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PII: S0723-2020(16)00038-2
DOI: http://dx.doi.org/doi:10.1016/j.syapm.2016.02.004
Reference: SYAPM 25755

To appear in:

Received date: 23-11-2015
Revised date: 26-2-2016
Accepted date: 28-2-2016

Please cite this article as: Y. Li, X. Li, Y. Liu, E.T. Wang, C. Ren, W. Liu, H. Xu, H. Wu, N. Jiang, Y. Li, X. Zhang, Z. Xie, Genetic Diversity and Community Structure of Rhizobia Nodulating *Sesbania cannabina* in Saline-Alkaline Soils, *Systematic and Applied Microbiology* (2016), http://dx.doi.org/10.1016/j.syapm.2016.02.004

Genetic Diversity and Community Structure of Rhizobia Nodulating *Sesbania cannabina* in Saline-Alkaline Soils

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Abstract

Sesbania cannabina is a plant that grows naturally along the seashores in Rudong County, China (RDC) and it has been introduced into the Yellow River Delta (YRD) as a pioneer plant to improve the saline-alkaline soils. In order to investigate the diversity of *S. cannabina* rhizobia in these soils, a total of 198 rhizobial isolates were characterized and phylogenetic trees were constructed based on data from multilocus sequence analysis (MLSA) of the housekeeping genes *recA*, *atpD* and *glnII*, as well as 16S rRNA. Symbiotic features were also studied by establishing the phylogeny of the symbiotic genes *nodA* and *nifH*, and by performing nodulation assays. The isolates had highly conserved symbiotic genes and were classified into nine genospecies belonging to the genera *Ensifer*, *Agrobacterium*, *Neorhizobium* and *Rhizobium*. A unique community structure was detected in the rhizobia associated with *S. cannabina* in the saline-alkaline soils that was characterized by five novel genospecies and four defined species. In addition, *Ensifer* sp. I was the predominant rhizobia in YRD, whereas *Ensifer meliloti* and *Neorhizobium huautlense* were the dominant species in RDC. Therefore, the study demonstrated for the first time that this plant strongly selected the symbiotic gene background but not the genomic background of its microsymbionts. In addition, biogeographic patterns existed in the rhizobial populations associated with *S. cannabina*, which were mainly correlated with pH and salinity, as well as the mineral nutrient contents. This study provided novel information concerning the interaction between soil conditions, host plant and rhizobia, in addition to revealing the diversity of *S. cannabina* rhizobia in

saline-alkaline soils.

Keywords: Interactions, *Sesbania*-rhizobia, Saline-alkaline soil, Phylogeny, Biogeography.

Introduction

The genus *Sesbania* is a member of the *Papilionoideae* and contains 70 flood-resistant species, mainly spread in tropical and subtropical areas [2, 9]. They form root- and/or stem-nodules with rhizobia via crack entry and determinate nodules under hydrogen conditions, however, they are also invaded by root hair infection and form both indeterminate and determinate nodules in non-flooded soil or vermiculite [7]. To date, nodulation of up to 40 *Sesbania* species has been reported after infection with symbiotic nitrogen-fixing bacteria, commonly called rhizobia [11], including *Neorhizobium huautlense* and other *Rhizobium, Mesorhizobium* and *Ensifer* (formerly *Sinorhizobium*) species associated with *Sesbania herbacea* [51, 52]; *Azorhizobium caulinodans* and *Bradyrhizobium* sp. with *S. rostrata* [12, 13, 15]; *A. doebereinerae* with *S. virgate* [16]; *Ensifer teranga* and *E. saheli* with *S. rostrate* and *S. cannabina* [12]; *Mesorhizobium plurifarium* with *S. punicea*, *S. sericea* and *S. herbacea*; *N. huautlense* with *S. sericea* and *S. exasperate* [48, 53]; *Rhizobium gallicum* with *S. sericea* and *S. sesban. S. sesban* was reported to have the broader spectrum of nodule-inducing rhizobial species from genera including *Rhizobium, Mesorhizobium*, *Ensifer* and *Allorhizobium* [6, 62].

Sesbania cannabina is an annual semi-shrub, found in Asian, African and Australian tropical regions where it is widely planted as green manure for improving soil fertility and reclaiming environments contaminated by heavy metals [2, 58]. Furthermore, *S.*

cannabina is believed to be strongly resistant to abiotic stresses, such as salinity, waterlogging, drought, and arid non-infertile conditions [34]. This species was described to form root nodules with *E. teranga*, *E. saheli*, *E. meliloti*, *N. huautlense*, *R. galegae* and *Allorhizobium undicola* related groups. [9, 12]. In China, *S. cannabina* has been planted as a wild plant in wet fields, hills and ditches of the Yangtze River Region, and it has been introduced into the recently formed Yellow River Delta (YRD) [60] in Shandong Province, where a high saline concentration is a problem for growing plants [60]. Since a massive number of wild *S. cannabina* plants have been observed in the seashore wetland and farm land along the Yellow Sea coasts, including Rudong County (RDC) in Jiangsu Province, *S. cannabina* has been introduced from RDC to YRD as a pioneer plant to improve the saline-alkaline soils.

Although *S. cannabina* along the seashores forms root nodules, no study has been published concerning the diversity of rhizobia associated with this plant in saline-alkaline soils. The chemical properties of saline-alkaline soils in both YRD and RDC may have strongly selected specific rhizobia to nodulate with *S. cannabina* in these environments, since the formation of legume-rhizobia symbiosis is affected by the interaction of factors related to the host plant, rhizobia and the environment [61]. Thus, the *Sesbania* rhizobial population in YRD and RDC may be different from those in other regions. Based on the background mentioned above, the aims of the present study were: 1) to identify and compare the community position of *Sesbania* rhizobia in YRD and RDC; and 2) to assess the geographic distribution of rhizobial

species correlated with different environmental factors in both regions.

