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ORIGINAL ARTICLE

Diversity and symbiotic divergence of endophytic and non-endophytic rhizobia of *Medicago sativa*

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Abstract

Knowledge of rhizobium diversity is helping to enable the utilization of rhizobial resources. To analyze the phenotypic and genetic diversity and the symbiotic divergence of rhizobia of *Medicago sativa*, 30 endophytic and non-endophytic isolates were collected from different parts of five alfalfa varieties in three geographic locations in Gansu, China. Numerical analyses based on 72 phenotypic properties and restriction fragment length polymorphism (RFLP) fingerprinting indicated the abundant phenotypic and genetic diversity of the tested strains. According to the phylogenetic analysis of 16S RNA, *atp*D, *gln*II, and *rec*A gene sequences, *Rhizobium* and *Ensifer* were further classified into four different genotypes: *Rhizobium radiobacter*, *Rhizobium* sp., *Rhizobium rosettiformans,* and *Ensifer meliloti*. The differences in architecture and functioning of the rhizobial genomes and, to a lesser extent, environment diversification helped explain the diversity of tested strains. The tested strains exhibited similar symbiotic feature when inoculated onto *M. sativa* cvs. Gannong Nos. 3 and 9 and Qingshui plants for the clustering feature of their parameter values. An obvious symbiotic divergence of rhizobial strains was observed in *M. sativa* cvs. Longzhong and WL168HQ plants because of the scattered parameter values. Their symbiotic divergence differed according to alfalfa varieties, which indicated that the sensitivity of different alfalfa varieties to rhizobial strains may differ. Most of the tested strains exhibited plant growth-promoting traits including phosphate solubilization and production of indole-3-acetic acid (IAA) when colonizing plant tissues and soil.

Keywords *Medicago sativa* · *Rhizobium* · Phenotypic diversity · Genetic diversity · Symbiotic divergence

Introduction

As an extensively planted forage species in arid and semi-arid areas, alfalfa (*Medicago sativa* L.) is characterized by its strong environmental adaptabilities and rich crude protein content (Benabderrahim et al. 2009). There is a symbiotic association between alfalfa and rhizobia in which the plant obtains nitrogen in the form of NH_4^+ transformed from atmospheric nitrogen in their root nodules induced by the rhizobia (Haag et al. 2013). And alfalfa provides environment and essential nutrients for the growth of rhizobia (Dudeja et al. 2011). Many studies have been done on rhizobium taxonomy. To date, 16 genera belonging to α-proteobacteria and four genera belonging to β-proteobacteria have been identified (Weir 2012; Dudeja et al. 2012; ICSP 2013; Berrada and Fikri-Benbrahim 2014).

The polyphasic taxonomy approach depending on phenotypic and genetic characteristics has been extensively adopted in rhizobium classification (Rai et al. 2012; Rasul et al. 2012; Rouhrazi and Khodakaramian 2015). The bacterial genus or species status can be achieved by 16S rRNA gene restriction fragment length polymorphism (RFLP)-PCR fingerprinting in combination with the analysis of several PCR products digested with endonuclease (Zhang 2012b). This approach has been recognized as a powerful and rapid method to determine the phylogenetic relationship of large numbers of legume root nodule isolates (Kalita and Malek 2010; Mierzwa et al. 2010; Shamseldin et al. 2013). The 16S rRNA sequence is highly conserved (Minoru and Doris 2015), and multilocus sequence typing (MLST) can provide highly robust data and

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meet the strict requirements for database construction (Rivas et al. 2009). In MLST, alleles of at least seven loci are identified by directly comparing nucleotide sequences rather than electrophoretic migration patterns of enzymes (Larsen et al. 2012). This method had been adopted in numerous rhizobium species taxonomy and is considered a significant improvement over others (Aserse et al. 2012, 2013; Zhang 2012b).

Compared with non-endogenous microorganisms, endophytes colonize inner plant tissues and have been detected in almost every plant studied (Cindy et al. 2002; Bacon and Hinton 2006). The bacterial endophytes isolated from nodules are found to be members of more than 129 species (belonging to 54 genera), among which *Bacillus*, *Pseudomonas*, *Agrobacterium*, and *Enterobacterium* are the most common dominant species (Samir et al. 2009). Some of them have the ability to promote growth (Senthilkumar et al. 2011; Tanuja and Mishra 2013; Beltrangarcia et al. 2014) as well as enhance abiotic (Miliute et al. 2015) and biotic (Pavlo et al. 2011) stress tolerance. There are previous studies concerning endophytic and non-endophytic rhizobium of legumes (Botha et al. 2004; Muresu et al. 2008; Deng et al. 2011; Aserse et al. 2012, 2013; Xu et al. 2014). We have known that soil type and plant genotype are the main factors affecting the diversity of rhizobial population (Leite et al. 2017). However, few reports are available regarding the diversity of endophytic bacteria colonizing inside the tissues (seed, root, stem, leaf, and flower) and non-endophytic ones obtained from soil of alfalfa varieties. The quantitative distribution (Li et al. 2009), migration dynamics (Zhang 2012a; Miao et al. 2017), stress tolerance (Qi 2006), and biodiversity (Qi 2004) of endophytic rhizobium isolated from seeds of alfalfa have been investigated. The relationship between the diversity of endophytic and non-endophytic rhizobia and their origins is still uncovered.

Diverse *Rhizobium* and *Ensifer* species were discovered to be symbionts of alfalfa, and they can form nitrogen-fixing nodules on most *M. sativa* varieties (Silva et al. 2007; Torres et al. 2016). In the current study, we identified and characterized these endophytic and non-endophytic strains. Since the signal factors such as flavonoids, which are produced by plant roots in response to nod factors, are the main plant determinants of host specificity (Cooper 2007). Here, we hypothesized that the symbiotic divergence of rhizobia on different *M. sativa* varieties differed according to plant varieties. Therefore, our primary goals in this study were as follows: (1) to investigate the phenotypic diversity of the endophytic and non-endophytic rhizobia using numerical analysis, (2) to investigate genetic diversity and taxonomic position using RFLP fingerprinting and analysis of partial 16S rRNA sequences, (3) to study in detail the phylogeny of *Rhizobium* and *Ensifer* strains using MLST of three protein coding housekeeping genes, (4) to screen bacterial strains for plant growth-promoting (PGP) activities, and (5) to test the symbiotic divergence of selected rhizobium isolates on five alfalfa varieties. This work may enable the exploration of rhizobia resources specific to alfalfa varieties and contribute to efficient development of rhizobia-alfalfa symbiosis.

Materials and methods

Site characteristics and sampling

Samples were collected from five alfalfa varieties growing naturally at one arid and two irrigated zones in Gansu, China, in May and August 2014 (Table 1). At the study sites, annual rainfall ranged from 158 to 320 mm, and annual mean air temperature ranged from 6.5 to 8.9 °C (Shi 2005). For each variety, healthy alfalfa plants were chosen randomly at the early blooming stage and uprooted along with the rhizospheric soils (Yao 2002). Field soils within 50 cm around an alfalfa plant to a depth of 20 cm were sampled. Alfalfa seeds were collected from uncut alfalfa plants in August 2014 and field-threshed manually. All the plants, soils, and seeds were sampled with five replications, placed in sterile plastic bags, labeled, and placed in an incubator containing ice packs and brought to the laboratory.

