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Two cultivated legume plants reveal the enrichment process of the microbiome in the rhizocompartments

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Abstract

The microbiomes of rhizocompartments (nodule endophytes, root endophytes, rhizosphere and root zone) in soya bean and alfalfa were analysed using high-throughput sequencing to investigate the interactions among legume species, microorganisms and soil types. A clear hierarchical filtration of microbiota by plants was observed in the four rhizocompartments – the nodule endosphere, root endosphere, rhizosphere and root zone – as demonstrated by significant variations in the composition of the microbial community in the different compartments. The rhizosphere and root zone microbial communities were largely influenced by soil type, and the nodule and root endophytes were primarily determined by plant species. Diverse microbes inhabited the root nodule endosphere, and the corresponding dominant symbiotic rhizobia belonged to *Ensifer* for alfalfa and *Ensifer–Bradyrhizobium* for soya bean. The nonsymbiotic nodule endophytes were mainly Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes. The variation in root microbial communities was also affected by the plant growth stage. In summary, this study demonstrated that the enrichment process of nodule endophytes follows a hierarchical filtration and that the bacterial communities in nodule endophytes vary according to the plant species.

Keywords: 16S rRNA gene amplicons, bacterial community, Illumina sequencing, nodule endophytes, rhizocompartments

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Introduction

As the driving force in the transformation of organic matter and nutrients, soil microorganisms play a vital role in the stability and sustainability of ecosystems (Kennedy & Smith 1995). Among plant-associated microorganisms, microbiota in the rhizosphere have attracted extensive attention (Bulgarelli *et al.* 2012; Lundberg *et al.* 2012; Philippot *et al.* 2013; Edwards *et al.* 2015). Inhabiting this narrow zone surrounding plant roots, an overwhelming number of microbes strongly affect the growth, nutrition and health of plants and even the structure of plant communities (Hoeksema *et al.* 2010; Maron *et al.* 2011; Ke & Miki 2015). In turn, plant roots substantially influence the growth and community structure of rhizospheric microbes by altering soil pH and structure and supplying carbon-rich exudates (Maron *et al.* 2011). In addition, rhizospheric microbes are influenced by the biotic and abiotic factors of the soil, including plant genotype (Lundberg *et al.* 2012), geographical location (Peiffer *et al.* 2013) and soil physicochemical characteristics (Fierer & Jackson 2006; Angel *et al.* 2010; Andrew *et al.* 2012; Inceoglu *et al.* 2012). Furthermore, some rhizospheric microbes can enter root tissues to form an endophyte microbiome, which may have a community composition substantially different from that of the rhizospheric microbiome (Gottel *et al.* 2011; Lundberg *et al.* 2012). Therefore, the root endophyte microbiome is not an opportunistic subset of the rhizospheric microbiome but may instead be selected by complex factors such as

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plant genotype and breed and plant growth stages (Bulgarelli *et al.* 2012; Lundberg *et al.* 2012; Peiffer *et al.* 2013). Edwards *et al.* (2015) found that the assembly of the rice root microbiome from the soil is a multistep process and that the rhizoplane plays a selective gating role. However, the interactions among nodule endophytes, rhizospheric microbes, plant species and soil type have not been sufficiently investigated.

Previous studies of the interactions between plant roots and microbial communities in the rhizosphere have found that nodule-forming legumes are different from other plants due to their symbiosis with diverse bacteria (rhizobia) in Alphaproteobacteria and Betaproteobacteria (Weir 2016). Specific associations between legume species and rhizobial species are determined by symbiotic genes on both sides but are also regulated by soil conditions (Mendes *et al.* 2014; Yan *et al.* 2014; Zhao *et al.* 2014; Saini *et al.* 2015). Depending on the soil conditions, soya bean plants can nodulate with various species in the genera *Bradyrhizobium*, *Ensifer* and *Rhizobium*, in which certain lineages of nodulation genes have been defined (Wu *et al.* 2011; Yan *et al.* 2014; Zhao *et al.* 2014). Alfalfa plants mainly nodulate with *Ensifer meliloti* strains with very similar nodulation genes in different geographical regions and soil types (van Berkum *et al.* 2006, 2010; Alias-Villegas *et al.* 2015). Cultivation methods have been used to reveal that root nodules are occupied not only by symbiotic bacteria but also by nonsymbiotic endophytic bacteria (Sturz *et al.* 1997; De Meyer *et al.* 2015; Saini *et al.* 2015), such as *Endobacter* (Ramirez-Bahena *et al.* 2013), *Paenibacillus* (Lai *et al.* 2015), *Burkholderia* (Diouf *et al.* 2007), *Herbaspirillum* (Hoque *et al.* 2011; Weiss *et al.* 2012), *Pseudomonas* (Deng *et al.* 2011), *Enterobacter* (Ibanez *et al.* 2009; Deng *et al.* 2011) and *Klebsiella* (Ibanez *et al.* 2009). The application of high-throughput sequencing of metagenomic DNA has revealed that leguminous species could significantly influence the diversity patterns of Rhizobiaceae communities inhabiting soils, root surfaces and nodules (Miranda-Sanchez *et al.* 2016). However, the diversity and specificity of nonsymbiotic microbes associated with legume roots, their interactions with the symbiotic microbes and their responses to soil conditions and plant growth remain unclear.

Therefore, the objectives of this study were as follows: (i) to investigate the hierarchical community compositions of microbes in the four rhizocompartments of legumes – nodule endosphere, root endosphere, rhizosphere and root zone (a narrow region outside the rhizosphere) – providing a spatial gradient to assist in observing the variation in the microbial community from the outer soil to the nodules; (ii) to clarify the structure and composition of legume nodule endophytes; and (iii) to illustrate the effects of legume species and soil types on the microbial community of each rhizocompartment.

