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Rhizobial biogeography and inoculation application to soybean in four regions across China

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Running head
Soybean rhizobia in China

Place(s) where the work was done
Six sites, four regions across China

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Abstract

Aims: The aim of the study was to survey rhizobial biogeography and to inoculate soybean with selected rhizobia in China to enhance symbiotic nitrogen fixation (SNF).

Methods and Results: Biogeography, genetic diversity and phylogeny of soybean rhizobia was surveyed. Inocula were prepared and applied to soybean. Results showed that *Bradyrhizobium elkanii* and *Ensifer fredii* were widely distributed in acid and alkaline soils, respectively. Available iron was detected as the first determinant for distribution of the two rhizobia; and the soybean varieties did not greatly affect the rhizobial compatibility. Geographic latitude and precipitation in June were the main geographic and climatic factors affecting the rhizobial distribution. Inoculation with selected rhizobia increased the nodule number, fresh weight, occupation ratio, seed protein content and soybean yields.

Conclusions: Selection and application of effective soybean rhizobia across China according to biogeography were clarified to promote the SNF, thereby improving soybean yield.

Significance and Impact of the Study: Rhizobial diversity and biogeography were evaluated systematically in six sites across China. Available iron and soil pH are found to be the most important determinants for the distribution of soybean rhizobia. Inoculation to soybean enhances SNF, positively correlating to the increase of soybean yield and seed protein content.

Key words: Soybean; Rhizobia; Biogeography; Inoculation; Diversity

Introduction

Soybean (*Glycine max*), is the most important oil crop and plant protein source and has been cultivated in China for more than 5,000 years. As the center of origin for soybean and its symbiotic rhizobia (Dowdle and Bohlool 1985), studies on the composition of indigenous rhizobia in different geographic regions in China are vital for sustainable agriculture. Full utilization of the SNF potential is of ecological and economic importance for obtaining sufficient nitrogen supply to support the growth of soybean and to increase the yield and seed protein content.

Soybean can form symbiotic nodules with various rhizobia in the genera *Bradyrhizobium* and *Ensifer* (formerly *Sinorhizobium*), e.g. *Bradyrhizobium japonicum* (Dowdle and Bohlool 1985), *B. elkanii* (Kuykendall et al. 1992), *B. yuanmingense* (Zhang et al. 2011) and *B. liaoningense* (Xu et al. 1995), *B. diazoefficiens* (Delamuta et al. 2013), *Ensifer fredii* (formerly *Sinorhizobium fredii*) (Chen et al. 1988), *E. sojae* (Li et al. 2011a) and *E. glycinis* (Yan et al. 2016). Soil pH and salinity have been found to be related to the ecological distribution of soybean-nodulating rhizobial species (Giongo et al. 2008; Man et al. 2008; Han et al. 2009; Li et al. 2011b; Zhang et al. 2011). Application of mineral or organic fertilization (Bizarro et al. 2011), different climate and soil property (Adhikari et al. 2012), have direct and indirect effects on soybean rhizobial population, composition and distribution. However, few studies focused on the ecological comparisons of rhizobial distribution associated with same/different soybean varieties grown in different or same ecological region.

Symbiotic effectiveness of inoculation with different rhizobial isolates on different soybean varieties under different native soils were not well understood (Argaw 2014). Compatibility of soybean variety BARC2 with *B. elkanii* genotype USDA61, as well as its Tn5 mutants, resulted in great difference of root nodule and shoot weight (Faruque et al. 2015; Tang et al. 2015). Amongst the soybean genotypes, one variety TGx showed better symbiotic effectiveness with *E. fredii* USDA 191; while another variety Gazelle displayed more symbiotic compatibility with *B. diazoefficiens* USDA 110 (Muthuri et al. 2014). However, few studies have investigated the symbiotic compatibility and adaptation of these two microbial associates grown in different soils or geographic regions.

Therefore, the first aim of this study was to systematically investigate and compare the community composition, geographic distribution, and phylogeny of the soybean rhizobia associating with three soybean varieties at six sites in four climate regions across China. Next, host specificity match of the soybean varieties with different rhizobia and adaptation of the rhizobial-soybean partners in different or the same soil(s) were compared. Further, inoculant preparation and inoculation to different soybean varieties in greenhouse and in field were carried out to enhance the SNF, crop yield as well as seed protein content.

Materials and methods

Experimental design

The whole experimental design of the current study was shown in Figure S1. Three soybean varieties were grown in six sites across China (Table S1). Soils were sampled before sowing to determine the number of indigenous rhizobia and to trap rhizobia using soybeans. Genetic

diversity of the rhizobia and their distribution correlated with soybean varieties, soil physiochemical properties, environments (greenhouse and field), geography and climates were analyzed and compared integratively. Symbiotic match among the soybean varieties and rhizobia were evaluated to select higher efficient strains used for inoculation to soybean in field. Nodule occupation ratio of inoculated strains was calculated. The soybean yield, seed protein and oil content were determined to compare the influence of inoculation to soybean. The details of the current work were described in the following.

Soybean varieties

Three soybean varieties: 1) Xudou 18, a medium-maturing cultivar with top-podding type growth habit (Wang et al. 2014); 2) Nansheng 270, another medium-maturing cultivar but with even-podding type growth habit (Yang et al. 2013); and 3) Heihe 43, a precocity cultivar with middle-podding type growth habit (Han et al. 2016), were selected to compare their symbiotic matching to rhizobia in soils of 6 field sites across China (Table S1). The first two varieties have 105 days growth period and were mainly grown around Shandong Province (warm temperate zone, site 3 and site 4), while Heihe 43 has 90 days growth period and is mainly grown in Heilongjiang Province (frigid temperate zone, site 1) (Table S1). All the seeds were purchased from seed companies.

