

Fungal community characteristics of the last remaining habitat of three paphiopedilum species in China

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Abstract

Background: *Paphiopedilum armeniacum*, *Paphiopedilum wenshanense* and *Paphiopedilum emersonii* are critically endangered wild orchids. The population is threatened, and the number of natural distribution sites has plummeted. Ex situ conservation and artificial breeding are the keys to maintaining the population to ensure the success of ex situ conservation and field return in the future. The habitat characteristics and soil nutrient information of the last remaining wild distribution sites of the three species were studied. ITS high-throughput sequencing was used to reveal the composition and structure of the soil fungal community, analyze its diversity and functional characteristics, and reveal its relationship with soil nutrients.

Results: The three species preferred to grow on low-lying, ventilated and shaded negative terrain with good water drainage. There were significant differences in soil alkali-hydrolyzed nitrogen and available phosphorus among the three species. There were 336 fungal species detected in the samples. On average, there were different dominant groups in the soil fungal communities of the three species. The functional groups of soil fungi in the habitats were dominated by saprophytic fungi and ectomycorrhizae, with significant differences in diversity and structure. The co-occurrence network of habitat soil fungi was mainly positive. Soil pH significantly affected soil fungal diversity in the habitats of the three *paphiopedilum* species. The study and analysis confirmed that the dominant groups of soil fungi were significantly correlated with soil nutrients.

Conclusion: The three species have similar habitat preferences, but there are significant differences in soil fungal composition, community structure and diversity. The functional group of fungi contains abundant saprophytic fungi, ectomycorrhizae, and a small amount of orchid mycorrhizae. The symbiotic relationships of the three species of soil fungi were harmonious, which was conducive to resistance to adverse environments. Soil environmental factors were significantly correlated with soil fungal communities, and pH significantly controlled fungal diversity. Our study on the habitat characteristics and soil fungal communities of the three wild *paphiopedilum* species laid a foundation for future ex situ conservation and field return work.

Background

Orchidaceae is one of the largest families of higher plants and one of the most evolutionarily morphologically rich plant families in nature. There are approximately 25,000-35,000 Orchidaceae species in 800 genera worldwide[1, 2]. Orchid organs are highly specialized, diverse, and highly adaptable to the environment and are widely distributed in various terrestrial ecosystems except for the poles and extreme arid desert regions[3, 4]. Orchids have highly specialized mycorrhizal communities and special pollination mechanisms [5] and are extremely sensitive to habitat changes [6, 7]. Orchids also have unique ornamental functions and medicinal value [8], making them “flagship” species in the ecological environment[9]. At present, due to severe anthropogenic activity and the impact of global climate change, wild orchids are facing great pressure to survive. All wild orchids in the world are protected by

the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and their trading and trading are explicitly prohibited [10]. *Paphiopedilum* belongs to the Orchidaceae family and has large, slip-shaped lips on its petals [11, 12]. As the flower shape is similar to that of the slippers of noble European women in the Middle Ages, *Paphiopedilum* is also known as the slipper orchid, which is the most ornamental group of orchaceae plants and enjoys a high reputation among international flower ornamentals. This topic has attracted the attention of many scientific researchers and flower lovers around the world [13]. Due to the ornamental value of *Paphiopedilum*, the wild population is subject to predatory mining and the smuggling trade [14]. In addition, it is difficult for this group of plants to reproduce in the wild, and the species and number of plants are rapidly reduced or even extinct [15]. At present, all *Paphiopedilum* species are listed on the CITES List and the IUCN Red List of endangered species, and in China, all of them are listed on the State Key Wild Plant Protection List, which is rare among the rare and endangered plant groups in the world [16]. Southwest China is the origin and differentiation center of *Paphiopedilum* and the distribution hotspot of *Paphiopedilum*. However, according to a recent survey of wild *Paphiopedilum* plants, the plants of *Paphiopedilum* in Southwest China are suffering devastating damage, especially *Paphiopedilum armeniacum* (*P. armeniacum*), *Paphiopedilum wenshanense* (*P. wenshanense*) and *Paphiopedilum emersonii* (*P. emersonii*), which are the most endangered[17]. At present, fewer than 10 natural distribution sites of these three *Paphiopedilum* species have been found, and the latter habitats are at risk of degradation and loss. These three species of *Paphiopedilum* are listed as small populations on the wild plant protection list in China, which means that there is a risk of extinction in the wild at any time, which also indicates the urgency and priority of protection.

Understanding the ecological habits of orchids is a prerequisite for scientific conservation work, especially for ex situ conservation of endangered species [18, 19]. In the natural environment, because the seeds of orchids are small, the germ is degraded and lacks nutrients, and the germination of seeds depends on the help of fungi, which can provide necessary resources such as carbon, nitrogen and phosphorus [20]. The entire growth cycle of orchids also depends on compatible mycorrhizal fungi in the soil, whether specialized or compatible. Considering the heavy dependence of orchids on mycorrhizal fungi, it can be predicted that their spatial distribution largely depends on the spatial distribution of microorganisms in the soil of the habitat, and the composition of fungi in the soil of the habitat is a key feature of the ecological habits of orchids [21, 22]. There is evidence that changes in mycorrhizal fungal communities driven by habitat conditions can directly or indirectly affect the distribution dynamics of terrestrial orchids [23, 24]. In addition, some seed introduction experiments showed that the seeds could germinate outside the densely distributed areas of orchids, indicating that orchid seeds may recruit fungi from the soil of the habitat to complete symbiosis, germination and growth [25, 26] and gradually form a microbial community with orchid roots as the distribution core. Therefore, the soil fungal community in a habitat may be the origin of orchid mycorrhizal fungi [27-29]. Moreover, locations close to orchid plant growth represent fungal hotspots for seed germination and new plant establishment. However, most of the current studies have focused on the endophytic fungi and rhizosphere fungal communities of orchids [30-33] while ignoring the fungal communities in habitat soil. This lack of understanding of the species

source and process of orchid mycorrhizal fungal community construction restricts the development of simulated environment construction for ex situ conservation. At present, due to the reduction in the number of remaining wild populations and the degradation of habitats, the supplementation of seedlings is difficult to guarantee, and the survival of wild populations is at stake. In this regard, a necessary and urgent solution is to construct a suitable habitat for the growth of *Paphiopedilum* in a good artificial environment, expand its population size, and ultimately return to the wild. To ensure success, it is crucial to fully reveal the soil fungal communities in the last remaining habitat of the three *Paphiopedilum* species, including how they change spatially and respond to the biotic environment.

In this study, *P. armeniacum*, *P. wenshanense*, *P. emersonii*, were used as research objects to comprehensively investigate the environmental characteristics of the remaining habitats, and soil samples were collected from the habitats of the three *Paphiopedilum* populations. The soil fungi in their habitats were determined based on high-throughput sequencing methods to clarify the fungal community composition structure, fungal functional groups, and fungal community diversity. The dynamic changes in fungi in the soil of *Paphiopedilum* habitat were revealed, and the response of the fungal community structure in the habitat to the biotic environment was initially explored, compensating for the lack of research on the habitat characteristics of the wild population of this group of species. In addition. This study can also provide a reference for ex situ conservation, artificial breeding environment selection and the construction of this group of plants.

Materials and methods

Sample collection

In this study, after fully investigating the natural distribution of three *Paphiopedilum* species, three natural distribution points with good growth and no man-made damage were selected for each species (Fig. 1). *P. armeniacum* is distributed in Nujiang Prefecture, Yunnan Province. *P. wenshanense* is distributed in Wenshan Prefecture, Yunnan Province. *P. emersonii* is located at the junction of Guizhou Province and Guangxi Province. First, the last habitat of *Paphiopedilum* was observed, and information on the latitude, longitude, altitude, slope, aspect and vegetation type was recorded. Sampling was carried out at 10 cm outside the dense distribution area of *Paphiopedilum* species. We set the direction of possible landing of seeds as the sampling direction and extracted 20 grams of repeated soil samples in 3 directions at 5 cm from the soil layer. Soil fungal samples from 27 habitats of three species were collected. Before sampling, the air environment was disinfected with 75% medical alcohol to remove the undecomposed litter. To collect representative soil samples, the soil was transferred to a 4 °C refrigerator within 12 hours after sampling to minimize DNA degradation. For meta-barcode analysis, a small amount of soil (< 250 mg) was added to BashingBead™ Lysis Tubes (Zymo Research, Cambridge Bioscience, Cambridge, UK) to preserve environmental DNA for the extraction and amplification process. Soil nutrient test samples According to the above sampling method, 200 g of soil (at a depth of 0 ~ 10 cm) from each habitat was collected, placed in a sampling bag and returned to the laboratory.

