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ORIGINAL ARTICLE

Genetic diversity of rhizobia associated with *Desmodium* species grown in China

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Abstract

Aims: Desmodia are leguminous plants used as important forage and herbal medicine in China. Little information is available about the nodule bacteria of *Desmodium* species. To understand the genetic diversity of rhizobia associated with *Desmodium* species grown in China, isolates from temperate and subtropical regions were obtained and analysed.

Methods and Results: A total of 39 rhizobial strains isolated from 9 *Desmodium* species grown in China were characterized by PCR-based 16S rDNA gene and 16S–23S rDNA intergenic spacer gene restriction fragment length polymorphism (RFLP) and 16S rRNA gene sequencing. The results showed high diversity among rhizobia symbiotic with *Desmodium* species. Most microsymbionts of *Desmodium* species belonged to *Bradyrhizobium* closely related to *Bradyrhizobium elkanii*, *Bradyrhizobium japonicum* and *Bradyrhizobium yuanmingense*. Several small groups or single strain were related to *Rhizobium*, *Sinorhizobium* or *Mesorhizobium*.

Conclusions: *Desmodium* species formed nodules with diverse rhizobia in Chinese soils.

Significance and Impact of the Study: These results offered the first systematic information about the microsymbionts of desmodia grown in the temperate and subtropical regions of China.

Introduction

The family Leguminosae comprises about 650 genera and more than 18 000 species, including annual herbs and woody perennials, which are distributed over a wide range of ecological conditions (Doyle 1994). The genus *Desmodium* belongs to Papilionoideae and Desmodiinae (Allen and Allen 1981). Many of its members play an important role in sustainable agriculture, forestry and forage production. For example, *Desmodium intortum* and *Desmodium heterocarpon* have been used as forages and shading plants in the fields, which can also suppress the growth of weeds (Khan *et al.* 2001). Moreover, the plants of various desmodia have served in folk medicine as febrifuges, remedies for dysentery and liver diseases and have been used in poultices and other decoctions to treat acne, ulcers, catarrh, abscesses and eye diseases (Allen and Allen 1981).

Desmodium species are widely distributed in the temperate and subtropical regions of China. All of these species are valuable forage and folk medicine and some are pioneer plants that can resist to xerothermic environment and grow in arid, barren regions. Desmodia formed nitrogen-fixing nodules with rhizobia, a kind of soil bacteria, which favour the legumes growing in diverse conditions. This nitrogen-fixing symbiosis has considerable agricultural and ecological importance in low-input sustainable agriculture and land reclamation. Some rhizobia isolated from *Desmodium* species grown in North America have been characterized and

identified as *Bradyrhizobium* related to *Bradyrhizobium elkanii* (Parker 1999,2002,2003). The microsymbionts of *Desmodium* species grown in other continents have not been systematically studied.

In this study, we isolated and characterized some rhizobial strains from *Desmodium* species grown in China. The aim of this work was to investigate their genetic diversity. The results revealed that the desmodia mainly nodulated with bradyrhizobial species and other small rhizobial groups or single strains related to *Rhizobium*, *Sinorhizobium* and *Mesorhizobium*.

Materials and methods

Bacterial strains and isolates

The bacterial isolates and reference strains in this study are listed in Table 1. The collection, isolation and nodulation on the original host of the rhizobia were performed using the standard procedures (Vincent 1970). The isolates were obtained from fresh root nodules of nine *Desmodium* species grown in the field of the temperate and subtropical regions of China. The isolates were purified by repeated streaking in plates of yeast-mannitol agar (YMA) (Vincent 1970). All strains were maintained on TY medium at 4°C and in 30% (w/v) glycerol at −70°C for long-term storage. All the isolates and reference strains were incubated on YMA medium at 28°C.

