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Diversity analysis and function prediction of rhizo- and endophytic bacterial communities of *Senecio vulgaris* L. (Asteraceae) in an invasive range

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Because increasing evidence has confirmed the importance of plant-associated bacteria for plant growth and productivity, it is believed that interactions between bacteria and alien plants play an important role in plant invasions. However, the diversity of bacterial communities associated with invasive plants is poorly understood. Therefore, we investigated the diversity of rhizo- and endophytic bacteria associated with the invasive annual plant *Senecio vulgaris* L (Asteraceae) based on bacterial 16S rRNA gene data obtained from 57 samples of four *S. vulgaris* populations in a subtropical mountainous area in central China. Significant differences in diversity were observed between plant compartments. Rhizosphere harbored much more bacterial OTUs and showed higher alpha diversity than the leaf and root endosphere. Bacterial community composition differed substantially between compartments and locations in relative abundance profiles, especially at phyla and family level. However, the top five phyla (Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Acidobacteria) comprised more than 90% of abundance in all the bacterial communities. And similar endophytic communities with a shared core set of bacteria were observed from different *S. vulgaris* populations. According to the function prediction based on the identification and abundance information of the OTU, bacteria characterized as plant pathogens, as well as those involved in ureolysis and nitrate reduction, were rich in endophytic communities. This study reveals the microbiomes and their putative function in the invasive *S. vulgaris* plants and is also the first step for future studies on the role of interactions between bacteria and alien plants in plant invasions.
Diversity analysis and function prediction of rhizo- and endophytic bacterial communities of *Senecio vulgaris* L. (Asteraceae) in an invasive range

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Abstract

Because increasing evidence has confirmed the importance of plant-associated bacteria for plant growth and productivity, it is believed that interactions between bacteria and alien plants play an important role in plant invasions. However, the diversity of bacterial communities associated with invasive plants is poorly understood. Therefore, we investigated the diversity of rhizo- and endophytic bacteria associated with the invasive annual plant Senecio vulgaris L (Asteraceae) based on bacterial 16S rRNA gene data obtained from 57 samples of four S. vulgaris populations in a subtropical mountainous area in central China.

Significant differences in diversity were observed between plant compartments: rhizosphere harbored much more bacterial OTUs and showed higher alpha diversity than the leaf and root endosphere. Bacterial community composition differed substantially between compartments and populations in relative abundance profiles, especially at phyla and family level. However, the top five phyla (Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Acidobacteria) comprised more than 90% of abundance in all the bacterial communities. And similar endophytic communities with a shared core set of bacteria were observed from different S. vulgaris populations. According to the function prediction based on the identification and abundance information of the OTU, bacteria characterized as plant pathogens, as well as those involved in ureolysis and nitrate reduction, were rich in endophytic communities.

This study reveals the bacteria and their putative function in the invasive S. vulgaris plants and is also the first step for future studies on the role of interactions between bacteria and alien plants in plant invasions.

Key words: invasive plant, bacterial community, plant-microbe interactions, endophytic bacteria, 16S rRNA gene
Introduction

With the development of globalization, the spread and outbreak of invasive species is occurring more frequently. Invasive plants can displace native species, destroy the structure and function of local plant communities, and further influence various animals or microbes inhabiting local communities, leading to decreased local or regional biodiversity and ultimately, unbalanced local ecosystems and loss of ecological function (Pysek et al., 2010; Blackburn et al., 2011). People worry that constant expansion of invasive plants reduces the uniqueness of local flora and even leads to global homogenization of species composition (Orians & Ward, 2010). To fundamentally control exotic plant invasion, it is essential to understand the mechanism of exotic plant invasion; accordingly, this topic has become one of the core studies of invasion ecology.

Some studies have shown that plants already have genetic characteristics in favor of invasion, known as the preadaptation hypothesis, which was supported by the observation of more biomass and higher root-stem ratios when compared with non-invasive plant species in the same genus under the same conditions (Van Kleunen et al., 2011). Hypotheses such as the Natural Enemies Release Hypothesis (ERH) (Keane & Crawley, 2002), Evolution of Increased Competition Ability (EICA) (Blossey & Notzold, 1995), Shifting Defense Hypothesis (Müller-Schärer et al., 2004; Joshi & Vrieling, 2005) and New Weapon Hypothesis (Callaway et al., 2008) explain the invasion mechanism based on the relationship between plants and aspects of their biotic environments, such as natural enemies or competitors.