Materials and Methods

Soil-nodule sampling and rhizobial isolation

Samples were collected from the root zone (0-20 cm depth) of *S. cannabina.* Five samples were taken from YRD (located in Bohai Gulf) and two were collected from the seashore wetland in RDC (in the Yellow Sea where the ancient estuary of the Yellow River was located 160 years ago), respectively. The sampled soils were air-dried, maintained in black plastic bags and transported to the laboratory. Five separate *S. cannabina* plants were uprooted and the root nodules were carefully collected and transferred into a sealed tube filled with silica gel particles for preservation until isolation. Nodules were rehydrated, surface sterilized, crushed and the nodule juice was then streaked onto yeast mannitol agar (YMA) plates in order to isolate the rhizobia with a standard protocol [7, 47]. All the inoculated plates were incubated at 28 ℃ for 3-7 days. Purification was achieved by isolation of colonies several times on the same medium until a pure culture was obtained. Pure cultures were then maintained at -80 ℃ in YM broth supplemented with 20% (v/v) glycerol.

Determination of soil chemical properties

The air-dried soil samples were passed through a 2 mm-mesh screen and used for determining the chemical properties. Soil pH was determined with a soil-water (1:2.5 w/v) suspension [14]. The organic carbon (OC) content of soil was measured by using the wet-oxidation method with $K_2Cr_2O_7$-concentrated H_2SO_4 [56]. The available

nitrogen (AN) was determined by means of quantifying the alkali-hydrolyzable N [40]. The available phosphorus (AP) content was determined by means of a colorimetry method [56]. The available potassium (AK) content was measured by means of NH_4OAc-extraction and the flame photometer method at a wavelength of 767 nm [40]. The total nitrogen (TN) content was measured by titration with a standard acid [30].

Phylogenetic analyses of house-keeping and symbiotic genes

Genomic DNA was extracted from each isolate by using the TIANGEN genomic DNA extraction kit (TIANGEN, China) for bacteria. All purified isolates were selected to amplify and sequence the *recA* gene (coding for DNA recombination and repair protein), using the primers recA41F/recA640R and the corresponding PCR protocol [49]. All the *recA* sequences were aligned by using Clustal W software and the similarity between each sequence pair was calculated using MEGA 5.05 software. Strains possessing identical *recA* sequences were identified as the same *recA* genotype. Subsequently, representative strains of different *recA* genotypes were selected for amplification and direct sequencing of the other housekeeping genes, 16S rRNA and symbiotic genes. The 16S rRNA genes were amplified and sequenced using the primers 27F/1492R and the corresponding PCR protocol [43]. The same strategy was adopted for housekeeping genes *atpD* and *glnII* using primers atpD255F/atpD782R and glnII12F/glnII689R, respectively [49]; *nodA* using the primer pair nodA1/nodA2 [18, 20]; *nifH* using primers nifHF/nifHR (the PCR product was approximately 800

bp) [23] or in some cases by using the primers ploF/ploR [32] generating a PCR product of approximately 480 bp. Nucleotide sequencing was performed by the Beijing AuGCT DNA-SYN Biotechnology Co., Ltd using the method of Sanger [39].

All the sequences obtained in this study were deposited in the GenBank database and were blasted in GenBank to search for homologous reference sequences. A phylogenetic tree was reconstructed for each gene by the neighbor-joining method [38] with Kimura's two-parameter model using MEGA version 5.05 [42]. The topology of the phylogenetic trees was evaluated by the bootstrap method with 1,000 replicates.

Multilocus sequence analysis (MLSA) is well known for providing data with greater discriminatory ability than analysis using a single gene [28, 50], and thus MLSA was performed with combined sequences of the three housekeeping genes (*recA*, *atpD* and *glnII*), and the sequence similarities between the tested and reference strains were calculated as described above. Genospecies were defined based on the MLSA relationship using a 97% sequence similarity threshold, as suggested previously [26].

Diversity evaluation and correspondence analyses

Sesbania rhizobial genospecies defined based on the MLSA results were used to evaluate the community structure and species richness. *Sesbania* rhizobial diversity, species richness, and evenness in different sampling locations were estimated by three common alpha ecological indices [21]: the Shannon-Wiener index (*H'*), which explains the species richness for a sample site, the Simpson index (*D*) showing the species dominance, and the Pielou index (*J*) indicating species evenness in a

community. The biodiversity indices for each sample were conducted in the Vegan package (version 1.17-4) and calculated using the R statistical language (version 3.1.2; http://www.r-project.org/) [44].

Redundancy analysis (RDA) [33], the canonical version of principal component analysis, was used to examine the multiple relationships between soil factors (available N, P and K, total nitrogen, organic carbon, salinity concentration and soil pH) and genospecies of *Sesbania* rhizobia in the sampling sites. The community data for rhizobia were pre-analyzed by detrended correspondence analysis (DCA) using CANOCO software 4.5 (Microcomputer Power, Ithaca, NY) [24]. In DCA, the length 2.996 of the gradient (first axis) demonstrated that RDA was the best method to evaluate the relationships between the soil characteristics and *Seabania* rhizobial genospecies, therefore, RDA (canonical correlation analysis) was applied to the data obtained in this study.

Symbiotic properties

Nodulation ability under laboratory conditions was examined for each of the representative strains using standard procedures [55]. Seeds of *S. cannabina* were surface sterilized and germinated on 0.6% agar plates at 28 ℃ in the dark for approximately 48 h. One germinated seedling was transferred to a Leonard jar filled with sterilized vermiculite, which was irrigated with nitrogen free nutrient solution and inoculated with the desired rhizobial inoculum. The inocula were prepared using bacteria grown in YM medium to the exponential phase, centrifuged and suspended at

approximately 10^8 cells mL^{-1} final concentration in distilled water, and 1 mL of the suspension was added to each jar using five replicates for each strain tested [1, 47, 59]. Controls were inoculated with 1 mL distilled water. All the plants were grown at 24 °C in an automated greenhouse with a daylight illumination period of 12 h [25]. Subsequently, all the plants were harvested and the effective (nitrogen fixing) root nodules were identified by the red color of root nodules and the dark-green leaves of the plants. Control plants remained small in size, did not bear nodules, and showed yellow leaves.

Results

Soil properties of the sampling sites

As shown in Table 1, all the soil samples were saline-alkaline with pH varying between 7.82 and 8.28, and salt concentrations varying from 0.12% to 0.43%. The content of the main mineral nutrients in dry soils were (in mg kg^{-1}) 24.27 to 112.0 for available N, 75.31 to 201.69 for available K, 1.76 to 43.57 for available P, 8.47 to 21.91 for organic carbon and 382.30 to 951.56 for total nitrogen. In comparison, available P, available K, OC and total nitrogen were more abundant in YRD soils, while salinity and pH values were greater in RDC samples (Table 1).

