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Original Article

Native rhizobia from reforested and natural forests and their symbiotic effectiveness with *Dalbergia cochinchinensis*

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Abstract

Native rhizobia are essential for biological nitrogen fixation and the establishment of leguminous trees in both reforested and natural forest ecosystems. We examined the diversity, phylogeny, and symbiotic performance of rhizobia associated with legumes in the Nongteng–Chakkarat National Reserved Forest in northeastern Thailand. Samples were collected from 10 forest plots representing different restoration histories. A total of 45 legume species and 156 rhizobial isolates were obtained. BOX-PCR fingerprinting revealed 55 distinct genetic profiles, indicating high genetic diversity. Phylogenetic analysis based on partial 16S rRNA gene sequences categorized effective isolates into the genera *Bradyrhizobium*, *Rhizobium*, and *Mesorhizobium*. Similar genotypes were present across various forest types and host species without clear clustering. Diversity indices were higher in long-term reforested deciduous dipterocarp forests. However, neither plot-level differences nor PERMANOVA indicated significant variation between natural and reforested forests. These patterns suggest that rhizobial assemblages were not strongly differentiated by forest type under the present sampling design and were more likely associated with host availability and local ecological context. Cross-inoculation assays showed that 21 isolates were capable of nodulating *Dalbergia cochinchinensis*. Native isolates significantly increased nodulation, nitrogenase activity (up to 1,326 nmol C_2H_4 h^{-1} g^{-1} nodule dry weight), chlorophyll content, and seedling biomass ($p < 0.05$). Total chlorophyll content was positively correlated with nodule number, nodule dry weight, nitrogenase activity, and plant biomass. This suggests a close association between symbiotic effectiveness and plant biomass. Among the tested isolates, 10Es1 and 9Pm1 consistently exhibited enhanced symbiotic performance. Native rhizobia displayed considerable genetic diversity and were widely distributed across forest types. However, only a limited number of isolates formed effective symbioses with *D. cochinchinensis*, suggesting functional filtering rather than broad symbiotic compatibility. This pattern highlights the importance of host compatibility and identifies several native strains as candidates for further evaluation as bioinoculants in legume-based forest restoration.

Keywords: Rhizobial diversity, Forest restoration, Host compatibility, Leguminous plants, Symbiotic effectiveness*, Dalbergia cochinchinensis*

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Introduction

Tropical forests have extensive degradation due to deforestation and changes in land use, resulting in reduced soil nitrogen and ecosystem productivity. Within these environments, biological nitrogen fixation (BNF) facilitated by symbiotic relationships between rhizobia and leguminous plants constitutes a significant mechanism for nitrogen recovery (Huang, 2024; Moura et al., 2020). The presence of native rhizobia in degraded and replanted forests has been linked to enhanced nodulation and early plant establishment, thus promoting the early stages of forest succession (Diabate et al., 2005). Consequently, these plant–microbe interactions are a key driver of ecosystem recovery in nitrogen-limited tropical systems.

Rhizobia vary widely in host range, allowing legumes to function as key sources of nitrogen and to enhance soil fertility and productivity in both low-input and managed ecosystems (Giordano et al., 2021). Rhizobial strains with greater host compatibility persist more effectively in disturbed environments. This advantage is especially evident in reforested areas where host availability and soil conditions are variable (Burghardt et al., 2022; Jiménez-Guerrero et al., 2021). Rhizobia–legume symbioses have been mostly studied in agricultural contexts (Castellano-Hinojosa et al., 2022; Taylor et al., 2020). However, their ecological distribution and functional performance in various tropical forest systems remain insufficiently understood. Reforested and fallow tropical forests can restore soil structure and function, strongly shaped by land-use history. The relative roles of environmental filtering and host-mediated selection in structuring rhizobial communities remain unclear.

Reforestation in Thailand has proceeded through natural regeneration and planned planting of native and non-native tree species, resulting in forests with varied management histories. The Nongteng–Chakkarat National Reserved Forest in northeastern Thailand has been shaped over decades by logging, human use, and restoration efforts since the 1980s (Royal Forest Department, 2016). Siamese rosewood (*Dalbergia cochinchinensis*) is a high-value and ecologically important leguminous tree widely used in forest restoration in Thailand. The species is classified as Vulnerable on the IUCN Red List (Barstow et al., 2022). However, symbiotic interactions between *D. cochinchinensis* and native rhizobia in reforested and natural forests of Southeast Asia are not well characterized. In particular, the extent to which native rhizobial communities support effective symbiosis under different forest conditions is still uncertain.

Understanding the diversity of native rhizobia provides a basis for selecting functionally effective strains to support sustainable forest restoration. This is especially relevant in ecosystems where legumes strongly influence nutrient cycling and overall recovery. Unlike previous studies focused on agricultural systems or single host species, this study integrates the rhizobia ecological distribution across reforested and natural forests. It further evaluates the functional effectiveness of symbiosis in *D. cochinchinensis*, a key species for forest restoration in Thailand. This integrative approach allows direct comparison between community-level patterns and strain-specific symbiotic performance across host and forest types within a single landscape.

We hypothesize that rhizobial communities are broadly distributed across forest types, while effective symbiosis with *D. cochinchinensis* is restricted to a subset of compatible strains. Therefore, ecological occurrence alone may not predict functional symbiotic effectiveness. The objectives of this study were to (i) assess rhizobial diversity in reforested and natural forests, (ii) investigate their ecological distribution and host-related patterns, and (iii) determine the symbiotic efficiency of selected isolates in enhancing nodulation, nitrogen fixation, and *D. cochinchinensis* growth.

Material and Methods

Study site and sampling design

The study was conducted in the Nongteng–Chakkarat National Reserved Forest, Nakhon Ratchasima Province, northeastern Thailand. The area encompasses a mosaic of forest types representing different restoration histories and management regimes, including long-term reforested deciduous dipterocarp forests, mixed deciduous forests, eucalyptus-associated forests, community-managed enrichment planting areas, and relatively undisturbed primary forests.

Ten sampling plots (20 × 80 m each) were set across four forest types. Plot locations, vegetation characteristics, restoration histories, and geographic coordinates are summarized in Table-1 and illustrated in Figure-1. Within each plot, leguminous plant species were surveyed, and root-associated samples were collected. When visible root nodules were present, nodules were excised directly from host

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plants. When nodules were absent or senescent, rhizospheric soil closely adhering to legume roots was collected for rhizobial trapping. All samples were placed in sterile bags, stored on ice, and processed within 24 h.

Table-1. Characteristics of the ten sampling plots in the Nongteng–Chakkarat National Reserved Forest, including forest type, vegetation description, restoration and land-use context, and geographic coordinates.

Plot	Forest Type	Description	Reforestation and land use	GPS coordinates
1	Reforested, Deciduous Dipterocarp Forest	Understory vegetation consists of *Vietnamosasa pusilla* and *V. ciliata*, interspersed with perennial trees (5–15 m).	Reforested since 1983, with periodic supplemental planting every 10 years. Adjacent to communities and a main road; locals use the forest for foraging and livestock grazing.	14.9722026835, 102.3037254408
2	Reforested, Deciduous Dipterocarp Forest	Dense ground cover of *V. pusilla* and *V. ciliata* interspersed with perennial trees (5–15 m).	Reforested since 1985; additional planting in 2010. Used by nearby communities for collecting forest products and grazing livestock.	14.96461836179, 102.2969573027
3	Natural, Mixed Deciduous Forest	Dense canopy with diverse ground vegetation.	Protected since 1983; No additional planting. Fenced to prevent disturbance. Strictly conserved area, undisturbed by human activity.	14.92901554773, 102.3151152928
4	Reforested, Mixed Deciduous – Eucalyptus Forest	Dominated by *Eucalyptus camaldulensis*, with canopy height around 15 m.	Eucalyptus plantation established 30 years ago, spaced 2 × 8 m. Previously disturbed area now managed for forest product collection and livestock access.	14.93266419897, 102.3318970836
5	Reforestation Forest	Replanted on formerly degraded land with sparse and regenerating vegetation.	Annual reforestation since 2016 under the Forest Target Plantation Project using *Acacia catechu*, *D. cochinchinensis*, and *Pterocarpus macrocarpus*. Used by locals for seasonal harvesting of edible shoots, and wild mushrooms.	14.89619719163, 102.3018046915
6	Reforested, Deciduous Forest	Characterized by scattered tree cover and open understory with	Managed by local community under the Forest Target Plantation Project, with ongoing enrichment planting	14.90909587078, 102.3666735122