Field site, location, climate and soil collected

The above three soybean varieties were grown in fields at six sites of four climate zones: Wudalianchi in the frigid temperate zone; Tongliao in the temperate zone; three sites of Feicheng, Linyi and Jining cities in the warm temperate zone; and Sanya in the tropic zone (Table S1). Tested parameter for the geography, climate, precipitation in June and soil type of

these six sites were summarized in Table S1. Soil types of these six sites includes black soil, gray meadow soil, cinnamon soil, sandy soil, brown loamy soil and coastal sandy soil, respectively. These four provinces (Heilongjiang, Inner Mongolia, Shandong and Hainan) accounted for over 83 % of the total soybean growing area in China.

Soil samples (5-20 cm in depth) were sampled around soybean rhizosphere from each field before sowing or sampled at the stage of mid-flowering, and were used for the subsequent analyses. No record was available previously for rhizobial inoculation in these sites.

Physicochemical analysis of soil sample

Parts of the sampled soils were air-dried and used for following physicochemical analyses: total nitrogen (TN), organic materials (OM), available nitrogen (AN), available phosphorus (AP), available potassium (AK), available molybdenum (AMo), available boron (ABo), available iron (AFe), potential of hydrogen (pH), soil salt (Sal), water-soluble calcium (SCa), water-soluble magnesium (SMg), water-soluble sodium (SNa), water-soluble bicarbonate ions ($SHCO_3$), water-soluble chloride ion (SCl), water-soluble potassium (SK), soluble aluminum (SAl), water-soluble sulfate ions (SSO_4), and electronic conductibility (EC) according to standard analytical techniques (Olsen 1954; Houba et al. 1986; Doran and Parkin 1994; Simonis 1996; Simonis and Setatou 1996) at the Beijing Academy of Agriculture and Forestry Sciences, China.

Counting and trapping of indigenous rhizobia in soil sample

The abundance of indigenous rhizobia in soil before sowing of the soybean was estimated by the most probable number (MPN) method (Doran and Parkin 1994). MPN values were calculated from standard tables (Doran and Parkin 1994). For trapping the rhizobia from soil, each soybean variety was sown in pots filled with mixture of sterilized vermiculite and sampled soil (1:1). The plants were irrigated with low N nutrient solution (Doran and Parkin 1994) and grown in greenhouse for 30 days. After 30 days the nodules were checked for efficient nodulation and were used to isolate rhizobia.

Isolation of rhizobia from root nodule

Nodules obtained from the plant roots grown with trapping method in greenhouse and nodules from each soybean variety grown in fields were used for rhizobial isolation with the standard method and yeast mannitol agar (YMA) medium (Vincent 1970). Further, nodule isolates were purified and were cultured in YM broth at 28 °C to obtain bacterial cells for extraction of DNA and BOX-PCR fingerprinting analysis.

BOX-PCR fingerprinting and *rpoB* gene phylogeny

To differentiate the nodule isolate and identify clones belonging to a same strain among the isolates, the high-resolution BOX-PCR fingerprinting technique was applied (Menna et al. 2009). Total extracted DNA (Terefework et al. 2001) from the nodule isolates were used as template to amply the repetitive sequences in the genome with BOX-A1R as primer and the PCR procedure described by Nick et al. (1999). Isolates representing distinct BOX-PCR fingerprinting separated by 1.5% (w v^{-1}) agarose gels were picked out for subsequent *rpoB*

gene (encoding for RNA polymerase β-subunit) analyses. The *rpoB* gene was applied followed by procedure of Mousavi et al. (2014) using primer pair of *rpoB*-F (5'-TCG CAG TTC ATG GAC CAG G-3') and *rpoB*-R (5'-GTA GCC GTT CCA GGG CAT G-3'). The obtained sequences and sequences of related rhizobia were aligned together using ClustalW and best-fit models of evolution were determined using MODELTEST integrated in MEGA7 (Kumar et al. 2016). The lowest Bayesian information criterion (BIC) score was chosen for a maximum likelihood (ML) phylogenetic analysis. Isolates sharing identical sequences were designated as a single genotype and over 97.7% sequence similarity was used as threshold to define species as suggested by Zhang et al. (2017).

Soybean-rhizobium specificity matching

The nitrogen fixation efficiency and specificity matching between three soybean varieties and different rhizobial isolates were evaluated through number and fresh weight of nodules, chlorophyll content of leaf, above-ground height and dry matter of each soybean plant grown in greenhouse. The leaf chlorophyll content was determined using a Soil and Plant Analyzer Development (SPAD) meter (model: 502 Plus). A higher SPAD value indicated a higher chlorophyll content, therefore a healthier plant. Each rhizobial isolate was cultured in 5 ml of YM broth with shaking up to the late exponential phase and was inoculated to the soybean varieties in sterile vermiculite moistened with low N plant nutrient solution (Doran and Parkin 1994). One ml (about 10^9 cells) of the culture was inoculated to a pre-germinated aseptic soybean seed in five replicates for each isolate. Seedlings without inoculation were included as control. Nodulation were measured after 30 days inoculation. The number and fresh weight of nodules, chlorophyll content, shoot height and weight were determined and

recorded. Strains with higher efficiency under greenhouse condition were selected for further evaluation in field.

Preparation of inoculant for field use

Rhizobial inoculant consisted of two parts: Part I was the mixture of rhizobial culture at late exponential phase (2.0×10^9 ml^{-1}) grown in YM broth (Vincent 1970) with addition of newly sterilized trehalose solution (used for protective agent against drought) in YM to a final concentration of 20% (w v^{-1}). Part II contained mixed components for enhancing nodulation and rhizobial adherence to the soybean seeds, i.e. fulvic acid, 500 ppm, sodium carboxymethylcellulose (CMC-Na), 1.5% (w v^{-1}); Jensen nitrogen-free mineral solution (Vincent 1970) (in g l^{-1}). Part II was spayed to soybean seeds (2 ml kg^{-1} seeds) before Part I was mixed with the seeds (3 ml kg^{-1} seeds; 10^6 rhizobial cells per seed). All rhizobial inoculations (prepared as above) were used to inoculate soybean seeds just before planting in order to maintain the viability of bacteria.

