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# Genetic diversity and symbiotic evolution of rhizobia from root nodules of *Coronilla varia*

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ABSTRACT

Ninety symbiotic rhizobial isolates from root nodules of *Coronilla varia* growing in the Shaanxi province of China were characterized. Combined with the results of RFLP patterns, six genotypes were defined among the rhizobial strains and they were divided into three genomic genera. These included *Mesorhizobium* sp., *M. alhagi*, *M. amorphae*, *M. metallidurans/M. gobiense* as the dominant group (86.7%), and *Rhizobium yanglingense* and *Agrobacterium tumefaciens* as the minor groups, according to analysis of the corresponding 16S rRNA, *nodC* and *nifH* genes. Five *nodC* types, which mainly grouped into the *Mesorhizobium* genus, were obtained from all the isolates examined, implying that *nodC* genes probably occurred from the native habitat through lateral transfer and long-term adaptation, finally evolving toward *M. alhagi*. Four different *nifH* types, displaying obvious differences compared to those of 16S rRNA and *nodC*, implied that possible lateral transfer of the symbiotic genes occurred between different genera. The association between soil components and the genetic diversity of the rhizobial population demonstrated that combined genotypes were positively correlated with the pH of soil samples.



## Introduction

Nitrogen is an indispensable nutrient element for plant growth and development. The rhizobia are free-living soil bacteria capable of forming nodules on plant roots or shoots within which they fix atmospheric nitrogen, with the fixed nitrogen being used for the benefit of the plant [9]. This symbiosis is a major contributor to the global nitrogen cycle, and since it is considered to be the most economic and eco-friendly resource for mankind [4,10,11], it has therefore received significant attention.

*Coronilla varia*, a perennial legume vine, is native to the European–Mediterranean area [8]. Since it has a developed root system, strong resistance and high nutritional value, as well as a beautiful color, it has been widely used for erosion control, soil rehabilitation, pasturage and roadside planting throughout the United States, Russia, Canada, and many other countries [31]. In the 1960s–70s, *C. varia* was introduced into China from Europe and the United States. In the subsequent years, this leguminous plant grew well and spread throughout the north, east and northwest of China, and it played an important role in the development of the rural economy and ecological environment.

However, few studies have been reported concerning the diverse rhizobia associated with *C. varia* [21]. Although a novel strain, *Rhizobium yanglingense*, was isolated and characterized from the root nodules of *C. varia* grown in Shannxi, Yangling, China by Tan et al. [32], the phylogenetic status of the *C. varia* rhziobia is still not clear or, in fact, how the mechanism of *C. varia*, as an exotic plant, adjusts itself to a new environment and specifically finds compatible symbionts. Therefore, this study focused on: (1) the genetic diversity and phylogenetic status of the rhizobial population associated with *C. varia*, (2) the evolution mechanism of its symbiotic genes, and (3) the relationship between the diversity of rhizobia and associated soil factors.

## Materials and methods

### *Field sites and root nodule sampling*

Root nodules were collected from cultivated *C. varia* grown at six sites in the middle region of Shaanxi province (Table 1). From each site, at least 15 plants were chosen randomly, and a certain distance interval between individual plants was set. Healthy root nodules of individual plants were excised, maintained on ice and transported to the laboratory.

Soils were also collected from each sampling site, and soil cores were sampled from ten locations at a depth of 20 and 5 cm from the tap roots. They were thoroughly mixed to obtain composite samples after being dried. Soil physicochemical characteristics such as pH, the organic matter content, total N, alkali-hydrolyzable

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**Table 1**
Geographic origins and genomic type of rhizobia isolated from root nodules of *C. varia*.