Soil nutrient analysis

The soil physiochemical properties were analyzed according to Bao and are described briefly as follows[34]. The pH was determined by the water:soil = 2.5:1 extraction pH meter (PHS-3G) method. The water content was determined by the 105 °C drying–weighing method. The organic carbon content was determined by the KCr2O7-H2SO4 external heating method. Total nitrogen was determined by the semimicro Kjeldahl method; total phosphorus was determined by the HClO4-H2SO4 digestion-molybdenum antimony colorimetric method. Total potassium was determined by NaOH melting-flame spectrophotometry. Available phosphorus was determined by a 0.03 mol/L NH4F-0.025 mol/L HCl extraction-molybdenum antimony anti-colorimetric method. Available potassium was extracted by a 1 mol/L neutral NH4OAc-flame photometer. Ammonium nitrogen and nitrate nitrogen were determined by a 2 mol/L KCl extraction-continuous flow analyzer. The soil enzyme activities were determined using an enzyme analysis kit (Yangling Xinhua Ecological Technology Co., Ltd., Shanxi, China).

Habitat soil fungal sequencing

DNA was extracted from the samples by the CTAB method, and the extracted DNA was detected by 1% agarose gel electrophoresis. PCR extraction was performed using barcode primers and high-fidelity enzymes. The following primer sequences were used: 5'-AAGCTCGTAGTTGAATTTTCG-3' and 5'-CCCAACTATCCCTATTAATCAT-3' [35, 36]. The PCR products were mixed and detected by 2% agarose gel electrophoresis. The quantified PCR products were subjected to Illumina HiSeq sequencing. First, Trimmomatic (version 0.33) [37] was used to filter the quality of the original data, and then Cutadapt (version 1.9.1)[38] was used to identify and remove the primer sequences. Subsequently, USEARCH (version 10) was used to splice the double-end reads and remove the chimeras (UCHIME, version 8.1) [39]. Chimeric sequences were removed to finally obtain high-quality sequences, and the characteristic sequences were taxonomically annotated using the simple Bayes classifier with UNITE as the reference database[40].

Data analysis

Soil nutrient difference analysis

The soil nutrients of the three *Paphiopedilum* species were statistically analyzed by SPSS 22.0. Univariate analysis of variance was used to analyze the means. When the variance analysis results of soil nutrients between different species were significant at the $p < 0.05$ level, the least significant difference (LSD) test was used to compare the mean values of the soil variables.

Analysis of fungal community structure in the habitat

FLASH (v.1.2.7) was used to merge forward and reverse double-ended sequences from the MiSeq platform. A paired-end reading was obtained for each sample based on a unique barcode sequence. QIIME software was used to remove data clutter, and 97% similarity was used as the standard to divide operational taxonomic units. Usearch was used to remove chimeras, and the RDP classifier Bayesian

algorithm was used to perform taxonomic analysis on representative OTU sequences. Species classification was performed using the fungal database Unite8.0/Fungi (Unite Release 8.0) for comparison and identification.

In this study, the abundance of fungal species was analyzed based on the Euclidean distance algorithm, and the FUNGuild online database platform was used to predict the function of soil fungi in three *Paphiopedilum* species. The Chao1 index, ACE index, Shannon index and Simpson index were used to represent the alpha diversity index, and a t test was used to determine significant differences. ANOSIM was used to verify the reliability of the species grouping. Beta diversity was analyzed by nonmetric multidimensional scaling (NMDS) calculated by the Bray–Curtis distance, and the results were subjected to the Adonis nonparametric variance test. The above analysis was implemented using the BioCloud platform (<https://www.biocloud.net/>).

To effectively reveal the symbiotic network relationships of the soil fungi in the three *Paphiopedilum* habitats, the soil fungal species data of the *Paphiopedilum* plant habitats were filtered under conditions of a relative abundance $\geq 0.1\%$ and at least three sampling points. Spearman correlation was performed on the filtered data set, and a correlation coefficient (ρ) ≥ 0.5 and $p < 0.01$ were selected to construct the network. The main topological attributes of the network were calculated by using the “igraph” package of R software, and the nodes were calculated and visualized with Gephi software.

The Mantel test was used to explore the effects of habitat soil nutrients on fungal diversity. db-RDA was used to reveal the effects of habitat soil nutrients on soil fungal community changes. Spearman correlation analysis was used to reveal the correlation between habitat soil nutrients and dominant habitat soil fungal groups. The analysis and mapping were achieved using the “vegan” package, “ggcor” package, and “corrplot” package of R software.

Results

Habitat information and soil nutrient characteristics

A habitat survey of three wild *Paphiopedilum* species revealed that (Table 1) *P. armeniacum* grows in mountain shrubs at an altitude of approximately 1700-2000 m, and the slope is usually 30-55°. *P. emersonii* is distributed in the evergreen and deciduous broad-leaved mixed forest at an altitude of approximately 300-800 m in karst low-mountain hills, with a very steep growth slope (75-90°). *P. wenshanense* is distributed in shrubs on normal landforms and karst landforms, with altitudes of approximately 1500-1600 m. The three species have similar habitats. They all like to grow on shady slopes such as those in the north and northwest and like to grow on negative terrain such as tree roots, stone pits and stone crevices. The soil nutrients of the three *Paphiopedilum* species were analyzed and tested by one-way analysis of variance (Table 2). The results showed that there was no significant difference in total nitrogen, total phosphorus, total potassium, organic matter, available potassium or pH among the three species. The content of alkali-hydrolyzable nitrogen in the soil of *P. emersonii* was

significantly greater than that in the soil of *P. wenshanense*. The available phosphorus in the soil of *P. emersonii* and *P. armeniacum* was significantly greater than that in the soil of *P. wenshanense*.

Table 1 Habitat information of three *Paphiopedilum* species

Specie	Altitude [m]	Geomorphology	Vegetational Form	Slope [°]	Aspect	Microhabitat
<i>P. armeniacum</i>	1776-2020	Normal Geomorphology	Shrubwood	30-55	North–West North	Tree Root Swallet
<i>P. emersonii</i>	389-835	Karst	Evergreen And Deciduous Broad Leaved Forest	75-90	North South East West	Stonewall
<i>P. wenshanense</i>	1525-1630	Karst–Normal Geomorphology	Shrubwood	30-50	Northwest	Tree Roots, Stone Crevices

Table 2 Differences in the soil nutrients in the habitats of the three *paphiopedilum* species

	<i>P.armeniacum</i>	<i>P.wenshanense</i>	<i>P.emersonii</i>
TN	5.19±1.28a	1.81±1.58a	7.88±5.06a
TP	0.62±0.24a	0.35±0.13a	0.57±0.13a
TK	5.18±2.23a	4.66±1.08a	3.19±0.76a
SOC	128.49±31.11a	43.18±37.63a	150.70±97.24a
AN	223.58±42.92ab	102.95±80.55b	316.74±85.20a
AP	4.1±2.19a	1.08±0.08b	5.04±1.30a
AK	138.67±45.24a	194.33±122.15a	70±41.04a
pH	6.76±1.046a	7.27±0.62a	7.77±0.32a

Note: The values are the means ± standard errors. Different lowercase letters in the same row indicate significant differences between horizontal gradients (P < 0.05). TN: total nitrogen; TP: total phosphorus; TK: total potassium; TOC: total organic carbon; AN: alkali-hydrolyzable nitrogen; AP: available phosphorus; AK: available potassium

Species dilution curve and Venn diagram

The dilution curve can truly reflect the sequencing depth of the sample sequence. As shown in Fig. 2a, at a similarity of 97%, the soil fungal dilution curves of the three *Paphiopedilum* species tended to decrease, indicating that the sample size could represent the soil fungal community in the plant habitat as a whole. A total of 2,161,515 pairs of reads were obtained from 27 fungal habitat samples. After

quality control and splicing of the double-ended reads, a total of 2,154,184 clean reads were generated. Each sample produced at least 79,308 clean reads, with an average of 79,785 clean reads. High-throughput sequencing analysis was performed based on the 97% similarity tag classification as an OTU standard, and a total of 1068 operable units were obtained. Among them, 452 unique OTUs were found in the soil of *P. emersonii* (Fig. 2b), followed by *P. wenshanense* (n=232) and *P. armeniacum* (n=211). There were 65 OTUs in the *P. wenshanense* and *P. armeniacum* habitat soils, 25 OTUs in the *P. armeniacum* and *P. emersonii* habitat soils, and 46 OTUs in the *P. emersonii* and *P. wenshanense* habitat soils; moreover, there were 37 common OTUs in the three *Paphiopedilum* habitat soils.

