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Biodiversity and biogeography of rhizobia associated with common bean (*Phaseolus vulgaris* L.) in Shaanxi Province

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ABSTRACT

The biodiversity and biogeography of rhizobia associated with bean in Shaanxi Province were investigated. A total of 194 bacterial isolates from bean nodules collected from 13 sampling sites were characterized based on phylogenetic analyses of the 16S rRNA gene, the housekeeping genes *recA*, *glnII* and *atpD*, and the symbiotic genes *nodC* and *nifH*. Fifteen genospecies belonging to the genera *Rhizobium*, *Agrobacterium*, *Ensifer*, *Bradyrhizobium* and *Ochrobactrum* were defined among the isolates, with *Rhizobium* sp. II, *Agrobacterium* sp. II, *E. fredii* and *R. phaseoli* being the dominant groups. Four symbiotic gene lineages corresponding to *Rhizobium* sp. I, *Rhizobium* sp. II, *R. phaseoli* and *B. liaoningense* were detected in the *nodC* and *nifH* sequence analyses, indicating different origins for the symbiotic genes and their co-evolution with the chromosome of the bacteria. Moreover, the *Ensifer* isolates harbored symbiotic genes closely related to bean-nodulating *Pararhizobium giardinii*, indicating possible lateral gene transfer from *Rhizobium* to *Ensifer*. Correlation of rhizobial community composition with moisture, temperature, intercropping, soil features and nutrients were detected. All the results demonstrated a great diversity of bean rhizobia in Shaanxi that might be due to the adaptable evolution of the bean-nodulating rhizobia subjected to the diverse ecological conditions in the area.

Introduction

Common bean (*Phaseolus vulgaris* L.) originated and was domesticated in the Andes and Mexico. It is an excellent food crop cultivated worldwide and represents 50% of leguminous grain products for direct human consumption. This plant was introduced from Latin America to China 600 years ago [42], and its wide cultivation has made China an important producer and one of the secondary centers of diversity for common bean [54]. Like other legumes, common bean is able to fix nitrogen by forming root and/or stem nodules with rhizobia symbiotic bacteria. Currently, different rhizobial species nodulating with common bean plants grown in various areas of the world have been described, including *R. etli* [36], *R. tropici* [27], *P. giardinii* and *R. gallicum* [1,32], R. *lusitanum* [41], *R. phaseoli* [35], *R. azibense* [30], *R. freirei* [9], *E. meliloti* [56], *E. americanus* [31], and *Bradyrhizobium* sp. [17], among others. Furthermore, *E. fredii*-like salt-tolerating bacteria and *Mesorhizobium* spp. have been isolated from common bean plants in Spain [18] and Brazil [16], respectively. The existence of biogeographic patterns in the common bean-nodulating rhizobia has been shown in previous studies, and was thought to be a result of interaction between environmental factors, host plants and the symbiotic bacteria [43].

Shaanxi Province in China is the center of the Asian section of the second Eurasian continental bridge with a total area of 205,800 km^2. It is an arid-humid transition zone with a continental climate divided into three geographic/ecological regions by the Beishan Mountains and Qinling Mountains. The northern Shaanxi (Plateau of Northern Shaanxi) is to the north of Beishan Mountains, and classifies as a temperate semi-arid area with an annual average temperature of 7–12 °C and an altitude of 800–1300 m. The central area of Shaanxi (Guanzhong Plain) is between the two mountain ranges, and is a sub-humid warm temperate region accompanied by high temperature and drought in the summer, with an altitude of 325–800 m. The region of southern Shaanxi, to the south of Qinling Mountains, has a typical subtropical climate with a higher than annual average temperature and precipitation than the other two regions. In addition, the soil types in Shaanxi Province are very

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diverse, with more than 400 soil species in total, and common bean is widely cultivated as one of the vegetables in all three regions. Based on the diverse climate and soil conditions, various rhizobia associated with common bean could be expected, which represent an excellent model for estimating the correlation between rhizobial diversification and specific environmental conditions. However, the diversity of rhizobia associated with common bean has not been studied thoroughly in Shaanxi.

Considering the existence of complex ecological conditions in Shaanxi and the strong effects of the ecological and geographical factors on the distribution/diversity of rhizobia, this study (i) investigated the diversity of common bean rhizobia in the three ecoregions of Shaanxi; and (ii) estimated the ecological drivers for the distribution of common bean rhizobial species.

Materials and methods

Sampling strategy and rhizobial isolation

Based on the geographical and climatic conditions, 13 sampling sites were selected (Fig. 1). A total of 8 sampling sites were chosen from N32° to N38° at E109°, and 5 sampling sites were chosen on the N34° line, ranging from E107° to E109°. The sites from N38° to N36° corresponded to the Shaanbei (northern) Region, N35° to N34° to the Guanzhong (central) Region, and N33° to N32° to the Shaannan (southern) Region (Supplementary Table S1). All the sampling sites were located in intercropping fields without a rhizobial inoculation history. At least 15 plants were randomly chosen from each site during July to August in 2010. Roots of common beans excavated from the soil were transported to the laboratory together with soil samples collected from the surface layer of the same sites at a depth of 0–20 cm. The soils were subsequently used for physiochemical characterization (pH, organic matter, available nitrogen, available phosphorus and available potassium) using routine methods. The geographical conditions (longitude, latitude, altitude, landforms, soil profile structure, groundwater level), climatic conditions (rainfall, average geothermal at a depth of 20 cm, annual effective accumulative temperature, monthly average temperature, monthly average relative humidity, sunshine duration), soil features (soil types, parent materials), cropping system (number of crops per annum) and intercropping (type of crop) for each sampling site were obtained from the Department of Agriculture and Meteorological Bureau of Shaanxi Province (Table S2).

