

# Phylogenetic relationships and diversity of $\beta$ -rhizobia associated with *Mimosa* species grown in Sishuangbanna, China

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In order to investigate the genetic diversity of rhizobia associated with various exotic and invasive species in tropical mainland China, 116 bacterial isolates were obtained from *Mimosa* root nodules collected from Sishuangbanna and Yuanjiang districts of Yunnan province. Isolated rhizobia were characterized by RFLP analysis of 16S rRNA genes, SDS-PAGE of whole-cell proteins and BOX-PCR. Most of the isolated strains were identified as  $\beta$ -rhizobia belonging to diverse populations of *Burkholderia* and *Cupriavidus*, and the phylogenetic relationships of their 16S rRNA gene sequences showed that they were closely related to one of four  $\beta$ -rhizobia species: *Burkholderia phymatum*, *B. mimosarum*, *B. caribensis* or *Cupriavidus taiwanensis*. Additionally, among the 116 isolates, 53 different whole-cell SDS-PAGE profiles and 30 distinct BOX-PCR genotypic patterns were detected, which demonstrated the genetic and phenotypic diversity found within these *Burkholderia* and *Cupriavidus* strains. To the best of our knowledge, this is the first report that  $\beta$ -rhizobia are extant and possibly widespread on the Chinese mainland and nodulate easily with *Mimosa* plants. We also find it especially interesting that this appears to be the first report from mainland China of *Cupriavidus* symbionts of *Mimosa*. These records enrich our knowledge and understanding of the geographical distribution and diversity of these bacteria.

## INTRODUCTION

Root-nodule bacteria classified within the class *Betaproteobacteria*, the so-called  $\beta$ -rhizobia, were isolated from *Mimosa* species by Chen *et al.* (2001). Subsequent studies on rhizobial strains isolated from mimosoid legumes led to the description of the species *Cupriavidus* (*Ralstonia*) *taiwanensis* (Chen *et al.*, 2001), *Burkholderia tuberum*, *B. phymatum* (Vandamme *et al.*, 2002), *B. nodosa*, *B. mimosarum* and *B. sabiae* (Chen *et al.*, 2006, 2007, 2008). It was also reported that *Burkholderia caribensis*, originally thought to be a non-symbiotic species, occurs in *Mimosa* root nodules in Taiwan (Chen *et al.*, 2003). *Mimosa pudica* was first introduced to

Taiwan in 1645 and became naturalized and subsequently invaded tropical and subtropical areas of mainland China (Wu *et al.*, 2003; Guan *et al.*, 2006), including Yunnan province in the south-west. *M. pudica* has been found to have a close relationship with *Rhizobium* and *Bradyrhizobium* strains in some subtropical regions of China, but only one strain belonging to the genus *Burkholderia* has been isolated from this plant (Liu *et al.*, 2007). Sishuangbanna district of Yunnan province is a tropical rainforest and is famous for its great biodiversity of plants and animals; however, its microbial diversity has not been well documented. Previous studies have shown that betaproteobacteria are the predominant nodulating strains isolated from *Mimosa* species (Chen *et al.*, 2003, 2005a, b; Barrett & Parker, 2005, 2006; Elliott *et al.*, 2007a, b; Andam *et al.*, 2007; Parker *et al.*, 2007); however, the  $\beta$ -rhizobia associated with *Mimosa* species grown widely in Sishuangbanna and elsewhere on the Chinese mainland have not been characterized. In order to obtain a better understanding of the specific symbiotic diversity of rhizobia

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SWF66003, SWF66029, SWF66044, SWF66049, SWF66117, SWF66131, SWF66166, SWF66194, SWF66232, SWF66247, SWF66286, SWF66289, SWF66304, SWF66322 and SWF66294 are FJ648690–FJ648692, FJ648694, FJ648696–FJ648705 and FJ751796, respectively.

**Table 1.** *Mimosa* rhizobia isolated in this study

Strains that are underlined were used in sequencing analysis. *Burkholderia* sp. CCBAU 65186a was described by Liu *et al.* (2007); details of other reference strains can be found in the respective species descriptions. ND, Not determined.