However, plants can also form mutualistic symbiotic relationships with other organisms. Land plants are colonized by microbiota in the rhizosphere, phyllosphere, and endophytic compartment (within the leaves and roots) (Rodriguez et al., 2008; Bulgarelli et al., 2012; Lundberg et al., 2012). It is well known that arbuscular mycorrhizal fungi (AMF) and root nodule bacteria form mutualistic symbioses with plants (Hardoin et al., 2015). Moreover, it was recently recognized that bacteria other than rhizobia are beneficial to plants. Such plant growth-promoting bacteria (PGPB) or plant growth-promoting rhizobacteria (PGPR) can stimulate plant growth, increase yield, reduce pathogen infection, and reduce biotic or abiotic stress without conferring pathogenicity (Compant et al., 2010; Pieterse et al., 2014). PGPR can produce growth-promoting substances such as IAA, GA3, zeatin, and ABA (Perrig et al., 2007). Many nitrogen-fixing bacteria in addition to *Rhizobium* species have been identified from plants (Gaby & Buckley, 2011).

Endophytic microbiome, which live within the tissues and organs of plants but do not cause plant infections (Rodriguez et al., 2009). Some PGPB are endophytic microbes that can enhance the tolerance of host plants to stressful environments, promote plant growth and improve plant protection (Bulgarelli et al., 2013). Moreover, unlike PGPR, endophytic PGPB can be propagated to the next generation of plants by seeds (Truyens et al., 2015). Accordingly, it can be inferred that endophytic bacteria can establish long-term symbiotic relationships with host plants and have an evolutionary impact on the adaptation of plant populations.

In recent years, several studies have suggested that endophytic bacteria play an important role in plant-invasion mechanisms. *Sorghum halepense*, an invasive plant that thrives on grassland with few nitrogen sources, contains endogenous nitrogen-fixing bacteria, which have improved the availability of resources in the soil (Rout & Chrzanowski, 2009; Rout et al., 2013). The effects of
rhizo- and endophytic bacteria on the invasion of exotic plants are species-specific and vary across environmental conditions (Long et al., 2008; Rout & Callaway, 2012; Dai et al., 2016). As people have done for fungal diversity in invasive plants (Shipunov et al., 2008; Mei et al., 2014), it is equally important to explore the diversity of bacteria associated with invasive plants to understand the plant-bacterial interactions that occur in the plant-invasion mechanism.

\textit{Senecio vulgaris} (Asteraceae), an annual or biennial herb, is treated as a weed in the United Kingdom, Western Europe, North America, Australia and New Zealand (Paul & Ayres, 1987; Müller-Schärer & Frantzen, 1996; Vitousek et al., 1996; Frantzen & Hatcher, 1997; Robinson et al., 2003; Figueroa et al., 2007). \textit{Senecio vulgaris} are small plants with short life cycles and a high self-crossing rate that can produce large numbers of seeds, which can germinate under the right conditions at any time; therefore, its ability to spread is very strong (Robinson et al., 2003). This species was introduced into northeast China in the 19th century, and it is now widely distributed and included in The Checklist of the Invasive Plants in China (Ndihokubwayo et al., 2016; Zhu et al., 2016; Cheng et al., 2017). \textit{Senecio vulgaris} grows well in ambient habitats, such as gardens, lawns and arable land, while it survives in stressful habitats such as roadside areas and waste facilities (Robinson et al., 2003). Bacteria might help \textit{S. vulgaris} resist heavy metals as well as to acquire nitrogen and phosphate in contaminated and oligotrophic environments.

In this study, we collected rhizosphere soil and plant samples of \textit{S. vulgaris} populations from four sites in the Shennongjia Forestry District, Hubei Province, China. We made the following hypotheses: (1) plant compartments and sampling locations determine the diversity and function of rhizosphere and endophytic bacterial communities associated with \textit{S. vulgaris} plants; (2) endophytic bacteria communities from different sites share core operational taxonomic units (OTUs); and (3) rhizosphere and endophytic bacteria have the potential to be beneficial to host plants. To test these hypotheses, we examined bacterial communities in the rhizosphere and leaf and root endospheres of \textit{S. vulgaris} populations using Illumina amplicon sequencing targeting the bacterial 16S rRNA gene region and through subsequent analyses. We also explored the functions of the OTUs, especially some of the top core endophytic bacterial OTUs of \textit{S. vulgaris} plants based on the Functional Annotation of Prokaryotic Taxa (FAPROTAX) database (Louca et al., 2016) and by review of previous studies.

\section*{Materials and Methods}

\subsection*{Sample collection and processing}

We aimed to examine bacterial communities in the rhizosphere, leaf and root endosphere of \textit{S. vulgaris} plants in four locations. Five quadrats were set in three locations and four quadrats were set in the fourth location. Thus, nineteen quadrats were set in our experiment. From each quadrat, we collected one rhizosphere, one root and one leaf endosphere sample. In total, we analyzed 57 samples.