Diversity and composition of rhizobial populations in YRD and RDC

A total of 198 bacterial strains were obtained in this study, including 166 from YRD and 32 from RDC (Table 1, detailed information available as Supplementary Table

S1). In the *recA* sequence analysis (Supplementary Fig. S1), 18 *recA* genotypes were identified among the isolates that were grouped into nine clusters (Table 1, detail available as Supplementary Fig. S1, and Tables S1, S2). The phylogenetic analyses of the 16S rRNA, *atpD* and *glnII* genes grouped the 18 representative isolates into eight rRNA lineages and nine specific lineages (Supplementary Figs. S2, S3 and S4, Table S2). Based on the MLSA tree (Fig. 1) and the concatenated sequence similarities, the representative strains were divided into nine genospecies (Table 1 and Table S2), corresponding to five novel groups and four defined species: I) strains YIC4009, YIC4027, YIC4031, YIC4032 and YIC4056 representing 121 isolates in five *recA* types formed *Ensifer* sp. I that showed 98.2-99.9% similarities among themselves and 92.8-93.8% similarities with the most related strain *E. sojae* CCBAU 05684^{T} in MLSA; II) Isolates YIC4071 and YIC5077 representing two *recA* types and 24 isolates were defined as *E. meliloti*, since 99.3% similarity occurred between the two isolates and 97.3%-97.7% similarities were detected with *E. meliloti* USDA1002^{T} using MLSA; III) A single isolate YIC5079 was grouped with *E. sesbaniae* CCBAU 65729^{T} at 99.9% similarity using MLSA and it was therefore identified as *E. sesbaniae*; IV) Isolates YIC4108 and YIC4261 representing two *recA* types and 21 isolates shared 98.9% similarity and were closely related to *E. sesbaniae* CCBAU 65729^{T} (94.3% and 94.4% similarity, respectively) with MLSA, and they were designated as *Ensifer* sp. II; V) The single isolate YIC4103 showed only 90.1% similarity with the most related reference strain *Rhizobium hainanense* CCBAU 57015^{T} in MLSA and was identified as *Rhizobium* sp.; VI) Isolate YIC4083,

representing 22 isolates, was closely related to *Neorhizobium huautlense* HAMBI 2409^T (99.1% similarity) with MLSA and was designated as this species; VII) A single isolate YIC4121 showed 94.7% similarity with the most related reference strain *Agrobacterium radiobacter* LMG 140^T in MLSA and was identified as *Agrobacterium* sp. I; VIII) Two single isolates YIC4072 and YIC4105 in *recA* analysis were grouped together with *Agrobacterium pusense* NRCPB10^T (99.5% and 99.6% similarity) in MLSA, and they were designated as *A. pusense*; IX) Isolates YIC4104, YIC4260 and YIC5082, representing five isolates, formed a group sharing only 95.3-95.8% similarities with *A. pusense* NRCPB10^T in MLSA and were identified as *Agrobacterium* sp. II.

Symbiotic properties and phylogenies of *nodA* and *nifH*

In phylogenetic analyses, *nodA* and *nifH* were successfully amplified and sequenced from 12 and 15 representative isolates, respectively. The obtained sequences showed close relationships with each other and with those of the previously described *Sesbania*-nodulating rhizobia in the phylogenetic trees of both *nodA* (Fig. 2) and *nifH* (Supplementary Fig. S5). In the *nodA* phylogenetic tree (Fig. 2), 11 representative strains showed 96.0% to 100% similarities to each other and 91.1% to 92.3% similarities with *N. huautlense* USDA4900, while *A. pusense* YIC4072 showed higher similarity (99.6%) to *Rhizobium* sp. IRBG74 (isolated from *Sesbania*). In the *nifH* phylogeny (Supplementary Fig. S5), similar relationships were observed, but all the isolates were grouped in a single clade that had 92.8%-100% similarities with each

other, and with *E. saheli* ORS609^T and *Rhizobium* sp. (*Sesbania*) IRBG74. Furthermore, all the representative isolates formed effective nodules with *S. cannabina*, which indicated they had different genomic backgrounds (Fig. 1), although their symbiotic gene backgrounds were stringently selected.

Distribution and diversity of *Sesbania* rhizobia in different sampling sites

In the present study, eight genospecies were isolated from YRD, including *Ensifer* sp. I, *E. meliloti*, *Ensifer* sp. II, *Rhizobium* sp., *N. huautlense*, *Agrobacterium* sp. I, *A. pusense* and *Agrobacterium* sp. II. *Ensifer* sp. I was predominant in YRD, accounting for 73% (121/166) of the local isolates. Only five genospecies were detected in RDC, including *Ensifer* sp. I, *E. meliloti*, *N. huautlense*, *E. sesbaniae* and *Agrobacterium* sp. II, in which *E. meliloti* (14/32, 44%) and *N. huautlense* (13/32, 41%) were dominant (Table 1). Among the seven sampling sites, the highest values for all three diversity indices were observed in Dongying and the lowest in Kenli2, while the two sites from RDC presented the second and third highest values for all cases (Table 1).

Correlation of soil properties and distribution of *Sesbania* rhizobia

The correlation between *Sesbania* rhizobial genospecies and soil factors is shown in Fig. 3. Based on the length of the arrows and the angles between them (Fig. 3), pH, salinity, AP, OC and TN were the main factors that determined the distribution of *Sesbania* rhizobia in this study. The pH values were positively correlated with *E. sesbaniae* but negatively influenced by *Ensifer* sp. I. The distributions of *E. meliloti*,

N. huautlense, *Rhizobium* sp., *Agrobacterium* sp. I and *A. pusense* were positively correlated with salinity and AN, and negatively affected by AP and AK. TN and OC were positive correlated with *Ensifer* sp. I, while AP positively influenced *Ensifer* sp. II.