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Plot	Forest Type	Description	Reforestation and land use	GPS coordinates
		signs of human management.	of native and economic species such as *Pterocarpus*, *Dalbergia*, and *Caesalpinia*. Moderate human activity for subsistence use.	
7	Natural, Deciduous Dipterocarp Forest	Mature forest dominated by *Xylia xylocarpa* and other native trees.	No planting interventions; left undisturbed for over 30 years. Protected old-growth forest with minimal human interference.	14.94634211126, 102.3632420075
8	Natural, DDF-MDF mixed	Primary Forest, Tall mature trees with low shrub density and minimal signs of disturbance.	Intact forest; no history of enrichment or planting. Limited access and occasional forest use.	14.96467513566, 102.3526500659
9	Natural, DDF-MDF mixed	Primary Forest with large canopy trees (>30 cm diameter); few understory species; contains lowland wet areas.	Not reforested; includes natural wetlands. Seasonal use for small-scale rice farming in natural wetlands area and medicinal plant collection.	14.9966427087, 102.3307154145
10	Natural, DDF-MDF mixed	Primary Forest , Intact canopy with large native trees.	No planting history; located in a remote area with limited accessibility. Occasionally accessed for non-timber forest products and herbal collection.	15.0030372316, 102.3425087479

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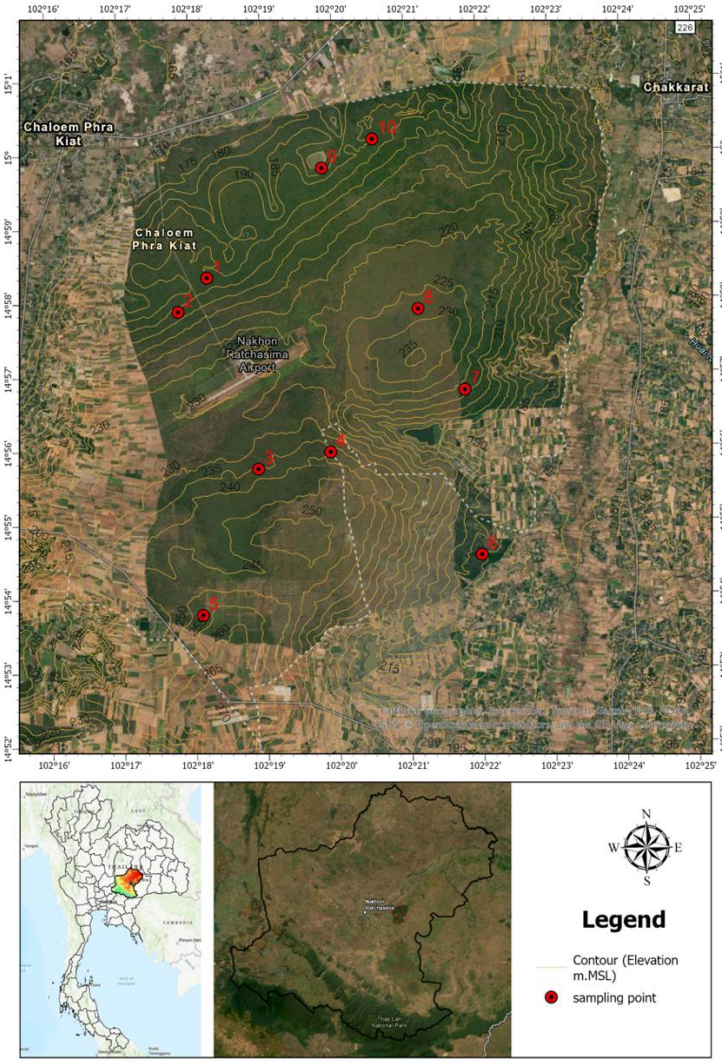

Figure-1. Map of the ten sampling plots in the Nongteng–Chakkarat National Reserved Forest.

Rhizobial trapping and isolation

Rhizobia were isolated from rhizospheric soils by a trap-plant approach with *Macroptilium atropurpureum* (siratro), a promiscuous legume host widely used for recovering symbiotically competent rhizobia. Siratro seeds were scarified with concentrated sulfuric acid for 10 min, thoroughly rinsed with sterile distilled water, and soaked overnight. Germination was carried out on sterile, moistened tissue paper in the dark at room temperature. Seedlings with radicles approximately 0.5–1.0 cm long were transplanted into pots containing collected field soil. Each pot contained four siratro seedlings and was placed on an individual tray to prevent cross-contamination. Plants were maintained under greenhouse conditions and irrigated with a nitrogen-free Hoagland nutrient solution (Somasegaran and Hoben, 1994). Nodules were collected after four weeks for rhizobial isolation.

Rhizobia were isolated from both field-collected nodules and siratro-induced nodules following the protocol of Somasegaran and Hoben (1994). Nodules were washed under running tap water, surface-sterilized in 95% ethanol for 10 s, followed by 3% sodium hypochlorite for 5 min, and rinsed at least five times with sterile distilled water. Sterilized nodules were crushed aseptically, and the contents were streaked onto yeast extract mannitol (YEM) agar supplemented with Congo red. Plates were incubated at 30 °C for up to 7 days. Distinct colonies were purified and maintained on YEM slants. In total, 156 rhizobial isolates were obtained and preserved at the

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Genomic fingerprinting by BOX-PCR

Genomic DNA was extracted using a commercial DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. Genetic diversity among the isolates was assessed using BOX-PCR fingerprinting with the BOXA1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′) (Smith et al., 2001). PCR products were amplified using standard procedures and analyzed on 2% agarose gels. Banding patterns were visualized under UV illumination and analyzed using UVibandmap software (Uvitec Cambridge, UK). Isolates showing the same, consistently reproducible patterns were grouped, whereas unique patterns were treated as genetically distinct. From these profiles, 55 representative isolates were selected for subsequent cross-inoculation tests and phylogenetic analyses.

Cross-inoculation assay with *D. cochinchinensis*

The ability of representative rhizobial isolates to nodulate *D. cochinchinensis* was evaluated using a pouch assay system. Seeds of *D. cochinchinensis* were surface sterilized with 95% ethanol for 1 min, 3% sodium hypochlorite for 10 min, and scarified with concentrated sulfuric acid for 10 min to break dormancy. Seeds were rinsed thoroughly and germinated on sterile moist tissue paper in the dark.

Sterile plastic growth pouches (5 × 8 inches) containing folded sterile straw paper and 50 mL nitrogen-free Hoagland nutrient solution were used. Germinated seedlings were inserted into the pouches so that roots extended between the paper layers. Each pouch was inoculated with 1 mL of bacterial suspension adjusted to approximately 10^7 CFU mL^{-1}. Uninoculated controls received sterile water. Plants were maintained under controlled conditions (12 h light/12 h dark, 25 °C). Each isolate was tested with three biological replicates. Nodulation was assessed after four weeks.

Nitrogenase activity assay

Rhizobial isolates that induced nodulation were further evaluated for nitrogen-fixing activity using Leonard's jar system. The upper compartment contained sterilized vermiculite (4–8 mm), and the lower compartment was filled with nitrogen-free Hoagland solution, connected by cotton wicks. Each seedling was inoculated with 1 mL of bacterial suspension (10^7 CFU mL^{-1}).

Plants were grown under controlled conditions (12 h light/dark, 28–30 °C). Nitrogenase activity was determined using the Acetylene Reduction Assay (ARA). Nodulated roots were incubated in airtight containers with 5% acetylene for 1 h, and ethylene production was measured using gas chromatography (SRI, Germany). After analysis, nodule dry weight was measured after oven drying and was used for calculating ARA, expressed as nmol C_2H_4 h^{-1} g^{-1} nodule DW.

16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA from effective isolates was amplified using 27F and 1492R primers. PCR products were purified and sequenced. Nucleotide sequences were aligned using CLUSTAL W and compared with reference sequences in the GenBank database using BLAST.

