Rhizobial Diversity and Nodulation Characteristics of the Extremely Promiscuous Legume *Sophora flavescens*

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Submitted 15 June 2015. Accepted 13 September 2015.

In present study, we report our extensive survey on the diversity and biogeography of rhizobia associated with *Sophora flavescens*, a sophocarpidine (matrine)-containing medicinal legume. We additionally investigated the cross nodulation, infection pattern, light and electron microscopies of root nodule sections of *S. flavescens* infected by various rhizobia. Seventeen genospecies of rhizobia belonging to five genera with seven types of symbiotic *nodC* genes were found to nodulate *S. flavescens* in natural soils. In the cross-nodulation tests, most representative rhizobia in class α -Proteobacteria, whose host plants belong to different cross-nodulation groups, form effective indeterminate nodules, while representative rhizobia in class β -Proteobacteria form ineffective nodules on *S. flavescens*. Highly host-specific biovars of *Rhizobium leguminosarum* (bv. *trifolii* and bv. *viciae*) and *Rhizobium etli* bv. *phaseoli* could establish symbioses with *S. flavescens*, providing further evidence that *S. flavescens* is an extremely promiscuous legume and it does not have strict selectivity on either the symbiotic genes or the species-determining housekeeping genes of rhizobia. Root-hair infection is found as the pattern that rhizobia have gained entry into the curled root hairs. Electron microscopies of ultra-thin sections of *S. flavescens* root nodules formed by different rhizobia show that the bacteroids are regular or irregular rod shape and nonswollen types. Some bacteroids contain poly- β -hydroxybutyrate (PHB), while others do not, indicating the synthesis of PHB in bacteroids is rhizobia-dependent. The extremely promiscuous symbiosis between *S. flavescens* and different rhizobia provide us a basis for future studies aimed at understanding the molecular interactions of rhizobia and legumes.

The association between leguminous plants and symbiotic nitrogen-fixing rhizobia is one of the most important symbioses and has attracted extensive studies. In rhizobia, the nodulation (*nod*) genes encoding for Nod factors are believed to be the determinants of rhizobial host specificity (Dénarié et al. 1996; Ferguson 2013). Based upon the differences in their *nod* genes, some rhizobial strains can only nodulate limited plant species, such as *Mesorhizobium muleiense* CCBAU 83963^T for *Cicer*

arietinum (Zhang et al. 2012), while other strains, such as *Sinorhizobium* sp. strain NGR234, have a broad host range and can nodulate with many different legumes, even the nonlegume *Parasponia andersonii* (Pueppke and Broughton 1999). Similarly, some legumes, like *Astragalus sinicus* (Wang et al. 2014), *Medicago truncatula* (Barker et al. 1990), and *Cicer arietinum* (Zhang et al. 2012) are hosts specific for a limited number of rhizobial species or even strains. In contrast, other legumes, such as common bean (*Phaseolus vulgaris*) (Michiels et al. 1998), *Acacia tortilis* (Ba et al. 2002), *Siratro* (*Macroptilium atropurpureum*) (Angus et al. 2013; Moulin et al. 2001), *Sophora alopecuroides* (Zhao et al. 2010), *Sarothamnus scoparius* (Sajnaga et al. 2001), and the nonlegume *Parasponia andersonii* (Op den Camp et al. 2012) are known as promiscuous hosts.

The legume genus *Sophora* contains about 50 species, in which the nodulation of about 20 species is documented (Sprent 2001), and fewer of them have been studied about their rhizobia in detail (Sprent 2001; Zhao et al. 2010). Previously, various rhizobia belonging to 11 genospecies in six genera harboring five *nodA* gene types have been isolated from the root nodules of *Sophora alopecuroides* (Han et al. 2008; Zhao et al. 2010). Most recently, Tan et al. (2015) studied 48 rhizobial isolates from the New Zealand native *Sophora* spp. and found that all isolates were classified into genus *Mesorhizobium*. These results demonstrate that *Sophora* spp. are a promiscuous host for diverse rhizobia.

Sophora flavescens, another species in genus *Sophora*, is a perennial medicinal plant native to northeastern Asia (Han et al. 2015). It is increasingly cultured in north China, since the nitrogen-containing sophocarpidine (matrine) in its roots acts as a traditional insecticide and an anticarcinogen (Han et al. 2015). Crow et al. (1981) reported that *S. flavescens* was a distinctive species and that the rhizobia isolated from *S. angustifolia* could nodulate *S. flavescens*, while others suggested that these two species were synonymous (Sprent 2001; 2009). Despite the nomenclature argument, the rhizobia associated with *S. flavescens* and their nodulation procedures have not yet been studied extensively. Three novel species, *Rhizobium sophorae*, *Rhizobium sophoriradicis*, and *Phyllobacterium sophorae*, were just recently recorded by our research group (Jiao et al. 2015a and b). Because of the wider distribution of *S. flavescens* than *S. alopecuroides*, it is possible that the genetic diversity of rhizobia is greater in the former than the latter.

In this study, we performed the first systematic analysis of the genetic diversity and distribution of rhizobia isolated from root nodules of *S. flavescens* grown in different eco-regions. Based on these results, we then carried out extensive

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*The e-Xtra logo stands for “electronic extra” and indicates that seven supplementary figures and one supplementary table are published online.

cross-nodulation tests under laboratory conditions to explore the symbiotic promiscuity of *S. flavescentis*. In addition, morphologies of the root nodule and nodule section, the infection process, and the presence of the bacteroids in the nodule cells were examined. Our studies revealed that *S. flavescentis* is an extremely promiscuous host for various rhizobia and it can serve as a good candidate for future studies on the symbiotic relationship between rhizobia and legumes.

RESULTS

Isolation of root-nodule bacteria and soil characterization.

In total, 269 pure rhizobial isolates were obtained from *Sophora flavescentis* nodules collected from three ecoregions (Supplementary Table S1). Except for three isolates (CCBAU 03429, CCBAU 03419, and CCBAU 71316), the remaining 266 isolates formed effective nodules on *S. flavescentis*, as evidenced by the bright green leaves and the red color of the nodule section (Supplementary Fig. S3).

The results of soil characteristics showed that the three ecoregions differed mainly in soil pH values. Ecoregion I in Shanxi province had a slightly alkaline soil (7.26 to 7.95), ecoregion II in Shaanxi province had an acidic soil (5.44 to 6.68), and ecoregion III in Liaoning province had an alkaline soil (8.36 to 8.59) (Table 1). In addition, ecoregion I had a much higher concentration of available potassium (AK) than the other two ecoregions. Ecoregion III had total nitrogen content, organic matter, available nitrogen, available phosphorus, available potassium, and total salt levels lower than those of the other two ecoregions (Table 1).

Genetic diversity and distribution of rhizobia associated with *S. flavescentis*.

Based on analysis of the *recA* gene sequence screening, 17 genotypes were identified and 35 representative isolates were further characterized with a multilocus sequence analysis (MLSA) of three housekeeping genes (*atpD*, *glnII*, and *recA*). In the maximum likelihood phylogenetic tree reconstructed with the MLSA data, these 35 representative isolates were

classified into 17 genospecies (Fig. 1; Table 2), belonging to genera *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Rhizobium*, and *Phyllobacterium*. Nine genospecies corresponding to *Sinorhizobium fredii*, *Mesorhizobium septentrionale*, *Bradyrhizobium elkanii*, *Rhizobium yanglingense*, *Mesorhizobium* sp. strain I, *R. sophorae*, *R. sophoriradicis*, *Mesorhizobium huakuii*, and *Rhizobium* sp. strain I were the major groups containing 12 to 46 isolates (Table 2). *Sinorhizobium fredii* was the most widely spread major genospecies occurring on eight sites (S3, S4, S7 to S11, and S18). Eight genospecies, including *Phyllobacterium sophorae*, *Bradyrhizobium* sp. strain I, *Mesorhizobium* sp. strain II, *R. mongolense*, *R. lusitanum*, *Rhizobium* spp. II, III, and V were minor groups containing 1 to 4 isolates (Table 2), which were found in one to three sampling sites.

