacteria by PCR-DGGE approach

The PCR-DGGE analysis incorporating 16S rDNA primer elucidated the structure of rice endophytic bacterial communities (Fig. 9). Almost all of the samples contained 2 major bands of DGGE-PCR products except from the reproductive stage of rice. In the seedling stage, the banding pattern showed only two identical major bands for samples from all types of soil. In the vegetative stage, the results were similar to those for the seedling stage except for roots. The DGGE-PCR products generated from root samples could not be detected clearly except in the samples from unfertilized plants, while the reproductive stage of rice seems to have a more distinctive microbial community structure than other stages, especially in plants grown in uncultivated soil. Sequences retrieved from the bands a–d (Fig. 9) were similar to some culturable strains. Band (a) showed highest similarity to *Enterobacter dissolvens*, band (b) to *Brevundimonas aurantiaca*, band (c) to *Pantoea agglomerans*, and band (d) to *Pseudomonas* sp.

4-3-4 Analysis of community structure of rice endophytic diazotrophic bacteria by PCR-DGGE

The *nifH* gene primer that is specific for diazotrophic species was used on the basis of nested PCR-DGGE (Fig. 10). Fertilizer amendment had an effect on the persistence of diazotrophs in roots at every growth stage.

In the vegetative stage, PCR-DGGE bands from stem samples could not be detected under any of the soil conditions examined. The *nifH* pattern in roots of this stage was similar to that in the seedling stage, but the *nifH* signature was more abundant in leaves of plants from unfertilized and uncultivated soils. Interestingly, the *nifH* signature in the reproductive stage had a different pattern from that of the seedling and the vegetative stages in leaf samples of all growth stage of rice and type of soil.

The endophytic population of diazotrophic bacteria did not change significantly in size during the different ontogenic stages of rice growth, nor was there any significant variation in the composition of the diazotrophic population over time. Plants from the fertilized and uncultivated soils had fewer *nifH* diversity patterns than those grown in the unfertilized paddy soil. No evidence of *nifH* diversity was found in roots of any plants from fertilized soil. Also, in the vegetative stage, *nifH* diversity was not found in stems under any conditions while it was found in leaves in every phase of growth.

4.4 Discussion

The population of viable endophytic diazotrophic bacteria in cultivated rice was affected by the type of soil, the growth stage of rice, and by the rice part. The single isolates from each diazotrophic consortium were shown to be capable of both the inhibition and promotion of N_2-fixation.

Endophytic bacteria in cultivated rice were found in greatest density in roots variation in density can be attributed to plant source, plant age, tissue type, time of sampling, and environment. Similar results were obtained by Zinniel *et al.* (2002). Generally, bacterial populations are larger in roots and decrease in the stems and leaves. The number of colonies of both *Gluconacetobacter diazotrophicus* and *Acetobacter peroxydans*, isolated from the surface sterilized roots and stems of rice varieties

cultivated in flooded fields in South India, was in the range of 10^2–10^3 CFU g^{-1} fresh weight of rice (Muthukumarasamy *et al.*, 2005). A similar study in South Korea indicates that *G. diazotrophicus* is naturally associated with the South Korean rice variety Hwsanbyeo in low numbers (10^4 CFU g^{-1} fresh tissue) (Muthukumarasamy *et al.*, 2007). Since nitrogen fixing ability could be detected in leaf consortia, it is not surprising that endophytic bacteria reside in the leaf or aerial parts of rice tissues, which are exposed to the air and to O_2 produced by photosynthesis. Sometimes, the plant-inhabiting endophytic bacteria probably proliferate in anoxic microzones produced by consortium or plant respiration, while they are under higher O_2 concentrations (Minamisawa *et al.*, 2004).

The mixed or consortium cultures exhibited the capability of N_2-fixation. This suggests the presence of nondiazotroph cultures, which facilitate N_2-fixation activity in consortium cultures. Similar results indicating the existence of anaerobic nitrogen-fixing consortia (ANFICOs) consisting of N_2-fixing clostridia and diverse nondiazotrophic bacteria in various nonleguminous plants were obtained by Minamisawa et al. (2004). They found that in a few ANFICOs, nondiazotrophic bacteria specifically induced nitrogen fixation by clostridia in mixed culture. This suggests that the presence of accompanying bacteria producing specific metabolites, as well as nondiazotrophic endophytes, induced/reduced N_2-fixation by the consortium.

Some of the more interesting culturable isolates were found to be closely related to *Enterobacter dissolvens*, *Brevundimonas aurantiaca*, *Pantoea agglomerans*, *Pseudomonas* spp., *Rheinheimera* sp., and *Enterobacteriaceae bacterium*. They were characterized as endophytic strains of wheat (Ruppel *et al.*, 1992), citrus plants (Araújo *et al.*, 2002), sugarcane (Loiret *et al.*, 2004), the rose wood tree (Rasolomampianina *et al.*, 2005), and rice (Xie *et al.*, 2006; Xie *et al.*, 2006).

From the results of genetic fingerprinting of endophytic bacterial communities (Fig. 9), it can be concluded that soil conditions have less effect than the stage of growth part of rice plants. However, some isolates such as those from leaves of all growth stages of plants grown in unfertilized soil, were not detected by the ARA procedure (Table 7), suggesting that these endophytic bacteria are nonculturable (or not yet cultured) endophytic bacteria from rice plants. The results from several bands matched with those from the bacterial isolates (culturable approach) and some bands did not match with any isolates.

Various studies have indicated that the abundance of total diazotrophs or of specific populations can be influenced by the amounts of organic matter (Limmer and Drake, 1998; Scott, 1998; Poly *et al.*, 2001; Green *et al.*, 2006). From our study, the differences among the bacterial density in the various types of soil may result from selection or adaptation of diazotrophs to distinct nitrogen environmental conditions. Moreover, the endophytic diazotrophic bacterial community in rice varied during plant development. Indeed, many of the endophytic bacteria were also present in leaves. Perhaps they may actually have been a net drain on the plant's resources.

In addition to fertilization with nitrogen, variations in the growth stage and part of rice plant and the environmental conditions caused large differences in the population structure of endophytic diazotrophs, as demonstrated in a culturable approach. Nitrogen fertilization has been reported as a leading repression factor of nitrogenase genes and inactivation of nitrogenase activity in most diazotrophic bacteria (Egener *et al.*, 1999; Fuentes-Ramirez *et al.*, 1999; Martin and Reinhold-Hurek, 2002).

It is widely recognized that endophytic diazotroph inoculum is capable of fixing N more efficiently than diazotrophs that remain in the rhizosphere or on the rhizoplane, this may be due to the fact that the plants directly provide the endophytic diazotroph bacteria

with their nutrient requirement so they do not need to compete with other soil microbes for scarce resources. In return for providing this niche, the bacteria provide fixed N and/or plant growth-promoting compounds to the host plant. In summary, there is reasonable evidence that these endophytic diazotrophic bacteria can be used as a biofertilizer for rice farming, thus providing an agricultural benefit which is of definite ecological and economic significance.

Chapter 5: Non-leguminous symbiosis; Preference of endophytic bradyrhizobia in different rice cultivars

5-1 Introduction

The plant infection and colonization by bradyrhizobia are not only found in leguminous plants but also in non-leguminous species such as rice (Chaintreuil *et al.*, 2000; Okubo *et al.*, 2013). Rice is the most important food crop in Asia. The high-yielding rice production requires huge amounts of nitrogen fertilizers. The biological nitrogen fixation (BNF) from rice root-associated bacteria has a great potential to improve the sustainable rice production. In addition, the rice-legumes rotational cropping systems are useful for rice production, since legumes can be planted after rice season and nitrogen can be provided from legumes to rice. Bradyrhizobia are well recognized for their ability to fix atmospheric dinitrogen into ammonia in the nodules of legumes, thus providing ammonia to host plants. The bradyrhizobia-legumes symbiosis has been reported to increase the legumes productivity for agricultural farms (Antoun *et al.*, 1998; Stephens and Rask, 2000; Abbasi *et al.*, 2008)

However, *Bradyrhizobium* has been well defined as the oligotrophic bacteria, which can survive under nutrients deprive condition (Crist *et al.*, 1984; Saito *et al.*, 1998). In addition, a renewed interesting endophytic diazotrophs in graminous plants have also arisen, because of their occurrence mainly within plant tissues. The endophytic diazotrophs actually performs plant growth promotion (Elbeltagy *et al.*, 2001). However, the relationships of endophytic *Bradyrhizobium* with rice cultivar and/or habitats have never been elucidated so far. Thus, the host preference for endophytic bradyrhizobia and rice growth promotion are the critical aspects to be determined before they were used in the fields.

To obtain the natural endophytic bradyrhizobia from rice, bradyrhizobial strains were isolated on the basis of the oligotrophic property. The siratro (*Macroptilium atropurpureum*) has been used as a standard host plant to screen the *nod*-containing *Bradyrhzobium* from homogenated rice tissues and soil samples (Lima *et al.*, 2009). A selective medium (BJSM) (Tong and Sadowsky, 1994) was also applied for *Bradyrhizobium* isolation.

5-2 Materials and Methods

5-2-1 Rice cultivars, soils sampling and endophytiv bradyrhizobial isolation

Seven different rice (*Oryza sativa* L.) cultivars and rice rhizospheric soil samples used in this study are listed in Table 8. Rice roots and paddy soils were collected and immediately transferred to the laboratory in polyethylene boxs at 4^{o}C to obtain endophytic bradyrhizobia. The roots and stems were chemically sterilized (3% sodium hypochlorite for 5 min followed by soaking in 70% ethanol for 5 min). The rice roots were thoroughly rinsed with sterilized distilled water at least five times and cut into 4 to 5-cm-long section. As a control to check superficial contamination for each individual plant, 200 μl of water from the final rinsed were spread on plate count agar (PCA) medium. No individual root samples resulting in contamination on PCA plates were retained for isolation of endophytic bradyrhizobia (Baldwin *et al.*, 1996). Sections were aseptically crushed by sterilized mortar and pestle containing 1 ml of sterilized water.

The strategies to obtain the endophytic bradyrhizobia were performed into three procedures (Fig. 11). Firstly, to obtain *nod* contained *Bradyrhizobium* strains, the 1 ml of roots homogenate solutions (as mentioned previously) was directly inoculated into germinated siratro (*M. atropurpureum*) seeds. Then, after 1 month, each nodule was crushed and streaked on arabinose-gluconate (AG) agar plates (Sadowsky *et al.*, 1987).

Secondly, the oligotrophic-based isolation was considered to reduce growth of other contaminants. The 1 ml of the roots homogenate was added into 50 ml of sterilized water for 1 month, and then 0.1 ml of solution was spread on BJSM plates. Finally, the 0.1 ml of roots homogenates was directly spread on BJSM plates (Fig. 11). In order to select different strains of bacteria, BOX-PCR was carried out for screening the redundant strains (Versalovic *et al.*, 1994). The bacterial strains showing the different BOX-PCR patterns were selected for bacterial identification by 16S rDNA sequencing (Table 9).

5-2-2 Phenotypic characteristics and nitrogen fixation assay

The color and morphology of bradyrhizobial colonies were observed during cultivation on HM agar medium (Cole and Elkan, 1973). In order to detect the photosynthetic pigments from the bradyrhizobial strains, they were grown aerobically at 30°C for 7 days under a 12-h-light/12-h-dark cycle. To determine bacterial pigment production, cell pellets were extracted in the dark with cold acetone-methanol (7:2 [vol/vol]) for 30 min (Lorquin *et al.*, 1997). Absorbance of the supernatant was observed at a wavelength range from 350 to 850 nm.

Acetylene reduction assay (ARA) was carried out to examine the nitrogen-fixing activity in free-living form of bradyrhizobial strains. The bacterial cultivation was done in a 21-ml test tube containing 7 ml of bacterial culture in HM medium, which was supplemented with L-arabinose and 0.005% yeast extract and incubated at 28°C for 5 days. The nitrogen-fixing activity of the bacterial cultures was examined by ARA as described previously (Prakamhang *et al.*, 2009).

5-2-3 The 16S rRNA, housekeeping genes and phylogenetic analyses

The genomic DNA of bradyrhizobial strains was prepared from the purified bacterial strains grown in HM broth (Piromyou *et al.*, 2011). The 16S rRNA gene was amplified using the primer pair fd1 and rp2 (Allardet-Servent *et al.*, 1993). The DNA

primers for housekeeping genes and other DNA primers used are listed in Table 10. Gene fragments were amplified using the Go Taq Flexi DNA polymerase kit (Promega, Mannheim, Germany). The PCR products were purified using the Wizard SV gel and PCR clean-up system (Promega, Germany) and sequenced using the same primers as those for the PCR. DNA sequencing was carried out by Macrogen, Inc. (Seoul, South Korea). The DNA sequences were generated and the most closely related sequences were obtained from the NCBI database. The nucleotide sequences were aligned using the CLUSTAL W program, and the phylogenetic tree of 16S rRNA and housekeeping genes sequences were constructed using tree the maximum likelihood method (Guindon and Gascuel, 2003). The confidence levels were estimated for 1,000 replicates. In comparison, phylogenetic trees were also reconstructed by the distance neighbor-joining method (Saitou and Nei, 1987) using the MEGA 4.1 package (Kumar *et al.*, 2008). DNA sequences of each gene from relative strains in the family *Bradyrhizobiaceae*, of other rhizobia, and of outgroups were obtained from the NCBI database.

5-3 Results

5-3-1 Bacterial isolation

The putative rice endophytic bacteria including bradyrhizobia were isolated from two different agricultural practice systems including rice monoculture and rice/legumes crop rotational systems along with the procedures illustrated in Fig 11. Sixty-two bacterial colonies were obtained from the samples of monoculture system from central part of Thailand, whereas, 91 colonies were isolated from samples of crop rotation system from every parts of Thailand. Total of the 153 bacterial colonies were screened for removing the redundant strains using BOXAIR1-PCR. The 98 strains showing different BOXAIR1 fingerprints (data not shown) were chosen for 16S rRNA identification. Only

6 strains were closely related to bradyrhizobial group, while other isolates were classified into four main groups including α-proteobacteria (*Agrobacterium* sp., *Methylobacterium* sp., and *Rhodopseudomonas* sp.) firmicutes, flavobacteria and β-proteobacteria (*Ralstonia* sp., *Burkholderia* sp. and *Acidovorex* sp.) (Fig. S3). In addition, rice endophytic bradyrhizobial strains (DOA1, DOA9 and SUTN9-2) isolated from *A. americana* L. (weeds grown in rice fields) (Noisangiam *et al.*, 2012) were also used in this study and three strains RP5, RP7 and WD16 from *O. sativa* L. ssp. *japonica* cv. Nipponbare in Japan were also used as putative Japanese rice endophytes representatives (Shinoda *et al.* unpublished).

The sterilized rice roots and shoots homogenate solutions were applied into three procedures to enhance the opportunity to acquire rice endophytic bradyrhizobia. Firstly, to obtain the *nod*-dependent bradyrhizobia, the nodulation test with siratro was conducted. Only an isolate SUT-R55 was obtained from root nodules of siratro. Secondly, in order to avoid growth of other contaminants, the oligotrophic isolation approach (10 % v/v of homogenate solution was incubated into sterilized water for 1 month) was applied for rice tissue prior to testing on BJSM medium and siratro nodulation. Most of strains, SUT-R3, SUT-PR9, SUT-PR48, SUT-R55 and SUT-PR64, can be isolated from this procedure. Finally, three strains, SUT-R3, SUT-R55 and SUT-R74, were also directly obtained from homogenate and grown on bradyrhizobial selective (BJSM) agar plates (Fig. 11).

The rice rhizospheric soils were also used for the bradyrhizobia isolation. The strains obtained from these soil samples were SUT-PR9 and SUT-R74, when cultivated in BJSM medium but not found from siratro nodulation test (Fig. 11). Most of putative rice endophytic bradyrhizobia from Thailand were found exclusively from crop rotation system, whereas no bradyrhizobial isolates were obtained from monoculture system (Table 9).

5-3-2 Phenotypic characteristics and phylogenetic analysis of 16S rRNA and housekeeping gene

To determine the relationships of bradyrhizobial strains among their members of the *Bradyrhizobiaceae*, the nucleotide sequences from various reference *Bradyrhizobium* members and *Rhodopseudomonas* species were used to construct the phylogenetic tree. *Mesorhizobium loti* ATCC33669 and *Bosea thiooxidans* DSM9653 were chosen as outgroup strains to the root of phylogenetic tree (Fig. 12). The phylogenetic tree could be divided into 4 major clusters, on the basis of 16S rRNA gene sequences similarity, cluster 1 comprises of bradyrhizobial strains SUT-PR64, SUT-PR48, SUT-PR9, RP5 and RP7. Only strains SUT-PR9, RP5 and RP7 were slightly separated from those mentioned strains. Strains SUT-PR48 and SUT-PR64 were closely related with *Bradyrhizobium* sp. ORS278, *Bradyrhizobium* sp. ORS285 and *Bradyrhizobium* sp. ORS375. The bradyrhizobial members in cluster 2 belong to various groups of *Bradyrhizobium* species (*Bradyrhizobium* sp., *B. yuanmingense*, *B. japonicum*, *B. liaoningense*, and *B. diazoefficiens*), *Bradyrhizobium* sp. S23321 (non-symbiotic strain), *Bradyrhizobium* sp. BTAi1 and *Rhodopseudomonas* members. Three stains from rice tissue including SUT-R3, SUT-R55, SUT-R74 and *A. americana* isolated strains (SUTN9-2, DOA1 and DOA9) belonged to this cluster, with strong bootstrap support (99%) separated from the cluster 1. In cluster 3 of phylogenetic tree, only *B. elkanii* species were located. Finally, strain WD16 was located in the cluster 4 of phylogenetic tree. The phylogenetic tree based on sequences of *dna*K, *rec*A, and *gln*B was also constructed. The taxonomic positions of the bradyrhizobial strains in the combination tree (Fig. S4) were almost concordant with their taxonomic positions in the 16S rRNA gene tree (Fig. 12). In the combination tree, the strains were clearly separated into two clusters. In order to construct

the correlation between phenotypic characteristics and phylogenetic positions, the photosynthetic pigment productions in bradyrhizobial strains were considered.

All of the strains formed typical slow-growing *Bradyrhizobium* colonies on HM agar plate (4-7 days). From total 9 strains, the strains SUT-PR48 and SUT-PR64 could synthesize the pink/orange pigments when cultured on yeast-mannitol agar and broth, whereas the pigments formation could not be detected from strains DOA1, DOA9, SUTN9-2, SUT-R3, SUT-PR9, SUT-R55, SUT-R74 including Japanese bradyrhizobial strains (Fig. S5).

The results from phylogenetic tree analyses were mainly congruent with pigment production characteristics as described before. On the basis of phylogenetic tree and phenotypic properties, therefore they might be separated into two main groups including PB and Non-PB groups. Nevertheless, the strains SUT-PR9, RP5, RP7 and WD16 were slightly separated from PB cluster.

5-3-3 Rice growth promotions

The effects of bradyrhizobial strains on rice biomass in Leonard's jar were compared among three rice cultivars. In this experiment, nitrogen source for plant at 0.1 mM NH_4NO_3 was applied into N-free medium to determine the rice growth promotion (Fig. 13).

Thai bradyrhizobial strains were promoted rice growth significantly higher than that of un-inoculated control, except strains SUT-R55 and SUT-R74, which were not able to promote rice growth when compared with un-inoculated control. Strains SUT-PR9 and SUT-PR64 could more highly promote rice biomass than other strains. Among the Japanese bradyrhizobial strains, only strain RP7 was able to promote higher rice biomass than un-inoculated control but with statistical insignificance difference. Generally, Thai bradyrhizobial strains could promote rice growth better than those of Japanese

bradyrhizobial strains especially when inoculated with Thai-rice cultivars, on the other hand, the rice growth promotion of cultivar Kasalash could not clearly be observed from all bradyrhizobial strains. Nevertheless, almost all of the bradyrhizobial strains (except WD16) could promote rice cultivar *O. sativa* Nipponbare significantly higher than un-inoculated control.

Bradyrhizobial isolates from *A. americana* nodules (DOA1, DOA9 and SUTN9-2) were phylogenetically intermixed in rice bradyrhizobia (Fig. 12). The inoculation of rice cultivar *O. sativa* Pathum Thani 1 with strains DOA1 and SUTN9-2 were also demonstrated and could significantly promote higher rice biomass than that of un-inoculated control as well. In contrast, the DOA9 did not show the effectiveness in rice growth promotion.