Strain(s)	n	Host plant	Sampling site	16S rRNA type	Pattern(s)*	
					SDS-PAGE	BOX-PCR
Reference strains						
<u>Burkholderia</u> sp. CCBAU 65186a	1	<i>M. pudica</i>	Mengzhe	I	54	32
<i>B. phymatum</i> LMG 21445 <sup>T</sup>	1	<i>Machaerium lunatum</i>	French Guiana	II	55	31
<i>B. tuberum</i> LMG 21444 <sup>T</sup>	1	<i>Aspalathus carnosa</i>	South Africa	ND	55	ND
<i>B. mimosarum</i> LMG 23256 <sup>T</sup>	1	<i>M. pigra</i>	Taiwan	ND	56	ND
<i>B. nodosa</i> LMG 23741 <sup>T</sup>	1	<i>M. scabrella</i>	Brazil	ND	57	ND
<i>B. caribensis</i> LMG 18531 <sup>T</sup>	1	Vertisol	Martinique	ND	58	ND
<i>B. sabiae</i> LMG 23245 <sup>T</sup>	1	<i>M. caesalpiniiifolia</i>	Brazil	ND	59	ND
<i>Cupriavidus taiwanensis</i> LMG 19424 <sup>T</sup>	1	<i>M. pudica</i>	Taiwan	ND	60	ND
<b><i>Burkholderia</i> sp. genotype I (protein group 1)</b>					(17)	(7)
SWF66001, SWF66003, SWF66085, SWF66301, SWF66303, SWF66030, SWF66017, SWF66004, SWF66013, SWF66031, SWF66008, SWF66009, SWF66014, SWF66050, SWF66016, SWF66021, SWF66028, SWF66034, SWF66018, SWF66020, SWF66027, SWF66058, SWF66059, SWF66023, SWF66024, SWF66033, SWF66036, SWF66040, SWF66046, SWF66048, SWF66052, SWF66045, SWF66049, SWF66056	34	<i>M. pudica</i>	Mengzhe	I	1–10	1–7
SWF66053, SWF66054, SWF66060, SWF66061, SWF66065, SWF66069, SWF66070, SWF66077, SWF66082, SWF66083	10	<i>M. pudica</i>	Mengban	I	1, 11–16	1–4, 6
SWF66221, SWF66222, SWF66238, SWF66239	4	<i>M. pudica</i>	Mengla	I	2, 10, 17	1, 3
SWF66240, SWF66244	2	<i>M. diplotricha</i>	Mengla	I	10	1
SWF66245, SWF66246	2	<i>M. diplotricha</i>	Menglun	I	10	2
<b><i>Burkholderia</i> sp. genotype I (protein group 2)</b>					(8)	(5)
SWF66117, SWF66118, SWF66120, SWF66122, SWF66123, SWF66125	6	<i>M. diplotricha</i>	Daluo	I	18–20	2
SWF66197, SWF66182, SWF66183, SWF66185, SWF66186, SWF66199	6	<i>M. pudica</i>	Bubeng	I	20, 21	3
SWF66201, SWF66204, SWF66206, SWF66207, SWF66216, SWF66225, SWF66226	7	<i>M. pudica</i>	Mengla	I	21	3, 4
SWF66086, SWF66087, SWF66088, SWF66089, SWF66074	5	<i>M. pudica</i>	Mengban	I	23	1, 4
SWF66257	1	<i>M. pudica</i>	Menglun	I	22	1
SWF66247	1	<i>M. diplotricha</i>	Menglun	I	24	8
SWF66304, SWF66307	2	<i>M. pigra</i>	Jinghong	I	25	2
<b><i>Burkholderia</i> sp. genotype II (protein group 3)</b>					(3)	(3)
SWF66029, SWF66044	2	<i>M. pudica</i>	Mengzhe	II	26, 27	9, 10
SWF66283, SWF66286	2	<i>M. pudica</i>	Jinghong	II	28	11
<b><i>Cupriavidus</i> sp. genotype III (protein group 4)</b>					(2)	(2)
SWF66076	1	<i>M. pudica</i>	Mengban	III	29	12
SWF66107	1	<i>M. pudica</i>	Daluo	III	30	13
<b><i>Cupriavidus</i> sp. genotype III (protein group 5)</b>					(16)	(11)

Table 1. cont.

Strain(s)	n	Host plant	Sampling site	16S rRNA type	Pattern(s)*	
					SDS-PAGE	BOX-PCR
SWF66109, SWF66131, SWF66133, SWF66139, SWF66140	5	<i>M. pudica</i>	Daluo	III	31, 32, 34–36	14–16
SWF66174, SWF66176	2	<i>M. pudica</i>	Bubeng	III	38, 41	16, 20
SWF66232	1	<i>M. pudica</i>	Mengla	III	42	20
SWF66256	1	<i>M. pudica</i>	Menglung	III	43	23
SWF66260, SWF66261, SWF66277	3	<i>M. pudica</i>	Jinghong	III	38, 43	14, 21, 24
SWF65027, SWF65028	2	<i>M. pudica</i>	Yuanjiang	III	45	17
SWF66276, SWF66291, SWF66292, SWF66314, SWF66316, SWF66317	6	<i>M. diplotricha</i>	Jinghong	III	33, 39, 40, 44, 46	16, 19, 22
SWF66166	1	<i>M. diplotricha</i>	Bubeng	III	37	18
SWF66248, SWF66224	2	<i>M. diplotricha</i>	Menglung	III	37	14
<b>Cupriavidus sp. genotype IV (protein group 6)</b>					(6)	(5)
SWF66194	1	<i>M. pudica</i>	Bubeng	IV	47	25
SWF66294, SWF66321, SWF66322, SWF66324	4	<i>M. pudica</i>	Jinghong	IV	48–51	26–28
SWF65033	1	<i>M. pudica</i>	Yuanjiang	IV	52	29
<b>Rhizobium sp. genotype V</b>						
SWF66289	1	<i>M. pudica</i>	Jinghong	V	53	30