The highest diversity index of Shannon-Weiner (H') was found on site S18 (1.68), followed by that on site S5 (1.58) and site S17 (1.22) (Table 2), and seven genospecies were found on each of these three sites. The lowest H' values (0) were found on sites S2, S9, S10, S14, and S15, with only one genospecies isolated on those sites. The other sampling sites had H' values ranging from 0.97 to 0.30. The values of Simpson's index (D) varied between 0.78 and 0 on 18 sampling sites and were well consistent with the H' values. Evenness index Pielou (J) varied from 0.97, in the case of sites S1 and S7, to 0.27, in the case of site S16. These results demonstrated that the diversity and genospecies composition of the rhizobial community associated with *S. flavescentis* varies on different sampling sites.

Soil factors influencing the geographic distribution of rhizobia.

According to the length of the arrows (the longer the arrow, the more highly related that this variable is to the genospecies distribution) and the angles between the arrow and the genospecies (the smaller, the more closely related) shown in Figure 2, the soil pH, total salt, and available potassium had an obviously positive correlation with the distribution of genospecies isolated from ecoregion I, but a negative correlation with the distribution of genospecies isolated from ecoregion II and III. Soil pH is one of the most important soil factors related to the distribution of

Table 1. Geographic information, climate, and soil characteristics of the 18 sampling sites

Ecoregion, ^a geographic origin, and sampling sites	Altitude (m)	GPS		Rainfall (mm) ^b	Soil characteristics ^c						
		Longitude	Latitude		TN	OM	AN	AP	AK	TS	pH
Ecoregion I: Shanxi province											
S1: Zhenxin town, Changzhi	1,224	113°03'08"E	36°02'24"N	411	1.18	16.7	70.0	5.9	436	0.75	7.95
S2: Zhenxin town, Changzhi	1,224	113°03'10"E	36°02'27"N	411	1.06	15.4	53.4	56.7	138	0.70	7.52
S3: Zhenxin town, Changzhi	1,224	113°03'09"E	36°02'30"N	411	0.82	16.3	42.0	19.9	122	0.83	7.76
S4: Wugu Mt., Changzhi	1,041	113°03'02"E	36°02'24"N	411	1.43	30.4	44.3	25.8	170	1.27	7.26
S5: Niusi town, Qinxian	993	112°03'30"E	36°54'36"N	606	0.89	11.4	58.5	14.8	273	0.85	7.75
S6: Niusi town, Qinxian	993	112°03'36"E	36°54'35"N	606	0.94	10.3	44.5	25.4	390	0.86	7.78
S7: Hanbei town, Wuxiang	1,225	113°11'56"E	36°45'40"N	560	1.01	23.0	103.0	33.4	398	0.91	7.76
S8: Hanbei town, Wuxiang	1,225	113°11'55"E	36°45'25"N	560	1.02	28.4	90.4	10.9	322	0.90	7.81
S9: Hanbei town, Wuxiang	1,225	113°11'57"E	36°45'32"N	560	0.90	17.7	50.9	20.8	470	0.73	7.91
S10: Hanbei town, Wuxiang	1,225	113°11'49"E	36°45'35"N	560	1.19	23.8	52.2	7.3	316	0.67	7.84
S11: Hanbei town, Wuxiang	1,225	113°11'50"E	36°45'31"N	560	1.06	20.9	57.3	16.7	562	0.80	7.91
Ecoregion II: Shaanxi province											
S12: Maping town, Luonan	1,066	110°04'10"E	34°12'06"N	700	1.27	18.4	52.7	23.1	93	0.41	5.80
S13: Maping town, Luonan	1,066	110°04'03"E	34°12'11"N	700	1.33	18.0	123.0	34.8	80	0.43	5.44
S14: Maping town, Luonan	1,050	110°03'59"E	34°12'06"N	700	1.43	20.2	89.3	25.5	110	0.45	5.57
S15: Youfang town, Luonan	1,109	110°02'44"E	34°13'14"N	700	1.06	13.0	83.5	18.9	65	0.53	6.68
Ecoregion III: Liaoning province											
S16: Heishui, Jianping	554	119°29'48"E	42°03'33"N	500	0.74	8.18	49.8	9.0	86	0.47	8.36
S17: Changlong, Jianping	526	119°23'16"E	41°58'41"N	500	0.47	5.6	36.6	1.6	40	0.64	8.59
S18: Yangshuling, Jianping	690	119°45'58"E	41°50'36"N	500	0.73	9.29	49.2	7.3	81	0.55	8.46

^a Ecoregion I: clay loam, semihumid, alkaline soil; ecoregion II: sandy loam, moist, acid soil; and ecoregion III: sandy loam, semiarid land, highly alkaline soil.

^b Mean annual rainfall (mm).

^c TN, total nitrogen content (g/kg); OM, organic matter (g/kg); AN, available nitrogen (mg/kg); AP, available phosphorus (mg/kg); AK, available potassium (K) (mg/kg); TS, total salt (g/kg); and pH, soil pH.

Fig. 1. Maximum likelihood phylogenetic tree based on the three concatenated genes (*atpD-glnII-recA*). The model GTR+G+I was used to construct the tree. Only bootstrap values greater than 50% are shown at branch nodes. Bar, 5% nucleotide substitution per site. Boldfaced strains were isolated from *Sophora flavescens*. R., M., B., S., and P. = *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Phyllobacterium*. T = type strain.

these rhizobia ($P = 0.0020$) (Fig. 2; Table 1). *R. yanglingense*, *R. mongolense*, and *Rhizobium* sp. strain IV were solely found in alkaline soil at ecoregion III, while *R. lusitanum*, *Mesorhizobium* sp. strains I and II, *M. huakuii*, *Bradyrhizobium elkanii* were only found in acidic soil at ecoregion II.

Diversity of symbiotic genes and cross nodulation.

Excepting 3 isolates CCBAU 03429, CCBAU 03419 and CCBAU 71316 that failed to nodulate *S. flavescentis*, the symbiotic genes *nodC* and *nifH* were amplified successfully from 266 isolates. As shown by the phylogenetic trees of *nodC* (Fig. 3) and *nifH* genes (Supplementary Fig. S2), the grouping results of *S. flavescentis* isolates were similar and 7 symbiotic gene types (I ~ VII) were distinguishable. The *nodC* type I was found in all of the three ecoregions, including all of the isolates in genospecies *M. septentrionale*. Several isolates defined as *R. mongolense*, *M. huakuii*, *Mesorhizobium* sp. strain I and sp. strain II, had *nodC* genes identical or very similar to those of the reference strain *M. septentrionale* CCBAU 03074 that originated from *Astragalus membranaceus*. Type II included the isolates of *Sinorhizobium fredii*, *M. septentrionale* and *P. sophorae* from ecoregion I, which were identical or very similar to those of reference strains for *Mesorhizobium* species associated with *Astragalus adsurgens*, *Glycyrrhiza pallidiflora* and *Caragana intermedia*. Type III was composed of isolates belonging to *Sinorhizobium fredii* from ecoregion I, and they

were identical to the reference strains in the same species that originated from soybean. Type IV was defined among the isolates of *R. sophorae*, *R. sophoriradicis* and *Rhizobium* sp. strain I from ecoregion I, whose symbiotic genes identical to those of some common bean (*Phaseolus vulgaris*)-nodulating rhizobia (bv. *phaseoli*). The *nodC* types V and VII were from the isolates of *R. yanglingense*, unique for ecoregion III, in which type V was similar to reference strains in the same species from *Gueldenstaedtia multiflora*/*Medicago ruthenica* while the type VII was a separated lineage. Type VI was specific for the isolates of *B. elkanii* from ecoregion II and their *nodC* and *nifH* genes were very similar to those of the soybean (*Glycine max*)-nodulating strain (*B. elkanii* USDA 76^T) in the same species.

