

Symbiotic efficiency and phylogeny of the rhizobia isolated from *Leucaena leucocephala* in arid–hot river valley area in Panxi, Sichuan, China

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Abstract In search of effective nitrogen-fixing strains for inoculating *Leucaena leucocephala*, we assessed the symbiotic efficiency of 41 rhizobial isolates from root nodules of *L. leucocephala* growing in the arid–hot river valley area in Panxi, China. The genetic diversity of the isolates was studied by analyzing the housekeeping genes 16S rRNA and *recA*, and the symbiotic genes *nifH* and *nodC*. In the nodulation and symbiotic efficiency assay, only 11 of the 41 isolates promoted the growth of *L. leucocephala* while the majority of the isolates were ineffective in symbiotic nitrogen fixation. Furthermore, one fourth of the isolates had a growth slowing effect on the host. According to the 16S rRNA and *recA* gene analyses, most of the isolates were *Ensifer* spp. The remaining isolates were assigned to *Rhizobium*, *Mesorhizobium* and *Bradyrhizobium*. The sequence analyses indicated that the *L. leucocephala* rhizobia had undergone gene recombination. In contrast to the promiscuity observed as a wide species distribution of the isolates, the results implied that *L. leucocephala* is preferentially nodulated by strains that share common symbiosis genes. The symbiotic efficiency was not connected to chromosomal background of the symbionts and isolates carrying a similar *nifH* or *nodC* showed totally different nitrogen fixation efficiency.

Keywords *Leucaena leucocephala* · Rhizobia · Housekeeping genes · Genetic diversity · Phylogeny · Symbiotic efficiency

Introduction

Rhizobia are well known for their ability to carry out symbiotic nitrogen fixation by forming nodules on the roots or occasionally on the stems of legumes. Owing to this ability, leguminous plants can grow in arid, infertile soils, acting as pioneer plants that stabilize soil and enhance fertility, thereby preventing water loss, soil erosion and desertification (Howieson et al. 2008; Sprent and Gehlot 2010).

Panxi (Panzhihua–Xichang) in the southwest of Sichuan province, China, is at the transitional zone from Qinhai–Tibet and Yunnan–Guizhou plateaus to Sichuan basin. The arid–hot river valley area in Panxi, characterized with xerothermic climate and abundant photothermal source, is the northernmost place with the Southeast Asia tropical climate (Zhao et al. 2011). Because of overcutting and shortage of fuel wood, Panxi area suffers from soil erosion and environmental deterioration. Therefore, improving environment and preserving water and soil by reforestation are imperative long-term tasks in this area.

Leucaena leucocephala is a fast-growing, tropical leguminous tree species in the subfamily *Mimosoideae*. It is referred as the miracle tree because of its exceptional capacity to produce biomass and protein (Somasegaran and Martin 1986). It is also used in agroforestry, soil improvement, preventing soil erosion, land reclamation and for firewood, timber, forage, and green manure (Moawad and Bohlool 1984; Somasegaran and Martin 1986). In its region of origin, Mexico, *L. leucocephala* is nodulated by a diverse selection of

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rhizobia, including *Ensifer* (*Sinorhizobium*), *Rhizobium*, and *Mesorhizobium* (Wang et al. 1999). The amount of nitrogen fixed by *L. leucocephala* rhizobium symbiosis is similar to or higher than that of crop legumes such as peanut and soybean, and most of the fixed nitrogen is returned to the soil in the leaf litter (Peoples et al. 1995). *L. leucocephala* was introduced into Panxi in the 1980s and 1990s as a pioneer species of planted forests. The planted *L. leucocephala* were not inoculated with rhizobia, and the indigenous *Leucaena* rhizobia in this area have not yet been investigated and described. We became aware that inoculants would be able to promote *L. leucocephala* development. Consequently, the first objective of this investigation was to isolate Panxi area *Leucaena* rhizobia and to find strains effective in plant growth promotion for inoculation. The second objective was to investigate the genetic diversity, the phylogeny and the taxonomic position of these isolates based on housekeeping genes 16S rRNA and *recA*, and on symbiosis related genes *nifH* and *nodC*.

Materials and methods

Isolation of nodule bacteria

Nodules were collected from lateral roots of *L. leucocephala* growing in 20 sites in the arid-hot river valley area in Panxi, China (Table 1). Bacteria were isolated from surface-sterilized nodules using the standard procedure and yeast extract-mannitol agar (YMA) medium (Vincent 1970). Single colonies were picked up and checked for purity by repeatedly streaking on YMA medium (Vincent 1970), and verified by colony morphology, absorption of Congo red (25 mg ml⁻¹) and Gram reaction. All the isolates were incubated on YMA slants at 28 °C and maintained at 4 °C for temporary storage and in 20 % glycerol at -70 °C for long-term storage.

In the restriction fragment length polymorphism (RFLP) analysis, 14 reference strains were included, as follows: *Bradyrhizobium japonicum* USDA6^T, *Bradyrhizobium yuanmingense* CCBAU10071^T, *Bradyrhizobium liaoningense* USDA3622^T; *Rhizobium mongolense* USDA1844^T, *Rhizobium hainanense* CCBAU 57015^T and *Rhizobium huautlense* LMG18254^T; *Ensifer/Sinorhizobium fredii* USDA205^T, *Ensifer adhaerens/Sinorhizobium morelense* LMG21331^T and *Ensifer/Sinorhizobium kostiense* HAM-BI1489^T; *Mesorhizobium ciceri* USDA3383^T and *Mesorhizobium plurifarium* LMG11892^T; and *Agrobacterium rubi* IAM13569^T, *Agrobacterium vitis* IAM14140^T and, *Allorhizobium undicola* LMG11875^T.

Nodulation and symbiotic efficiency assays

The nodulation ability and the symbiotic efficiency of the isolates were tested by plant inoculation assay on their

original host plant (Table 1). *L. leucocephala* seeds were surface sterilized, placed on sterile Whatman filter paper, and germinated in sterile water in Petri dishes for 3–5 days. The seedlings were transplanted into glass bottles containing Jensen's solution with 0.003 % of Ca(NO₃)₂ as starter nitrogen in all inoculation assays, including the uninoculated controls. The fast- and slow-growing isolates were grown in YEM broth (Vincent 1970) for 5 and 7 days, respectively. The seedlings were inoculated with 1.5 ml of the culture containing ca 10¹⁰ bacterial cells per milliliter and grown under a 16 h light and 8 h dark regime at 25 °C (Chen et al. 2003). All inoculation assays were done in triplicate. After 56 days, the plants were harvested, and the nodule numbers and plant shoot dry weights were determined. The data was analyzed by one-way analysis of variance, with the mean values compared using SPSS15.0 least significant difference (LSD) analysis ($P=0.05$).

