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Full Length Research Paper

Multilocus sequence analysis of the Rhizobia from five woody legumes in southern China

Junkun Lu[1], Lihua Kang[1], Xinhua He[2] and Daping Xu[1]*

[1]Research Institute of Tropical Forestry, Chinese Academy of Forestry, Guangzhou, Guangdong 510520, China..
[2]State Centre of Excellence for Ecohydrology and School of Plant Biology, University of Western Australia, Crawley, WA 6009, Australia.

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A total of 11 rhizobial strains from five woody legumes (*Acacia confusa*, *Dalbergia odorifera*, *Dalbergia fusca*, *Erythrophleum fordii* and *Pterocarpus macarocarpus*) in Southern China were identified, using 16S rRNA gene sequence analysis and multilocus sequence analysis (MLSA) of three housekeeping genes (*recA*, *glnII* and *dnaK*). Among the 11 isolated rhizobia, seven were classified as *Agrobacterium tumefaciens*, *Bradyrhizobium elkanii*, *Ensifer adhaerens* and *Rhizobium multihospitium* and four were within the genus *Rhizobium* that might be novel species when considering their clustering independently in topology tree and relatively low *recA* gene sequence similarities (<94%) to the recognized species. In each gene phylogeny, the 16S rRNA gene was found to be incongruent with each house-keeping gene, indicated that this gene should not be used as a single marker in rhizobial taxonomy. In addition, more robust topology trees were produced through the analysis of a concatenation of three housekeeping genes than through each housekeeping gene and 16S rRNA gene trees, with high bootstrap support (≥55%). Overall, our results support that the MLSA has higher discrimination potential in rhizobial taxonomy than the 16S rRNA gene and is suitable to estimate phylogenetic relationships among rhizobia species.

Key words: Multilocus sequence analysis, 16S rRNA, rhizobia and woody legume.

INTRODUCTION

Southern China is one of the most important hardwood-timber produce regions in China. The biodiversity of precious woody legumes is exceptional in this region and many of them, such as *Dalbergia* species (e.g. *Dalbergia balansae*, *Dalbergia fusca*, *Dalbergia mimosoides*, *Dalbergia obtusifolia*, *Dalbergia odorifera* etc), are precious because of the quality and beauty of the heartwood (Gao et al., 1994; Lin et al., 2009). However, such precious heartwoods are seriously over-exploited due to the high demand by the furniture and construction industries with the recent economic boom in China. As a result, investment has triggered large scale woody legume plantations in southern China.

Legumes are able to establish mutualistic symbiosis with rhizobia to fix atmospheric dinitrogen into ammonia, a primary source of nitrogen for plant growth, which reduces the need for chemical N fertilizer in agriculture and agro-forestry (Roux et al., 2009; Peoples et al., 1995; Zahran, 1999). Studies of tropical or subtropical woody legume species have revealed an unexpected diversity of symbionts as various new species of rhizobia were observed in southern China (Chen et al., 1997; Gao et al., 1994; Lin et al., 2009). However, detailed information regarding taxonomic features of native rhizobia in southern China is not available.

The 16S rRNA gene has been widely applied to rhizobial taxonomy as a molecular marker (Kwon et al.,

*Corresponding author. E-mail: gzfsrd@163.com. Tel: +8620 87031626. Fax: +86 020 87031622.

Table 1. List of primers, and sizes of PCR products.

Gene	Primer	Sequence(5'-3')	Size (bp)	Reference
16S rRNA	1492R 27F	AGA GTT TGA TCA TGG CTC AG TAG GGT TAC CTT GTT ACG ACT T	1450	DeLong (1992)
recA	recA-41F recA-640R	TTC GGC AAG GGM TCG RTSA TG ACA TSA CRC CGA TCT TCA TGC	508	Islam et al. (2008)
glnII	glnII-12F glnII-689R	YAA GCT CGA GTACAT YTG GCT TGC ATG CCS GAG CCG TTC CA	637	Islam et al. (2008)
dnaK	dnaK1466F dnaK1777R	AAG GAR CAN CAG ATC CGC ATC CA TAS ATS GCC TSR CCR AGC TTC AT	305	Ribeiro et al. (2009)

Degeneracy is indicated by standard conventions: M, A/C; N, A/C/G/T; R, A/G; S, C/G; Y, C/T.

2005; Silva et al., 2005; Willems, 2006). However, and horizontal gene transfer, make it marker difficult to discriminate closely related species with this marker (van Berkum et al., 2003; Young and Haukka, 1996). Multilocus sequence analysis (MLSA), which uses sequences of multiple protein-coding genes for genotypic characterization of bacteria, has firstly proposed as an alternative phylogenetic marker for evaluating prokaryotic species (Gevers et al., 2005). Since then MLSA has been broadly applied in different genera of rhizobia (Martens et al., 2007, 2008; Ribeiro et al., 2009; Vinuesa et al., 2008).

An effective N_2-fixing rhizobium is critical to establish N_2-fixation in woody legumes ensuring quality seedlings in nursery stocks, successful outplanting in the fields and optimum tree growth (Lal and Khanna, 1996). The present work was undertaken to characterize rhizobia isolates from a range of woody legumes in southern China, first by sequence analysis of the 16S rRNA gene, and then by the MLSA of three housekeeping genes, *recA* (encoding the recombinase A protein), *glnII* (encoding a glutamine synthetase) and *dnaK* (encoding the heat-shock protein Hsp70). Phylogenies of each housekeeping genes and concatenated sequences were also examined and compared to that of their 16S rRNA gene.

MATERIALS AND METHODS

Collection of rhizobia and culture condition

Naturally occurring root nodules were collected from four rose wood species (*D. odorifera*, *D. fusca*, *Erythrophleum fordii* and *Pterocarpus macarocarpus*) and one woody legume *Acacia confusa*, at four plantation sites in subtropical or tropical regions of Southern China (Guangdong, Guangxi and Hainan Province). The collected nodules were surface sterilized by washing for 1 min in 70% (v/v) ethanol followed by 3 min in 3% (v/v) sodium hypochlorite and finally rinsed with sterilized distilled water. The sterilized nodules were crushed into sterilized water and the suspension was streaked onto yeast extract-mannitol (YM) agar (Vincent, 1970). Single colonies with white, translucent, mucoid and convex were limitations including slow evolution, genetic recombination appearance picked after 3 to 7 days incubation at 28°C.