In the cross nodulation tests, 54 strains representing different species of the rhizobia in the classes of alpha-Proteobacteria (α -rhizobia) and beta-Proteobacteria (β -rhizobia) were selected to inoculate *S. flavescentis* (Fig. 3 and Table 3). Most test strains, including the highly host-specific *Rhizobium leguminosarum* bv. *viciae* and *R. etli* bv. *phaseoli*, could effectively form nodules on *S. flavescentis*. Another highly host-selective strain, *R. leguminosarum* bv. *trifolii* LMG 8820^T, could nodulate *S. flavescentis* too, but the symbiosis were inefficient. The following 11 strains, *Bradyrhizobium japonicum* USDA 6^T, *B. diazoefficiens* USDA 110^T, *B. yuanmingense* CCBAU 10071^T, *Bradyrhizobium* sp. strain ORS278, *B. oligotrophicum* LMG 10732^T, *Ochrobactrum lupini* LMG 22726^T, *M. muleiense* CCBAU 83963^T, *M. amorphae*

Table 2. Representative genospecies identified based on multilocus sequence analysis (MLSA) similarities and their distribution in each sampling site and ecoregion

		Number of isolates in different ecoregions and sites ^c																		
CCBAU no. ^a	Genospecies ^b	Ecoregion I (n = 105)											Ecoregion II (n = 81)				Ecoregion III (n = 83)			Total
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	
03470	<i>R. sophoriradicis</i> (100.0%)	2	2		12														4	20
03386	<i>R. sophorae</i> (100.0%)					3	2					11					1	5	3	25
03360	<i>Rhizobium</i> sp. strain I (95.8%)	3			2	2		2	1			2								12
03429	<i>Rhizobium</i> sp. strain II (95.5%)					1														1
03419	<i>Rhizobium</i> sp. strain III (94.8%)					1												2		3
11560	<i>Rhizobium</i> sp. strain IV (94.5%)																	1	1	2
71316	<i>R. lusitanum</i> (99.5%)													2						2
11559	<i>R. mongolense</i> (96.0%)																1	2	1	4
11536	<i>R. yanglingense</i> (97.8%)																27	1	1	29
03373	<i>S. fredii</i> (99.2%)			5	20			3	6	2	8	1							1	46
71358	<i>Mesorhizobium</i> sp. strain I (95.4%)												1			28				29
71303	<i>Mesorhizobium</i> sp. strain II (94.5%)												2							2
71320	<i>M. huakuui</i> (96.4%)												11	8						19
03405	<i>M. septentrionale</i> (97.1%)			1		9	1											24	6	41
71325	<i>B. elkanii</i> (96.5%)														6	23				29
03416	<i>Bradyrhizobium</i> sp. strain I (94.5%)					1														1
03422	<i>P. sophorae</i> (100%)					2												2		4
	Total	5	2	6	34	19	3	5	7	2	8	14	14	16	23	28	29	37	17	269
Diversity indexes ^d	Shannon-Wiener index (<i>H'</i>)	0.67	0.00	0.45	0.85	1.58	0.64	0.67	0.41	0.00	0.00	0.66	0.66	0.97	0.00	0.00	0.30	1.22	1.68	/
	Simpson index (<i>D</i>)	0.48	0.00	0.28	0.53	0.72	0.44	0.48	0.24	0.00	0.00	0.36	0.36	0.59	0.00	0.00	0.13	0.55	0.78	/
	Pielou index (<i>J</i>)	0.97	Null	0.65	0.77	0.81	0.92	0.97	0.59	Null	Null	0.60	0.60	0.89	Null	Null	0.27	0.63	0.86	/

^a CCBAU no.) Three isolates (03429 [from site S5], 03419 [from site S5], 71316 [from site S13]) could not nodulate *Sophora flavescentis* in the nodulation tests in laboratory conditions and the *nodC* gene could not be amplified.

^b MLSA similarity to known species. $n = 17$. *R.*, *M.*, *B.*, *S.*, and *P.* = *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Phyllobacterium*.

^c Nodules collected in S1 to S11 were from Shanxi province; S12 to S15 were from Shaanxi province; and S16 to S18 were from Liaoning province.

^d The Shannon-Wiener index (H') is the diversity considering the species richness in a community; the Simpson index (D) shows the species dominance in a community; the Pielou index (J) shows the species evenness in a community. / = no data.

ATCC 19665^T, *R. galegae* HAMBI 1174^T, *Burkholderia nodosa* LMG 23741^T and *Burkholderia phyatum* STM 815^T could not infect *S. flavescens* (Fig. 3; Table 3). *Sinorhizobium meliloti* USDA 1002^T, CCBAU 01199, and 1021 could occasionally nodulate, but they fix nitrogen effectively on *S. flavescens* (Fig. 3; Table 3). The β -rhizobia *Burkholderia* sp. strain ICMP 19430 and ICMP 19869, *Burkholderia tuberum* LMG 21444^T, and *Cupriavidus taiwanensis* LMG 19424^T could nodulate *S. flavescens*, but the nitrogen fixation was inefficient and the nodules were white, small, and spherical, indicative of deficient function (Supplementary Figs. S4 and S7).

The isolates that originated from *S. flavescens* belonging to *nodC* types VI (*B. elkanii* CCBAU 71325 and CCBAU 71331), type IV (*R. sophorae* CCBAU 03468, CCBAU 03386 and *R. sophoriradicis* CCBAU 03470), type II (*M. septentrionale* CCBAU 03399), and V (*R. yanglingense* CCBAU 11536) could nodulate effectively on *Glycine max*, *Phaseolus vulgaris*, *Arachis hypogaea*, *Astragalus membranaceus*, and *Caragana intermedia*, respectively (Table 3). While the isolates in types I (*Mesorhizobium* sp. strains I CCBAU 71358 and CCBAU 71303 and *M. septentrionale* CCBAU 03405) and II (*P. sophorae* CCBAU 03422) could not nodulate either *Astragalus sinicus* or *A. membranaceus* (Table 3).

Infection pattern, morphology, and efficiency of nodules.

Cellular curl, branch, or swell could be visible on the tips of root hairs of *S. flavescens* inoculated with the representative rhizobium (*Sinorhizobium fredii* CCBAU 45436 or *R. yanglingense* CCBAU 01603) under light-field microscopy (Fig. 4B through E). Aggregation on the bifurcating site, formation of the infection pocket or thread were observed clearly on and in the root hairs infected by *lacZ*-labeled rhizobia (strains CCBAU 45436 or CCBAU 01603) (Fig. 4F through H). Nodule primordium was found after 15 days of inoculation (Fig. 4I through J).

Most nodules were found on lateral roots and only a few on the taproots. Most reference strains and isolates (like *M. septentrionale* CCBAU 03399) formed effective nitrogen-fixation nodules with red color in the nodule section, while reference strain *M. huakuii* 7653R formed low-efficiency nodules on the plants that showed yellowish leaves. Significant differences ($P < 0.05$) for chlorophyll content were observed between the plants inoculated with strains CCBAU 03399 and 7653R. Sphere-like and deficient nodules were formed by β -rhizobia of *Burkholderia tuberum* LMG 21444^T, *Burkholderia* spp. ICMP 19869 and ICMP 19430 (not shown), and *Cupriavidus taiwanensis* LMG 19424^T on *S. flavescens*, while most α -rhizobia formed effectively indeterminate

Fig. 2. Biplot showing relationships between the 17 genospecies and soil factors in three ecoregions, drawn using CANOCO software. AN = available nitrogen, AP = available phosphorus, AK = available potassium, OM = organic materials, TS = total salt. The arrows and triangles show soil factors and genospecies, respectively. The circles (○), squares (□), and diamonds (◇) show sampling sites in the three ecoregions I, II, and III. Triangles (△) show the distribution of genospecies. The longer the arrow is, the greater the influence the soil factor has on the distribution of the genospecies; the smaller the angle between the arrow and the line (not drawn) linking the triangle and original point (O), the closer relationship the soil factor and the specific rhizobial genospecies have.