\*Numbers in parentheses indicate the number of different patterns found within each protein group.

associated with *Mimosa* species, we used 16S rRNA gene sequencing, BOX-PCR, whole-cell protein SDS-PAGE and nodulation tests to characterize root-nodule bacteria from *M. pudica*, *M. diplotricha* and *M. pigra* in Sishuangbanna and the neighbouring district of Yuanjiang. The objectives of this study were to test whether  $\beta$ -rhizobia have any relationships with *Mimosa* species from Yunnan, China, and, if so, what is the composition of the  $\beta$ -rhizobia on different *Mimosa* species in these districts. Additionally, we sought to identify the phenotypic and genetic diversity among the various nodule bacteria and the phylogenetic relationships between strains isolated in Yunnan and previously reported  $\beta$ -rhizobia.

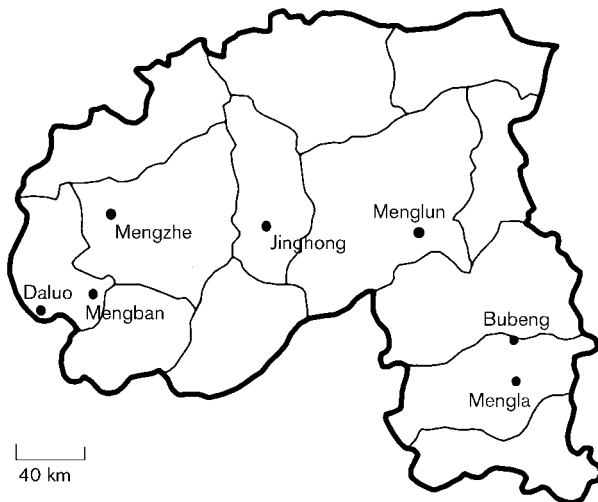
## METHODS

**Bacterial strains and culture conditions.** Root nodules were collected from plants of three *Mimosa* species at eight sites in Yunnan province (Table 1), which generally have ferrosol and latosol soil types of pH 5.0–6.0 with high soil organic matter (2–3.5%), limited total phosphorus (0.04–0.2%) and high total potassium (0.5–3.0%). Of the eight sampling sites, seven were in Sishuangbanna district (Fig. 1) and the eighth was in Yuanjiang district, which is located 200 km north-east of Sishuangbanna. Two fields in each site were selected and three to five plants in each field were uprooted. Three to five nodules were selected randomly from each plant, surface-sterilized and used in rhizobia isolation experiments following routine methods with YMA medium (Vincent, 1970). *B. phymatum* LMG 21445<sup>T</sup>, *B. tuberum* LMG 21444<sup>T</sup> (Vandamme *et al.*, 2002), *B. mimosarum* LMG 23256<sup>T</sup>, *B. nodosa* LMG 23741<sup>T</sup>, *B. sabiae* LMG 23245<sup>T</sup>, *B. caribensis* LMG 18531<sup>T</sup> (Chen *et al.*, 2007, 2008), *C. taiwanensis* strains LMG 19424<sup>T</sup> and LMG 19425 (Chen *et al.*, 2001) and *Burkholderia* sp. CCBAU 65186a (Liu *et al.*, 2007) were used as reference strains. All isolates were grown at 28 °C and stored at –80 °C in YEM broth with 20% (w/v) glycerol.

**RFLP analysis of the amplified 16S rRNA gene.** The 16S rRNA gene of each isolate was amplified by PCR with primers P1 and P6 (Tan *et al.*, 1997) and the PCR products were digested separately with the restriction endonucleases *Hae*III, *Rsa*I, *Hinf*I and *Msp*I (Laguerre *et al.*, 1994). Restriction fragments were separated by electrophoresis in 3% (w/v) agarose gels containing 0.5 µg ethidium bromide ml<sup>–1</sup> and visualized under UV light (Laguerre *et al.*, 1994). Normalized restriction patterns obtained from electrophoresis were used in a cluster analysis (Tan *et al.*, 1997) and strains with identical patterns in the four digestions were assigned to a single rRNA type.

