

Diversity of Endophytic Fungal Community in *Huperzia Serrata* from Different Ecological Areas and Their Correlation with Hup A Content

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Abstract

Hup A has attracted considerable attention as an effective therapeutic candidate drug used for the treatment of Alzheimer's disease. However, till now, very few comprehensive studies are available on the biological diversity and structural composition of endophytic fungi and the effects of endophytic fungi on the Hup A accumulation in *H. serrata*. In this research, the composition and diversity of fungal communities were deciphered comparative sequence analysis of the ITS2 region of the fungal rRNA genes based on high-throughput sequencing technology. The correlation between endophytic fungal community and Hup A content was investigated as well. Results revealed that the richness as well as diversity of endophytic fungi was various according to different tissues and different ecological areas. The endophytic fungal communities of *H. serrata* exhibit specie-specific, ecological-specific and tissue-specific characteristic. This study will provide realistic basis and theoretical significance for understanding the biological diversity and structural composition of endophytic fungal communities in *H. serrata*, as well as providing novel insights into the interaction between endophytic fungi and Hup A content.

Introduction

Huperzine A (HupA) is a highly active alkaloid extracted and isolated from the herbal plant *Huperzia serrata*, and has been successfully used to treat myasthenia gravis and Alzheimer's disease (AD) as a potent, reversible, and selective acetylcholinesterase (AChE) inhibitor (Liu et al., 1986; Jianget al., 2003; Ma et al., 2005; Baie et al., 2007). Unfortunately, the HupA content in wild *H. serrata* is relatively low and the content of the compound in the dried herb is less than 0.025% (Bialeret al., 2007; Xie et al., 2016). Furthermore, *H. serrata* grows very slowly and has a long-life cycle and a small yield can be obtained, as well as hardly to be cultivated under natural conditions currently (Ferreira et al., 2016). Thus, the current yield of wild *H. serrata* is far from meeting the increasing demand for HupA in clinical treatment. This also led to the scarcity of raw materials and an overexploitation of wild resources. As a result, *H. serrata* is currently listed as a Level II nationally protected plant species in China (Yang et al., 2021).

Endophytic fungi reside within the tissues of almost all living plants, without causing any apparent harm or pathogenic infection to their host, and play critical roles in plant growth, development, fitness improving stress resistance, protecting plants from phytopathogens and promoting nutrient absorption (Park et al., 2012; Ginting et al., 2013; Tian et al., 2020). Furthermore, some endophytic fungi established an interaction with their hosts plants and co-evolve with them. They can produce valuable secondary metabolites like as their hosts, regarding as a novel and important source of bioactive compounds with potential biotechnological applications in the context of agriculture, food, cosmetics and medicine (Strobel et al., 2003; Huang et al., 2015; Santos et al., 2020). In recent years, there are many studies reports on the isolation of Hup A-producing endophytic fungi from different *Huperiaceae* (Deshmukhet al., 2018; Sang et al., 2020). This offers an alternative method to reduce the immense plant harvest related logistics needed to meet the increasing demand for the Hup A.

High-throughput sequencing (HTS) technology has the advantages of culture-free, large amount of information providing fast and accurate microbial diversity analysis method for microbes, including bacteria, fungi and arbuscular mycorrhizal fungi (AMF) in various ecosystems (Antje et al., 2012; Liu et al., 2017). Previous studies have reported that the diversity and composition of endophytic fungi in cultivated *H. serrata*, and relevance to the production of HupA (Fan et al., 2020; Cui et al., 2021). However, few researches have been conducted on the analysis of the diversity and composition of endophytic fungi within *H. serrata* from different ecological areas and their correlation with Hup A content.

In this study, a high-throughput sequencing method was employed to sequence the ITS region of fungal ribosome RNA (rRNA) genes to investigate the fungal community diversity, composition in root, stem and leaf samples in *H. serrata* from two different ecological areas. The correlation between HupA and endophytic fungal community was also being investigated. Our results will provide new insights for further research on the fungal community of *H. serrata* and their interactions with Hup A production. This could help elucidate both the ecological function of endophytic fungi and lay a foundation for further research on *H. serrata*.

Experimental Materials And Methods

The natural populations of *H. serrata* materials were collected from two ecological areas, Chengdu city, Sichuan Province, China (30°34'N, 103°64' E) and Qixianhu Wetland Park, Jinhua city, Zhejiang Province, China (28°88'N, 120°55'E), respectively. These two ecological areas almost have similar latitudes and climate, but the distance is more than 2,000 km. In order to maximize the chance of obtaining endophytic fungi of *H. serrata*, more than 3-years old wild *H. serrata* were collected, which can produce mature spores. Three plants were randomly selected and merged into one sample, and three biological replicates were prepared for each sample. After removing all sporangia, the samples were marked as follows: Chengdu root (marked as CDR_1, CDR_2, CDR_3), stem (marked as CDS_1, CDS_2, CDS_3) and leaf (marked as CDY_1, CDY_2, CDY_3); Qixianhu Wetland Park root (marked as QXR_1, QXR_2, QXR_3), stem (marked as QXS_1, QXS_2, QXS_3) and leaf (marked as QXY_1, QXY_2, QXY_3), respectively.

The whole plant was dug up with roots and soil, kept moist, put in a sterile self-sealing bag and placed in a refrigerator at 4 °C. And the material was treated within 24 hrs. The whole plant of fresh *H. serrata* is continuously washed with tap water until no impurities such as sediment can be seen. After rinsing, tissue surface of materials was dried by the aseptic filter paper.

All the tissues of different plants and different sections were surface-sterilized using the following washing steps: 70% (v/v) ethanol for 1 min, 2% (v/v) sodium hypochlorite solution for 5 min, 2.5% (w/v) sodium thiosulfate for 5 min, and rinsing the samples five times with sterile water. And then quickly transport to the liquid nitrogen, and stored at - 80 °C for DNA extraction.

Dna Extraction, Amplicon, And High-throughput Sequencing

All samples were well grounded in liquid nitrogen, and 100 mg of each sample was transported to a 2 ml centrifuge tube. Total DNA was extracted using the E.Z.N.A. soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instruction. The DNA was checked on 1% agarose gel, and DNA concentration and purity were determined with DeNovix DS-11 spectrophotometer (DeNovix Scientific, USA).

The conserved intergenic transcribed spacer 2 (ITS2) region of the fungal rRNA genes was amplified with the primers ITS1FI2 (5'-GTGARTCATCGAATCTTTG-3') and ITS2 (5'-TCCTCCGCTTATTGATATGC-3') (Karlsson et al., 2014). The PCR mixture (25 μ L) contained 1 μ L of DNA template (50ng μ L⁻¹), 12.5 μ L Phusion Hot start flex 2X Master Mix (Biolabs, New England), 2.5 μ L forward and reverse primers. The PCR reaction program was as follows: 98°C for 30 s, followed by 32 cycles at 98°C for 10s, 54°C for 30s, and 72°C for 45s, and a final extension at 72°C for 5min.

The resulting products were verified using electrophoresis on 2.0% agarose gels and purified using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified with a Qubit instrument (Invitrogen, USA). The amplicon pools were prepared for sequencing, and the size and quantity of the amplicon library were assessed using an Agilent 2100 Bioanalyzer (Agilent, USA) and the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA), respectively. The libraries were sequenced on NovaSeq PE250 platform at LC-Bio Technology Co., Ltd, Hang Zhou (Hangzhou, China) according to the manufacturer's recommendations.

Sequence Processing And Data Analysis

Cutadapt software (v1.9) were used to assign and truncate paired-end reads based on their unique barcodes and primer sequences. Paired-end reads were merged using the PEAR R software (v0.9.6). Fqtrim software (v0.94) was used to perform quality filtering of the raw tags to obtain clean tags. Chimeric sequences were filtered using the Vsearch software (v2.3.4). After dereplication, the feature table and the feature sequence were obtained using DADA2, which were further clustered into operational taxonomic units (OTUs) with 97% pairwise identity.