Phylogenetic relationships were inferred using the Maximum Likelihood method implemented in MEGA version 12, with 1,000 bootstrap replicates. Sequences were deposited in GenBank under accession numbers PV687327–PV687347.

Greenhouse evaluation of plant growth promotion

Greenhouse experiments were conducted to evaluate the effects of selected rhizobial isolates on *D. cochinchinensis* seedling growth. Surface-sterilized seeds were germinated and transplanted into polybags filled with a sterile sand: burnt rice husk mixture (2:1, v/v). The experiment was arranged in a randomized design with three biological replicates per treatment, which were used for all measurements.

Each seedling was inoculated with 1 mL of bacterial suspension ($OD_{600} \approx 0.5$), while the control plants were treated with sterile water. Plants were watered daily with nitrogen-free Hoagland solution. After 60 days, plant biomass and chlorophyll content were measured.

Determination of chlorophyll content

Chlorophyll was extracted from fresh leaf tissue (100 mg) using 80% acetone. Absorbance was measured at 645 and 663 nm using a spectrophotometer, and

chlorophyll a, chlorophyll b, and total chlorophyll contents were calculated according to Arnon (1949). Chlorophyll concentrations were expressed on a fresh weight basis.

Statistical analysis

Rhizobial diversity across forest plots was assessed using the Shannon–Wiener (H′) and Simpson (D) diversity indices based on isolate abundance associated with legume hosts. Non-parametric Kruskal–Wallis tests were used to evaluate differences in diversity indices among plots. Rhizobial community composition was analyzed using multivariate statistical approaches based on Bray–Curtis dissimilarity. Presence–absence data of BOX-PCR groups (G01–G55) across forest sites were used to construct the community matrix. Differences in community composition between forest types (natural vs. reforested) were tested using permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations. Non-metric multidimensional scaling (NMDS) was performed to visualize patterns of community similarity among sites. Ordinations were based on Bray–Curtis distances, and stress values were used to evaluate the goodness-of-fit of the ordination. Forest type was used as a grouping factor, and 95% confidence ellipses were fitted to illustrate group dispersion. All multivariate analyses were conducted using PAST (version 5.2).

Plant growth, nodulation, nitrogenase activity, and chlorophyll data were analyzed by one-way analysis of variance (ANOVA). When significant effects were detected, mean comparisons were performed using Duncan's multiple range test at $p < 0.05$. All univariate statistical analyses were conducted using SPSS version 26.0 (IBM Corp., Armonk, NY, USA). Results are presented as mean ± standard deviation (SD).

Pearson correlation analysis was conducted using individual replicate values to evaluate relationships between total chlorophyll content and symbiotic parameters (nodule number, nodule dry weight, nitrogenase activity, and plant biomass). Linear regression analysis was performed to assess relationship strength, and coefficients of determination (R2) and significance levels (*p*-values) were calculated using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). For graphical presentation, mean values of three replicates were used for each isolate.

Results

Legume richness and rhizobial recovery across forest types

Legume richness across the ten forest plots ranged from 6 to 22 species (Figure-2; Table-2). Long-term reforested deciduous dipterocarp forests (Plots 1 and 2) showed the highest richness and yielded the most rhizobial isolates. Recently restored plots (5 and 6) and primary forest plots (8–10) showed lower richness and fewer isolates. Papilionoideae was the most common subfamily among all plots, followed by Mimosoideae and Caesalpinioideae. Herbaceous legumes such as *Clitoria macrophylla*, *Phyllodium pulchellum*, and *Tephrosia purpurea* were widespread across forest types. Woody legumes with high ecological value, such as *D. cochinchinensis* and *Pterocarpus macrocarpus*, were mostly found in reforested and community-managed plots. Plots with higher legume richness yielded more rhizobial isolates, indicating that host availability and forest management shape rhizobial recovery. Restored forests with sustained legume recruitment maintain more diverse rhizobial communities.

Genetic diversity of rhizobial isolates revealed by BOX-PCR fingerprinting

A total of 156 rhizobial isolates were obtained from field-collected nodules and siratro trap plants across all sampling plots. BOX-PCR fingerprinting revealed high genomic diversity among these isolates, with 55 distinct fingerprint profiles identified (Figure-3). The number of unique BOX profiles per plot generally corresponded to legume richness and isolate recovery but did not show clear clustering by forest type or host plant species. Similar BOX PCR profiles across multiple forest plots and host legumes indicate that genetically similar rhizobia occur in diverse ecological settings, with their broad distribution showing little clustering by site or host.

Figure-2. Representative vegetation structure and canopy characteristics of the ten forest sampling plots (Plots 1–10), showing variation in forest types and restoration conditions.

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Table-2. Leguminous plant species recorded across the ten forest sampling plots and the number of rhizobial isolates obtained from each host species. Presence of species is indicated by "/", and numbers in parentheses represent isolate counts.

Leguminous plant	Code	Subfamily	Number of plots observed	Sampling plots									
				1	2	3	4	5	6	7	8	9	10
Abrus precatorius L.	Ap	Papilionoideae	2		/ (5)				/ (2)				
Acacia catechu (L.f.) Willd	Acc	Mimosoideae	3	/	/ (1)		/ (3)						
Acacia comosa Gagnep.	Ac	Mimosoideae	2	/ (1)		/ (2)							/
Afzelia xylocarpa (Kurz) Craib	Ax	Caesalpinioideae	2		/					/			
Albizia lebbeck (L.) Benth.	Al	Mimosoideae	3					/		/		/	
Albizia myriophylla	Am	Mimosoideae	1			/							
Albizia odoratissima (L.f.) Benth.	Ao	Mimosoideae	2	/ (8)	/ (2)								
Alysicarpus vaginalis	Av	Papilionoideae	2	/	/								
Bauhinia purpurea L.	Bp	Caesalpinioideae	1										/
Bauhinia saccocalyx Pierre	Bs	Caesalpinioideae	4	/	/					/			/
Cajanus scarabaeoides (L.)	Cs	Papilionoideae	3				/	/ (2)		/			
Cassia fistula Linn.	Cf	Caesalpinioideae	2		/ (1)				/				
Centrosema pubescens	Cp	Papilionoideae	1		/								
Clitoria macrophylla Wall.	Cm	Papilionoideae	7		/ (2)	/ (1)	/ (2)			/	/ (2)	/ (1)	/
Crotalaria alata Buch.-Ham.	Ca	Papilionoideae	5		/		/ (1)	/	/	/ (1)			
Crotalaria pallida	Cp	Papilionoideae	1								/		
Crotalaria verrucosa L	Cv	Papilionoideae	1		/								
Dalbergia cochinchinensis Pierre	Dc	Papilionoideae	2			/		/					
Dalbergia dongnaiensis Pierre	Dd	Papilionoideae	2		/						/		
Dalbergia nigrescens Kurz.	Dn	Papilionoideae	2									/	/
Derris scandens	Ds	Papilionoideae	2							/		/	
Desmodium triflorum (L.) DC.	Dt	Papilionoideae	1					/ (2)					
Desmodium velutinum	Dv	Papilionoideae	5	/ (2)		/ (3)				/ (6)	/ (5)		/ (3)
Dialium cochinchinense Pierre	Dc	Caesalpinioideae	2	/		/ (4)							
Eriosema chinense	Ec	Papilionoideae	1				/						
Erythrophleum succirubrum Gagnep.	Es	Caesalpinioideae	4	/ (1)	/ (1)		/ (1)						/ (1)
Hegnera obcordata (Miq.)	Ho	Papilionoideae	1			/ (4)							
Indigofera hirsuta Linn.	Ih	Papilionoideae	2		/ (3)		/ (1)	/					
Macroptilium atropurpureum (DC.)	Ma	Papilionoideae	4	/ (8)	/ (3)	/ (2)		/ (2)					
Macroptilium lathyroides	Ml	Papilionoideae	2	/ (5)			/						
Millettia leucantha Kurz	Mle	Papilionoideae	1							/			
Mimosa putida	Mp	Mimosoideae	5	/	/ (5)	/ (3)	/ (2)		/				
Mucuna pruriens DC.	Mpt	Papilionoideae	1			/							
Peltophorum pterocarpum (D.C.)	Ppt	Caesalpinioideae	1					/					
Phyllodium elegans (Lour.) Desv.	Pe	Papilionoideae	4			/ (2)	/ (4)			/ (5)	/ (1)		
Phyllodium pulchellum (L.) Desv.	Pp	Papilionoideae	6		/	/	/ (2)	/ (2)	/				/ (1)
Pterocarpus integrum Craib.	Pi	Caesalpinioideae	1								/		
Pterocarpus macrocarpus Kurz	Pm	Papilionoideae	7	/	/ (1)	/	/ (1)	/		/		/ (1)	
Senna garrettiana (Craib)	Sg	Caesalpinioideae	1										/
Senna occidentalis	Sa	Caesalpinioideae	1						/				
Sindora siamensis Teijsm.	Ss	Caesalpinioideae	4		/ (5)				/ (4)	/ (3)		/ (5)	