**Sequence analyses of the 16S rRNA gene.** For sequence analysis, 16S rRNA genes of strains representing each rRNA type were amplified using primers P1 and P6 (Tan *et al.*, 1997) and sequenced directly (Tan *et al.*, 1997) at the China Autolab Biology Company, Beijing. Two cloned PCR products were sequenced independently for strains SWF66131, SWF66166 and SWF66232 because of apparent sequence heterogeneity among rRNA operons. Multiple alignments were performed with CLUSTAL\_X (Thompson *et al.*, 1997) using the acquired sequences and related sequences extracted from the GenBank database by using the BLAST program. Sequence similarities were estimated by using the MEGA 4.1 package (Kumar *et al.*, 2004). Neighbour-joining phylogenetic trees were constructed with Jukes–Cantor distances and were bootstrapped with 1000 pseudoreplicates. Trees were visualized by using the TreeView program (Page, 1996).

**Analysis of protein electrophoresis patterns and BOX-PCR analyses.** In order to discriminate strains within each rRNA type, SDS-PAGE of whole-cell proteins and BOX-PCR were performed. Strains were incubated overnight in 5 ml YMA medium (Vincent,



**Fig. 1.** Map of Sishuangbanna, China, showing the locations of the seven sampling sites where nodules were collected. The eighth sampling site was located in Yuanjiang district.

1970) at 28 °C with agitation. Whole-cell proteins were extracted from cell cultures and used in SDS-PAGE according to Tan *et al.* (1997). Protein patterns were visualized by silver staining (Tan *et al.*, 1997) and compared between strain pairs to obtain the similarity coefficient  $S_D$  [ $S_D = 2c/(a+b)$ , where  $c$  is the number of bands shared by the two strains and  $a$  and  $b$  are the numbers of protein bands recorded in each strain]. A UPGMA (Sneath & Sokal, 1973) dendrogram was constructed by using the MVSP 3.1 software (Kovach Computing Services) and similar profiles were combined into one phenotype.

DNA was subjected to a PCR with the primer BOX-AIR (5'-CTACGGCAAGGCGACGCTGACG-3') (Versalovic *et al.*, 1994) using the protocol described by Gao *et al.* (2001). An aliquot of 5  $\mu$ l PCR product for each sample was separated by electrophoresis in 1.5% agarose gel (15 cm long) at 150 V for 4 h. Patterns were visualized under UV light after staining with ethidium bromide. Strains or isolates with different patterns were assigned to distinct BOX-PCR types. A dendrogram was constructed and distances were measured as described for protein electrophoresis pattern analysis.

**Nodulation assays.** Nodulation tests were carried out using the glass tube method described previously (Liu *et al.*, 2007) with seedlings of the original *Mimosa* hosts, *M. pudica* and *M. diplotricha* and *M. pigra*. Inoculated seedlings were planted in glass tubes (20  $\times$  180 mm), with filter-paper supports, containing a nitrogen-free plant nutrition solution (Vincent, 1970) and were grown under aseptic conditions in growth chambers at 25 °C with a photoperiod of 16 h. Nodulation was observed after 4 weeks of growth and nitrogen-fixation ability of the nodules was determined using the closed acetylene reduction assay described by James & Crawford (1998). SDS-PAGE of whole-cell proteins was performed to identify the bacteria reisolated from nodules.

## RESULTS

### RFLP and sequence analysis of the amplified 16S rRNA gene

A total 116 isolates were used for RFLP analysis. Five distinct genotypes were detected among the tested strains