All samples were collected in April of 2016 in Shennongjia Forestry District, Hubei Province (Figure 1). In Shennongjia, the annual temperature is 12°C, annual precipitation ranges from 800 to 2500 mm, and the elevation ranges from 398 to 3105 m above sea level. In March and April
2016, the daily minimum temperature in Shennongjia was often below 10°C (Figure S1). The vertical vegetation spectrum along sampling sites consisted of mixed deciduous and evergreen broad-leaved forest (1000–1700 m) and deciduous forest (1600–2100 m).

We sampled the *S. vulgaris* population in a waste disposal facility and a roadside area. At each sampling point, we set four or five square quadrats with an area of 1 m×1 m. The distance between each quadrat was greater than 5 m. In each quadrat, more than three healthy *S. vulgaris* plants were gently pulled out of the ground, and soil around the roots was shaken off. We then put these plants into a sterile plastic bag, which was subsequently sealed and stored at 4°C until return to the laboratory, at which time the samples were treated immediately. All plants from one quadrat were pooled as one sample.

We put the roots of *S. vulgaris* from one quadrat into a 50 ml centrifuge tube, after which they were rinsed with sterile water and centrifuged for 5 min at 2000 g. The supernatant was then discarded, while the rhizosphere soil was stored at -80°C until DNA extraction. Healthy and undamaged leaves and roots were randomly selected, washed with ultrapure water, soaked and oscillated for 1 min with 70% alcohol, then washed for 1 or 5 min with 1% sodium hypochlorite solution (leaves for 1 min and roots for 5 min), and finally rinsed 4 times with sterile water.

Next, 0.1 mL of the final wash was spread on trypticase soy agar (TSA) plates to check for contamination (Siciliano & Germida, 1999).

Plant tissue was macerated with a sterile pestle and mortar with liquid nitrogen and 0.25–0.3 g of finely ground material of soil or plant tissue were used for DNA extraction. We extracted DNA with the MOBIO Power Soil DNA Isolation Kit (MO-BIO, Carlsbad, CA, USA) according to the manufacturer’s protocols.

**PCR amplification and next-generation sequencing**

We used 16S rRNA gene amplicons to determine the diversity of the bacterial communities in each of the samples. For polymerase chain reaction (PCR), we used primers 799F (5’-AACMGAGGTAGATCCCG-3’) and 1193R (5’-ACGTACGCCACCTTCC-3’), which were designed to specifically amplify the V5, V6, and V7 hypervariable regions of the 16S rRNA gene of bacterial DNA while excluding amplification of chloroplast DNA from plants as suggest in some previous studies (Chelius & Triplett, 2001; Bulgarelli et al., 2012; Bodenhausen et al., 2013; Beckers et al., 2016). PCR reactions were conducted with a Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Briefly, the same volume of 1× loading buffer (contained SYB green) was mixed with PCR products, then electrophoresed on 2% agarose gel for detection. Samples with a bright main strip between 400–450 bp were chosen for further experiments. PCR products mixed in equidensity ratios were purified with a Qiagen Gel Extraction Kit (Qiagen, Germany) and sequencing libraries were generated using a TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations. In addition, index codes were added to the libraries. The library quality was assessed using a Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an IlluminaHiSeq2500 platform and 250 bp paired-end reads were generated. Sequencing was conducted at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China).
Sequence data treatment

Paired-end reads were assigned to samples based on their unique barcode, truncated by cutting off the barcode and primer sequence and then merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/). Quality filtering of the raw tags was performed under specific filtering conditions to obtain high-quality clean tags according to the QIIME (V1.7.0, http://qiime.org/index.html) quality-controlled process. The tags were compared with those in a reference database (Gold Database, http://drive5.com/uchime/uchime_download.html) using the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) to detect chimera sequences, which were removed to yield the effective tags.

Sequence analyses were performed with the Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/), and sequences with ≥97% similarity were assigned to the same OTU. Representative sequences for each OTU were then screened for further annotation. For each representative sequence, the GreenGene Database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) was employed based on the RDP classifier (Version 2.2, http://sourceforge.net/projects/rdp-classifier/) algorithm to annotate taxonomic information.

To investigate the phylogenetic relationships of different OTUs and the differences in the dominant species among samples (groups), multiple sequence alignment was conducted using the MUSCLE software (Version 3.8.31, http://www.drive5.com/muscle/). OTUs abundance information were normalized using a standard sequence number corresponding to the sample with the lowest number of sequences.

Selection of core bacterial OTUs in the endosphere

The core OTUs were manually selected based on the average relative abundance and the relative frequency of each OTU per compartment. We first ranked the OTUs from highest relative abundance to lowest, then selected a certain number of top OTUs that collectively comprised about 80% of the total abundance of the bacterial community. This is similar to the Pareto concept (the 80–20 rule) applied in microbiological community analysis as suggested by Werner et al. (2011). After their identification, we plotted average relative abundance and frequency of the core OTUs across each sample type.