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Leguminous plant	Code	Subfamily	Number of plots observed	Sampling plots									
				1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Tephrosia purpurea (L.) Pers.	Tp	Papilionoideae	6	/	/		/ (3)	/	/	/			
Tephrosia vestita Vogel.	Tv	Papilionoideae	1		/ (3)								
Uraria crinita Desv.	Uc	Papilionoideae	2	/ (5)			/ (3)						
Xylia xylocarpa (Roxb.) Taub.	Xx	Mimosoideae	6		/		/(1)		/	/	/		/
Number of plants				**14**	**22**	**13**	**15**	**11**	**9**	**14**	**7**	**6**	**10**
- Mimosoideae				4	4	3	3	1	2	2	1	1	2
- Caesalpinioideae				3	5	1	1	1	3	3	1	1	4
- Papilionoideae				7	13	9	11	9	4	9	5	4	4
Number of bacterial isolates				**30**	**32**	**21**	**24**	**8**	**6**	**15**	**8**	**7**	**5**

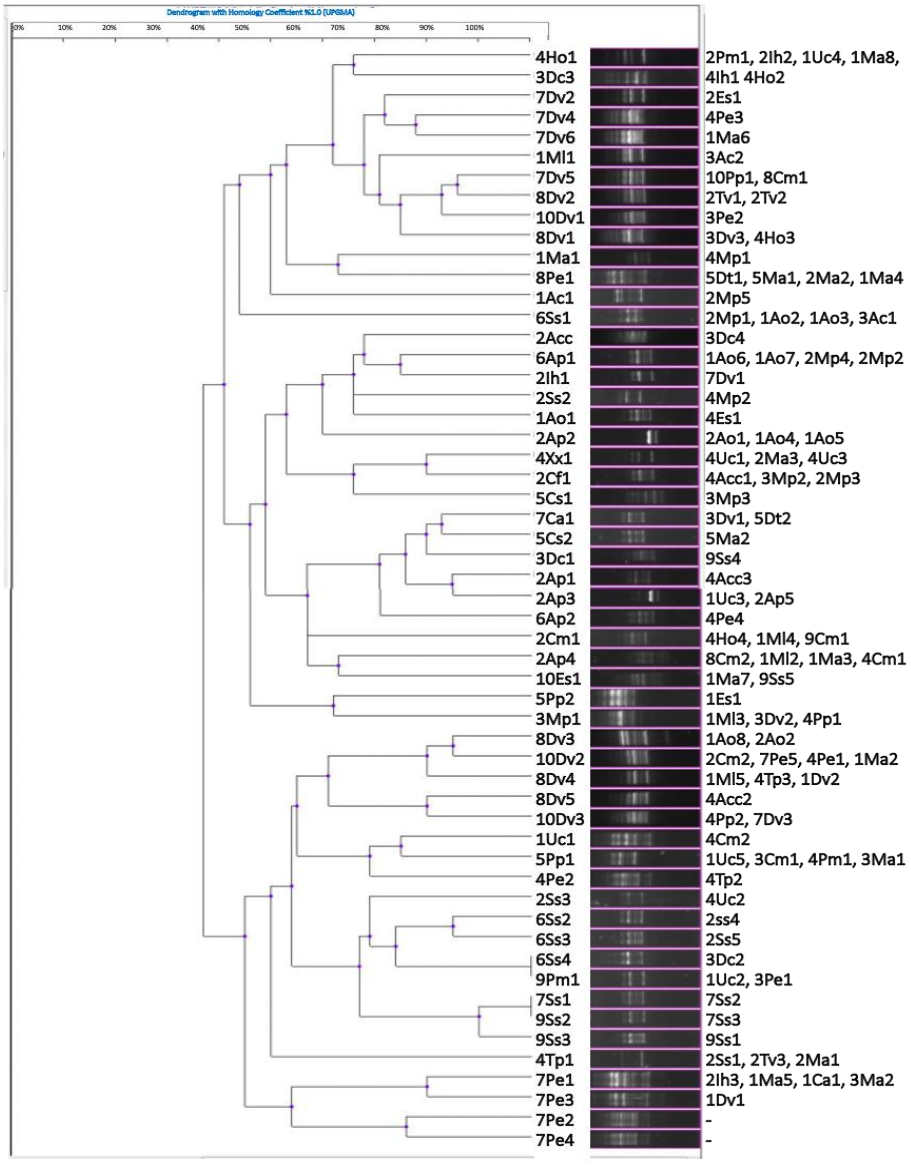

Figure-3. BOX-PCR fingerprinting of rhizobial isolates using the BOXA1R primer. Representative isolates (e.g., 4Ho1) were selected from each cluster to reduce redundancy, and isolates on the right share identical or highly similar banding patterns.

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Phylogenetic affiliation of effective rhizobial isolates

Of the 55 genetically distinct isolates, 21 successfully induced nodulation in *D. cochinchinensis* and were selected for phylogenetic analysis based on partial 16S rRNA gene sequences. Phylogenetic reconstruction grouped these isolates into three major genera: *Bradyrhizobium, Rhizobium* and *Mesorhizobium* (Figure-4).