Field experiment design

Two to five days before sowing, the experimental sites were prepared by ploughing, harrowing and levelling, and received ammonium sulphate-based fertilizer compounds (225 kg ha^{-1}, N : P_2O_5 : K_2O = 15 : 15 : 15) as basic fertilizer (except Linyi site where one ton of fermented cow dung used). The usage of the chemical fertilizer was far lower than the routine dosage (300~450 kg ha^{-1} N) applied by local farmers and few of them use rhizobial inoculants to soybean because the unavailability from local markets. The experimental design was a split plot in a randomized complete block design with three replications. The field in

each site was ridged and divided into 2 m × 5 m plots. Each experimental plot consisted of one or two inoculations and one uninoculated control. Subplot treatments comprised three soybean varieties. Each soybean variety was planted in 10 cm distance between plants, 50 cm distance between rows. The crop was irrigated in time when there was no effective precipitation, kept free from weeds, and observed weekly in order to prevent the possible pests or diseases.

Agronomic data and crop yield

At the mid-flowering (50% flowering) stage, ten soybean plants from each replicate were uprooted to determine their growth and document the nodulation. Number and weight of nodules per plant were counted and there fresh weight was weighted. At the time of harvest, the measured yield area was 4 m^2 with 3 repeats. Seed moisture determined and the yield was adjusted into kg ha^{-1} : Adjusted yield = 100 − MC/100 −13.5 × unadjusted yield (Birru 1979). Where MC is moisture content of soybean seeds, and 13.5 is the standard moisture content (in percent) of soybean seeds. The grain protein and oil content [g 100 g^{-1} seed] were measured using FOSS near-infrared grain analyzer (Mode: Infratec TM1241, FOSS).

Climatic data

Current and imminent climatic data were derived from measurements of altitude, temperature and precipitation, and 19 bioclimatic variables using the WorldClim data (http://www.worldclim.org/) (Hijmans et al. 2005; Suwannatrai et al. 2017).

Statistical analysis

Soybean rhizobial diversity, species richness, evenness in greenhouse and field were estimated by three popular alpha ecological indexes (Hill et al. 2003): the Shannon-Wiener index (*H´*), representing species richness in a community; the Simpson index (*D*) and the Pielou evenness index (*J*), showing the species dominance and evenness, respectively, in a community. Nodule occupation ratio (NOR) was calculated according to the equation described by Zhang et al. (2014). The principal component analysis (PCA) was applied to examine the relationship between the soil factors (including soils sampled before sowing and at the stage of mid-flowering) and the distribution of rhizobial genospecies (obtained from greenhouse and field sites) using CANOCO software 5.0 (Šmilauer and Lepš 2014). Grey relational analysis (Dai et al. 2014) was used to calculate the concrete correlation value between the soil factors and the distributions of rhizobial genospecies. After removing identical influence factors, forward selection analysis (Blanchet et al. 2008) was applied to test the following correlations among the spatial (longitude, latitude and altitude), edaphic (TN, AN, AK, pH and AFe), climatic (prec6, bio3), rhizobia (*B. elkanii*, *E. fredii*, *B. japonicum*, *B. huanghuaihaiense*, *B. daqingense*, *B.* sp. I and *Ensifer* sp.) and soybean varieties under greenhouse or field conditions. The growth and yield data among the treatments were compared using Duncan's new multiple range test.

Results

Soil characteristics of the sampling sites

Total 17 soil physiochemical properties (Table S2) were determined before sowing the soybean. Instantly, acid soils with pH 6.41, 5.20, 6.54 with relatively high AFe and AK were detected in Wudalianchi, Linyi and Sanya, respectively, despite their different geographic locations and soil types. Among these three sites, the soil in Wudalianchi had higher nutrient contents like TN, OM, AP and AK, while the highest AK (306 mg kg^{-1}), SK (71.7 mg kg^{-1}) and SSO_4 (127 mg kg^{-1}) were detected in Sanya soil. The soils in the remaining three sites were neutral to slightly alkaline with pH from 6.98-7.60, despite their different soil types. Various contents of nutrients were detected among the alkaline soils, but the soil in Feicheng presented greater contents of nutrients of TN, OM, AP, AK, AFe, as well as higher values of Sal and EC. In addition, 11 physicochemical characters in rhizosphere of three soybean cultivars were measured at the stage of mid-flowering of soybean in the field (Table S3). Organic material (OM) increased greatly at Linyi site because of the usage of fermented cow dung. There was no significant difference between the soil data obtained before sowing and at the mid-flowering stage of soybean.

Diversity of rhizobia trapped under greenhouse condition

The numbers of indigenous rhizobia in these six sites ranged from $1.47 \pm 0.40 \times 10^5$ to $7.20 \pm 2.42 \times 10^5$ cell per gram soil using the MPN method (Table S4). Total 483 isolates were obtained from the root nodules of the three soybean varieties grown in soils collected from the six different sites under greenhouse condition. The number of isolates varied from 61 to 96 in the case of Feicheng soil and Tongliao soil, respectively (Table S5). A total of 44

BOX-PCR fingerprinting profiles (not shown) were obtained and their representing strains were clustered into 8 genospecies at similarity of 97.7% of the *rpoB* gene sequences, corresponding to *E. fredii*, *B. elkanii*, *B. japonicum*, *B. huanghuaihaiense*, *B. yuanmingense*, *B. liaoningense*, *B. diazoefficiens* and an unidentified *Bradyrhizobium* sp. I (Figure 1, Table S5 and Figure S2A).

