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Biodiversity and Biogeography of Rhizobia Associated with Soybean Plants Grown in the North China Plain▽†

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As the putative center of origin for soybean and the second largest region of soybean production in China, the North China Plain covers temperate and subtropical regions with diverse soil characteristics. However, the soybean rhizobia in this plain have not been sufficiently studied. To investigate the biodiversity and biogeography of soybean rhizobia in this plain, a total of 309 isolates of symbiotic bacteria from the soybean nodules collected from 16 sampling sites were studied by molecular characterization. These isolates were classified into 10 genospecies belonging to the genera *Sinorhizobium* and *Bradyrhizobium*, including four novel groups, with *S. fredii* (68.28%) as the dominant group. The phylogeny of symbiotic genes *nodC* and *nifH* defined four lineages among the isolates associated with *Sinorhizobium fredii*, *Bradyrhizobium elkanii*, *B. japonicum*, and *B. yuanmingense*, demonstrating the different origins of symbiotic genes and their coevolution with the chromosome. The possible lateral transfer of symbiotic genes was detected in several cases. The association between soil factors (available N, P, and K and pH) and the distribution of genospecies suggest clear biogeographic patterns: *Sinorhizobium* spp. were superdominant in sampling sites with alkaline-saline soils, while *Bradyrhizobium* spp. were more abundant in neutral soils. This study clarified the biodiversity and biogeography of soybean rhizobia in the North China Plain.

Soybean (*Glycine max* L.) is a major legume crop in the world, representing 50% of the global crop legume area and 68% of global legume production (11). It also plays a very important role in sustainable agriculture and in the economy for many countries because of its great nitrogen-fixing ability, which is acquired from its symbiosis with rhizobia in root nodules. In China, the soybean was domesticated approximately 4,000 years ago (29), and the region between 34 and 35°N, corresponding to the North China Plain or Huang-Huai-Hai (HHH) Plain, which refers to the downstream regions of the Huang (Yellow) River, Huai River, and Hai River (Fig. 1), was suggested to be the area of the origination of soybean based upon a comparative study of seed protein contents of cultivated (*G. max*) and wild (*Glycine soja* Sieb. and Zucc.) soybeans from different latitudes in China (46). Geographically and climatically, the North China Plain is a transit area between the northeastern, northwestern, and southern regions of China. Therefore, the North China Plain may have served as a diversification center of soybean rhizobia as estimated by Lie et al. (21), and the soybean rhizobial communities in different sites of this plain may vary in relation to their geographic locations or relative distances from other regions, since biogeographic patterns have been found in some rhizobia (22, 39), including soybean rhizobia (10, 23).

As the largest alluvial plain of eastern Asia, the North China Plain is based on the deposits of the Yellow River, Huai River, Hai River, Luan River, etc. The Yellow River flows through the middle of the plain into Bohai Gulf. The plain covers an area of about 409,500 km^2, most of which is less than 50 m above sea level, has an average precipitation of about 500 to 800 mm from north to south, and includes three types of geographic zones: (i) alluvial fans in the north part, where the soils are fertile loam with neutral pH; (ii) alluvial plains in the central and main part of the plain, where the level of underground water is high and the soils are saline-alkaline with high clay content; and (iii) coastal plains and the delta of the Yellow River, where the soils are highly saline and mainly composed of clay. Currently, the North China Plain is the second largest region for soybean production in China, having 2,700,000 ha for soybean planting annually, which are mainly in the alluvial fans and plains. The diverse soil and climate conditions, vast area of soybean culture, transit geographic location, and the fact that it is the original center of soybean production make the North China Plain a good model for studying the biogeography and determinants of soybean rhizobia. However, the soybean rhizobia in this plain have not been sufficiently studied, although microsymbionts isolated from other regions have been studied extensively, including classification (6, 16) and the determination of phylogeny (32) and genotypic characteristics (24, 47).

To date, rhizobia nodulating with soybean in Xinjiang (a geographically isolated region surrounded by deserts and high mountains) and in subtropical and tropic regions of China have been systematically studied (4, 5, 10, 23, 47, 48), and diverse soybean rhizobia belonging to *Bradyrhizobium*, *Mesorhizobium*,

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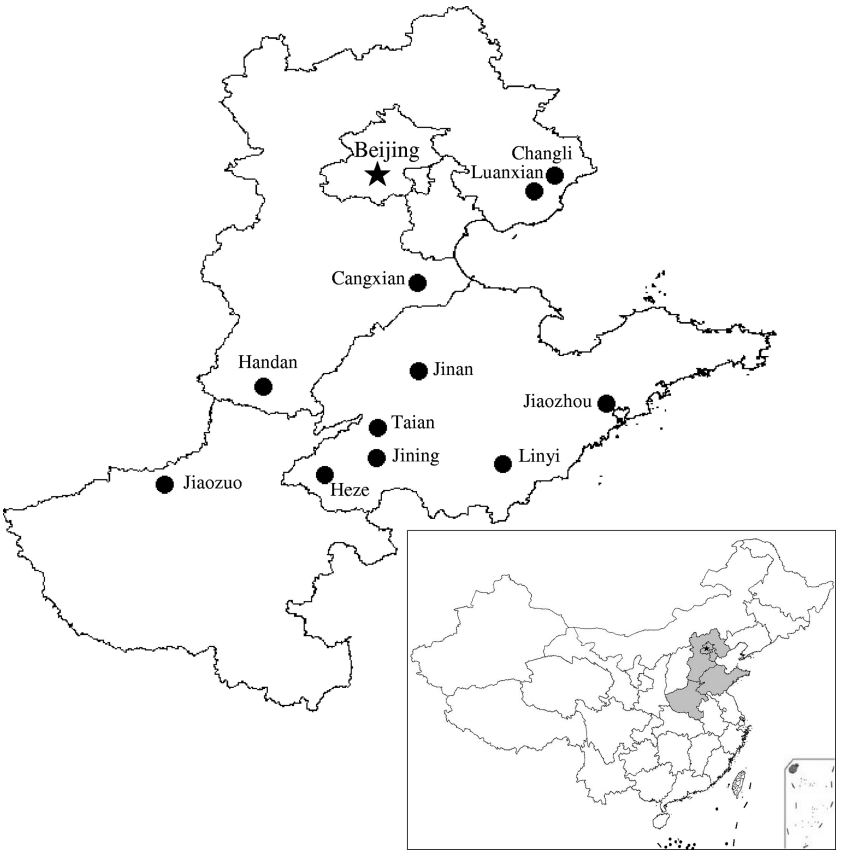

FIG. 1. Map of the Huang-Huai-Hai (HHH) Plain showing the sampling sites (●). ⋆, Capital of China, Beijing. The corresponding position of the HHH Plain in China is shown in the inset. The two maps were created using DIVA-GIS software (http//www.diva-gis.org), and the sampling sites were added according to GPS records.

and *Sinorhizobium* have been reported in these studies (4, 5, 10, 23, 47, 48). In addition, biogeographic patterns have been observed in the soybean rhizobia (10, 20, 23). Since the geographical distribution of symbiotic bacteria was affected by biological factors like the host plants (44) and animals (14) and by soil factors (10), similarly to the free-living bacteria (9), diverse soybean rhizobia adapted to the local conditions should be expected in the North China Plain.