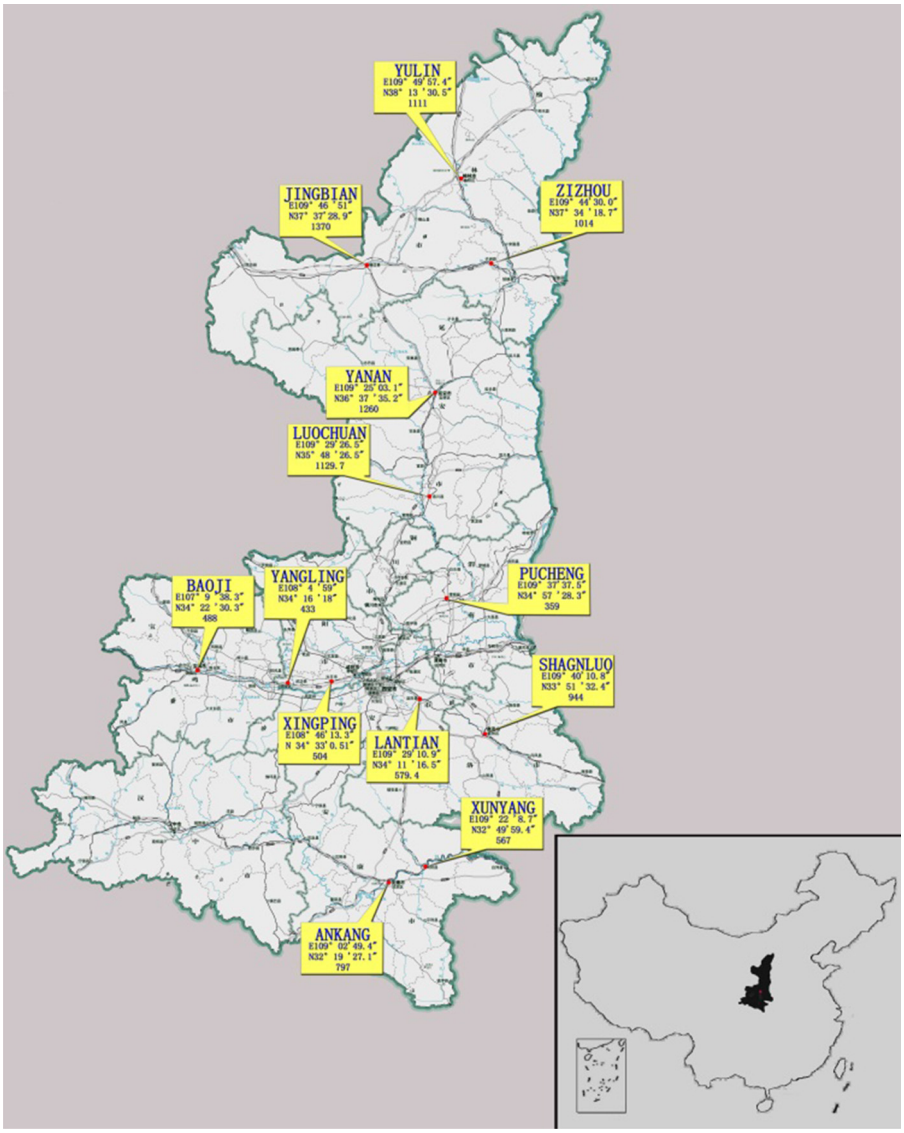


Fig. 1. Map of Shaanxi Province showing the sampling sites (·). The corresponding position of Shaanxi Province in China is shown in the inset. The two maps were created using DIVA-GIS software (http://www.diva-gis.org), and the sampling sites were added according to GPS records.

In this study, healthy and complete nodules randomly selected from the bean roots were sterilized by immersion in 95% (v/v) ethanol for 30 s and 0.1% $HgCl_2$ for 5 min, and then rinsed six times with sterile distilled water [44]. The surface-sterilized nodules were crushed separately in sterilized microtubes and the nodule fluid was streaked onto yeast-mannitol agar (YMA) plates [44]. The inoculated plates were incubated at 28 °C for 3–14 days and the single bacterial colonies were purified by repeatedly streaking on the same medium. Pure cultures were maintained on YMA slants at 4 °C for short-term storage or in YM broth supplied with 20% (w/v) glycerol at −80 °C for long-term storage. All the rhizobial isolates and their relevant information are listed in Supplementary Table S1.

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

Genomic DNA was extracted from each rhizobial isolate using the CTAB method [50] and was used as template for amplifying various housekeeping genes and symbiotic genes. Primers P1 and P6 [40] were used for amplifying the 16S rRNA gene, as described previously [49]. The products were digested separately with the restriction endonucleases *Msp*I, *Hinf*I, *Hae*III and *Hha*I at 37 °C for 3 h. The restriction fragments were separated and visualized by electrophoresis in 2% (w/v) agarose gels containing 0.5 μg mL^{-1} ethidium bromide [47]. Subsequently, isolates sharing the same RFLP patterns were designated as a single rRNA type.

Sequence analyses of 16S rRNA, recA, glnII, atpD, nifH and nodC genes

Based on the results of RFLP analysis of the 16S rRNA gene, representative isolates of different types were chosen for the phylogenetic analysis. The 16S rRNA gene was amplified as mentioned above. The partial housekeeping genes *recA*, *glnII* and *atpD* were amplified using primer pairs *recA*41F/*recA*640R, *glnII*12F/*glnII*689R and *atpD*255F/*atpD*782R, respectively, as described previously [45]. The fragments of the *nifH* (approximately 800 bp) and *nodC* (approximately 700 bp) genes were amplified with the primer pairs *nifH*F/*nifH*R and *nodC*F540/*nodC*R1160, respectively [22]. The PCR products were purified and directly sequenced by the Beijing AuGCT DNA-SYN Biotechnology Co., Ltd. The closely related sequences acquired in this study were aligned by BLAST from the GenBank database. The alignment of the acquired sequences and closely related sequences was generated by ClustalW in the MEGA package version 5 [39]. The index of substitution saturation (I_{ss} and $I_{ss.c}$) was calculated with DAMBE [51] for evaluating the quality of sequences. After trimming to the same length, housekeeping gene sequences were concatenated, and analysis of the sensitivity of the concatenated sequences was undertaken. Incongruent length difference (ILD) tests between the gene partitions were performed with the PAUP*4.0b10 program in order to evaluate whether the data sets containing different evolutionary histories were incongruent [11,12]. The phylogenetic trees of the 16S rRNA, housekeeping and symbiotic genes, as well as multilocus sequence analysis (MLSA) (concatenated sequences of *recA*, *glnII*, and *atpD*), were reconstructed with the neighbor-joining method using Kimura's 2-parameter model in MEGA version 5 [39]. Genospecies were defined based on the MLSA relationships, as previously suggested [6,25,30], by taking the grouped results of defined species as a reference.