According to the species annotation (Table 3), a total of 336 fungal species belonging to 11 phyla, 30 classes, 74 orders, 157 families, and 272 genera were identified. A total of 230 species of fungi belonging to 10 phyla, 26 classes, 62 orders, 127 families, and 202 genera were identified in the soil of *P. emersonii*. A total of 138 species of fungi belonging to 9 phyla, 21 classes, 44 orders, 86 families, and 116 genera were identified in the soil of *P. armeniacum*. A total of 145 species of fungi belonging to 7 phyla, 21 classes, 52 orders, 98 families, and 126 genera were identified in the soil of *P. wenshanense*.

Table 3 Number of fungal species in the soil of three *Paphiopedilum* species

Species	Phylum	Class	Order	family	Genus	Species	OTUS
<i>P.emersonii</i>	10	26	62	127	202	230	560
<i>P.armeniacum</i>	9	21	44	86	116	138	338
<i>P.wenshanense</i>	7	21	52	98	126	145	380
Total	11	30	74	157	272	336	1068

Fungi composition and functional group composition in the habitat soil of three *paphiopedilum* species

The relative abundance of fungal groups in the habitat soil of the three *Paphiopedilum* species at the phylum and genus levels is shown in Fig. 3. The dominant group of fungi in the soil of *P. wenshanense* was Calcarisporiellomycota, and no obvious dominant group was found in the soil of *P. armeniacum*. There were dominant fungal groups, such as Kickxellomycota, Entorrhizomycota, Olpidiomycota and Rozellomycota, in the *P. emersonii* habitat. At the genus level, there were unclassified_Sordariomycetes, *Sebacina*, unclassified_Basidiomycota, *Boletus*, unclassified_Boletaceae, *Archaeorhizomyces* and other dominant fungi in the habitat soil of *P. wenshanense*. Unclassified _ Thelephoraceae, *Hygrocybe*, unclassified_Serendipitaceae, unclassified_Ascomycota, *Tomentella* and unclassified_Agaricomycetes were found in the soil fungal habitat of *P. armeniacum*. Acremonium, unclassified_Fungi, unclassified_Chaetothyriales, Apodus, unidentified, unclassified_Hypocreales and other soil fungi were found in the soil of *P. emersonii*.

Based on the ecological role of fungi in the environment, the functional classification and annotation of soil fungi in the soil of three *Paphiopedilum* species were carried out by using the FUNGuild

microecological tool. The functions of soil fungi can be divided into three types according to the nutritional mode: saprophytic nutrition, symbiotic nutrition and pathological nutrition. In the soil of the three *Paphiopedilum* species, the saprophytic and symbiotic nutrient types were dominant (Fig. 4a). In particular, the relative abundance of the two nutritional fungi in the soil of *P. armeniacum* accounted for 98%. It also accounts for more than 80% of the habitat of *P. wenshanense* and *P. emersonii*. The fungal functional groups were further divided into 10 categories by environmental resource absorption (Fig. 4b). These included Undefined Saprotroph, Ectomycorrhizal, Undefined-Biotroph, Soil Saprotroph, Fungal Parasite, Wood Saprotroph, Plant Saprotroph, Animal Pathogen, Plant Pathogen and Orchid Mycorrhizal. Among them, undefined saprophytic fungi and ectomycorrhizal fungi account for a large proportion of the three *Paphiopedilum* species in the soil and are two types of fungal functional groups that play important ecological roles. Undefined saprophytic fungi, ectomycorrhizal fungi, and undefined trophic fungi were dominant in the habitat soil fungal functional group of *P. armeniacum*, accounting for more than 95% of the relative abundance, and some orchid mycorrhizal and animal pathogenic fungi. The main fungal functional groups of *P. wenshanense* in the soil habitat were saprophytic fungi, animal pathogens, plant parasitic fungi, plant saprophytic fungi, wood saprophytic fungi, plant pathogens and other dominant functional groups. The soil fungal functional groups of *P. emersonii* were also rich, and their relative abundances were relatively similar.

Diversity analysis

The alpha diversity analysis of the habitat soil fungal communities of the three *Paphiopedilum* species revealed no significant differences in the ACE and Chao1 indices, indicating that there was no significant difference in the community abundance of the habitat soil fungi among the three species of *Paphiopedilum*. The Simpson index and Shannon index of *P. emersonii* were significantly greater than those of *P. armeniacum* and significantly greater than those of *P. wenshanense* (Fig 5). In addition, there was no significant difference in the four diversity indices between *P. armeniacum* and *P. wenshanense*.

To clarify the overall differences in the soil fungal community structure among the three *Paphiopedilum* species, the beta diversity was analyzed via nonmetric multidimensional scaling (NMDS) based on the Bray–Curtis distance (based on fungal abundance and species presence or absence). Prior to this, to verify the reliability of species as a grouping unit, we used permutational MANOVA. The results showed that the differences in the habitat soil fungal community structure among the three *Paphiopedilum* species were significantly greater than the intraspecific differences, indicating that the grouping results were reliable (Figure 6). The R values were 0.214 and 0.388 at the phylum and genus levels, respectively, indicating that the grouping method explained 21.4% and 38.8% of the sample differences, respectively.

Figure 7 shows the results of the NMDS analysis, and the ordination axis was set to 2. The stress values (strees) of the soil fungal community in the soil of the three *Paphiopedilum* species at the phylum and genus classification levels were less than 0.2, indicating that the results have explanatory significance. The stress values at the phylum and genus levels were 0.0071 and 0.159 (Fig. 7), respectively, indicating

that the differences in the habitat soil fungal community structure of the three *Paphiopedilum* species were more obvious at the phylum level.

Soil fungal co-occurrence network of three *Paphiopedilum* species

To study the potential interactions between the soil fungi in the three *Paphiopedilum* species and the changes in the co-occurrence network, an OTU-level co-occurrence network of the soil fungi of the three *Paphiopedilum* species was constructed based on random matrix theory. The same threshold ($r > 0.6$, $p < 0.01$) was used to construct the co-occurrence network, and the changes in the co-occurrence network were compared and analyzed. As shown in Fig. 8, there were 74 nodes and 606 edges in the habitat soil fungal co-occurrence network of *P. emersonii*, of which 93.56% were positively correlated and only 6.44% were negatively correlated (Fig. 8A). There were 77 nodes and 479 edges in the co-occurrence network of soil fungi in the habitat of *P. armeniacum*. The proportion of positively correlated edges was 70.56%, and the proportion of negatively correlated edges was 29.44% (Fig. 8B). The aggregation of the soil fungal co-occurrence network in the *P. wenshanense* habitat was the smallest, with only 53 nodes and 183 nodes. The proportion of positive correlation edges was 87.43%, and the proportion of negative correlation edges was 12.57% (Fig. 8C). This study revealed that the co-occurrence network of the soil fungi of *P. emersonii* and *P. armeniacum* had a high degree of modularity and a large proportion of positive effects, indicating that the fungal co-occurrence network included modules that resisted changes in the external environment. This symbiotic model may help maintain community structure to resist adverse environmental conditions and contribute to the effective degradation of organic matter.