| Strain | rRNA type[a] | *nodC* type[a] | *nifH* type[a] | Sampling sites |
|---|---|---|---|---|
| CCNWSX582,583,584,585,586,588,589,590,591,593,594,596,597,598,599 | A(aaaa) | A(aaaa) | A(aaaa) | I Xianyang, Shaanxi 108°42′22.66″E 34°18′34.23″N |
| CCNWSX601,602,604,605,606,607,608,609,610,611,612,613,614,617 | A | A | A | II Caijiapo,Shaanxi 107°35′56.93″E 34°18′28.02″N |
| CCNWSX615 | E(ebbb) | — | — | |
| CCNWSX618,619,620,621,622,623,625,626,628,629 | A | A | A | III Meixian, Shaanxi 107°42′15.46″E 34°12′33.66″N |
| CCNWSX634,644 | E | — | A | IV Xingping, Shaanxi 108°30′06.46″E |
| CCNWSX635,636,638,639,641,642,645 | A | A | A | |
| CCNWSX637 | E | — | C(accb) | |
| CCNWSX646,652,653,654,655 | B(baac) | B(babb) | B(bbba) | |
| CCNWSX649 | E | — | — | |
| CCNWSX650 | D(cabb) | D(cbaa) | C | |
| CCNWSX651 | C(daad) | B | B | |
| CCNWSX647,656,657,659,660,664,667 | C | B | B | V Zhouzhi,Shaanxi 108°04′52.82″E 34°09′40.96″N |
| CCNWSX648 | A | A | A | |
| CCNWSX658,663,668,669,670 | D | D | C | |
| CCNWSX661,665,666 | B | B | B | |
| CCNWSX662 | E | — | — | |
| CCNWSX672, 673 | F(aaae) | E(dcdd) | D(aada) | VI Yangling, Shaanxi 108°04′31.34″E 34°16′52.53″N |
| CCNWSX674,675,678,679,689,690,692 | A | A | A | |
| CCNWSX680,681,685,686,687,691 | A | C(aaae) | A | |

[a] The rRNA, nodC and nifH types marked with capital letters were defined based on the RFLP patterns (marked with small letters in parentheses) corresponding to each of the amplified genes restricted separately with the endonucleases HinfI, MspI, HaeIII and HhaI. Identical patterns are designated by the same letter.

nitrogen, total P, soluble phosphor, total K and soluble potassium were analyzed.

*Rhizobial isolation and plant nodulation tests*

A total of 2–3 fresh, full and healthy root nodules were picked from each plant. Nodules were surface sterilized with 95% alcohol for 30 s and 0.1% $HgCl_2$ for 5 min, then rinsed 6 times with sterile distilled water to thoroughly eliminate $HgCl_2$, and they were subsequently crushed and streaked with aseptic forceps onto yeast–mannitol agar (YMA) plates. Bacteria isolated from nodules were incubated and purified by repeatedly streaking on YMA medium incubated at 28 °C, and single colonies were picked and checked for purity by cellular morphology and microscopic examination. Only one single strain was isolated from each plant, and a total of 90 strains were obtained (Table 1). The nodulation abilities of the isolated strains were verified by plant inoculation assay, according to the protocols reported by Zhao et al. [41].

*Extraction of total DNA and RFLP-PCR*

All isolates were incubated at 28 °C in TY (tryptone–yeast) broth culture with shaking at 150 rpm. In the cell logarithmic growth phase, a 3 mL TY culture was centrifuged at 8000 g for 5 min and the pellet was washed three times by suspension in 1 mL 10 mM tris–HCl (pH 8.0) and centrifugation. Total genomic DNA was extracted from each of the rhizobial isolates following the protocol of Terefework et al. [34].

The 16S rRNA, *nodC* and *nifH* genes were amplified from the genomic DNA with the following primers, respectively: (1) 16S rRNA-P1 (5′-AGAGTTTGATCCTGGCTCAGAAC GAACGCT-3′) [33] and P6 (5′-TACGGCTACCTTGTTACGACTTCACCCC-3′), (2) *nodC*-nodCF (5′-AYGTHGTYGAYGACGGTTC-3′) and nodCI (5′-CGYGACAGCCANTCKCTATTG-3′) [25] and (3) *nifH*-nifHF (5′-TACGGNAARGGSGGNATCGGCAA-3′) and nifHI (5′-AGCATGTCYTCSAGYTCNTCCA-3′) [25]. The aliquot of the PCR products was digested separately with restriction endonucleases *Hha*I, *Hinf*I, *Hae*III and *Msp*I, according to the production guide. The restricted fragments were separated by electrophoresis in 2% (w/v) agarose gels at 75 V for 4 h and photographed under UV light after staining with 0.5 μg $mL^{-1}$ ethidium bromide for 15 min. The RFLP patterns obtained from the four endonucleases were combined for comparison, and strains with identical combined RFLP patterns were designated as a genotype.

