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Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2018-0158.R3
Manuscript Type:	Article
Date Submitted by the Author:	18-Sep-2018
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Keyword:	<i>Medicago sativa</i> , Endophytic bacteria, Rhizobium, Genetic diversity, Symbiotic divergence
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

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Genetic diversity and symbiotic efficiency difference of endophytic rhizobia of *Medicago sativa*

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Abstract

Research on rhizobium diversity has paved the way for diversification of rhizobial germplasm resources. Seventy-three endophytic bacterial isolates were collected from seven tissues of five alfalfa varieties in three geographic locations in Gansu, China, and restriction fragment length polymorphism (RFLP) fingerprinting of 16S rRNA and analysis of concatenated sequence of three housekeeping genes *atpD*, *glnII* and *recA*, and symbiotic genes *nodC* and *nifH* were used for strain identification. Results showed that the endophytic strains were genetically diverse at different taxonomic levels, and *Ensifer meliloti* (31) and *Agrobacterium radiobacter* (12) are common *Medicago sativa* endophytic bacteria in Gansu, China. The *nifH* genes (97%–98% sequence identity) of *E. meliloti* strains were more diverse than their *nodC* genes (99%–100% sequence identity), although they evolved from a common ancestor. The dispersion degree of symbiotic phenotypes of *E. meliloti* strains on *M. sativa* cvs. Gannong No. 3, Gannong No. 9, and Qingshui was much less than that on *M. sativa* cvs. Longzhong and WL168HQ. This suggested that the symbiotic efficiency of *E. meliloti* strains on the former three alfalfa varieties was similar, and that on the later two was discrepant. Their symbiotic efficiency differed primarily according to alfalfa varieties, to a lesser extent, tested strains, indicating the difference of sensitivity of different alfalfa varieties to rhizobial strains.

Keywords

Medicago sativa; Endophytic bacteria; *Rhizobium*; 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). Rhizobia are beneficial soil bacteria, the symbiotic nitrogen fixation process between alfalfa and rhizobia promotes plant growth and enhances soil fertility (Liu et al. 2011; Haag et al. 2013). To date, 16 genera belonging to *Alphaproteobacteria* and four genera belonging to *Betaproteobacteria* have been identified (Weir 2010; Dudeja et al. 2012; Berrada and Fikri-Benbrahim 2014).

Plant-associated bacteria that inhabit the interior of plant tissues such as flowers, fruits, leaves, stems, roots, nodules, and seeds, without triggering damage to the plant, are known as endophytic bacteria (Cindy et al. 2002; Bacon and Hinton 2006). Endophytic bacteria have been obtained from nodules of a wide range of legumes species (Deng 2016; Zhong et al. 2017). These bacterial endophytes are found to be members of more than 129 species (belonging to 54 genera), among which *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Agrobacterium*, *Enterobacter* are the most common dominant genera (Suneja et al. 2016). Rhizobia can also colonize plant tissues, and there are previous studies concerning 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). There are also reports on endophytic rhizobium trapped from seeds (Qi 2004; Qi and Shi 2007), flowers, fruits, leaves, stems, and roots (Li et al. 2009; Frascella et al. 2012; Zhang 2012a; Miao et al. 2017) of alfalfa. However, few specific reports are available regarding the diversity and symbiotic divergence of endophytic bacteria colonizing the tissues (seed, root, stem, leaf and flower) of alfalfa varieties.

Endophytic bacteria have the ability to promote growth (Tanuja et al. 2013; Beltrangarcia et al. 2014) as well as enhance abiotic (Miliute et al. 2015) and biotic (Pavlo et al. 2011) stress tolerance. Some of them are also capable of producing plant growth-promoting phytohormones such as auxin phytohormone indoleacetic acid (IAA), and solubilizing mineral phosphate (Aserse et al. 2013). There are several endophytic bacteria that have been used as inoculants to promote nodulation, nitrogen fixation, and yield (Liu et al. 2010).

The polyphasic sorting approach depending on genetic characteristics has been extensively adopted in rhizobium taxonomy (López et al. 2009; Rai et al. 2012; Rasul et al. 2012; Rouhrazi et al. 2015). The bacterial genus or species status can be achieved by 16S rRNA gene 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; Vinay 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 meet the strict requirements for database construction (Rivas et al. 2009). In MLST, the nucleotide sequences of multiple loci are directly compared rather than comparing electrophoretic migration patterns of enzyme digestion products (Larsen et al. 2012). This method has been adopted in numerous studies on rhizobium species taxonomy and is considered a significant improvement over others (Aserse et al. 2012, 2013; Zhang 2012b).

Therefore, our primary goals in this study were as follows: (1) to investigate genetic diversity and taxonomic position of the endophytic bacteria using RFLP fingerprinting and analysis of partial 16S rRNA sequences; (2) to study in detail the phylogeny of *Agrobacterium* and *Ensifer* strains by using MLST of three protein coding housekeeping genes *atpD*, *glnII*, *recA*, and analysis of symbiotic genes *nifH* and *nodC*; (3) to screen bacterial strains for plant growth-promoting (PGP) activities; (4) to test the symbiotic divergence of selected endophytic rhizobium isolates on five alfalfa varieties.

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 °C to 8.9 °C (Shi 2005). For each variety, healthy alfalfa plants were chosen randomly at the early blooming stage. Alfalfa seeds were collected from uncut alfalfa plants in August 2014 and field-threshed manually. All the plants 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 endophytic bacterial strains

Alfalfa plant roots were firstly washed with sterile distilled water, then dried, and divided into six parts: fresh nodule, leaf, stem, flower, 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⁻¹) iodine disinfection for three min, then thoroughly rinsed with sterile distilled water five times and dried under aseptic condition. Seeds were surface-sterilized by immersion in 0.45–0.55% (w v⁻¹) iodine for two min (Huo 2014).