Bacterial function prediction

Based on the identification and abundance information of the OTU, we predicted its metabolically and ecologically relevant functions using the FAPROTAX database and quantified every functional groups (Louca et al., 2016). We then illustrated the metabolic structure of the bacterial communities using a heatmap based on the standard and average data of the relative abundance of OTUs associated with each function group annotated by FAPROTAX for each of the 12 sampling groups (3 plant compartments × 4 sampling locations).

Statistical analyses

Analyses of alpha and beta diversity were performed based on the output normalized data. We calculated the Shannon diversity (H') index using the BiodiversityR, while Venn diagrams were
plotted with the ‘venn.diagram’ function of the VennDiagram package. Differences in the bacterial alpha diversities between compartments and locations were compared by two-way ANOVA using the ‘aov’ function. Multiple comparisons of means between compartments were accomplished using Tukey Contrasts. Nonmetric multidimensional scaling (NMDS) was performed using the ‘Mass’ and ‘vegan’ packages. Permutational ANOVAs (PERMANOVAs) were conducted with the ‘adonis’ function in the ‘vegan’ package as described by Desgarennes et al. (2014). All analyses were conducted using R v.2.15.2 (R Foundation for Statistical Computing; available at http://www.R-project.org).

Results

Alpha-diversity of bacterial communities

Of 3,046,898 high-quality reads that we obtained, we used the 2,620,319 sequences that remained after removing OTUs not classified as bacteria or matching chloroplasts, mitochondrial or Viridiplantae for further analysis. The average length of the sequences was 375 nt. Because of contamination from chloroplasts, less sequences were obtained from leaf samples than from root and soil samples. However, all samples showed high-coverage (>10,000 usable reads); therefore, we used all samples (Table S1). In total, 554,085 reads were annotated to 34 bacterial phyla, 518,579 reads were annotated to 275 bacterial family and 165,219 reads annotated to 246 species (Table S2).

The majority of bacterial OTUs identified in the leaf and root endosphere were also present in the rhizosphere. Moreover, 289 OTUs were detected solely in the aboveground tissues, which was a considerably small number and only 6.6% of all identified OTUs. Additionally, only 160 and 69 OTUs were exclusively observed in leaves and roots, representing 12.4% and 4.6% of the leaf and root communities (Figure 2a). The percentage of OTUs shared between locations was 33%, 17% and 9% for rhizosphere, root and leaf samples, respectively (Figure 2b–d).

The levels of microbial diversity differed significantly among compartments. Alpha diversity measured by the Shannon (H’) index was affected by compartments, but not by locations. Specifically, H’ decreased significantly from the soil to the root and leaf endospheres (Figure 3; Table 1–2).

Bacterial community composition

Across all samples, we detected a total of 34 distinct bacterial phyla, among which the top ten phyla comprised an average of > 98% bacteria abundance in all samples, and the top five (Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Acidobacteria) comprised an average of > 90% of the bacterial abundance (Figure 4). Bacterial community composition differed substantially between compartments and locations in relative abundance profiles at the phylum level (Table 1), and the same pattern was found at the OTU level as well (Figure 5). Samples from different compartments differed from one another in relation to the relative abundance of the five dominant phyla; specifically, rhizosphere bacterial communities were enriched for Acidobacteria; root endosphere samples had lowest abundance of Actinobacteria and leaf endosphere samples had highest abundance of Firmicutes, but depleted levels of
Proteobacteria and Bacteroidetes (Figure 4; Table 2).

The bacterial community composition differed significantly between compartments at the family level. Rhizosphere bacterial communities had higher abundances of Flavobacteriaceae and Comamonadaceae, while Oxalobacteraceae and Pseudomonadaceae were most abundant in the root endosphere and Caulobacteraceae and Pseudomonadaceae were enriched in the leaf endosphere (Figure 6).

Core bacterial OTUs in root and leaf endospheres

From the 1,284 OTUs in leaf endosphere, we identified 36 OTUs with > 0.70 relative frequency as core OTUs that collectively comprised about 80.28% of the leaf endophytic bacterial communities. The endosphere bacterial communities were dominated by a few bacterial phyla or orders including Alpha-, Beta-, Gammaproteobacteria, Actinobacteria, Firmicutes (Bacilli) and Bacteroidetes (Flavobacteria, Table 3). The top five OTUs in the leaf endosphere were Brevundimonas diminuta (Alphaproteobacteria), Exiguobacterium sibiricum (Bacilli), Pseudomonas sp. (OTU7, Gammaproteobacteria), OTU6 (Alcaligenaceae, Betaproteobacteria), and Pseudomonas viridiflava (Gammaproteobacteria, Figure 7a; Table S3).

