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Diversity and phylogeny of rhizobia associated with *Desmodium* spp. in Panxi, Sichuan, China

Kai Wei Xu[a,*,1], Lan Zou[a,1], Petri Penttinen[b,1], Xiangzhong Zeng[c], Ming Liu[a], Ke Zhao[a], Cuiping Chen[a], Yuan Xue Chen[a,**], Xiaoping Zhang[a,***]

[a] Department of Microbiology, College of Resources, Sichuan Agricultural University, Chengdu 625014, China
[b] Department of Environmental Sciences, University of Helsinki, Helsinki Fin-00014, Finland
[c] Soil and Fertilizer Institute, Sichuan Academy of Agricultural Sciences, Chengdu 610066, China

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ABSTRACT

Thirty-four rhizobial isolates were obtained from root nodules of four wild *Desmodium* species growing in Panxi, Sichuan, China. According to the combined ARDRA and IGS-RFLP (CACAI) cluster analysis, *Rhizobium*, *Pararhizobium* and *Mesorhizobium* isolates outnumbered *Bradyrhizobium* isolates. In general, the isolates representing the same species from the same site clustered together. Furthermore, the four *Desmodium* species were all nodulated by more than one rhizobial species. AFLP and phenotypic analyses showed that the 34 isolates represented at least 32 distinct strains. None of the strains were found from more than one site or host, indicating a high degree of rhizobial diversity in Panxi. In the multilocus sequence analysis, the isolates were assigned to *Pararhizobium giardinii*, *Bradyrhizobium japonicum*, *Mesorhizobium septentrionale*, and to undescribed species of the genera *Rhizobium*, *Bradyrhizobium* and *Agrobacterium*.

Introduction

Desmodium spp. are leguminous plants in the subfamily *Papilionoideae*, tribe *Desmodieae*, that are widely distributed in temperate and subtropical regions, and they are used in traditional folk medicine as antipyretic and anti-inflammatory agents, as well as forage plants [1,8,10]. The genus includes pioneer species that resist the xerothermic environment and grow in arid, barren sites. *Desmodium* species that form nitrogen-fixing symbiosis with rhizobia play an important role in sustainable agriculture [1,12] and a total of 13 species, including *Desmodium oldhamii* (synonym *Hylodesmum oldhamii*), have been found in Sichuan, China [5]. Most of the *Desmodium*-nodulating rhizobia described to date belong to the genus *Bradyrhizobium*, however, this legume is also nodulated by strains representing the *Rhizobium*, *Ensifer* and *Mesorhizobium* genera [6,8,20–22].

Panxi (Panzhihua-Xichang) in the southwest of Sichuan Province is located in South-Central China, and is one of the 25 biodiversity hotspots that hosts over 3500 endemic plants [19]. The diversity of plant-associated bacteria is plausibly multifold since the plants in this area also host numerous bacterial species [39,40]. Due to the over-felling of trees, the arid-hot river valleys in Panxi suffer from soil erosion. Leguminous pioneer species (e.g. *Desmodium* spp.) are valuable in reclaiming degraded soils. Previously, we characterized rhizobia nodulating introduced *Leucaena leucocephala* trees in Panxi, and found that the species distribution of nodulating strains was different from those in other areas [35,36]. Thus, it was reasonable to suppose that the *Desmodium* spp. in this area also hosted different rhizobia from those in the Americas and in other parts of China [6,8,20–22]. Therefore, in order to reveal the diversity of rhizobia nodulating *Desmodium* species in Panxi, they were isolated from the nodules of four *Desmodium* spp. growing in six locations, and the genetic and phenotypic characteristics of the isolates were assessed.