Materials and methods

Soil collection and preparation

Farmland soil samples representing the main Chinese soil types – chernozem (CH) (Mollisol) in Heilongjiang Province (46°24′10.2″N, 125°21′59.5″E), cinnamon soils (CI) (Alfisol) in Shaanxi Province (34°4′14.88″N, 108°36′8.28″E) and red earth (RE) (Acrorthox) in Jiangxi Province (28°21′41.5″N, 115°55′0.80″E) – were collected separately. Surface soils (0–20 cm) were collected in sterile plastic zip-lock bags, transported to the laboratory under environmental temperature and stored at 4 °C before further processing. The soil samples were passed through a sterile 2-mm sieve (Lundberg *et al.* 2012). The soil physicochemical properties were analysed using routine methods (Page 1982) and included pH, organic matter, total phosphorus (TP), total nitrogen (TN), available nitrogen (AN), available phosphorus (AP) and available potassium (AK) (Table S1, Supporting information).

Plant cultivation

This experiment was performed with a factorial design as shown in Fig. 1. Soya bean and alfalfa were grown separately in three types of soil (CI, CH and RE) and represented grain legumes and forage legumes, respectively.

After surface sterilization (Edwards *et al.* 2015), the seeds were germinated at 28 °C in the dark for 2 days on a water agar medium. Surface-sterilized seeds and water from the final rinse were placed on LB plates and YMA plates (De Meyer *et al.* 2015) for 5 days of cultivation at 28 °C to ensure that no viable microbes were left on the seed. Pregerminated seeds, three for soya bean and eight for alfalfa, were aseptically transplanted into pots (diameter × height: 18 × 11 cm) that were cleaned with 75% ethanol and filled with soil. A total of 162 pots (two legumes species × three soils × three periods × three replicates (six pots) + three soils × three periods × six pots (without plants)) were randomly placed in a glasshouse to ensure relatively stable culture conditions with 16 h of light at 25 °C and 8 h of dark at 20 °C at a relative humidity of 45%. Pots without plants served as controls. All pots were watered every 2 days with distilled water.

Sampling strategy

Samples were collected at three time points: Period 1, Period 2 and Period 3, that is 25, 40 and 55 days after sowing, respectively. These three periods corresponded to the vegetative growth stage, flowering stage and

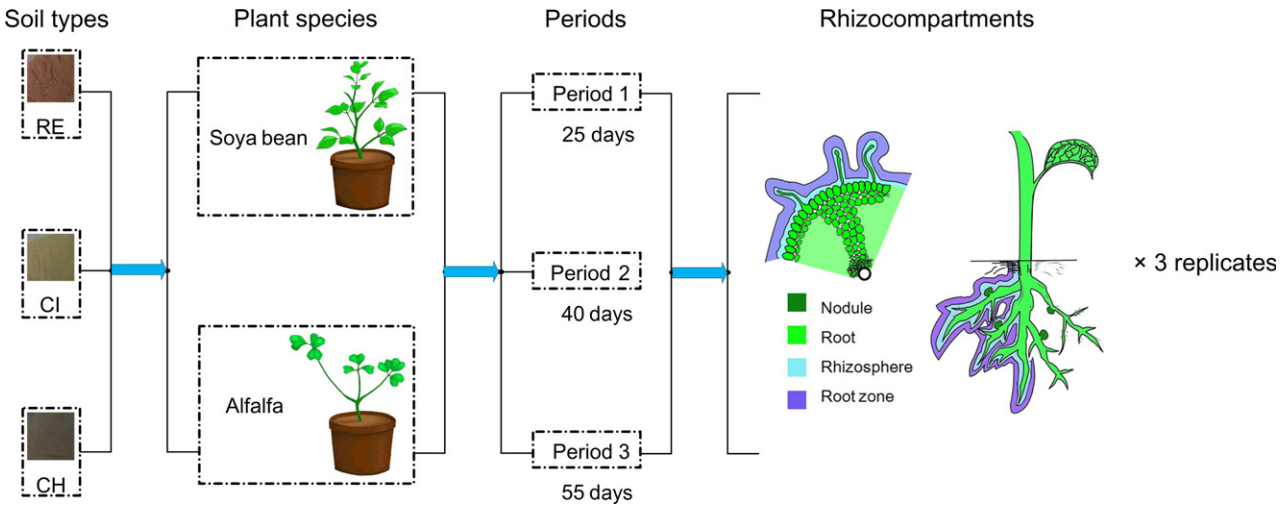

Fig. 1 The flow chart of the experimental design and the sketch of four rhizocompartments, which include nodule endosphere, root endosphere, rhizosphere soil and root zone soil. The RE, CI and CH were abbreviations for red earth, cinnamon soil and chernozem, respectively. [Colour figure can be viewed at wileyonlinelibrary.com]

podding stage for soya bean, respectively, but were all within the vegetative growth stage for alfalfa. Each time, the samples consisted of four rhizocompartments: nodule, root, rhizosphere soil and root zone soil (Fig. 1).

Plant roots, rhizospheric soils and root zone soils were collected according to the method described by Bulgarelli *et al.* (2012). The detailed sampling procedure is shown in the Supporting Information (SI). Due to the small number of root nodules observed on the plants, nodules from all the plants in two pots were compiled as one replicate to ensure that enough DNA could be extracted. Finally, a total of 234 DNA samples (two plant species × three soils × three periods × four rhizocompartments × three replicates + three bulk soil (no plant cultivation) × three periods + three bulk soil (before transplantation) × three replicates) were used for PCR amplification of the V4-V5 regions and the subsequent metagenomic sequencing (Fig. 1).

Sample preparation and metagenomic DNA extraction

We compared two washing procedures to ensure that endophytes and rhizospheric microbes could be completely separated. Roots after washing according to the method described by Bulgarelli *et al.* (2012) and roots rinsed only with double-distilled water were both photographed using scanning electron microscopy (Bulgarelli *et al.* 2012). No bacteria were observed on the root surface when using the first washing method (Fig. S1, Supporting information).