To isolate the endophytic bacteria, surfaced-disinfected nodules were individually crushed, and the crushed material was streaked onto YMA plates. All other sterilized tissues (1 g) were triturated with 2 mL of sterile distilled water using a mortar and pestle. The triturate of 0.2 mL (5 min, centrifuged at 2000 × g) was diluted to 10⁻⁴ (w v⁻¹) with sterile distilled water. Then, 0.2 mL of the 10⁻⁴ (w v⁻¹) dilution was 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 short-term use, and transferred monthly to new YMA plates to keep them viable. The morphological characteristics and Gram reaction of the strains were determined before their storage at –80 °C for long-term use in an automated Tube (Liconic Instruments, Lichtenstein) (Safronova and Tikhonovich 2012).

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). The purity and concentration of the DNA samples were measured by spectrophotometry (Bio-Rad, Hercules, CA, USA).

PCR amplification

The gene fragments of 16S rRNA, *atpD*, *glnII*, *recA*, *nifH* and *nodC* were amplified using Taq Master Mix (Dye Plus) (Vazyme Biotech, Nanjing, China) and sequenced as described (Mishra et al. 2012; Zhang 2012b). Primers used for amplification were obtained from the literature and synthesized by Sangon Biotech (Weisburg et al. 1991; Laguerre et al. 2001; Sarita et al. 2005; Vinuesa et al. 2005). The primers and PCR conditions are listed in Table 2.

16S rRNA gene RFLP-PCR analysis

16S rRNA RFLP-PCR-amplified products (3 µL) were then digested with 5U endonucleases (*AluI*, *HaeIII*, *HinfI* and *MspI*, respectively) and 1 µL buffer solution at 37 °C for 12 h, with ddH₂O added to 10 µL. The enzyme-digested restriction fragments were electrophoretically separated in 3% agarose gels in TAE at 200 V for 40 min. All isolates listed in Table 3 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).

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 97%–100% identity to *M. sativa* isolates were retrieved from EzBioCloud. The phylogeny of the *Agrobacterium* and *Ensifer* strains was further studied using *atpD*, *glnII*, *recA*, *nifH* and *nodC* 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, *atpD*, *glnII*, *recA*, *nodC* and *nifH* gene sequences was aligned using ClustalW (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 two-parameter model (1980). The stability of relationships was assessed by performing bootstrap analysis of the NJ data based on 1000 replications (Felsenstein 1985).

Phosphate solubilization and indole-3-acetic acid (IAA) production

The potential plant growth-promoting activity of the tested *Agrobacterium* and *Ensifer* strains was evaluated by screening them for the phosphate solubilization and production of 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 Shi et al. (2007) 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. One plate of four dots represented one replicate 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 Li et al. (2011). 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.

Symbiotic efficiency of bacterial isolates

This study was performed at the College of Grassland Science, GSAU, in May 2015. Five varieties, *M. sativa* cvs. Gannong No. 3, Gannong No. 9, Longzhong, Qingshui, and WL168HQ, were tested. The symbiotic efficiency of *Agrobacterium* and *Ensifer* 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⁻¹) 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 condition. 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 (10⁹ 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. 2011), nodule nitrogenase activity (Zaied et al. 2009), compound leaf number, shoot height, root length, shoot and root fresh weight, and chlorophyll content (Miao et al. 2017), and dried at 65 °C for 72 h in a stove to determine shoot and root dry weight (Sánchez et al. 2014).

Statistical analyses

Shannon diversity indexes were calculated as described by Ke et al. (2017). 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

Trapping of *M. sativa* bacteria from plant tissues

We trapped 73 endophytic bacterial isolates from seven plant tissues of five alfalfa varieties in three geological areas (Table 1). It is notable that trapping in flower (two), stem (two), seed (one), and leaf (zero) gave significantly less isolates compared with that in root nodule (41), epidermis (18) and stele (nine).

The 73 purified isolates were distributed into 27 ribotypes by 16S rRNA PCR-RFLP using four restriction enzymes. Assignment of each profile to a rhizobial species was then performed by sequencing of the partial 16S rRNA gene (1400 bp long) from a representative strain (see Table 4 and Fig. 1). A 16S rRNA gene phylogenetic tree (Fig. 1) was built to infer the evolutionary relationships among representative strains of our collection, including selected reference strains (*E. medicae* WSM419^T and *R. ipomoeae* shin9-1^T). These isolates were distributed into 14 species of 13 genera and all of them could be assigned to a described species based on the percent of 16S rRNA nucleotide identity (Table 3).

Distribution of species and ribotypes across plant tissues

The distribution of 16S rRNA PCR-RFLP types in plant tissues was presented in Table 4. A variable number of species were sampled depending on the different plant tissues. Root nodule hosted three species of *Medicago* rhizobia (*E. meliloti*, *A. radiobacter*, *P. catacumbae*) and five species of non-rhizobia (*P. soli*, *B. toyonensis*, *L. fusiformis*, *P. granadensis*, *A. pittii*). Epidermis and stele harbored two genotypes of *E. meliloti* and *A. radiobacter*, and three genotypes of *E. ludwigii*, *P. soli* and *L. fusiformis*. Besides, the former tissue hosted species *P. catacumbae*, *L. adecarboxylata* and *P. simiae*, and the latter one hosted species *V. paradoxus* and *E. mexicanum*. Flower (*L. fusiformis* and *E. gerundensis*) and stem (*A. radiobacter* and *P. granadensis*) harbored two genotypes, while seed (*A. radiobacter*) hosted only one.

