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 influence the quality and quantity of the medicinal plant's bioactive compounds through specific fungus-host interactions. Nevertheless, due to the paucity of information, the composition of endophytic fungal communities and the mechanism by which effective ingredients regulate endophytic fungal communities in roots remains unclear.

Methods. In this study, we collected root and soil samples (depth range: 0-20, 20-40, and 40-60 cm) of three *Glycyrrhiza* species (*Glycyrrhiza uralensis*, *Glycyrrhiza inflata,* and *Glycyrrhiza glabra*). Glycyrrhizic acid and liquiritin content were determined using high-performance liquid chromatography (HPLC), and total flavonoid content was determined using ultraviolet spectrophotometry. High-throughput sequencing technology was employed to explore the composition and diversity of the endophytic fungal community in different root segments of three *Glycyrrhiza* species. Furthermore, soil samples were subjected to physicochemical analyses.

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

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physicochemical properties of the soil regulated the endophytic fungal community composition and diversity of medicinal licorices.

Introduction

Glycyrrhiza species are widely grown perennial herbs in arid and semi-arid regions (Zhang et al., 2005). Three *Glycyrrhiza* species stipulated in Chinese Pharmacopeia, namely dried root and rhizome of are *Glycyrrhiza uralensis*, *Glycyrrhiza inflata*, and *Glycyrrhiza glabra*. Its dried roots and rhizomes are widely used as herbal medicines in eastern and western countries (Rizzato, Scalabrin et al., 2017). A wide variety of effective ingredients, such as triterpene saponins, polysaccharides, and flavonoids (Tianshui Niu, 2009), are extracted from the roots of *Glycyrrhiza* (Wang, Yang et al., 2015). Glycyrrhizic acid, the chief triterpene saponin (Li-Ping, Cui-Ai et al., 2010), had demonstrated anti-inflammatory (Schr?Felbauer, Raffetseder et al., 2009), antiviral, and immunoregulatory effects (Baba and Shigeta, 1987; Crance, Scaramozzino et al., 2003). Liquiritin is a major component of flavonoids that mainly exerts anti-inflammatory (Yina, a et al., 2018), antioxidant, and antibacterial effects (Weidner, Kordala et al., 2009; Antolak, Czyzowska et al., 2016). Due to its medicinal and economic value, the medicinal licorice plant has become a major research hotspot. The majority of the studies on licorice plants are focused on improving licorice content in licorice plants and discerning their ecological characteristics.

As per the conventional view, the quality and quantity of the bioactive compounds extracted from medicinal plants are primarily influenced by the genetic background of the medicinal plants, the ecological environment of the plant, and soil nutrients (Ncube, Finnie et al., 2012; Han, Jia et al., 2013). However, recent studies (S., H. et al., 2002; Huang, Cai et al., 2007; Shah, Rather et al., 2016; He, Cui et al., 2020) have shown that endophytic fungi substantially influences the quality and quantity of bioactive compounds in medicinal plants through specific fungus-host interactions.

Endophytes, in particular, endophytic fungi, are one of the most crucial components of plant's micro-ecosystems (Min, Ling et al., 2016). Endophytic fungi form a symbiotic relationship with its host plant, and it inhabits and grows in different healthy tissues of the host plant, including stems (Vaz, Mota et al., 2009), leaves (Hernawati, Wiyono et al., 2011), and roots (Radi?, Likar et al., 2014). Endophytic fungi sequester carbohydrates and other nutrients from the host plant for its own growth (Singh and Mukerji, 2006) and, in exchange, confer multiple benefits to host plants. Endophytic fungi can promote the growth of host plants by increasing levels of growth hormones, such as gibberellin, indoleacetic acid, abscisic acid, and zeatin (Zhang, Wang et al., 1999). It also enhances the resistance of host plants to environmental stress by increasing the production of biologically bioactive compounds (Zhao, Shan et al., 2011;

Ratnaweera, Silva et al., 2015). For instance, endophytic fungi promote plant growth and abiotic stress resistance in wheat plants (Farhana, Alam et al., 2019). Besides, endophytic fungi increase the accumulation of secondary metabolites, such as paclitaxel and deoxypodophyllotoxin in the host plant (Firáková, ?Turdíková et al., 2007), thereby affecting the quantity and quality of bioactive compounds of medicinal plants.

Endophytic fungi have demonstrated high biodiversity and are widely distributed in a myriad of terrestrial and aquatic plants (Saikkonen, Faeth et al., 1998). Endophytic fungi were isolated from multiple plants species, which includes important cash crop species (Pimentel, Glienke-Blanco et al., 2006), such as soybean, and medicinal plant species (Liu, Wenhong et al., 2017; Coutlyne, Thierry et al., 2018), such as *Dendrobium officinale* and *Sceletium tortuosum*. However, it is noteworthy that the rapid development of high-throughput sequencing technology and bioinformatics has enabled the identification of a plethora of novel fungal species (Taylor, Hollingsworth et al., 2014). Previous studies based on high-throughput sequencing technology have speculated that there are around 5.1 million fungal species, the majority of which are symbionts (Blackwell, 2011). Currently, only a small proportion of endophytic fungi could be isolated and identified, and the majority of the medicinal plant' endophytic fungi could not be cultured on routinely used media (Kivlin, Lynn et al., 2017). Therefore, it is indispensable to detect the endophytic fungal community in medicinal plants by adopting non-conventional culture methods. Modern molecular technology, specifically Illumina high-throughput sequencing technology, had comprehensively and accurately detected the diversity of endophytic fungal communities in medicinal plants (Berg, 2009; Kathrin, Blumenstein et al., 2015). Next-generation sequencing, a high-throughput sequencing technique, is a more robust and accurate characterization technique for the microbial community than 18S rDNA-based non-culture methods and conventional culturing methods.

Numerous studies (Karliński, Rudawska et al., 2010) have shown that the host's genetic background (genotype or species) determines the composition of endophytic fungi. Soil fertility and the ecological environment, which directly affect the content of bioactive compounds in medicinal plants, showed indirect effects on the composition and structure of the endophytic fungal community (Min, Ling et al., 2016). However, so far, there is insufficient information on the composition of endophytic fungi in the root of medicinal licorice of different genetic backgrounds (species) and soil environmental factors that affect the community structure of endophytic fungi in the root of medicinal licorice plants. Thus, in this study, we investigated the distribution and composition of endophytic fungal species of three distinct medicinal licorices at three different root depths using high-throughput sequencing and explored their relationship with effective ingredients in the root of host plants and soil' physicochemical properties. The outcomes of this study will enhance researchers' understanding of the environmental and host

factors that influence endophytic fungi and the symbiotic relationship between endophytic fungi and medicinal plants. This study provides reference data for licorice growth for commercial and medicinal use.

Materials & Methods

Sample collection: The roots and rhizospheric soils samples (all samples were 0-20 cm, 20-40 cm, and 40-60 cm, respectively) of three *Glycyrrhiza* plants (*Glycyrrhiza uralensis, Glycyrrhiza inflata,* and *Glycyrrhiza glabra*) were collected during August-September, 2019 from specimens growing at three distinct sites in three different eco-regions of Xinjiang province, China. The geographical location of sampling points and soil' physical and chemical properties are shown in Table S1. To increase the statistical significance of the study we randomly selected three healthy medicinal licorices plants from each geographical location as per 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 (0-20 cm), middle (20-40 cm), and lower (40-60 cm). Roots of each segment were equally divided into two parts: one part was used to determine the effective ingredients of the *Glycyrrhiza* root samples and placed in a sterile plastic bag, and the second part of the sample was put into a sterile bag and shipped to the laboratory in ice boxes for microbial characterization. The soil and root materials from each eco-region were collected as described above.

