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Rhizobia symbiosis of seven leguminous species 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 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.

KEY WORDS: Legume-rhizobia symbiosis, 16S rRNA gene, Stable nitrogen and Carbon isotope ratios.

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 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.*,

Table 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

2003). Among the seven species, the bacterial symbionts of *A. vaginalis*, *C. zanzibarica*, *M. pudica* and *S. cannabina* in Taiwan have been reported (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 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 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* (Table 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.

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°C 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 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, 2004).

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 Fig. 2.

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 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

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 (Fig. 1A-D), while *T. repens*, *M. pudica* and *C. zanzibarica* produced indeterminate nodules with no branch, few branches and many branches, respectively (Fig. 1E-G). In addition, the lenticels (as white stripes) were observed on the nodules of *A. vaginalis*, *D. triflorum*, *D. heterophyllum* (Fig. 1A-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 (Fig. 1L 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 slow-growing phenotype, forming visible colonies on YEM plates after 5-7 days at 30°C. 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 (Fig. 2). These isolates were separated into two distinct groups, belonging to alpha- and beta-rhizobia. In alpha-rhizobia group, a

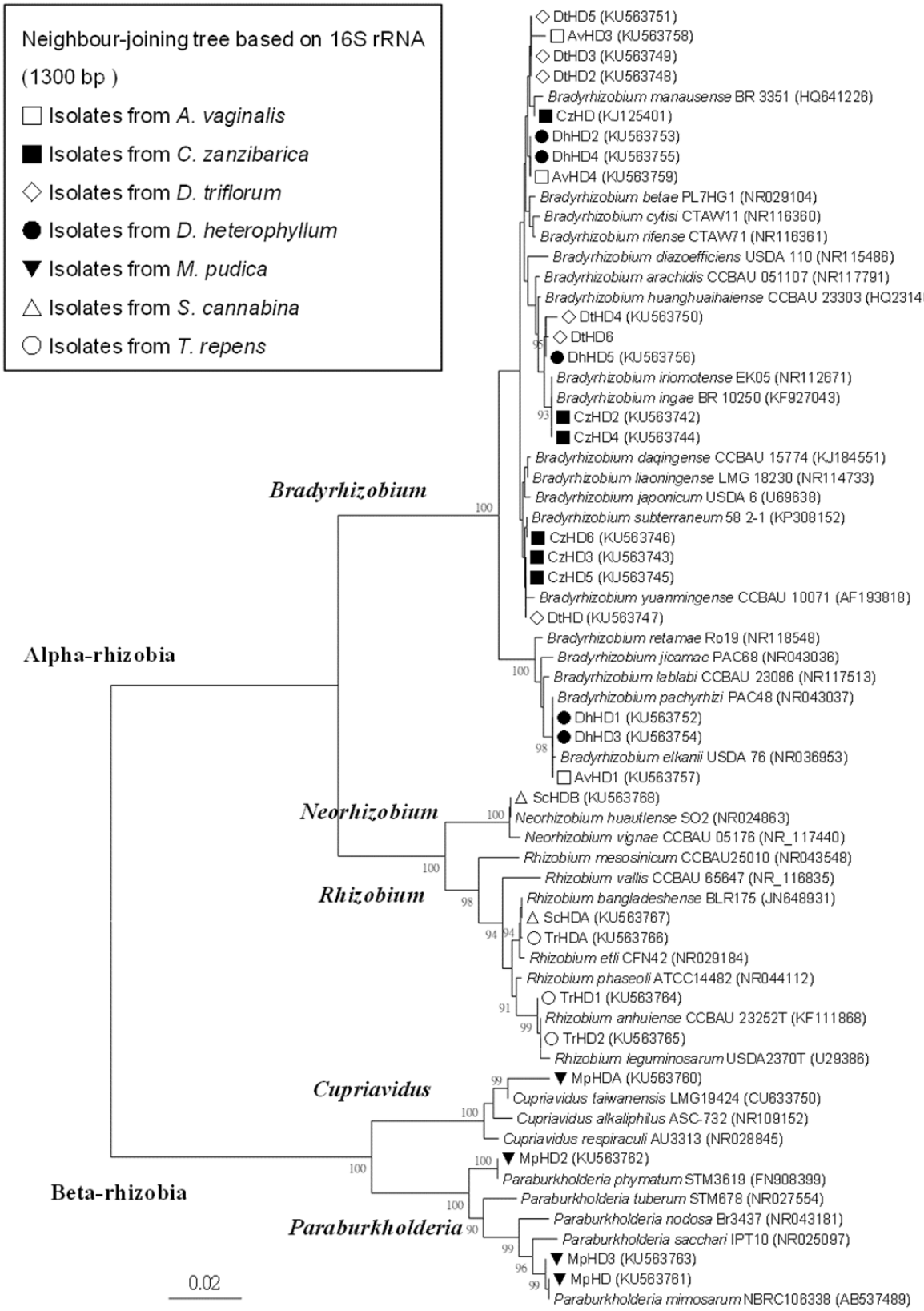

Fig. 2: Neighbor-joining tree based on 16S rRNA gene sequences of the nodule symbionts isolated from seven legumes growing along riverbank of Xindian River in Northern Taiwan. Only bootstrap values > 90 are shown at the internodes. The scale bar represents 2 % nucleotide substitutions.