Amongst all genotypes isolated, the most abundant was *E. meliloti* (31 strains), followed by *A. radiobacter*

(12), *L. fusiformis* (10), *P. soli* (six), *P. catacumbae* (two), *B. toyonensis* (two), *E. ludwigii* (two), and *P. granadensis* (two). The other genotypes each had only one isolate (Table 3 and 4). The ribotype *L. fusiformis* (NM1rt1) was isolated from the highest number of tissues (four). Ribotypes *E. meliloti* (AGal), *A. radiobacter* (DRhy) and *P. soli* (KJ1oq1) were isolated from three tissues. Genotypes *P. catacumbae* (QO1tv1), *E. ludwigii* (GH1ln1) and *P. granadensis* (ON1su1) were found for two isolates in two different tissues, with the QO1tv1 found in nodule and epidermis, the GH1ln1 in epidermis and stele, and the ON1su1 in nodule and stem, respectively. Other ribotypes were found only in one tissue, such as *E. meliloti* (AHbm, AGbl, AGba1, EGbl, FGbl), *A. radiobacter* (DMgs, DRdy), *B. toyonensis* (LK1pr1), and *A. pittii* (R1T1y1a2) in nodule, *E. meliloti* (AHbn), *A. radiobacter* (DNht, DPfv), *L. adecarboxylata* (ML1qs1), and *P. simiae* (UN1wp1) in epidermis, *A. radiobacter* (DKeq, DLfr), *V. paradoxus* (HI1np1), and *E. mexicanum* (S1U1z1b2) in stele, *E. gerundensis* (TQ1vx1) in flower, and *A. radiobacter* (DQkz) in seed (Table 4).

Shannon diversity indexes were calculated for each tissue and compared (excluding seed from which too few strains could be trapped). At the species level, root epidermis ($H=1.75$) and stele ($H=1.61$) were the most diverse, and root nodule ($H=1.05$), flower ($H=1.04$) and stem ($H=1.04$) were intermediate. The Shannon diversity indexes of strains in epidermis and stele were statistically different from that in nodule, flower and stem.

Intra-species variability of isolates

The intra-species variability of each species sampled could be assessed by the distribution across plant tissues of each 16S rRNA PCR-RFLP types (Table 4). For *E. meliloti* and *A. radiobacter* species, seven and eight PCR-RFLP types could be distinguished (EM1-EM7; RR1-RR8), respectively, with one type dominating the isolates (EM1 for *E. meliloti*, RR6 for *A. radiobacter*). Except for PCR-RFLP type EM1 and RR6, type EM2-EM7 and RR1-RR5, RR7, RR8 were found to be tissue specific. For *P. catacumbae* and 11 non-rhizobia species, only one RFLP type was found for each species (probably due to a low number of isolates of these species).

At the intra-species level, Shannon indexes were $H=2.20$ (stele), 1.99 (epidermis), 1.93 (nodule), 0.70 (flower) and 0.70 (stem). Diversity indexes in flower and stem were statistically different from that in other three plant tissues. *A. radiobacter* was the most diverse species ($H=1.81$), *E. meliloti* ($H=1.00$) followed. Endophytic bacteria populations in each tissue (epidermis, stele, nodule and flower) were compared on the basis of their

genetic composition using Chi-square.

Phylogeny of *Ensifer* and *Agrobacterium*: concatenated sequence analysis of three housekeeping genes

Based on the phylogenies of *atpD*, *glnII* and *recA* genes of representative strains (15) of each 16S PCR-RFLP type, the evolutionary relationship of housekeeping genes among the *Ensifer* and *Agrobacterium* was inferred. The length of the fragments was 475 bp for *atpD*, 556 bp for *glnII*, and 532 bp for *recA*. The topologies of the phylogenetic trees based on *atpD* (Fig. 2a), *glnII* (Fig. 2b), and *recA* (Fig. 2c) showed that the phylogenetic statuses of some isolates were unstable, and that their evolutionary branches differed between the three trees. Therefore, phylogenetic analysis of these individual housekeeping gene sequences was not sufficiently accurate to represent the taxonomic statuses of the tested isolates (Zhang 2012b).

A NJ phylogenetic tree was constructed based on the combined *atpD*, *glnII* and *recA* gene sequences (in total 1563 bp in length). Within the *Ensifer* lineage (Fig. 3), all isolates grouped with the type strain *E. meliloti* LMG 6133^T with sequence similarity >97%. These isolates included WLP2 and LP3 from root epidermis, and G9L4, LL7, G3L7, G3L3 and G3L4 from root nodule. Strains belonged to *Agrobacterium* lineage were closely related to *A. radiobacter* LMG 140^T (98%–100% sequence similarity) although dispersed in different clades. Isolates G3P2, WLG2, WLL2, and WLN3 clustered in a branch, which was sister to that comprising LP4, G3G1, G3G2, G3L1 and *A. radiobacter* LMG 140^T. These eight strains were isolated from root epidermis (G3P2 and LP4), stele (WLG2, G3G1 and G3G2), nodule (WLL2 and G3L1), and seed (WLN3).

Amplification of *nodC* and *nifH* gene fragments and analysis of their phylogenies

The fragments of *nodC* and *nifH* genes sized about 800 bp and 440 bp were amplified for all *Ensifer* (31) and *Agrobacterium* (12) isolates. We could not amplify *nodC* and *nifH* genes for the *Agrobacterium* isolates. To infer the origin and evolutionary relationship of symbiotic genes among the *Ensifer*, the phylogenetic trees based on *nodC* and *nifH* genes of representative strains of the seven 16S PCR-RFLP types were established (Fig. 4). All *nodC* gene sequences obtained from *Ensifer* were 99%–100% identical to each other. In the *nodC* gene tree (Fig. 4a), all *Ensifer* test strains formed an independent branch and were most closely related to *E. meliloti* CCBAU 75245^T (>99% sequence identity). Similar to the *nodC* gene tree, all strains in the *nifH* gene tree (Fig. 4b) clustered together with *E. meliloti* CCBAU 75245^T, with a 97%–98% sequence identity to one another.