RFLP of 16S rDNA and 16S–23S intergenic spacer genes

DNA extracted as described by Terefework *et al.* (2001) was used as templates to amplify the 16S rDNA and intergenic spacer (IGS). 16S rDNA was amplified using the universal primers P1 (5′-AGA GTT TGA TCC TGG CTC AGA ACG AAC GCT-3′, corresponding to positions 8–37 in *Escherichia coli* 16S rRNA) and P6 (5′-TAC GGC TAC CTT GTT ACG ACT TCA CCC C-3′, positions 1479–1506) as described and the methods were performed as described (Tan *et al.* 1997). The IGS between the 16S and 23S rDNAs was amplified by PCR as described by Laguerre *et al.* (1996) using the primers FGPS1490-72 derived from the 3′ end of the 16S rDNA (5′-TGC GGC TGG ATC CCC TCC TT-3′) and FGPL132-38 from the 5′ end of the 23S rRNA (5′-CCG GGT TTC CCC ATT CGG-3′). Aliquots (8–10 μl) of PCR products were digested with 5 U of the restriction endonucleases *Hinf*I, *Hae*III, *Rsa*I and *Msp*I [Promega, SABC (Sino-American Biotechnology Company), China] for 16S rDNA as recommended previously (Laguerre *et al.* 1994) and 5 U for *Hae*III, *Rsa*I, *Msp*I, *Alu*I and *Dde*I for the 16S–23S IGS. The restriction fragments were separated and visualized by horizontal electrophoresis in 3% (w/v) agarose gel containing ethidium bromide (Wang *et al.* 1999). The RFLP patterns were recorded and used in a clustering analysis using the S_j coefficient and UPGMA method (Sneath and Sokal 1973).

16S rDNA sequencing

The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Tiangen, China) according to the manufacturer's instruction and sequenced directly as reported previously (Hurek *et al.* 1997). The sequences were compared with those of related species in GenBank database. All sequences were aligned using Clustal X software (Thompson *et al.* 1997) and a neighbour-joining phylogenetic tree was reconstructed with the PHYLIP package with distances estimated using Jukes–Cantor model. A bootstrap analysis using 1000 replications was performed.

Cross-nodulation tests

Seeds of *Glycine max*, *Phaseolus vulgaris*, *Leucaena leucocephala*, *Amorpha fruticosa* and *Lespedeza cuneata* were surface-disinfested using a standard procedure (Vincent 1970). Each germinated seed was inoculated with 0·1 ml of log-phase culture (about 10^8 cells). Five duplicates were used and seedlings without inoculation were included as a blank control. The nodulation of seedlings was observed after 4–6 weeks of growth. The effectiveness of nodules was estimated by the presence of red colour (leghemoglobin) inside the nodules.

Results

Analysis of 16S rDNA and 16S–23S rDNA IGS RFLP

Of the 50 nodulate isolates obtained, 39 have the ability of nodulation and fixing nitrogen on the original host and were studied further. Nearly, full-length 16S rDNA and the 16S–23S rDNA IGS region of 39 rhizobial isolates as well as the eight type strains were amplified by PCR. All the isolates produced a single band. PCR products obtained by primers P1 and P6 were about 1500 bp. A total of 21 rDNA genotypes were obtained among the isolates and the reference strains (Table 1) and the isolates were found in 15 rDNA types that divided into four major groups. A total of 29 strains were variously assigned to the *Bradyrhizobium* branch, 6 to *Rhizobium*, 1 to *Mesorhizobium* and 3 to the *Sinorhizobium* lineage.

Bands amplified by the primer pair FGPS1490 and FGPL132 ranged from 1000 to 1200 bp. To investigate further the genetic diversity among the 39 rhizobia isolated from *Desmodium* spp. in different regions of

Table 1 Nodule isolates from *Desmodium* species and reference strains used in this study