Isolation of bacterial strains

Alfalfa plant roots were first brushed to collect rhizospheric soils, then washed with sterile distilled water, dried, and divided into six parts: fresh nodules, leaves, stems, flowers, root epidermis, and root stele, using a scalpel. One gram of each tissue was placed in a sterilized flask and surface-sterilized by immersion in 0.45–0.55% (*w*/*v*) iodine disinfection for 3 min, then thoroughly rinsed with sterile distilled water five times and dried under aseptic conditions. Seeds were surface-sterilized by immersion in 0.45–0.55% (*w*/*v*) iodine for 2 min (Huo 2014).

To isolate the endophytic bacteria, surfaced-disinfected nodules were individually crushed, and the crushed material was streaked onto yeast mannitol agar (YMA) plates. All other sterilized tissues (1 g) were triturated with 2 mL of sterile distilled water using a mortar and pestle. As for the non-endophytic bacteria, soil suspensions were obtained by mixing 10 g of sifted rhizosphere and field soils into 90 mL of sterile distilled water followed by shaking for 1 h. The triturate and soil suspensions of 0.2 mL (5 min, centrifuged at 2000×*g*) were diluted to 10^{-4} (*w*/*v*) with sterile distilled water. Then, 0.2 mL of the 10^{-4} (*w*/*v*) dilutions were plated onto YMA plates, with four replications. After incubation at 28 °C for 5–7 days, individual colonies were selected and purified by repeated streaking on YMA plates. The strains were maintained on YMA plates and stored at 4 °C for further use and transferred monthly to new YMA plates to keep them

Table 1 Details of bacteria and sampled sites

Endophytic rhizobia	Non-endophytic rhizobia	Isolation spots on plant	Alfalfa variety	Site prospected	Geographical position (latitude, longitude, altitude)	Soil texture	Annual rainfall (mm)
G3G1, G3G2 G3P2		Stele Epidermis	*Medicago sativa* cv. Gannong No. 3	Wuwei, Gansu, China	E 102° 50′, N 37° 52′, 1650 m	Gray brown desert	158
G3L1, G3L2, G3L3		Nodule					
	G3T1, G3T2	Rhi-soil[a]					
	G9TT1, G9TT2, G9TT4, G9TT5	F-soil	*M. sativa* cv. Gannong No. 9				
LP3, LP4 LL1, LL2		Epidermis Nodule	*M. sativa* cv. Longzhong	Huining Gansu, China	E 105° 06′, N 34° 40′, 1760 m	Loess sandy loam	300
	LT2, LT3	Rhi-soil					
QL2		Nodule	*M. sativa* cv. Qingshui				
WLP2 WLL2, WLL3, WLL4, WLL5		Epidermis Nodule	*M. sativa* cv. WL168HQ	Lanzhou, Gansu, China	E 105° 41′, N 34° 05′, 1517.3 m	Loess loam	320
WLG1, WLG2		Stele					
WLN3		Seed					
WLJ3		Stem					
	WTT4, WTT6	F-soil					

[a] Rhi-soil for soil adhering to roots and f-soil for soil within 50 cm around the alfalfa trunks

viable. The morphological characteristics and Gram reaction of the strains were determined.

Numerical analysis of phenotypic characteristics

All isolates were phenotypically identified based on (1) utilization of individual carbon substrates (malic acid, inositol, creatine, mannitol, sucrose, glucose, D-fructose, lactose, succinic acid, fumaric acid) and nitrogen substrates (L-tryptophan, glycine, arginine, histidine, and phenylalanine); (2) tolerance to different concentrations (5, 50, 100, and 300 μg mL^{-1}) of antibiotics (erythromycin, kanamycin, gentamycin, chloramphenicol, streptomycin, neomycin, and ampicillin) and dyes (methyl red, methyl green, bromine thymol blue, bromophenol blue, Congo red, methylene blue, neutral red, malachite green, sodium nitrite, and bromine methyl green); (3) growth at varying salt concentrations (1, 2, 4, and 6% NaCl), pH (5, 9, and 11), and temperatures (8, 37, and 40 °C) as described by Zhang (2009); and (4) biochemical traits, including amylolysis, gelatin hydrolysis, bromothymol blue reaction (BTB), sulfureted hydrogen and 3-keto-lactose, Voges-Proskauer test, catalase activity and citrate utilization, which were assessed as described (Rai et al. 2012). Further, indole production was examined using the method of Egamberdieva and Kucharova (2009). All tests were carried out with three replications. Bacterial growth was assessed after 5–7 days of incubation at 28 °C, and only isolates showing growth in all replicates were considered to show positive reaction. For isolates that presented different results between replicates, the test was repeated to verify the characteristics.

The potential plant growth-promoting activity of the tested strains was evaluated by screening them for the phosphate solubilization and production of indole-3-acetic acid (IAA). The ability of phosphate solubilization was detected by dotting a single colony from each bacterial culture grown on YMA medium on solid Monkina organic (EYPC) and Pikovskaya's (PKO) inorganic medium, which were prepared as described by Qi (2006) and Li et al. (2011), respectively. The plates were incubated at 28 °C for 7–10 days and observed for clear solubilization halo around colonies. Each plate had four dots and each strain had three replications. IAA production was detected from bacterial cultures grown in YEM broth supplemented with 100 mg L^{-1} tryptophan as described by Egamberdieva and Kucharova (2009). The development of a pink color after 15 min of incubation of a mixture of bacterial supernatants and Salkowski reagent indicated the production of IAA.

Cluster analysis based on phenotypic characteristics was performed. For each isolate, phenotypic features were scored as 1 for a positive reaction and 0 for a negative one. Similarity matrices from the binary data were derived with the Similarity for Qualitative Data Program (SIMQUAL) in Numerical Taxonomy and Multivariate Analysis System for Personal Computer (NTSYS-PC) version 2.0 (Rohlf 1993). Estimates

for similarity were based on the Nei and Li (1979) correlation coefficient (Sneath and Sokal 1973). Matrices of similarity were analyzed using the unweighted pair group method with arithmetic averages clustering method (UPGMA).

Template DNA preparation

A single colony from each YMA plate was transferred to 50 mL of liquid YM medium and shaken at 150 rpm, 25 °C, for 12 h (Beringer 1974). Each proliferated bacterium in the YM culture medium was used to extract DNA following the manufacturer's instructions of Ezup column bacterial genomic DNA extraction kit (SK8256) (Sangon Biotech, Shanghai, China) following the manufacturer's instructions. The purity and concentration of the DNA samples were measured by spectrophotometry (Bio-Rad, Hercules, CA, USA).

PCR amplification

Primers P1 (5′-AGA GTT TGA TCC TGG CTC AGA ACG AAC GCT-3′) and P6 (5′-TAC GGC TAC CTT GTT ACG ACT TCA CCC C-3′) were used for PCR amplification of a 1400-bp segment of the 16S rRNA gene (Weisburg et al. 1991). Primers *atp*D 255F (5′-GCT SGG CCG CAT CMT SAA CGT C-3′) and *atp*D 782R (5′-GCC GAC ACT TCM GAA CCN GCC TG-3′) were used for amplifying the 475-bp fragment of *atp*D; primers *gln*II 12F (5′-YAA GCT CGA GTA CAT YTG GCT-3′) and *gln*II 689R (5′-TGC ATG CCS GAG CCG TTC CA-3′) were used for amplifying the 556-bp fragment of *gln*II; primers *rec*A 41F (5′-TTC GGC AAG GGM TCG RTS ATG-3′) and *rec*A 640R (5′-ACA TSA CRC CGA TCT TCA TGC-3′) were used for amplifying the 532-bp fragment of *rec*A (Vinuesa et al. 2005). All primers were synthesized by Sangon Biotech (Shanghai, China).

