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台灣入侵植物南美豬屎豆及其根瘤菌之共生關係

Symbiotic relationship between an invasive legume, *Crotalaria zanzibarica*, and its root-nodulating rhizobia in Taiwan

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本論文係 黃承泰 君（學號 F99b44021）在國立臺灣大學生態學與演化生物學研究所完成之博士學位論文，於民國 107 年 4 月 23 日承下列考試委員審查通過及口試及格，特此證明

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摘要

南美豬屎豆 (*Crotalaria zanzibarica* Benth.) 是台灣歸化豆科植物中分布最廣的一種，為多年生的木本植物，常見於道路旁、河岸及廢耕地。野外觀察發現南美豬屎豆普遍具有根瘤，因此推測與固氮菌(根瘤菌)共生有助其在貧瘠棲地建立族群，是讓此植物能夠在台灣廣泛分布的原因之一。然而，南美豬屎豆在台灣的共生根瘤菌未曾被研究。本論文探討南美豬屎豆與根瘤菌之間的共生關係，以了解此外來植物在台灣的共生根瘤菌之多樣性及可能來源，並檢驗不同根瘤菌株對植物的共生表現。

從種植在台大溫室的南美豬屎豆根瘤中分離出具多形態的菌株 CzR2，分析此菌株之 6 個管家基因 (*atpD*、*dnaK*、*glnII*、*gyrB*、*recA* 和 *rpoB*) 序列，結果顯示 CzR2 屬於 *Bradyrhizobium arachidis*。CzR2 在游離狀態時受到甘露醇或果糖誘導會產生多形態細胞，此現象首次在根瘤菌中被發現。CzR2 在與南美豬屎豆共生時也產生多形態的類菌體，然而有些類菌體其染色體具多倍體，有別於游離細胞的單倍體及二倍體。此結果顯示 CzR2 與南美豬屎豆共生時其染色體有內複製的現象。

調查新店溪沿岸的南美豬屎豆及常見的六種共域豆科植物，這些植物具有不同的根瘤(有限及無限)和類菌體(膨大及非膨大)形態。由分離菌株的 16S rRNA 序列分析，得知南美豬屎豆、蠅翼草、異葉山螞蝗和鍊夾豆皆與 *Bradyrhizobium* 建立共生，與田菁共生的根瘤菌為 *Neorhizobium* 和 *Rhizobium*，白花菽草則為 *Rhizobium*，含羞草的共生根瘤菌為 *Cupriavidus* 和 *Paraburkholderia*。雖然這些植物具有不同的共生特徵，其葉片都具有類似的穩定性氮同位素比值($\delta^{15}N$)，其值約為 -1 ‰；且這些植物根瘤的 $\delta^{15}N$ 皆為正值(3.7-7.3 ‰)，其中無限根瘤比有限根瘤普遍具有較高的 $\delta^{15}N$ 數值。

以多基因序列 (*dnaK-glnII-recA-rpoB*) 分析從台灣北、中、南三河岸南美豬屎豆族群所分離出之 59 株根瘤菌株，以及其他共域豆科植物族群所分離出之 54 株根瘤菌株，結果顯示這些菌株皆屬於 *Bradyrhizobium* 且可區分成 21 個支序群。同

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時比較分析其他共域豆科的根瘤菌群時，發現某些菌群似乎對南美豬屎豆有專一性，然而某些菌群則廣泛出現在多種植物的根瘤中。多數南美豬屎豆根瘤菌具有代表美洲起源的 *nodA* 共生基因，其次為亞洲起源及世界廣布型，顯示此物種在台灣能夠與來自不同地理區的的根瘤菌建立共生。

藉由溫室實驗比較接種不同菌株對南美豬屎豆的影響，結果發現南美豬屎豆與不同菌株共生時產生不同的根瘤且類菌體形態亦有所不同，顯示根瘤及類菌體形態亦受到共生菌株的影響，不全受到宿主決定；南美豬屎豆植株生物量及植株總氮量在不同菌株接種處裡下有顯著差異，然而，南美豬屎豆植株生物量分配、氮含量、穩定性同位素比值和共生效率(總生物量變化/總根瘤生物量變化)受不同接種菌株的影響並不顯著。

綜合野外調查和溫室實驗結果，證實南美豬屎豆能夠與多樣 *Bradyrhizobium* 菌種建立有效共生，顯示其為廣適性宿主，此特徵有助於該物種擴散到不同地點時能找到相容根瘤菌。另一方面，來自南美洲的外來根瘤菌對於南美豬屎豆在台灣與根瘤菌建立共生中扮演重要角色。

關鍵詞: 入侵植物、南美豬屎豆、豆科與根瘤菌共生、慢生型根瘤菌、共生特徵

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Abstract

Crotalaria zanzibarica Benth., a perennial shrub native to Africa, is the most widely-distributed naturalized legume in Taiwan. The plant, commonly distributed along roadsides and riverbanks, and in abandoned fields, established symbiosis with rhizobia forming root nodules. Root nodules are capable of fixing nitrogen. Accordingly, the symbiosis with nitrogen-fixing rhizobia might help *C. zanzibarica* colonizing nutrient-poor habitats. In this dissertation, I studied the symbiotic relationship between rhizobia and this legume, aiming to understand the diversity and possible origins of the symbiotic rhizobia and the beneficial effects of the symbiosis to *C. zanzibarica*.

A rhizobial strain, designed as CzR2, was isolated from the nodules of *C. zanzibarica* grown in a greenhouse. This strain displayed pleomorphism, cell size ranging from 2 to 10 μm, in free-living state when cultivated in standard YEM medium which significantly differs from any known rhizobia. Based on the analysis of *atpD-dnaK-glnII-gyrB-recA-rpoB* gene set, CzR2 belongs to *Bradyrhizobium arachidis*. Results of further experiments revealed that pleomorphism in this strain in its free-living state could be induced by mannitol, or fructose, but not by glucose. Accordingly, the pleomorpism is substrate-dependent. CzR2 in its free-living state contained haploid and diploid cells, while that in symbiosis with *C. zanzibarica* was elongated with polyploidy, suggesting the occurrence of genomic endo-reduplication.

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Legume-rhizobia symbioses of *C. zanzibarica* and six common legume species growing sympatrically along Xindian riverbank were investigated in Chapter 2. I found that these legumes form either determinate or indeterminate types of root nodules and harbored swollen or non-swollen bacteroids. Based on the 16S rRNA sequences, the symbionts of these legumes were classified as *Bradyrhizobium, Neorhizobium*, *Rhizobium*, *Cupriavidus* and *Paraburkholderia.* Irrespective of their possessing of diverse symbiotic traits and nodule symbionts, the seven legume species had similar and consistently negative leaf $\delta^{15}N$ values (mean of -1.2 ‰), and showed ^{15}N enrichment (varying from 3.7 to 7.3 ‰) in their nodules. In addition, variations in the values of leaf $\delta^{13}C$ (varying from -29 to -34‰) among the seven legumes were measured, indicating their photosynthetic water use efficiencies were different. The results also suggested that *C. zanzibarica* could be nodulated by diverse rhizobia.

To compare the symbionts of *C. zanzibarica* and sympatric legumes growing along three distant riverbanks in Taiwan, I collected 59 isolates from this plant and 54 isolates from coexisting legumes. Based on the multilocus sequence analysis of concatenated *dnaK-glnII-recA-rpoB* gene sequences, the *C. zanzibarica* isolates were highly diverse, belonging to 14 clades and varied among sampling sites, which can be either phylogenetically similar to or distinct from the isolates of coexisting legumes. The majority of *C. zanzibarica* isolates had *nodA* genes of American origin, following by

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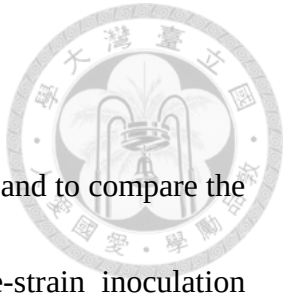

Asian origin, while others might be cosmopolitan.

To confirm the field isolates are able to nodulate *C. zanzibarica* and to compare the effects of symbionts on growth of this plant, I conducted single-strain inoculation experiment and investigated growth response, nodulation response, symbiotic efficiency and nitrogen relationship of *C. zanzibarica* inoculated with six rhizobial strains. The greenhouse inoculation experiment revealed that nodule and bacteroid morphologies in *C. zanzibarica* were rhizobial strain-dependent. Furthermore, *C. zanzibarica* plants showed significant variation in total plant biomass and nitrogen accumulation among the strains inoculated, while there was very little variation in biomass allocation, nitrogen content, $\delta^{15}N$ value and symbiotic efficiency among these tested plants.

Results of the greenhouse experiments and field investigations indicated that *C. zanzibarica* was capable of forming effective symbiosis with diverse rhizobia, which might confer the plant the ability of colonizing various habitats and contribute to its widely distribution in Taiwan.

Keywords: invasive plant, *Crotalaria zanzibarica*, legume-rhizobia symbiosis, *Bradyrhizobium*, symbiotic traits

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LIST OF ABBREVIATIONS

Culture medium

HM	HEPES-MES
YEM	Yeast extract mannitol

Phylogenetic analysis

NJ	Neighbor-joining
ML	Maximum likelihood
BI	Bayesian inference
MLSA	Multilocus sequence analysis

Plant physiology

$\delta^{13}C$	Carbon stable isotope ratio
$\delta^{15}N$	Nitrogen stable isotope ratio
WUE	Water use efficiency
C_{mass}	Carbon content on dry weight basis
N_{mass}	Nitrogen content on dry weight basis

Sampling site

XD River	Xiandan River (新店溪)
DJ River	Dajia River (大甲溪)
GP River	Gaoping River (高屏溪)

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General Introduction

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As a consequence of globalization, many plant species were intentionally or accidentally transported into new ecosystems by human activities. Some of these exotic plants can colonize and invade into native communities in the introduced areas, disturbing the structure and function of ecosystems. Therefore, plant invasions have become one of the major threat to global biodiversity (Vitousek *et al*., 1997; Pimentel *et al*., 2005) and received much attention from the researchers.

Taiwan, with no exception, has 608 naturalized plant species which comprise 12% of the total flora on this island (Wu *et al*., 2010). The family Fabaceae (Leguminosae) is one of the dominant families (79 species) among naturalized plants in Taiwan (Wu *et al*., 2010) and also the most contributor of the naturalized plants in the world (Pyšek, 1998). In addition to their prevalence, naturalized legumes could have impacts on ecosystems. Some naturalized legumes increased the soil nitrogen and altered the growth rate of other plant species, thus interfered the structure and function of plant communities (Vitousek, 1990; Yelenik *et al*., 2004). Wu *et al.* (2003) provided the background information (such as origin, habit, life form and minimum residence time) of naturalized legumes in Taiwan which was a starting point for evaluating the leguminous plant invasion in Taiwan. However, one of the most remarkable features of leguminous plants, the capability to form mutualistic symbiosis with rhizobia, is still poorly investigated in Taiwan. This thesis aims to understand the symbiotic relationship between the naturalized legume and its nodulating rhizobia in Taiwan.

In this chapter, I gave a brief introduction about the definitions of plant invasions, legume taxonomy, rhizobial symbionts of legumes, and studies of legume-rhizobia symbiosis in Taiwan. A summary of the objectives in this thesis was presented in the last section of this chapter.

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1. Terminology of plant invasions

In 1958, Elton published the classic book 'The Ecology of Invasions by Animals and Plants' which first exploited the field of invasion ecology. However, the term 'invasive species' was not strictly defined in Elton's book, and its meaning was not consistent among the published literatures, causing considerable confusion and misuse. Some researchers suggested that exotic species which causing in economic and/or environmental damages or harm to human are considered invasive species (Cronk and Fuller, 1995; Mack, 1997). Some researchers defined the invasive species as exotic species which spread over large areas in the introduced sites (Richardson *et al*., 2000a; Williamson, 1997).

The terminology used in this proposal followed the definitions of Richardson *et al*. (2000a). The authors suggested that the spread of plants were limited by several barriers, including major geographical, environmental, reproductive and dispersal barriers., Plants introduced by human activities across major geographical barriers into new habitats are 'alien plants' (synonym: exotic plants). Alien plants which overcome the local environmental barriers in their introduced sites and sustain populations over many generations without human intervention, are referred as 'naturalized plants'. If the naturalized plants could spread into new areas away from (> 100 m) their introduced sites, they are then defined as 'invasive plants'. In addition, 50-80% of invasive plants which cause obviously negative impacts on native biodiversity are further categorized as 'environmental weeds'. About 10% of invasive plants which are capable of changing the nature of ecosystem over considerable areas (such as nitrogen-fixer, sand stabilizers, fire promoters/suppressors...etc.) are referred as 'transformers'.

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2. Legumes of the world and in Taiwan

The family Leguminosae (Fabaceae), one of the largest plant families, contains more than 18,000 species throughout the world (Lewis *et al*., 2005; Sprent, 2009). Papilionoideae is the largest subfamily of legumes, and most of its members are able to form nodules with symbiotic rhizobia (Sprent, 2007, 2009). Lavin *et al*. (2005) subdivided the major nodulating legumes within Papilionoideae into seven distinct groups, Genistoids, Dalbergioids, Mirbelioids, Indigoferoids, Millettioids, Robinioids and Inverted Repeat Loss clade (IRLCs) (**Figure A**), based on their fossil records and molecule data. According to the book 'Legume Nodulation: A Global Perspective' published by Sprent (2009), the global distribution of these papilionoid groups were distinctive. Crotalarieae and Genisteae, the two largest tribes in Genistoids, are mainly distributed in Africa and Mediterranean regions, respectively. Dalbergioids are largely in Central and South Americas. Millettioid is a pan-tropical group and world-wide spread. Mirbelioids are endemic to South Africa and Australia. Indigoferoid legumes are mostly distributed in Africa, considerable numbers are also found in Asia and Australia. Most of the genera in Robinioids and IRLCs are

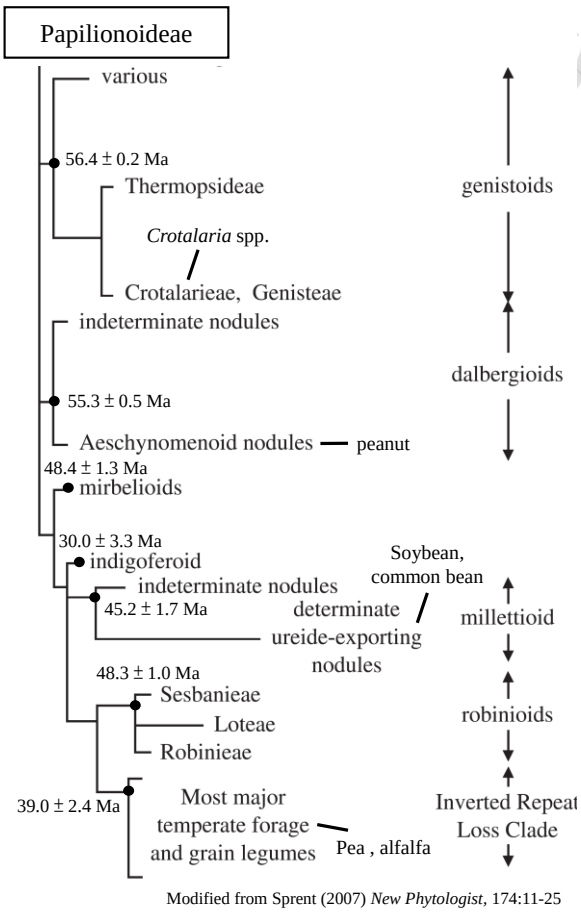

Figure A. Phylogeny of the nodulating groups in Papilionoideae. Age estimates are shown at the nodes of each group based on the report of Lavin *et al*. (2005). Ma = million years. The important legumes belonged to these groups are indicated.

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distributed in temperate regions.

Taiwan, a sub-tropical Pacific island, has 214 legume species, including 160 native species and 54 naturalized species (Huang, 1993; Wu *et al*., 2003). Thus, about 25% of the legume plants in Taiwan are introduced. Most of legumes in Taiwan belong to the subfamily Papilionoideae (including 140 native and 44 naturalized species). Among these papilionoid legumes, 127 species (106 native and 21 naturalized) belong to Milletioids, 26 species (20 native and 6 naturalized) to Genistoids, 16 species (4 native and 12 naturalized) to IRLCs, 11 species (7 native and 4 naturalized) to Dalbergioids and 4 species (3 native and 1 naturalized) to Robinioids.

3. Rhizobia play an important role in legume ecology

Rhizobia, comprising phylogenetically diverse soil bacteria, are able to induce nodules, a tumor-like structure hosting the nitrogen-fixing rhizobia, on stems or on roots of legume plants. Based on the 16S rRNA gene sequences, currently known rhizobia are divided into two classes, Alphaproteobacteria and Betaproteaobacteria, referred as alpha- and beta-rhizobia, respectively. The legume-rhizobia symbiosis is commonly found in Mimosoideae and Papilionoideae, but rarely in other subfamilies (Sprent, 2009). Mimosoideae usually establishes symbiosis with beta-rhizobia (such as *Burkhoderia* and *Cupriavidus*), whereas Papilionoideae is commonly nodulated by alpha-rhizobia (Sprent, 2007, 2009; Gyaneshwar *et al*., 2011). Alpha-rhizobia are highly diverse, including the classical rhizobia, such as *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium* and *Azorhizobium* (Young and Haukka, 1996), and the non-classical genus, *Methylobacterium* (Sy *et al*., 2001). The host specificity of alpha-rhizobia vary among genera, for examples *Bradyrhizobium* is a common

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symbiont of Milletioid, Genistoid and Dalbergioid legumes, while *Rhizobium* spp. mainly nodulate IRLCs (Sprent, 2007, 2009).

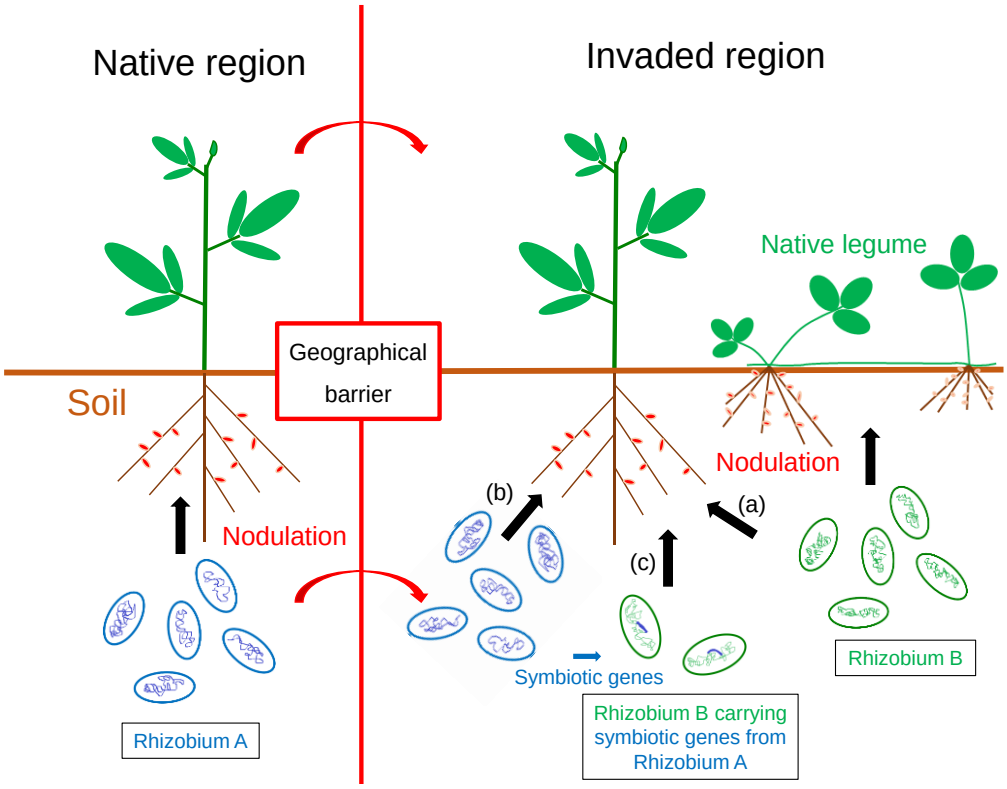

Figure B. Three scenarios may contribute to the finding of exotic legume for its compatible rhizobia in the invaded areas. (a) Exotic legume might recruit the local rhizobia from native legumes, (b) exotic rhizobia might be introduced accompanying the introduction of exotic legume, and (c) exotic legume might be associated with the rhizobia which are phylogenetically similar to local rhizobia but carrying the symbiotic genes from exotic rhizobia.

Rhizobia have been well known to improve legume yields in agriculture. Besides, rhizobia could also be critical factors affecting legume distributions in natural ecosystems. It has been reported that the growth of exotic legumes was limited by scarcity of compatible rhizobia when they were originally introduced to new habitats (Parker *et al.*, 2006; Richardson *et al.*, 2001b). Several scenarios allowing exotic legumes to find their nodule symbionts in the introduced areas have been reported (**Figure B**). For their successful establishment, the exotic legumes might recruit the

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indigenous rhizobia in the introduced areas (Parker *et al*., 2006). Alternatively, the exotic legumes and their nodule symbionts might have co-introduced from native ranges into invaded regions (Rodríguez-Echeverría, 2010; Crisóstomo *et al*., 2013). For example, Horn *et al*. (2014) reported that an invasive legume, *Cytisus scoparius* in North America obtained its compatible *Bradyrhizobium* symbionts from heterogeneous ancestry. Some were associated with native rhizobia from native legumes of North America. Some utilized exotic rhizobia of European *C. scoparius* (the ancestral population). Besides, the authors also found that some symbionts of *C. scoparius* were phylogenetically similar to native rhizobia but displayed European originated symbiotic genes.

4. Studies of legume-rhizobia symbiosis in Taiwan

Some of *Mimosa* plants are notorious invasive plants in the world (Binggeli, 1996). Among them, *Mimosa pudica* is listed as the world's worst weed (Holm *et al*., 1991) and also as one of the 20 major environmental weeds in Taiwan (Chiang *et al*., 2003). Chen *et al*. (2003) examined 190 nodule symbionts isolated from *M. pudica* and its congener, *M. dipliotricha* distributed in 14 sites across Taiwan Island. Based on 16S rRNA gene sequences, 93% of isolates belonged to *Ralstonia taiwanensis* (later renamed *Cupriavidus taiwanensis*). In contrast, *M. pigra*, a newly naturalized plant in Taiwan (Wu *et al*., 2010), was mainly nodulated by *Burkholderia* (Chen *et al*., 2005). Barrett and Parker (2005) reported that *M. pudica* and *M. pigra* growing in their native ranges (Panama) were commonly associated with *Burkholderia* strains. These results suggested that naturalized legumes in Taiwan could utilized the compatible rhizobia distinct from or similar to those symbionts in their original regions.

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Sesbania cannabina, belonging to Robinioid group, has been used as a common green manure in Taiwan and is also on the list of the 20 major environmental weeds in Taiwan (Chiang *et al*., 2003). Chen and Lee (2001) investigated 18 nodule symbionts isolated from *S. cannabina* grown in the sugarcane fields, locating in the southern part of Taiwan. According to amplified 16S rDNA restriction analysis (ARDRA) and 16S rDNA sequencing, 18 isolates were separated into four genotypes which belonged to *Sinorhizobiun* and *Rhizobium*. Furthermore, the symbiotic genes (*nifH*) of these isolates were also diverse. This result indicated that naturalized *S. cannabina* plants in Taiwan were nodulated by phylogenetically diverse rhizobial community even though they were grown in adjacent fields.

Hung *et al*. (2005) reported the nodule symbionts of seven native legumes, including six Millettioid legumes and one Genistoid, growing in the central part of Taiwan. Among the 83 isolates they examined, most of strains belonged to *Rhizobium*, *Bradyrhizobium* and *Agrobacterium*, whereas one *Sinorhizobium* strain and one *Burkholderia* strain were also isolated.

Taken together, the aforementioned studies revealed that rhizobia associated with legumes in Taiwan were highly diverse. However, most of these studies used the 16S rRNA gene analysis and phenotypic traits to characterize the isolated rhizobia. It has been suggested that the 16S rRNA genes and phenotypic traits of rhizobia are variable. In addition, the origin of these rhizobia and the specific relationship between these rhizobia and their legume hosts were rarely studied.

5. Objectives and studies of this study

Crotalaria plants, containing around 600 species mainly native to Africa, were

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introduced to many countries as green manures or fodders and became the common exotic legumes found throughout the world (Polhill, 1982). Nine naturalized *Crotalaria* species were recorded in Taiwan, including *C. bialata* Schrank, *C. incana* L., *C. lanceolata* E. Mey, *C. linifolia* L. f., *C. micans* Link, *C. pallida* Ait., *C. spectabilis* Roth., *C. triquetra* Dalzell and *C. zanzibarica* Benth. (Wu *et al*., 2010). *C. zanzibarica*, first documented in 1934, has been evaluated as a naturalized species with the highest invasiveness in Taiwan (Wu *et al*., 2003, 2005). This plant mainly distributed in the nutrient-poor habitats, such as roadsides, riverbanks and abandoned fields, commonly formed root nodules (Wu *et al*., 2005). In a preliminary study, I cultivated *C. zanzibarica*, neither inoculated nor fertilized, in a greenhouse of NTU and found some individuals formed root nodules and looked much healthier than the others without such structures. I therefor suspected that the nitrogen-fixing symbiosis of *C. zanzibarica* might contribute to its successful colonization and widely distribution in Taiwan. Despite the fact that *Crotalaria* is the largest Genistoid legume and spreads worldwide (Sprent, 2009), the studies on its symbiotic characteristics are relatively few. In addition, the symbiotic relationship between legumes and nitrogen-fixing rhizobia in Taiwan were also rarely exploited. The observation and literature survey invoked my interests in studying the relationship between *C. zanzibarica* and its symbionts.

In this thesis, I focused on the symbiotic characteristics of naturalized *C. zanzibarica* in Taiwan, and the specific objectives are: (1) to investigate the diversity, possible origin and host specificity of rhizobia associated with *C. zanzibarica*, (2) to document the symbiotic related traits of *C. zanzibarica*, including nodule type, bacteriod morphology, nitrogen content and nitrogen stable isotope, and (3) to understand how rhizobial strains collected from divergent sources varied in their

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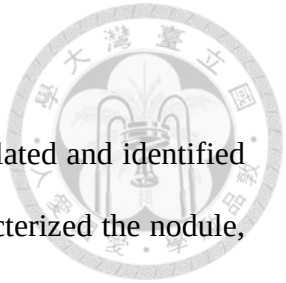

symbiotic performance on *C. zanzibarica* plant. To begin with, I isolated and identified the nodule symbiont of greenhouse-grown *C. zanzibarica* and characterized the nodule, bacteroid and symbiosome features. The results are presented in **Chapter 1**.

Following the results of **Chapter 1**, in order to understand whether field-growing and greenhouse-grown *C. zanzibarica* had similar symbiotic characteristics, I investigated *C. zanzibarica* growing along Xindian riverbank in the northern part of Taiwan. In addition, to further examine whether *C. zanzibarica* shared nodule symbionts with sympatric legumes along Xindian riverbank, several co-existing legumes were also analyzed. Results were presented in **Chapter 2**.

After investigating the Xindian population, I extended the investigation into two other *C. zanzibarica* populations growing along Dajia and Gaoping riverbanks, locating in the central and southern parts, respectively, of Taiwan. In **Chapter 3**, I reported the phylogeny and possible origins of rhizobia isolated from these three *C. zanzibarica* populations.

In previous studies (**Chapter 1 and 2**), I found that plants of *C. zanzibarica* cultivated in a greenhouse of NTU and growing along the three riverbank were nodulated by different *Bradyrhizobium* strains and displayed distinctive bacteroid sizes. In **Chapter 4**, I conducted the single-strain inoculation experiment to confirm the symbiotic relationship.

A figure illustrating the main theme of each chapter in this study is shown in **Figure C**.

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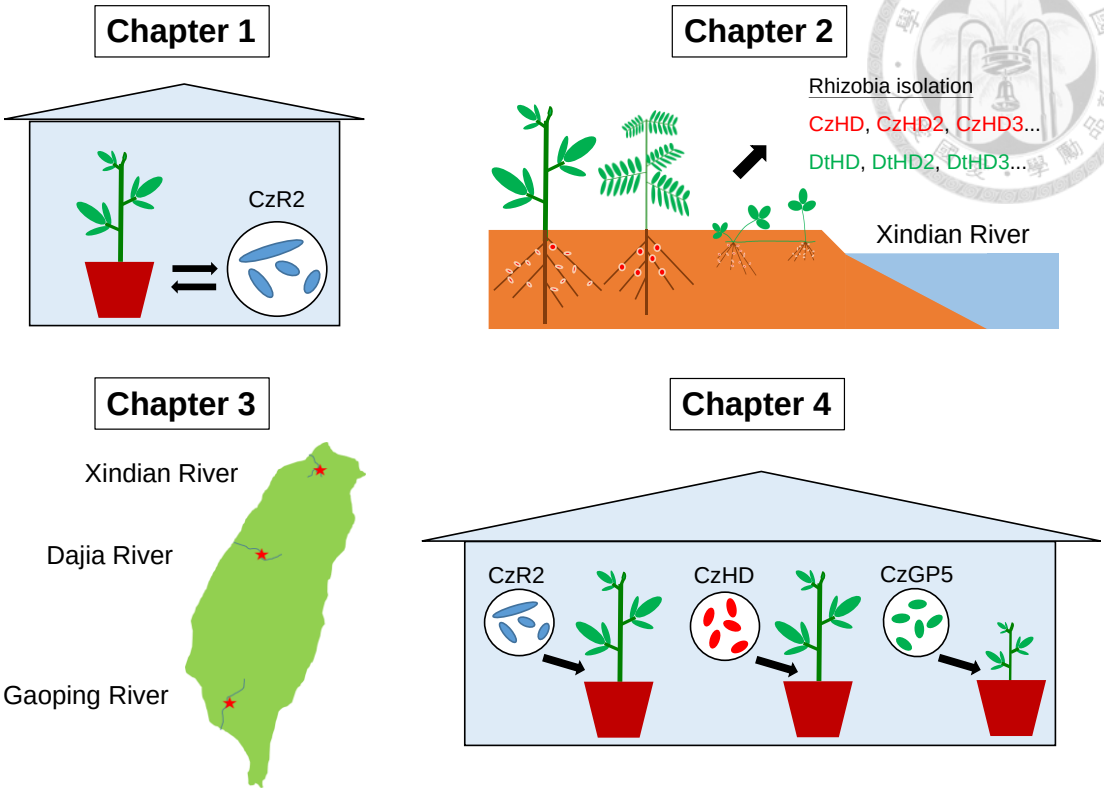

Figure C. Illustration of the context of the four main chapters in this dissertation.

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Chapter 1

C. zanzibarica cultivated in the greenhouse was nodulated by a peculiar strain, CzR2

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Abstract

Crotalaria zanzibarica is an exotic, widely-distributed leguminous plant in Taiwan. To understand traits contributing to its successful invasion, I cultivated this plant in a greenhouse. Without fertilizer and rhizobia added, the plants still formed root nodules and had high leaf nitrogen contents (5-8%). A rhizobial strain, designed as CzR2, was isolated from these nodules. This strain displayed pleomorphism, cell size ranging from 2 to 10 μm, in free-living state when cultivated in standard YEM medium which significantly differs from any known rhizobia. In this study, I identified and studied the characteristics of the strain, and observed, the nodules formed by the strain. The objective of the study was to understand the relationship between *C. zanzibarica* and its rhizobial symbionts.

Results of multilocus sequence analysis of *atpD*, *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB* genes confirmed that CzR2 belongs to *Bradyrhizobium arachidis*. CzR2 was uniformly rod-shaped in basal HM medium but it displayed pleomorphic cells when mannitol or fructose was added which indicates its unusual morphology in YEM medium is caused by the gradient mannitol. No infection thread was found but highly pleomorphic bacteriods and a symbiosome containing several bacteroids were often observed under FM and TEM observations of the indeterminate nodules of *C. zanzibarica* formed by the inoculation of CzR2. Results of the flow cytometric analysis revealed that CzR2 cells in YEM medium and in nodules of *C. zanzibarica* had two and three peaks of relative DNA contents, respectively. It suggests that elongated cells of CzR2 in free-living state might result from cell-cycle delay while those in symbiotic state from genomic endo-reduplication.

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Introduction

Soil bacteria that establish symbiotic relationships with legumes and induce tumor-like, N_2-fixing structure (nodule) on roots are referred to as rhizobia. Rhizobia have been shown to improve legume yields in agriculture (Thies *et al*., 1991). Most of the research of legumes-rhizobia symbioses focused on the legume crops and model legumes, such as *Glycine*, *Pisum*, *Medicago* and *Lotus*, but relatively rare in other legumes (Doyle and Luckow, 2003). Besides the agricultural benefits, rhizobia could also play a critical role in affecting legume distributions in natural ecosystems. It has been reported that the growth of exotic legumes were limited by scarcity of compatible rhizobia when they were first introduced to new habitats (Richardson *et al*., 2000b; Park *et al*., 2006). For their successful establishment, the naturalized legumes ("naturalized plants" in this report are exotic plants that can sustain populations over many generations without human intervention, defined by Richardson *et al*. (2000a) should acquire their symbionts from newly occupied habitats. Although legumes are the most contributors of the naturalized plants of the world (Pyšek, 1998), much less is known about their compatible rhizobia in their introduced regions and how specific these symbionts are to host plants.

The genus *Crotalaria*, containing around 600 species distributed in tropical and subtropical regions, is a member of the subfamily Papilionoideae and most of its members are native to Africa (Polhill, 1982). Some species of *Crotalaria* were introduced to countries as green manures or fodders (Polhill, 1982). Despite the fact that *Crotalaria* is a large genus and spread worldwide, studies on its symbiotic rhizobia are relatively few. According to the limited literatures, *Bradyrhizobium* strains are the most common symbionts isolated from *Crotalaria* species (Samba *et al*., 1999; Liu *et al*.,

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2007; Aserse *et al*., 2012). However, some nonclassical rhizobial strains, such as *Methylobacterium* (Sy *et al*., 2001) and *Burkhoderia* (Liu *et al*., 2007), have also been reported to nodulate *Crotalaria* spp..