(rod-shape or bifurcated) nodules on *S. flavescentis*. Different parts showing the growth of the infection, nitrogen-fixation, and senescence zones were observed clearly in the section of effective nodules induced by α -rhizobium *M. septentrionale* CCBAU 03399 (Supplementary Fig. S5). The root nodules formed by β -rhizobia were much smaller (about 2 mm in diameter) than those formed by α -rhizobia. No more than half of the plant cells within a nodule were infected by β -rhizobium (e.g., *Burkholderia tuberum* LMG 21444^T), while most of the plant cells within a nodule were full of α -rhizobium (e.g., *M. septentrionale* CCBAU 03399). Yellowish leaves of the plants inoculated with β -rhizobia were visualized and chlorophyll content of the leaves was only 23 to 25 $\mu\text{g}/\text{cm}^2$ (SPAD 502), lower than in the α -rhizobia-inoculated plants (approximately 30 $\mu\text{g}/\text{cm}^2$).

Bacteroids in the nodules.

Observation of mature nodules (35 days old) through electron microscopy indicated that the bacteroids in nodule cells of *S. flavescentis* were regular or irregular rods with lengths $<3\ \mu\text{m}$ and widths $<1.6\ \mu\text{m}$ (Fig. 5; Supplementary Fig. S6). Some rhizobia, such as *Sinorhizobium* sp. strain NGR 234, *B. elkanii* USDA76^T, and *Sinorhizobium meliloti* CCBAU 01199, synthesized large amounts of poly- β -hydroxybutyrate (PHB) inside the bacteroids; while other strains, like *M. loti* NZP 2213^T, *M. septentrionale* CCBAU 03399, and *B. arachidis* CCBAU 051107^T only synthesized a few PHB granules (Fig. 5). One to 10 bacteroids could be embedded in a peribacteroid membrane (PBM) inside a host plant cell (Fig. 5). Some bacteroids inside the intercellular walls were not embedded in PBM and, therefore, had a free existence (Fig. 5D, E and F). The volume of peribacteroid space (PBS) varied in nodules induced by different rhizobia on *S. flavescentis*. The bacteroids of *Mesorhizobium loti* NZP 2213^T presented an intracellular membrane system (Fig. 5H). Only a few bacteroids were found in the ineffective nodules formed by β -rhizobium *Burkholderia tuberum* LMG 21444^T, which existed singly (Fig. 5I) and were accompanied by starch grains inside the nodule cells (Fig. 5I). In addition, incomplete PBM surrounding the bacteroids was observed in some *Burkholderia*-induced nodules (Fig. 5J).

DISCUSSION

Diversity of rhizobia nodulating *Sophora flavescentis*.

In the present study, rhizobial diversity and nodule characteristics of *S. flavescentis* were thoroughly studied. The identification of 17 genospecies belonging to five genera (Table 2) with seven very different *nodC* types (Fig. 3) among the nodule isolates indicated that this plant has a less stringent requirement for either the genomic backgrounds or the symbiotic gene backgrounds of the rhizobia, which suggested a third group of legumes, in addition to the two groups classified by Laguerre et al. (2003), i.e., legumes strongly selected for both the genomic background and symbiotic background and legumes that strongly preferred the symbiotic gene background but not the genomic background. All the *S. flavescentis*-nodulating rhizobia, no matter their hosts of origin, showed the existence of *nodC*, suggesting that symbiosis between this plant and the rhizobia is a nod factor-dependent procedure, similar to most of the other legumes found so far (Madsen et al. 2010).

Previously common bean (*Phaseolus vulgaris*) has been believed to be one of the most promiscuous hosts for rhizobia (Dall'Agnol et al. 2014; Verástegui-Valdés et al. 2014), which nodulated with 16 rhizobial species in genera *Rhizobium* and *Sinorhizobium*, but all the bean-nodulating rhizobia harbored very similar symbiotic genes, even biovars *phaseoli* and *mediterraneum* were differentiated (Verástegui-Valdés et al. 2014). The other promiscuous hosts were members of genus *Acacia*,

which can establish symbiosis with several species in genera *Mesorhizobium* and *Sinorhizobium* (Ba et al. 2002) and Siratro (*Macroptilium atropurpureum*), which is often used to test the capability of nodulation of different rhizobia (Angus et al. 2013; Moulin et al. 2001). Recently, *Sophora alopecuroides* was reported as a promiscuous host for 10 rhizobial species in the genera *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Phyllobacterium*, with five divergent types of symbiotic genes (Zhao et al. 2010). In addition, Han et al. (2008) identified three strains isolated from *S. alopecuroides* as *B. elkanii* and *R. tropici*-related genospecies. Compared with the three legumes mentioned above, the rhizobia of *S. flavescentis* showed the following differences. The first remarkable difference was that *S. flavescentis* presented a much larger promiscuous property by nodulating with rhizobia in 17 genospecies of five genera in the fields (Table 2) and with 20 more species originated from other legume species in laboratory conditions (Table 3). The second difference was that the rhizobial community composition of *S. flavescentis* was uniquely characterized by nine major groups in genera *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, and *Rhizobium* as well as eight minor groups in genera *Phyllobacterium*, *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium*, including seven putative novel species (Table 2; Fig. 1). These results differentiated *S. flavescentis* clearly from *S. alopecuroides*, common bean, and *Acacia* spp. for their different rhizobial composition.

Geographic patterns of *S. flavescentis* rhizobia.

Geographic patterns have been observed previously in rhizobia associated with *Caragana* spp. (Lu et al. 2009) and with soybean (Zhang et al. 2011). This pattern is also observed in the rhizobia associated with *S. flavescentis* (Table 2; Supplementary Fig. S1). Soil factors were revealed as determinants for the distribution and community composition of rhizobia (Fig. 2), similar to previous results (Lu et al. 2009; Zhang et al. 2011). The distribution of *Sinorhizobium fredii* mainly in ecoregion I (Table 2) demonstrated its adaptation to high-salinity soils (Table 1), which is similar to the previous study on soybean rhizobia (Han et al. 2009; Li et al. 2011; Zhang et al. 2011). The limited distribution of *B. elkanii* to ecoregion II evidenced its adaptation to acid soils (Table 2), which is also similar to previous observations for soybean rhizobia (Li et al. 2011; Zhang et al. 2011). Previously, Weir et al. (2004) and Tan et al. (2015) found that *Sophora* spp. in New Zealand nodulated with *Rhizobium leguminosarum*, *Mesorhizobium loti*, and other *Mesorhizobium* species. According to the results in the current study as well as others, it is clear that the *Sophora* species, especially *S. flavescentis*, preferred different rhizobial species in different soils. The capability of nodulation with extremely diverse rhizobia offered *S. flavescentis* a wide range of microsymbionts for selection and help in adapting to various environments.

Evolution of symbiotic genes and nodulation of *S. flavescentis*.

The phylogenetic relationships in the *nodC* gene tree (Fig. 3) and the *nifH* gene tree in the present study demonstrated a coevolution of the nodulation genes and the nitrogen-fixation genes in the *S. flavescentis*-nodulating rhizobia. Previously, it has been estimated that the biogeographic patterns of rhizobia are determined by their adaptation to soil environments and the selection of host legumes for the rhizobial genospecies and symbiotic genes (Zhang et al. 2012; Zheng et al. 2013). But the symbiotic genes in rhizobia associated with *Glycine max* (Zhang et al. 2012), *S. alopecuroides* (Zhao et al. 2010), and *S. flavescentis* involved in the present study (Fig. 3) were diverse, indicating that the specific selection of the symbiotic genes was not stringent for these plant species. This estimation was further

supported by the fact that many rhizobial strains harbored diverse symbiotic genes and originated from distinct legumes in the tribes Galegeae, Phaseoleae, Viciae, Crotalariae, Trifolieae, Mimoseae, and Aeschynomeneae could nodulate with *S. flavescens* under laboratory conditions (Fig. 3; Table 3). These results demonstrated that the biogeographic patterns of the *S. flavescens*–nodulating rhizobia may be mainly determined by environmental adaptation and less affected by the selection of symbiotic gene background by the host plant. Further surveys are needed to clarify the mechanism for this phenomenon.