Alpha diversity and beta diversity were calculated by QIIME2 software, which the same number of sequences were extracted randomly through reducing the number of sequences to the minimum of some samples, and the relative abundance (X fungi count/total count) is used in fungi taxonomy. Alpha diversity and Beta diversity were analyzed by QIIME2 process, and pictures were drawn by R (v3.5.2). The sequence alignment of species annotation was performed by QIIME2 plugin feature-classifier, and the alignment database was RDP and unite (Bolyen et al., 2019).

Quantification of Hup A in *H. serrata* by HPLC

The Hup A extracts were prepared by method as reported previously (Shu et al., 2019). In Brief, The *H. serrata* plant samples were dried at 60 °C for 24 h, pulverized, and precisely weighted in 2 g. The powder

was dissolved in 40 mL pH = 3.0 HCl and stirred for 10 h at room temperature, then sonicated at 50 °C for 1 h. To the filtered solution, 20 mL HCl (pH = 3.0) was added and extracted. After centrifuging again all the extracts were collected and adjusted the pH value to 9.0 with $\text{NH}_3 \cdot \text{H}_2\text{O}$ to pH 9.0. Then, the same volume of chloroform was added to the extracts and extracted twice. After that, the chloroform layers were dried by the rotary evaporator and evaporated into dryness at low pressure. Then, obtained residues were dissolved in 10 mL of methanol after evaporation and purified through a filter syringe (0.45 μm) to perform HPLC. Each sample was extracted and quantified with three replications.

Hup A content was measured by HPLC using a Waters Alliance e2695 (Waters, WATERS corporation, MA, USA) with a Waters SunFire C18 column (4.6 mm $\times$ 250 mm, 5 μm ; WATERS corporation, MA, USA). The temperature of the column compartment was maintained at 25°C. The flow rate was 1.0 mL/min using 80 mM ammonium acetate (pH 6.0)–methanol (65:35, v/v) as the mobile phase. Samples of 20 μL were loaded for detection at 310 nm (Zhi et al., 2011; Shaohua et al., 2014). The Hup A contents in the samples were quantified by using the standard curve generated from the Hup A standard (purchased from Chengdu Desite Biotechnology Co. Ltd., Chengdu, China), which is linear in the range of 5.2–52 $\mu\text{g/mL}$ and with $r^2 = 0.9999$.

Results

Analysis of the sequencing data

A total of 1,434,573 effective tags of fungal samples were obtained after filtering low-quality and other unsuitable sequences. The number of effective tags per sample ranged from 72,677 to 84,744, and the average number of clean reads was 79,699 per sampled group. The sequence lengths of all samples are mainly concentrated in 200-300bp and 300-400bp, accounting for 89.9% and 10.0% respectively. The quality of the sequencing data was evaluated mainly through the statistics of sequence number, sequence length, GC content, Q20 and Q30 quality values, effective ratio, and other parameters in each sample (Table 1).

Table 1
The statistics and quality evaluation of the sequencing data.

Sample	Raw_Tags	Valid Tags	Q20(%)	Q30(%)	GC(%)	Valid(%)
CDR_1	87444	84744	99.31	97.35	52.54	96.91
CDR_2	85883	81799	99.10	96.50	53.74	95.24
CDR_3	80058	75929	99.31	97.21	53.11	94.84
CDS_1	85964	83838	99.44	97.65	52.09	97.53
CDS_2	82868	79893	99.51	97.88	54.59	96.41
CDS_3	82394	80274	99.47	97.80	55.60	97.43
CDY_1	81011	79162	99.52	97.90	54.67	97.72
CDY_2	83619	81254	99.71	98.58	55.33	97.17
CDY_3	85349	83548	99.49	97.75	53.94	97.89
QXR_1	80931	75692	99.17	96.78	55.47	93.53
QXR_2	82683	77556	99.67	98.31	54.46	93.80
QXR_3	81931	75342	99.48	97.72	52.79	91.96
QXS_1	85104	82224	99.59	98.09	55.67	96.62
QXS_2	83886	72677	99.60	98.25	53.26	86.64
QXS_3	81085	78158	98.82	95.87	56.20	96.39
QXY_1	84574	82311	99.54	97.98	56.57	97.32
QXY_2	84803	81718	99.57	98.06	55.37	96.36
QXY_3	80351	78454	99.11	96.66	54.11	97.64

The rarefaction curves, displaying the relationship between the number of reads and operational taxonomic units (OTUs) in each sample, exhibited a stable plateau with the increase of the sample size (Fig. 1A), indicating that the sequencing depth and the number of OTUs were sufficient for each sample to represent the fungal communities and continue with further analyses. The rarefaction curves also showed that leaf samples had the highest abundance of fungal species, while root samples had the lowest. The Good's coverage values for the eighteen samples ranged from 98.2–100% (Fig. 1B), also indicating that the sequencing data confidently reflected the structure of the endophytic fungi community of the samples.

Taxonomic Analysis Of Endophytic Fungi

A total of 2,521 operational taxonomic units (OTUs) was yielded in six groups, and 1,829 and 1,288 OTUs were totally detected in ChengDu samples and Qixianhu samples, respectively. Moreover, a total of 140 OTUs and 102 OTUs were common to ChengDu samples and Qixianhu samples, respectively (Fig. 2A, Fig. 2B). As depicted in the petal diagram, 25 common OTUs were present in each sample's, indicating that there may be great differences in endophytic fungi between the two areas due to different ecological conditions (Fig. 2C).

The taxonomic distribution of endophytic fungi in the roots, stems and leaves of *H. serrata* was displayed in Fig. 3. After screening out rare OTUs, the remaining OTUs represented 9 phyla, 40 classes, 102 orders, 228 families, and 430 genera, respectively. At the level of phyla, the operational taxonomic units (OTU) were assigned into 8 known fungal, which were *Ascomycota*, *Basidiomycota*, *Zygomycota*, *Glomeromycota*, *Chytridiomycota*, *Olpidiomycota*, *Mucoromycota*, *Mortierellomycota*. According to the results of multiple sequence alignment of features feature sequences of these phyla, the evolutionary tree of feature sequences is constructed (Fig. 3A). Among them, the predominant phylum was *Ascomycota* (54.34%, 41.14%-62.30%), followed by *Basidiomycota* (41.51%, 32.42%-57.17%), *Fungi_unclassified* (1.83%, 0.49%-3.34%), *Zygomycota* (0.58%, 0.15%-3.41%) and *Glomeromycota* (0.62%, 0%-3.52%). *Basidiomycota*, *Ascomycota*, *Zygomycota* and *Olpidiomycota* were found in all tested samples. Otherwise, all the *Glomeromycota* were found in the root samples and the stem samples, but not found in leaf samples from two sites. *Chytridiomycota* and *Mucoromycota* were only found in the roots samples from Chengdu (CD), but not found in Qixianhu (QX). In addition, *Mucoromycota* and *Mortierellomycota* were only found in the root samples and leaf samples from ChengDu (CD), respectively. At the genus level, a total of 430 distinct fungal genera were identified, and the compositions and proportions of the genera was significantly different among in different tissues and different ecological areas. The genus *Ascomycota* was the most abundant in leaf and stem samples (CDY, CDS, QXY, QXS), with relative abundances ranging from 21.45–28%. While, *Ascomycota* genus was relative low abundance in root samples, with the relative abundance were 0.77% and 1.67% in CDR and QXR, respectively. And *Piskurozyma* genus showed similar features. In contrast, *Cladophialophora* and *Mycena* genera were more abundant in root samples than in leaf and stem sample. It is remarkable to mention that *Sebacina* was the second dominant genus in ChengDu samples (CDR, CDS and CDY), which account for 18.54%, 15.74% and 11.76%, respectively, but it was hardly detected in Qixianhu samples (QXR, QXS and QXY). (Fig. 3B).