Some tendencies could be found in the distribution of species in the sites. Only the *Bradyrhizobium* species were trapped from the three acid soils (4 or 3 species in each) that contained medium to high AFe (54.1 - 155 mg kg^{-1}) and only the *E. fredii* populations were detected in the alkaline soils (pH 7.59 - 7.60) with low AFe (8.75 - 8.94 mg kg^{-1}). While both *E. fredii* and three *Bradyrhizobium* species were isolated from the neutral soil (pH 6.98) with mediate AFe (61.9 mg kg^{-1}). Furthermore, the five abundant rhizobial species were common for all the three soybean varieties, and only the minor groups of *B. yuanmingense* (3 isolates), *Bradyrhizobium* sp. (1 isolate) and *B. liaoningense* (1 isolate) were specific to Nansheng 270 and Heihe 43.

Under greenhouse conditions, the highest Shannon–Wiener's (*H′*) index, Simpson's (*D*) index and Pielou's evenness (*J*) index were detected in the rhizobial populations of Nansheng 270 (1.62, 0.66 and 0.81), Heihe 43 (1.50, 0.67 and 0.94), and Xudou 18 (1.39, 0.61 and 0.88) in Feicheng soil sample, where *E. fredii*, *B. elkanii*, *B. yuanmingense*, *B. diazoefficiens* were detected. The lowest *H′* values (0.00) and *D* index (0.000) were found in Jining and Tongliao sites despite of the varieties (Table S5), because only *E. fredii* was isolated from the nodules there.

Nodule occupation ratio (NOR) under greenhouse condition

As shown in Table S5, under greenhouse condition, all the rhizobial isolates trapped from soil samples of Jining and Tongliao belonged to *E. fredii*. In soils from Linyi, Feicheng and Sanya sites, *B. elkanii* was the predominant rhizobia in most cases, with NOR of 41.18% to 95.65% for the three soybean varieties. *B. diazoefficiens* was the dominant rhizobium for soybean Heihe 43, with 41.18% NOR in soil of Feicheng site. However *B. japonicum* was the dominant rhizobium for soybean Heihe 43, with 95.45% NOR in soil of Wudalianchi site. These four higher NOR rhizobia, *E. fredii*, *B. elkanii*, *B. japonicum* and *B. diazoefficiens*, were used for further determining the symbiotic efficiency with soybean varieties under greenhouse condition. The other four rhizobial species, *B. huanghuaihaiense*, *B. yuanmingense*, *Bradyrhizobium* sp. I and II had lower NOR, ranging from 4.35% to 13.64% were not used further.

Screening for efficient rhizobia under greenhouse conditions

Total 47 representative strains of the four higher NOR rhizobial species, *E. fredii*, *B. elkanii*, *B. japonicum* and *B. diazoefficiens*, were used to inoculate soybeans grown in sterilized vermiculite under greenhouse condition. Number, fresh weight of nodules, chlorophyll content, above-ground height and dry matter of each soybean were shown in Figure S3 I – VI. Total 22 strains were selected out for their higher symbiotic efficiency and were used further in field tests. The 22 strains used for soybean varieties Xudou 18, Heihe 43 and Nansheng 270, respectively, were *B. elkanii* XD18-11, *B. japonicum* HH43-49 and *B. elkanii* NS270-4 used in Wudalianchi site; *E. fredii* XD18-18, *E. fredii* HH43-17 and *E. fredii* NS270-1 in Tongliao site; *E. fredii* XD18-16 (and *B. elkanii* XD18-5), *E. fredii* HH43-22 (and *B. elkanii* HH43-6), *E. fredii* NS270-3 (and *B. elkanii* NS270-20) in Feicheng site; *B. elkanii* XD18-31, *B. elkanii* HH43-20 and *B. elkanii* NS270-21 in Linyi site; *E. fredii*

XD18-3 and *E. fredii* XD18-11 in Jining site (for the three soybean varieties); *B. elkanii* XD18-1 (and *B. elkanii* XD18-25), *B. elkanii* HH43-9, *B. elkanii* NS270-2 (and *B. elkanii* NS270-19) in Sanya site, respectively (Table S6).

Diversity and NOR of soybean rhizobia in fields

The 22 strains were cultured in the laboratory and the inoculants were prepared and used to inoculate the soybeans in sites in the fields. At the stage of mid-flowering of soybean, root nodules were sampled and total 921 numbers of isolates, including 368 from uninoculated control, were obtained (Table S6). These isolates were identified as 75 strains by BOX fingerprinting (not shown) and 7 genospecies by *rpoB* sequence analysis at the 97.7% sequence similarity, corresponding to the two dominant groups of *E. fredii* (457 isolates), *B. elkanii* (445 isolates), and the five minor groups of *Ensifer* sp., *B. japonicum*, *B. huanghuaihaiense*, *B. daqingense*, *B. diazoefficiens* representing by 1 to 3 isolates each (Table S6, Figure 1, and Figure S2B).

From the uninoculated treatments, four groups, *E. fredii*, *B. elkanii*, *B. japonicum* and *B. huanghuaihaiense* were isolated. Among them, the dominant rhizobia were *E. fredii* in Jining site (100% NOR), Feicheng site (73.08%-78.57% NOR) and Tongliao site (80.77%-100% NOR); and *B. elkanii* in Linyi site (70%-100% NOR), Wudalianchi site (78.57%-100% NOR) and Sanya site (100% NOR) in nodules of the three soybean varieties. The lowest Shannon–Wiener's (*H'*) and Simpson's index (*D*) with 0 were detected in Jining and Sanya, despite the soybean varieties, where only *E. fredii* and *B. elkanii* were isolated, respectively. The highest or secondary high diversity index (*H'*, *D* and *J*) was detected in Feicheng soil for

all the three soybean varieties where *E. fredii*, *B. elkanii* and *B. japonicum* were isolated. In addition, the rhizobial population of Linyi soil (for soybean Heihe 43, uninoculated) presented the highest diversity (1.36 and 0.51, respectively) (Table S6), including groups of *E. fredii*, *B. elkanii*, *B. japonicum* and *B. huanghuaihaiense*.