Relationships between habitat soil fungi and soil nutrients of three *paphiopedilum* species

The correlation between soil nutrients and the effect on fungal alpha diversity was analyzed by the Mantel test. The results showed that (Fig. 9A) there were some significant correlations between the soil nutrient factors. The soil total nitrogen content was significantly positively correlated with organic carbon, alkali-hydrolyzable nitrogen and available phosphorus, and total phosphorus was significantly positively correlated with available phosphorus. Available potassium was significantly negatively correlated with total phosphorus, alkali-hydrolyzable nitrogen and available phosphorus, and the pH was significantly negatively correlated with total potassium. The soil pH significantly affected the Shannon index and Simpson index of the soil fungi. Redundancy analysis was performed with soil nutrients using the fungal groups with the ten most dominant fungal taxa as response variables. The results showed that the first axis (Fig 9B) explained 60.41% of the variance. The second axis explained 20.29% of the variance. A total of 80.70% of the changes in the horizontal direction of the fungal dominant groups were explained, of which total nitrogen, organic carbon, and alkali-hydrolyzed nitrogen were the most important factors. Further Spearman correlation analysis was used to reveal the associations between soil nutrients and dominant fungal groups (Fig. 10). In the *P. emersonii* habitat, total potassium was significantly negatively correlated with Rozellomycota, Mortierellomycota and Ascomycota and significantly positively correlated with Basidiomycota. Alkaline nitrogen was significantly negatively correlated with unclassified fungi and Chytridiomycota. The pH was significantly positively correlated

with unclassified fungi and Chytridiomycota. In the *P. armeniacum*, Rozellomycota, Basidiomycota, and Ascomycota habitats, all nutrient factors except total potassium were significantly correlated. The abundance of Rozellomycota was negatively correlated with available potassium and positively correlated with available potassium. Basidiomycota were positively correlated with available potassium and negatively correlated with the other factors. The abundance of Ascomycota was also negatively correlated with available potassium and positively correlated with the other phyla. There was a negative correlation between unclassified fungi and total potassium. In the *P. wenshanense* habitat, unclassified fungi, Basidiomycota, Ascomycota and Chytridiomycota were significantly correlated with total nitrogen, total phosphorus, organic carbon, alkali-hydrolyzed nitrogen, available phosphorus and available potassium but were not significantly correlated with the remaining factors. The abundance of Mortierellomycota was significantly negatively correlated with organic carbon and available potassium, and the abundance of Glomeromycota was significantly negatively correlated with pH.

Discussion

Ex situ conservation, as an important measure for reducing the risk of orchid extinction, has always played a key role in biodiversity conservation [41,42]. However, the success of ex situ conservation depends on the understanding of the ecological habits of species and the mastery of the ecological and biological characteristics of the living environment of species. In particular, for species such as orchids that are highly dependent on fungal symbiosis, revealing the characteristics of their habitat soil environment is a necessary prerequisite for successful ex situ conservation. Although some studies have been carried out on the habitat of wild orchids, most of these studies have been based on the distribution pattern, habitat preference and habitat evaluation of orchids, ignoring the specific habitat characteristics of orchids [43-46]. In this study, targeted sampling methods were used to study the habitat of wild populations of *Paphiopedilum* species. The habitat characteristics, soil nutrients and soil fungal microbial community structures of three rare and endangered *Paphiopedilum* species were revealed, and the relationships among them were explored. This information will be helpful for the construction of the simulated environment and site selection for field return in future ex situ conservation processes.

The results showed that the three species of *Paphiopedilum* preferred to grow in low-lying areas with high vegetation canopy density and ventilation, and the relative humidity of the habitat was relatively high, which made the *Paphiopedilum* species more resistant to arid climatic conditions; however, this negative terrain may also make it difficult for the population to spread. The soil physical and chemical properties of the three species of *Paphiopedilum* were not significantly different except for the significant differences in available nitrogen and available phosphorus, which may be related to the strong topographic heterogeneity in this area. A survey revealed that the three species of *Paphiopedilum* are distributed in a narrow area in the mountainous areas of Southwest China. Southwest China has both karst and nonkarst landforms, diverse soil-forming bedrock, an interwoven distribution of soil types, and a changeable climate and space-time heterogeneity. [47] The complexity of this environmental background has caused variations in soil nutrients in the habitats of *Paphiopedilum* species, which may

lead to large differences in soil nutrients at different distribution points within the same species [48-50]. The soil fungal community is not only the main factor that directly affects plant growth and distribution but also the key bridge that indirectly affects plant growth and distribution. Its composition and structure are often controlled by environmental conditions[51,52].

In this study, the number of soil-specific OTUs in the *P. emersonii* habitat was greater than that in the other two species of *Paphiopedilum*, indicating that the soil-specific fungi in the *P. emersonii* habitat were more diverse and that the habitat characteristics of this growth area were related. [31] A survey revealed that the habitat of *P. emersonii* was mainly humus soil, which itself contains abundant fungi. However, *P. wenshanense* and *P. armeniacum* are distributed in high-elevation areas of Yunnan (1500-2000 m above sea level), which has a subtropical mountain monsoon climate characterized by long sunshine and low relative humidity, which limits the diversity of soil fungi. The soil composition and structure of the fungal communities of the three *Paphiopedilum* species differed at the phylum and genus levels, and their dominant fungal groups appeared. The differences in composition may be due to two reasons: on the one hand, the mycorrhizal characteristics of different *Paphiopedilum* species can screen out fungal groups that are symbiotic with *Paphiopedilum* species and change the fungal community structure of the habitat soil through alternate, antagonistic and competitive methods during the growth process [53-55]. On the other hand, there are differences in the growth environments of different *Paphiopedilum* species. In particular, the environmental heterogeneity caused by geographical distribution is a direct environmental factor affecting the composition of fungal communities in habitats[56,57]. To adapt to the environment, fungi adopt different nutritional methods, which is a survival strategy adopted by fungi to adapt to different living conditions [58,59]. In this study, saprophytic fungi and ectomycorrhizal fungi were dominant in the soil of the three *Paphiopedilum* species, providing a good material basis and symbiotic fungal resources for plant survival. It is generally believed that the diversity of fungal functional groups in the soil is related to the complexity of the environment [60,61], which may be related to the special habitat preferences of *Paphiopedilum* species. This group of plants loves to environments with high vegetation canopy density, good ventilation and appropriate shading and often grows in rock joints or humus layers near the bases of other woody plants. This habitat itself breeds abundant saprophytic fungi and ectomycorrhizal fungi. It plays an ecological role in plant growth promotion and nutrient cycling [62-65]. Ectomycorrhizal groups may provide the initial impetus for the germination of *Paphiopedilum* seeds and are also the main source of heterotrophic fungi in the early stage of *Paphiopedilum* species. Saprophytic fungi accelerate the circulation of habitat materials, soften the soil texture, and increase water permeability and air permeability [66,67] so that nutritional conditions and habitat characteristics are more conducive to the growth of *Paphiopedilum* species. We also found a small amount of orchid mycorrhizal fungi in the habitat soil. [68] indicated that when the habitat soil fungal community provides the original material for orchid mycorrhizal fungi, mycorrhizal fungi will also have a place in the habitat soil with the growth of *Paphiopedilum*, and this result will also be conducive to the germination of orchid seeds. [69,70].

The alpha diversity index is an important measure of community characteristics in ecology and biological sciences. This study revealed that the habitat soil fungal diversity indices of three

Paphiopedilum species were consistent with the results of detecting the number of fungal-specific OTUs, reflecting the consistency between fungal community diversity and the number of fungal-specific OTUs. Beta diversity emphasizes the change in community structure along a gradient or the direction of variation within a specific gradient range [71,72]. This study also confirmed that there are obvious differences in the habitat soil fungal community structure of the three *Paphiopedilum* species. Although the three *Paphiopedilum* species have similar genetic relationships [15], this may be due to differences in their historical geographical distributions and habitat conditions, which leads to changes in the soil fungal community structure in their habitats and reflects differences in habitat selection among the three *Paphiopedilum* species. A microbial co-occurrence network is a powerful means to reveal the coexistence relationship between microorganisms. This study revealed that the soil fungal habitat of the three *Paphiopedilum* species was dominated by positive effect symbiosis, and the proportion of negative effect symbiosis was very small, which is completely different from the results of biological co-occurrence network analysis of other research objects [73,74]. We speculate that the construction of soil fungal communities in these habitats of the three *Paphiopedilum* species has reached a mature level, and many fungi can coexist harmoniously and play a biological role together [75-77], which is also reflected in the diversity of habitat soil fungal functional groups of the three *Paphiopedilum* species. Of course, we speculate that this positive effect-based microbial coexistence model enables soil microorganisms to better resist the stress of adverse environments to provide nutrition for *Paphiopedilum* species in extreme environments[78]. This speculation needs to be further verified.