PCR amplification of 16S rRNA, *atp*D, *gln*II, and *rec*A genes were performed in 25-μL reaction mixtures containing 13 μL 2× Taq Master Mix (Dye Plus) (Vazyme Biotech, Nanjing, China), 1 μL of each primer, 2 μL of purified template DNA, and 8 μL of sterile ultrapure water. The temperature profile for amplification of 16S rRNA gene was as follows: initial denaturation at 95 °C for 5 min, 30 cycles of 30 s at 94 °C, 1 min at 58 °C, 2 min at 72 °C; final extension at 72 °C for 10 min; and holding at 4 °C (Weisburg et al. 1991). For PCR amplification of three housekeeping genes, *atp*D, *gln*II, and *rec*A, the following temperature profile was adopted: initial denaturation at 95 °C for 5 min; 30 cycles of 1-min denaturation at 94 °C, 1 min annealing at 56 °C (for *rec*A and *atp*D) or 58 °C (for *gln*II), and 1-min extension at 72 °C; final extension is at 72 °C for 1 min; and hold at 4 °C (Vinuesa et al. 2005). PCR products were electrophoresed on 0.8% agarose gel (Sigma) in Tris-acetate-EDTA (TAE) at 100 V for 30 min, purified using the PureLink™ Quick kit (Invitrogen) per the manufacturer's instructions, and sequenced (with the primers described above) by Sangon Biotech (Shanghai, China).

16S rRNA gene RFLP-PCR analysis

16S rRNA RFLP-PCR-amplified products (3 μL) were then digested with 5 U *Alu*I, *Hae*III, *Hinf*I, and *Msp*I endonucleases; 1 μL buffer solution at 37 °C for 12 h; with ddH_2O added to 10 μL. The enzyme-digested restriction fragments were electrophoretically separated in 3% agarose gels in TAE at 200 V for 40 min (Wand et al. 1998). All isolates listed in Table 2 were used in PCR tests, and each reaction was repeated at least twice. Two fast-growing rhizobial reference strains were used: *Rhizobium ipomoeae* shin9-1^T and *Ensifer medicae* WSM419^T. They were obtained from the Key Laboratory of Grassland Ecosystems of the Ministry of Education, College of Grassland Science, Gansu Agricultural University, Lanzhou, China. The gel images were analyzed with the BioNumerics 7.0 software (Applied Maths, Kortrijk, Belgium). Cluster analysis based on genotypic fingerprinting was performed as described in numerical analysis of phenotypic characteristics. For each isolate, the presence or absence of each band in the results of the 16S rRNA RFLP-PCR were determined and designated 1 if present or 0 if absent.

Sequence analysis

The tested strains were identified based on 16S rRNA gene sequence similarities by searching on the EzBioCloud's Identify service website (https://www.ezbiocloud.net/) (Yoon et al. 2017). The 16S rRNA sequences for recognized bacterial (type) strains that showed high percentage identity to *M. sativa* isolates were retrieved from EzBioCloud. The phylogeny of the *Rhizobium* and *Ensifer* strains were further studied using *atp*D, *gln*II, and *rec*A gene sequences, by comparing them with closely related reference species and type strains retrieved from the NCBI Assembly Database (www.ncbi.nlm.nih.gov/assembly). Each of the 16S rRNA, *atp*D, *gln*II, and *rec*A gene sequences was aligned using Clustal W (Thompson et al. 1994) in MEGA version 6.0 (Tamura et al. 2011). Phylogenetic trees were generated using the neighbor-joining (NJ) method (Saitou and Nei 1987) based on Kimura's (1980) two-parameter model. The stability of relationships was assessed by performing bootstrap analysis of the NJ data based on 1000 replications (Felsenstein 1985).

Symbiotic efficiency of bacterial isolates

This study was performed at the College of Grassland Science, GSAU, in May 2015. Five varieties, *M. sativa* cv. Gannong No. 3, *M. sativa* cv. Gannong No. 9, *M. sativa* cv. Longzhong, *M. sativa* cv. Qingshui, and *M. sativa* cv.

Table 2 Characteristics distinguishing tested isolates

Distinctive characteristics	Groups[a] and their reactions					
	A (10)[b]	B (2)	C (8)	D (1)	E (9)	F (2)
Application of sole carbon and nitrogen source (1%)						
D-Fructose	+[c] (9)[d]	+	+(7)	+	+	+
L-tryptophan	+(6)	+(1)	+(4)	–	+(2)	–
Glycine	+	+	+(5)	–	+(1)	–
Arginine	+	+	+	–	+(4)	+
L-Histidine	+	+	+	–	+(3)	+
Phenylalanine	+	+	+(7)	–	+(2)	–
Tolerance to dyes (1%)						
Bromothymol blue	+	+	+	+	+	+(1)
Methyl red	+(8)	+(1)	+	+	+	+(1)
Bromphenol blue	+(9)	+	+(6)	+	+	+
Methylene blue	+	+	+(4)	–	+(6)	–
Malachite green	+	+	+	+	+(7)	+
Tolerance to NaCl (%), pH, temperature (°C)						
NaCl (6%)	+(5)	+	+(2)	–	+(1)	+
pH 11.0	+	+	+(5)	+	+	–
8 °C	+(9)	+	+(1)	–	+(1)	–
37 °C	+	+	+	–	+	+
40 °C	+	+(1)	+	–	+	+
Tolerance to antibiotics						
Erythromycin [b][e]	+	+	+	–	+(8)	–
Erythromycin [c]	+(9)	+	+(2)	–	+(7)	–
Erythromycin [d]	+(3)	+	–	–	+(1)	–
Chloromycetin [d]	+(9)	+	+	–	+	+(1)
Kanamycin [a]	+(9)	+	+	+	+	–
Kanamycin [b]	+(3)	–	+(1)	–	+	–
Tolerance to antibiotics						
Kanamycin [c]	+(4)	+	–	–	+(7)	–
Kanamycin [d]	+(4)	+(1)	–	–	+(8)	–
Ampicillin [b]	+	+	+(7)	+	+	–
Ampicillin [c]	+(9)	+	+(4)	+	+	–
Ampicillin [d]	+	+	+(7)	+	+	–
Neomycin [a]	+	+	+	+	+	+(1)
Neomycin [b]	+	+(1)	+(1)	+	+	–
Neomycin [c]	+(9)	+	+(4)	+	+	+(1)
Neomycin [d]	+(7)	+	+(3)	+	+	+(1)
Streptomycin [a]	+	+	+(6)	+	+	+
Streptomycin [b]	+(6)	+	+(3)	+	+(6)	+
Streptomycin [c]	+(9)	+	+(7)	+	+	+
Streptomycin [d]	+(9)	+	+(6)	+	+	+(1)
Gentamycin [a]	+(9)	+	+(7)	+	+	–
Gentamycin [b]	+(4)	+	–	–	+	–
Gentamycin [c]	–	+	–	–	+	–
Gentamycin [d]	–	+(1)	–	–	+(6)	–
Others						
Amylolysis	+	–	+(3)	+	+(7)	+
Gelatin hydrolysis	+(8)	–	+(6)	–	+(4)	+(1)
Voges-Proskauer test	+(5)	–	–	–	–	–
Indole production	+(3)	–	+(5)	–	+(4)	–
BTB acid production	+	+(1)	+	+	+	–
Citrate utilization	+	–	+(3)	–	+(2)	–
3-Keto-lactose production	+(1)	–	+(2)	–	+(1)	–
Phosphate solubilization	+	+	+(6)	–	+(2)	–
Indole-3-acetic acid (IAA) production	+(6)	–	+(3)	+	+(3)	–