Only the *nodC* types represented by the *Cicer*-nodulating strains *Mesorhizobium ciceri* UPM-Ca7^T and *M. muleiense* CCBAU 83963^T, *Amorpha*-nodulating strain *M. amorphae* ACCC 19665^T, soybean-nodulating strains *B. japonicum* USDA 6^T, *B. diazoefficiens* USDA 110^T, and *Lespedeza*-nodulating strain *B. yuanmingense* CCBAU 10071^T could not nodulate *S. flavescens* in the cross-nodulation tests. Therefore, the phylogenetic relationships of *nodC* could not accurately reflect the host specificity of rhizobia in some cases, although Laguerre et al. (2001) used this relationship to estimate the host range for rhizobia. It is interesting that the soybean-nodulating strains *Sinorhizobium fredii* CCBAU 45436 (*nodC* type III) and *B. elkanii* USDA 76^T (*nodC* type VI) could nodulate *S. flavescens* efficiently, but *B. japonicum* USDA 6^T and *B. diazoefficiens* USDA 110^T (belonging to a distinct *nodC* type) could not. In another case, the strains *M. qingshengii* CCBAU 33460^T and *M. huakuii* 7653R originated from *Astragalus sinicus* and harbored identical *nodC* genes, but the former could nodulate with *S. flavescens* effectively while the latter one formed less-efficient nodules. These cases demonstrate that other genes might also affect the nodulation preference of *S. flavescens*.

In the present study, four β -rhizobial strains belonging to genera *Burkholderia* and *Cupriavidus* formed inefficient nodules (Fig. 5), demonstrating that the affinity between the β -rhizobia and *S. flavescens* has not been well developed. The *Burkholderia* spp. may be more related in their adaptation to infertile, acidic soils and in their symbioses with legumes that are similarly adapted to these climatic and edaphic conditions (Garau et al. 2009). More β -rhizobia need to be surveyed for nodulation efficiency with *S. flavescens* in different soil conditions in the future.

The discordance between the phylogenetic tree for *nodC* (Fig. 3) and the phylogenetic tree inferred from MLSA (Fig. 1) implies that lateral transfer of the symbiotic genes might have happened across some strains and species, such as between *M. temperatum* SDW 018^T and *Sinorhizobium fredii* CCBAU 03373, *M. septentrionale* SDW 014^T and *P. sophorae* CCBAU 03422^T, among *R. sophoriradicis* CCBAU 03470^T, *R. sophorae* CCBAU 03386^T, and *R. vallis* CCBAU 65647^T, and among *Mesorhizobium* sp. strain I CCBAU 71358, *Mesorhizobium* sp. strain II CCBAU 71303, and *M. huakuii* CCBAU 71320 (Fig. 3). The existence of lateral gene transfer of *nodC* among different rhizobia may be another reason that *S. flavescens* could nodulate with such diverse rhizobia.

Although diverse rhizobia with different symbiotic gene types were detected in the microsymbionts of *S. flavescens*, the presence and relative predominance of *nodC* type I in all three ecoregions as well as their high similarity to that of three *Mesorhizobium* strains (with representative isolates of ICMP 19551, ICMP 19558, and ICMP 19567 originating from *Sophora* spp. grown in New

Zealand [Tan et al. 2015]) implies that type I *nodC* is the preferable symbiotic genotype for *S. flavescens*. The failure of cross nodulation of the representative strains in the branch of *nodC* type I (CCBAU 03405, CCBAU 71320, CCBAU 71358, and CCBAU 71303) on *Astragalus membranaceus*, *A. sinicus*, and *Caragana intermedia* (Table 3) further supports the fact that there are distinctly symbiotic genotypes held by these strains.

Infection pattern and nodule morphology of *S. flavescens*.

Previous reports have shown the relationship between the rhizobial spectrum of legume species and the infection mechanisms (Liu et al. 2014; Madsen et al. 2010; Op den Camp et al. 2012; Sajnaga et al. 2001). In our current study, only root-hair infection (RHI) was clearly observed on *S. flavescens* (Fig. 4). Considering the results of the promiscuous associations of soybean and common bean (both are RHI patterns), it is possible that the diversity of rhizobia associated with a legume is not related to the infection pattern but to the other determinants (Yang et al. 2010).

Generally, nodules of legumes can be classified as indeterminate and determinate forms depending on the persistent or transient apical distribution of nodule meristem (Sprenst 2009). Indeterminate nodules are more common in legumes, but all the examined members of the tribes Desmodieae, Phaseoleae, and Psoraleae and some members of the Loteae produce determinate nodules. Two legume species, *Sesbania rostrata* (Fernández-López et al. 1998) and *Dipogon lignosus* (Liu et al. 2014), were capable of forming both indeterminate and determinate nodules. In the present study, root nodules of *S. florescence* formed by α -rhizobia were indeterminate and no determinate nodules were observed. In addition, the fact that strain *Bradyrhizobium arachidis* CCBAU 051107^T originated from peanut (Wang et al. 2013), induced determinate nodules on peanut and indeterminate nodules on *S. flavescens* further confirmed the control of nodule morphology by host plants.

Bacteroids inside the nodule cells of *S. flavescens*.

In contrast to the relatively low bacteroid counts in the symbiosome of soybean (Margaret et al. 2013) and *Astragalus sinicus* (Lei et al. 2014), *S. flavescens* can contain 10 or more bacteroids in a single symbiosome in the effective nodules. This might imply that bacterial division inside the *S. flavescens* symbiosomes was not well controlled. Future studies will be focused on determining if this phenomenon is related to a wide spectrum of microsymbionts.

Spherical and swollen large bacteroids were observed in peanut nodules (Bal et al. 1985; Okubo et al. 2012; Oono and Denison 2010), but *Bradyrhizobium arachidis* CCBAU 051107^T, a strain originated from peanut (Wang et al. 2013), presented only nonswollen bacteroids in nodules of *S. flavescens* (Fig. 5F). In addition, all other bacteroids in nodules formed by different rhizobia on *S. flavescens* were nonswollen. This finding provides further evidence that the differentiation of bacteroids into swollen or nonswollen types is determined by the host plant (Oono and Denison 2010).

Most PBS formed by *Mesorhizobium loti* NZP 2231^T is limited by the PBM and the bacteroids being in close contact (Fig. 5), while the PBS formed by *M. septentrionale* CCBAU 03399, *Sinorhizobium* sp. strain NGR 234, and *B. arachidis*

Fig. 3. Maximum likelihood phylogenetic tree based on *nodC* genes. The model T92+G+I was used to construct the tree. Only bootstrap values greater than 50% are shown at branch nodes. Bar, 5% nucleotide substitution per site. A thick vertical bar indicates effective nodules; circles (○), no nodule; triangles (△), not stable, but effective nodules; and diamonds (◇), less-effective or inefficient nodules. The strains with boldfaced names were isolated from *Sophora flavescens*. Host plants of origin of these rhizobia were added after the accession number of each strain. Ecoreg. = ecoregion, *R.* = *Rhizobium*, *B.* = *Bradyrhizobium*, *S.* = *Sinorhizobium*, *M.* = *Mesorhizobium*, and T = type strain.