The fungal community, which plays an important role in the soil environment, is also affected by soil nutrients [79-82]. This study revealed the effects of habitat-related nutrient changes on the soil fungal community structure of three *Paphiopedilum* species. The Mantel test revealed that the pH had a significant controlling effect on the diversity of the soil fungal communities of the three *Paphiopedilum* species. Soil pH controls diversity by directly affecting the survival, competition, growth and reproductive efficiency of soil fungi [83]. Moreover, the soil pH is also an indirect manifestation of differences in comprehensive environmental conditions [84]. Liu *et al.* confirmed that the soil pH is an important predictor of soil fungal groups in Southwest China [85]. In future ex situ conservation of *Paphiopedilum* species, attention should be given to monitoring the soil pH. This study also revealed the relationships between these dominant fungal groups and soil nutrients, and the compositions of the dominant habitat soil fungal groups of the three *Paphiopedilum* species were complex. There are Rozellomycota, Olpidiomyota, Mortierellomycota, Kickxellomycota, Glomeromycota, Entorrhizomycota, Chytridiomycota, Calcarisporielomycota, Basidiomycota, Ascomycota and other fungal groups. Most of the groups were significantly associated with soil nutrients, which is similar to previous research results[86,87]. That is, fluctuations in soil properties will cause changes in fungal community structure. Moreover, as an important microorganism in soil, fungi play a vital role in the material and energy cycle of soil systems and the improvement of soil structure through the decomposition of organic matter and the release of nutrients. This shows that soil nutrients are also a factor worth considering in future ex situ conservation.

Conclusion

The results of this study showed that the three species of *Paphiopedilum* grew in low-lying, shaded and ventilated places, and their habitat characteristics were highly heterogeneous. There was no significant difference in soil nutrients among the different species, but the soil nutrients at different distribution points of the same species had strong variability. The analysis of the soil fungal community structure among the habitats revealed that the soil fungal community composition significantly differed among the three *Paphiopedilum* species, but in the *P. emersonii* habitat soil, more unique OTUs and fungal species were detected. Similarly, the soil fungal functional groups of the three *Paphiopedilum* species were similar, and they were mainly composed of saprophytic, symbiotic and pathotrophic symbiotic fungi. These included UndefinedSaprotroph, Ectomycorrhizal, Undefined-Biotroph, Soil Saprotroph, Fungal Parasite, Wood Saprotroph, and Plant Saprophytic fungi. There were significant differences in the soil fungal communities among the three *Paphiopedilum* species. The Simpson index and Shannon index of *P. emersonii* were significantly greater than those of the other two species. The results of microbial co-occurrence network analysis showed that the symbiosis of soil fungi in the three *Paphiopedilum* species was mainly positive, and *P. emersonii* had a greater degree of symbiosis network modularity. Nutrients significantly affected the Shannon and Simpson indices of the three *Paphiopedilum* species. Soil nutrients represented a total of 80.70% of the horizontal changes in the dominant soil fungal groups of the three *Paphiopedilum* species. The main groups of soil fungi in each habitat of *Paphiopedilum* species were significantly correlated with soil nutrients, indicating that soil nutrients and soil fungal communities interacted with each other.

Declarations

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Availability of data and materials

Raw amplicon sequence data related to this study were deposited in the NCBI Sequence Read Archive (NCBI SRA) under Bioproject PRJNA888962. Given that the study's subject (*P. armeniacum*, *P. emersonii*, *P. wenshanense*) belongs to rare or endangered species in the IUCN standard. We collected their habitat soil with the permission of the local conservation authority, and there was no damage to the plants.

Ethics approval and consent to participate

We complied with all relevant institutional, national and international guidelines in experimental research and field studies on plants. Material sampling done with permission by the Department of Wildlife Conservation and Nature Reserve Management of the National Forestry and Grassland Administration of China. The identification of the three species was completed by Professor An Mingtai. The habitat soil

voucher specimens were collected in the Biodiversity Conservation and Research Center of Guizhou University, and the voucher specimens numbers were XH-2022,WS-2022, BH-2022.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Authors' contributions

Li Tian wrote the main manuscript text and Feng Liu and Yang Zhang prepared figures 1-3. Mingtai An reviewed the manuscript.

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Figures

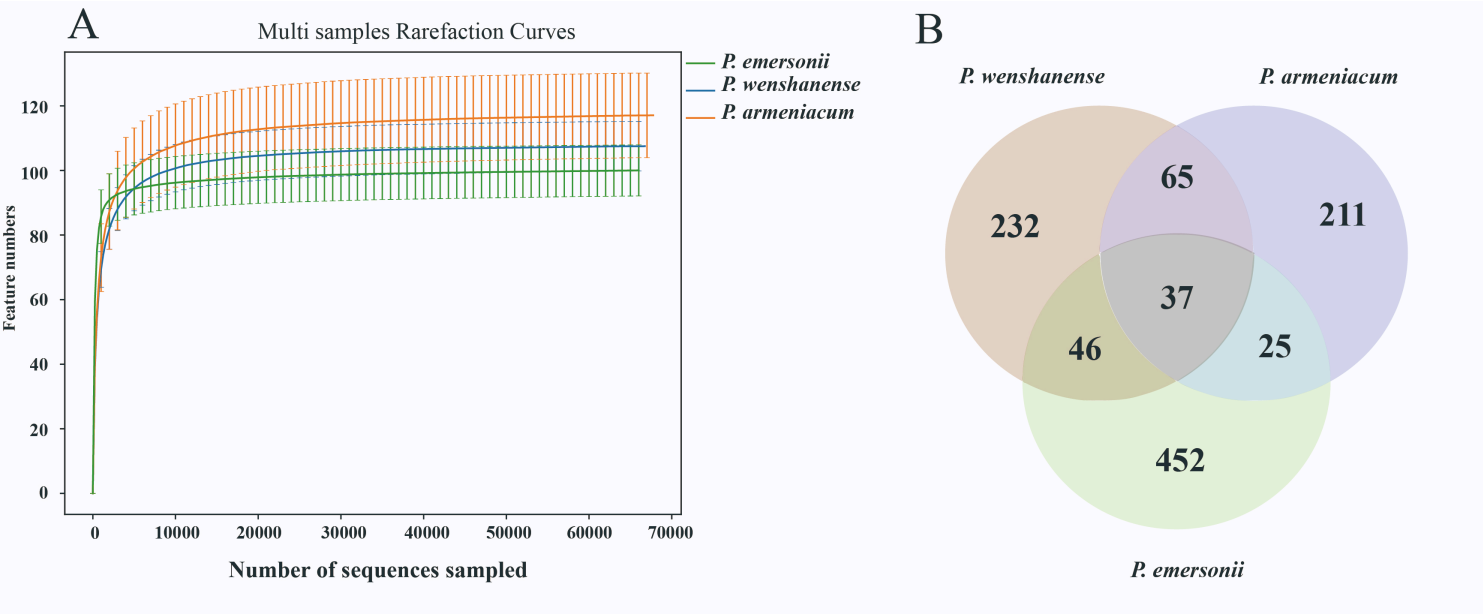


Figure 2

Sequencing results of soil fungi in three *Paphiopedilum* habitats. (A) Dilution curves of soil fungi in three *Paphiopedilum* habitats. (B) Venn diagram showing the number of unique and common OTUs of the

three species.