Surface sterilization: To remove the interference of other microbes, the surface of the licorice root was disinfected and sterilized in the laboratory as described previously (Saude, Hurtado-Gonzales et al., 2008). The samples from the final rinse solution were cultured using the potato dextrose agar (PDA) plate for 72 h at 28°C. No fungal growth was observed on the PDA media, which suggested that root samples were effectively surface-sterilized (Cui, Vijayakumar et al., 2018). All root samples were labeled and immediately placed on ice and stored at liquid nitrogen until total DNA extraction.

Physicochemical analysis of the soil: For the physicochemical analysis of rhizospheric soil, the soil samples were air-dried and sieved (2 mm mesh), and the physicochemical analysis was performed as described previously by Bao et al. (Bao, 2008). Soil pH (soil: distilled water in 1: 5 ratio) was measured using a pH meter, and 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 the acid digestion method. 0.01 M calcium chloride extraction method was used to determine the soil nitrate-nitrogen (SNN) and soil ammonium nitrogen (SAN) levels. The available phosphorus (SAP) content was measured by the sodium bicarbonate extraction method (molybdenum-

antimony colorimetry). The available potassium (SAK) content was determined by the ammonium acetate extraction method using atomic absorption spectrometry.

Determination of effective ingredients of *Glycyrrhiza* plant root: The *Glycyrrhiza* root samples were dried to constant weight, powdered using mortar and pestle, and sieved through 60-mesh. To analyze the effective ingredients, 0.2 g of sieved root powder samples were extracted using chromatographic methanol (71% concentration) in the ultrasonic bath (250 W, 40 kHz). The levels of effective ingredients, i.e., glycyrrhizic acid (GIA) and liquiritin (LI), were measured by high-performance liquid chromatography (HPLC, Agilent-1260 Infinity, USA), as described previously (Dang, Zhang et al., 2020). Agilent ZORBAX SB-C18 column (150 mm × 4.6 mm, 5 μm), DAD detector, and a mobile phase (chromatographic methanol: ultra-pure water: 36% glacial acetic acid = 71:28:1; acetonitrile: 0.5% glacial acetic acid = 1:4; 5 μL injection volume; 1.0 mL•min-1 elution rate) were used in the HPLC analysis. For calibration purposes, the reference materials of GIA and LI were: (CAS#1405-86-3) and LI (CAS#551-15-5), respectively, from Solarbio. The total flavonoid (GTF) content in root was measured by ultraviolet spectrophotometry at 334 nm with the liquiritin standard (CAS#551-15-5) from Solarbio as the control.

DNA extraction and library construction: Total genomic DNA was extracted from 0.5 g of root samples using the DNA Quick Plant System kit (Tiangen, China) as per the manufacturer's protocol. The concentration and integrality of extracted DNA were detected using a NanoDrop2000 (Thermo Fisher Scientific, USA) and 1% agarose gel electrophoresis, respectively. After determining the final concentration, DNA samples were diluted to 1 ng/μL with sterile distilled water, and 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 (David, Berry et al. 2011). To ensure amplification efficiency and accuracy, all PCR reactions were performed 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, 30 cycles (95°C/30 s, 55°C/30 s, 72°C/30 s), and 72°C/5 min. PCR products were mixed with 1X loading buffer (containing SYBR green) in equidensity ratios and visualized on 2% agarose gel electrophoresis. The target sequences were purified using GeneJET^{TM} Gel Extraction Kit (Thermo Scientific). The DNA libraries were constructed using TruSeq ® DNA PCR-Free Sample Preparation Kit (Illumina, USA) as per the manufacturer's instruction, and the quality was assessed on the Qubit ® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. ITS sequencing was carried out with the Illumina platforms (HiSeq2500) at the Beijing Compass Biotechnology Co., Ltd. (Beijing, China).

Bioinformatics analysis and statistical analysis: Cutadapt (Liu, Zhenshan et al., 2016) software was employed to assign the single-end reads to the respective samples based on the unique barcode, and single-end reads were truncated by cutting off the barcodes and primer sequences. Before the subsequent analysis, a total of 2,199,148 raw sequences were filtered by using Cutadapt software to remove the influence of the non-microbiota community, including chloroplast and mitochondrial sequences. Cutadapt software specific filtering conditions were used for strict quality control in order to generate high-quality clean reads. Clean reads were obtained by comparison with the reference database (Unite database) (Haas, Gevers et al., 2011) using the UCHIME algorithm to detect and remove chimeric sequences.

UPARSE software (Martin, 2011) (Version 7.0.1001) was used to cluster the clean reads into the same operational taxonomic units (OTUs) with ≥ 97% similarity. The clean reads with the highest frequency were used as the representative sequence of each OTU. The classification information for each representative sequence was annotated through the Unite database based on the BLAST algorithm using QIIME software (Version 1.9.1). To decipher the phylogenetic relationship among 27 samples, MUSCLE (Version 3.8.31) software was employed for multiple sequence alignment. The OTU abundance information was normalized by the standard sequence number corresponding to the minimum sequence sample (54,262 reads for sample D.2.1).

Alpha diversity analysis based on output normalized data were used to study the complexity of species diversity in a sample using six indices (observed-species, Shannon, Simpson, Chao1, ACE, and good-coverage) (Li, Zhang et al., 2013). All indices in the samples were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 3.6.1).

To evaluate differences in sample species complexity, the beta diversity analysis of output-normalized data was used, which was based on weighted Unifrac and calculated using 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 3.6.1) was also used for 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 (Pearson coefficient, r) was performed for the effective ingredients and the physicochemical properties of the soil, with the significance level set to 0.05. ANOVA was performed with SPSS (Version19.0) (IBM Inc., Armonk, USA) and displayed with GraphPad Prism 5. The statistically significant differences were determined by ANOVA, followed by Bonferroni's statistical test for multiple comparisons, and the significance level was set to 0.05.

Results

Differences in levels of effective ingredients in *Glycyrrhiza* roots

The effective ingredients of *Glycyrrhiza* roots and physicochemical properties of soil are presented in Table S2. The results of two-way ANOVA showed that the levels of the effective ingredients, i.e., glycyrrhizic acid (GIA), liquiritin (LI), and total flavonoid (GTF), were not significantly affected by the interaction between root depth (0-20 cm, 20-40 cm, and 40-60 cm) 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 Figure 1, LI content in *Glycyrrhiza uralensis* (W) root was significantly higher than *Glycyrrhiza inflata* (D) ($P < 0.05$) and *Glycyrrhiza glabra* (G) roots ($P < 0.05$) (Figure 1a).

As per the Pearson correlation analysis, levels of effective ingredients were significantly correlated with the physicochemical properties of the soil (Table 1). GIA content in *Glycyrrhiza* level was significantly and positively correlated to the available potassium (SAK) and water content of the soil (SWC) ($r > 0$; $P < 0.05$); however, LI level in root was significantly and negatively correlated to SAK and total salt (TS) content of the soil ($r < 0$; $P < 0.05$).

Sequencing of *Glycrrhiza* root's endophytic fungi

A total of 2,118,633 effective sequences were identified by sequencing the root samples from three *Glycyrrhiza* (*Glycyrrhiza uralensis*, *Glycyrrhiza glabra*, and *Glycyrrhiza inflata*) species using Illumina HiSeq sequencing and after filtering out low-quality and short sequence reads. The sequencing results of each sample are listed in Supplementary Table S4. The effective sequences were clustered into OTUs with 97% identity, and a total of 1,063 OTUs were obtained. Out of the total effective sequences, 91.53% 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 (Figure S1a). The rarefaction curves showed that the number of OTU in each sample increased gradually with the sequence quantity, which validated that the sequencing data was adequate for the analysis (Figure S1b).