DNA extraction and PCR amplification

Total genomic DNA was extracted from the stationary phase of bacterial batch cultures in YM liquid media as described by Chen et al. (1993). Each reaction contained, in a volume of 25 µl : 20 pmol each primer, 200 µM dNTPs (BBI, Ontario, Canada), 1 U Dream Taq DNA polymerase (Fermentas, Ontario, Canada), approximately 25 ng genomic DNA, and 2.5 µl Dream Taq polymerase buffer. Amplifications were carried out in a DNA thermal cycler 2720 (ABI, Carlsbad, USA) with the following program: initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 1 min, 55°C for 50 s and 72°C for 1 min·kb^{-1}; and final extension step at 72°C for 7 min. Primers for amplification and sequence of 16S rRNA, *recA*, *glnII* and *dnaK* genes are listed in Table 1.

Sequencing

Gel extraction kits (Sangon, Shanghai, China) were used for the purification of PCR products following the manufacture's instructions. Nucleotide sequences were determined with the BigDyeterminator v3.1 using ABI-PRISM3730 Genetic Analyzer (ABI, Carlsbad, USA). Sequences were submitted to GenBank database (http://blast.ncbi.nlm.nih.gov/Blast) to seek significant alignments and gene accession numbers are listed in Table 2.

Phylogenetic analysis

Gene sequence alignment was performed with the Clustal_X version 1.83 (Thompson et al., 1997). Evolutionary trees were generated using the MEGA software version 3.1 (http://www.megasoftware.net) with the default parameters, Kimura's two-parameter (K2P) distance model, and neighbour-joining algorithm. Statistical supports for tree nodes were evaluated by performing bootstrap analyses of the data based on 1,000 resamplings. Thirteen reference/type strains, the obtained isolates and a list of the source, sampling sites with the origin of the obtained isolates are provided in Table 2.

Table 2. Isolates obtained and reference/type strains used in this study.

Strain	Source	16S rRNA	*recA*	*glnII*	*dnaK*	Reference
Fast-growing isolates						
1-2	*D. odorifera* (one-year-old), Guangxi, China	HM151906[a]	HM063998	HM063977	HM063971	
3	*D. odorifera* (one-year-old), Guangxi, China	HM151908	HM064000	HM063979	HM063969	
2-1	*D. odorifera* (one-year-old), Guangxi, China	HM151907	HM063999	HM063978	HM063968	
GZ	*D. odorifera* (two-year-old), Guangdong, China	HM151909	HM064006	HM063983	HM063966	
8211	*D. odorifera* (two-year-old), Hainan, China	FJ870550	HM064002	HM063986	HM063967	
8213	*D. odorifera* (two-year-old), Hainan, China	FJ870552	HM064003	HM063980	HM063972	
8203	*D. odorifera* (two-year-old), Hainan, China	FJ870556	HM064001	HM063976	HM063965	
TW	*A. confusa* (three-year-old), Guangdong, China	HM151912	HM064008	HM063985	HM063974	
HHT	*D. fusca* (two-year-old), Guangdong, China	HM151910	HM064007	HM063984	HM063973	
Slow-growing isolates						
GM	*E. fordii* (two-year-old), Guangdong, China	HM151911	HM064005	HM063982	HM063970	
DG	*P. macarocarpus* (three-year-old), Guangdong, China	HM151913	HM064004	HM063981	HM063975	
Reference strains						
R. etli CFN 42^T	*P. vulgaris*, Mexico	EU488751	EU488824	EU488776	EU488768	Ribeiro et al. (2009)
R. tropici type A CFN 299^T	*P. vulgaris*, Brazil	EU488741	EU488817	EU488777	EU488773	Ribeiro et al. (2009)
R. tropici type B CIAT899^T	*P. vulgaris*, Columbia	EU488752	EU488815	EU488791	EU488764	Ribeiro et al. (2009)
R. hainanense CCBAU 57015^T	*D. sinuatum*, Hainan, China	U71078	HM047132	GU726294	JF279696	
R. mesosinicum CCBAU 41044	*D. balansae*, Hunan, China	AY395697	EU034028	DQ310809	JF279695	Lin et al. (2009)
R. mulithospitium 83401^T	*H. halodendron*, Xinjiang, China	EF035074	EF490029	EF490040	HM142766	Han et al. (2008)
E. meliloti LMG 6133^T = (USDA 1002^T)	*M. sativa*, USA	X67222	AM182133	DQ767676	AM182089	Martens et al. (2007)
E.medicae A 321^T	*M. truncatula*, France	L39882	AM182135	AF169592	AM182091	Martens et al. (2007)
E.adhaerens LMG 20582^T (= Lc04)	Agricultural soil, Belgium	AJ420775	AJ505596	HM997095	AM182107	Martens et al. (2007)
B. elkanii USDA 76^T	*Glycine max*, USA	U35000	AY591568	AY599117	AY328392	Islam et al. (2008)
B. japonicum USDA 110	*Glycine max*, Japan		BA000040			Kaneko et al. (2002)
R. rhizogenes LMG 150^T(=LMG 152)	*P. vulgaris*	X67224	AM182126	AY929470	AM182082	Martens et al. (2007)
A. tumefaciens C58	Plant pathogen and diazotrophic bacterium [b], USA		AE007869			Wood et al. (2001)

The designation $^{(T)}$ indicates type strains. [a] Sequences in bold were determined and corresponding sequences were derived from the complete genome. [b] It was reported that this strain could fix nitrogen in a free-living condition (Kanvinde and Sastry, 1990).