5-4 Discussion

Prior to understanding the behavior of bradyrhizobia as endophyte in rice, the advantages of oligotrophic-based isolation were considered to reduce the growth of other contaminants, while the bradyrhizobia still survive under nutrients deprived (oligotrophic) condition. The incubation of homogenates solution as low as 1 ml into large amount of water as 50 ml for 1 month was mimiced the oligotrophic condition. Therefore, almost of rice oligotrophic endophytic brydyrhizobia in this study were obtained by this method except SUT-R74. Proportion of bradyrhizobial strains and other bacterial endophytes on the basis of culture-dependent approach showed less amount of bradyrhizobia in rice tissues (6 bradyrhizobial strains from 98 bacterial strains). It seems that the putative endophytic bradyrhozobia may not be dominant species in cultivated rice. This may support the previous report, which revealed that proteobacteria are the major endophytic bacteria in rice tissue. Among proteobacteria, alpha- and gamma-

proteobacteria are stably dominate in the shoots. Besides, the bacterial strains *Burkholderia*, *Bradyrhizobium*, and *Methylosinus* are also found in rice tissue. These endophytic bacteria became much more abundant in the low nitrogen root microbiome (Ikeda *et al.*, 2014).

The bradyrhizobial isolates were successfully obtained from rice/legume crop rotation system. In contrast, the endophytic bradyrhizobia could not be recovered from monoculture system. The data were congruent with Guong *et al.* (2012), which revealed that *Bradyrhizobium* sp. and *Herbaspirillum* sp. instantly colonize the interior of rice roots when grown in rotation with a legume crop. Besides, the bacterial community from rice/legumes crop rotation system is significantly different and higher than those in rice monoculture. Therefore, the appropriate crop rotations provide a feasible practice to maintain the equilibrium in soil microbial environment. These observations raise the hypothesis that the agricultural practice conditions may drive the evolution of bradyrhizobia-host association. This hypothesis may be also gained more supportive evidences since *A. americana* bradyrhizobial strains (DOA1, DOA9 and SUTN9-2) perform rice endophytic properties. Furthermore, the bradyrhizobial strains nodulating *A. americana* have never been detected in the paddy soil (Table 9). Therefore, the bradyrhizobial strains nodulating *A*. *americana* can survive during without genuine host and reform nodules in the following year. They may proliferate their cells in rice tissues even though *A*. *americana* cannot grow well each year during December until July the following year (tentatively eight months). Actually, *A*. *americana* is the leguminous weed grown in rice field for only four months a year (during July until December). Therefore, these results may be a part of evidence that confirms the bradyrhizobia-host evolution.

Plant growth promotion properties of rhizobia have been previously reported for various cereals such as wheat, maize and rice (Webster *et al.*, 1997; Yanni *et al.*, 1997;

Yanni *et al.*, 2001; Prévost *et al.*, 2012). Most of Thai isolated strains and especially, SUTN9-2 are significantly increase the rice biomass. On the other hand, all Thai rice growth was not promoted by Japanese strains (strains RP5, RP7 and WD16) (Fig. 13). Moreover among three rice cultivars, only *O. sativa* L. ssp. *indica* cv. Pathum Thani 1 responded positively only to putative Thai rice endophytic bradyrhizobia, by which this phenomenon was not found in Japanese rice cultivars. These results implied that the rice cultivar is one of the factors that control the compatibility of rice-bacteria association. Besides, this phenomenon displayed the bradyrhizobial host preference between Thai endophytic bradyrhizobial strains and Thai rice cultivars. This scenario is confirmed by previous report, that rice genotypes mainly affected the bacterial association in rice tissues (Sasaki *et al.*, 2013).

Chapter 6: Applications: PGPR coinoculated with *Bradyrhizobium diazoefficiens* USDA110 and *B. japonicum* THA6 on soybean symbiosis and its potential of field application and Carrier for Rhizobial Inoculant Production

6-1 Introduction

Bradyrhizobium plays a special role in the nitrogen cycle of agroecosystems by infecting the roots of soybean (*Glycine max*) and forming dinitrogen-fixing nodules. In this way, significant amounts of nitrogen are fixed and transferred to the plant, reducing the need for nitrogen fertilizer. However, there is a number of factors which impede the nodulation on soybean root. Several abiotic and biotic factors can inhibit the formation of the N_2-fixing symbiosis between rhizobia and leguminous plants.

Enhancement of legume nitrogen fixation by coinoculation of rhizobia with some plant growth promoting rhizobacteria (PGPR) is an alternative approach to improve the nitrogen availability in sustainable agricultural production systems. Some PGPR strains are capable of promoting growth of leguminous plants, nodulation, and nitrogen fixation when coinoculated with rhizobia. Examples of these are *Azospirillum* (Aung *et al.*, 2013), *Azotobacter* (Wu *et al.*, 2012), *Bacillus* (Atieno *et al.*, 2012), *Serratia* (Zahir *et al.*, 2011), and *Streptomyces* (Tokala *et al.*, 2002). Rhizobia and PGPR share common microhabitats in the root and rhizosphere soil interface so they must interact during the root colonization process. The effect of *Rhizobium*–PGPR coinoculation has been observed in different symbiotic and plant growth parameters. Compared to single *Rhizobium* inoculation, coinoculation of *Rhizobium* spp. and *Azospirillum* spp. can enhance the number of root hairs, the amount of flavonoids exuded by the roots, and the number of nodules formed (Remans *et al.*, 2008).

Furthermore, Using rhizobial inoculant is a clean technology for sustainable agriculture. Rhizobial inoculants have been used as an environmental friendly source of nitrogen fertilizer for several decades to reduce putting chemical nitrogen fertilizer into the soil, as well as the cost of legume production. There are several forms of rhizobial inoculant available in the market, including solid or liquid forms that can maintain the survival of effective rhizobia at the level of 10^8 cells/g for at least 6 months (Stephens and Rask, 2000). However, solid inoculants, especially those using peat-based carrier, are still popular for biofertilizer inoculant production because peat could support the survival of bacteria in long term storage (Okon and Labandera-Gonzalez, 1994; Kishore *et al.*, 2005). In addition, peat is limited in many countries including Thailand, so it is necessary to find carriers that are locally available for commercial scale production. However, the quality of inoculant is varied according to the physicochemical and biological properties of material as well as the sterilization method applied to carrier (Khavazi *et al.*, 2007; Swelim *et al.*, 2010). Since contaminant microorganism is the main problem that affects the quality and shelf-life of rhizobial inoculant, sterilized carrier is necessary to be accomplished prior injection of the pure culture of rhizobia into carrier.

Commercial rhizobial carriers are normally sterilized by gamma irradiation or autoclaving, depending on the accessibility and availability of instrument. Mechanism of sterilization by autoclaving is based on the wet killing of microorganisms under high temperature (121ºC) and high pressure (15 ponds/inch^2) for a period of time, depending on the size and the composition of material. However, the problem of spore forming microorganisms contaminant remains in the carrier after autoclaving is the main problem of using this sterilization method. While the mechanism of sterilization by gamma irradiation is due to the direct breakdown of double strand DNA, or occurring from the ionized water molecules form free radicals and disrupt biological system in the cell (Block, 2001).

Therefore, the objective of this research is to select the appropriate PGPR and evaluate an increase of soybean (*Glycine max*) yield via nodule nourishment mediated by coinoculation PGPR with *B. diazoefficiens* USAD110 and *B. japonicum* THA6 compared to single inoculation. In addition, the other aimed of this study to find out the process of carrier preparation to achieve lowest dose rate that effectively sterilized carrier by gamma irradiation. As well as the strategy of killing geminated spore forming microorganism by the application of tyndallization approach with autoclaving sterilization was also determined in order to minimize the contaminating microorganisms in the compost and peat for using as rhizobial carrier in inoculant production.

6-2 Materials and methods

6-2-1 Bacterial strains

Bradyrhizobium diazoefficiens USDA110 and *B. japonicum* THA6, which are commercially used in rhizobial inoculants production for soybean in Thailand, were obtained from the Department of Agriculture, Bangkok, Thailand. *B. diazoefficiens* USDA110 and *B. japonicum* THA6 were cultured in yeast extract-mannitol (YEM) broth (Somasegaran and Hoben, 1994). The PGPR isolates were originally isolated from soybean rhizosphere soil as described by Piromyou et. al. (2011) from 30 soybean fields in 11 provinces of Northern and North-Eastern part of Thailand.

The PGPR on N-free LG plates (Lipman, 1904) were incubated for 2 days at 30^{o}C, and bacteria representative of the predominant morphologically distinct colonies present on the plates was selected for further analysis. All 285 bacterial isolates were screened against both *B. diazoefficiens* USDA110 and *B. japonicum* THA6 by antimicrobial spot test. Each bradyrhizobial culture was swab over YEM agar plates and after the plates were dried, 20 µl of each strains of PGPR suspension was spotted onto bradyrhizobia

agar plate. After 4-day cultivation at 30°C, the plates were daily examined for growth inhibition by observing the clear zones. Only PGPR isolates which did not show any clear zone on both USDA110 and THA6 agar plate were selected for further coinoculation assay. All bacterial cultures were maintained by periodic transferred and stored in the refrigerator for further studies.

6-2-2 Effect of PGPR inoculation dose

One ml of the bacterial broth culture (10^6 Colony Forming Unit (CFU) ml^{-1} $seed^{-1}$) was inoculated onto each seed according to the following treatments. For the single inoculation treatment, the seedlings were inoculated separately with 10^6 CFU ml^{-1} $seed^{-1}$ of S141, S222, USDA110, and THA6. For the coinoculation treatment, the USDA110 or THA6 culture at 10^6 CFU ml^{-1} $seed^{-1}$ (as recommended using for soybean cultivation in Thailand) was mixed in a ratio of 1:1 with PGPR isolate S141 or S222 at five inoculum doses; 10^3, 10^4, 10^5, 10^6 and 10^7 CFU ml^{-1} $seed^{-1}$. In the control treatment, the cell suspensions were replaced by sterilized distilled water. The plants were cultivated in growth chambers using modified Leonard's jar assemblies and harvested at 45 days after inoculation (DAI). The nodule number, nodule dry weight, and plant dry weight were measured. Nitrogenase activity was measured by the Acetylene Reduction Assay (ARA) (Somasegaran and Hoben, 1994).

6-2-3 Coinoculation effect of bradyrhizobia and PGPR on soybean

6-2-3-1 Leonard's jar experiment

The soybean (Glycine max (L.) Merrill) cv. Chiang Mai 60 is a recommended commercial line for use under Thai field condition. Seeds of soybean were cultivated in growth chambers using modified Leonard's jar assemblies (Blauenfeldt et al., 1994). Seeds were surface sterilized and gnotobiotically germinated on wet tissue paper (Somasegaran and Hoben, 1994). The early stationary phase of PGPR and bradyrhizobia

cultures were centrifuged (4,000 ×g for 5 min) and washed with sterilized 0.85% (w/v) NaCl to remove the excess media, and the cell pellet was resuspended in 0.85% (w/v) NaCl solution. A preliminary Leonard's jar experiment was conducted to evaluate the coinoculation effects of *B. diazoefficiens* USDA110 and *B. japonicum* THA6 with 45 PGPR isolates on soybean. For the single inoculation, the seedlings were inoculated separately with 1 ml seed^{-1} of 10^6 CFU ml^{-1} of PGPR or *B. japonicum,* and mixed in a ratio of 1:1 for coinoculation treatment. In the control treatment, the cell suspensions were replaced by 0.85% (w/v) NaCl solution. Plants were grown in growth chamber at 25°C light room under 16/8 h light/dark photoperiod. During the experiment, the plants were watered regularly with N-free nutrient solution (Zhang *et al.*, 1996). The experiment was laid out with five replicates for each treatment. Plants were sampled at 45 DAI and the nodule number, nodule dry weight, and plant dry weight were recorded. The top 12 PGPR that can promote soybean growth were selected for further PGPR characterization and identification studies. Only 2 PGPR isolates which showed the highest capability of growth promoting were selected for further experiments.

6-2-3-2 Field experiment

Single and coinoculation inocula were prepared as described in Leonard's jar experiment by 9 different treatments as follow: (1) Control; Non inoculation, (2) USDA110; single inoculation with *B. diazoefficiens* USDA110, (3) THA6; single inoculation with *B. japonicum* THA6, (4) S141; single inoculation with *Bacillus subtilis* strain S141, (5) S222; single inoculation with *Staphylococcus* sp. strain S222, (6) U110+S141; coinoculation with *B. diazoefficiens* USDA110 and *B. subtilis* strain S141, (7) U110+S222; coinoculation with *B. diazoefficiens* USDA110 and *Staphylococcus* sp. strain S222, (8) THA+S141; coinoculation with *B. japonicum* THA6 and *B. subtilis* strain S141, and (9) THA+S222; coinoculation with *B. japonicum* THA6 and *Staphylococcus*

sp. strain S222. The experiment was performed in two sites. Site 1 was Organic Farm located in Suranaree University of Technology (SUT) campus at Nakhon Ratchasima province, Thailand (14° 52' 16.11"N and 102° 1' 31.95" E) which has history of legume cultivations but no history of chemical fertilizer application. Site 2 was rice paddy field at Buriram province (14° 34' 3.63" N and 102° 32' 38.93" E) which has a history of chemical fertilizer application but no history of legume cultivations. Each field site was laid out under a completely randomized design (CRD) by dividing into nine treatments (as mentioned above), and each treatment consisted of 3 replicates making a total of 27 plots according to 9 different treatments as mentioned above. Inoculum containing approximately 1 ml bacteria (10^6 CFU $seed^{-1}$) was poured over the seed in each row prior to covering the seeds with soil. The regular agricultural practices were done except chemical fertilizer application and pesticide spraying. The plants were harvested at 100 DAI. Seed yield was taken from the two middle rows of each plot. Pods were removed and threshed by hand and seed yield was determined.

6-2-4 16S rRNA gene analysis

The selected PGPR were identified at species level based on the full length sequence analysis of 16S rRNA gene. The genomic DNA was extracted following the method of Prakamhang et al. (2009). The 16S rRNA universal primers 27F and 1492R were used to amplify approximately 1.5-kb internal region of the 16S rRNA gene (Weisburg *et al.*, 1991). The nucleotide sequence of purified PCR products was analyzed at the Macrogen Service Center (Seoul, Korea). The DNA sequences were generated and the most closely related sequences were obtained from the NCBI database.

6-2-5 The statistical analysis

Data from each experiment were first submitted to tests of normality and homogeneity of variances for each variable and then to analysis of variance (ANOVA).

When confirming a statistically significant value in the F-test ($p \leq 0.05$), a post hoc test (Duncan's multiple-range test at $p \leq 0.05$) was used as a multiple comparison procedure (Duncan, 1955) by SPSS® software for WINDOWS™, Version 14.0; SPSS, Chicago, IL).

6-2-6 Carrier materials

Two types of carrier, peat and compost, were used throughout the experiments. Peat was derived from Department of Agriculture (DOA), Thailand, while compost was obtained from Suranaree University of Technology. Compost was made from the mixture of agricultural wastes, such as cassava peel, filter cake, chicken dung and cow dung. The pH of peat and compost was 4.5 and 7.49, respectively. Both peat and compost were first milled and passed through a 100-mesh sieve, before the pH of peat was adjusted to nearly 7.0 by using $CaCO_3$. Peat and compost were sent to Soil Laboratory, School of Plant Production Technology, Suranaree Univeristy of Technology, Thailand to determine the physical and chemical characteristics of carriers according to the standard method.

6-2-7 Carrier sterilization procedures

6-2-7-1 Sterilization by gamma irradiation

Carrier packages were sterilized with gamma irradiation at Thailand Irradiation Center, Thailand Institute of Nuclear Technology (Nakhon Na-yok, Thailand). Cobolt-60 was used as a source of radiation. The packages were sterilized with different doses of gamma irradiation at 5, 10, 15, 20, and 25 kGy. The dosage received at various depths and locations were measured by using dosimeter to ensure the amount of radiation doses. The gamma irradiated packages were delivered to Suranaree University of Technology within a week for determination of the number of microbial contaminants in carrier after gamma sterilization.

6-2-7-2 Sterilization by autoclaving

Carriers were moistened to 10% before packing 80 g of each carrier into 5×8 (inches×inches) of PE bag. The tyndallization approach was applied for carrier sterilization. The autoclave was operated for 2 times in a row at 121ºC for 60 min, while the waiting period after each time of autoclaving was varied at 18 and 24 h.

6-2-8 Microbial contaminants enumeration

The number of microbial contaminants in sterilized carrier package was determined at 1 week after sterilization by aseptically removing 10 g of carrier from each bag, and carrier was diluted in 90 ml sterilized water, and shaken at 200 rpm for 30 min. The sample was 10-fold serial diluted in sterilized water and plated in duplicate onto plate count agar (PCA) and potato dextrose agar (PDA) for bacteria and molds enumeration, respectively. Colonies were counted after incubation at 28ºC for 5 days, and calculated as log number of colony forming unit (cfu)/g dry weight carrier. The torn carrier packages were excluded from the experiment.

6-2-9 Inoculant preparation and storage

Bradyrhizobium sp. PRC008, obtained from DOA, Thailand has normally been used as an inoculum strain for inoculant production by DOA. This strain was recommended to use with mungbean (*Vigna radiata* L.) to reduce the use of chemical nitrogen fertilizer. In this study, PRC008 was cultured in a yeast extract mannitol (YEM) broth until late log phase. The culture was diluted 10 times, and 20 ml of diluted culture were aseptically injected into the bag containing 80 g of sterilized carrier with 20% of moisture content. Bags were thoroughly kneaded by hands, and the final moisture content of carrier was 40% at this step. Then, bags were incubated at 28ºC for 1 week and then left at room temperature (28-30ºC) for 6 months.

6-2-10 Enumeration of rhizobial cells by plant infection count

The number of effective *Bradyrhizobium* sp. PRC008 was determined every month for 6 months after the injection by using plant infection count based on the principle assumptions underlying the most-probable-number (MPN) method (Somasegaran & Hoben, 1994).

6-3 Results

6-3-1 Screening of PGPR

Out of 285 soybean rhizosphere soil bacterial isolates, only 45 PGPR isolates did not show antimicrobial activity or clear zone on both Bradyrhizobia USDA110 and THA6 agar plates. All 45 PGPR isolates were tested with soybean in Leonard's jar trails, and only 12 PGPR showed significant capability of promoting nodule number, nodule dry weight and plant dry weight when coinoculated with both bradyrhizobia when compared to single inoculation on soybean at 45 DAI (P<0.05) (Fig. 14).

Variations in nodule number at 45 DAI as consequence of different microbial inoculation and coinoculation were presented in Figure 14A. The highest mean number of nodules per plant was recorded in USDA110+S222 treatment (49.00 nodules plant^{-1}) followed by THA6+S141 (46.75 nodules plant^{-1}) when compared with all other treatments and control. Enhancement in number of soybean nodule obtained from coinoculated with USDA110 and S141 or S222 was higher than those of single coinoculation with USDA110 by 63.1 and 76.6%, respectively. Similar trend was also found in case of THA6 and its both coinoculation S141 and S222 by 90.8 and 54.1%, respectively. The data on the nodule dry weight were presented in Figure 14B. At 45 DAI, significantly higher nodule dry weight of 102.8 mg plant^{-1} was recorded in USAD110+S222 treatment followed by USDA110+S141 (100.43 mg plant^{-1}).

Coinoculation of USDA110 with S222 and S141 enhanced nodule dry weight of soybean compared with single inoculation with USDA110 by 148.1 and 102.8%, respectively. Soybean nodule dry weight was significantly improved when coinoculated THA6 with S141 and S222 by 150.1 and 141.2%, respectively.

Coinoculation USDA110+S141 treatment showed significant maximum plant dry weight (1,400.5 mg plant^{-1}) (Fig. 14C) followed by USDA110+S222 treatment (1,377.9 mg plant^{-1}). Coinoculation of USDA110 with S141 and S222 enhanced soybean plant dry weight compared with USDA110 single inoculation by 39.3 and 37.1%, respectively. Similar results were also found in case of THA6, in which soybean plant dry weight was increased when coinoculated THA6 with S141 (40.4%) and S222 (50.1%) when compared with single inoculation of THA6.

Among the coinoculation treatments, S141 and S222 performed significantly highest in all plant growth parameters when coinoculated with USDA110 and THA6. Therefore, S141 and S222 were selected for further experiments.