The inoculation of rhizobia increased significantly the NOR of the induced strains in the nodules (Figure 2B and Table S6) especially for the strains in two species of *E. fredii* and *B. elkanii*. Only one exception existed that the induced strain to soybean variety Heihe 43 in Wudalianchi site was *B. japonicum* strain HH43-49, while the dominant strain in the nodules was another strain of *B. elkanii*, with a NOR of 100% (Table S6).

Distribution of rhizobia affected by soil and climatic factors

The correlations between soil factors and distributions of the rhizobial genospecies (gsp.) were estimated by both correspondence analysis (Figure S4A, S4B) and grey correlation matrix (Table S7). Under the greenhouse condition, the eigen values were 0.6407 for axis 1 and 0.1267 for axis 2, which explained 76.74% variation (cumulative) (Figure S4A). According to the lengths of the arrows and the angles among them, the dominant species *E. fredii* showed strong positive correlations with contents of $SHCO_3$ and ABo, and then pH value and SMg, SNa; but strong negative correlation with AFe and AK contents (Figure S4A). The distribution of another dominant species *B. elkanii* was most positively correlated with AFe and AK contents but negatively correlated with $SHCO_3$ content and pH value. Distributions of the minor genospecies, *B. japonicum*, *B. huanghuaihaiense* and *B. liaoningense* were positively correlated with the soil SK and AK contents, but negative with

SCa, SMa, SNa, ABo, $SHCO_3$ and pH value. *B. yuanmingense* and *B. diazoefficiens* tended to be positive associated with Sal and AP contents.

Under the field conditions, the eigen values were 0.7464 for axis 1 and 0.1133 for axis 2, that explained 85.97% of the variation (cumulative) (Figure S4B). The pH value and $SHCO_3$ content in soil were positively and AFe was negatively correlated, as the most important factors, with the distribution of *E. fredii*, as well as with the distributions of *Ensifer* sp. and *B. daqingense.* In contrast, distributions of *B. elkanii* was positively correlated with high content of AFe, but negatively correlated with pH value and ABo content. *B. huanghuaihaiense* and *B. diazoefficiens* were positively, and *B. japonicum* was negatively correlated with AN, AK and SCa contents.

The grey relational coefficients under greenhouse conditions (Table S7) supported the results of correlation analysis, but some inconsistencies existed. For example, the highest and the second highest coefficients 0.6650 and 0.5520 between *E. fredii* and $SHCO_3$ and between *E. fredii* and SNa demonstrated their high positive correlations (Table S7). But this value between *E. fredii* and pH was not so high (0.3555). Similar cases were also found in the grey relational coefficients for the field data (Table S8).

The forward selection analysis results discovered the influence of environmental factors (edaphic, spatial and climatic) and soybean varieties on the rhizobial community structure (Figure S4C), that showed 0.8661 of the eigen value for axis 1, and 0.0074 for axis 2. They explained 87.35% variation of the distribution of rhizobia in spatial, edaphic, climatic and

variety environmental factors. Again, AFe and pH were the most important factors positively and negatively correlated with the distribution of *E. fredii*, and their effects were opposite for *B. elkanii*. A statistical analysis showed that significant correlations existed both between biological and environmental variables (Figure 2B to 2F, and Table S9). The AFe and pH content in soil were significant positively correlated with the distribution of rhizobia (R^2 = 0.710, p = 0.001 for AFe, and R^2 = 0.676, p = 0.001 for pH).

The latitude factors were positively correlated with the distribution of rhizobia (R^2 = 0.091, p = 0.035), However, the relevance between the distribution of rhizobia and geographic altitude (R^2= 0.044, p = 0.145) and longitude (R^2 = 0.025, p = 0.263) were not closely related. The prec5, 6, 8, 9, 10, 11, 12 and bio 2, 3, 12, 13, 16 of the climatic factors were positively correlated with the distribution of rhizobia. Of these climatic factors, precipitation in June (prec6, data in Table S1) affected the rhizobial distribution significantly (R^2 = 0.374, p = 0.001) (Figure 2, Table S9, Figure S4C). The soybean varieties, Xudou 18 (R^2 = 0.001, p = 0.931), Heihe 43 (R^2 = 0.006, p = 0.635) and Nansheng 270 (R^2 = 0.006, p = 0.674) were not close correlated with the distribution of rhizobia.

Soybean nodulation, yield, protein and oil content

A total of 22 rhizobial strains were used in the field experiments to evaluate their influences on soybean nodulation, growth and yield. The fresh nodule weights of all the soybean varieties were significantly higher in the inoculated treatments than those in the uninoculated controls in all the six sites (Figure S5 I - VI). Among all the treatments, nodule number and

fresh weight were significantly correlated with protein content at $p = 0.0127$ and $p = 0.0096$, respectively; however, they were not significantly correlated with seed yield or oil content in seeds (Figure 3).

Most of the inoculation treatments significantly ($p = 0.05$ and /or $p = 0.01$) increased the soybean yield ranging from 4.96% to 31.67% compared with that of the uninoculated controls (Figure 3 and Table 1). The only exceptions were Heihe 43 inoculated with *E. fredii* strain HH43-17 in Tongliao and Xudou 18 inoculated with *B. elkanii* strain XD18-1 in Sanya, which increased 8.55% and 6.27% of the yield, respectively, though were not significant (Table 1). The maximum increase in grain yield (31.67% higher than non-inoculation control) was registered for the strain XD18-3-11 inoculated to Xudou 18 in Jining site, followed closely by strain HH43-11 inoculated to Heihe 43 in Jining site with a 31.57% increase (Table 1). Xudou 18 and Nansheng 270 had no mature grains at the Wudalianchi site due to their longer growth period, and the yield was not included in the statistics. Soybean protein content was significantly increased in 20 of the 27 available inoculation combination, ranging from 0.16% to 7.80% (Table 1). The effects of inoculants on the oil contents varied from –9.21% to +2.13% among the cultivar/inoculant combinations compared to the uninoculated controls, with 13 increased and 14 decreased (Table S10).

