Differences in the endophytic fungal community and effective ingredients in root of three *Glycyrrhiza* species in Xinjiang, China

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Abstract

Background. Endophytic fungi have played an important role in influencing the quality and quantity of bioactive compounds of medicinal plants through specific fungus-host interactions. Nevertheless, there is little information about the composition of endophytic fungal communities of medicinal licorices, and the mechanism by which effective ingredients regulate endophytic fungal community in root is still unclear.

Methods. In this study, we collected root and soil samples at a range of depths (0–20, 20–40, and 40–60 cm) of three *Glycyrrhiza* species (*Glycyrrhiza uralensis*, *Glycyrrhiza inflata* and *Glycyrrhiza glabra*). We examined the content of effective ingredients (glycyrrhizic acid, liquiritin and total flavonoids) based on high-performance liquid chromatography, and using high-throughput sequencing technology to explore the composition and diversity of endophytic fungal community in different roots segments of three *Glycyrrhiza* species. Furthermore, soil samples were subjected to analyses of soil physicochemical properties.

Results. Results showed that the liquiritin content was not affected by the root depth (0-20cm, 20-40cm and 40-60cm), but was significantly affected by the main effect species (*Glycyrrhiza uralensis, Glycyrrhiza inflata, Glycyrrhiza glabra*) ($P < 0.05$). In *Glycyrrhiza* root, a total of 8 phyla and 140 genera were annotated, among them, the phylum Ascomycota and Basidiomycota, and the genera *Fusarium*, *Paraphoma* and *Helminthosporium* were significantly dominant. Spearman correlation analysis revealed liquiritin content was accountable for the differences in the diversity of endophytic fungal community. Furthermore, distance-based redundancy analysis (db-RDA) showed that soil physicochemical properties (available potassium and ammonium nitrogen), and the root factor (liquiritin and water content) were the main contributing factors to the variations in the overall structure of endophytic fungal community. Our results clarified the effective ingredients content and soil physicochemical can regulate the endophytic fungal community composition and diversity of medicinal licorices.

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Introduction

Glycyrrhiza species are perennial herbs with widely grows in arid and semi-arid regions [1]. There are three different original plants of *Glycyrrhiza* stipulated in Chinese Pharmacopeia, namely dried root and rhizome of *Glycyrrhiza uralensis*, *Glycyrrhiza inflata* and *Glycyrrhiza glabra* its dried roots and rhizomes is one of the most commonly used herbs medicines in both Eastern and Western countries [2]. A wide variety of effective ingredients can be extracted from root [3], mainly include triterpene saponins, polysaccharides and flavonoids [4]. Glycyrrhizic acid, the richest content of triterpene saponins [5], is the important pharmacological effective ingredients with anti-inflammatory [6], antiviral and immune regulation [7, 8] and other biological effects. Liquiritin is a major component of flavonoids that mainly exerts anti-inflammatory [9], antioxidant and antibacterial [10, 11]. Because of its medicinal and economic value, medicinal licorices plant has become the research direction of medicinal licorices to improve the content of licorice herbal medicine and understand its ecological characteristics.

The traditional view widely believes that the quality and quantity of the bioactive compounds extracted from medicinal plants are largely affected by the genetic background of the related plant, the ecological environment in which the plant lives, and soil nutrients [12, 13]. However, in recent years, some studies [14, 15] have shown that endophytic fungi have played a very important role in influencing the quality and quantity of bioactive compounds of medicinal plants through specific fungus-host interactions.

Endophytes, especially endophytic fungi, are one of the most important components in plant micro-ecosystems [16]. Endophytic fungi can form symbiotic relationships with host plants, on the one hand, which can present and grow in different healthy tissues of living plants, including stems [17], leaves [18] and roots [19]. Endophytic fungi, on the other hand, can extract carbohydrates and other nutrients from the host plant for their own growth [20]. In return, host plants may receive benefits from endophytic fungi associations. First, endophytic fungi can promote the growth of host plants by increasing hormones, including Gibberellin, Indoleacetic acid, Abscisic acid, Zeatin [21]. Second, endophytic fungi can enhance the resistance of host plants to environmental stress by producing biologically bioactive compounds [22, 23], such as, endophytic fungi of wheat can promote plant growth and abiotic stress resistance [24]. Last but not least, endophytic fungi can promote the accumulation of secondary metabolites of the host plant [25], such as paclitaxel and deoxypodophyllotoxin, thereby affecting the quantity and quality of bioactive compounds of medicinal plants.

Endophytic fungi have great biodiversity and are widely distributed in various terrestrial and aquatic plants species [26], and numerous studies have shown that endophytic fungi can be isolated from various plants species, ranging from important cash crop species [27] such as soybean, to medicinal plant species [28, 29], such as *Dendrobium Officinzle* and *Sceletium Tortuosum*. However, it should be noted that, with the rapid development of high-throughput sequencing technology and bioinformatics, a large number of undiscovered fungi have been discovered [30]. Previous studies based on high-throughput sequencing technology have speculated that there are as many as 5.1 million fungal species, most of which are involved in

plant-endophytic interactions [31]. At present, only a small part of endophytic fungi are isolated and identified, and most of the endophytic fungi in medicinal plant cannot be purely cultured on the existing medium [32]. Therefore, it is necessary to detect the endophytic fungi community in medicinal plants by adopting non-culture methods. Modern molecular technology, especially Illumina high-throughput sequencing technology, is an emerging technology in recent years, which can comprehensively and accurately detect the diversity of endophyte communities in medicinal plants [33, 34]. The high-throughput sequencing technique of next-generation sequencing is a more robust and accurate microbial community characterization technique compared to 18S rDNA-based non-culture methods and conventional culturing methods.

Numerous studies [35] have shown that the host genetic background (genotype or species) determine the composition of endophytic fungi. Meanwhile, soil fertility and ecological environment directly affect the content of bioactive compounds of medicinal plants, which will indirectly affect the composition and community structure of endophytic fungi [16]. However, for now, there is little information about the composition of endophytic fungi in the root of medicinal licorices at different genetic backgrounds (species), and soil environmental factors affecting the community structure of endophytic fungi in the root of medicinal licorice are still unclear. Therefore, in this study, we investigated the distribution and composition of endophytes fungal species of three medicinal licorices at three root depths through high-throughput sequencing and explored their relationship with host plants' effective ingredients and soil physicochemical properties. The results will enhance researchers' understanding about the environmental and host factors that influence endophytic fungi and the friendly relationship between endophytic fungi and medicinal plants, thus providing reference information for licorice growing for commercial medicinal purposes.

Materials & Methods

Sample collection: The roots and rhizosphere soils samples (all samples were 0-20cm, 20-40cm and 40-60cm, respectively) of three *Glycyrrhiza* (*Glycyrrhiza uralensis, Glycyrrhiza inflata* and *Glycyrrhiza glabra*) were collected from August to September in 2019 from specimens growing at 3 distinct sites in 3 eco-regions in Xinjiang province, China; the geographical location of sampling points and soil physical and chemical properties are shown in Table S1. In addition, to ensure that the experiment was representative, we randomly selected three medicinal licorices plants in good growth condition from each geographical location according to the five-point sampling method, and all root samples were cut with sterile scissors. The roots of each plant were divided into three depth segments: upper (0-20cm), middle (20-40cm), and lower (40-60cm), and the roots of each segments are equally divided into two parts: one part was placed into a ziplocked bag for the determination of the effective ingredients in the root, while the other part was placed into a sterile bag and transported on a piece of ice to the laboratory in preparation for the microbe determination. The soil and root materials from each eco-regions were collected as described above, and all the samples were labeled by combination with letters and numbers, with the first letter representing the species (W, G and D: *Glycyrrhiza uralensis*, *Glycyrrhiza glabra* and *Glycyrrhiza inflata*, respectively), the second letter representing the root

depth (1, 2 and 3: 0-20cm, 20-40cm, 40-60cm), and the third number representing the replicate number. For example, W.1.3 represents the third repetition of *Glycyrrhiza uralensis* at 0-20cm.