[a] Cluster analysis of phenotypic features identified six groups (A–F) at 73% similarity

[b] Number of isolates in the group

[c] a–d mean the concentration of antibiotics is 5, 50, 100, and 300 μg mL^{-1} , respectively

[d] + Strains were positive, − strains were negative;

[e] Number of reactions in which strains were positive

WL168HQ, were tested. The symbiotic efficiency of rhizobium isolates was determined by inoculating seedlings of the corresponding *M. sativa* varieties grown on sterilized sand (sieved with a 2-mm sieve, pH 7, autoclaved at 121 °C for 26 min) in a 40 × 250 mm glass test tube which was plugged with a tampon wrapped in gauze. Seeds were surface-sterilized using the methods described above and germinated on 0.8% (*w*/*v*) water agar at 28 °C for 24 h before being transplanted into a glass test tube loaded with 200 g of sterilized sand under aseptic conditions. Each glass tube contained seven germinated seeds. Each treatment was prepared in triplicate, and three tubes without inoculated bacteria served as controls. Plants were transferred to an illuminated incubator and cultivated under the conditions described by Li et al. (2009) and fed with 4 mL of sterilized Hoagland nutrient solution on the seventh day (Hoagland and Arnon 1950). Rhizobial suspension (10^9 cells mL^{-1}) of each isolate was prepared using the method of Li et al. (2009). When the first true leaves of alfalfa seedlings emerged, each glass test tube was inoculated with 4 mL of rhizobial suspension (Li et al. 2009), and then fed with sterilized Hoagland N-free nutrient solution once a week (Huo 2014). The tampon was not removed until the plant height exceeded that of the test tube.

Thirty days after inoculation, the plants were harvested for nodule number, effective nodule weight, nodule diameter (Miao et al. 2017), nodule grade (Li et al. 2010), nodule nitrogenase activity (Zaied et al. 2009), compound leaf number, shoot height, root length, shoot and root fresh weight, and dried at 65 °C for 72 h in a stove to determine shoot and root dry weight (Sánchez et al. 2014), chlorophyll content (Miao et al. 2017), and crude protein content (Shetta and Alshahrani 2016).

Statistical analyses

In the strain efficiency tests, a completely randomized design model was applied to analyze all parameters. Statistical analysis was performed using one-way ANOVA by Duncan's multiple range test at 5% probability with SPSS 19.0 (SPSS Inc.) (Boukhatem et al. 2012). Given the strong correlations between several parameters, a principal component analysis (PCA) of standardized values of these parameters was conducted using SPSS 19.0 to identify the primary axes of covariation (Tang et al. 2016).

Results

Origins of bacteria

Referring to Berger's bacterial identification manual and rhizobium morphological properties, of 78 bacterial isolates purified, only 30 were selected (Table 1). There were 20 endophytic bacteria and 10 non-endophytic bacteria isolated from the rhizosphere and field soils. Endophytic bacteria were isolated from root epidermis (four), nodule (ten), root stele (four), stem (one), and seed (one). No endophytic rhizobia were isolated from foliage and flower. All bacterial cultures on YMA medium produced round, smooth, pearl-white, mucous, convex colonies that are typical for root nodule bacteria. After 24 h of incubation, the diameter of the colonies ranged from 4 to 6 mm. The bacteria cells were motile rods and Gram-negative.

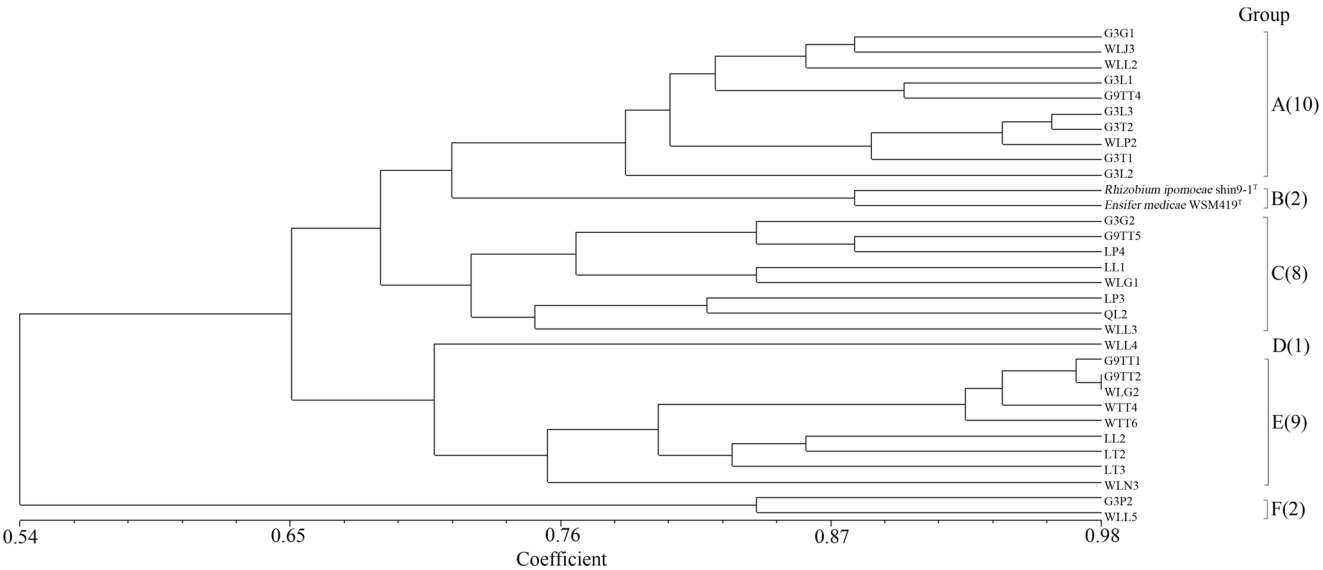

Fig. 1 Dendrogram showing the phenotypic similarities among the alfalfa isolates and the reference strains of *Rhizobium ipomoeae* shin9-1^T and *Ensifer medicae* WSM419^T. The unweighted pair group method with arithmetic averages (UPGMA) method was used for the cluster analysis

Numerical taxonomy analysis of phenotypic characteristics

A total of 32 bacterial strains comprising 30 *M. sativa* isolates and two reference strains (*R. ipomoeae* shin9-1^T and *E. medicae* WSM419^T) were analyzed for their phenotypic properties. The resulting dendrogram of the cluster analysis showed six main groups with a 73% similarity (Fig. 1). Groups A and C–E comprised a total of 28 isolates, group B consisted of the two reference strains, and group F comprised two isolates (G3P2 and WLL5), which were clearly separated from the other isolates. All the isolates shared 26 phenotypic properties; 48 distinctive phenotypic characteristics are listed in Table 2. In group A, ten isolates shared 20 positive characters and two negative tests. Two reference isolates in group B shared 33 positive and 8 negative characteristics. The eight isolates in group C shared 12 positive and 7 negative characteristics. Isolate WLL4 in group D displayed 22 positive and 26 negative characteristics. In group E, the nine isolates were positive in 24 characteristics and negative in one test. Finally, the isolates in group F: G3P2 and WLL5 displayed 12 positive and 28 negative characteristics. The isolates in each group presented different properties. Isolates in groups A, C, and E presented 26, 29, and 23 distinctive characteristics, respectively. Isolates in group B had seven distinctive characteristics, and those in group F had eight distinctive properties.