Nodulation tests

Representative isolates of different RFLP types were chosen for verifying their nodulation ability with common bean. The common bean seeds were surface sterilized with NaClO, pre-germinated on sterilized wet filter paper, sown and inoculated (10^8 CFU per seed) in Leonard jars filled with sterilized vermiculite, according to the protocol of Vincent [44]. The plants were grown in a growth cabinet, programmed for a 16 h/8 h light/dark and 28 °C/20 °C day/night cycle, with 50% relative humidity. The nodules were collected after six weeks and the effectiveness of nodules for nitrogen fixation was estimated from the pink color (leghemoglobin) of the nodules and the dark green color of the leaves, as compared to the control plants (without inoculation).

Data analysis

The Shannon–Wiener, Simpson, richness and evenness indices were applied in order to estimate common bean rhizobial diversity, species richness and evenness in different sampling sites calculated by the vegan package for R [33]. Correlation analyses between environmental variables and rhizobial groups were conducted with the SPSS 19.0 package [5]. To evaluate the relationship between genospecies abundance and environmental factors, non-parametric multivariate methods were used. Detrended canonical analysis (DCA) was firstly used to choose a linear or unimodal ordination model for analysis. The length of gradient (first axis) was 2.308 below 3 by DCA, suggesting that the linear model was recommended but the unimodal methods could also be used. Canonical correspondence analysis (CCA) was used to analyze the correlation of genospecies abundance and environmental factors by CANOCO 5 (Microcomputer Power, Ithaca, NY) [23]. In addition, the envfit function was used with the vegan package in R [33] in order to evaluate the influence of each environmental factor on species distribution.

Results

Isolation and sequence analyses of the 16S rRNA gene

A total of 194 isolates were obtained from the 13 sampling sites (Table S1). The amplicons of 16S rRNA genes were approximately 1500 bp for all the isolates. According to the restriction patterns, 20 rRNA genotypes were defined among the isolates (Table 1), in which genotypes L, C, E and M were dominant with 35, 33, 27 and 20 isolates (18.0%, 17.0%, 13.9% and 10.3%), respectively, and they were distributed in most of the sampling sites. The minor types A, R, K, P, S and O were also distributed between various sampling sites.

Species identification of the isolates

Partial fragments of *atpD* (480 bp), *glnII* (900 bp) and *recA* (500 bp) were successfully amplified from all the representative isolates. Based on the grouping results of reference strains in the MLSA phylogenetic tree (Fig. 2), 15 genospecies were identified with similarities higher than 97% for the concatenated sequences (Table 1). Isolates SX1557 (rRNA type A), SX1674 (rRNA type M) and SX1579 (rRNA type K), representing 46 isolates, were defined as *Agrobacterium* sp. I, II and III, respectively. They were distantly related to *A. radiobacter* (91.3–94.9% similarities) and were supported by bootstrap values of 58−99%. Isolates of rRNA types B, I and P were defined as *Rhizobium* sp. I, and they showed 93.8–95.0% similarities to reference strains of *R. vallis* and *R. phaseoli*. Isolates in rRNA types C, L and N were defined as *Rhizobium* sp. II and they had similarities of 93.3–93.7% with the *R. phaseoli* type strain. Isolate SX1556 (rRNA type D) was distantly related to *R. yanglingense* (94.4%) and was considered as *Rhizobium* sp. III. Isolates of rRNA type O showed 94.8% similarity to *R. leguminosarum* and were defined as *Rhizobium* sp. IV. The rRNA type J isolate had 98.7%

Table 1
Classification richness of genospecies at different latitudes.

Identification (relative species abundance)[a]	Representative isolate	rRNA type (number of strains)	Nodulation ability	*nifH/nodC*		Richness of genospecies at each latitude (N)							Most related bacterium (MLSA similarity, %)
				PCR	type	32°	33°	34°	35°	36°	37°	38°	
Agrobacterium sp. I (7.7%)	SX1557	A (15)	–	–/–	–/–	3		4	4	2	2		*A. radiobacter* (91.3)
Agrobacterium sp. II (10.3%)	SX1674	M (20)	–	–/–	–/–	3	2	6			4	5	*A. radiobacter* (94.9)
Agrobacterium sp. III (5.7%)	SX1579	K (11)	–	+/+	I/I	2	5	3		1			*A. radiobacter* (94.3)
Rhizobium sp. I (8.3%)	SX1648	B (2)	+	+/+	I/I		1	1					*R. vallis* (95.0)
	SX1652	P (9)	+	+/+	I/I			9					*R. vallis* (95.0)
	SX1706	I (5)	+	+/+	I/I	3						2	*R. phaseoli* (93.8)
Rhizobium sp. II (35.6%)	SX1587	C (33)	+	+/+	I/I	2		13	9	7	2		*R. phaseoli* (93.3)
	SX1532	L (35)	+	+/+	I/I	3	3	8	3	3	7	8	*R. phaseoli* (93.7)
	SX1617	N (1)	+	+/+	I/I			1					*R. phaseoli* (93.3)
Rhizobium sp. III (0.5%)	SX1556	D (1)	+	+/+	I/I						1		*R. yanglingense* (94.4)
Rhizobium sp. IV (3.1%)	SX1627	O (6)	+	+/+	I/I	2	1	3					*R. leguminosarum* (94.8)
R. phaseoli (10.3%)	SX1713	R (12)	+	+/+	I/I	9	3						*R. phaseoli* (97.0)
	SX1630	S (8)	+	+/+	I/I	3		5					*R. phaseoli* (97.5)
Pararhizobium giardinii (0.5%)	SX1555	J (1)	+	+/+	I/II						1		*R. giardinii* (98.7)
E. fredii (13.9%)	SX1597	E (27)	+	+/+	II/II			18	1	4	4		*E. fredii* (99.4)
E. kummerowiae (1.0%)	SX1613	F (2)	+	+/+	I/II			1	1				*E. kummerowiae* (97.4)
Ensifer sp. I (0.5%)	SX1660	Q (1)	+	+/+	II/II			1					*E. fredii* (94.0)
Ensifer sp. II (0.5%)	SX1647	H (1)	+	+/+	I/II			1					*E. morelense* (93.3)
B. liaoningense (0.5%)	SX1676	G (2)	+	+/+	I/III			2					*B. liaoningense* (99.7)
Ochrobactrum anthropi (0.5%)	SX1601	T (2)	–	–/–	–				2				*O. anthropic* (97.0)