Discussion

As shown in Table 1, all the sampling sites had saline-alkaline soils, however, the lower pH and salinity values, and greater AP, AK, OC and TN contents differentiated the YRD soils from the RDC soils. The consistent phylogenetic relationships of the isolates based on the *recA* sequence analysis and MLSA (Fig. 1), as well as the consistent genus/species affiliation of the isolates defined by both methods (Fig. 1, Table 1), demonstrated that *recA* could be an adequate molecular marker for screening the phylogenetic relationships among a large number of isolates. In addition, *recA* showed higher resolution than *atpD* and *glnII* (Fig. S3, S4 and Table S2). The MLSA for genospecies definition and the 16S rRNA gene for genus definition have been applied to studies of other rhizobia [5]. In this current study, the definition of nine genospecies in *Agrobacterium*, *Ensifer*, *Neorhizobium* and *Rhizobium* demonstrated that *S. cannabina* formed symbiosis with distinct rhizobia. Previously, three lineages in *Ensifer* (*Sinorhizobium*) and one group close to *N. huautlense* were detected from nodules of *S. cannabina* in rotation with rice [9], while strains related to *R. tropici*, *R. etli*, *E. saheli*, *A. rubi* and *N. huautlense* were detected from this plant growing in the arid river valley of Jinshajiang (upstream of the Yangtze River) in China [22]. Compared with the previous reports, the results of the present study revealed much

280 greater diversity at the genospecies level, and the super relative abundance of *Ensifer*
281 genospecies could be a characteristic differentiating the rhizobial community
282 associated with *S. cannabina* in saline-alkaline soils from those in rice fields [57] and
283 in the river valley [22]. It seems that the *Ensifer* species are quite adapted to the
284 saline-alkaline soils, since soybean and common bean also form symbiosis with
285 *Ensifer* species in this type of soil [19, 46].

286 Biogeographic patterns of rhizobia have been found for the microsymbionts of
287 soybean, *Caragana* spp., faba bean, and *Cicer arietinum* [3, 8, 19, 27, 45, 59]. In
288 addition, the interactions between rhizobia and their legume hosts are affected by their
289 genetic background and environment factors such as soil pH and salinity [3, 8, 19, 59].
290 Although different numbers of genospecies were detected from the YRD (9 species)
291 and RDC (5 species) regions, the diversity indices in the RDC samples were higher
292 than those in 4 of the 5 YRD samples, except for the Dongying sample (Table 1). The
293 variation in species richness between these two regions might be caused by the
294 sample size, since there were 166 isolates from YRD compared to 32 isolates from
295 RDC (Table 1). Furthermore, the higher diversity indices in the Dongying, Rudong 1
296 and Rudong 2 sites might be related to their fertility level, since lower AK, AP, and
297 OC contents and higher pH values were detected in these three sites (Table 1). In
298 addition, they were the principle factors regulating different rhizobial species,
299 positively or negatively (Fig. 3), which was similar to the results of previous studies
300 [4, 36, 37, 61].
301

The universal distribution of *Ensifer* sp. I in all the sampling sites demonstrated its wide ability for adaptation to the saline-alkaline conditions; however, the lower dominance in Dongying, Yongan, Rudong 1 and Rudong 2 implied their sensitivity to higher alkaline conditions (Table 1, Fig. 3). The distribution in five sites and dominance of *E. meliloti* in Dongying and Rudong 1 showed it to be another species adapted to the saline-alkaline conditions (Table 1, Fig. 3). *Ensifer* sp. II were only isolated from three of the YRD sites but were dominant in Yongan, implying their great nodulation ability in lower salinity and slight alkaline conditions (Table 1, Fig. 3). *N. huautlense* was found in Dongying, Kenli 1 and Rudong 1, with high dominance in the latter, which revealed similar adaptation ability to *E. meliloti*. Therefore, the community structure of rhizobia nodulated with *S. cannabina* in the saline-alkaline soils varied and was dominated by *Ensifer* sp. I, *E. meliloti*, *Ensifer* sp. II or *N. huautlense*, according to the nutrient and pH-salinity levels.

Among the nine genospecies identified in the present study, *N. huautlense* [9, 29, 51, 52], *E. sesbaniae* [54], and *E. meliloti* [9] have been reported as microsymbionts for *S. cannabina* or other *Sesbania* species growing in different regions. Meanwhile, the other genospecies represented a novel record as microsymbionts of *E. sesbaniaes. A. pusens*e was reported for non-symbiotic bacteria in the rhizosphere of chickpea [31], therefore, the identification of two strains as *A. pusense* in this study showed that this species also contained symbiotic members. In addition, the other five isolates belonging to *Agrobacterium* sp. I and *Agrobacterium* sp. II were also closely related to *A. pusense* by MLSA (Fig. 1). Therefore, since *Ensifer* sp. I, *Ensifer* sp. II and

Rhizobium sp. also represented potential novel groups, the nodules of *S. cannabina* might be a source of novel microsymbiont genospecies.

The close phylogenetic relationships of the *nodA* and *nifH* genes (Fig. 2 and Supplementary Fig. S5), despite the genospecies, indicated that lateral gene transfer might have occurred between the rhizobial communities studied and that *S. cannabina* had a strong preference for the symbiotic gene background. Horizontal transfer occurs at a low frequency in nature, but it is an important mechanism of adaptation and evolution for bacteria [17, 41]. The close relationships of the *nodA* and *nifH* genes between the representative isolates and the other *Sesbania*-nodulating reference strains *Agrobacterium* sp. SIN-1 [12, 35], *N. huautlense* S02^{T}, *E. saheli* ORS 609^{T} [12, 35] and *Agrobacterium* sp. IRBG74 [10-11] implied the same origin and recent diversification of the symbiotic genes in the *Sesbania*-nodulating rhizobia. Since effective nodulation with *S. cannabina* was confirmed for all the representative isolates, the failure to amplify *nodA* and/or *nifH* sequences in several isolates, such as YIC4056 and YIC4260, was unexpected and the reason was unknown.

In conclusion, a unique community structure of rhizobia associated with *S. cannabina* in saline-alkaline soils was detected, and it was characterized by five putative novel groups and four defined species in the genera *Agrobacterium*, *Ensifer*, *Neorhizobium* and *Rhizobium*. *Ensifer* sp. I, *Ensifer* sp. III, *E. meliloti* and *N. huautlense* were the predominant groups in different sites depending on the fertility level, pH values and salinity. The *Sesbania*-nodulating rhizobia harbored similar symbiotic genes (*nodA*

and *nifH*), indicating that *S. cannabina* had stringently selected the symbiotic gene background for its microsymbionts and that the symbiotic genes of *S. cannabina*-nodulating rhizobia not only had the same origin but had also recently diversified. In addition, lateral gene transfer may have occurred between the rhizobia tested. As the first systematic survey of *Sesbania* rhizobia in saline-alkaline soils, this study improved the knowledge concerning the diversity and biogeography of rhizobia nodulating with this plant, and demonstrated the possible evolution of novel rhizobia under host and environmental selection.