Surface sterilization: At the same time, to remove the interference of other microorganisms, we carried out disinfection and sterilization process on the surface of licorice root in the laboratory: firstly, the roots were carefully rinsed off the surface of the soil under running water and then rinsed with distilled water. Secondly, to surface disinfection the roots after wiped with filter paper were soaked with 75% ethanol for 30 s, followed by washed with sterile distilled water, then soaked in 5% sodium hypochlorite for 5 min, and finally air-dried under sterile conditions after washed five times with sterile distilled water [36]. The samples from the last rinse solution were inoculated onto a potato dextrose agar (PDA) plate and no fungi growth confirmed that the surface sterilization was successful [37]. All root samples were labeled and as soon as possible placed on ice and stored at liquid nitrogen until total DNA extraction.

Soil physicochemical: For the physicochemical analysis of rhizosphere soil, the soil samples were air-dried and sieved (2 -mm mesh), and were detected according to the methods described by the Bao et al [38]. Soil pH (1: 5= soil: distilled water) was measured using a pH meter. Soil Water content (SWC) was measured by weighing. The content of organic matter (SOM) and total salt (TS) were measured by external heating with potassium dichromate and atomic absorption spectrometry, respectively. The total nitrogen (STN), total phosphorus (STP) and total potassium (STK) content were determined by acid digestion method. Using 0.01 M calcium chloride extraction to determined nitrate nitrogen (SNN) and ammonium nitrogen (SAN) contents. The available phosphorus (SAP) content was measured by sodium bicarbonate extraction (molybdenum-antimony colorimetry). The available potassium (SAK) content was determined by ammonium acetate extract method (atomic absorption spectrometry).

Determination of effective ingredients of *Glycyrrhiza* plant root: After drying to a constant weight, the root samples were ground into a powder with a mortar and pestle and passed through a sieve (60-mesh). For the analysis of effective ingredients, an aliquot (0.2 g) of powdered root sample was extracted with 71% chromatographic methanol in an ultrasonic bath (250 W, 40 kHz) at room temperature. The content of effective ingredients (glycyrrhizic acid (GIA) and liquiritin (LI)) were determined by high performance liquid chromatography (HPLC, Agilent-1260 Infinity, USA) using an Agilent ZORBAX SB-C18 column (150 mm × 4.6 mm, 5 μm) according to previous study [39]. Reference materials of GIA (CAS#1405-86-3) and LI (CAS#551-15-5) were purchased from Solarbio and used for calibration purposes. The total flavonoid (GTF) content in root was measured by ultraviolet spectrophotometry at 334 nm with the liquiritin standard (CAS#551-15-5) as the control.

DNA extraction and library construction: Total genome DNA was extracted from 0.5g root samples using the DNA Quick Plant System kit (Tiangen, China) according to the manufacturer's protocol, and the concentration and quality of DNA were detected using a NanoDrop2000 (Thermo Fisher Scientific, USA) and 1% agarose gel electrophoresis, respectively. After the final concentration was diluted to 1 ng/μL with sterile distilled water, each PCR product was used as template DNA. The ITS (Internal Transcribed Spacer) rDNA genes of

the ITS1 region were amplified using specific primers (ITS5-1737F 5'-GGAAGTAAAAGTCGTAACAAGG-3'and ITS2-2043R 5'-GCTGCGTTCTTCATCGATGC-3') with barcodes [40]. To ensure amplification efficiency and accuracy, all PCR reactions were carried out with Phusion ® High-Fidelity PCR Master Mix and GC Buffer (New England Biolabs). The temperature regime for PCR reactions was as follows: 95 °C/3 min, 95 °C/30 s, 55 °C/30 s, 72 °C/30 s, 72 °C/5 min, and PCR amplification was prepared with 30 cycles. PCR products were mixed with 1×loading buffer (containing SYBR green) in equidensity ratios, and then were detected by 2% agarose gel electrophoresis. Using GeneJETTM Gel Extraction Kit (Thermo Scientific) to purify target sequences. The libraries were constructed using a TruSeq ® DNA PCR-Free Sample Preparation Kit (Illumina, USA) according to the manufacturer's recommendation, and index codes were added. The library quality was assessed on the Qubit ® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, amplicon sequencing was performed using the Illumina HiSeq2500 platforms at the Beijing Compass Biotechnology Co., Ltd. (Beijing, China).

Bioinformatics analysis and statistical analysis: Single-end reads was assigned to samples using Cutadapt [41] software based on their unique barcode and truncated by cutting off the barcode and primer sequence. To avoid the influence of non-microbiota sequences (such as, chloroplast and mitochondrial sequences), the raw sequences were further quality filtering by Cutadapt software to remove non-microbiota taxa before subsequent analysis. Then raw reads were subjected to a strict quality controlled process under specific filtering conditions using Cutadapt software to obtain high-quality clean reads. Clean reads were obtained by comparison with the reference database (Unite database) [42] using UCHIME algorithm to detect and remove chimeric sequences.

UPARSE software [43] (UPARSE v7.0.1001) was used to cluster the effective tags of all samples into the same operational taxonomic units (OTUs) with $\geqslant$97% similarity, and taking the sequence with the highest frequency as the representative sequence of each OTU. The taxonomic information for each representative sequence was annotated using the Unite database basing on Blast algorithm which was calculated by QIIME software (Version 1.9.1), and multiple sequence alignment was conducted using MUSCLE (Version 3.8.31) software to study the phylogenetic relationship of the representative sequences of OTUs among the 27 root samples. OTU abundance information was normalized using a standard sequence number corresponding to the sample with the least sequences (54,262 reads for sample D.2.1). Subsequent analysis of alpha diversity and beta diversity were performed based on this output normalized data.

Alpha Diversity analysis was used to study the complexity of species diversity in a sample through six indices (observed-species, Shannon, Simpson, Chao1, ACE, and Good-coverage) [44]. All indices in the samples were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3).

Beta diversity analysis was used to evaluate differences in sample species complexity, which based on weighted Unifrac was calculated by QIIME software. The Un-weighted Pair-group Method with Arithmetic Mean (UPGMA) clustering analysis was conducted by QIIME

software (Version 1.7.0). In addition, R software (Version 2.15.3) was also used to rarefaction curve generation, Wilcoxon rank sum test, Metastat statistical test, Spearman correlation analysis of heat maps and Distance-based Redundancy Analysis (db-RDA). Pearson correlation analysis was run among the effective ingredients and the soil physicochemical properties. Two-way ANOVA was performed with SPSS 19.0 (IBM Inc., Armonk, USA), and displayed with GraphPad Prism 5.

Results

Differences in root effective ingredients contents

The effective ingredients and physicochemical properties of samples were presented in Table S2. The results of two-way ANOVA showed that the content of the effective ingredients (glycyrrhizic acid (GIA), liquiritin (LI) and total flavonoid (GTF)) were not significantly affected by the interaction effect between root depth (0-20cm, 20-40cm and 40-60cm) and plant species (*Glycyrrhiza uralensis*, *Glycyrrhiza inflata*, and *Glycyrrhiza glabra*) ($P > 0.05$) (Table S3). However, the content of LI was significantly affected by the main effect plant species ($P < 0.05$) (Table S3 and Figure 1). As shown in the Figure 1, the contents of LI in root of W were significantly higher than those in D ($P < 0.05$), and the contents of LI in root of W were significantly higher than those in G ($P < 0.05$) (Figure 1a).

Pearson correlation analysis showed that the content of effective ingredients was significantly correlated with soil physicochemical properties (Table 1). GIA content in root had a very significant positive correlation with available potassium (SAK) and soil water content (SWC) ($r > 0$; $P < 0.05$), but LI content in root had a very significant negative correlation with SAK and total salt (TS) content ($r < 0$; $P < 0.05$).

Root Endophytic fungal sequencing results

In the root of the three *Glycyrrhiza* (*Glycyrrhiza uralensis*, *Glycyrrhiza glabra*, and *Glycyrrhiza inflata*), a total of 2,118,633 effective sequences were obtained after filtering out low-quality and short sequence reads. The sequencing results of each sample were listed in Supplementary Table S4. The effective sequences were clustered into OTUs with 97% identity, and a total of 1,063 OTUs were obtained, among them, 91.53% of the effective sequences were assigned to the Kingdom level, 59.27% to the phylum level, 54.37% to the class level, 53.72% to the order level, 46.19% to the family level, 38.01% to the genus level, and 23.52% to the species level by the Illumina HiSeq (Figure S1a). The rarefaction curves showed that the number of OTU in each sample increased gradually with quantity of sequence, thus confirming that the amount of sequencing data was adequate (Figure S1b).