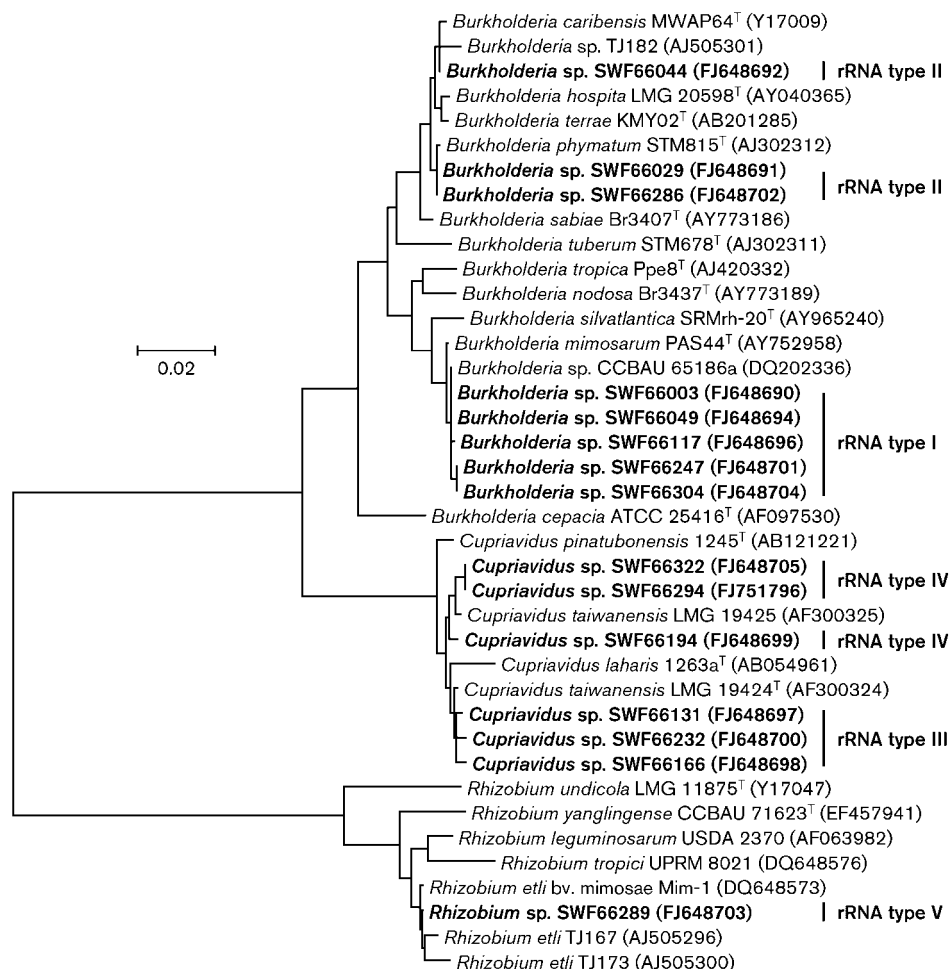
(Table 1). rRNA type I contained 80 strains and clustered with reference strain *Burkholderia* sp. CCBAU 65186a. Among these, 67 strains were isolated from *M. pudica* nodules from five sites in Yunnan identified as Mengzhe, Mengban, Menglun, Bubeng and Mengla. Eleven strains were isolated from *M. diplotricha* from the Menglun, Mengla and Daluo sites. The remaining two strains were isolated from *M. pigra* at the Jinghong site (Sishuangbanna Tropical Flower Garden). rRNA type II contained four strains, all isolated from *M. pudica* nodules from the Mengzhe and Jinghong sites, and grouped with reference strain *B. phymatum* LMG 21445<sup>T</sup>. rRNA types III and IV contained 25 and six strains, respectively, and were isolated from *M. pudica* and *M. diplotricha* at all sites, except Mengzhe. One strain made up rRNA type V, isolated from *M. pudica* at Jinghong.

A 1450 bp portion of the 16S rRNA gene was sequenced from 15 isolates representing the five rRNA types. A BLAST search showed that each of the five genotypes was related to one of the three bacterial genera *Burkholderia*, *Cupriavidus* and *Rhizobium*. In the phylogenetic analysis (Fig. 2), the five strains of rRNA type I and *Burkholderia* sp. CCBAU 65186a grouped with *B. mimosarum* PAS44<sup>T</sup>, sharing similarities of 99.7–99.8%. Three isolates of type II clustered into two subgroups, strains SWF66029 and SWF66286 clustered with *B. phymatum* STM815<sup>T</sup>, while strain SWF66044 grouped with *B. caribensis* MWAP64<sup>T</sup> and Taiwan strain *Burkholderia* sp. TJ182. Six strains of types III and IV were related to *C. taiwanensis* strains LMG 19424<sup>T</sup> and LMG 19425 with similarities of 99.0–99.7%. The lone type V strain, SWF66289, had 99.8% similarity to *Rhizobium etli* bv. *mimosae* strain Mim-1 and 99.7% similarity to *R. etli* TJ167 from amongst the Taiwanese strains.

### SDS-PAGE of whole-cell proteins and BOX-PCR analysis

The rRNA type I strains showed 25 protein profiles and eight BOX-PCR genotypes, the rRNA type II strains revealed three protein profiles and three BOX-PCR genotypes, the rRNA type III strains showed 16 protein profiles and 11 BOX-PCR genotypes and the rRNA type IV strains exhibited six protein profiles and five BOX-PCR genotypes (Table 1).

In the cluster analysis, the 53 protein patterns grouped into six clusters plus a single strain, all showing at least 80% similarity (Fig. 3). The *Burkholderia* isolates were found in groups 1 (rRNA type I), 2 (rRNA type I) and 3 (rRNA type II) and the *Cupriavidus* isolates were clustered in groups 4 (rRNA type III), 5 (rRNA type III) and 6 (rRNA type IV). These groups were consistent with the definition of rRNA types, but revealed more detailed heterogeneity in the bacterial populations. Reference strains *B. phymatum* LMG 21445<sup>T</sup>, *B. tuberum* LMG 21444<sup>T</sup> (Vandamme *et al.*, 2002), *B. mimosarum* LMG 23256<sup>T</sup>, *B. nodosa* LMG 23741<sup>T</sup>, *B. caribensis* LMG 18531<sup>T</sup> (Chen *et al.*, 2007, 2008), *B. sabiae*



**Fig. 2.** Neighbour-joining tree based on 16S rRNA gene sequences showing phylogenetic relationships among the *Mimosa* rhizobia. Accession numbers are given in parentheses. Strains in bold were isolated and sequenced in this study. Bar, 2% substitutions per site.