The top 9 classes were selected to make a heatmap clustering, which further indicated that species distributions differed greatly across the three tissue samples and different ecological areas. The heatmap representation of the results showed that the root samples, i.e., CDR and QXR clustered together, exhibiting a relatively similar community structure (Fig. 3D). The top 30 genera (i.e., those with relative abundance > 1%) were also selected to make a heatmap clustering (Figure. 3d). At the genus level, *Auricularia* and *Mycena* were the dominant genus in root sample (CDR and QXR), while *Mortierella*, *Pestalotiopsis*, *Cladophialophora* *Agaricomycetes*, and *Herpotrichiellaceae* were more abundant in the QXR samples than in CDR samples. The results of fungal communities showed that CDR and QXR samples clustered together, as did CDS and QXS, CDY and QXY, exhibiting a relatively similar community

structure. The results hinted that the origin of endophytic fungi in roots is different from that in leaves and stems.

Alpha diversity analyses of the endophytic fungal communities in *H. serrata*. from different ecological areas

Alpha diversity analyses, including Shannon, Simpson, Chao1 and ACE indices, were conducted by Wilcoxon rank-sum test, characterize differences in fungal community abundances and diversities in different groups. The results of the alpha diversity analysis of the fungal communities indicated that the Simpson index of the six samples was no significant (Fig. 4A). Specifically, the Shannon index of CDS and CDY samples was significantly higher than CDR samples ($P < 0.05$) (Fig. 4B). The Chao1 index of the CDS samples were significantly higher than CDR and QXR, and QXR samples was significantly lower than except five samples ($P < 0.05$) (Fig. 4C). The ACE index of the CDR samples was significantly lower than CDS and CDY samples and the ACE index of QXR samples was significantly lower than QXS and QXY ($P < 0.05$) (Fig. 4D).

Beta diversity analyses of the endophytic fungal communities in *H. serrata*. from different ecological areas

To evaluate differences in endophytic fungal composition among different samples in *H. serrata*, beta diversity analysis was performed. A principal coordinate analysis (PCoA) based on the unweighted Unifrac distance matrix was conducted to show the relationship between the different *H. serrata*. samples (Fig. 5). In the PCoA result, the first axis explained 21.95% of the data's variability and the second axis explained 16.8%. And the results revealed that the structures of the fungal communities of the stem and leaf samples (CDS and CDY, QXS and QXY) clustered near each other, while the endophytic fungal communities in roots (CDR and QXR) was distinctly separated from those of the stems and leaves (CDS and CDY, QXS and QXY) (Fig. 5A). Moreover, the endophytic fungal communities of two root samples (CDR and QXR) from different nature populations were distinctly separated. Similarly, the structures of the fungal communities of stem and leaf samples from two nature populations were also separated. Similar results were found for Non-metric multidimensional scaling (NMDS) analysis, hinting that the endophytic fungal communities from *H. serrata* displayed a strong separation based on the plant tissues and natural populations (Fig. 5B).

UPGMA tree was conducted by unweighted unifrac method based on the genus level. Important distinctions were found in the composition of fungal communities in two area's root, stem, and leaf samples. Two different clusters were observed, the endophytic fungal communities from leaf and stem samples clustered together (CDS, CDY QXS and QXY), while the root sample clustered alone and distinctly separated from those of the stem and leaf samples (CDR and QXR). Otherwise, the endophytic fungal communities of the leaf samples and stem samples in same ecological areas (CDS to CDY, QXS to QXY) were more like than that of different ecological areas (CDS to QXS, CDY to QXY) (Fig. 5C). The results suggest that the endophytic fungal communities of the root sample might have specie-specific and those of the leaf and stem samples probably have ecological specificity.

Linear discriminant analysis effect size (LEfSe) analysis was employed to determine different taxon abundances of endophytic fungi among the six tissues from two ecological areas. Concerning the different tissues and ecological areas, a total of 53 biomarkers were employed to discern significant differences with an LDA score greater than 3.0. The LEfSe analysis showed more taxa with statistically significant abundance in CDs followed by CDR and CDY in Chengdu samples with an LDA score greater than 3.0 at the genus level. The CDY samples contain more *Tremellales_unclassified* and *Tremellomycetes_unclassified* CDS samples contain more *Piskurozyma*, *Strelitziana*, *Pleosporales_unclassified*, *Spizellomycetaceae_unclassified*, *Rhinocladiella*, *Halosphaeriaceae_unclassified*, *Tremella* and *Veronaea*. And *Auricularia*, *Gliocladium*, *Ilyonectria*, *Cotylidia*, *Clavulinopsis*, *Olpidiaceae_unclassified* and *Chaetothyriaceae_unclassified* are more abundant in CDR (Fig. 6A). On the other hand, more taxa with statistically significant abundance in QXR followed by QXY and QXS as well. At the genus level, *Auricularia*, *Glomus*, *Rhizophagus* and *Glomeraceae_unclassified* were significantly enriched in QXR samples, while *Eurotiomycetes_unclassified*, *Tremellales_unclassified* and *Strelitziana* were more abundant in QXY samples. And *Septobasidium* and *Teichosporaceae_unclassified* were significantly enriched in QXS samples (Fig. 6b).

Correlation Analysis Between Fungal Endophytes Diversity And Hup A Content

The content of Hup A in different samples was quantified by high performance

liquid chromatography (HPLC) (Fig. 7). The retention time of standard Hup A was 17.989 min and the HPLC spectrum of Hup A standard is shown in Fig. 7A. The results showed that the content of Hup A in roots (CDR and QXR) was significantly lower than that in stems and leaves. The Hup A content from Qixianhu samples was significantly higher than that from ChengDu samples, hinting that Hup A content might have variety specificity (Fig. 7B). The correlation between the top 30 OTUs of endophytic fungal community and Hup A content is depicted using Pearson heat map (Fig. 8). There were 7 genera (*Fungi_unclassified*, *Pestalotiopsis*, *Rhodotorula*, *Ascomycota_unclassified*, *Cyphellophora*, *Sporobolomyces* and *Trichomeriaceae_unclassified*) were significantly and positively correlated to Hup A content of Chengdu samples ($CI \geq 0.95$), while, there were 7 genera (*Mortierella*, *Russula*, *Auricularia*, *Mycena*, *Tomentella*, *Chaetothyriales_unclassified* and *Cladophialophora*) were significantly and negatively correlated to Hup A content of Chengdu samples ($CI \leq -0.95$) (Fig. 8A). On the other hand, there were 10 genera (*Carlosroseea*, *Ascomycota_unclassified*, *Sporobolomyces*, *Fungi_unclassified*, *Trichomeriaceae_unclassified*, *Basidiomycota_unclassified*, *Chaetothyriales_unclassified*, *Bionectria*, *Phialophora* and *Trechispora*) were significantly and positively correlated to Hup A content of Qixianhu samples ($CI \geq 0.95$), and there were 7 genera (*Fungi_unclassified*, *Pestalotiopsis*, *Rhodotorula*, *Ascomycota_unclassified*, *Cyphellophora*, *Sporobolomyces* and *Trichomeriaceae_unclassified*) were significantly and negatively correlated to Hup A content of Chengdu samples ($CI \leq -0.95$) (Fig. 8B). In which, there are 6 genera (*Ascomycota_unclassified*, *Cyphellophora*, *Fungi_unclassified*, *Sporobolomyces*

and *Trichomeriaceae_unclassified*) were significantly and correlated to Hup A content in all two areas, whereas, there are 6 genera (*Auricularia*, *Cladophialophora*, *Cryptococcus*, *Mortierella* and *Mycena*) were significantly and negatively correlated to Hup A content of in all two areas. These genera which showed positively or negatively correlated Hup A content of in all two areas may probably have species specific. However, those genera which showed positively or negatively correlated Hup A content of in only one area may probably have eco-environmental specificity.