DNA extraction and PCR-RFLP analysis of the 16S rRNA gene

The subsequent DNA analyses were done on the 41 isolates that successfully nodulated *L. leucocephala* (Table 2). Total genomic DNA was obtained after lysozyme-sodium dodecyl sulfate lysis, followed by phenolchloroform extraction and ethanol precipitation as described by Li et al. (2009).

16S rRNA gene fragments were amplified using 20 pmol of each primer P1 and P6 (Li et al. 2009) in 50 µl of amplification buffer (250 µM of each dNTP, 10 mM Tris-HCl [pH 8.3], 1.5 mM MgCl₂, 50 mM KCl and 2.5 U Taq DNA polymerase), with about 50 ng of total DNA as template. The amplification was performed using a Bio-RAD MyCycler™ with the following temperature profile: an initial denaturation at 92 °C for 3 min; 30 cycles of denaturation at 94 °C for 3 min, annealing at 58 °C for 1 min and extension at 72 °C for 2 min; and a final extension at 72 °C for 6 min, with a final soak at 4 °C. The size of the amplification products was verified by electrophoresis in 1 % agarose gel.

Aliquots (5 µl) of 16S polymerase chain reaction (PCR) products were individually digested with restriction endonucleases *MspI*, *HinfI*, *HaeIII*, and *TaqI* (MBI, Fermentas; 5 U per reaction) as specified by the manufacturer in a total volume of 10 µl. The fragments were separated by electrophoresis in 2.0 % agarose at 120 V for 3 h. Gels were stained with ethidium bromide and photographed with Gel Document System. The band patterns of RFLP analysis were converted into a two-dimensional binary matrix through a binary scoring system and the similarities were evaluated by simple matching coefficient. Dendrograms were constructed from the distance matrix by UPGMA clustering algorithm in NTSYS-pc (2.1) program (Rohlf

Table 1 Bacterial isolates from *L. leucacephala* and summary of nucleotide identity of the 16 S rRNA, *recA*, *nifH* and *nodC* sequences of the representative isolates

Isolates ^a	Sampling site ^b	Altitude (m)	Clade in phylogenetic tree			Phylogenetic affiliation ^c			
			rRNA	<i>recA</i>	<i>nifH</i>	<i>nodC</i>	<i>recA</i>	<i>nifH</i>	<i>nodC</i>
SCAU190, SCAU191	SCAU191	790	rRNA-E2	<i>recA</i> -E2	<i>nifH</i> -E	<i>nodC</i> -E	<i>E. mexicanus</i> HAMB1 2910 ^T (99.5 %)	<i>E. americanum</i> CFNEI 156 ^T (93.8 %)	<i>E. melioli</i> bv. <i>mediterraneanse</i> LILM4H41 (90.0 %)
SCAU192, SCAU194	SCAU194	920	rRNA-E2	<i>recA</i> -E2	<i>nifH</i> -E	<i>nodC</i> -E	<i>E. mexicanus</i> HAMB1 2910 ^T (99.7 %)	<i>E. americanum</i> CFNEI 156 ^T (93.8 %)	<i>E. melioli</i> bv. <i>mediterraneanse</i> LILM4H41 (90.3 %)
SCAU195, SCAU196	SCAU196	720	rRNA-E2	<i>recA</i> -E2	<i>nifH</i> -E	<i>nodC</i> -E	<i>E. mexicanus</i> HAMB1 2910 ^T (99.1 %)	<i>E. americanum</i> CFNEI 156 ^T (93.7 %)	<i>E. melioli</i> bv. <i>mediterraneanse</i> LILM4H41 (89.9 %)
SCAU197, SCAU198	SCAU198	1,280	rRNA-E2	<i>recA</i> -E2	<i>nifH</i> -E	<i>nodC</i> -E	<i>E. mexicanus</i> HAMB1 2910 ^T (99.2 %)	<i>E. americanum</i> CFNEI 156 ^T (93.8 %)	<i>E. melioli</i> bv. <i>mediterraneanse</i> LILM4H41 (90.3 %)
SCAU199, SCAU200	SCAU200	720	rRNA-E2	<i>recA</i> -E2	<i>nifH</i> -E	<i>nodC</i> -E	<i>E. mexicanus</i> HAMB1 2910 ^T (99.7 %)	<i>E. americanum</i> CFNEI 156 ^T (93.7 %)	<i>E. melioli</i> bv. <i>mediterraneanse</i> LILM4H41 (89.9 %)
SCAU201, SCAU202A, SCAU203	SCAU203	1,170	rRNA-M	<i>recA</i> -M	<i>nifH</i> -M2	<i>nodC</i> -M2	<i>M. plurifarium</i> LMG 11892 ^T (99.6 %)	<i>M. robiniae</i> ACCC 14543 ^T (99.1 %)	<i>M. robiniae</i> ACCC 14543 ^T (99.1 %)
SCAU204	SCAU204	1,110	rRNA-R	<i>recA</i> -R	–	–	<i>R. huaulense</i> LMG18254 ^T (99.9 %)	<i>R. huaulense</i> LMG18254 ^T (99.8 %)	NO
SCAU205, SCAU206, SCAU207	SCAU207	1,230	rRNA-B	<i>recA</i> -B	<i>nifH</i> -B	<i>nodC</i> -B	<i>B. japonicum</i> USDA 6 ^T (99.6 %)	<i>B. japonicum</i> USDA 3622 ^T (95.1 %)	<i>B. japonicum</i> USDA 6 ^T (98.1 %)
SCAU208, SCAU209, SCAU210, SCAU211	SCAU211	1,480	rRNA-E2	<i>recA</i> -E2	<i>nifH</i> -E	<i>nodC</i> -E	<i>E. mexicanus</i> HAMB1 2910 ^T (99.6 %)	<i>E. americanum</i> CFNEI 156 ^T (93.8 %)	<i>E. melioli</i> bv. <i>mediterraneanse</i> LILM4H41 (90.3 %)
SCAU212	SCAU212	1,440	rRNA-E2	<i>recA</i> -E2	<i>nifH</i> -E	<i>nodC</i> -E	<i>M. plurifarium</i> LMG 11892 ^T (99.6 %)	<i>M. plurifarium</i> LMG 11892 ^T (94.0 %)	<i>M. plurifarium</i> LMG 11892 ^T (89.6 %)
SCAU213	SCAU213	1,930	rRNA-E1	<i>recA</i> -E1	<i>nifH</i> -E	<i>nodC</i> -E	<i>E. sabehi</i> LMG 7837 ^T (99.4 %)	<i>E. americanum</i> CFNEI 156 ^T (93.8 %)	<i>E. melioli</i> bv. <i>mediterraneanse</i> LILM4H41 (90.3 %)
SCAU214, SCAU215, SCAU216, SCAU217, SCAU218	SCAU218	1,360	rRNA-E1	<i>recA</i> -E1	<i>nifH</i> -E	<i>nodC</i> -E	<i>E. fredii</i> USDA205 ^T (99.2 %)	<i>E. xinjiangense</i> LMG 17930 ^T (95.0 %)	<i>E. melioli</i> bv. <i>mediterraneanse</i> LILM4H41 (90.3 %)
SCAU219, SCAU220, SCAU221	SCAU221	1,070	rRNA-E3	<i>recA</i> -E3	<i>nifH</i> -E	<i>nodC</i> -E	<i>E. melioli</i> LMG 6133 ^T (99.7 %)	<i>E. americanum</i> CFNEI 156 ^T (96.7 %)	<i>E. melioli</i> bv. <i>mediterraneanse</i> LILM4H41 (92.1 %)
SCAU222, SCAU223	SCAU223	1,080	rRNA-E1	<i>recA</i> -E1	<i>nifH</i> -E	<i>nodC</i> -E	<i>E. xinjiangense</i> LMG 17930 ^T (95.0 %)	<i>E. americanum</i> CFNEI 156 ^T (93.7 %)	<i>E. melioli</i> bv. <i>mediterraneanse</i> LILM4H41 (90.3 %)
SCAU224	SCAU224	1,100	rRNA-E1	<i>recA</i> -E1	<i>nifH</i> -E	<i>nodC</i> -E	<i>E. xinjiangense</i> LMG 17930 ^T (95.0 %)	<i>E. americanum</i> CFNEI 156 ^T (93.8 %)	<i>E. melioli</i> bv. <i>mediterraneanse</i> LILM4H41 (90.3 %)
SCAU225	SCAU225	1,200	rRNA-E1	<i>recA</i> -E1	<i>nifH</i> -E	<i>nodC</i> -E	<i>E. xinjiangense</i> LMG 17930 ^T (95.0 %)	<i>E. americanum</i> CFNEI 156 ^T (93.8 %)	<i>E. melioli</i> bv. <i>mediterraneanse</i> LILM4H41 (90.3 %)
SCAU226	SCAU226	1,200	rRNA-E1	<i>recA</i> -E1	<i>nifH</i> -E	<i>nodC</i> -E	<i>E. xinjiangense</i> LMG 17930 ^T (95.0 %)	<i>E. americanum</i> CFNEI 156 ^T (93.8 %)	<i>E. melioli</i> bv. <i>mediterraneanse</i> LILM4H41 (90.3 %)
SCAU227, SCAU228	SCAU228	1,230	rRNA-E1	<i>recA</i> -E1	<i>nifH</i> -E	<i>nodC</i> -E	<i>E. xinjiangense</i> LMG 17930 ^T (95.0 %)	<i>E. americanum</i> CFNEI 156 ^T (93.8 %)	<i>E. melioli</i> bv. <i>mediterraneanse</i> LILM4H41 (90.3 %)
SCAU229, SCAU230, CAU231	CAU231	1,250	rRNA-E1	<i>recA</i> -E1	<i>nifH</i> -E	<i>nodC</i> -E	<i>E. xinjiangense</i> LMG 17930 ^T (95.0 %)	<i>E. americanum</i> CFNEI 156 ^T (93.8 %)	<i>E. melioli</i> bv. <i>mediterraneanse</i> LILM4H41 (90.3 %)