Discussion

Abundance and diversity of soybean rhizobia in China

Previously, it has been revealed that the rhizobial abundance and diversity were affected by both the land use and crop managements and the abundance of soybean rhizobia varied from lower number (6.1×10^2 cell g^{-1} soil) in maize monoculture soil to higher number (6.8×10^6 cell g^{-1} soil) in soil of maize/soybean/wheat rotation supplied with chemical fertilizer (Yan et al. 2014). In the present study, high contextual abundance of rhizobia (about 10^5 cells g^{-1} soil) was detected in soils of these six sites (Table S4), which tailored the long soybean culture history because these abundances correspond to the continued soybean monoculture field and field with maize-soybean-wheat rotation without fertilizer supply (Yan et al. 2014). Though the existence of higher indigenous soil rhizobia, the nodule numbers per plant increased significantly because of our inoculation, meaning the inoculation still could enhance the rhizobial numbers in soybean rhizospheric soils, therefore promoting the SNF.

As the center of origin for soybean, various rhizobia with clear biogeographic patterns associated to this crop, have been isolated from China. Up to date, at least nine *Bradyrhizobium* species have been reported (Zhang et al. 2011), including *B. daqingense, B. diazoefficiens, B. elkanii, B. huanghuaihaiense, B. japonicum, B. liaoningense, B. yuanmingense, Bradyrhizobium* spp. I and III. In addition, three *Sinorhizobium/Ensifer* species including *S. fredii* (Chen et al. 1988), *E. sojae* (Li et al. 2011a), *E. glycinis* (Yan et al. 2016) have been confirmed to be soybean's nitrogen-fixing microsymbionts in China. In our current study, of the 8 *Bradyrhizobium* species and 2 *Sinorhizobium/Ensifer* species, we

found another two unidentified *Bradyrhizobium* species and one novel *Ensifer* species, i. e. *E. shofinae* published most recently (Chen et al. 2017), meaning the distinct differentiation and specialization of soybean rhizobia in specific soils.

In addition, the trapping procedures in greenhouse and in fields did not affect the rhizobial composition at the species level, especially the dominant groups, which confirmed in the previous report (van Cauwenberghe et al. 2016). In the present study, the two rhizobial species *B. elkanii* and *E. fredii* were found predominantly not only in the greenhouse but in the field soils. Therefore, great consistency existed between the rhizobial community composition from greenhouse and field conditions. Only the minor species *E. shofinae* and *B. daqingense* isolated in the Jining field were not trapped in the greenhouse; while *B. yuanmingense* and two *Bradyrhizobium* spp. were trapped in greenhouse from soils of Feicheng, Sanya and Wudalianchi but not isolated from fields. The differences in the minor groups might be the random cases. Therefore, the plant trapping procedure might have the same value with the field isolation in the selection of rhizobial inoculants.

It was observed interestingly that, in the near-neutral soil at Feicheng site, *B. elkanii* and *B. diazoefficiens* were always more dominant than that of *E. fredii* for the three soybean varieties grown under the greenhouse conditions. However, inoculation with *E. fredii* in the same field site caused the higher nodule occupy rate of *E. fredii* than that of *B. elkanii*. This finding revealed again that the dominant indigenous, locally-adapted competitive rhizobia might be the most efficient microsymbionts, therefore could be selected as candidates for rhizobial inoculants.

Factors affecting biogeography of soybean rhizobia

Environmental stresses such as drought, salinity, coldness, freezing and high temperatures affect plant growth and generate a threat to agriculture (Mahajan and Tuteja 2005), as well as to the survival of rhizobia applied to leguminous seed or soil. The soil factors affected survival, persistence and diversity of rhizobia (Hungria and Vargas 2000), and further influenced the nodulation and SNF properties. The survival and diversity of rhizobia in soil has been shown to be affected by organic matter content (Yan et al. 2014), drought (Sinclair et al. 2007), salinity/alkalinity (Youseif et al. 2014), pH (Giongo et al. 2008), available nitrogen (Wongphatcharachai et al. 2015), available phosphate (Han et al. 2009), available potassium (Yan et al. 2014), and other microelements (e.g. Fe, Cu, and Zn) (Giri et al. 2007). In our current study, we firstly found that available iron (AFe) content and pH value in soil were significantly correlated with the biogeography of soybean rhizobia. Besides, we reported the positive correlations between water-soluble bicarbonate ions ($SHCO_3$) content and distribution of *E. fredii*; between soil salt (Sal) content and distribution of *B. diazoefficiens*.

Regarding to the climate factors, the geographic latitude positively affected the soybean rhizobial biogeography. At the same latitude (the Feicheng, Linyi and Jining sites), however, distributions of *Bradyrhizobium* and *Ensifer* were closely related respectively with soil acid and alkaline pH values, differing from the rhizobial distribution in USA that only different *Bradyrhizobium* species were found (Shiro et al. 2013). The precipitation in June (prec6, Table S1) was also positively correlated with soil pH value and the rhizobial distribution (Figure 2, Table S9). The higher precipitation in June, the lower of soil pH, and the exclusive distribution of *Bradyrhizobium*, in Sanya and Linyi sites, were observed clearly. Conversely,

the lower precipitation in June, the higher of soil pH, and the exclusive distribution of *Ensifer*, in Tongliao and Jining sites. The genomic characterization of these two rhizobial genera may explained the adaptation of them in acid or alkaline soils respectively (Tian et al. 2012). Because the easier measurement of soil pH value, it is suggested that we should consider the soil pH as the first indicator for selection of suitable soybean rhizobial species used for inoculation in different soils. In addition, the indispensability of iron (Fe) element for nitrogenase and SNF, but the lower content of available iron (AFe) in alkaline soils, it is recommended to supply iron nutrition in these soils to enhance SNF.