Acknowledgements

This work was financed by the Strategic Priority Research Program of the Chinese Academy of Sciences (Grant No. XDA11020403), the Key Research Program of the Chinese Academy of Sciences (Grant No. KZZD-EW-14), the National Natural Science Foundation of China (31370108 and 31570063), the One Hundred-Talent Plan of the Chinese Academy of Sciences (CAS), and the Yantai Science and Technology Project (2013JH021). Entao Wang was supported by the projects SIP 20150597 and 20140124 authorized by IPN, Mexico.

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539

540 **Figure Legends**

541 **Figure 1.** MLSA phylogenetic tree based on concatenated sequences of *recA* (393 nucleotides, nt),
542 *atpD* (401 nt) and *glnII* (518 nt). The tree was constructed by the neighbor-joining method using
543 MEGA version 5.05. Bootstrap values greater than 50% are shown at the nodes.

544

545 **Figure 2.** Phylogenetic tree based on *nodA* sequences of representative rhizobia strains. The tree
546 was constructed by the neighbor-joining method using MEGA version 5.05. Bootstrap values
547 greater than 50% are shown at the nodes. Strains in bold typeface were also positive in *nifH*
548 amplification.

549

550 **Figure 3.** RDA biplot of the 10 genospecies and their soil factors from sampling sites in YRD and
551 RDC by CANOCO. AN, available N; AP, available P; AK available K; Salinity, salinity
552 concentration; OC, organic carbon; TN, total N. Canonical correspondence analyses (CCA) were
553 used to evaluate the influence of soil chemistry characteristics on the distribution of *Sesbania*
554 rhizobia. The length of the arrow indicates increasing influence and the smaller the angle between
555 the two arrows indicates closer relationships.

556

557 **Table 1.** Relevant properties of soil samples and the distribution of different rhizobia genotypes

Properties	Soil samples						
	Xianhe	Dongying	Kenli1	Kenli2	Yongan	Rudong1	Rudong2
GPS	N38.15 E118.45	N37.26 E118.42	N37.45 E118.59	N37.46 E118.58	N37.34 E118.49	N32.28 E121.11	N32.27 E121.19
Physiochemical properties							
Salinity (%)	0.30	0.34	0.23	0.35	0.15	0.43	0.39
pH	7.85	7.98	7.86	7.82	7.91	8.16	8.28
AN (mg kg^{-1})	67.20	39.20	24.27	84.00	61.6	112.00	33.60
AK (mg kg^{-1})	136.00	120.00	160.00	136.00	201.69	75.31	75.31
AP (mg kg^{-1})	25.46	9.71	12.77	18.25	43.57	4.36	1.76
OC (g kg^{-1})	13.29	10.84	13.87	21.91	14.15	11.76	8.47
TN (mg kg^{-1})	793.37	800.24	476.54	951.56	654.20	420.56	382.30
Fertility level (N/P/K)*	4/2/3	5/4/3	6/3/2	4/3/3	4/1/1	3/5/4	5/6/4
Rhizobial distribution (representative strain)							
Ensifer sp. I (YIC4031, 4027, 4032, 4009, 4056)	34	12	31	37	4	1	1
E. meliloti (YIC4071, 5077)	1	8	0	1	0	13	1
E. sesbaniae (YIC5079)	0	0	0	0	0	0	1
Ensifer sp. II (YIC4261, 4108)	0	1	0	1	19	0	0
Agrobacterium sp. I (YIC4121)	0	1	0	0	0	0	0
N. huautlense (YIC4083)	0	4	5	0	0	13	0
Sesbanirhizobium sp. (YIC4103)	0	1	0	0	0	0	0
A. pusense (YIC4072, 4105)	0	2	0	0	0	0	0
Agrobacterium sp. II (YIC5082, 4260, 4104)	0	1	1	0	1	0	2
Total strain number	36	30	37	39	24	27	5
Diversity index#							
H´	0.25	1.62	0.52	0.24	0.62	0.82	1.05
D	0.11	0.74	0.28	0.10	0.34	0.53	0.64
J	0.23	0.78	0.47	0.22	0.56	0.75	0.96

558 *According to the China national standard, level 1=very rich, 2=rich, 3=moderate, 4=poor, 5=very poor,

559 6=extremely poor (http://www.soil17.com/news_more/1663.html)

560 #*H´*, Shannon-Wiener index; *D*, Simpson index; *J*, Pielou index.