The majority of isolates (17/23) exhibited the traits of at least two plant growth regulators (PGRs) (Table 3). Phosphate solubilization was the most common PGR property, with 20/23 and 13/23 being capable of organic and inorganic phosphate solubilization, respectively (Table 3). Isolates LL2 and WLL3 displayed the strongest and weakest ability to solubilize organic phosphate, respectively, with a halo ratio (*D*:*d*) reaching 1.73 and 1.01, respectively. These two isolates, along with LL1, WLG1, LT3, LP4, and G9TT5 solubilized organic phosphate but not inorganic phosphate. In the isolates that could solubilize inorganic phosphate, WLP2 had the highest *D*:*d* of 1.76. Significant differences were observed in the inorganic phosphate solubilization *D*:*d* of isolates WLP2 and G3G2 and that of the reference strain *R. ipomoeae* shin9-1^T. IAA production was a less common PGR activity among the tested isolates (14/23). High IAA production was observed in two *Ensifer* isolates (G3L3 and WLP2) and seven *Rhizobium* isolates (G3G2, G3T1, G9TT4, G9TT5, LP4, LT3, and WTT1). Two other *Ensifer* isolates (G3T2 and LL2) presented moderate productions of IAA. *Ensifer* isolate G3L2, and two *Rhizobium* isolates (WLL4 and WTT6) displayed low production of IAA.

RFLP analysis of the 16S rRNA gene

To elucidate the phylogeny and taxonomy position of the 30 *M. sativa* isolates, fingerprinting of 16S rRNA was done by RFLP with the individual application of four nucleases (*Alu*I, *Hae*III, *Hinf*I, and *Msp*I). The resulting 16S rRNA patterns were analyzed. *Msp*I was the most discriminating restriction enzyme for these bacteria and was able to differentiate 15 restriction types (Table 4). *Hae*III and *Hinf*I produced 12 and 11 DNA profiles, respectively. *Alu*I produced the fewest restriction types ($n = 4$). The 16S rRNA-RFLP patterns of the tested isolates were compared with those of *E. medicae* WSM419^T and *R. ipomoeae* shin9-1^T. The 16S rRNA-RFLP profiles of the reference strains obtained in two independent

Table 3 IAA production and phosphate solubilization capacity of different strains

Strain	Organic phosphate solubilization halo ratio (*D*:*d*)[a]	Inorganic phosphate solubilization halo ratio (*D*:*d*)	IAA production
G3G1	1.42 ± 0.088ab[b]	1.46 ± 0.087ab	–[c]
G3G2	1.14 ± 0.103bc	0.89 ± 0.155b	+++
G3L1	1.39 ± 0.076ab	1.25 ± 0.037ab	–
G3L2	1.18 ± 0.020b	1.28 ± 0.065ab	+
G3L3	0.94 ± 0.136bc	1.28 ± 0.035ab	+++
G3T1	1.11 ± 0.080bc	1.21 ± 0.038ab	+++
G3T2	1.07 ± 0.003bc	1.22 ± 0.044ab	++
G9TT4	1.10 ± 0.021bc	1.36 ± 0.017ab	+++
G9TT5	1.06 ± 0.003bc	*N*[d]	+++
LL1	1.34 ± 0.030ab	*N*	–
LL2	1.73 ± 0.597a	*N*	++
LP4	1.06 ± 0.003bc	*N*	+++
LT3	1.07 ± 0.015bc	*N*	+++
WLG1	1.21 ± 0.012b	*N*	–
WLJ3	1.38 ± 0.112ab	1.25 ± 0.003ab	–
WLL2	1.26 ± 0.029b	1.32 ± 0.033ab	–
WLL3	0.67 ± 0.051c	*N*	–
WLL4	*N*	*N*	+
WLP2	1.07 ± 0.005bc	1.76 ± 0.582a	+++
WTT1	*N*	*N*	+++
WTT6	*N*	*N*	+
Rhizobium ipomoeae shin9-1^T	1.22 ± 0.028b	1.07 ± 0.384b	–
Ensifer medicae WSM419^T	1.20 ± 0.035b	1.20 ± 0.013ab	–

[a] *D* indicated the diameter of phosphate solubilization halo, *d* indicated the diameter of strain colony

[b] Mean ± standard errors were obtained from three replicates per strain tested. Mean ± standard errors followed by different letters in a same column are significantly different according to the Duncan multiple range test at $P = 0.05$

[c] – no IAA production activity, + low IAA production activity, ++ moderate IAA production activity, +++ high IAA production activity

[d] *N* indicated that the strains were incapable of solubilizing phosphate

Table 4 RFLP analysis of PCR-amplified 16S rRNA and concatenated housekeeping gene groups of 18 representative strains

Representative strain	Restriction types[a] of amplified 16S rRNAs digested with				16S rRNA RFLP patterns[b]	16S rRNA RFLP clusters[c]	Group[d] concatenated
	*Alu*I	*Hae*III	*Hinf*I	*Msp*I			
WLP2	A	E	a	l	I (7)	i	3
G3L3	A	F	b	m	II	i	3
LP3	A	F	b	n	III	i	3
WTT6	B	G	c	o	IV	ii	2
G3G1	D	I	e	q	VII	v	1a
G3G2	D	J	f	r	VII	iv	1a
G3L1	D	K	g	s	IX	v	1d
G3P2	D	L	h	t	X	iv	1d
G3T1	D	M	i	u	XI	v	1d
G9TT1	D	N	f	v	XII (5)	iv	1b
LT2	D	O	h	w	XIII	iv	1d
LT3	D	P	j	x	XIV	iv	1c
WG2	D	P	h	y	XV (5)	iv	1d
WL2	D	P	d	y	XVI	iv	1d
WN3	D	M	k	z	XVII	v	1d
WTT4	D	J	d	u	XVII	iv	1d
Rhizobium ipomoeae shin9-1^T	A	H	d	p	V	iii	—[e]
Ensifer medicae WSM419^T	C	E	a	l	VI	i	–

[a] Letters (A–J, a–z) refer to 16S rRNA RFLP pattern types of tested strains detected with each restriction enzyme

[b] Roman numerals refer to 16S rRNA RFLP patterns of tested strains identified by the combined analysis of 16S rRNA restriction profiles obtained with endonucleases *Alu*I, *Hae*III, *Hinf*I, and *Msp*I

[c] Cluster refer to the results gained from Fig. 2

[d] Group based on the phylogenetic tree of concatenated sequence of *atp*D, *gln*II, and *rec*A genes

[e] Reference type strains *Rhizobium ipomoeae* shin9-1^T and *Ensifer medicae* WSM419^T were not included in the concatenated analysis of *atp*D, *gln*II, and *rec*A gene sequences

experiments were highly similar (data not presented). Based on the combined RFLP patterns of 16S rRNA gene, 18 distinct 16S rRNA genotypes were distinguished (16 among the 30 *M. sativa* isolates and 2 in the reference species) (Table 4). Among the *M. sativa* isolates, the most widespread genotype was that designated as I, which comprised seven isolates. Two other genotypes (XII and XV) occurred less frequently in alfalfa symbionts (five isolates each). There were 15 genotypes specific to a single *M. sativa* isolate (Table 4). None showed the 16S rRNA restriction patterns identical to that of reference strains.