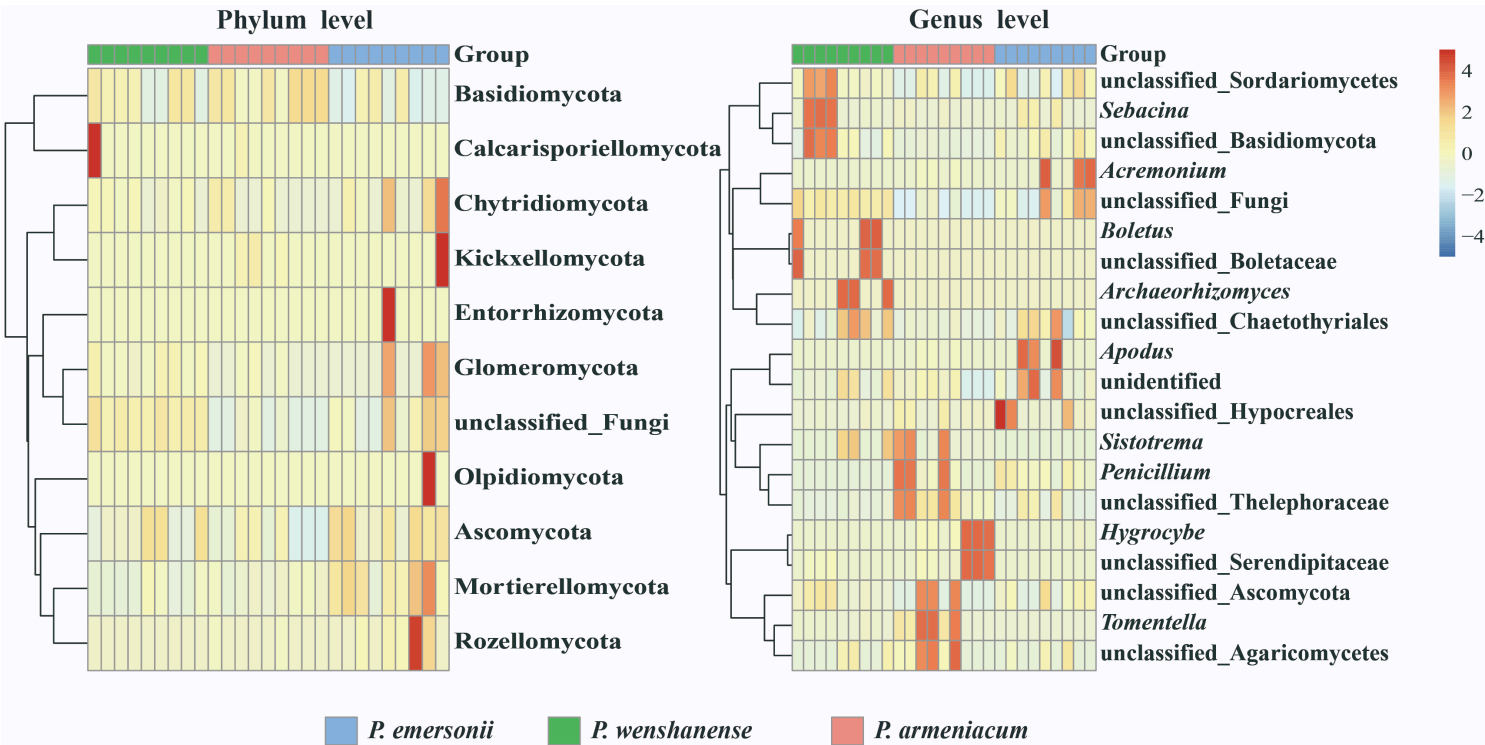


Figure 3

The main species composition of the soil fungal communities of the three *Paphiopedilum* species

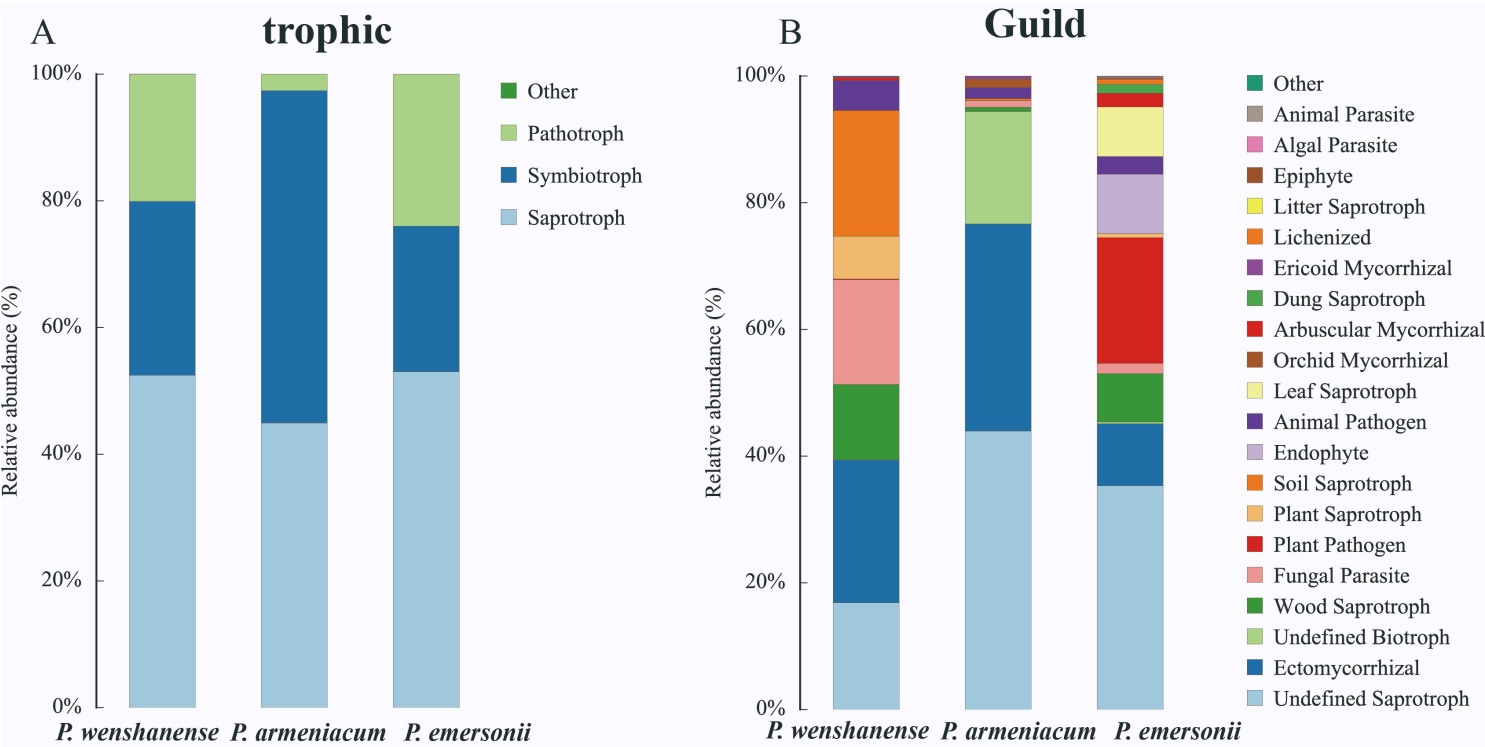


Figure 4

Composition ratios of the soil fungal functional groups of the three *Paphiopedilum* species. (A) Commented on a nutritional basis. (B) Annotation by way of absorbing environmental resources.