Similarly, from the 1,543 OTUs, we identified 30 OTUs as core root endophytic bacteria, the four most abundant being OTU3 (Oxalobacteraceae, Betaproteobacteria), Pseudomonas sp. (OTU7), Pseudomonas viridiflava and Duganella sp. (OTU15, Betaproteobacteria, Figure 7b; Table S4). With the exception of three OTUs, all core root endophytic bacteria were present with =1.00 relative frequency, and these OTUs collectively comprised about 79.62% of the root endophytic bacterial communities.

Bacterial function prediction

In this study, 63 function groups were represented, indicating that any one of these groups was associated with at least one OTU identified from the samples. Overall, 1,269 of 4,902 OTUs (25.89%) were assigned to at least one function group, while 3,633 (74.11%) could not be assigned to any group. Additionally, several OTUs were assigned to multiple functional groups.

We found that the metabolic functional structure of bacterial communities was quite different among samples from different plant compartments. Moreover, samples from the same plant compartments showed similar metabolic functional structures (Figure 8). Samples from rhizosphere soil were distinct in that they contained abundant OTUs involved in nitrogen metabolic pathways, plastic degradation, and arsenate detoxification (Figure S2b), while root endosphere samples were more closely related to nitrogen and methanol (or methylal) metabolic pathways (Figure S2c). Interestingly, leaf samples differed from others in that they contained OTUs related to animal parasites, plants and human pathogens (Figure S2d).

Twenty-eight of the 60 core OTUs were functionally annotated, among which 22 were annotated by FAPROTAX and six according to previous studies. Quite a few OTUs were predicated being associated with the ability to reduce nitrate and ureolysis, while a few were classified as plant or human pathogens, and two might have been able to conduct methanol oxidation (Table S5-6).
Discussion

Difference between plant compartments and sampling locations

We determined that bacterial communities associated with *S. vulgaris* were primarily influenced by plant compartments, where the alpha diversity was significantly decreased in the root and leaf endospheres compared with the rhizosphere soil (Figure 3; Table 1–2). These findings were consistent with observations from many plants such as *Agave* species (Coleman-Derr et al., 2015), rice (Edwards et al., 2015) and poplar trees (Beckers et al., 2016). Our study and others provided evidence that soil is a potential reservoir for endophytic bacteria. Microbial diversity declines sequentially from the rhizosphere to roots and leaves, which suggests increasingly stronger competition among microorganisms as the habitat becomes more tightly defined (Müller et al., 2016).

The rhizosphere bacteria and those in the root and leaf endospheres were clearly distinct from one another. Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes dominated the rhizosphere and endosphere of *S. vulgaris* plants. However, the relative abundance of Proteobacteria and Firmicutes increased, while that of Acidobacteria decreased from the rhizosphere to the endosphere. These findings are consistent with observations from other plants such as rice (Edwards et al., 2015), maize (Liu et al., 2017), grapes (Zarraonaindia et al., 2015), agave (Coleman-Derr et al., 2015), *Brassica stricta* (Wagner et al., 2016), *Oxyria digyna*, and *Saxifraga oppositifolia* (Kumar et al., 2017). Taken together, these results indicate that there may be some factors that shape the structure of endophytic bacteria acting in different environments and host species. Bulgarelli et al. (2012) suggested that such factors included the physicochemical properties of plant cell walls and metabolites from active plant cells. Moreover, Bulgarelli et al. (2013) put forward a two-step selection model in which rhizodeposition and convergent host genotype-dependent selection drives the community shift in the rhizosphere and endophyte microbiota differentiation. Obviously, this plant selection process can explain the differentiation between the bacteriome in the endosphere and in soil.

We also found that bacterial communities associated with *S. vulgaris* were influenced by the sampling locations. This kind of influence lies in the difference between climate and soil physicochemical properties between locations. Moreover, *S. vulgaris* plants often differed between locations, which could also affect bacterial communities. Recent studies have demonstrated that plant host-specific traits, including broad morphological characteristics (Kembel et al., 2011) and specific genetic pathways and gene products (Horton et al., 2014; Lebeis et al., 2015), can have significant effects on microbiome composition and diversity.

Core bacterial OTUs in root and leaf endospheres

When only the profile of the endophytic bacterial OTUs was considered, there were great differences between locations (Figure 2). However, when the abundance of the OTUs was considered, *S. vulgaris* plants from different locations were found to share the same core OTUs in the leaf and root endospheres. These core OTUs accounted for much less than 20% of the total OTUs, but 80% of the abundance of the endophytic bacterial communities. These findings demonstrated that the core endophytic bacteriome was consistent across hosts of the same species grown in different locations, as has been observed in *Arabidopsis* (Bulgarelli et al., 2012;
The dominating phyla or order, including Alpha-, Beta-, and Gammaproteobacteria, Actinobacteria, Firmicutes (Bacilli) and Bacteroidetes (Flavobacteria) also tend to dominate the endophytic bacteriomes of other plants reviewed by Haroim et al. (2015), Müller et al. (2016) and Finkel et al. (2017).