Differences in alpha diversity

The alpha diversity index of each group is demonstrated in Table S5. Alpha diversity indices, Shannon and Chao1, deciphered the diversity and richness of microbial communities in *Glycyrrhiza* root samples. A higher index value denotes higher species diversity and distribution. The Shannon index of the W1 (4.910) sample was the highest, and that of the D1 (3.393) sample was the lowest. Moreover, the D1 root sample showed the lowest Chao1 (238.678) and ACE (253.105) values, while the D3 root sample showed the highest Chao1 (356.317) and ACE (355.694) values. As per Wilcoxon rank-sum analysis, the Shannon index showed significantly different distribution between W and D samples, especially at 0-20 cm at the root depth (Figure 2a). The Shannon index in the W1 root sample was significantly higher than the D1 root sample ($P < 0.05$). Furthermore, the Chao1 index in sample D increased gradually with decreasing root

depths, and as per the Wilcoxon rank-sum test, sample D'Chao1 index value was significantly affected by root depth (Figure 2b). Specifically, the Chao1 index value of D3 sample was significantly higher than the D1 sample ($P < 0.01$), and that of the D2 root sample was significantly higher than the D1 root sample ($P < 0.05$).

Differences in beta diversity

To evaluate differences in microbial community composition among *Glycyrrhiza* root samples, beta diversity analysis was performed. The Unweighted Pair-Group Method with Arithmetic (UPGMA) cluster analysis was performed to discern similarity in the composition of endophytic fungal community among different *Glycyrrhiza* root samples. The UPGMA clustering results were integrated with the relative abundance of species at the phyla and taxon levels in each group. The UPGMA cluster tree outcomes based on Weighted Unifrac distances showed that G3, G2, G1, W1 samples and W3, D2, W2, D1 samples were clustered together (Figure 3a). Meanwhile, to discern the difference in the beta diversity between different groups of samples, a Wilcoxon rank-sum test based on Weighted Unifrac distances was constructed (Figure 3b). The outcomes of this test showed that there were significant differences in beta diversity between the W and D group of samples, which was consistent with the 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). It indicated that endophytic fungal community composition differed significantly between different species and different root depths in medicinal licorice plants.

Differences in endophytic fungal community composition in medicinal licorice

Based on OTU sequences and the Unite database, total sequences were annotated into 8 phyla, 23 classes, 53 orders, 102 families, 140 genera, and 141 species. The most abundant endophytic fungal phyla in all the nine groups are enumerated in Figure 4a. Ascomycota phyla were found to be the most dominant phyla among all the samples, accounting for 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 samples, respectively. In addition, Basidiomycota accounted for 21.348%, 28.440%, 10.631%, 12.523%, 6.749%, and 5.110% of relative abundance in D2, D3, G2, G3, W2, and W3 samples, respectively. The relative abundance of Ascomycota phyla decreased with increasing root depth. To determine the differences at the phylum level in different groups of root samples, a MetaStat statistical test based on species abundance was conducted. As per the outcomes of this test, the relative abundance of Ascomycota in sample D showed significant differences in distribution at different root depths (Figure 4b). Specifically speaking, the relative abundance of Ascomycota in the D1 sample (91.821%) was significantly higher than the D3 sample (39.956%) (Figure 4b).

At genus level, the top 10 dominant fungal genera based on relative abundance in each group (Figure 4c) were *Fusarium* (D1: 27.907%, G1: 23.944%, G2: 31.071%, G3: 25.381%, W1: 19.253%, W3: 18.215%), *Paraphoma* (D1: 27.738%, D3: 23.937%, W3: 13.980%), *Helminthosporium* (D1: 26.567%, G1: 25.124%, W1: 8.224%, W2: 17.408%), *Sarocladium* (D2: 3.326%, G1: 16.547%, G2: 17.243%, G3: 21.897%, W1: 4.218%), *Cladosporium* (D2: 6.446%, D3: 2.721%, W3: 15.174%). Furthermore, *Cadophora* (13.200%) and *Psathyrell*a (10.917%) were found to be the most dominant genera in D2 sample, *Tomentella* (14.472%) in D3 sample, and *Conocybe* (12.068%) 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, Agaricomycetes were found to be the dominant class; Hypocreales, Pleosporales, Thelephorales dominant order; Nectriaceae, Phaeosphaeriaceae, Massarinaceae dominant family; *Fusarium-solani, Paraphoma-radicina, Sarocladium-kiliense* dominant species.

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

Spearman correlation analysis demonstrated that dominant endophytic fungi phyla were significantly correlated to effective ingredients and physicochemical properties of the soil (Table S7). Ascomycota showed a highly significant but negative correlation with RWC ($r < 0$, $P < 0.01$); conversely, Basidiomycota showed a highly significant but positive correlation with RWC ($r > 0$; $P < 0.01$). Besides, a significant and positive correlation was observed between Olpidiomycota and GIA ($r > 0$; $P < 0.05$); Mortierellomycota and STK, SWC, SAK ($r > 0$; $P < 0.05$); Rozellomycota and SOM, STK, RWC ($r > 0$; $P < 0.05$). However, Mucoromycota showed significant and positive correlation with SOM ($r > 0$; $P < 0.01$), but it showed significant and negative correlation with STP ($r < 0$, $P < 0.01$).

As depicted in Figure 5, dominant fungal genera were found to be significantly correlated to effective ingredients and soil physicochemical properties. A significant and positive correlation was found between *Fusarium* and LI content ($P < 0.05$), *Helminthosporiu*m, and PH ($P < 0.05$). *Sarocladium* showed a significant negative correlation with SOM, STN, and SNN ($P < 0.05$). *Paraphoma* showed a significant and positive correlation with SAN ($P < 0.05$) but a significant negative correlation with SAK, TS, and SWC ($P < 0.05$). *Conocybe* showed a significant positive correlation with SWC but a significant negative correlation with SAN ($P < 0.05$).

Correlation of effective ingredients with physicochemical properties of soil and endophytic fungal community in the *Glycyrrhiza* roots

Spearman correlation analysis showed that the LI content was significantly and positively correlated with the alpha diversity index ($r > 0$, $P < 0.05$) (Figure 6). Besides, LI content showed

a highly significant and positive correlation with the Shannon index, Simpson index, and Chao1 index ($P < 0.05$). It indicated that the LI content led to the differences in the diversity of the endophytic fungal community in medical licorice roots in this study.

Distance-based redundancy analysis (db-RDA) based on the Bray-Curtis distance showed that the effective ingredients and physicochemical properties of the soil had significant effects on the differences in the endophytic fungal community (Figure 7). The differential distribution of endophytic fungal community was restricted primarily to the first and second ordination axes and explained 16.23%, 13.89% of the total variability, respectively (Figure 7). Out of all the soil' environmental factors, SAK content affected the differences of the endophytic fungal community most significantly ($r^2 = 0.329$, $P < 0.01$), followed by SAN ($P < 0.05$). Among the root factors, RWC most significantly affected the difference of endophytic fungal communities ($r^2 = 0.247$, $P < 0.05$), followed by LI content ($P < 0.05$) (Figure 7, Table S8). As per the outcomes 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 the endophytic fungal community in the roots of the medicinal plants in the current study.