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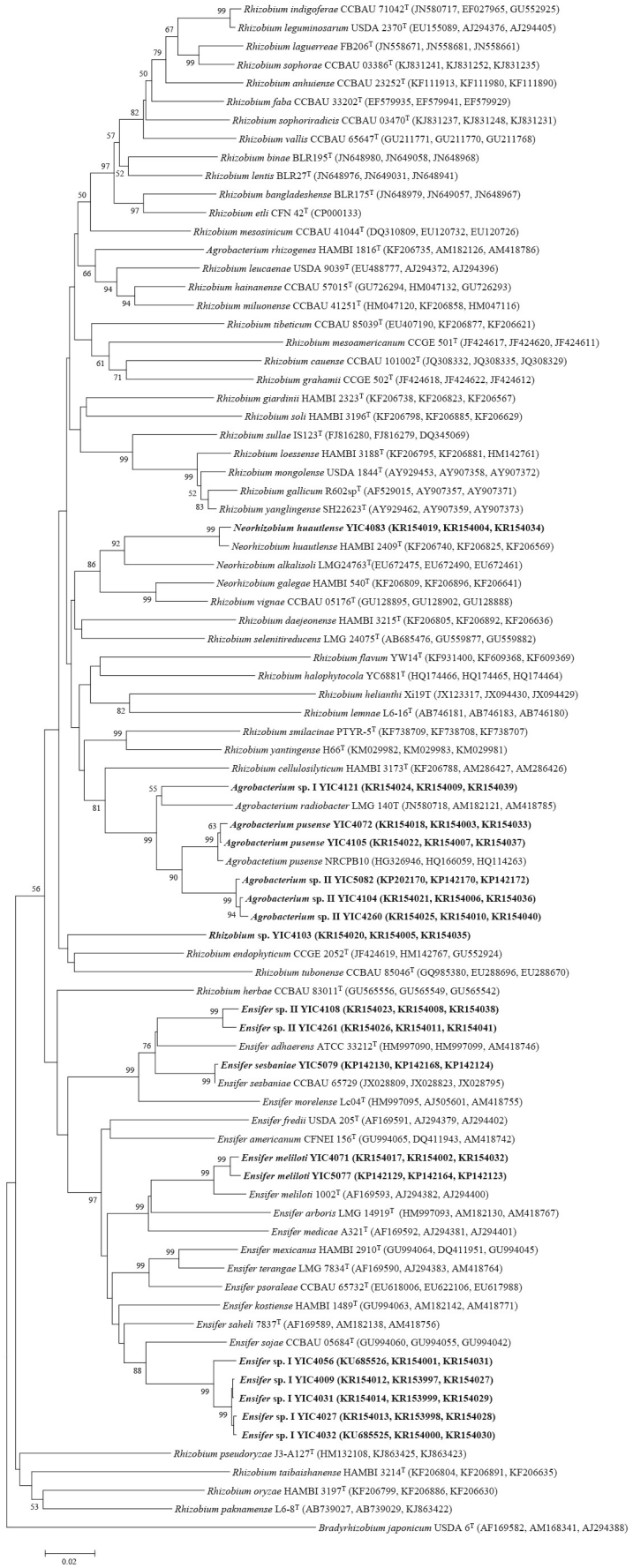
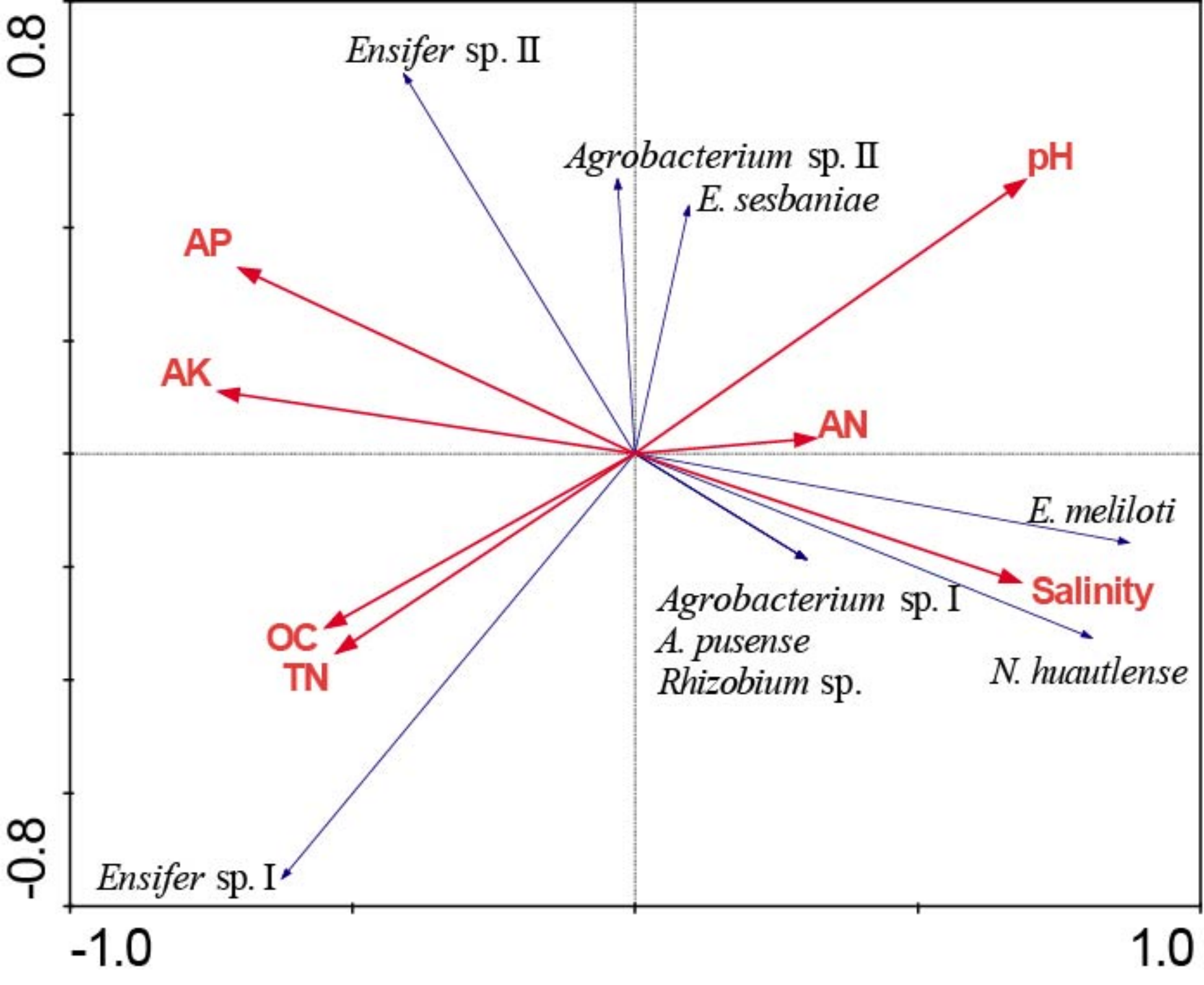
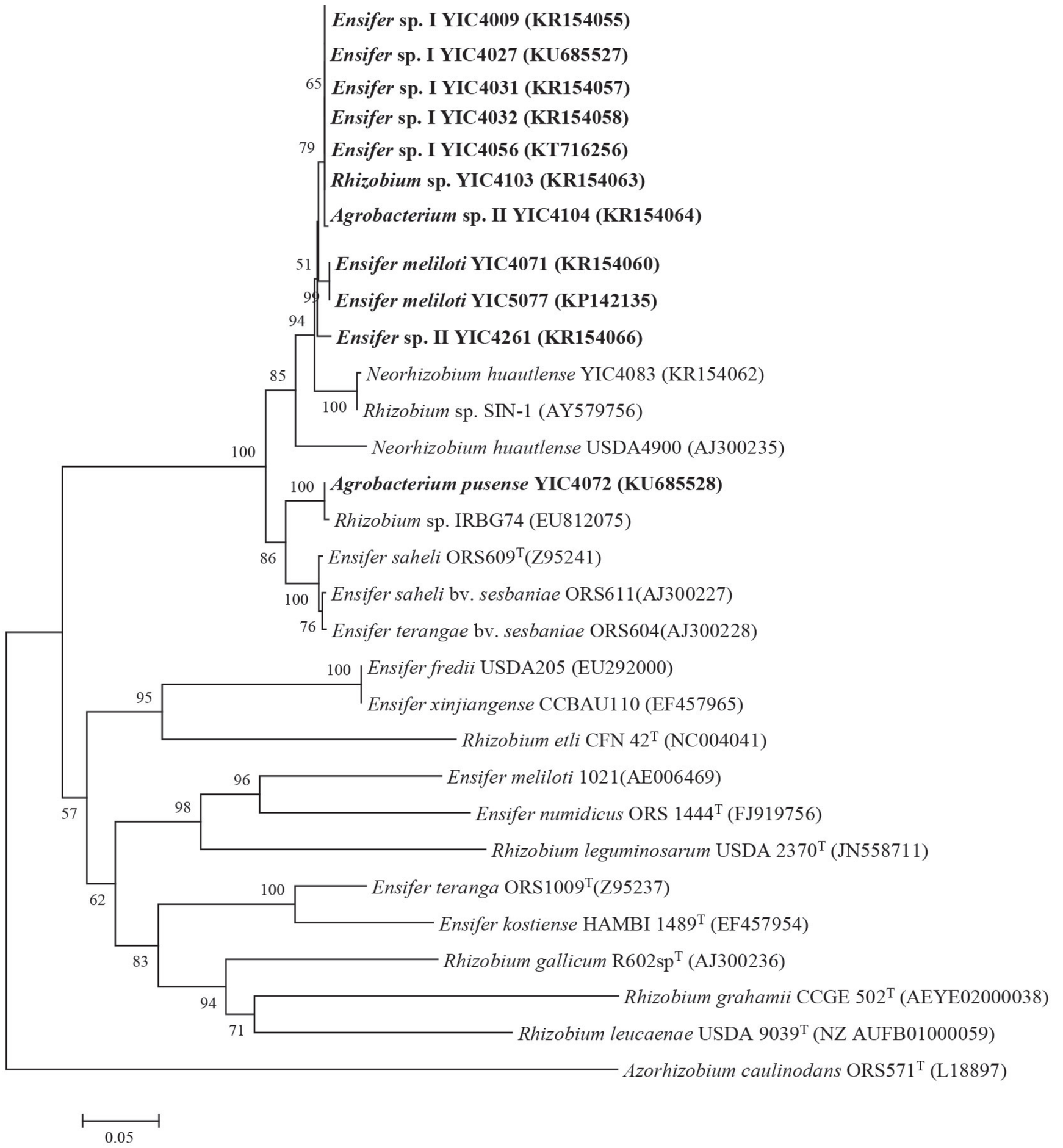