[a] Relative species abundance measured by dividing the number of isolates in the genospecies by the total number of isolates in the ecoregion.

similarity to the type strains of *P. giardinii*, and was identified as this species. The isolates of rRNA types R and S were defined as *R. phaseoli* according to their high similarities (97.0% and 97.5%, respectively) with the type strain. The isolates of rRNA types E, F, G and T were considered as *E. fredii*, *E. kummerowiae*, *B. liaoningense* and *O. anthropi*, respectively, based on their similarities with the type strains. Isolates SX1613 (rRNA type F) and SX1647 (rRNA type H) were distantly related to *E. fredii* (94.0% similarity) and *E. morelense* (93.3% similarity), respectively, and were identified as *Ensifer* sp. I and *Ensifer* sp. II.

Generally, genus *Rhizobium* was the most dominant group and accounted for 57.8% of the isolates, in which *Rhizobium* sp. II was the major group with 35.6%, followed by *R. phaseoli* (10.3%), *Rhizobium* sp. I (8.3%), *Rhizobium* sp. IV (3.1%), and *Rhizobium* sp. III (0.5%). *Agrobacterium* was the second most abundant genus (23.7%), comprising 7.7%, 10.3% and 5.7% for *Agrobacterium* sp. I, *Agrobacterium* sp. II and *Agrobacterium* sp. III, respectively. *Ensifer* (15.9%) was the third most abundant group, including *E. fredii* (13.9%), *E. kummerowiae* (1.0%), *Ensifer* sp. I (0.5%) and *Ensifer* sp. II (0.5%). *P. giardinii* (0.5%), *B. liaoningense* (0.5%) and *O. anthropi* (0.5%) were detected as minor groups.

Sequence analyses of symbiotic genes nodC and nifH and the nodulation test

Except for SX1557 (type A, *Agrobacterium* sp. I), SX1674 (type M, *Agrobacterium* sp. II) and SX1601 (type T, *O. anthropi*), the *nodC* and *nifH* genes were amplified from all the other representative isolates. In the *nodC* gene phylogenetic tree (Fig. 3), the representative isolates formed three clades. Clade I contained eleven isolates representing all the *Rhizobium* genotypes (rRNA types B, S, P, R, K, D, N, L, C, O, I) and *Agrobacterium* sp. III (rRNA type K), as well as the common bean-nodulating reference strains *R. vallis*, *R. etli* and *R. phaseoli*, in which the *nodC* genes of rRNA types B, P and S were more similar to those of *R. etli* and *R. phaseoli*, while the *nodC* genes of rRNA types C, D, I, K, L, N, O and R were more similar to that of *R. vallis*. The *Ensifer* isolates SX1597 (type E), SX1660 (type Q), SX1647 (type H), SX1613 (type F) and *P. giardinii* SX1555 (type J) formed Clade II and shared *nodC* sequences that were very similar to those of soybean-nodulating reference strains *E. fredii*, *E. sojae* and *E. saheli*. The *B. liaoningense* isolate SX1676 (type G) and a soybean-nodulating reference strain *B. yuanmingense* formed Clade III. In the *nifH* gene phylogenetic tree (Supplementary Fig. S10), the representative isolates formed two clades. Two *Ensifer* isolates, SX1597 (type E) and SX1660 (type Q), as well as reference strains of soybean-nodulating *E. sojae* and several other *Ensifer* species formed Clade II, while all the remaining representative isolates formed Clade I together with common bean-nodulating reference strains for *R. vallis*, *R. etli* and *R. phaseoli*.

In the nodulation test, most of the representatives could form nodules successfully on the roots of common bean, except *Agrobacterium* sp. I SX1557 (type A), *Agrobacterium* sp. II SX1674 (type M), *Agrobacterium* sp. III SX1579 (type K), and *Ochrobactrum* sp. SX1601 (type T). An average of eleven nodules was observed on each inoculated plant and most of the nodules were located on the lateral roots. Most nodules were pink with diameters of approximately 0.4 cm, indicating that the nodules were effective for nitrogen fixation.

Distribution and diversity of common bean rhizobia in different geographic regions

The Shannon–Wiener index (H) ranged from 0.970 (N38°) to 2.098 (N34°), and the Simpson's index (D) varied from 0.581 (N36°) to 0.834 (N34°) between the sampling sites. The Shannon–Wiener index (H) showed statistically significant correlation with the soil physiochemical features, the cropping systems and the annual accumulation of effective temperature. Nevertheless, the Simpson's index (D) was significantly related to the annual effective temperature, average geothermal at a depth of 20 cm, and the average monthly rainfall. Moreover, the richness (S) showed the least number of species in the sampling sites at N38° and the highest at N34°, which was correlated with soil features, thickness of the plow layer, cropping systems, soluble K content, and average monthly relative humidity. On the contrary, bean rhizobial diversity had the highest species evenness at N33° ($J = 0.915$) and the least at N35° ($J = 0.773$), which were also significantly correlated with soil features, cropping systems and average monthly relative humidity (Tables 2 and 3, Supplementary Table S2).