Discussion

In the current study, the composition and diversity of endophytic fungal communities at different root depth range (0-20 cm, 20-40 cm, and 40-60 cm) of three *Glycyrrhiza* species (*Glycyrrhiza uralensis*, *Glycyrrhiza glabra*, and *Glycyrrhiza infl*ata) were investigated using high-throughput sequencing technology. Thus, a highly accurate and substantial amount of data was procured than previous studies based on conventional technology (Tie, Hu et al., 2010; Jiang-Tao, Xiao-Xia et al., 2013; Palak, Arora et al., 2019). As per the alpha and beta diversity analysis of endophytic fungal community, fungal communities between *Glycyrrhiza uralensis* and *Glycyrrhiza inflata* showed significant differences at different root depths (0-20 cm, 20-40 cm, and 40-60 cm) (Figure 2 and Figure 3). It indicated that the host plant's genotype and ecological region contributed to the differences in endophytic fungal communities. Numerous studies (Saikkonen, W?Li et al., 2004) have demonstrated that the adaptation of the endophytic fungal community primarily relies on adapting host plants to the ecological environment. It implies that host plants substantially influence the colonization and distribution of the endophytic fungal communities. The interaction between fungus and host plant is often considered dynamic where orientation is determined by subtle differences in the expression of fungal genes in response to the host or, conversely, by the host's recognition and response fungus. Thus, slight genetic differences in the two genomes control the symbiosis (Moricca and Ragazzi, 2008).

Furthermore, the current study also showed that root depth significantly affected the richness and composition of the endophytic fungal community (Figure 2 and Figure 3). It

indicated that ecologically different fungi might represent certain ecological regions (root depth). It might be a crucial factor that should be taken into account while inoculating endophytic fungi into the host plants. We speculated that this could be correlated to root respiration and soil C content. Root respiration, which accounts for 60% of total soil respiration, regulates the metabolism of roots and soil microbes and is considered a significant contributor to the terrestrial carbon budget (Pregitzer, Laskowski et al., 1998). Also, the C content in unstable soil varies significantly at different soil depths (de Graaff, Jastrow et al., 2014). Moreover, Noah Fierer et al. (Fierer, Schimel et al., 2003) demonstrated that the vertical distribution of the specific microbial species was correlated mainly to decreased carbon availability with increasing soil depth.

In this study, we employed high-throughput sequencing to determine the composition of endophytic fungal communities at different taxonomic levels (phylum, class, older, family, genus, and species) (Figure 4a, Figure 4c, and Table S6). The results indicated that 27 samples of medicinal licorice roots contained a peculiar microbiome. For example, Ascomycota was the dominant phylum in all samples, followed by Basidiomycota, which was in line with previous studies (Stephenson, Tsui et al., 2013; Tan, Zhou et al., 2018). The phylum Ascomycota, the largest phylum of fungi, entails a highly diverse population and plays a vital role in genetics (Wallen and Perlin, 2018), ecology (Belnap and Lange, 2005), and phylogeny (López-Giráldez, Crous et al., 2009). For instance, Ascomycota produces large numbers of spores through both asexual and sexual reproduction. To disperse ascospores, asci act as small water cannon and sprays spores into the air. Spores are also spreads multiple phytopathogenic and saprophytic fungi (Trail, 2010). Most of the members of Ascomycota are saprophylaxis and plays an important role in organic matter decomposition in the soil. In this process, the dominant fungal community assimilates root exudates for organic matter degradation (Mylène, Patricia et al., 2018). He et al. (He, Cui et al., 2020) showed that inoculation of licorice plants with dark-colored septogenic endophyte (DSE), conidia, or sterile ascomycetes increased root biomass, uptake of nitrogen (N) and phosphorus (P) by roots, and concentration of glycyrrhizin and glycyrrhizic acid.

Moreover, outcomes of this study indicated that the relative abundance of Ascomycota gradually decreased with the increasing root depths (Figure 4b), in line with the previous study by Ko, Daegeun et al. (Ko, 2015). Based on this data, we investigated the correlation between Ascomcycota in *Glycyrhizza* roots and root depth. We observed that the relative abundance of Ascomycota in *Glycyrrhiza inflata* differed significantly with root depth. However, the relative abundance of Ascomycota in *Glycyrrhiza uralensis* and *Glycyrrhiza glabra* did not differ significantly with root depth. It indicated that specific endophytes proliferate preferentially in certain ecological regions and play different ecological roles than other endophytes. Concisely,

in addition to soil depth, the relative abundance of endophytes was also correlated to the genotype of the host plant species. These findings were in line with the study on host genotype and soil conditions on the ectomycorrhizal community of poplar clones (Karliński, Rudawska et al., 2013).

However, the primary limitation of the generalization of the current study's outcomes is that three samples of *Glycyrrhiza* plant roots were collected from distinct geographical areas. As it is challenging to procure three distinct *Glycyrrhiza* species from the same habitat, to a certain extent, physicochemical properties of the soil can represent the environmental factors of *Glycyrrhiza* species. Therefore, in this study, we investigated the effect of the root as well as soil factors.

Numerous studies (Da-Cheng, Hao et al., 2015; Li and Wu, 2018) reported that the accumulation of effective ingredients in medicinal licorice roots is affected by multiple factors. In this study, the LI content was affected more by the plant species than root depth (Table S3). LI content in *Glycyrrhiza uralensis* root was significantly higher than in *Glycyrrhiza inflata* and *Glycyrrhiza glabra* (Figure 1a), in line with a previous study (Zhang, Yang et al., 2018). We speculate that it might be correlated to the expression of specific functional genes, which might be strongly correlated to the content of effective ingredients, such as glycyrrhizic acid and liquiritin in the root of the licorice species. As per the previous studies (Winkel-Shirley 2002, 2017; Mochida, Sakurai et al., 2017), key functional genes, such as chalcone synthase gene, 3-Hydroxy-3-methylglutary CoA reductase (*HMGR*), and squalene synthase (*SQS*), regulate the transcription of glycyrrhizic acid and liquiritin. However, further in-depth analysis is required to characterize the expression of functional genes of effective ingredients. The current study provides a theoretical basis for the developmental strategies related to the improvement of *Glycyrrhiza uralensis* cultivation. The content of effective ingredients in *Glycyrrhiza* root samples may result from the interaction between plants and their environment during plant growth. Thus, the accumulation of effective ingredients in root is influenced by its ecological environment. In this study, GIA, GTF, and LI content showed a positive correlation with soil total nitrogen (STN) ($r > 0$) (Table 1). Thus, we speculate that the majority of the soil nutrients can promote the accumulation of effective ingredients; however, certain soil nutrients, such as soil total potassium (STK), are an exception. Potassium activates multiple enzyme systems and increases stress resistance in plants (Wang and Wu, 2017). In this study, STK was found to be negatively correlated ($r < 0$) to the GIA, GTF, and LI content, in line with the previous study (Liu, Li et al., 2020). In addition, soil available potassium (SAK) was found to be significantly and positively correlated to GIA level but significantly and negatively correlated to LI level (Table 1). In this study, we speculated that the utilization mechanism of soil nutrients by effective ingredients might be entirely different. However, the underlying mechanism for

potassium mediated regulation of effective ingredients remains unclear. Thus, this study may provide platform data for an in-depth analysis. In general, these soil factors exhibit habitat-specific characteristics for regulating the effective ingredients in licorice roots.

In recent years, a growing number of studies (Stegen, Lin et al., 2012; Edwards, Johnson et al., 2015; Nuccio, Anderson-Furgeson et al., 2016) reported that the dynamics of the microflora is driven to a large extent by environmental factors, such as soil characteristics (pH, nitrogen, phosphorus, and potassium) and climatic conditions (rainfall and temperature). In line with these reports, our study showed that LI, RWC, SAN, and SAK content were the major contributing factors to the variations in the overall structure of the endophytic fungal community (Figure 7 and Table S8). In addition, we found that the LI content in *Glyrrhiza* root was significantly and positively correlated to the diversity of endophytic fungal community (Shannon and Simpson index) ($P < 0.05$) (Figure 6). Liquiritin (LI), an essential component of flavonoids, confers clinical efficacy to the medicinal licorices and serves as an important quality index for determining the quality of medicinal licorices. Flavonoids synthesis in host plants is induced when the symbiotic fungus is acted upon by purified signaling molecules secreted from the same fungal cells during colonization. Chen et al. (Meilan, Chen et al., 2017) demonstrated that stem biomass, root biomass, and liquiritin content in the root of host plants increased significantly when inoculated with fungi *Glomus mosseae*, *Glycyrrhiza uralensis*.