Based upon the background knowledge mentioned above, we performed the present study to reveal the community composition of soybean rhizobia and to estimate the rhizobial distribution in correlation with different ecological factors in the North China Plain.

MATERIALS AND METHODS

Nodule sampling and isolation of rhizobia. Root nodules of soybean were collected from 16 sampling sites in the major soybean production areas of the North China Plain (Fig. 1 and Table 1) during July to August of 2008. In general, the 4 sites in Hebei Province are classified as the temperate region, while the 2 sites in Henan and 10 sites in Shandong are located in subtropic regions according to their latitude and climate characteristics. All of the sampling sites were located in cultivated soybean fields without rhizobial inoculation history. In eight sites, 24 cultivars of soybean were sampled, of which 7 were found in two or four sites. In these cases, two plants were sampled for each cultivar in each site, but nine plants were sampled for cultivar Yudou 25 in the site of Jiaozuo1 for checking the effect of sampling size on the estimation of rhizobia diversity. In another eight sites in which the soybean cultivars were not recorded, five plants were sampled from each site. Roots of soybean plants excavated from soil were washed immediately with tap water to eliminate attached soils. Healthy and complete nodules dissected from roots were maintained at environmental temperature in plastic tubes filled with dehydrated silica gel for transportation. For rhizobium isolation, five dehydrated nodules randomly selected from each plant were immersed in sterile deionized-distilled water (ddH_2O) overnight at 4°C and were surface sterilized by immersion in 95% ethanol for 30 s and then in 0.2% mercuric chloride for 4 to 5 min (depending on the nodule diameter). They then were washed six times with sterile ddH_2O (41). The surface-sterilized nodules were crushed separately in sterilized microtubes, and the nodule juice was stroked on plates of yeast-mannitol agar (YMA) (41), which were incubated at 28°C for the isolation of the rhizobia. The obtained bacterial colonies were purified by being repeatedly struck on the same medium. The nodulation capability of each isolate was tested by inoculating seedlings of the soybean cultivar Zhonghuang 13 (a popular cultivar in the HHH Plain) grown in Leonard jars filled with vermiculite as reported previously (41). Cultures of pure isolates were maintained on YMA slants at 4°C for short-term storage or in YM broth supplied with 20% (wt/vol) glycerol at −80°C for long-term storage.

Soil sampling and characterization. Soil (about 50 g) was sampled from the root zone (0 to 20 cm in depth) when the nodules were collected. Air-dried soil samples were ground and passed through 2-mm mesh screens for determining the chemical properties. Contents of available N, available P, and available K and the pH of soil were analyzed at the Plant Nutrient and Resource Research Institution, Beijing Academy of Agriculture and Forestry Sciences, using methods described previously (1, 13, 26, 34).

Amplification and RFLP of 16S rRNA gene. Total template DNA was extracted from each isolate and reference strain using the GUTC method described by Terefework et al. (38). Primers P1 and P6 (37) were used for the amplification of the 16S rRNA gene. The PCR amplification was performed with the procedure of Weisburg et al. (45). Amplification products were digested separately with each of the restriction endonucleases MspI, HinfI, HaeIII, and AluI (5 U per reaction) at 37°C for 6 h. The restriction fragments were separated and

TABLE 1. Characteristics of soil factors, number of strains in genospecies, and diversity indexes of soybean rhizobia from 16 sampling sites in the HHH Plain (23.5 to 40°N)

Site	Characteristics of soil factors[a]				No. of strains in genospecies[b]:										Sum[c]			Index[d]		
	A_N	A_P	A_K	pH	S1	S2	S3	B1	B2	B3	B4	B5	B6	B7	S	B	Spp	*H′*	*D*	*J*
Hebei (36.6–39.6°N, temperate region)																				
Cangxian	69.5	6.6	210	7.94	4		16					4			20	4	3	0.87	0.50	0.79
Changli	80.2	91.2	597	7.67	1				4				1		1	5	3	0.87	0.50	0.79
Luanxian	32.1	5.6	54.8	7.13				3	5				2	1	0	11	4	1.24	0.68	0.89
Handan	56.6	41.6	290	8.16	4		1								5	0	2	0.50	0.32	0.72
Henan (35.9–36.25°N, subtropic region)																				
Jiaozuo1	97.2	19.2	373	7.81	39					4	2	1			39	7	4	0.57	0.27	0.41
Jiaozuo2	106	28.2	218	7.88	11							1			11	1	2	0.29	0.15	0.41
Shangdong (30.65°–36.65°N, subtropic region)																				
Heze1	60.1	15.3	120	7.95	21	1									22	0	2	0.18	0.09	0.27
Heze2	95.2	39.6	362	8.07	9										9	0	1	0.00	0.00	N[e]
Jinan	83.3	24.1	245	7.97	34			1	1			4		2	34	8	5	0.72	0.33	0.45
Jining	64.0	8.4	96.6	8.12	46										46	0	1	0.00	0.00	N
Jiaozhou	85.2	23.2	106	6.91				4	4						0	8	2	0.69	0.50	1.00
Linyi	57.9	14.0	108	7.73				8	3	4	4		1	1	0	21	6	1.57	0.76	0.87
Taian1	60.1	25.8	180	8.09	4							1			4	1	2	0.50	0.32	0.72
Taian2	67.2	28.0	248	8.09	6										6	0	1	0.00	0.00	N
Taian3	103.0	37.0	396	7.85	24							4			24	4	2	0.41	0.24	0.59
Taian4	134.0	57.1	476	7.69	8			6	1		3				8	10	4	1.19	0.66	0.86
Total (ratio[f])					211 (68.3)	1 (0.3)	17 (5.5)	22 (7.1)	18 (5.8)	8 (2.6)	9 (2.9)	15 (4.9)	4 (1.3)	4 (1.3)	229 (74.1)	80 (25.9)				

[a] A_N, available N; A_P, available P; A_K, available K. The unit of measurement is mg kg^{-1}.
[b] S1, *S. fredii*; S2, *Sinorhizobium* sp. I; S2, *Sinorhizobium* sp. II; B1, *B. elkanii*; B2, *B. japonicum* USDA 6^T; B3, *B. japonicum* USDA 110; B4, *B. liaoningense*; B5, *B. yuanmingense*; B6, *Bradyrhizobium* sp. I; B7, *Bradyrhizobium* sp. II.
[c] S, total number of strains in the genus *Sinorhizobium*; B, total number of strains in the genus *Bradyrhizobium*; Spp, number of genomic species in each sampling site.
[d] *H′*, Shannon-Weiner's index; *D*, Simpson's index; *J*, Pielou's evenness index.
[e] N, null.
[f] Ratios are given as percentages.

visualized by electrophoresis in 2.5% (wt/vol) agarose gels containing 0.5 μg ml^{-1} ethidium bromide (43). The restriction fragment length polymorphism (RFLP) patterns (with fragments of more than 100 bp) were combined and used in cluster analysis with the Dice coefficient and the method of unweighted pair grouping with mathematic averages (UPGMA) by using the Gelcompar II software package (Applied Maths, Belgium) (40). Isolates sharing the same RFLP patterns were designated a single rRNA type.