The core OTUs in leaves belonged to 19 families (Table S3), while those in roots belonged to 10 families (Table S4). We compared these dominant families with those reported in previous studies. Dominant families in *S. vulgaris* roots substantially overlapped with those reported as a core set of *Arabidopsis thaliana*, *Salicornia europaea* and *Helianthus annuus*: Oxalobacteraceae and Flavobacteriaceae were found as core members of the root microbiome in six studies, while Comamonadaceae were observed as core taxa of the root microbiome in seven different studies.

In relation to leaf endophytic bacteria, *A. thaliana* shared the abundant leaf taxa at the family level, while *Sequoia sempervirens* and *Sequoia dendrongiganteum* shared few leaf taxa with *S. vulgaris* (Table 4). The comparison indicated that although the host effect on the structure of endophytic bacteria communities was strong, taxa similarity could be observed at the phylum, order or even the family level.

In leaf and root bacterial communities of *S. vulgaris*, there were several dominant genera; namely, *Brevundimonas*, *Pseudomonas*, *Exiguobacterium*, *Sphingomonas*, *Flavobacterium*, *Rhizobium*, *Massilia*, and *Duganella*. Among these, *Pseudomonas* and *Rhizobium* have been thoroughly investigated as plant-associated genera. *Pseudomonas* are known to occupy numerous ecological niches, including the rhizospheres and endospheres of many plants. For instance, 21 *Pseudomonas* strains were isolated from the roots of *Populus deltoides* (Jun et al., 2015), and 12 *Pseudomonas* strains showed promising growth-promoting effects when applied to lettuce in the field (Cipriano et al., 2016). *Massilia* and *Duganella* are Burkholderiales, which are well known for their biodegradative capacities and antagonistic properties toward multiple soil-borne fungal pathogens (Benítez & Gardener, 2009; Chebotar et al., 2015). Finally, the genus *Flavobacterium* comprises a significant fraction of endophytic microbiomes in a broad range of plant species, indicating a specialized capacity to proliferate in plant environments and suggesting a role in plant function (Kolton et al., 2016).

We also identified some cold-resistant bacteria as core bacterial OTUs in root and leaf endospheres of *S. vulgaris*. These bacteria included *Sphingomonas aerolata*, *Sphingomonasfaeni*, *Exiguobacterium sibiricum* and OTU 3. Isolates of two *Sphingomonas* species (*S. aerolata* and *S. faeni*) showed psychrotolerant traits (Busse et al., 2003). *Exiguobacterium sibiricum* is one of 14 known *Exiguobacterium* spp. (Vishnivetskaya et al., 2009). Strains of this species isolated from the Siberian permafrost could grow well at low temperature (e.g., 4°C) and had remarkable tolerance to repeated freeze-thawing cycles (Vishnivetskaya et al., 2007). OTU3 (Oxalobacteraceae), which may have been from members of the *Duganella*, *Rugamonas* or *Janthinobacterium* genus, was highly abundant in root samples (Figure 7b; Table S4). *Janthinobacterium lividum* was observed in the endosphere of two native perennial plants, *Oxyria digyna* and *Saxifraga oppositifolia*, in three Arcto-Alpine regions (Kumar et al., 2017). *Janthinobacterium* spp. were reported to be thriving in extreme cold, dry, and high solar ultraviolet (UV) radiation environments and to manifest strong antimicrobial activity (Koo et al.,...
When our plants were collected in April of 2016, in Shennogjia, we found that *S. vulgaris* was one of the weeds that emerges in early spring, and that the daily minimum temperature was often below 10°C (Figure S1). Therefore, it is not surprising that the cold-resistant bacteria are present in the endosphere of *S. vulgaris* plants in this region, and it is possible that they could facilitate host growth under cold conditions.

**Bacterial function prediction**

Corresponding to the structural differences between plant compartments, bacterial communities from different compartments also differed relative to functional grouping. This functional fraction based on the plant microenvironment has also been observed in other plants, including *Espeletia* species in an Andean high-mountain ecosystem (Ruiz-Pérez et al., 2016). Similar to PICRUST (Langille et al., 2013) and Geochip (He et al., 2010), FAPROTAX classifies bacterial function based on metabolomic traits. Moreover, FAPROTAX adds annotations according to the ecological relationship between bacteria and eukaryotes (plants, animal and humans). Thus, we may see that more human, animal and plant pathogens were harbored in the *S. vulgaris* leaf endosphere than that in the other compartments (Fig 7, Table S5–6). However, care should be taken when drawing this conclusion because the properties of pathogenicity may depend on many factors, including plant and microbial genotype, microbial numbers, and quorum sensing or environmental conditions (Hardoim et al., 2015).