Effect of inoculation on soybean yield, protein and oil

Increase of yields and seed protein content by inoculation of *Bradyrhizobium* strains have been observed in soybean (Getachew et al. 2017). Positive correlations between soybean yield/protein content and nodule number/weight were clearly observed after successful inoculation with *Bradyrhizobium* or *Ensifer* strains, in our current study (Figure 3, Table 1). Different strains in *Bradyrhizobium* and *Ensifer* had different increase on the soybean yields/protein contents when they were inoculated to a common soybean variety in neutral soil (at Feicheng site), but the *Bradyrhizobium* strains showed more efficiency in SNF with the soybean variety. For different strains in *B. elkanii* or *E. fredii*, they showed different increase on the soybean yields/protein contents, meaning the importance of selection of excellent strains symbiotically matching to a specific soybean variety. The oil content of soybean was not closely related to the inoculation. In some cases, the oil content increased, but in other cases, it decreased, even in a same soil, but with inoculation of different rhizobial strains. Therefore, some other environmental factors may affect the content and composition of soybean seed oil (Song, et al., 2016).

Overall, regardless of soybean varieties, available Fe content (AFe) is the first soil predictor for the community composition of soybean rhizobia at the country scale of China. Rhizobial species belonging to the genera of *Bradyrhizobium* and *Ensifer* (formerly *Sinorhizobium*) are the main microsymbionts for soybean in higher AFe content with acid or neutral soils and lower AFe content with alkaline soils, respectively. Two species, *B. elkanii* and *E. fredii* are the most predominant rhizobial species selected by the three soybean varieties grown in different sites across China. Though higher numbers of native rhizobia in soils reaching to 10^5 cells per gram soil, inoculation of effective strains still could enhance soybean nodulation and seed yield. The survey, selection of effective rhizobia, and the formulation of better inoculants carried out in this study are especially recommended to be adopted in order to ensure the highest SNF in sustainable agriculture across China.

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Conflict of interest

No conflict of interest and relevant ethical statements declared.

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Figure Legends

Figure 1. Combination of phylogenetic tree and rhizobial genospecies richness matrix. The *rpoB* phylogenetic tree was constructed using JC69 model and 1000 bootstrap. Data of rhizobial genospecies richness were from Supplementary Table S5 and Table S6 online. Boldfaced strains were newly isolated from soybean nodules sampled from greenhouse or from field and their accession numbers for *rpoB* gene in GenBank were included in parentheses. Bar, 10% estimated phylogenetic divergence. Scale, corresponding to the numbers of rhizobial genospecies.

Figure 2. Rhizobial ratio influenced by different factors.

The coefficient of determination (R^2) of each factor was estimated. Significance of R^2 (P value) was assessed by the constrained analysis of principal coordinates. The width of each arrow is proportional to the strength of the relationship between rhizobial ratio and different factors. (A) Rhizobial ratio, (B) Rhizobial inoculation, (C) Geographic regions, (D) Soybean variety, (E) Soil factor, (F) Climatic factor.

Abbreviation for soil factor: AFe, available iron (mg kg^{-1}); pH, potential of hydrogen; ABo, available boron (mg kg^{-1}); EC, electrical conductivity (S m^{-1}); SCa, water-soluble calcium(mg kg^{-1}); OM, organic matter (g kg^{-1}); AP, available phosphorus (mg kg^{-1}). Abbreviation for climatic factor: bio2 (◦C), mean diurnal range, mean of monthly air temperature $t_{max} - t_{min}$; bio3, isothermality, (bio2/bio7) × 100%; bio12 (mm), annual precipitation; bio13 (mm), precipitation of wettest month; bio16 (mm), precipitation of wettest quarter. Prec5~12 (cm), mean monthly precipitation summary in May to December. Significant level: *, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.001$.

Figure 3. The regression analysis among the root nodule number, nodule fresh weight, seed yield, seed protein and oil of soybean grown in the field.

The width of each path arrow is proportional to the strength of the relationship. Correlation coefficient value (r) is labeled along the arrow. *, significant correlation at $p < 0.05$; **, significant correlation at $p < 0.01$. "–" before the data, negative correlation. Negative and positive correlations were shown using dashed and solid line, respectively.

Table 1. Effect of rhizobial inoculation on soybean yield and protein.

Site	Cultivar	Inoculated strain †	Yield (kg·ha^{-1})	Increased (%)	Protein content (g 100g^{-1} seed)	Increased (%)
Wudalianchi	Xudou 18	Control	/		/	
		B. elkanii XD18-11	/		/	
	Heihe 43	Control	2196.91 ± 58.74 bA		41.64 ± 0.07 bB	
		B. japonicum HH43-49	2305.89 ± 18.52 aA	4.96	41.92 ± 0.05 aA	0.67
	Nansheng 270	Control	/		/	
		B. elkanii NS270-4	/		/	
Tongliao	Xudou 18	Control	4233.45 ± 56.96 bA		43.35 ± 0.02 bB	
		E. fredii XD18-18	4516.20 ± 102.57 aA	6.68	44.06 ± 0.07 aA	1.64
	Heihe 43	Control	1916.85 ± 77.80 aA		41.13 ± 0.01 aA	
		E. fredii HH43-17	2080.65 ± 75.65 aA	8.55	41.51 ± 0.57 aA	0.92
	Nansheng 270	Control	3096.60 ± 49.24 bB		41.16 ± 0.05 aA	
		E. fredii NS270-1	3346.20 ± 66.22 aA	8.06	41.61 ± 0.57 aA	1.09
Feicheng	Xudou 18	Control	2974.20 ± 113.91 bB		44.13 ± 0.03 bA	
		E. fredii XD18-16	3315.15 ± 68.16 aA	11.46	44.20 ± 0.05 abA	0.16
		B. elkanii XD18-5	3377.47 ± 175.27 aA	13.56	44.24 ± 0.03 aA	0.25
	Heihe 43	Control	1179.74 ± 67.97 bB		41.25 ± 0.02 cB	
		E. fredii HH43-22	1439.22 ± 60.56 aA	21.99	41.40 ± 0.06 bAB	0.36
		B. elkanii HH43-6	1512.26 ± 23.04 aA	28.19	41.55 ± 0.07 aA	0.73
	Nansheng 270	Control	2315.92 ± 37.94 bB		41.76 ± 0.01 bB	
		E. fredii NS270-3	2550.56 ± 47.32 aA	10.13	42.08 ± 0.21 aAB	0.77
		B. elkanii NS270-20	2667.89 ± 110.53 aA	15.20	42.26 ± 0.07 aA	1.20
Linyi	Xudou 18	Control	3034.20 ± 38.89 bA		42.30 ± 0.14 bB	
		B. elkanii XD18-31	3231.15 ± 115.03 aA	6.49	42.85 ± 0.06 aA	1.30
	Heihe 43	Control	1097.85 ± 58.40 bA		42.08 ± 0.21 bB	
		B. elkanii HH43-20	1216.80 ± 44.71 aA	10.83	43.02 ± 0.09 aA	2.23
	Nansheng 270	Control	2380.95 ± 79.64 bB		41.38 ± 0.17 aA	
		B. elkanii NS270-21	2620.80 ± 27.68 aA	10.07	41.95 ± 0.35 aA	1.38

