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Comparative analysis of root-associated fungal diversity and community composition of two *Satyrium* species

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Abstract

Root-associated endophytic fungi (REFs) residing within the host plant can significantly impact on the distribution and abundance of host species. The diversity and community composition of endophytic fungi connected to the roots of two terrestrial orchid species with contrasting distribution patterns, remain little explored. Therefore, we used amplicon sequencing method to explore and analyze the diversity and community composition of fungi associated with the roots of two *Satyrium* Sw. species, including the widely distributed *Satyrium nepalense* D. Don. and the narrowly distributed *Satyrium yunnanense* Rolfe. Our study has illuminated that REFs communities did not show significant difference according to the distribution pattern of host species statistically, because the *S. yunnanense* was rich in different endophytic fungi than *S. nepalense.* Anyway, *S. yunnanense* was dominated by uncul-tured_hygrocybe and *S. nepalense* was dominated by uncultured_fungus species separately. The result of beta-diversity indicated that there were significant differences in fungal com-munities between the two sample species with (P=0.003) value. It provides the strong evidence against the null hypothesis. Similarly, Linear discriminant analysis Effect Size (LEfSe) and Linear Discriminant Analysis (LDA) scored the two sample species as crucial by different biomarkers separately and significantly over the sample groups *Satyrium nepalense* (SN) and *Satyrium yunnanense* (SY) for their distribution and population stability. Therefore, the diversity and community composition of endophytic fungi are found to be independent on distribution pattern of host alone.

Keywords Amplicon sequencing · Distribution patterns · Fungal diversity and community composition · REFs · *Satyrium s*pecies

Introduction

The Orchidaceae is one of the most diverse and second-biggest families of flowering plants, with over 30,000 species. However, habitat loss, climate change, and anthropogenic issues are currently posing serious risks to the Orchidaceae (Koju et al. 2023). For successful conservation of orchids, we should know about their distribution pattern and influencing factors over these pattern (Wen et al. 2022). The "endophytic fungi", one of the major influential factors in orchid distribution, refers to fungi that live in plant tissues for the entirety or a part of their life cycle by establishing a mutually beneficial symbiotic relationship with their host plant without causing any disease or adverse effect (Hyde et al. 2019; Patchett et al. 2021). An in-depth examination of fungal diversity is necessary to understand the relationship between plants and fungal associations. Culture-based methods are difficult to identify the relevant fungi because most of the species are uncultivated and visually similar, which could lead to taxonomically discriminatory outcomes (Cruz et al. 2011; Weiß et al. 2016; Bajpai et al. 2019). However, molecular ecology develops through Sanger sequencing and high-throughput sequencing (HTS) technologies can substantially improve our understanding of plant microbiota (Müller et al. 2016; Nilsson et al. 2019; Toussaint et al. 2020). Fungal symbionts are the main variables affecting woodland orchids dispersion throughout the environment (Hemrova et al. 2019).

According to (Zhang et al. 2023) species that are limited to uncommon environments are likely to be found only in a small number of locations, whereas species that use the most common habitat are likely to be ubiquitous. Microbial communities can improve plants environmental adaptability and fitness by contributing to plant protection against abiotic stress or pathogens. Plants and their microbial communities strongly influence each other within the given environmental conditions (Fitzpatrick et al. 2020). Keystone species in microbial communities have a major impact on community composition and microbiome performance (Banerjee et al. 2018). Metagenomics data identify the specific organisms, clades, OTUs, and their relative abundances between two or more groups of samples and proposed as potential biomarkers (Chang et al. 2022). They inducing changes in population and communities, and potentially impacts in a future population (Lemos et al. 2022).

The relative abundance (effect size) of biomarkers among the groups is analyzed using the effect size approach and linear discriminant analysis (LDA) (Segata et al. 2011). Similarly, Orchid mycorrhizal fungi (OMF) are the key microbes for the conservation of orchid species (Pandey et al. 2013; Rasmussen et al. 2009). The patchy distribution, heterogeneous abundance and spatiotemporal variability of OMF have a crucial effect on the local distribution and population dynamics of host orchids (Li et al. 2021). The variety of fungi resides in orchid roots as non-mycorrhizal endophytes predating the evolutionary transitions to a tight mycorrhizal symbiosis (Selosse et al. 2022). Some orchids continue their association with the same fungi and others change partners as they progress from seed germination to adult stages (Ventre Lespiaucq et al. 2021). Mycorrhizal dependency has been increasingly recognized as an important factor influencing both the distribution and abundance of orchid populations (McCormick et al. 2018). This study tries to perform a comprehensive study on the diversity and community composition of endophytic fungi associated with the roots of two orchid species from the same genus *Satyrium* Sw. with different distribution patterns.

Satyrium nepalense is a terrestrial orchid distributed from the Indian sub-continent to South central China. In Nepal, it is distributed across the central, western, and eastern parts of Nepal between the elevation of 1000-3200m (Raskoti 2009). *Satyrium yunnanense* is a native species of China distributed in South West Sichuan, North West and

Central Yunnan (IPNI 2024). The geographical distribution of *Satyrium* was mainly influenced by environmental variables, in current climatic situation the suitable habitat area for *S. yunnanense* was higher than *S. nepalense* in the context of China (Ouyang et al. 2022). Orchids with a limited number of fungi have restricted distribution while orchids that associate with a large number of fungi or with fungi have a broad distribution (Waud et al. 2016; Davis et al. 2015; and Tesitelova et al. 2015).

On the basis of this statement, this study is carried out to know a new perspective on fungi diversity and community composition of endophytic fungi associated with the roots of two orchid species from the same genus but different distribution patterns. Still now, no any similar work over this genus. Because of this, we collected root and tubers of two species from four populations on a local scale to analyze the endophytic fungi through amplicon sequencing method and analyzed their diversity and composition. We tested the hypothesis (#H1) The root-associated endophytic fungal communities of orchids differing between the host and their distribution pattern and proposed to answer these questions 1) Are the root-associated endophytic fungal diversity and community composition of these two orchids significantly different? 2) What are the dominant fungal species of these two species? 3) Are the biomarkers for each species significantly different? Ultimately, it provides the theoretical basis and reference for the comprehensive protection of sample orchids in their natural habitat.

Materials and Methods

Study species and sampling procedure

T wo orchid species from same genus *Satyrium* were chosen as the sample species. We have selected two sites (<100km apart) and four populations for sample collection. Five individuals (whole plant) from each population were collected during the vegetative period in July 2023. Both the roots and tubers were used for endophytic fungi isolation without missing any associated fungi (Johnson et al. 2023). The randomly selected sample species were at least one meter apart from each other to avoid the potential effects of disturbance and cloned individuals. Any way they were nearly co-occurrence with each other but from different population. The collected roots and tubers were washed with distilled water and sterilized with 75% ethanol for (5-10) minutes and stored at (4℃) for up to one-week maximum for further processing. The location and general description of the sampling sites as below;

Table. 1 (The location and general description of sampling sites and Samples)

Map of study area/ Sample and sampling species

DNA isolation and processing

After complete pre-treatment of samples (roots and tuber) both were prepared for DNA extraction from each sample. Genomic DNA was extracted and purified using the Fast DNA Spin kit for Plant and Animal Tissues as described by the manufacturer (MP Biomedicals CA, USA). To amplify the fungal internal transcribed spacer 2 (ITS2) regions, by selecting better PCR amplification primers for terrestrial species with corresponding region of IT3F_ITS4OFR are, the forward primer ITS3F: GCATCGATGAAGAACGCAGC, and the reverse primer ITS4OFR:

GTTACTAGGGAATCCTTGTT (Tylor and McCormick 2008). PCR amplification was performed includes 10µl of 2×ProTaq buffer; 0.8 µl of forward primer (5 µm); 0.8µl of reserve primer (5µm);10ηg/µl of template DNA; and 20µl of ddH_2O. The PCR amplification program was as follows: 95℃ for 3 min, 35 cycles; denaturation at 95℃ for 30 secs, annealing temperature at 55℃ for 30 seconds; extension at 72℃ for 45 secs. Finally, stable extension at 72℃ for 10 minutes in ABI Gene Amp® 9700 thermal cycler (Shanghai Major Bio-Pharma Technology Co. Ltd., China). Amplicons were extracted from 2% agarose gels and purified using the (Nano Drop 2000, USA). The sequenced data were further analyzed through Divisive Amplicon Denoising Algorithm (DADA2) was used to filter for quality, trim and infer amplicon sequence variants (ASVs) were classified to genus or higher level taxonomically (Callahan et. al. 2016).