Discussion

In this study, ITS amplicon sequencing of endophytic fungi in *H. serrata* from two ecological areas was conducted using a high-throughput sequencing technique based on fungal ITS1-ITS2 sequences. A total of 2521 fungal OTUs was detected from six tissue samples of *H. serrata* derived two ecological areas. Therefore, endophytic fungal communities can exhibit different distributions in different tissues of a single plant. The compositions endophytic fungi of the root, stem and leaf communities in *H. serrata* was analyzed at the phylum and genus levels. At the phylum level, the endophytic fungi of *H. serrata* included members of the *Ascomycota*, *Basidiomycota*, *Zygomycota*, *Glomeromycota*, *Chytridiomycota*, *Olpidiomycota*, *Mucoromycota*, *Mortierellomycota*, and a small number of unidentified fungi. Of these groups, the *Ascomycota* and *Basidiomycota* were the most abundant overall, and the *Chytridiomycota* was rather abundant in leaf and stem samples than in root samples. The results are similar to previous reports that dominant endophytic fungi communities in *H. serrata* and are consistent with other reports of endophytic fungi in medicinal plants (Fan et al., 2020; Cui et al., 2021; Zhou et al., 2021; Katoch et al., 2014).

At the genus level, a total of 430 genera were identified, and the compositions and proportions of the genera was significantly different in different tissues from two ecological areas. The genus *Ascomycota* and *Piskurozyma* were more abundant in leaf and stem sample (CDY, CDS, QXY, QXS) than in root samples (CDR and QXR). On the contrary, *Cladophialophora* and *Mycena* were more abundant in root samples than in leaf and stem samples. These genera displayed tissue-specific characteristic. The *Sebacina* was the second dominant genus in ChengDu samples (CDR, CDS and CDY), but it was hardly detected in Qixianhu samples (QXR, QXS and QXY) (Fig. 3). The same results were also found in *Russula* and *Mycena* genus. Other studies showed that the *Sebacina* genera could be detected in all detected samples in Hunan Province (Fan et al., 2020). It has reported that the endophytic fungus *Sebacina* genera could increase the survival of regenerated tobacco plantlets and to increase the root and shoot biomass of *Zea mays*, tobacco, *Petroselinum crispum*, *Spilanthes calva* and *Withania somnifera* (Varma et al., 1999; Barazani et al., 2007). The *Sebacina* genera showed different distribution characteristics, hinting that *Sebacina* may probably have ecological-specific rather than species-specific. On the other hand, *Agaricomycetes*, *Pleosporales*, *Trechispora* and *Rhodotorula* were more abundant in Qixianhu samples than in Chengdu samples. These genera also showed ecological-specific characteristic. In previous study, Fan et al. (Fan et al., 2000) report that *Cladosporium*, *Oidiodendron*, *Phyllosticta*, *Sebacina* and *Ilyonectria* were dominant genera. And in another report, Cui et al. (Cui et al., 2021) reported that *Cladophialophora* is the dominant genera in endophytic fungal strains based on potato dextrose agar (PDA) medium cultured

method. The composition and frequency of endophytic fungal taxa varied greatly among different areas, which might be related to altitude, exposure, related vegetation type of tissue and phase of tissue, as well as mean annual temperature of different production areas, rhizosphere soil and climate (Huang et al., 2015; Rai et al., 2011; Verma et al., 2007; Li et al., 2020). Furthermore, traditional microbial research methods, such as isolation and culture, construction of clone library, are restricted by various factors, which usually leads to underestimation of microbial community composition and diversity (Liu et al., 2017; U'Ren et al., 2012; Chen et al., 2020). The application of high-throughput sequencing technology in plant rhizosphere and in vivo microbial research effectively avoids the disadvantages of traditional methods and is considering an effective method for fine microbial research (Villalobos-Flores et al., 2021).

The results of the alpha diversity analysis of the fungal communities indicated that there were no significant differences in the Simpson among six groups. Furthermore, there were also no significant differences in the Simpson, Chao1, ACE and Shannon indices among same organs from different ecological areas, i.e. root (CDR and QXR), stem (CDS and QXS) and leaf (CDY and QXY). While, Shannon indices showed that the diversity of endophytic fungi in Chengdu root sample (CDR) was lower than that of rest five groups (CDS, CDY, QXR, QXS and QXY). And Chao1, and ACE indices showed that the diversity of endophytic fungi in root samples was the lowest among all samples. In total, the diversity and abundance of endophytic fungal community in the roots of *H. serrata* was lower than that of in the leaves and stems. The distribution of endophytic fungal taxa varied greatly among different plant tissues. Alpha diversity analysis (Chao1, Ace, Shannon, Simpson indices) revealed that the richness and diversity of the endophytic fungal communities in different tissues differed significantly ($p < 0.05$) with greater richness and diversity in leaf sample, followed by stem and roots, respectively (Fig. 4). In previous reports, the leaves of the medicinal plant *Mansoa alliacea* was reported harbored greater colonization of endophytic fungi than stems (Kharwar et al., 2011). The similar results have also been reported by other studies (Li et al., 2011; da Silva et al., 2020; Zhou et al., 2021).

Beta diversity analyses are usually used to compare microbial community composition among environmental gradients (Chen et al., 2021; Rivest et al., 2019). In our study, the stem and leaf samples from the same areas (CDS and CDY, QXS and QXY) grouped together based on similarity in endophytic fungal community composition and diversity (Fig. 5). However, the root samples from two areas were more similar than they were to its stem and leaf samples. The communities of endophytic fungi within the stems were similar to the communities within leaves at all taxonomic levels. This is also a reasonable agreement with other studies of different plants that contain a higher species richness as well as diversity in the leaves and stems than in the roots (Ma et al., 2005; Tian et al., 2021).

Several studies reported that the Hup A compound is produced by many types of endophytic fungi, including *Aspergillus*, *Colletotrichum*, and *Fusarium* strains, which offers an alternative method to reduce the burden on wild *H. serrata* plant and meet the increasing demand for the compound (Sang et al., 2020). Our report revealed that the content of Hup A was different in all detected organs of *H. serrata*. In summary, the highest content was found in the leaves and stems, while the roots had slightly smaller

quantities, whereas the Hup A contents are various among different area. The results are consistent with those of previous results (Ferreira et al., 2016; Bialer et al., 2007). Furthermore, the Hup A content in Qixianhu samples was slightly higher than those of in Chengdu sample. Pearson correlation analysis showed that there are 6 genera were positively correlated with Hup A content in the two areas, and 6 genera were negatively correlated with Hup A content in the two areas, indicating that these genera have species specificity, while others genera may have ecological environment specificity. Up to now, totally about 13 fungal genera have been reported to produce Hup A (Sang et al., 2020; Le et al., 2020; Cruz-Miranda et al., 2020). Of which 8 genera were found in our report and account for 61.5% of them. Remain 5 of the reported Hup A-producing endophytic fungal genera could not be detected, which probably because the planting environment and developmental stage of host plant affecting the endophytic fungi community. Thus, our study sets the ground for new insights into the diversity and composition of endophytic fungi in *H. serrata*, as well as the interaction between endophytic fungi community and Hup A production.

Declarations

Author Contributions

B.P., Q. J. and D.W. developed the concept, obtained financial support, and designed the lab experiments. B.P., Y. Z., D. Y., L. Q., N. M. and H. S. performed experiment. B.P. and D.W. wrote the paper. Q. J. and Z. L. edited the paper. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All raw sequencing data in this article are available at the National Center for Biotechnology Information (NCBI) <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA798846>, SRA accession: SAMN25128121-SAMN25128138

Conflict of Interest

The authors declare no conflict of interest.