No. of isolates and strains	Host	Geographic origin	16S genotypes (PCR–RFLP)	IGS groups (PCR–RFLP)
Isolates from *Desmodium* spp.				
Bradyrhizobium sp. I (*Bradyrhizobium elkanii*)				
CCBAU35095	*Desmodium caudatum*	Fujian*	1	5
CCBAU51017	*D. caudatum*	Guangdong*	1	5
CCBAU51021	*Desmodium heterocarpon*	Guangdong	1	5
CCBAU51187	*D. heterocarpon*	Guangdong	1	5
CCBAU51317	*D. heterocarpon*	Guangdong	1	5
CCBAU51410	*D. heterocarpon*	Guangdong	1	5
CCBAU23174	*Desmodium microphyllum*	Anhui*	1	5
CCBAU23010	*Desmodium elegans*	Anhui	1	5
CCBAU41039, CCBAU41134, CCBAU41139, CCBAU41142	*Desmodium racemosum*	Hunan*	1	5
CCBAU41153	*Desmodium sequax*	Hunan	1	5
CCBAU33229, CCBAU33179	*D. elegans*	Jiangxi*	1	5
CCBAU65184	*Desmodium gangeticum*	Yunnan*	1	5
CCBAU43063, CCBAU43082	*D. elegans*	Hubei*	2	5
CCBAU43139	*D. racemosum*	Hubei	2	5
Bradyrhizobium sp. II (*Bradyrhizobium yuanmingense*)				
CCBAU43140	*D. racemosum*	Hubei	12	3
CCBAU33221	*D. racemosum*	Jiangxi	9	3
CCBAU23230	*Desmodium fallax*	Anhui	10	3
Bradyrhizobium sp. III (*Bradyrhizobium japonicum*)				
CCBAU23013	*D. microphyllum*	Anhui	3	1
CCBAU43169	*D. elegans*	Hubei	7	4
CCBAU43174	*D. racemosum*	Hubei	8	4
CCBAU23238	*D. heterocarpon*	Anhui	3	1
CCBAU65222	*Desmodium triflorum*	Yunnan	4	4
CCBAU65199	*D. sequax*	Yunnan	3	4
CCBAU65200	*D. sequax*	Yunnan	6	4
Mesorhizobium sp. I CCBAU13090	*D. microphyllum*	Jilin*	20	Single
Rhizobium sp. I				
CCBAU13070, CCBAU13093	*D. microphyllum*	Jilin	17	6
CCBAU33220	*D. racemosum*	Jiangxi	17	6
Rhizobium sp. II				
CCBAU65255, CCBAU65256	*D. sequax*	Yunnan	16	8
Rhizobium sp. III CCBAU23084	*D. microphyllum*	Anhui	13	Single
Sinorhizobium sp.				
CCBAU33036	*D. racemosum*	Jiangxi	19	7
CCBAU33037	*D. racemosum*	Jiangxi	19	7
CCBAU53044	*D. sequax*	Guangxi*	19	9
Reference strains				
Bradyrhizobium elkanii USDA76^T	*Glycine max*	US	1	5
Bradyrhizobium japonicum				
USDA6^T	*Glycine max*	Japan	3	2
B15	*Glycine max*		3	2
USDA110	*Glycine max*		5	
Bradyrhizobium yuanmingense				
CCBAU10071^T	*Lespedeza*	Beijing*	11	3
CCBAU10073	*Lespedeza*	Beijing	11	3
Bradyrhizobium liaoningense USDA3622^T	*Glycine max*	Liaoning*	3	Single
Mesorhizobium loti NZP2213^T	*Lotus*	New Zealand	21	Single
Rhizobium leguminosarum USDA2370^T	*Pisum sativum*	US	15	Single
Rhizobium hainanense CCBAU57015^T	*Desmodium*	Hainan*	14	Single
Sinorhizobium fredii USDA205^T	*Glysine soja*	Henan*	18	9

*Chinese provinces or cities.