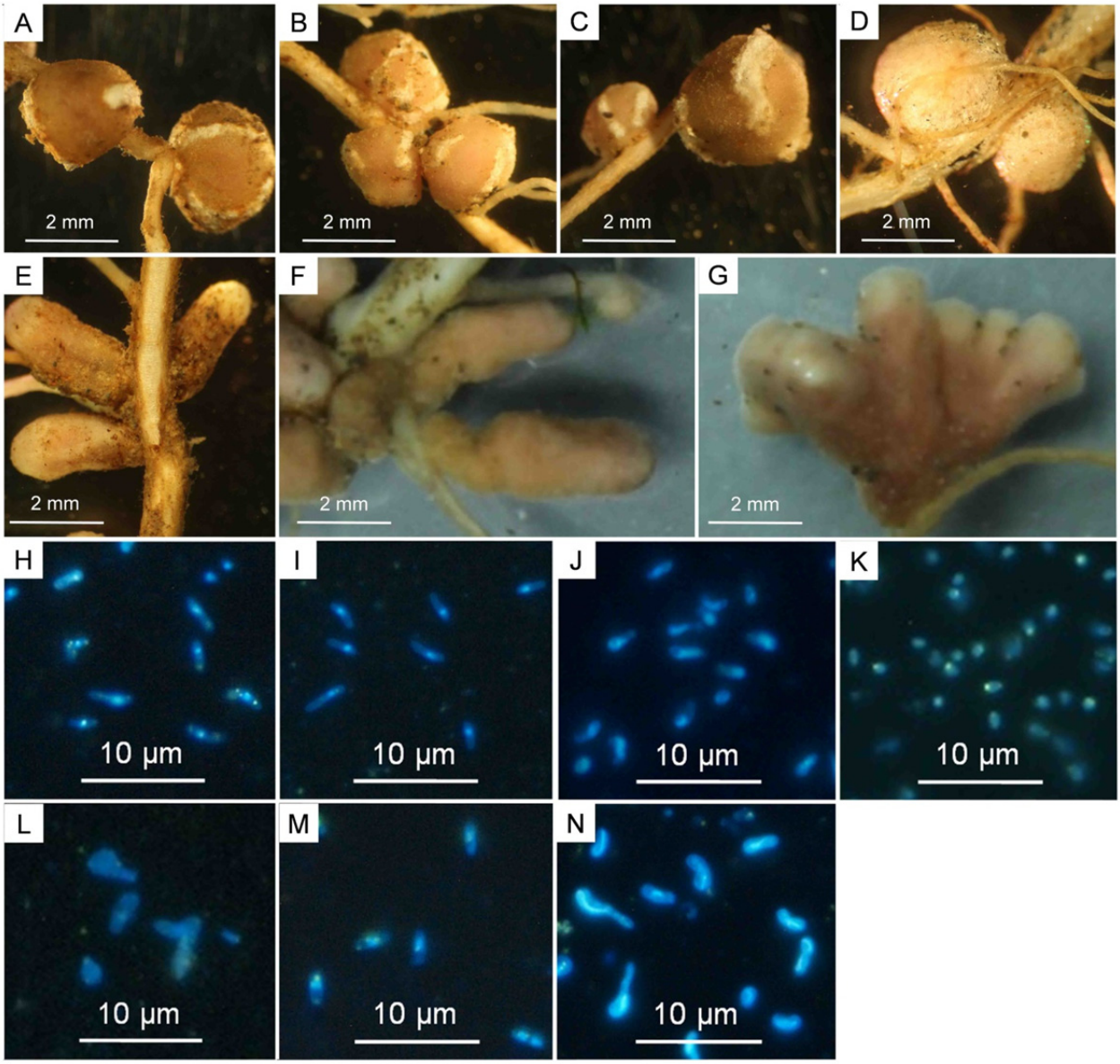

Fig. 1: These pictures showed the root nodules (**A-G**) and extracted bacteroids (**H-N**), stained with DAPI, of ***Alysicarpu vaginalis*** (**A**, **H**), ***Desmodium triflorum*** (**B**, **I**), ***D. heterophyllum*** (**C**, **J**), ***Sesbania cannabina*** (**D**, **K**), ***Trifolium repens*** (**E**, **L**), ***Mimosa pudica*** (**F**, **M**) and ***Crotalaria zanzibarica*** (**G**, **N**) growing along riverbank of Xindian River in Northern Taiwan.

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 (Fig. 2). The latest two species have been recently separated from genus *Burkholderia* and reclassified as members of the *Paraburkholderia* (Sawana *et al.* 2014).

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

Table 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‰.

Table 2. 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).

Table 3 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).

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 (Fig. 3).

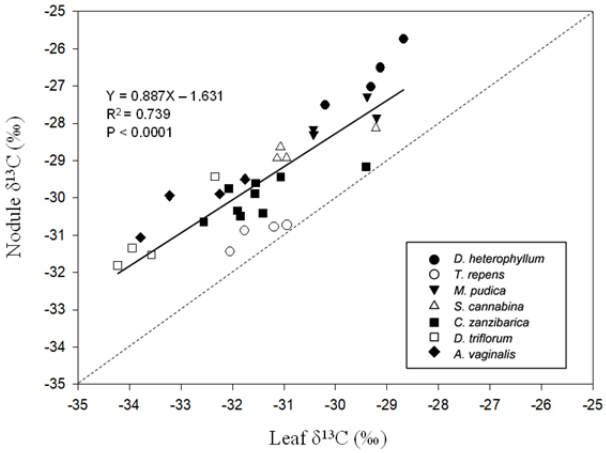

Fig. 3. Correlations between leaf and nodule $\delta^{13}C$ values among the seven legume species coexisting along riverbank of Xindian River in Northern Taiwan. Dash line represents 1:1 relationship between leaf and nodule $\delta^{13}C$ values.

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.* 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* (Fig. 2). 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* (Fig. 2). 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. 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 (Fig. 2). 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 (Fig. 2). 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 (Fig. 2). 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*, *Paraburkholderia phymatum* and *P. mimosarum* (Fig. 2). 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 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 1). It is reported that the morphology of the bacteroids was determined by host plants (Haag *et al.*, 2013)

As shown in Table 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), 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 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). 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 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 *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 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 $\delta^{13}C$ (Fig. 3) 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 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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