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Figures

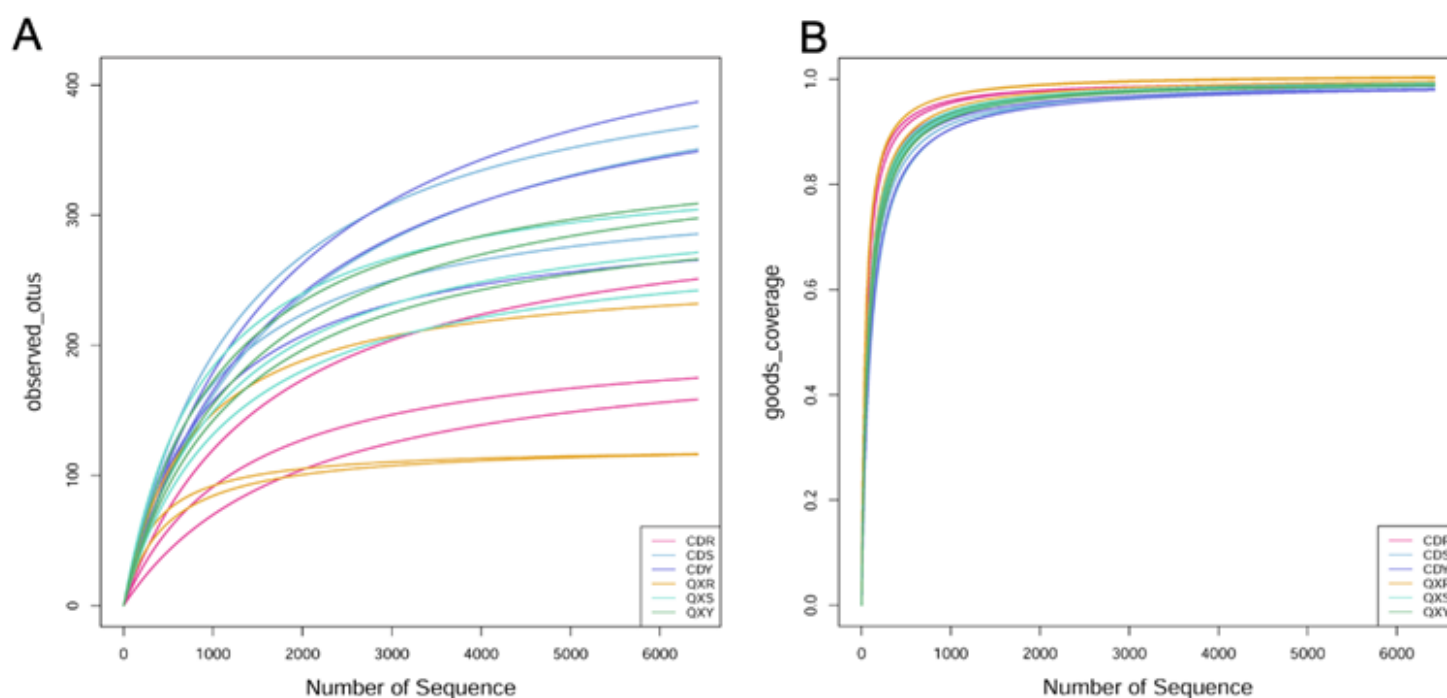


Figure 1

Rarefaction curves based on the ITS2 sequences of endophytic fungi from each group. Each group was comprised of 3 replicates ($n = 3$). CDR, CDS and CDY represent root sample, stem sample and leaf sample from Chengdu respectively; QXR, QXS and QXY represent root sample, stem sample and leaf sample from Qixianhu respectively.

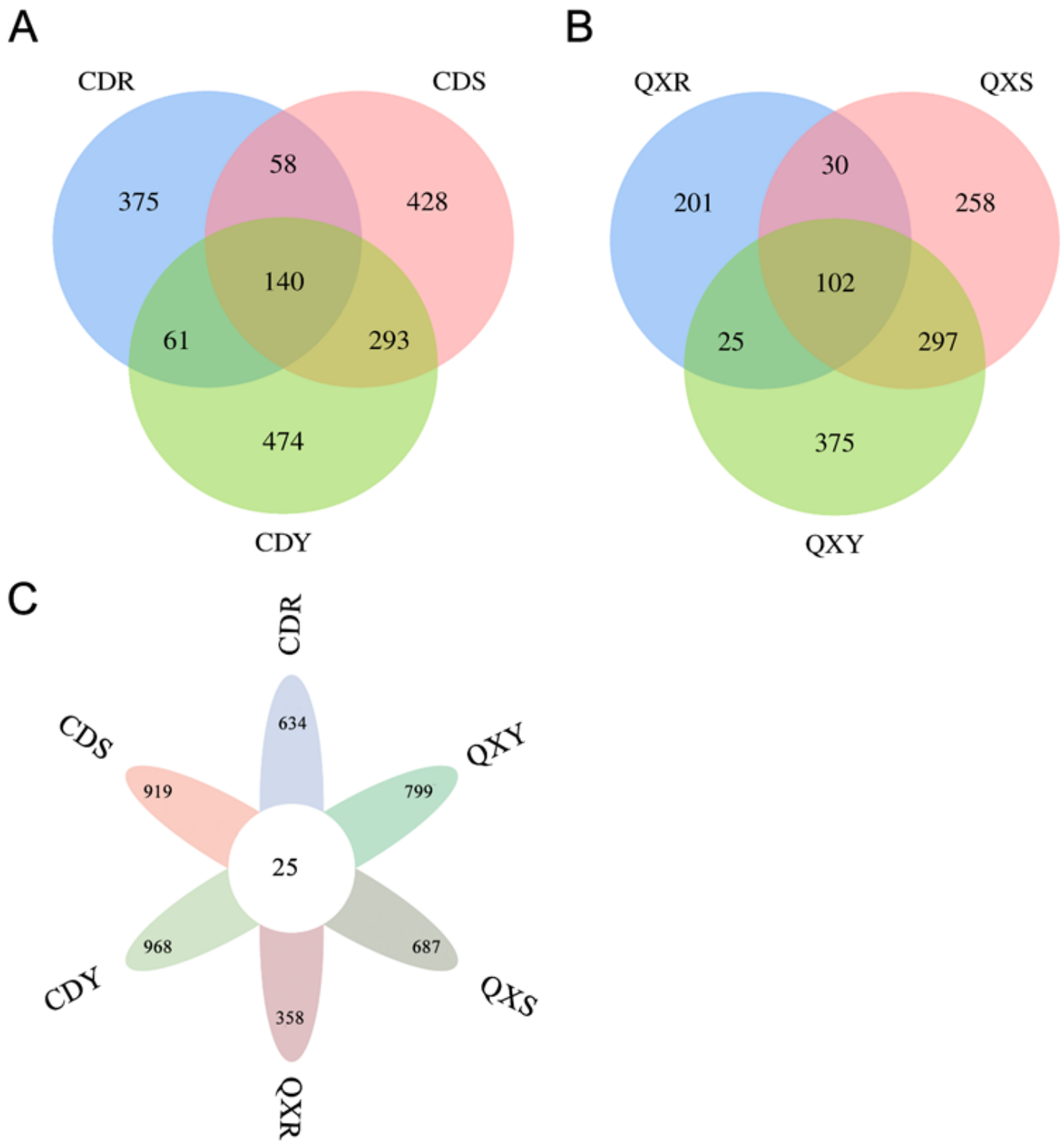


Figure 2

Distribution difference of endophytic fungi in six group from two different ecological areas. Description: The Venn diagram (a and b) and petal diagram (c) based on operational taxonomic units (OTU), which represent common or unique OTUs to a given group.