Crotalaria has been introduced into Taiwan for more than 80 years, and is the largest genus of the naturalized legumes of the island (Wu *et al*., 2003). *Crotalaria zanzibarica* is a perennial leguminous shrub, native to Africa, and becomes the most widely-distributed naturalized legume in Taiwan (Wu *et al*., 2005). This plant, mainly distributed along roadsides and riverbanks, and in abandoned fields, commonly established symbiosis with rhizobia forming root nodules. *C. zanzibarica*, despite being evaluated as a species of wide distribution and of highest invasiveness among the naturalized legume in Taiwan (Wu *et al*., 2003, 2005), the relationship between its distribution and the symbiotic rhizobia has not been addressed. In a preliminary study, I grew *C. zanzibarica*, neither inoculated nor fertilized, in a greenhouse and found some individuals formed root nodules and looked much healthier than the others without such structures. In addition, the nodules and leaves of the plant accumulated unusually high nitrogen contents, 6-9% and 5-8%, respectively (Huang and Kao, unpublished). The results prompted us to study the symbiotic relationship between the plant and its root-nodulating rhizobia.

In this study, a peculiar rhizobium, with pleomorphism both in YEM medium and in symbiotic state, from root nodules of greenhouse-grown *C. zanzibarica* was isolated. Based on its slow-growing phenotype, I suspected that this isolate as a species of *Bradyrhizobium*. In this study, I analyzed 16S rRNA gene sequence and several housekeeping and symbiotic genes of this rhizobium to elucidate its species identity. The phenotypic variations, in particular the causes of pleomorphism in YEM medium,

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and symbiotic characteristics with *C. zanzibarica* of the rhizobium were also investigated.

Materials and methods

Isolation of rhizobia from root nodules of C. zanzibarica

Nodules of *C. zanzibarica* grown in a greenhouse of National Taiwan University (Taipei City, Taiwan) were collected. Fresh nodules were surface sterilized by immersion in 0.5% SDS for 1 min, then 70% ethanol for 5 min, and washed three times by sterile deionized-distilled water (DDW). Nodule suspension was obtained by crushing the nodule in DDW and spread onto YEM agar plate (grams per litter: NaCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 0.5; $FeSO_4 \cdot 7H_2O$, 0.005; mannitol, 10.0; yeast extract, 0.4; agar, 15.0; the pH was adjusted to 6) placing at 30℃. When putative rhizobial colonies were visible, single colony was picked and checked for unity by repeated streaking on YEM agar plate. A pure culture was obtained and designated as strain CzR2. For comparative purpose, a reference strain, *Bradyrhizobium japonicum* USDA 110 was purchased from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). Pure cultures of each strains were stored in 15% glycerol-YEM broth at -80℃.

Molecular identification of strain CzR2

Total genomic DNA was extracted from the pure culture of strain CzR2 grown in YEM broth until the late exponential phase of growth. Extraction of the DNA was performed by using Geneaid DNA Mini kit (Geneaid). The 16S rRNA genes were amplified by using bacterial universal primers (Marchesi *et al*., 1998).

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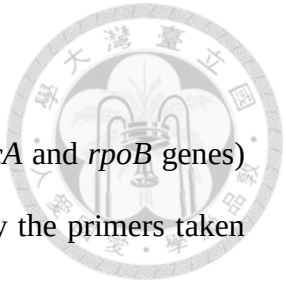

The other six housekeeping genes (*atpD*, *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB* genes) and three symbiotic genes (*nodA*, *nodZ* and *nifH*) were amplified by the primers taken from published literatures (Menna and Hungria, 2011; Stępkowski *et al*, 2005; Parker, 2012). Sequencing primers of all genes were the same primers used for PCR amplification.

The aforementioned sequences and reference sequences of housekeeping genes were aligned by using the CLUSTALW program (Thompson *et al*, 1994). Phylogenetic identification of strain CzR2 was conducted by analyzing 16S rRNA and combined *atpD-dnaK-glnII-gyrB-recA-rpoB* genes dataset, respectively. Maximum likelihood (ML) phylogenies and model tests were both performed by using software MEGA version 6 (Tamura *et al*., 2013). The best-fit models for ML analyses were T92+G+I for 16S rRNA gene and GTR+G+I for *atpD-dnaK-glnII-gyrB-recA-rpoB* genes dataset. The topology of the trees was evaluated by bootstrapping with 1,000 replications. All the sequences obtained in this study were deposited in GenBank database. The GenBank accession numbers of strain CzR2 generated in this study are KJ125399 (16S rRNA), KU315329 (*atpD*), KU000974 (*dnaK*), KU000975 (*glnII*), KU315331 (*gyrB*), KU001035 (*recA*), KU001095 (*rpoB*), KJ125402 (*nodA*), KJ135034 (*nodZ*) and KJ135031 (*nifH*).

Cellular morphology and growth dynamic in YEM medium

Both CzR2 and the reference strain were grown on YEM agar medium at 30°C for 7 days. Single colony of each strain was picked and stained with DAPI (4,6-diamidino-2-phenylindole; Sigma-Aldrich) at 50 μg/ml for 10 min at 25 ℃. The cell samples were examined by the microscopy (BX51, Olympus, Japan) under a bright

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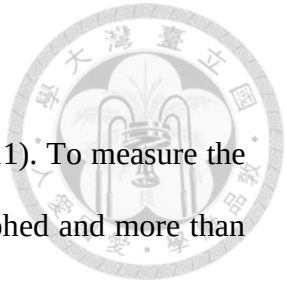

or fluorescent field, following the method reported by Liu et al. (2011). To measure the bacterial cell size, at least ten random fields of view were photographed and more than 1,000 cells of each sample were examined by using ImageJ 1.48 software.

For constructing the growth curves, strain CzR2 and USDA 110 were inoculated into 3 ml of fresh YEM broth (five replicates for each strain) and grown at 30 ℃, 200 rpm on a shaking table. The cell growth was evaluated as the number of CFU ml^{-1} and OD_{600} measurement. CFU ml^{-1} was counted every 24 hours and OD_{600} value was measured every eight to ten hours. The mean generation times were calculated from the exponential phase of growth based on the OD_{600} measurement. The cell size was also measured every 24 hours during cell growth.

Substrate tests in HM medium

Because YEM media containing yeast extract is a complex medium, the defined medium, HM (HEPES-MES) medium (Cole and Elkan, 1973) was used to conduct the substrate tests. Strain CzR2 were grown on the HM basal plates and these media comprised of different substrates (0.04% or 0.3% yeast extract, 0.5% mannitol, 0.5% fructose and 0.5% glucose, respectively) at 30°C for 7 days.

To compare the growth response among different substrates, I also inoculated strain CzR2 into 3 ml HM basal broth and the broths added 0.04% yeast extract, 0.5% mannitol, 0.5% fructose or 0.5% glucose. In all substrate tests, strain USDA 110 was also included for comparative purposes. Additionally, *B. arachdis* CCBAU 51107^{T} was tested its morphology grown in YEM, HM basal broths and HM broths with different substrates.

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Morphological features of CzR2 infected nodules

Seeds of *C. zanzibarica* were sterilized using 5% SDS and 10 mM NaCl for 5 min, then 70% ethanol for 30 min and finally drilled by sterilized needle to break seed coat. The sterilized seeds were germinated in a Petric dish at room temperature for 2 days. After germination, seedlings were transferred into 1L glass pots filled with a sterilized mixture of peat soil, vermiculite and perlite in a proportion of 5:2:2, respectively. Plants were irrigated with distilled water regularly. After 4-5 days of growing in the pots, plants in pots were inoculated either with 600 μl of strain CzR2 at exponential phase in YEM broth or with a YEM liquid media without rhizobial culture as a negative control. All the seedlings were grown in a humidity controlled (70%) growth camber with a 12 hours of light period (photosynthetic active radiation of 100~150 μmol m^{-2} s^{-1}, at 28℃) and a 12 hours dark period (at 25℃). When plants were 40 days old, they were transferred from the growth camber to a glasshouse and grown in the 2 L plastic pots in natural daylight. For histological observation, branched nodules from two months old plants were fixed by FPGA (formalin: propionic acid: glycerol 95%: EtOH: H_20=1:1:3:7:8) and sectioned to a thickness of 15 μm by using a microslicer DTK-1000. Nodule sections were stained with 1% toluidine blue O (TBO), and then examined by light microscopy.

Observations of symbiotic CzR2 (bacteroids) under fluorescent and transmission electron microscopies

Nodule samples were collected from mature *C. zanzibarica* plants (two months old) which were inoculated by strain CzR2 and grown in a NTU greenhouse. For FM observation, bacteroids were extracted from fresh nodules and stained with DAPI,

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following the aforementioned protocol. For TEM observation, nodule samples were fixed with 2.5% glutaraldehyde, dehydrated in 15-100% acetone series, and then, embedded with Spurr's resin. Ultrathin sections (80 nm) were conducted by using an ultramicrotome (PowerTome XL) and analyzed on a transmission electron microscope (Hitachi H-7650) at 100 kV.

DNA content of free-living and symbiotic CzR2

Free-living CzR2 was grown in YEM broth for 7 days and symbiotic bacteroids were extracted from mature root nodules of *C. zanzibarica* (67 days old). These cells were fixed in 90% ethanol for 16 h at 20°C, then washed twice with PBS followed by centrifugation for 2 min at 4,000 rpm. Pelleted cells were stained with propidium iodide (PI)-RNase staining buffer solution (BD Biosciences) for 30 min at room temperature. For each flow cytometry experiment, the DNA content was measured in a population of 20,000 cells with a Cytomics FC500 analyzer (Beckman Coulter Ltd.). Data analysis was performed with CXP software (Beckman Coulter Ltd.).

Results

Phylogenetic identification of strain CzR2 by using 16S rRNA gene and MLSA of atpD, dnaK, glnII, gyrB, recA and rpoB genes

In the phylogenetic tree of 16S rRNA gene (**Figure 1-1**), strain CzR2 was grouped together with four *Bradyrhizobium* type strains, and all of them had nearly identical sequences. Strain CzR2 showed 99.92% similarity to *B. huanghuaihaiense* CCBAU 23303^{T}, 99.77% similarity to *B. arachidis* CCBAU 51107^{T} and 99.39% similarity to both *B. iriomotense* EK05^{T} and *B.ingae* BR1025^{T}.

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Maximum likelihood analysis of the concatenated six housekeeping genes (*atpD*, *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB*) revealed that strain CzR2 and four CCBAU strains formed a well-supported group (**Figure 1-2**). These four CCBAU strains were reported that they belonged to a novel species, *Bradyrhizobium arachidis* (Wang et al., 2013). Comparison of these six genes among related strains, strain CzR2 was most similar (99.27% identity) to the type stain of *B. arachidis*, CCBAU 51107, which is peanut symbiont isolated from Hebei, China.

Similarities of symbiotic genes between strain CzR2 and CCBAU 51107

Beside the housekeeping genes, I also compared the symbiotic gene sequences between strain CzR2 and CCBAU 51107. These two strain had 98.71% similarity in *nodA* gene (460 bp) and 99.85% similarity in *nifH* gene (648 bp). The *nodZ* gene sequence of CCBAU 5117 was not available in GenBank.

Comparison of cellular morphology and growth dynamic between strain CzR2 and USDA 110 in YEM medium

Strain CzR2 and USDA 110 both showed slow-growing phenotype which formed detectable colonies on YEM plate after 5-6 days of incubation. The cellular morphology of these two strains was observed by optical microscopy. As shown in **Figure 1-3**, strain CzR2 was highly various in cell size (1.4 to 10.9 μm in length) and branched cells were occasionally observed (1-2 % in examined cells). On the other hand, USDA 110 was uniformly rod-shaped and the cell length was from 1.3 to 3.4 μm under the same growth condition.

The mean length of strain CzR2 dramatically increased from 3.2 to 5.0 μm during

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exponential growth phase (**Figure 1-4**), about 3% of these cells were branched. In contrast, the cell size of USDA 110 was consistent (about 2 μm) in any growth phases (data not shown).

Effect of substrates on the morphology of free-living strain CzR2

Strain CzR2 was uniformly rod-shaped on the basal HM plate. When 0.04 or 0.3% yeast extract were added, it remained uniformly rod-shaped. In contrast, when it was grown on the HM plate with 0.5% mannitol displaying pleomorphic cells. Furthermore, the pleomorphism of strain CzR2 can also be induced by adding fructose, but not by glucose (**Figure 1-5**). On the other hand, the cells of USDA 110 grown with any tested substrates were uniformly rod-shaped.

Symbiotic characteristics of strain CzR2 in C. zanzibarica

Strain CzR2 can induce *C. zanzibarica* to form visible nodules (1 mm) at 7 dpi (days post inoculation). The nodules of seedlings at 35 dpi became branched, and that of plants at 56 dpi displayed multi-lobed morphology (**Figure 1-6 ABC**). On the other hand, the control plants never formed any nodule in the growth chamber. Nodule sections showed that all plant cells in the infection zone were uniformly infected and no infection thread was formed (**Figure 1-6 DE**).

Bacteroids extracted from the multi-lobed nodules of *C. zanzibarica* were highly pleomorphic and some were longer than 10μm in length (**Figure 1-7A**). TEM images of nodule section also showed that strain CzR2 displayed pleomorphism inside the host cells. Besides, symbiosomes commonly comprised multiple bacteroids (**Figure 1-7B, C**).

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The results of flow cytometric analysis were shown in **Figure 1-8**. Symbiotic CzR2 isolated from nodules of *C. zanzibarica* showed three peaks (1C, 2C and 3C). On the other hand, free-living CzR2 was composed of two peaks (1C and 2C).

Discussions

In this study, I isolated a rhizobial strain, CzR2, from roots of greenhouse-grown *C. zanzibaria* and found this strain displayed bacteroid-like morphology in free-living states. To my knowledge, this is the first report that free-living rhizobia showed pleomorphism in standard YEM medium. Based on the MLSA of six housekeeping genes, strain CzR2 can be classified as *Bradyrhizobium arachidis*. Strain CzR2 and *B. arachidis* CCBAU 51107^{T} were nearly identical (>99% similarity) in the both housekeeping and symbiotic gene sequences. Wang et al. (2013) reported that *B. arachidis* is a peanut symbiont in China, with rod-shape of 1.30 to 1.97 μm in length, and its generation time was 8.8 hours in YEM broth. In consistent with their report, strain CzR2 is able to nodulate peanut (data not shown). However, cell length (**Figure 1-3**) and generation time (ca. 30.2 hours, data not shown) of CzR2 were significantly different from those of *B. arachidis* CCBAU 51107^{T} reported by Wang et al. (2013). To elucidate the discrepancy, I purchased and cultured *B. arachidis* CCBAU 51107^{T} (LMG 26795), deposited in Belgian Coordinated Collections of Microorganisms, and confirmed this type strain also displayed pleomorphism in standard YEM medium (data not shown).

Bacteria usually maintained their morphological uniformity due to equal cell-division (Young, 2006). A pure bacterial culture displaying diverse cell shape and size (called pleomorphism) was considered as the product of old culture (composing

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ageing and dying cells) or contaminants (Wainwright, 1997). In this study, strain CzR2 in YEM broth gradually increased its cell size at the early exponential phase (**Figure 1-4**), when cells were young. Even when CzR2 was sub-cultured repeatedly or re-isolated from legume hosts, pleomorphism was still present. In contrast, reference strain USDA 110 never showed such unusual morphology under the same culture conditions. Taken together, these results confirm that pleomorphic phenotype of CzR2 is a genuine feature rather than artifact.

It has been reported that some bacteria produced filamentous cells in response to specific environmental cues. Steinberger et al. (2002) reported that cells of *Pseudomonas aeruginosa* grown in media with low nutrient was significantly elongated but their width remained unchanged. As consequence, the ratio of cell surface area to cell volume was increased which might enhance the absorbance of nutrients. In addition, several pathogenic bacteria produced elongated cells when phagocytes or antibiotics existed, hence reduced the chance of being killed (Justice et al., 2008). Elongated cells of free-living rhizobia have also been reported when they were cultured in the medium with dicarboxylate or high concentration of yeast extraction (Reding and Lepo, 1989; Skinner et al., 1977). In this study, I showed that fructose and mannitol can induce strain CzR2 from uniformly rod-shaped cells to highly pleomorphic and elongated cells (**Figure 1-5**), which has not been found before. The biological roles of this unusual phenomenon are currently understudy.

When inoculated with CzR2, *C. zanzibarica* produced multi-lobe indeterminate nodules (**Figure 1-6C**), in consistence with the specific nodule type (crotalarioid) described by Corby (1988). Furthermore, the infection zone of nodules only contained infected cells and infection thread was never observed (**Figure 1-6DC**), suggesting that

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nodulation process of *C. zanzibarica* was not though root-hair infection pathway. These results supported the idea that the nodulation of genistoid legumes were commonly by crack and epidermal infections, and few infected cells divided repeatedly to form uniform infected zone (Sprent, 2007). However, distinctive features were found inside the nodule cells of the *C. zanzibarica* inoculated by CzR2, for example, symbiosomes commonly contained multiple bacteroids with pleomorphism (**Figure 1-7**). In the nodules of pea, each symbiosome usually harbors a single but pleomorphic (such as swollen, elongated, or branched) bacteroid. In contrast, in the nodules of soybean, each symbiosome usually contains multiple bacteroids of uniformly rod-shape (Haag et al., 2013; Mergaert et al., 2006; Oono et al., 2009).

Although strain CzR2 formed filamentous cells in both free-living and symbiotic states, flow cytometric analysis revealed that those cells had different DNA contents (**Figure 1-8**). It suggests that elongated cells of strain CzR2 in free-living state might result from cell-cycle delay while those in symbiotic state from genomic endo-reduplication. The results that adding fructose to HM medium can induce cell elongation of CzR2 (**Figure 1-5**) suggest that cellular morphology of strain CzR2 in symbiotic state might be also regulated by fructose.

In conclusion, the rhizobium CzR2 isolated from nodules of *C. zanzibarica* belongs to a *Bradyrhizobium arachidis* strain. Pleomorphism of this strain in the free-living state can be in induced by adding mannitol, fructose, but not by glucose, to the HM medium, thus, it is substrate dependent. CzR2 in free-living contain haploid and diploid cells while when symbiotic with *C. zanzibarica* were elongated and with polyploidy, indicating the occurrence of genomic endo-reduplication. In this *C. zanzibarica*-CzR2 symbiosis, symbiosomes commonly contained multiple, pleomorphic

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bacteroids which is also an unusual feature.

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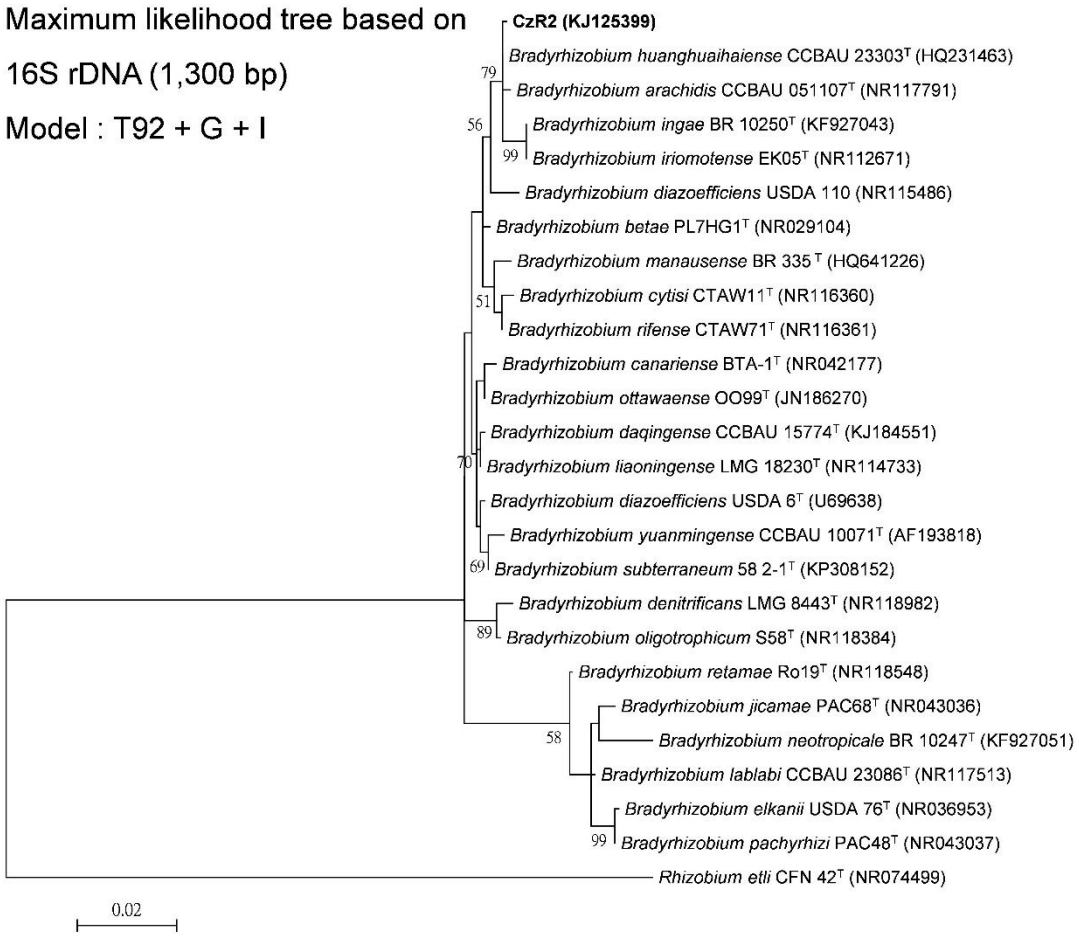

Figure 1-1 Phylogeny of strain CzR2 based on 16S rRNA genes. Maximum likelihood tree based on partial 16 rRNA genes (1,300 bp) showing the relationships among strain CzR2 and defined *Bradyrhizobium* species. Only bootstrap value > 50 are shown at the internodes. The scale bar represents 2 % nucleotide substitutions.

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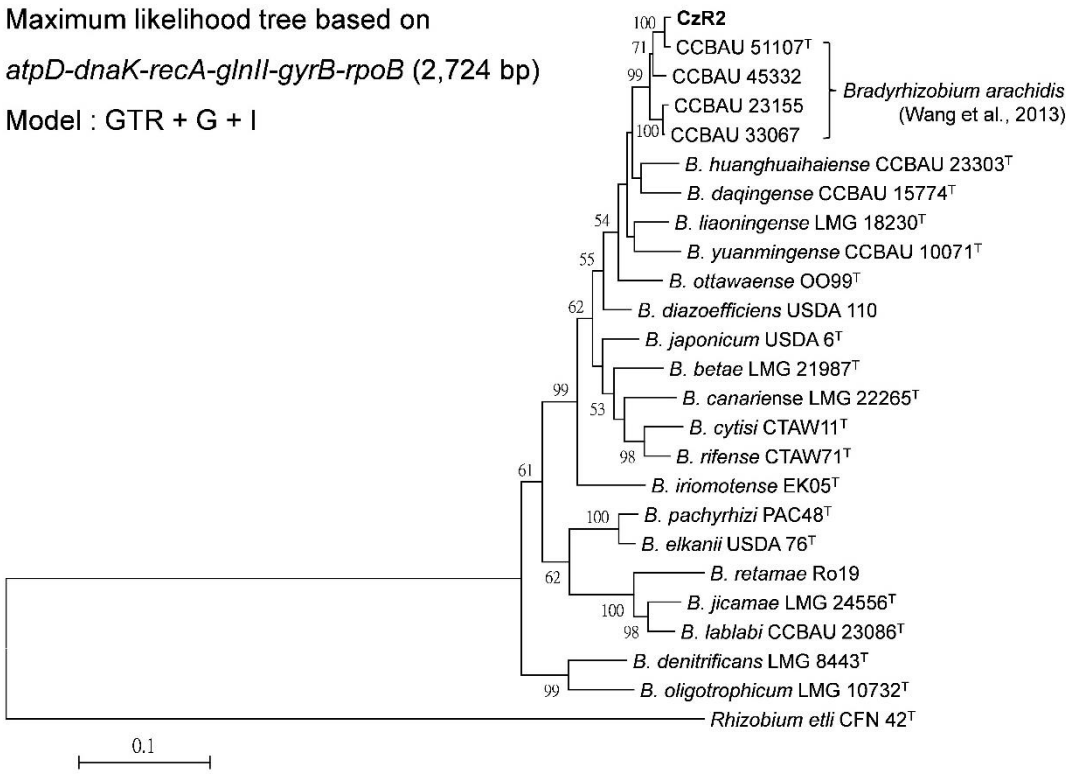

Figure 1-2 Phylogeny of strain CzR2 based on the six housekeeping genes. Maximum likelihood tree based on concatenated *atpD-dnaK-glnII-gyrB-recA-rpoB* gene sequences (2,724 bp) showing the relationships among strain CzR2 and defined *Bradyrhizobium* species. Only bootstrap value > 50 are shown at the internodes. The scale bar represents 10 % nucleotide substitutions.

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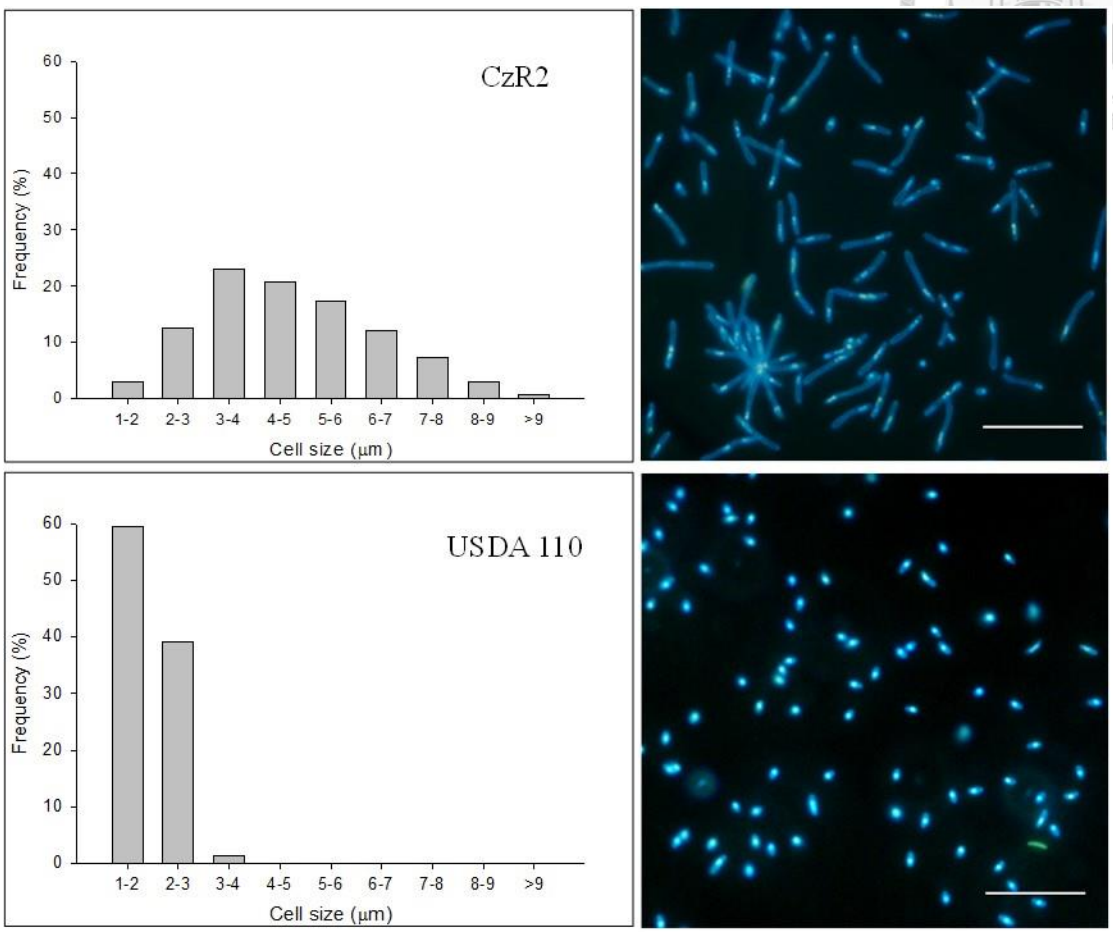

Figure 1-3 Cellular morphology of strain CzR2 and USDA 110 on YEM plates. Size variation and distribution of strain CzR2 (Top) and USDA 110 (down) grown on YEM plate for 7 days. In each strain, at least 1,000 cells were examined. The pictures showed these cells stained by DAPI. Bar, 10 μm.

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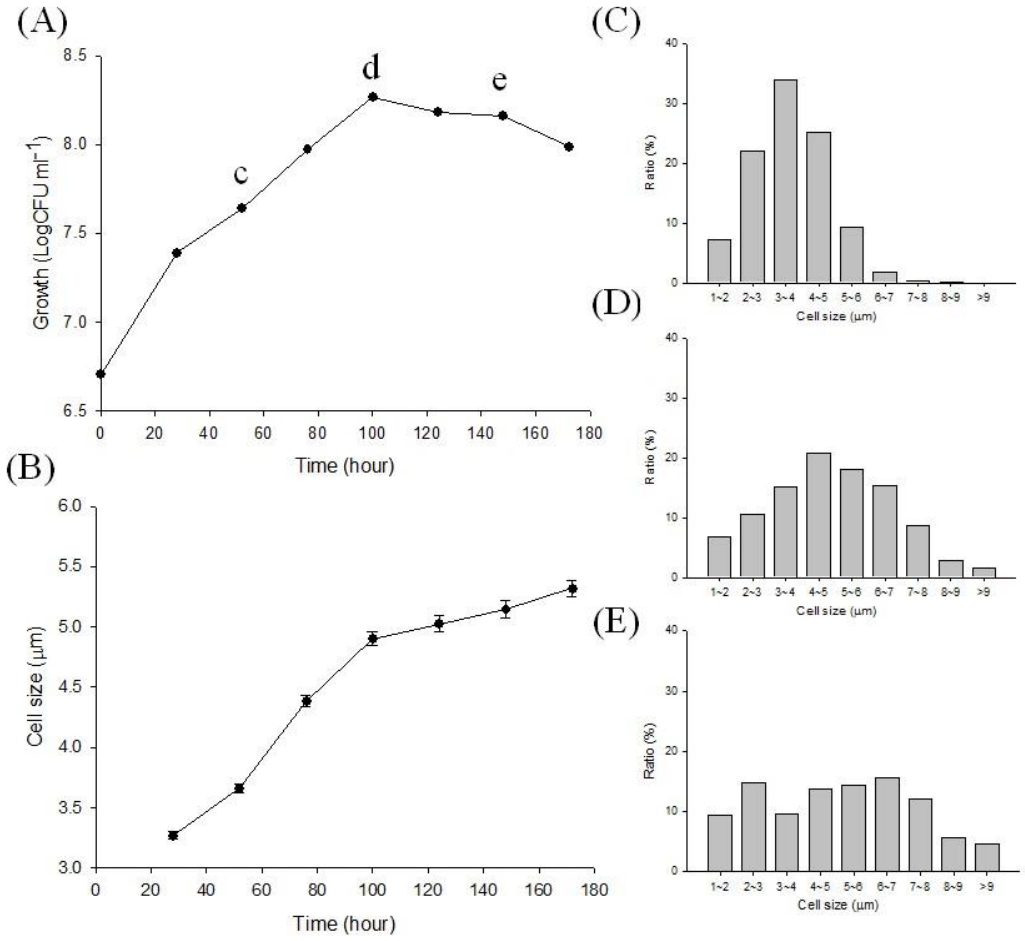

Figure 1-4 Cell size variation during growth kinetics of strain CzR2 grown in YEM broth. Growth was evaluated as the number of Log CFU ml^{-1} (A) and mean cell size (B), error bars in (B) indicate standard errors (n >1000). Distribution and frequency of cells with different size were also calculated in the exponential phase (C), transition phase (D) and stationary phase (E).

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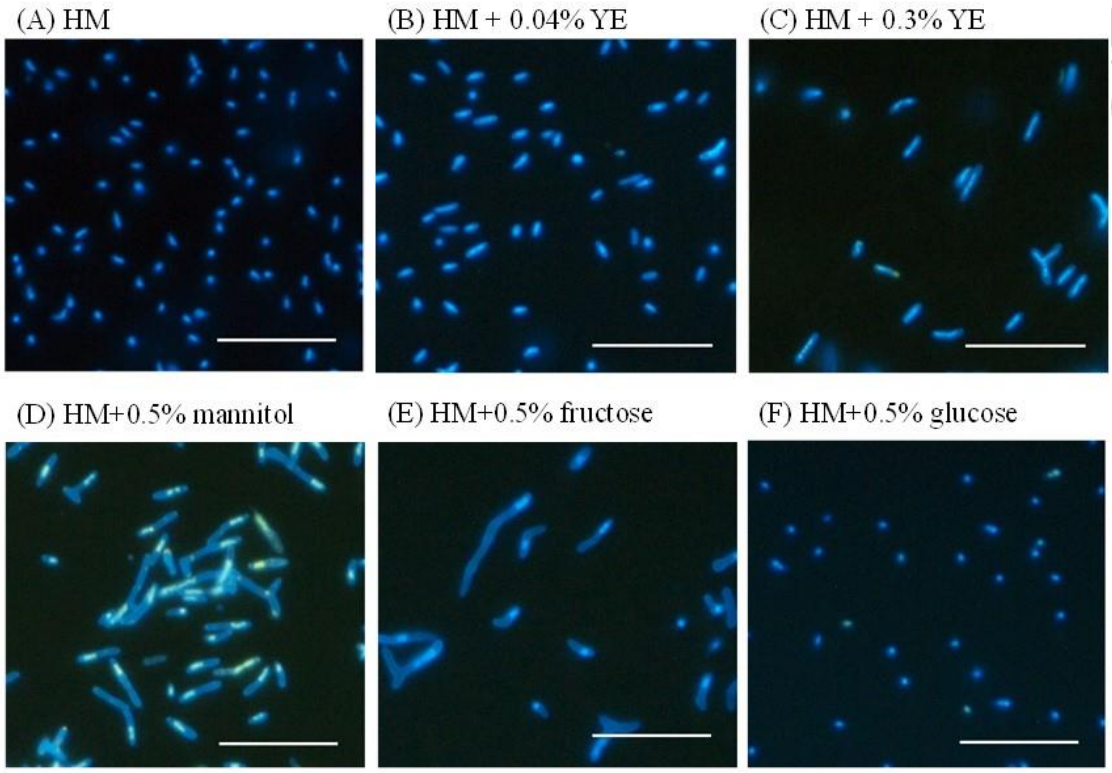

Figure 1-5 Morphology of strain CzR2 grown with different substrates. CzR2 was cultivated on the plates of HM (HEPES-MES) basal medium (A), HM+0.04% yeast extract (YE) (B), HM+0.3% YE (C), HM+0.5% mannitol (D) HM+0.5% fructose (E) and HM+0.5% glucose (F) for 14 days. Cells were stained with DAPI, bar = 10 μm.