Differences in Alpha diversity

The alpha diversity index of each group was shown in Table S5. Some indexes (Shannon and Chao1) respectively reflected the diversity and richness of microbial communities in samples, the greater the index, the higher the species diversity, the richer the distribution. The Shannon index of the W1 (4.910) sample was the highest. In contrast, that of the D1 (3.393) sample was the lowest. Moreover, we found that D1 had the lowest Chao1 (238.678) and ACE (253.105), while the D3 sample had the highest Chao1 (356.317) and ACE (355.694), respectively.

Meanwhile, the results based on Wilcoxon rank sum test showed that the Shannon index was significantly different distribution between W and D, especially 0-20cm at the root depth (Figure 2a). Specifically, the Shannon index in W1 sample was significantly higher than D1 sample ($p < 0.05$). Furthermore, the Chao1 index in D increased gradually with the downward movement of root depths, and based on Wilcoxon rank sum test showed that the Chao1 index in D was significantly affected by root depth (Figure 2b). Specifically, the Chao1 index in D3 sample was significantly higher than D1 sample ($p < 0.01$); D2 sample was significantly higher than D1 sample ($p < 0.05$).

Differences in Beta diversity

Beta diversity analysis was used to evaluate differences in microbial community composition among the samples. The Unweighted Pair-Group Method with Arithmetic (UPGMA) cluster analysis was performed to study similarity in the composition of endophytic fungal community among different samples, and the clustering results were integrated with species relative abundance at phyla taxon level in each group. As shown in Figure 3a, the results of UPGMA cluster tree based on Weighted Unifrac distances showed that samples from G3, G2, G1 and W1 were clustered together, and samples from W3, D2, W2 and D1 were clustered together (Figure 3a). Meanwhile, for the difference analysis between the beta diversity, a Wilcoxon rank sum test based on Weighted Unifrac distances was constructed (Figure 3b), and the results showed that there were significant differences in beta diversity between W and D, which was consistent with UPGMA cluster tree. Specifically, there were significant differences in beta diversity between D1 and D2 samples ($P < 0.05$), D3 and W3 samples ($P < 0.05$), and D1 and W1 samples ($P < 0.01$) (Figure 3b), which indicated there were significant differences in endophytic fungal community composition in roots of medicinal licorices between different species and different root depth.

Differences in root fungi community composition

According to the OTUs sequence and Unite database, a total of 8 phyla, 23 classes, 53 orders, 102 families, 140 genera and 141 species were annotated. The endophytic fungal phyla with the greatest abundance from nine groups were enumerated in Figure 4a. Ascomycota dominated the observed sequences at the phylum level, representing 91.821%, 60.558%, 39.956%, 79.651%, 62.305%, 54.241%, 82.176%, 81.928% and 80.290% of the total number of species in D1, D2, D3, G1, G2, G3, W1, W2 and W3, respectively. In addition, Basidiomycota occupied a large part of the relative abundance in D2 (21.348%), D3 (28.440%), G2 (10.631%), G3 (12.523%), W2 (6.749%) and W3 (5.110%), respectively. Meanwhile, our results showed that the relative abundance of Ascomycota gradually decreased with the downward movement of root depths. For the difference analysis at the Phylum classification level, a MetaStat statistical test based on species abundance was constructed, and the results showed that the relative abundance of Ascomycota in D significant difference distribution at different root depth (Figure 4b). Specifically speaking, the relative abundance of Ascomycota at D1 sample (91.821%) was significantly higher than D3 sample (39.956%) (Figure 4b).

In terms of genus, we listed the top 10 dominant fungal genera in each group in Figure 4c: *Fusarium* was found to be the predominant genus in D1 (27.907%), G1 (23.944%), G2 (31.071%), G3 (25.381%), W1 (19.253%) and W3 (18.215%). Meanwhile, the abundance of *Paraphoma* was high in the D1, D3 and W3 samples, accounting for 27.738%, 23.937% and 13.980%, respectively. *Helminthosporium* occupied a large part of the relative abundance in D1 (26.567%), G1 (25.124%), W1 (8.224%) and W2 (17.408%), respectively. *Sarocladium* occupied a large part of the relative abundance in D2 (3.326%), G1 (16.547%), G2 (17.243%), G3 (21.897%) and W1 (4.218%), respectively, the abundance of *Cladosporium* was high in D2 (6.446%), D3 (2.721%) and W3 (15.174%). *Cadophora* (13.200%) and *Psathyrell*a (10.917%) were found to be the most dominant in D2 sample. *Tomentella* (14.472%) was found to be the most dominant in D3 sample. *Conocybe* (12.068%) was found to be the most dominant in G3 sample (Figure 4c).

At the same time, details of the composition of the top 10 dominant fungi at other classification levels (Class, Older, Family and Species) were listed in Table S6. Specifically speaking, Sordariomycetes, Dothideomycetes and Agaricomycetes were dominate at the class taxonomic level; the dominant species at the order taxonomic level are Hypocreales, Pleosporales, Thelephorales; the dominant species at the family taxonomic level are Nectriaceae, Phaeosphaeriaceae, Massarinaceae; the dominant species at the species taxonomic level are *Fusarium-solani, Paraphoma-radicina, Sarocladium-kiliense*.

The relationship between the dominant phylum and genus of endophytic fungi and the effective ingredients and soil physicochemical properties

Spearman correlation analysis showed that there was a significant relationship between dominant endophytic fungi phylum and effective ingredients and soil physicochemical properties (Table S7). Specifically, Ascomycota showed a very significant negative correlation with RWC ($r < 0$, $P < 0.01$); Basidiomycota showed a very significant positive correlation with RWC ($r > 0$; $P < 0.01$); Olpidiomycota showed a significant positive correlation with GIA ($r > 0$; $P < 0.05$); Mortierellomycota showed a significant positive correlation with STK, SWC and SAK ($r > 0$; $P < 0.05$); Mucoromycota showed a very significant positive correlation with SOM ($r > 0$; $P < 0.01$), but a significant negative correlation with STP ($r < 0$, $P < 0.01$); Rozellomycota showed a significant positive correlation with SOM, STK and RWC ($r > 0$; $P < 0.05$).

At the same time, as shown in Figure 5, there was a significant relationship between the dominant fungi genus and effective ingredients and soil physicochemical properties. Specifically, *Fusarium* showed a significant positive correlation with LI content ($P < 0.05$); *Paraphoma* showed a significant positive correlation with SAN ($P < 0.05$), but a significant negative correlation with SAK, TS and SWC ($P < 0.05$); *Helminthosporiu*m showed a significant positive correlation with PH ($P < 0.05$); *Sarocladium* showed a significant negative correlation with SOM, STN and SNN ($P < 0.05$); *Conocybe* showed a significant positive correlation with SWC, but a significant negative correlation with SAN ($P < 0.05$).

Relationships among effective ingredients and soil physicochemical properties and root endophytic fungi communities

Spearman correlation analysis showed that the content of LI was significantly positive correlated with alpha diversity index ($r > 0$, $P < 0.05$) (Figure 6). As shown in Figure 6, the content of LI had a very significant positive correlation with Shannon index, Simpson index and Chao1 index ($P < 0.05$), which indicated that the content of LI was accountable for the differences in the diversity of endophytic fungal community in this study.

Distance-based redundancy analysis (db-RDA) based on the Bray–Curtis distance showed that the effective ingredients and soil physicochemical had significant effects on the differences of endophytic fungal community (Figure 7). The differential distribution of endophytic fungal community was mainly restricted in the first and second ordination axes, and the first ordination axis, the second ordination axis were explained 16.23%, 13.89% of the total variability, respectively (Figure 7). Specifically, among the soil environment factors, SAK content was identified as the factor that most significantly affects the differences of endophytic fungal community ($r2 = 0.329$, $P < 0.01$), followed by SAN ($P < 0.05$). Among the root factors, the RWC was explained the difference of endophytic fungal communities in roots to the greatest extent ($r2 = 0.247$, $P < 0.05$), followed by LI content ($P < 0.05$) (Figure 7, Table S8). According to results of the db-RDA analysis, the SAN, SAK, RWC, and LI content were the major factors contributing to the variations in the overall structure of endophytic fungal community in this study.