Amplification and RFLP of 16S-23S IGS. The analysis of the internally transcribed spacer (IGS) sequences has been used to identify closely related species (37). In the present study, the ribosomal IGS between 16S and 23S rRNA genes was amplified using the primers FGPS6 and 23S-38 (25) with the PCR protocol of Rasolomampianina et al. (28). The PCR products of IGS were digested separately with each of the restriction endonucleases MspI, HinfI, and HaeIII (5 U per reaction) at 37°C for 6 h. The restriction fragments were separated, visualized, and clustered as described above for the RFLP analysis of 16S rRNA genes. Isolates sharing the same RFLP patterns were designated a single IGS type.

Sequence analyses of 16S rRNA, *recA*, *glnII*, *atpD*, *nifH*, and *nodC* genes. Based on the results of RFLP analyses of 16S rRNA genes and 16S-23S IGS, representative isolates of different clusters were chosen for the analysis of multiple gene sequencing. The 16S rRNA gene was amplified with the same primers and procedures used in RFLP analysis. Partial housekeeping genes *recA*, *glnII*, and *atpD* were amplified using primer pairs recA41F/recA640R, glnII12F/glnII689R, and atpD255F/atpD782R, respectively, with protocols described by Vinuesa et al. (42). A fragment of the *nifH* gene (about 800 bp) was amplified with primer pair nifHF/nifHR and the protocol of Laguerre et al. (17). A fragment of the *nodC* gene (about 700 bp) was amplified with primer pair nodCF540/nodCR1160 using the protocol of Sarita et al. (33). All of the acquired nucleotide sequences were deposited in the GenBank database, and the accession numbers are individually specified in the corresponding phylograms. The sequences acquired in this study and the related sequences obtained from the GenBank database by BLASTn searching were aligned, and phylogenetic trees were constructed using the software MEGA 4.0.1 (36) with the neighbor-joining method and the Kimura two-parameter model for the 16S rRNA gene, *nodC*, and *nifH* and for multilocus sequence analysis (MLSA) (combined sequences of *recA*, *glnII*, and *atpD*). The phylogenetic trees were bootstrapped with 1,000 bootstrap replications.

Data analyses. To estimate the community structure and species richness of soybean rhizobia, genospecies were defined based mainly upon the MLSA in this study. Soybean rhizobial diversity, species richness, and evenness in different sampling sites were estimated by three popular alpha ecological indexes (12): the Shannon-Wiener (*H'*) index and Simpson (*D*) index to represent diversity considering both the species richness and evenness in a community, and the Pielou (*J*) index to show the species evenness in the community. These indexes of biodiversity were implemented in the Vegan package (version 1.17-4; http://ftp.ctex.org/mirrors/CRAN/) and calculated by the R statistical language (version 2.12.0; http://www.r-project.org/). The program Bioenv (7) was used to find the best subset of environmental variables, so that the Euclidean distances of scaled environmental variables have the maximum (rank) correlation with community dissimilarities in biodiversity and the best subset of environmental variables, which also is implemented in the Vegan package using the R statistical language. Redundancy analysis (RDA) (27), the canonical version of principal component analysis (PCA), was used to examine the multiple relationships between soil factors (available N, P, and K and soil pH) and genospecies of soybean rhizobia of 16 sampling sites in the North China Plain. Community data of rhizobia (Table 1) were preanalyzed by detrended correspondence analysis (DCA) using CANOCO software 4.5 (Microcomputer Power, Ithaca, NY) (18); the length of gradient (first axis) was 3.872, so the data were analyzed by RDA.

Correspondence analysis also was performed to estimate the correlation among soybean cultivars and rhizobial genospecies by using SPSS software (PASW statistics 18.0; IBM Corporation). Twenty-four cultivars were treated as 24 separate levels for the cultivar variable, while the nine rhizobial genospecies (except of *Bradyrhizobium* sp. II) were used as levels for species variable.

Nucleotide sequence accession numbers. The GenBank database numbers of the sequences determined in the course of this work are listed in Fig. 2 to 4 as described in the figure legends.

RESULTS

Isolation of root nodule bacteria. A total of 309 pure rhizobial isolates were obtained from 625 soybean nodules, including 229 fast-growing (colonies of ≥2 mm in diameter in 3 to 5 days), acid-producing bacteria and 80 slow-growing (colonies of ≤1 mm in diameter in 7 days), alkali-producing bacteria. All of them formed effective nodules on the soybean cultivar Zhonghuang 13, as evidenced by the red color of nodules and the healthy plants (dark-green leaves). Isolation was not successful for 316 nodules, because (i) no growth was obtained in the nodule juice after 20 days of incubation in several cases, and (ii) in other cases, the isolates did not induce nodules on soybean in the nodulation test and were not included in the subsequent analyses. The numbers of isolates from different sampling sites are shown in Table 1, and detailed information is available in Table S1 in the supplemental material.

Soil characteristics. The results of soil characterization are presented in Table 1. In general, most of the soil samples were slightly alkaline, with pH 7.67 to 8.16; only two samples, from Luanxian (pH 7.13) and Jiaozhou (pH 6.91), were neutral. The contents of the main mineral nutrients in dry soil were (in mg kg^{-1}) 32.1 to 134.0 for available N, 5.6 to 91.2 for available P, and 54.8 to 597.0 for available K.

RFLP analyses of 16S rRNA gene and IGS. In the PCR-RFLP analysis of the 16S rRNA gene, five rRNA types (rrs types) were identified among the 309 isolates (see Table S1 in the supplemental material), which were clustered into two groups corresponding to the genera *Bradyrhizobium* and *Sinorhizobium*. These rRNA types were identical to those of reference strains for *B. yuanmingense* (type I, containing 15 isolates), *B. japonicum-B. liaoningense* (type II, containing 43 isolates), *B. elkanii* (type III, containing 22 isolates), *S. fredii* USDA 194 (type IV, containing 24 isolates), and *S. fredii* USDA 205^T (type V, containing 205 isolates). The IGS-RFLP analysis revealed greater genetic diversity and higher resolution among isolates than were obtained by RFLP of 16S rRNA genes. A total of 17 IGS types (patterns) were obtained among the isolates, which were grouped into 12 IGS clusters (A through L) (see Table S1 in the supplemental material) at a similarity level of 94% based on the cluster analysis. *Bradyrhizobium* comprised five IGS clusters (A through E), and *Sinorhizobium* contained seven IGS clusters (F to L). Isolates of rRNA type I all were identified as IGS cluster A. Isolates of rRNA type II were divided into three clusters (B, C, and D), which comprised 26, 8, and 9 isolates, respectively. rRNA type III was regarded as IGS cluster E (22 isolates). Isolates of rRNA type IV were divided into IGS clusters F, G, and I, containing 1, 17, and 6 isolates, respectively. rRNA type V was divided into four IGS clusters (H, J, K, and L), which comprised 85, 5, 23, and 92 isolates, respectively (see Table S1).