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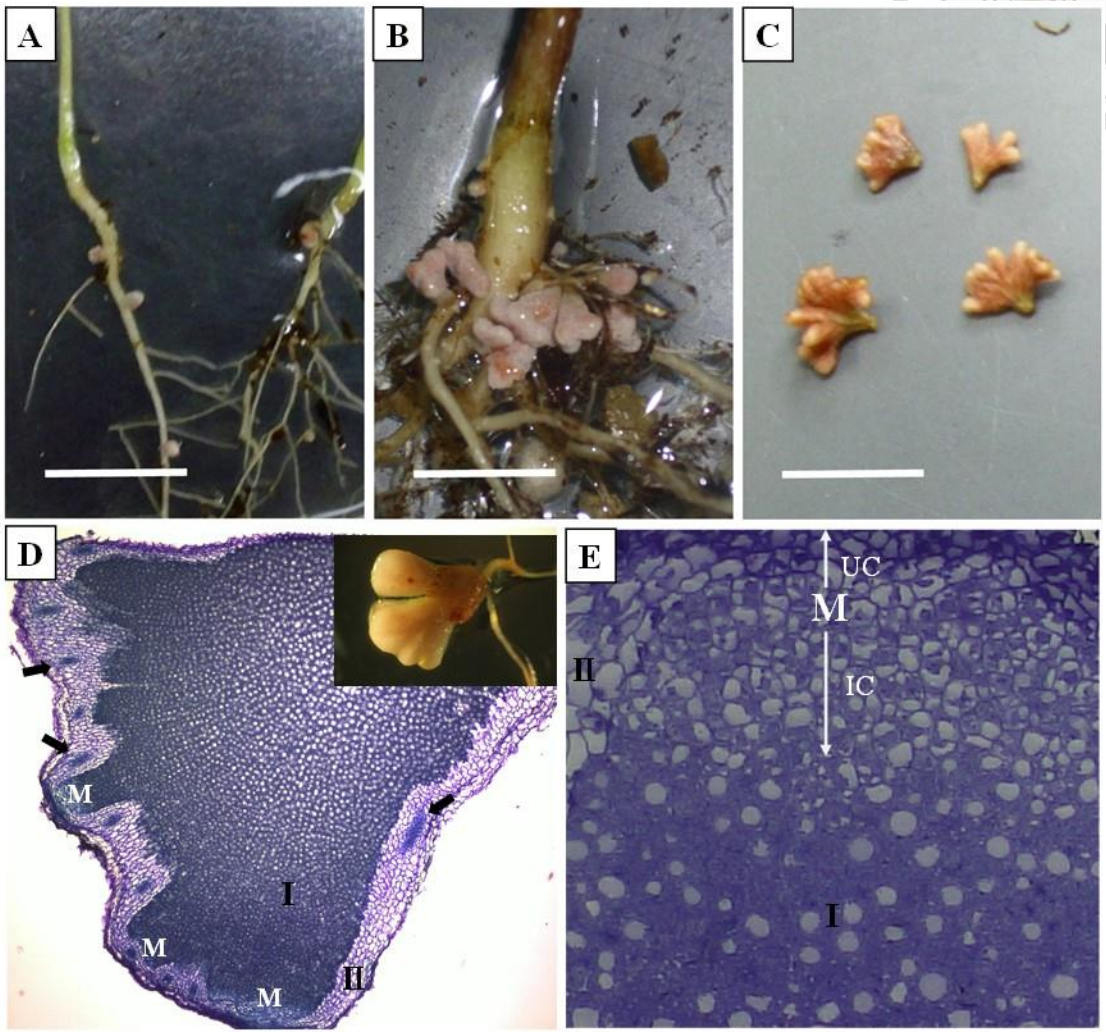

Figure 1-6 Nodulation characteristics of *C. zanzibarica* infected by strain CzR2. The nodules of *C. zanzibarica* inoculated by strain CzR2 were at 7 dpi (days post inoculation) (A), 35 dpi (B) and 56 dpi (C), bar = 1 cm. Nodules were longitudinally sectioned and stained with toluidine blue O (D and E). The darkly-stained infection zone (I) were surrounded by cortex (Ⅱ) and vascular bundles (black arrow). The apical nodule meristem (M) showed two distinct cell groups, uninfected cells (UC) and infected cells (IC).

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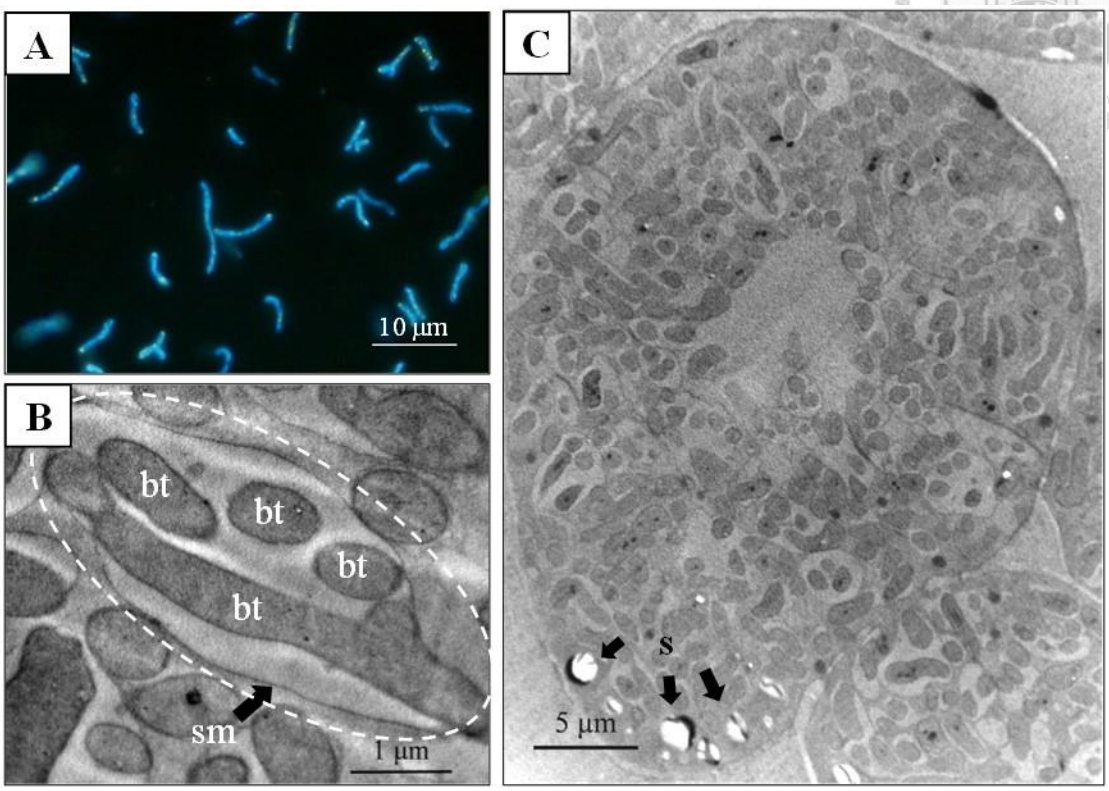

Figure 1-7 Cellular morphology of strain CzR2 symbiotic with *C. zanzibarica*. (A) Bacteroids of strain CzR2 stained with DAPI displayed pleomophism. (B) TEM images of root nodule of *C. zanzibarica* showed that a single symbiosome (dashed circle) was encompassed by the symbiosome membrane (sm) and harbored multiple bacteroids (bt). (C) Infected cells were filled with bacteroids except for central region and starch grains (s) scattered on the periphery.

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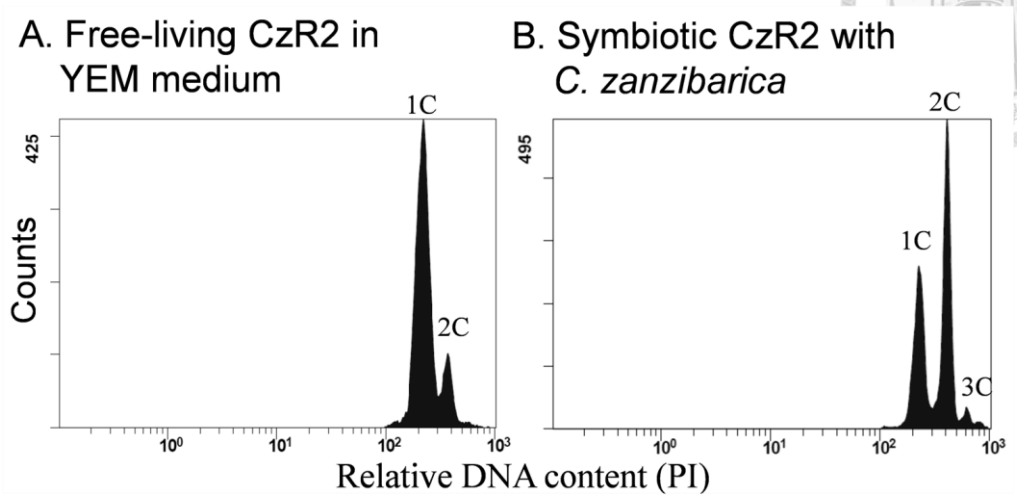

Figure 1-8 Flow cytometry analyses of the DNA contents in free-living and symbiotic CzR2. Cells were stained with propidium iodide (PI). Free-living CzR2 (A) was grown in YEM broth and sampled from late log phase, and bacteroids (B) were extracted from the nodules of 67 days old *C. zanzibarica*. The x axis shows fluorescence levels, indicating the DNA contents and the y axis shows cell counts. In each experiment, 20,000 cells were analyzed.

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Chapter 2

Rhizobia symbiosis of *C. zanzibarica* and coexisting legumes growing along Xindian riverbank of Northern Taiwan

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Abstract

Legume-rhizobia symbioses of seven leguminous species growing along Xindian riverbank of Northern Taiwan were investigated in this study. These legumes form either determinate or indeterminate types of root nodules. The determinate nodules of *Alysicarpus vaginalis, Desmodium. triflorum, D. heterophyllum, Sesbania cannabina* and the indeterminate nodules of *Mimosa pudica* harbored bacteroids of morphological uniformity (length of 1-3 μm), while the indeterminate nodules of *Crotalaria zanzibarica* and *Trifolium repens* contained bacteroids of highly pleomorphism (size varying from 1 to 5 μm). The enclosed bacteria were isolated from respective nodules, and twenty slow-growing and nine fast-growing rhizobial isolates were recovered. The slow-growing isolates were classified to the genus *Bradyrhizobium* based on the 16S rRNA sequences, whereas the fast-growing rhizobia comprise four genera, *Neorhizobium, Rhizobium, Cupriavidus* and *Paraburkholderia.* Results of stable isotope analyses revealed that the seven leguminous species had similar and consistently negative $\delta^{15}N$ values in leaves (mean of -1.2 ‰), whereas the values were positive (varying from 3.7 to 7.3 ‰) in the nodules. *These values* were significantly higher in the indeterminate nodules than those in the determinate ones. In addition, variations in the values of leaf $\delta^{13}C$ (varying from -29 to -34‰) among the seven legumes were

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measured, indicating their photosynthetic water use efficiencies were different. This is the first field survey to report the rhizobial diversity and the nutrient relationships of sympatric legume in Taiwan.

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Introduction

Nitrogen, one of the most important nutrients for plant growth and reproduction, is often limited in the ecosystems (Vitousek and Howarth, 1991). Some plants evolved symbiosis with bacteria capable of nitrogen fixation and overcame the limitation. Consequently, the nitrogen availability and the primary productivity of the ecosystems are improved by the symbiotic activity (Vitousek *et al.*, 2002). Because the interaction plays an important role in affecting primary productivity and is of great application in agriculture, the symbiotic relationship has received much attention from the researchers worldwide.

Most leguminous plants are capable of fixing atmospheric N_2 via symbiosis with rhizobia (Sprent, 2001, 2009). There were 217 legume species, including 148 native species and 59 exotic species, recorded in Taiwan (Huang, 1993; Wu *et al*., 2003). Despite the fact that legume is one of the largest plant families in Taiwan (Huang, 1993) and the importance of the symbiotic relationship in contribution to the nitrogen availability and primary productivity of ecosystems, the bacteria symbionts have been investigated only in few species of leguminous plants in Taiwan (Chen *et al*., 2000, 2003, 2005; Chen and Lee, 2001; Hung *et al*., 2005; Huang *et al*., 2016). Field survey of root-nodulating rhizobia and concomitantly measurements of nutrient of sympatric

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leguminous species are lacking in Taiwan.

Xindian River is located in Northern Taiwan. The soil along riverbank is mainly sandy and frequently subjected to disturbances, such as flooding and human activity. Despite this, leguminous plants are prevalent in this habitat. The nitrogen-fixing symbiosis between the leguminous plants and the soil rhizobia might provide these plants competitive advantages in the nitrogen-poor and arid habitats. Seven leguminous species were commonly observed in this area, including three native species (*Alysicarpus vaginalis*, *Desmodium triflorum* and *D. heterophyllum*) and four exotic species (*Crotalaria zanzibarica*, *Mimosa pudica*, *Sesbania cannabina* and *Trifolium repens*) (Table 1). These species belong to distantly related legume groups, including Genistoids (*C. zanzibarica*), Milletioids (*A. vaginalis*, *D. triflorum*, and *D. heterophyllum*), Robinioids (*S. cannabina*), Inverted Repeat-lacking clade (*T. repens*) and Mimosoids (*M. pudica*) (Lewis *et al*., 2005; Sprent, 2007). In addition, the geographic origins of these legumes are also disparate. *A. vaginalis*, *D. triflorum*, and *D. heterophyllum* are native species in Taiwan, while *C. zanzibarica*, *S. cannabina*, *T. repens* and *M. pudica*, are exotic and originated from Africa, Asia, Europe and America, respectively (Wu *et al*., 2003). Among the seven species, the bacterial symbionts of *A. vaginalis*, *C. zanzibarica*, *M. pudica* and *S. cannabina* in Taiwan have been reported

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(Chen and Lee, 2001; Chen *et al*., 2003; Hung *et al*., 2005; Huang *et al*., 2016) but not the other three species. However, the reported symbionts were mostly isolated from central and southern Taiwan, except that of *C zanzibarica* was recently isolated from a greenhouse in northern Taiwan (Huang *et al*., 2016). These phylogenetically distant legume species originated from disparate geographic sources in combination with the heterogeneous soil conditions of the riverbank might result in novel symbiotic properties.

Two major types of nodules are classified by their growth. Determinate nodules usually have a round shape and are short-lived (lasting for days to weeks), while indeterminate nodules may have few or many branches and last for several months (Sprent, 2001, 2007). Within the nodules, the rhizobia differentiate into nitrogen-fixing bacteroids. The morphology of bacteroids is either similar to or different from that of free-living bacteria (short rod, about 1μm long). For example, bacteroids in nodules of pea display pleomorphism, such as swollen, elongated, or branched (Mergaert *et al*., 2006), while those in nodules of soybean uniformly rod-shaped (Oono *et al*., 2009). The swollen bacteroids might optimize nitrogen-fixing efficiency (Oono and Denison, 2010).

Stable isotopes techniques have been widely used in ecological studies (Peterson

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and Fry, 1987). The nitrogen isotope ratio ($\delta^{15}N$) of individual plants is often used to assess the forms of nitrogen source, i.e. NO_3^-, NH_4^+ or N_2 (Robinson, 2001). In general, foliar $\delta^{15}N$ values of non-N_2-fixing plants vary widely (could be positive or negative values), while N_2-fixing legumes often display consistently negative foliar $\delta^{15}N$ values (Virginia and Delwiche, 1982; Sprent *et al*., 1996). In contrast to the leaves, nodules of legume plants commonly have positive and variable $\delta^{15}N$ values which might depend on their nodule symbionts and reflect the nitrogen fixing activities (Shearer *et al*., 1982; Steele *et al*., 1983; Wanek and Arndt, 2002). The carbon isotope ratio, $\delta^{13}C$, can be used to identify photosynthetic pathway. In addition, $\delta^{13}C$ of C3 plants is a proxy of water use efficiency (WUE) (Farquhar *et al*., 1982). Studies have shown that increases in N supply improve WUE hence enhance plant productivity (Brueck, 2008). It is also found that water use efficiency (WUE) was positively related to leaf nitrogen content for woody nitrogen fixing plants (Adams *et al*., 2016). The analyses of nitrogen and carbon isotopes could provide information of nitrogen sources and water use efficiency of the sympatric plants.

In this study, we analyzed 16S rRNA genes to classify the genus of rhizobial symbionts, examined nodule and bacteroid morphologies, and analyzed nitrogen and carbon contents and $\delta^{15}N$ and $\delta^{13}C$ values, of seven leguminous plants growing

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sympatrically along the bank of Xindian River in northern Taiwan. The objective of the study is to understand the diversity of rhizobial symbionts associated with the seven leguminous plant and the nutrient and water relationships of the host plants.

Materials and methods

Sampling site and plant materials

Xiandan (XD) River is located in northern Taiwan. The dominant leguminous species, *A. vaginalis*, *C. zanzibarica*, *D. triflorum*, *D. heterophyllum*, *M. pudica*, *S. cannabina* and *T. repen* (**Figure 2-1 and Table 2-1**) co-existing in an area about 10 × 150 m along the XD riverbank (24°98′ N, 121°52′ E), was investigated in May 2013, March and June 2014. Six individuals of each legume species were surveyed, and 1-2 nodules per individual were collected for rhizobial isolation. Mature leaves and all available nodules were collected from additional individuals of the seven legumes species (4 to 9 individuals per species) for nitrogen and carbon contents, and $\delta^{15}N$ and $\delta^{13}C$ analyses. For comparison purpose, leaves of a non-legume species, *Bidens pilosa* var. *radiata*, growing about 100 m away from the legume community were also analyzed.

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Rhizobial isolation and bacteroid morphology

Fresh nodules were surface sterilized by immersion in 0.5% SDS for 1 min, then 70% ethanol for 5 min, and washed three times by sterile deionized-distilled water (DDW). Nodule suspension was obtained by crushing the nodule in DDW and spread onto yeast extract-mannitol (YEM) plate (Vincent, 1970). A single of putative rhizobium was isolated from each nodule sample and checked for the unity by repeated streaking on YEM plate. For observation of bacteroid morphology, the nodule suspension were stained with DAPI (4,6-diamidino-2-phenylindole; Sigma-Aldrich, St. Louis, MO, USA) at 50 μg/ml for 10 min at 25℃ and examined by a fluorescent microscopy (BX51, Olympus, Tokyo, Japan).

Phylogenetic analysis of nodule symbionts by using 16S rRNA genes

Total genomic DNA was extracted from the pure cultures of each rhizobial isolates grown in YEM broth until the late exponential phase of growth. Extraction of the DNA was performed by using Geneaid DNA Mini kit (Geneaid Biotech, New Taipei, Taiwan). PCR amplification of 16S rRNA genes were performed by using bacterial universal primer pairs, 27F-1492R (Marchesi *et al*., 1998) and Taq DNA Polymerase 2x Master Mix RED (Ampliqon, Copenhagen, Denmark). PCR products were first checked on

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1.5% agarose gel and purified with Gel/PCR DNA fragments extraction kit (Geneaid Biotech, New Taipei, Taiwan). Sequencing reactions were performed by using the ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequences assembled and quality checked were conducted by using BioEdit 7.2.5 (Hall, 1999).

To analyze the phylogenetic relationships between the isolates and defined rhizobial species, the 16S rRNA sequences of reference strains which are highly similar with the isolates were download from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) based upon BLAST results. The 16S rRNA gene sequences of isolates and reference strains were then aligned by MUSCLE program as implemented in MEGA version 6 (Tamura *et al*., 2013). The Kimura's 2-parameter distance correction model was used to reconstruct neighbor-joining (NJ) phylogenetic trees by software MEGA6. The topology of the tree was evaluated by bootstrapping with 1,000 replications. The GenBank accession numbers of the 16S rRNA gene sequences generated in this study are shown in **Figure 2-3**.

Nitrogen and carbon contents, and $\delta^{15}N$ and $\delta^{13}C$ values analyses

Leaf and nodule samples were washed with distilled water then dried at 60 °C in an oven for three days. Dried samples were ground to a homogenized powder with a

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mortar and pestle. A 2 mg of ground material was loaded into a tin capsule for further analysis (Kao, 2010). Nitrogen content (N_{mass}, mg g^{-1}) and carbon content (C_{mass}, mg g^{-1}) was determined with an elementary analyzer (FlashEA 1112 series, Thermo Fisher Scientific, Italy). Stable nitrogen isotope ratio ($\delta^{15}N$) was determined by an isotope ratio mass spectrometer (DeltaV Advantage, Finnigan Mat, Germany) and calculated as: $\delta^{15}N$ (‰) = $[(R_{sample} / R_{standard}) - 1]*1000$, where R is the ratio of ^{15}N to ^{14}N. Stable carbon isotope ratio ($\delta^{13}C$) was calculated as: $\delta^{13}C$ (‰) = $[(R_{sample} / R_{standard}) - 1]*1000$, where R is the ratio of ^{13}C to ^{12}C (Ehleringer and Osmond, 1989).The standards for $\delta^{15}N$ and $\delta^{13}C$ are atmospheric N_2 and Pee Dee Belemnite, respectively.

Statistical analysis

To determine whether variables (leaf/nodule N, C contents, $\delta^{15}N$ and $\delta^{13}C$ values) were significantly different among seven legumes and *B. pilosa*, one way analysis of variance (ANOVA) was conducted by using the software SAS 9.4 (SAS inst. Inc. USA). If the null hypothesis was rejected after the analysis of ANOVA, then SNK (Student-Newman-Keuls) test was used for multiple comparisons.

Results

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Nodule types and morphology of bacteroids

Fig. 1 shows the morphology of nodules collected from field-growing legumes. *A. vaginalis*, *D. triflorum*, *D. heterophyllum* and *S. cannabina* formed determinate nodules (**Figure 2-2A-D**), while *T. repens*, *M. pudica* and *C. zanzibarica* produced indeterminate nodules with no branch, few branches and many branches, respectively (**Figure 2-2E-G**). In addition, the lenticels (as white stripes) were observed on the nodules of *A. vaginalis*, *D. triflorum*, *D. heterophyllum* (**Figure 2-2A-C**).

Bacteroids isolated from the nodules of *A. vaginalis*, *D. triflorum*, *D. heterophyllum*, *S. cannabina* and *M. pudica* displayed morphological uniformity with 1-3 μm in length (Fig. 1H-K and M). In contrast, those of *C. zanzibarica*, and *T. repens* were highly pleomorphic (included rod-shaped, branched and club-like cells) and varied in size from 1 to 5 μm (**Figure 2-2L and N**).

16S rRNA gene phylogeny of rhizobial isolates

A total of 29 putative rhizobial isolates, 3 from *A. vaginalis*, 6 from *C. zanzibarica*, 6 from *D. triflorum*, 5 from *D. heterophyllum*, 4 from *M. pudica*, 2 from *S. cannabina* and 3 from *T. repens*, were recovered from the nodules of the seven legume species. The isolates from *A. vaginalis*, *C. zanzibarica*, *D. triflorum*, and *D. heterophyllum* displayed

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slow-growing phenotype, forming visible colonies on YEM plates after 5-7 days at 30℃. In contrast, the isolates from *M. pudica*, *S. cannabina* and *T. repens* formed detectable colonies after 1-2 days, indicating that they were fast-growing strains.

The 1,300 bp of 16S rRNA gene sequences were used to analyze the relationship among the 29 isolates and defined genus of the strains (**Figure 2-3**). These isolates were separated into two distinct groups, belonging to alpha- and beta-rhizobia. In alpha-rhizobia group, a total of 20 isolates from *A. vaginalis*, *C. zanzibarica*, *D. triflorum* and *D. heterophyllum* were situated within genus *Bradyrhizobium*. One isolate, ScHDB, from *S. cannabina* belongs to *Neorhizobium*, a novel genus recently been separated from *Rhizobium* (Mousavi *et al*. 2014). Additionally, one isolate from *S. cannabina* (ScHDA) and three isolates from *T. repens* (TrHDA, TrHD1 and TrHD2) were grouped together with *Rhizobium* strains. In beta-rhizobia group, four isolates from *M. pudica* are closely related to *Cupriavidus taiwanensis* (MpHDA), *Paraburkholderia phymatum* (MpHD2) and *P. mimosarum* (MpHD and MpHD3), respectively (**Figure 2-3**). The latest two species have been recently separated from genus *Burkholderia* and reclassified as members of the *Paraburkholderia* (Sawana *et al.* 2014).

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Nitrogen and carbon contents, $\delta^{13}C$ and $\delta^{15}N$ analyses

Table 2-2 shows the nitrogen content and $\delta^{15}N$ values of nodules and leaves. Variations in nodule and leaf N contents and $\delta^{15}N$ of nodules were found among the seven leguminous plants, but similar $\delta^{15}N$ values were found in their leaves. In comparison of nodules and leaves of the same plants, in general, nodules had higher N contents and more positive $\delta^{15}N$ values than leaves. In comparison to legume plants, *B. pilosa* had significantly more positive and variable leaf $\delta^{15}N$ values, ranging from -0.3‰ to +2.1‰.

The carbon and $\delta^{13}C$ contents of nodules and leaves are shown in Table 3. All legume plants had similar C content in the nodules (about 43%), while their leaf C content varied from 42% to 47%. In each of the legume species, nodules had significantly more positive $\delta^{13}C$ values than leaves, the enrichment was approximate 1.5‰. The relationship between leaf and nodule $\delta^{13}C$ values among seven legume species was significantly positive (**Figure 2-4**).

Discussion

Few rhizobial species have been isolated from Taiwan soils in previous studies (Chen *et al*., 2000, 2003, 2005; Chen and Lee, 2001; Hung *et al*., 2005; Huang *et al*.

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2016). However, the natural diversity of nodulating bacteria in field growing, sympatric leguminous species has not been reported in Taiwan. This is the first study conducted to explore the diversity of nodulating rhizobia associated with sympatric leguminous plants in the field of Taiwan and the results provide rhizobial diversity at genus level. The survey recovered five genera of nodulating bacteria, *Bradyrhizobium, Neorhiobium, Rhizobium, Cupriavidus* and *Paraburkholderia*, associated with seven sympatric leguminous species growing along the riverbank of northern Taiwan. Most of isolates (20 out of 29) in our survey belong to the genus *Bradyrhizobium* (**Figure 2-3**). The result confirms previous report that *Bradyrhizobium* is the most abundant and prevalent rhizobial genus contributing to the major symbiont of tropical and sub-tropical legume taxa (Sprent, 2007, 2009). Strains of *Bradyrhizobium* were isolated from the nodules of *A. vaginalis, C. zanzibarica, D. triflorum* and *D. heterophyllum* (**Figure 2-3**). A more precise classification of these strains into discrete species is hampered by the exceptional conservation of the 16S rRNA gene sequence in the genus *Bradyrhizobium* (Rivas *et al*., 2009; Azevedo *et al*., 2015). Among the *Bradyrhizobium* spp. reported in Taiwan, *B. arachidis* was recently isolated from the nodules of *C. zanibarica* grown in a greenhouse (Huang *et al*., 2016) and *B. japonicum* from nodules of *A. vaginalis* growing in central Taiwan (Hung *et al*., 2005). Strains associated with *D. heterophyllum* and *D.*

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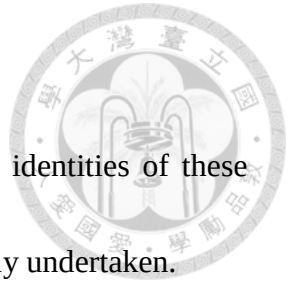

triflorum in Taiwan have not been reported. To reveal the species identities of these isolates, analyses combined multiple housekeeping genes are currently undertaken.

Rhizobium strains were isolated from *S. cannabina* and *T. repens* in this study (**Figure 2-3**). Chen and Lee (2001) reported that *S. cannabina* growing in the southern part of Taiwan were nodulated by *Rhizobium* and *Sinorhizobium* (*Ensifer*) strains. Although we did not identify any *Sinorhizobium* strain in the nodules of *S. cannabina*, we isolated a *Neorhizobium* strain (**Figure 2-3**). These results revealed that *S. cannabina* can establish symbiosis with rhizobia of *Rhizobium*, *Neorhizobium* and *Sinorhizobium* in Taiwan. In addition, there was no report with respect to rhizobia establish symbiosis with *T. repens* in Taiwan, but *Rhizobium* is known to establish symbiosis with *T. repens* in China (Liu *et al*., 2007). Among the seven legume species investigated, *M. pudica*, an invasive plant in Taiwan, is the only species that establishes symbiosis with beta-rhizobia (**Figure 2-3**). This plant is known to establish symbiosis with both *Cupriavidus* and *Paraburkholderia* strains in Taiwan (Chen *et al*., 2003), while its nodule symbionts were restricted to *Paraburkholderia* in the native regions (Barrett and Parker, 2005). In consistent with the previous report by Chen *et al.* (2013), the isolates from *M. pudica* growing along Xindian riverbank had highly similar 16S rRNA gene sequences with several beta-rhizobia, including *Cupriavidus taiwanensis*,

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Paraburkholderia phymatum and *P. mimosarum* (**Figure 2-3**). The results revealed that these genera of beta-rhizobia co-existed in this habitat and only established symbiosis with *M. pudica* but not with other 6 species of legume.

Morphologies of nodule and bacteroid are two of the most noticeable traits in legume-rhizobia symbiosis. These traits are related to the evolution of the symbiosis. For example, indeterminate nodules and non-swollen bacteroids are considered ancestral traits, while determinate nodules and swollen bacteroids are derived (Doyle, 2011; Oono *et al*., 2010). In this study, the seven legume species formed either indeterminate or determinate nodules. Within each group of the legume, formed either indeterminate or determinate nodules, plants were nodulated by phylogenetically distant rhizobia (**Table 2-1**). This result confirms that the formation of nodule types is dependent on the host plant not on the specific rhizobia (Oono *et al*., 2010). The relationship between the nodule types and the morphologies of the enclosed bacteroids are not discreet, the determinate nodules of *A. vaginalis*, *D. triflorum*, *D. heterophyllum*, *S. cannabina* harbored exclusively non-swollen bacteroids, while the indeterminate nodules of *C. zanzibarica* and *T. repens* harbored swollen but that of *M. pudica* harbored non-swollen bacteroids (**Table 2-1**). It is reported that the morphology of the bacteroids was determined by host plants (Haag *et al*., 2013)

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As shown in **Table 2-2**, the 7 leguminous species had similar and consistently negative foliar $\delta^{15}N$ and their foliar $\delta^{15}N$ differed significantly from that of the non-N_2-fixing *B. pilosa* var. *radiata* (with variable foliar $\delta^{15}N$), strongly suggested that these leguminous plants depend on the same nitrogen source (from atmospheric N_2) differing from the sources (NH_4 and/or NO_3 in soil) utilized by the non-N_2-fixing plant. These legume with symbiotic bacteria in root nodules can fix atmospheric nitrogen (N_2), and this would give them an advantage in low soil nitrogen (N) habitats. Since the soil of riverbank is commonly nutrient-poor, and this result might explain the prevalence of legume plants along the bank of Xindian River. In contrast to the slightly depletion of ^{15}N in their leaves, the seven legumes surveyed in this study all showed ^{15}N enrichment in their nodules (**Table 2-2**), regardless induced by distinct rhizobial symbionts (fast- or slow-growing rhizobia). Though Bergersen *et al*. (1986) reported that slow-growing rhizobial strains and fast-growing strains induces *Lupinus* plants produced ^{15}N enriched nodules and little or no ^{15}N enriched nodules, respectively. Explanations for the enrichment of ^{15}N in nodules have been suggested, including denitrification in nodules preferentially releasing ^{14}N (Shearer *et al*. 1980), importation from phloem of ^{15}N enriched amino acids into nodules (Bergersen *et al*. 1988), exported of ^{15}N depleted ureide from nodules (Shearer *et al*. 1982), or diffusion of NH_3 from bacteroids causing

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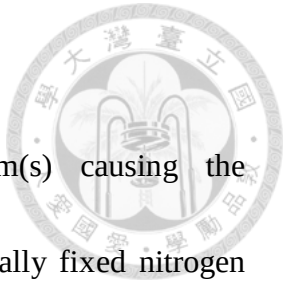

discrimination (Yoneyama *et al*. 1991). However, mechanism(s) causing the phenomenon have not been revealed unequivocally. The symbiotically fixed nitrogen can be assimilated and exported via aminde or ureide pathway depending on host species (Sprent, 2001). Among the seven leguminous species *C. zanzibarica*, *M. pudica*, *S. cannabina* and *T. repens* are aminde exporters while *A. vaginalis*, *D. triflorum* and *D. heterophyllum* are ureide exporters (Sprent, 2001). Even so, no significant difference was found neither in the nodule $\delta^{15}N$ values nor in the leaf $\delta^{15}N$ values between the two groups. Interestingly, the mean $\delta^{15}N$ value of the indeterminate nodules of *C. zanzibarica*, *M. pudica* and *T. repens* was significantly higher than that of the determinate nodules of *A. vaginalis*, *D. triflorum*, *D. heterophyllum* and *S. cannabina* (**Table 2-2**). It is possible that $\delta^{15}N$ of nodule is also affected by nodule age. Accordingly, indeterminate nodules, with longer life span, might accumulate more ^{15}N thus resulting in higher $\delta^{15}N$ values than determined nodules.