Materials and methods

Isolation of nodule bacteria

Nodules were collected from the roots of four wild *Desmodium* spp. in Panxi, Sichuan, China (Table 1). Nodules were surface sterilized by immersing in 95% ethanol for 5 min and in 0.1% $HgCl_2$ for 5 min, and they were then rinsed approximately 8 times

* Corresponding author. Tel.: +86 28 8629 0982; fax: +86 28 8629 0983.
** Corresponding author. Tel.: +86 28 8629 1179; fax: +86 28 8629 0983.
*** Corresponding author. Tel.: +86 835 2882710; fax: +86 835 2883166.
E-mail addresses: xkwei@sicau.edu.cn (K.W. Xu), cyxue2002@aliyun.com (Y.X. Chen), zhangxiaopingphd@126.com (X. Zhang).
[1] These three authors contributed equally to this work.

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Table 1
Bacterial isolates in this study and their phylogenetic relationships.

Isolates[a]	Host[b]	Origin[c]	Altitude (m)	RFLP[d]			Most related species (sequence similarity, %)[e]					Genospecies identified
				rrs type	IGS type	CACAI group	16S	*recA*	*atpD*	*glnII*	Combined	
SCAUd1	Ds	HL	1280	1	a	R1						
SCAUd2	Ds	HL	1280	1	a	R1	Rv(99.7)	Rv(93.4)	Re(94.8)	Re(94.7)	Re(93.8)	R. spI
SCAUd3	Ds	HL	1280	1	a	R1						
SCAUd4	Ds	HL	1280	1	a	R1						
SCAUd5	Ds	LP	1610	2	b	M						
SCAUd6	Ds	LP	1610	2	b	M	Ma(100)	Ma(96.4)	Ms(96.7)	Ms(98.2)	Ms(97.1)	Ms
SCAUd7	Ds	LP	1610	2	c	M						
SCAUd8	Ds	LP	1610	2	c	M						
SCAUd9	Ds	LP	1610	2	d	M						
SCAUd10	Ds	LP	1610	2	d	M						
SCAUd11	Ds	LP	1610	2	c	M						
SCAUd12	Ds	LP	1610	2	c	M						
SCAUd13	Ds	LP	1610	2	c	M						
SCAUd14	Ds	LP	1610	2	c	M						
SCAUd15	Ds	LP	1610	2	c	M						
SCAUd16	Ds	XL	1470	3	e	P						
SCAUd17	Ds	XL	1470	3	e	P						
SCAUd18	Ds	XL	1470	3	e	P						
SCAUd19	Ds	XL	1470	3	e	P	Pg(100)	Pg(99.0)	Pg(99.4)	Pg(97.8)	Pg(98.7)	Pg
SCAUd20	Ds	XL	1470	3	e	P						
SCAUd21	De	DL	2290	3	e	P						
SCAUd22	De	DL	2290	4	f	B1						
SCAUd23	De	DL	2290	4	f	B1						
SCAUd24	De	DL	2290	4	f	B1	Bc(99.7)	Bj(98.2)	Bj(97.0)	Bj(98.0)	Bj(97.5)	Bj
SCAUd25	De	DL	2290	4	f	B1						
SCAUd26	Dg	XP	1230	5	g	B2						
SCAUd27	Dg	XP	1230	5	h	B2						
SCAUd29	Dg	XP	1230	5	j	B2	Bg(99.7)	Bl(95.8)	Bd(96.2)	Bj(95.8)	By(94.5)	B. sp
SCAUd30	Do	FP	1110	6	k	R2						
SCAUd31	Do	FP	1110	6	k	R2	Rs(98.4)	Rm(92.0)	Ry(92.5)	Rs(93.6)	Ry(93.0)	R. spII
SCAUd28	Dg	XP	1230	7	i	A						
SCAUd32	Do	FP	1110	7	l	A	Ar(99.7)	Ar(93.4)	Ar(94.8)	Ar(95.4)	Ar(94.3)	A. sp
SCAUd33	Do	FP	1110	7	m	A						
SCAUd34	Do	FP	1110	7	l	A						