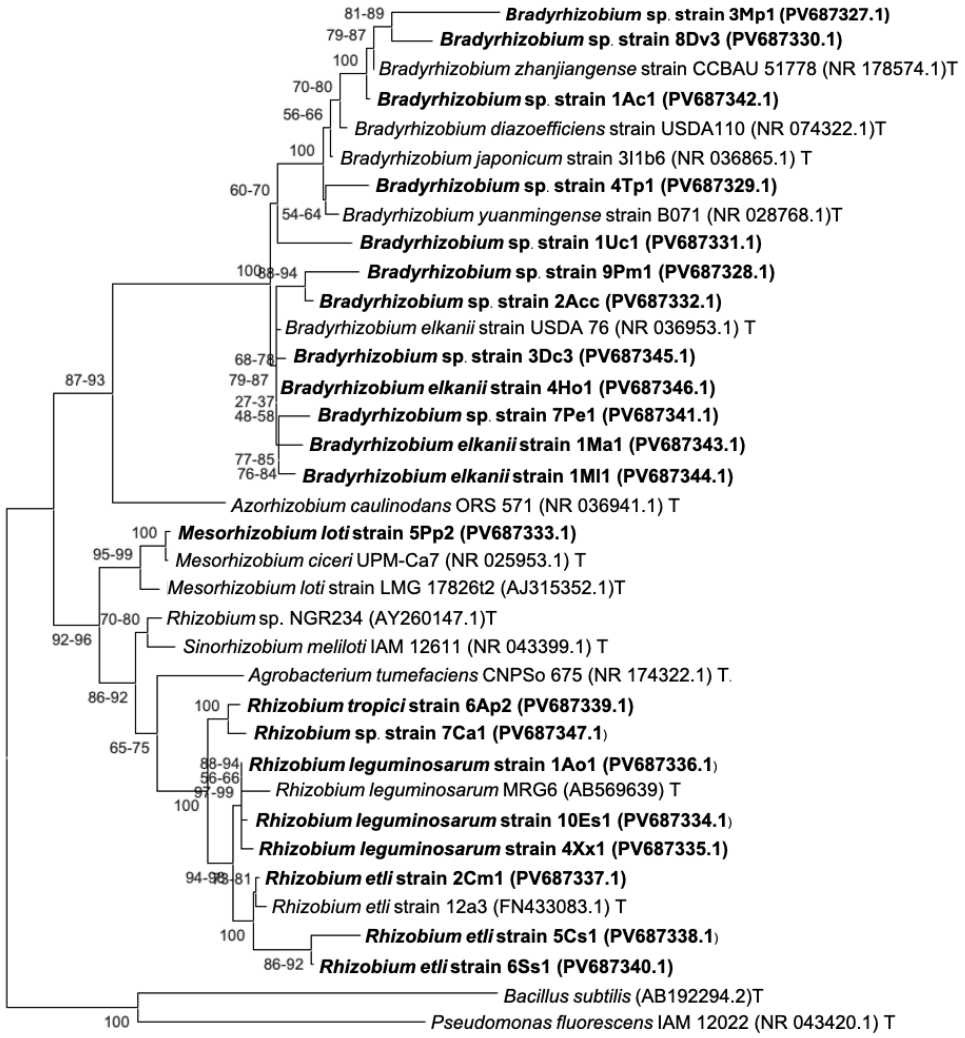

Figure-4. Maximum Likelihood phylogenetic tree based on partial 16S rRNA gene sequences of rhizobial isolates nodulating *D. cochinchinensis*. Bootstrap values (>50%) from 1,000 replicates are shown at branch nodes. Isolates obtained in this study are shown in bold, and reference strains retrieved from GenBank are indicated with superscript "T".

Bradyrhizobium was the most common group, with isolates closely related to *B. diazoefficiens*. These were found in different forest types and on both trees and herbaceous plants. *Rhizobium* isolates showed high genetic diversity and broad host associations, including *Phyllodium, Tephrosia*, and *Crotalaria*. A single isolate (5Pp2) clustered with *Mesorhizobium loti*, indicating minor but notable taxonomic diversity. Phylogenetic patterns showed no clear association with forest type or host, with similar lineages occurring across multiple ecological settings.

Rhizobial diversity patterns across forest plots

Rhizobial community composition showed no significant differences between natural and reforested forests using PERMANOVA analysis (p = 0.081). This result was supported by NMDS ordination, which revealed considerable overlap between the two forest types, with no clear separation of the communities (Figure-5). Sites from both forest types overlapped in ordination space, with broadly overlapping 95% confidence ellipses, indicating similar community composition.

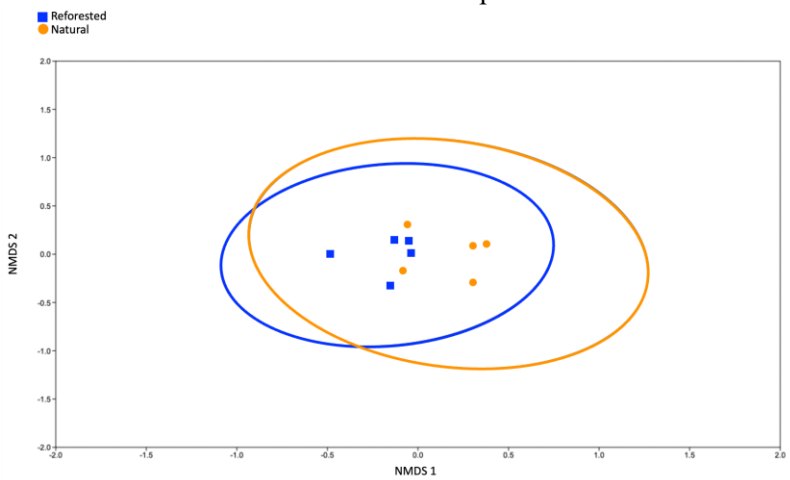

Figure-5. Non-metric multidimensional scaling (NMDS) ordination based on Bray–Curtis dissimilarity showing rhizobial community composition across forest types. Blue squares indicate reforested forests, and orange circles indicate natural forests. Ellipses represent 95% confidence intervals for each group.

Shannon–Wiener and Simpson indices were used to assess alpha diversity across forest plots (Figure-6). Although numerical values were higher in long-term reforested and community-managed forests, the Kruskal–Wallis test showed no significant differences among plots ($p > 0.05$).

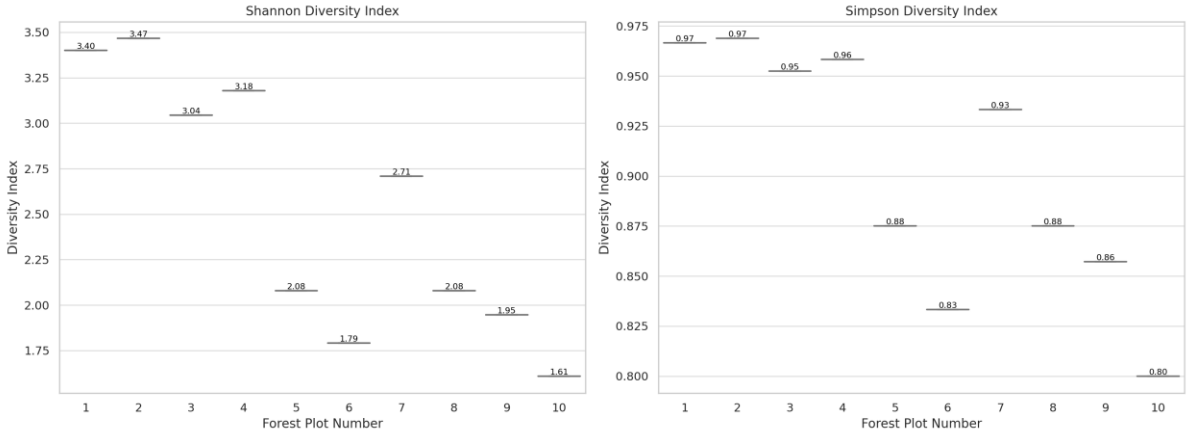

Figure-6. Shannon–Wiener (H′) and Simpson (D) diversity indices of rhizobial isolates across the ten forest plots. Boxplots represent median values, interquartile ranges, and data dispersion among plots.

Nodulation capacity and nitrogenase activity in *D. cochinchinensis*

Cross-inoculation testing showed that 21 of the 55 isolates (38%) induced nodulation in *D. cochinchinensis* (Table-3), while the uninoculated control showed no nodulation. Nodule number ranged from 2.33 to 19.00 per plant, with isolates 9Pm1, 10Es1, and 1Ml1 producing the highest nodule counts. Nodule dry weight also differed significantly among isolates ($p < 0.05$), with 10Es1, 9Pm1, and 4Tp1

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producing the highest nodule biomass. Nitrogenase activity ranged from 11.80 to 1,326.54 nmol C_2H_4 h^{-1} g^{-1} nodule DW, with 10Es1 and 9Pm1 showing the highest activity, followed by 4Tp1 ($p < 0.05$). No nitrogenase activity was detected in the uninoculated control. Variation in nodule number and nitrogenase activity was observed among isolates, including those from the same host species.

Table-3. Effects of selected rhizobial isolates on nodulation and nitrogenase activity of *D. cochinchinensis* grown in Leonard's jar systems.