Metagenomic DNA was extracted from the soil samples (0.5 g for each) using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, CA, USA) and from plant tissues (roots and nodules, 0.5 g each) with a DNA secure Plant Kit (Tiangen Biotech, Beijing, China) according to the manufacturers' instructions. The soil humus, which can inhibit the subsequent PCR procedure, was removed by adding 20% (w/v) polyvinylpolypyrrolidone after lysis. The DNA concentration and purity were estimated using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and electrophoresis in 1% (w/v) agarose gel (Trujillo *et al.* 2010).

Sequencing of 16S rRNA genes and bioinformatics analyses

The hypervariable V4-V5 region of the 16S rRNA gene was amplified using the primers F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R926 (5′-CCGYC AATT YMTTTRAGTTT-3′) (Peiffer *et al.* 2013). Each DNA sample was amplified in triplicate, and no-template controls were included in all steps of the process. The total volume of the PCR mixture was 50 μL, which consisted of 0.5 μL of each primer (50 pmol), 5 μL of 2.5 mmol dNTP mixture, 5 μL of 10 × *Ex* Taq buffer (20 mmol Mg^{2+}; TaKaRa Inc., Dalian, China), 0.25 μL of *Ex* Taq DNA polymerase (TaKaRa) and 1 μL of the DNA template. The cycle conditions for the PCR amplification were as follows: initial denaturation at 94 °C for 3 min; followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 30 s; and an extension step at 72 °C for 5 min after cycling was complete.

Three replicates of purified PCR amplicons representing one DNA sample were combined into a single tube in equimolar ratios and then sequenced using an Illumina Miseq 250PE at Macrogen Inc. (http://www.macrogen.com, Seoul, South Korea) for 500 cycles using two

lanes of the flow cell. The PCR products were gel-purified using a QIAquick Gel Extraction Kit (Qiagen). The concentration of each amplicon was determined using the Quant-iT PicoGreen dsDNA reagent kit (Life Technologies, Merelbeke, Belgium).

The 16S rRNA sequences were denoised (homopolymer error correction) using DENOISER V0.91 software (Edwards *et al.* 2015). Chimeric sequences were identified and removed using USEARCH (Edgar *et al.* 2011). The sequences were then assigned to each sample with a 12-bp barcode using a script derived from the QIIME (Quantitative Insights into Microbial Ecology) pipeline (Caporaso *et al.* 2010). High-quality sequences from all samples were clustered into OTUs at 97% sequence similarity (OTU_{97}) using the default QIIME pipeline UCLUST. The representative sequence in each OTU_{97} was assigned to taxonomic groups using the RDP classifier with an 80% confidence threshold (Edwards *et al.* 2015).

Statistical analysis

Analyses were performed on the normalized data set. The alpha diversity index, analysis of similarities (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) were calculated or performed with 999 permutations using the 'Vegan' package v2.3-0 of R version 3.1.1 (Oksanen *et al.* 2007). Wilcoxon tests (Mann–Whitney *U*-test) were employed to compare the alpha diversity between the communities in different samples using the R package 'Stats' v3.1.1. Principal coordinate analysis (PCoA) of the UniFrac distance was performed using the R package 'Ape' v3.4. The 'EdgeR' package v3.8.6 was used to identify significantly altered OTU_{97} with environmental changes. Weighted and unweighted UniFrac distances (WUF and UUF) from all samples were calculated using QIIME. ANOSIM was performed to test the difference between rhizocompartment microbiomes based on the Bray–Curtis similarity matrix. PERMANOVA and PCoA based on WUF and UUF were performed. GraPhlAn (graphical phylogenetic analysis) was performed following the process described by Asnicar *et al.* (2015). Statistical analyses of the co-occurrence network were carried out in the R environment, and network visualization was carried out using the interactive platform Gephi (Bastian *et al.* 2009).

Results

In this experiment, soya bean and alfalfa were grown in three types of soil and sampled at three sampling periods. The composition and structure of microbial communities in the four rhizocompartments were compared to estimate their responses to different abiotic and biotic factors.

Variation of alpha diversity of microbiomes in relation to environmental factors

After quality control, a total of 11 691 935 DNA sequences were obtained with a size of 333–492 bp and were classified into 34 phyla. In general, taxon numbers at all levels (from genus to phylum) were similar among the microbiomes in the bulk soils, root zone soils and rhizosphere soils and between the microbiomes for root endosphere and root nodule endosphere; however, these numbers were significantly greater in the soils than in endospheres (Table S2, Supporting information). A similar trend was also found in the evenness, richness and diversity indexes, except in the case of coverage values (Table S2, Fig. S2, Supporting information). The Shannon–Weiner index and richness significantly increased in the order of nodule < root < rhizosphere < root zone soil (Figs 2a and S3, Tables S3 and S4, Supporting information).

Our results showed that there were significant differences in the Shannon–Weiner indexes of the microbiomes of root endosphere, rhizosphere and root zone samples among the soil types (Table S3, Supporting information). The Shannon–Weiner indexes of nodule samples were not significantly different among the soil types (Table S3, Supporting information). The species richness in all rhizocompartments was significantly different among the soil types (Table S4, Supporting information). Furthermore, there were significant differences in the Shannon–Weiner index and species richness between the microbiomes associated with soya bean and alfalfa (Tables S3 and S4, Supporting information). The Shannon–Weiner indexes of microbiomes in the nodule and root endosphere of soya bean were greater than those of alfalfa. However, the opposite was true for the rhizosphere and root zone samples (Table S3, Supporting information). The Shannon–Weiner indexes of microbiomes in the root zone and root endosphere significantly changed over time, while the microbiomes in other rhizocompartments did not change over time (Table S3, Supporting information). The species richness of microbiomes in the rhizocompartments of the two plants was not significantly different among sampling times (Table S4, Supporting information).