There were abundant OTUs involved in nitrogen metabolic pathways, including ureolysis and nitrate reduction. Six endophytic bacteria belonging to four genera (*Pseudomonas, Flavobacterium, Rhizobium* and *Xanthomonas*) isolated from burley tobacco had strong abilities to reduce nitrate and nitrite, and they are also observed in the *S. vulgaris* endospheres. These endophytic bacteria can be used to reduce tobacco-specific nitrosamines (TSNA), which are carcinogens found in the tobacco plant (Zhu et al., 2004). The six endophytic bacteria may have a close affinity to bacteria involved in nitrogen metabolic pathways, and we may isolate endophytic bacteria from these four genera and investigate whether they were related to nitrogen metabolic pathways in future studies.

The FAPROTAX annotates the dominant endophytes *B. diminuta* and *R. leguminosarum* as plant pathogens; however, some studies offer evidence suggesting that they may also be beneficial to host plants. Singh et al. (2016) applied *B. diminuta* to rice and found it helped reduce arsenic accumulation, and that it produced IAA to obtain soluble phosphate and promote the growth of rice. Moreover, *R. leguminosarum* biovar. Phaseoli isolated from sludge-treated soil was found to form root nodules in white clover (*Trifolium repens*) (Chaudri et al., 1992; Chaudri et al., 1993). Purchase et al. (1997) found that *R. leguminosarum* were resistant to heavy metals, especially to cadmium, and that they could effectively conduct nitrogen fixation. In addition, Chabot et al. (1996) showed that *R. leguminosarum* promoted the growth of maize and lettuce via phosphate solubilization.

When studying the plant bacteriome, it is important to know whether a certain bacterium has plant growth-promoting traits (PGPT), such as the ability to produce indole acetic acid (IAA), hydrogen cyanide, siderophore, and ACC deaminase, the ability to fix nitrogen or solubilize phosphate, and antifungal activity. Because large culture collections are available for controlled experimentation, the function of plant-associated bacteria is becoming more accessible, and it is
anticipated that databases focusing on the PGPT diversity of plant bacteria will soon be available.

Conclusions

Bacterial 16S rRNA gene data obtained from rhizosphere soil and root and leaf endosphere samples in four *S. vulgaris* populations in a subtropical mountainous area revealed significant structural and functional differences between bacterial communities from different plant compartments and populations. However, similar endophytic communities formed from a shared core set of bacteria were acquired, despite a distance of over 100 km and an elevation range of 1,200–1,800 m. As expected, we observed heavy metal-resistant, phosphate-solubilizing and nitrogen-fixing bacteria, such as *B. diminuta* and *R. leguminosarum*, in *S. vulgaris* at relatively high abundance. However, the presence of cold-resistant bacteria was unexpected. The presence of these kind of bacteria might be important to the ability of *S. vulgaris* to adapt to harsh environments. Future studies should be conducted to isolate these endophytes in *S. vulgaris* plants and test their function *in vitro* and *in vivo*.

Acknowledgements

We are very thankful to colleges in Biological Department of CUG for their kind technical assistant.

Reference


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Rout ME, Callaway RM. 2012. Interactions between exotic invasive plants and soil microbes in the rhizosphere suggest that ‘everything is not everywhere’. *Annals of Botany* 110:213-222


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Figure 1 (on next page)

The map of four sampling locations in Shennongjia, Hubei Province, China
Venn diagrams of shared OTUs (number of OTUs) across three compartments of *Senecio vulgaris* plants and four sampling locations.

L=leaf endosphere, R=root endosphere, RS=rhizosphere; 1–4 represent the four sampling locations.
Figure 3 (on next page)

Estimated Shannon index (H’) in the bacterial communities of each compartment of *Senecio vulgaris* plants and sampling location
Figure 4 (on next page)

Phylum-level relative abundance plots of the bacterial communities associated with each compartment of *Senecio vulgaris* plants and sampling location.
Nonmetric multidimensional scaling (NMDS) plots for Bray–Curtis distances of the bacterial communities associated with each compartment of *Senecio vulgaris* plants and sampling location.
Figure 6 (on next page)

Family-level relative abundance plots of bacterial communities associated with each compartment of Senecio vulgaris plants and sampling locations.
**Figure 7** (on next page)

Relative frequency versus relative abundance of core bacterial operational taxonomic units (OTUs) in the root and leaf endospheres of *Senecio vulgaris* plants

OTUs: 1=Brevundimonas diminuta, 2=Exiguobacterium sibiricum, 3=Pseudomonas spp., 4=an undefined species from Alcaligenaceae, 5=Pseudomonas viridiflava, 6=an undefined species from Oxalobacteraceae, and 7=Duganella spp.
(a) Leaf endosphere