Isolates	Nodule number (plant^{-1})		Nodule dry weight (g plant^{-1})		ARA in Leonard's jar (nmol C_2H_4 h^{-1} g^{-1} nodule DW)	
Uninoculated control	0.00 ± 0.00	k	0.000 ± 0.000	h	0.00 ± 0.00	m
1Ac1	4.00 ± 1.73	ij	0.028 ± 0.012	defgh	172.62 ± 16.45	ij
1Ao1	2.33 ± 1.53	jk	0.008 ± 0.007	gh	11.80 ± 1.72	lm
1Ma1	4.00 ± 1.00	ij	0.030 ± 0.013	defgh	242.92 ± 26.49	hi
1Ml1	18.33 ± 3.06	a	0.144 ± 0.050	a	1,048.12 ± 56.10	c
1Uc1	8.33 ± 2.31	defg	0.044 ± 0.014	cdefg	647.92 ± 5.99	f
2Acc	2.33 ± 0.58	jk	0.010 ± 0.007	fgh	72.37 ± 2.53	klm
2Cm1	6.33 ± 1.15	efghi	0.063 ± 0.008	cd	715.21 ± 32.30	ef
3Dc3	2.67 ± 0.58	jk	0.017 ± 0.006	efgh	18.86 ± 1.10	lm
3Mp1	5.33 ± 0.58	ghij	0.035 ± 0.007	defgh	118.60 ± 5.88	jk
4Ho1	9.00 ± 2.00	defg	0.053 ± 0.013	cde	98.51 ± 2.93	jkl
4Tp1	16.33 ± 2.08	ab	0.116 ± 0.030	ab	1,187.40 ± 51.06	b
4Xx1	10.00 ± 2.65	cde	0.060 ± 0.017	cd	523.00 ± 22.94	g
5Cs1	12.00 ± 3.61	cd	0.084 ± 0.027	bc	900.80 ± 8.48	d
5Pp2	2.33 ± 1.15	jk	0.011 ± 0.008	fgh	11.82 ± 3.31	lm
6Ap2	4.33 ± 2.08	hik	0.026 ± 0.013	defgh	21.74 ± 1.83	lm
6Ss1	6.00 ± 1.73	fghij	0.037 ± 0.012	defgh	48.87 ± 3.08	klm
7Ca1	9.33 ± 2.08	def	0.067 ± 0.019	cd	733.42 ± 15.58	e
7Pe1	8.00 ± 1.73	efgh	0.051 ± 0.017	cdef	540.86 ± 17.97	g
8Dv3	13.33 ± 2.08	bc	0.081 ± 0.019	bc	295.36 ± 4.82	hi
9Pm1	19.00 ± 2.65	a	0.148 ± 0.040	a	1,236.77 ± 135.71	b
10Es1	18.67 ± 3.79	a	0.128 ± 0.040	a	1,326.54 ± 152.05	a

Values represent means ± SD (n = 3). Different letters within each column indicate significant differences among treatments at $p < 0.05$.

Effects of rhizobial inoculation on plant growth and chlorophyll content

Greenhouse experiments showed that effective rhizobial inoculation significantly enhanced seedling growth of *D. cochinchinensis* (Table-4). The highest dry biomass occurred in seedlings inoculated with isolates 9Pm1 (5.28 ± 0.86 g), 10Es1 (5.22 ± 0.94 g), and 4Tp1 (4.95 ± 0.09 g), consistent with their high nitrogenase activity.

Table-4. Effects of rhizobial inoculation on biomass production and chlorophyll content of *D. cochinchinensis* under greenhouse conditions.

Isolates	Total Chlorophyll (mg g^{-1} FW)	Chlorophyll a/b ratio	Plant fresh weight (g $plant^{-1}$)	Plant dry weight (g $plant^{-1}$)
Uninoculated Control	2.19 ± 0.04 ghi	1.62 ± 0.10 g	0.70 ± 0.29 h	0.23 ± 0.11 i
1Ac1	2.02 ± 0.02 j	2.57 ± 0.42 cd	1.79 ± 0.02 fgh	0.66 ± 0.03 ghi
1Ao1	2.16 ± 0.07 hij	1.72 ± 0.06 fg	1.20 ± 0.07 gh	0.58 ± 0.04 hi
1Ma1	2.27 ± 0.08 fghi	1.56 ± 0.16 g	1.37 ± 0.04 gh	0.48 ± 0.03 hi
1Ml1	2.61 ± 0.11 c	2.70 ± 0.47 bcd	10.28 ± 0.77 b	4.25 ± 0.32 b
1Uc1	2.13 ± 0.04 ij	2.65 ± 0.29 bcd	3.97 ± 0.04 d	1.66 ± 0.05 d
2Acc	2.18 ± 0.14 ghi	1.81 ± 0.41 fg	1.37 ± 0.17 gh	0.47 ± 0.02 hi
2Cm1	2.30 ± 0.08 fgh	1.95 ± 0.07 efg	3.30 ± 0.20 def	1.21 ± 0.07 def
3Dc3	2.20 ± 0.05 ghi	1.67 ± 0.06 g	1.16 ± 0.07 gh	0.34 ± 0.06 i
3Mp1	2.16 ± 0.04 hij	1.72 ± 0.05 fg	2.31 ± 0.03 efg	0.89 ± 0.07 fgh
4Ho1	2.45 ± 0.01 de	2.28 ± 0.24 de	6.39 ± 0.13 c	2.60 ± 0.03 c
4Tp1	3.19 ± 0.17 a	2.96 ± 0.16 abc	11.62 ± 2.02 ab	4.95 ± 0.09 a
4Xx1	2.33 ± 0.07 efg	1.95 ± 0.03 efg	4.05 ± 0.07 d	1.69 ± 0.04 d
5Cs1	2.42 ± 0.04 def	2.87 ± 0.14 abc	7.25 ± 0.07 c	3.04 ± 0.08 c
5Pp2	2.26 ± 0.04 ghi	1.85 ± 0.11 fg	1.36 ± 0.06 gh	0.29 ± 0.02 i
6Ap2	2.24 ± 0.12 ghi	1.78 ± 0.21 fg	1.99 ± 0.03 efgh	0.68 ± 0.04 ghi
6Ss1	2.22 ± 0.04 ghi	1.70 ± 0.07 fg	2.99 ± 0.14 def	1.12 ± 0.15 efg
7Ca1	2.24 ± 0.08 ghi	1.78 ± 0.02 fg	3.37 ± 0.23 de	1.58 ± 0.06 de
7Pe1	2.33 ± 0.03 efg	2.13 ± 0.30 ef	3.35 ± 0.08 de	1.38 ± 0.06 def
8Dv3	2.53 ± 0.11 cd	2.58 ± 0.30 cd	7.06 ± 0.07 c	2.96 ± 0.09 c
9Pm1	2.79 ± 0.11 b	3.15 ± 0.11 a	12.02 ± 1.65 a	5.28 ± 0.86 a
10Es1	3.10 ± 0.09 a	3.06 ± 0.36 ab	11.57 ± 2.71 ab	5.22 ± 0.94 a

Values represent means ± SD (n = 3). Different letters within each column indicate significant differences among treatments at $p < 0.05$.

Pearson correlation analysis revealed that total chlorophyll content was strongly and positively correlated with nodule number ($R^2 = 0.70$, $p < 0.001$), nodule dry weight ($R^2 = 0.68$, $p < 0.001$), nitrogenase activity ($R^2 = 0.61$, $p < 0.001$), and plant biomass ($R^2 = 0.52$, $p < 0.001$) (Figure-7). These results demonstrate a consistent positive association between nodulation, nitrogen fixation, and plant physiological performance.