Variation in microbial community composition in response to environmental factors

The microbiomes of root zone and rhizosphere soils were found to belong to 30–31 phyla, which is similar to the number in the bulk soils (29 phyla). Root zone and rhizosphere microbiomes were dominated by Proteobacteria (31.16%, 40.43%), Acidobacteria (13.91%, 8.43%), Bacteroidetes (13.47%, 12.92%) and Actinobacteria (12.35%, 18.42%), although the relative abundances

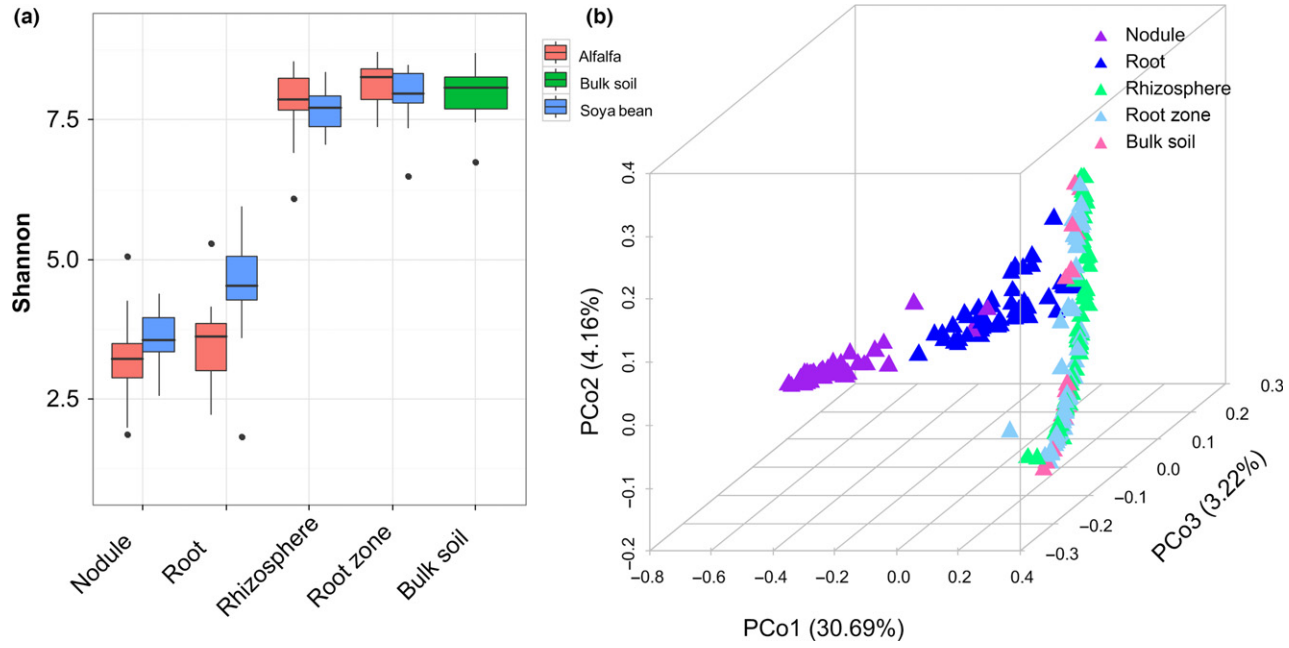

Fig. 2 The boxplot of Shannon–Weiner index of the microbial community colonized in bulk soil and the soya bean and alfalfa rhizocompartments (a) and the dot plot of PCoA of all samples using Weighted UniFrac distance metric (b). [Colour figure can be viewed at wileyonlinelibrary.com]

varied (Fig. 3). The root and root nodule endosphere microbiomes were composed of 28 and 27 phyla, respectively. The main difference between the root and the root nodule was the super-dominance of Proteobacteria (59.32%) in the nodules, while Proteobacteria (23.96%), Actinobacteria (14.59%) and Bacteroidetes (3.39%) were the main groups in the roots (Fig. 3).

The analysis of similarities (ANOSIM) revealed no significant difference in microbial communities among bulk soil before planting and at the three sampling stages (R = 0.1432, P = 0.136). ANOSIM revealed a striking difference between the structure of bulk soil and root zone soil microbial communities (R = 0.1254, P = 0.012). The PCoA of all samples based on WUF and UUF confirmed that the endophytic microbiomes clearly separated from the soil microbiomes, as shown in Fig. 3 (Figs 2b and S3, Supporting information). PERMANOVA also indicated that the microbiomes in the four rhizocompartments were significantly different (WUF: 66.57%, P = 0.001; UUF: 72.32%, P = 0.001; Table S5, Supporting information). Even when rhizobia were removed, the structure and composition of nodule endophytes and root endophytes remained significantly different (R = 0.239, P = 0.001) based on ANOSIM.

To illustrate the influence of plant species and soil type, PCoA was performed on each rhizocompartment based on WUF and UUF (Fig. S3, Supporting information). Nodule and root samples belonging to different plant species generally separated into two groups – soya bean and alfalfa – along PCA axis 1 (Fig. S3, Supporting information). Rhizosphere and root zone samples from distinct soils separated from each other (Fig. S3, Supporting information).

Furthermore, PERMANOVA was performed to confirm the PCoA results (Table S5, Supporting information). Nodule endophytes, root endophytes and rhizospheric microbiomes were significantly different between soya bean and alfalfa. The influence of plant species on the structure and composition of the microbiome increased from the outer layer (root zone) to the inner layer (nodule) (Table S5, Supporting information). Root endophytes, rhizospheres and root zone microbiota were significantly different from each other among different soil types. The influence of soil type exhibited an opposite trend compared with that of plant species (Table S5, Supporting information). The structure and composition of root endophytes were significantly affected by plant growth, while other rhizocompartments were not significantly different among different sampling stages (Table S5, Supporting information).