*Sequencing and phylogenetic analysis of 16S rRNA, nodC and nifH genes*

Based on the PCR-RFLP profiles, the 16S rRNA, *nodC* and *nifH* genes of the strains representing different genotypes were directly sequenced from PCR products. The sequences obtained were blasted in the GenBank database to find those of related sequences. All the obtained and related sequences were aligned with Clustal X 1.81 software, and the similarity of each pair of sequences was computed with DNAMAN 6.0 software. The phylogenetic trees based on sequences of the three genes were constructed, respectively, by the neighbor-joining method in the MEGA 4.1 package.

*Diversity and correlation analysis*

To estimate the genetic diversity of the rhizobial population associated with *C. varia* grown in different geographic origins, four popular ecological indexes were used: the Richness index, Simpson index, Shannon–Wiener index and Pielou index. The Richness index represented the Richness index; the Simpson index ($D$): $D = 1 - \sum P_i^2$, with $P_i$ being the proportion of rhizobial strains of genotype i; the Shannon–Wiener index ($H'$): $H' = -\Sigma P_i \ln P_i$; and the Pielou index ($E$): $E = H'/H_{max}$, $H_{max} = \ln S$, where $S$ represented

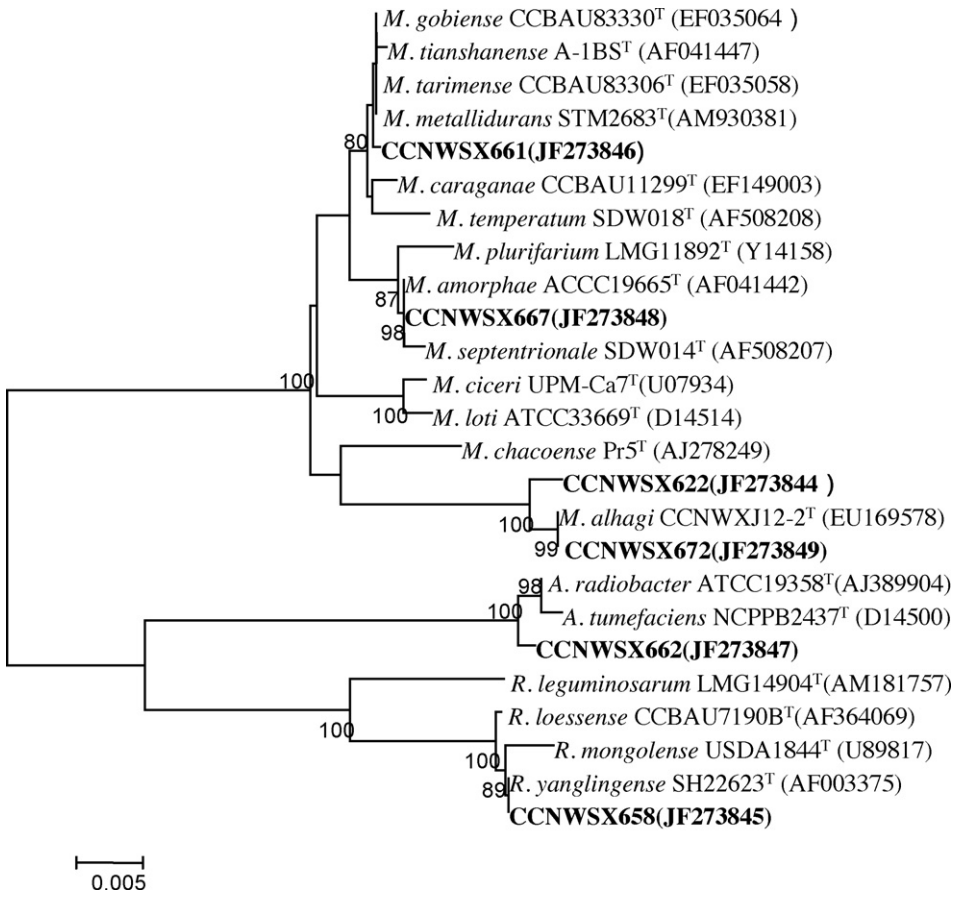


**Fig. 1.** Phylogenetic tree based on 16S rRNA gene sequences using the neighbor-joining method. The figures on the branches indicate the reliabilities. GenBank accession numbers are in parenthesis. The strains in boldface were used for 16S rRNA gene sequencing. The scale bar indicates the number of substitutions per site.