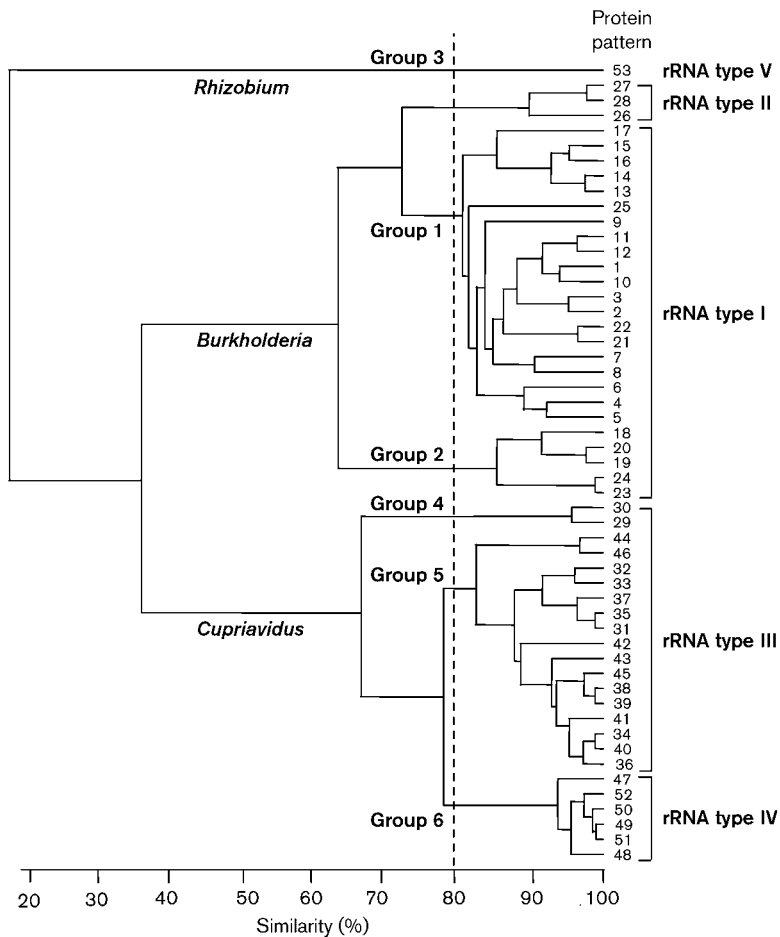
LMG 23245<sup>T</sup> (Chen *et al.*, 2008) and *C. taiwanensis* LMG 19424<sup>T</sup> (Chen *et al.*, 2001) were also included in this analysis (not included in Fig. 3); from the protein profiles we found that the *Burkholderia* and *Cupriavidus* isolates from Yunnan were distinct from the examined type strains (Fig. 4). The dendrogram of representative isolates with reference strains based on protein patterns revealed that the new isolates were obviously different from related reference strains, such as the type II strain SWF66029 and *B. phymatum* LMG 21445<sup>T</sup> (58% identity), another type II strain SWF66044 and *B. caribensis* LMG 18531<sup>T</sup> (77%), the type I strain SWF66049 and *B. mimosa* LMG 23256<sup>T</sup> (73%), the type III strains SWF66166 and SWF66232 and *C. taiwanensis* LMG 19424<sup>T</sup> (<75%) and the type IV strains SWF66194 and SWF66322 and *C. taiwanensis* LMG 19424<sup>T</sup> (67%).

In the BOX-PCR cluster analysis, the isolates in rRNA type I formed a group with a similarity of 77%. Two groups with three BOX-PCR patterns each and one group with

two BOX-PCR patterns (corresponding to rRNA types III and IV) were found. Another eight BOX-PCR patterns representing rRNA types III or IV were ungrouped (dendrogram not shown). With respect to phylogenetic relationships, these groupings demonstrated that the genetic diversity was great in the bacterial populations of *Burkholderia* and *Cupriavidus* strains; the *Cupriavidus* strains showed more genetic diversity than the *Burkholderia* strains. From comparisons of BOX-PCR profiles between isolates and reference strains such as *B. phymatum* LMG 21445<sup>T</sup> and *B. tuberum* LMG 21444<sup>T</sup>, we also found that the Yunnan *Burkholderia* and *Cupriavidus* isolates were clearly distinguished from representative type strains (Fig. 5).