In this study, the thickness of the plow layer varied between 15 cm and 40 cm at different sampling sites. Soil samples from sites at N34° to N38° were slightly alkaline (pH 7.30–8.53), and soil

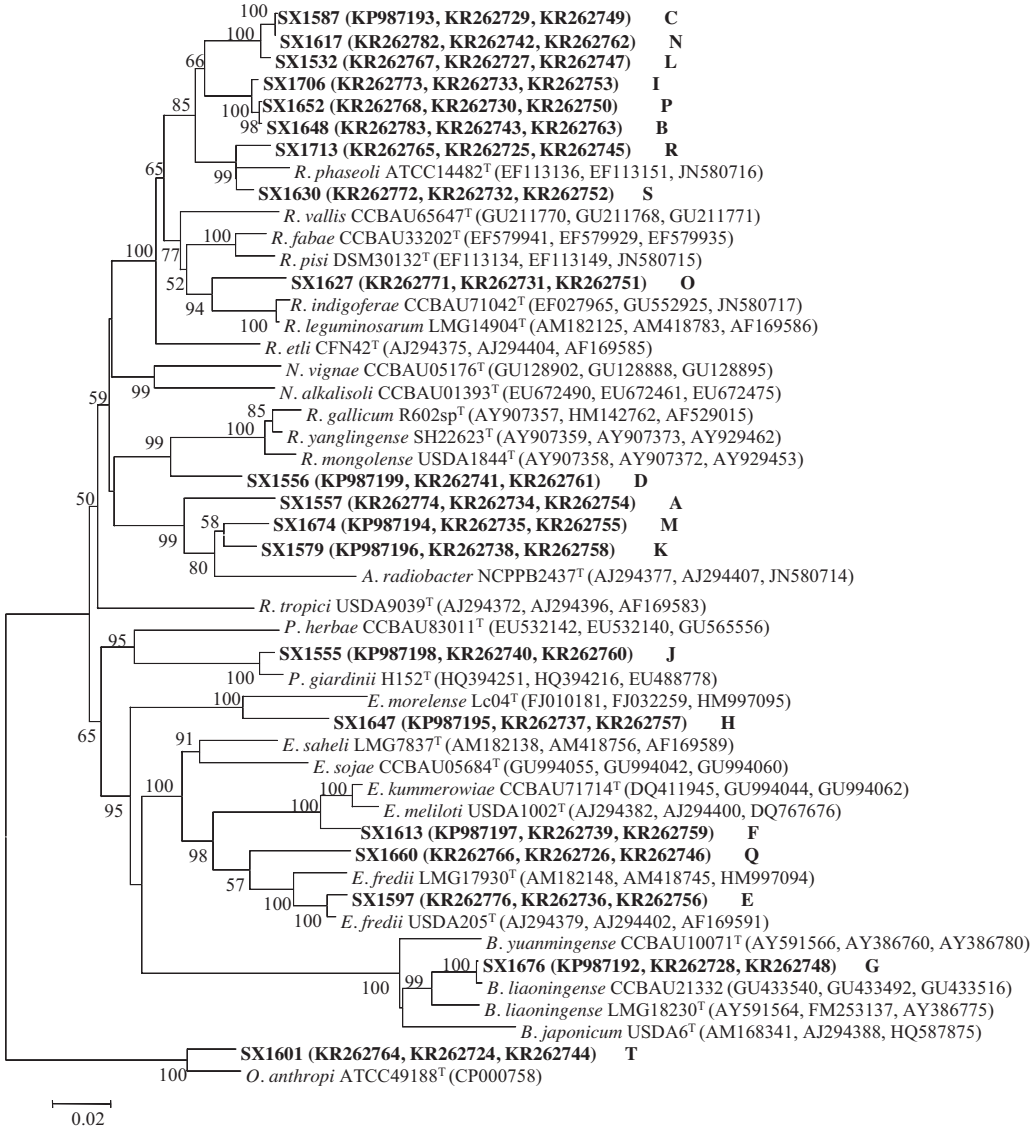


Fig. 2. Phylogenetic tree based on the concatenated sequences of *recA*, *atpD* and *glnII* using the neighbor-joining method. The numbers on the branches are the bootstrap values indicating the reliabilities. GenBank accession numbers are shown in parenthesis. Bold typeface is used to mark the representative strains obtained in this study. The scale bar indicates the number of substitutions per site.

samples from sites at N32° to N33° were slightly acid (pH 6.43–6.80). The contents of the main mineral nutrients in dry soils were 28.2–184.0 mg kg^{-1} for available nitrogen, 4.2–73.4 mg kg^{-1} for available phosphorus, 68.0–669.6 mg kg^{-1} for available potassium, and 0.98–19.4 g kg^{-1} for organic matter. In DCA, the length of gradient (first axis) was 2.9 SD units, indicating that the redundancy analysis (RDA) was recommended. In comparison to the results of the permutation test, CCA was used to illustrate the relationship between species abundance and soil physicochemical properties (Fig. 4). The analysis by envfit (Fig. 4B) showed that pH (r^2 = 0.7855, p = 0.001), available nitrogen (r^2 = 0.5870, p = 0.022) and available potassium (r^2 = 0.5644, p = 0.029) were the most important environmental variables for determining the distribution of rhizobial species in Shaanxi. In Fig. 4A, the distributions of *Rhizobium* sp. I, *Rhizobium* sp. II, *Ensifer* sp. I, *O. anthropi*, and *B. liaoningense* were mainly affected by pH; *P. giardinii* and *E. kummerowiae* were influenced by the thickness of plow layer; *Agrobacterium* sp. I, *Ensifer* sp. II, and *E. fredii* were distributed corresponding to the contents of available potassium and available phosphorus; the distributions of *Rhizobium* sp. III, *Rhizobium* sp. IV, *Agrobacterium* sp. III and

Table 2
Diversity indices for the sampling sites at each latitude.