the Richness index [22,29]. Correlation analyses were conducted with the SPSS 12.0 package.

## Results

### *Plant nodulation tests*

The nodulation ability was confirmed for all the isolates by nodulation tests on their original host plant. Expect for CCNWSX615, 634, 637, 644, 649 and 662, all the other 84 isolates could form nodules on the roots of *C. varia*, which indicated that nodules were effective for nitrogen fixation.

An average of six nodules could be observed on each inoculated plant, and most of the nodules were located on the lateral roots, whereas a few were on both the taproots and lateral roots. The majority of nodules were spherical and short rod shaped, with a size of approximately 0.4 mm, and they were white or pink in color.

### *Analysis of 16S rRNA genes*

According to the RFLP fingerprints of the 16S rRNA genes, six different genotypes were obtained from the total of 90 isolates (Table 1). Genotype A, containing 60 isolates, was the most dominant of all the genotypes, and accounted for 66.7% of all the isolates examined. In addition, genotype A was isolated from all the sampling sites and was the unique genotype from sites I and III. On the other hand, genotype F contained two isolates distributed only at site VI and was the least dominant type. Furthermore, genotypes B, C and D were only isolated from sites IV and V.

The phylogenetic tree based on the alignment of nucleotide sequences of the 16S rRNA gene from representative strains and reference strains is shown in Fig. 1. The tree shows that all the representative strains were grouped into three clades represented by genera *Mesorhizobium*, *Rhizobium* and *Agrobacterium*. CCNWSX622, 661, 667, 658, 662 and 672 were the representative strains of genotypes A, B, C, D, E and F, respectively. Both CCNWSX622 and 672 were closest to *M. alhagi* CCNWXJ12-2$^T$, since they shared the highest similarity of 99.6% and 100%, respectively. However, CCNWSX622 was grouped into different sub-clades with *M. alhagi* CCNWXJ12-2$^T$. CCNWSX661, representing genotype B, formed a sub-clade at 99.9% similarity with several reference strains of *M. gobiense*, *M. tianshanense*, *M. tarimense* and *M. metallidurans*, and with both *M. metallidurans* STM2683$^T$ and *M. gobiense* CCBAU83330$^T$. The genotype C representative strain, CCNWSX667, was defined as *M. amorphae*, since it shared a similarity of 100% with the reference type strain *M. amorphae* ACCC19665$^T$. CCNWSX662, representing genotype E, formed a sub-clade with the reference strains of *Agrobacterium* sp. and was most closely related to *A. tumefaciens* NCPPB2437$^T$ at a similarity of 99.6%. CCNWSX658 represented genotype D and was grouped in the same clade with the reference strains of *Rhizobium* sp., which shared the highest similarity of 100% with *R. yanglingense* SH22623$^T$.

### *Analysis of nodC genes*

*NodC* genes were amplified from 84 of the 90 rhizobial isolates, and were represented by five different types (Table 1). Type A contained 54 isolates and accounted for 64.29% of all the isolates