6-3-2 Determination for dose of *Bradyrhizobium* and PGPR

There were differences among PGPR doses for all soybean parameters including plant dry weight (Fig. 15A), nodule number (Fig. 15B) nodule dry weight (Fig. 15C), and also nitrogen fixing activity (Fig. 15D). The results of the controls and single inoculation of each PGPR were not different from each other on soybean plant dry weight. All inoculant doses (10^3 - 10^7 CFU ml^{-1} seed^{-1}) of single inoculation of USDA110 and THA6 or coinoculation treatments promoted higher plant dry weight than those obtained from uninoculated control or single inoculation of PGPR treatments. Soybean nodule number, nodule dry weight, and N_2-fixing activity of single inoculation of bradyrhizobia and all coinoculation treatments were significantly different ($P < 0.05$) among the bacterial concentrations tested. In case of USDA110 with S141 and S222, the inoculum dose at

10^6:10^6 CFU ml^{-1} $seed^{-1}$ increased plant biomass by 115.5 and 65.8%, nodule number by 64.85 and 113.3%, nodule dry weight by 127.0 and 133.9%, and ARA activity by 98.5 and 114.8%, respectively. In case of THA6, the optimum coinoculation dose was also 10^6 CFU ml^{-1} $seed^{-1}$ for S141 and S222. This dose increased plant biomass by 78.6 and 48.7%, nodule number by 38.0 and 23.4%, nodule dry weight by 67.0 and 142.5%, and ARA activity by 69.9 and 128.8%, respectively. Thus the optimum coinoculation dose was at 10^6 CFU ml^{-1} $seed^{-1}$ for USDA110 and THA6 with 10^6 CFU ml^{-1} $seed^{-1}$ for both PGPR isolates S141 and S222.

6-3-3 Effects of coinoculation on soybean grain yield under field condition

In order to evaluate the effects of coinoculation on nodule number, nodule dry weight, and soybean seed yield under field condition, the experiments were carried out at two different locations, Buriram province and SUT Organic Farm field. At Buriram site, the number of soybean nodule obtained from coinoculations of USDA110 and S141 and S222 treatments was higher than those of single inoculation with USDA110 by 47.4 and 58.5%, respectively. Similar trend was also found in case of THA6 both coinoculations of S141 and S222 by 66.7 and 59.1%, respectively (Fig. 16A). At SUT Organic Farm site, the nodule numbers obtained from coinoculations of USDA110 and S141 and S222 treatments were significantly increased and higher than those of single inoculation with USDA110 by 63.1 and 72.7%, respectively. Parallel trend was also found in case of THA6 coinoculation with S141 and S222 by 47.7 and 37.2 %, respectively (Fig. 16B). The coinoculations of USDA110 with S141 and S222 increased the nodule dry weight per plant when compared to the single inoculation with USDA110 by 48.3 and 40.1%, respectively. In case of THA6, the coinoculations with S141 and S222 increased the nodule dry weight by 40.8 and 37.5%, respectively (Fig. 16C). Moreover, at SUT Organic Farm site, the nodule dry weights per plant of coinoculations of USDA110 with

S141 and S222 were higher than those of USDA110 single inoculation by 48.3 and 40.1%, respectively. For THA6, the coinoculations with S141 and S222 induced the nodule dry weight by 40.8 and 37.5%, respectively (Fig. 16D). The highest soybean seed yield was noticed in USDA110+S141 (2,811.70 kg ha^{-1}) followed by THA6+S222 (2,732.45 kg ha^{-1}) at Buriram site (Fig. 16E). The seed yields from coinoculations with USDA110 and S141 and S222 treatments were increased by 28.4 and 23%, respectively. In case of THA6, the coinoculation with S141 and S222 induced the seed yield by 25.2 and 27.3%, respectively. Furthermore, all coinoculation treatments were on par with each other. At SUT Organic Farm site, the highest seed yield was noticed in THA6+S141 (2,868.87 kg ha^{-1}) followed by USDA110+S141 (2,748.23 kg ha^{-1}) as shown in Figure 16F. The seed yields in coinoculation with USDA110 and S141 and S222 treatments were increased by 9.8 and 1.4%, respectively. In case of THA6, the coinoculation with S141 and S222 induced the seed yield by 43.6 and 27.5 %, respectively.

6-3-4 Physicochemical characteristics of compost

In order to determine whether the compost had similar properties to peat, the physical and chemical characteristics of compost and peat were determined. The results are shown in Table 11. Peat and compost were much different in pH, organic matter, nitrogen, and phosphorus contents. The pH of compost was readily in neutral range, which would be suitable for survival of rhizobia, while acidity of peat must be neutralized by using $CaCO_3$.

Although compost used in this study has several characteristics that indicate its potential as a good carrier for rhizobial inoculant production, the final decision is based on rhizobial cell survival during storage. Thus, the appropriate sterilization methods using gamma irradiation and autoclaving with tyndallization approach, as well as

rhizobial survival test in the compost as carrier, were elucidated below by comparing with peat carrier.

6-3-5 Factors affecting the sterilization efficiency by using gamma irradiation

Sterilization using gamma irradiation with peat or compost, which have complex carbon-based structure and usually contain high level of contaminating load need to determine the appropriate dose rate and other factors that influence sterilization efficiency. The compost with 10, 20, and 30% moisture contents could be efficiently sterilized by gamma radiation at dose rate of 10, 15, and 25 kGy, respectively (Fig. 17). There were no bacteria or molds found in carrier at one week after irradiation when 10% moisture content was applied with gamma radiation at dose rate of 10 kGy, while at the same dose rate the number of molds more than 10 cfu/g and bacteria more than 10^4 cfu/g were detected in the carriers with 20 and 30% moisture content, respectively. It is interesting that even high thrash dose rate of 20 kGy was applied, the number of bacteria more than 10^3 cfu/g remained in the carrier with 30% moisture (Fig. 17). These results revealed the influence of moisture content on gamma irradiation efficiency.

6-3-6 Sterilization of carriers by autoclaving with tyndallization approach

In this study, carriers with 10% moisture were autoclaved at 121ºC for 60 min two times, while the holding period after each time of autoclaving was varied at 18 and 24 h. Microbial vegetative cells were killed during each time of heat challenge, while the spore forming microorganisms were allowed to germinate during the holding period, and their vegetative cells were eliminated by the second autoclaving. The results showed that molds at 10^7-10^8 and 10^5-10^6 cfu/g carrier were completely eliminated in peat and compost, respectively by autoclaving two times with 18 h holding period between each time (Fig. 17). Nevertheless, bacterial cell around 100 cfu/g carrier still remained in both carriers by using this method. On the other hand, bacteria and molds were found to

survive in peat and compost when 24 h holding period was applied. The amount of bacteria and molds remained in range of 10-100 cfu/g peat, which was similar to the number of bacterial cell remain in compost. However, a number of living molds in the compost was higher than that of living bacteria (100-1000 cfu/g carrier). It is possible that the germination and spore forming period of bacteria or fungi are different according to their species, thus 24 h holding time would activate some of microorganism species to fall into spore forming period. Moreover, the response of different species to surrounding environment, such as heat, moisture, pH, and nutrition in each carrier would be the factors that affect the sterilization by using tyndallization approach.

6-3-7 Survival of Bradyrhizobium sp. PRC008 in sterilized carriers

To investigate the possibility of using compost instead of peat, and sterilization methods using gamma irradiation or autoclaving with tyndallization process to minimize the contaminant in carriers, *Bradyrhizobium* sp. PRC008 was injected into both peat and compost derived from both sterilization methods in order to determine the survival of cell during long term storage at room temperature. The number of cells in the treatments was monitored every month by using MPN method in order to check the nodulation activity of bradyrhizobia after storage. The initial number of bradyrhizobia in both carriers after injection was 10^6 cells/g carrier (Fig. 19). The results showed that bradyrhizobia could grow and survive well in peat as carrier regardless of sterilization methods. The number of cells increased from 10^6 to 10^8 cells/g after one month, and remained more than 10^8 cells/g at 6 months storage (Fig. 19), which is high enough for effective competition and nodulation of mungbean seed. Thus, the number of contaminants remaining in peat after sterilization by gamma irradiation or autoclaving did not affect bradyrhizobial growth. However, it was not successful to use compost as carrier for bradyrhizobial inoculant. The number of cells increased only 1 magnitude from 10^6 to 10^7 cells/g at one month

storage, and sharply decreased at two months storage with no significant difference between gamma irradiation and autoclaving sterilization methods (Fig. 19). These results indicated an inappropriate condition for bradyrhizobial growth and survival in the compost.

6.4 Discussion

In this study, the PGPR isolates were preliminary isolated from soybean rhizosphere soil using LG N-free medium in order to obtain the most abundant root-adhering bacteria (Piromyou *et al.*, 2011). Enhancement of nitrogen fixation is the ultimate goal of any *Rhizobium*-legume symbiosis. Therefore, all the PGPR selection experiments were carried out in N-free conditions. These might result in more chances for PGPR to persist and provide some nitrogen to plants via nitrogen fixation. We hypothesized that increase in nodule number, nodule dry weight, and plant dry weight of soybean due to coinoculation with *Bradyrhizobium* and PGPR could be attributed to a greater nitrogen fixation.

The most effective PGPR isolates in this study were S141 and S222, which performed significantly highest in all plant parameters. However, the coinoculation of both bradyrhizobia with S222 was not obviously different from single inoculation of bradyrhizobia in the field experiment at Buriram site. This might be because of the effect of indigenous rhizobia or rhizospheric bacteria on PGPR such as indigenous microbial community structure (Piromyou *et al.*, 2011), competitive colonization, and rhizosphere eubacterial community structures of soybean (Aung *et al.*, 2013). Moreover, the soybean yield trend on the SUT Organic Farm site was obviously increased, especially when coinoculated with THA6+S141. This might also depend on the soil property such as legume planting history or the chemical fertilizer application history. Moreover, both soil

nitrogen and symbiotic nitrogen are required for the optimum soybean production. These results suggested that the availability of adequate amount of plant nutrient in soil could support the soybean production. Moreover, the organic matter available can promote the dispersal and activity of applied PGPR.

On the other hand, for rhizobial inoculant production. The organic matter of peat was 4.4 times higher than in the compost. The amount of organic matter may contribute to longer survival of rhizobial cells in the carrier. Khavazi *et al.* (2007) reported that the mixing of perlite with high organic matter containing materials, such as sugarcane bagasse or malt residue could increase the number of bradyrhizobial cell survival at six months storage. Although the organic matter of compost was lower than peat, compost had higher nutrient contents. The high nutrient content was one of the key characteristics of good carrier (Smith, 1992). Compost in this study was made from the mixture of cassava peel, filter cake, chicken dung, and cow dung. However, not all sources of compost material could be used as carrier.

The gamma ray is an effective method for sterilization, since the ionizing radiation could interact directly with charged particles such as DNA, and cause double strand break with matter to cell survival. Moreover, the charged particles and ions destroy the cellular integrity, resulting in spore inactivation and loss of viability (Helfinstine *et al.*, 2005). Ionized water molecules could also form free radicals and disrupt biological systems (Hansen and Shaffer, 2001).

The survivors were mostly spore-forming bacteria and encapsulated microorganisms. The main bacterial species identified in the carriers by Yardin *et al.* (2000) were *Bacillus* spp. and actinomycetes, even carriers were exposed to gamma radiation at dose rate more than 50 kGy. Although gamma irradiation could not be ensured to kill all of microbial contaminants, the significant reduction of contaminants

load could allow high number of rhizobia to remain in the carrier after long term storage when compared with using unsterilized carrier.

The approach of tyndallization process was applied in this study in order to destroy microbial cell and spore forming microorganisms which were present in peat or compost. Tyndallization process was based on thermal inactivation of microorganisms and spores with a heat treatment for double or triple times in the row (Valero and Salmeron, 2003; Gould, 2006).

In this study the tyndallization process failed to reduce contamination in compost. The tyndallization-like process using two steps heating separated by holding period under anaerobic condition failed to kill spore-forming *Bacillus cereus* and *B. subtilis* in dairy product (Brown *et al.*, 1979). Since compost used in this study was derived from the mixture of cassava peel, filter cake, chicken dung, and cow dung, it should be note that not all types of compost could be used as carrier. As reported by Albareda *et al.* (2008) , the compost made from grape bagasse also caused sharply reduces the number of rhizobia. Cassava peels contain toxic compound in form of cyanogenic glucosides (Oboh, 2006). It could be possible that some level of toxic compound might remain in the compost used in this experiment. An inappropriate condition might also be the result from some spore-forming microorganisms that still remains in the compost after sterilization. These spore-forming microorganisms may produce toxin or compete for nutrients available in carrier and finally reduce the number of bradyrhizobia.

Chapter 7: General Discussion

This is the first study to investigate deeply of the information on the genetic and functional diversity of rhizobia nodulating *A. americana* L in Thailand. The results from this study explore some novel knowledge. The *A. americana* nodulating isolated strains were non photosynthetic bacteria and most of them were assigned to the genus *Bradyrhizobium*. These strains were divided into 2 groups *nod*-containing and divergent *nod*-containing strains. The divergent *nod*-containing strains and photosynthetic bradyrhizobia shared close *nifH* sequence similarity and an ability to fix nitrogen in the free-living state. Surprisingly, the *A. americana* nodulating strains could nodulate *Aeschynomene* that belong to different cross inoculation (CI) groups including *A. afraspera* and *A. indica*. This is the first discovery of bradyrhizobia (non-photosynthetic and *nod*-containing strain) originating from CI-group 1 nodulating roots of *A. indica* (CI-group 3). Moreover, one of these strains, *Bradyrhizobium* sp. DOA9 showed a broad host range, being able to colonize and efficiently nodulate the roots of most plants tested of the dalbergioid, millettioid, and robinoid tribes. In addition, DOA9 carried two replicons, a chromosome (cDOA9) and a single megaplasmid (pDOA9) larger than 352 kb. Interestingly, genes for nodulation (*nodA*, *B*, *C*) and nitrogen fixation (*nifH*) were localized on the megaplasmid. These results suggested that *Bradyrhizobium* sp. DOA9 has the unusual combination of a broad host range, bacteroid differentiation, and symbiosis-mediating replicons. Moreover, the discovery in this study raises several novelties about rhizobia-legumes symbiosis, especially on *Aeschynome* species that initiate nodule formation without nodulation factors (NFs) (Giraud et al. 2007). If the simple mode is used for invasion instead of the complex one via root hair curling and infection thread formation, it is possible to transfer these bacteria to non- leguminous

plants.

Rice is the most important food croup in Asia. The high-yielding rice production requires huge amounts of nitrogen fertilizers. The biological nitrogen fixation (BNF) from rice root-associated bacteria has a great potential to improve the sustainable rice production. On the other hand, the rice endophytic *Bradyrhizobium* discovery was scarcely reported. Some previous study indicated that photosynthetic *Bradyrhizobium* strains induce N_2-fixing nodules on stems and roots of the genus *Aeschynomene* (Molouba *et al.*, 1999), and also form a natural endophytic association with the wild rice species *Oryza breviligulata* (Chaintreuil *et al.*, 2000). However, the non photosynthetic strains isolated from *A. america* showed the rice endophytic bacteria characteristics. These strains promoted rice growth when compared with control (without inoculation of bradyrhizobium). Therefore, the discovery in this study raise several novelties about rhizobia-legumes symbiosis and also rhizobia-non legumes symbiosis. These informations come from *Bradyrhizobium* strains isolated from only one model, *A. americana.* Most of all, it is benefit to investigate the symbiotic diversity in the topics (southeast Asia), where the legume diversity is very high.

Since using rhizobial inoculant is a clean technology for sustainable agriculture. Rhizobial inoculants have been used as an environmental friendly source of nitrogen fertilizer for several decades. However, there are a number of factors that impede the nodulation on legumes. The coinoculation with bradyrhizobia and PGPR leads to an increased number of the most active nodules and plant yield as well as a greater nitrogen fixation. Finally, The carrier material had a significant influence on rhizobial inoculant quality. Nevertheless, the efficient sterilization processes using gamma irradiation and autoclaving with tyndallization approach were elucidated and could be used for sterilization of peat based carrier.

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Figure legends

Fig. 1 Neighbor-joining trees based on sequences of 16S rRNA genes showing classification of the *Bradyrhizobium* strains isolated from nodules of *A. americana*. Bootstrap values are expressed as percentages of 1,000 replications. The bar represents 1 estimated substitution per 100 nucleotide positions. The evolutionary distances were computed using the Kimura two-parameter method and are shown in the units representing the number of base substitutions per site.

Fig. 2 Maximum likelihood tree based on combined sequences of housekeeping genes (*dnaK*, *recA*, and *glnB*), showing classification of divergent nod-containing *Bradyrhizobium* strains isolated from nodules of *A. americana*. Bootstrap value is expressed as percentages of 1,000 replications. The bar represents 1 estimated substitution per 100 nucleotide positions.

Fig. 3 Examples of (A–E) root colonization, (F–J) nodule morphology, and (K–O) thin sections of nodules inoculated with GUS-tagged DOA9 in various legumes. (A, F, and K); *Aeschynomene americana*, (B, G, and L); *Stylosanthes hamate*, (C, H, and M); *Indigofera tinctoria,* (D, I, and N) *Macroptilium atropurpureum,* (E, J, and O) *Lotus japonicus*.

Fig. 4 Light microscopic observations of *Oryza sativa* ssp. *indica* seedlings inoculated with GUS-tagged *Bradyrhizobium* sp. DOA9. (A) 1 day after inoculation. (B) 5 days after inoculation. (C) Close-up view of root hair and (D) close-up view of root tissue at 5 days after inoculation.

Fig. 5 Examples of differential interference contrast (DIC) views of *Bradyrhizobium* sp. DOA9 bacteroids. (A) Free-living. (B–F) Bacteroids isolated from (B) *Indigofera*

tinctoria, (C) *Macroptilium atropurpureum*, (D) *Aeschynomene americana*, (E) *Aeschynomene afraspera*, and (F) *Arachis hypogaea*.

Fig. 6 Examples of shapes of (left-hand column) free-living DOA9 bacteria and (other columns) resident bacteroids isolated from nodules of (B, F, J) *Arachis hypogaea*, (C, G, K) *Aeschynomene afraspera*, and (D, H, L) *Lespedeza* sp. DIC, differential interference contrast microscopy; DAPI, fluorescence microscopy with 4′,6-diamidino-2-phenylindole stain; PI, fluorescence microscopy with propidium iodide stain.

Fig. 7 Determination of *Bradyrhizobium* sp. DOA9 replicons and symbiosis genes. (A) Megaplasmid profiles: lane 1, *Mesorhizobium loti* MAFF303099 (208 and 352 kb); lane 2, DOA9; (B–E) Southern blot hybridization signals of nodulation and N-fixation genes on megaplasmid and chromosome from DOA9 under low stringency conditions: (B) *nodA*; (C) *nodB*; (D) *nodC*; (E) *nifH*.

Fig. 8 Determination of copy numbers of (A) *nodA*, (B) *nodB*, (C) *nodC*, and (D) *nifH* by Southern blot hybridization. *Bradyrhizobium* sp. DOA9 genomic DNA was digested with the restriction enzymes shown, and the blot was hybridized with probes for *nodA* (from *B. yuanmingense* SUTN6-2), *nodB* (from *B. canariense* SUTN7-2), *nodC* (from *B. japonicum* USDA110), and *nifH* (from *B. yuanmingense* SUTN6-2).

Fig. 9 PCR-DGGE analysis of 16S rRNA gene banding patterns from rice endophytic bacteria in samples from plants grown in different types of soil (fertilized paddy soil, unfertilized paddy soil and uncultivated forest soil). Arrows show excised and sequenced bands (a–d). Azt., Azotobacter sp.; Asp., Azospirillum sp.; R, root; S, stem; L, leaf.

Fig. 10 Nested PCR-DGGE of *nifH* banding patterns from rice endophytic bacteria. R, root; S, stem; L, leaf; types of soil.

Fig. 11 Scheme for the isolation of endophytic bradyrhizobial strains: Name of strains with "P" means photosynthetic bradyrhizobial strain while without "P" indicating the non-photosynthetic bradyrhizobial strain.

Fig. 12 Neighbor-joining trees based on sequences of 16S rRNA genes showing classification of the photosynthetic bradyrhizobia (PB) and non-photosynthetic bradyrhizobia (Non-PB) strains isolated from rice tissues. Bootstrap values are expressed as percentages of 1,000 replications. The bar represents 1 estimated substitution per 100 nucleotide positions. The evolutionary distances were computed using Kimura two-parameter method and are shown in the units representing the number of base substitutions per site.

Fig. 13 The effects of putative rice endophytic bradyrhizobium on rice biomass in Leonard's jar were compared among three rice cultivars (*O. sativa* L. ssp. *indica* cv. Pathum Thani 1, *O. sativa* L. ssp. *indica* cv. Kasalash and *O. sativa* L. ssp. *japonica* cv. Nipponbare). The clear box represent; *A. americana* isolated strains, light gray box; Thai strain, and dark gray box; Japanese strain.