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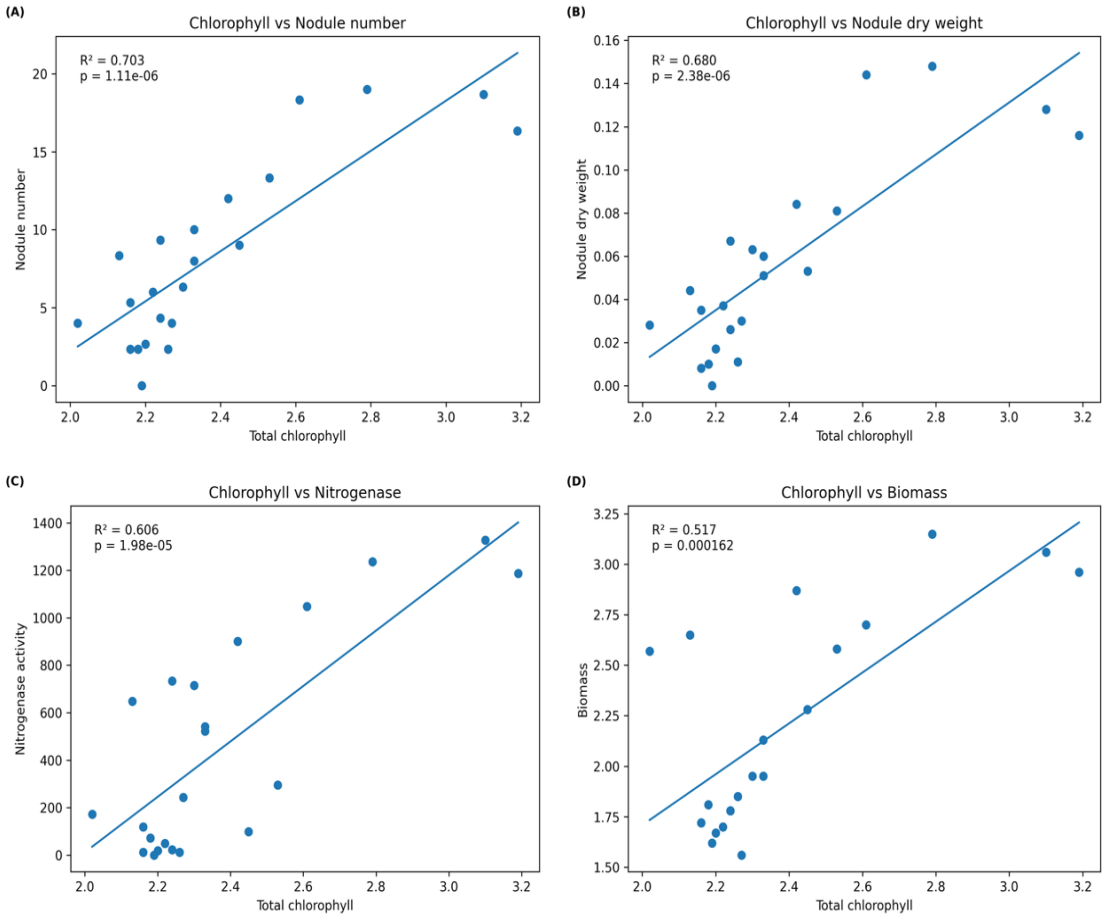

Figure-7. Pearson correlation analysis between total chlorophyll content and (A) nodule number, (B) nodule dry weight, (C) nitrogenase activity, and (D) plant biomass of *D. cochinchinensis* inoculated with different rhizobial isolates. Values are based on three biological replicates per treatment, with significant correlations ($p < 0.001$).

Total chlorophyll content was significantly higher in inoculated plants, suggesting enhanced photosynthetic ability linked with symbiotic nitrogen fixation. Inoculated plants also had higher chlorophyll a/b ratios, which suggests more efficient nitrogen allocation. Consistently, inoculated seedlings demonstrated superior overall growth performance compared to non-inoculated controls (Figure-8). These findings confirm that native rhizobial isolates are highly effective symbionts of *D. cochinchinensis*, promoting nodulation, nitrogen fixation, and plant growth.

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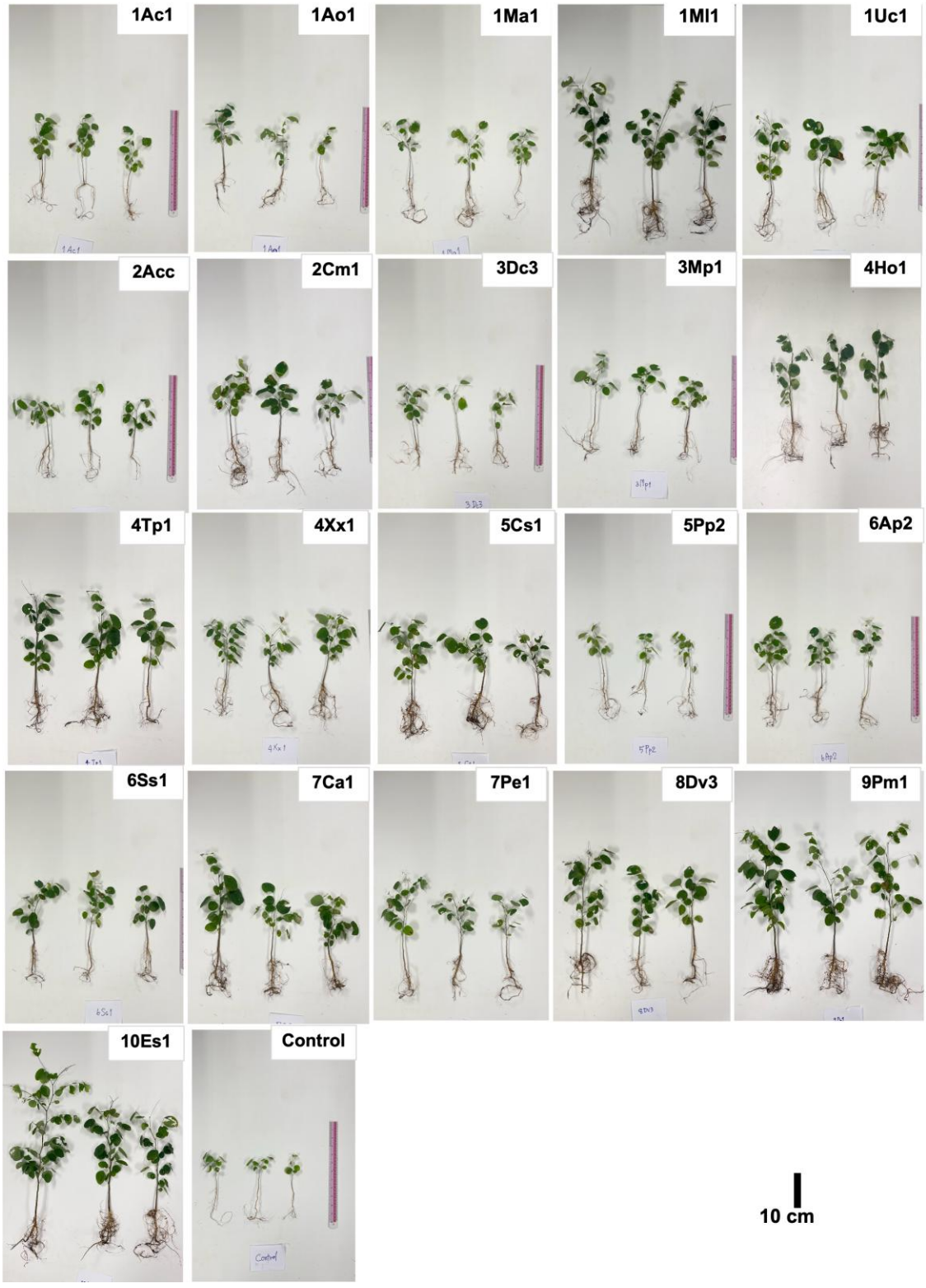

Figure-8. Growth performance of *D. cochinchinensis* seedlings inoculated with selected native rhizobial isolates under greenhouse conditions. Scale bar = 10 cm.

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Discussion

In this study, rhizobial isolates from natural and restored forests in northeastern Thailand showed high genetic diversity and substantial variation in symbiotic performance. Field-based sampling combined with molecular and functional analyses revealed differences in community-level distribution patterns and strain-level symbiotic effectiveness across forest types. Total chlorophyll content was positively correlated with nodulation, nitrogenase activity, and plant biomass, indicating a close association between symbiotic effectiveness and plant physiological performance.

The absence of significant differences in rhizobial community composition between natural and reforested forests suggests that forest type alone might not have a strong influence on these communities. This observation is consistent with prior research indicating that soil microbial communities can undergo recovery and convergence during ecosystem restoration (Barber et al., 2017; Mackay et al., 2016; Ormeño-Orrillo et al., 2012). Consequently, these findings imply that rhizobial assemblages are influenced not only by forest type but also by the availability of hosts and microenvironmental variation.

Genetic profiling showed that similar rhizobial genotypes occur across multiple forest types and hosts, with no clear clustering by site or host. These patterns are consistent with ecologically flexible rhizobia rather than strictly host- or site-specific lineages. However, broad distribution does not necessarily imply broad symbiotic compatibility, as only a subset of isolates nodulated *D. cochinchinensis*. This observation reinforces the distinction between ecological occurrence and functional compatibility. Similar patterns have been reported in plantation and reforestation systems, where some rhizobial lineages, particularly *Bradyrhizobium*, persist across sites, whereas others increase mainly with compatible legume hosts (Sene et al., 2012).