The 16S rRNA gene fingerprint profiles showed that all tested isolates were genetically distinct and diverse (Fig. 2). The strains were included in five RFLP groups with an 80% similarity level. Cluster i contained nine isolates and the reference strain *E. medicae* WSM419^T. Clusters ii and iii contained strain WTT6 and the reference strain *R. ipomoeae* shin9-1^T, respectively. Cluster iv included 16 isolates and were further subdivided into three subclusters at an 88% similarity level (Fig. 2). Cluster v consisted of four isolates.

Sequence analysis of the 16S rRNA gene

In order to clarify the taxonomic status and phylogenetic relations of the alfalfa isolates and other rhizobia, nearly full-length 16S rRNA genes (1400-bp long) of 18 representative bacteria in each 16S rRNA RFLP pattern were amplified by PCR and sequenced (Fig. 3). By the 16S rRNA partial sequence analysis, the 18 representative bacteria were included in three groups (1–3) related to *Rhizobium* ($n = 14$) and *Ensifer* (n = 4). Twelve isolates (WLL2, WTT4, WLG2, G3P2, WLN3, G3T1, G3G1, G3L1, G3G2, G9TT1, LT3, and LT2) were phylogenetically close to *Rhizobium radiobacter* ATCC 19358^T with 16S rRNA sequence similarities ranging from 89.73 to 98.72%. Strain WTT6 was closely related to *Rhizobium*

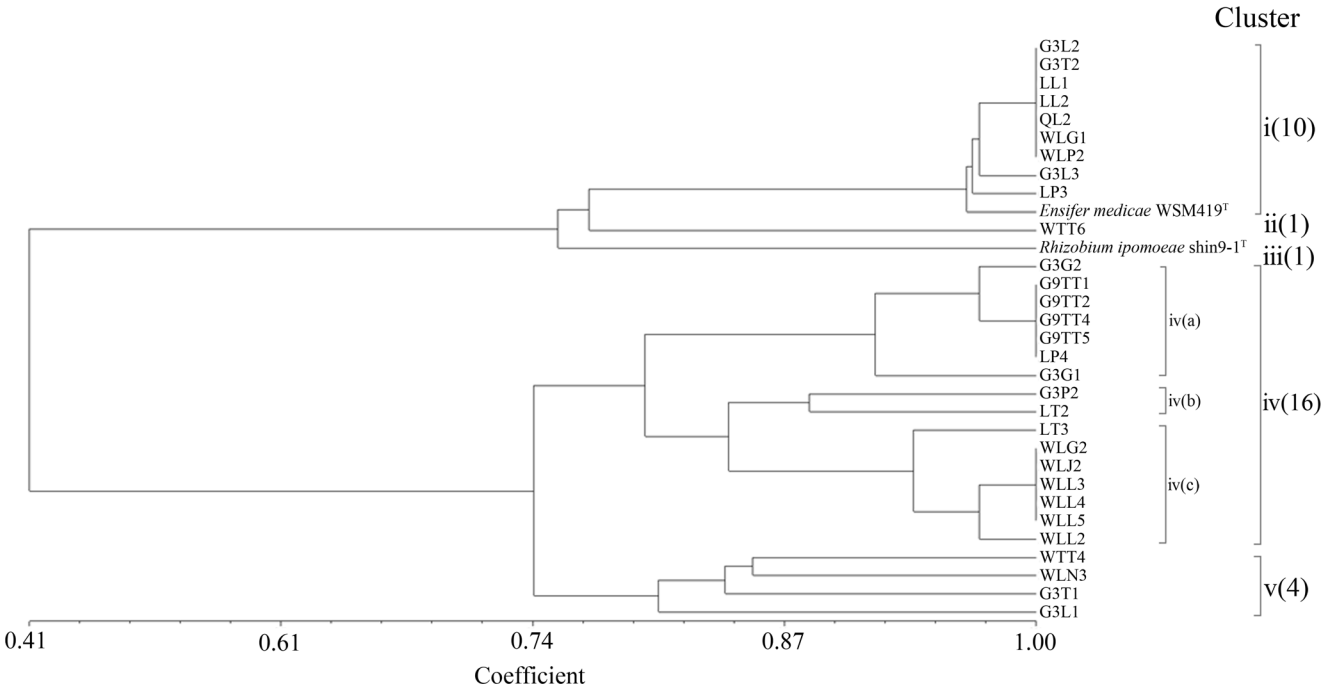

Fig. 2 Dendrogram based on the UPGMA cluster analysis of RFLP patterns of 16S rRNA showing phylogenetic relationship of *M. sativa* symbionts and reference strains

rosettiformans W3^T at a phylogenetical sequence similarity level of 97.1%. Strain WLP2 (98.42%), LP3 (97.26%), and G3L3 (96.1%) were related to *Ensifer meliloti* LMG 6133^T.

Concatenated sequence analysis of housekeeping genes *atp*D, *gln*II, and *rec*A

The phylogenetic tree of the concatenated sequences of *atp*D, *gln*II, and *rec*A housekeeping genes is shown in Fig. 4. The topology of the MLST tree was fundamentally congruent with the tree constructed with 16S rRNA gene sequences. Similar to the 16S rRNA gene tree, the 18 representative strains were consistently grouped (1–3) in the concatenated tree with high bootstrap support (92–100%) at each branch. The 12 *R. radiobacter* isolates were further assigned to four groups (1a–d). Thus, the tight relationships between the test strains G3G1 and G3G2 and *R. radiobacter* HAMBI 1814 in group 1a; strain G9TT1 and *R. radiobacter* CCBAU 73262 in group 1b; strain LT3 and *R. radiobacter* CCBAU 75221 in group 1c; strain WTT6 and *R. rosettiformans* W3^T in group 2; and strains LP3, WLP2, and G3L3 and *E. meliloti* LMG 6133^T in group 3 were confirmed by concatenated sequence analysis. The concatenated tree showed also the unique position of the eight strains (G3T1, WLN3, LT2, G3L1, WLL2, G3P2, WLG2, and WTT4) in group 1d, suggesting that the strains in this group might represent a new *Rhizobium* species.

Principal component and symbiotic divergence analyses

A principal component analysis (PCA) of 14 variables was used to gauge the correlations among the variables of the first two principal axes associated with the first two principal components (Fig. 5). PCA axis 1 accounted for 51.04% of the overall variance in the standardized variables, with axis 2 explaining 13.69% of the standardized variance. The standardized values of all 14 parameters were applied to create a scatter diagram with each color representing an alfalfa variety. The parameter values of *M. sativa* cvs. Gannong Nos. 3 and 9 and Qingshui plants inoculated with the 32 isolates clustered together (Fig. 5). Compared to the parameter values of *M. sativa* cv. Gannong No. 9, which gathered in the second quadrant, those of *M. sativa* cvs. Gannong No. 3 and Qingshui plants mainly clustered in the third quadrant. The parameter values of *M. sativa* cvs. Longzhong and WL168HQ plants predominantly scattered in the first and fourth quadrants. This indicated a small degree of symbiotic divergence of the isolates in *M. sativa* cvs. Gannong Nos. 3 and 9 and Qingshui. The symbiotic efficiency of the isolates in *M. sativa* cvs. Longzhong and WL168HQ diverged to a large extent.