Fig. 14 Nodule number per plant (A) nodule dry weight per plant (B) and plant dry weight (C) of soybean after single and coinoculated between *B. diazoefficiens* USDA110 or *B. japonicum* THA6 with 12 selected PGPR isolates at 45 DAI. Treatments are represented by Control (non-inoculated); USDA110 (*B. diazoefficiens* USDA110); THA6 (*B. japonicum* THA6); + (coinoculation treatment). Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

Fig. 15 Effects of inoculation dose between *B. diazoefficiens* USDA110 or *B. japonicum* THA6 at 10^6 cells $seed^{-1}$ with various dose of PGPR isolates S141 and S222 on soybean plant dry weight (A) nodule number per plant (B) and nodule dry weight per plant (C) and N_2-fixing activity (nmol C_2H_4 g^{-1} nodule dry weight) (D). The numbers at the x-axis

symbolize the varied inoculation doses of PGPR are 10^3 - 10^7 CFU ml^{-1} $seed^{-1}$ and the coinoculation ratio between Bradyrhizobia at 10^6 CFU ml^{-1} $seed^{-1}$ and varies inoculation dose of PGPR from 10^3 – 10^7 CFU ml^{-1} $seed^{-1}$. Treatments are represented by Control (non-inoculated); U110 (*B. diazoefficiens* USDA110); THA6 (*B. japonicum* THA6); + (coinoculation treatment). Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

Fig. 16 Effects of coinoculation between *B. diazoefficiens* USDA110 or *B. japonicum* THA6 with PGPR isolates S141 and S222 at 10^6:10^6 cells $seed^{-1}$ on nodule number per plant (A-B), nodule dry weight per plant (C-D), and soybean seed yield (E-F) performed at Buriram site (panel A, C, and E) and SUT Organic Farm site (panel B, D, and F), respectively. Data represent the means of nine experiments, each with three replicates. Values represent mean ± SD (n=3). Within treatment, means labeled with different letters are statistically different at $P<0.05$.

Fig. 17 Number of microbial contaminants, bacteria (grey bars) and molds (white bars) remaining in compost with moisture contents of 10, 20, and 30%, and sterilization by gamma irradiation at dose rates of 0 (non gamma), 5, 10, 15, 20, and 25 kGy.

Fig. 18 Number of microbial contaminants, bacteria (grey bars) and molds (white bars) remaining in (A) peat and (B) compost without sterilization (non autoclave) and with sterilization by autoclaving two times with the holding period of 18 h (2-18) and 24 h (2-24) between each time of sterilization.

Fig. 19 Number of *Bradyrhizobium* sp. PRC008 surviving in peat with autoclave sterilization (close square), peat with gamma irradiation (open square), compost with autoclave sterilization (close triangle), and compost with gamma irradiation (open triangle) after storage for six months at room temperature.

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Kamonluck Teamtisong

TABLE 1 Strains and plasmids used in this study, sampling sites, and relevant characteristic

Strain	Geographical origin (province in Thailand)	Relevant characteristic(s)	Source or reference
Strains			
*Bradyrhizobium*sp.			
SUTN1-2	Pitsanulok	*A. americana* nodule isolate	This study
SUTN1-3	Pitsanulok	*A. americana* nodule isolate	This study
SUTN1-4	Pitsanulok	*A. americana* nodule isolate	This study
SUTN1-6	Pitsanulok	*A. americana* nodule isolate	This study
SUTN1-7	Pitsanulok	*A. americana* nodule isolate	This study
SUTN1-8	Pitsanulok	*A. americana* nodule isolate	This study
SUTN1-9	Pitsanulok	*A. americana* nodule isolate	This study
SUTN1-12	Pitsanulok	*A. americana* nodule isolate	This study
SUTN1-13	Pitsanulok	*A. americana* nodule isolate	This study
SUTN2-1	Pitsanulok	*A. americana* nodule isolate	This study
SUTN2-2	Pitsanulok	*A. americana* nodule isolate	This study
SUTN2-3	Pitsanulok	*A. americana* nodule isolate	This study
SUTN2-4	Pitsanulok	*A. americana* nodule isolate	This study
SUTN3-1	Uttradit	*A. americana* nodule isolate	This study
SUTN4-1	Uttradit	*A. americana* nodule isolate	This study
SUTN4-3	Uttradit	*A. americana* nodule isolate	This study
SUTN5-1	Uttradit	*A. americana* nodule isolate	This study
SUTN5-3	Uttradit	*A. americana* nodule isolate	This study
SUTN5-5	Uttradit	*A. americana* nodule isolate	This study
SUTN5-6	Uttradit	*A. americana* nodule isolate	This study
SUTN6-1	Uttradit	*A. americana* nodule isolate	This study
SUTN6-2	Uttradit	*A. americana* nodule isolate	This study
SUTN7-1	Chiangmai	*A. americana* nodule isolate	This study
SUTN7-2	Chiangmai	*A. americana* nodule isolate	This study
SUTN8-2	Chiangmai	*A. americana* nodule isolate	This study
SUTN8-3	Chiangmai	*A. americana* nodule isolate	This study
SUTN9-1	Lampang	*A. americana* nodule isolate	This study
SUTN9-2	Lampang	*A. americana* nodule isolate	This study
SUTN9-3	Lampang	*A. americana* nodule isolate	This study
SUTN9-4	Lampang	*A. americana* nodule isolate	This study
SUTN9-5	Lampang	*A. americana* nodule isolate	This study
DOA1	Lopburi	*A. americana* nodule isolate	This study
DOA2	Lopburi	*A. americana* nodule isolate	This study
DOA3	Lopburi	*A. americana* nodule isolate	This study
DOA4	Lopburi	*A. americana* nodule isolate	This study
DOA6	Lopburi	*A. americana* nodule isolate	This study
DOA7	Lopburi	*A. americana* nodule isolate	This study
DOA8	Lopburi	*A. americana* nodule isolate	This study
DOA9	Lopburi	*A. americana* nodule isolate	This study
DOA10	Lopburi	*A. americana* nodule isolate	This study
BTAi1		Photosynthetic bacterium isolated from	Eaglesham and

Strain	Geographical origin (province in Thailand)	Relevant characteristic(s)	Source or reference
		A. indica nodule	Szalay, (1983)
ORS278		Photosynthetic bacterium isolated from *A. sensitive* nodule	Molouba, *et.al* (1999)
ORS285		Photosynthetic bacterium isolated from *A. afraspera* nodule	Molouba, *et.al* (1999)
B. japonicum USDA110		Wild type, soybean isolated	Jordan, (1982)

TABLE 2 Primers used in this stud

Target gene	Primer name	Primer sequence (5′→3′)	Reference or description of design	Annealing temp used(°C)
16S rRNA	fD1	AGAGTTTGATCCTGGCTCAG	Weisburg *et al.* (1991)	55
	rP2	ACGGCTACCTTGTTACGACTT		
dnaK	TSdnaK4	GGCAAGGAGCCGCAYAAGG	Nzoué *et al.* (2009)	53
	TSdnaK2	GTACATGGCCTCGCCGAGCTTCA		
glnB	TSglnBF	AAGCTCGAGTACATCTGGMSCTCGACGG	Nzoué *et al.* (2009)	53
	TSglnBR	SGAGCCGTTCCAGTCGGTGTCG		
recA	recA41F	TTCGGCAAGGGMTCGRTSATG	Vinuesa *et al.* (2005)	53
	recA640R	ACATSACRCCGATCTTCATGC		
nodA	nodAF28	GAAGGATCTTCTGGGCGCG	Designed from *nodA* of *B. japonicum* USDA110 (NC_004463)	45
	nodAR627	CTCAGGCCCGTTACGATCG		
	nodAYF46	GCTCAAGTGCAGTGGAGCCTTC	Designed from *nodA* of *B. yuanmingense* CCBAU10071 (AM117557)	53
	nodAYR595	CCGGCCATTCGCTTATCGAGCG		
nodB	nodBF26	CTGTCCGCTGCGACTACGC	Designed from *nodB* of *B. japonicum* USDA110 (NC_ 004463)	47
	nodBR625	CGCGCCGTTGTAGTGCTGG		
nodC	nodCF	AYGTHGTYGAYGACGGTTC	Laguerre *et al*. (2001)	41
	nodCI	CGYGACAGCCANTCKCTATTG		

Table 3 Nodulation test on various leguminous plants and genotypic characterization by Southern hybridization of nodulation genes

	Nodulation on[a]:						*nod* gene hybridization[b]		
Bradyrhizobium strains	*A. americana*	*G. max*	*A. hypogaea*	*V. radiate*	*M. atropurpureu*	*S. rostrata*	*nodA*	*nodB*	*nodC*
Divergent *nod*-containing strains									
SUTN1-2	+	0	+	+	+	0	YL	CL	JL
SUTN1-3	+	0	+	+	+	0	Y	CL	JL
SUTN1-4	+	0	+	+	+	0	YL	CL	JL
SUTN1-6	+	0	+	+	+	0	YL	CL	JL
SUTN1-8	+	0	+	+	+	0	YL	CL	J
SUTN1-12	+	0	+	+	+	0	J	CL	JL
SUTN1-13	+	0	+	+	+	0	YL	CL	JL
SUTN2-1	+	0	+	+	+	0	YL	CL	J
SUTN2-2	+	0	+	+	+	0	YL	CL	JL
SUTN2-3	+	0	+	+	+	0	YL	J	JL
SUTN2-4	+	0	+	+	+	0	YL	CL	JL
SUTN4-3	+	0	+	+	+	0	YL	CL	J
SUTN5-6	+	0	+	+	+	0	YL	CL	JL
DOA1	+	0	+	+	+	0	YL	CL	JL
DOA2	+	0	+	+	+	0	YL	CL	JL
DOA3	+	0	+	+	+	0	YL	CL	JL
DOA4	+	0	+	+	+	0	YL	CL	JL
DOA6	+	0	+	+	+	0	N	CL	JL
DOA7	+	0	+	+	+	0	YL	CL	JL
DOA8	+	0	+	+	+	0	N	CL	JL
DOA9	+	0	+	+	+	0	N	CL	JL
DOA10	+	0	+	+	+	0	YL	CL	JL
nod-containing strains									
SUTN1-7	+	0	+	+	+	0	J	J	J
SUTN1-9	+	0	+	+	+	0	J	J	J
SUTN3-1	+	0	+	+	+	0	J	J	J
SUTN4-1	+	0	+	+	+	0	J	J	J
SUTN5-1	+	0	+	+	+	0	J	J	J
SUTN5-3	+	0	+	+	+	0	J	J	J
SUTN5-5	+	0	+	+	+	0	J	J	J
SUTN6-1	+	0	+	+	+	0	J	J	J

	Nodulation on[a]:						*nod* gene hybridization[b]		
Bradyrhizobium strains	*A. americana*	*G. max*	*A. hypogaea*	*V. radiate*	*M. atropurpureu*	*S. rostrata*	*nodA*	*nodB*	*nodC*
SUTN6-2	+	0	+	+	+	0	J	J	J
SUTN7-1	+	0	+	+	+	0	J	J	J
SUTN7-2	+	0	+	+	+	0	J	J	J
SUTN8-1	+	0	+	+	+	0	J	J	J
SUTN8-2	+	0	+	+	+	0	J	J	J
SUTN9-1	+	0	+	+	+	0	J	J	J
SUTN9-2	+	0	+	+	+	0	J	J	J
SUTN9-3	+	0	+	+	+	0	J	J	J
SUTN9-4	+	0	+	+	+	0	J	J	J
SUTN9-5	+	0	+	+	+	0	J	J	J
USDA110	0	+	+	+	+	0	J	J	J
nod-independent strains									
BTAi1	0	0	+	0	0	0	N	N	N
ORS278	0	0	+	0	0	0	N	N	N

[a] +, positive with effective nodule; 0, negative.

[b] Southern blot hybridization signals are presented in Fig. S1 in the supplemental material. J, signal obtained from *B. japonicum* USDA110 under a stringent condition; YL, signal obtained from strain SUTN6-2 (99% 16S rRNA similarity to *B. yuanmingense*) under a low-stringency condition; CL, signal obtained from strain SUTN7-2 (99% 16S rRNA similarity to *B. canariense*) under a low-stringency condition; JL, signal obtained from *B. japonicum* USDA110 under a low-stringency condition and from strains SUTN6-2 and SUTN7-2 under a stringent condition; N, not detected.

Table 4 Nodulation by *Bradyrhizobium* sp. DOA9 and bacteroid morphology in various legumes.

Plants	Nodulation[a]	Nodule type	Bacteroid
Papilionoideae			
Genistoids			
Lupinus polyphyllus	(+)	determinate	unswollen
Crotalaria juncea	+	determinate	unswollen
Dalbergioids			
Aeschynomene americana (a local Thai variety)	+	determinate	unswollen
Aeschynomene indica (ecotype Tottori, Japan)	–	–	–
Aeschynomene indica (ecotype Tomeshi, Japan)	–	–	–
Aeschynomene indica (a local Thai variety)	–	–	–
Aeschynomene afraspera	+	determinate	unswollen
Aeschynomene evenia	–	–	–
Arachis hypogaea	+	determinate	swollen
Stylosanthes hamata	(+)	determinate	swollen
Millettioids			
Glycine max (cv. SJ5)	–	–	–
Macroptilium atropurpureum	(+)	determinate	unswollen
Vigna radiata	+	determinate	unswollen
Desmodium sp.	+	determinate	unswollen
Lespedeza sp.	(+)	determinate	unswollen
Indigofera tinctoria	+	determinate	unswollen
Robinioids			
Lotus japonicus (ecotype Miyagi, Japan)	(+)	determinate	unswollen
Sesbania rostrata	–	–	–

Plants	Nodulation[a]	Nodule type	Bacteroid
IRLC (inverted repeat–lacking clade)			
Medicago truncatula	–	–	–
Medicago sativa	(+)	determinate	unswollen
Trifolium repens (white clover)	–	–	–
Mimosoideae			
Mimoseae			
Mimosa pudica	–	–	–
Leucaena leucocephala	(+)	determinate	unswollen
Neptunia natans	–	–	–
Ingeae			
Samanea saman	(+)	determinate	unswollen

[a] +, positive with effective nodule; (+), positive with ineffective nodule; –, negative.

Table 5 Chemical and physical properties of the soils used for rice cultivation.

Origin	pH	Soil Texture				Organic matter	Phosphorus	Potassium	Calcium	Magnesium
		%Sand	%Silt	%Clay	Texture	%	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})
Paddy soil	7.8	65	16	19	SL	0.9	39	70	3520	420
Forest soil	7.7	51	14	35	SC	2	11	330	7600	1180

Texture; SL = sandy loam soil, SC = sandy clay soil.

Table 6 Quantification of bacteria isolated from fresh rice tissue (means of three replicates, ± standard error)*.

Sample	CFU g^{-1} fresh weight	Sample	CFU g^{-1} fresh weight	Sample	CFU g^{-1} fresh weight
Root					
SFR	$1.09 \pm 0.09 \times 10^6$	VFR	$5.10 \pm 0.10 \times 10^5$	RFR	$2.65 \pm 0.43 \times 10^5$
SNR	$2.25 \pm 0.05 \times 10^6$	VNR	$3.51 \pm 0.09 \times 10^6$	RNR	$1.81 \pm 0.15 \times 10^5$
SUR	$1.11 \pm 0.09 \times 10^6$	VUR	$1.42 \pm 0.09 \times 10^6$	RUR	$1.27 \pm 0.12 \times 10^6$
Stem					
SFS	$1.02 \pm 0.02 \times 10^5$	VFS	$0.11 \pm 0.11 \times 10^4$	RFS	$2.11 \pm 0.12 \times 10^4$
SNS	$3.03 \pm 0.98 \times 10^5$	VNS	$1.39 \pm 0.03 \times 10^4$	RNS	$1.51 \pm 0.08 \times 10^5$
SUS	$2.28 \pm 0.09 \times 10^5$	VUS	$3.71 \pm 0.15 \times 10^3$	RUS	$2.43 \pm 0.18 \times 10^4$
Leaf					
SFL	$2.40 \pm 0.26 \times 10^3$	VFL	$2.10 \pm 0.13 \times 10^3$	RFL	$1.66 \pm 0.11 \times 10^3$
SNL	$2.72 \pm 0.08 \times 10^4$	VNL	$3.78 \pm 0.09 \times 10^3$	RNL	$2.58 \pm 0.09 \times 10^3$
SUL	$6.06 \pm 0.92 \times 10^3$	VUL	$1.28 \pm 0.11 \times 10^3$	RUL	$2.62 \pm 0.35 \times 10^3$

Abbreviations: First letter is growth stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is type of soil (F; fertilized, N; unfertilized, U; uncultivated soil), and third letter is rice part (R; root, S; stem, L; leaf).

* The endophytic bacterial population was significantly affected ($P \leq 0.05$) by the type of soil ($F_{2,54} = 709.68$), the growth stage of rice ($F_{2,54} = 552.87$), the rice part ($F_{2,54} = 5985.467$), and by the interaction of these three factors ($F_{8,54} = 404.45$).

Table 7 Nitrogen fixation activity and putative identity based on 16S rRNA gene full length sequence analysis of some consortium cultures and single culturable N_2-fixing endophytic bacteria from rice*.

Consortium/Single isolate	N_2-fixing activity	Putative identity
SFS2-2(consortium)	2,201.67[d]	
SFS2-2(1)	1,738.50[c]	99% *Enterobacter dissolvens* LMG 2683
SFS2-2(2)	7,395.70[h]	98% *Brevundimonas aurantiaca*
VNS3-1(consortium)	5,840.70[e]	
VNS3-1(1)	18.34[a]	99% *Pantoea agglomerans* WAB1927
VNS3-1(2)	23,319.17[i]	99% *Enterobacter dissolvens* LMG 2683
RUL2-2(consortium)	949.24[b]	
RUL2-2(1)	6,123.88[f]	99% *Pseudomonas sp.* BWDY-42
RUL2-2(2)	6,355.38[g]	99% *Enterobacteriaceae bacterium* ZHS050721

N_2-fixing activity = Ethylene concentration (nmol C_2H_4 $h^{-1}tube^{-1}$).

Abbreviations: First letter is growth stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is type of soil (F; fertilized, N; unfertilized, U; uncultivated soil), and third letter is rice part (R; root, S; stem, L; leaf).

* Values followed by the same letter are not significantly different at $P \leq 0.05$.

Table 8 Rice cultivar used in this study, geographical origin and relevant characteristics.

Rice cultivars	Geographical origin (province in Thailand/Japan)	Relevant characteristics	Source or reference
O. sativa* L. ssp. *Indica			
Khao Dowk Mali 105	Chonburi/Thailand	Photosensitivity, lowland rice	Sommai Lertna [a]
Leuang Yai 148	Chiangmai/Thailand	Photosensitivity, lowland rice	Sommai Lertna [a]
Lu Ni	Nakhonratchasima/Thailand	Photosensitivity, floating rice	Sommai Lertna [a]
Pathum Thani 1	Pathumthani/Thailand	Non-photosensitivity, lowland rice	Sommai Lertna [a]
Sang Yod Phatthalung	Phatthalung/Thailand	Photosensitivity, lowland rice	Sommai Lertna [a]
Kasalash	Japan	Non-photosensitivity, lowland rice	Kiwamu Minamisawa [b]
O. sativa* L. ssp. *Japonica			
Nipponbare	Japan	Non-photosensitivity, lowland rice	Kiwamu Minamisawa [b]

[a] Nakornratchasima Rice Research Center, Phimai, Nakhonratchasima, 30110, Thailand

[b] Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan

Table 9 Strains used in this study, sampling site and relevant characteristics.