^a The isolates in bold face were used in 16S rRNA, *recA*, *nifH*, and *nodC* genes sequencing

^b Sampling sites: 1, 3, and 4 Hulukou Town Ningnan County Liangshan Prefecture (LP); 6 and 7 Pisha Town Ningnan County LP; 8 and 9 Puji Town Puge County LP; 11 and 15 Yinjiang Town East District Panzhuhua City (PC); 14 and 16 Tongzilin Town Yanbian County PC; 2 Jinxing Town Ningnan County LP; 5 Dachong Town Huidong County LP; 10 Xiaoliao Town Xichang City LP; 12 Jinjiang Town Renhe District PC; 13 Dalongtan Town Renhe District PC; 17 Yakou Town Miyi County PC; 18 Baima Town Miyi County PC; 19 Wangqiu Town Miyi County PC; 20 Xijei Town Miyi County PC

^c The percentages are sequence similarities to the closely related species or the closest type strain
NO no obtained

Table 2 Symbiotic traits and 16S rRNA gene RFLP characteristics of rhizobia isolated from *L. leucocephala*

Isolates no.	Symbiotic traits		PCR-RFLP of 16S	
	Plant shoot dry weights (g/plant) ^a	No. of nodules (per plant)	16S rRNA gene RFLP patterns ^b	16S rRNA genotype ^c
SCAU223	0.0756±0.0115 ↑*	9.7	AAAA	I
SCAU221	0.0753±0.0112 ↑*	12.7	AAAA	I
SCAU202A	0.0687±0.0206 ↑*	7.3	ADDD	IV
SCAU225	0.0657±0.0054 ↑*	12.0	AAAA	I
SCAU191	0.0652±0.0030 ↑*	5.7	AAAA	I
SCAU196	0.0649±0.0069 ↑*	7.3	AAAA	I
SCAU195	0.0621±0.0032 ↑*	5.0	AAAA	I
SCAU217	0.0592±0.0084 ↑*	9.0	AABA	IX
SCAU198	0.0589±0.0024 ↑*	7.3	AAAA	I
SCAU214	0.0583±0.0048 ↑*	2.3	AAAA	I
SCAU _{non4}	0.0567±0.0060 ↑*	0.0	–	–
SCAU229	0.0561±0.0021 ↑*	16.7	AAAA	I
SCAU204	0.0540±0.0067 ns	21.3	AAAA	I
SCAU201	0.0530±0.0022 ns	4.3	AAAA	I
SCAU199	0.0527±0.0045 ns	14.0	AAAA	I
SCAU215	0.0515±0.0045 ns	5.3	AGDC	VIII
SCAU222	0.0511±0.0039 ns	6.3	AAAA	I
SCAU207	0.0510±0.0015 ns	12.7	AAAA	I
SCAU212	0.0507±0.0013 ns	7.7	AAAA	I
SCAU220	0.0495±0.0054 ns	13.3	AAAA	I
SCAU190	0.0493±0.0012 ns	3.3	AAAA	I
SCAU203	0.0489±0.0094 ns	4.3	CEEE	V
SCAU205	0.0461±0.0032 ns	2.0	ACDD	III
NC	0.0458±0.0029	0.0	–	–
SCAU208	0.0457±0.0056 ns	16.0	DFCE	VI
SCAU206	0.0454±0.0045 ns	5.3	AAAA	I
SCAU224	0.0454±0.0050 ns	7.7	AABA	IX
SCAU _{non1}	0.0437±0.0076 ns	0.0	–	–
SCAU _{non2}	0.0431±0.0038 ns	0.0	–	–
SCAU200	0.0430±0.0019 ns	9.7	AAAA	I
SCAU219	0.0425±0.0021 ns	11.3	AAAA	I
SCAU _{non3}	0.0423±0.0040 ns	0.0	–	–
SCAU211	0.0406±0.0028 ns	6.7	EAGE	VII
SCAU230	0.0381±0.0020 ns	7.3	AAAA	I
SCAU192	0.0376±0.0039 ns	4.6	BBAB	II
SCAU194	0.0370±0.0096 ns	8.0	AAAA	I
SCAU210	0.0353±0.0031 ↓*	16.7	EAGE	VII
SCAU216	0.0345±0.0044 ↓*	14.3	AABA	IX
SCAU213	0.0340±0.0040 ↓*	8.7	AAAA	I
SCAU209	0.0330±0.0015 ↓*	5.3	DFCE	VI
SCAU197	0.0303±0.0042 ↓*	2.0	AAAA	I
SCAU226	0.0296±0.0052 ↓*	3.0	AAAA	I
SCAU218	0.0287±0.0033 ↓*	9.0	AAAA	I
SCAU228	0.0275±0.0045 ↓*	5.3	AAAA	I
SCAU231	0.0248±0.0036 ↓*	15.3	FFCE	X
SCAU227	0.0221±0.0072 ↓*	3.0	AAAA	I