ASV analysis and abundance curve

Amplicon sequencing is a high-throughput targeted sequencing approach that allows sequencing up to thousands of genetic regions of interest in a single run (De Filippis et al. 2017). Therefore, we used this method to analyze the different taxonomical groups of fungi and their numbers through sequencing results. We used the rank abundance curve to visualize species richness and evenness via two-dimensional plots (abundance rank in the x-axis and relative abundance in y-axis). The width and large range of the curve on the horizontal axis represent the higher abundance of that species, and a smoother curve reflects the evenness of the species. Similarly, a flatter curve more uniform distribution of species. The horizontal axis represents the level of sample species and the vertical axis represents the relative abundance of species (such as ASV).

Community diversity analysis

Alpha diversity, refers to the diversity of species within a specific area, community or ecosystem (Jha et al. 2023). Alpha diversity is used to explore the diversity of microbial communities within the sample and the richness and evenness of species between communities using different statistical indices (Baczkowski et al. 1998). Alpha diversity was analyzed by using diversity index, Index group difference test and curve analysis methods separately. Where diversity index used to insights the species richness and evenness within individual microbiome sample (community). Similarly, index group different test helps to set the information about species richness, diversity and coverage in selected two group of samples. Rarefaction curve method was used for comparing the species richness among habitats and also estimate the sampling depth either sufficient or not by displacing them in two dimensional plots. Similarly, Beta diversity, refers to the diversity of species between two communities or ecosystem and comparing the number of unique species to each community or population.

We used sample level clustering analysis and non-metric multidimensional scaling. NMDS analyses was used to analyze the rank-order correlation between the sample's population and reflected the multidimensional space in the form of points and the degree of difference between samples. The closer the sample points are more similar in species composition and vice-versa.

Community composition analysis

Different analysis methods (Venn plot analysis, Bar plots analysis and Community heat map) were used to analyze the species composition between sample communities with different taxonomic levels. Venn-plot analysis

visually organizes the information to display the number of shared and unique species to the corresponding sampling units (inter-species, intra-species and sample population). Similarly, the bar-plot visually displays the proportion of species (different taxonomic level) in different sample column with different colors. The community heat map visually displays the distribution of top-dominated fungal species (taxonomic level) across sample population. It also predicts the activity status of species numerically represented by color gradients. The species composition influence on ecosystem functioning and stability of host and its associate.

Species difference analysis

The significance level of species abundance difference obtains the species from multiple groups as microbial biomarkers, which may play a key role in environmental change process, ecosystem functioning and stability. The linear discriminants analysis effect size methods (Segata et al. 2011) was used to determine particular microbial taxa associated with sample groups. Linear discriminant analysis (LDA) was used to measure the rank features according to their effect size among the sample groups by the help of LDA score (Li et al. 2018). It also helps to determine the diversity and composition of biomarkers species and their correlation according to the distribution pattern of host species.

Data Analysis/Results

Fungal composition in the roots of sample species

In this study, a total of 20 roots along with tuber were studied from four populations of the two species (*S. nepalense* and *S. yunnanense*) in Chenggong district of Yunnan Province of China. Through the amplicon sequencing method, roots associated fungi were analyzed up to different taxonomic levels. We have obtained 1301373 and 55901613 bases of optimized sequences with an average length of 427 base pairs. The numbers of ASVs were different according to the sampling species (**Information table.1**). In total 33620 fungal ASVs were analyzed, where 285 ASVs only identified including 4 phyla, 21 class, 42 order, 61 families, 81 genera, and 103 fungal species. The *S. yunnanense* was richer in species diversity than *S. nepalense* but there was no significant difference in ASV number. Any way, 212 and 209 ASVs were analyzed from roots of *S. nepalense* and *S. yunnanense* respectively. Similarly, we analyzed the fungal diversity, community composition and species differences analysis according to Majorbio Cloud: A one-stop, comprehensive bioinformatics platform for multiomics analyses (Ren et al. 2022).

Community diversity analysis

In this study, we analyzed the alpha and beta diversity of fungal community. The alpha diversity was analyzed using the Sobs and Simpson diversity index over different taxonomic levels. The diversity of different taxonomic levels of each sample population was displayed through a bar chart, which displayed the size of the diversity index of different samples. The horizontal axis represents the sample name, and the vertical axis represents the observed value of a certain index type with different colors. Alpha diversity index, inter-group different test was done on different taxonomic levels between sample species. One-way ANOVA (P=0.932) and student t-test (P=0.506) were done for the Sobs index over the sample population and sample species separately.

Similarly, the rarefaction curve method was used for comparing species richness among the population and species. It estimates whether the sampling depth was sufficient or not by displacing them in the graph. Sobs index was

used to display the dilution curve on ASV level overall sample population. When the curve tends towards a flat end, it indicates that the sequencing data volume was reasonable.

Fig. 1 Diversity index (Simpson index and Sobs index)

Fig. 2 Bar-plot displayed the diversity of fungal ASVs between sample species and sample population

Fig. 3 Dilution curves and Sample depth

The beta-diversity analysis, analyzed the level of similarity between the samples. The high beta-diversity index indicates a low level of similarity and low beta-diversity index indicates a high level of similarity. NMDS analysis was used to study the beta-diversity between the sample species and population. NMDS analysis showed the difference in species composition between sample species or sample population. The distance algorithm (bary-curtis) was used to reflect the degree of clustering of sample community. The value (stress< 0.2) indicates that the graph has certain explanatory significance and it statistically analyzed by ANOSIM treatment to present the significant difference in fungi community between the control and treatment groups, but there were no any significant differences in stress value with or without treatment. If the order of sample points was relatively concentrated means, there were less different in endophytic fungi communities in similar species. Similarly, the order of sample points was relatively dispersed. It indicates that there was some difference in the endophytic fungi communities in the same or different species according to their respective habitat.

Fig. 4 NMDS analysis between sample species on ASV level with and without treatment.

Fig. 5 NMDS analysis between intra-specific species on ASV level A-(SN1 and SN2) B-(SY1 and SY2)

Fig. 6 NMDS analysis between sample population on ASV level with or without treatment.