Phylogeny of 16S rRNA gene and housekeeping genes. In the phylogenetic tree of the 16S rRNA gene (Fig. 2), 28 isolates representing different IGS clusters were grouped into the genera *Bradyrhizobium* and *Sinorhizobium*. The isolates of rRNA type I (IGS cluster A, represented by strain CCBAU 25575) and type III (IGS cluster E, represented by strains CCBAU 25551 and CCBAU 051156) were most similar to *B. yuanmingense* and *B. elkanii*, respectively. Isolates of rRNA type II (IGS clusters B, C, and D) were close to *B. japonicum*, *B. liaoningense*, *B. canariense*, and *B. iriomotense* reference strains. IGS clusters F and G were two lineages related to, but different from, the reference strains for *S. fredii* and *S. saheli-S. sojae*, while isolates of IGS clusters H through L were similar to *S. fredii*.

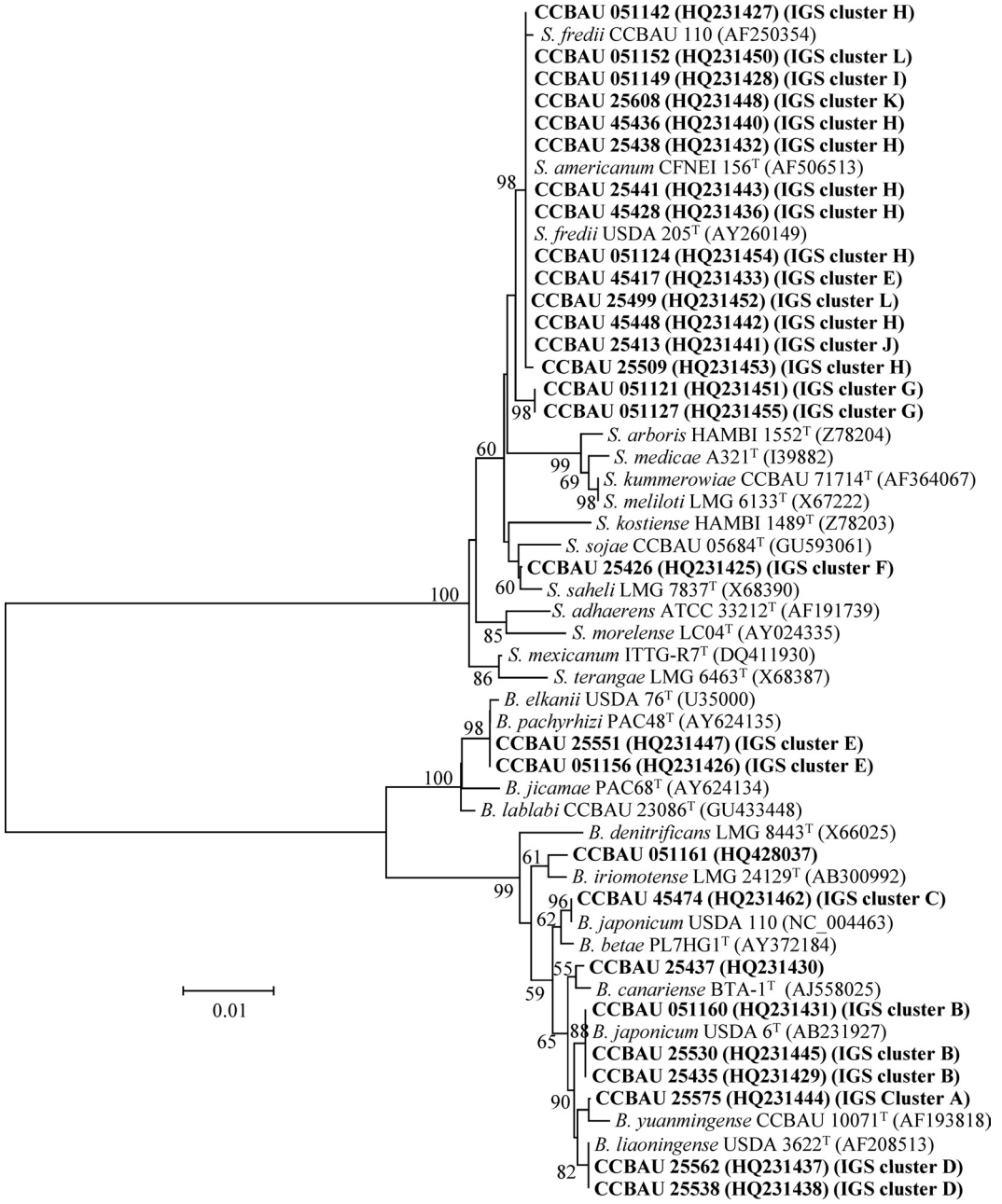

FIG. 2. Phylogenetic tree of 16S rRNA gene sequences showing the relationships between the representative isolates (in boldface) and the reference strains for defined rhizobial species. GenBank accession numbers in boldface were newly determined as a result of this study. The neighbor-joining (NJ) tree was derived from a 16S rRNA gene sequence distance matrix (Kimura two parameter). Bootstrap confidence levels of ≥50% are indicated at the internodes. The scale bar represents 1% nucleotide substitutions.

Based on the MLSA sequence similarities between the isolates and reference strains (Table 2) and the MLSA phylogenetic tree (Fig. 3), 10 genospecies were identified, including *S. fredii*, *Sinorhizobium* sp. I and sp. II, *B. elkanii*, *B. japonicum* (USDA 6^T), *B. japonicum* Ia (USDA 110), *B. yuanmingense*, *B. liaoningense*, and *Bradyrhizobium* sp. I and sp. II.

Sequence analyses of symbiotic genes *nodC* and *nifH*. In the phylogenetic tree of *nodC* genes (Fig. 4A), the representative isolates formed four clades. All of the isolates belonging to *Sinorhizobium* formed a lineage harboring sequences identical or very similar to those of reference strains for *S. fredii*, *S. sojae*, and *S. saheli*. The representative isolates of *Bradyrhizobium* genospecies were divided into three clades corresponding to *B. elkanii*, *B. yuanmingense*, and *B. japonicum-B. liaoningense.* These grouping results were consistent in general with the relationships revealed in the MLSA, except for the cases of *Bradyrhizobium* sp. II CCBAU 25437 and sp. I CCBAU 051161, the representatives for two lineages distinct from *B. japonicum* and *B. liaoningense* in MLSA that had a *nodC* gene identical to those of *B. japonicum*. The phylogenetic tree of

TABLE 2. Similarities of the housekeeping gene sequences between the new isolates and the reference strains