The $\delta^{13}C$ values of leaves of the sympatric legume varied from -29‰ to -34‰ (**Table 2-3**), indicating the legume species sampled in this study belong to C3 plants (O'Leary, 1988). The leaf $\delta^{13}C$ values in C3 plants is known to reflect the ratio of intercellular to ambient concentration of CO_2 (Ci/Ca), which is affected by both stomatal conductance (CO_2 diffusion) and photosynthesis (CO_2 consumption) (Farquhar

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et al., 1982). Hence, leaf $\delta^{13}C$ value in C3 plants is often used as a proxy of photosynthetic water use efficiency (Farquhar *et al*., 1982). The more positive $\delta^{13}C$ value showed the higher WUE. Variation in $\delta^{13}C$ values of the seven leguminous indicates that they had different WUE. In addition, five of the seven leguminous plants had significantly more positive $\delta^{13}C$ values than their neighbor, the non-legume *B. pilosa* var. *raidiata* (**Table 2-3**). Water use efficiency was found positively related to leaf N content (on leaf area basis, N_{area}) for woody nitrogen fixing plants (Adam *et al*., 2016). The seven legumes also had significant differences in leaf N content (on dry weight basis, N_{mass}). However, because we did not measure specific leaf area thus cannot convert the N_{mass} (measured in this study) into N_{area}. Therefore, we cannot tell whether the differences in $\delta^{13}C$ values of the leguminous species were attributed by their differences in leaf N_{area}. The significantly positive linear relationship between leaf $\delta^{13}C$ and nodule $\delta^{13}C$ (**Figure 2-4**) provides the evidence that nodule derived C from leaves. However, nodules had consistently more positive $\delta^{13}C$ values than leaves. Two processes, transportation of ^{13}C enriched carbon compounds from shoot to nodules and/or emission of ^{13}C depleted CO_2 during respiration, might be responsible for the difference (Werth and Kuzyakov, 2010).

In conclusion, the seven leguminous species co-existing along Xindian riverbanks

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had species-specific nodule type, indeterminate or determinate, induced by diverse rhizobia. A total of 29 rhizobial isolates belonging to *Bradyrhizobium*, *Neorhizobium*, *Rhizobium*, *Cupriavidus* and *Paraburkholderia* were obtained from these root nodules. The seven legume species had similar and consistently negative leaf $\delta^{15}N$ values suggested that these legume plants depend on atmospheric N_2. In contrast, these leguminous plants had differences in leaf $\delta^{13}C$ indicating differences in photosynthetic water use efficiency.

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Table 2-1 Characteristics of the seven leguminous species growing sympatrically along riverbank of Xindian River in Northern Taiwan

Legume species	Phylogenetic group	Growth habit	Life form[1]	Origin	Nodule type[2]	Bacteroid[3]	Rhizobial symbionts
Alysicarpus vaginalis	Milletioid	Herb	Per	Native	D	NS	*Bradyrhizobium*
Crotalaria zanzibarica	Genistoid	Shrub	Ann or Per	Africa	I	S	*Bradyrhizobium*
Desmodium triflorum	Milletioid	Herb	Per	Native	D	NS	*Bradyrhizobium*
Desmodium heterophyllum	Milletioid	Herb	Per	Native	D	NS	*Bradyrhizobium*
Mimosa pudica	Mimosoid	Herb	Ann or Bien	America	I	NS	*Cupriavidus/ Paraburkholderia*
Sesbania cannabina	Robinioid	Herb	Ann	India	D	NS	*Neorhizobium/ Rhizobium*
Trifolium repens	IRLC	Herb	Per	Europe	I	S	*Rhizobium*

[1]Ann: annual; Per: perennial; Bien: biennial; Ann or Bien: annual or biennial; Ann or Per: annual or perennial.
[2] D: determinate; I: indeterminate
[3] S: swollen; NS: non-swollen

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Table 2-2 Nitrogen contents and ^{15}N signatures of seven legumes growing along XD riverbank. Nitrogen contents (N_{mass}, mg g^{-1}, mean ± SD, n = 4~9) and stable nitrogen isotopes ratio ($\delta^{15}N$, ‰, mean ± SD, n = 4~9) of nodule and leaf samples collected from the seven legumes, *Alysicarpus vaginalis*, *Crotalaria zanzibarica*, *Desmodium triflorum*, *D, heterophyllum*, *Mimosa pudica*, *Sesbania cannabina* and *Trifolium repens* growing sympatrically along riverbank of Xindian River in Northern Taiwan. The non-legume species, *Bidens pilosa* var. *radiata* growing along the same riverbank was also analyzed.

	N_{mass} (mg g^{-1})		$\delta^{15}N$ (‰)	
	Nodules	Leaves	Nodules	Leaves
Legume				
Alysicarpus vaginalis (n = 4)	56±5^{bc}*	32±3^{b}	4.3±1.0^{b}**	-1.0±0.1^{b}
Crotalaria zanzibarica (n = 9)	63±5^{b}*	55±8^{a}	7.3±1.2^{a}**	-1.3±0.2^{b}
Desmodium triflorum (n = 4)	48±6^{c}*	28±3^{b}	3.7±0.3^{b}**	-1.4±0.1^{b}
Desmodium heterophyllum (n = 4)	55±6^{bc}*	33±2^{b}	4.2±0.4^{b}**	-1.6±0.3^{b}
Mimosa pudica (n = 4)	52±7^{c}*	27±2^{b}	6.7±1.5^{a}**	-1.0±0.3^{b}
Sesbania cannabina (n = 4)	51±2^{c}	51±6^{a}	4.3±1.3^{b}**	-1.1±0.1^{b}
Trifolium repens (n = 4)	78±7^{a}*	53±5^{a}	5.9±0.8^{ab}**	-0.8±0.5^{b}
Non-legume				
Bidens pilosa (n = 4)	N.A.	32±1^{b}	N.A.	1.0±1.2^{a}

Mean within each column followed by different letters differed significantly (ANOVA).
*: significant differences between nodule N_{mass} and leaf N_{mass} (paired t-test).
**: significant differences between nodule $\delta^{15}N$ and leaf $\delta^{15}N$ (paired t-test).

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Table 2-3 Carbon contents and ^{13}C signatures of seven legumes growing along XD riverbank. Carbon contents (C_{mass}, mg g^{-1}, mean ± SD, n = 4~9) and stable carbon isotopes ratio ($\delta^{13}C$, ‰, mean ± SD, n = 4~9) of nodule and leaf samples collected from the seven legumes, *Alysicarpus vaginalis*, *Crotalaria zanzibarica*, *Desmodium triflorum*, *D, heterophyllum*, *Mimosa pudica*, *Sesbania cannabina* and *Trifolium repens* growing sympatrically along riverbank of Xindian River in Northern Taiwan. The non-legume species, *Bidens pilosa* var. *radiata* growing along the same riverbank was also analyzed.

	C_{mass} (mg g^{-1})		$\delta^{13}C$ (‰)	
	Nodules	Leaves	Nodules	Leaves
Legume				
Alysicarpus vaginalis (n = 4)	439±6*	422±4^{c}	-30.1±0.7^{c}**	-32.8±0.9^{cd}
Crotalaria zanzibarica (n = 9)	439±22	470±7^{a}*	-30.0±0.5^{c}**	-31.5±0.8^{bc}
Desmodium triflorum (n = 4)	444±27	436±3^{b}	-31.0±1.1^{c}**	-33.5±0.8^{d}
Desmodium heterophyllum (n = 4)	448±11	459±3^{a}	-26.7±0.7^{a}**	-29.3±0.6^{a}
Mimosa pudica (n = 4)	411±2	456±3^{a}*	-27.9±0.4^{b}**	-29.9±0.6^{a}
Sesbania cannabina (n = 4)	417±5	438±7^{b}*	-28.7±0.3^{b}**	-30.6±0.8^{ab}
Trifolium repens (n = 4)	439±13	443±9^{b}	-30.9±0.3^{c}**	-31.5±0.4^{bc}
Non-legume				
Bidens pilosa (n = 4)	N.A.	411±18^{c}	N.A.	-33.7±0.6^{d}

Mean within each column followed by different letters differed significantly (ANOVA).
*: significant differences between nodule C_{mass} and leaf C_{mass} (paired t-test).
**: significant differences between nodule $\delta^{13}C$ and leaf $\delta^{13}C$ (paired t-test).

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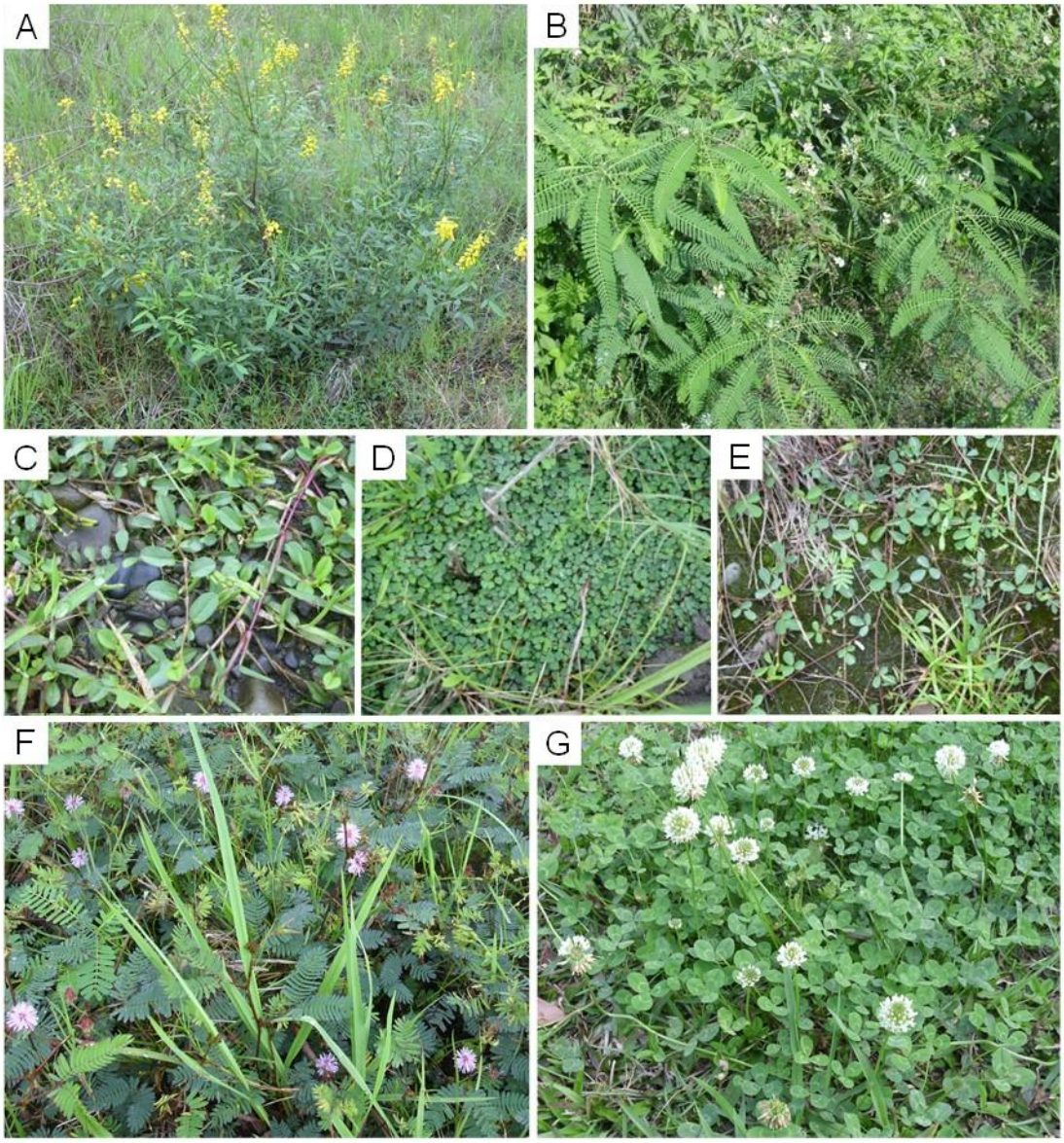

Figure 2-1 The common legumes growing along Xindian riverbank. These pictures showed *C. zanzibarica* (A), *Sesbania cannabina* (B), *Alysicarpu vaginalis* (C), *Desmodium triflorum* (D), *D. heterophyllum* (E), *Mimosa pudica* (F), and *Trifolium repens* (G) growing along Xindian riverbank.

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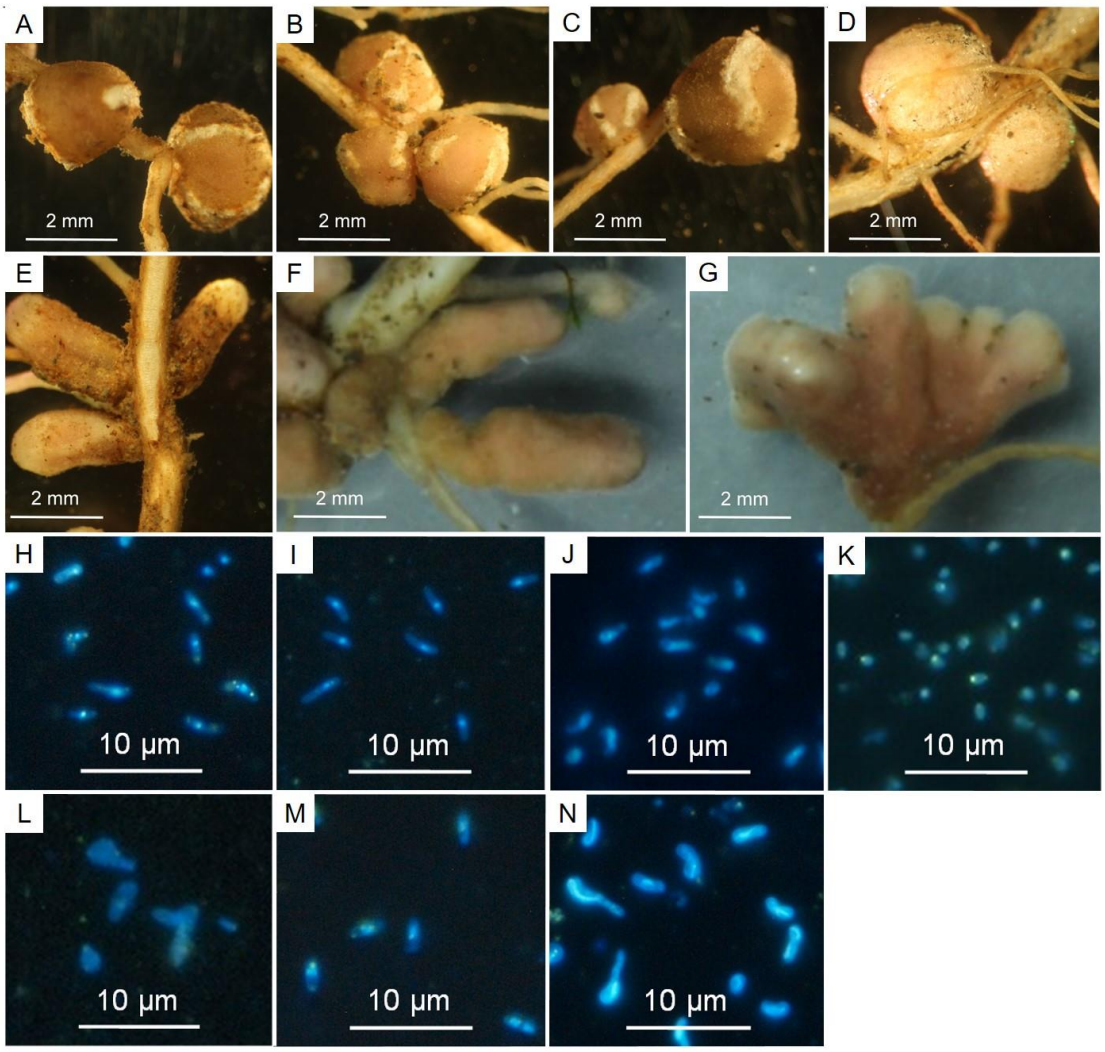

Figure 2-2 Nodule and bacteroid morphologies of common legumes growing along Xindian riverbank. These pictures showed the root nodules (A-G) and extracted bacteroids (H-N), stained with DAPI, of *Alysicarpu vaginalis* (A, H), *Desmodium triflorum* (B, I), *D. heterophyllum* (C, J), *Sesbania cannabina* (D, K), *Trifolium repens* (E, L), *Mimosa pudica* (F, M) and *Crotalaria zanzibarica* (G, N).

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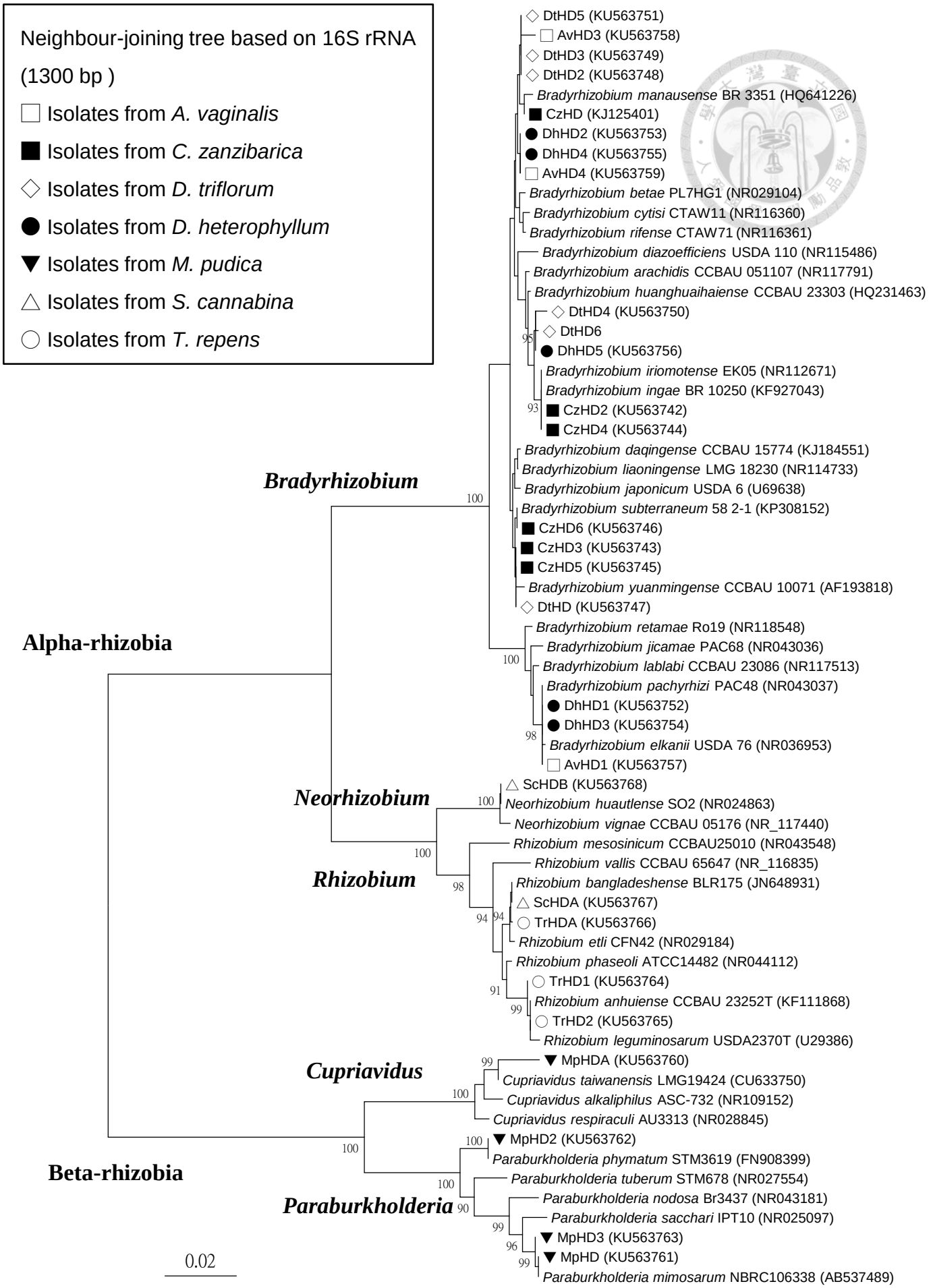

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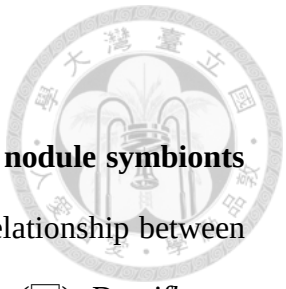

Figure 2-3 Phylogenetic tree of 16S rRNA gene sequences of the nodule symbionts isolated from seven legumes growing along XD riverbank. The relationship between the isolates from *C. zanzibarica* (■), *S. cannabina* (△), *A. vaginalis* (□), *D. triflorum* (◇), *D. heterophyllum* (●), *M. pudica* (▼), and *T. repens* (○) and the defined strains was built based on 16S rRNA gene sequences (1300 bp). This tree was constructed by using the neighbor-joining method and Kimura two parameter model. Only bootstrap values > 90 are shown at the internodes. The scale bar represents 2 % nucleotide substitutions.

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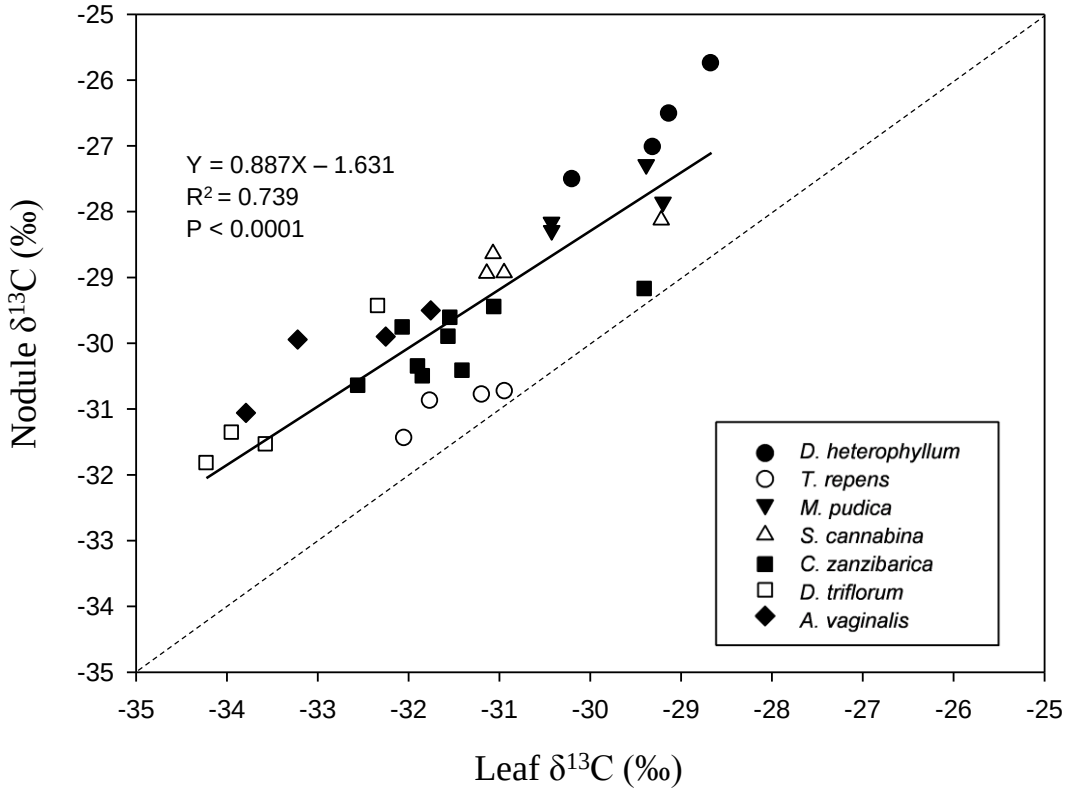

Figure 2-4 Correlations between leaf and nodule $\delta^{13}C$ values among the seven legume species coexisting along riverbank of Xindian River in Northern Taiwan.

Dash line represents 1:1 relationship between leaf and nodule $\delta^{13}C$ values.

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Chapter 3

Phylogenetic analyses of *Bradyrhizobium* symbionts associated with invasive *C. zanzibarica* and its coexisting legumes in Taiwan

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Abstract

The genetic diversity and identification of *Bradyrhizobium* symbionts of *Crotalaria zanzibarica*, the most widely-distributed invasive legume in Taiwan, and other sympatric legume species growing along riverbanks of Taiwan were evaluated. This is the first study investigating the diversity of *Bradyrhizobium* symbionts in Taiwan. In total, 59 and 54 *Bradyrhizobium* isolates were obtained from *C. zanzibarica* and its coexisting legume species, respectively. Based on the multilocus sequence analysis (MLSA) of concatenated four housekeeping genes (*dnaK-glnII-recA-rpoB* gene sequences, 1901 bp), these isolates displayed 53 unique haplotypes, grouping into 21 clades. Eleven of these clades are congruent to already defined *Bradyrhizobium* species, while other clades are not congruent to any defined species. The *C. zanzibarica* isolates belong to 14 MLSA clades, six of which overlapped with the isolates of coexisting legumes. According to the sequences of their symbiotic *nodA* genes (555 bp) the isolates were classified into three known *nodA* clades, III.2, III.3 and VII and were further clustered into 10 groups. The *C. zanzibarica* isolates were clustered into 8 *nodA* groups, five of which overlapped with the isolates from coexisting legumes. The *nodA* genes of the isolates from native species were dominated by Asian origin, while those of *C. zanzibarica* by American origin. In conclusion, *C. zanzibarica* is a promiscuous host capable of recruiting diverse *Bradyrhizobium* symbionts, some of which are phylogenetically similar to the symbionts of coexisting legumes in Taiwan. However, there is no evidence that *C. zanzibarica* established symbiosis with more genetically diverse *Bradyrhizobium* communities than its coexisting legumes.

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Introduction

Bradyrhizobium, a major symbiont of legume taxa distributed in tropical and sub-tropical regions, is the most abundant genus among the known rhizobial genera (Sprent 2007, 2009) and its symbiotic relationship with legume has been widely studied. However, these studies have been mostly conducted in continental areas and relatively few on island regions. Among the limited reports, the genetic diversity of symbiotic rhizobia in islands was generally found more diverse than that in continental area. For example, Parker and Rousteau (2014) reported that *Bradyrhizobium* symbionts on the Caribbean island (Guadeloupe) were extremely diverse, which might be generated by multiple colonization events and substantial horizontal gene transfer of symbiotic genes.

Taiwan, a sub-tropical Pacific island, has more than 200 legume species (Huang, 1993), among which the milletoid clade is the most dominant group (127 species), followed by the genistoid clade with 26 species. About a quarter to one third (50-70 species) of the legume species recorded on the island are naturalized (Wu *et al*., 2003, 2010). The exotic legumes and their nodule symbionts could be co-introduced from native regions into invaded areas (Rodríguez-Echeverría, 2010; Crisóstomo *et al*., 2013; Horn *et al*., 2014). Thus, the introduced legume species might play important roles in affecting the soil rhizobia community of the island. *Crotalaria zanzibarica* Benth., a perennial leguminous shrub native to Africa, was first recorded in 1934 in Taiwan. After its introduction, the species became the most widely-distributed naturalized legume and has been evaluated as a naturalized legume with the highest invasiveness among the naturalized legumes in Taiwan (Wu *et al*., 2010). Factors contributing to its widely distribution in Taiwan have not been studied. This plant can be found growing along roadsides, in abandoned fields, but mainly along riverbanks, where there is low soil

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fertility. Legume-rhizobia symbiosis has been suggested as one of the critical factors helping exotic legumes colonize novel ranges (Richardson *et al*., 2000b; Parker *et al*., 2006; Klock *et al*., 2015). The capability of forming effective symbioses with diverse rhizobia further plays an important role in increasing the distribution of widespread legume species (Thrall *et al*., 2000; Klock *et al*., 2015). Accordingly, *C. zanzibarica* might have the ability to recruit diverse local rhizobia after its introduction into Taiwan hence establishes symbiosis with more genetically diverse rhizobia communities than other exotic legume plants. Alternatively, exotic rhizobia might have been introduced accompanying the introduction of this plant into Taiwan. Consequently, the symbiotic relationship allows *C. zanzibarica* to thrive in the nitrogen-poor habitats, such as riverbanks.

In previous studies, I investigated nodule symbionts of *C. zanzibarica* and demonstrated that this plant was nodulated by *Bradyrhizobium* in the greenhouse condition and in riverbank of Northern Taiwan (Huang *et al*., 2016, 2018). This study expands previous studies investigating the genetic diversity and identification of *Bradyrhizobium* symbionts of *C. zanzibarica* growing along banks of three major rivers of Taiwan. Though *Bradyrhizobium* is known to be the most common rhizobium nodulating milletioid and genistoid legumes (Sprent, 2007, 2009), the two most dominant legume groups in Taiwan, the diversity and origins of *Bradyrhizobium* symbionts have not been investigated in field growing legumes. To infer the possible sources of the *Bradyrhizobium* symbionts of *C. zanzibarica* and to increase our understanding of the diversity of *Bradyrhizobium* in Taiwan, I also sampled native and other exotic legumes, growing adjacent to *C. zanzibarica*, known to form symbiosis with *Bradyrhizobium*. To the best of our knowledge, this is the first study investigating

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the diversity of *Bradyrhizobium* symbionts of legumes in Taiwan.

According to the aforementioned background, the objectives of the study were to investigate the diversity of *Bradyrhizobium* symbionts in Taiwan and to trace possible sources of the *Bradyrhizobium* symbionts of *C. zanzibarica*. Specifically, phylogenetic relationships and taxonomic identities of the isolates were analyzed utilizing the multilocus sequence analysis (MLSA) technique with four housekeeping genes, *dnaK*, *glnII*, *recA* and *rpoB* genes. To infer the possible geographic sources and potential hosts of the isolates, the phylogeny of the symbiotic *nodA* gene from these isolates was also studied.

Materials and methods

Sampling sites, nodule collection and rhizobial isolation

Xiandan (XD, 24°98′N, 121°52′E), Dajia (DJ, 24°25′N, 120°82′E) and Gaoping (GP, 22°77′N, 120°45′E) Rivers are located in Northern, Central and Southern Taiwan, respectively. Along the bank of each of the three rivers, I selected one sampling area (about 10 × 150 m) containing a population of *C. zanzibarica*. Soils of the three sites were mostly sandy with slight acidity (pH 5.8 to 6.3) and low electronic conductivity (0.04 to 0.08 S/cm). Legume plants were prevalent but the composition of the legume communities differed among the three sampling sites. In addition to analyzing the nodule symbionts of *C. zanzibarica*, I also investigated those of the sympatric legume species, occurring at a distance less than 1 m from *C. zanzibarica* plants, known to associate with *Bradyrhizobium*. In total, 11 legume species, including plants of distinct tribes and originating from disparate sources, were sampled (**Table 3-1**).

At each of the three sampling sites, ten to fourteen individuals of *C. zanzibarica*

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were excavated, and 2-5 nodules per individual were collected. For the coexisting legumes, nodules (1-3 nodules per individual) were collected from six individuals of each species. A total of 199 root nodules (92 from *C. zanzibarica* and 107 from other legumes) were collected for rhizobial isolation.

Fresh nodules were surface sterilized by immersion in 0.5 % SDS for 1 min, 70 % ethanol for 5 min, and finally washed three times by sterile deionized-distilled water (DDW). Nodule suspension was prepared by crushing the nodule in DDW and spread onto yeast extract mannitol (YEM) plate (Vincent, 1970). A single rhizobial isolate was obtained from each nodule and checked for the unity by repeated streaking on YEM plate.

Amplification, sequencing and DNA polymorphism of five genetic markers

Total genomic DNA of each pure rhizobial culture, grown in YEM broth at late exponential phase, was extracted with Geneaid DNA Mini kit (Geneaid Biotech, New Taipei, Taiwan). The primer pairs and PCR conditions for amplifications of the four housekeeping genes (*dnaK*, *glnII*, *recA* and *rpoB*) and the symbiotic gene, *nodA* are listed in Supplementary Table S1. PCR mixtures were made up with Taq DNA Polymerase 2x Master Mix RED (Ampliqon, Copenhagen, Denmark) following by the standard protocol. PCR products were first checked on 1.5% agarose gel and purified with Gel/PCR DNA fragments extraction kit (Geneaid Biotech, New Taipei, Taiwan). All sequencing reactions were performed by using the ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA). For checking the quality, chromatogram of each sequence was examined by using BioEdit 7.2.5 (Hall, 1999). GenBank accession numbers of the five genes sequences of these isolates are provided in

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Supplementary Table S2.

Sequences for each of the five genes were aligned by using MUSCLE (Edgar, 2004) in MEGA version 6 (Tamura *et al*., 2013). To analyze the sequence polymorphisms, haplotype diversity (*Hd*) and nucleotide diversity (π, the average number of nucleotide differences per site) for each of the five genes was estimated by using software DnaSP5.10 (Librado and Rozas, 2009). Additionally, nucleotide diversity for synonymous and nonsynonymous substitutions of each of the five genes was also calculated.

Phylogenetic identification of Bradyrhizobium *isolates by using four housekeeping genes*

For species identification, the *dnaK*, *glnII*, *recA* and *rpoB* gene sequences of type strains of all 39 validly published *Bradyrhizobium* species were download from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/). However, due to lack of *rpoB* gene sequences, four type strains of *B. erythrophlei*, *B. ferriligni*, *B. ganzhouense* and *B. lupini* were excluded from the phylogeny reconstruction. In the preliminary analysis, *B. oligotrophicum*, *B. denitrificans*, *B. jicamae*, *B. lablabi*, *B. retamae*, *B. icense*, *B. valentinum* and *B. paxllaeri* are not closely related to our isolated strains (data not shown). For brevity, these 8 species were also excluded from further analysis. Therefore, a total of 27 type strains of *Bradyrhizobium* species were used as the reference strains. The GenBank accession numbers of the four housekeeping genes of the reference strains are listed in Supplementary Table S3. The homologous sequences from *Rhodopseudomonas palustris* BisB5 (NC_007958) were used as an outgroup for each of the gene phylogenies.