Latitude	Geographic region	Shannon–Wiener (H)	Simpson (D)	Richness (S)	Evenness (J)
N38°	Shaanbei	0.970	0.587	3	0.883
N37°	Shaanbei	1.509	0.730	6	0.842
N36°	Shaanbei	1.071	0.581	4	0.773
N35°	Shaanbei	1.158	0.585	5	0.720
N34°	Guanzhong	2.098	0.834	13	0.817
N33°	Shaannan	1.640	0.782	6	0.915
N32°	Shaannan	1.717	0.773	7	0.882

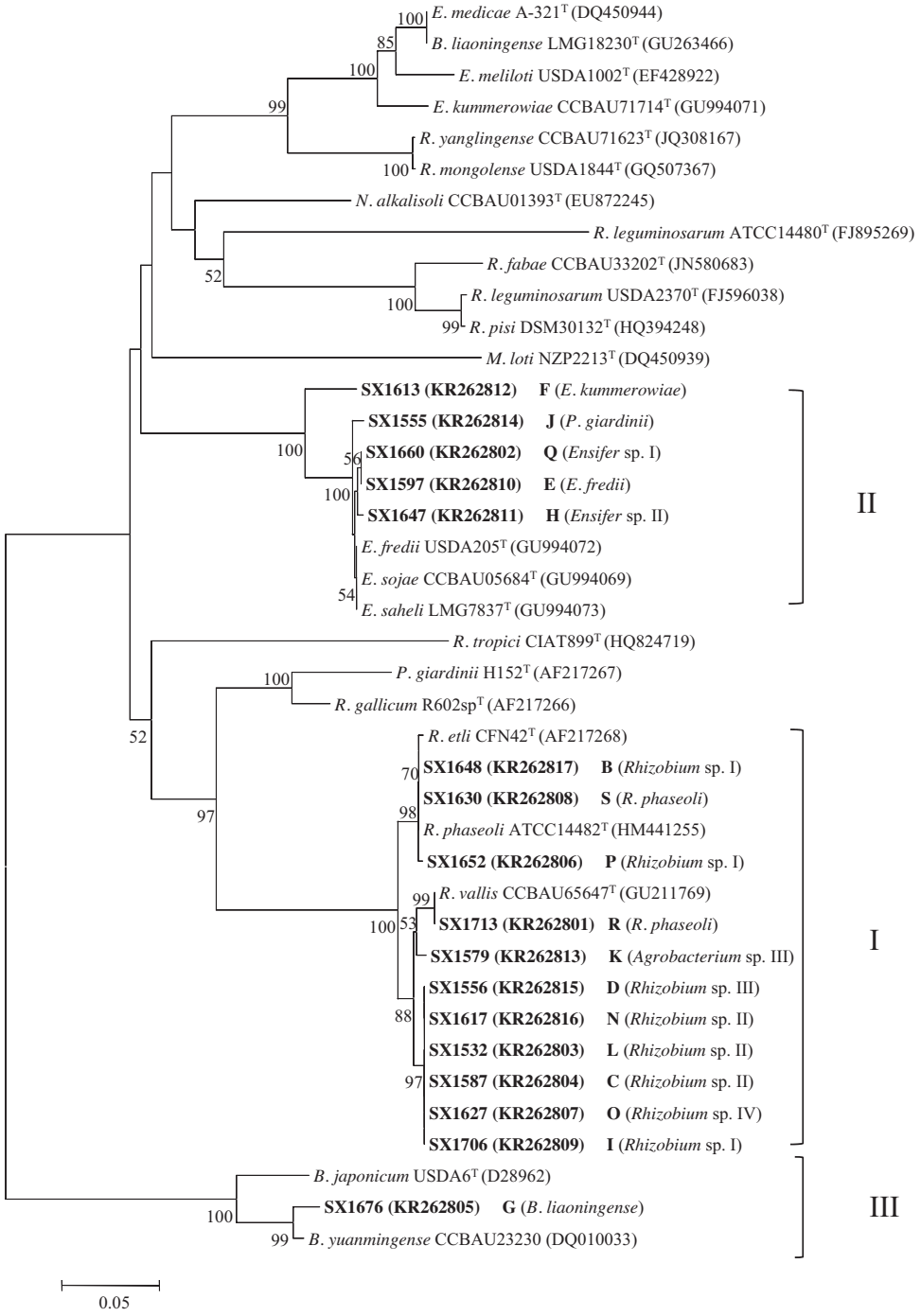


Fig. 3. Phylogenetic tree based on *nodC* sequences using the neighbor-joining method. The numbers on the branches are the bootstrap values indicating the reliabilities. GenBank accession numbers are shown in parenthesis. Bold typeface is used to mark the representative strains obtained in this study. The scale bar indicates the number of substitutions per site.

R. phaseoli were affected by available nitrogen and organic matters. These results demonstrated that the diversity and species composition of common bean rhizobia varied dramatically between different sampling sites and were closely related to environmental factors.

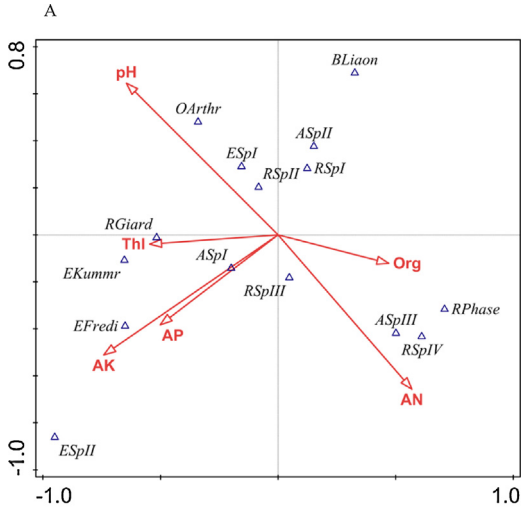
Discussion

Among the 15 genospecies defined in the present study (Fig. 2 and Table 1), *Agrobacterium* sp. I, *Agrobacterium* sp. II and *O. anthropi* could be identified as non-symbiotic nodule endophytes, as documented in several other studies [20,24,53], based on the failure to amplify symbiotic genes and nodulation of the host. The failure of *Agrobacterium* sp. III SX1579 nodulation on common bean was unexpected because it harbored the *nodC* and *nifH* genes that are similar to the soybean-nodulating *Rhizobium* strains (Table 1). These conflicting results may demonstrate that nine *Agrobacterium* sp. III SX1579 symbiotic genes were acquired recently but they were not stably maintained or expressed. However, we still considered this isolate as a symbiotic bacterium. The identification of 12 genomic species as symbiotic bacteria in this study, especially the seven unidentified species within *Agrobacterium*,

Table 3
Correlation between the diversity indices and environmental factors.