Discussion

In this study, we investigated the composition and diversity of endophytic fungal communities in different root depth (0-20cm, 20-40cm and 40-60cm) of three *Glycyrrhiza uralensis* (*Glycyrrhiza uralensis*, *Glycyrrhiza glabra*, and *Glycyrrhiza infl*ata) using high-throughput sequencing technology, which provides a large amount of data with more accuracy than that obtained in previous studies using traditional technology [45-47]. We obtained the composition of endophytic fungal communities at different taxonomic levels (phylum, class, older, family, genus and species) by high-throughput sequencing (Figure 4a, Figure 4c and Table S6). The results showed that there was a specific microbiome in 27 samples of tree medicinal licorices, and the relative abundance of endophytic fungi was correlated with the host plant species and root depth. For example, Ascomycota was the dominant phylum in all samples, followed by Basidiomycota, which result consistent with previous studies [48, 49]. The phylum Ascomycota, as the largest phylum of fungi, has diverse populations and plays an important role in genetics [50], ecology [51] and phylogeny [52]. Such as, the Ascomycota produce large numbers of spores through both asexual and sexual reproduction. Asci can act as small water cannon, spraying spores into the air. Dispersal process of ascospores, spores is important for dissemination of many fungal plant diseases and for the dispersal of many saprophytic fungi [53].

Moreover, our results showed that the relative abundance of Ascomycota gradually decreased with the downward movement of root depths (Figure 4b), which was consistent with the results of Ko, Daegeun et al [54]. On this basis, we found that the relative abundance of Ascomycota in *Glycyrrhiza inflata* had a significant difference at different root depth, but

Glycyrrhiza uralensis and *Glycyrrhiza glabra* were not significant difference, indicating that some endophytes may preferentially proliferate in a certain ecological region and play different ecological roles from other endophytes. Overall, in addition to soil depth, the relative abundance of endophytes was also related to the genotype of the host plant species. This was consistent with the results of host genotype and soil conditions on ectomycorrhizal community of poplar clones by Karliński, Leszek et al. [55].

Alpha Diversity and Beta Diversity analysis of endophytic fungal community showed significant differences in root depths (0-20cm, 20-40cm and 40-60cm) between *Glycyrrhiza uralensis* and *Glycyrrhiza inflata* (Figure 2 and Figure 3), which indicated that both genotype and ecological region of host plants contributed to the differences of endophytic fungal community. Meanwhile, numerous studies [56] have shown that the adaptation of endophytic fungal community largely depends on the adaptation of host plants to the ecological environment, which indicated that host plants largely determine the colonization and distribution of endophytic fungal community. The relationship between fungus and host plant were also often considered as a flexible interaction, with orientations determined by subtle differences in the expression of fungal genes in response to the host, or conversely, by the host's recognition and response to the fungus. Thus, slight genetic differences in the two genomes control the symbiosis [57].

Furthermore, our results showed that the root depth had a significant effect on the richness and composition of endophytic fungal community (Figure 2 and Figure 3), which indicated that different ecological types of endophytic fungi may represent certain ecological regions (different root depth), these should become an important consideration factor that endophytic fungi inoculation. We speculated that this is related to root respiration and soil C content. On the one hand, root respiration, accounts for 60% of total soil respiration, can regulates the metabolism of roots and related microorganisms, and is an important part of terrestrial carbon budget [58]; on the other hand, the content of C in unstable soil varies greatly between different soil depths [59]. Moreover, Noah Fierer et al. [60] demonstrated that the vertical distribution of the specific microbial species was largely related to the decrease in carbon availability with soil depth.

However, one weakness in this study was that the samples of three *glycyrrhiza* species were collected from areas which differed in geographical environment. Since it is rare to find three *glycyrrhiza* species in the same habitat, to a certain extent, the soil physicochemical properties can represent the environmental factors in which three *glycyrrhiza* species were growing. Therefore, in this study, in addition to root factor, also included the effects of the soil factors.

Numerous studies [61, 62] showed the accumulation of effective ingredients in medicinal licorice roots is affected by various factors. In this study, the content of LI were more affected by main effect plant species than main effect root depth (Table S3), among them, the content of LI in root of *Glycyrrhiza uralensis* were significantly higher than those in *Glycyrrhiza inflata* and *Glycyrrhiza glabra* (Figure 1a), which is consistent with the results of Zhang et al. [63]. We speculate that this is related to the expressions of some functional genes that are closely associated with the content of effective ingredients including glycyrrhizic acid and liquiritin in

root of licorice species. Some studies [64-66] have shown that key functional genes, such as chalcone synthase gene, 3-Hydroxy-3-methylglutary CoA reductase (HMGR) and squalene synthase (SQS), are involved in transcriptional level regulation process in glycyrrhizic acid and liquiritin biosynthesis. Although further studies are required to characterize the expression of functional genes of effective ingredients, this study provides a theoretical basis for the development of strategies to expand the *Glycyrrhiza uralensis* cultivation. On the other hand, the content of effective ingredients is the result of the interaction between plants and their growing environment, therefore, the accumulation of effective ingredients in root is influenced by the ecological environment of its. In this study, GIA, GTF and LI content had a positive correlation with soil total nitrogen (STN) ($r > 0$)(Table 1), indicating that soil nutrients can promote the accumulation of effective ingredients, but not all soil nutrients, such as soil total potassium (STK), have such a function. Although potassium can be involved in many enzyme activation systems in plants and improve plant stress resistance [67], the content of GIA, GTF and LI were negatively correlated with STK ($r < 0$) in this study, which is consistent with the results of Liu et al [68]. In addition, soil available potassium (SAK) had a significant positive correlation with GIA, but had a significant negative correlation with LI (Table 1), indicating that the utilization mechanism of soil nutrients by effective ingredients is completely different. Although the mechanism by which available potassium regulate effective ingredients is still unclear, this discovery may form the basis of further in-depth research. In general, these soil factors exhibit habitat specific characteristics are related to the regulation of effective ingredients in root of licorice.

In recent years, a growing number of studies [69-71] have demonstrated that the dynamics of the microflora is driven to a large extent by environmental factors including soil characteristics (pH, nitrogen, phosphorus and potassium) and climate condition (rainfall and temperature). Consistent with these reports, our results showed that LI, RWC, SAN and SAK content were the major factors contributing to the variations in the overall structure of endophytic fungal community (Figure 7 and Table S8). In addition, we found that the content of LI in root had a very significant positive correlation with diversity of endophytic fungal community (Shannon and Simpson index) ($P < 0.05$) (Figure 6). Liquiritin (LI), the main effective ingredients of flavonoids, is one of the material basis for clinical efficacy and an important index of the quality of medicinal licorices. Flavonoids can be specifically induced by symbiotic fungus to respond to purified signaling molecules from these organisms when the fungus colonizes. Chen et al. [72] demonstrated that with inoculation of fungi *Glomus mosseae*, *Glycyrrhiza uralensis* plants significantly increased stem and root biomass and liquiritin content in the main root.

Meanwhile, our results showed that soil physicochemical and effective ingredients had a significant effect on composition of endophytic fungal communities (such as phylum and genus) (Figure 5 and Table S7), which showed that there is an interaction among endophytic fungal community, root and soil factor. This suggests that we may be able to alter the fungal composition by altering soil factors [73], thereby promoting the accumulation of effective

ingredients in plants [74]. In the case of medicinal licorices, Wei Xie et al. [75] shown that P addition and arbuscular mycorrhizal (AM) inoculation could improve plant growth and facilitated glycyrrhizic acid and liquiritin accumulation in *Glycyrrhiza uralensis*. Meanwhile, Y. Orujei et al. [76] also shown that two species of arbuscular mycorrhizal fungi (AMF) were successful inoculation, the increase in the growth rate and the accumulation of effective ingredients in licorice roots (*Glycyrrhiza glabra*) were observed compared to control. In general, this study clarified the ecological role of non-biological factor (soil and root) in the endophytic fungal community of medicinal licorices, which provided useful an information for the development of strategies to improve the production and quality of medicinal licorices, although further studies are required to characterize the functions of these endophytic fungi.

Conclusions

In this study, numerous endophytic fungal communities were detected in roots of *Glycyrrhiza* based on high-throughput sequencing. Furthermore, we identified significant differences in the relative abundance of Ascomycota among root depth. Furthermore, the analysis of alpha diversity and beta diversity were showed that the endophytic fungal community structure and composition differed among the species and root depth in medicinal licorices. Moreover, the SAN, SAK, RWC, and LI content were the major factors contributing to the variations in the overall structure of endophytic fungal community in this study. This study clarified the ecological role of non-biological factor (soil and root) in the endophytic fungal community of medicinal licorices, which may provide theoretical basis for the synthesis of bioactive compounds and rational utilization of medicinal plants in production practice.