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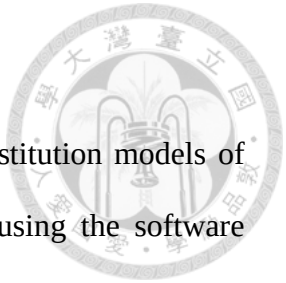

Maximum likelihood (ML) phylogenetic trees and best fit substitution models of these four housekeeping genes were reconstructed and evaluated using the software MEGA6. The best substitution models of four gene sequences were listed as the following. The Tamura-Nei model plus Gamma rate distribution (TN93 + G) was for *dnaK* sequences; The Tamura-Nei model plus Gamma rate distribution and invariant site (TN93 + G + I) was for *glnII* sequences; The Tamura 3-parameter model plus Gamma rate distribution and invariant site (T92 + G + I) was for both *recA* and *rpoB* sequences. To visualize the conflicting phylogenetic signals among the four housekeeping genes, I concatenated *dnaK*, *glnII*, *recA* and *rpoB* gene sequences of 113 isolates and performed NeighborNet analysis by using SplitsTree v4.13.1 (Huson and Bryant, 2006). Based on a network for the concatenated dataset (**Supplementary Figure S1**), multiple reticulations indicated that horizontal gene transfer of housekeeping genes might occur among these isolates, which was consistent with previous findings (Andam and Parker, 2008; Koppell and Parker, 2012). Due to single gene phylogenies usually failing provide sufficient information for taxonomic conclusions of *Bradyrhizobium* (Menna *et al*., 2009; Rivas *et al*., 2009; Azevedo *et al*., 2015), the concatenated sequence for the *dnaK*, *glnII*, *recA* and *rpoB* genes was also used to reconstruct an ML tree, as has been suggested from previous studies.

An ML tree based on the concatenated *dnaK-glnII-recA-rpoB* gene dataset was reconstructed under the model General Time Reversible plus Gamma rate distribution and invariant site (GTR + G + I). To test the strength of the phylogeny of all ML trees, the bootstrap method based on 1000 replicates was used. Approximate likelihood ratio test (aLRT) was used to assess robustness of ML tree in PhyML3.0 (Guindon *et al*., 2010).

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In addition, the concatenated *dnaK-glnII-recA-rpoB* gene tree was assessed by Bayesian Inference (BI) with MrBayes version 3.2.2 (Ronquist et al., 2012) using the nucleotide substitution model GTR + G + I. For analysis, I used 3,000,000 Markov Chain Monte Carlo (MCMC) generations and trees were sampled every 250 generations. Posterior probabilities were calculated by sampling 250 post-burnin trees.

nodA *phylogeny and possible origin of isolates*

In order to infer the possible origin of the isolates, the *nodA* gene sequences of reference strains similar to those of the isolates were download from NCBI GenBank based upon BLAST results. In addition, the sampling information (host and geographic origin) of each reference strain was also retrieved from NCBI GenBank and published literatures. The *nodA* gene sequence from *Methylobacterium nodulans* ORS2060 (AF266748) was used as the outgroup. The *nodA* phylogeny among the isolates and reference strains was assessed using both ML and BI analyses. The ML tree was reconstructed under T92 + G model with 1,000 bootstrap replicates. Bayesian analysis was assessed by using the Hasegawa–Kishino–Yano model plus Gamma rate (HKY + G) and run for 1,000,000 MCMC generations, sampling every 250 generations with a relative burnin of 25%.

Results

DNA polymorphism patterns of Bradyrhizobium *isolates*

A total of 113 putative rhizobial isolates were obtained from the root nodules of 11 legume species growing along the three riverbanks (**Table 3-1**). Among these isolates, 59 were collected from *C. zanzibarica*. All isolates formed visible colonies on YEM

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plates after 5-7 days of incubation at 30℃, consistent with the slow-growing phenotype of *Bradyrhizobium*.

Partial sequences of the four housekeeping genes, *dnaK* (224 bp), *glnII* (539 bp), *recA* (527 bp) and *rpoB* (611 bp), were obtained from all 113 isolates. Among these isolates, eight isolates (CzHD6, ZAHD2, CzDJD1, CzDJG1, CmDJA1, AaGPB1, CeGPD and CeGPC) did not form specific PCR product of *nod*B-D box, consequently, the partial *nodA* gene (555 bp) was only sequenced in 105 isolates. Table 2 shows the haplotype (*Hd*) and nucleotide diversity (π) of the *C. zanzibarica* isolates and those of isolates from other coexisting legumes at each of the sampling sites. In general, haplotype diversity calculated with the five genetic markers are relatively similar within the analyzed groups, while among the five markers, symbiotic *nodA* gene displayed much higher nucleotide diversity than the four housekeeping genes. This result was not only due to high synonymous substitution of *nodA* gene, but also caused by considerable variation in non-synonymous sites of this gene (**Table 3-2**). The level of DNA polymorphism of isolates from *C. zanzibarica* was either similar to or lower than that of isolates from other coexisting legumes at the same sampling site. Hence, there is no evidence that *C. zanzibarica* established symbiosis with more genetically diverse *Bradyrhizobium* communities than its coexisting legumes.

Phylogenetic analysis and taxonomic identification by using four housekeeping genes

Thirty-two, 46, 41 and 43 haplotypes of *dnaK*, *glnII*, *recA* and *rpoB* genes, respectively, were identified among the 113 isolates. The ML trees reconstructed individually with each of the four housekeeping genes (**Supplementary Figures S2 to S5**) rarely provided well-supported resolution among the haplotypes of isolates and the

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27 named *Bradyrhizobium* taxa. On the other hand, concatenated *dnaK-glnII-recA-rpoB* gene sequences (1901 bp) of the 113 isolates displayed 53 unique multilocus haplotypes, designed as MLSA-hap 1-53. ML analysis of the combined sequences dataset produced more robust and well-supported phylogeny for these *Bradyrhizobium* isolates (**Figure 3-1**). The resulting phylogenetic tree contained two major clades (A and B), including the type strains of *B. japonicam* and *B. elkanii*, commonly referred to as *B. japonicam* and *B. elkanii* lineages, respectively (Vinuesa *et al*., 2008; Rivas *et al*., 2009). Most of the isolates in this study were situated in *B. japonicam* lineages (Clade A), and further split into 18 terminal clades (defined as A.1-A.18). Only 8 isolates, all collected from the legume hosts of tribe Desmodieae, belonged to the *B. elkanii* lineages (Clade B.1-B.3). Several clades formed well-supported groups (ML aLRT confidence test values > 0.7, ML bootstrap support > 70% and Bayesian inference posterior probability > 0.8) with named *Bradyrhizobium* strains. Accordingly, Clade A.1-A.5 were classified as *B. yuanmingense*, Clade A.7 as *B. liaoningense*, Clade A.12 as *B. daqingense*, Clade A.15 as *B. arachidis*, Clade A.18 as *B. manausense*, Clade B.1 as *B. pachyrhizi* and Clade B.2 as *B. elkanii*. Other clades were situated outside the defined strains. These clades might represent novel genospecies.

The number of isolates belonging to each of the 21 MLSA clades varied from 1 to 35 (**Table 3-3**). Among the 21 clades, 14 were limited to a single sampling site, 6 were found in two sampling sites, and only one clade, clade A.3, extended across all three sites. Clade A.3 was the most abundant clade containing 31% of the isolates. At the XD site, most of the isolates from *C. zanzibarica* and its coexisting legumes were separated into non-overlapping clades, with one exception of clade A.8. At the DJ site, 3 clades, A.2, A.3 and A.10 were shared by *C. zanzibarica* and its coexisting legumes. In contrast,

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all the four clades (A.1, A.2, A.3 and A.12) found in GP site were shared by *C. zanzibarica* and its coexisting legumes (**Table 3-3**).

nodA *gene phylogeny and possible origins*

ML analyses of the *nodA* gene sequences of the present isolates and reference strains produced a well-resolved tree with three major clades (**Figure 3-2**). Each clade contained previous published *Bradyrhizobium nodA* gene sequences, and thus, the three clades could be identified as *nodA* sub-clades III.3, III.2 and clade Ⅶ (Moulin *et al*., 2004; Stępkowski *et al*., 2005, 2007). More than half of the present isolates (64 out of 105 isolates) belonged to a highly diverse clade, *nodA* sub-clade III.3, and were further divided into 8 groups (assigned as III.3a-h). The *nodA* Group III.3a included 27 isolates from diverse sources, including isolates from three sampling sites and from both native and exotic legumes. This *nodA* group clustered with the reference strains originating from Thailand, India, Japan and China (**Figure 3-2**), indicating these *nodA* gene sequences were prevalent in Asia. In both *nodA* Group III.3c and III.3d, all isolates were collected from *C. zanzibarica* and were exclusively grouped together with multiple peanut symbionts in China (**Figure 3-2**). On the other hand, isolates in the *nodA* Group III.3b, III.3e and III.3g were mainly *C. zanzibarica* symbionts and clustered with the reference strains from distant geographic regions, hence their specific origins could not be resolved (**Figure 3-2**). One *C. zanzibarica* isolate, strain CzDJC1 was not grouped together with any reference strain, forming *nodA* Group III.3f (**Figure 3-2**). In *nodA* Group III.3h, all isolates belonged to *B. elkanii* lineage (Clade B, **Figure 3-1**) and clustered with *B. elkanii* USDA76 and reference strains from diverse sources (**Figure 3-2**).

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In addition, 38 isolates were classified as *nodA* sub-clade III.2 strains which were dominant by *C. zanzibarica* isolates (26 isolates) and were found in all three sampling sites (**Figure 3-2** and **Table 3-4**). Consistent with previous reports, all reference strains in *nodA* sub-clade III.2 were from the Americas (Moulin *et al*., 2004; Stępkowski *et al*., 2005, 2007). Particularly, the *nodA* gene sequences of these isolates were nearly identical to that of strain NC92, which was a peanut symbiont originated in Bolivia (Urtz and Elkan, 1996). Furthermore, it had been reported that the strains belonged to *nodA* clade VII exclusively originated from Central and South America (Stępkowski *et al*., 2007), however, our data showed that 3 isolates from *Desmodium* spp. and reference strains from Australia and China were also situated in this clade (**Figure 3-2**).

The *nodA* groups of isolates from *C. zanzibarica* and other legumes were rarely, partially and totally overlapping in XD, DJ and GP sites, respectively (**Table 3-4**).

Discussion

C. zanzibarica *was nodulated by diverse* Bradyrhizobium *genospecies*

Aserse *et al*. (2012) reported that several *Crotalaria* spp. growing in their native regions (Ethiopia) were associated with diverse *Bradyrhizobium* symbionts. A non-classical rhizobium, *Methylobacterium nodulans*, was also reported to nodulate native *Crotalaria* plants in Senegal (Jourand *et al*., 2004). In this study, I found that the 59 isolates collected from exotic *C. zanzibarica* in Taiwan all belonged to the genus *Bradyrhizobium*, in contrast, I did not find any *Methylobacterium* isolate. Thirty-four *C. zanzibarica* isolates in MLSA clades A1-A5 formed a well-supported group with *B. yuanmingense* 10071^{T} (ML bootstrap support = 77% and BI posterior probabilities = 1, **Figure 3-1**). Accordingly, *B. yuanmingense* appears to be a dominant species of *C.*

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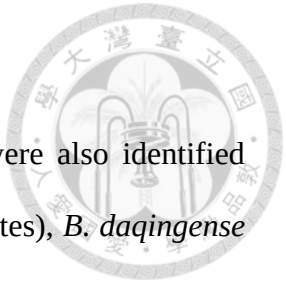

zanzibarica symbionts in Taiwan. Besides, several other species were also identified among the *C. zanzibarica* isolates, including *B. liaoningense* (2 isolates), *B. daqingense* (4 isolates), *B. arachidis* (1 isolate) and *B. manausense* (4 isolates). Among the defined species, *B. yuanmingense* (Yao *et al*. 2002) and *B. liaoningense* (Xu *et al*., 1995) were first described in China and had been reported as legume symbionts widely across distant geographic regions, such as Africa (Aserse *et al*., 2012; Grönemeyer *et al*. 2014), America (Koppell and Parker, 2012), Asia (Vinuesa *et al*., 2008; Noisangiam *et al*., 2012) and Australia (Stępkowski *et al*., 2012). *B. arachidis* (Wang *et al*., 2013), *B. daqingense* (Wang *et al*., 2013) and *B. manausense* (Silva *et al*. 2014) were identified more recently, and thus, information about these species is relatively limited. Although most of the *C. zanzibarica* isolates (45 out of 59 isolates) analyzed in this study were closely related to defined species, the remaining 14 isolates in MLSA clades A.8, A.9, A.10, A.11, A.14 and A.16 might represent novel *Bradyrhizobium* genospecies (**Figure 3-1**). Particularly, clade A.10, comprising 8 isolates collected from two distant *C. zanzibarica* populations and two isolates from *Crotalaria micans* and *Indigofera spicata*, is a strongly supported clade and distinct from any known species. Thus, the genospecies, clade A.10, might be well adapted to diverse soil conditions and legume hosts in Taiwan, and is therefore worth further analyzing for proposal of a novel species.

Multiple *Bradyrhizobium* lineages were observed in all three sampling sites (Table 3). In some cases, *C. zanzibarica* and its coexisting native legumes shared symbionts with the same genomic background in clades A.1 (in GP), A.2 (in DJ and GP), A.3 (in DJ and GP), A.8 (in XD) and A.10 (in DJ). Besides, the isolates of *C. zanzibarica* and its coexisting exotic legumes also overlapped in clades A.1 (in GP), A.2 (in DJ), A.3 (in GP), A.10 (in DJ) and A.12 (in GP). The results imply that *C. zanzibarica* has the ability

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of recruiting diverse local rhizobia, some phylogenetically similar to the rhizobia of the coexisting legumes, after its introduction into Taiwan. Thought it has been reported that invasive plants could established symbiosis with more genetically diverse rhizobia communities (Thrall *et al*., 2000; Klock *et al*., 2015), but there is no evidence that *C. zanzibarica* established symbiosis with more genetically diverse *Bradyrhizobium* communities than its coexisting legumes (**Table 3-2**).

The analysis of nodA *genes reveal that* C. zanzibarica *symbionts in Taiwan might have originated from multiple geographic sources.*

Symbiotic *nodA* gene provides information about geographic sources and potential hosts of *Bradyrhizobium*, and thus, this marker is widely used to trace the origins of the nodule symbionts (Moulin *et al*., 2004; Steenkamp *et al*., 2006; Stępkowski *et al*., 2007, 2012; Rodríguez-Echeverría, 2010). Analysis of the sequences of *nodA* gene of the isolated *Bradyrhizobium* strains suggested that the sources of symbiont utilized by *C. zanzibarica* could be grouped into the following four categories.

1. From Asia: Taiwan Island is relatively small (ca. 36,000 km^2) and of young geographic age (ca. 5 million yeas) (Sibuet and Hsu, 1997). Thus, the indigenous communities in this island most likely have migrated from the proximate continental regions. Given the results that *nodA* gene sequences of the Taiwanese isolates in Group III.3a were identical or nearly identical to those of the isolates collected from China, India and Thailand (**Figure 3-2**) and that *nodA* gene sequences of the major isolates obtained from the native legumes (12/21) growing in the three distant sampling sites (**Table 3-4** and **3-5**) were in Group III.3a, it is highly possible that III.3a strains in this study are putative local rhizobia in Taiwan. Accordingly, the result that *C. zanzibarica*

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shared the III.3a strains with its coexisting native legumes in all three sites (**Table 3-4**) support the hypothesis that *C. zanzibarica* is capable of recruiting local rhizobia. In addition, the III.3a strains not only nodulated *C. zanzibarica* but also nodulated other exotic legumes in DJ and GP sites (**Table 3-4** and **3-5**), indicating the importance of these strains for nodulating exotic legumes in Taiwan.

2. Specifically from China: The *nodA* Groups III.3c and III.3d were two closely related groups, and exclusively comprised the isolates collected from *C. zanzibarica* growing along XD riverbank (**Table 3-4**). The *nodA* gene sequences of these isolates were highly similar to those of several strains isolated from peanut in China (**Figure 3-2**), suggesting the III.3c and III.3d strains might specifically have originated from China.

3. From America: Interestingly, the sub-clade III.2, referred to as the "Pan-American" subgroup (Stępkowski *et al*., 2007), was also identified in this study. To the best of our knowledge, this is the first report to show that *nodA* III.2 strains were isolated from legume plants growing outside the Americas. Most of the *nodA* III.2 strains collected in this study shared the same genomic background, belonging to the most prevalent MLSA clade, clade A.3 (**Table 3-3**), with a few exception of clade A.1 (**Figure 3-2**). Given the fact that clade A.3 strains were commonly found among the *C. zanzibarica* isolates (**Table 3-3**), I thus concluded that a peculiar *Bradyrhizobium* lineage carrying symbiotic gene of American origin has been widespread and contribute to the major symbiont of exotic *C. zanzibarica* in Taiwan. Since *C. zanzibarica* is a common green manure cultivated in America (Hanelt, 2001), it is likely that some symbionts accompanied this plant were introduced from America into Taiwan. However, the III.2 strains were also commonly found among the nodules of other exotic American legumes coexisting along GP riverbank (**Table 3-4**). Thus, it is also possible that not *C. zanzibarica* but other

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exotic legume(s) hosting rhizobia of American origin were introduced into Taiwan, and *C. zanzibarica* subsequently utilized these exotic rhizobia as its nodule symbionts. It is noteworthy that two isolates collected from the native legumes were also found carrying these exotic symbiotic genes (**Table 3-4**). The result highlights the potential threat of invasional meltdown in legume-rhizobia symbiosis (Rodríguez-Echeverría, 2010) in Taiwan.

4. With no specific origin: In some cases, no clear correlations between the *nodA* groups and the geographic origins was observed. For example, Group III.3b was closely related to strains collected from Asia and Africa; Group III.3e was closely related to strains collected from America and Australia; Group III.3g was closely related to strains collected from Asia, Africa and America (**Figure 3-2**). These results were consistent with previous finding that *nodA* sub-clades III.3 is known to comprise cosmopolitan *Bradyrhizobium* distributed in tropical and subtropical regions (Moulin *et al*., 2004; Stępkowski *et al*., 2007). Among Groups III.3b, III.3e and III.3g, most of the isolates (16/22) were collected from *C. zanzibarica*, while the isolates collected from the coexisting legumes were relatively few, especially, only one isolate was from native legume (**Table 3-5**). This result indicated *C. zanzibarica* could also recruit certain nodule symbionts which might have spread worldwide. Additionally, a single *C. zanzibarica* isolate (CzDJC1) formed an aberrant branch, Group III.3f, which potentially represented unique Taiwanese strain; however, more sampling of this strain is needed to support this conclusion.

It has been widely reported that phylogenetic trees reconstructed using symbiotic genes (such as *nodA* and *nifD*) and housekeeping genes in *Bradyrhizobium* usually show conflicting evolutionary histories, due to the prevalence of horizontal gene transfer

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(Stępkowski *et al*., 2007, 2012; Koppell and Parker, 2012). The occurrence of horizontal gene transfer would result in phylogenetically distant rhizobia carrying same symbiotic genes, and being competitive for nodulation of certain legume hosts (Parker, 2012; Horn *et al*., 2014). In this study, I also found that the topology of symbiotic gene tree was incongruent with that of housekeeping gene tree. For example, the *nodA* genes of isolates belonged to MLSA clade A.1 were separated into three distinct *nodA* groups, Ⅲ.3a, Ⅲ.3b and Ⅲ.2, while *nodA* Group Ⅲ.3a comprised isolates from diverse MLSA clades A.1, A.2, A.3, A.4, A.6, A.8 and A.11 (**Figure 3-2**). These results are consistent with previous reports suggesting that horizontal transfer of symbiotic genes commonly occur among *Bradyrhizobium* symbionts (Stępkowski *et al*, 2005; Menna and Hungria, 2011; Parker, 2012).

The results that *C. zanzibarica* isolates situated in the diverse *nodA* groups III.3a, III.3c, III.3d, III.3g and III.2 all shared nearly identical sequences (similarity 98-100%) with reference strains isolated from *Arachis hypoogaea* (peanut) grown in the distant geographic sources (China and Bolivia) are striking. (**Figure 3-2**). It implies that *C. zanzibarica* and peanut might belong to the same cross-inoculation group which share nodule symbionts. Peanut is known to be a common legume crop with a long cultivation history in Taiwan. Therefore, it seems likely that the peanut plantation might also play important roles in facilitating the colonization of *C. zanzibarica* in this island. I have selected some drought and/or high temperature resistant *Bradyrhizobium* isolates from *C. zanzibarica* (unpublished). For agricultural application, the symbiotic performance of peanut plants, inoculated with these isolates, in drought and/or high temperature conditions will be evaluated.

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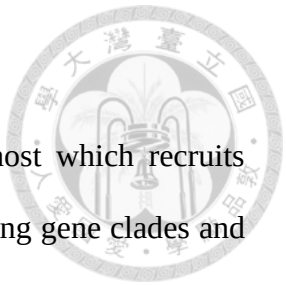

In conclusion, *C. zanzibarica* appears to be a promiscuous host which recruits diverse *Bradyrhizobium* symbionts belonging to multiple housekeeping gene clades and *nodA* gene groups. However, there is no evidence that *C. zanzibarica* established symbiosis with more genetically diverse *Bradyrhizobium* communities than its coexisting legumes. According to the analysis of *nodA* genes, the symbiont acquisition of this invasive legume in Taiwan are highly complex. (1) This plant shared the local rhizobia (or rhizobia prevailed in Asiatic regions) with coexisting native and exotic legumes. (2) The peanut symbionts originated from China exclusively nodulated *C. zanzibarica*. (3) The exotic rhizobia carrying symbiotic genes of American origin were commonly found among the isolates of *C. zanzibarica* and its coexisting exotic legumes, but rarely in native legumes. (4) *C. zanzibarica* recruited certain putative cosmopolitan symbionts which were relatively few in other coexisting legumes. Taken together, being able to associate with diverse *Bradyrhizobium* symbionts from multiple origins might be one of the key factors contributing to the successful invasion of *C. zanzibarica* in Taiwan.

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Table 3-1 Legume host and sampling sites of 113 *Bradyrhizobium* isolates analyzed in this study.

Legume host (origin[a])	List of isolates collected from the three sampling sits		
	Xindian riverbank (XD)	Dajia riverbank (DJ)	Gaoping riverbank (GP)
Tribe Crotalarieae			
Crotalaria zanzibarica (Africa)	CzHD, CzHD2, CzHD3, CzHD4, CzHD5, CzHD6, CzHDA2, CzHDA4, CzHDB2, CzHDD1, CzHDE1, CzHDE2, CzHDF1, CzHDF2, CzHDF3, CzHDF4, CzHDF5, ZAHD2, ZAHD3, ZBHD3, ZBHD4	CzDJA1, CzDJA2, CzDJA3, CzDJB1, CzDJB2, CzDJB3, CzDJC1, CzDJC2, CzDJC3, CzDJD1, CzDJD2, CzDJE1, CzDJE2, CzDJF1, CzDJG1, CzDJG2, CzDJG3, CzDJI1, CzDJI2, CzDJJ1, CzDJK	CzGP1, CzGP2, CzGP5, CzGPA1, CzGPA2, CzGPB2, CzGPB3, CzGPB4, CzGPB5, CzGPC1, CzGPC2, CzGPC3, CzGPC4, CzGPC5, CzGPD, CzGPE1, CzGPE2
Crotalaria micans (America)	N.A.	CmiDJA1, CmiDJA2, CmiDJA3	N.A.
Tribe Desmodieae			
Alysicarpus vaginalis (Native)	AvHD1, AvHD3, AvHD4	N.A.	AvGPA, AvGPC2, AvGPD1
Desmodium heterophyllum (Native)	DhHD1, DhHD2, DhHD3, DhHD4, DhHD5	N.A.	N.A.
Desmodium triflorum (Native)	DtHD, DtHD2, DtHD3, DtHD4, DtHD5, DtHD6	N.A.	N.A.
Desmodium tortuosum (Asia)	N.A.	DtoDJA1, DtoDJA2, DtoDJB1, DtoDJD1, DtoDJD2	N.A.
Tribe Dalbergieae			
Aeschynomene americana (America)	N.A.	N.A.	AaGPA1, AaGPA2, AaGPB1, AaGPD2
Tribe Indigofereae			
Indigofera spicata (Native)	N.A.	IsDJA1, IsDJA2, IsDJC, IsDJD	N.A.
Tribe Phaseoleae			
Macroptilium atropurpureus (America)	N.A.	MaDJA1, MaDJA2, MaDJB1, MaDJD1, MaDJD2	MaGPA1, MaGPA2, MaGPB, MaGPC1, MaGPD1, MaGPD3
Calopogonium mucunoides (America)	N.A.	N.A.	CmGP1, CmGP2, CmGP6
Centrosema pubescens (America)	N.A.	N.A.	CeGPA, CeGPB, CeGPC, CeGPD, CeGPE1, CeGPE2, CeGPE4

a: Wu et al., 2003

N.A.: The legume species was neither absent nor rarely found in the sampling site.

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Table 3-2 DNA polymorphism for *Bradyrhizobium* isolates collected from three riverbanks in Taiwan. The genetic diversity of given group are estimated using number of haplotype (*h*), haplotype diversity (*Hd*) and nucleotide diversity (π, all sites, π_T; synonymous sites, π_S; nonsynonymous sites, π_N).

Loci		XD isolates		DJ isolates		GP isolates	
		from *C. zanzibarica*	from others	from *C. zanzibarica*	from others	from *C. zanzibarica*	from others
Housekeeping gene		*n* = 21	*n* = 14	*n* = 21	*n* = 17	*n* = 17	*n* = 23
dnaK (224 bp)	*h/Hd*	12/0.924	8/0.912	6/0.557	9/0.897	4/0.669	8/0.818
	π_T	0.060	0.056	0.016	0.068	0.021	0.021
	π_S	0.161	0.173	0.059	0.196	0.089	0.081
	π_N	0.029	0.021	0.002	0.030	0.000	0.002
glnII (539 bp)	*h/Hd*	14/0.957	8/0.901	7/0.714	13/0.963	7/0.794	14/0.937
	π_T	0.046	0.064	0.024	0.068	0.031	0.036
	π_S	0.153	0.173	0.080	0.198	0.100	0.116
	π_N	0.013	0.031	0.007	0.029	0.010	0.011
recA (527 bp)	*h/Hd*	12/0.933	8/0.912	6/0.557	11/0.949	6/0.691	12/0.874
	π_T	0.047	0.056	0.024	0.062	0.026	0.032
	π_S	0.175	0.185	0.087	0.211	0.100	0.122
	π_N	0.003	0.011	0.002	0.011	0.000	0.000
rpoB (611 bp)	*h/Hd*	13/0.948	9/0.934	7/0.714	12/0.963	7/0.794	13/0.933
	π_T	0.040	0.050	0.018	0.058	0.018	0.018
	π_S	0.138	0.148	0.070	0.163	0.074	0.075
	π_N	0.008	0.018	0.002	0.023	0.000	0.000
Symbiotic gene		*n* = 19	*n* = 14	*n* = 19	*n* = 16	*n* = 17	*n* = 20
nodA (555 bp)	*h/Hd*	10/0.918	9/0.923	5/0.532	12/0.958	7/0.860	12/0.921
	π_T	0.138	0.163	0.116	0.140	0.142	0.144
	π_S	0.346	0.411	0.300	0.361	0.359	0.369
	π_N	0.065	0.074	0.051	0.061	0.063	0.063

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Table 3-3 A summary of the number of isolates based on MLSA, from *C. zanzibarica* (Cz) and its coexisting native (CN) and exotic (CE) legumes in each of the three sampling sites (XD, DJ and GP) in Taiwan. These isolates belonged to each of the MLSA clades in phylogeny tree reconstructed with the combined sequences for *dnaK*, *glnII*, *recA* and *rpoB* genes (Figure 3-1).

Site	XD			DJ			GP			Total
Host	Cz	CN	CE	Cz	CN	CE	Cz	CN	CE	
MLSA clade										
A.1		1					3	1	8	13
A.2				2	1	4	1	1		9
A.3	3			14	1		9	1	7	35
A.4					1	1				2
A.5	2									2
A.6		4								4
A.7	2									2
A.8	1	3								4
A.9	1									1
A.10	5			3	1	1				10
A.11				1						1
A.12							4		5	9
A.13		3								3
A.14	1			1						2
A.15	1					1				2
A.16	1									1
A.17						1				1
A.18	4									4
B.1		2								2
B.2						1				1
B.3		1				4				5

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Table 3-4 A summary of the number of isolates based on *nodA* genes, from *C. zanzibarica* (Cz) and its coexisting native (CN) and exotic (CE) legumes in each of the three sites (XD, DJ and GP) in Taiwan. These isolates belonged to each of the *nodA* groups in phylogeny tree reconstructed with partial sequence of *nodA* gene (Figure 3-2).

Site	XD			DJ			GP			Total
Host	Cz	CN	CE	Cz	CN	CE	Cz	CN	CE	
nodA group										
Ⅲ.3a	1	8		3	2	5	2	2	4	27
Ⅲ.3b							5		3	8
Ⅲ.3c	2									2
Ⅲ.3d	4									4
Ⅲ.3e	6			2	1	1				10
Ⅲ.3f				1						1
Ⅲ.3g	3					1				4
Ⅲ.3h		3				5				8
Ⅲ.2	3			13	1		10	1	10	38
Ⅶ		3								3

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Table 3-5 The possible origins inferred by *nodA* gene sequences of isolates collected from *C. zanzibarica* and other native/exotic legumes in this study.

Possible geographic origin	*nodA* group	Isolates from *C. zanzibarica* (n = 55)	Isolates from others (n = 50)	
			Native legumes (n = 21)	Naturalized legumes (n = 29)
Asia	Ⅲ.3a	6	12	9
	Ⅲ.3c	2		
	Ⅲ.3d	4		
America	Ⅲ.2	26	2	10
Cosmopolitan	Ⅲ.3b	5		3
	Ⅲ.3e	8	1	1
	Ⅲ.3g	3		1
	Ⅲ.3h		3	5
	Ⅶ		3	
Not detemined	Ⅲ.3b	1		

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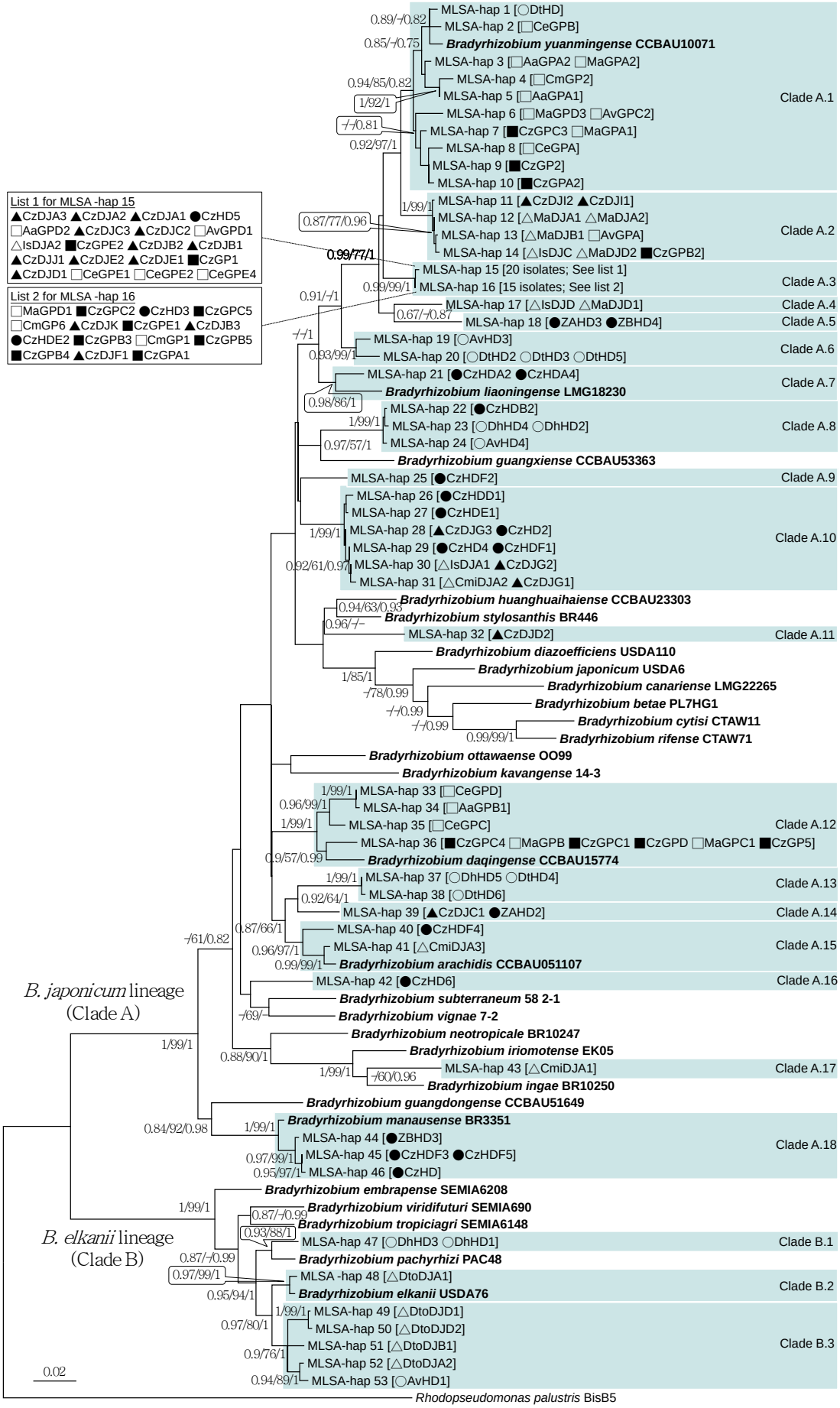

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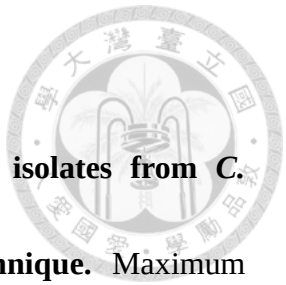

Figure 3-1 Phylogenetic identification of the *Bradyrhizobium* isolates from *C. zanzibarica* and its coexisting legumes by using MLSA technique. Maximum likelihood (ML) phylogeny tree based on the combined sequences (1901 bp) for the genes *dnaK*, *glnII*, *recA* and *rpoB*, showing the relationships among 53 haplotypes found among 113 isolates and 27 type strains of defined *Bradyrhizobium* species (bold). Only ML aLRT confidence test values >0.6, ML bootstrap support >60% and BI posterior probabilities values >0.6 are shown at each node. The symbols correspond to the sampling sites and plant hosts: circle, Xindian; triangle, Dajia; square, Gaoping. Filled symbols indicate isolates from *C. zanzibarica*, while open symbols represent the isolates from other coexisting legumes. Clade designations (A.1–18 and B.1-3) for the isolates in this study are indicated on the right. The scale bar indicates 2% nucleotide substitutions of the genes.