Community composition analysis

In total 33620 fungal ASVs were analyzed and 285 were identified in our study. The 285 ASVs include with 4 phyla, 21 class, 42 order, 61 families, 81 genera, 103 fungal species. The primarily composed four phyla, Unclassified_k_fungi, Basidiomycota, Ascomycota and Mucoromycota were dispersed with an average relative abundance of 38.94%, 35.43%, 23.50%, and 2.10% respectively, across all sample population. We studied the root-associated endophytic fungal communities between inter-specific and intra-specific along with sample population of two sites. The number of ASVs were different according to the species and habitat. Venn-plot analysis displayed the number of unique and common taxonomic group of fungi between the two or multiple sample groups. The number associated with overlapping part represents the number of species shared by multiple groups, and the number associated with non-overlapping parts represents the number of species (taxonomic level) unique to the corresponding group **(Information table. 2).**

This type of analysis helps to analyzed the correlation between similar species and dissimilar species along with their associated fungi communities. Comparatively, *S. yunnanense* was rich in species composition than *S. nepalense*. But in intra-specific species composition analysis, the ASVs composition were different according to the sampling habitat and sites.

Fig. 7 The composition of ASV numbers according to the sample species and population.

The bar diagram compares the data or categories between x-axis and y-axis, the ordinate represents the sample groups and abscissa is the proportion of fungal taxonomic level in sample columns with different colors. The length of the bar column represents the proportion of species or different taxonomical level of fungi. It helps to determine the community composition between fungi and sample species. However, *S. yunnanense* was rich in species composition either sample species or sample population. The relative abundance of same species of endophytic fungi from single host also differ according to the host habitat **(Information table. 3)**. The availability of OMF family Ceratobasidiaceae was habitat specific, because it is available on two populations from same site.

Fig. 8 Bar-plot shown the relative abundance fungal species associated with sample species and sample population.

Community heat map or clustering heat map visually displayed the distribution of top dominated species or taxonomic level of root-associated endophytic fungi across all samples **(Information table. 4)**. In this study the horizontal axis represents the sample name and the vertical axis represents the certain taxonomic level of fungi. Certain color gradient is used to show the proportion of species, the color gradient is representing with positive and negative numeral values. According to the color gradient those variables which showed the positive correlation are usually represented by warm colors like red and orange. Negative correlation was usually represented by cool colors such as blue or green. Those group having with warm colors and positive value are more or highly active in the community and light color and negative value carrying taxonomic group are less active in the community. The top ten dominated families of endophytic fungi analyzed by the heat map were Hygrophoraceae, Unclassified_k_fungi, Unclassified_p_Basidiomycota, Nectriaceae, Ceratobasidiaceae, Trichosporonaceae, Unclassified_c_Saccharomycetes, Mrakiaceae, Pezizaceae, and Unclassified_p_Ascomycota. The positive correlation and their availability may influence in diversity and distribution pattern of host along with respective endophytes.

Fig. 9 Correlation between the families of endophytic fungi and sample population (Individuals).

Species differences analysis

The effect size approach of linear discriminant analysis following (Segata et al. 2011) was employed to identify certain microbiological species as biomarkers linked to sample groups. About the sample groups, LEfSe was utilized to identify the characteristics of species with notable abundance **(Information table. 5)**. Additionally, characteristics were ranked based on the relative difference (effect size) across groups using linear discriminant analysis (LDA). Multi-group comparison was done all-against-all strategy from taxonomic level family to species with a significance value ($P<0.05$). The threshold on algorithmic LDA score for discriminative features was set at 4. The LDA score for each biomarker is obtained computing the logarithm (base 10) of this value induces the ranking of biomarker relevance for robustness and additionally supported by bootstrapping. Certain species exhibiting strong correlations also belonged to the ones contributing most to the difference between groups (*S. nepalense* and *S. yunnanense*) as reveled by LEfSe analysis. Any way LEfSe provided the convenience for identifying good biomarkers that characterize statistical difference among biological groups (Chang et al. 2022).

Fig. 10 Species differences analysis through (LEfSe-Cladogram) and (LDA-bar chart)

Discussion

In the current study, we focused on root-associated endophytic fungal diversity and community composition associated with two orchid species having different distribution pattern through the amplicon sequencing method.

According to (Zhang et al. 2023), plant species found in uncommon environments are likely to be found only in a small area, whereas species that use the most common habitat have broad distribution. Similarly, OMF may restrict terrestrial orchid distributions at local scales, but at broad geographic scales, terrestrial orchids are not controlled by OMF (Li et al. 2021). The role of root-associated endophytic fungi for their host distribution because fungus symbionts are the main variables affecting how terrestrial orchids disperse throughout the landscape (Hemrova et al. 2019). The fungi community near the roots is relatively stable (Huang et al. 2022). So, we find the dominated species of endophytic fungi associated with sample orchids as an indicators and compared the root-associated endophytic fungal communities associated with two orchid species and insight the effect of host distribution pattern on REFs diversity and community composition.

Orchid-fungal diversity and reintroduction

The identification of orchid root associated fungi has been improved through the amplicon sequencing method. The individual samples had different ASV numbers among them, 285 were identified up to different taxonomic levels. The roots of single orchid can colonize two or more fungi species (Tylor et al. 2003). There was no significant difference in endophytic fungi diversity statistically between sample species according to their distribution pattern, but *S. yunnanense* was slightly rich in species diversity. The dominant endophytic fungi were more stable, and these may be key fungi influencing the growth and development of their host. In this study both sample species were dominated by different fungal species separately. Individual populations had maximum number of unique ASVs and only limited were shared by them. So, the fungi diversity not only depend on the host plant, but different biotic and abiotic components associated with host and host habitat may influence their distribution and composition. Here, the dominant family of endophytic fungi from all sample population were (Hygrophoraceae, Unclassified_k_fungi, Uncalssified_p_Basidiomycota, Nectriaceae, Ceratobasidiaceae, Trichosporonaceae, Saccharomyceties, Mrakiaceae, Pezizaceae, Unclassified_p_Ascomycota) according to their abundance proportion. Among them Ceratobasidiaceae one of the family of OMF only found in specific habitats but not related to species-specific. It means that edaphic factors also responsible in fungi distribution.

Where microbial communities harbor keystone taxa, that have a significant influence on community composition and microbiome performance irrespective of their abundance (Banerjee et al. 2018). Multiple fungal taxa simultaneously associate with orchid species (Selosse, M.A. and Roy, M. 2009), the association range is narrow and involves only a few OTUs (Vogt-Schilb et al. 2020). It has been suggested that mycorrhizal specificity might be influenced by environmental conditions (Jacquemyn et al. 2016). Different fungal taxa are present in the same orchid species across different sites but how various biotic and abiotic factors contribute to fungal specificity is still not well known (Mennicken et al. 2024). So, here we conclude that endophytic fungi were not distributed according to the distribution pattern of their host only. The diversity and distribution of REFs will be influenced by biotic and abiotic factors. Similarly, dominated fungal species act as a biomarker for their host distribution and population stability. This study suggests the further study including about soil nutrients and host chemical profiles associated with host and their influence on fungi distribution. Similarly, the knowledge about the species-specific fungal symbionts and other influencing factors for fungal distribution play a crucial role in knowing the status of many orchids and REFs around the world and is useful for successive preservation and reintroduction of them.

Primer selection in High throughput sequencing

According to (Tylor and McCormick 2008), ITS1-OF and ITS4-OF were the first developed specific primers for studying orchid mycorrhizae diversity by amplifying the full ITS based on the sequences of Basidiomycetes fungi including *Tullasnela* species. (Waud et al. 2014) suggested that ITS3_ITS4OF and ITS86F_ITS4OF primers were targeting the ITS2 sub-region of ITS, which is better for orchid root samples. ITS2 can produce more operational taxonomic units and higher phylogenetic richness than the other sub-region ITS1, most researcher prefer it for fungal identification through HTS platform (Mello et al. 2011; Nilsson et al. 2019). For the identification of mycorrhizal fungal communities in the root of terrestrial orchids and surrounding soil, the ITS3_ITS4OF was frequently used (Esposito et al. 2016; Duffy et al. 2019). Similarly, ITS86F/ITS4 was used for the detection of mycorrhizal partners from epiphytic orchids (Izuddin et al. 2019; Jacquemyn et al. 2021).