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Table 1(on next page)

Pearson correlation coefficient of the content of bioactive compounds with soil physicochemical properties

Description: the values are the Pearson's correlation coefficients. ** means $P<0.01$; * means $P<0.05$.

Table 1. Pearson correlation coefficient of the content of bioactive compounds with soil physicochemical properties

	GIA	GTF	LI	SOM	STN	STP	STK	SNN	SAN	SAP	SAK	TS	PH	SWC
GIA	1.000	0.419*	0.294	-0.146	0.121	0.158	-0.107	-0.144	-0.337	-0.121	0.463*	-0.069	0.263	0.609**
GTF	0.419*	1.000	0.172	-0.142	0.132	0.345	-0.121	0.048	-0.008	0.034	0.151	-0.217	0.000	0.160
LI	0.294	0.172	1.000	-0.031	0.183	0.251	-0.294	0.070	0.239	-0.216	-0.415*	-0.403*	0.058	-0.183
SOM	-0.146	-0.142	-0.031	1.000	0.274	-0.527**	0.238	0.532**	0.248	0.400*	-0.176	0.136	-0.229	-0.327
STN	0.121	0.132	0.183	0.274	1.000	0.455*	-0.249	0.416*	0.333	0.415*	-0.022	0.166	-0.236	-0.300
STP	0.158	0.345	0.251	-0.527**	0.455*	1.000	-0.465*	-0.198	0.245	-0.045	-0.034	-0.119	0.090	-0.033
STK	-0.107	-0.121	-0.294	0.238	-0.249	-0.465*	1.000	0.167	-0.326	0.211	0.247	0.182	-0.020	0.156
SNN	-0.144	0.048	0.070	0.532**	0.416*	-0.198	0.167	1.000	0.267	0.736**	-0.011	0.489**	-0.284	-0.411*
SAN	-0.337	-0.008	0.239	0.248	0.333	0.245	-0.326	0.267	1.000	0.076	-0.566**	-0.066	-0.315	-0.641**
SAP	-0.121	0.034	-0.216	0.400*	0.415*	-0.045	0.211	0.736**	0.076	1.000	0.364	0.723**	-0.253	-0.172
SAK	0.463*	0.151	-0.415*	-0.176	-0.022	-0.034	0.247	-0.011	-0.566**	0.364	1.000	0.556**	0.063	0.750**
TS	-0.069	-0.217	-0.403*	0.136	0.166	-0.119	0.182	0.489**	-0.066	0.723**	0.556**	1.000	-0.062	0.238
PH	0.263	0.000	0.058	-0.229	-0.236	0.090	-0.020	-0.284	-0.315	-0.253	0.063	-0.062	1.000	0.446*
SWC	0.609**	0.160	-0.183	-0.327	-0.300	-0.033	0.156	-0.411*	-0.641**	-0.172	0.750**	0.238	0.446*	1.000

Description: the values are the Pearson's correlation coefficients. ** means $P<0.01$; * means $P<0.05$.

Figure 1

Effect of main effect plant species on the effective ingredients of licorice roots

Ordinate is the content of LI (a), GIA (b) and GTF (c); abscissa is the group name: D, G and W: *Glycyrrhiza inflata, Glycyrrhiza glabra* and *Glycyrrhiza uralensis*, and the mark * is significance test ($p < 0.05$).

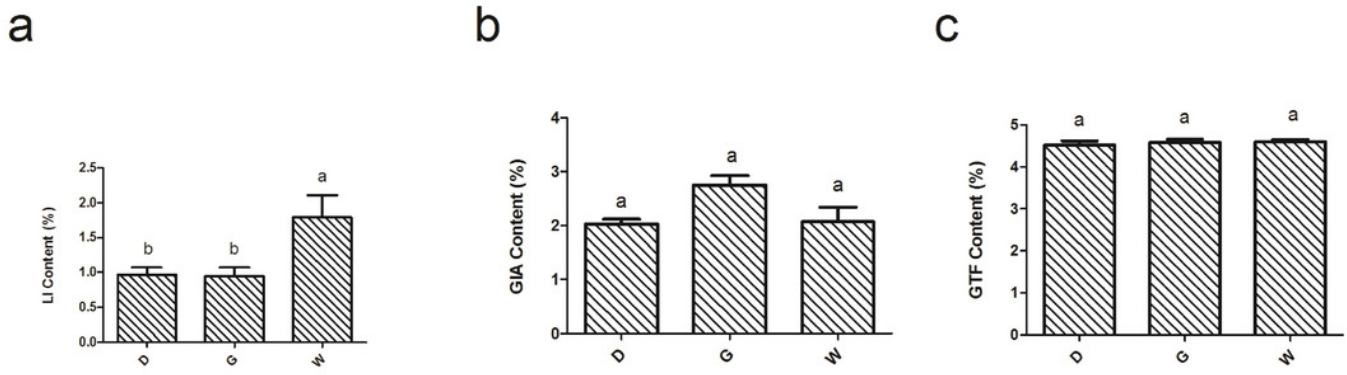


Figure 2

The significance test of the differences of Alpha Diversity

Ordinates are Shannon index (a) and Chao1 index (b), respectively. Abscissa is the group name: D, G and W: *Glycyrrhiza inflata, Glycyrrhiza glabra* and *Glycyrrhiza uralensis*; 1, 2 and 3: root depth 0-20cm, 20-40cm, and 40-60cm, respectively. The mark * is significance test $p < 0.05$.