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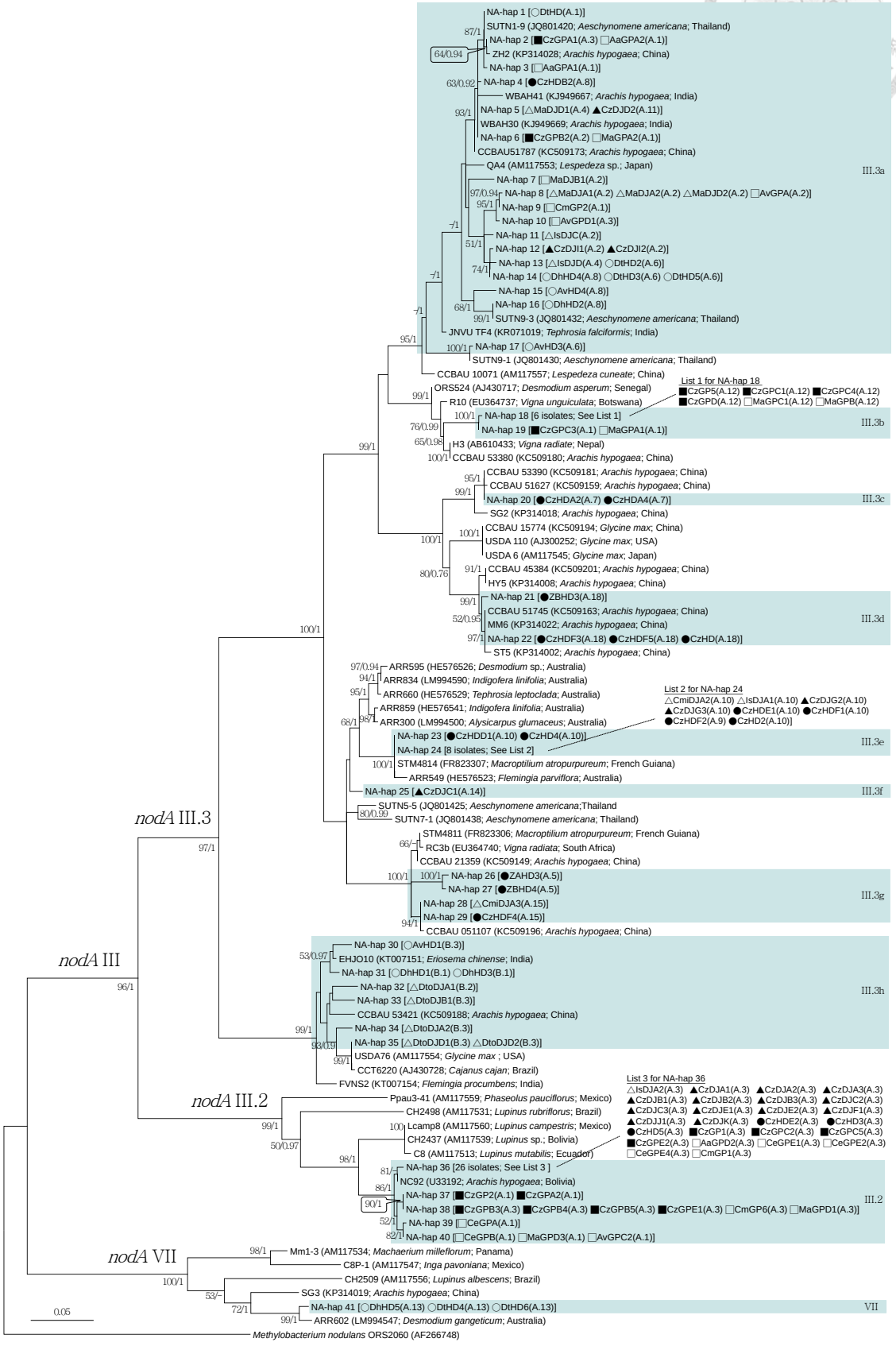

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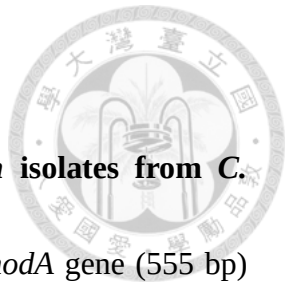

Figure 3-2 The *nodA* gene phylogenies of the *Bradyrhizobium* isolates from *C. zanzibarica* and its coexisting legumes. ML phylogeny of partial *nodA* gene (555 bp) showing the relationships among Taiwanese *Bradyrhizobium* isolates and reference strains. The three known *nodA* clades, sub-clades III.3, III.2 and clade Ⅶ are indicated at the respective nodes. The symbols indicate the sampling site and plant host of each isolate (see **Figure 3-1**). For each of the Taiwanese *Bradyrhizobium* isolates, the MLSA clade (see **Figure 3-1**) are provided in parentheses. In addition, the *nodA* groups to which these isolates belong are indicated on the right. For each of the reference strains, GenBank accession number, host and geographic origin are listed in parentheses. ML bootstrap support (>50%) and BI posterior probabilities values (>0.7) are indicated at each node. The scale bar indicates 5% nucleotide substitutions of *nodA* gene.

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Chapter 4

Evaluation the growth of *C. zanzibarica* inoculated by *Bradyrhizobium* strains

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Abstract

In previous chapters, I reported that cultivated *C. zanzibarica* plants were nodulated by a single strain, *B. arachidis* CzR2 in a greenhouse, whereas field-growing *C. zanzibarica* established symbiosis with diverse *Bradyrhizobium* lineages, including *B. liaoningense*, *B. daqingense*, *B. arachidis*, *B. manausense* and several undefined genospecies. To confirm the field isolates are able to nodulate *C. zanzibarica* and to evaluate effects of symbionts on growth of this plant, I conducted single-strain inoculation experiment and investigated growth response, nodulation response, symbiotic efficiency and nitrogen relationship of *C. zanzibarica* inoculated with six rhizobial strains. The growth of *C. zanzibarica* neither inoculated nor nitrogen fertilized, and that of *C zanzibariba* fertilized with NO_3/NH_4 but no inoculation, were also measured.

The reference plants without inoculation and nitrogen fertilizer grew poorly and accumulated little biomass. All inoculated strains induced nodules on roots of *C. zanzibarica*, however, the nodulated plants showed significant variation in total plant biomass and nitrogen accumulation among the six inoculants. In contrast, there was very little variation in biomass allocation among these tested plants. Although strain CzR2 displayed larger bacteroids inside the nodules, there was no evidence that this strain was more symbiotically benefit to *C. zanzibarica* than other tested strains. In general, more nodule formation on roots of *C. zanzibarica* commonly translated into higher total plant biomass. This result indicates that symbiotic rhizobia are critical factors for growth of *C. zanzibarica* in the nitrogen deficient environment.

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Introduction

Nitrogen is one of the most important nutrients limiting plant growth. Symbiosis with nitrogen-fixing rhizobia provides leguminous plants advantage in low soil nitrogen (N) habitats and hence increases the niches of these plants. However, for exotic legume species, there is no guarantee that they could find compatible rhizobia in the newly arrived habitats. Therefore, the ability of establishing symbiotic relationship with local rhizobia might be a critical trait for successful invasion of exotic legumes.

Thrall *et al*. (2000) investigated *Acacia*-rhizobia symbiosis in Australia, and reported that common *Acacia* species performed equally well when inoculated with certain rhizobial strains, while rare *Acacia* species showed significant variation in performance when inoculated with the same tested strains. Accordingly, widespread *Acacia* species displayed less degree of symbiotic specificity than rare *Acacia* species in Australia. Similar results were reported by Klock *et al*. (2015), in which they found that invasive Australian *Acacia* species had positive symbiotic performance inoculated with a wider range of rhizobial strains than naturalized and non-invasive species. These studies supported the idea that promiscuity of legume hosts might facilitate their wide distribution.

Crotalaria zanzibarica, a widely-distributed legume, has been evaluated as a species of the highest invasiveness among the naturalized legume in Taiwan (Wu *et al*., 2003). I hypothesized that being able to form effective symbioses with diverse rhizobia might contribute to its successful invasion in Taiwan. To test the hypothesis, I have isolated nodule-forming bacteria from roots of *C. zanzibarica* growing along riverbanks and found that field-growing *C. zanzibarica* plants were nodulated by diverse *Bradyrhizobium* strains. However, the symbiotic relationship and the effect of the

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rhizobial inoculation on the growth of *C. zanzibarica* have not been confirmed. In this study, I compared the growth of *C. zanzibarica* without fertilization, fertilized with NO_3/NH_4, or inoculated with diverse strains of greenhouse and field isolated *Bradyrhizobium*.

For those plants with inoculation, I compared their nodulation response, growth response, symbiotic efficiency and nitrogen relationship by using single-strain inoculation experiment.

Materials and methods

Inoculation experiment

About 250 seeds collected from a population of *C. zanzibarica* growing along the riverbank (24°98′ N, 121°52′ E) of Xiandan River were surface sterilized with 70% ethanol for 10 min, and then germinated in Petric dishes at room temperature. After germination (ca. 2 days), seedlings were transferred into 8 plastic containers (18 cm x 14 cm x 8 cm, 25-30 seedlings in each container) filled with sterilized mixture of peat soil, vermiculite and perlite (5:2:2). To avoid contamination by potential rhizobia in the greenhouse, all seedlings were first grown in a growth chamber (30/25℃, light/dark and 12/12hr). When seedlings were 4 days old, seedlings in the 6 containers were treated with individual rhizobial strain grown in YEM broth, while seedlings in the other 2 containers were treated with blank YEM broth as controls. In the rhizobia-inoculated groups, each seedling was inoculated with 200 μl (OD_{600}=1.5) of one tested rhizobial strain. The species identities and source information of the 6 rhizobial strains were listed in **Table 4-1**. All rhizobia inoculated seedlings were irrigated with distilled water regularly and fertilized with N-free Hoagland solution every week. In addition to

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receive irrigation, seedlings of one control group were fertilized with 15 mM NH_4NO_3 every week (designed as N^+ control), while those of the control group were not fertilized with any N-containing solution (designed as N^- control).

After 11 days of growing, seedlings were transferred from the growth chamber to a greenhouse. In the greenhouse, Seedlings in each treatment were transplanted into six 2L plastic pots (three seedlings per pot) filled with sterilized mixture. Accordingly, there were 18 seedlings for each of the treatment. The location of plastic pots on the bench were rotated every 4-5 days, and plants were grown under natural daylight. The experiment was conducted from May to June 2016.

Plant harvest

Plants were harvested three times. Five to six plants in each of the eight treatments were harvested at ages of 27, 34 and 41 DAS (days after sowing). The N^- control plants displayed yellow leaves and dropped leaves at 30-35 DAS. Therefore, results of plants of N^- control were not analyzed. Besides, one individual (of 41 DAS) in N^+ control formed root nodules and was also excluded from further analyses. During each harvest, one individual was rooted from each pot. Shoot, root, and nodule of each individual were separated carefully and washed with distilled water. Nodule numbers were counted and nodule sizes were measured. All plant materials were dried at 60 °C in an oven for three days and then weighted.

In addition, I have amplified and sequenced the *nodZ* genes from nodule suspension of each treatment to confirm the rhizobial strain within the nodules.

Nitrogen content and stable isotope ratio

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Dried samples were ground into a homogenized powder with a mortar and pestle. A 2 mg of ground material was loaded into a tin capsule for further analysis. Nitrogen content (N%) was determined with an elementary analyzer (FlashEA 1112 series, Thermo Fisher Scientific, Italy). The total nitrogen accumulation of an individual was calculated by following formula: (shoot biomass)*(shoot N content, % dry weight) + (root biomass)*(root N content, % dry weight) + (nodule biomass)*(nodule N content, % dry weight). Because N^- control plants were too small, only whole plant nitrogen content was measured.

Results

Nodulation response across rhizobial strains

All inoculated plants formed visible nodules on roots after 7 days of inoculation (data not shown). In contrast, only one individual received the control treatment (more than 30 seedlings) was found nodulated, indicating the level of cross-contamination was very low.

The morphology of nodules induced by different *Bradyrhizobium* strains was distinctive. For example, nodules induced by strain CzR2 and CzDJG3 appeared more reddish than those by other tested strains (**Figure 4-1**). The reddish color might be resulted from the accumulation of leghemoglobin located between the cortex and infection zone (**Supplementary Figure S6**).

The number and biomass of nodules harvested from roots of 41 DAS plants inoculated with individual strain of *Bradyrhizobium* were shown in **Figure 4-2A**. Roots of *C. zanzibarica* inoculated by CzR2, CzHD, CzHD3, CzDJG3 and CzGP3 produced an average of 25 nodules per plant, while plants inoculated by DtHD2 produced only

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about 10 nodules per individual. Significant differences was also found in nodule biomass among plants inoculated with different *Bradyrhizobium* strains (**Figure 4-2B**). Plants inoculated with CzGP5 and DtHD2 had significantly less nodule biomass than those with CzHD, CzDJG3 and CzHD3.

Plant growth and biomass allocation

Significant variations in plant growth among the eight treatment plants were found (**Figure 4-3A**). The N^- control plants had lowest biomass and showed yellow leaves, indicating the nitrogen sources from the seeds and matrix were not sufficient for seedling growth. In contrast, plants with NH_4NO_3 fertilization or inoculated with the *Bradyrhizobium* strains accumulated significantly more biomass than N^- plants after 41 days of sowing. In general, NH_4NO_3 fed plants (N^+) accumulated more biomass than inoculated plants. Among the rhizobial treatments, plants inoculated with CzDJG3 accumulated the highest biomass, following by CzHD3, CzHD and CzR2. Growth responses of plants inoculated with CzGP5 and DtHD2 were relatively poor.

Despite the fact that total plant biomass varied among plants inoculated with different rhizobial strains, the ratios of above/below ground biomass ratio were relatively consistent among these plants at 34 and 41 DAS (**Figure 4--3B**).

Symbiotic efficiency

Significantly positive, linear relationships were found between nodule biomass and total plant biomass of *C. zanzibarica* inoculated with different *Bradyrhizobium* strains (**Figure 4-4**). The slope of the relationship represents symbiotic efficiency (Oono and Denison, 2010). Similar symbiotic efficiency (slopes of 11.6 to 13.1) was found

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among *C. zanzibarica* inoculated with CzHD3, CzR2, CzGP5, DtHD2 and CzDJG3, while significantly lower slope was found in plants inoculated with CzHD (8.3, $P < 0.001$).

Nitrogen accumulation and $\delta^{15}N$ values

Table 4-2 shows the nitrogen concentration (% dry weight) of shoot, root and nodule of *C. zanzibarica* under different treatments at 41 DAS. The nodulated plants and NH_4NO_3-fed plants (N^+) had similar shoot nitrogen concentration (about 4%), while shoot and root $\delta^{15}N$ values between these two treatments differed significantly, indicating their nitrogen sources were distinct. Because the nodulated plants had consistent nitrogen concentration on shoot (4-5%), root (1-2%) and nodule (6-7%) across the six symbionts, greater plant biomass indicates high total plant nitrogen accumulation. Since N^- plants only accumulated negligible nitrogen (0.16 mg per plant), the total plant nitrogen of nodulated plants (ranging from 5.5 to 12.7 mg per plant) were mainly through biological N_2 fixation. The whole plant $\delta^{15}N$ values of nodulated plants were close to zero, which further confirmed that these plants utilized atmospheric N_2 ($\delta^{15}N = 0‰$) as the major nitrogen source.

Discussion

Analyses of the diversity of soil rhizobia and their effects on legume hosts (nodulation and growth) could provide information for understanding the distribution patterns of legume plants in natural ecosystem (Burdon *et al*., 1999; Thrall *et al*., 2000; Parker, 2006). Although several studies investigated the diversity of nodule symbionts associated with non-crop legumes in Taiwan (Chen *et al*., 2000, 2003, 2005; Chen and

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Lee, 2001; Hung *et al*., 2005; Huang *et al*., 2016, 2018), there has been little research focused on the host performance inoculated with diverse rhizobial isolates. The present study confirmed different plant performance in response to inoculation by different *Bradyrhizobium* strains.

All the six rhizobial strains used in this study were able to induce root nodules on *C. zanzibarica*, while the significant variation in total plant biomass were found among the host plants inoculated with diverse strains (**Figure 4-3A**). Perhaps not surprisingly, DtHD2 (isolated from *Desmodium triflorum*) had poor effectiveness (defined here as the biomass/nitrogen accumulation of host plants) and induced significantly fewer nodules than other *C. zanzibarica* isolates (**Figure 4-1A**). This result might partially explain the previous finding in **Chapter 3**, in which I found that *C. zanzibarica* usually did not share nodule symbionts with its coexisting *Desmodium* plants. Interestingly, DtHD2 and CzHD3 are two closely related strains, both belonging to *B. yuanmingense*, despite this, DtHD2-inoculated plants only produced about half as much biomass as CzHD3-inoculated ones (**Figure 4-3A**). In contrast, the plants inoculated with phylogenetically distant strains CzHD (*B. manausense*), CzHD3 (*B. yuanmingense*) and CzDJG3 (unknown genospecies) performed equally well, implying that the species identity of rhizobia is not a reliable predictor of plant performance in *C. zanzibarica* when the symbiosis occurs.

Among the isolates collected from wild *C. zanzibarica* populations, the symbiotic variation was also observed. CzGP5 (collected from Gaoping riverbank) showed significantly poorer growth and nodulation responses on *C. zanzibarica* than strains from Xindian riverbank (CzHD and CzHD3) and from Dajia riverbank (CzDJG3). Similar to this result, Burdon *et al*. (1999) reported that some *Acacia* plants displayed

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significantly differences in growth responses when they were inoculated with "local" (collected from the same population as the legume species) and "foreign" (collected from the same *Acacia* species growing in different regions). However, because I only used the seeds of *C. zanzibarica* collected from Xindian riverbank. Therefore, I cannot tell whether CzGP5 always performed poorly among *C. zanzibarica* populations or this strain could show high symbiotic effectiveness to its local host population.

Besides the diverse genetic background of rhizobia, the morphological variation in their symbiotic stage (bacteroid) could also affect the plant performance. Oono and Denison (2010) demonstrated that swollen bacteroids are more symbiotically benefit to legume hosts than non-swollen ones. The following reasons might result in this phenomenon. First, elongated and branched bacteroids may display polar localization and enhance the nutrient exchange (Young, 2006). Second, swollen bacteroids commonly have more DNA content due to genomic endo-reduplication (Mergaert *et al*., 2006), which potentially increase the nitrogen fixation per cell. Third, symbiosome normally harbored single swollen bacteroid, whereas multiple non-swollen bacteroids per symbiosome were commonly observed, which may lead to some bacteroids did not contact host cytoplasm directly. Although CzR2 displayed significantly larger bacteroids than other isolates, this strain did not have higher symbiotic efficiency to *C. zanzibarica* (**Figure 4-4**). In the preliminary study, I compared CzR2 and CzHD3 bacteroids in this legume, and demonstrated that CzR2 neither had more DNA content nor showed single bacteroid per symbiosome (**Supplementary Figures S7 and S8**). These results might partially explain why CzR2 is not more beneficial to *C. zanzibarica* than other strains.

In conclusion, results of this study confirmed that *C. zanzibarica* can establish effective symbiosis with phylogenetically diverse *Bradyrhizobium* strains isolated from

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roots of greenhouse-grown and field-growing *C. zanzibarica*. However, total plant biomass in *C. zanzibarica* varied across the tested symbionts, the differences in total plant biomass were attributed to their differences in total nodule biomass. Therefore, rhizobial strains capable of inducing more nodules are likely more benefit to this legume.

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Table 4-1 Profile of *Bradyrhizobium* strains used in this study.

Strain	Species identity	Host	Sampling site
CzR2	*B. arachidis*	*Crotalaria zanzibarica*	NTU greenhouse
CzHD	*B. manausense*	*Crotalaria zanzibarica*	XD riverbank
CzHD3	*B. yuanmingense*	*Crotalaria zanzibarica*	XD riverbank
CzDJG3	*B.* genospecies	*Crotalaria zanzibarica*	DJ riverbank
CzGP5	*B. daqingense*	*Crotalaria zanzibarica*	GP riverbank
DtHD2	*B. yuanmingense*	*Desmodium trifolium*	XD riverbank

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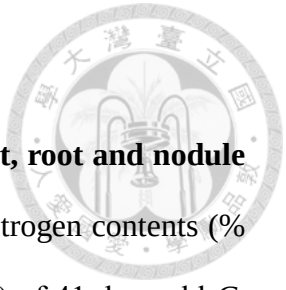

Table 4-2 Nitrogen concentration and stable isotope ratio of shoot, root and nodule of *C. zanzibarica* under different treatments. This table showed nitrogen contents (% dry weight, mean ± SD,) and stable nitrogen isotopes ratio (δ^{15}N ‰) of 41 days old *C. zanzibarica* plants inoculated with rhziobial strains CzR2, CzHD, CzHD3, CzDJG3, CzGP5 and DtHD2. Two control groups, N^+ (with NH_4NO_3) and N^- (without NH_4NO_3) were also analyzed. There were 5 to 6 replicates in each treatment.

	Shoot		Root		Nodule		Whole plant		
	N%	δ^{15}N ‰	N%	δ^{15}N ‰	N%	δ^{15}N ‰	Total N (mg)	δ^{15}N ‰	biomass:N
Inoculant									
CzR2 (*n* = 6)	4.6±0.2	-1.3±0.1^{bc}	1.7±0.0^{b}	-1.1±0.1^{a}	6.4±0.6^{b}	4.1±0.5^{c}	8.8±2.3^{ab}	-0.7±0.1^{bc}	22.4±1.0^{b}
CzHD (*n* = 6)	4.7±0.2	-1.9±0.2^{d}	1.8±0.1^{b}	-1.2±0.2^{a}	7.4±0.3^{a}	6.8±0.5^{b}	10.7±2.3^{a}	-0.4±0.2^{ab}	21.0±0.8^{b}
CzHD3 (*n* = 6)	4.7±0.2	-1.4±0.1^{c}	1.8±0.1^{b}	-1.2±0.1^{a}	7.4±0.3^{a}	7.9±0.5^{a}	11.6±4.2^{a}	-0.3±0.1^{a}	21.4±1.0^{b}
CzDJG3 (*n* = 6)	4.5±0.4	-1.1±0.2^{b}	1.8±0.1^{b}	-0.9±0.4^{a}	7.3±0.2^{a}	4.9±0.6^{c}	12.7±3.8^{a}	-0.3±0.2^{a}	22.3±1.5^{b}
CzGP5 (*n* = 5)	4.3±0.4	-1.1±0.1^{b}	1.7±0.1^{b}	-0.8±0.2^{a}	6.3±0.2^{b}	4.3±0.6^{c}	5.9±2.0^{b}	-0.5±0.2^{ab}	23.6±1.1^{ab}
DtHD2 (*n* = 6)	4.3±0.3	-1.1±0.1^{b}	1.7±0.1^{b}	-0.8±0.2^{a}	7.6±0.3^{a}	4.6±0.4^{c}	5.5±1.3^{b}	-0.3±0.2^{a}	23.3±1.5^{ab}
N+ (*n* = 5)	4.2±1.0	-0.7±0.4^{a}	2.4±0.3^{a}	-2.7±0.4^{b}	N.A.	N.A.	13.1±2.4^{a}	-0.9±0.3^{c}	26.0±5.1^{a}
N- (*n* = 5)							0.16±0.05	-1.0±0.5	121.5±8.5

Mean within each column followed by different letters differed significantly (ANOVA, $P < 0.05$).

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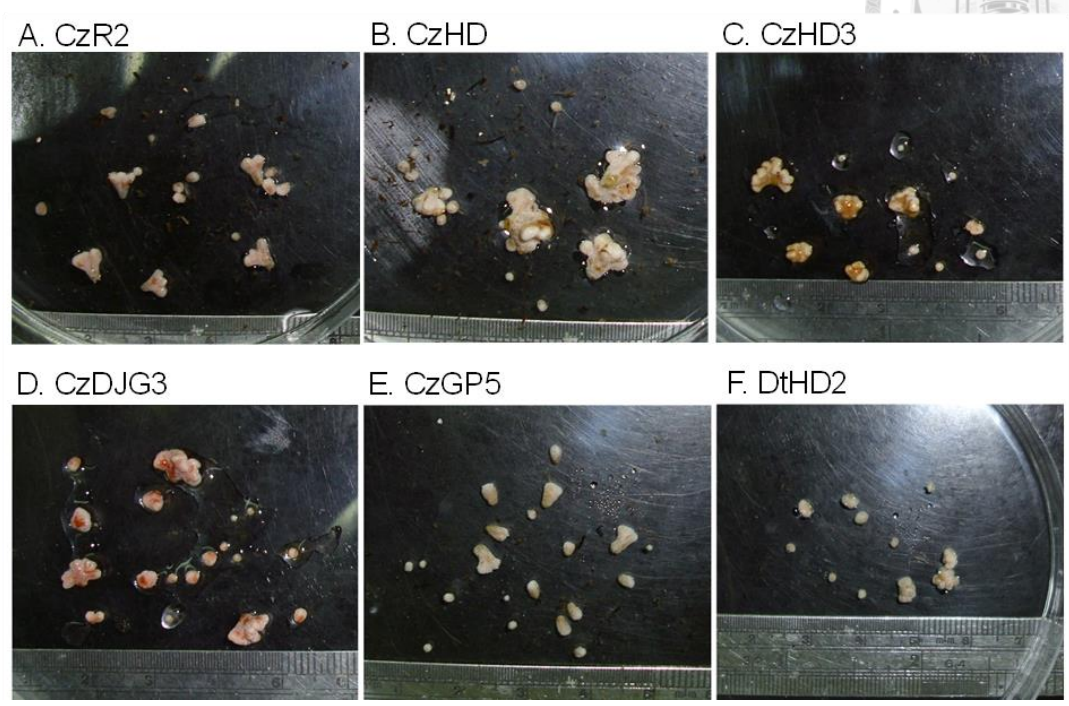

Figure 4-1 Nodule morphology of *C. zanzibarica* inoculated with different rhizobial strains at 41 DAS.

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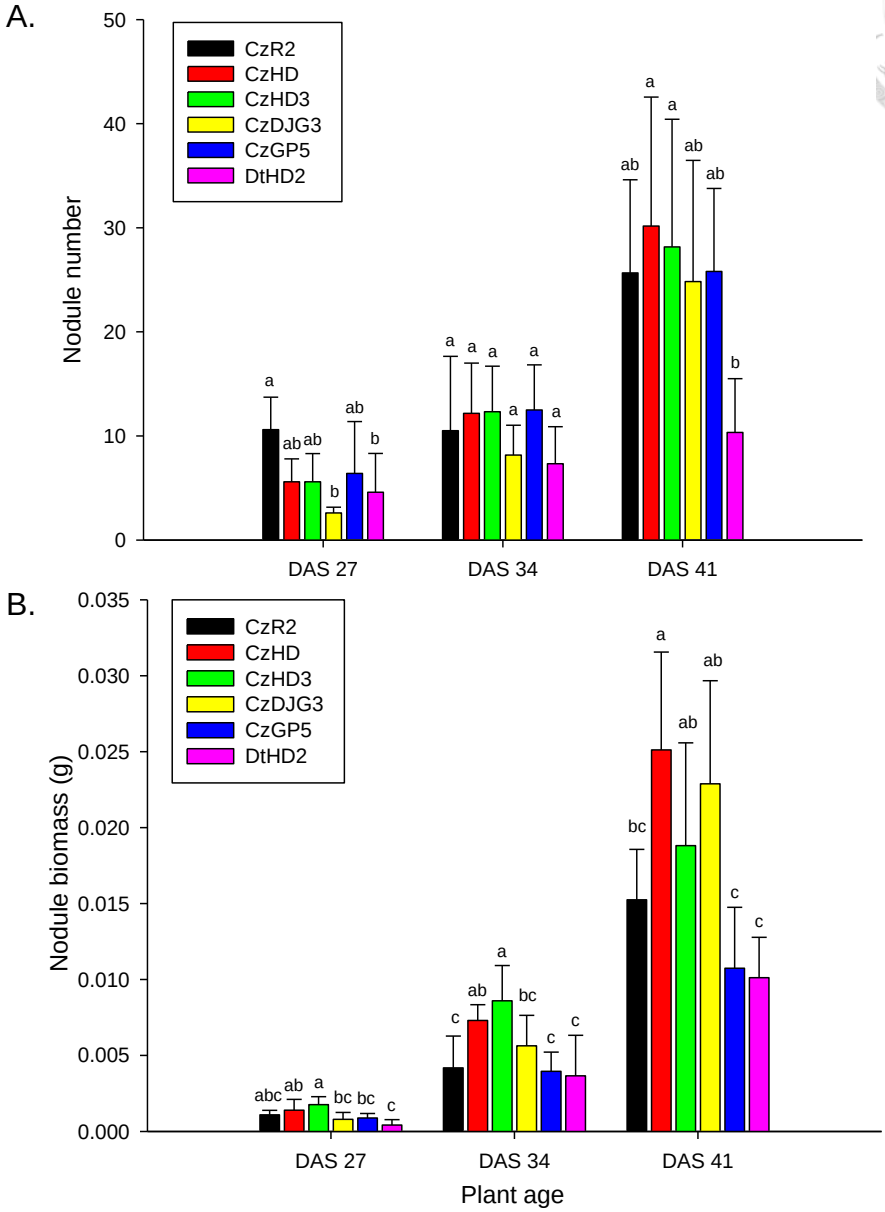

Figure 4-2 Nodulation response for *C. zanzibarica* inoculated with six rhizobial strains. Nodule number (A) total nodule biomass (B) of plants nodulated by different strains during 27, 34, and 41 DAS. Bars with different letters mean significant difference among the treatment at each plant ages.

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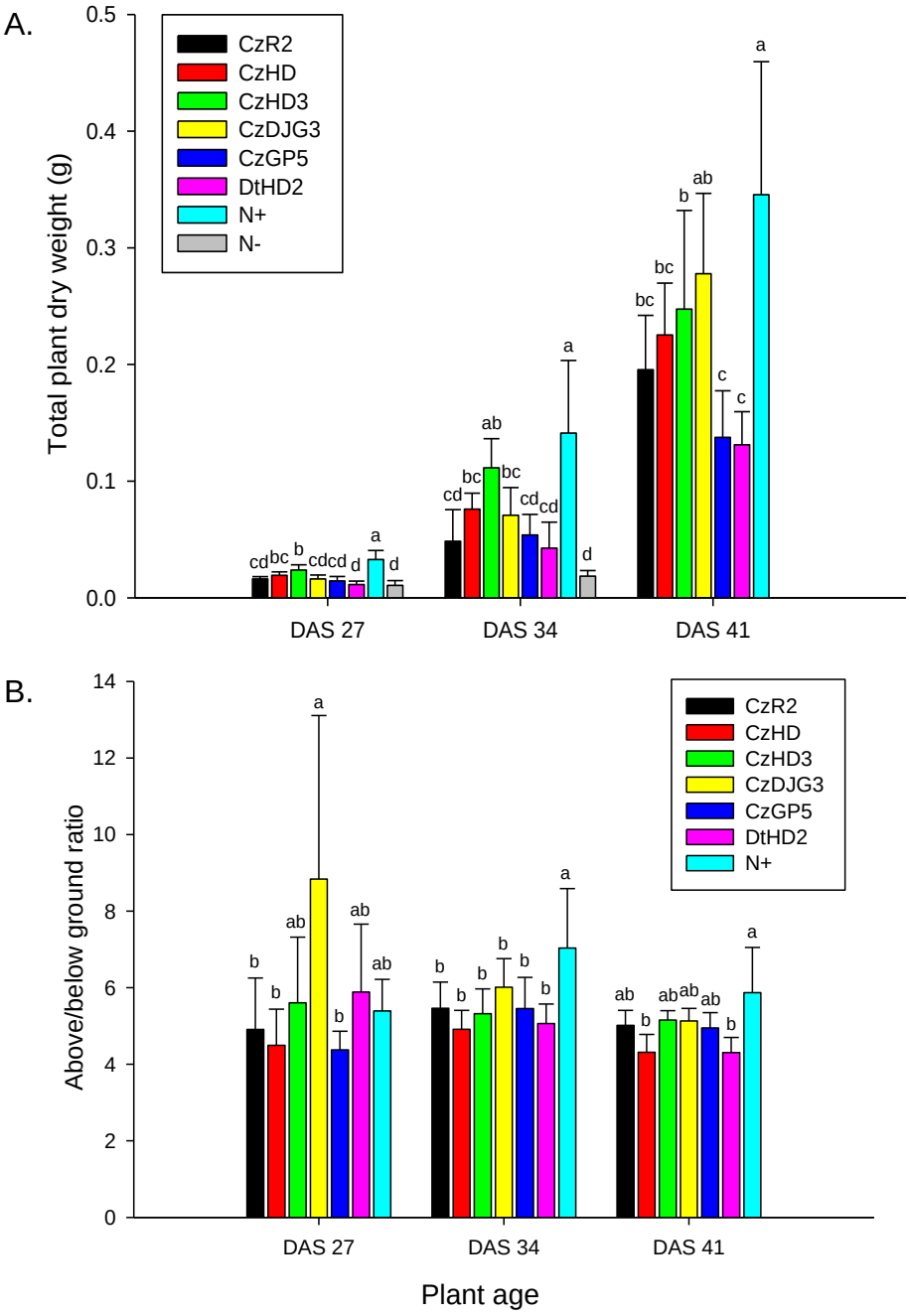

Figure 4-3 Variation in growth of *C. zanzibarica* under different treatments. These figures showing total plant biomass (A) and above/below-ground ratio (B) of plants inoculated with the six strains and non-inoculated plants (N^+/N^-, with/without nitrogen fertilizer). Plant age is indicated as DAS (day after sowing). Different letters mean significant difference among the treatment at each plant ages.