Based on the above, we have used ITS3F: GCATCGATGAAGAACGCAGC, as a forward primer, and ITS4OFR: GTTACTAGGGAATCCTTGTT as a reverse primer, and produced maximum ASVs. Where 33620 ASVs were represented by 1301373 sequences. Out of them 38.94% from Unclassified_k_fungi, 35.43% from Basidiomycota, 23.50% from Ascomycota, and nearly 2.10% from Mucoromycota. The number of ASVs from different phylum like Unclassified_k_fungi, Basidiomycota, Ascomycota and Mucoromycota were 111, 101, 67 and 6 respectively.

Orchid mycorrhizal fungi diversity

Orchid mycorrhizal fungi have been reported from at least 17 families of Basidiomycetes and 5 families of Ascomycetes (Li et al. 2021). Anyway OMF mostly belongs to the phylum Basidiomycota, are traditionally grouped under the name rhizoctonias, including Tulasnellaceae, Ceratobasidiaceae as well as Serendipitaceae. OMF associated with orchids exhibiting high mycorrhizal specificity tend to have a wide distribution (Swift et al. 2019; Thixton et al. 2020). Here also reported 7 percent of Ceratobasidiaceae family along with other Tulasnellaceae, and Serendipitaceae. They were not species- specific but their distribution influenced by habitat and other hidden components associated with host and host habitat. In species wise data analysis, endophytic fungi in family level the relative abundance of Ceratobasidiaceae family in *S. nepalense* was (0.082) and (0.043) in *S. yunnanense.* Similarly, in population wise data analysis the availability of OMF family Ceratobasidiaceae only in specific habitat (same sites but different populations) of host with different proportion. The relative abundance of Ceratobasidiaceae in one population of *S. nepalense* was (0.164) and one population of *S. yunnanense* was (0.107). This type of availability OMF may influence the distribution pattern of host species and their associate fungi also.

Orchids non-mycorrhizal fungi (ONF) diversity

Root-associated endophytic fungi frequently related to Orchid Non-mycorrhizal Fungi (ONF). They are beneficial for host orchids in physiological and ecological aspects by producing bioactive compounds which are beneficial for orchids by improving their resistance over abiotic stress. The ONF that frequently occurs in orchids roots, in order to determine their potential physiological and ecological advantages. Active compounds produced by some ONF may prove beneficial for orchids by improving their resistance to abiotic stresses and promoting their adaptability to different environmental conditions (Ma et al. 2015) or by protecting against herbivores and pathogens. Here we identified four different groups of endophytic fungi like Unclassifird_k_fungi, Basidiomycota, Ascomycota

and Mucoromycota in different proportion. *S. nepalense* and *S. yunnanense* are two species with different distribution patterns. Where only two families (Unclassified_k_fungi and Hygrophoraceae) of endophytic fungi covered more than 50% of fungi availability.

Among them in *S. nepalense* carried out Unclassified_k_fungi and Hygrophoraceae with 37.78 % and 13.99% respectively. But in *S. yunnanense* Hygrophoraceae occupied 41.86 % and Unclassified_k_fungi occupied 16.28 % of fungal availability. The remaining percentages was sharing by both species of OMF and ONF in different proportions. ONF provide the nutrients to their host plant after decomposed local substrates to balance the endophytic fungi composition, population stability and host distribution (Tedersoo et al. 2009; Waterman et al. 2011; Herrera et al. 2019).

Difference and adaptability of root-associated endophytic fungi in different habitats.

The diversity and coexistence of orchid species are influenced indirectly by the composition, structure, and richness of OMF communities. The OMF community composition related to the same orchid species dispersed across several habitats, distinct orchid species coexisting in the same habitat, or varying individuals of the majority of orchid species in the same habitat may differ to some extent (Martos et al. 2012; Jacquemyn et al. 2014, 2015a, 2021; Pecoraro et al. 2018; Duffy et al. 2019). In our study, we selected four populations from to sites having with different habitat. The distribution pattern of two species is different. They mutually shared some endophytic fungi with different proportion. In all population, only two families of endophytic fungi occupied more than 50% of fungal availability. In individual population the relative abundance of two dominated families were highly fluctuated according to the habitat. According to our study, the availability and stability of dominated families of endophytic fungi were determined by host and host habitat. Anyway, the relative abundance of Hygrophoraceae was more and Ceratobasidiaceae was less in *S. yunnanense* but in *S. nepalense* the relative abundance of Unclassified_k_fungi and Ceratobasidiaceae both were more.

Finally, the availability of ONF related Hygrophoraceae and less availability of Ceratobasidiaceae may bring the obstacle in distribution mechanism for *S. yunnanense*, so future study including the effect of Hygrophoraceae and Ceretobasidiaceae on seed germination and development of host associate because OMF communities indirectly affect the coexistence and widespread distribution of orchid species. The dominated endophytic fungi also determine their host coexistence and distribution (Waud et al. 2017).

Host distribution pattern and fungi diversity

Orchid populations depend on the distribution of orchid mycorrhizal fungi (McCormick et al. 2018). The orchid mycorrhizal fungi distribution shows no correlation to compactible orchid distribution (Voyron et al. 2017). Most fungal endophytes are generalist and superficially colonizing all plant taxa and globally distributed mainly in boreal forest (Suryanarayan et al. 2018; U'Ren et al. 2019). That was the main issue about the distribution of orchids and related endophytes. Two sample species from single genus *Satyrium* having with different distribution patterns. Where, only two families (Hygrophoraceae and Unclassified_k_fungi) of endophytic fungi occupied more than 50% of fungi availability in both. The narrow distributed orchid species associated with high species richness than broadly distributed orchid. Single family of Hygrophoraceae occupied 41.86% and the relative abundance of the family of OMF only 0.053, which is comparatively less than next orchid species. So, we can say that the diversity of endophytic

fungi does not depend on distribution pattern of host orchid in local level. The above result did not support our hypothesis (#H1), where other biotic and abiotic factors may directly or indirectly influence in fungi diversity, composition and distribution.

Endophytic fungi act as biomarkers and host distribution

LEfSe analysis showed that on (family, genus to species) level, the biomarker demonstrating significant difference between *S. nepalense* and *S. yunnanense*. There was no any common species between two sample groups having LDA score more than 4 and p value ($P< 0.05$). It means that sample groups significantly influenced by different endophytic fungi separately. In sample group *S. nepalense* four different families and their individual species (Uncultured_fungus, *Alternaria*_sp, Uncultured_saccharomycetes, and Cutaneotrichosporon_curvatum) influenced as indicator species but in another sample group *S. yunnanense*, two families of endophytic fungi (Hygrophoraceae and Nectriaceae) were dominated and three species (Uncultured_hygrocybe, *Fusarium*_sp, and Neonectria_sp_nwa_besc_196c1) from these families were act as indicator species. The number of families with high LDA score and significance p-value play an important role in distribution of host species.