NC uninoculated control
 Because SCAU_{non1}, SCAU_{non2}, SCAU_{non3}, SCAU_{non4} did not nodulate *L. leucocephala*, they were not included in the genetic diversity studies

(↑) increase, (↓) decrease, and *ns* nonsignificant difference in plant shoot dry weight according to the LSD test (at **p*=0.05 level)

^aData presented as average± standard deviation from three replicates

^bEach letter refers to a restriction pattern obtained with enzymes *Hae*III, *Hinf*I, *Msp*I, and *Taq*I, respectively

^cThe 16S rRNA genotypes of *Leucaena* rhizobia represent combination of restriction patterns obtained by enzymes used (*Hae*III, *Hinf*I, *Msp*I, and *Taq*I)

1990). Isolates sharing the same RFLP patterns were classified as an rRNA genotype.

16S rRNA, *recA*, *nifH*, and *nodC* gene sequencing

Based on the symbiotic effectiveness of the isolates and on the PCR-RFLP, 15 representative isolates were chosen for sequencing of the 16S rRNA, *recA*, *nifH*, and *nodC* genes. The 16S rRNA gene was amplified as for 16S RFLP. The *recA* gene was amplified as described using primers *recAF1* and *recAR1*, with the exception of SCAU209 and SCAU211 whose *recA* was amplified using *recAF2* and *recAR2* (Gaunt et al. 2001). The *nifH* gene was amplified using primers *nifHF* and *nifHI* as described (Laguerre et al. 2001). Five forward primers, *nodCF*, *nodCFu*, *nodCF2*, *nodCF4*, and *nodCFn*, and the reverse primer *nodCR* were used for amplification and sequencing of the *nodC* gene (Laguerre et al. 2001). The amplification products of 16S rRNA, *recA* and *nodC* genes were directly purified and sequenced (Invitrogen, Shanghai, China). The purified *nifH* gene fragments were cloned into a pMD19-T vector (Takara, Dalian, China) transformed into competent *Escherichia coli* DH5 α cells and examined with M13 primers according to the manufacturer's instructions before sequencing at Invitrogen, Shanghai, China. The quality of the sequences was verified by sequencing both strands.

The sequences of representative isolates and the reference sequences were aligned using ClustalW as implemented in Mega4.0 (Tamura et al. 2007). Phylogenetic trees were constructed using the Neighbor-joining method in MEGA program version 4.0 (Tamura et al. 2007), and confidence level determined by 1,000 replicates bootstrapping. The percentage similarity of the genes was estimated using DNAMAN (version 6.0; Woffelman 1994). Isolates were assigned to genus or species with the highest sequence similarity with the type strain. The sequences obtained in this study were submitted to the GenBank sequence database under the accession numbers JQ362359–JQ362389, JF330102–JF330106, JF330109–JF330110, HQ538614, HQ538616, HQ538622, HQ538618–HQ538620, and JX073914–JX073927.

Results

Symbiotic effectiveness of *L. leucocephala* rhizobia

When searching for effective strains for inoculating *L. leucocephala* in Panxi, altogether 45 isolates were obtained from *L. leucocephala* root nodules. From them, 41 isolates that were able to nodulate *L. leucocephala* plants were called *Leucaena* rhizobia. Four isolates were not considered as rhizobia because they did not form nodules on their

original host (Table 2). However, one of the four non-nodulating isolates, isolate SCAUon4 increased plant shoot dry weight significantly (Table 2), indicating that some endophytic non-nodulating bacteria are able to promote plant growth.

Plant shoot dry weight was consistent with plant shoot fresh weight (data not shown), and correlation ($r=0.96^{**}$) was significant at the 0.01 level (two-tailed). Only 11 of 41 *Leucaena* rhizobia promoted the growth of *L. leucocephala* significantly (56.1–75.6 mg of shoot dry weight/plant) as compared to the uninoculated control (45.8 mg of shoot dry weight/plant; Table 2). Hence, the 11 isolates were good candidates and among them isolates SCAU223 and SCAU221 the best candidates for inoculating *L. leucocephala* in this area (Table 2). Over two thirds of the tested rhizobial isolates were ineffective in symbiotic nitrogen fixation. Strikingly, 10 *Leucaena* rhizobial isolates could be considered as parasitic because plant shoot dry weight decreased significantly (22.1–35.3 mg of shoot dry weight/plant) as compared to the uninoculated control. On the average, both the effective nitrogen fixing and parasitic isolates formed from two to 17 nodules per plant (Table 2).