Discussion

In this study, bacterial isolates were obtained from nine plant parts of five *M. sativa* varieties in three different ecological

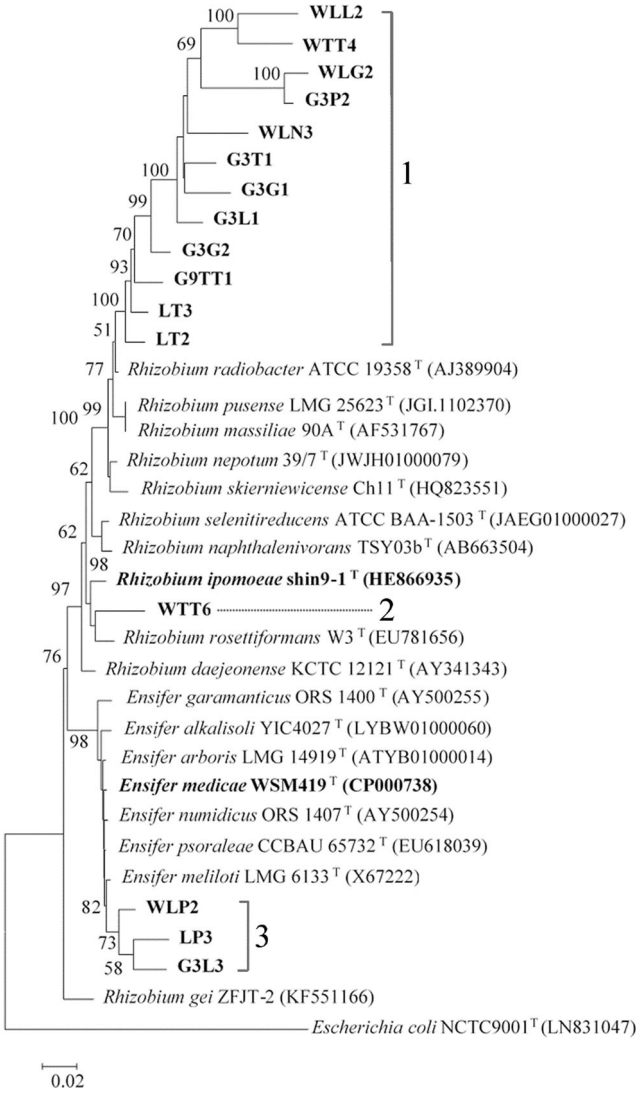

Fig. 3 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of 18 representative strains (in bold) and related taxa. Reference type strains are indicated with superscripted T. Numbers at nodes indicate bootstrap percentages (based on 1000 resampled datasets). Only node supports higher than 50% are shown. The length unit of 0.02 indicates 2% of the genetic distance

areas in Gansu, China. Based on the 16S rRNA gene sequence, 30 bacterial isolates (38%) were assigned to the genera *Rhizobium* and *Ensifer*. Isolates from the nodule, epidermis, stele, stem, and seed accounted for 33, 13.3, 13.3, 3.3, and 3.3% of the total, respectively. The low success rate of endophytic rhizobia isolation could be explained by the very low density of nodulating bacteria in wild alfalfa plants (Zhang 2012a). The composition of endogenous microbial populations depends mostly on plant and bacterial genotypes, as well as biotic and abiotic environmental factors (Miliute et al. 2015). Microenvironment divergences among distinct tissues in term of aeration, bio-enzymes, chemical components, structure, nutrient supply, and moisture, may also affect the presence of endogenous rhizobia in various ecological niches (Arnold 2007).

In our work, numerical taxonomy, 16S rRNA RFLP-PCR, 16S rRNA sequence, and MLST of *atp*D, *gln*II, and *rec*A were used in a combined manner in order to probe rhizobia diversities in the different tissues of alfalfa. Cluster analysis based on 72 variable phenotypic features clustered the isolates into five main groups (except group E in which two reference strains were included). Each group contained strains isolated from different geological areas, alfalfa varieties, and plant parts and reacted differently in some properties, indicating the abundant phenotypic diversity of the tested strains. 16S rRNA gene sequencing and MLST with *atp*D, *gln*II, and *rec*A were used successfully to classify *Bradyrhizobium* and *Ensifer* strains into different taxa (Zhang 2012b). Similarly, in this work, strains identified as *Rhizobium* and *Ensifer* with MLST were classified into three genomic species representing *R. radiobacter*, *Rhizobium* sp., *R. rosettiformans*, and *E. meliloti* (Figs. 3 and 4) (Wang et al. 2011). The presence of the new *Rhizobium* species (*Rhizobium* sp.) is probably because of the lack of some reference sequences for *atp*D, *gln*II, or *rec*A genes in the database. The genomic species were clustered in five RFLP clusters, confirming their distinct taxonomic positions. There were 3 16S rRNA RFLP patterns (I, II, and III) among the nine *E. meliloti* isolates, while 12 patterns were identified among the 20 *R. radiobacter* strains. This indicated that isolates in same genera were genetically diverse. Generally, the RFLP clusters depicted in Fig. 1 were formed according to the taxonomic affiliation of the strains, supporting previous reports (Aserse et al. 2012, 2013). Meanwhile, the RFLP clusters were formed inconsistent with the geological origin, alfalfa variety, or plant part, indicating that these factors might not determine the composition of the bacterial genomes.

The tested strains in individual phenotypic groups were clustered into different genetic groups in some cases, indicating that bacteria of the same phenotype differed in genotype and vice versa (Nandwani and Dudeja 2009). The diversity of rhizobial populations is affected by soil type and plant genotype, individual plants within a variety, nodulation variants of the same variety, and soil management regimes (Leite et al. 2017). Rhizobial communities originating from regions with an endemic legume presence can contain dozens of physiologically and genetically diverse strains (Depret and Laguerre 2008; Sachs et al. 2009; Wielbo et al. 2010, 2011). Consistent with prior reports (Kuklinsky-Sobral et al. 2004; Li et al. 2008), isolates in this study showed different abilities of organic and inorganic phosphate solubilization and IAA production. It is possible that environment diversification, such as soil versus an endosymbiotic environment, is one of the reasons for maintaining a considerable metabolic differentiation (Silva et al. 2007), and architecture and functioning of rhizobial genomes may account for intra-diversification of local