Conclusion

Our comprehensive investigation showed that the root-associated endophytic fungi (REFs) communities were not significantly different according to the distribution patterns of host species statistically. It reveals that the diversity and composition of root-associated endophytic fungi associated with *S. yunnanense* only support in local scale distribution. According to the LEfSe analysis three species (Uncultured_hygrocybe, *Fusarium*_sp, and *Neonectria*_sp) from two families (Hygrophoraceae and Nectriaceae) having with high LDA score and significance ($P<0.05$) value act as indicator species in *S. yunnanense* but in *S. nepalense* four species (Uncultured_fungus, *Alternaria*_sp, Uncultured_saccaromycetes, and Cutaneotrichosporon_curvatum) from four different families (Unclassicified_k_fungi, Pleosporaceae, Unclassified_c_Saccaromycetes and Trichosporonaceae) act as the indicator species and influenced in host distribution and vice-versa. Similarly, the availability of OMF family Ceratobasidiaceae was comparatively higher in *S. nepalense* than *S. yunnaense* which may primarily influence in cosmopolitan distribution. However, our current limited data cannot provide strong evidence in favor the fungal diversity and composition between the two geographically distinct orchid species of single genus *Satyrium* Sw. globally. The present study provides a base for host based endophytic fungal diversity and describe the complete picture of fungal diversity in local level. These results extend the understanding of assembly mechanism and interspecific relationship in root-associated endophytic communities not only derived by host orchid in local level but other biotic and abiotic factors are equally responsible for their ecological resilience and potential biotechnological application or conservation of endophytic fungi and their associated host.

Supplementary Information The following supporting information can be downloaded at: https://doi.org/xx.xxxx

Information table. 1 Sample species and ASVs detail.

Information table. 2 Group level and ASVs numbers.

Information table. 3 Sample population and endophytic fungi.

Information table. 4 Sample population and top ten dominated families of endophytic fungi.

Information table. 5 Species different analysis through LEfSe-Clodogram and LDA-bar chart).

Table. S1 ASV-associated with sample species

Table. S2 Information about ASVs

Table. S3 Phylum-wise distribution of fungi

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Author contribution Investigation and sampling by T.L.; Conceptualization and Validation; Writing manuscript by B.P. and Review and Editing by R.A. Similarly, Visualization; Project Administration and Funding Acquisition by J.G.

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Data availability Additional supporting information is available online in the Supplementary Information section of the article.

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Table and figure notes:

Table. 1 (The location and general description of sampling sites and Samples)

Sites Name	Site-A (Chenggong district, Yunnan Province, China	Site- B Chenggong district, Yunnan Province, China
Name of species	*Satyrium nepalense* D. Don. (SN) *Satyrium yunnanense* Rolfe. (SY)	*Satyrium yunnanense* Rolfe. (SY) *Satyrium nepalense* D. Don. (SN)
Population	Populations (A and B)	Populations (C and D)
Number of samples	Population A=5/Population B=5	Population C=5/Population D=5
Latitudes	A=25°, 07', 35.03" N B=25°, 07', 21.83"N	C=24°, 48', 27.03" N D=24°, 48', 12.00"N
Longitudes	A=102°, 42', 21.68" E B=102°, 42', 17.42"E	C=102°, 53', 46.52" E D=102°, 53', 35.16"E
Elevation	A=2261m.a.s.l. B=2272m.a.s.l.	C=2416m.a.s.l. D=2436m.a.s.l.
Mean annual precipitation	5.2 Inches (5.1 to 5.3In)[a]	5.2 Inches (5.1 to 5.3In)[a]
Mean annual temperature	72°F (55°F to 82°F)[a]	72°F (55°F to 82°F)[a]
Soil nature	Red soil with less litter	Red soil along with plant litter
Vegetation	Sloppy grassland with pine forest	Grassland with pine forest

[a] low and high monthly temperature in the sampling sites. #Source-https://weatherspark.com>China>Yunnan

Fig. 1 Plot **(A)** Simpson index showed the species richness and relative abundance, where higher the value in Simpson index determine the less diversity in read values. Similarly, Plot **(B)** Sobs index shown the number of read taxonomic units on the basis of individual sample. The height of the bar indicates the number of read ASVs associated with sample.

Fig. 2 Bar plot **(A)** displayed the diversity of fungal ASVs between the sample population with (P=0.944) which means that sets of data were identical and no significance change has been observed. Similarly, **(B)** displayed the diversity between two sample species on fungal families with (P=0.506). Which also indicated that there was no significantly change observed statistically.

Fig. 3 The dilution curves shown the depth of read sampled, according to Sobs index reflect the species richness in given sample and our sampling depth was sufficient.

Fig. 4 NMDS analysis between sample species on ASV level **(A)** represent the control group and **(B)** represent the treatment group but there was no any significant different in stress (0.167) value. Points of different colors, represent sample species from different groups; the closer points were more similar in species composition and disperse points were dissimilar in species composition.

Fig. 5 NMDS analysis between intra-specific species on ASV level. **(A)** great representation of data from two populations of *S. nepalense* with stress value (0.063). **(B)** the good representation of data from two populations of *S. yunnanense* with stress value (0.112). Points of different colors, represent sample species from different groups, the closer points were more similar in species composition and disperse points were dissimilar in species composition.

Fig. 6 NMDS analysis between sample population on ASV level. Points of different colors, represent sample species from different population; the closer points are more similar in fungal composition than disperse once. **(A)** represent the control group and **(B)** represent the treatment group but there was no any significant different in stress (0.167) value any way this stress value indicated the good representation of data.

Fig. 7 According to the diagram **(A)** and (**B**) the composition of ASV numbers were different in between similar species, which were differ according to sampling habitat. Similarly, diagram **(C)** 122 unique ASVs only in *S. nepalense* and 146 unique ASVs in *S. yunnanense* remaining 17 ASVs were common from both species. **(D)** mentioned the distribution pattern of fungal ASVs related to sample population. Where only 4 ASVs were shared by all sample groups. The sample species *S. yunnanense* was rich in species composition than *S. nepalense* in ASV level.

Fig. 8 Bar plot **(A)** The relative abundance of uncultured_hygrocybe, and uncultured_fungus species was higher in *S. yunnanense* and *S. nepalense* respectively**. (B)** In sample population the relative abundance of fungal species was also same as sample species in plot A, but the relative abundance of OMF species was specific to habitat.

Fig. 9 The Unclassified_k_fungi and Hygrophoraceae two families were highly dominated in both sample species with positive correlation values. The *S. nepalense* from different population was positively correlated with dominated families of endophytic fungi comparatively than *S. yunnanense*. The positively correlated orchids and endophytic fungi were mutually support to each other for their growth, development, composition and distribution.

Fig. 10 LEfSe analysis (**A)** The cladogram showed that the degree of microbial species with significance differences in two groups; Blue and Green bars indicate different groups with the classification level of (family, genus and species) shown from inside to outside. The blue and green nodes in the phylogenetic tree represent endophytic fungal species that play a significant role in the sample species *S. nepalense* and *S. yunnanense* respectively. **(B)** Different taxonomic level of endophytic fungi with significant difference that have an LDA score greater that the estimated value with default score is 4. The length of the histrogram represent the LDA score; the degree of influence of species with significant difference both sample species SN and SY.