Table 3. Cross nodulation tests on *Sophora flavescens* and other legumes^a

Strains (<i>nodC</i> type)	Original host (tribe)	<i>Sophora flavescens</i>		<i>Caragana intermedia</i>		<i>Astragalus membranaceus</i>		<i>Astragalus sinicus</i>		<i>Glycine max</i>		<i>Phaseolus vulgaris</i>		<i>Arachis hypogaea</i>	
		Nod	Nif	Nod	Nif	Nod	Nif	Nod	Nif	Nod	Nif	Nod	Nif	Nod	Nif
<i>Bradyrhizobium</i> spp.															
<i>elkanii</i> USDA 76 ^T	<i>Glycine max</i> (Paseoleae)	+	F							O	O				
<i>elkanii</i> CCBAU 71325 (VIB)	<i>Sophora flavescens</i> (Sophoreae)	O	O							+	F				
<i>elkanii</i> CCBAU 71331 (VIC)	<i>Sophora flavescens</i> (Sophoreae)	O	O												
<i>diazoefficiens</i> USDA 110 ^T	<i>Glycine max</i> (Paseoleae)	–								O	O				
<i>japonicum</i> USDA 6 ^T	<i>Glycine max</i> (Paseoleae)	–								O	O				
<i>liaoningense</i> LMG 18320 ^T	<i>Glycine max</i> (Paseoleae)	–													
<i>arachidis</i> CCBAU 051107 ^T	<i>Arachis hypogaea</i> (Aeschynomeneae)	+	F											O	O
<i>yuanmingense</i> CCBAU 10071 ^T	<i>Lespedeza cuneata</i> (Desmodieae)	–													
<i>oligotrophicum</i> LMG 10732 ^T	Soil, symbiosis with <i>Aeschynomene indica</i>	–													
sp. strain ORS278	<i>Aeschynomene sensitive</i> (Aeschynomeneae)	–													
<i>Rhizobium</i> spp.															
<i>yanglingense</i> CCBAU 01603	<i>Caragana intermedia</i> (Galegeae)	+	F	O	O										
<i>yanglingense</i> CCBAU 71623 ^T	<i>Gueldenstaedtia multiflora</i> (Galegeae)	+	F												
<i>fabae</i> CCBAU 33202 ^T	<i>Vicia faba</i> (Vicieae)	+	F												
<i>leguminosarum</i> bv. <i>viciae</i> USDA 2370 ^T	<i>Pisum sativum</i> (Paseoleae)	+	F												
<i>galegae</i> HAMI 540 ^T	<i>Galega orientalis</i> (Galegeae)	–													
<i>tropicici</i> CIAT 899 ^T	<i>Phaseolus vulgaris</i> (Paseoleae)	+	F									O	O		
<i>pisi</i> DSM 30132 ^T	<i>Pisum sativum</i> (Paseoleae)	+	F												
<i>laguerreae</i> FB206 ^T	<i>Vicia faba</i> (Vicieae)	+	F												
<i>vallis</i> CCBAU 65647 ^T	<i>Phaseolus vulgaris</i> (Paseoleae)	+	F									O	O		
<i>phaseoli</i> ATCC 14482 ^T	<i>Phaseolus vulgaris</i> (Paseoleae)	+	F									O	O		
<i>etli</i> bv. <i>phaseoli</i> CFN42 ^T	<i>Phaseolus vulgaris</i> (Paseoleae)	+	F									O	O		
<i>leguminosarum</i> bv. <i>trifolii</i> LMG 8820 ^T	<i>Trifolium pratense</i> (Trifolieae)	+	◇												
<i>sophorae</i> CCBAU 03468 (IV)	<i>Sophora flavescens</i> (Sophoreae)	O	O									+	F		
<i>sophorae</i> CCBAU 03386 (IV)	<i>Sophora flavescens</i> (Sophoreae)	O	O									+	F		
<i>sophoriradicis</i> CCBAU 03470 (IV)	<i>Sophora flavescens</i> (Sophoreae)	O	O									+	F		
<i>yanglingense</i> CCBAU 11536 (V)	<i>Sophora flavescens</i> (Sophoreae)	O	O	+	F										
<i>Sinorhizobium</i> spp.															
<i>meliloti</i> USDA 1002 ^T	<i>Medicago sativa</i> (Trifolieae)	±	F												
<i>meliloti</i> 1021	<i>Medicago sativa</i> (Trifolieae)	±	F												
<i>meliloti</i> CCBAU 01199	<i>Medicago sativa</i> (Trifolieae)	±	F												
sp. strain NGR 234	<i>Lablab purpureus</i> (Paseoleae)	+	F												
<i>fredii</i> CCBAU 45436	<i>Glycine max</i> (Paseoleae)	+	F							O	O				
<i>fredii</i> CCBAU 03392 (III)	<i>Sophora flavescens</i> (Sophoreae)	O	O							+	F				
<i>Mesorhizobium</i> spp.															
<i>loti</i> NZP 2213 ^T	<i>Lotus corniculatus</i> (Loteae)	+	F												
<i>muleiense</i> CCBAU 83963 ^T	<i>Cicer arietinum</i> (Cicereae)	–													
<i>huakuii</i> CCBAU 2609 ^T	<i>Astragalus sinicus</i> (Galegeae)	–						O ^b							
<i>huakuii</i> 7653R	<i>Astragalus sinicus</i> (Galegeae)	+	◇					O	O						
<i>qingshengii</i> CCBAU 33460 ^T	<i>Astragalus sinicus</i> (Galegeae)	+	F					O	O						
<i>amorphae</i> ACCC 19665 ^T	<i>Amorpha fruticosa</i> (Amorpheae)	–													
<i>tianshanense</i> USDA 3592 ^T	<i>Glycyrrhiza pallidiflora</i> (Galegeae)	+	F												
<i>temperatum</i> CCBAU 01578	<i>Caragana intermedia</i> (Galegeae)	+	F	O	O										
<i>amorphae</i> CCBAU 01583	<i>Caragana intermedia</i> (Galegeae)	+	F	O	O										
<i>septentrionale</i> CCBAU 03074	<i>Astragalus membranaceus</i> (Galegeae)	+	F			O	O								
<i>septentrionale</i> CCBAU 03399 (IIA)	<i>Sophora flavescens</i> (Sophoreae)	O	O	+	F	+	F	–							
<i>amorphae</i> CCBAU 03405 (IA)	<i>Sophora flavescens</i> (Sophoreae)	O	O	–		–		–							
<i>huakuii</i> CCBAU 71320 (IB)	<i>Sophora flavescens</i> (Sophoreae)	O	O	–		–		–							
sp. strain I CCBAU 71358 (IB)	<i>Sophora flavescens</i> (Sophoreae)	O	O	–		–		–							
sp. strain II CCBAU 71303 (IB)	<i>Sophora flavescens</i> (Sophoreae)	O	O	–		–		–							
<i>Ochrobactrum</i> sp.															
<i>lupini</i> LMG 22726 ^T	<i>Lupinus albus</i> (Lupininae)	–													
<i>Phyllobacterium</i> sp.															
<i>sophorae</i> CCBAU 03422 ^T (IIB)	<i>Sophora flavescens</i> (Sophoreae)	O	O	–		–		–							
<i>Burkholderia</i> spp.															
<i>tuberum</i> LMG 21444 ^T	<i>Aspalathus carnosa</i> (Crotalariaeae)	+	◇												
sp. strain ICMP 19869	<i>Dipogon lignosus</i> (Phaseoleae)	+	◇												
<i>nodosa</i> LMG 23741 ^T	<i>Mimosa scabrella</i> (Mimoseae)	–													
<i>phymatum</i> STM815 ^T	<i>Machaerium lunatum</i> (Dalbergieae)	–													
sp. strain ICMP 19430	<i>Dipogon lignosus</i> (Phaseoleae)	+	◇												
<i>Cupriavidus</i> sp.															
<i>taivanensis</i> LMG 19424 ^T	<i>Mimosa pudica</i> (Mimoseae)	+	◇												

^a Nod, nodulation; Nif, nitrogen fixation; +, strains could nodulate on *S. flavescens*; –, strains could not nodulate on *S. flavescens*; ±, strains form effective nodules on some plants; F, strains form effective nodules; ◇, strains form less effective or ineffective nodules; O, original host plants. Effectiveness for nitrogen fixation was visually evaluated by comparing color of leaves and nodule section between inoculated and noninoculated plants.

^b Although *Mesorhizobium huakuii* CCBAU 2609^T was isolated from *Astragalus sinicus*, it could not nodulate the host plant.

Fig. 4. Light micrograph of root hair, infection thread, and nodule primordium of *Sophora flavescens* inoculated by different rhizobia. **A**, Uninoculated root hairs at 6 days. **B** and **C**, Root hairs, 6 days after inoculation (dai) with *Sinorhizobium fredii* CCBAU 45436. **D** and **E**, Root hairs, 6 dai with *Rhizobium yanglingense* CCBAU 01603. **F**, Aggregating cells of the *lacZ*-labeled *Sinorhizobium fredii* CCBAU 45436 surrounding the tip of a biforked root hair, 10 dai. **G**, Infection pocket formed by the *lacZ*-labeled *R. yanglingense* CCBAU 01603, 10 dai. **H**, Infection thread induced by *lacZ*-labeled *Sinorhizobium fredii* CCBAU 45436, 11 dai. **I** and **J**, Nodule primordium induced by *lacZ*-labeled *Sinorhizobium fredii* CCBAU 45436 and *R. yanglingense* CCBAU 01603, respectively, both at 15 dai. Bars: 40 μm in A, B, D, E, and H; 20 μm in C, F, and G; and 60 μm in I and J.