Phosphate solubilization and IAA production of *Ensifer* and *Agrobacterium*

A certain number of isolates (13/43) exhibited the traits of at least two plant growth regulators (PGRs) (Table 5). IAA production was the most common PGR property, and 23/31 strains were capable of producing IAA. High IAA production was observed in four *Ensifer* isolates (G3L3, WLP2, G9L8 and G3L5) and two *Agrobacterium* isolates (G3G2 and LP4). Five *Ensifer* isolates (LL2, LL6, LL5, LL10 and G9L6) showed moderate production of IAA. *Ensifer* isolates G3L2, LL11, G3L12, QL5, G3L4, QL4, G3L10, G9L4, G3L6, G9L3, G3L8 and one *Agrobacterium* isolate (WLL3) had low IAA production ability.

Phosphate solubilization capacity was a less common PGR activity among the tested strains (17/31), with 17/31 and 8/31 being capable of organic and inorganic phosphate solubilization, respectively (Table 5). Isolates LL11 and LP4 showed the highest and weakest capacity of solubilizing organic phosphate, with halo ratios (D/d) reaching 4.01 and 1.06, respectively. These two strains, along with LL1, LL2, WLL2, WLL3, G9L5, LL6, and LL7, were only capable of organic phosphate solubilization and could not solubilize inorganic phosphate. In the strains that could solubilize inorganic phosphate, WLP2 had the highest D/d of 1.46. Significant differences existed between the inorganic phosphate solubilization D/d of strain G3G2 and that of other strains.

Symbiotic efficiency analysis

In our experiments, tested endophytic isolates were inoculated on the seedlings bred with seeds sampled simultaneously with the isolates. No nodules could be observed from the plants inoculated with *Agrobacterium* strains after 45 days of growth. The *Ensifer* strains were much more effective (1–45, 0.6–2 mm, pink nodules per plant) on five alfalfa varieties than uninoculated controls, with average nodule nitrogenase activity reaching $5.3 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ (Table S1–S5). The plants of five *M. sativa* varieties inoculated with *Ensifer* strains gave higher compound leaf number (average 8.6 per plant), shoot height (11.3 cm), root length (7.8 cm), shoot ($313.3 \text{ mg} \cdot \text{plant}^{-1}$) and root ($152.2 \text{ mg} \cdot \text{plant}^{-1}$) fresh weight, shoot ($70.5 \text{ mg} \cdot \text{plant}^{-1}$) and root ($18.6 \text{ mg} \cdot \text{plant}^{-1}$) dry weight, and chlorophyll content ($2.2 \text{ mg} \cdot \text{g}^{-1}$) than uninoculated controls (Table S1–S5).

In terms of alfalfa varieties, all the 13 symbiotic parameters of *M. sativa* cvs. Gannong No. 3, Gannong No. 9, and Qingshui showed much lower mean squares compared with that of *M. sativa* cvs. Longzhong and WL168HQ (Table S6). This result suggested that endophytic isolates had similar symbiotic efficiency with *M.*

sativa cvs. Gannong No. 3, Gannong No. 9, and Qingshui. In contrast, the symbiotic efficiency of strains on *M. sativa* cvs. Longzhong and WL168HQ varied widely, with obvious dispersion degree observed among strains (Table S6). There were no differences in the symbiotic phenotypes of the strains depending on where they were isolated.

A principal component analysis (PCA) of 13 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 52.42% of the overall variance in the standardized variables, with axis 2 explaining 29.30% of the standardized variance. The standardized values of all 13 parameters were applied to make a scatter diagram with each color representing an alfalfa variety. The parameter values of *M. sativa* cvs. Gannong No. 3, Gannong No. 9, and Qingshui plants inoculated with the 31 *Ensifer* isolates clustered together (Fig. 5), which mainly ranged from -1 to 1 in PCA axis 1 and -2 to 2.5 in PCA axis 2. Compared with these three alfalfa varieties, that of *M. sativa* cvs. Longzhong and WL168HQ plants dispersed greatly, and ranged from -1.5 to 4 in PCA axis 1. The green dots (*M. sativa* cv. Longzhong) and light blue dots (*M. sativa* cv. WL168HQ) on the right side of the PCA (1 to 4 in PCA axis 1) represented *Ensifer* strains G3L2, G3L3, LL1, LL2, LP3, WLG1 (green dots) and LL1, LL2, QL2, WLG1, WLP2 (light blue dots). In the PCA axis 2, all the parameter values of these two alfalfa varieties inoculated with the 31 *Ensifer* isolates ranged from -2.5 to 3.5, except for some values (green dots at the bottom of the PCA representing strains LL8 and G3L8) of *M. sativa* cv. Longzhong which scattered between -4 and -5. This indicated a small degree of symbiotic divergence of the isolates in *M. sativa* cvs. Gannong No. 3, Gannong No. 9, and Qingshui. And symbiotic efficiency of the isolates in *M. sativa* cvs. Longzhong and WL168HQ diverged to a large extent.

Discussion

Based on the 16S rRNA gene sequence, 73 endophytic bacterial isolates obtained from nodule (41), epidermis (18), stele (nine), flower (two), stem (two), and seed (one) represented taxonomically diverse bacteria (Table 4). RFLP fingerprinting results showed the presence of high genetic diversity among strains within a genus or species. Putative *Ensifer* and *Agrobacterium* strains were further studied with sequence analyses of protein-coding housekeeping and symbiotic genes. Some *Ensifer* and *Agrobacterium* strains showed different features of PGRs (Tables 4). Symbiotic effects of rhizobium strains on *M. sativa* cvs. Longzhong and WL168HQ

were more divergent than that on *M. sativa* cvs. Gannong No. 3, Gannong No. 9 and Qingshui (Fig. 5).