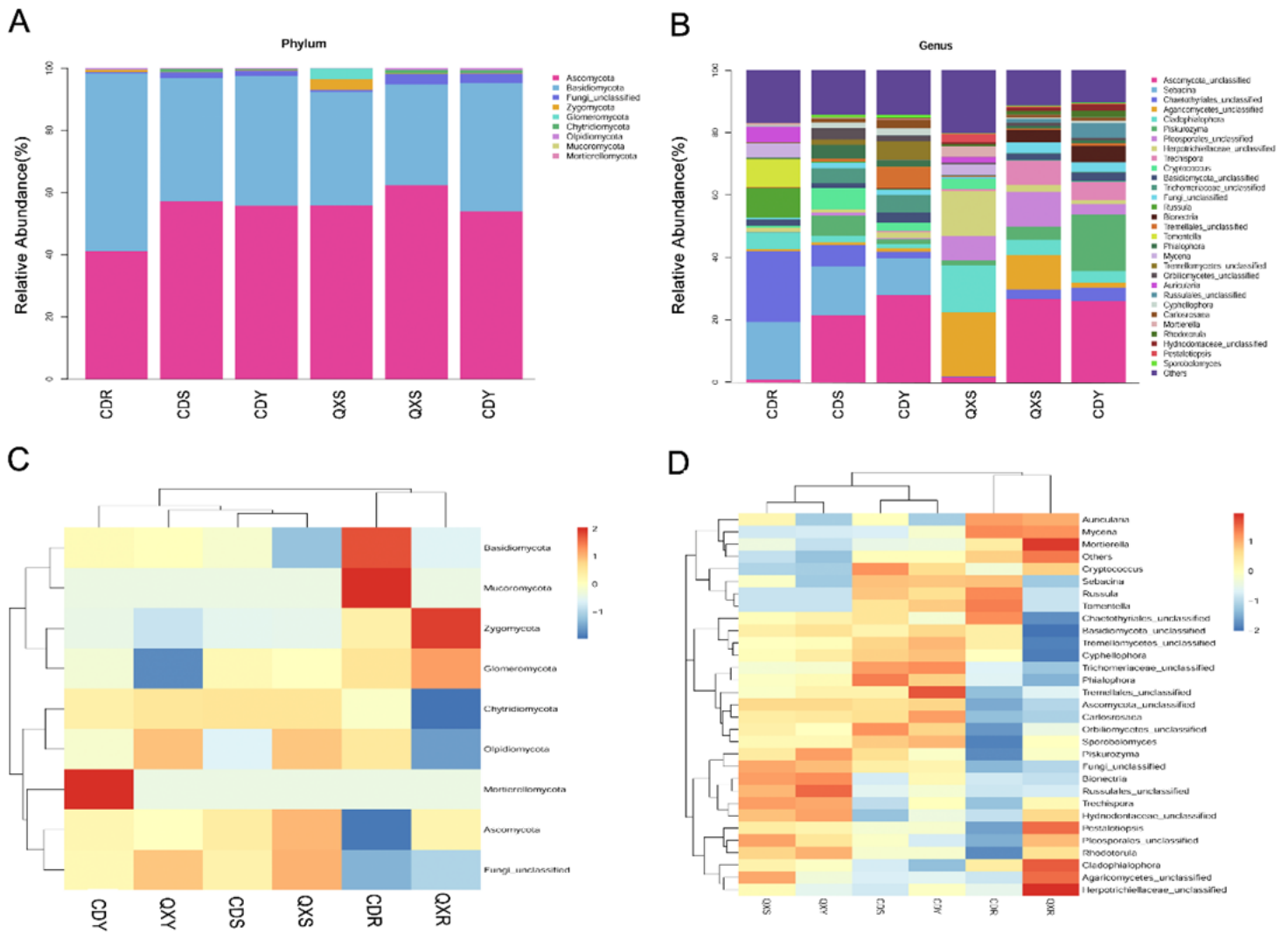


Figure 3

The relative abundance and heatmap of endophytic fungi in the six groups at different taxonomy levels. (a): Relative abundance of fungi at the phylum level. (b): Relative abundance of fungi at the genus level with a relative abundance of more than 1%. (c) and (d) The heatmap of shows the absolute abundance of taxa for endophytic fungi at the phylum and genus level, respectively.

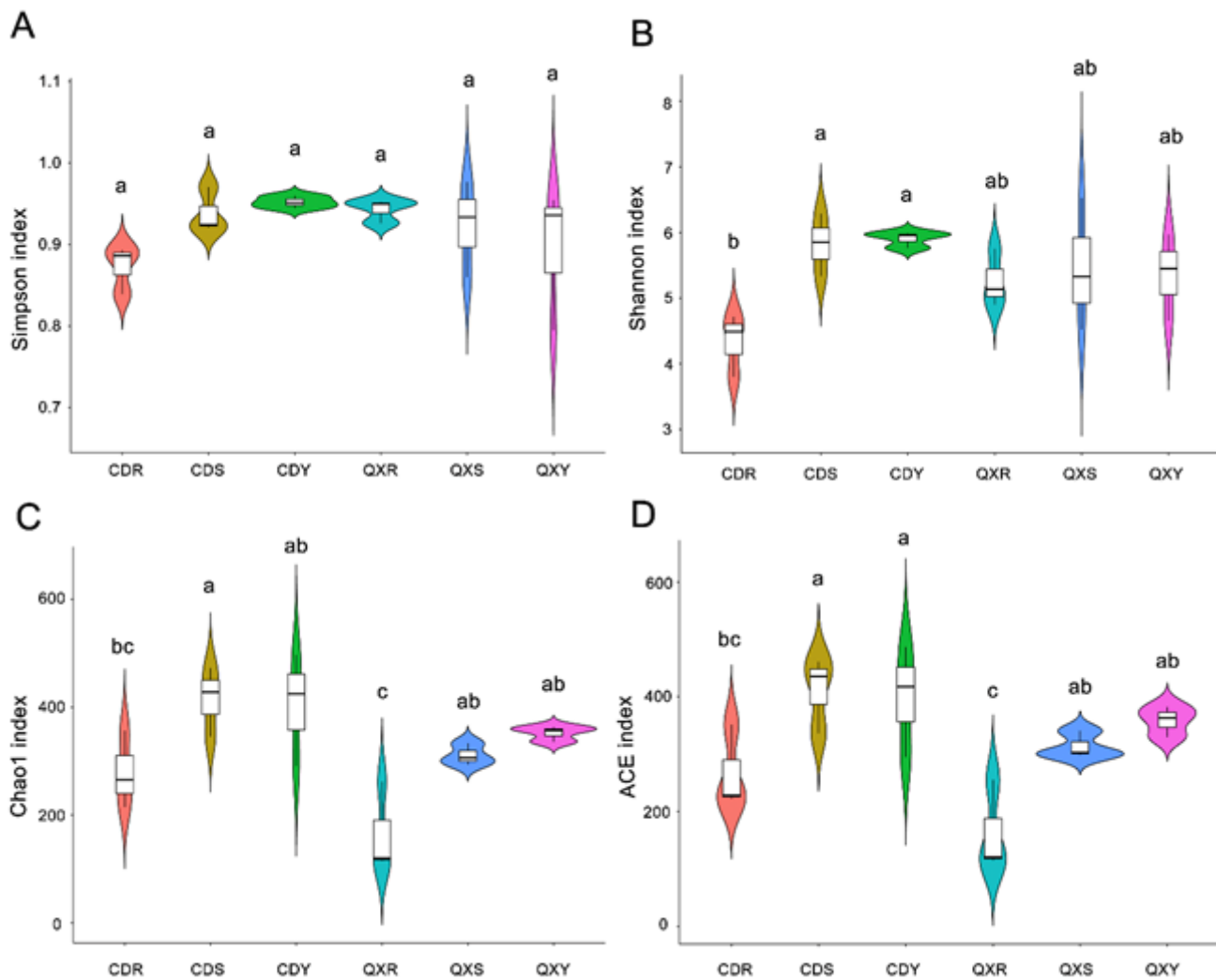


Figure 4

Violin of the alpha diversity indices of the endophytic fungal communities of *H. serrata*.