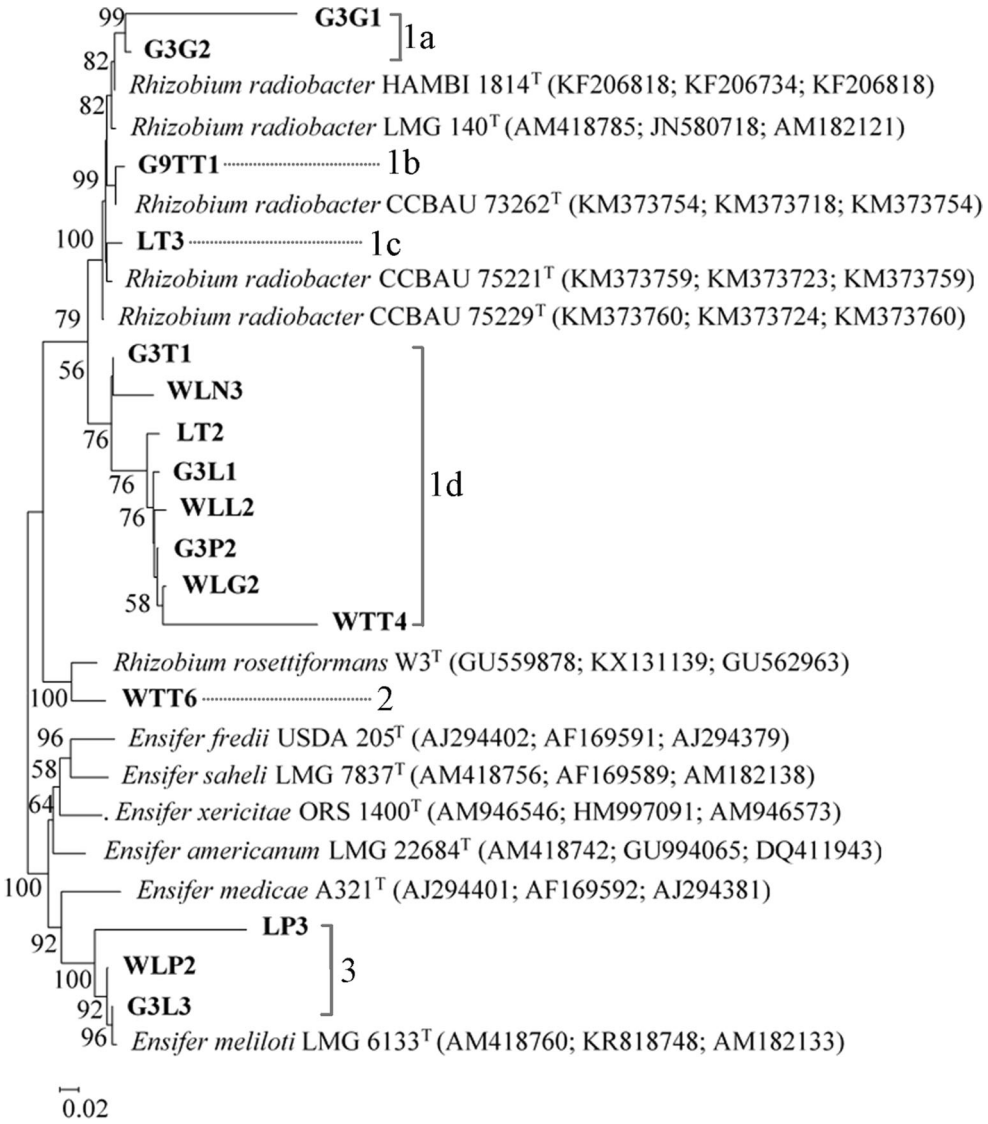

Fig. 4 Neighbor-joining phylogenetic tree based on concatenated sequences of *atp*D, *gln*II, and *rec*A genes showing the relationships of 18 representative strains (in bold) and related taxa. Reference type strains are indicated with superscripted T. Numbers at nodes indicate bootstrap percentages (based on 1000 resampled datasets). Only node supports higher than 50% are shown. The length unit of 0.02 indicates 2% of the genetic distance

rhizobial populations (Wielbo 2012). These possibilities could also explain why there was no direct link between the origin of the strain and its diversity.

The genetic diversity of rhizobia has symbiosis-related phenotypic consequences (Wielbo 2012). Even though the tested strains were diverse phenotypically and genetically, their symbiotic divergence was not obvious in all five alfalfa varieties. The tested isolates had similar symbiotic efficiency with *M. sativa* cvs. Gannong Nos. 3 and 9 and Qingshui for their clustering features. The symbiosis between the tested strains and these three alfalfa varieties showed a strong variety effect. This supported the hypothesis that rhizobial symbiotic efficiency differed according to alfalfa varieties, rather than tested strains. In contrast, the symbiotic efficiency of strains on *M. sativa* cvs. Longzhong and WL168HQ plants varied widely. Obvious differences were observed among symbiotic efficiency of strains. Leite et al. (2017) reported that for the rhizobia-host interaction, the plant genotype has a greater influence on the selection of the bacterial symbiont. Another study described significant symbiotic differences of strains in a group of plant genotypes susceptible to bacterial molecular signals (Wielbo 2012). There may be sufficient plant flavonoid-dependent recognition between strains and plants of *M. sativa* cvs. Longzhong and WL168HQ plants. Genetic analyses of *Rhizobium* populations isolated from different alfalfa varieties in this study provide reasonable support for a hypothesis of coevolution of *Rhizobium*-legume symbiosis, which occurs in centers of genetic diversification of plant hosts (Martínez-Romero 2009; Doyle 2011).

In this study, tested endophytic and non-endophytic rhizobia displayed extremely pronounced phenotypic and genetic diversities. There was no direct relationship between the origin of a strain and its diversity. The most plausible explanation for rhizobial diversity seems to be differences in architecture and functioning of rhizobial genomes and, to a lesser

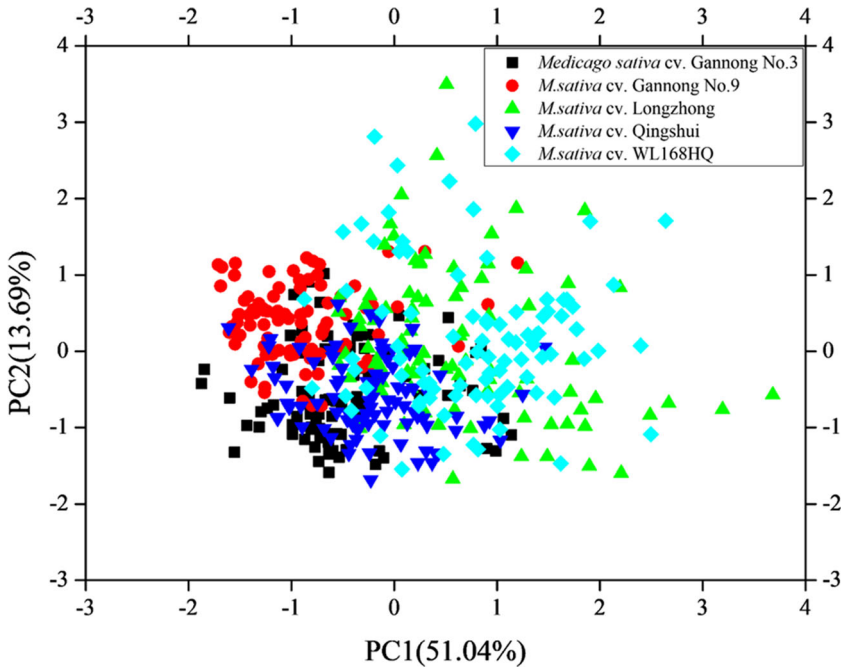

Fig. 5 Scatter diagram based on the standardized values of all 14 parameters. Parameters including nodule number, effective nodule weight, nodule diameter, nodule grade, nodule nitrogenase activity, compound leaf number, shoot height, root length, shoot and root fresh weight, shoot and root dry weight, chlorophyll content, and crude protein content of five alfalfa varieties plants were determined after 45 days of growth. All of the determined values were applied to conduct a principal component analysis (PCA)

extent, environment diversification. An obvious symbiotic divergence of rhizobial strains was observed in *M. sativa* cvs. Longzhong and WL168HQ plants. The sensitivity of different alfalfa varieties to rhizobial strains may differ. Most of the examined endophytes and non-endophytes have the potential for promoting plant growth. Thus, further testing is required to uncover the effects of isolation parts on the population composition, plant growth, and symbiotic efficiency of endophytic and non-endophytic *Rhizobium*.

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