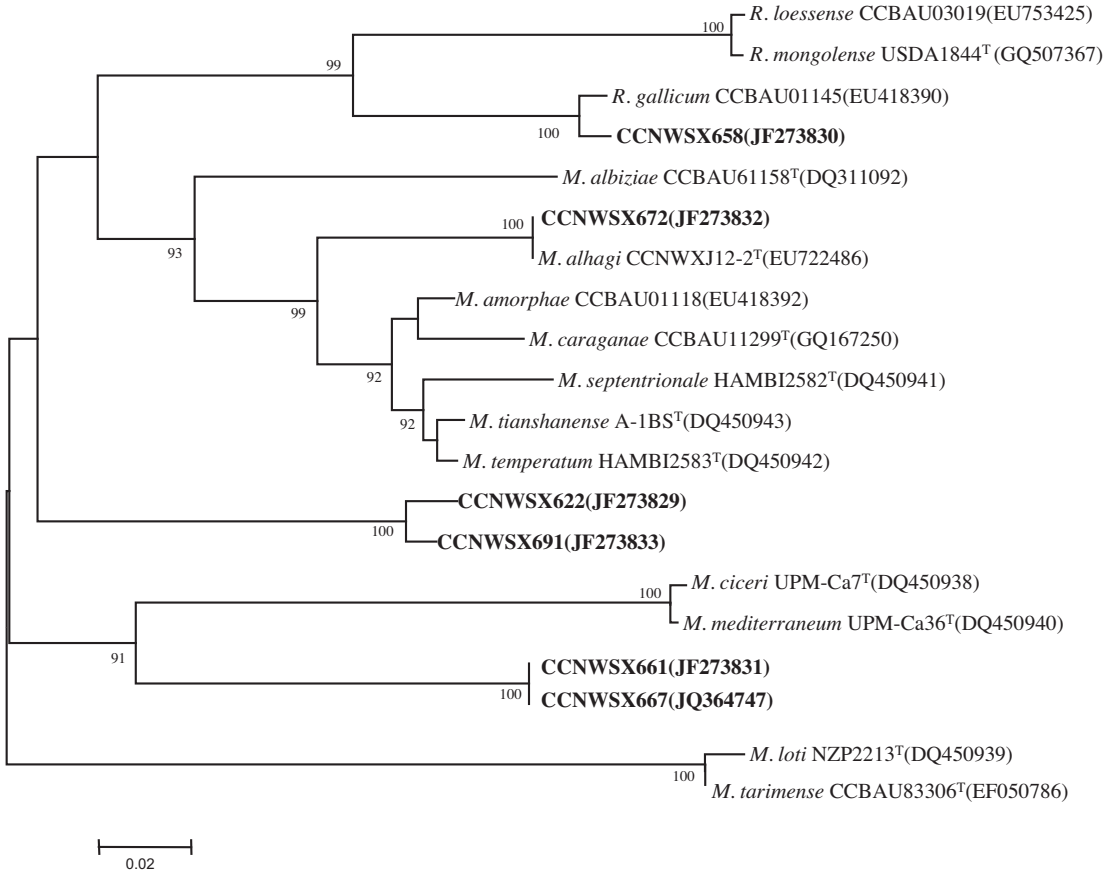


**Fig. 2.** Phylogenetic tree based on *nodC* gene sequences using the neighbor-joining method. The figures on the branches indicate the reliabilities. GenBank accession numbers are in parenthesis. The strains in boldface were used for *nodC* gene sequencing. The scale bar indicates the number of substitutions per site.

examined. Moreover, these isolates were found to be distributed in all six sampling sites. Type B contained 16 isolates and accounted for 19.05% of all the isolates, whereas types C and D each contained six isolates, and only two isolates belonged to type E.

The phylogenetic tree based on the alignment of nucleotide sequences of *nodC* from representative strains and reference strains is shown in Fig. 2. The tree shows that all the representative strains were divided into two groups represented by the genera *Mesorhizobium* and *Rhizobium*. The *Mesorhizobium* group, accounting for 92.86% of all the examined strains, contained four types, A, B, C and E, whereas the *Rhizobium* group contained only type D. CCNWSX622 and 691, representing types A and C, respectively, formed an independent clade with 98.8% similarity, but they differed greatly from other reference strains of the *Mesorhizobium* group. The type B representative strains CCNWSX661 and 667, belonging to the genus *Mesorhizobium*, were related to reference strains *M. mediterraneum* UPM-Ca36$^T$ and *M. ciceri* UPM-Ca7$^T$ of the same clade with approximately 81% similarity. CCNWSX658, the representative strain of type D, formed a sub-clade with *R. gallicum* CCBAU01145 at 99.0% similarity. CCNWSX672, representing type E, formed one sub-clade with *M. alhagi* CCNWXJ12-2$^T$ at 100% similarity.