Variation of microbes in different rhizocompartments

The OTUs varied significantly between the rhizocompartments and bulk soils. The OTU counts from bulk soil were taken as a control with a P-value cut-off of 0.05. Adjusted P-values for multiple comparisons were calculated using Benjamini and Hochberg's algorithm.

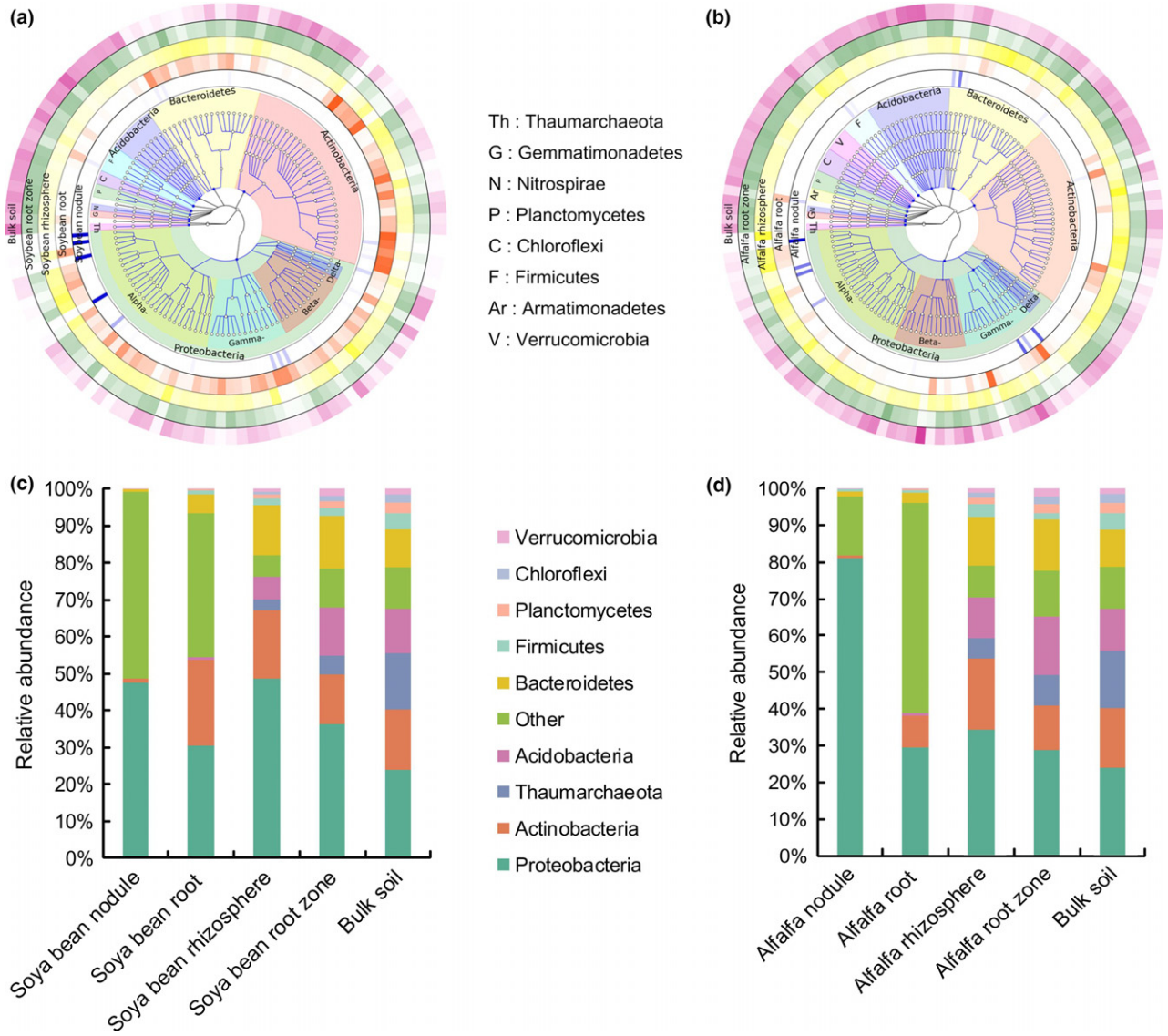

Fig. 3 (a) The GraPhlAn (Graphical Phylogenetic Analysis) of the soya bean (a) and alfalfa (b) rhizocompartments. The GraPhlAn was constructed based on the phylogenetic tree of the microbes in all the samples. The rings around the phylogenetic tree are the heatmaps of microbial community of samples, which include the nodule endosphere, root endosphere, rhizosphere, root zone and bulk soil. The heatmaps are based on genus level. The deeper the colour of heatmap is, the greater the abundance of microbe is. c, d, Mean relative abundances (%) of dominant lineages (phylum level) in the rhizocompartments (nodule, root, rhizosphere and root zone) and bulk soil of soya bean (c) and alfalfa (d). [Colour figure can be viewed at wileyonlinelibrary.com]

Compared to that of other rhizocompartments, the composition and structure of microbial communities of the root zone soil were more similar to those of the bulk soil, as shown in the Venn diagram (Fig. 4). Compared with microbiomes in bulk soils, 60, 146, 299 and 269 significantly enriched OTUs and 939, 1698, 4136 and 5772 significantly depleted OTUs were detected in root zone soils, rhizosphere soils, the root endosphere and the nodule endosphere, respectively (Fig. 4).