Meanwhile, our results showed that physicochemical factors of the soil and effective ingredients had a significant effect on the composition of endophytic fungal communities at phylum and genus levels (Figure 5 and Table S7). It demonstrated the interaction between endophytic fungal community, root factors, and soil factors. Thus, it indicated that fungal composition could be altered by altering soil factors (Liu, Jinshan et al., 2018), promoting the accumulation of effective ingredients in plants (Han, Jia et al., 2013). Wei Xie et al. (Xie, Hao et al., 2019) showed that in medicinal licorice, P addition and arbuscular mycorrhizal (AM) inoculation improved plant growth and facilitated glycyrrhizic acid and liquiritin accumulation in *Glycyrrhiza uralensis*. Meanwhile, Y. Orujei et al. (Orujei, Shabani et al., 2013) also showed arbuscular mycorrhizal fungi (AMF) inoculation enhanced the growth rate and accumulation of effective ingredients in licorice roots (*Glycyrrhiza glabra*) as compared to control.

In general, the current study unraveled the ecological role of non-biological factors (soil and root) in the endophytic fungal community composition of medicinal licorices. Besides, this study provides crucial information for the developmental strategies related to improving the production and quality of medicinal licorice plants. However, further in-depth studies are required to characterize the functions of the endophytic fungi.

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Table 1(on next page)

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

the values are Pearson's correlation coefficients (significance level was set at 0.05). The correlation coefficient r of Pearson is between -1 and 1, r < 0 means negative correlation, r > 0 means positive correlation, r = 0 means no linear correlation. ** means P<0.01; * means P<0.05. Abbreviations: GlA, glycyrrhizic acid; GTF, total flavonoid; LI, liquiritin; SOM, soil organic matter; STN, soil total nitrogen; STP, soil total phosphorus; STK, soil total potassium; SNN, soil nitrate nitrogen; SAN, soil ammonium nitrogen; SAP, soil available phosphorus; SAK, soil available potassium; TS, total salt; PH, soil pH; SWC, soil water content.

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 Pearson's correlation coefficients (significance level was set at 0.05). The correlation coefficient r of Pearson is between -1 and 1, r < 0 means negative correlation, r > 0 means positive correlation, r = 0 means no linear correlation. ** means P<0.01; * means P<0.05.

Abbreviations: GlA, glycyrrhizic acid; GTF, total flavonoid; LI, liquiritin; SOM, soil organic matter; STN, soil total nitrogen; STP, soil total phosphorus; STK, soil total potassium; SNN, soil nitrate nitrogen; SAN, soil ammonium nitrogen; SAP, soil available phosphorus; SAK, soil available potassium; TS, total salt; PH, soil pH; SWC, soil water content.

Figure 1

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

Ordinate is the content of liquiritin (a), glycyrrhizic acid (b) and total flavonoid (c); abscissa is the group name: D, G and W: *Glycyrrhiza inflata, Glycyrrhiza glabra* and *Glycyrrhiza uralensis*, and the mark * is significance test ($p < 0.05$). Bars (mean with standard error) with different lowercase letters indicated significant difference ($P < 0.05$) assessed by one-way analysis of variance followed by Bonferroni's test for multiple comparisons.

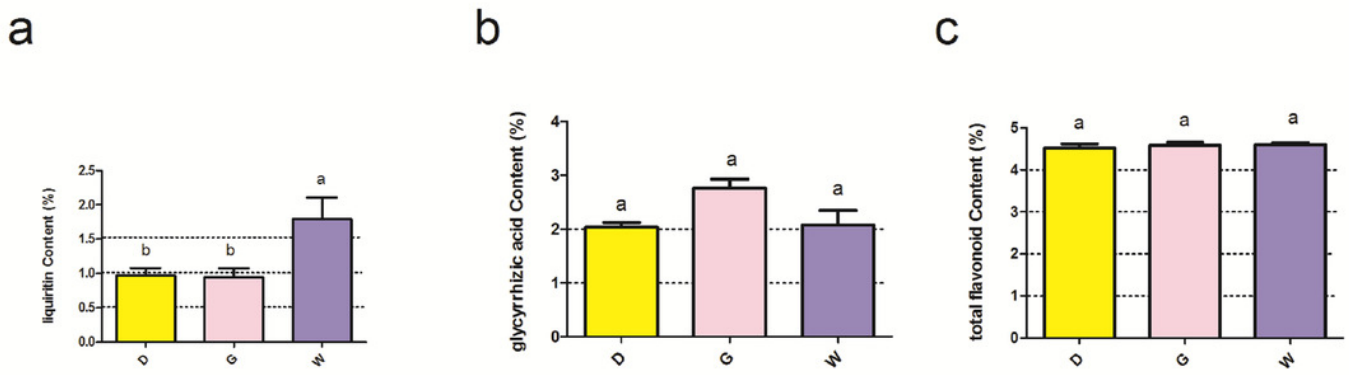


Figure 2

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 $p < 0.05$.