Strain	Geographical origin (province in Thailand/Japan) and coordination	Relevant characteristics and source of isolation	Source or reference
Strains			
Bradyrhizobium sp.			
SUT-R3	Chiang Mai 19° 13' 11.3"N, 98° 50'51.7"E	Rice root isolate (rice with crop rotation)	This study
SUT-PR9	Uttaradit 17° 39' 1.9"N, 100° 8' 34.2"E	Rice root isolate/paddy soil isolate (rice with crop rotation)	This study
SUT-PR48	Surin 14°39'41.3"N, 103°17'09.5"E	Rice root isolate (rice with crop rotation)	This study
	Prachuap Khiri Khan 12°36'43.4"N, 99°51'40.5"E	Rice root isolate (rice with crop rotation)	
SUT-R55	Lampang 18° 49' 42.5"N, 99° 56' 37.2"E	Rice root isolate (rice with crop rotation)	This study
SUT-PR64	Khonkaen 16° 14' 13.5"N, 102° 31' 31.1"E	Rice root isolate (rice with crop rotation)	This study
SUT-R74	Chiangrai 19° 22' 31.7"N, 99° 30' 5.9"E	Rice root isolate/paddy soil isolate (rice with crop rotation)	This study
DOA1	Lopburi	*A. americana* nodule isolate (paddy crop)	Noisangiam *et.al.*(2012)
DOA9	Lopburi	*A. americana* nodule isolate (paddy crop)	Noisangiam *et.al.*(2012)
SUTN9-2	Lampang	*A. americana* nodule isolate (paddy crop)	Noisangiam*et.al.*(2012)
WD16	Kashimadai city, Miyagi prefecture, Japan	Rice root isolate (rice monoculture)	Unpublished Ryo Shinoda
RP5	Kashimadai city, Miyagi prefecture, Japan	Rice root isolate (rice monoculture)	Unpublished Ryo Shinoda
RP7	Kashimadai city, Miyagi prefecture, Japan	Rice root isolate (rice monoculture)	Unpublished Ryo Shinoda

Table 10 Sequences of DNA primers for PCR

Target gene	Primer name	Primer sequence (5'→3')	Reference or description of design	Annealing temperature used (C^o)
16S rRNA	fD1	AGAGTTTGATCCTGGCTCAG	(Okubo *et al.*, 2013)	55
	rP2	ACGGCTACCTTGTTACGACTT		
BOXA1R	BOXA1R	CTACGGCAAGGCGACGCTGAC	(Stephens and Rask, 2000)	63
nodA	nodAF28	GAAGGATCTTCTGGGCGCG	(Abbasi *et al.*, 2008)	45
	nodAR627	CTCAGGCCCGTTACGATCG		
	nodAF25-ORS285	GTATGCTGGGAGAGTGATCTCG	(Abbasi *et al.*, 2008)	46
	nodAR584-ORS285	GGCCCATTCCTCTCAATCGTTG		
nodB	nodBF26	CTGTCCGCTGCGACTACGC	(Abbasi *et al.*, 2008)	47
	nodBR625	CGCGCCGTTGTAGTGCTGG		
	nodBF73-ORS285	CTGACATTTGACGATGGGCCCG	(Abbasi *et al.*, 2008)	53
	nodBR622-ORS285	GCCCATGAAGAGCTGGGATCAG		
nodC	nodCF195	CGCCGAATGTCTGGAGTCG	(Abbasi *et al.*, 2008)	45
	nodCR1394	CCTGAGTCATCAGCCGACC		
	nodCF197-ORS285	TGGCGTGCCTAGAGTCGATTGC	(Abbasi *et al.*, 2008)	53
	nodCR1196-ORS285	CACGGTGATTTGCGCGACAACC		
nifH	nifHF	TACGGNAARGGSGGNATCGGCAA	(Saito *et al.*, 1998)	48
	nifHI	AGCATGTCYTCSAGYTCNTCCA		

Sequences of DNA primers for PCR

Abbasi, M.K., A. Majeed, A. Sadiq and S.R. Khan. 2008. Application of bradyrhizobium japonicum and phosphorus fertilization improved growth, yield and nodulation of soybean in the sub-humid hilly region of azad jammu and kashmir, pakistan. *Plant production science*, 11(3): 368-376.

Okubo, T., S. Fukushima, M. Itakura, K. Oshima, A. Longtonglang, N. Teaumroong, H. Mitsui, M. Hattori, R. Hattori and T. Hattori. 2013. Soil oligotrophic bacterium agromonas oligotrophica (bradyrhizobium oligotrophicum) is a nitrogen-fixing symbiont of aeschynomene indica as suggested by genome analysis. *Applied and environmental microbiology*: AEM. 00009-00013.

Saito, A., H. Mitsui, R. Hattori, K. Minamisawa and T. Hattori. 1998. Slow‐growing and oligotrophic soil bacteria phylogenetically close to bradyrhizobium japonicum. *FEMS Microbiology Ecology*, 25(3): 277-286.

Stephens, J. and H. Rask. 2000. Inoculant production and formulation. *Field Crops Research*, 65(2): 249-258.

Table 11 Physical and chemical properties of peat and compost

Materials	pH	Organic matter (%)	N (%)	P (%)	K (%)	Initial Moisture (%)
Peat	4.5	62.23	1.19	1.73	0.5	8.07
Compost	7.49	14.13	2.19	3.22	0.77	6.78

Peat was adjusted to pH 7.0 by using $CaCO_3$ before using in the experiments.

Table S1. Symbiotic phenotype of *Bradyrhizobium* sp. DOA9 with various legumes.

Plants	Strain	No. of root Nodules/plant	Plant dry weight g/plant	Fixation activity[c] nmol /g plant DW
C. juncea	No inoculum	0.00	135.00±1.13	0.00
	DOA9	9.56±7.51	249.66±66.21*	0.03±0.04
A. americana	No inoculum	0.00	5.30±0.32	0.00
	DOA9	7.78±4.60	13.81±2.75**	1,070.53±490.35
A. afraspera	No inoculum	0.00	152.85±16.34	0.00
	DOA9	36.83±5.48	431.33±51.02**	1.50±0.22
A. hypogaea	No inoculum	0.00	933.60±11.96	0.00
	DOA9	67.00±11.52	1,538.18±116.88**	0.91±0.18
S. hamata	No inoculum	0.00	7.03±1.65	0.00
	DOA9	11.00±1.87	6.48±1.16	1,144.17±219.74
M. atropurpureum	No inoculum	0.00	56.93±6.04	0.00
	DOA9	17.33±4.13	46.72±10.96	98.03±16.20
V. radiata	No inoculum	0.00	284.20±2.90	0.00
	DOA9	16.14±1.40	363.80±36.41**	4.81±0.77

Desmodium sp.	No inoculum	0.00	821.50±10.85	0.00
	DOA9	57.00±9.42	1,427.17±105.76**	0.80±0.17
Lepedeza sp.	No inoculum	0.00	13.03±1.20	0.00
	DOA9	5.63±1.94	9.50±2.26	395.67±189.73
I. tinctoria	No inoculum	0.00	11.53±0.95	0.00
	DOA9	9.56±1.35	30.13±4.24**	72.42±20.61
L. japonicus	No inoculum	0.00	7.45±0.54	0.00
	DOA9	2.58±1.63	7.63±0.48	248.17±139.13
L. leucocephala	No inoculum	0.00	152.50±16.12	0.00
	DOA9	1.33±0.58	135.12±13.16	27.40±22.22
S. saman	No inoculum	0.00	355.75±20.00	0.00
	DOA9	67.50±14.55	428.07±52.43	237.85±77.50

The number of nodules, plant dry weight and acetylene reduction assay was measured 35 days after inoculation.

** and * are mean significant difference of plant dry weight in each plant host at 1% and 5%, respectively according to t-test.

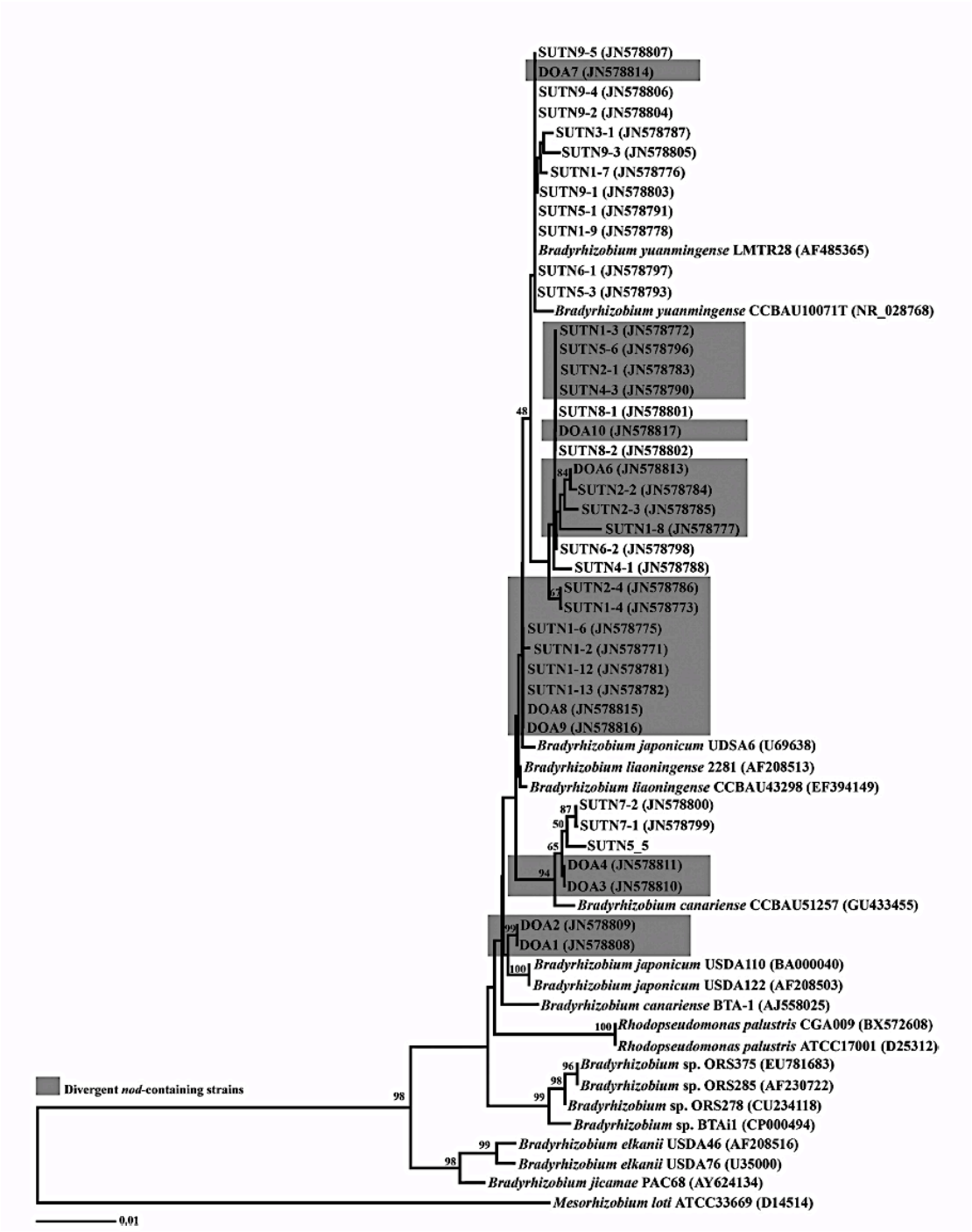

Fig. 1 Neighbor-joining trees based on sequences of 16S rRNA genes showing classification of the *Bradyrhizobium* strains isolated from nodules of *A. americana*. Bootstrap values are expressed as percentages of 1,000 replications. The bar represents 1 estimated substitution per 100 nucleotide positions. The evolutionary distances were computed using the Kimura two-parameter method and are shown in the units representing the number of base substitutions per site.

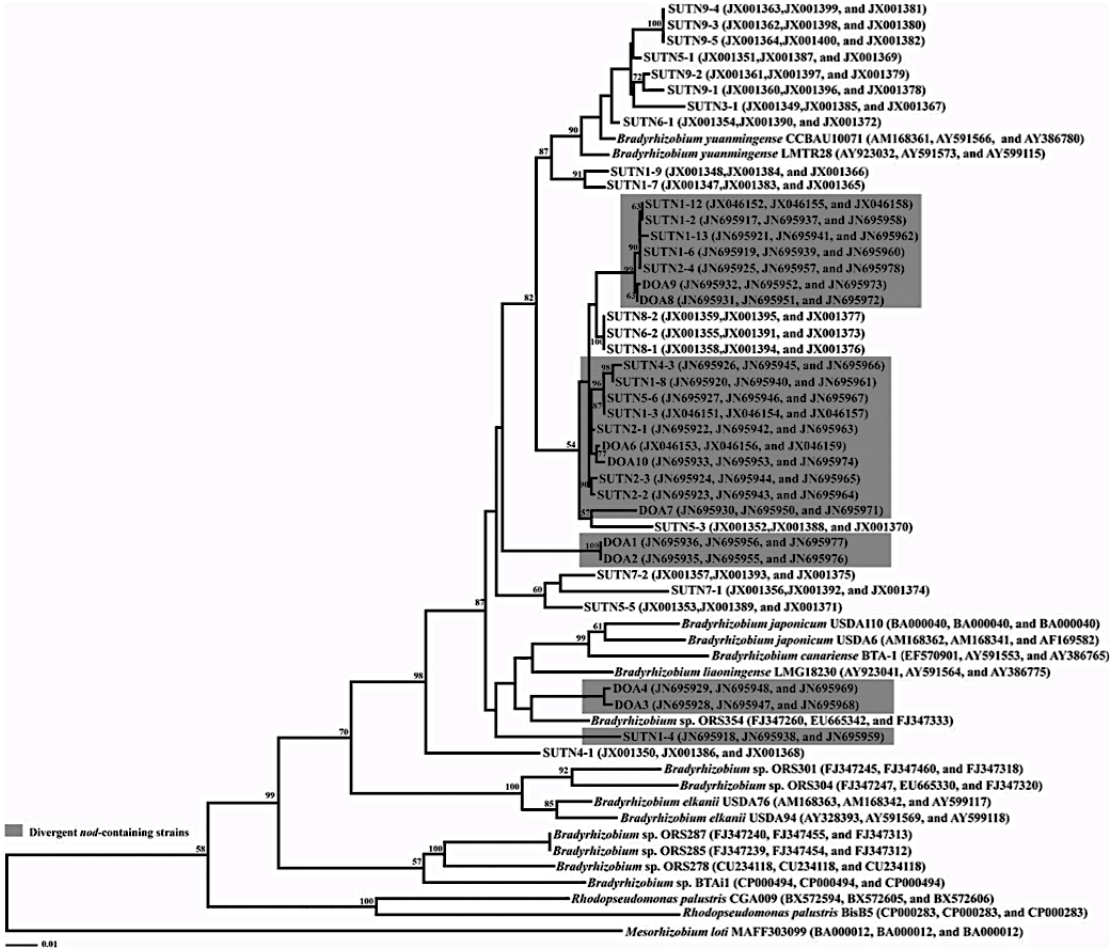

Fig. 2 Maximum likelihood tree based on combined sequences of housekeeping genes (*dnaK*, *recA*, and *glnB*), showing classification of divergent *nod*-containing *Bradyrhizobium* strains isolated from nodules of *A. americana*. Bootstrap value is expressed as percentages of 1,000 replications. The bar represents 1 estimated substitution per 100 nucleotide positions.

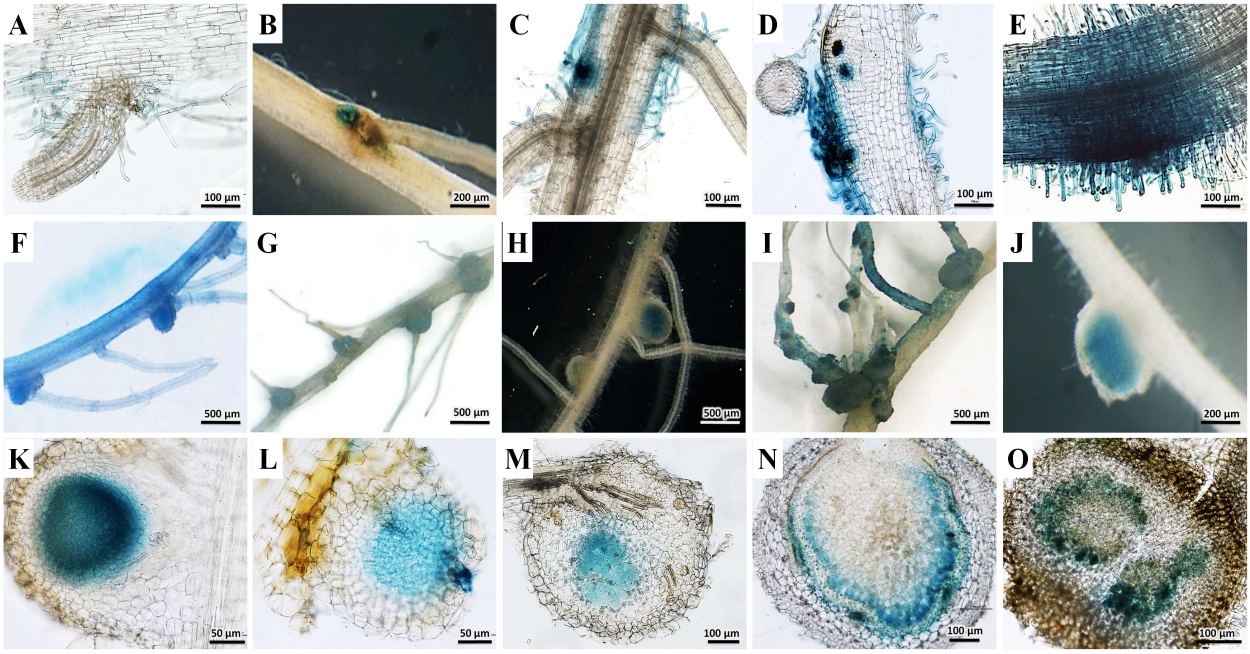

Fig. 3 Examples of (A–E) root colonization, (F–J) nodule morphology, and (K–O) thin sections of nodules inoculated with GUS-tagged DOA9 in various legumes. (A, F, and K); *Aeschynomene americana*, (B, G, and L); *Stylosanthes hamate*, (C, H, and M); *Indigofera tinctoria,* (D, I, and N) *Macroptilium atropurpureum,* (E, J, and O) *Lotus japonicus*.

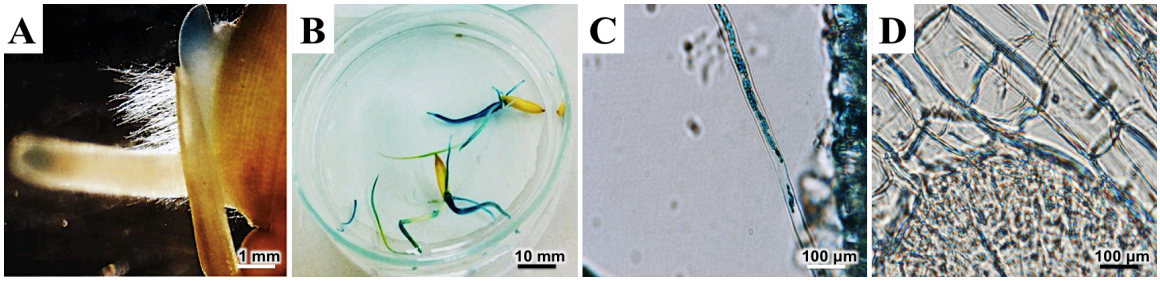

Fig. 4 Light microscopic observations of *Oryza sativa* ssp. *indica* seedlings inoculated with GUS-tagged *Bradyrhizobium* sp. DOA9. (A) 1 day after inoculation. (B) 5 days after inoculation. (C) Close-up view of root hair and (D) close-up view of root tissue at 5 days after inoculation.

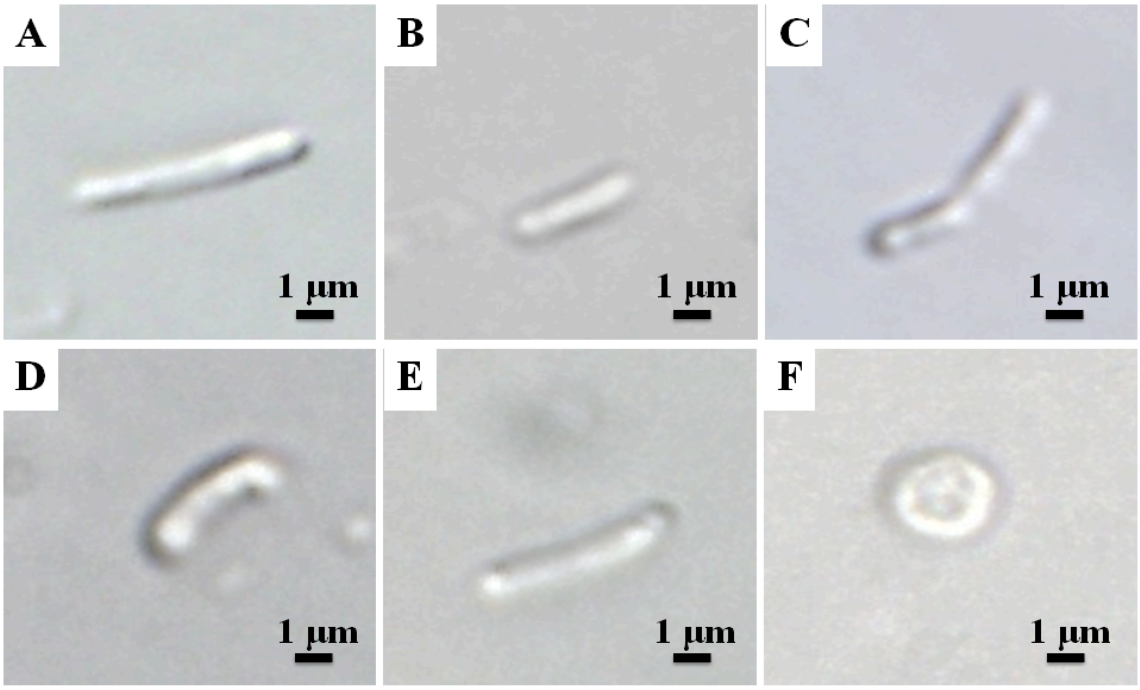

Fig. 5 Examples of differential interference contrast (DIC) views of *Bradyrhizobium* sp. DOA9 bacteroids. (A) Free-living. (B–F) Bacteroids isolated from (B) *Indigofera tinctoria*, (C) *Macroptilium atropurpureum*, (D) *Aeschynomene americana*, (E) *Aeschynomene afraspera*, and (F) *Arachis hypogaea*.