Some abundant OTUs overlapped among compartments (Fig. 4); of the 60 enriched OTUs in the root zone soils, 10, 5 and 1 were also significantly enriched in the rhizosphere, root endosphere and nodule endosphere, respectively. Of the 146 significantly enriched OTUs in the rhizosphere, 58 and 9 were also significantly enriched in the root and nodule endosphere, respectively. Finally, of the 299 significantly enriched OTUs in the root endosphere, 120 were also significantly enriched in the nodule endosphere (Fig. 4). These data indicated that more significantly enriched OTUs were shared between the nodule endosphere and root endosphere than with the rhizosphere. A substantial component of the OTUs

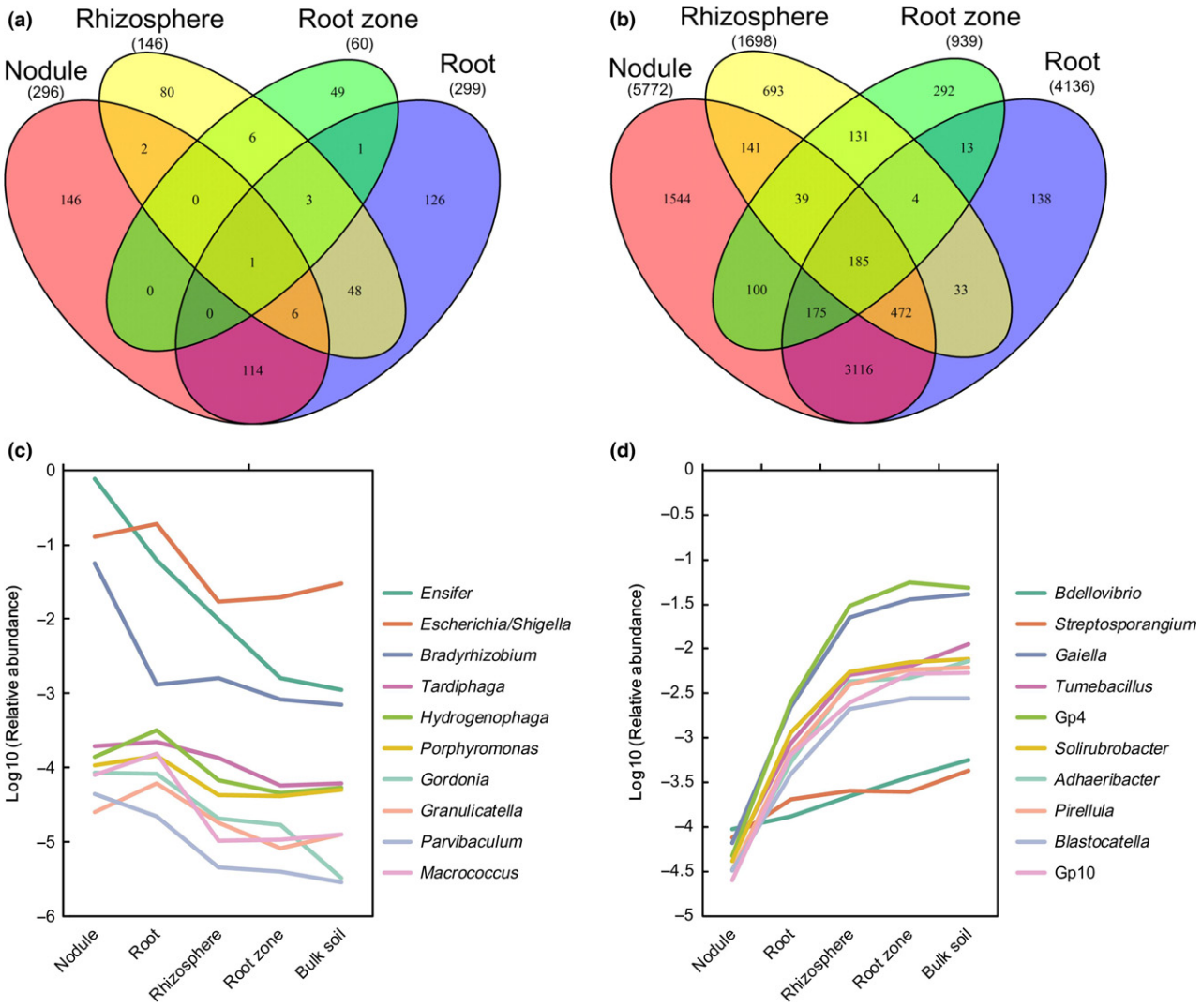

Fig. 4 Venn diagrams of the significantly enriched (a) and depleted (b) OTUs of the microbiome colonized in rhizocompartments compared with the bulk soil. Substantial components of significantly enriched microbes in nodule (c) compared with bulk soil, root zone soil and rhizosphere soil. Substantial components of significantly depleted microbes in nodule compared with bulk soil, root zone soil, rhizosphere soil and root (d). [Colour figure can be viewed at wileyonlinelibrary.com]

significantly enriched in nodules belonged to *Ensifer*, *Bradyrhizobium*, *Escherichia*, *Tardiphaga*, *Hydrogenophaga*, *Porphyromonas*, *Gordonia* and other genera (Fig. 4).

The depleted OTUs were mainly members of Proteobacteria, Actinobacteria Acidobacteria, Bacteroidetes and Firmicutes. Nearly all of the significantly depleted OTUs (3948/4136) in the root endosphere were also significantly depleted in the nodule endosphere (Fig. 4). Of the 1698 OTUs significantly depleted in the rhizosphere, 694 and 837 were also significantly depleted in the root endosphere and in the nodule endosphere, respectively (Fig. 4). Of the 939 OTUs significantly depleted in the root zone soil, 359 were also significantly depleted in the rhizosphere. A substantial number of the OTUs significantly depleted in nodule and root belonged to *Bdellovibrio*, *Streptosporangium*, *Gaiella*, *Tumebacillus*, Gp4, *Solirubrobacter* and other genera (Fig. 4).