### *Analysis of nifH genes*

*NifH* genes were amplified from 87 of the 90 rhizobial isolates and four different *nifH* types were obtained (Table 1). Type A, accounting for 71.26% of all the isolates examined, was the most dominant type, and all the 62 isolates it contained were distributed in all six sampling sites. Both types B and C, distributed in sites IV and V, contained 17 isolates, accounting for 19.54% and 6 isolates, respectively, whereas only two isolates belonged to type D.

Fig. 3 shows the phylogenetic tree constructed based on the alignment of *nifH* nucleotide sequences from representative strains and reference strains. In the *nifH* tree, all representative strains were grouped into the genera *Mesorhizobium*, *Agrobacterium* and *Rhizobium*. CCNWSX622 and 691, the representative strains of type A, formed a single clade with *A. tumefaciens* GZ12-1 at 99.0% similarity. Both CCNWSX667 and 661, representing type B, shared the same similarity (88.7%) with *M. ciceri* UPM-Ca7$^T$ and *M. mediterraneum* UPM-Ca36$^T$. The type C representative strain CCNWSX658 belonged to the *Rhizobium* clade and had the same similarity of 99.0% with *R. yanglingense* SH22623$^T$, *R. mongolense* USDA1844$^T$ and *R. loessense* CCBAU05026. The type D representative strain CCNWSX672 formed a single clade with *M. alhagi* CCNWXJ12-2$^T$ at 100% similarity.

### *16S rRNA gene analysis of C. varia rhizobia from different geographic origins*

As Table S1 shows, the diversity indexes of sampling sites IV and V had a relatively high level. The Richness indexes of these two sampling sites were both 5, whereas the Simpson index, Shannon–Wiener index and Pielous index for site IV showed the highest level, followed by site V. However, sampling sites I and III had the lowest diversity indexes, with a Richness index of 1, while the others were 0.

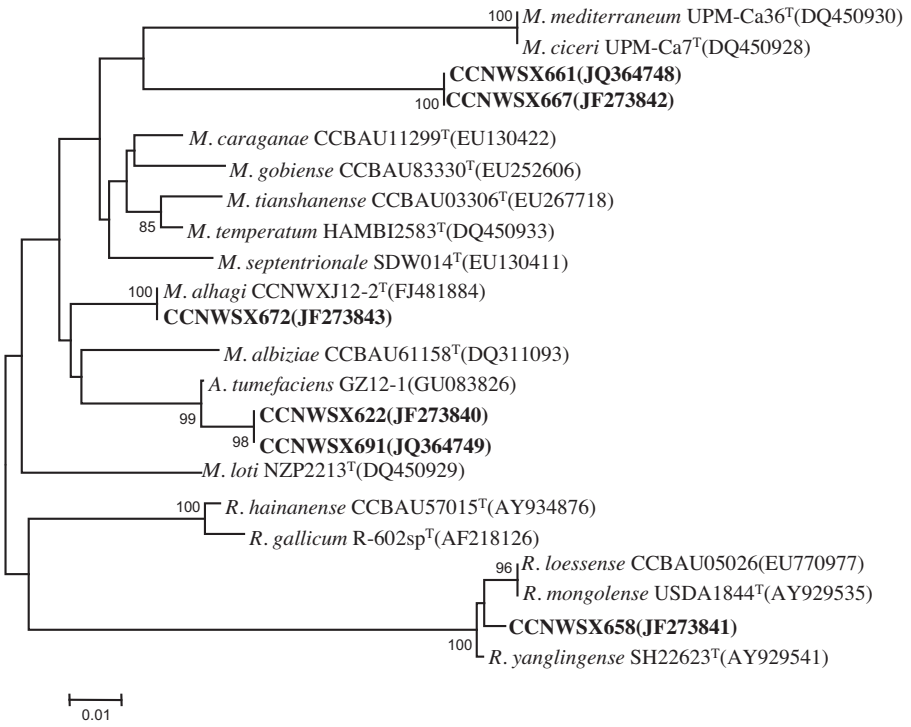


**Fig. 3.** Phylogenetic tree based on *nifH* gene sequences using the neighbor-joining method. The figures on the branches indicate the reliabilities. GenBank accession numbers are in parenthesis. The strains in boldface were used for *nifH* gene sequencing. The scale bar indicates the number of substitutions per site.