Materials and Methods

Map of study sites / Sample and sampling species

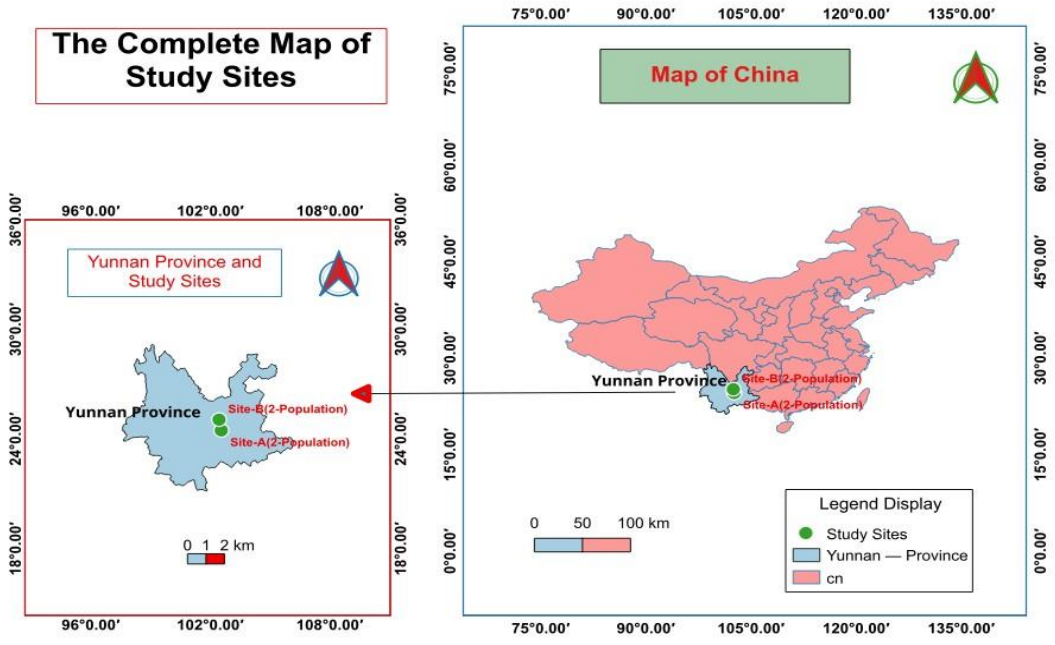
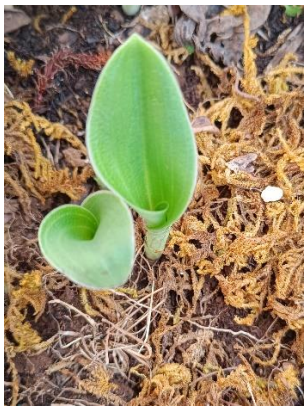


Photo plates. Sample species and sample



(A) Satyrium yunnanense Rolfe.

(B) *Satyrium nepalense* D. Don.

(C) Sample of root and tuber

Data Analysis/Results

Community diversity analysis

Fig. 1 Diversity index (Simpson index and Sobs index)

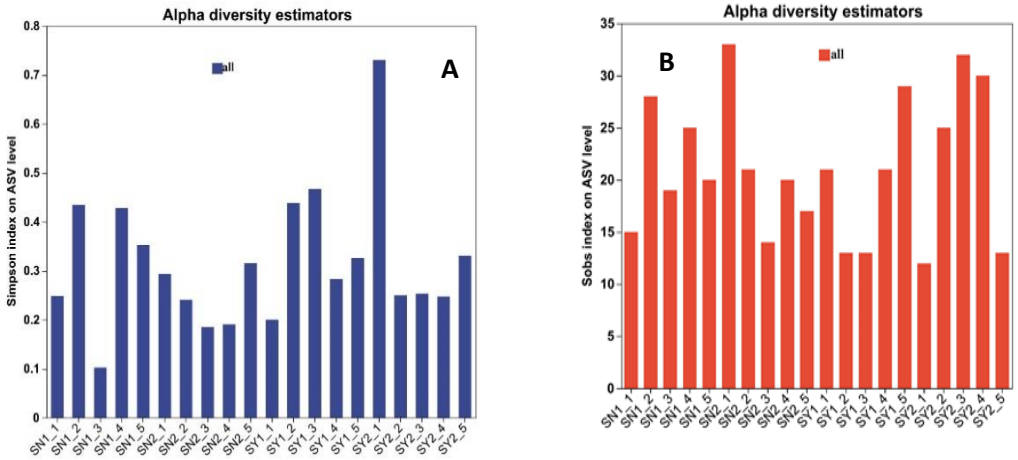


Fig. 2 Bar-plot displayed the diversity of fungal ASVs between sample species and sample population

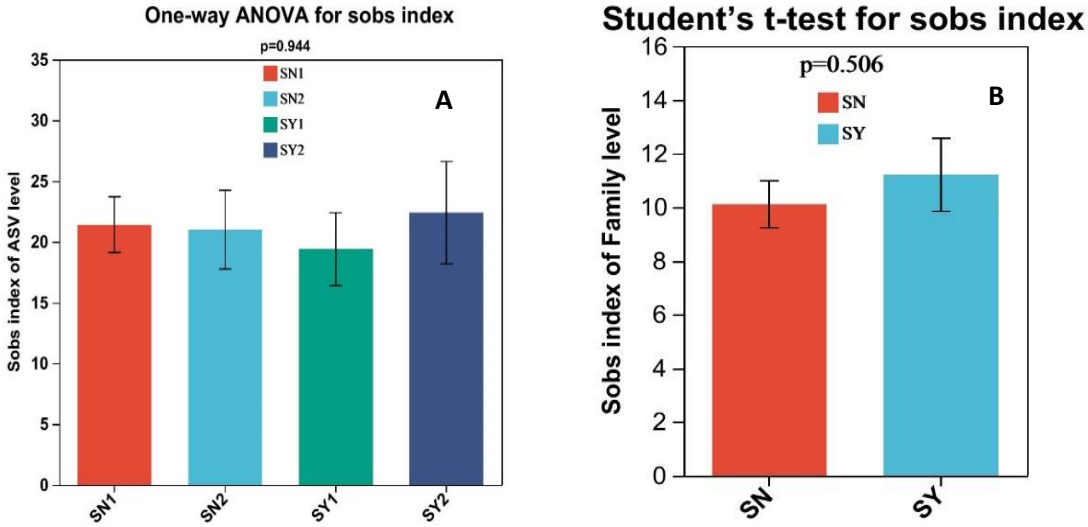


Fig. 3 Dilution curves and Sample depth

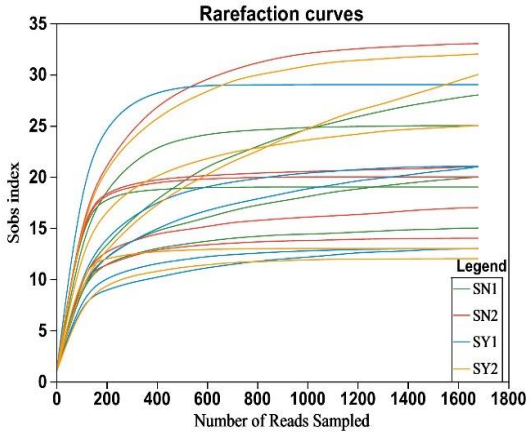


Fig. 4 NMDS analysis between sample species on ASV level with and without treatment.