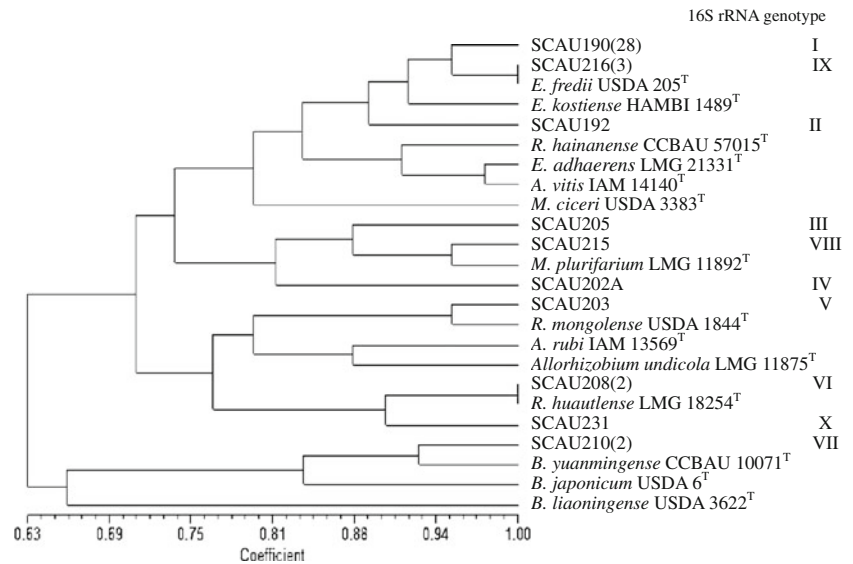
RFLP analyses of the 16S rRNA gene

To preliminary estimate the taxonomic positions of isolates, their 16S rRNA RFLP restriction profiles were compared with the restriction profiles of 14 reference strains that represented different rhizobial species and genera (Table 2). A dendrogram was constructed by UPGMA algorithm based on the combined restriction patterns (Fig. 1). This analysis resulted in six, seven, five, and five different restriction patterns of 41 *Leucaena* rhizobia for *HaeIII*, *HinfI*, *MspI*, and *TaqI*, respectively. Restriction patterns were arbitrarily identified by letters and used to assign the isolates into RFLP groups (Table 2). For the 41 isolates analyzed, 10 distinct genotypes were distinguished. Genotype I comprising 28 isolates was the most frequent 16S rRNA genotype (Table 2). According to the 16S RFLP analysis, *Leucaena* rhizobia were distributed into four genera. *Ensifer* (32 isolates) was the dominant genus. The remaining isolates were assigned as *Rhizobium* (four isolates), *Mesorhizobium* (three isolates), and *Bradyrhizobium* (two isolates).

Phylogenetic analyses of 16S rRNA and *recA* genes

To get more detailed information on the diversity of the *L. leucocephala* rhizobia, two housekeeping genes (16S rRNA and *recA*) of 15 representative isolates were sequenced. Amplification of the 16S rRNA and *recA* genes was successful, resulting in a single fragment of about 1,500 and 500 bp, respectively (data not shown).

Fig. 1 UPGMA dendrogram constructed based on the RFLP analysis of PCR-amplified 16S rRNA genes of the isolates and reference strains using four restriction endonucleases (*MspI*, *HinfI*, *HaeIII*, and *TaqI*). Genotype I to X (indicated on the right) are described in Table 2. Numbers in parentheses show the number of isolates with the same 16S rRNA genotype



The assignments of the 15 representative isolates as defined by 16S rRNA gene were consistent with 16S RFLP at the genus level (Figs. 1 and 2). In the phylogenetic analyses of the 16S rRNA gene, the 15 representative strains were grouped

into four main groups corresponding to *Ensifer*, *Rhizobium*, *Mesorhizobium*, and *Bradyrhizobium* or six clades in detail (Fig. 2). Most isolates (11/15) were assigned to genus *Ensifer*, and were distributed over three distinct clades. Four isolates

Fig. 2 Phylogenetic tree of 16S rRNA gene sequences showing the relationships between the representative isolates and the reference strains for defined rhizobial species. Bootstrap values >50 % are given at the branching points. The scale bar indicates the number of substitutions per site. Sequence accession numbers are given in parentheses. The representative isolates are shown in **bold**. ↑*, ↓*, and *ns* indicates an increase, a decrease or a non-significant difference in plant shoot dry weight according to the LSD test (at **p*=0.05 level), respectively