### *Correlation analysis between the genetic diversity of C. varia rhizobia and soil factors*

The physicochemical characteristics of soil from six sampling sites are shown in Table S2. The results of the correlation analysis showed that, although the soil pH, total P content and total K content were positively correlated with the diversity of 16S rRNA, the pH of the soil showed a significant correlation ($P < 0.05$). The diversity of the 16S rRNA gene, within a certain range, tended to be higher as the pH of the soil increased. However, the content of organic matter, total N, alkali–hydrolyzable nitrogen, soluble phosphorous and soluble potassium were all negatively correlated with the diversity of the 16S rRNA gene, although the correlation was not significant (Table S3).

## Discussion

### *Genetic diversity of C. varia rhizobia*

Based on 16S rDNA PCR-RFLP and nucleotide sequence analysis, a total of 90 isolates obtained from Shaanxi province were found to be distributed among the three genera *Mesorhizobium*, *Rhizobium* and *Agrobacterium*, including six different species of *Mesorhizobium* sp., *M. alhagi*, *M. morphae*, *M. metallidurans*/*M. gobiens*e, *R. yanglingense* and *A. tumefaciens*, which indicated a great genetic diversity. The results obtained provided the following information: (1) although *R. yanglingense* was the first rhizobial strain isolated from root nodules of *C. varia* in an earlier study [32], only six isolates were characterized in this study as *R. yanglingense*, which accounted for 6.67% of the isolates obtained. The *Mesorhizoium* group was the most dominant, since it contained 78 isolates that accounted for 86.7% of those obtained, (2) *Mesorhizoium* sp., represented by four species, was the dominant rhizobia group that could form symbiotic association with *C. varia*. The *Mesorhizoium* sp. group of genotype A, accounting for 66.7%, was the dominant species that was distributed among all six sampling sites, and was most closely related to *M. alhagi*. In addition, the species were divided into different sub-clades, indicating that this group of strains might represent novel species. However, multilocus sequencing and DNA–DNA hybridization would have to be carried out before confirming this conclusion. *M. alhagi* and *M. amorphae* were isolated for the first time from *Alhagi sparsifolia* [38] and *Amorpha fruticosa* [36], respectively, as reported in previous studies. Thus, the identification of rhizobial strains isolated from *C. varia* in this study as *M. alhagi* and *M. amorphae* expanded their host ranges.

*C. varia* grows well in the northwest of China, although many other plants are hardly able to survive because of drought stress and poor soil. The success of the legume plant may be attributed largely to its ability to form a nitrogen-fixing symbiosis with rhizobia [27,30], although one of the most critical factors is to find compatible rhizobia in new soil in order to form the symbiotic relationship [23,24]. Symbiotic promiscuity grants an advantage in colonizing new soils because promiscuous legumes are more likely to find compatible symbionts in new areas, whereas specific legumes are not [26]. In this study, carried out only in the middle regions of Shaanxi province, *C. varia* was shown to gain access to and form symbiotic relationships with various common species of rhizobia, such as *M. alhagi*, *M. amorphae*, and *R. yanglingense*. This relationship is also one of reasons why this symbiotic legume has a competitive advantage and can adapt better to these environments, and is widespread throughout many regions and countries around the world.