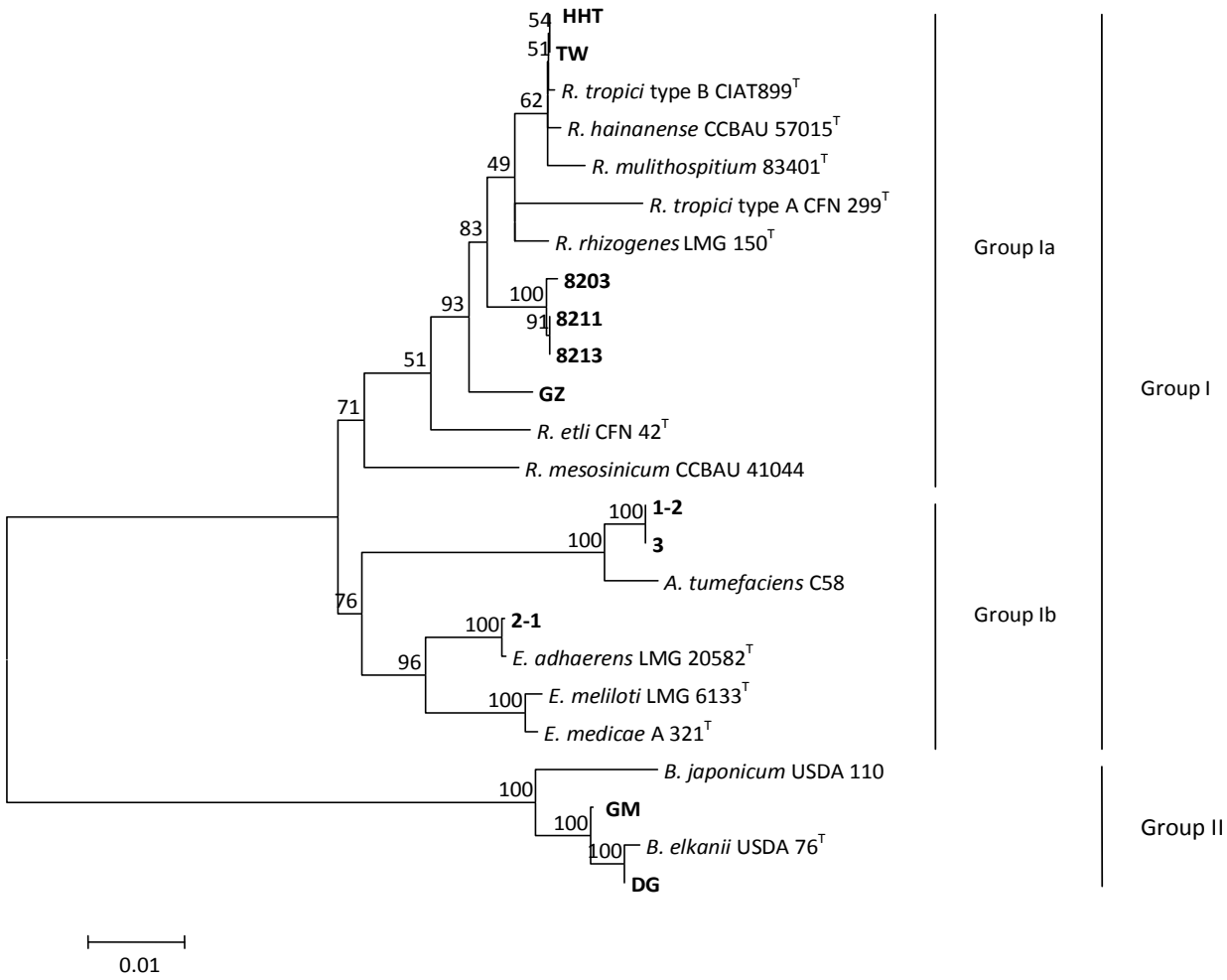

Figure 1. Neighbour-joining phylogeny for 16S rRNA genes of indigenous strains of woody legumes in southern China and reference/type strains (T). The tree was generated using MEGA version 3.1 with K2P distance model, with 1,000 samplings in bootstrap analyses. Bar, 1% nucleotide substitutions. *R*., *Rhizobium*, *E*., *Enisifer* and *B*., *Bradyrhizobium*.

RESULTS

Isolation of root nodule rhizobia from woody legumes

A total of 11 microsymbionts were isolated. These isolates produced 2 to 10 mm diameter, circular, convex, mucilaginous, semitranslucent, white colonies within 3 to7 days on YM medium incubated at 28°C. Nine of these isolates were fast-growing and two were slow-growing bacteria indicate these in Table 2.

Analysis of 16S rRNA gene sequences

A full-length 16S rRNA sequences (approx. 1450 bp) were obtained from all 11 isolates. A phylogenetic tree, based on the similarity of the 16S rRNA sequences of these isolates and reference/type strains, was constructed using the K2P model for estimating phylogenetic distances and the neighbour-joining algorithm (Figure 1). The phylogenetic tree produced two main groups: Group I included fast-growing bacteria (*Agrobacterium, Ensifer* and *Rhizobium*), while Group II included the slow-growing bacteria (*Bradyrhizobium*) (Figure 1).

Isolates HHT and TW clustered with *R. tropici* type B stain CIAT 899, *Rhizobium hainanense* type strain CCBAU 57015 and *Rhizobium multihospitium* type strain 83401, sharing 99.8, 99.6 and 99.9% sequence identity, respectively.

Isolates 8203, 8211 and 8213 clustered together in a branch, separate from other *Rhizobium* type strains. The levels of 16S rRNA sequence identity between these isolates and the *Rhizobium tropici* type A strain CFN 299, *R. multihospitium* type strain 83401, *R. hainanense* type strain CCBAU 57015, *R. tropici* type B strain CIAT 899 and *Rhizobium rhizogenes* type strain LMG 150 were 97.3, 98.5, 98.4, 98.5 and 98.0%, respectively.