(A) Control group **(B)** Treatment group

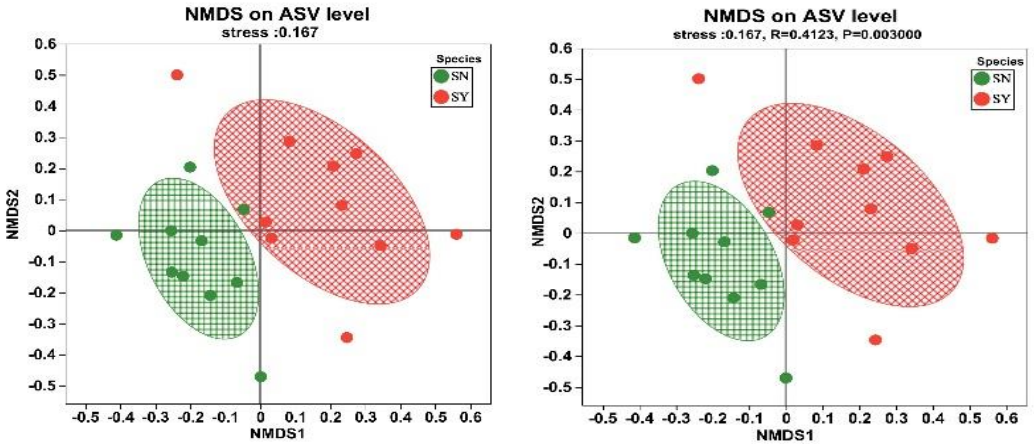


Fig. 5 NMDS analysis between intra-specific species on ASV level

(A) NMDS between SN1 and SN2 **(B)** NMDS between SY1 and SY2

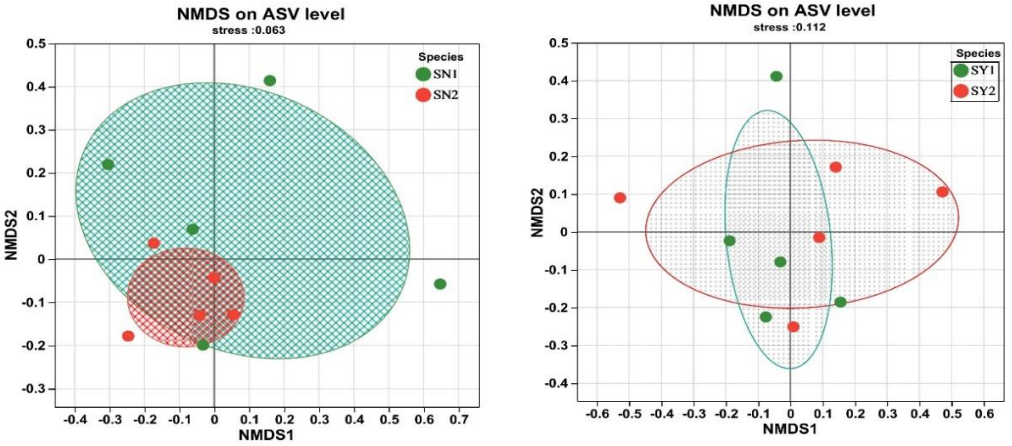
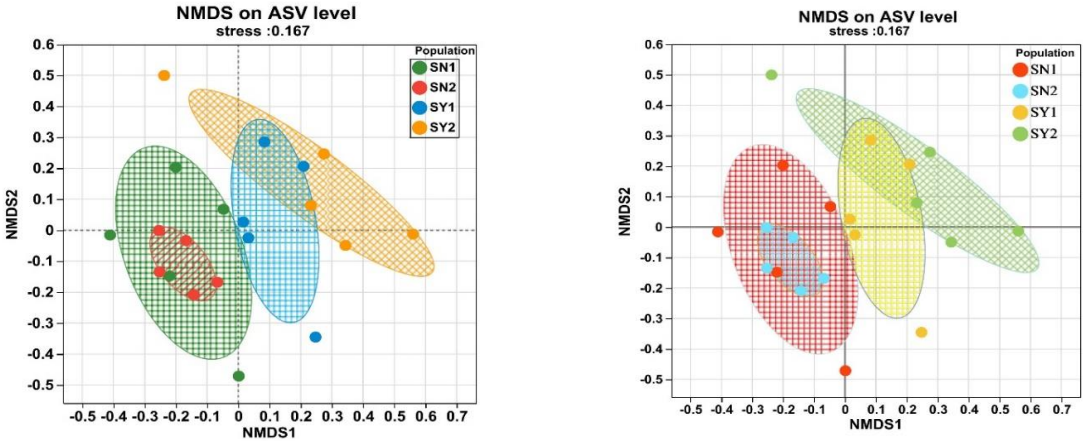


Fig. 6 NMDS analysis between sample population on ASV level with or without treatment.

(A) Control group **(B)** Treatment group



Community composition analysis

Fig. 7 The composition of ASV numbers according to the sample species and population.

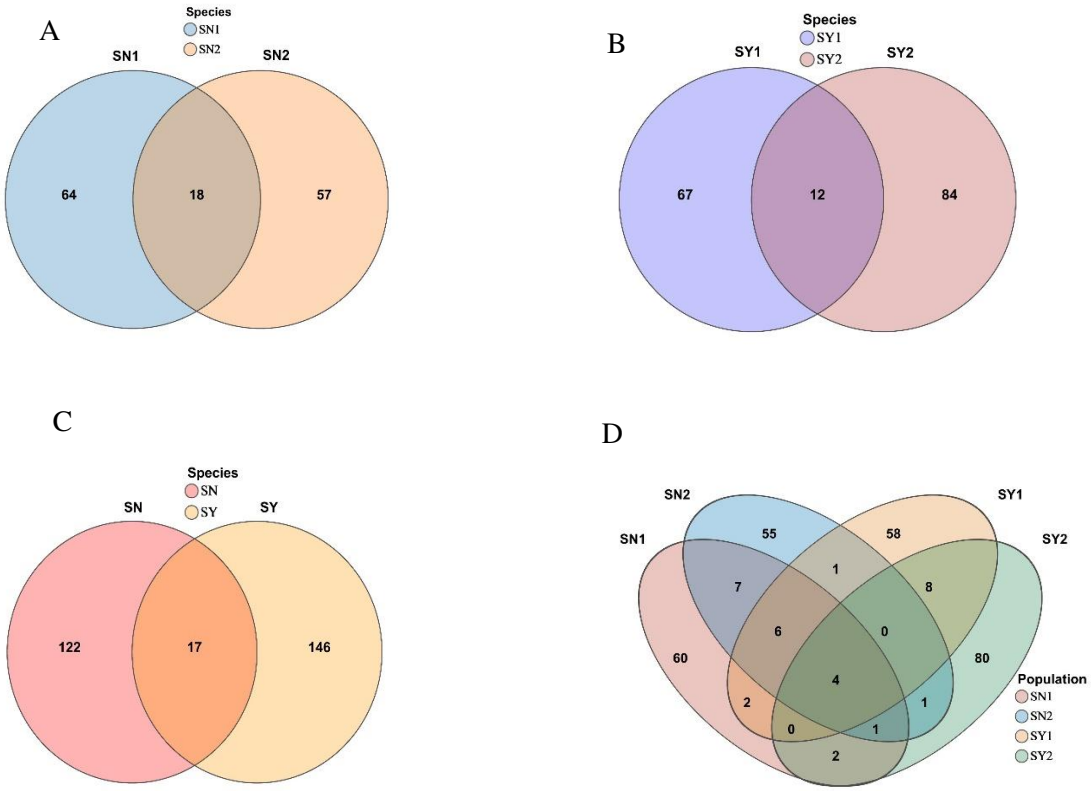
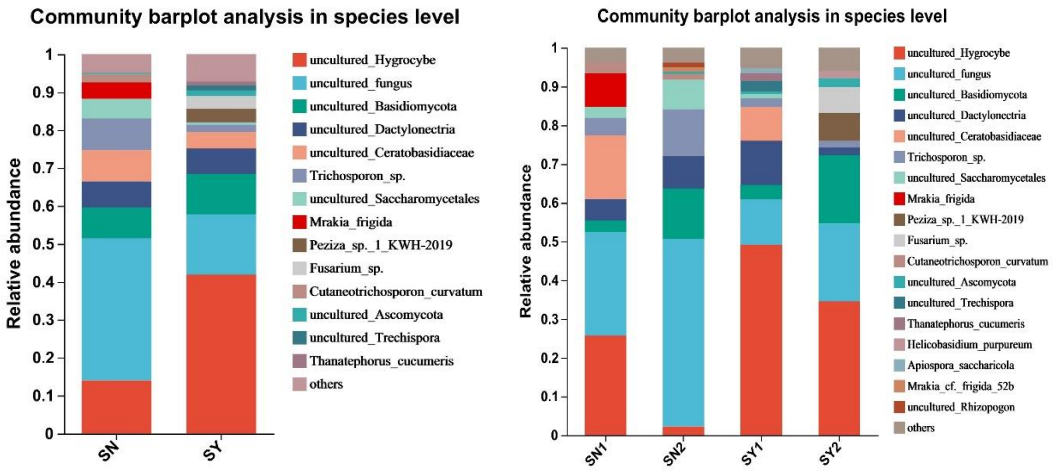
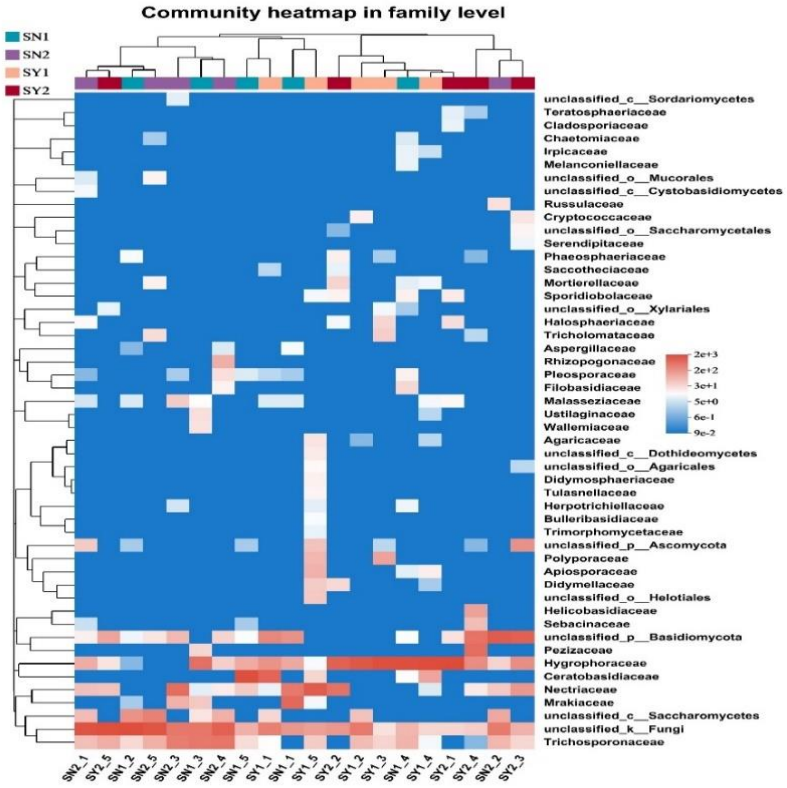