Continued to Table 1.

Site	Cultivar	Inoculated strain †	Yield (kg·ha^{-1})	Increased (%)	Protein content (g/100 g seed)	Increased (%)
Jining	Xudou 18	Control	2974.20 ± 109.03 cC		43.13 ± 0.02 cC	
		E. fredii XD18-3	3315.15 ± 10.51 bB	11.46	44.25 ± 0.04 aA	2.60
		E. fredii XD18-11	3377.47 ± 56.60 bB	13.56	44.18 ± 0.20 aA	2.43
		E. fredii XD18-3-11	3916.20 ± 77.96 aA	31.67	43.59 ± 0.04 bB	1.07
	Heihe 43	Control	1078.30 ± 89.27 bB		41.42 ± 0.15 cC	
		E. fredii HH43-3	1300.67 ± 60.90 aA	20.62	41.49 ± 0.16 cC	0.17
		E. fredii HH43-11	1418.77 ± 44.03 aA	31.57	42.00 ± 0.05 bB	1.40
		E. fredii HH43-3-11	1303.83 ± 70.81 aA	20.92	44.65 ± 0.05 aA	7.80
	Nansheng 270	Control	2204.40 ± 114.34 cB		41.44 ± 0.13 bA	
		E. fredii NS270-3	2621.40 ± 121.09 aA	18.92	41.78 ± 0.48 abA	0.82
		E. fredii NS270-11	2411.66 ± 90.04 bAB	9.40	41.91 ± 0.31 abA	1.13
		E. fredii NS270-3-11	2660.67 ± 80.85 aA	20.70	42.39 ± 0.32 aA	2.29
Sanya	Xudou 18	Control	1585.35 ± 75.38 bA		45.00 ± 0.12 cB	
		B. elkanii XD18-1	1684.80 ± 46.18a bA	6.27	45.67 ± 0.05 bAB	1.49
		B. elkanii XD18-25	1856.40 ± 127.28 aA	17.10	46.23 ± 0.39 aA	2.73
	Heihe 43	Control	840.45 ± 26.88 bA		39.74 ± 0.05 bA	
		B. elkanii HH43-9	998.40 ± 63.75 aA	18.79	39.83 ± 0.01 aA	0.23
	Nansheng 270	Control	1606.80 ± 81.08 bB		40.16 ± 0.03 cC	
		B. elkanii NS270-2	1895.40 ± 68.59 aA	17.96	41.49 ± 0.02 aA	3.31
		B. elkanii NS270-19	1936.35 ± 39.31 aA	20.51	40.50 ± 0.07 bB	0.85

Note: /, no data available because these soybean varieties do not mature in those sampling site.

† Strains numbered with -3 or -11 had identical, and strains numbered with -3-11 was a mixture of 3 and 11 strains in Jining site.

The data among the treatments were compared using Duncan's new multiple range test, different capital letters and small letters after the value respectively show significantly different at the 0.05 and 0.01 probability levels.

Supplementary Files

Figure S1. Experimental design of the current study.

Figure S2. Maximum likelihood (ML) phylogenetic trees based on *rpoB* genes of rhizobial isolates isolated from root nodules of soybean grown in the greenhouse (A) or in the field (B).

Figure S3 I - VI. Symbiotic performance of selected rhizobia inoculated to different soybean varieties grown in vermiculite under greenhouse condition. Rhizobia were trapped from soils of Wudalianchi (I), Tongliao (II), Feicheng (III), Linyi (IV), Jining (V) and Sanya (VI).

Figure S4. Correspondence analysis.

Figure S5 I - VI. Effects of rhizobial inoculation on the nodulation of different soybean varieties grown in the field of Wudalianchi (I), Tongliao (II), Feicheng (III), Linyi (IV), Jining (V) and Sanya (VI) sites.

Table S1. Geographical information and climate zone of the six experimental field sites in China.

Table S2. Chemical characteristics of soil samples from different sites before sowing and experiment.

Table S3. Soil characteristics of sampling sites for mid-flowering of soybean under field conditions.

Table S4. Numbers of indigenous soybean rhizobia in soil of the six sampling sites before sowing determined by most probable number (MPN).

Table S5. Community composition and abundance of rhizobia trapped in greenhouse by three soybean varieties from soil samples of different sites.

Table S6. Community composition and abundance of rhizobia associated three soybean varieties grown in different fields.

Table S7. Grey correlation matrix between the rhizobial species and soil characteristics (soil sampled before sowing) under greenhouse conditions.

Table S8. Grey correlation matrix between the rhizobial species isolated from nodules of soybean grown in the field and soil characteristics (soil sampled at mid-flowering stage of soybean) under field conditions.

Table S9. The explanation variation of the distribution of rhizobia in environmental factors.

Table S10. Effect of rhizobial inoculation on soybean oil content.