Simpson (a), Chao1 (b), ACE (c) and Shannon (d) indices. Each violin represents the distribution of diversity present in three replicates ($n=3$). Different lower-case letters represented a significant difference ($P < 0.05$) was assessed by one-way ANOVA followed by the Duncan's multiple range test.

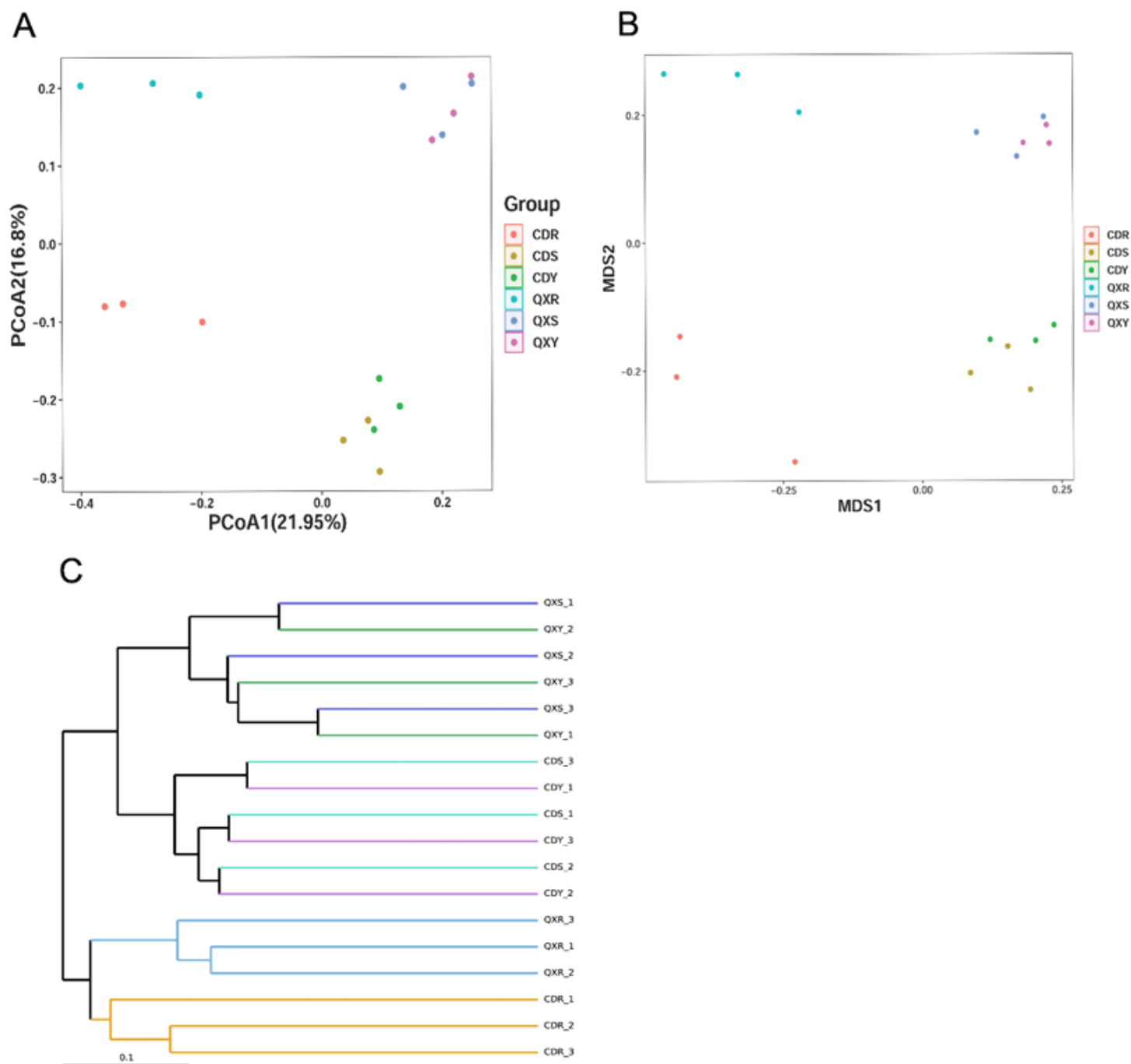


Figure 5

Beta diversity analysis of endophytic fungal community based on unweighted UniFrac distance for the *H. serrata* samples. a. Principal coordinate analysis (PCoA) plot. b. Non-Metric Multi-Dimensional Scaling (NMDS) analysis. c. UPGMA tree of different fungal community structures at the genus level in the different samples.

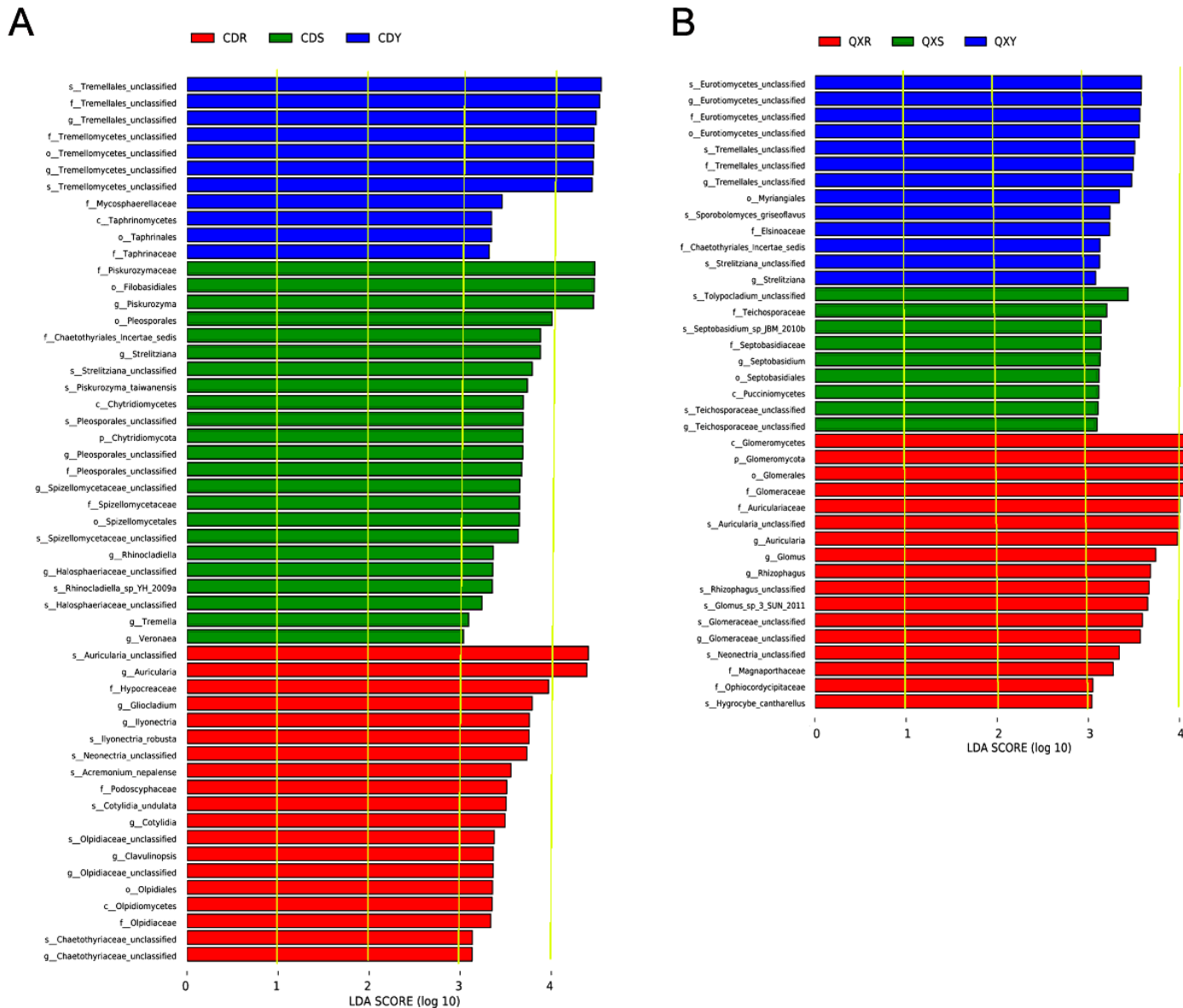
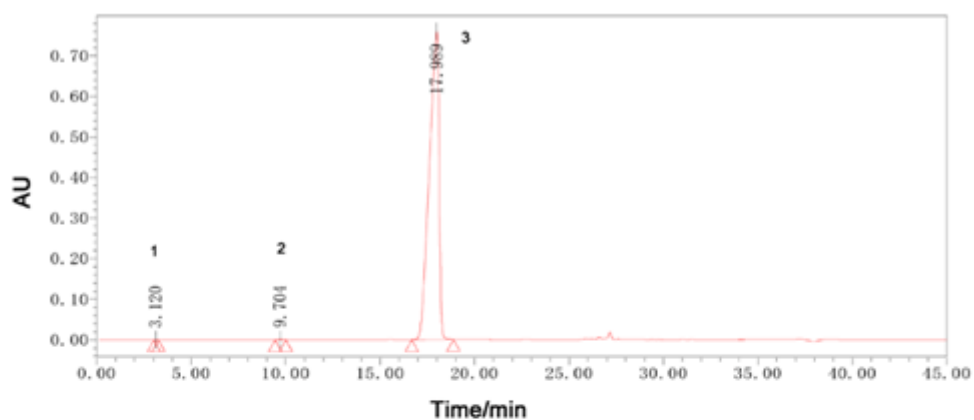