Alphaproteobacteria* may constitute a predominant part of endophytic bacterial isolates of *M. sativa

This study aimed to, with five *M. sativa* varieties as trap hosts, uncover the genetic diversity of endophytic bacterial isolates in plant tissues of Gansu, China. As the main site for rhizobia colonization, nodule hosted the most endophytic bacteria (56%) in this study, followed by epidermis (25%) and stele (12%). The strains obtained from flower, stem and seed accounted for 7% of the total isolates. Interestingly, no bacterial isolates were detected here in the plant aerial parts (leaves), which were reported harbor complex, highly variable bacterial communities, and plant-specific (Mengoni et al. 2009).

Four classes of strains were presented in the plant tissues under study. *Alphaproteobacteria* strains were dominant in nodule and epidermis (families *Rhizobiaceae* and *Phyllobacteriaceae*). They were also observed in stele, stem and seed (*Rhizobiaceae*). *Gammaproteobacteria* strains were found in epidermis (*Enterobacteriaceae* and *Pseudomonadaceae*), nodule and stem (*Pseudomonadaceae*), stele and flower (*Enterobacteriaceae*). *Bacillales* strains were abundant in epidermis (*Planococcaceae*), and they were also found in nodule (*Planococcaceae* and *Bacillaceae*), stele (*Planococcaceae* and *Exiguobacteriaceae*) and flower (*Planococcaceae*). *Betaproteobacteria* was found only in stele (*Comamonadaceae*), but a low presence in other niches cannot be excluded. The *M. sativa* endophytic bacteria sampled revealed two species of *Pseudomonas* (*P. granadensis* and *P. simiae*), and one species of *E. meliloti*, *A. radiobacter*, *P. catacumbae*, *E. ludwigii*, *V. paradoxus*, *P. soli*, *B. toyonensis*, *L. adecarboxylata*, *L. fusiformis*, *E. gerundensis*, *A. pittii* and *E. mexicanum*, indicating that the diversity of species assemblages was independent of the plant tissues. In agreement with Frascella et al. (2012), in the experiments reported here, root nodule, epidermis and stele, and aerial parts of *M. sativa* plants harbored distinct bacterial communities with specific signatures at the class, family and species levels. Besides, the most diversified rhizobial populations were discovered on plant root surface (as compared to the soil or nodule), and strains in epidermis and stele were more diverse than that in nodule both at the species and intra-species levels, which were consistently found by Sachs and colleagues (2009).

***E. meliloti* and *A. radiobacter* are common *M. sativa* endophytic bacteria in Gansu, China**

Within *Alphaproteobacteria* the most sampled species was *E. meliloti* (31 strains), which comprised 43% of all

isolates and was highly diversified (in terms of RFLP types) in nodule (six haplotypes) but strangely not in epidermis (two haplotypes) and stele (one haplotype). We chose to name all these strains *E. meliloti* because they were phylogenetically grouped in the same clade with *E. meliloti* LMG 6133^T (in both the 16S rRNA gene and concatenated sequence analysis of three housekeeping genes). Interestingly, this ‘large’ *E. meliloti*-like species was detected in the population structure study of this symbiotic partner in soil and plant aerial tissues (Frascella et al. 2012) as well as in *M. sativa* nodules in the report of Tindall (2008). It was found in belowground (root nodule, root epidermis and root stele) rather than aboveground environments (flower, stem, leave and seed), suggesting that the interplay of colonization of multiple environments (root nodule, epidermis, stele, maybe other plant tissues) by this species was much less complex, and that population structure might be slightly influenced by random drift (Frascella et al. 2012).

Agrobacterium radiobacter was the second most sampled species in *Alphaproteobacteria*, which represented 16% of all sampled strains with 12 isolates, and was found in nodule, epidermis, stele, stem and seed. It contained the most diversity, with eight RFLP types identified in nodule, stele, epidermis, stem and seed. All these 12 strains were phylogenetically grouped with *A. radiobacter* ATCC 19358^T in 16S rRNA gene, while several subgroups could be observed in phylogenetically trees of single housekeeping gene and concatenated sequence analysis of three housekeeping genes. This species thus seemed to be a well-adapted symbiont of *M. sativa* in Gansu, for its widespread distribution in the areas of origin and rich diversity in the genus *Medicago*.

Symbiotic genes of endophytic rhizobium *E. meliloti*

The *nodC* and *nifH* allele histories of *E. meliloti* of *M. sativa* from Gansu, China were slightly different, although the relationships mirrored the relationships of 16S rRNA and concatenated sequence of three housekeeping genes *atpD*, *glnII*, and *recA*. The high sequence identity of *nodC* (99%–100%) and *nifH* (97%–98%) genes of *E. meliloti* suggested that these genes evolved from a common ancestor (Laguerre et al. 2001), although their *nifH* genes were more diverse than *nodC* genes. This kind of clustering results in the phylogeny of *nodC* and *nifH* genes could be explained by purifying selection and diversifying selection, respectively. Our result was consistent with the phylogeny of *nodC* and *nifH* genes from *Rhizobium* symbionts (*R. phaseoli*) and *Mesorhizobium* symbionts (*M. amorphae*, *M. septentrionale* and *M. ciceri*), in which 99%–100% *nodC* and *nifH* gene sequence identities presented between strains (Aserse et al. 2013; Gnat et al. 2015). In spite of this, all the representative

strains in this study were related to *E. meliloti* both in the phylogeny of *nodC* and *nifH* genes. As with beta-rhizobia, the symbiotic genes of alpha-rhizobia *Ensifer* and *Rhizobium* are assembled in operons carried by symbiotic plasmids (Velázquez et al. 2005; Amadou et al., 2008; Mishra et al. 2012) and they are supposed to have coevolved jointly. In the present study, no *nodC* and *nifH* genes were amplified from the *A. radiobacter*, which was in accordance with the fact that no symbiotic genes existed in *A. radiobacter* (Liu et al. 2010; Aserse et al. 2013).