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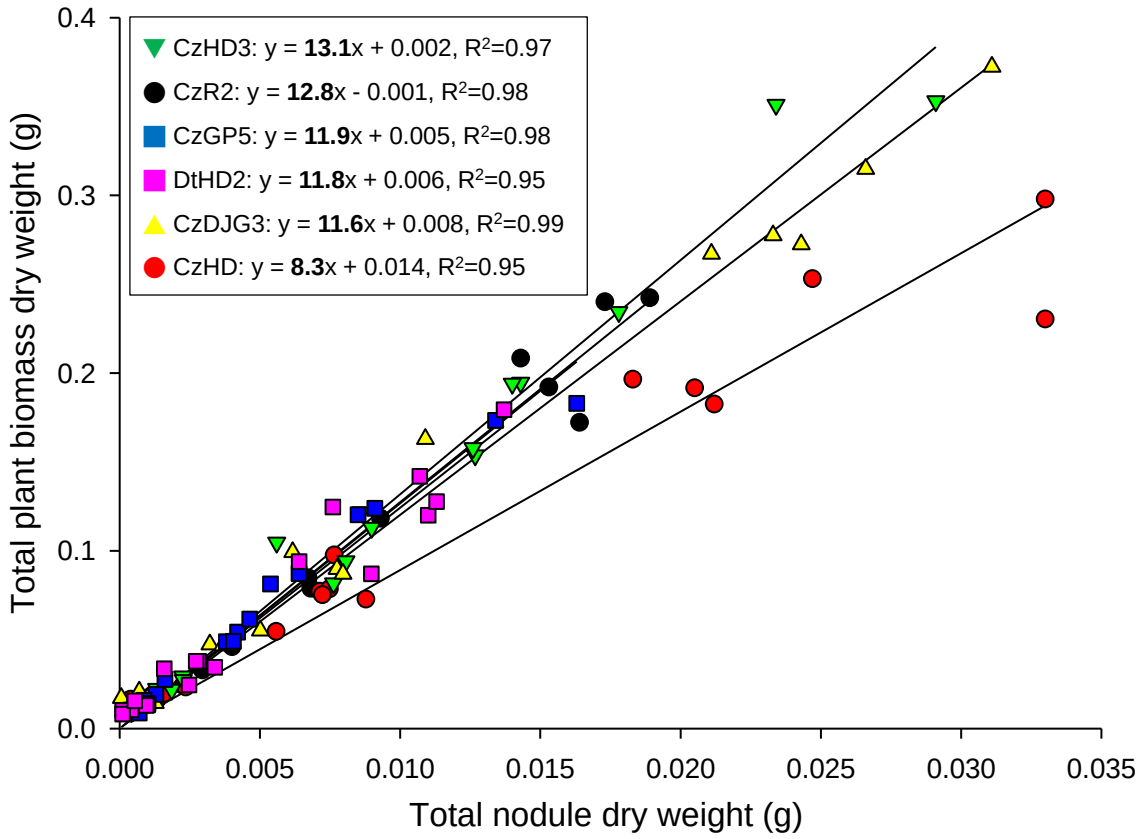

Figure 4-4 Relationship between total plant dry weight and total nodule dry weight in *C. zanzibarica* inoculated with six rhizobial strains. Different symbols represent plants inoculated with different strains of rhizobia.

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Conclusions

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In this dissertation, I characterized the symbiotic traits and investigated the diversity of nodule symbionts of *Crotalaria zanzibarica*, a naturalized legume with the highest invasiveness in Taiwan.

Followings are important findings of the study.

1. *C. zanzibarica* normally produces multi-lobed, indeterminate nodules, harboring swollen bacteroids. The nodulated *C. zanzibarica* plants, greenhouse-grown or field-growing, accumulated high nitrogen contents in both leaves (4-6%, dry weight) and nodules (5-7%, dry weight); however, negative $\delta^{15}N$ values were observed in leaves (ca. -1.2 ‰), whereas the nodules showed ^{15}N enrichment ($\delta^{15}N$ = 7-9 ‰).

2. A peculiar strain, *Bradyrhizobium arachidis* CzR2 which displayed pleomorphism, polar differentiation and rosette-like aggregation in YEM medium, was isolated from greenhouse-grown *C. zanzibarica*. On the other hand, diverse *Bradyrhizobium* strains belonging to multiple housekeeping gene clades and *nodA* gene groups were isolated from field-growing *C. zanzibarica*. These field isolates included possibly indigenous rhizobia in Taiwan, rhizobia specifically originated from China or America, and putative cosmopolitan rhizobia.

3. The growth of *C. zanzibarica* was significantly promoted by inoculation of rhizobia isolated from field-growing *C. zanzibarica*, indicating the beneficial effect of the symbiosis. In conclusion, being able to form symbiosis with diverse *Bradyrhizobium* symbionts from multiple origins might be one of the key factors contributing to the successful invasion of *C. zanzibarica* in Taiwan.

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Future works

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Given the limited number of rhizobial isolates and legume hosts I investigated in this study, the distribution patterns of *Bradyrhizobium* throughout Taiwan Island and the importance of these *Bradyrhizobium* symbionts in natural/agricultural systems remain unclear. Therefore, I propose following three major directions for future works.

1. To establish database of indigenous rhizobia in Taiwan

All *Bradyrhizobium* isolates in this study were collected from a greenhouse and the riverbanks, where have been likely influenced by human activities. To excavate the native *Bradyrhizobium* populations in Taiwan, legume plants from remote areas, such as mountainous regions and national parks, should be sampled. With this sampling protocol, the unique Taiwanese strains may be identified.

2. To monitor the invasion of exotic rhizobia in Taiwan

A *Bradyrhizobium* lineage carrying the symbiotic genes of American origin was isolated from the three distant sampling riverbanks (**Chapter 3**) which indicates its widespread in Taiwan. Since this lineage was also found in the nodules of native legumes, this exotic rhizobium has potential affecting native legume communities and local *Bradyrhizobium* populations in Taiwan. Therefore, a long-term monitor of such exotic rhizobia is necessary for maintaining biodiversity in Taiwan.

3. To evaluate the effect of the isolated *Bradyrhizobium* symbionts on peanut

In **Chapter 3**, I found that the symbiotic genes of most *Bradyrhizobium* isolates in this study were highly similar to those of peanut symbionts. Furthermore, preliminary

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tests revealed that certain isolates are drought or high temperature tolerant (data not shown). It is therefore worth further evaluation of the growth of peanut plants, inoculated with these rhizobia, in drought and/or high temperature conditions.

doi:10.6342/NTU201800782

Literature cited

Adams MA, Turnbull TL, Sprent JI, Buchmann N (2016) Legumes are different: Leaf nitrogen, photosynthesis, and water use efficiency. Proc. Natl. Acad. Sci. 113:4098-4103

Appunu C, N'Zoue A, Moulin L, Depret G, Laguerre G (2009) *Vigna mungo, V. radiata and V. unguiculata* plants sampled in different agronomical–ecological–climatic regions of India are nodulated by *Bradyrhizobium yuanmingense*. Syst. Appl. Microbiol.32:460-470

Andam CP, Parker MA (2008) Origins of *Bradyrhizobium* nodule symbionts from two legume trees in the Philippines. J. Biogeogr. 35:1030-1039

Aserse AA, Räsänen LA, Aseffa F, Hailemariam A, Lindström K (2012) Phylogenetically diverse groups of *Bradyrhizobium* isolated from nodules of *Crotalaria* spp., *Indigofera* spp., *Erythrina brucei* and *Glycine max* growing in Ethiopia. Mol. Phylogenet. Evol. 65:595-609

Azevedo H, Lopes FM, Silla PR, Hungria M (2015) A database for the taxonomic and phylogenetic identification of the genus *Bradyrhizobium* using multilocus sequence analysis. BMC Genomics 16(Suppl 5):S10

Barrett CF, Parker MA (2005) Prevalence of *Burkholderia* sp. nodule symbionts on four mimosoid legumes from Barro Colorado Island, Panama. Syst. Appl. Microbiol. 28:57-65

Bergersen FJ, Turner GL, Amarger N, Mariotti F, Mariotti A (1986) Strain of *Rhizobium lupini* determines natural abundance of ^{15}N in root nodules of *Lupinus* spp. Soil. Biol. Biochem. 18:97-101

Bergersen FJ, Peoples MB, Turner GL (1988) Isotopic discrimination during the accumulation of nitrogen by soybeans. Aust. J. Plant Physiol. 15:407-420

Binggeli P (1996) A taxonomic, biogeographical and ecological overview of invasive woody plants. J. Veg. Sci. 7:121-124

Brueck H (2008) Effects of nitrogen supply on water-use effeciency of higher plants. J. Plant Nutr. Soil Sci. 171:210-219

Burdon JJ, Gibson AH, Searle SD, Woods MJ, Brockwell J (1999) Variation in the effectiveness of symbiotic associations between native rhizobia and temperate Australian *Acacia*: within-species interactions. J. Appl. Ecol. 36:398-408

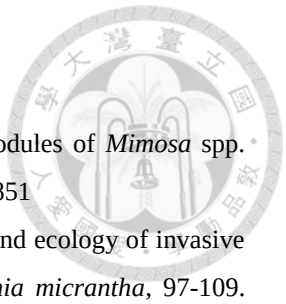
Chen WM, Lee TM, Lan CC, Cheng CP (2000) Characterization of halotolerant rhizobia isolated from root nodules of *Canavalia rosea* from seaside areas. FEMS Microbiol. Ecol. 34:9-16

Chen WM, Lee TM (2001) Genetic and phenotypic diversity of rhizobial isolates from sugarcane-*Sesbania cannabina*-rotation fields. Biol. Fertil. Soils 34:14-20

Chen WM, Moulin L, Bontemps C, Vandamme P, Bena G, Boivin-Masson C (2003) Legume symbiotic nitrogen fixation by β-Proteobacteria is widespread in nature. J. Bacteriol. 185:7266-7272

Chen WM, James EK, Chou JH, Sheu SY, Yang SZ, Sprent JI (2005) Beta-rhizobia from *Mimosa* pigra, a newly discovered invasive plant in Taiwan. New Phytol. 168:661-675

doi:10.6342/NTU201800782



Chen WM et al. (2006) *Burkholderia mimosarum* sp. nov., isolated from root nodules of *Mimosa* spp. from Taiwan and South America. Int. J. Syst. Evol. Microbiol. 56:1847-1851

Chiang MY, Hsu LM, Yuan CI, Chen FY, Chiang YJ (2003) The harmful effect and ecology of invasive plants in Taiwan The Harmful Effect and Field Management of *Mikania micrantha*, 97-109. Weed Science Society of the Republic of China and Hualien District Agricultural Research and Extension Station, Council of Agricultural Executive Yuan, Hualien

Cole MA, Elkan GH (1973) Transmissible Resistance to Penicillin G, Neomycin, and Chloramphenicol in *Rhizobium japonicum*. Antimicrob. Agents Chemother. 4:248-253

Corby HDL (1988) Types of rhizobial nodules and their distribution among the Leguminosae. Kirkia 13:53-124

Crisóstomo JA, Rodríguez-Echeverría S, Freitas H (2013) Co-introduction of exotic rhizobia to the rhizosphere of the invasive legume *Acacia saligna*, an intercontinental study. Appl. Soil Ecol. 64:118-126

Cronk QCB, Fuller JL (1995) Plant Invaders: The Threat to Natural Ecosystems. Chapman & Hall, London.

Dart PJ, Mercer FV (1966) Fine structure of bacteroids in root nodules of *Vigna sinensis*, *Acacia longifolia*, *Viminaria juncea*, and *Lupinus angustifolius*. J. Bacteriol. 91:1314-1319

Doyle JJ, Luckow MA (2003) The rest of the iceberg. Legume diversity and evolution in a phylogenetic context. Plant Physiol. 131:900-910

Doyle JJ (2011) Phylogenetic perspectives on the origins of nodulation. Mol. Plant-Microbe Interact. 24:1289-1295

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput Nucleic Acids Res. 32:1792-1797

Elliott GN et al. (2007) Nodulation of *Cyclopia* spp. (Leguminosae, Papilionoideae) by *Burkholderia tuberum.* Ann. Bot. 100:1403–1411

Grönemeyer JL, Kulkarni A, Berkelmann D, Hurek T, Reinhold-Hurek B (2014) Rhizobia indigenous to the Okavango region in sub-Saharan Africa: diversity, adaptations, and host specificity. Appl. Environ. Microbiol. 80:7244-7257

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0.". Systematic Biology 59:307-321

Gyaneshwar P et al. (2011) Legume-nodulating betaproteobacteria: Diversity,host range, and future prospects. Mol. Plant-Microbe Interact. 24:1276-1288

Haag AF et al. (2013) Molecular insights into bacteroid development during *Rhizobium*–legume symbiosis. FEMS Microbiol Rev 37:364-383

Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for

doi:10.6342/NTU201800782

Windows 95/98/NT. Nucleic Acids Symposium Series 41:95-98

Hanelt P (2001) Leguminosae. In: Hanelt P and Institute of plant genetics and crop plant research (ed) Mansfeld's encyclopedia of agricultural and horticultural crops (excepts ornamentals). Springer-Verlag, Berlin

Holm LG, Pancho JV, Herberger JP, Plucknett DL (1991) A geographical atlas of world weeds. Krieger Publisher Company, Malabar, Florida, USA

Horn K, Parker IM, Malek W, Rodríguez-Echeverría S, Parker MA (2014) Disparate origins of *Bradyrhizobium* symbionts for invasive populations of Cytisus scoparius (Leguminosae) in North America. FEMS Microbiol. Ecol.:1-10

Huang TC (1993) Flora of Taiwan, vol. 3. 2nd edn. Editorial Committee of the Flora of Taiwan, Department of Botany, National Taiwan University, Taipei, Taiwan.

Huang CT, Liu CT, Chen SJ, Kao WY (2016) Phylogenetic identification, phenotypic variation and symbiotic characteristics of a peculiar rhizobium, strain CzR2, isolated from *Crotalaria zanzibarica* in Taiwan. Microbes Environ.

Huang CT, Liu CT, Kao WY (2018) Rhizobia symbiosis of seven leguminous species growing along Xindian riverbank of Northern Taiwan. Taiwania 68:7-15

Hung MH, Bhagwath AA, Shen FT, Devasya RP, Young CC (2005) Indigenous rhizobia associated with native shrubby legumes in Taiwan. Pedobiologia 49:577-584

Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. 23:254-267

Kalita M, Stpękowski T, Łotocka B, Małek W (2006) Phylogeny of nodulation genes and symbiotic properties of *Genista tinctoria* bradyrhizobia. Arch Microbiol 186:87-97

Kohl DH, Reynolds PHS, Shearer G (1989) Distribution of ^{15}N within pea, lupin, and soybean nodules. Plant Physiol. 90:420-426

Koppell JH, Parker MA (2012) Phylogenetic clustering of *Bradyrhizobium* symbionts on legumes indigenous to North America. Microbiology 158:2050-2059

Lavin M, Herendeen PS, Wojciechowski MF (2005) Evolutionary rates analysis of Leguminosae implicates a rapid diversification of lineages during the tertiary. Syst. Biol. 54:575-594

Lewis G, Schrire B, Mackinder B, Lock M (2005) Legumes of the world. London: Royal Botanic Gardens Kew.

Librado P, Rozas J (2009) DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451-1452

Liu XY, Wang ET, Li Y, Chen WX (2007) Diverse bacteria isolated from root nodules of *Trifolium*, *Crotalaria* and *Mimosa* grown in the subtropical regions of China. Arch Microbiol 188:1-14

Liu CT et al. (2011) Involvement of the Azorhizobial chromosome partition gene (parA) in the onset of bacteroid differentiation during *Sesbania rostrata* stem nodule development. Appl. Environ.

doi:10.6342/NTU201800782

Microbiol. 77:4371-4382

Justice SS, Hunstad DA, Cegelski L, Hultgren SJ (2008) Morphological plasticity as a bacterial survival strategy. Nature 6:162-168

Klock MM, Barrett LG, Thrall PH, Harms KE (2015) Host promiscuity in symbiont associations can influence exotic legume establishment and colonization of novel ranges. Divers. Distrib. 21: 1193-1203.

Marchesi JR et al. (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. 64:795-799

Mack RN (1997) Plant invasions: Early and continuing expressions of global change. Springer-Verlag, Berlin.

Melkonian R et al. (2014) The geographical patterns of symbiont diversity in the invasive legume *Mimosa pudica* can be explained by the competitiveness of its symbionts and by the host genotype. Environ. Microbiol. 16:2099-2111

Menna P, Barcellos FG, Hungria M (2009) Phylogeny and taxonomy of a diverse collection of *Bradyrhizobium* strains based on multilocus sequence analysis of the 16S rRNA gene, ITS region and *glnII*, *recA*, *atpD* and *dnaK* genes. Int. J. Syst. Evol. Microbiol. 59:2934-2950

Menna P, Hungria M (2011) Phylogeny of nodulation and nitrogen-fixation genes in *Bradyrhizobium*: supporting evidence for the theory of monophyletic origin, and spread and maintenance by both horizontal and vertical transfer. Int. J. Syst. Evol. Microbiol. 61:3052-3067

Mergaert P et al. (2006) Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium*–legume symbiosis. Proc. Natl. Acad. Sci. 103:5230-523

Moulin L, Bena G, Boivin-Masson C, Stępkowski T (2004) Phylogenetic analyses of symbiotic nodulation genes support vertical and lateral gene co-transfer within the *Bradyrhizobium* genus. Mol. Phylogenet. Evol. 30:720-732

Muñoz V, Ibanez F, Tonelli ML, Valetti L, Anzuay MS, Fabra A (2011) Phenotypic and phylogenetic characterization of native peanut *Bradyrhizobium* isolates obtained from Córdoba, Argentina. Syst. Appl. Microbiol. 34:446-452

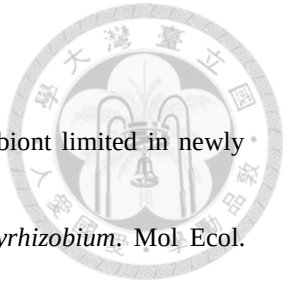
Noisangiam R et al. (2012) Genetic diversity, symbiotic evolution, and proposed infection process of *Bradyrhizobium* strains isolated from root nodules of *Aeschynomene americana* L. in Thailand. Appl. Environ. Microbiol. 78:6236-6250

Oono R, Denison RF, Kiers ET (2009) Controlling the reproductive fate of rhizobia: how universal are legume sanctions? New Phytol. 183:967-979

Oono R, Denison RF (2010) Comparing symbiotic efficiency between swollen versus nonswollen rhizobial bacteroids. Plant Physiol. 154:1541-1548

Oono R, Schmitt I, Sprent JI, Denison RF (2010) Multiple evolutionary origins of legume traits leading to extreme rhizobial differentiation. New Phytol. 187:508-520

doi:10.6342/NTU201800782



Parker MA, Malek W, Parker IM (2006) Growth of an invasive legume is symbiont limited in newly occupied habitats. Divers. Distrib. 12:563-571

Parker MA (2012) Legumes select symbiosis island sequence variants in *Bradyrhizobium*. Mol Ecol. 21:1769-1778

Peterson BJ, Fry B (1987) Stable isotopes in ecosystem studies. Annu. Rev. Ecol. Syst. 18:293-320

Pimentel D, Zuniga R, Morrison D (2005) Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecol. Econ. 52:273-288

Polhill RM (1982) *Crotalaria* in Africa and Madagascar., Balkema, A. A., Rotterdam, The Netherlands.

Pyšek P (1998) Is there a taxonomic pattern to plant invasions? Oikos 82:282-294

Reding HK, Lepo JE (1989) Physiological characterization of dicarboxylate-induced pleomorphic forms of *Bradyrhizobium japonicum*. Appl. Environ. Microbiol. 55:666-671

Richardson DM, Pyšek P, Rejmánek M, Barbour MG, Panetta FD, West CJ (2000a) Naturalization and invasion of alien plants: concepts and definitions. Divers. Distrib. 6:93-107

Richardson DM, Allsopp N, D'Antonio CM, Milton SJ, Rejmanek M (2000b) Plant invasions - the role of mutualisms. Biol. Rev. 75:65-93

Rivas R, Martens M, Lajudie Pd, Willem A (2009) Multilocus sequence analysis of the genus *Bradyrhizobium*. Syst. Appl. Microbiol. 32:101-110

Robinson D (2001) $\delta^{15}N$ as an integrator of the nitrogen cycle. Trends Ecol. Evol. 16:153-162

Rodríguez-Echeverría S (2010) Rhizobial hitchhikers from Down Under: invasional meltdown in a plant–bacteria mutualism? J. Biogeogr. 37:1611–1622

Ronquist F et al. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61:539-542

Sajnaga E, Małek W, Łotocka B, Stpkowski T, Legocki A (2001) The root-nodule symbiosis between *Sarothamnus scoparius* L. and its microsymbionts. Antonie van Leeuwenhoek 79:385–391

Samba R, De Lajudie P, Gillis M, Neyra M, Barreto M, Dreyfus B (1999) Diversity of rhizobia nodulating *Crotalaria* spp. from Senegal. Symbiosis 27:259-268

Shearer G, Kohl D, Harper JE (1980) Distribution of ^{15}N among plant parts of nodulating and non-nodulating isolines of soybeans. Plant Physiol. 66:57-60

Shearer G et al. (1982) ^{15}N abundance of nodules as an indicator of N metabolism in N_2-fixing plants. Plant Physiol. 70:465-465

Skinner FA, Roughley RJ, Chandler MR (1977) Effect of yeast extract concentration on viability and cell distortion in *Rhizobium* spp. J. Appl. Bacteriol. 43:287-297

Sibuet JC, Hsu SK (1997) Geodynamics of the Taiwan arc-arc collision. Tectonophysics 274:221-251

Silva FV et al. (2014) *Bradyrhizobium manausense* sp. nov., isolated from effective nodules of *Vigna unguiculata* grown in Brazilian Amazonian rainforest soils. Int. J. Syst. Evol. Microbiol. 64:2358-2363

doi:10.6342/NTU201800782

Sprent JI (2001) Nodulation in legumes. London: Royal Botanic Gardens Kew.

Sprent JI (2007) Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodulation. New Phytol. 174:11-25

Sprent JI (2008) 60Ma of legume nodulation. What's new? What's changing? J. Exp. Bot. 59:1081-1084

Sprent JI (2009) Legume nodulation: a global perspective. Wiley-Blackwell, Oxford, United Kingdom

Steenkamp ET, Stępkowski T, Przymusiak A, Botha WJ, Law IJ (2008) Cowpea and peanut in southern Africa are nodulated by diverse *Bradyrhizobium* strains harboring nodulation genes that belong to the large pantropical clade common in Africa. Mol. Phylogenet. Evol. 48:1131-1144

Steinberger RE, Allen AR, Hansma HG, Holden PA (2002) Elongation correlates with nutrient deprivation in *Pseudomonas aeruginosa* unsaturated biofilms Microb. Ecol. 43:416-423

Stępkowski T, Moulin L, Krzyżańska A, McInnes A, Law IJ, Howieson J (2005) European origin of *Bradyrhizobium* populations infecting *Lupins* and *Serradella* in soils of Western Australia and South Africa. Appl. Environ. Microbiol. 71:7041-7052

Stępkowski T et al. (2007) Diversification of lupine *Bradyrhizobium* strains: Evidence from nodulation gene trees. Appl. Environ. Microbiol. 73:3254-3264

Stępkowski T, Watkin E, McInnes A, Gurda D, Gracz J, Steenkamp ET (2012) Distinct *Bradyrhizbium* communities nodulate legumes native to temperate and tropical monsoon Australia. Mol. Phylogenet. Evol. 63:265-277

Sy A et al. (2001) Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J. Bacteriol. 183:214-220

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30:2725-2729

Thies JE, Singleton PW, Bohlool BB (1991) Modeling symbiotic performance of introduced rhizobia in the field by use of indices of indigenous population size and nitrogen status of the soil. Appl. Environ. Microbiol. 57:29-37

Thrall PH, Burdon JJ, Woods MJ (2000) Variation in the effectiveness of symbiotic associations between native rhizobia and temperate Australian legumes: interactions within and between genera. J. Appl. Ecol. 37:52-65

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680

Unkovich M (2013) Isotope discrimination provides new insight into biological nitrogen fixation. New Phytol. 198:643-646

Urtz BE, Elkan GH (1996) Genetic diversity among *Bradyrhizobium* isolates that effectively nodulate peanut (*Arachis hypogaea*). Can. J. Microbiol. 42:1121-1130

Van de Velde W et al. (2010) Plant peptides govern terminal differentiation of bacteria in symbiosis.

doi:10.6342/NTU201800782

Science 327:1122-1126

Vincent JM (1970) A manual for the practical study of the root-nodule bacteria., International biological programme handbook 15. Blackwell Scientific Publications, Oxford, United Kingdom.

Vinuesa P et al. (2008) Multilocus sequence analysis for assessment of the biogeography and evolutionary genetics of four *Bradyrhizobium* species that nodulate soybeans on the asiatic continent. Appl. Environ. Microbiol. 74:6987-6996

Virginia RA, Delwiche CC (1982) Natural ^{15}N abundance of presumed N_2-fixing and non-N_2-fixing plants from selected ecosystems. Oecologia 54:317-325

Vitousek PM (1990) Biological invasions and ecosystem processes: towards an integration of population biology and ecosystem studies Oikos 57:7-13

Vitousek PM, Howarth RW (1991) Nitrogen limitation on land and in the sea: how can it occur? Biogeochemistry 13:87-115

Vitousek PM, Mooney HA, Lubchenco J, Melillo JM (1997) Human domination of Earth's ecosystems. Science 277:494-499

Vitousek PM et al. (2002) Towards an ecological understanding of biological nitrogen fixation. Biogeochemistry 57:1-45

Wainwright M (1997) Extreme pleomorphism and the bacterial life cycle: a forgotten controversy. Perspectives in Bio. Med. 40:407-414

Wanek W, Arndt S (2002) Difference in delta(15)N signatures between nodulated roots and shoots of soybean is indicative of the contribution of symbiotic N(2) fixation to plant N. J. Exp. Bot. 53:1109-1118

Wang JY et al. (2013) *Bradyrhizobium daqingense* sp. nov., isolated from soybean nodules. Int. J. Syst. Evol. Microbiol. 63:616-624

Wang R et al. (2013) *Bradyrhizobium arachidis* sp. nov., isolated from effective nodules of *Arachis hypogaea* grown in China. Syst. Appl. Microbiol. 36:101-105

Williamson M (1997) Biological Invasions. Chapman& Hall, London.

Wu SH, Chaw SM, Rejmánek M (2003) Naturalized Fabaceae (Leguminosae) species in Taiwan: the first approximation. Bot. Bull. Acad. Sin. 44:59-66

Wu SH, Rejmánek M, Grotkopp E, Ditomaso JM (2005) Herbarium records, actual distribution, and critical attributes of invasive plants: genus *Crotalaria* in Taiwan. Taxon 54:133-138

Wu SH, Yang TYA, Teng YC, Chang CY, Yang KC, Hsieh CF (2010) Insights of the latest naturalized flora of Taiwan: change in the past eight years. Taiwania 55:139-159

Xu LM, Ge C, Cui Z, Li J, Fan H (1995) *Bradyhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. Int. J. Syst. Bacteriol. 45:706-711

Yao ZY, Kan FL, Wang ET, Wei GH, Chen WX (2002) Characterization of rhizobia that nodulate legume species of the genus *Lespedeza* and description of *Bradyrhizobium yuanmingense* sp.

doi:10.6342/NTU201800782

nov. Int. J. Syst. Evol. Microbiol. 52:2219-2230

Yelenik SG, Stock WD, Richardson DM (2004) Ecosystem level impacts of invasive *Acacia saligna* in the South African fynbos. Restor. Ecol. 12:44-51

Yoneyama T, Uchiyama T, Yazaki J (1991) Ontogenetic change of nitrogen accumulation and natural ^{15}N abundance in pea and faba bean with special reference to estimate of N_2 fixation and ^{15}N enrichment of nodules. J. Mass Spectrom Soc. Jpn. 39:267-276

Young JPW, Haukka KE (1996) Diversity and phylogeny of rhizobia. New Phytol. 133:87-94

Young KD (2006) The selective value of bacterial shape. Microbiol. Mol. Biol. Rev. 70:660-703

Zhang YF et al. (2008) *Bradyrhizobium elkanii*, *Bradyrhizobium yuanmingense* and *Bradyrhizobium japonicum* are the main rhizobia associated with *Vigna unguiculata* and *Vigna radiata* in the subtropical region of China. FEMS Microbiol. Lett. 285:146-154

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Supplementary materials

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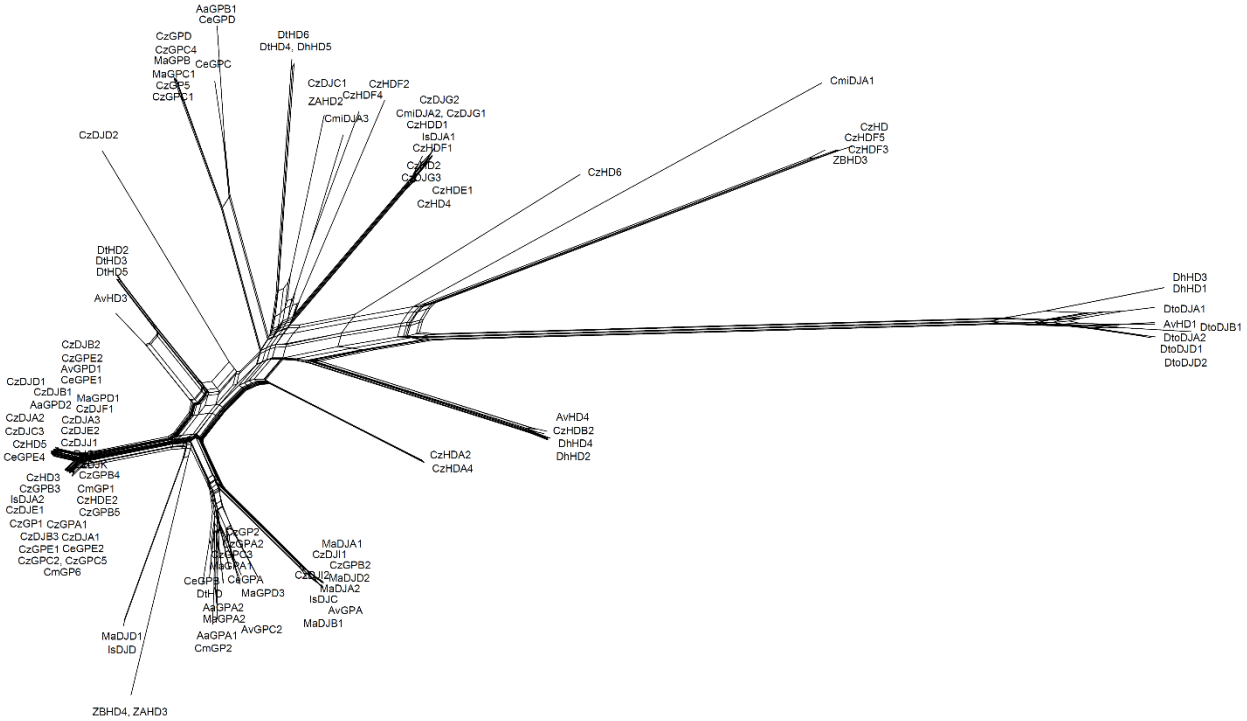

Supplementary Figure S1 NeighborNet graph for concatenated *dnaK, glnII, recA* and *rpoB* gene sequences for the 113 *Bradyrhizobium* isolates.

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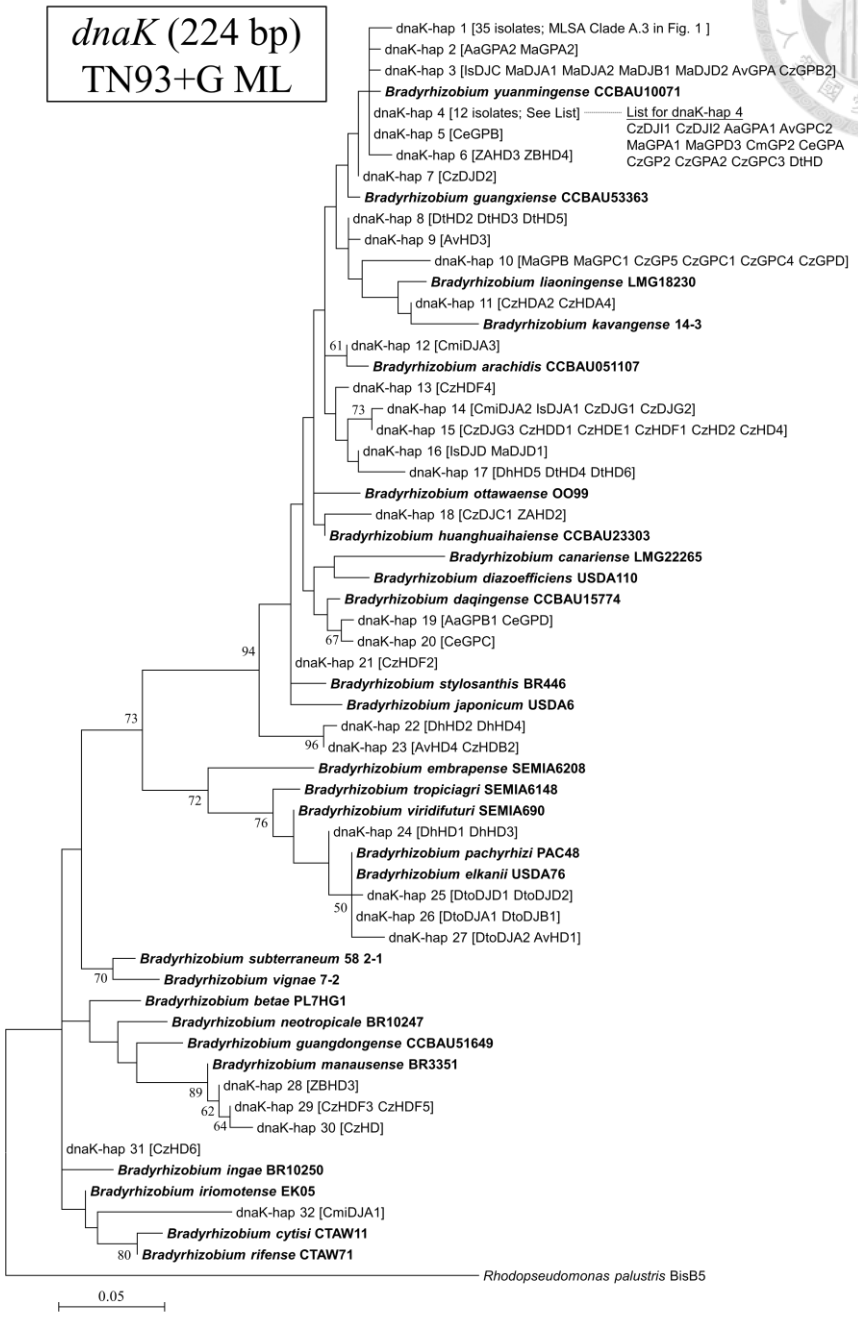

Supplementary Figure S2 *dnaK* phylogeny of the 113 *Bradyrhizobium* isolates and reference strains.