Environmental factors	Shannon–Wiener (*H*)	Simpson (*D*)	Richness (*S*)	Evenness (*J*)
Longitude	0.428	0.549	0.239	−0.008
Latitude	0.428	0.549	0.239	−0.008
Altitude	0.421	0.542	0.233	−0.004
Parent materials of soil	0.066	0.278	−0.220	0.358
Soil species	0.080	0.226	−0.087	0.242
Soil profile structure	0.675*	0.527	0.761**	−0.704**
Groundwater level	−0.082	−0.250	0.092	−0.295
Thickness of plow layer	0.517	0.426	0.573*	−0.486
Cropping systems	0.486	0.545	0.395	−0.139
Intercropping	0.572*	0.413	0.666*	−0.688**
pH	−0.192	−0.398	0.073	−0.296
Organic matter (g kg^{-1})	0.164	0.323	−0.016	0.241
Alkali hydrolyzable N content (mg kg^{-1})	0.060	0.252	−0.163	0.367
Soluble P content (mg kg^{-1})	0.436	0.345	0.536	−0.404
Soluble K content (mg kg^{-1})	0.440	0.294	0.581*	−0.537
Annual rainfall	0.165	0.313	−0.041	0.193
Annual effective accumulative temperature	0.560*	0.607*	0.479	−0.194
Average geothermal at a depth of 20 cm	0.483	0.581*	0.361	−0.044
Average monthly temperature	−0.018	0.141	−0.215	0.325
Average monthly rainfall	0.450	0.570*	0.309	0.020
Average monthly relative humidity (%)	−0.522	−0.244	−0.778**	0.873**
Average sunshine duration	0.389	0.283	0.423	−0.473

−, negative correlation.
* Significant difference at $p < 0.05$ level.
** Significant difference at $p < 0.01$ level.



B

	CCA1	CCA2	r2	*P*
pH	0.68044	-0.7328	0.7855	0.001 ***
Org	-0.90977	0.41512	0.2504	0.212
AN	-0.72829	0.68527	0.587	0.022 *
AP	0.97229	0.23377	0.3559	0.119
AK	0.90557	0.42419	0.5644	0.029 *
Thl	0.99996	-0.00864	0.226	0.288

Fig. 4. (A) Biplot of CCA by R for the 12 symbiotic genospecies and their influential soil factors from sampling sites. AN, available nitrogen; AK, available potassium; AP, available phosphorus; Thl, thickness of plow layer; Org, organic matter. *ASpI*, *Agrobacterium* sp. I; *ASpII*, *Agrobacterium* sp. II; *ASpIII*, *Agrobacterium* sp. III; *BLiaon*, *Bradyrhizobium liaoningense*; *ESpI*, *Ensifer* sp. I; *ESpII*, *Ensifer* sp. II; *EFredi*, *E. fredii*; *EKummr*, *E. kummerowiae*; *RSpI*, *Rhizobium* sp. I; *RSpII*, *Rhizobium* sp. II; *RSpIII*, *Rhizobium* sp. III; *RSpIV*, *Rhizobium* sp. IV; *RGiard*, *Pararhizobium giardinii*; *RPhase*, *R. phaseoli*; *OArthr*, *O. anthropi*. (B) Environmental vectors or factors fitted to an ordination plot. The values in columns CCA1 and CCA2 represent the cosine of angles between environmental factors and ordinations, respectively. The column r2 indicates the impact of environmental factors for distribution of species. '***', $p < 0.001$; '**', $p < 0.01$; '*', $p < 0.05$. AP, available phosphorus; AK, available potassium; Org, organic matter; AN, available nitrogen; Thl, thickness of plow layer

Ensifer and *Rhizobium*, revealed a great diversity of common bean rhizobia in Shaanxi and enlarged our knowledge of the diversity of common bean rhizobia. Previously, *R. etli*, *R. gallicum*, *R. leguminosarum*, *R. lusitanum*, *R. phaseoli*, *R. vallis*, *R. leucaenae*, *R. tropici*, *R. mesoamericanum*, *R. freirei*, *R. azibense*, *E. meliloti*, *E. americanus*, *Bradyrhizobium* sp. and *P. giardinii* have been reported as microsymbionts of common bean in different regions [43], but such a greatly diverse rhizobial community has not been reported in any previous studies. Except for the seven novel or unidentified genospecies, *E. fredii* and *E. kummerowiae* were also new records for common bean rhizobia in the present study. Therefore, the communities of common bean rhizobia in Shaanxi Province were clearly different from those reported in other regions.

Previously, distinct rhizobial species associated with common bean in different regions have been reported [28]. The predominant rhizobia of common bean were *R. etli* in the Americas [36], *E. meliloti*/*E. americanus* in alkaline-saline soils [31,43,56], *P. giardinii* and *R. gallicum* in France [1], *R. lusitanum* in Portugal [41], and *R. etli* and *R. leguminosarum* in an agricultural-forestry ecosystem of north-eastern China [48], although the common bean rhizobia were super dominated by *Rhizobium* sp. II, followed by *E. fredii*, *R. phaseoli*, *Agrobacterium* sp. III and *Rhizobium* sp. I. These differences could be due to interactions between environmental factors, the rhizobia and the common bean plants, as estimated previously [43] and subsequently discussed. The great diversity of rhizobia isolated from bean plants in Shaanxi might be due to the selection of common bean and the diverse ecological conditions acting on the rhizobia throughout the long history of cultivation, as proposed in other studies [46].