[a] Representative isolates for sequencing in **bold**.
[b] Ds: *Desmodium sequax*; De: *Desmodium elegans*; Dg: *Desmodium gangeticum*; Do: *Desmodium oldhamii* (synonym *Hylodesmum oldhamii*).
[c] HL: Hulukou Town Ningnan County, Liangshan Prefecture; LP: Luoji Town, Puge County, Liangshan Prefecture; XL: Xiaoliao Town, Xichang City, Liangshan Prefecture; DL: Dajing Town, Xichang City, Liangshan Prefecture; XP: Xijei Town, Miyi County, Panzhihua City; FP: Fangtian Town, Miyi County, Panzhihua City.
[d] *rrs* and IGS genotypes of rhizobial isolates represent the combination of restriction patterns obtained by the enzymes used (*Msp*I, *Hin*fI, *Hae*III, and *Taq*I).
[e] Combined: multilocus sequence analysis of combined *recA*, *atpD*, and *glnII*. Ar: *Agrobacterium radiobacter* NCPPB 2437^T; Bc: *Bradyrhizobium canariense* BTA-1^T; Bd: *B. diazoefficiens* USDA110^T; Bg: *B. ganzhouense* RITF806^T; Bj: *B. japonicum* USDA6^T; Bl: *B. liaoningense* LMG 18230^T; By: *B. yuanmingense* CCBAU10071^T; Ma: *Mesorhizobium amorphae* ICMP 15022^T; Ms: *M. septentrionale* SDW014^T; Pg: *Pararhizobium giardinii* H152^T; Re: *Rhizobium etli* CFN 42^T; Rm: *R. mongolense* USDA 1844^T; Rs: *R. sullae* USDA IS123^T; Rv: *R. vallis* CCBAU 65647^T; Ry: *R. yanglingense* CCBAU 71623^T.

in sterile water for 5 min per time [35]. Nodule bacteria were isolated using yeast mannitol agar (YMA) medium [11,31]. Single colonies were picked and checked for purity by repeatedly streaking on YMA medium, and they were verified by colony morphology, absorption of Congo red (25 mg mL^{-1}) and the Gram stain 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. For RFLP analysis, six reference strains were included: *Rhizobium hainanense* CCBAU 57015^T, *Rhizobium mongolense* USDA1844^T, *Bradyrhizobium yuanmingense* CCBAU10071^T, *Bradyrhizobium japonicum* USDA6^T, *Mesorhizobium plurifarium* LMG11892^T and *Agrobacterium rubi* IAM13569^T.

Nodulation assays

There is no commercial source for *Desmodium sequax*, *Desmodium elegans*, *Desmodium gangeticum* and *Desmodium oldhamii* seeds, and the *D. gangeticum* seeds collected from Panxi did not germinate. However, the Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences, Hainan, China, provided us with a small quantity of *D. gangeticum* and *Desmodium velutinum* seeds. Nevertheless, due to the low germination percentage of the seeds, it was only possible to perform *D. gangeticum* and *D. velutinum* nodulation tests with 12 and 27 isolates, respectively. The nodulation tests were conducted as described previously [8,35]. The seedlings were inoculated with 1.0 mL of a culture containing approximately 10^9 bacterial cells mL^{-1}. All inoculation assays, including the uninoculated controls, were carried out in triplicate and the root nodulating ability of the isolates was evaluated after approximately 42 days.

Restriction fragment length polymorphism (RFLP)

Genomic DNA was extracted from the isolates and reference strains using the GUTC method [30]. For ARDRA (amplified rDNA restriction analysis), the 16S rRNA gene was amplified using the P1 and P6 primers, as previously described [35]. For IGS-RPLP, 16S-23S rRNA intergenic spacer (IGS) fragments were amplified using the primers pHr and p23SR01, as previously described [36]. Amplification products (5 μL) were individually digested with the restriction endonucleases *Hin*fI, *Msp*I, *Hae*III and *Taq*I (MBI, Fermentas; 5 U per reaction), as specified by the manufacturer, in a total volume of 10 μL. The fragments obtained were separated by electrophoresis in 2.0% agarose containing 0.5 μg mL^{-1} ethidium bromide at 120 V for 3 h and visualized as previously described [35]. Cluster analysis