Symbiotic divergence of *E. meliloti* was obvious in different alfalfa varieties

Genetic diversity of rhizobia is correlated with symbiosis-related phenotypic consequences (Wielbo 2012). In the present study, no nodules were obtained from the plants inoculated with *Agrobacterium* strains. This was consistent with previous reports that *Agrobacterium* strains could not induce nodules on their host of origin (Lajudie et al. 1999). We could see from the green and light blue dots represented by *Ensifer* strains on the right side of PCA that strains G3L2, G3L3, LL1, LL2, LP3, QL2, WLG1, WLP2 were much more effective than others, and symbiotic phenotypes of 31 endophytic isolates with *M. sativa* cvs. Gannong No. 3, Gannong No. 9, and Qingshui were less divergent than that with *M. sativa* cvs. Longzhong and WL168HQ. The symbiosis between the tested strains and *M. sativa* cvs. Gannong No. 3, Gannong No. 9, and Qingshui showed a strong variety effect. Their symbiotic efficiency differed primarily according to alfalfa varieties, to a lesser extent, tested 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.

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. IAA may improve phytostimulation, by which the bacteria can pass defense mechanisms of the plants easily (Aserse et al. 2013). This might explain the reason why the production of the IAA was common among our endophytic strains. It was interesting to note that strains from nodules showed moderate and low ability of secreting IAA. Strains here were more capable of dissolving organic phosphate. A possible explanation may be the symbiotic property of the

rhizobia of alfalfa enhanced the bind between strains and organism, which strengthened the ability of strains to dissolve nutrient elements of the organism (Qi 2006). Most of our endophytes showed one or more of the plant growth-promoting traits *in vitro*, indicating their potential for promoting plant growth. Thus, further testing is required to uncover the effects of alfalfa variety and isolation parts on the population composition, plant growth, and symbiotic efficiency of endophytic rhizobia.

Acknowledgements

This work is supported by the National Natural Science Foundation of China (NSFC), Fund No. 31562666. Grateful appreciation is made to the Key Laboratory of Grassland Ecosystem of Ministry of Education of Gansu Agricultural University for providing sampling sites and alfalfa plants. Authors declare that they have no conflicts of interest, and this article does not contain any studies with human participants or animals performed by any of the authors.

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616 **Tables****Table 1** Details of bacteria and sampled sites

Isolate code	Isolation spots on plant	<i>Medicago sativa</i> variety	Site prospected	Geographical position (latitude, longitude, altitude)	Soil texture	Annual rainfall (mm)
G3G1 ^a , G3G2, G3G3, G3G4	Stele	<i>M. sativa</i> cv. Gannong No. 3	Wuwei, Gansu, China	E 102°50',	Gray brown desert	158
G3P2, G3P3	Epidermis			N 37°52',		
G3L1- G3L13	Nodule			1650 m		
G9G1, G9G2	Stele	<i>M. sativa</i> cv. Gannong No. 9				
G9P1, G9P2, G9P3	Epidermis					
G9L2-G9L8	Nodule					
LH2	Flower	<i>M. sativa</i> cv. Longzhong	Huining, Gansu, China	E 105°06',	Loess sandy loam	300
LG1	Stele			N 34°40',		
LP1, LP2, LP3, LP4, LP5	Epidermis			1760 m		
LL1-LL11	Nodule					
QH1	Flower	<i>M. sativa</i> cv. Qingshui				
QP1, QP2, QP3, QP4, QP5	Epidermis					
QL1, QL2, QL3, QL4, QL5	Nodule					
WLN3	Seed	<i>M. sativa</i> cv. WL168HQ	Lanzhou, Gansu, China	E 105°41',	Loess loam	320
WLJ1, WLJ2	Stem			N 34°05',		
WLG1, WLG2	Stele			1517.3 m		
WLP1, WLP2, WLP4	Epidermis					
WLL1-WLL5	Nodule					

^a G3-strain, strains isolated from *M. sativa* cv. Gannong No. 3; G9-strain, strains isolated from *M. sativa* cv. Gannong No. 9; L-strain, strains isolated from *M. sativa* cv. Longzhong; Q-strain, strains isolated from *M. sativa* cv. Qingshui; WL-strain, strains isolated from *M. sativa* cv. WL168HQ.

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Table 2 Primers used for amplification and sequencing of 16S rRNA, *atpD*, *glnII*, *recA*, *nodC* and *nifH* genes

Target gene	Primer name	Primer sequence (5'-3')	References
16S rRNA	P1	AGAGTTTGATCCTGGCTCAGAACGAACGCT	Weisburg et al. 1991
16S rRNA	P6	TACGGCTACCTTGTTACGACTTCACCCC	Weisburg et al. 1991
<i>atpD</i>	<i>atpD</i> 255F	GCTSGGCCGCATCMTSAACGTC	Vinuesa et al. 2005
<i>atpD</i>	<i>atpD</i> 782R	GCCGACACTTCMGAACCNGCCTG	Vinuesa et al. 2005
<i>glnII</i>	<i>glnII</i> 12F	YAAGCTCGAGTACATYTGCT	Vinuesa et al. 2005
<i>glnII</i>	<i>glnII</i> 689R	TGCATGCCSGAGCCGTTCCA	Vinuesa et al. 2005
<i>recA</i>	<i>recA</i> 41F	TTCGGCAAGGGMTCGRTSATG	Vinuesa et al. 2005
<i>recA</i>	<i>recA</i> 640R	ACATSACRCCGATCTTCATGC	Vinuesa et al. 2005
<i>nodC</i>	<i>nodC</i> 540	TGATYGAYATGGARTAYTGGYT	Sarita et al. 2005
<i>nodC</i>	<i>nodC</i> 1160	CGYGACARCCARTCGCTRTTG	Sarita et al. 2005
<i>nifH</i>	<i>nifH</i> F	TACGGNAARGGSGGNATCGGCAA	Laguerre et al. 2001
<i>nifH</i>	<i>nifH</i> R	AGCATGTCYTCSAGYTCNTCCA	Laguerre et al. 2001