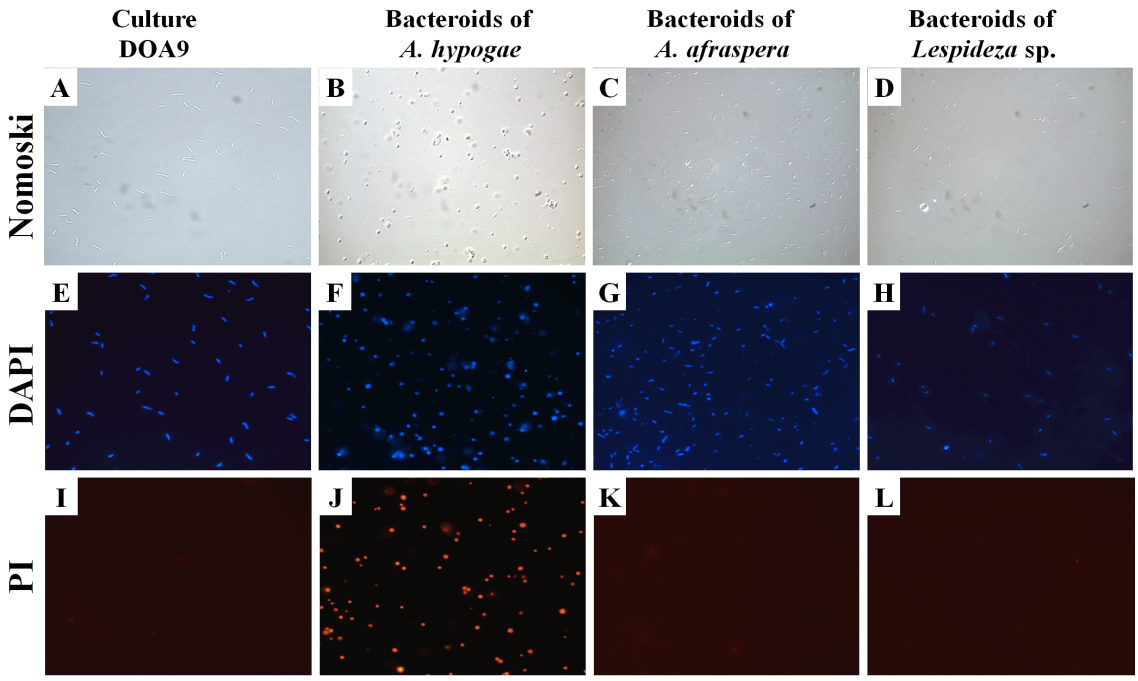

Fig. 6 Examples of shapes of (left-hand column) free-living DOA9 bacteria and (other columns) resident bacteroids isolated from nodules of (B, F, J) *Arachis hypogaea*, (C, G, K) *Aeschynomene afraspera*, and (D, H, L) *Lespedeza* sp. DIC, differential interference contrast microscopy; DAPI, fluorescence microscopy with 4′,6-diamidino-2-phenylindole stain; PI, fluorescence microscopy with propidium iodide stain.

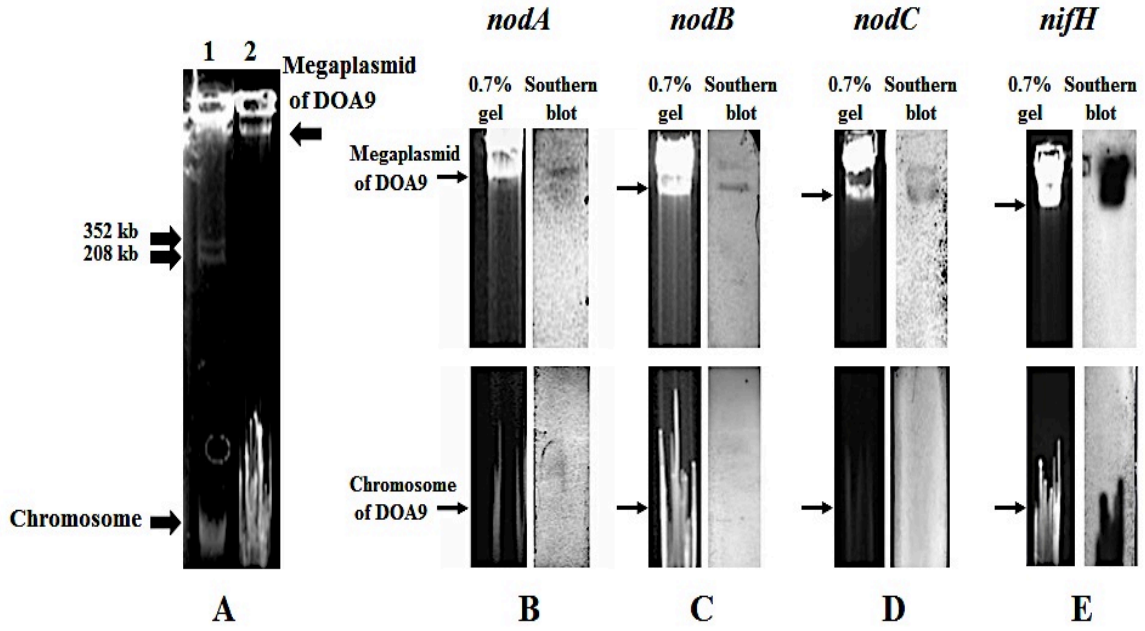

Fig. 7 Determination of *Bradyrhizobium* sp. DOA9 replicons and symbiosis genes. (A) Megaplasmid profiles: lane 1, *Mesorhizobium loti* MAFF303099 (208 and 352 kb); lane 2, DOA9; (B–E) Southern blot hybridization signals of nodulation and N-fixation genes on megaplasmid and chromosome from DOA9 under low stringency conditions: (B) *nodA*; (C) *nodB*; (D) *nodC*; (E) *nifH*.

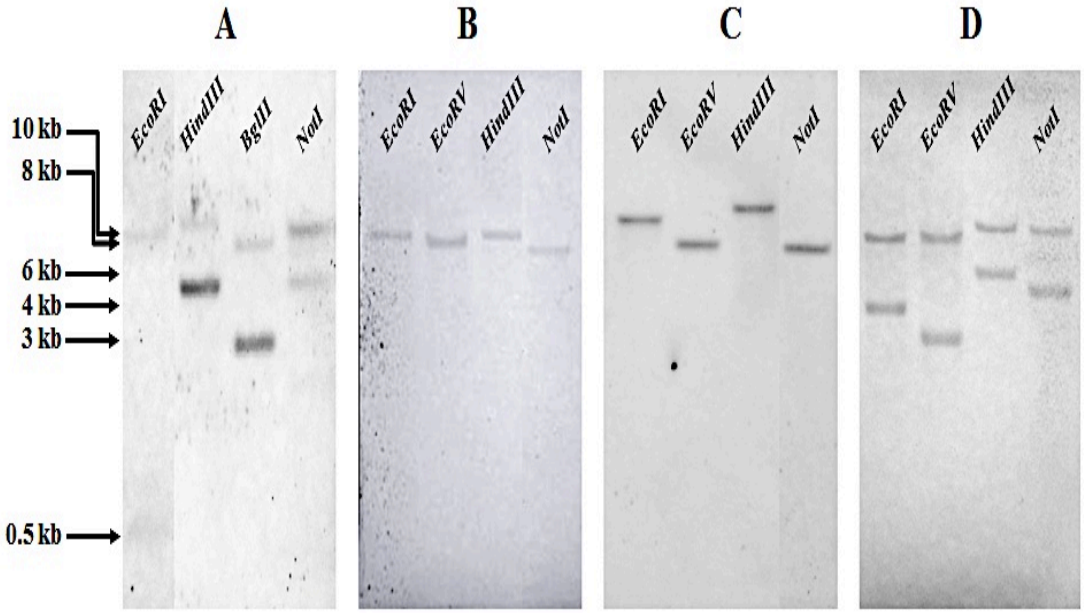

Fig. 8 Determination of copy numbers of (A) *nodA*, (B) *nodB*, (C) *nodC*, and (D) *nifH* by Southern blot hybridization. *Bradyrhizobium* sp. DOA9 genomic DNA was digested with the restriction enzymes shown, and the blot was hybridized with probes for *nodA* (from *B. yuanmingense* SUTN6-2), *nodB* (from *B. canariense* SUTN7-2), *nodC* (from *B. japonicum* USDA110), and *nifH* (from *B. yuanmingense* SUTN6-2).

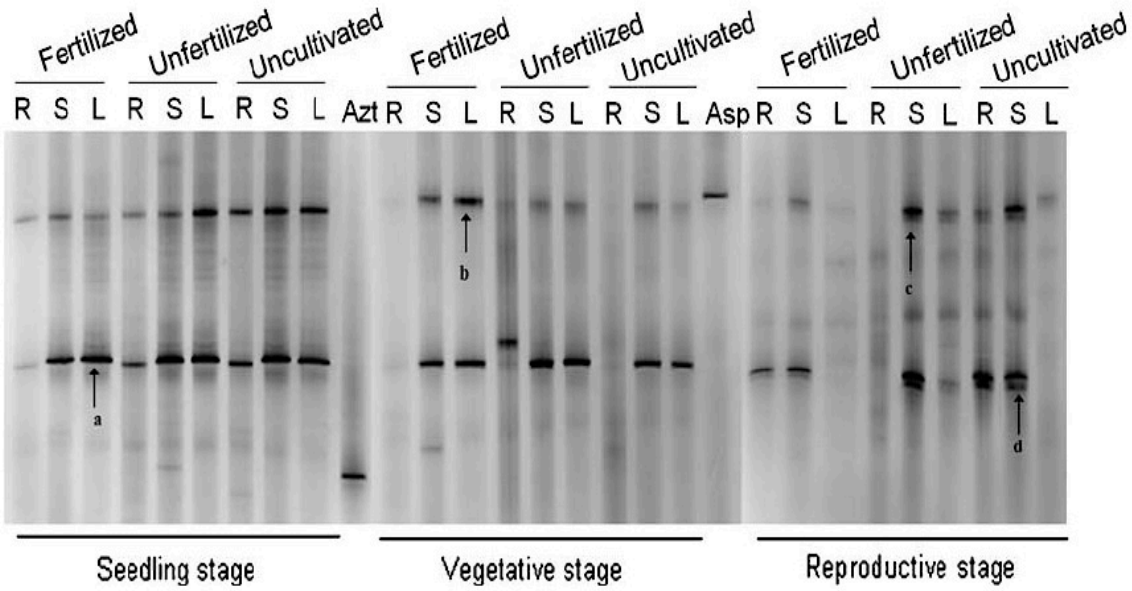

Fig. 9 PCR-DGGE analysis of 16S rRNA gene banding patterns from rice endophytic bacteria in samples from plants grown in different types of soil (fertilized paddy soil, unfertilized paddy soil and uncultivated forest soil). Arrows show excised and sequenced bands (a–d). Azt., Azotobacter sp.; Asp., Azospirillum sp.; R, root; S, stem; L, leaf.

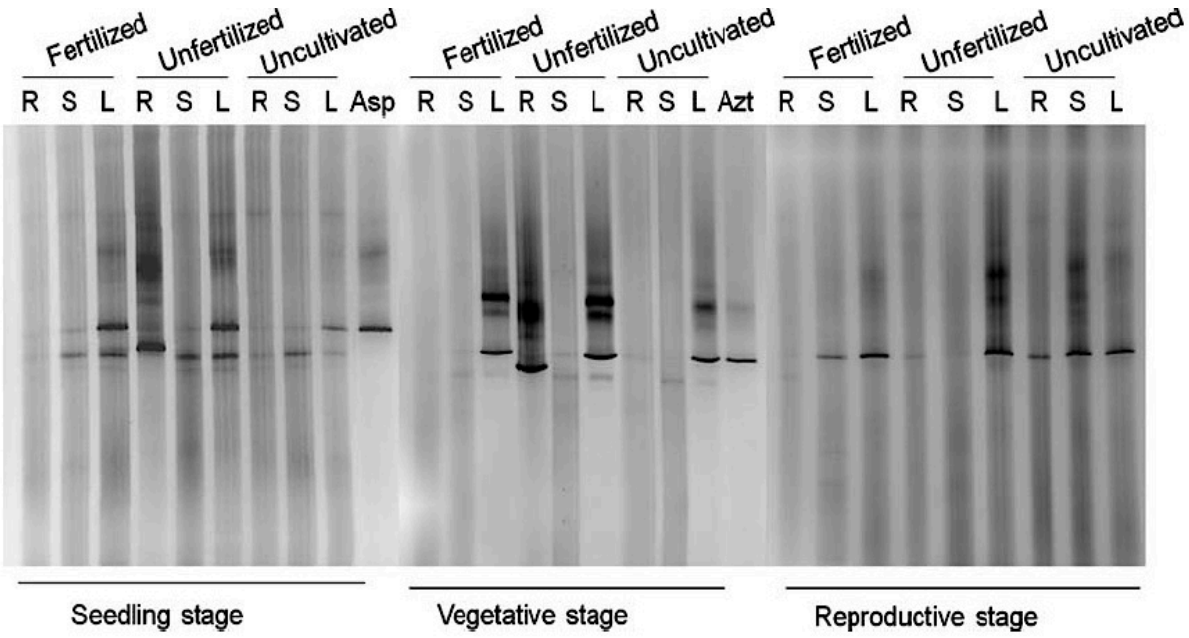

Fig. 10 Nested PCR-DGGE of *nifH* banding patterns from rice endophytic bacteria. R, root; S, stem; L, leaf; types of soil.

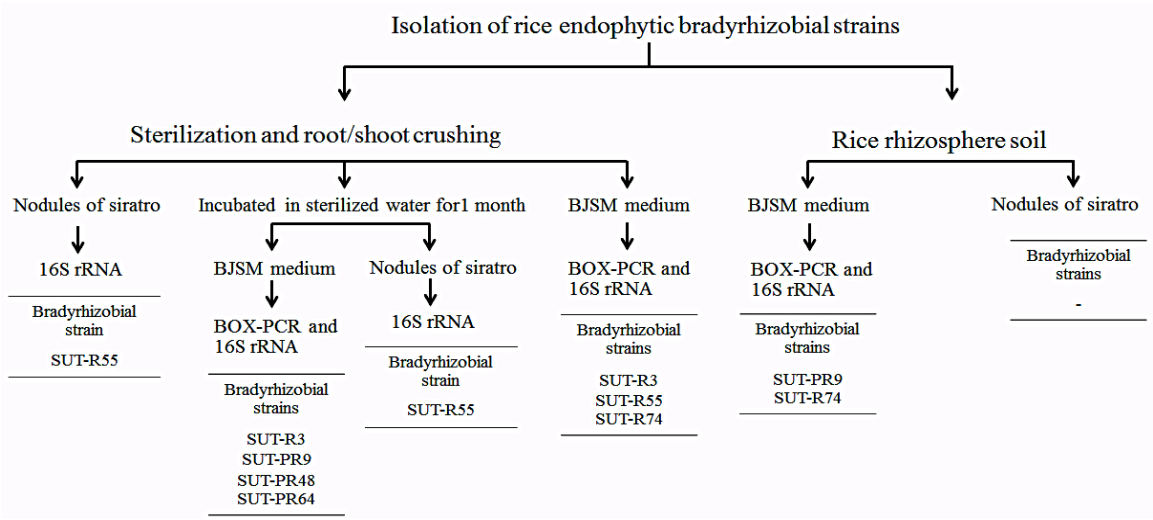

Fig. 11 Scheme for the isolation of endophytic bradyrhizobial strains: Name of strains with "P" means photosynthetic bradyrhizobial strain while without "P" indicating the non-photosynthetic bradyrhizobial strain.

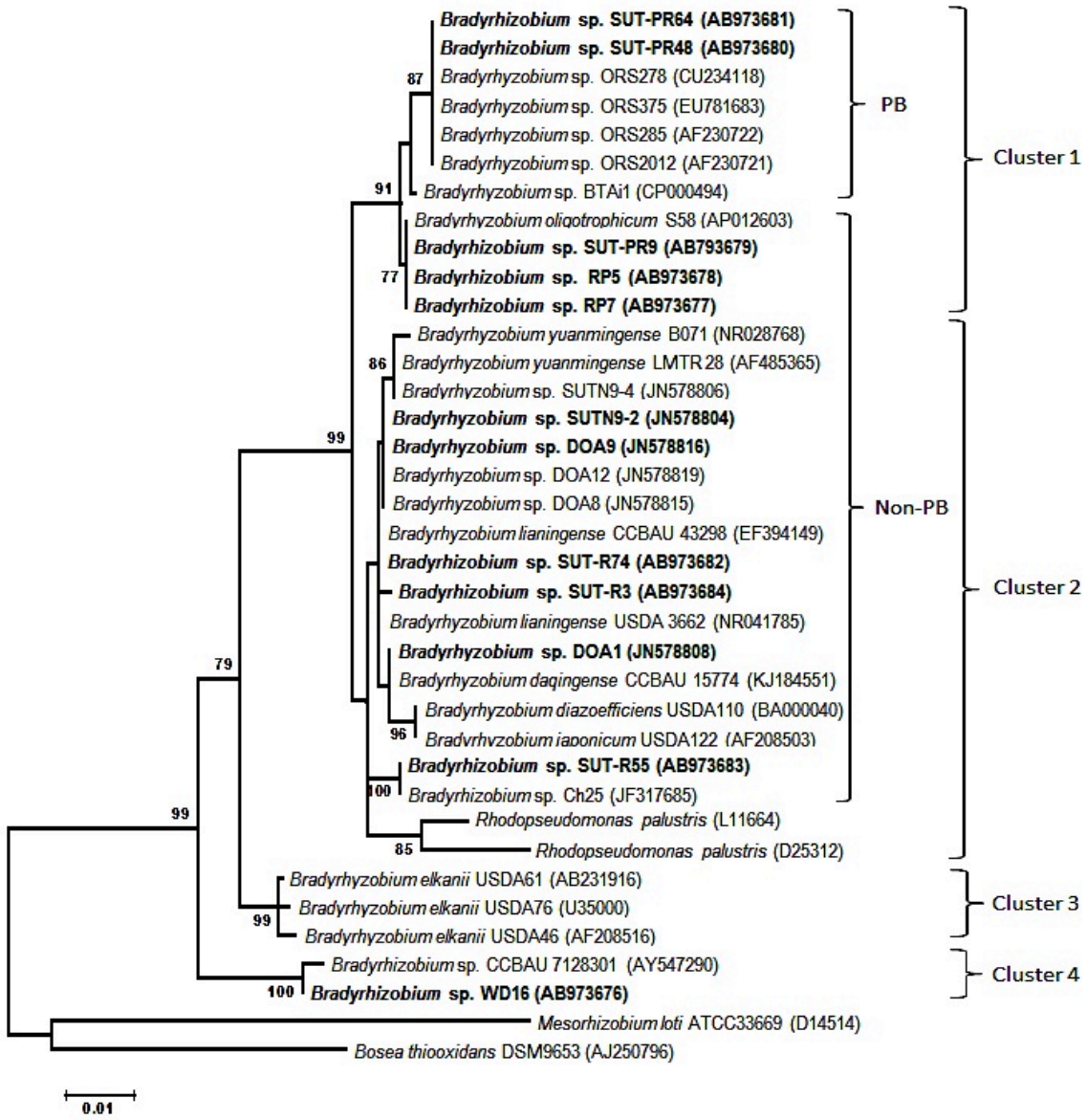

Fig. 12 Neighbor-joining trees based on sequences of 16S rRNA genes showing classification of the photosynthetic bradyrhizobia (PB) and non-photosynthetic bradyrhizobia (Non-PB) strains isolated from rice tissues. Bootstrap values are expressed as percentages of 1,000 replications. The bar represents 1 estimated substitution per 100 nucleotide positions. The evolutionary distances were computed using Kimura two-parameter method and are shown in the units representing the number of base substitutions per site.