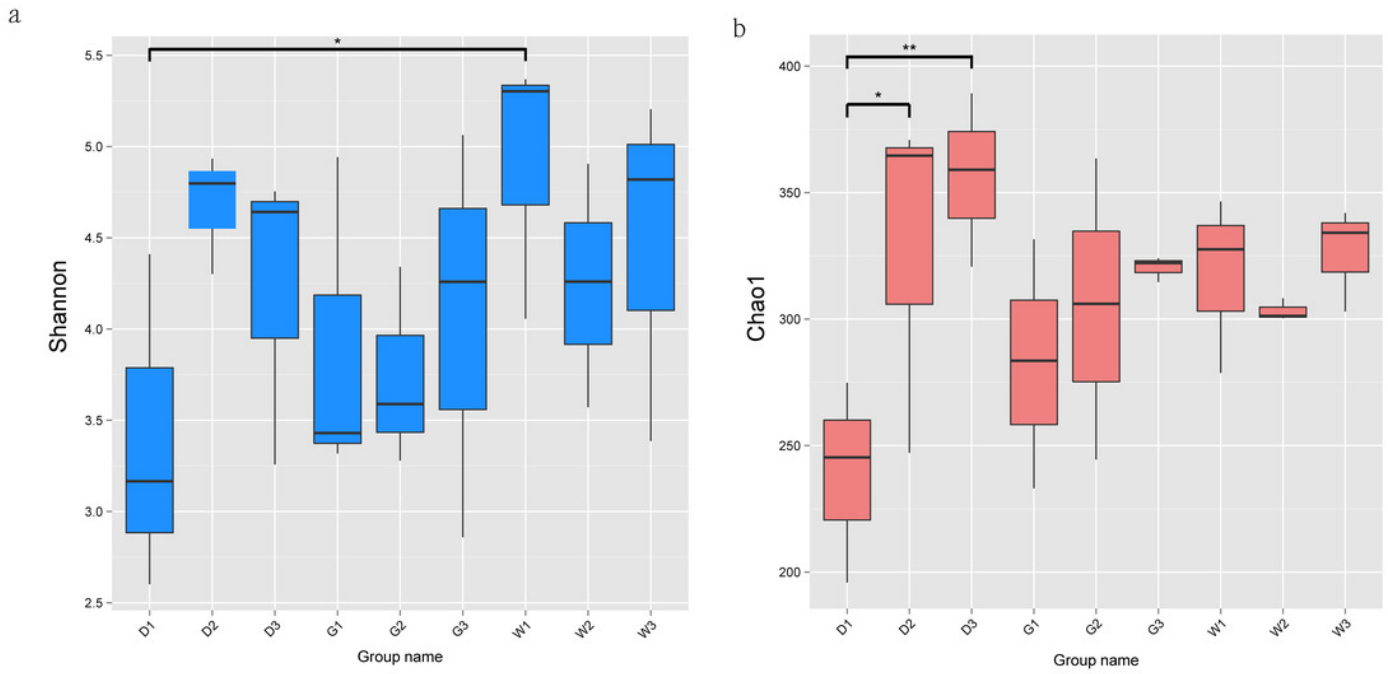
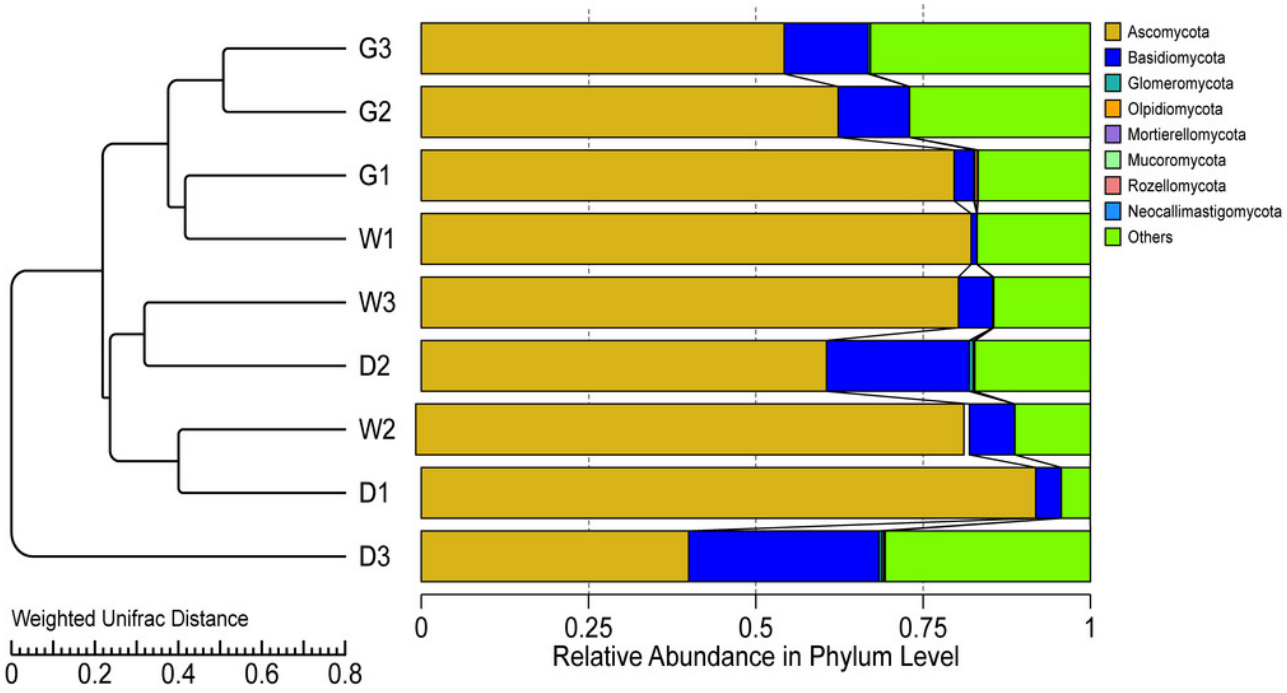


Figure 3

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

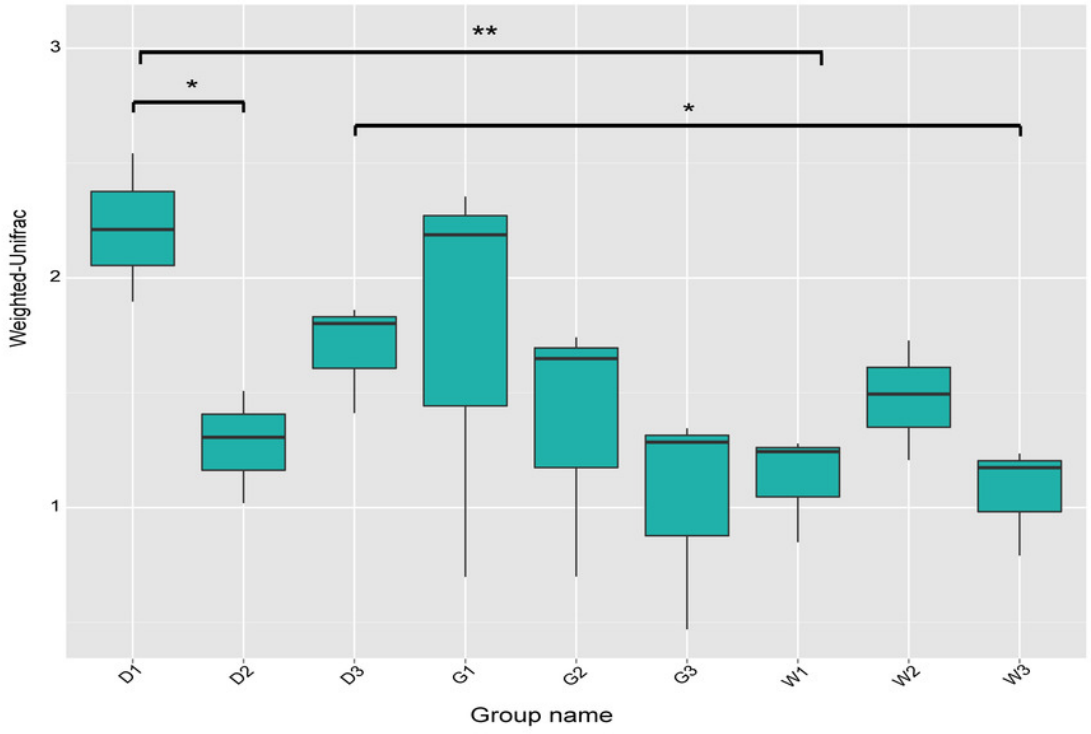

**
*
*
Weighted-Unifrac
3
2
1
D1
D2
D3
G1
G2
G3
W1
W2
W3
Group name

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.