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Table 3 Taxonomic affiliation of endophytic bacteria

Phylogenetic affiliation ^a	Number of strains	Number of 16S-RFLP ribotypes ^b	16S rRNA gene sequence most related type strain ^c	Nucleotide identity to the type strain ^c
<i>Ensifer meliloti</i>	31	7	<i>E. meliloti</i> LMG 6133 ^T	99%–100%
<i>Agrobacterium radiobacter</i>	12	8	<i>A. radiobacter</i> ATCC 19358 ^T	97%–98%
<i>Phyllobacterium catacumbae</i>	2	1	<i>P. catacumbae</i> CSC19 ^T	98%
<i>Enterobacter ludwigii</i>	2	1	<i>E. ludwigii</i> EN-119 ^T	99%
<i>Variovorax paradoxus</i>	1	1	<i>V. paradoxus</i> NBRC 15149 ^T	99%
<i>Psychrobacillus soli</i>	6	1	<i>P. soli</i> NHI-2 ^T	97%–99%
<i>Bacillus toyonensis</i>	2	1	<i>B. toyonensis</i> BCT-7112 ^T	99%
<i>Leclercia adecarboxylata</i>	1	1	<i>L. adecarboxylata</i> NBRC 102595 ^T	99%
<i>Lysinibacillus fusiformis</i>	10	1	<i>L. fusiformis</i> NBRC 15717 ^T	98%–100%
<i>Pseudomonas granadensis</i>	2	1	<i>P. granadensis</i> F-278770 ^T	99%
<i>Pseudomonas simiae</i>	1	1	<i>P. simiae</i> Oli ^T	100%
<i>Erwinia gerundensis</i>	1	1	<i>E. gerundensis</i> EM595 ^T	99%
<i>Acinetobacter pittii</i>	1	1	<i>A. pittii</i> CIP 70.29 ^T	100%
<i>Exiguobacterium mexicanum</i>	1	1	<i>E. mexicanum</i> 8N ^T	97%

^a Phylogenetic affiliation was based on 16S rRNA gene phylogeny;^b Number of 16S-RFLP ribotypes was based on the results of 16S rRNA gene RFLP-PCR analysis;^c 16S rRNA gene sequence most related type strains and nucleotide identity to the type strain were obtained from EzBioCloud's Identify service website.

Table 4 Distribution of species and 16S-RFLP ribotype in plant tissues

Species ^a	Representative strains	16S-RFLP ribotype ^b	16S-RFLP								
			type ^b	Nodule	Epidermis	Stele	Flower	Stem	Seed	Leave	All
<i>Ensifer meliloti</i>	WLP2	AGal	EM1	21 ^c	1	1					23
<i>E. meliloti</i>	G3L3	AHbm	EM2	1							1
<i>E. meliloti</i>	LP3	AHbn	EM3		1						1
<i>E. meliloti</i>	G9L4	AGbl	EM4	3							3
<i>E. meliloti</i>	LL7	AGba1	EM5	1							1
<i>E. meliloti</i>	G3L4	EGbl	EM6	1							1
<i>E. meliloti</i>	G3L7	FGbl	EM7	1							1
<i>Agrobacterium radiobacter</i>	G3G1	DKeq	RR1			1					1
<i>A. radiobacter</i>	G3G2	DLfr	RR2			1					1
<i>A. radiobacter</i>	G3L1	DMgs	RR3	1							1
<i>A. radiobacter</i>	G3P2	DNht	RR4		1						1
<i>A. radiobacter</i>	LP4	DPfv	RR5		1						1
<i>A. radiobacter</i>	WLG2	DRhy	RR6	3		1		1			5
<i>A. radiobacter</i>	WL2	DRdy	RR7	1							1
<i>A. radiobacter</i>	WLN3	DOkz	RR8						1		1
<i>Phyllobacterium caticumbae</i>	LL3	QO1tv1	-	1	1						2
<i>Enterobacter ludwigii</i>	G3G3	GH1ln1	-		1	1					2
<i>Variovorax paradoxus</i>	G3G4	HI1np1	-			1					1
<i>Psychrobacillus soli</i>	QP1	KJ1oq1	-	1	4	1					6
<i>Bacillus toyonensis</i>	G9L2	LK1pr1	-	2							2
<i>Leclercia adecarboxylata</i>	G9P1	ML1qs1	-		1						1
<i>Lysinibacillus fusiformis</i>	G9P2	NM1rt1	-	2	6	1	1				10
<i>Pseudomonas granadensis</i>	WLJ1	ON1su1	-	1				1			2
<i>Pseudomonas simiae</i>	QP4	UN1wp1	-		1						1
<i>Erwinia gerundensis</i>	QH1	TQ1vx1	-				1				1
<i>Acinetobacter pittii</i>	G3L11	R1T1y1a2	-	1							1
<i>Exiguobacterium mexicanum</i>	LG1	S1U1z1b2	-			1					1
Total				41	18	9	2	2	1	0	73

^a Species assignment was based on 16S rRNA gene phylogeny;
^b 16S-RFLP ribotype and 16S-RFLP type were based on the results of 16S rRNA gene RFLP-PCR analysis;
^c Numbers indicate distribution of 16S-RFLP types across plant tissues.