### *Transfer and evolution of symbiotic genes*

In the collection of rhizobia, the sequence analyses of symbiotic genes *nodC* and *nifH* did not correlate well with their corresponding 16S rRNA gene. In the 16S rRNA phylogenetic tree, genotype A had a high similarity with *M. alhagi*, whereas it was closest to *Agrobacterium* in the *nifH* tree. However, in the *nodC* tree, this group was divided into *nodC* types A and C, which formed a distinct branch

that showed a great difference with other reference strains of the genus *Mesorhizobium*. In addition, the strains in genotype B were closest to *M. metallidurans/M. gobiense*, whereas genotype C and *M. amorphae* were on the same branch in the 16S rRNA phylogenetic tree, and yet they belonged to the branch with *M. mediterraneum/M. ciceri* in both the *nodC* and *nifH* trees. This indicated that lateral gene transfer may have occurred occasionally between different rhizobial taxa for the symbiotic genes in the *C. varia* rhizobia, and similar results have also been reported in earlier studies [7,12,15]. Most researchers believe that gene transfer probably played an important role during evolution of symbiosis [5,15,18]. However, in the 16S rRNA phylogenetic tree, genotype D showed a similarity as high as 100% with *R. yanglingense*, although the lack of a *nodC* gene sequence for this representative strain did not allow us to conclude whether gene transfer had occurred for this gene.

The gene transfer existing in rhizobial strains, to some extent, is the driving force for genetic diversity, as well as adaptability [1,15,16]. The nod genes are unique to rhizobia, and the *nod*C gene, coding for N-acetylglucosaminyl transferase, has been widely used as a phylogenetic marker for studying the diversity and evolution of rhizobia [13,18]. From the phylogeny analysis of the *nodC* gene, the genus *Mesorhizobium* had four *nodC* types (A, B, C and E) that were distributed into three sub-groups. Except for type E that had 100% similarity with *M. alhagi*, types A, B and C, which represented the majority of strains, were found to be different from known local rhizobia. However, type B formed a single clade with *M. mediterraneum/M. ciceri* with a similarity of approximately 81%. *M. mediterraneum/M. ciceri* has been known to form a symbiotic relationship with *Cicer arietinum* and they have strong specificity [19,20]. In addition, *Coronilleae* and *Cicereae* are phylogenetically closely related and they are both from the same geographic origin in the Mediterranean region. Although such regions are a long way from China, the *nodC* genes were still on the same branch in this study. Therefore, it is assumed that these *nodC* genotypes might have developed in the Shaanxi rhizobial bacteria by lateral transfer when *C. varia* was introduced from its native region. Actually, this assumption has been confirmed by earlier studies on other plants such as *Robinia pseudoacacia* [2,37,39]. However, *nodC* genes have changed gradually and diversely in order to adapt better to a change of environment and different climate factors. In addition, there is a possibility that *nodC* types A, B and C finally evolved into type E carrying the same *nodC* gene as *M. alhagi*, although further studies would be needed to confirm this.

### *Soil pH and rhizobia diversity*

The rhizobia are soil microorganisms that can associate with legumes directly or indirectly. Since the soil system is complicated, the type of soil, temperature, humidity, pH, fertility and cultivation method can affect the distribution and diversity of the rhizobial population [14,22,28]. In this study, since the six sample sites were close to areas with similar soil types, temperature and cultivation methods, our attention focused on the relationships between rhizobia diversity and soil pH or fertility. Based on the experimental results, it was found that the diversity of the 16S rRNA gene was positively correlated with the pH of the soil (Table S3). Nevertheless, the mechanism of rhizobia diversity affected by soil pH is still not clear, and therefore it is assumed that there are several possible explanations: (1) soil pH can affect the survival and persistence of the rhizobial population. Brockwell et al. [6] reported nearly a $10^{-3}$ decrease in the number of *Sinorhizobium meliloti* in soils with a pH < 6.0 compared to those with a pH > 7.0, (2) soil pH can affect the molecular signal exchange between legumes and rhizobia. The exudation of flavonoid compounds from clover roots required for *nod* gene induction in *R. leguminosarum* bv. *trifolii* was reduced when the plants were grown at a pH < 5.0 [3]. In addition, low pH can affect the production and excretion of nodulation factors in strains of *R. leguminosarum* bv. *trifolii* [17] and (3) soil pH also has an influence on the nodulation competitiveness of rhizobia. The ratio of fast-growing and slow-growing rhizobia varies greatly under different soil pH conditions. The fast-growing rhizobial population is obviously dominant in alkaline soil [40], which has recently been confirmed also from the perspective of genomics [35].

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2012.10.004.

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