Representative strain(s) (CCBAU[a] no.)	IGS cluster	Closest species	Similarity (%)	Genospecies identified
25575	A	*B. yuanmingense* CCBAU 10071^T	98.5	*B. yuanmingense*
25435, 051160, 25530	B	*B. japonicum* USDA 6^T	97.5~99.4	*B. japonicum* USDA 6^T
051161	B	*B. liaoningense* USDA 3622^T	94.8	*Bradyrhizobium* sp. I
25437	B	*B. liaoningense* USDA 3622^T	94.6	*Bradyrhizobium* sp. II
45474	C	*B. japonicum* USDA 110	100	*B. japonicum* Ia USDA 110
25562, 25538	D	*B. liaoningense* USDA 3622^T	100	*B. liaoningense* USDA 3622^T
25551, 051156	E	*B. elkanii* USDA 76^T	99.7~100	*B. elkanii*
25426	F	*Sinorhizobium fredii* USDA 205^T	93.6	*Sinorhizobium* sp. I
051121, 051127	G	*S. americanum* CFNI 156^T	96.4	*Sinorhizobium* sp. II
051149	I	*S. fredii* USDA 205^T	98.9~100	*S. fredii*
25413	J	*S. fredii* USDA 205^T	98.9~100	*S. fredii*
25608	K	*S. fredii* USDA 205^T	98.9~100	*S. fredii*
45417,25441, 051152	L	*S. fredii* USDA 205^T	98.9~100	*S. fredii*
45436, 25438, 25009, 45428, 25499, 051142, 45448, 051124	H	*S. fredii* USDA 205^T	98.9~100	*S. fredii*

[a] Culture Collection of Beijing Agricultural University.

nifH genes (Fig. 4B) showed topology similar to that of *nodC* genes.

Distribution and diversity of soybean rhizobia in different sampling sites. In general, *S. fredii* is the superdominant species (occupied 68.28% of total isolates), followed by *B. elkanii* (7.12%), *B. japonicum* (USDA 6^T) (5.83%), *Sinorhizobium* sp. II (5.50%), and *B. yuanmingense* (4.85%). The relative abundance for genospecies *Sinorhizobium* sp. I, *B. japonicum* Ia (USDA 110), *B. liaoningense*, and *Bradyrhizobium* sp. I and sp. II varied from 0.32 to 2.91% (Table 1). All of the isolates from the sites of Jiaozhou, Luanxian, and Linyi belonged to *Bradyrhizobium*, while isolates from Heze1, Heze2, Taian2, Jining, and Handan all were *Sinorhizobium* species. Isolates from the other sampling sites comprise both *Bradyrhizobium* and *Sinorhizobium* (Table 1 and Fig. 5).

The diversity index (Table 1) of Shannon-Weiner (H') for Linyi was the highest (1.57), followed by that for Luanxian (1.24) and Taian4 (1.19). The lowest value (0) was found in Heze2, Jining, and Taian2, because only one species was isolated there. The remaining sampling sites had H' values between 0.87 and 0.18. The values of Simpson's index (D) varied between 0.76 and 0 in the 16 sampling sites and were very consistent with the H' values. The evenness index, Pielou (J), varied from 0.27 in the case of Heze1 to 1.0 in the case of Jiaozhou. These results demonstrated that the diversity and species composition of soybean rhizobial communities varied dramatically between different sampling sites (Table 1 and Fig. 5).

Correlation among soil characters or soybean cultivars and distribution of soybean rhizobia. In the DCA to test the models of species response to environmental variables, the length of the gradient (first axis) was 3.872, demonstrating that both the linear model and unimodal model are suitable. After further model tests, redundancy analysis (RDA) proved to be the best method. The results of RDA demonstrated that soil pH and available N explained the largest fraction of variation of soybean rhizobia in the North China Plain (RDA axis 1, 52.1%; $P = 0.004$). Nearly 61% of the variation in species ($P = 0.002$) was explained by environmental variables of soil pH and available N, P, and K (Fig. 6). The analysis by Bioenv proved that soil pH was the best subset of environmental variables to explain the distribution of species (Spearman rank correlation coefficient of 0.45), while the climate (subtropical and temperate regions) seems not to contribute to the biogeography of soybean rhizobia in the North China Plain.

According to the lengths of the arrows and the angles among them (Fig. 6), we could observe that high pH has strong positive correlation with the distribution of *S. fredii*, *Sinorhizobium* sp. I, *Sinorhizobium* sp. II, *B. yuanmingense*, and *B. japonicum* group Ia (USDA 110); strong negative correlation with the distribution of *B. elkanii* and *B. japonicum* (USDA 6^T group); and no significant correlation with the distribution of *B. liaoningense*, *Bradyrhizobium* sp. I, and *Bradyrhizobium* sp. II.

Contents of available K and P had slight effects on the distribution of soybean rhizobia, because the arrows representing them were short. Based upon the direction of the arrows, the effects of K were the same as those of pH, and the effects of P were in contrast to those of pH.

Since the angle between the content of N and pH was almost 90°, these two factors had independent effects on the distribution of soybean rhizobia in the sampling area. According to the results shown in Fig. 6, high nitrogen content in soil was positively correlated with the distribution of *S. fredii*, *Sinorhizobium* sp. I, *B. liaoningense*, and *B. yuanmingense* and negatively correlated with *B. japonicum* (USDA 6^T), *Bradyrhizobium* sp. I and sp. II, and *Sinorhizobium* sp. II, while having almost no effects on *B. elkanii* and *B. japonicum* Ia (USDA 110).

In the correlation analysis between the soybean cultivars and genospecies of rhizobia (correspondence figure not shown), only cultivar Handou 5 and *Sinorhizobium* sp. II had a close correlation. The remaining soybean cultivars and rhizobial genospecies did not show a close relationship, which meant that these soybean cultivars did not select their rhizobial partners strictly. And because only one isolate of *Sinorhizobium* sp. II was involved in the correlation analysis, the influence of soybean cultivars on rhizobial populations in the North China Plain was not significant.

DISCUSSION

In the present study, the soybean rhizobia were isolated from 16 sites, which represented the main soybean planting