### Nodulation assays

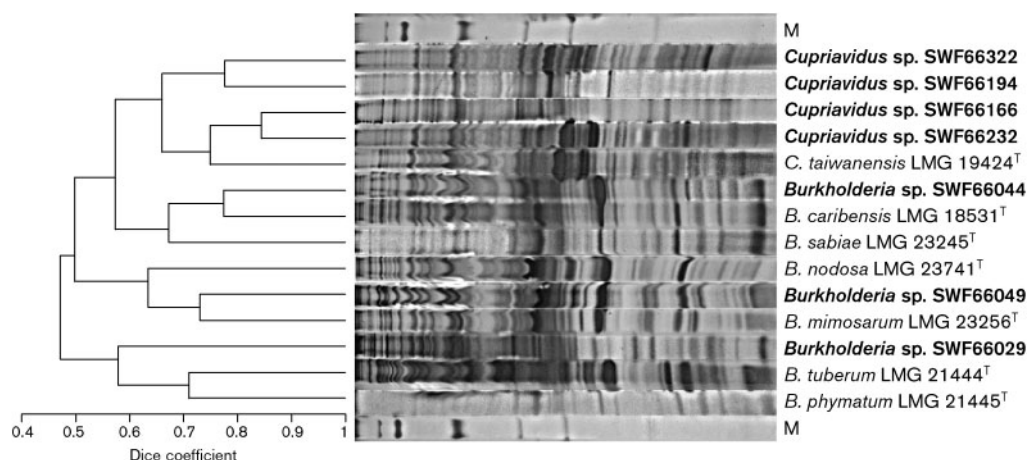
One to ten isolates representing each rRNA type were chosen for nodulation assays. On *Mimosa* plants, all 26 representative isolates formed nodules, with three to nine



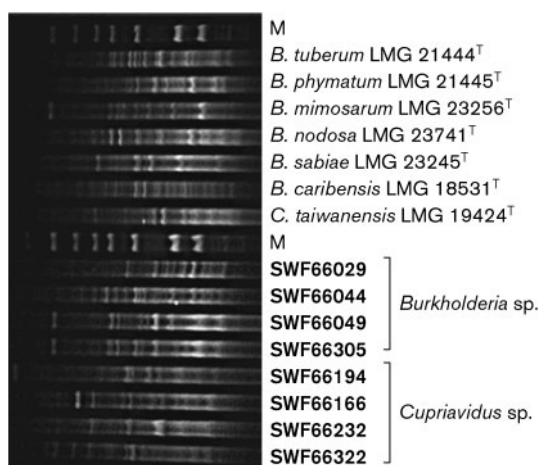
**Fig. 3.** Dendrogram showing genetic diversity of *Mimosa* rhizobia revealed by SDS-PAGE of whole-cell proteins. The Dice coefficient and UPGMA method were used for cluster analysis.

nodules per plant. Six nodules from each of five selected strains growing on *M. pudica* showed nitrogenase activity, as measured by acetylene reduction assays. The 25

betaproteobacteria isolates caused effective nodules, with acetylene reduction activity, and nodule occupation of inoculated isolates was confirmed by whole-cell protein



**Fig. 4.** Dendrogram based on whole-cell protein electrophoretic profiles of root-nodule isolates of *Burkholderia* and *Cupriavidus* and reference strains of *B. phymatum*, *B. tuberum*, *B. mimosarum*, *B. nodosa*, *B. sabiae*, *B. caribensis* and *C. taiwanensis*. M, Markers.



**Fig. 5.** Electrophoretic BOX-PCR profiles of root-nodule *Burkholderia* and *Cupriavidus* isolates and reference strains of *B. phymatum*, *B. tuberum*, *B. mimosarum*, *B. nodosa*, *B. sabiae*, *B. caribensis* and *C. taiwanensis*. M, Markers.

SDS-PAGE of reisolated bacteria. Only the *Rhizobium* strain formed inefficient nodules and showed no acetylene reduction activity.

## DISCUSSION

In the present study, the results of 16S rRNA gene RFLP analysis clearly demonstrate that  $\beta$ -rhizobia belonging to the genera *Burkholderia* and *Cupriavidus* are the main nitrogenous bacteria associated with *Mimosa* species in Sishuangbanna and Yuanjiang districts of Yunnan, and the lone *Rhizobium* isolate was found not to be effective in forming nodules in its original host, *M. pudica*. To our knowledge, this is the first report that  $\beta$ -rhizobia are common symbionts of *Mimosa* grown in tropical regions of mainland China; especially noteworthy is that these symbionts include *Cupriavidus* strains, recorded here for the first time in mainland China. The grouping results of protein SDS-PAGE and BOX-PCR patterns indicate that these  $\beta$ -rhizobia represent genetically diverse populations.