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Table 5 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±0.088cd ^b	1.46±0.087a	- ^c
G3G2	1.14±0.103cd	0.89±0.155b	+++
G3L1	1.39±0.076cd	1.25±0.037a	-
G3L2	1.18±0.020cd	1.28±0.065a	+
G3L3	1.08±0.010d	1.28±0.035a	+++
LL1	1.34±0.030cd	N ^d	-
LL2	1.73±0.597c	N	++
LP4	1.06±0.003d	N	+++
WLG1	1.21±0.012cd	1.25±0.003a	-
WLJ2	1.38±0.112cd	1.32±0.033a	-
WLL2	1.26±0.029cd	N	-
WLL3	1.51±0.116cd	N	+
WLP2	1.07±0.005d	1.29±0.036a	+++
G9L4	1.61±0.056cd	N	-
LL6	3.05±0.341b	N	++
LL11	4.01±0.005a	N	+
LL7	3.02±0.117b	N	-
G9L8	N	N	+++
G3L12	N	N	+
QL5	N	N	+
G3L4	N	N	+
LL5	N	N	++
QL4	N	N	+
LL10	N	N	++
G3L5	N	N	+++
G3L10	N	N	+
G9L4	N	N	+
G3L6	N	N	+
G9L3	N	N	+
G3L8	N	N	+
G9L6	N	N	++

^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 N indicated that the strains were incapable of solubilizing phosphate;

^d - no IAA production activity, + low IAA production activity, ++ moderate IAA production activity, +++ high IAA production

activity.

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Figure legends

Fig. 1 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of 27 representative strains, two reference strains *E. medicae* WSM419^T and *R. ipomoeae* shin9-1^T (in bold), and related taxa. Reference type strains are indicated with superscripted T. Gene sequence accession numbers of tested strains are shown within *parenthese*. 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

Fig. 2 Neighbor-joining phylogenetic trees based on sequences of *atpD* (a), *glnII* (b), and *recA* (c) genes showing the relationships of 15 representative strains of *Ensifer* and *Agrobacterium* (in bold) and related taxa. Reference type strains are indicated with superscripted T. Gene sequence accession numbers of tested strains are shown within *parenthese*. Numbers at nodes indicate bootstrap percentages (based on 1000 resampled datasets). Only node supports higher than 50% are shown. The length units of 0.01 and 0.02 indicate 1% and 2% of the genetic distance

Fig. 3 Neighbor-joining phylogenetic tree based on concatenated sequences of *atpD*, *glnII*, and *recA* genes showing the relationships of 15 representative strains of *Ensifer* and *Agrobacterium* (in bold) and related taxa. Reference type strains are indicated with superscripted T. Gene sequence accession numbers of tested strains are shown within *parenthese*. 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

Fig. 4 Neighbor-joining phylogenetic trees based on sequences of *nodC* (a) and *nifH* (b) genes showing the relationships of seven representative strains of *Ensifer* (in bold) and related taxa. Reference type strains are indicated with superscripted T. Gene sequence accession numbers of tested strains are shown within *parenthese*. Numbers at nodes indicate bootstrap percentages (based on 1000 resampled datasets). Only node supports higher than 50% are shown. The length units of 0.2 and 0.02 indicate 20% and 2% of the genetic distance

Fig. 5 Scatter diagram based on the standardized values of all 13 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, and chlorophyll content of five alfalfa varieties plants were determined after 45 days of growth. All of the determined values were applied

656 to conduct a principal component analysis (PCA). Each dot represents two standardized values (in PC1 and PC2)
657 for one repetition of the 31 isolates (with three repetitions for each strain)

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64x92mm (300 x 300 DPI)

Fig. 2 Neighbor-joining phylogenetic trees based on sequences of *atpD* (a), *glnII* (b), and *recA* (c) genes showing the relationships of 15 representative strains of *Ensifer* and *Agrobacterium* (in bold) and related taxa. Reference type strains are indicated with superscripted T. Gene sequence accession numbers of tested strains are shown within parentheses. Numbers at nodes indicate bootstrap percentages (based on 1000 resampled datasets). Only node supports higher than 50% are shown. The length units of 0.01 and 0.02 indicate 1% and 2% of the genetic distance

225x104mm (300 x 300 DPI)

Fig. 3 Neighbor-joining phylogenetic tree based on concatenated sequences of *atpD*, *glnII*, and *recA* genes showing the relationships of 15 representative strains of *Ensifer* and *Agrobacterium* (in bold) and related taxa. Reference type strains are indicated with superscripted T. Gene sequence accession numbers of tested strains are shown within parentheses. 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

81x88mm (300 x 300 DPI)

Fig. 4 Neighbor-joining phylogenetic trees based on sequences of *nodC* (a) and *nifH* (b) genes showing the relationships of seven representative strains of *Ensifer* (in bold) and related taxa. Reference type strains are indicated with superscripted T. Gene sequence accession numbers of tested strains are shown within parentheses. Numbers at nodes indicate bootstrap percentages (based on 1000 resampled datasets). Only node supports higher than 50% are shown. The length units of 0.2 and 0.02 indicate 20% and 2% of the genetic distance

132x56mm (300 x 300 DPI)

Fig. 5 Scatter diagram based on the standardized values of all 13 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, and chlorophyll 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). Each dot represents two standardized values (in PC1 and PC2) for one repetition of the 31 isolates (with three repetitions for each strain)

231x181mm (300 x 300 DPI)