Rhizobium indigoferae CCBAU 71042^{T} (JN580717, EF027965, GU552925)
Rhizobium leguminosarum USDA 2370^{T} (EU155089, AJ294376, AJ294405)
Rhizobium laguerreae FB206^{T} (JN558671, JN558681, JN558661)
Rhizobium sophorae CCBAU 03386^{T} (KJ831241, KJ831252, KJ831235)
Rhizobium anhuiense CCBAU 23252^{T} (KF111913, KF111980, KF111890)
Rhizobium faba CCBAU 33202^{T} (EF579935, EF579941, EF579929)
Rhizobium sophoriradicis CCBAU 03470^{T} (KJ831237, KJ831248, KJ831231)
Rhizobium vallis CCBAU 65647^{T} (GU211771, GU211770, GU211768)
Rhizobium binae BLR195^{T} (JN648980, JN649058, JN648968)
Rhizobium lentis BLR27^{T} (JN648976, JN649031, JN648941)
Rhizobium bangladeshense BLR175^{T} (JN648979, JN649057, JN648967)
Rhizobium etli CFN 42^{T} (CP000133)
Rhizobium mesosinicum CCBAU 41044^{T} (DQ310809, EU120732, EU120726)
Agrobacterium rhizogenes HAMBI 1816^{T} (KF206735, AM182126, AM418786)
Rhizobium leucaenae USDA 9039^{T} (EU488777, AJ294372, AJ294396)
Rhizobium hainanense CCBAU 57015^{T} (GU726294, HM047132, GU726293)
Rhizobium miluonense CCBAU 41251^{T} (HM047120, KF206858, HM047116)
Rhizobium tibeticum CCBAU 85039^{T} (EU407190, KF206877, KF206621)
Rhizobium mesoamericanum CCGE 501^{T} (JF424617, JF424620, JF424611)
Rhizobium cauense CCBAU 101002^{T} (JQ308332, JQ308335, JQ308329)
Rhizobium grahamii CCGE 502^{T} (JF424618, JF424622, JF424612)
Rhizobium giardinii HAMBI 2323^{T} (KF206738, KF206823, KF206567)
Rhizobium soli HAMBI 3196^{T} (KF206798, KF206885, KF206629)
Rhizobium sullae IS123^{T} (FJ816280, FJ816279, DQ345069)
Rhizobium loessense HAMBI 3188^{T} (KF206795, KF206881, HM142761)
Rhizobium mongolense USDA 1844^{T} (AY929453, AY907358, AY907372)
Rhizobium gallicum R602sp^{T} (AF529015, AY907357, AY907371)
Rhizobium yanglingense SH22623^{T} (AY929462, AY907359, AY907373)
***Neorhizobium huautlense* YIC4083 (KR154019, KR154004, KR154034)**
Neorhizobium huautlense HAMBI 2409^{T} (KF206740, KF206825, KF206569)
Neorhizobium alkalisoli LMG24763^{T}(EU672475, EU672490, EU672461)
Neorhizobium galegae HAMBI 540^{T} (KF206809, KF206896, KF206641)
Rhizobium vignae CCBAU 05176^{T} (GU128895, GU128902, GU128888)
Rhizobium daejeonense HAMBI 3215^{T} (KF206805, KF206892, KF206636)
Rhizobium selenitireducens LMG 24075^{T} (AB685476, GU559877, GU559882)
Rhizobium flavum YW14^{T} (KF931400, KF609368, KF609369)
Rhizobium halophytocola YC6881^{T} (HQ174466, HQ174465, HQ174464)
Rhizobium helianthi Xi19T (JX123317, JX094430, JX094429)
Rhizobium lemnae L6-16^{T} (AB746181, AB746183, AB746180)
Rhizobium smilacinae PTYR-5^{T} (KF738709, KF738708, KF738707)
Rhizobium yantingense H66^{T} (KM029982, KM029983, KM029981)
Rhizobium cellulosilyticum HAMBI 3173^{T} (KF206788, AM286427, AM286426)
***Agrobacterium* sp. I YIC4121 (KR154024, KR154009, KR154039)**
Agrobacterium radiobacter LMG 140T (JN580718, AM182121, AM418785)
***Agrobacterium pusense* YIC4072 (KR154018, KR154003, KR154033)**
***Agrobacterium pusense* YIC4105 (KR154022, KR154007, KR154037)**
Agrobactetium pusense NRCPB10 (HG326946, HQ166059, HQ114263)
***Agrobacterium* sp. II YIC5082 (KP202170, KP142170, KP142172)**
***Agrobacterium* sp. II YIC4104 (KR154021, KR154006, KR154036)**
***Agrobacterium* sp. II YIC4260 (KR154025, KR154010, KR154040)**
***Rhizobium* sp. YIC4103 (KR154020, KR154005, KR154035)**
Rhizobium endophyticum CCGE 2052^{T} (JF424619, HM142767, GU552924)
Rhizobium tubonense CCBAU 85046^{T} (GQ985380, EU288696, EU288670)
Rhizobium herbae CCBAU 83011^{T} (GU565556, GU565549, GU565542)
***Ensifer* sp. II YIC4108 (KR154023, KR154008, KR154038)**
***Ensifer* sp. II YIC4261 (KR154026, KR154011, KR154041)**
Ensifer adhaerens ATCC 33212^{T} (HM997090, HM997099, AM418746)
***Ensifer sesbaniae* YIC5079 (KP142130, KP142168, KP142124)**
Ensifer sesbaniae CCBAU 65729 (JX028809, JX028823, JX028795)
Ensifer morelense Lc04^{T} (HM997095, AJ505601, AM418755)
Ensifer fredii USDA 205^{T} (AF169591, AJ294379, AJ294402)
Ensifer americanum CFNEI 156^{T} (GU994065, DQ411943, AM418742)
***Ensifer meliloti* YIC4071 (KR154017, KR154002, KR154032)**
***Ensifer meliloti* YIC5077 (KP142129, KP142164, KP142123)**
Ensifer meliloti 1002^{T} (AF169593, AJ294382, AJ294400)
Ensifer arboris LMG 14919^{T} (HM997093, AM182130, AM418767)
Ensifer medicae A321^{T} (AF169592, AJ294381, AJ294401)
Ensifer mexicanus HAMBI 2910^{T} (GU994064, DQ411951, GU994045)
Ensifer terangae LMG 7834^{T} (AF169590, AJ294383, AM418764)
Ensifer psoraleae CCBAU 65732^{T} (EU618006, EU622106, EU617988)
Ensifer kostiense HAMBI 1489^{T} (GU994063, AM182142, AM418771)
Ensifer saheli 7837^{T} (AF169589, AM182138, AM418756)
Ensifer sojae CCBAU 05684^{T} (GU994060, GU994055, GU994042)
***Ensifer* sp. I YIC4056 (KU685526, KR154001, KR154031)**
***Ensifer* sp. I YIC4009 (KR154012, KR153997, KR154027)**
***Ensifer* sp. I YIC4031 (KR154014, KR153999, KR154029)**
***Ensifer* sp. I YIC4027 (KR154013, KR153998, KR154028)**
***Ensifer* sp. I YIC4032 (KU685525, KR154000, KR154030)**
Rhizobium pseudoryzae J3-A127^{T} (HM132108, KJ863425, KJ863423)
Rhizobium taibaishanense HAMBI 3214^{T} (KF206804, KF206891, KF206635)
Rhizobium oryzae HAMBI 3197^{T} (KF206799, KF206886, KF206630)
Rhizobium paknamense L6-8^{T} (AB739027, AB739029, KJ863422)
Bradyrhizobium japonicum USDA 6^{T} (AF169582, AM168341, AJ294388)
0.02