The levels of 16S rRNA sequence identity between

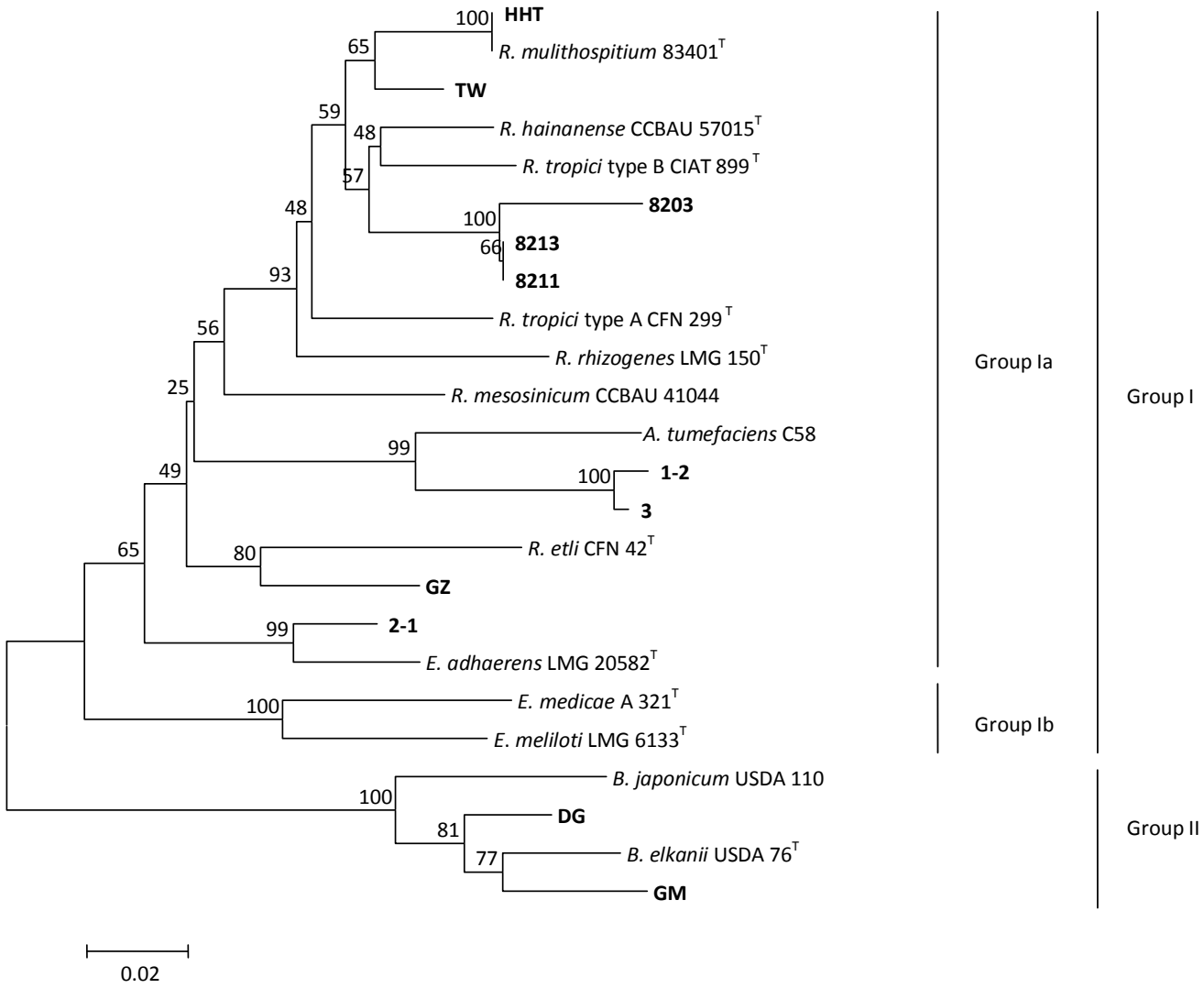

Figure 2. Neighbour-joining phylogeny for *recA* genes of indigenous strains of woody legumes in southern China and reference/type strains (T). Other details as Figure 1. Bar, 2% nucleotide substitutions.

isolate GZ and 6 *Rhizobium* reference/type strains ranged from 96.7 to 98.6%, and were more similar with *R. multihospitium* type strain 83401 in the gene sequence. Thus, isolate GZ formed a differentiated branch within Group Ia (Figure 1).

Isolates 1- 2 and 3 appeared to be closely related to the *A. tumefaciens* cluster, showing 98.8 and 98.4% sequence identity. On the other hand, isolate 2-1 exhibited 99.5% sequence identity to *E. adhaerens* type strain LMG20582, and 97.8% sequence identity to both *E. medicae* type strain A 321 and *E. meliloti* type strain LMG 6133.

Strain DG and GM which were grouped with *B. japonicum* USDA 110 and *Bradyrhizobium elkanii* type strain USDA 76 to compose Group II. The two strains shared the highest sequence identity with *B. elkanii* type strain USDA 76 (99.8 and 99.0%, respectively).

Analyses of individual gene and concatenated sequence of housekeeping genes

The three housekeeping genes, *recA*, *glnII* and *dnaK*, were applied to perform a multilocus sequence analysis. Neighbour-joining trees were constructed for each gene (Figures 2 and 4), as well as for the concatenated sequences of all three genes (Figure 5). These strains clearly formed two groups in each tree, in line with the tree based on the 16S rRNA, except for the dividing into three groups in the analysis of *glnII* (Figure 3).