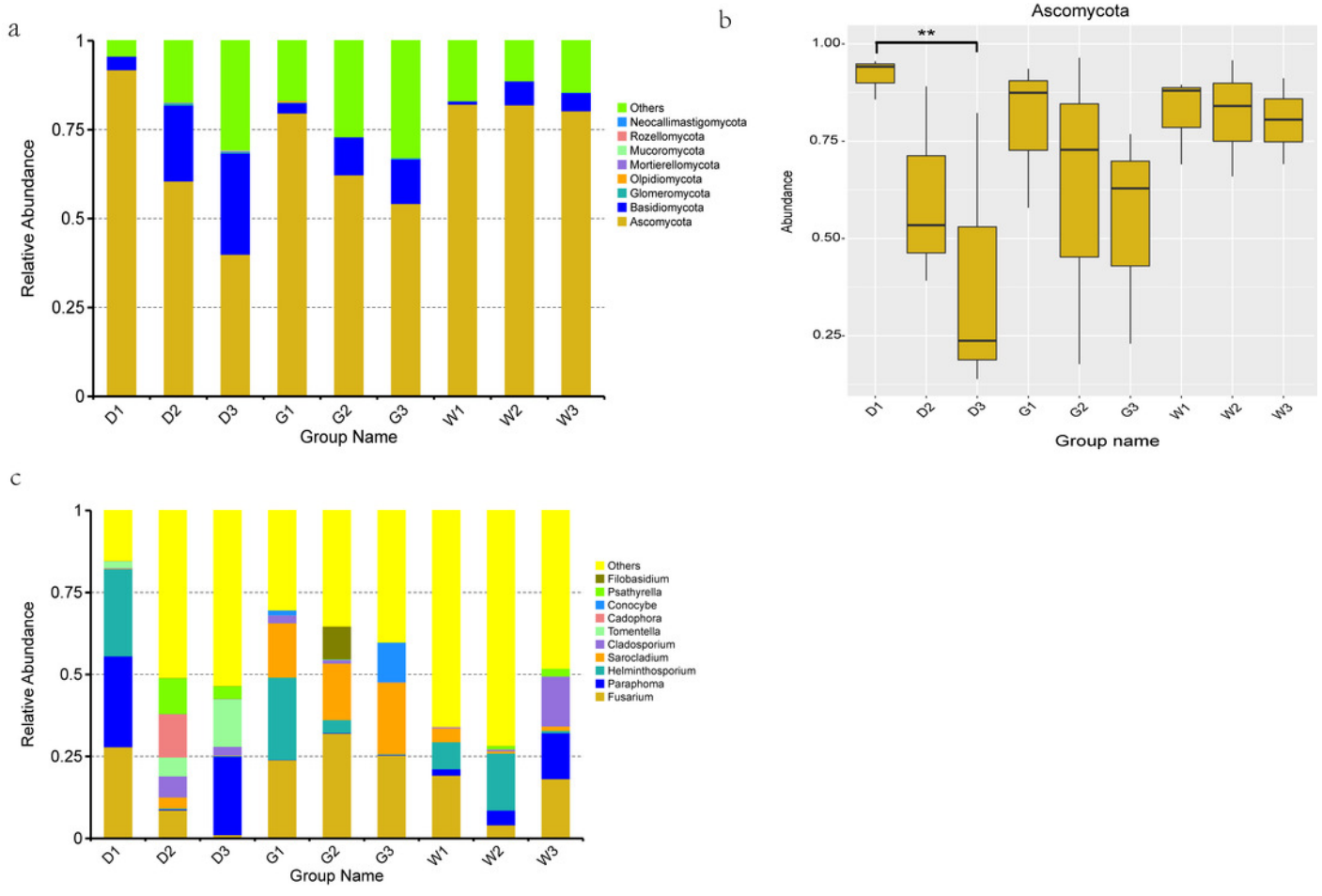


Figure 5

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 ($p < 0.05$). Abbreviations: SOM, soil organic matter; STN, soil total nitrogen; STP, soil total phosphorus; STK, soil total potassium; SNN, soil nitrate nitrogen; SAN, soil ammonium nitrogen; SAP, soil available phosphorus; SAK, soil available potassium; TS, total salt; PH, soil pH; SWC, soil water content; RWC, root water content; GlA, glycyrrhizic acid; GTF, total flavonoid; LI, liquiritin.

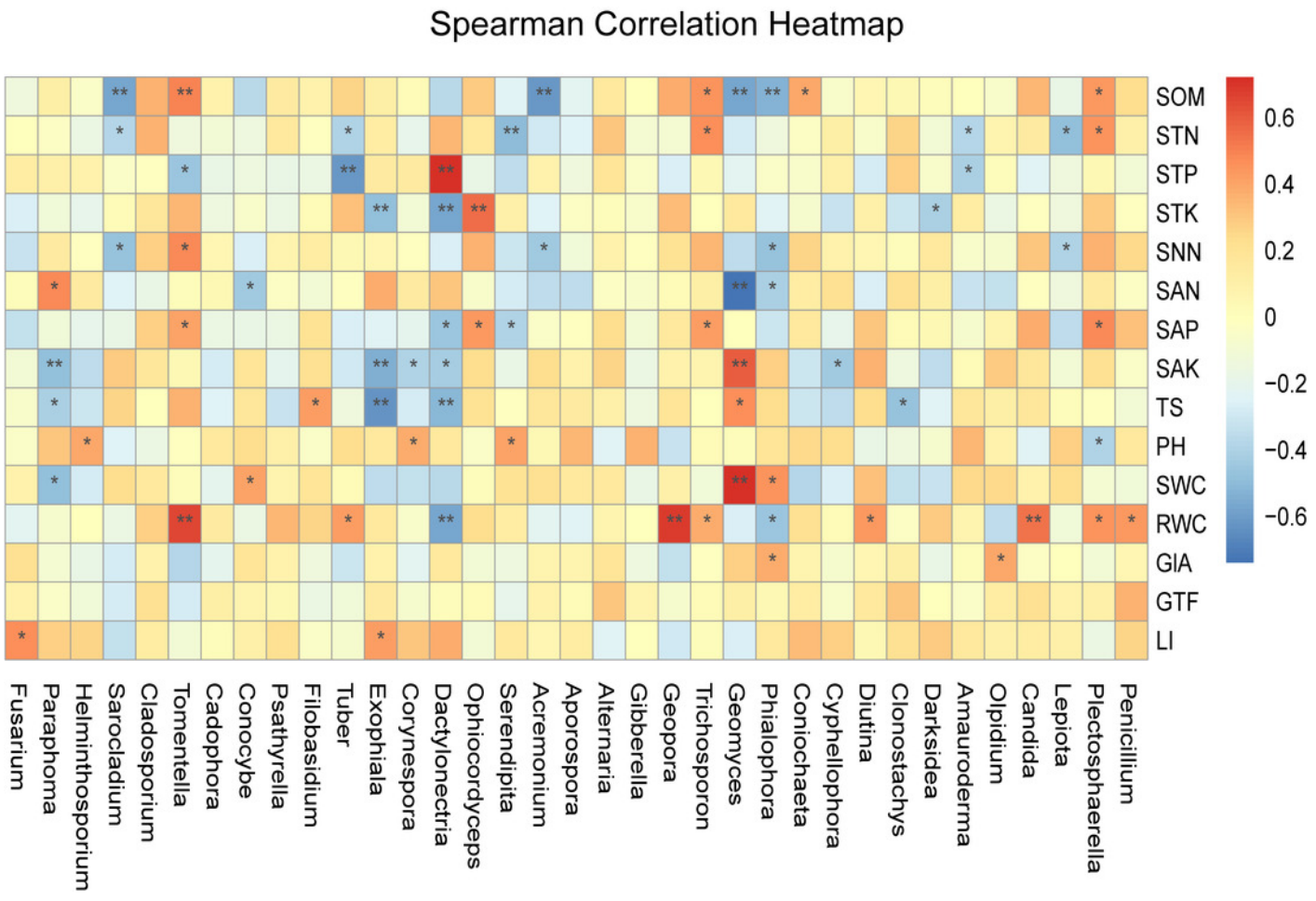


Figure 6

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 ($p < 0.05$). Abbreviations: SOM, soil organic matter; STN, soil total nitrogen; STP, soil total phosphorus; STK, soil total potassium; SNN, soil nitrate nitrogen; SAN, soil ammonium nitrogen; SAP, soil available phosphorus; SAK, soil available potassium; TS, total salt; PH, soil pH; SWC, soil water content; RWC, root water content; GlA, glycyrrhizic acid; GTF, total flavonoid; LI, liquiritin.