These results suggested that each rhizocompartment possesses a unique filtration function. Among the compartments, the nodule endosphere had the strongest filtration function. In addition, the influence of plant roots on soil was not restricted only to rhizospheric microbiome but also to the root zone microbiome because the filtration function was initiated at the root zone.

Co-occurrence network analyses also indicated the filtration function. The co-occurrence patterns were based on strong and significant correlations between OTUs (nonparametric Spearman's, $P < 0.01$ and $R > 0.6$). Low-abundance OTUs were eliminated from the OTU table if they did not have a total of at least 100 counts across

all the samples in the experiment. A comparison of highly connected OTUs among rhizocompartments is presented in Fig. S4 (Supporting information). The co-occurrence network analyses demonstrated that the highly connected OTUs varied in different rhizocompartments (Fig. S4, Supporting information). The networks for the rhizosphere and root zone OTUs corresponded to 15 phyla, which were more complex than those for the nodule and root endospheres in both soya bean and alfalfa (Fig. S5, Supporting information). These results also demonstrated that the root and nodule endospheres possess a strong filtration function.

The composition of nodule endophytes

At the phylum level, Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes were the main nodule endophytes (Fig. 2), with Proteobacteria accounting for 95.88% and 96.68% of these four phyla for soya bean and alfalfa, respectively (Fig. S6, Supporting information). At the order level, Rhizobiales and Enterobacteriales occupied the greatest proportion of Proteobacteria for soya bean and alfalfa. The most abundant genera were *Ensifer* and *Bradyrhizobium* in soya bean nodules, and the most abundant genus was *Ensifer* in alfalfa nodules (Fig. S6, Supporting information). In the Venn diagram based on bacterial genus (Fig. S7, Supporting information), the community compositions of nodule and root endophytes of alfalfa were the same; 439 genera in total were detected. For soya bean, 325 genera were identified in the nodule endosphere, and 311 of these overlapped with those in the root endosphere (Fig. S7, Supporting information).

The phylum Actinobacteria accounted for 2.29% and 0.67% of the population in soya bean and alfalfa nodules, respectively. In the phylum Bacteroidetes, orders Sphingobacteriales, Bacteroidales, Flavobacteriales, Ohtaekwangia and Cytophagales were detected. In the phylum Firmicutes, orders Bacillales, Selenomonadales, Lactobacillales and Clostridiales were found in the nodules of both plants, while Erysipelotrichales was only present in alfalfa nodules.

Discussion

It is widely accepted that the root nodules of legume plants form a special niche for nitrogen fixation mainly colonized by rhizobia. The present study demonstrated that diverse non-nitrogen fixation microbes are present in root nodules, despite the dominant existence of symbiotic nitrogen fixation bacteria. These findings confirmed the coexistence of nitrogen-fixing bacteria and other bacteria in the nodules, as shown by the culture-dependent methods, but the present study greatly expanded the diversity of nodule endophytes. It has also been demonstrated that these diverse endophytes in the nodules and roots are filtered by plants from the bulk soil microbiome.

The filtration function of rhizocompartments

Clear hierarchical filtration effects for microbes were observed in the four rhizocompartments following a spatial gradient in the order nodule > root > rhizosphere > root zone (Figs 2–4). Such filtration effects have been previously reported in the root endosphere, rhizoplane and rhizosphere of rice (Edwards *et al.* 2015); the root endosphere and rhizosphere of *Arabidopsis* (Lundberg *et al.* 2012); and nodule/bulk soil rhizobial communities of *Acacia* and *Phaseolus vulgaris* (Miranda-Sanchez *et al.* 2016). This filtration was related to the effects of roots on nutrient supply and the physicochemical features of the rhizosphere as well as to the change in environment from soil to endosphere (Gottel *et al.* 2011). Consistent with the results of Miranda-Sanchez *et al.* (2016), our results demonstrated that the nodule bacteria (rhizobia) undergo a hierarchical filtration through bulk soil to the root by legume plants. In addition, our study indicated that both nonsymbiotic and symbiotic nodule endophytes are subjected to hierarchical filtration.

Previously, plant root exudates and plant genotype have been proposed to play predominant roles in the selection of rhizospheres and plant endosphere microbiota (Edwards *et al.* 2015; Miranda-Sanchez *et al.* 2016), and the changes in microbiota between the nearby rhizocompartments might also be related to specified metabolic functions (Young *et al.* 2014). Therefore, the bacterial community in each rhizocompartment might be shaped by the specific niche associated with it. In the present study, the discovery that soil type has a major effect on the soil (rhizosphere and root zone) microbiome and that the plant species had major effects on the endophytic microbiomes supports the suggestion that niche-related selection of functional groups of microbes causes the filtration in rhizocompartments. Therefore, the filtration effects between the rhizocompartments were not due to the physical barriers but, rather, to niche characteristics. That a smaller number of co-occurring phyla were found in the inner rhizocompartments than in the outer ones also supported this suggestion.

For both soya bean and alfalfa plants, although the enriched OTUs in the nodule endosphere had little overlap with those in rhizosphere and in the root zone soil (Fig. S8, Supporting information), half of the total enriched OTUs from the nodule endosphere were shared with those from the root endosphere (Fig. S8, Supporting information). Moreover, the vast majority of microbes depleted in the nodule endosphere were also depleted in the root endosphere (Fig. S8, Supporting information). These data suggested that the root may play a gating role

that restricts microbes from permeating into the nodule endosphere, which is supported by the dynamic change of the network (Fig. S4, Supporting information).