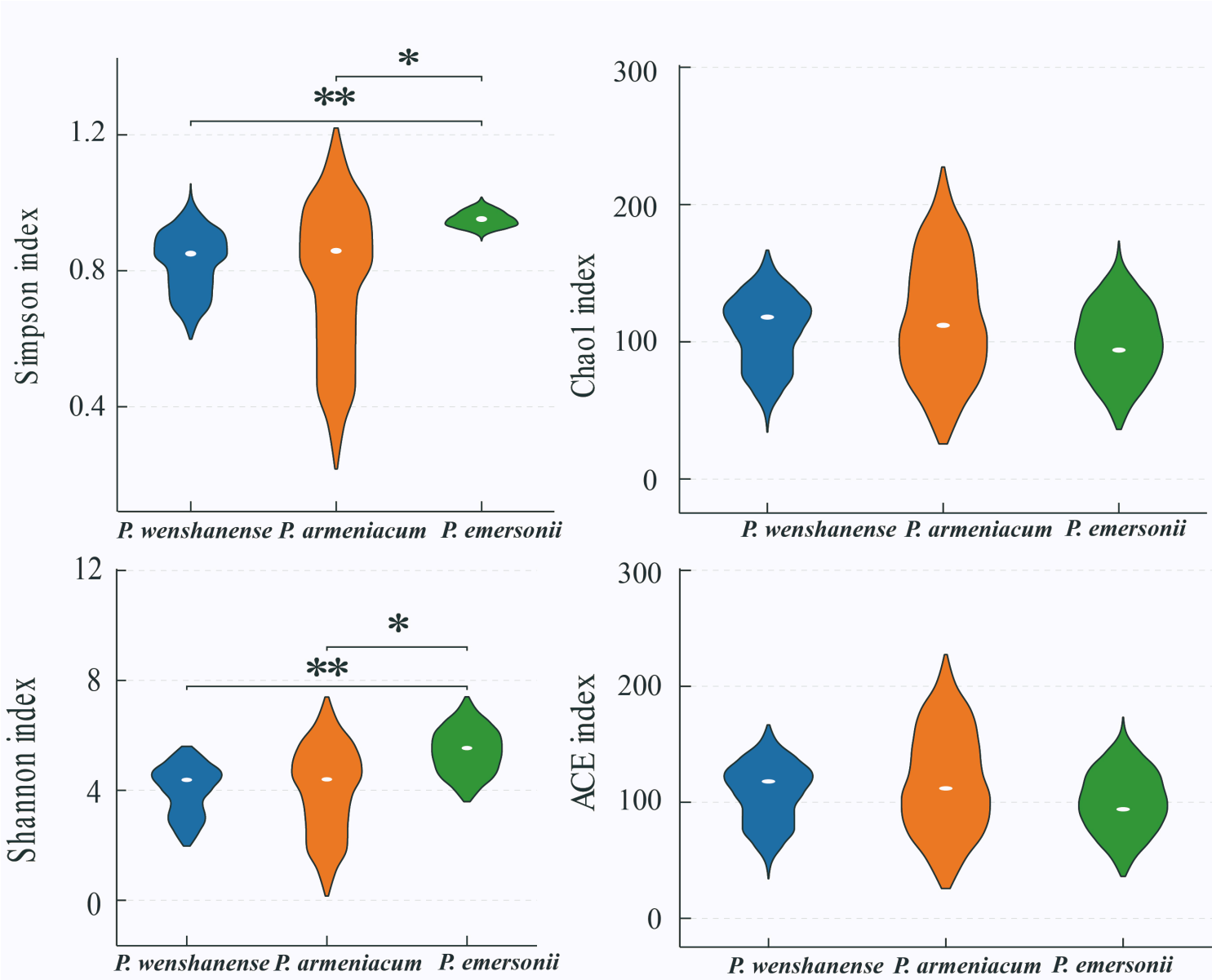


Figure 5

The alpha diversity of fungi in the habitats of three *Paphiopedilum* species. *and* * indicate that there were significant differences between diversity indices (p <0.05).

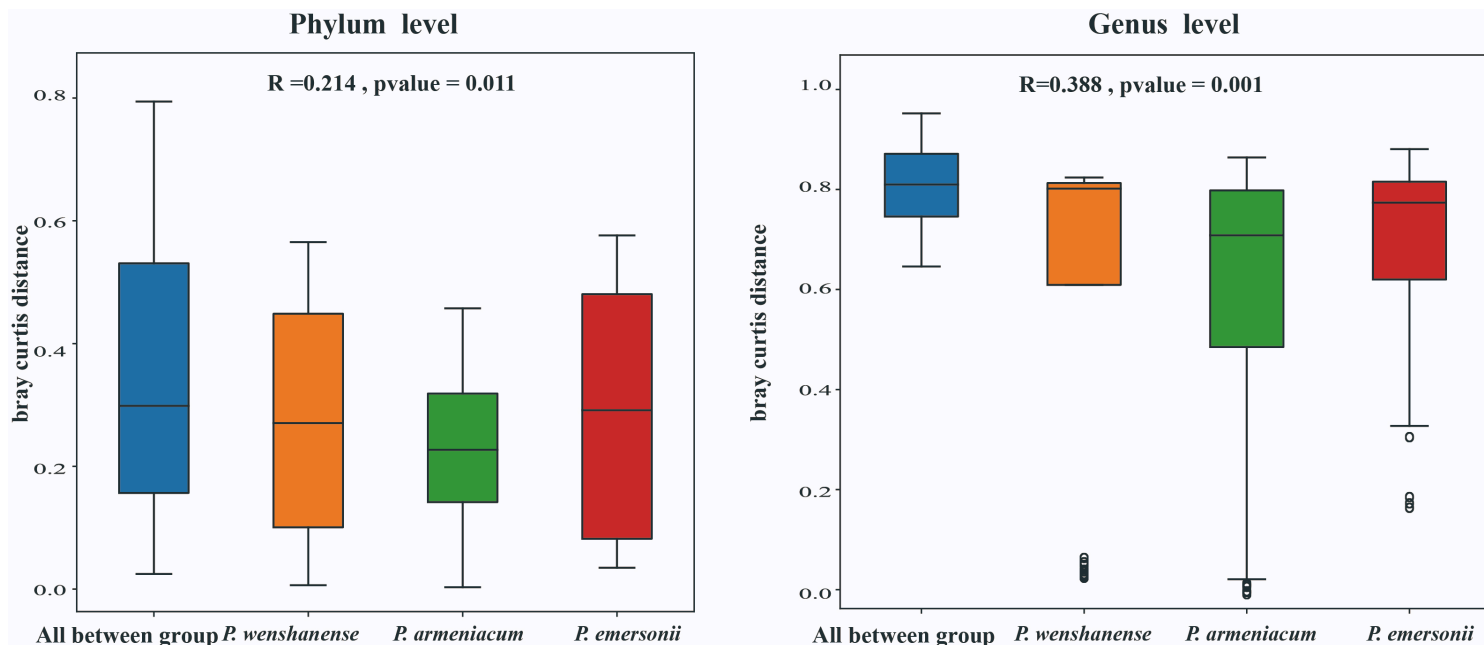


Figure 6

MANOVA analysis at the (a) gate level and (b) genus level

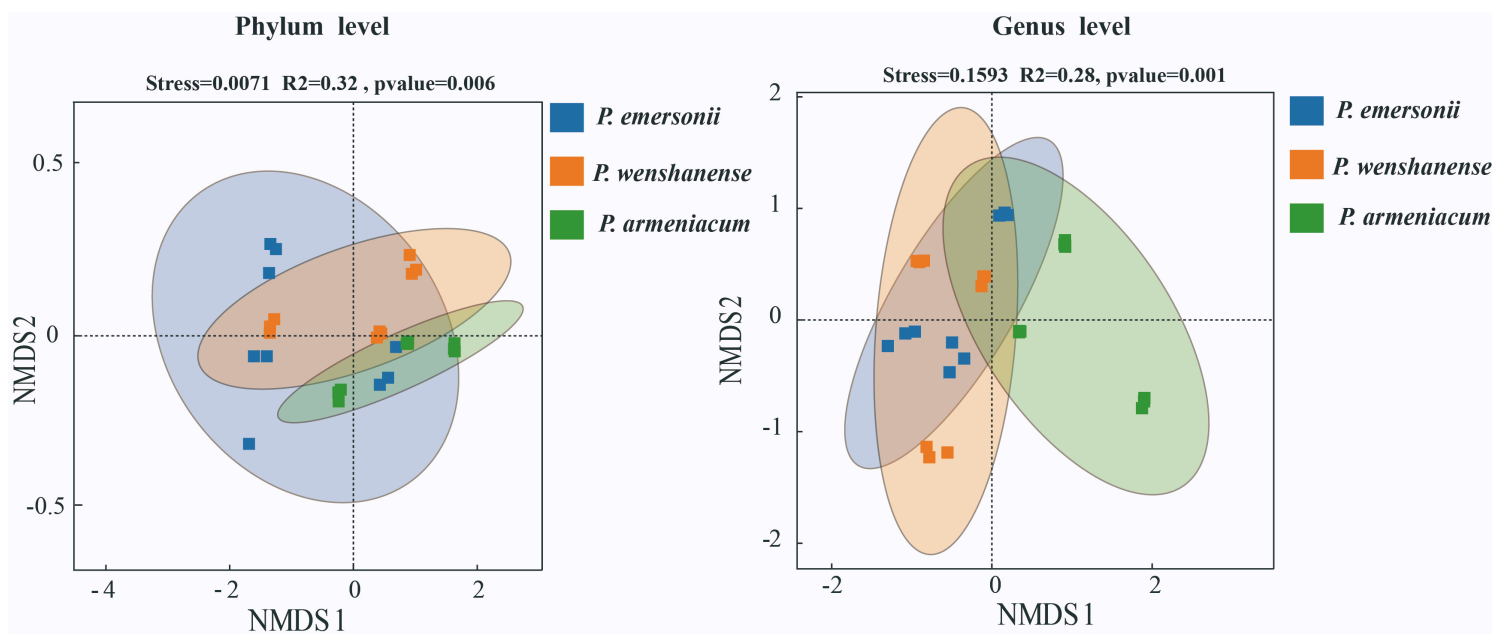


Figure 7

Nonmetric multidimensional scaling analysis (NMDS) at the (a) gate level and (b) genus level. Each point in the figure represents a sample; different colors represent different groups; the elliptical ring represents a 95% confidence ellipse. When the stress is less than 0.1, it can be considered a good sort; when the stress is less than 0.05, it has good representativeness. It is generally believed that when the stress is less than 0.2, NMDS analysis has a certain reliability. The closer the sample is to the coordinate diagram, the greater the similarity.