Isolates TW and HHT were clustered in a branch together with *R. multihospitium* type strain 83401, with high bootstrap values (65 to 100%) in both trees (Figures 2, 3 and 5), except for the *dnaK* tree, in which the isolate TW clustered with *R. hainanense* type strain CCBAU 57015 (Figure 4). However, the two isolates showed

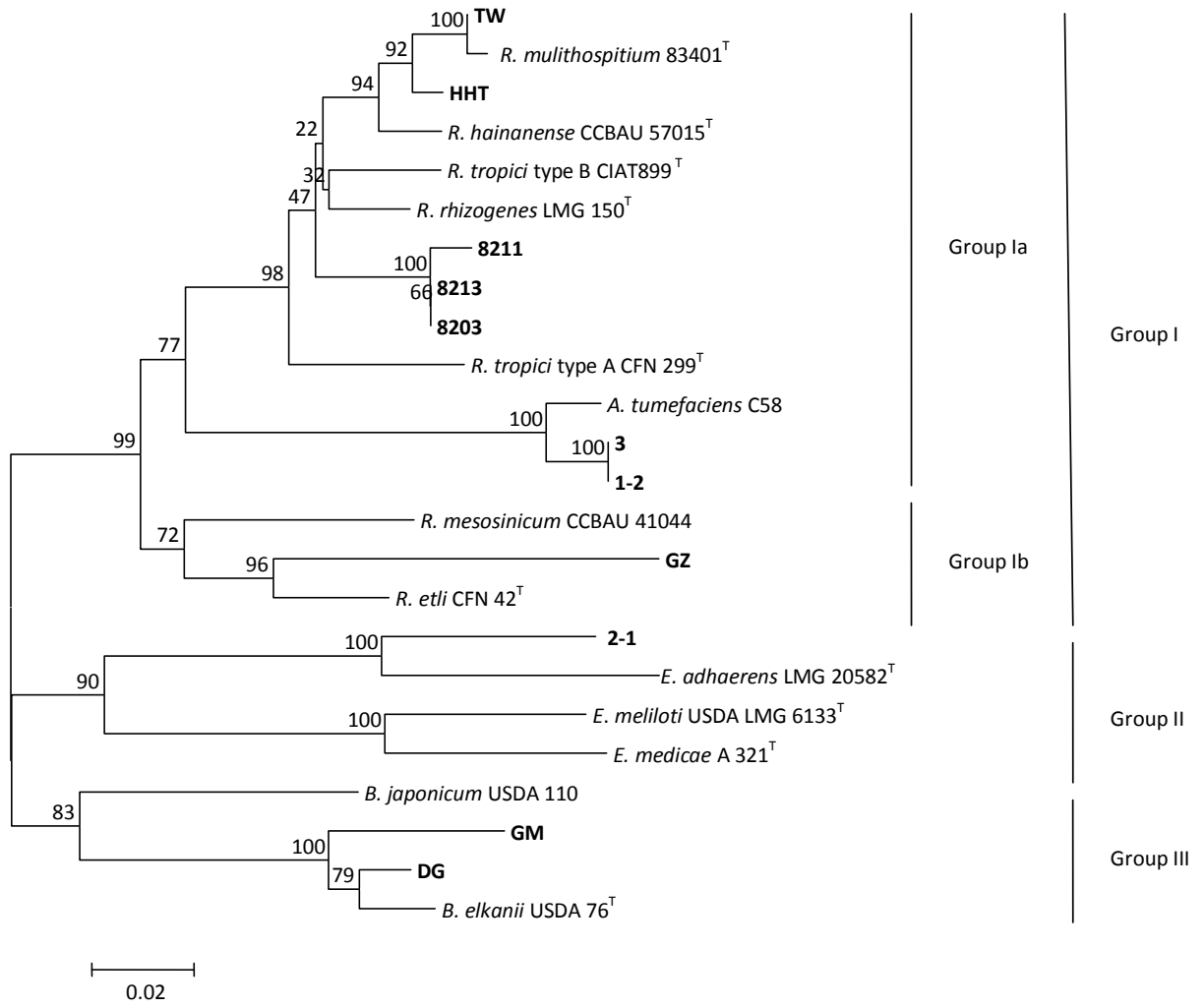

Figure 3. Neighbour-joining phylogeny for *glnII* genes of indigenous strains of woody legumes in southern China and reference/type strains (T). Other details as Figure 1. Bar, 2% nucleotide substitutions.

concatenated tree. In the *recA* tree, the three strains were grouped with *R. hainanense* type strain CCBAU 57015 and *R. tropici* type B strain CIAT 899 (Figure 2). Their *recA* sequences were more related to the *R. hainanense* type strain CCBAU 57015 (95.1, 92.7 and 95.0% similarity, respectively). In the *glnII* tree, the three isolates were clustered independently within Group Ia (Figure 3), and they were also closely related to the *R. hainanense* type strain CCBAU 57015 (95.8, 95.5 and 94.5% similarity, respectively) in *glnII* gene sequence. In the case of the *dnaK* tree, the three strains were grouped with the isolates TW and HHT, *R. hainanense* type strain CCBAU 57015 and *R. multihospitium* type strain 83401 within Group Ia (Figure 4), thus sharing the highest sequence similarity with *R. tropici* type B strain CIAT 899 (95.3, 95.7and 95.3%, respectively). For the concatenated tree, the three strains clustered with isolates TW and HHT, *R. hainanense* type strain CCBAU 57015, *R. multihospitium* type strain 83401 and *R. tropici* type B strain CIAT 899 forming a subgroup (Figure 5), in good agreement with each housekeeping gene tree except with *glnII*.

Isolates 3 and 1- 2 were associated with *A. tumefaciens* C58 and formed a clade with good bootstrap values (98 to 100%) in each individual housekeeping gene and concatenated trees. The level of each gene sequence identity between the *A. tumefaciens* C58 and isolates 3 and 1- 2 varied from 90.1 to 97.9%.

Isolate GZ, another *D. odorifera* isolate, was clustered with *R. etli* type strain CFN 42 and *Rhizobium mesosinicum* strain 41044 on the basis of *glnII*, *dnaK* and concatenated sequence analysis (Figure 3 and 5), thus isolate GZ only clustered with *R. etli* type strain CFN 42 in the *recA* gene analysis (Figure 2). This isolate showed more similar sequence identity with *R. etli* type strain CFN42 and *R. mesosinicum* strain 41044 in each housekeeping gene analysis (91.1 and 91.9% for *recA*; 93.1 and 89.4% for *glnII*; 91.9 and 93.5% for *dnaK*, respectively).