The community composition of nodule endophytes

Using high-throughput sequencing, the present study provided a directory of the nodule endophytes of soya bean and alfalfa. The detection of 1818 OTUs corresponding to 435 genera in 21 phyla in soya bean nodules and 3783 OTUs in 523 genera in 26 phyla in alfalfa nodules (Table S2, Supporting information) greatly improved our knowledge of the diversity of endophytic microbes in the nodules. Previously, few genera were identified as endophytic bacteria in the nodules of different legumes based on isolation methods (Sturz *et al.* 1997; Moulin *et al.* 2001; De Meyer *et al.* 2015; Saini *et al.* 2015). Therefore, our results demonstrated that the root nodules of legumes are a habitat harbouring bacteria far more diverse than previously known.

Although the great diversity of nodule endophytes was a surprise, the super-dominance of *Ensifer* and *Bradyrhizobium* in soya bean nodules and *Ensifer* in alfalfa nodules was expected (Fig. S6, Supporting information) because of the known symbiotic bacteria associated with these two plants (van Berkum *et al.* 2010). The existence of OTUs corresponding to *Rhizobium* and *Mesorhizobium*, which are possible microsymbionts for other legumes, in soya bean and alfalfa nodules indicated that the symbiotic rhizobia might also be endophytes in the nodules of legumes other than their hosts (Gage 2002; Peix *et al.* 2014). In addition, the dominant nodule endophytes, *Ensifer* and *Bradyrhizobium*, were relatively rare in bulk soil. This phenomenon was also observed in *Phaseolus vulgaris* (Miranda-Sanchez *et al.* 2016). The detection of Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes as the main phyla in the nodules of both plant species demonstrated the common preference of the nodules for the endophytes (Fig. S6, Supporting information), and this common preference was also evident at the genus level (Fig. S6, Supporting information). Compared with the culture-dependent analyses (Sturz *et al.* 1997; Mhamdi *et al.* 2005; Li *et al.* 2008; De Meyer *et al.* 2015), the common nodule endophytes *Agrobacterium* and *Bacillus* were not abundantly detected in the nodules analysed in the present study, indicating that the isolation-mediated procedure cannot precisely reveal the community composition and abundance of nodule endophytes.

Some species in the genera *Burkholderia*, *Achromobacter*, *Rhodococcus*, *Pseudomonas*, *Azospirillum*, *Azoarcus* and *Bacillus* detected in the nodules have been reported to possess the ability to promote plant growth (Deng *et al.* 2011; Gaiero *et al.* 2013, Saini *et al.* 2015). In addition, co-inoculating nodule endophytes with rhizobia can promote plant yield and nodulation (Rajendran *et al.* 2008). These previous results suggest possible functions of the nonsymbiotic endophytes in the nodules. Furthermore, the previous detection of symbiotic nitrogen-fixing functions in some species of the genera *Methylobacterium* (Sy *et al.* 2001), *Devosia* (Rivas *et al.* 2002), *Blastobacter* (Van Berkum & Eardly 2002), *Ochrobactrum* (Ngom *et al.* 2004), *Shinella* (Li *et al.* 2008), *Pseudomonas* and *Burkholderia* (Moulin *et al.* 2001) and the detection of sequences related to these genera might be indirect evidence that they have acquired symbiotic genes from the symbiotic bacteria in the nodules as proposed in the related studies. The significant differences between the composition of nonrhizobia nodule endophytes and the root endophytes may suggest that the nonrhizobia nodule endophytes are not only passengers in the nodule but are also essential members possessing biological functions. This possibility warrants further studies of gene transcription and translation levels.

Conclusion

This study found that legume nodules are a habitat for diverse bacteria and demonstrated that the enrichment of legume nodule endophytes is based on the plant species. Distinct bacterial communities in different rhizocompartments followed a hierarchical filtration function. Furthermore, the microbial communities of the rhizosphere and root zone were mainly influenced by soil type and the nodule and root endophytes were primarily determined by plant species. The variation of microbial communities in the rhizocompartments was also affected by the plant growth stage.

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Conflict of interest

The authors declare no conflict of interest.

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G.W. and X.X. conceived and designed the experiments, and W.C. participated in the design of experiments. X.X. performed all experiments with help from W.C., L.Z., J.Y., S.J. and Y.B. X.X. analysed experimental results. X.X. and W.C. wrote the manuscript. E.W. assisted with manuscript preparation and revised the manuscript.

Data accessibility

The 16S rDNA Illumina libraries obtained from the sequencing company were deposited at the NCBI's small read archive (SRA) in BioProjectID PRJNA325735, with Accession no. SRP076750, and run number SRR3714933 – SRR3714936.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 The rhizosphere soil washing method employed in the sampling procedure.

Fig. S1 Photos of root surface by scanning electron microscope.

Fig. S2 Rarefaction curves of all samples.

Fig. S3 Boxplot of species richness and dot plots of PCoA of samples using UUF and WUF between different factors.

Fig. S4 Networks of co-occurring OTUs colonized in rhizocompartments based on correlation analysis. A connection stands for strong (Spearman's $r > 0.6$) and significant ($P < 0.01$) correlation. Each node represents an OTU. Different colors of nodes represent OTUs belonged to different phyla. The size of each node is proportional to the number of connections.

Fig. S5 Histograms of the nodes in each rhizocompartment co-occurrence network corresponding to phylum.

Fig. S6 Pie charts of the relative abundance of nodule endophytes.

Fig. S7 Venn diagrams of four rhizocompartments based on genus.

Fig. S8 Venn diagrams of the enriched and depleted OTUs of the microbiota colonized in soybean and alfalfa rhizocompartments compared with bulk soil.

Table S1 Soil micronutrient analysis.

Table S2 General features of the high-throughput sequencing results.

Table S3 Pairwise comparisons of Shannon-Weiner index between different factors.

Table S4 Pairwise comparisons of species richness between different factors.

Table S5 PERMANOVA analysis of the rhizocompartment microbial community composition based on WUF and UUF.