Relative frequency

Relative abundance

(b) Root endosphere

Relative frequency

Relative abundance
Figure 8 (on next page)

Functional community structure of bacterial communities associated with each compartment of Senecio vulgaris plants and sampling locations

L = leaf endosphere, R=root endosphere, RS=rhizosphere; 1-4 represent the four sampling locations.
Table 1 (on next page)

Effect of plant compartments and sampling locations on diversity and structure of bacterial communities in rhizo- and (leaf or root) endosphere of *Senecio vulgaris* plants

**P < 0.001; **P < 0.01; *P < 0.05.**
### (a) Two – way ANOVA test (Shannon index as independent variable)

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<th>Df</th>
<th>F value</th>
<th>P value</th>
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<td>2.47</td>
<td>0.07*</td>
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<tr>
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<td>209.40</td>
<td>0.001***</td>
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<td>0.50</td>
<td>0.81</td>
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<tr>
<td>Residuals</td>
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### (b) Permutational ANOVAs (relative abundance of the top 10 phyla as independent variable)

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<th>F value</th>
<th>P value</th>
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<td>0.022*</td>
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<td>Residuals</td>
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Table 2 (on next page)

Results of the multiple comparisons of diversity and relative abundance of the top five phyla of bacterial community from different compartments of *Senecio vulgaris* plants

***P < 0.001; **P < 0.01; *P < 0.05
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<td>t. value</td>
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<td>17.12 ***</td>
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<td>Rhizosphere - Root endosphere</td>
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<td>17.85 ***</td>
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<td>(b) Acidobacteria</td>
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<td></td>
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<tr>
<td>Root endosphere-Leaf endosphere</td>
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<td>0.00</td>
<td>11.78 ***</td>
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<td>0.00</td>
<td>11.26 ***</td>
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<td>(c) Actinobacteria</td>
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<td>Root endosphere-Leaf endosphere</td>
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<td>0.01</td>
<td>-5.71 ***</td>
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<td>0.01</td>
<td>6.23 ***</td>
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<td>(d) Bacteroidetes</td>
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<td>(e) Firmicutes</td>
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<td>3.41 **</td>
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Table 3 (on next page)

The bacterial taxa dominating in the endosphere of Senecio vulgaris plants *

*This table summarized the taxa information of the core OTUs in endosphere of S. vulgaris plants, details of the core OTUs can be seen in Table S2-3; /=unidentified taxa
<table>
<thead>
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<td></td>
<td></td>
<td></td>
<td>Pseudomonadales</td>
<td>Pseudomonas</td>
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<td><strong>Only in leaves</strong></td>
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<td>Actinobacteria</td>
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<td><strong>Only in roots</strong></td>
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</table>
Table 4 (on next page)

Dominant bacterial families in the root and leaf endosphere of *Senecio vulgaris* plants reported as core members in previous studies

\(^a\) Dominant family in root endosphere of *S. vulgaris*, \(^b\) Dominant family in leaf endosphere of *S. vulgaris* (Figure 6). √ corresponds to bacterial families present as core members. *Arabidopsis thaliana*, Barely and Rice are based on Müller *et al.* and references in there (2016); *Vitis* spp. based on Samad *et al.* (2017); *Oxyria digyna* and *Saxifraga oppositifolia* based on Kumar *et al.* (2017); *Populus tremula*, and *Populus alba* based on Beckers *et al.* (2016); *Salicornia europaea* based on Zhao *et al.* (2016); *Helianthus annuus* based on Leff *et al.* (2016); *Sequoia sempervirens* and *Sequoia dendrongiganteum* based on Carrell & Frank (2015).
<table>
<thead>
<tr>
<th>Family</th>
<th>Root endosphere</th>
<th>Leaf endosphere</th>
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<tr>
<td>Caulobacteriaceae&lt;sup&gt;a, b&lt;/sup&gt;</td>
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<td>Pseudomonadaceae&lt;sup&gt;a, b&lt;/sup&gt;</td>
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<td>√</td>
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<td>Sphingomonadaceae&lt;sup&gt;a, b&lt;/sup&gt;</td>
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<td>Oxalobacteriaceae&lt;sup&gt;a, b&lt;/sup&gt;</td>
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<td>√</td>
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<td>Flavobacteriaceae&lt;sup&gt;a, b&lt;/sup&gt;</td>
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<td>Propionibacteriaceae&lt;sup&gt;b&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup> Representative species include: *A. thaliana*, *P. tremula, P. alba*, *H. annuus*.<br>
<sup>b</sup> Representative species include: *O. digyna, S. oppositifolia*, *S. europaea*, *S. sempervirens, S. giganteum*.