From the results of RFLP analysis of amplified 16S rRNA genes, the  $\beta$ -rhizobia isolates were divided into two *Burkholderia* genotypes and two *Cupriavidus* genotypes (Table 1). Genotypes within *Burkholderia* and *Cupriavidus* had different distributions among the three sampled *Mimosa* species. For *M. diplotricha* and *M. pigra*, all the *Burkholderia* isolates belonged to genotype I, whereas 95 % of the isolates from *M. pudica* were genotype I; genotype II was isolated only from *M. pudica*. With respect to *Cupriavidus* genotypes, *M. diplotricha* was associated with genotype III only, whereas *M. pudica* harboured similar numbers of genotype III and IV isolates. The close relationships observed from 16S rRNA gene sequencing between the Yunnan *Mimosa* rhizobia and reference strains

isolated from other regions demonstrate that the Yunnan  $\beta$ -rhizobia might share an ancestor with  $\beta$ -rhizobia from other regions, as estimated for Taiwanese *Burkholderia* and *Cupriavidus* strains (Chen *et al.*, 2005a). In China, diverse rhizobia associated with legumes from different geographical regions, including tropical regions (Gao *et al.*, 1994; Chen *et al.*, 1997), have been found in the last three decades, and most of them are  $\alpha$ -rhizobia. To date, symbiotic *Burkholderia* and *Cupriavidus* strains have been isolated in China from *Mimosa* species only. Considering that  $\beta$ -rhizobia are widely distributed in the world in association with *Mimosa* and several other legumes, such as *Cyclopia* and *Rhynchosia* (Elliott *et al.*, 2007b; Garau *et al.*, 2009), it is clear that the  $\beta$ -rhizobia have co-evolved with their hosts.

In the present study, three to five nodules from each individual plant were isolated and the results indicated that a few plants harboured members of more than one bacterial genus and that *Burkholderia* and *Cupriavidus* isolates occupied different nodules on one root system. However, most root systems formed nodules with either *Burkholderia* or *Cupriavidus* isolates and not both. These results demonstrate that symbiotic *Burkholderia* and *Cupriavidus* bacteria coexist in some soils, and that nodulation of the host by one bacterium might inhibit successful nodulation by other bacteria, implying a competitive relationship, possibly influenced by the host plant. The results of cross-nodulation tests also support this suggestion, since *Burkholderia* isolates are the most competitive symbionts of *Mimosa* (Elliott *et al.*, 2009) under nitrogen-limited conditions, and different host species have a preference for different bacteria. For example, *M. pigra* showed a preference for *Burkholderia* isolates, whereas *M. diplotricha* and *M. pudica* are less selective (Chen *et al.*, 2005b; Elliott *et al.*, 2007a, 2009). We also found that *Cupriavidus* strains were found more often than *Burkholderia* strains with *M. pudica*, as seven sites supported *Cupriavidus* strains and only five sample sites yielded *Burkholderia* isolates. This distribution may indicate that *Cupriavidus* strains are more competitive than *Burkholderia* isolates in Sishuangbanna and Yuanjiang. The local soils have high soil organic matter (2–3.5 %) and high total potassium levels (0.5–3.0 %) but limited total phosphorus (0.04–0.2 %). It is possible that *Cupriavidus* strains prefer higher nutrient levels. In our isolation procedures, we frequently isolated enterobacteria intermingled with various *Cupriavidus* strains.

The *Rhizobium* strain SWF66289, similar to *R. etli*, was isolated from *M. pudica* but was unable to induce nodules on its original host in nodulation tests. A similar result was observed previously from a *Sinorhizobium* strain isolated from *Mimosa* plants (Chen *et al.*, 2003). Elliott *et al.* (2009) have shown recently that some *Rhizobium* strains can nodulate *Mimosa* and increase the nitrogen status effectively. Perhaps the failure of *Rhizobium* strain SWF66289 to induce nodules on its original host resulted from limited nitrogen nutrition in our nodulation tests. In a future

study, we hope to resolve some of these questions about this *Rhizobium* strain isolated from *Mimosa*.

Our results enrich our knowledge of the geographical distribution and diversity of these bacteria. Some of the isolates in this study were distinct from reference strains in their SDS-PAGE and BOX-PCR profiles. In future, we will carry out DNA–DNA hybridizations, fatty acid methyl ester analysis and extensive biochemical characterization to characterize these new isolates further.

In conclusion, we observe that  $\alpha$ -rhizobia and different  $\beta$ -rhizobia coexist in association with *Mimosa* species in these ferrosol and latosol soils in Sishuangbanna and Yuanjiang districts of Yunnan province. Additionally, we believe that  $\beta$ -rhizobia are more successful and effective nitrogenous symbionts of *Mimosa* species under these conditions. We also conclude that both the soil type and host species affect the composition of rhizobia populations and their abundance in nodules of *Mimosa* species.

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