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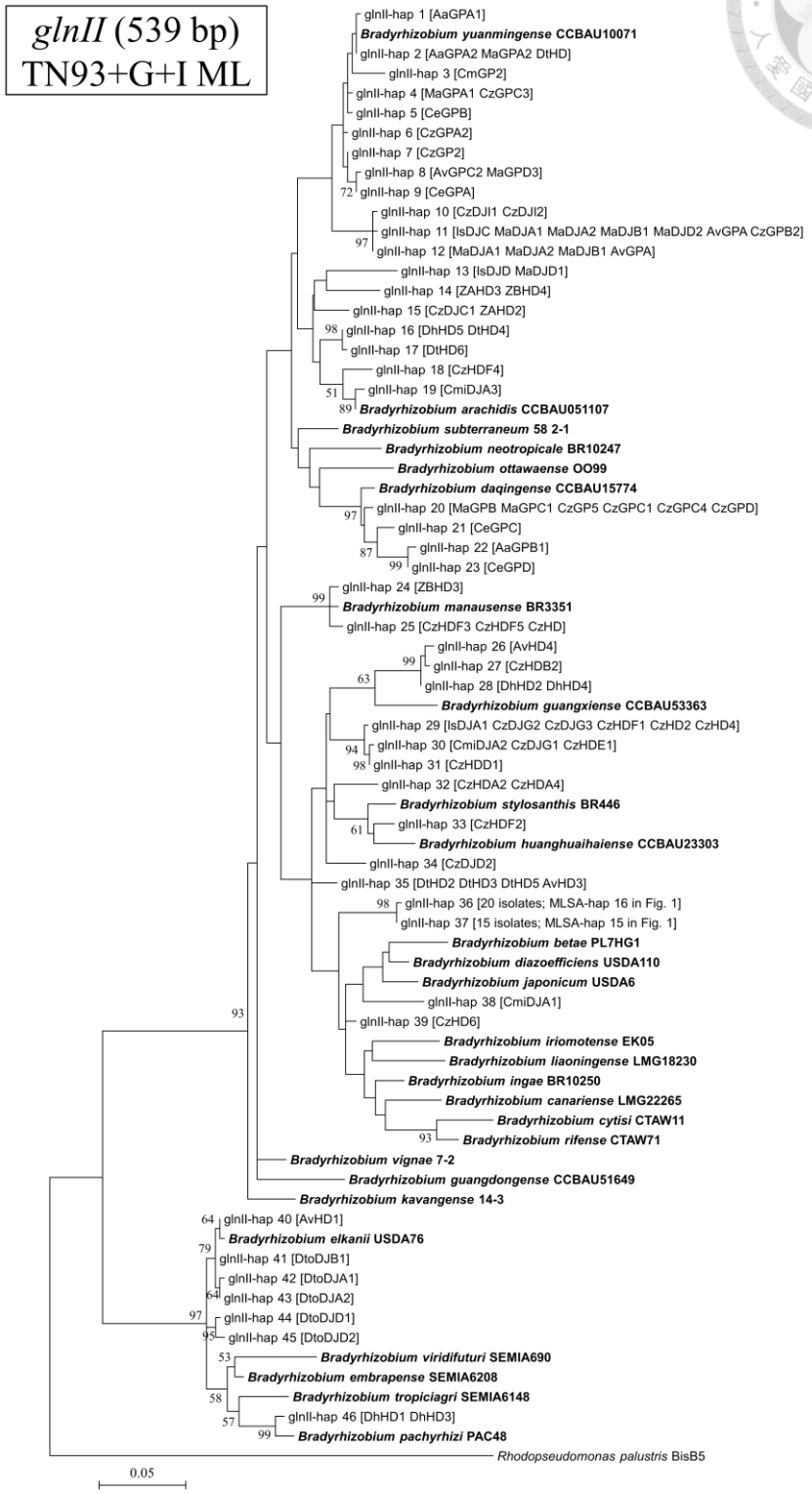
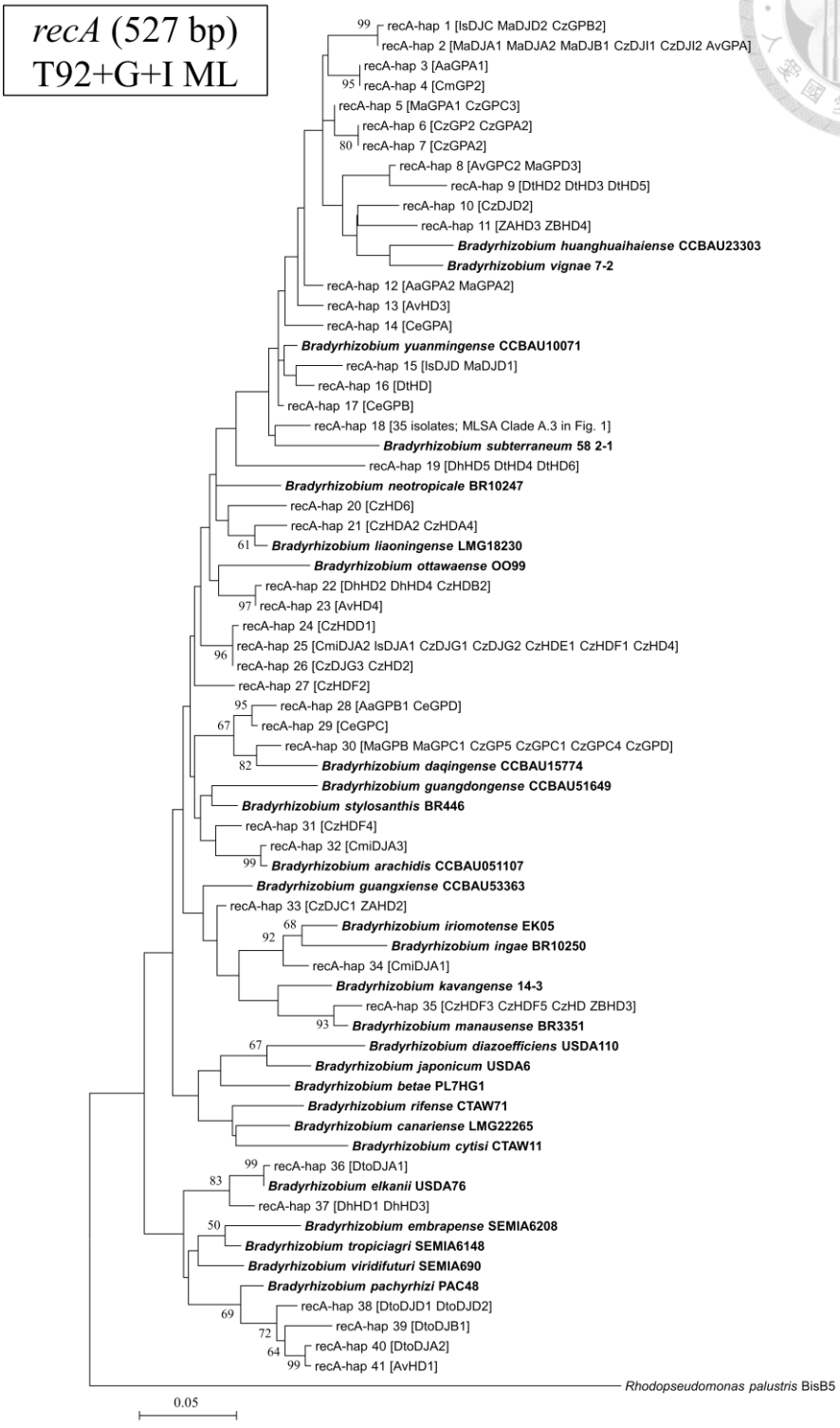


Supplementary Figure S3 *glnII* phylogeny of the 113 *Bradyrhizobium* isolates and reference strains.

doi:10.6342/NTU201800782



Supplementary Figure S4 *recA* phylogeny of the 113 *Bradyrhizobium* isolates and reference strains.

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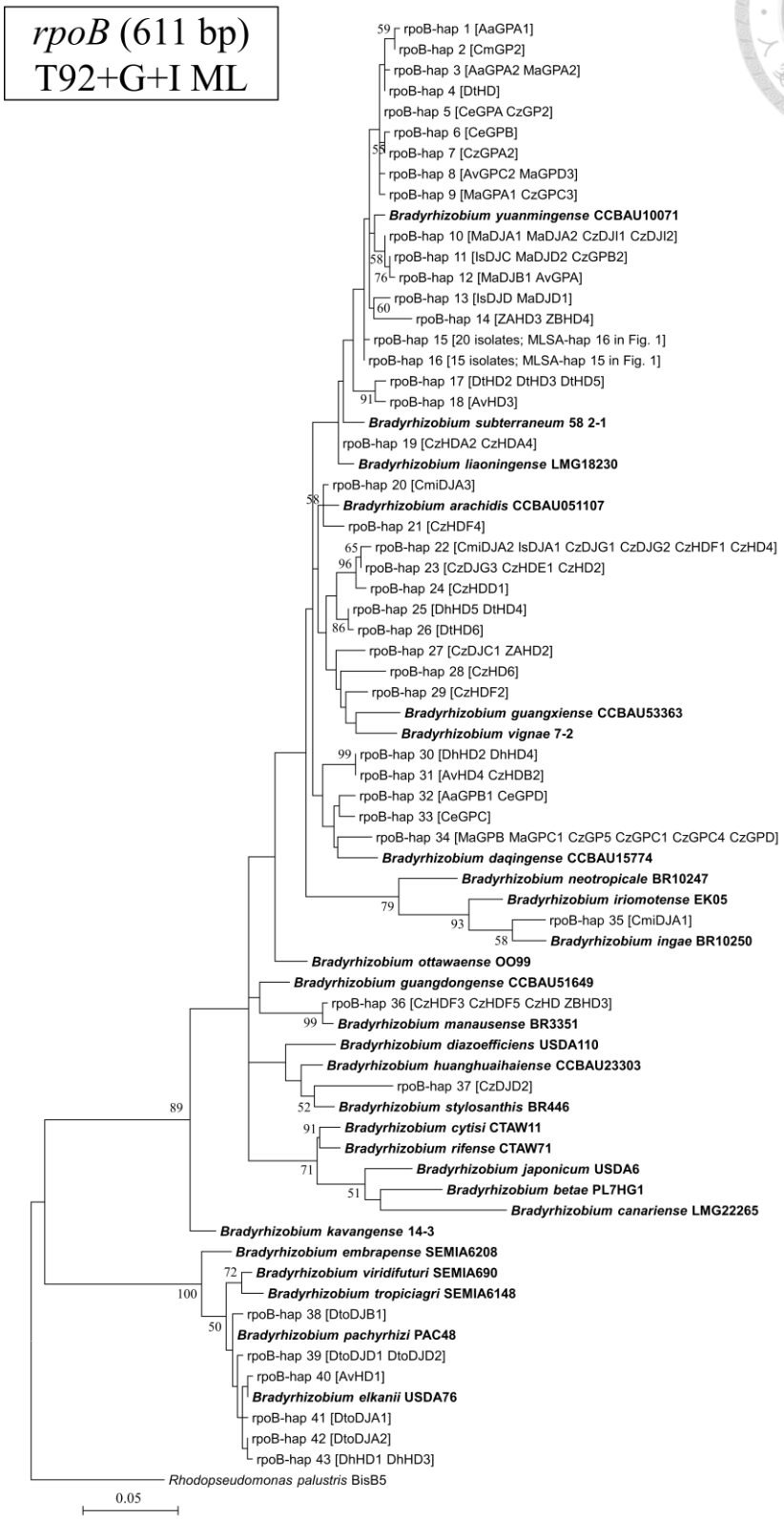
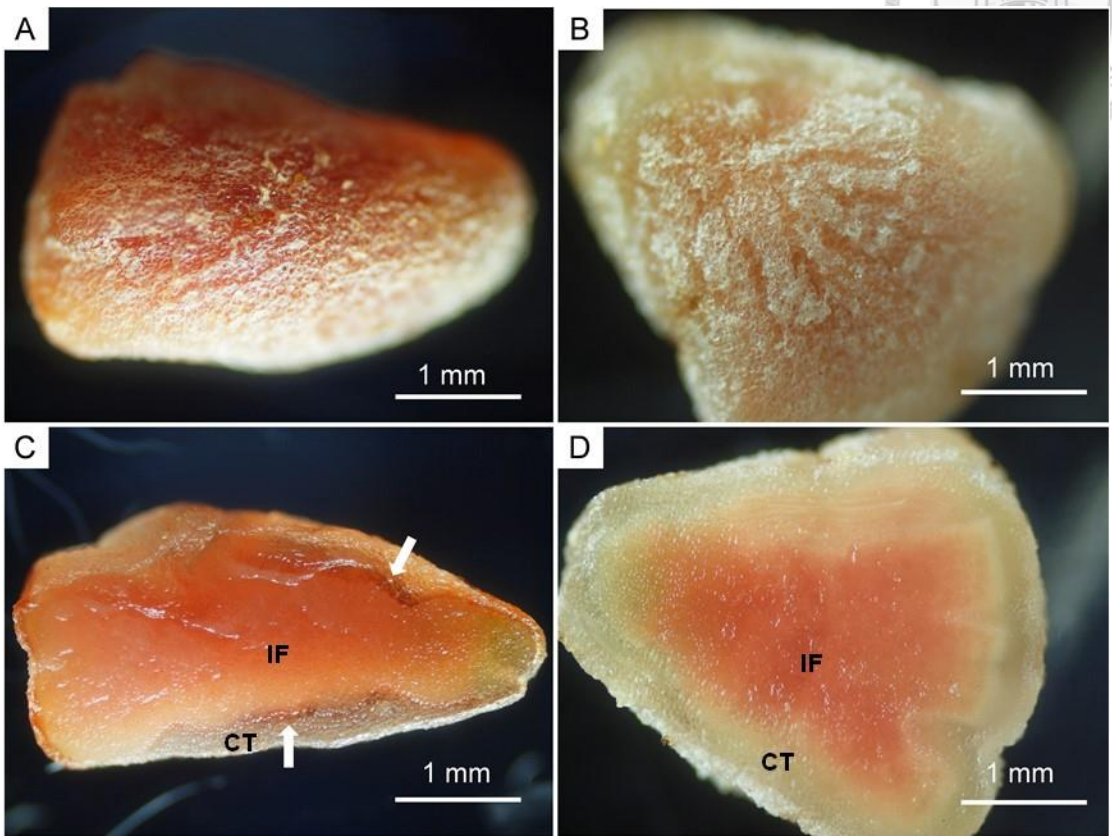


Supplementary Figure S5 *rpoB* phylogeny of the 113 *Bradyrhizobium* isolates and reference strains.

doi:10.6342/NTU201800782



Supplementary Figure S6 Nodule sections of *C. zanzibarica* inoculated with strain CzR2 and CzHD. The nodules infected by CzR2 (A) were more reddish than that infected by CzHD (B). Longitudinal section through nodules showed that CzR2-infected nodules (C) might accumulate leghemoglobin (arrows) between the cortex (CT) and infection zone (IF) which was not found in CzHD-infected nodules (D).

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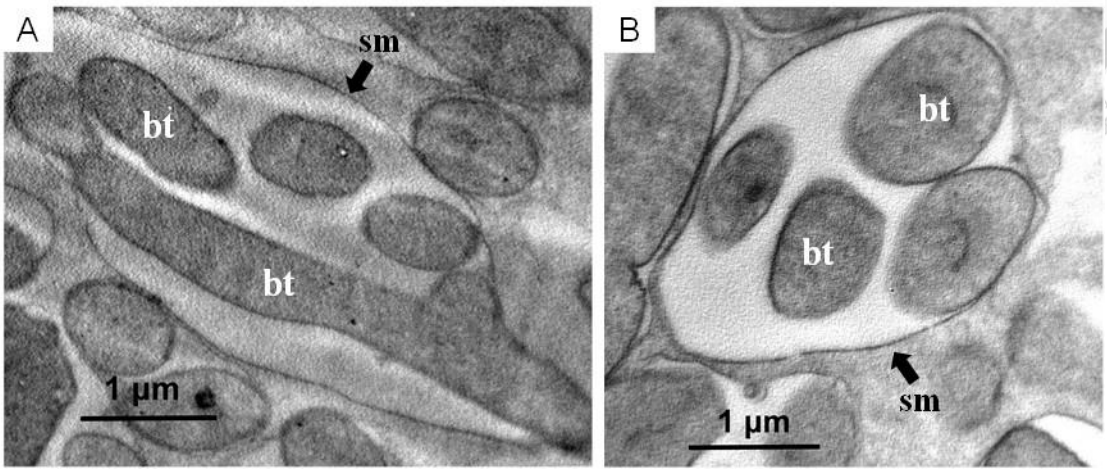

Supplementary Figure S7 Symbiosomes of symbiotic CzR2 and CzHD3 with *C. zanzibarica*. TEM images of sections of root nodule of *C. zanzibarica* induced by CzR2 (A) and CzHD3 (B) both showed that multiple bacteroids (bt), in a single symbiosome, were encompassed by the symbiosome membrane (sm).

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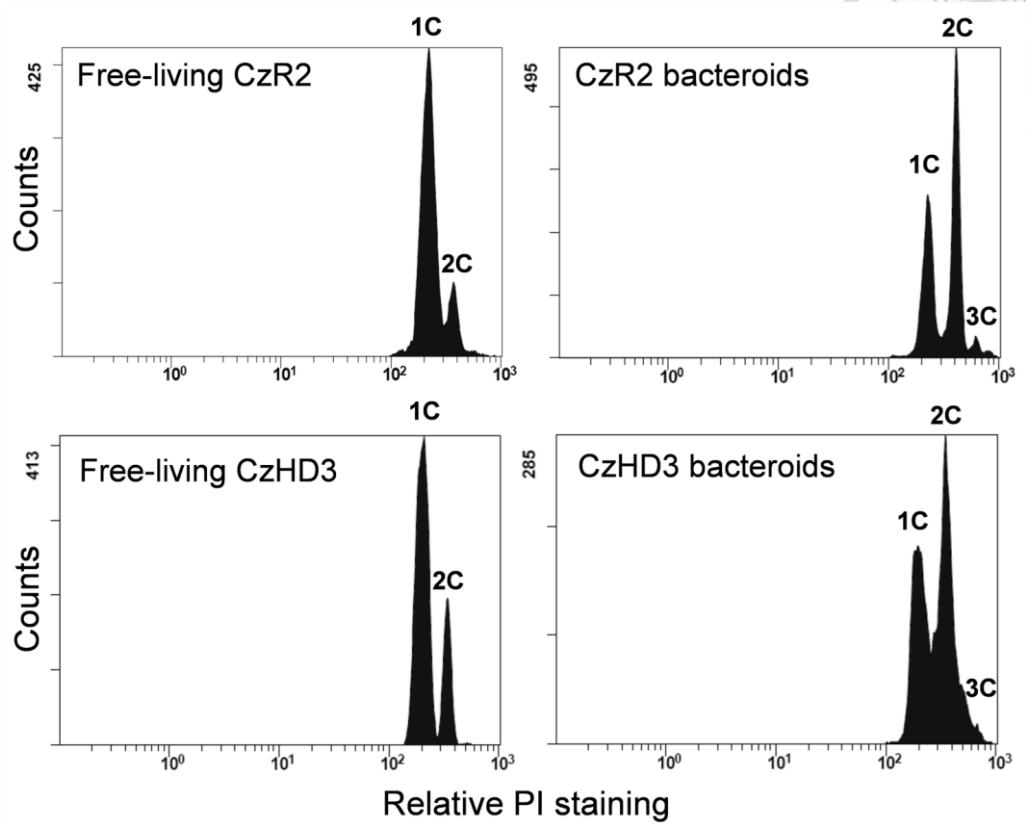

Supplementary Figure S8 Relative DNA contents of CzR2 and CzHD3 in free-living and symbiotic states. Cells were stained with propidium iodide (PI). Populations of free-living cells of CzR2 and CzHD3 grown in YEM broth, at late log phase, were sampled. Bacteroids were extracted from the nodules of *C. zanzibarica* after 62 days of being inoculated with CzR2 and CzHD3. The x axis shows fluorescence levels, indicating the DNA contents and the y axis shows cell counts. In each experiment, 20,000 cells were analyzed.

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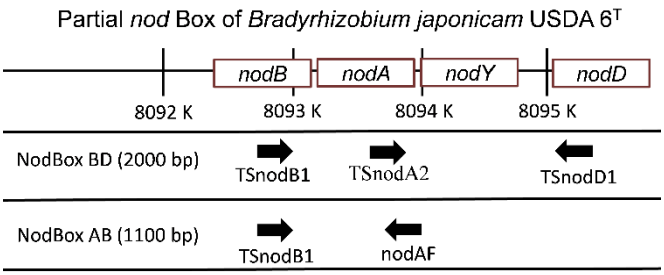
Supplementary Table S1 Primers used and PCR cycling conditions. In this study, we used two different methods to sequence *nodA* gene. Complete *nodA* gene sequences (633 bp) can be obtained from the PCR product of *nodB-D* Box by using TSnodB1 and TSnodA2 as the sequencing primes (see the illustration below Table S1). Alternatively, we designed an additional primer (nodAF) for amplification of *nodA-B* box and sequencing the PCR products, and thus, about 560 bp *nodA* gene sequences can be obtained.

Primer	Target gene (product size)	Sequence (5'–3')	PCR cycling	Reference
For the four housekeeping genes				
BRdnaKf	dnaK (400 bp)	TTCGACATCGACGCSAACGG	2 m 95 ℃, 30x(45 s 95℃, 30 s 58℃, 1 m 72 ℃), 7 m 72℃	Menna et al. (2009)
BRdnaKr		GCCTGCTGCKTGTACATGGC		
TSglnIIf	glnII (600 bp)	AAGCTCGAGTACATCTGGCTCGACGG		Stepkowski et al. (2005)
TSglnIIr		SGAGCCGTTCCAGTCGGTGTCG		
TSrecAf	recA (600 bp)	CAACTGCMYTGCGTATCGTCGAAGG		
TSrecAr		CGGATCTGGTTGATGAAGATCACCATG		
rpoBF	rpoB (700 bp)	CGTATCGCGGYTCCTGGCTC		Parker (2012)
rpoBR		CGAGGCGCATGTTCATCTTGAC		
For *nodA* gene				
TSnodD1	nodB-D Box (2000 bp)	CAGATCNAGDCCBTTGAARCGCA	2 m 95 ℃, 30x(45 s 95℃, 30 s 55℃, 2 m 72 ℃), 7 m 72℃	Moulin et al. (2004)
TSnodB1		AGGATAYCCGTCGTGCAGGAGCA		
TSnodA2	Sequencing	GCTGATTCCAAGBCCYTCVAGATC		
nodAF	nodA-B Box (1100 bp)	GGAAGCTCCCATGAACATTGC	2 m 95 ℃, 30x(45 s 95℃, 30 s 55℃, 1 m 20 s 72 ℃), 7 m 72℃	This study
TSnodB1		AGGATAYCCGTCGTGCAGGAGCA		



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Supplementary Table S2 GenBank accession numbers of 113 isolates in this study.

Isolate	GenBank acession number				
	dnaK	*glnII*	*recA*	*rpoB*	*nodA*
CzHD	KU000915	KU000976	KU001036	KU001096	KJ125404
CzHD2	KU000916	KU000977	KU001037	KU001097	KT989788
CzHD3	KU000917	KU000978	KU001038	KU001098	KT989789
CzHD4	KU000918	KU000979	KU001039	KU001099	KT989790
CzHD5	KU000919	KU000980	KU001040	KU001100	KT989791
CzHD6	KU000920	KU000981	KU001041	KU001101	
ZAHD2	KU000921	KU000982	KU001042	KU001102	
ZAHD3	KU000922	KU000983	KU001043	KU001103	KT989792
ZBHD3	KU000923	KU000984	KU001044	KU001104	KT989793
ZBHD4	KU000924	KU000985	KU001045	KU001105	KT989794
CzHDA2	KU000925	KU000986	KU001046	KU001106	KT989795
CzHDA4	KU000926	KU000987	KU001047	KU001107	KT989796
CzHDB2	KU000927	KU000988	KU001048	KU001108	KT989797
CzHDD1	KU000928	KU000989	KU001049	KU001109	KT989798
CzHDE1	KU000929	KU000990	KU001050	KU001110	KT989799
CzHDE2	KU000930	KU000991	KU001051	KU001111	KT989800
CzHDF1	KU000931	KU000992	KU001052	KU001112	KT989801
CzHDF2	KU000932	KU000993	KU001053	KU001113	KT989802
CzHDF3	KU000933	KU000994	KU001054	KU001114	KT989803
CzHDF4	KU000934	KU000995	KU001055	KU001115	KT989804
CzHDF5	KU000935	KU000996	KU001056	KU001116	KT989805
CzDJA1	KU000936	KU000997	KU001057	KU001117	KT989806
CzDJA2	KU000937	KU000998	KU001058	KU001118	KT989807
CzDJA3	KU000938	KU000999	KU001059	KU001119	KT989808
CzDJB1	KU000939	KU001000	KU001060	KU001120	KT989809
CzDJB2	KU000940	KU001001	KU001061	KU001121	KT989810
CzDJB3	KU000941	KU001002	KU001062	KU001122	KT989811
CzDJC1	KU000942	KU001003	KU001063	KU001123	KT989812
CzDJC2	KU000943	KU001004	KU001064	KU001124	KT989813
CzDJC3	KU000944	KU001005	KU001065	KU001125	KT989814
CzDJD1	KU000945	KU001006	KU001066	KU001126	
CzDJD2	KU000946	KU001007	KU001067	KU001127	KT989815

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CzDJE1	KU000947	KU001008	KU001068	KU001128	KT989816
CzDJE2	KU000948	KU001009	KU001069	KU001129	KT989817
CzDJF1	KU000949	KU001010	KU001070	KU001130	KT989818
CzDJG1	KU000950	KU001011	KU001071	KU001131	
CzDJG2	KU000951	KU001012	KU001072	KU001132	KT989819
CzDJG3	KU000952	KU001013	KU001073	KU001133	KT989820
CzDJI1	KU000953	KU001014	KU001074	KU001134	KT989821
CzDJI2	KU000954	KU001015	KU001075	KU001135	KT989822
CzDJJ1	KU000955	KU001016	KU001076	KU001136	KT989823
CzDJK	KU000956	KU001017	KU001077	KU001137	KT989824
CzGP1	KU000957	KU001018	KU001078	KU001138	KT989825
CzGP2	KU000958	KU001019	KU001079	KU001139	KT989826
CzGP5	KU000959	KU001020	KU001080	KU001140	KT989827
CzGPA1	KU000960	KU001021	KU001081	KU001141	KT989828
CzGPA2	KU000961	KU001022	KU001082	KU001142	KT989829
CzGPB2	KU000962	KU001023	KU001083	KU001143	KT989830
CzGPB3	KU000963	KU001024	KU001084	KU001144	KT989831
CzGPB4	KU000964	KU001025	KU001085	KU001145	KT989832
CzGPB5	KU000965	KU001026	KU001086	KU001146	KT989833
CzGPC1	KU000966	KU001027	KU001087	KU001147	KT989834
CzGPC2	KU000967	KU001028	KU001088	KU001148	KT989835
CzGPC3	KU000968	KU001029	KU001089	KU001149	KT989836
CzGPC4	KU000969	KU001030	KU001090	KU001150	KT989837
CzGPC5	KU000970	KU001031	KU001091	KU001151	KT989838
CzGPD	KU000971	KU001032	KU001092	KU001152	KT989839
CzGPE1	KU000972	KU001033	KU001093	KU001153	KT989840
CzGPE2	KU000973	KU001034	KU001094	KU001154	KT989841
DhHD1	KU896489	KU896503	KU896531	KU896545	KU896517
DhHD2	KU896490	KU896504	KU896532	KU896546	KU896518
DhHD3	KU896491	KU896505	KU896533	KU896547	KU896519
DhHD4	KU896492	KU896506	KU896534	KU896548	KU896520
DhHD5	KU896493	KU896507	KU896535	KU896549	KU896521
DtHD	KU896494	KU896508	KU896536	KU896550	KU896522
DtHD2	KU896495	KU896509	KU896537	KU896551	KU896523
DtHD3	KU896496	KU896510	KU896538	KU896552	KU896524

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DtHD4	KU896497	KU896511	KU896539	KU896553	KU896525
DtHD5	KU896498	KU896512	KU896540	KU896554	KU896526
DtHD6	KU896499	KU896513	KU896541	KU896555	KU896527
AvHD1	KU896500	KU896514	KU896542	KU896556	KU896528
AvHD3	KU896501	KU896515	KU896543	KU896557	KU896529
AvHD4	KU896502	KU896516	KU896544	KU896558	KU896530
CmiDJA1	KY694798	KY694839	KY694880	KY694921	
CmiDJA2	KY694799	KY694840	KY694881	KY694922	KY694761
CmiDJA3	KY694800	KY694841	KY694882	KY694923	KY694762
DtoDJA1	KY694801	KY694842	KY694883	KY694924	KY694763
DtoDJA2	KY694802	KY694843	KY694884	KY694925	KY694764
DtoDJB1	KY694803	KY694844	KY694885	KY694926	KY694765
DtoDJD1	KY694804	KY694845	KY694886	KY694927	KY694766
DtoDJD2	KY694805	KY694846	KY694887	KY694928	KY694767
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IsDJA2	KY694807	KY694848	KY694889	KY694930	KY694769
IsDJC	KY694808	KY694849	KY694890	KY694931	KY694770
IsDJD	KY694809	KY694850	KY694891	KY694932	KY694771
MaDJA1	KY694810	KY694851	KY694892	KY694933	KY694772
MaDJA2	KY694811	KY694852	KY694893	KY694934	KY694773
MaDJB1	KY694812	KY694853	KY694894	KY694935	KY694774
MaDJD1	KY694813	KY694854	KY694895	KY694936	KY694775
MaDJD2	KY694814	KY694855	KY694896	KY694937	KY694776
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AaGPA2	KY694816	KY694857	KY694898	KY694939	KY694778
AaGPB1	KY694817	KY694858	KY694899	KY694940	
AaGPD2	KY694818	KY694859	KY694900	KY694941	KY694779
AvGPA	KY694819	KY694860	KY694901	KY694942	KY694780
AvGPC2	KY694820	KY694861	KY694902	KY694943	KY694781
AvGPD1	KY694821	KY694862	KY694903	KY694944	KY694782
MaGPA1	KY694823	KY694864	KY694905	KY694946	KY694784
MaGPA2	KY694824	KY694865	KY694906	KY694947	KY694785
MaGPB	KY694825	KY694866	KY694907	KY694948	KY694786
MaGPC1	KY694826	KY694867	KY694908	KY694949	KY694787
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MaGPD3	KY694828	KY694869	KY694910	KY694951	KY694789
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CmGP6	KY694831	KY694872	KY694913	KY694954	KY694792
CeGPA	KY694832	KY694873	KY694914	KY694955	KY694793
CeGPB	KY694833	KY694874	KY694915	KY694956	KY694794
CeGPC	KY694834	KY694875	KY694916	KY694957	
CeGPD	KY694835	KY694876	KY694917	KY694958	
CeGPE1	KY694836	KY694877	KY694918	KY694959	KY694795
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CeGPE4	KY694838	KY694879	KY694920	KY694961	KY694797

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Supplementary Table S3 GenBank accession numbers of 27 type strains of *Bradyrhizobium* species.

Species	Type strain	GenBank acession number			
		dnaK	*glnII*	*recA*	*rpoB*
Bradyrhizobium arachidis	CCBAU 051107	KJ560556	KF962689	KF962707	JX437682
Bradyrhizobium betae	PL7HG1	AY923046	FJ970431	FJ970378	GU562860
Bradyrhizobium cytisi	CTAW11	KF532219	GU001594	GU001575	JN186288
Bradyrhizobium daqingense	CCBAU 15774	KF962684	KF962690	HQ231270	JX437676
Bradyrhizobium diazoefficiens	USDA 110	CP000133			
Bradyrhizobium elkanii	USDA 76	AY328392	AY599117	KF532941	EF190188
Bradyrhizobium huanghuaihaiense	CCBAU 23303	KF962686	KF962691	KF962709	JX437679
Bradyrhizobium iriomotense	EK05	JF308944	AB300995	AB300996	HQ587646
Bradyrhizobium japonicum	USDA 6	NC017249			
Bradyrhizobium jicamae	PAC68	JN207408	FJ428204	HM590776	HQ587647
Bradyrhizobium liaoningense	LMG 18230	AY923041	AY386775	FM253180	EF190181
Bradyrhizobium oligotrophicum	S58	KF962688	JQ619233	JQ619231	KF962713
Bradyrhizobium ottawaense	OO99	JF308816	HQ587750	HQ587287	HQ587518
Bradyrhizobium pachyrhizi	PAC48	KF532217	FJ428201	HM590777	HQ587648
Bradyrhizobium rifense	CTAW71	JQ945187	GU001604	GU001585	KC569468
Bradyrhizobium yuanmingense	CCBAU 10071	AY923039	AY386780	AY591566	EF190174
Bradyrhizobium neotropicale	BR10247	KJ661693	KJ661700	KJ661714	KF983829
Bradyrhizobium ingae	BR10250	KF927055	KF927067	KF927061	KF927073
Bradyrhizobium manausense	BR3351	LJYG01000000			
Bradyrhizobium viridifuturi	SEMIA 690	LGTB00000000			
Bradyrhizobium kavangense	14-3	KR259949	KM378446	KM378399	KM378311
Bradyrhizobium guangxiense	CCBAU 53363	KC508974	KC509033	KC509279	KC509328
Bradyrhizobium guangdongense	CCBAU 51649	KC508964	KC509023	KC509269	KC509318
Bradyrhizobium embrapense	SEMIA 6208	LFIP02000000			
Bradyrhizobium tropiciagri	SEMIA 6148	LFLZ00000000			
Bradyrhizobium vignae	7-2	KR259951	KM378443	KM378374	KM378308
Bradyrhizobium stylosanthis	BR 446	LVEM01000000			

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