Fig. 5. Electron microscope of nodule section showing the characteristics of bacteroids of different rhizobia. All the sections were from zone III (i.e., the N_2 -fixation zone). **A**, *Bradyrhizobium elkanii* USDA 76^T; **B**, *Mesorhizobium septentrionale* CCBAU 03399; **C**, **D**, and **H**, *Mesorhizobium loti* NZP 2213^T; **E**, *Sinorhizobium* sp. strain NGR 234; **F**, *Bradyrhizobium arachidis* CCBAU 051107^T; **G**, *Sinorhizobium meliloti* CCBAU 01199; **I** and **J**, *Burkholderia tuberum* LMG 21444^T. Bac, bacteroid in symbiosome; Bac*, bacteroid in intercellular wall; HCW, host cell wall; IS, intercellular space; ED, electron-dense material; CV, cytoplasmic vacuole; PP, electron-opaque polyphosphate inclusions; RS, ring and space surrounding an electron-opaque inclusion; PBS, peribacteroid space; ESB, empty symbiotic bubble; PBM, peribacteroid membrane; S, starch grains in plant cells; and IPM, incomplete peribacteroid membrane.

CCBAU 051107^T are bigger (Fig. 5). Regardless of the volume and size of PBS, all of these rhizobia fix nitrogen effectively on *S. flavescentis*. The bigger PBS makes *S. flavescentis* and some rhizobia good candidates for studying pH changes in the PBS and the transportation from PBS to plant cytoplasm across the PBM. The existence of bacteroids inside the intercellular spaces (Fig. 5D, F and G) without PBM suggests the movement of rhizobia through spaces between plant cells is ongoing.

Synthesis of starch grains and disrupted PBM were reported in ineffective nodules of *Phaseolus vulgaris* infected by a C₄-dicarboxylic acid mutant of *Rhizobium leguminosarum* bv. *phaseoli* (Lafontaine et al. 1990). The incomplete PBM surrounding the bacteroid of *Burkholderia tuberum* and the accumulation of starch grains (Fig. 5I) further explain the ineffectiveness of nodules and the incompatibility between *Burkholderia* spp. and *S. flavescentis*.

PHB inclusion in the nodule of *S. flavescentis*.

The existence and accumulation of PHB in bacteroids is considered to stabilize cellular redox conditions (Anderson and Dawes 1990) and may provide energy and a carbon source to the bacteroids when they were released from nodules to soil (Ratcliff et al. 2008). PHB accumulation in bacteroids of *S. flavescentis* nodules varied for different rhizobial strains. *B. elkanii* USDA 76^T, *Sinorhizobium* sp. strain NGR 234, and *Sinorhizobium meliloti* CCBAU 01199 synthesized a great amount of PHB within bacteroids, while *M. loti* NZP 2213^T and *M. amorphae* CCBAU 03399 synthesized few PHB (Fig. 5). However, the *S. flavescentis* inoculated with all these strains fix nitrogen efficiently, which is consistent with previous reports that strains unable to synthesize PHB or glycogen are still able to form nodules and fix nitrogen (Wang et al. 2007). So, the synthesis of PHB in bacteroids of *S. flavescentis* is strain-dependent and may not be always stringently related to the efficiency of nitrogen fixation.

Conclusion and remarks.

Sophora flavescentis is an extremely promiscuous legume for rhizobial symbionts in natural environments as well as under laboratory conditions. It has no strong preference for either the chromosomal or symbiotic gene backgrounds of rhizobia. Some narrow host-range rhizobia, e.g., different biovars of *Rhizobium leguminosarum*, *Mesorhizobium huakuii*, and *M. loti* could nodulate and fix nitrogen efficiently on *S. flavescentis*. A clear biogeographic pattern exists for *S. flavescentis*-nodulating rhizobia belonging to different species and distinct symbiotic gene types; however, this pattern is possibly affected by the soil conditions. Typical RHI is found in the rhizobial entry to *S. flavescentis*. Most α -rhizobia form functionally indeterminate nodules, while representative β -rhizobia induce inefficient nodules on *S. flavescentis*. In addition, initial records indicate as many as 10 bacteroids could be included in a single symbiosome and that the PHB synthesis in bacteroids of *S. flavescentis* is strain-dependent.

Based on the results obtained in this study, it seems worthwhile to investigate i) whether the *nod* genes (or Nod factors) play the same role in invasion via RHI; ii) how the development of bacteroids of a rhizobial species (e.g., *Bradyrhizobium arachidis*) in the swollen (*Arachis hypogaea*) or nonswollen (*S. flavescentis*) host plants differ; iii) whether the number of bacteroids in the symbiosome and the synthesis of PHB affect the nitrogen fixation of the nodules; and iv) how the legume selects distinct rhizobia in different environments.

MATERIALS AND METHODS

Nodule sampling and isolation of rhizobia.

Root nodules of cultured *Sophora flavescentis* were collected from 18 sampling sites in the provinces of Shanxi, Shaanxi,

and Liaoning, corresponding to three different ecoregions (I, II, and III) of China (Table 1; Fig. 2), in July through August of 2012 and 2013. At least five plants were surveyed and more than three nodules were collected per plant grown in cultivated *S. flavescentis* fields without rhizobial inoculation history. Sampling, transportation, and isolation of root-nodule bacteria followed the methods of Zhang et al. (2011), except for the replacement of 0.2% mercury bichloride (HgCl₂) with 2.5% sodium hypochlorite (NaClO) and the altering of sterilization time to 8 min for the surface-sterilization of nodules. Culture on plates or in broth of yeast-mannitol (YM) medium (Vincent 1970) and the storage methods were the same as described by Zhang et al. (2011).

Soil sampling and characterization.

Soil was sampled and treated for determining chemical properties following the methods of Zhang et al. (2011) and Yan et al. (2014). Contents of available N, P, K, and pH of soil factors were analyzed in the Plant Nutrient and Resource Research Institution, Beijing Academy of Agriculture and Forestry Sciences (Beijing), using the standard methods (Yan et al. 2014).

Estimating genetic diversity of rhizobia by MLSA of housekeeping and symbiotic genes.

Total DNA was extracted from the bacterial culture in TY broth (tryptone, 5 g; yeast extract, 3 g; CaCl₂, 0.6 g; distilled water, 1 liter; pH 7.0) using the GuTC (guanidinium-Tris-CDTA buffer with celite) method (Terefework et al. 2001). The *recA* gene was amplified in all rhizobial isolates, using primers *recA41F* and *recA640R* and the polymerase chain reaction (PCR) procedure described by Vinuesa et al. (2005). The PCR products were sequenced directly using the *recA41F* primer. All of the *recA* sequences acquired in this study were used to determine their identities with Clustal W software (Larkin et al. 2007). Isolates sharing identical sequences were designated as a single genotype. Based on the grouping results from *recA* gene analysis, representative isolates of different genotypes were chosen to amplify the other two housekeeping genes, *glnII* and *atpD*, using primer pairs *glnII12F* and *glnII689R* and *atpD255F* and *atpD782R*, respectively, with protocols described by Vinuesa et al. (2005). Two symbiotic genes, *nifH* and *nodC*, were amplified separately using the primer pairs *nifHF* and *nifHR* (Laguette et al. 2001) and *nodCF540* and *nodCR1160* (Sarita et al. 2005), following the corresponding protocols. All the PCR products were sequenced using the corresponding forward primers. The acquired sequences and the related sequences obtained from the GenBank database by BLASTn searching were aligned and a maximum-likelihood phylogenetic tree was constructed, using the software MEGA 6.0 (Tamura et al. 2013). The phylogenetic trees were bootstrapped with 1,000 replications.