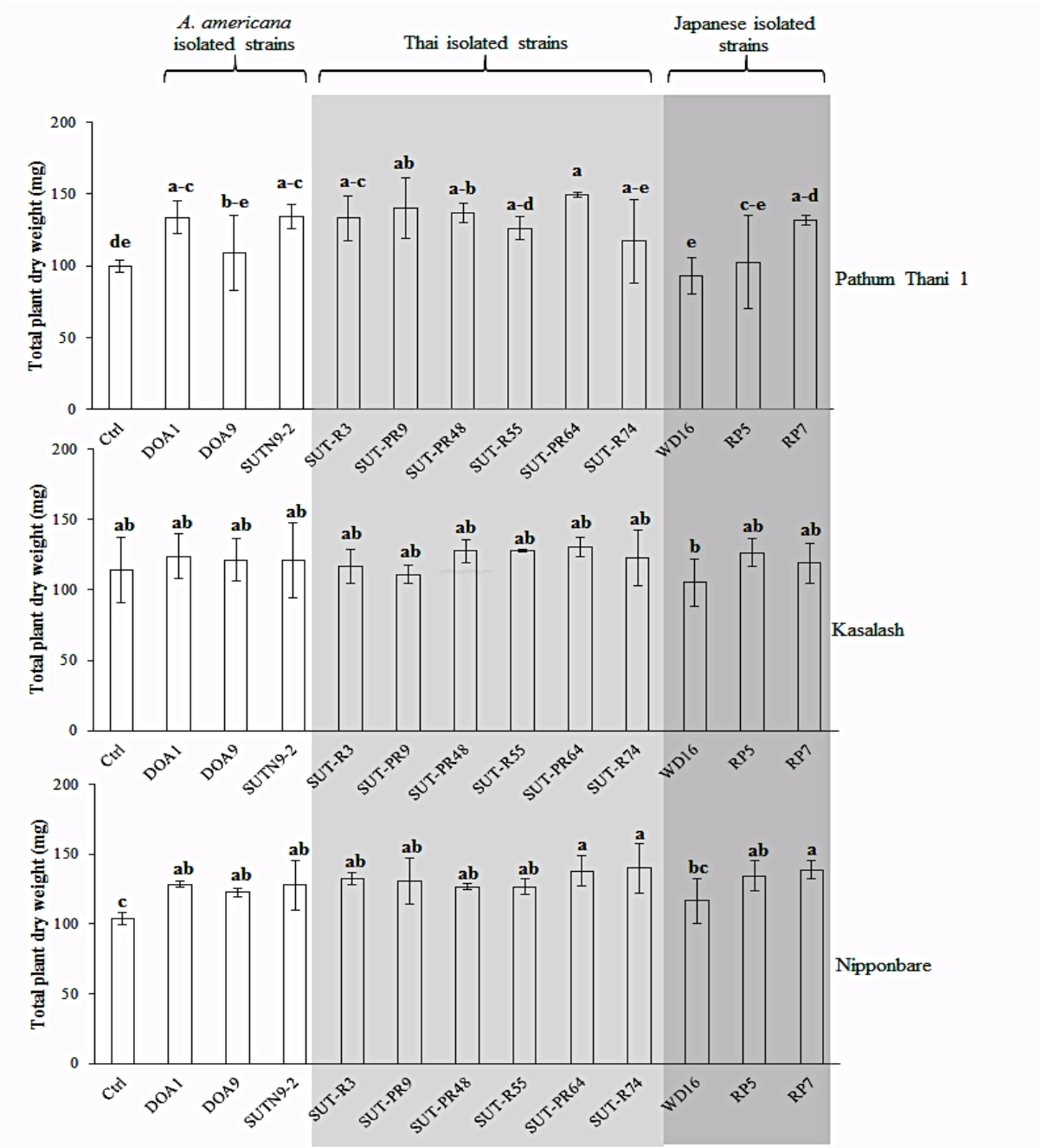

Fig. 13 The effects of putative rice endophytic bradyrhizobium on rice biomass in Leonard's jar were compared among three rice cultivars (*O. sativa* L. ssp. *indica* cv. Pathum Thani 1, *O. sativa* L. ssp. *indica* cv. Kasalash and *O. sativa* L. ssp. *japonica* cv. Nipponbare). The clear box represent; *A. americana* isolated strains, light gray box; Thai strain, and dark gray box; Japanese strain.

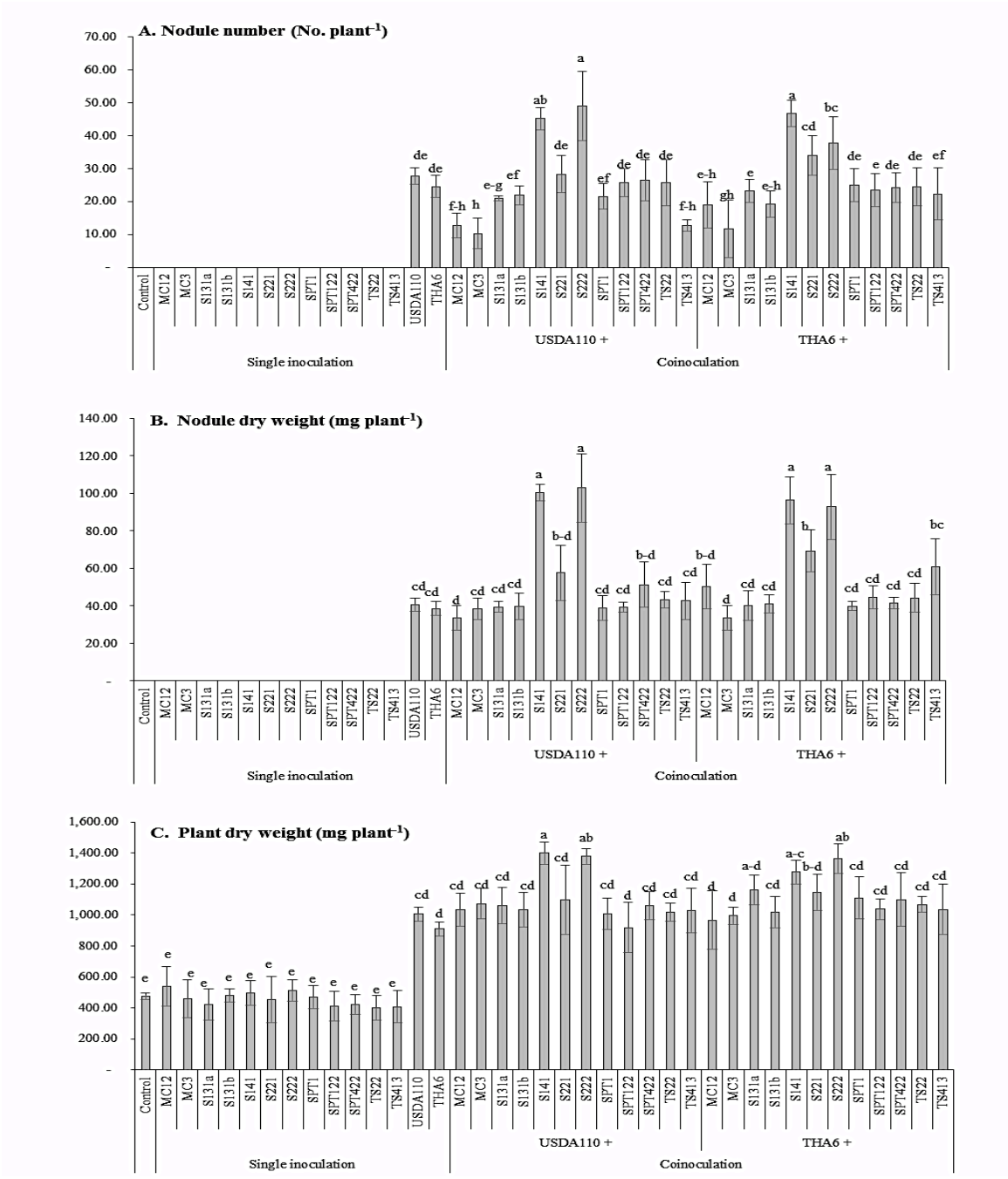

Fig. 14 Nodule number per plant (A) nodule dry weight per plant (B) and plant dry weight (C) of soybean after single and coinoculated between *B. diazoefficiens* USDA110 or *B. japonicum* THA6 with 12 selected PGPR isolates at 45 DAI. Treatments are represented by Control (non-inoculated); USDA110 (*B. diazoefficiens* USDA110); THA6 (*B. japonicum* THA6); + (coinoculation treatment). Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

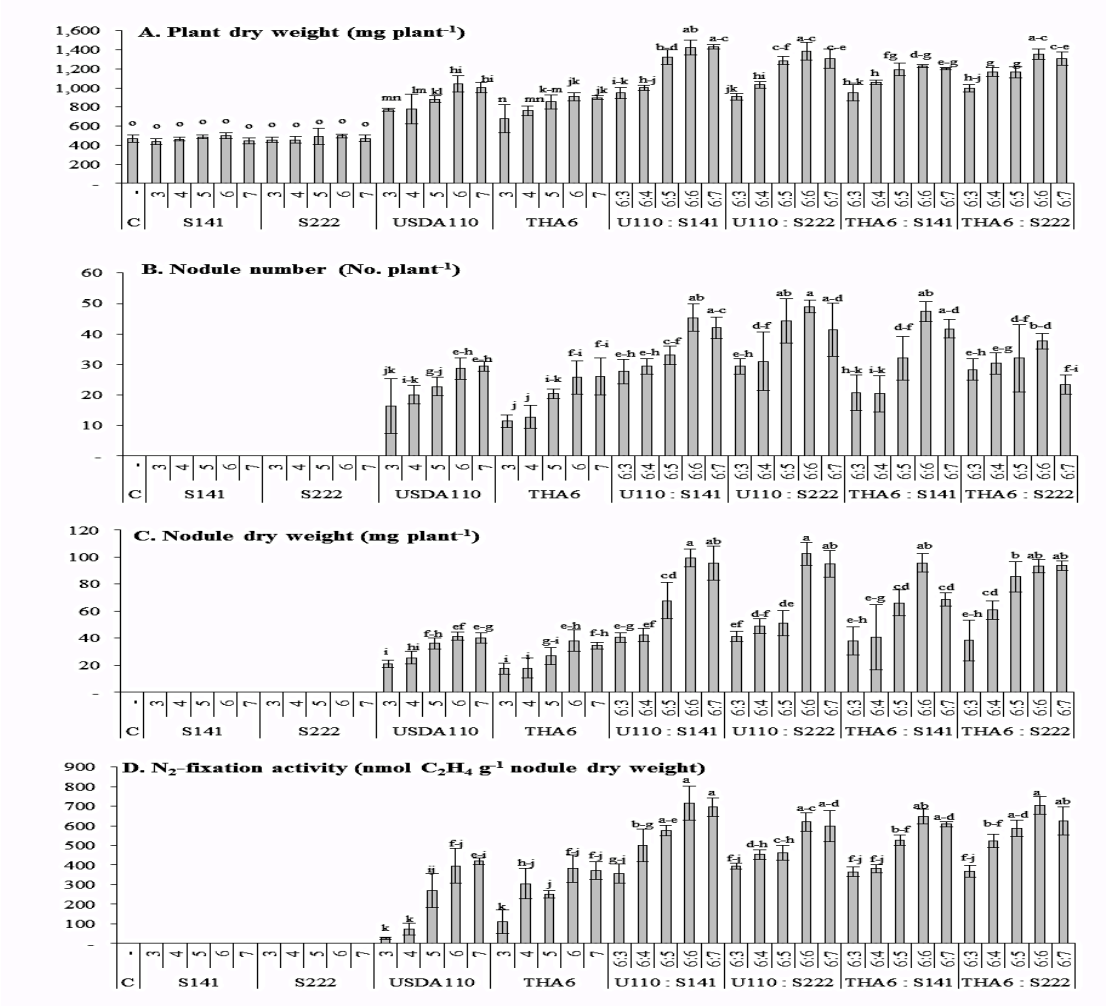

Fig. 15 Effects of inoculation dose between *B. diazoefficiens* USDA110 or *B. japonicum* THA6 at 10^6 cells $seed^{-1}$ with various dose of PGPR isolates S141 and S222 on soybean plant dry weight (A) nodule number per plant (B) and nodule dry weight per plant (C) and N_2-fixing activity (nmol C_2H_4 g^{-1} nodule dry weight) (D). The numbers at the x-axis symbolize the varied inoculation doses of PGPR are 10^3 - 10^7 CFU ml^{-1} $seed^{-1}$ and the coinoculation ratio between Bradyrhizobia at 10^6 CFU ml^{-1} $seed^{-1}$ and varies inoculation dose of PGPR from 10^3 – 10^7 CFU ml^{-1} $seed^{-1}$. Treatments are represented by Control (non-inoculated); U110 (*B. diazoefficiens* USDA110); THA6 (*B. japonicum* THA6); + (coinoculation treatment). Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

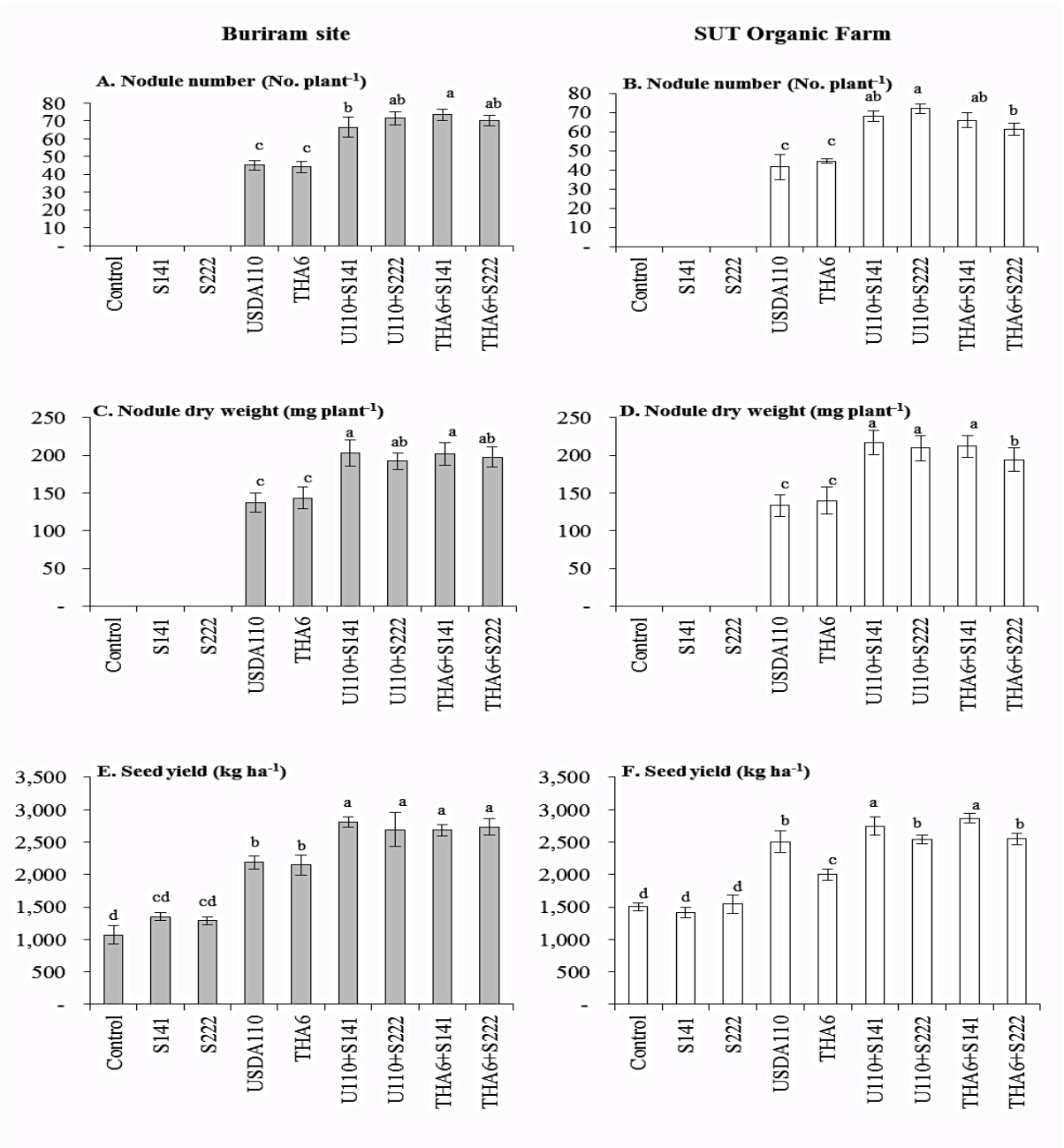

Fig. 16 Effects of coinoculation between *B. diazoefficiens* USDA110 or *B. japonicum* THA6 with PGPR isolates S141 and S222 at 10^6:10^6 cells seed^{-1} on nodule number per plant (A-B), nodule dry weight per plant (C-D), and soybean seed yield (E-F) performed at Buriram site (panel A, C, and E) and SUT Organic Farm site (panel B, D, and F), respectively. Data represent the means of nine experiments, each with three replicates. Values represent mean ± SD (n=3). Within treatment, means labeled with different letters are statistically different at $P<0.05$.

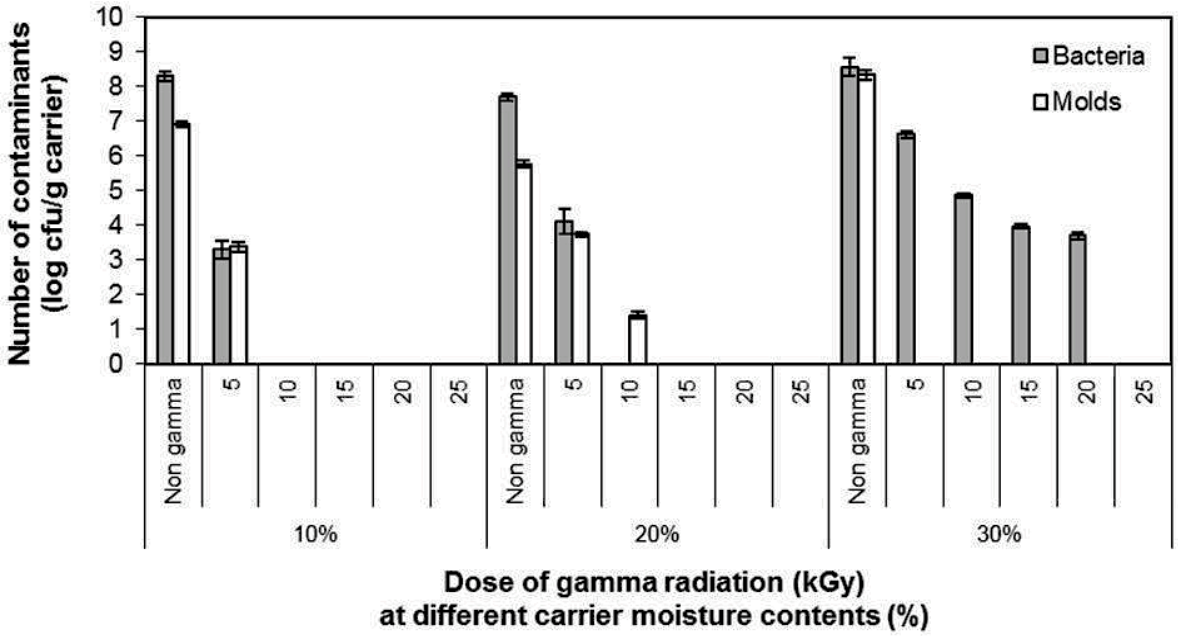

Fig. 17 Number of microbial contaminants, bacteria (grey bars) and molds (white bars) remaining in compost with moisture contents of 10, 20, and 30%, and sterilization by gamma irradiation at dose rates of 0 (non gamma), 5, 10, 15, 20, and 25 kGy.