Figure 6

Linear discriminant analysis effect size (Lefse) analysis of differentially abundant taxonomic clades in fungal communities from Chengdu(a) and Qixianhu(b) with an LDA score higher than 3.0. The figure shows the taxa with an LDA score greater than 3.0. The length of the horizontal bars represents the effect size for each taxon (LDA score), and different colors represent different grouped taxa.

A



B

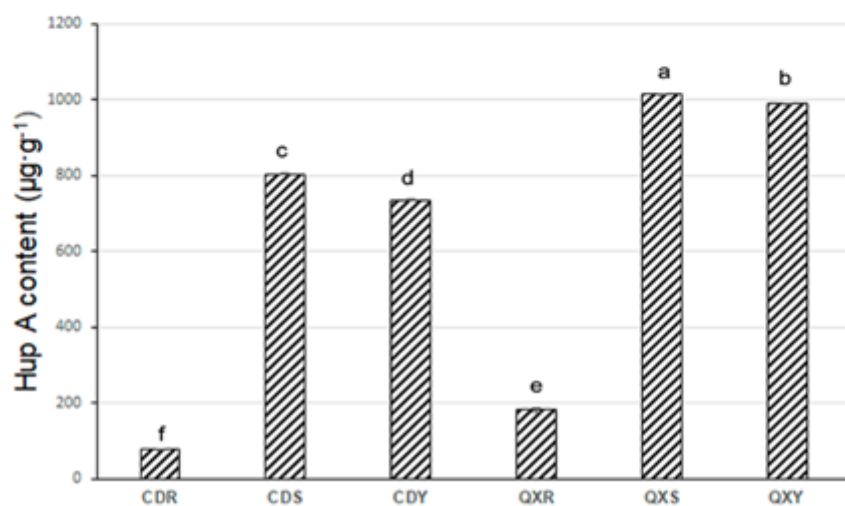


Figure 7

HPLC analysis of the Hup A standard (a) and Hup A contents in different samples. The retention time for Hup A standard was 17.989 min. Different letters on the bars indicate significant differences among all means in different treatments using a one-way ANOVA followed by the Duncan's multiple range test ($P < 0.05$).

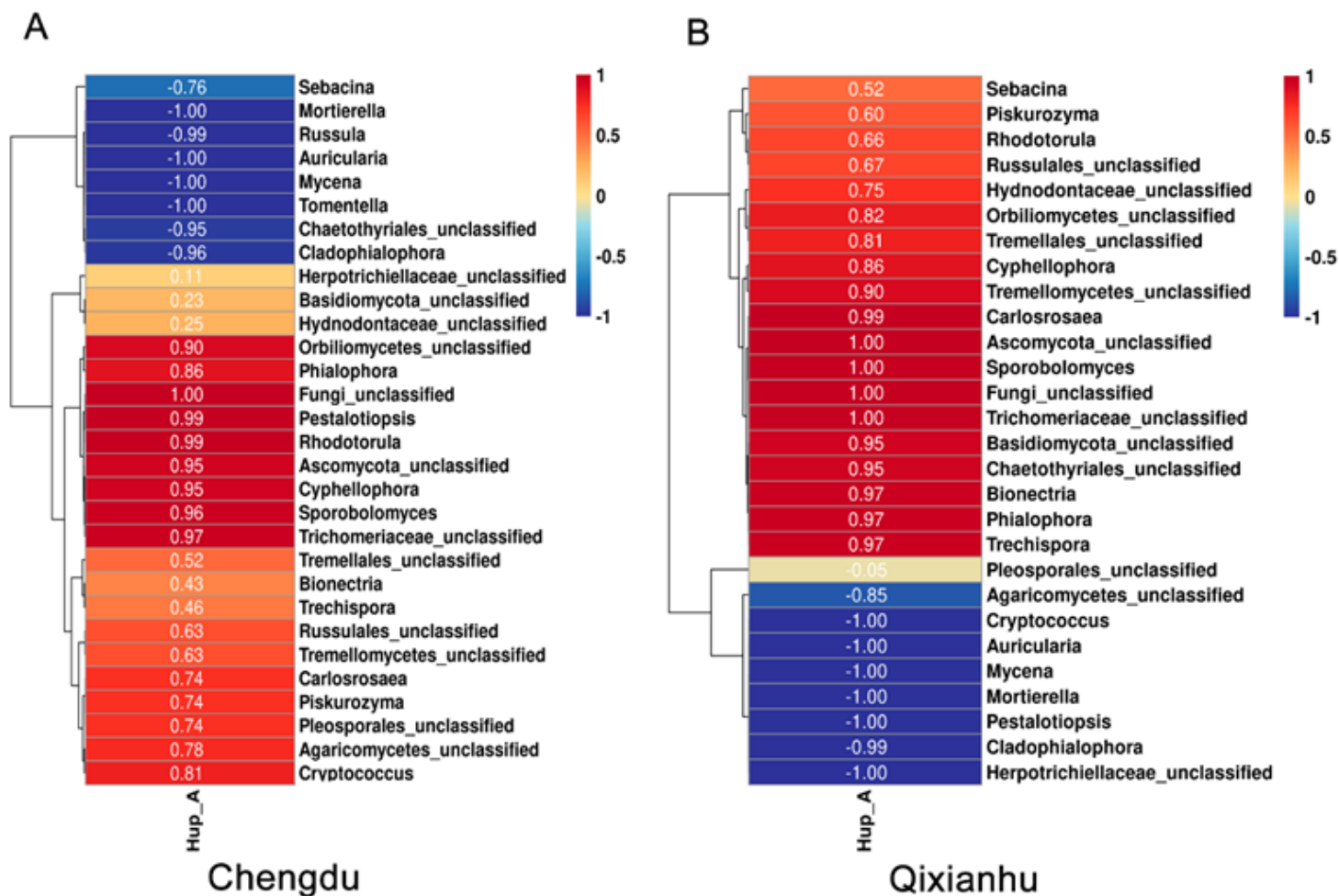


Figure 8

The correlation of Hup A content with endophytic fungi community from Chegndu samples(a) and Qixianhu samples(b).The correlation index (CI) was shown infigures.