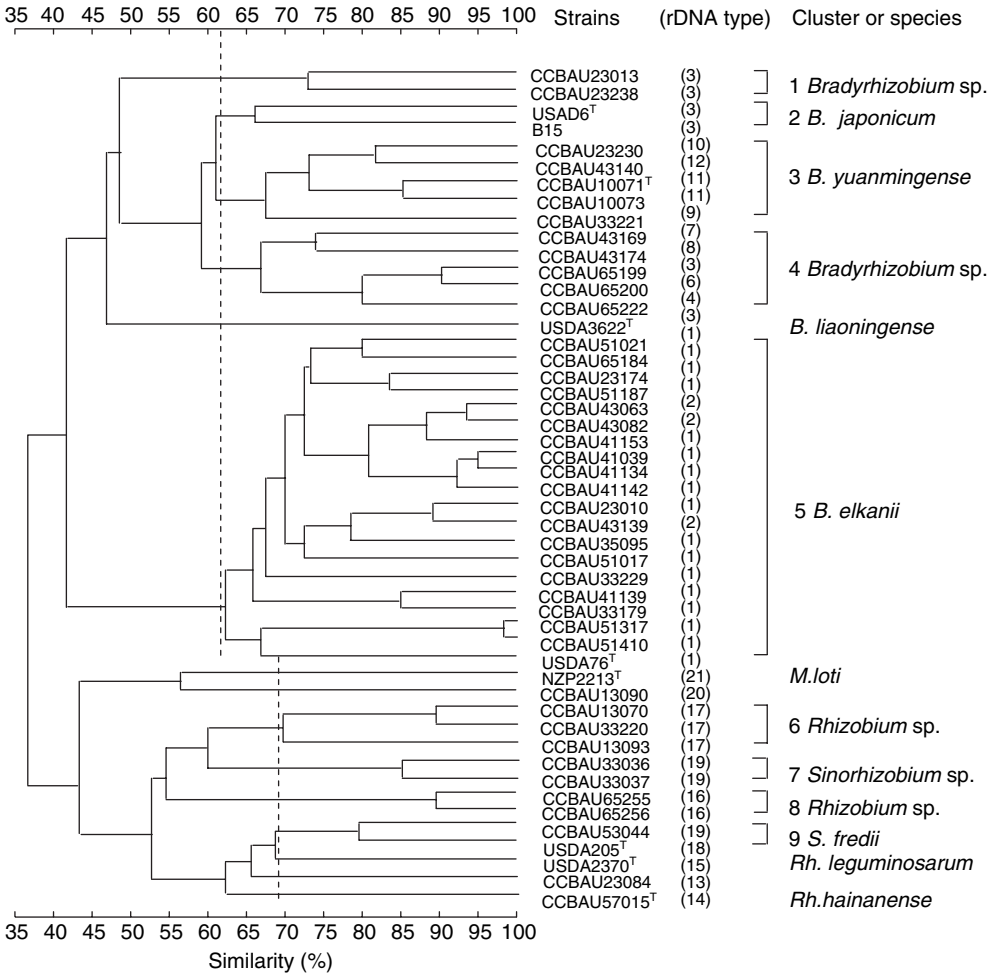

Figure 1 Genetic relationships among the rhizobia isolated from *Desmodium* and reference strains revealed by the PCR–RFLP analysis of 16S–23S spacer. Dendrogram was constructed using the UPGMA method (Sneath and Sokal 1973). Nine clusters were defined among the strains at the similarity level of 62% (for bradyrhizobia) and 69% (for fast-growing rhizobia) as shown by the dashed lines.

China, we analysed the 16S–23S rDNA IGS by PCR–RFLP with five enzymes *Hae*III, *Rsa*I, *Msp*I, *Alu*I and *Dde*I. Each strain or isolate has its own restriction pattern (Fig. 1). Most of the test bacteria were divided into nine groups (Table 1). The bradyrhizobial isolates were in groups 1 through 5. The group 5 was the largest one including the type strain for *B. elkanii* and 19 isolates, which had two genotypes in PCR–RFLP of 16S rDNA gene. The fast-growing rhizobial isolates and reference strains formed groups 6 to 9. The reference strains for *Rhizobium leguminosarum*, *Rhizobium hainanense*, *Bradyrhizobium liaoningense* and for *Mesorhizobium loti*, as well as isolates CCBAU23084 and 13090 were single branches (Table 1). These results revealed that the isolates were genetically diverse belonging to distinct rhizobial groups.

16S rRNA gene sequences and the phylogeny

In this work, the 16S rRNA genes of every representative isolate for each IGS groups (Fig. 1) and the ungrouped isolates CCBAU23084 and CCBAU13090 were sequenced.