Among the two *nifH* and three *nodC* types detected in this study (Fig. 3 and Supplementary Fig. S10), *nifH* and *nodC* type I were similar to those reported for common bean rhizobia related to *R. etli* and they could be identified as symbiovar (sv.) phaseoli [6,22], but the remaining types were only detected in Shaanxi Province or other regions of China. In previous studies, the symbiotic genes *nodC*, *nodA* and *nifH* in the common bean-nodulating *Ensifer* species were different from those of *R. etli* and they were designated as sv. mediterranense [31,43,56]. However, the *Ensifer* isolates in the present study mainly harbored symbiotic genes similar to those of the soybean-nodulating *Ensifer* species (Fig. 3), while

the *B. liaoningense* SX1676 isolate harbored a *nodC* gene similar to the soybean-nodulating bradyrhizobial species (Fig. 3), but a *nifH* gene similar to sv. phaseoli (Supplementary Fig. S10) [46]. These results indicated the possibility that common bean plants in Shaanxi have adopted some soybean microsymbionts that are popular and well adapted to the local environment. Undoubtedly, the findings in the present study enlarged the diversity at both the symbiotic gene level and the species level for the common bean-nodulating rhizobia, and further demonstrated the common bean plants as promiscuous hosts for rhizobia.

In most cases, the phylogenetic lineages of symbiotic genes (Fig. 3 and Supplementary Fig. S10) grouped the isolates according to their genus definition of the rhizobia, demonstrating that they had coevolved with chromosome genes. Therefore, vertical transfer might be the main way to maintain symbiotic genes in common bean rhizobia, as described previously in the soybean rhizobia [26,55]. However, the detection of similar symbiotic genes in *Agrobacterium* sp. III SX1579 and the rhizobia in sv. phaseoli raises the possibility that lateral gene transfer may have occurred in the evolution of common bean rhizobia involved in this study, which is similar to the observation in previous studies on *Mesorhizobium* [38]. Furthermore, the presence of a *nodC* gene similar to the soybean-nodulating *Ensifer* species (Fig. 3), and a *nifH* gene of sv. phaseoli (Supplementary Fig. S10) in isolates *P. giardinii* SX1555, *E. kummerowiae* SX1613 and *Ensifer* sp. II SX1647 demonstrated the possibility that *nif* and *nod* genes may be separately transferred and recombined in some cases. Other evidence for this estimation could be the isolate *B. liaoningense* SX1676, which harbored the *nifH* of sv. phaseoli, but the *nodC* of soybean-nodulating bradyrhizobia (Fig. 3 and Supplementary Fig. S10). These data also confirmed the hypothesis that *Rhizobium* and *Ensifer* had common ancestors with the intragenomic transfer recipient (ITR) plasmid [52]. Although lateral gene transfer was not frequently observed among the symbiotic bacteria in the present study or in previous studies [26,55], it might be an important mechanism for enhancing the biodiversity of rhizobia.

The unique community composition of common bean rhizobia in Shaanxi Province, compared with previous studies [2,4,6,13,37,43,46], demonstrated the presence of biogeographic patterns in the bean rhizobia. According to the results of this current study (Fig. 4), the diversity of these rhizobia was highly influenced by moisture (average monthly relative humidity and average monthly rainfall), temperature (average geothermal at a depth of 20 cm and annual effective accumulative temperature), intercropping, soil feature (soil profile structure and thickness of the plow layer) and nutrients (soluble K content) (Table 3). However, the humidity and the soil profile structure/thickness of plow layer were not recorded in previous studies [43], and their real functions for regulating the rhizobial community need further study. Moreover, the rhizobia possessed higher diversity in the sampling sites located at latitude N34° (corresponding to the region of Guanzhong Plain), indicating that the environmental conditions in this region have accelerated the diversification of rhizobial species. The correlation between soil profile structure and rhizobial diversity might be explained by the great impact of the soil water content, as well as the ratio of nitrogen, phosphorus, potassium, manganese and calcium in the soil [8,14], since the soil profile structure is a determinant for the depth of underground water [10], which is closely related to plant growth and soil salinization [3,19,21]. As to the thickness of the plow layer, its effects on rhizobia may be related to the alternation of nutrient content and the oxidation-reduction potential. Previously, it has been reported that a high underground water level of 8000–8500 mm and a deep plow layer favored *E. fredii* [29], and the diversity and composition of *Bradyrhizobium* communities were altered with a change of plants and land use [34]. All of these correlations between common bean rhizobia and environmental factors showed that the diversity and distribution of rhizobia were significantly shaped by the environmental factors, as revealed previously [7]. However, the full evaluation and explicit assertions of these correlations are still insufficient, although the common bean rhizobia have been extensively studied in the world [29].

In the current study (Fig. 4), soil pH as a main determinant for distribution of *Rhizobium* sp. I, *Rhizobium* sp. II, *Ensifer* sp. I, *O. anthropi* and *B. liaoningense*, as well as the contents of available potassium and phosphorus as main regulators for distribution of *Agrobacterium* sp. I, *Ensifer* sp. II, and *E. fredii*, were similar to the results in several previous studies [15,29,31,56]. However, the effects of the thickness of the plow layer on distribution of *P. giardinii* and *E. kummerowiae*, and of available nitrogen and organic matter contents on distribution of *Rhizobium* sp. III, *Rhizobium* sp. IV, *Agrobacterium* sp. III and *R. phaseoli* were novel observations.

In conclusion, a total of 194 rhizobia isolated from common bean in Shaanxi Province showed great diversity and biogeographic patterns. These isolates were classified into 15 genospecies belonging to the genera *Rhizobium*, *Agrobacterium*, *Ensifer*, *Bradyrhizobium* and *Ochrobactrum*, with *Rhizobium* sp. I, *Rhizobium* sp. II and *E. fredii* predominating. The three *nodC* and two *nifH* types symbiotic gene types were detected within the isolates, of which two *nodC* types (type II, III) were not recorded previously. Possible lateral gene transfer of *nodC-nifH* was detected among the isolates of *Agrobacterium*, *Bradyrhizobium*, *Rhizobium* and *Ensifer*. The environmental factors, such as moisture, temperature, intercropping, soil and nutrients, were correlated with the diversity and distribution of the rhizobial species. Soil pH, available nitrogen and available potassium were the main factors explaining the distribution of identified rhizobial species. The revelation of several novel or unidentified rhizobial species and two novel records of symbiotic gene types demonstrated that the common bean plants had developed specific symbiosis with the local bacteria due to adaptation of the symbiotic genes via lateral gene transfer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2016.02.001.

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