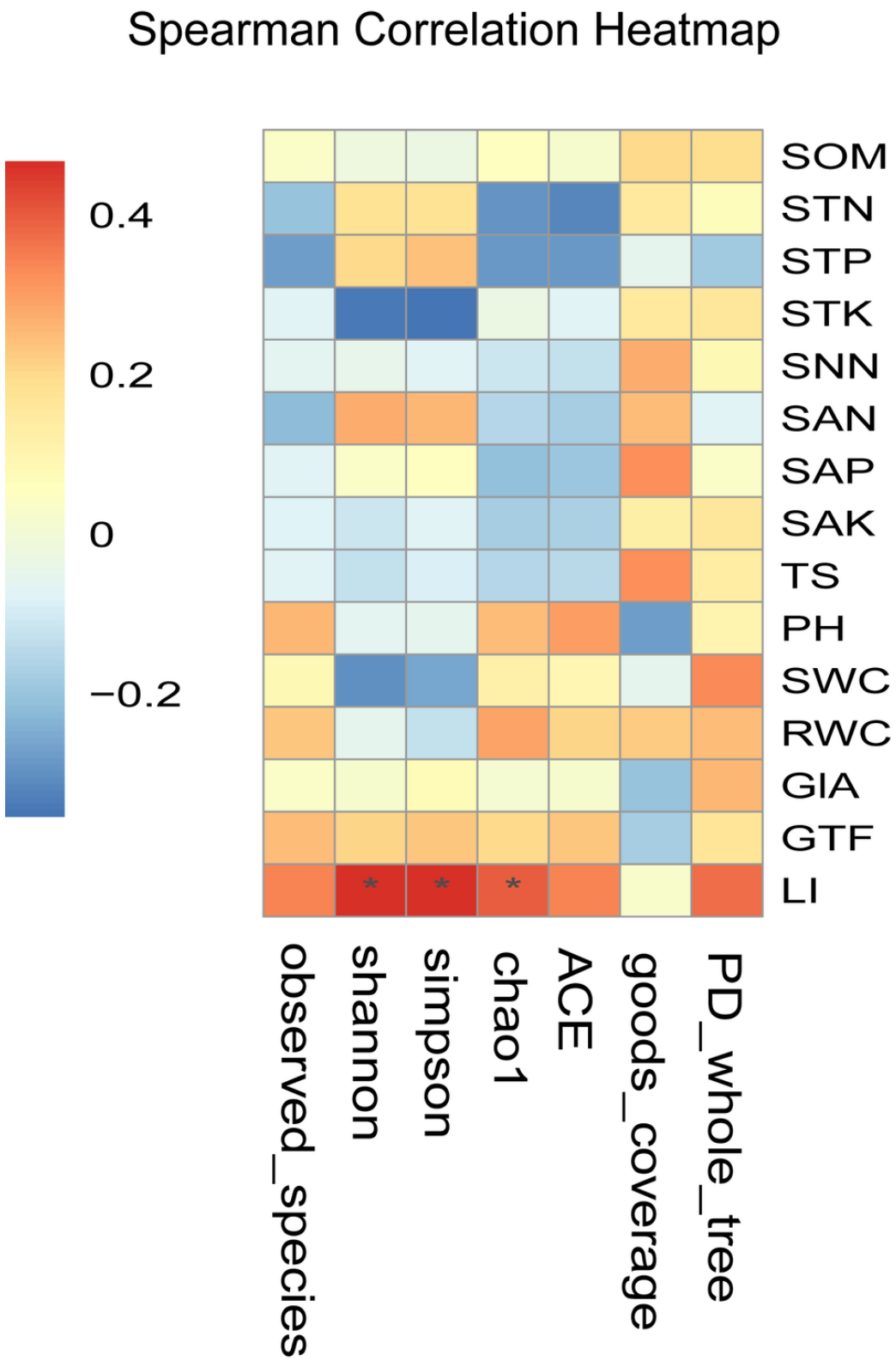
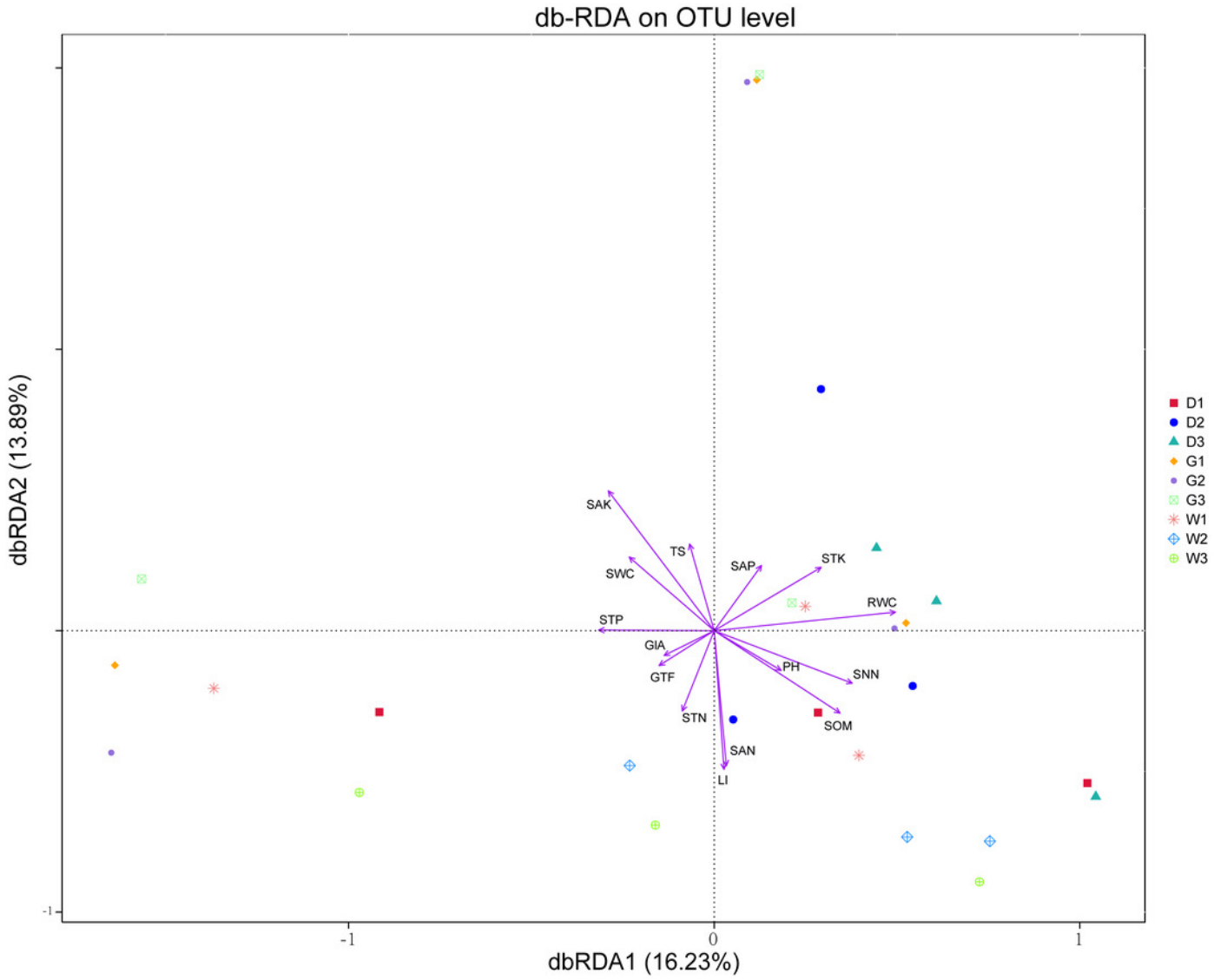

Spearman Correlation Heatmap
0.4
0.2
0
−0.2
SOM
STN
STP
STK
SNN
SAN
SAP
SAK
TS
PH
SWC
RWC
GIA
GTF
LI
*
*
*
observed_species
shannon
simpson
chao1
ACE
goods_coverage
PD_whole_tree

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. Abbreviations: SOM, soil organic matter; STN, soil total nitrogen; STP, soil total phosphorus; STK, soil total potassium; SNN, soil nitrate nitrogen; SAN, soil ammonium nitrogen; SAP, soil available phosphorus; SAK, soil available potassium; TS, total salt; PH, soil pH; SWC, soil water content; RWC, root water content; GlA, glycyrrhizic acid; GTF, total flavonoid; LI, liquiritin.


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