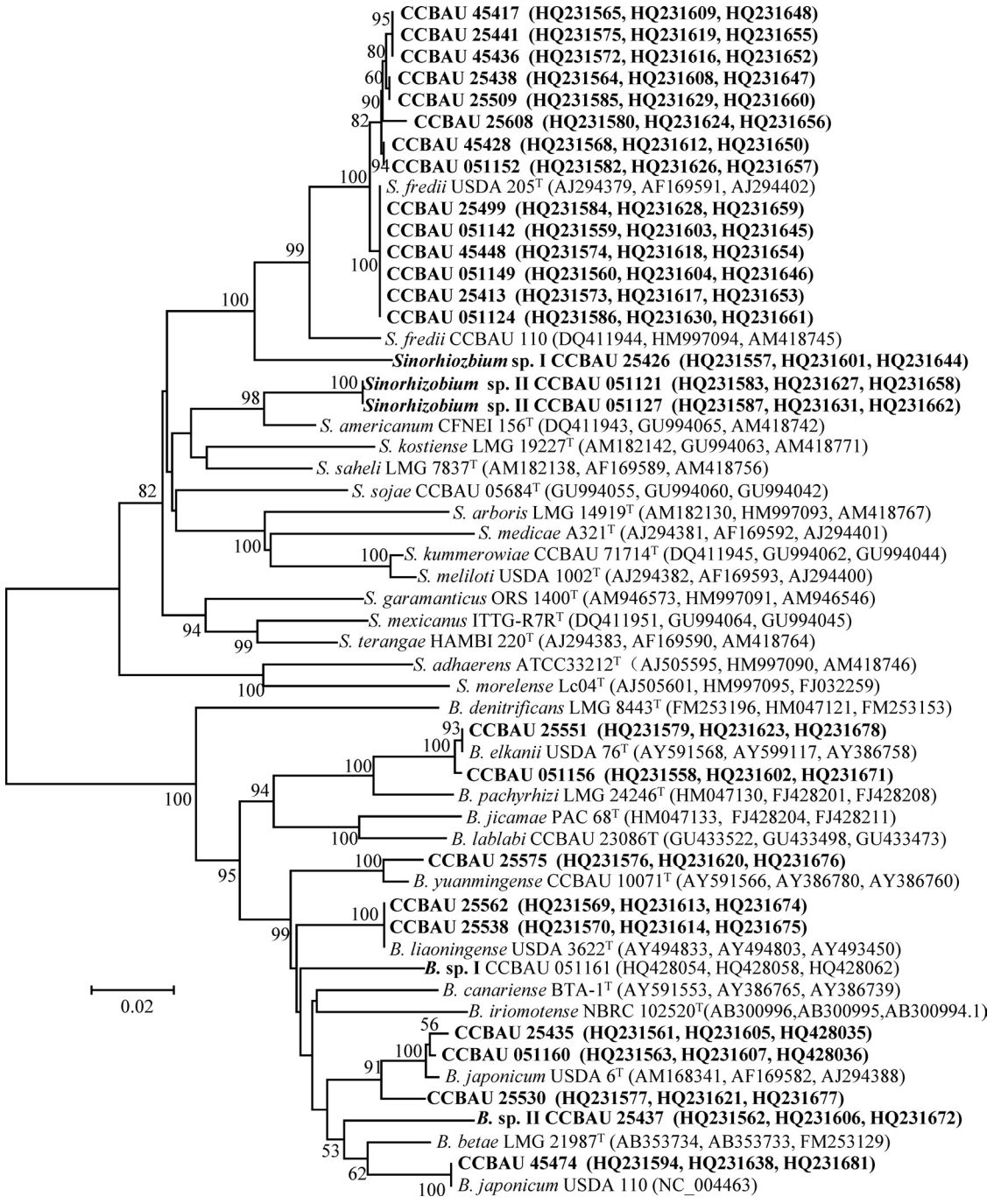

FIG. 3. Phylogenetic tree of MLSA based on concatenated sequences of *recA* (375 nucleotides [nt]), *glnII* (519 nt), and *atpD* (359 nt). Taxa and GenBank accesion numbers in boldface were newly determined as a result of this study. Bootstrap confidence levels of ≥50% are indicated at the internodes. The bar indicates 2% nucleotide divergence.

areas in the North China Plain. According to the soil characteristics presented in Table 1, the major proportion of the sampling sites had alkaline soil, and only two had neutral soil. This proportion is well reflected in the composition of soil types in the plain, as mentioned in the introduction. Based upon the National Norma of China, soil fertility was divided into six levels (http://www.soil17.com/news_more/1663.html). The soils in the 16 sampling sites covered levels 1 through 5 (very high to low) for available N, levels 1 through 4 for available P, and levels 1 through 5 for available K. These variations among the sampling sites demonstrated that the sampling sites were good representatives for the soil types in the plain.

In the present study, the operational taxonomic units (OTUs) were defined according to the consensus of grouping results in RFLP analyses of 16S rRNA genes and IGS, as well

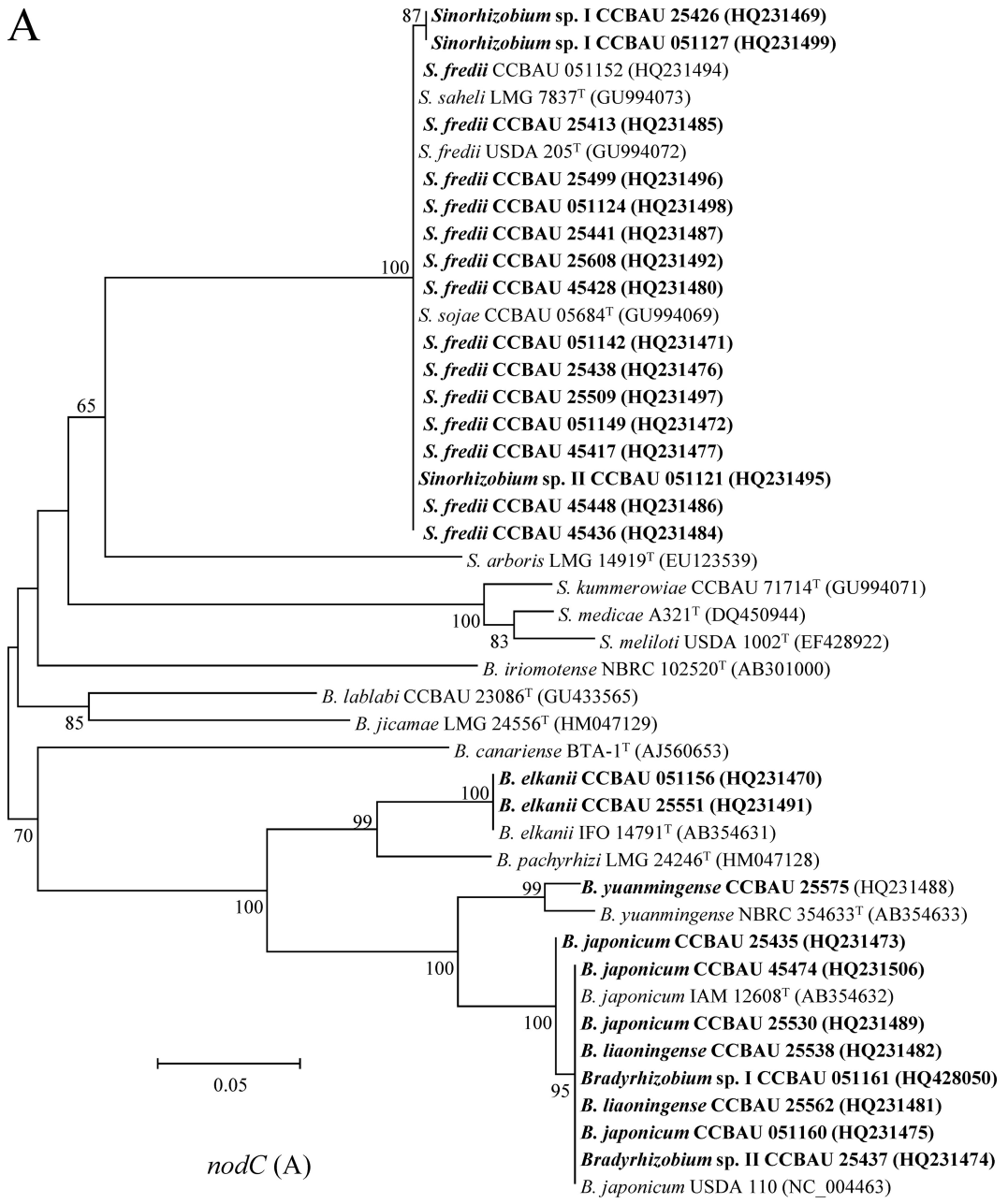

FIG. 4. Phylogenetic tree of *nodC* (A) and *nifH* (B) gene sequences showing the relationships between the representative strains (in boldface) and the related rhizobial species. GenBank accession numbers in boldface were newly determined as a result of this study. The neighbor-joining dendrograms were derived from a sequence distance matrix (Kimura two parameter). Bootstrap confidence levels of ≥50% are indicated at the internodes. Scale bars represent 5 or 2% nucleotide divergence.