The sequences were aligned and compared with the 16S rDNA sequences of other members of the family Rhizobiaceae available in the GenBank database. In the reconstructed phylogenetic tree (Fig. 2), the slow-growing isolates CCBAU23013 (group 1), 23230 (group 3), 65199 (group 4) and CCBAU23174 (group 5) were all in the branch of *Bradyrhizobium* with more than 99% similarity related to *Bradyrhizobium japonicum*, *Bradyrhizobium yuanmingense*, *B. liaoningense* and *B. elkanii*, respectively. Isolates CCBAU33220 and CCBAU65255 were included in the *Rhizobium* phylogenetic branch, most closely

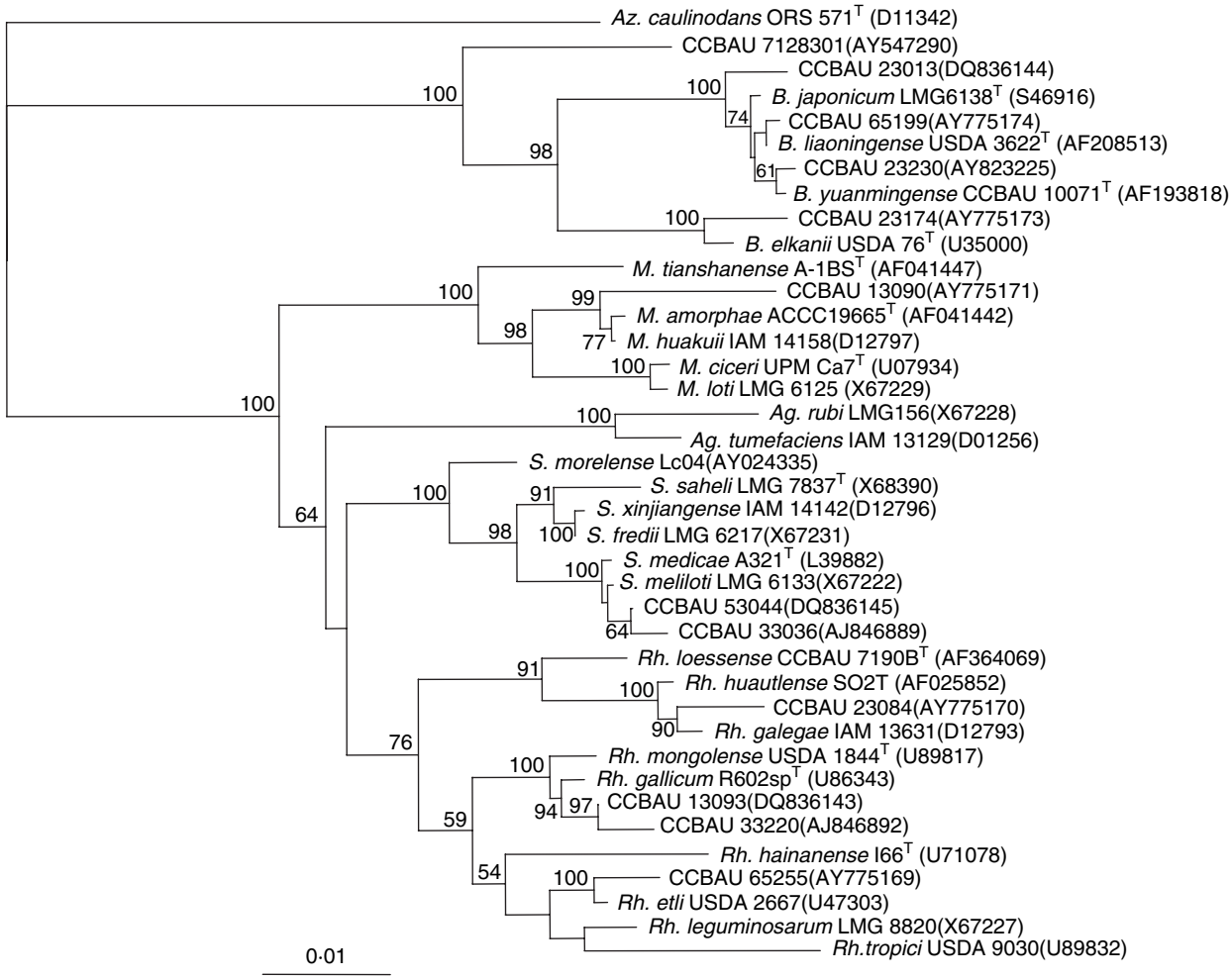

Figure 2 Phylogenetic tree of 16S rDNA showing the relationships among the rhizobial isolates and related species. The tree was reconstructed using the neighbour-joining method. Bootstrap confidence levels greater than 50% are indicated above the nodes. GenBank accession numbers are shown in parentheses.

related to *Rhizobium gallicum* and *Rhizobium etli* (with 99·7% and 99·3% similarity, respectively). The single strain CCBAU23084 was also located in the *Rhizobium* branch, which closely related to *Rhizobium galegae* of 99·6% similarity. The other two ungrouped strains, CCBAU33036 and CCBAU13090 were included in *Sinorhizobium* and *Mesorhizobium* phylogeny branches, which had a high similarity with *Sinorhizobium meliloti* and *Mesorhizobium amorphae*, respectively. The phylogenetic relationships obtained by the sequencing of 16S rDNA and by the PCR-based RFLP of 16S and 16S–23S rDNA IGS had a good correspondence to each other.

Nodulation tests

To know the symbiotic properties of the rhizobial isolates from *Desmodium* spp., the fast-growing strains and nine slow-growing strains chosen from three bradyrhizobial genetic groups (CCBAU23174, 41039, 41142, 23230, 43140, 33221, 65199, 65222, 23013) were checked for cross-nodulation. All the tested rhizobia failed to nodulate with *A. fruticosa* and *L. cuneata*. The fast-growing strains were all symbiotic with *P. vulgaris* and formed efficient nodules. There were only two isolates (CCBAU65255 and 65256), which nodulated with *L. leucocephala*. Several slow-growing strains can nodulate with *G. max* with ineffective nodules and *P. vulgaris* with effective nodulation.