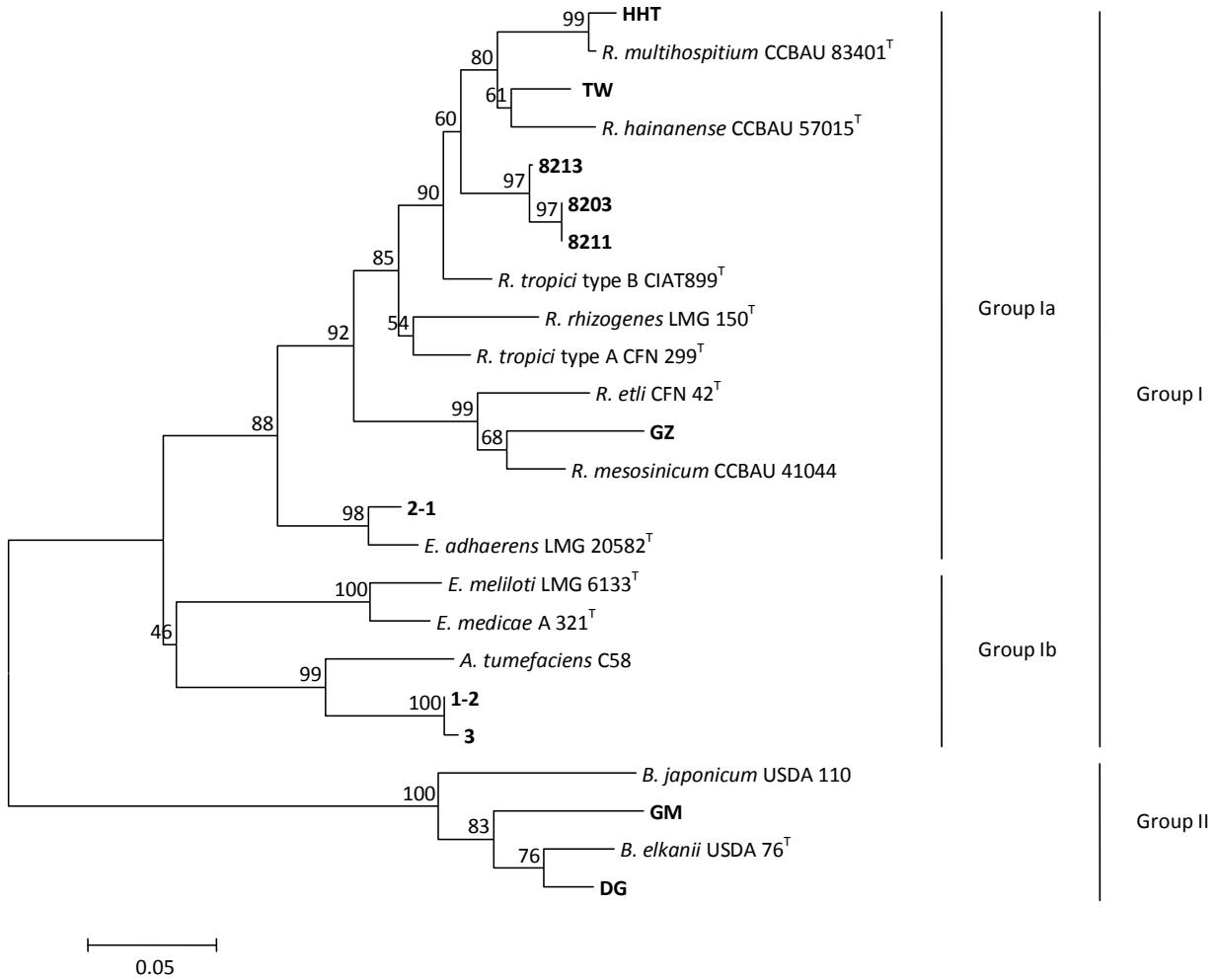

Figure 4. Neighbour-joining phylogeny for *dnaK* genes of indigenous strains of woody legume in southern China and reference/type strains (T). Other details as Figure 1. Bar, 5% nucleotide substitutions.

Although, isolate 2-1 was grouped particularly with *E. adhaerens* type strain LMG20582 with high bootstrap values (99 to 100%), it was separated from another two *Ensifer* species in the *recA* and *dnaK* phylogenies (Figures 2 and 4). The sequence identity between isolate 2-1 and *E. adhaerens* type strain LMG20582 was 96.2% for *recA*, 91.1% for *glnII*, and 97.1% for *dnaK*.

Bradyrhizobium species formed a monophylectic group for all trees, including two test isolates (DG and GM) and two type/reference strains (*B. elkanii* type strain USDA 76 and *B*. *japonicum* USDA 110), with high bootstrap support (64 to 100%) (Figures 2 and 5). In addition, isolates DG and GM were clustered robustly with the *B. elkanii* type strain USDA 76 in both trees, which were congruent in the topologies based on 16S rRNA. Sequences of the same gene for the test strains and *B. elkanii* type strain USDA 76 were similar, and the sequence similarity was 95.3 and 95.0% for *recA*, 97.8 and 95.7% for *glnII*, and 94.7 and 92.4% for *dnaK*, respectively, strongly suggesting that they belong to the same species.

Lastly, all mentioned clusters were supported by higher bootstrap values (≥55%) in the concatenated tree than in the single-gene trees (≥22% for recA tree, ≥25% for *ginII* tree, and ≥46% for *dnaK* tree) indicating the robustness of this approach (Figure 5).

DISCUSSION

In the present study, we characterized 11 rhizobia from five species of woody legumes in southern China, including three endemic and precious leguminous species of *D. odorifera*, *D. fusca* and *E. fordii*. The distribution of these symbiotic isolates among the genera *Agrobacterium* (2 isolates), *Bradyrhizobium* (2 isolates), *Ensifer* (1 isolate) and *Rhizobium* (6 isolates) was supported by both the analysis of 16S rRNA gene and the MLSA of three housekeeping genes. This is the first report that strains of *Rhizobium* sp. constitute the predominant symbionts for multiple woody legume species in southern