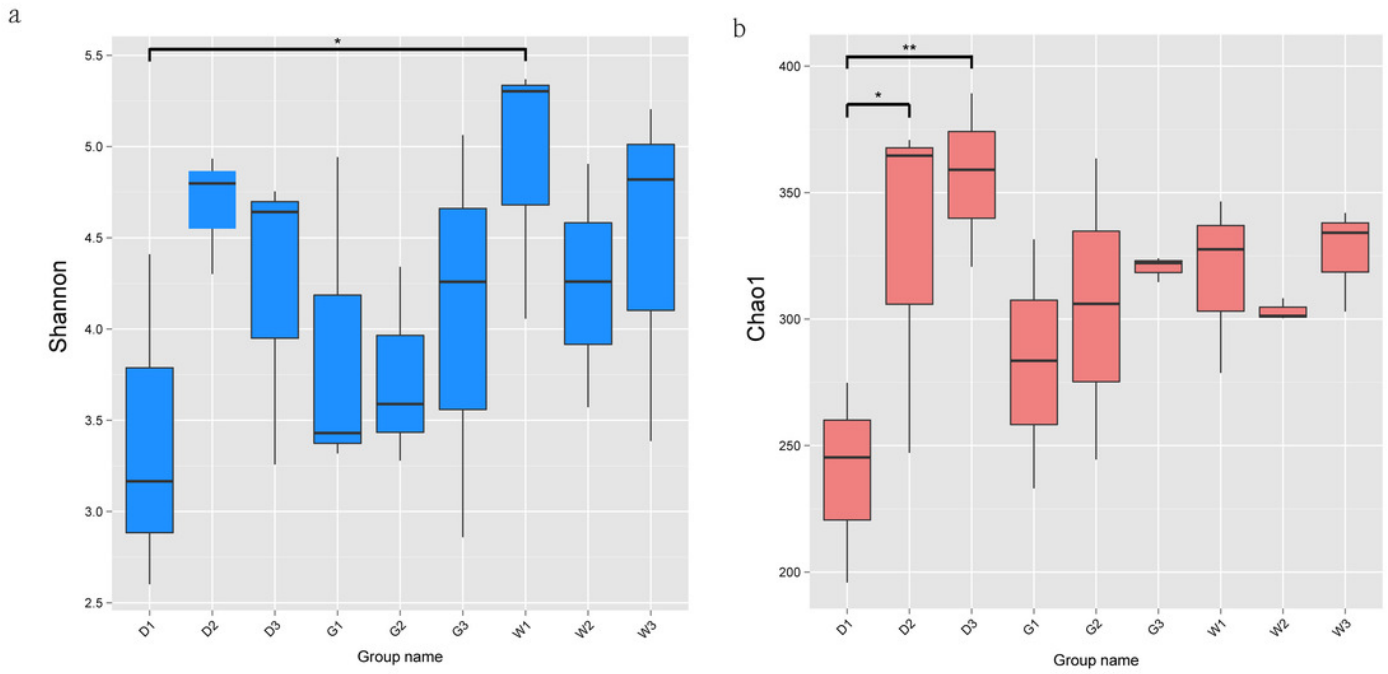
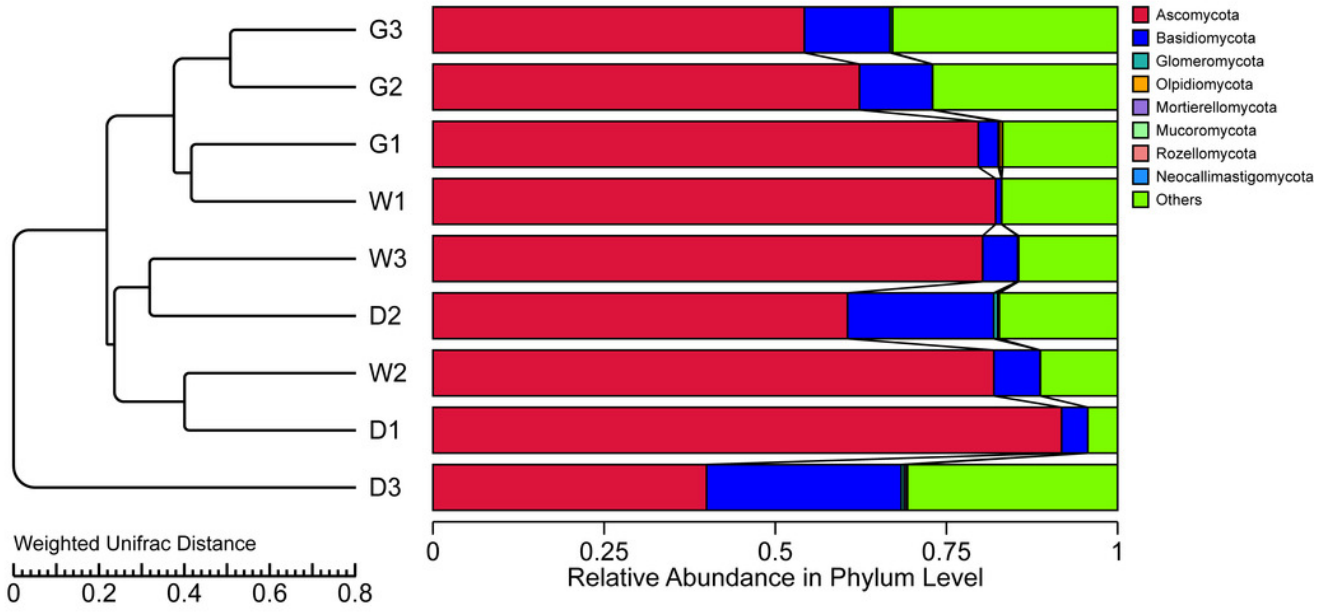


Figure 3

Unweighted Pair-Group Method with Arithmetic (UPGMA) clustering tree base on the weighted unifrac distance (a); and the significance test of the differences of Beta Diversity (b).

a: The left is the UPGMA cluster tree structure, and the right is the distribution of relative abundance of each sample at the phylum level; b: Ordinate is the Beta diversity; Abscissa is the group name: D, G and W: Glycyrrhiza inflata, Glycyrrhiza glabra and Glycyrrhiza uralensis; 1, 2 and 3: root depth 0-20cm, 20-40cm, and 40-60cm, respectively. The mark * is significance test $p < 0.05$.

a


G3
G2
G1
W1
W3
D2
W2
D1
D3
Ascomycota
Basidiomycota
Glomeromycota
Olpidiomycota
Mortierellomycota
Mucoromycota
Rozellomycota
Neocallimastigomycota
Others
Weighted Unifrac Distance
0
0.2
0.4
0.6
0.8
0
0.25
0.5
0.75
1
Relative Abundance in Phylum Level

b

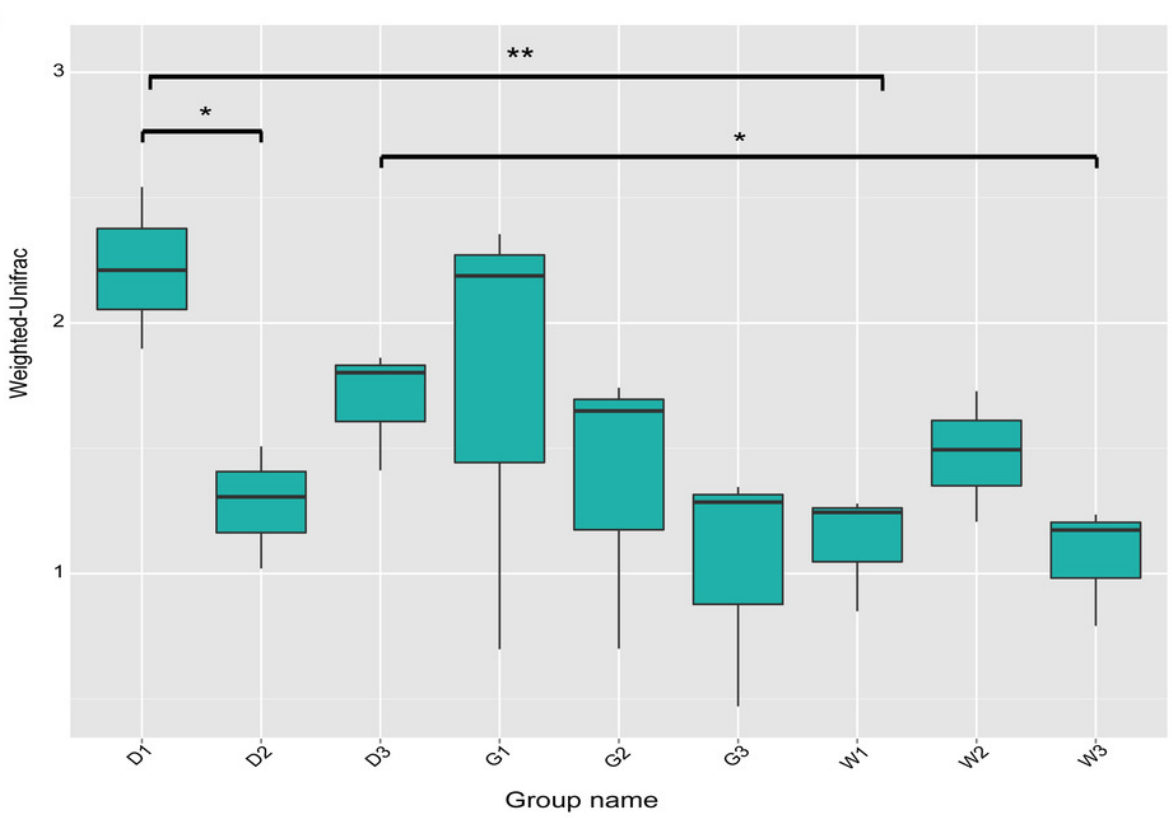

**
*
*
Weighted-Unifrac
3
2
1
D1
D2
D3
G1
G2
G3
W1
W2
W3
Group name

Figure 4

Histograms of relative abundance of the top 10 endophytic fungi at the phyla (a) level of taxonomy and difference analysis at the Phylum classification level (b), Histograms of relative abundance of the top 10 endophytic fungi at the genera (c) level.

Ordinate is the relative abundance; others refers to are sequences with less or not be annotated. Abscissa is the group name: D, G and W: *Glycyrrhiza inflata*, *Glycyrrhiza glabra* and *Glycyrrhiza uralensis*; 1, 2 and 3: root depth 0-20cm, 20-40cm, and 40-60cm, respectively. ** means P<0.01.