Discussion

In this study, 39 rhizobial isolates from root nodules of herbaceous or shrub species in the genus *Desmodium* grown in China were characterized with 16S rDNA genes

and 16S–23S spacer PCR–RFLP and 16S rRNA gene sequencing. We found high genetic diversity among the rhizobial strains.

Slow-growing bacteria closely related to *B. elkanii* were described as microsymbionts of different *Desmodium* species mainly in North America (Parker 1999,2002,2003). However, the slow-growing isolates from nodules of *Desmodium* species grown in Hainan Province, a tropical region of China, were classified as *B. japonicum* by numerical taxonomy and DNA–DNA hybridization (Gao *et al.* 1994). In contrast, some fast-growing rhizobia isolated from *Desmodium triquetrum*, *Desmodium heterophyllum* and *Desmodium gyroides* were a unique group (Gao *et al.* 1994), classified as *Rh. hainanense* in a subsequent work (Chen *et al.* 1997). In this study, rhizobia associated with desmodia from temperate and subtropical regions of China were closely related to *B. elkanii* and *B. japonicum* with an aggregate similarity of 68% of rhizobial population. There are two small groups and a single strain CCBAU23084 belonging to *Rhizobium* but not related to the species of *Rh. hainanense*. Moreover, three isolates were closely related to *Sinorhizobium* and one located in the *Mesorhizobium* lineage.

Results obtained from cross-nodulation tests showed that all the isolates can nodulate with *P. vulgaris*, suggesting that *P. vulgaris* is a non-selective host for nodulation (Michiels *et al.* 1998). Bradyrhizobial strains symbiotic with *P. vulgaris* in Chinese soil have been reported (Han *et al.* 2005). However, the symbiotic mechanisms of these isolates need further investigation.

A collection of *Desmodium* spp. nodulating bacteria with high diversity has been characterized in this study. Nevertheless, further research is needed to collect more strains associated with *Desmodium* spp. and analysis of their symbiotic genes may help explain the basis of this diversity.

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