as sequence analyses of 16S rRNA and three housekeeping genes (Fig. 2 and 3), which have been used to distinguish other known diazotrophic organisms (30) and soybean rhizobial species (3). With these methods, 10 genospecies within the genera *Sinorhizobium* and *Bradyrhizobium* were defined among the soybean rhizobia (Tables 1 and 2; also see Table S1 in the supplemental material). Most of these genospecies, like *S. fredii*, *B. elkanii*, *B. japonicum*, *B. japonicum* Ia (USDA 110), *B. liaoningense*, and *B. yuanmingense*, have been reported as soybean rhizobia previously (2, 10, 23), but the definition of *Bradyrhizobium* sp. I and sp. II and *Sinorhizobium* sp. I and sp. II demonstrated that there may be novel rhizobial species associated with soybean in the North China Plain. The finding of novel soybean rhizobia implies that the diversity of rhizobia is far from fully evaluated, since the soybean rhizobia have been extensively studied in the world.

The community composition of soybean rhizobia in the North China Plain is quite different from those reported in other regions in China (10, 23) and in India (2). The soybean rhizobia in the North China Plain were characterized by the

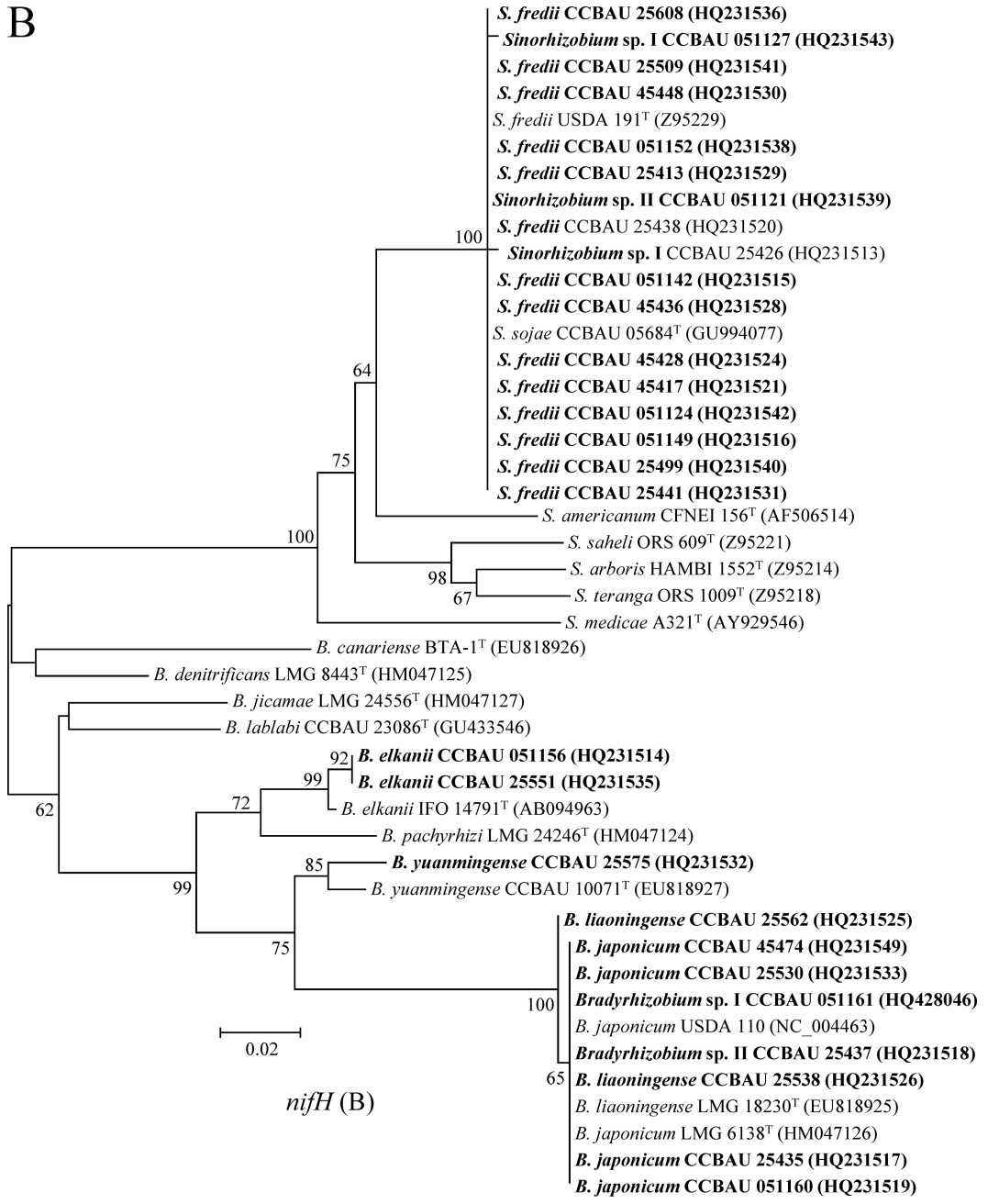

FIG. 4—*Continued.*

predominance of *S. fredii* (68.28%) followed by *B. elkanii* (7.12%) and the other eight genospecies (0.32 to 5.83%). In the subtropical and tropic regions of China, which has acidic soils in general, the most abundant soybean rhizobia was *B. japonicum*, followed by *B. elkanii* and several genospecies of *Bradyrhizobium* (23), while *S. fredii* was isolated only in some zones (4). In Xinjiang, which has saline alkaline soils, both *S. fredii* (45%) and *B. liaoningense* (43%) were the predominant soybean rhizobia, while *B. yuanmingense*, *B. japonicum*, and *Rhizobium* strains were the minor groups. In India, the soybean rhizobia in alkaline soils were *Bradyrhizobium* spp. (38%), *B. yuanmingense* (36%), and *B. liaoningense* (26%). Considering the recently described soybean rhizobial species *Sinorhizobium sojae* (19), at least 11 species were found in root nodules of soybean grown in the North China Plain. Therefore, the soybean rhizobia in the North China Plain were more diverse than those in other regions, which might be related to the long history of soybean cultivation and the diverse soil conditions in this region.