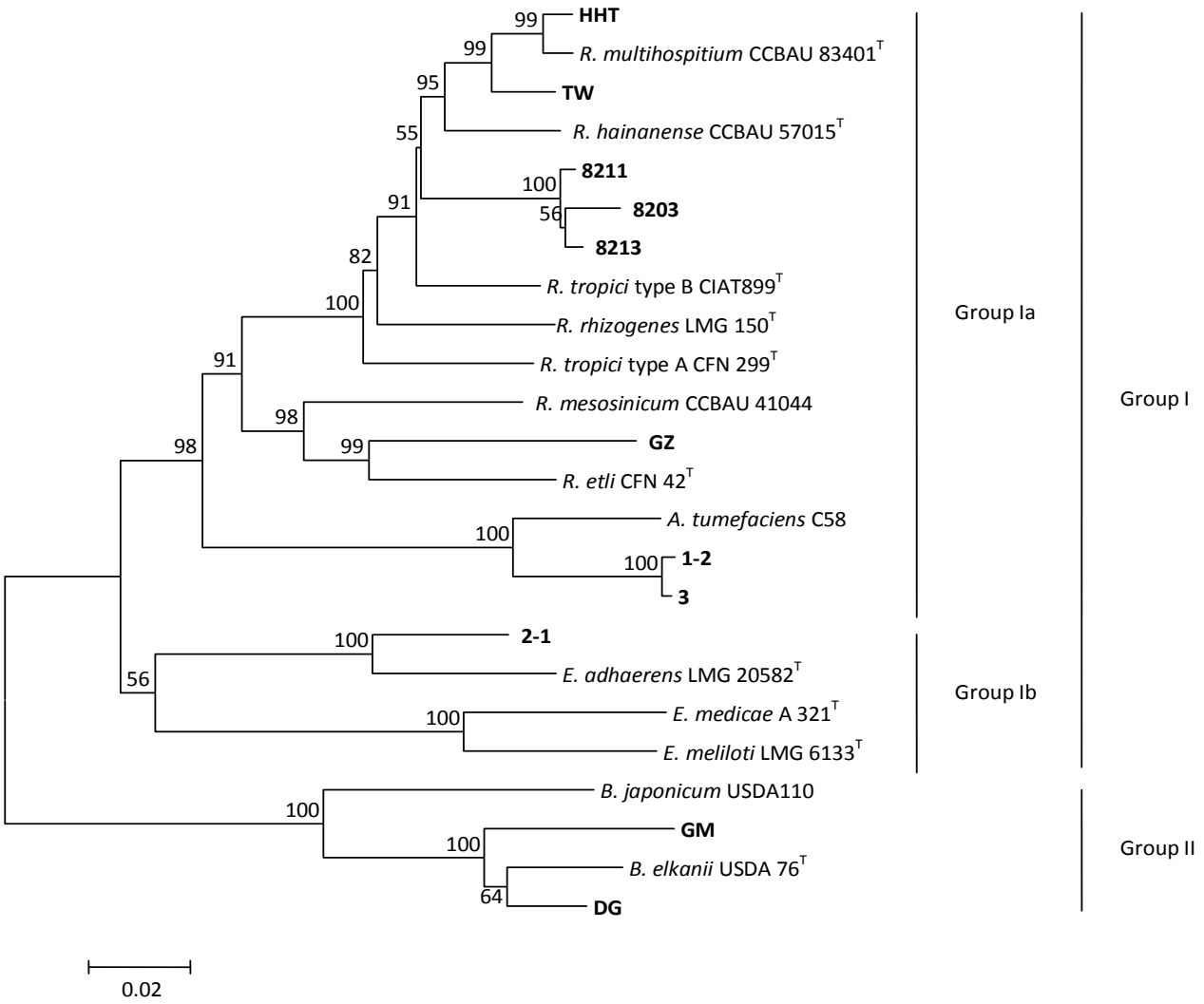

Figure 5. Neighbour-joining phylogeny for concaternated genes *recA* + *glnII* +*dnaK* of indigenous strains of woody legumes in southern China and reference/type strains $^{(T)}$. Other details as Fig. 1. Bar, 2% nucleotide substitutions.

China. Actually, rhizobial diversity of woody legume is poorly documented, especially for rose wood. Rasolomampianina et al. (2005) have isolated seven different bacterial genera from nodules of rose wood legume trees (*Dalbergia* spp.) endemic to Madagascar, which are *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, *Azorhizobium* and *Phyllobacterium* from *Alphaproteobacteria*, and *Burkholderia* and *Ralstonia* from *Betaproteobacteria*, revealing that a wide range of rhizobial species can established effective symbioses with *Dalbergia* species. Gao et al. (1994) isolated three strains from three rose wood species (*D. obtusifolia*, *D. odorifera* and *D. balansae*) native to Hainan Island, China, belonging to *Bradyrhizobium* based on numerical taxonomy and DNA relatedness. More recently, Lin et al. (2009) proposed a novel species *R. mesosinicum* from root nodules of three different woody legume trees (including genera *Albizia*, *Kummerowia* and *Dalbergia*), indicating an unexpected biodiversity of the nodulating rhizobia associated with woody legumes in China.

The MLSA has been verified to be a useful alternative to 16S rRNA sequence analysis and DNA-DNA hybridization for elucidation of the rhizobial taxonomy (Martens et al., 2008; Ribeiro et al., 2009). First of all, MLSA can offer enough and accurate genetic information of rhizobia (Martens et al., 2007, 2008; Ribeiro et al., 2009).

Housekeeping genes not only have faster evolution rate than 16S rRNA gene, but also conserve enough to reserve genetic information (Martens et al., 2007, 2008; Ribeiro et al., 2009). Secondly, housekeeping genes are able to minimize the shortcomings (e.g. sequence mosaicism), which is brought about from genetic recombination and horizontal gene transfer among 16S rRNA and symbiotic genes (Gevers et al., 2005). In addition, it is easier to amplify the housekeeping genes than the symbiotic genes since we were unable to

amplified *nifH* or *nodA* gene from most isolates in this study, except the two *Bradyrhizobium* isolates. This limited us to obtain their further symbiotic information of these isolates. Nowadays, MLSA has been successfully applied to studies with several genera of prokaryotes, including *Nocardia* (Mc Taggart et al., 2010), *Vibrio* (Thompson et al., 2008), *Rhizobium* (Ribeiro et al., 2009), *Bradyrhizobium* (Menna et al., 2009; Vinuesa et al., 2008) and *Ensifer* (Martens et al., 2007, 2008).