To estimate the community structure and species richness of *S. flavescentis* rhizobia, genospecies were defined based upon the result of MLSA with the concatenated *recA*, *atpD*, and *glnII* sequences, using the average nucleotide identity of 96% as threshold of genospecies (Ji et al. 2015). Rhizobial diversity, species richness, and evenness in different sampling sites (S1 to S18) were estimated by three popular alpha ecological indexes (Hill et al. 2003), i.e., the Shannon-Wiener (*H'*) and Simpson (*D*) indexes to represent diversity considering both the genospecies richness and evenness, respectively, in a community (here the sampling sites) and the Pielou index (*J*) to represent genospecies evenness in the community. These indexes of biodiversity, implemented in the CRAN *vegan* package (version 1.17-4), were calculated by the R statistical language (R Core Team 2014).

Correspondence analysis among the rhizobial genomic species, ecoregions, and soil characters.

Redundancy analysis (RDA) (Rao 1964), the canonical version of principal component analysis, was used to look for possible relationships between the soil factors and different rhizobial distributions in the 18 sampling sites (Table 1). Community data for rhizobial genospecies (Table 2) were preanalyzed by detrended correspondence analysis (DCA), using CANOCO software 4.5 (Microcomputer Power, Ithaca, NY, U.S.A.) (Lepš and Šmilauer 2003). In the DCA, the models of species response to environmental variables and the length of the gradient (first axis) were 4.709, so both the linear model and the unimodal model are suitable. After further model tests, RDA proved to be the best method.

GenGIS software was used to merge geographic, ecological, and phylogenetic biodiversity data in a single interactive visualization and analysis environment (Parks et al. 2009). Four files, including the China map (in shapefile format), the geographic location of the sampling sites (in comma-separated values [.csv] format), the statistic ratio (Table 2) of genospecies in each site (in .csv format) and the hierarchical cluster dendrogram of the ratio of genospecies in each site (in Newick format) were prepared and were introduced in sequence to GenGIS software, following the GenGIS manual.

Nodulation tests.

The nodulation capability of all the isolates was tested on the host plant *S. flavescentis*. To study the symbiotic promiscuity of *S. flavescentis*, representative strains belonging to different rhizobial species in classes α - and β -Proteobacteria with different *nodC* types and whose leguminous hosts in different cross-nodulation groups were tested for nodulation capability and symbiotic effectiveness on *S. flavescentis* (Table 3). These strains were obtained from CCBAU or from other culture collections (Table 3). Some representative strains isolated from *S. flavescentis* were also used to inoculate other related legumes (Table 3). Seeds of *S. flavescentis* were immersed in concentrated sulphuric acid (H_2SO_4) for 20 to 30 min. After discarding the H_2SO_4 , 95% ethanol was added to rinse the seeds. Then, seeds were surface-sterilized using 2.5% sodium hypochlorite (NaClO) for 15 min. After being rinsed in sterilized distilled water seven times, the seeds were transferred onto a water-agar (0.8%, wt/vol) plate for germination for 3 days. The germinated seedlings were transplanted into the upper layer of a Leonard jar containing sterilized vermiculite moistened with nitrogen-free nutrient solution (Vincent 1970). A 1-ml bacterial suspension cultured to logarithmic phase was added to the rhizosphere of the seedlings. The noninoculated plants were used as negative control. Plants were moved to the greenhouse with the rotation of light for 16 h at 25°C and dark for 8 h at 23°C. Nodulation was checked 45 days after inoculation. The color of the mature root-nodule section (red or white) and the leaves of the plant (bright green or yellow green) were observed, to evaluate plant growth and nitrogen-fixing efficiency. Chlorophyll content of the leaves was measured, using a chlorophyll meter (Spad 502 Plus, Konica Minolta, Inc., Osaka, Japan) to estimate the symbiotic effectiveness of the nodules indirectly.

Microscopy observation.

For observing the nodule structure under bright-field microscopy, samples were prepared as follows. Three nodules were excised from *S. flavescentis* plants and were fixed with freshly prepared 4% paraformaldehyde and 0.25% glutaraldehyde in 10 mM sodium phosphate buffer (pH 6.8) supplemented with 100 mM NaCl. Nodules in 1% toluidine blue liquor were placed in vacuum conditions for 20 to 30 min to improve infiltration and staining. Specimens were washed in

the same buffer and were dehydrated in a graded ethanol series (75, 90, and 100%, vol/vol). Then, they were infiltrated and embedded in JB-L5-tissue (Junjie Ecoelectronics, Wuhan, China). Semithin slides (4 to 5 μm thickness) were cut, using a LEICA Microm RM2016 microtome (Wetzlar, Germany), and were photographed using Olympus U-HSCBM bright-field microscopy.

Observation of bacteroids inside the nodules was performed using a JEM-1230 transmission electron microscope (TEM). Root nodules were fixed in 2.5% glutaraldehyde for more than 2 h, were washed with 0.1 M phosphate buffer three times, were fixed in 1% osmium for 2 h, and were dehydrated in a graded acetone (30, 50, 70, 80, 90, and 100%, vol/vol, for 5 to 15 min each). Then, nodules were embedded in SPURR epoxy resin, were polymerized, and were sliced longitudinally into ultrathin slices, using a LEICA UC6 ultra microtome. After being stained with both uranyl acetate and lead citrate, the ultrathin slice was observed with TEM.

Observation of the infection process.

To observe the infection process, two representative strains, *R. yanglingense* CCBAU 01603 and *Sinorhizobium fredii* CCBAU 45436 (both are resistant to nalidixic acid [NA], 30 $\mu\text{g ml}^{-1}$), were labeled with the *lacZ* reporter gene by tri-parental mating as previously described (Hoekema et al. 1983; Vadawale et al. 2011). Briefly, *Escherichia coli* DH5 α with the *lacZ* reporter plasmid PGD926 (tetracycline resistant, 20 $\mu\text{g ml}^{-1}$) (Ditta et al. 1985) as the donor strain, *E. coli* DH5 α with pRK2013 (kanamycin resistant, 50 $\mu\text{g ml}^{-1}$) (Ditta et al. 1980) as the helper strain, and the rhizobial strain CCBAU 01603 (or CCBAU 45436) as the recipient strain were incubated in Luria-Bertani (for *E. coli* at 37°C) or TY (for rhizobia at 28°C) broth to the potential phase. Cultures were collected by centrifugation, were resuspended in 1 ml of TY broth, and were mixed in a ratio of 3:2:10 (vol:vol:vol) on TY agar for mating for 24 h at 28°C. The mating culture was spread onto the YM agar containing two antibiotics, tetracycline and NA, for selecting the conjugants.

After germination on water-agar plates for 3 days, seedlings of *S. flavescentis* were transferred to vermiculite-containing Leonard jars as described above. Three days later, the seedlings were inoculated with 1 ml of *lacZ*-labeled logarithmic growing conjugants in YM broth containing corresponding antibiotics. At an interval of 3 days, plants were taken out to observe the rhizobial infection and nodule development. Plant roots inoculated with the *lacZ*-labeled strain CCBAU 01603 (or CCBAU 45436) were fixed and were stained using standard procedures (Marchetti et al. 2010). Samples were observed using a bright-field microscope (Olympus BX53 microscope with DP72 capture).

ACKNOWLEDGMENTS

Many thanks to X. Liu, W. Liu, M. Andrews, F. John, M. Sadowsky, C. Verecke, Y. G. Li, and L. Wang for providing us with reference strains or plasmids. Thanks to Z. Guan and S. Wu for their help on collecting nodules in the fields. Appreciations go to M. Y. Li, A. Zimmerman and T. Godfrey for their improvement for the English of the manuscript. Thanks to D. X. Yang for his help on polishing the photos. This research was supported by China Natural Foundation (No. 31270052), Chinese Universities Scientific Fund (2015TC001) and Northern China Standardized Plant Base Optimization and Upgrading and Series Products Comprehensive Development of *Sophora flavescentis* (No. 2011BA107B03).

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