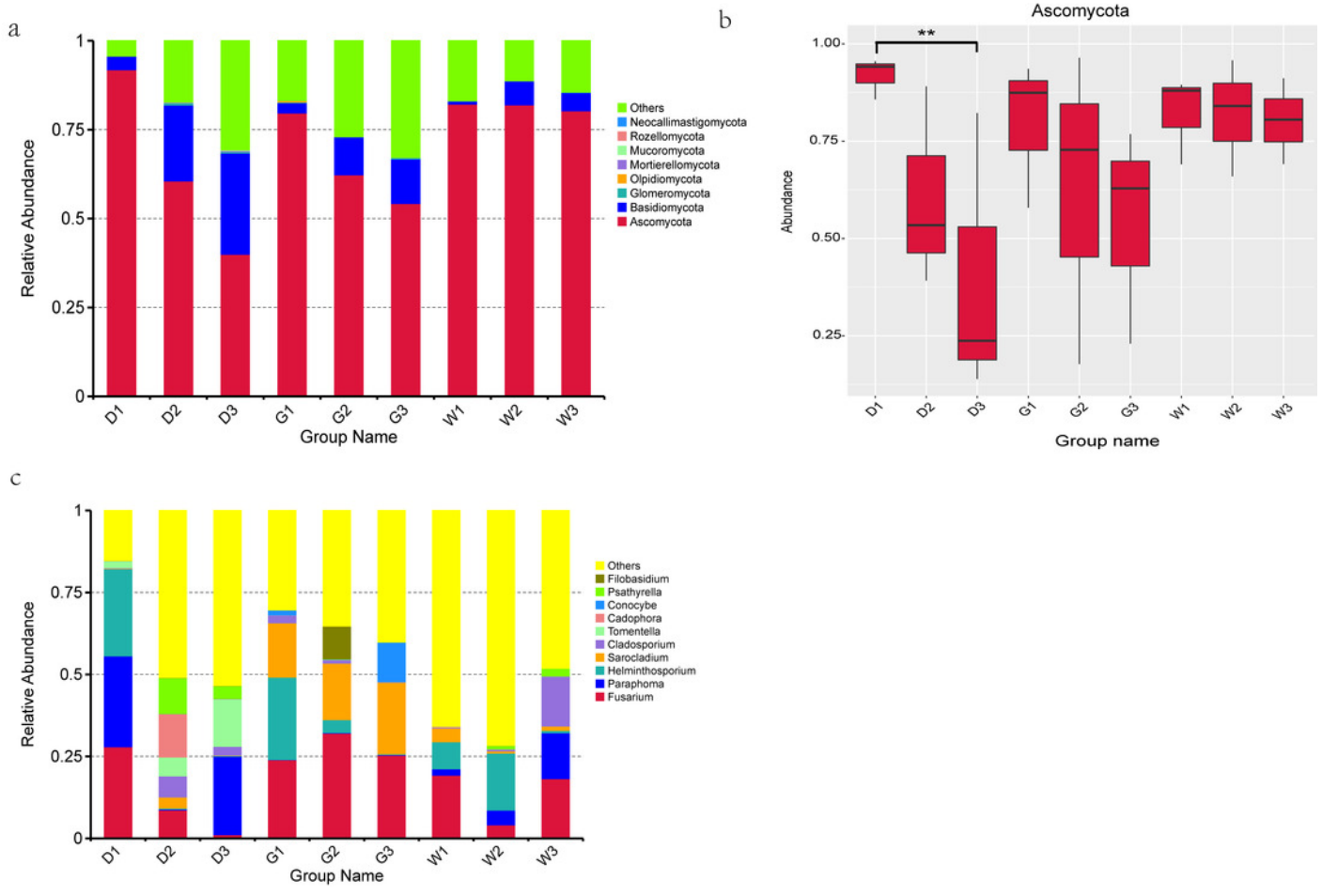


Figure 5

Heatmaps of Spearman correlation analysis

Ordinate is the information of environmental factors, and abscissa is the information of species at the genera level of taxonomy. The correlation coefficient r of Spearman is between -1 and 1, r < 0 is negative correlation, r > 0 is positive correlation, and the mark * is significance test (p < 0.05).

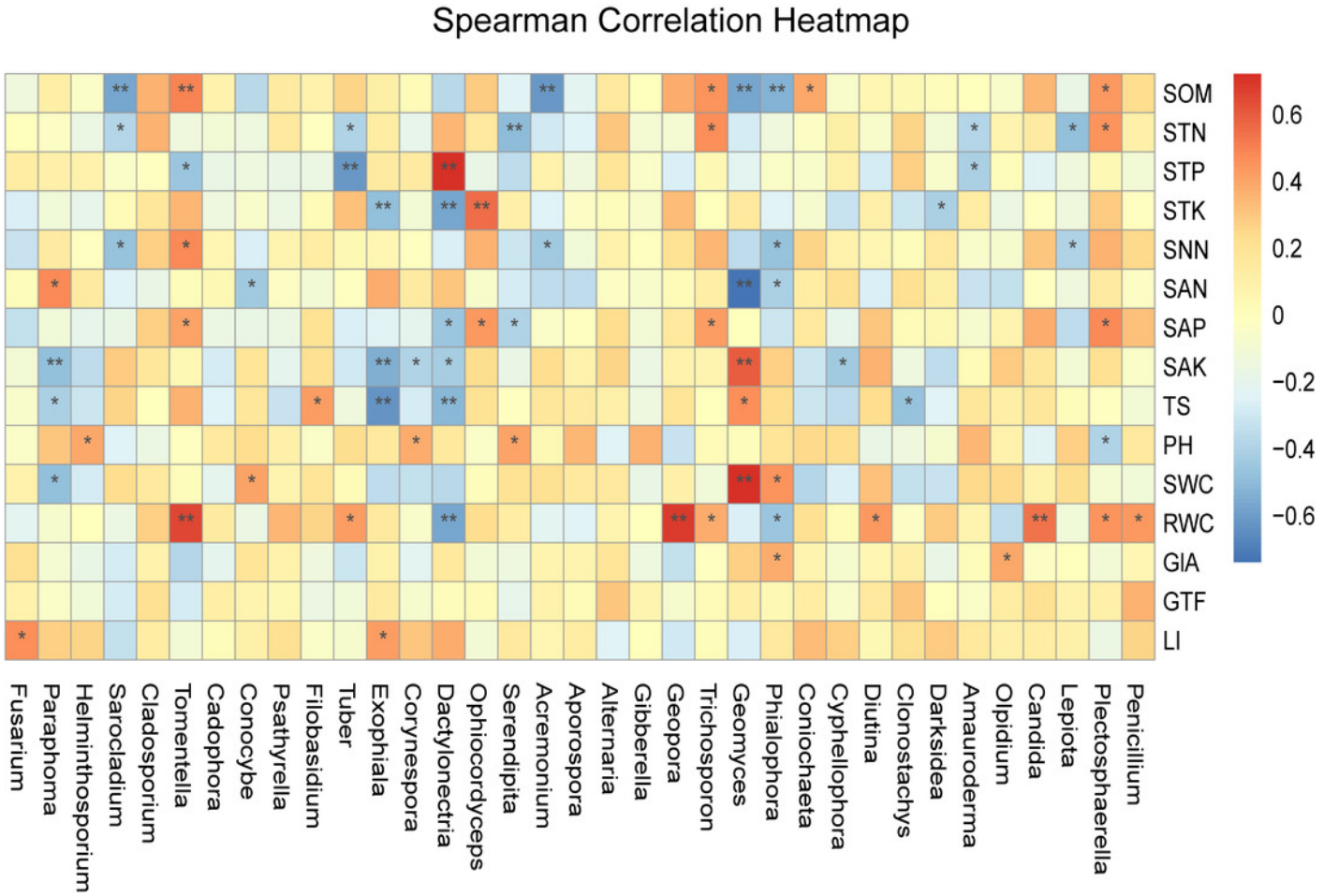


Figure 6

Heatmaps of Spearman correlation analysis

Ordinate is the information of environmental factors, and abscissa is the information of alpha diversity indexes. The correlation coefficient r of Spearman is between -1 and 1, $r < 0$ is negative correlation, $r > 0$ is positive correlation, and the mark * is significance test ($p < 0.05$).