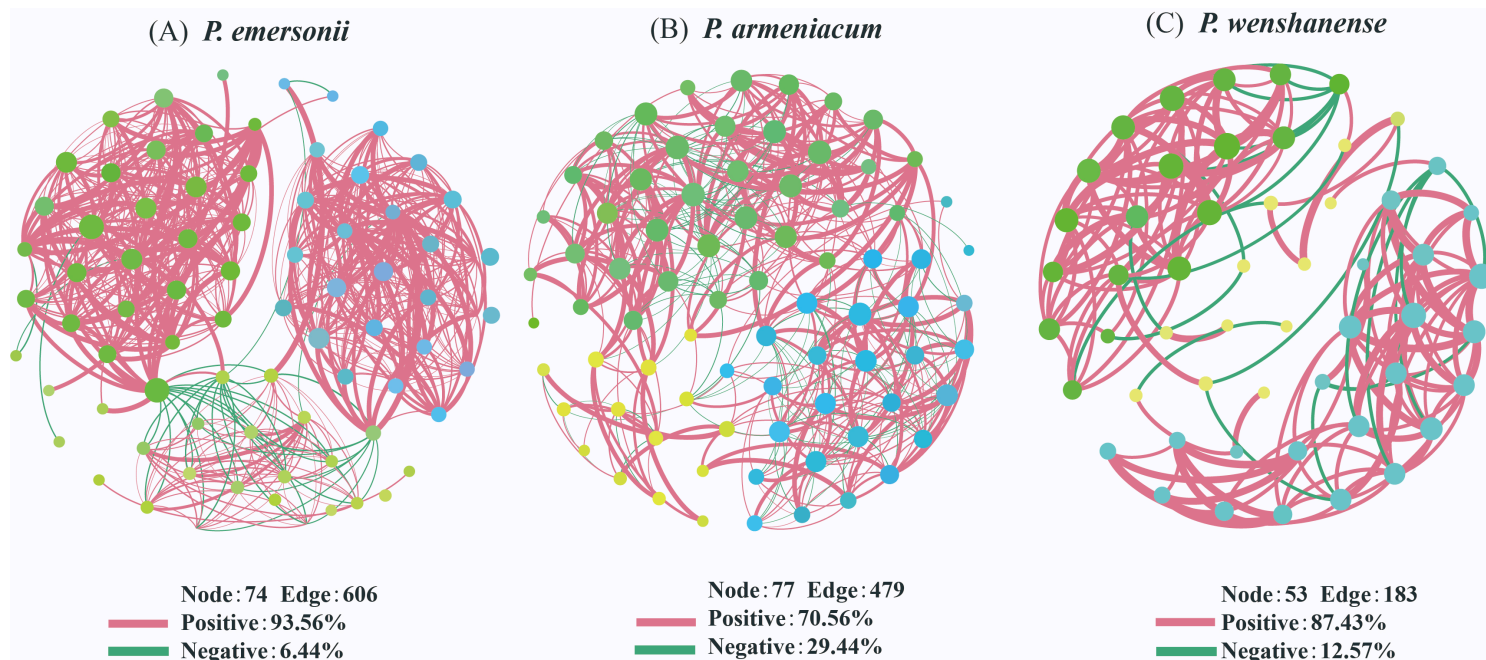


Figure 8

Soil fungal co-occurrence network of three *Paphiopedilum* species in habitats. Each node represents an OTU, and the connection of the nodes represents a strong correlation (Spearman's $r > 0.6$), $p < 0.01$ adjusted by fdr . The edges between the nodes represent a significant correlation between the two OTUs. The red edge indicates a positive correlation, and the green edge indicates a negative correlation.

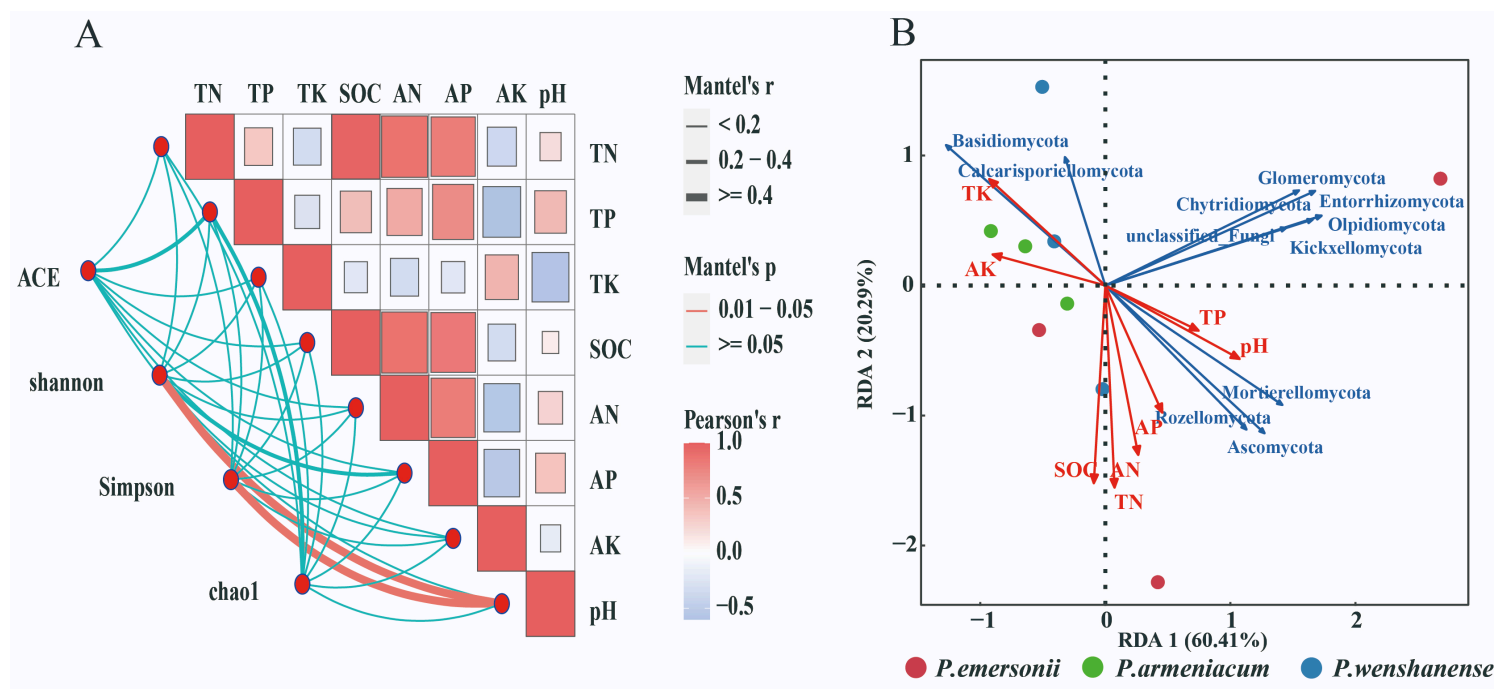


Figure 9

(A) Mantel test of alpha diversity and soil nutrients. (B) Redundancy analysis of the main soil fungal groups and soil nutrients.

Supplementary Files

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