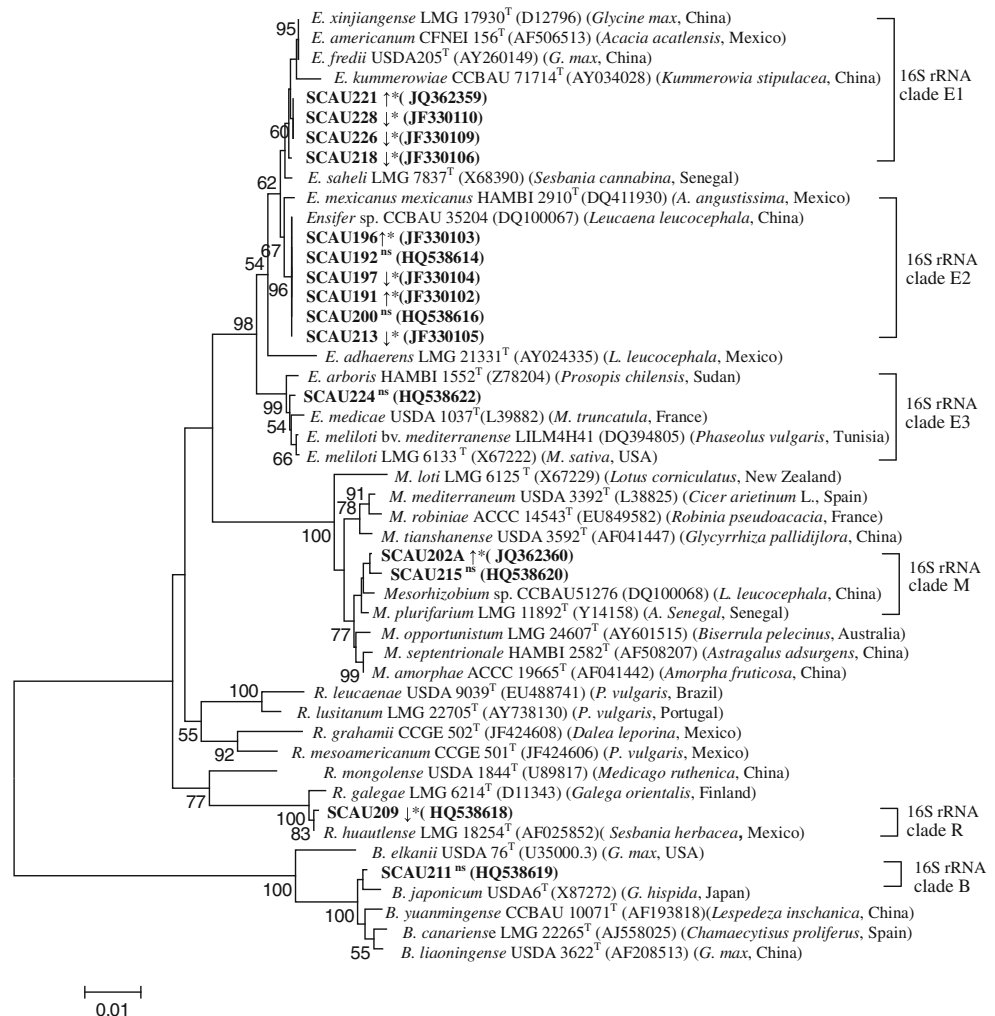
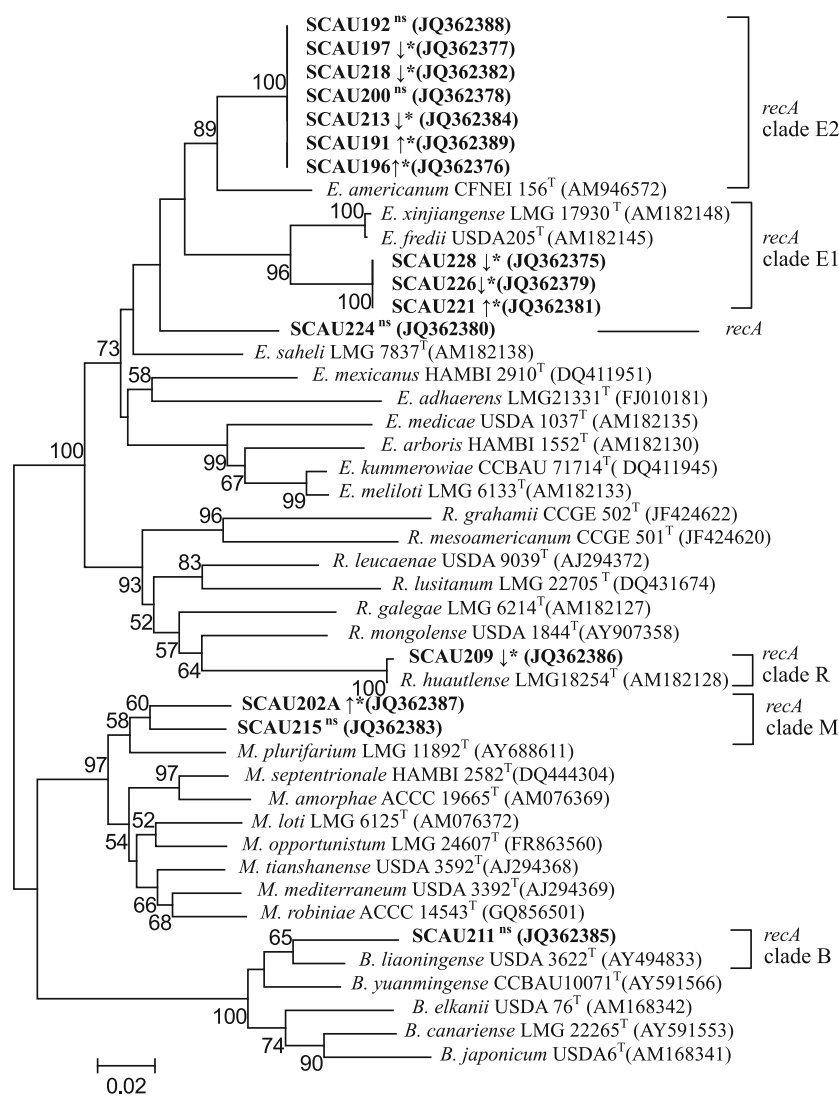


Fig. 3 Phylogenetic tree of *recA* gene sequences showing the relationships between the representative isolates and the reference strains for defined rhizobial species. Bootstrap values >50 % are given at the branching points. The scale bar indicates the number of substitutions per site. Sequence accession numbers are given in parentheses. The representative isolates are shown in *bold*. ↑*, ↓*, and *ns* indicates an increase, a decrease or a non-significant difference in plant shoot dry weight according to the LSD test (at * $p=0.05$ level), respectively



clustered together with *Ensifer xinjiangense* LMG 17930^T, *E. fredii* USDA205^T, *Ensifer americanum* CFNEI 156^T, and *Ensifer kummerowiae* CCBAU 71714^T, and represented a distinct phylogenetic branch (clade E1). Six isolates clustered together with *Ensifer mexicanus* HAMBI 2910^T with similarities ranging from 99.1 to 99.7 % (clade E2; Table 1, Fig. 2). SCAU224 clustered with *Ensifer meliloti* LMG 6133^T, *Ensifer medicae* USDA 1037^T, and *Ensifer arboris* HAMBI 1552^T (clade E3). In the *Mesorhizobium* group, SCAU202A and SCAU215 each shared 99.6 % similarity and clustered together with *M. plurifarium* LMG 11892^T (Table 1, Fig. 2; clade M). SCAU209 formed a distinct clade (clade R) with *Rhizobium huautlense* LMG18254^T (99.9 % sequence similarity) in the *Rhizobium* group. SCAU211 clustered together with *Bradyrhizobium japonicum* USDA6^T (99.6 % sequence similarity) in *Bradyrhizobium* group (clade B; Table 1, Fig. 2).

In the phylogenetic analysis of the *recA* gene, the 15 representative isolates were grouped into four main groups *Ensifer*, *Rhizobium*, *Mesorhizobium*, and *Bradyrhizobium* or

six clades in detail as in the 16S rRNA tree (Figs. 2 and 3). The *recA* phylogeny was consistent with the 16S rRNA phylogeny at the genus and species level in *Rhizobium* and *Mesorhizobium* (Figs. 2 and 3, Table 1). However, the topology of the *recA* tree was different from that of the 16S rRNA tree for the *Ensifer* group. In the *recA* clade E1, three isolates had 100 % *recA* sequence similarity and formed a sister clade to *E. xinjiangense* LMG 17930^T and *E. fredii* USDA205^T. SCAU218, a member of 16S rRNA clade E1, had 100 % *recA* sequence similarity with the six isolates from 16S rRNA clade E2. Together, they formed *recA* clade E2 with the closest type strain *E. americanum* LMG CFNEI 156^T. SCAU224 had 92.2 % similarity with the closest type strain *E. americanum* species and was the most divergent branch in the genus. Like in the 16S rRNA tree, SCAU202A and SCAU215 clustered together with *M. plurifarium*, and SCAU209 with *R. huautlense* LMG18254^T (Fig. 3). SCAU211 formed a clade (clade B) with the nearest type strain *B. liaoningense* USDA 3622^T (95.1 %; Table 1, Fig. 3).