Fig. 8 Bar-plot shown the relative abundance fungal species associated with sample species and sample population.



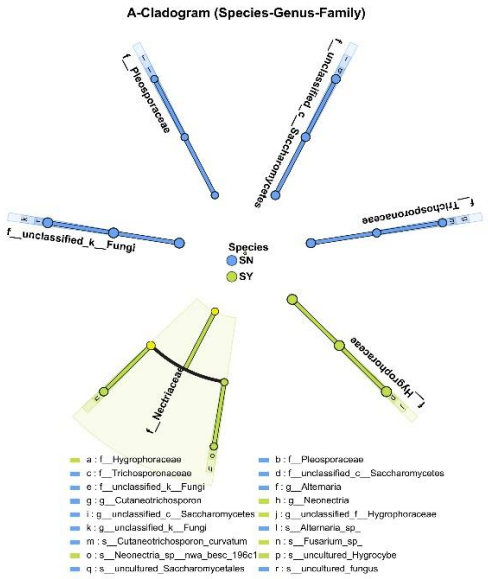
A-Relationship between fungi species and sample species. B-Relationship between fungi species and sample population.

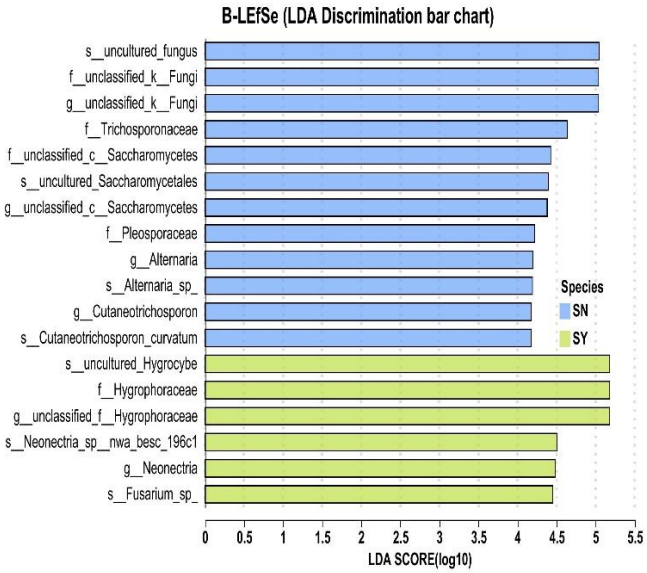
Fig. 9 Correlation between the families of endophytic fungi and sample population (Individuals).



Species differences analysis

Fig. 10 Species differences analysis through (LEfSe-Cladogram) and (LDA-bar chart)


A-Cladogram (Species-Genus-Family)
f__Pleosporaceae
f__unclassified_c__Saccharomycetes
f__unclassified_k__Fungi
f__Trichosporonaceae
Species
SN
SY
f__Hygrophoraceae
f__Nectriaceae
a : f__Hygrophoraceae
b : f__Pleosporaceae
c : f__Trichosporonaceae
d : f__unclassified_c__Saccharomycetes
e : f__unclassified_k__Fungi
f : g__Alternaria
g : g__Cutaneotrichosporon
h : g__Neonectria
i : g__unclassified_c__Saccharomycetes
j : g__unclassified_f__Hygrophoraceae
k : g__unclassified_k__Fungi
l : s__Alternaria_sp_
m : s__Cutaneotrichosporon_curvatum
n : s__Fusarium_sp_
o : s__Neonectria_sp__nwa_besc_196c1
p : s__uncultured_Hygrocybe
q : s__uncultured_Saccharomycetales
r : s__uncultured_fungus


B-LEfSe (LDA Discrimination bar chart)
s__uncultured_fungus
f__unclassified_k__Fungi
g__unclassified_k__Fungi
f__Trichosporonaceae
f__unclassified_c__Saccharomycetes
s__uncultured_Saccharomycetales
g__unclassified_c__Saccharomycetes
f__Pleosporaceae
g__Alternaria
s__Alternaria_sp_
g__Cutaneotrichosporon
s__Cutaneotrichosporon_curvatum
s__uncultured_Hygrocybe
f__Hygrophoraceae
g__unclassified_f__Hygrophoraceae
s__Neonectria_sp__nwa_besc_196c1
g__Neonectria
s__Fusarium_sp_
Species
SN
SY
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
LDA SCORE(log10)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ASVassociatedwithsamplespecies.xlsx
- InformationaboutASVs.xlsx
- Informationtables.docx
- Phylumwisedistributionoffungi.xlsx