Several gene sequences from isolates TW and HHT have a very high similarity to *R. multihospitium* type strain 83401. Moreover, the latter strain is verified to be capable of nodulating *Robinia pseudoacacia* and *Halimodendron halodendron* (Han et al., 2008). According to our nodulation tests, these two tested strains nodulate not only their original hosts, but also *D. odorifera*. Further work will be needed to clarify the nodulation capacity of these strains on various host plants.

Three *Rhizobium* strains (8211, 8213 and 8203) isolated from the Hainan Island, southern China, seem to be closely related to *R. hainanense* type strain CCBAU 57015 in the *recA* and *glnII* gene sequence analysis, and *R. tropici* type B strain CIAT 899 in the 16S rRNA and *dnaK* gene analysis. Surprisingly, these three isolates and *R. hainanense* type strain CCBAU 57015 were also isolated from the Hainan Island (Chen et al., 1997; Gao et al., 1994). Nevertheless, we assume that these strains may represent a novel *Rhizobium* species because of their clustering independently in all phylogenetic trees.

In the 16S rRNA sequence analysis, the strain GZ, which was also isolated from *D. odorifera* in Guangdong Province, Southern China, has 96.7 to 98.6% identity with the recognized *Rhizobium* species, and forms a single clade distant from other reference/type strains. In contrast, in all trees of housekeeping loci analysis except with *recA*, strain GZ was grouped with *R. elti* CFN 42 and *R. mesosinicum* strain 41044, and was confirmed with a bootstrap of 98 to 99% on the concatenated tree (Figure 5). However, strain GZ was most similar to *R. mesosinicum* strain 41044 for *recA* and *dnaK* sequence analysis, and coincidently, *R. mesosinicum* strain 41044 was isolated from *Dalbergia balansae*, another *Dalbergia* species native to Southern China (Lin et al., 2009). Thus, strain GZ had low sequence identity with all recognized species from the *recA* gene analysis (≤91.9%), and this was incongruent with the suggestion that strains of the same species should have at least 94% *recA* gene sequence similarity (Thompson et al., 2005), suggesting that this strain properly belongs to a novel species within *Rhizobium* genus.

Strains 1-2, 3 and 2-1 were isolated from *D. odorifera* in Guangxi Province, China. Both gene sequence analysis, including the 16S rRNA and three housekeeping loci, showed those strains 1-2 and 3 were phylogenetically closely related to *A. tumefaciens* strain C58, and strain 2-1 was more closely related to *E. adhaerens* type strain LMG 20582, although the grouping pattern of these *D. odorifera* isolates showed some variation in different phylogenetic trees. *A. tumefaciens* was konwn to fix nitrogen in a free-living condition (Kanvinde and Sastry, 1990) and form N_2-fixing nodules on legumes harboring other *Rhizobium* plasmids (Martinez et al., 1987; Pankhurst et al., 1983). Interestingly, we could provide evidence on the nodulation capacity of strains 1 to 2 and 3 based on inoculation test, which were capable of nodulating their original host *D. odorifera*, suggesting the presence of *Agrobacterium* in the nodules of *D. odorifera* was not opportunistic (Unpublished data). Therefore, the future work will be needed to amplify the *nodA* gene of strains and verify the gene of these two *Agrobacterium* strains whether obtaining from other rhizobia through lateral-gene transfer.

The 16S rRNA and three housekeeping sequences analysis of two *Bradyrhizobium* isolates identified them as *B. elkanii*. We also searched for the presence of *nodA* and *nifH* gene, and sequence comparisons indicated that *nodA* and *nifH* genes of the two *Bradyrhizobium* isolates are highly similar with *B. elkanii* type strain USDA 76. Gao et al. (1994) identified *Bradyrhizobium* strains among nodule isolates from three *Dalbergia* species growing in previously unexplored environments in Hainan Island. In this study, we could not isolate any *Bradyrhizobium* strain from two *Dalbergia* species (*D. fusca* and *D. odorifera*), although we identified for the first time *Bradyrhizobium* strains in the nodules of *E. fordii* and *P. macarocarpus*, which are taxonomically distant from *Dalbergia* species. In fact, *Bradyrhizobium* is proved to be the dominant microsymbiont of most tropical legume trees (Parker, 2003; Rasolomampianina et al., 2005).

Another important observation from our results is that, according to 16S rRNA and MLSA of three housekeeping genes, the phylogenetic signal of the MLSA are not congruent with that of the 16S rRNA gene used. The 16S rRNA analysis divided *Rhizobium* and *Agrobacterium* into different clades (Figure 1), but the MLSA of each housekeeping genes and concatenated sequence were in good agreement with that the species of these two genera do not form two separate clades (Gaunt et al., 2001). In the 16S rRNA phylogeny, we could not obviously distinct the isolates HHT and TW should be closely related to which reference/type strains, thus these two test strains were clearly grouped with *R. multihospitium* 83401 in each housekeeping gene and concatenated trees. Similarly, even though GZ formed an independent phylogenetic clade in the 16S rRNA tree, it was grouped with the *R. elti* type strain CFN 42 or *R. elti* type strain CFN 42 and *R. mesosinicum* strain 41044 in each of housekeeping gene and concatenated sequence trees, which were more similar to GZ in both housekeeping gene sequences. As a consequence, all these results strongly suggested that the MLSA were more discriminative than the 16S rRNA gene for rhizobial taxonomy.

Our results showed that the MLSA effectively established phylogenetic relationships in tested isolates

with the comparison of reference/type strains, and 16S rRNA should always be combined with data from other genes to accurately assess phylogenetic relationships for rhizobial taxonomy. In addition, the genetic diversity reported in this study lends strong support to the suggestion that several strains classified currently as *Rhizobium* might fit into new species. Polyphasic taxonomic work is under way in our lab so as to confirm this hypothesis. Finally, the obtained rhizobia isolates will be further screened for effective N_2-fixing isolates to be used in woody legume plantations.

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