This pattern reflects the ecological adaptability of rhizobia and the significant role of host plant identity in structuring rhizobial communities. The presence of diverse legume hosts across forest types likely promotes overlapping assemblages, reducing the effect of forest type on community composition. Together, these observations suggest that rhizobial community assembly is driven more by host-mediated selection than by forest type–associated environmental filtering. However, the influence of soil physicochemical factors was not assessed in this study and could also affect community structure. In addition, BOX-PCR profiling and partial 16S rRNA gene sequencing primarily reflect genetic and taxonomic similarity rather than host compatibility mechanisms, representing a limitation also reported in previous studies (Castellano-Hinojosa et al., 2022; Taylor et al., 2020).

Although isolate recovery and genetic diversity tended to be higher in some reforested deciduous dipterocarp forests, particularly in long-term restored sites, this pattern was not consistent across all forest types. This suggests that ecosystem age alone does not determine rhizobial community composition, and that host availability and land-use history may shape rhizobial communities (Sansupa et al., 2021; Yang et al., 2021). Reforestation can enhance soil microbial functional capacity, especially genes involved in carbon, nitrogen, and phosphorus cycling, even when overall microbial diversity remains unchanged (Liu et al., 2025a).

The diversity observed here reflects only the nodulating and symbiotically compatible rhizobia captured by the siratro trapping approach, which excludes non-nodulating or host-incompatible strains (Bünger et al., 2021; Mendoza-Suárez et al., 2021). This filtering process could make community patterns across different sites more similar, possibly hiding smaller ecological differences. Therefore, the similarity between forest types likely reflects the functionally compatible rhizobia rather than total soil rhizobial diversity.

Although many rhizobial taxa were found across the forest sites, only 38% of distinct isolates nodulated *D. cochinchinensis*, indicating that compatible symbionts represent only a subset of the rhizobial community. Similar strain-specific patterns have been reported in other tropical timber legumes. *Bradyrhizobium* is one of the few lineages that form strong and effective symbioses with host plants (Bünger et al., 2021; Mendoza-Suárez et al., 2021; Zuñiga-Orozco et al., 2025). Although many rhizobia occur in the surrounding vegetation and soils, successful nodulation and nitrogen fixation are often limited to these specific groups (Bünger et al., 2021). Native rhizobial inoculation in tropical legumes promotes growth and nitrogen fixation in certain host species, but the results vary across legume species and strains within the same ecosystem (Zuñiga-Orozco et al., 2025). These findings highlight the distinction between ecological presence and functional symbiotic

effectiveness among rhizobia (Mendoza-Suárez et al., 2021). Only a subset of rhizobia possesses the genetic and physiological capacity for effective symbiosis with *D. cochinchinensis*. These results show that community-level diversity does not reliably predict symbiotic effectiveness, supporting the distinction between ecological occurrence and functional performance.

Among nodule-forming isolates, nodule number, nitrogenase activity, and plant growth parameters differed widely. Isolates 10Es1, 9Pm1, and 4Tp1 showed higher acetylene reduction activity (Table-3), along with increased chlorophyll content and biomass (Table-4), suggesting enhanced nitrogen fixation and improved seedling growth under nitrogen-limited conditions (Taylor and Komatsu, 2024). Chlorophyll content correlated positively with nodulation and nitrogenase activity. These findings support a strong association between symbiotic effectiveness and plant physiological performance. The declining strength of correlations from nodulation to biomass indicates a biologically coherent gradient, in which early symbiotic processes have a stronger effect on plant physiology than later growth responses. The lack of a consistent relationship between nodule number and nitrogenase activity further reflects variation in symbiotic efficiency among isolates. Similar trends have been reported in other *Dalbergia* species, with effective rhizobial partnerships improving seedling development and physiological function (Dhiman et al., 2022; Lu et al., 2017). The inconsistency between nodulation and nitrogen fixation indicates that symbiotic effectiveness depends on strain-specific functional traits rather than nodulation capacity alone. Hence, widespread rhizobial occurrence does not guarantee effective symbiosis with *D. cochinchinensis*. This pattern aligns with reforestation studies reporting enhanced nitrogen-cycling capacity but simplified microbial networks, suggesting a selective functional optimization (Liu et al., 2025a).

Effective native rhizobial strains from restored and natural forests can support legume establishment and improve nitrogen acquisition in degraded areas. A recent study shows that restoration success depends more on the recovery of key microbial functions than on increases in overall microbial diversity (Liu et al., 2025a; Saltonstall et al., 2025). Native rhizobia have demonstrated greater performance compared to commercial inoculants in legume growth and N_2 fixation (Castellano-Hinojosa et al., 2022), and their use has been recommended to reduce nitrogen fertilizer inputs (Abd-Alla et al., 2023). Native isolates showed high symbiotic efficacy with *D. cochinchinensis*, supporting their potential as bioinoculants for legume-based forest restoration. In particular, isolates 10Es1, 9Pm1, and 4Tp1 were identified as strong candidates, and the integrative approach used here provides a practical framework for identifying locally adapted rhizobia for restoration efforts.

The gap between the common presence of rhizobia and their actual function suggests that naturally occurring rhizobia might not always help legumes grow well. Therefore, successful restoration depends on finding and using highly effective and compatible strains. Environmental changes and competition with native rhizobia may limit inoculant persistence and effectiveness in the field. Therefore, restoration approaches should combine both community composition and strain-level functional performance (Taylor and Komatsu, 2024).

Limitations and future research directions

The absence of soil physicochemical data limits inference about the drivers of rhizobial community structure. As soil properties are known determinants of rhizobial distributions, their inclusion may clarify the weak compositional differences observed across forest types. Furthermore, using partial 16S rRNA sequences limited taxonomic resolution to the genus- level. Future studies using multilocus sequence typing and whole-genome sequencing would enable more accurate strain-level identification (Liu et al., 2025b; Shopina et al., 2024). These approaches would also allow the identification of key symbiotic genes, including nodulation (*nod*) and nitrogen fixation (*nif*) genes, which may explain differences in host compatibility and symbiotic effectiveness among isolates. Metagenomic and functional gene analyses may further clarify how rhizobial inoculation affects microbial interactions and nutrient cycling during forest recovery (Liu et al., 2025a; Pereira et al., 2025; Saltonstall et al., 2025). Long-term field studies are required to assess inoculant persistence, competitiveness, and ecological impacts, which are critical for applying laboratory findings to restoration practice.

Conclusions

Rhizobial populations in reforested and natural forests of northeastern Thailand showed high genetic diversity and marked variation in symbiotic efficiency. Although closely related rhizobia were distributed across forest types, community composition was not significantly differentiated by forest type and was more likely associated with host availability and local ecological context. These results suggest that rhizobial community assembly is driven primarily by host-mediated selection rather than forest type alone.

The presence of rhizobia did not confirm functional compatibility, as nodulation of *D. cochinchinensis* was limited to a subset of isolates, demonstrating strong functional filtering. Strains 10Es1, 9Pm1, and 4Tp1 consistently outperformed others by enhancing nodulation, nitrogenase activity, chlorophyll content, and seedling biomass. In addition, positive relationships among chlorophyll content, nodulation parameters, nitrogenase activity, and plant biomass suggest a close linkage between symbiotic performance and plant physiological responses.

Overall, successful legume-based forest restoration requires not only rhizobial presence but also the selection of highly effective and compatible strains. Integrating community-level patterns with functional performance offers a robust basis for identifying locally adapted rhizobial inoculants. However, naturally occurring rhizobia may not consistently support host establishment, and the effectiveness of selected strains under field conditions remains uncertain. Field-based validation under variable environmental conditions is required to confirm the persistence, competitiveness, and ecological performance of candidate strains in restoration settings.

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Contribution of Authors

Somwatcharajit R: Investigation, data collection, laboratory work, data curation, formal analysis and writing – original draft.

Sookruksawong S: Methodology, greenhouse experiment, data analysis and interpretation and manuscript editing.

Klinchan R: Field survey coordination, site access, sampling support and resources.

Prakamhang J: Conceptualization of study, supervision, statistical analysis, manuscript revision and final approval.

All authors read and approved the final draft of the manuscript.

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