0.8
-0.8
-1.0
1.0
Ensifer sp. II
Agrobacterium sp. II
E. sesbaniae
pH
AP
AK
AN
E. meliloti
Salinity
Agrobacterium sp. I
A. pusense
Rhizobium sp.
N. huautlense
OC
TN
Ensifer sp. I


Ensifer sp. I YIC4009 (KR154055)
Ensifer sp. I YIC4027 (KU685527)
65
Ensifer sp. I YIC4031 (KR154057)
Ensifer sp. I YIC4032 (KR154058)
79
Ensifer sp. I YIC4056 (KT716256)
Rhizobium sp. YIC4103 (KR154063)
Agrobacterium sp. II YIC4104 (KR154064)
51
Ensifer meliloti YIC4071 (KR154060)
99
Ensifer meliloti YIC5077 (KP142135)
94
Ensifer sp. II YIC4261 (KR154066)
85
Neorhizobium huautlense YIC4083 (KR154062)
100
Rhizobium sp. SIN-1 (AY579756)
Neorhizobium huautlense USDA4900 (AJ300235)
100
100
Agrobacterium pusense YIC4072 (KU685528)
Rhizobium sp. IRBG74 (EU812075)
86
Ensifer saheli ORS609T(Z95241)
100
Ensifer saheli bv. sesbaniae ORS611(AJ300227)
76
Ensifer terangae bv. sesbaniae ORS604(AJ300228)
100
Ensifer fredii USDA205 (EU292000)
95
Ensifer xinjiangense CCBAU110 (EF457965)
Rhizobium etli CFN 42T (NC004041)
96
Ensifer meliloti 1021(AE006469)
57
98
Ensifer numidicus ORS 1444T (FJ919756)
Rhizobium leguminosarum USDA 2370T (JN558711)
100
Ensifer teranga ORS1009T(Z95237)
62
Ensifer kostiense HAMBI 1489T (EF457954)
83
Rhizobium gallicum R602spT (AJ300236)
94
Rhizobium grahamii CCGE 502T (AEYE02000038)
71
Rhizobium leucaenae USDA 9039T (NZ AUFB01000059)
Azorhizobium caulinodans ORS571T (L18897)
0.05