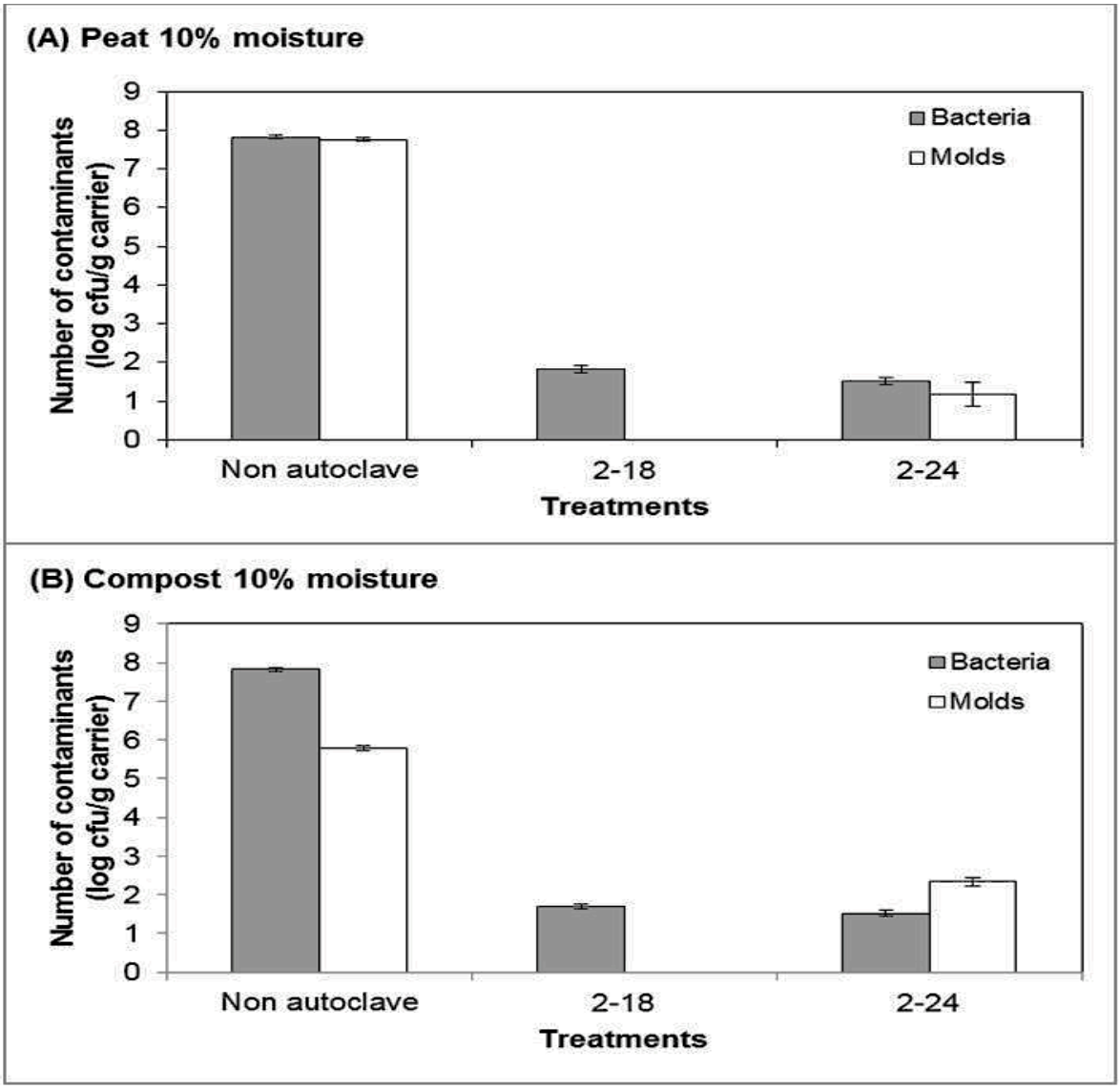

Fig. 18 Number of microbial contaminants, bacteria (grey bars) and molds (white bars) remaining in (A) peat and (B) compost without sterilization (non autoclave) and with sterilization by autoclaving two times with the holding period of 18 h (2-18) and 24 h (2-24) between each time of sterilization.

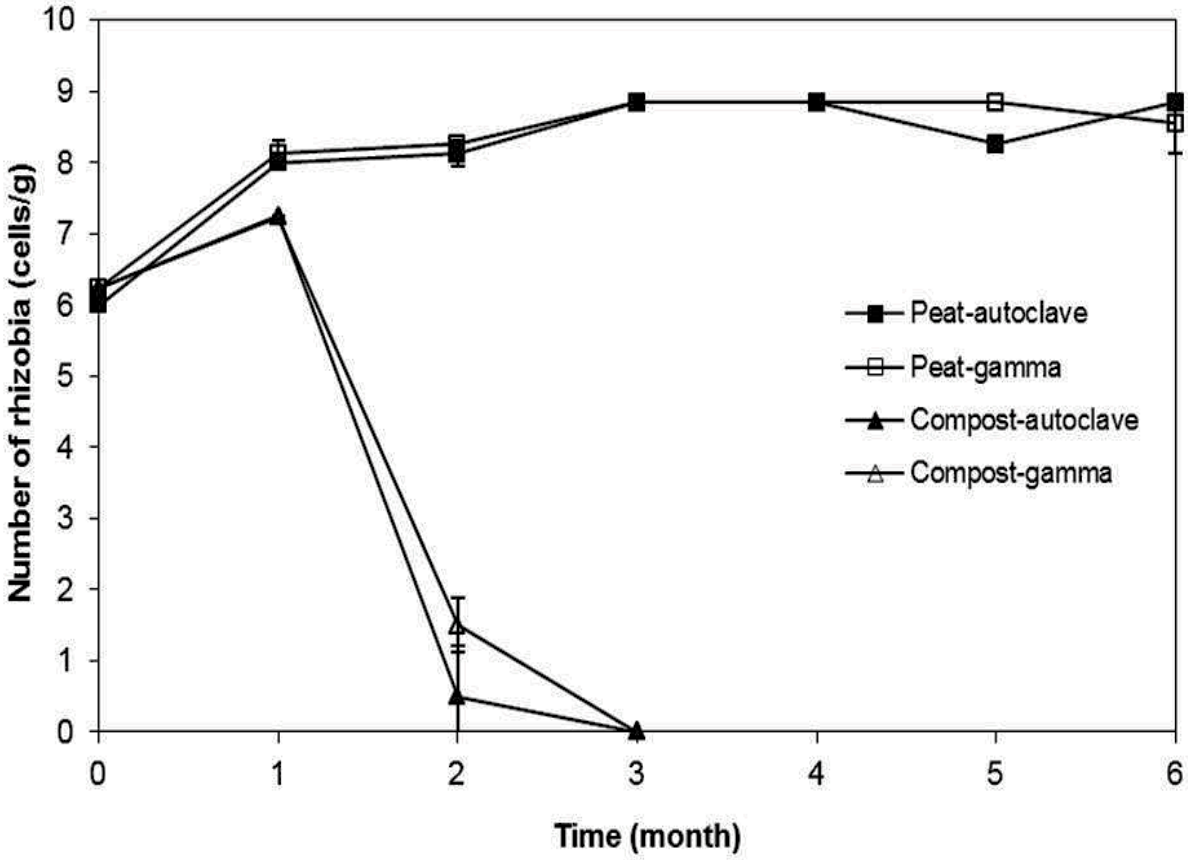

Fig. 19 Number of *Bradyrhizobium* sp. PRC008 surviving in peat with autoclave sterilization (close square), peat with gamma irradiation (open square), compost with autoclave sterilization (close triangle), and compost with gamma irradiation (open triangle) after storage for six months at room temperature.

Supplemental materials

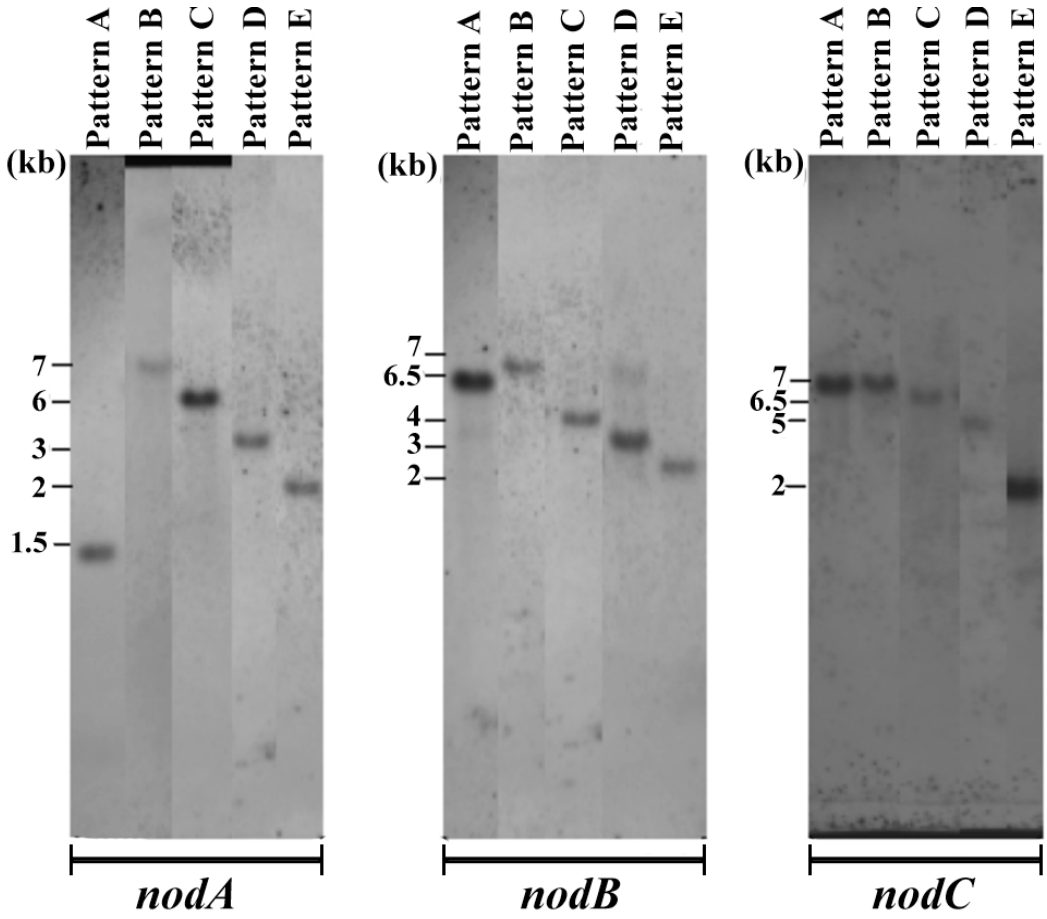

Fig. S1 Southern blot hybridization of genomic DNA showing a representative sample of the different patterns obtained from *A. americana* isolates; lists of the bacterial strains showing the different Southern blot hybridization patterns of each *nod*gene.

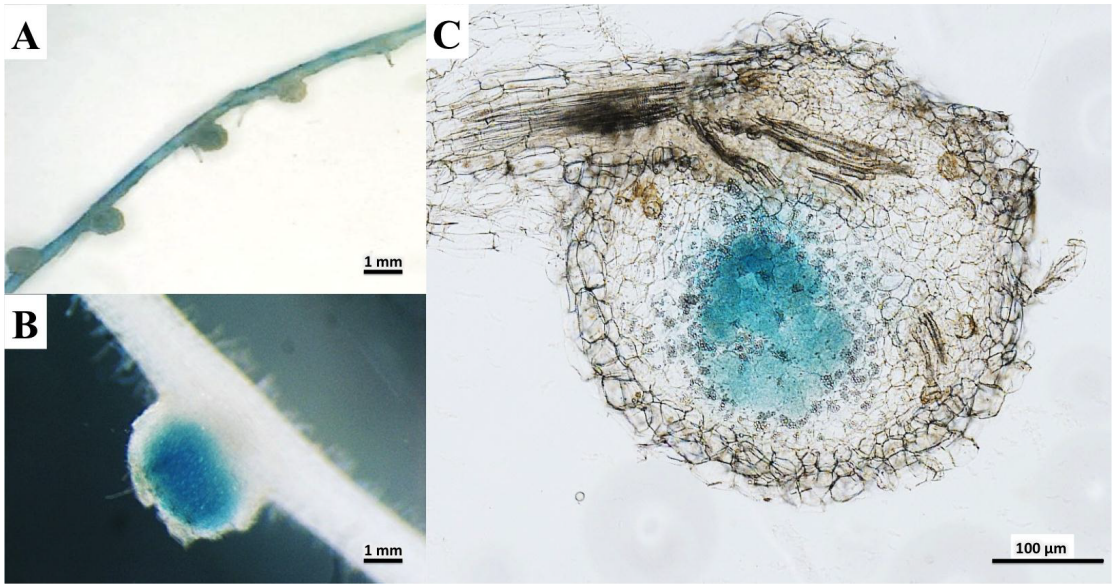

Fig. S2 GUS-tagged *Bradyrhizobium* sp. DOA9 induced ineffective nodules on *Lotus japonicus*. (A, B) Nodules. (C) Thin section of nodule.

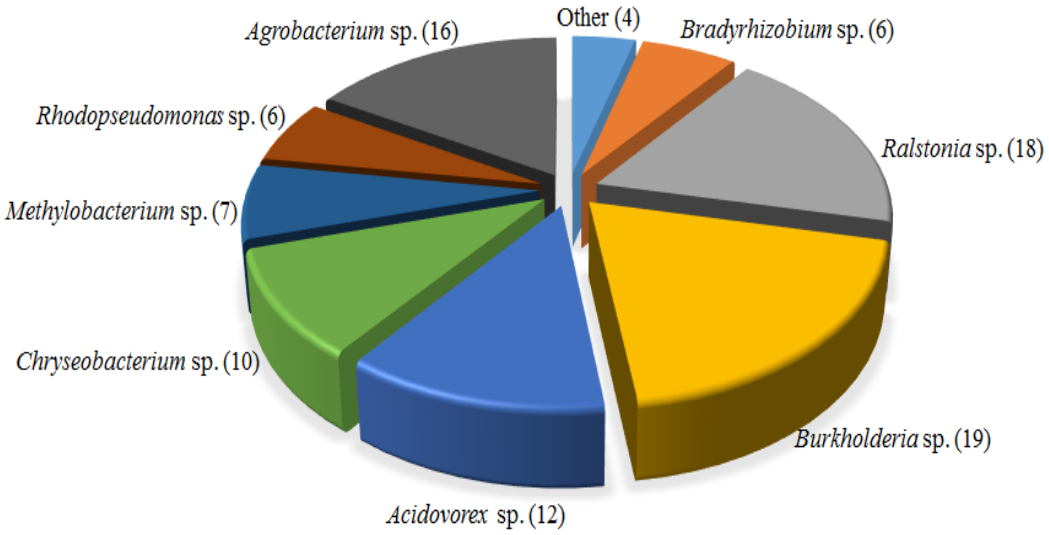

Fig. S3 Rice endophytic bacteria: number was represented different BOX-PCR pattern as same genus.

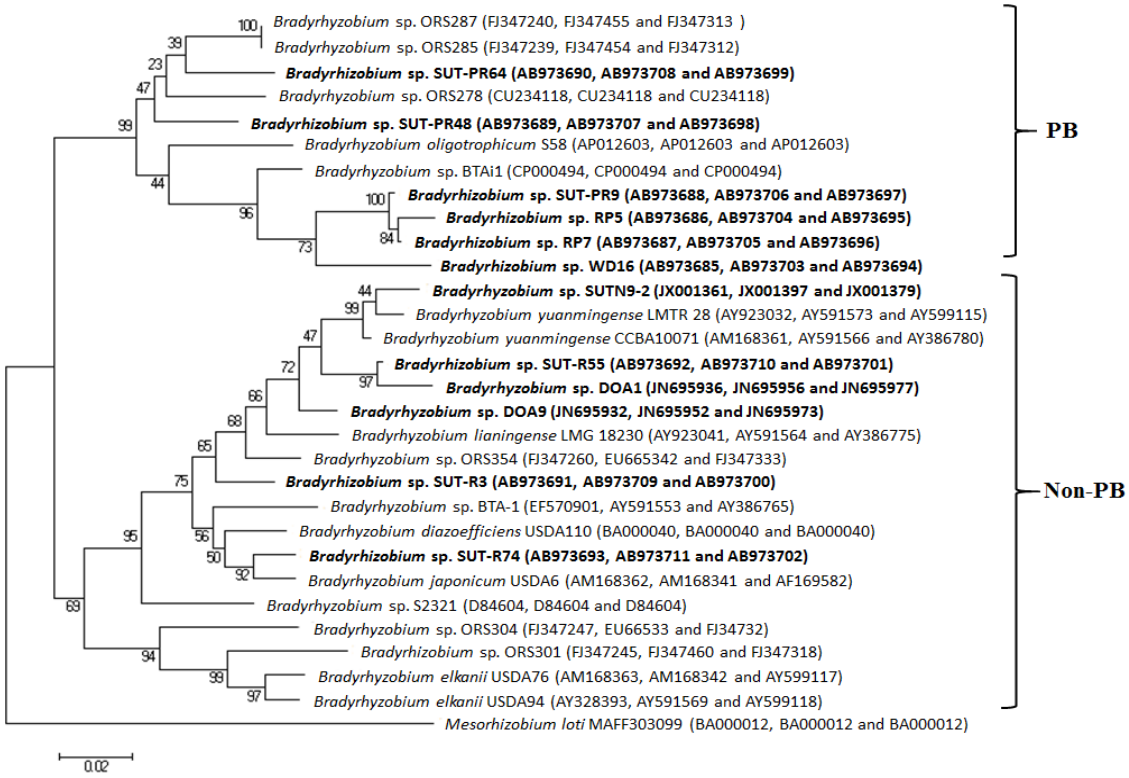

Fig. S4 Maximum likelihood tree based on combined sequences of housekeeping genes (*dna*K, *rec*A and *gln*B), showing classification of photosynthetic bradyrhizobia (PB) and non-photosynthetic bradyrhizobia (Non-PB) strains isolated from rice tissues. Bootstrap values are expressed as percentages of 1,000 replications. The bar represents 1 estimated substitution per 100 nucleotide positions.

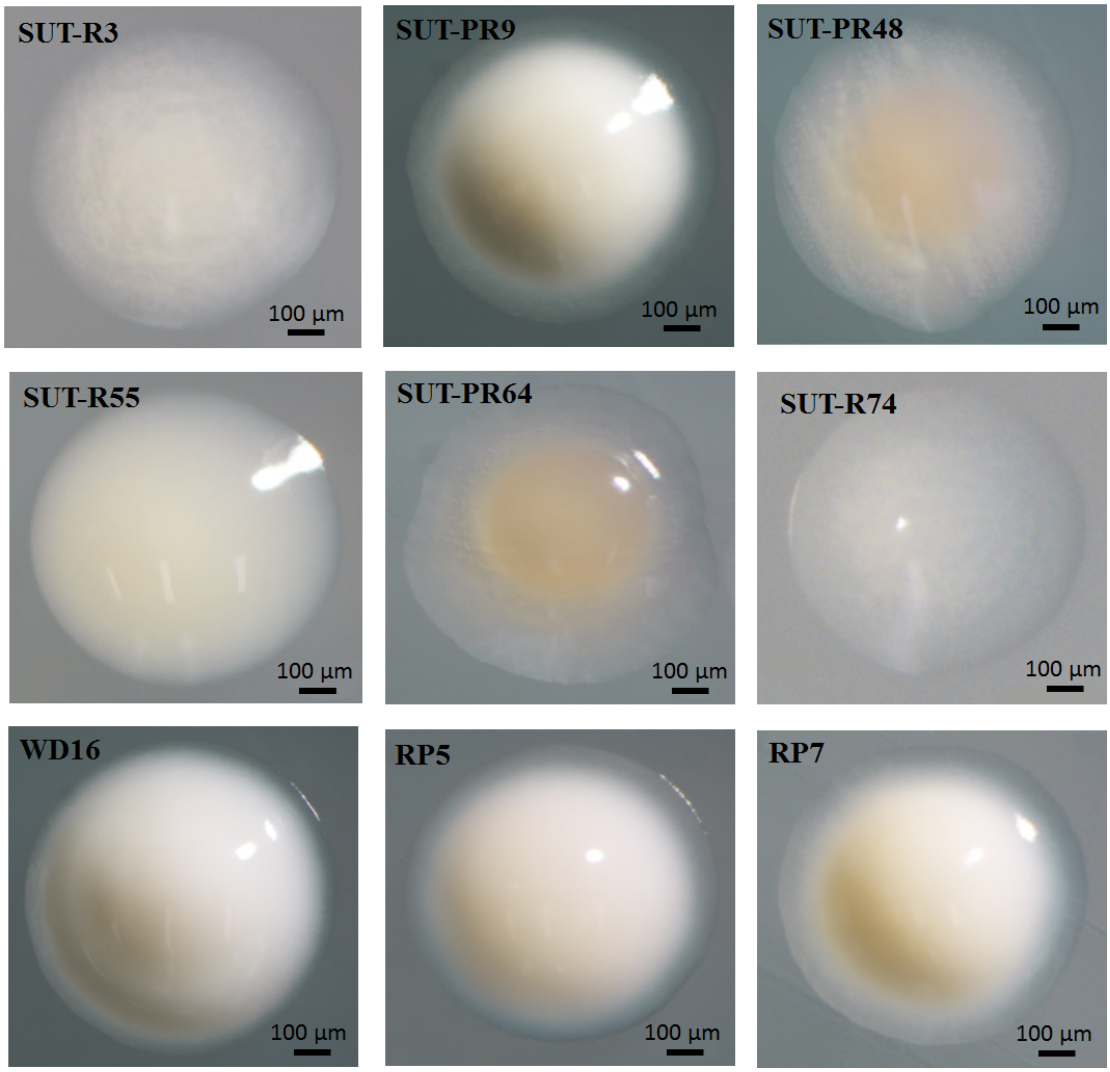

Fig. S5 Phenotypic colonies of all bradyrhizobial isolated strains