Phylogenetic analysis of *nifH* and *nodC* genes

To characterize the in vivo observed symbiotic properties on gene level, two symbiotic genes (*nifH* and *nodC*) of 15 representative isolates were sequenced. The *nifH* and *nodC* genes from SCAU209, assigned as *R. huautlense* in the 16S rRNA and *recA* phylogeny, failed to be amplified. For the other 14 representative isolates, amplification of the *nodC* using the primer pair nodCF/nodCR resulted in a single fragment of about 900 bp (data not shown). The 737 bp sequences of the *nifH* gene were obtained after removal of the primer sequences. The topology of the *nodC* phylogenetic tree (Fig. 4) was very similar to that of the *nifH* tree (Fig. 5). As in the 16S rRNA and *recA* trees, the isolates grouped with *Ensifer*, *Mesorhizobium*, and *Bradyrhizobium* in both the *nifH* and *nodC* trees. Even though the 11 *Ensifer* isolates formed three clades in both the 16S rRNA and *recA* trees, they formed only one clade in both the *nifH* and *nodC* trees, suggesting that they harbored very similar or same symbiotic genes.

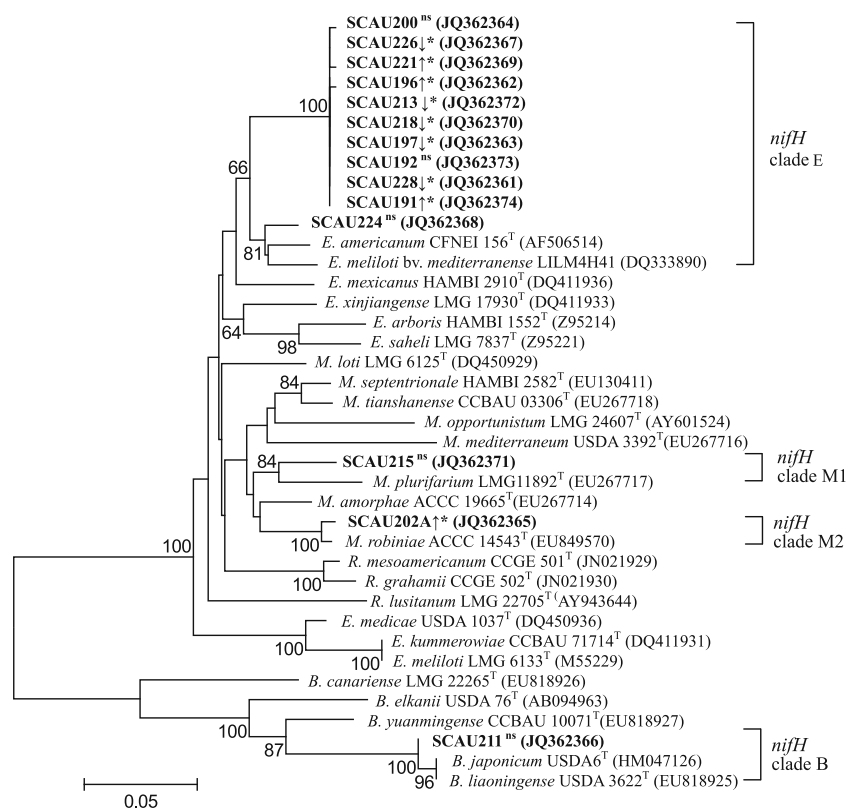
Discussion

In search of rhizobial strains for inoculating *L. leucocephala*, we investigated the symbiotic efficiency of isolates from root nodules of *L. leucocephala* growing in the arid-hot river valley area in Panxi, China. Due to the rhizobial

diversity, effective nitrogen-fixing strains are isolated even from soils with no history of cultivation of the host plant (Cardoso et al. 2012). *L. leucocephala* is considered as a promiscuous host (Wang et al. 1999, 2006). It is nodulated with both fast- and slow-growing rhizobia, yet again ineffective nodulation is common (Bala and Giller 2001). We found that only 11 out of 41 *L. leucocephala* rhizobia were efficient in nitrogen fixation (Figs. 1, 2, and 3; Table 2), making them appropriate candidates to be used as inoculants in this area. While the majority of the isolates were ineffective in symbiotic nitrogen fixation, 10 isolates could be considered as parasites with a growth slowing effect on the host. However, it cannot be ruled out that the isolates inefficient in nitrogen fixation do not bring about other benefits, e.g., they might give resistance to plant diseases.

To find explanations for the diversity in nitrogen fixation efficiency, we assessed the genetic diversity of the isolates. The molecular systematics of rhizobia is mainly based on sequence data for the 16S rRNA gene (Binde et al. 2009). Because of the high level of sequence conservation and occasional lateral gene transfer and recombination, it is important to determine the phylogenies of other housekeeping genes, e.g., *recA*, for rhizobial species assignments (van Berkum et al. 2003). In general, the *recA* phylogeny is consistent with the 16S rRNA gene phylogeny (Zhao et al. 2010) as it was in this study at genus and species level in *Rhizobium* and *Mesorhizobium* (Figs. 2 and 3; Table 1). However, the closest matches of the 16S rRNA and *recA*

Fig. 4 Phylogenetic tree of *nifH* gene sequences showing the relationships between the representative isolates and the reference strains for defined rhizobial species. Bootstrap values >50 % are given at the branching points. The scale bar indicates the number of substitutions per site. Sequence accession numbers are given in parentheses. The representative isolates are shown in bold. †*, ‡*, and ns indicates an increase, a decrease or a non-significant difference in plant shoot dry weight according to the LSD test (at * $p=0.05$ level), respectively



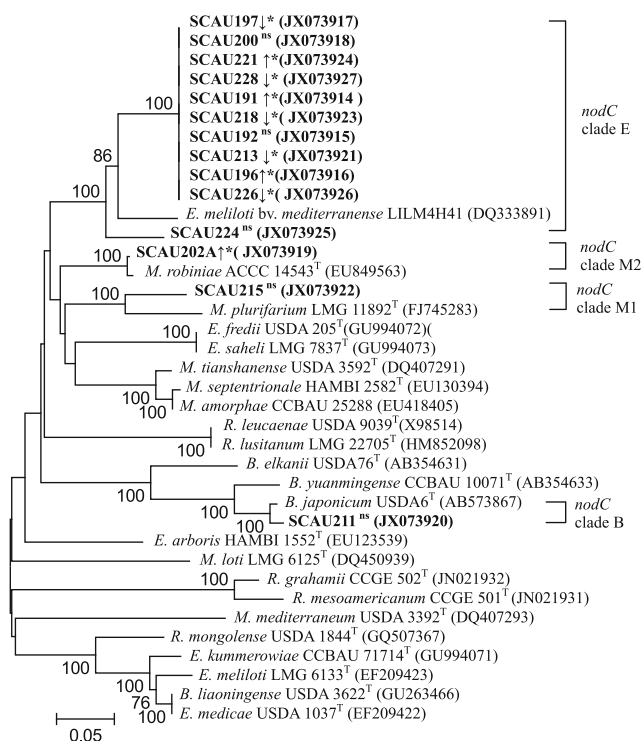


Fig. 5 Phylogenetic tree of *nodC* gene sequences showing the relationships between the representative isolates and the reference strains for defined rhizobial species. Bootstrap values >50% are given at the branching points. The scale bar indicates the number of substitutions per site. Sequence accession numbers are given in parentheses. The representative isolates are shown in bold. †, ‡, †*, ‡*, and ns indicates an increase, a decrease or a nonsignificant difference in plant shoot dry weight according to the LSD test (at **p*=0.05 level), respectively

of all the *Ensifer* isolates and the *Bradyrhizobium* isolate were discordant, indicating that the *Ensifer* and *Bradyrhizobium* isolates might represent new species and that lateral transfer of housekeeping genes might occur in interspecies of *Ensifer* and *Bradyrhizobium*.