The diversity of soybean rhizobia in the North China Plain also was revealed by the sequence analyses of symbiotic genes (Fig. 4). As the original center of soybean production, the soybean rhizobia in the North China Plain harbored all the known types of symbiotic genes in soybean rhizobia reported

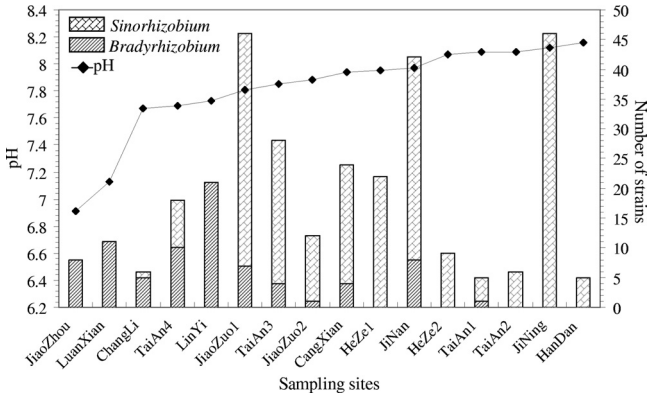

FIG. 5. Influence of soil pH in different sampling sites of the HHH Plain on the distribution and relative abundance of *Sinorhizobium* and *Bradyrhizobium* associated with soybean.

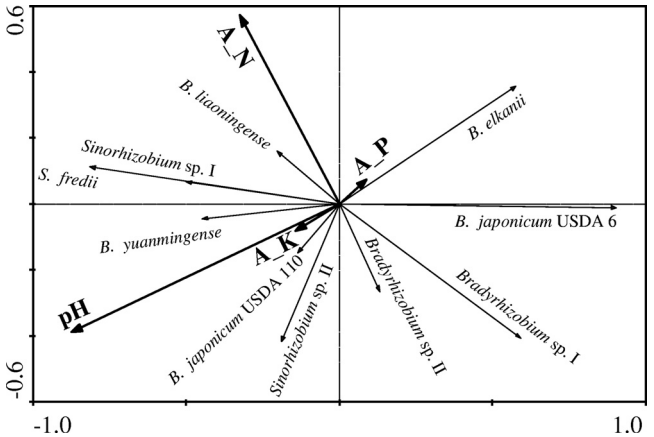

FIG. 6. Biplot of the RDA on the 10 genospecies and their soil factors from sampling sites in the HHH Plain by CANOCO. A_N, available N; A_P, available P; A_K, available K. Canonical correspondence analyses (CCA) were used to evaluate influence. The longer the arrow is, the greater the influence it has; the smaller the angle is between two arrows, the closer their relationship.

previously (2, 10). The four phylogenetic lineages of symbiotic genes (*S. fredii*, *B. japonicum*, *B. yuanmingense*, and *B. elkanii*) found in the soybean rhizobia demonstrated again that vertical transfer is the main form to maintain symbiotic genes in soybean rhizobia (10, 23), and that they have divergent origins and have coevolved with chromosome genes. Similarly to previous studies (10, 19), several lateral transfers of symbiotic genes were detected in the present study, because identical *nodC* and *nifH* genes were shared by isolates of *Sinorhizobium* sp. I and sp. II and *S. fredii* and by *Bradyrhizobium* sp. I and sp. II and *B. japonicum* (Fig. 4). Horizontal transfer happened rarely, but it is an important mechanism to form novel species and to improve the biodiversity of soybean rhizobia. These results also demonstrated that the relationships between the symbiotic genes and housekeeping genes in soybean rhizobia are rather stable, although the symbiotic genes only related to their host range (17) and are located on transferable elements (symbiotic plasmids or islands) (35).

The study of the biogeography of plants and animals at continental and local scales started centuries ago, but similar studies of bacteria were impossible at that time (9), because they are too small to see by the naked eye and the definition of bacterial species is difficult. Both these difficulties now have been overcome by the development of microscopy and molecular methods. In the present study, the rhizobial community composition varied in the sampling sites in the North China Plain, as demonstrated by the presence or absence and relative abundance of the rhizobial genospecies (see Table S1 in the supplemental material) and by the diversity indexes (Table 1).

The unique community composition of soybean rhizobia in the major soybean-producing areas of the North China Plain, together with previous studies of soybean rhizobia (10) in Xinjiang in the subtropical regions of China (23) and in alkaline soils in India (2), evidenced the existence of biogeography in soybean rhizobia (Table 1 and Fig. 5). Although analyses by CANOCO (Fig. 6) and R statistical language revealed the content of available N as a main factor correlating to the distribution of *B. liaoningense* and *Bradyrhizobium* sp. I and sp. II, more study was needed to verify this correlation, because it was not reported previously.

The soil pH as the main ecological factor to determine the distribution of different soybean rhizobia observed in the present study was similar to findings of previous reports for soybean rhizobia (2, 10) and for other soil bacterial and fungal communities (8, 31). The data reported previously (2, 10, 23) and in the present study demonstrated that (i) *B. elkanii* and *B. japonicum* (USDA 6^T) were common in acidic and neutral soils, and high pH (>8.0) greatly decreased or eliminated their nodule occupation in fields; (ii) *Sinorhizobium* species were the predominant soybean rhizobia in saline-alkaline soils; and (iii) *B. yuanmingense*, *B. liaoningense*, and *B. japonicum* Ia (USDA 110) were more resistant to alkaline soils than *B. elkanii* and *B. japonicum* (USDA 6^T) but may be more sensitive to salinity than the *Sinorhizobium* spp. (Fig. 5 and 6).

The slight correlation between the content of available P and the distribution of soybean rhizobia was similar to previous observations (10). The slight correlation of K content was not found in the previous study (10), and it is possible that this effect partially reflects the soil salinity, as revealed in the study on soybean rhizobia in Xinjiang (10).

Previously, effects of legume cultivar, including on the diversity and composition of symbiotic bacteria, has been reported, including that for soybean rhizobia (15). During the collection of nodules, at least 24 soybean cultivars were involved, but no apparent difference was found among the rhizobial populations associated with distinct cultivars. *S. fredii* was isolated from almost all of the cultivars, and only several *Bradyrhizobium* strains were isolated from them. It seems that the diversity and distribution of soybean rhizobia were determined mainly by the soil characteristics, and effects of cultivars were not important in the samples. This observation might be related to the fact that all of these cultivars were selected to fit the local conditions, especially the saline-alkaline soils, which are different from the laboratory conditions in the study of Israel et al. (15).

In the case of cultivar Yudou 25, the nodules were collected from two sites with different intensities: 37 isolates from Jiaozuo1, including 31 of *S. fredii* and 6 of *Bradyrhizobium* spp., and 4 isolates of *S. fredii* from Jiaozuo2. These results demonstrated that the species richness might be un-

derestimated in some sites when the sample size (number of isolates) was small, since these two sites have similar soil conditions.

In conclusion, 10 genospecies within the genera of *Sinorhizobium* and *Bradyrhizobium*, including two novel genospecies in each, were detected from the nodules of soybean grown in the North China Plain, the putative center of origin for soybean in China. The rhizobial community was more diverse than those detected in other regions and were characterized by the absolute predominance of *S. fredii*, followed by *B. elkanii* and other *Bradyrhizobium* and *Sinorhizobium* species. The biodiversity indexes also indicated a unique distribution and composition of soybean rhizobial communities in this plain. The geographic distribution of these rhizobial species is highly influenced by the soil pH. The present study is the first systematic assessment of *G. max* microsymbionts in the North China Plain and contributes to clarifying the biogeography of soybean rhizobia, providing a comprehensive illustration of how these species are distributed across the country.

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