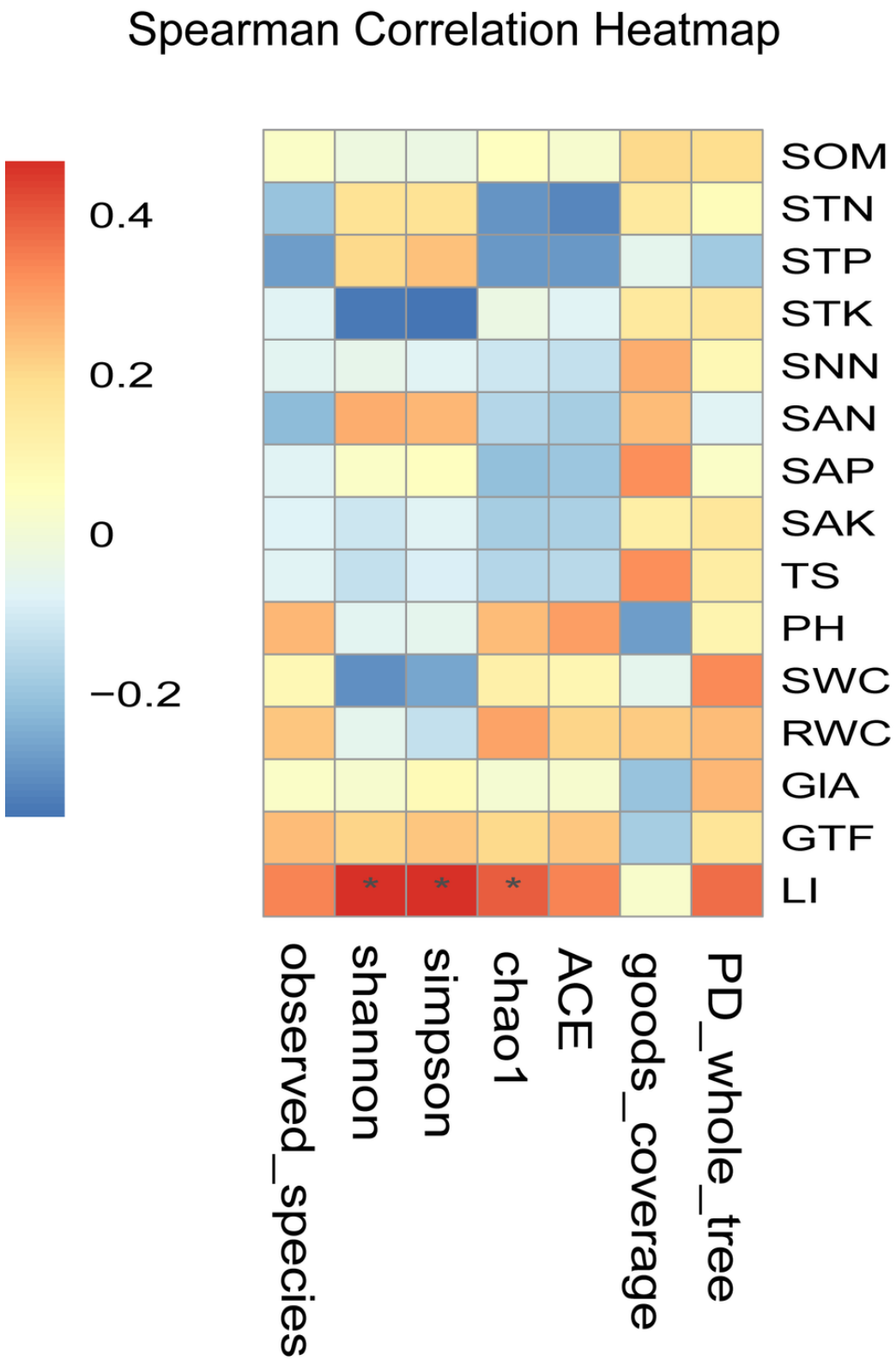
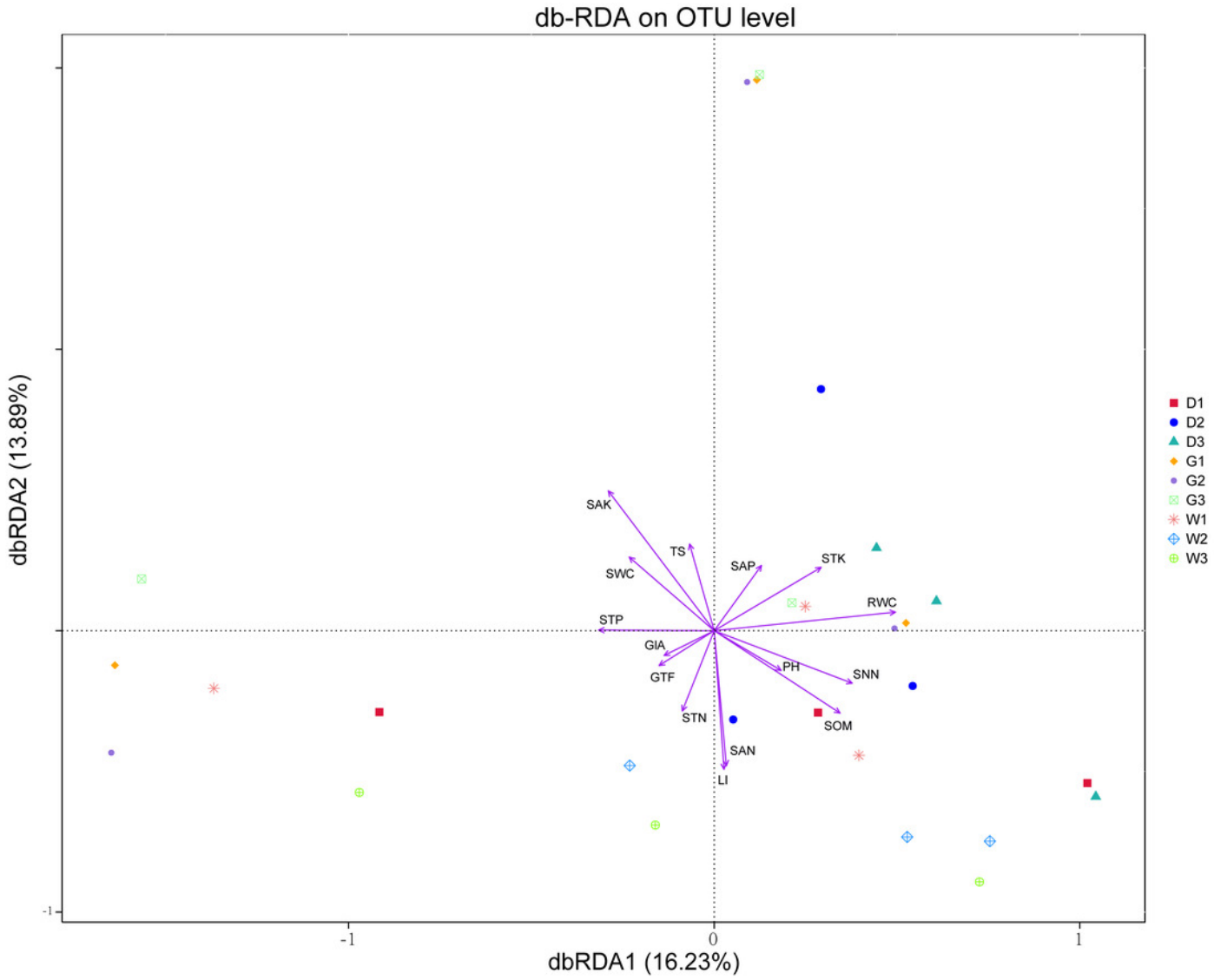

Spearman Correlation Heatmap
0.4
0.2
0
−0.2
SOM
STN
STP
STK
SNN
SAN
SAP
SAK
TS
PH
SWC
RWC
GIA
GTF
LI
*
*
*
observed_species
shannon
simpson
chao1
ACE
goods_coverage
PD_whole_tree

Figure 7

Distance-based redundancy analysis (db-RDA) for all groups

Environmental factors are generally represented by arrows. The length of the arrow line represents the degree of correlation between a certain environmental factor and community and species distribution, and the longer the arrow, the greater the correlation. When the angle between the environmental factors is acute, it means that there is a positive correlation between the two environmental factors, while when the angle is obtuse, there is a negative correlation.


db-RDA on OTU level
dbRDA2 (13.89%)
dbRDA1 (16.23%)
-1
0
1
SAK
TS
SWC
SAP
STK
RWC
STP
GIA
GTF
PH
SNN
STN
SOM
SAN
LI
D1
D2
D3
G1
G2
G3
W1
W2
W3