The *nodC* gene is unique and symbiosis-specific to rhizobia and essential for the synthesis of the Nod factor (Debellé et al. 2001). The *nifH* gene is essential for nitrogen fixation by rhizobia inside the nodule but also for diazotrophs that fix nitrogen in the free-living state (Raymond et al. 2004). Little polymorphism was found between *nifH* and *nodC* genes sequence of *L. leucocephala* *Ensifer* symbionts (Figs. 4 and 5). In contrast to the promiscuity observed as a wide species distribution of the isolates, *L. leucocephala* was nodulated by strains that shared common symbiosis genes. This might reflect host specificity towards this plant by strains that produce essentially identical Nod factors, as has been suggested in other studies (Wei et al. 2009).

L. leucocephala originates from Mexico, and it is most effectively nodulated by *Ensifer* strains (Bala and Giller 2006). We found that 10 out of 11 effective nitrogen-fixing isolates from Panxi belonged to the genus *Ensifer* (Figs. 1, 2, and 3; Table 2). The symbiotic efficiency of rhizobia is

not connected to the chromosomal background of the symbiont, and strains carrying a similar *nifH* may show totally different efficiency in nitrogen fixation (Mnasri et al. 2009). These phenomena were observed in our study, too (Table 2; Figs. 2, 3, and 4). For instance, even though the 16S RNA, *recA*, *nifH*, and *nodC* sequences of the six strains in rRNA clade E2 were similar, only two of them were significantly effective in fixing nitrogen. Similarly, only one out of the three strains in rRNA clade E1 was effective. According to a model proposed by Li et al. (2012), nodulating strains are divided into true and sporadic symbionts. Almost all strains of a true symbiont species nodulate effectively, while within the sporadic symbiont species there is large variation in effectiveness and nodulation ability of the strains. Contrary to the promiscuous hosts *Glycyrrhiza* and *Phaseolus vulgaris* (Li et al. 2012; Aserse et al. 2012), *L. leucocephala* seems to be nodulated by sporadic symbionts only. Likewise, genetically close symbionts of *Acacia seyal* show variation in effectiveness (Diouf et al. 2010), calling upon the questions if these trees should be considered as truly promiscuous hosts and whether this a characteristic feature of other promiscuous tropical leguminous trees, too.

Introduced legumes are likely to carry their symbionts into the new environment where the symbionts interact with native rhizobia (Weir et al. 2004). In this study, the 16S rRNA gene sequences of all *Ensifer* isolates had higher similarity to strains not isolated from *L. leucocephala* than to *E. adhaerens* LMG21331^T, a Mexican *L. leucocephala* isolate (Fig. 2). Despite the 16S rRNA and *recA* diversity, the *Ensifer* isolates harbored similar symbiosis genes (*nifH* and *nodC*; Figs. 4 and 5; Table 1). The incongruence indicates possible horizontal transfer of symbiotic genes as previously reported for the *Mesorhizobium*, *Ensifer*, *Rhizobium*, and *Bradyrhizobium* species (Laranjo et al. 2008; Li et al. 2011; Aserse et al. 2012) and implies that the symbiotic genes which introduced *L. leucocephala* symbionts carried were transferred into indigenous *Ensifer* strains.

Invasive *Mimosa* spp., mimosoid legume woody shrubs and herbs originating in South America, are preferentially nodulated by β -rhizobia (Liu et al. 2012). Liu et al. (2012) isolated *Mimosa* spp. β -rhizobia in China and found that all the isolates fixed nitrogen effectively, contrary to the small percentage of efficient isolates among the *L. leucocephala* nodulating rhizobia in this study. The Chinese *Mimosa* spp. symbionts are closely related to South American strains likely to being brought to China with the host, i.e., to strains with a ca. 50 myears of coexistence with the host (Liu et al. 2012), whereas the *L. leucocephala* isolates seemed to be indigenous rhizobia that had received their symbiotic genes laterally during the last 30 years, a relatively short time frame for the partners to adapt to each other. The differences in nitrogen fixation efficiency between the seemingly similar isolates tell us that the isolates possessed diversity

outside the characterized gene range. It may be asked whether a promiscuous host like *L. leucocephala* ever poses such strict requirements for the nodulating strains that an uninoculated plant would host only effective isolates. If the answer is no, it gives further motivation to search for effective inoculant strains.

The species distribution of nodulating strains is different at different sites (Bala and Giller 2006; Liu et al. 2012). Wang et al. (2006) isolated the same rhizobial genera from *L. leucocephala* growing in subtropical China. However, most of the strains isolated by Wang et al. (2006) were *Mesorhizobium* spp., whereas in Panxi *L. leucocephala* had a preference for *Ensifer* strains. Since soil conditions affect the nodulating species distribution (Bala and Giller 2006; Liu et al. 2012), the dominance of *Ensifer* strains in arid environments (Romdhane et al. 2006; Benata et al. 2008; Gehlot et al. 2012) and the difference in species distribution between Wang et al. (2006) and our results might be due to the soil properties in the sampling areas. This suggests that when choosing inoculants, strains adapted to local soil conditions should be preferred.

In conclusion, we showed that in the arid–hot river valley area of Panxi *L. leucocephala* is nodulated by indigeneous rhizobia belonging to genera *Ensifer*, *Rhizobium*, *Mesorhizobium*, and *Bradyrhizobium*. Molecular characterization of the 41 isolates revealed that *Ensifer* was the predominant genus associated with *L. leucocephala* in this area. Because the *Ensifer* isolates had undergone gene recombination, their precise species assignment was not possible. This ambiguity can be clarified by analyzing more housekeeping genes. The search for effectively nitrogen-fixing inoculant strains is justified since most of the indigenous *L. leucocephala* nodulating isolates were ineffective; furthermore, one fourth of the isolates had a growth slowing effect on the host. As in earlier studies on rhizobia isolated from various legumes (Cardoso et al. 2012; Mnasri et al. 2009), the genetic background of the isolates was diverse and not connected to their symbiotic performance, necessitating the use of traditional plant growth tests in screening effective inoculants.

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