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Characteristics of Rhizosphere Soil Fungal Communities of *Cypripedium macranthos* Sw. at Different Latitudes in Heilongjiang Province

Jiawei Qian [1,†], Shang Dong [1,2,†], Jiale Liu [1], Mengsha Li [3,*] and Liqiang Mu [1,*]

[1] College of Forestry, Northeast Forestry University, Harbin 150040, China; qianjiawei0126@163.com (J.Q.); 18204582945@139.com (S.D.); ljiale1204@163.com (J.L.)
[2] Yichun Branch of Heilongjiang Academy of Forestry, Yichun 153000, China
[3] Institute of Nature and Ecology, Heilongjiang Academy of Science, Harbin 150086, China
* Correspondence: lms19861004@163.com (M.L.); mlq0417@163.com (L.M.); Tel.: +86-139-4608-3125 (M.L.); +86-133-5168-8417 (L.M.)
† These authors contributed equally to this work.

Abstract

In recent years, due to over-excavation and destruction of the living environment, *Cypripedium macranthos* Sw. (commonly known as 'big pocket flower') has been in an endangered state. It is crucial to investigate the rhizosphere soil fungal community characteristics of *C. macranthos* to restore its population. In this study, we collected rhizosphere soils from *C. macranthos* populations along the latitudinal gradient of 44°–49° in Heilongjiang Province, China, and analysed the diversity and composition of *C. macranthos* rhizosphere soil fungal communities using high-throughput sequencing technology to investigate the diversity and community composition of *C. macranthos*. The results showed that 4228 OTUs were obtained by clustering based on a 97% similarity level. Alpha diversity results showed that Shannon diversity indices and Simpson diversity indices decreased with an increasing latitudinal gradient. The principal coordinate analysis (PCoA) results showed that the rhizosphere soil of *C. macranthos* at different locations differed in composition. It is important to reveal the characteristics of rhizosphere soil fungal communities at the molecular level, across varying latitudes, to conserve *C. macranthos*.

Keywords: *Cypripedium macranthos*; rhizosphere soil; high-throughput sequencing; fungal communities; soil property

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Academic Editor: Ipek Kurtboke

Received: 10 July 2025
Revised: 11 August 2025
Accepted: 13 August 2025
Published: 15 August 2025

Citation: Qian, J.; Dong, S.; Liu, J.; Li, M.; Mu, L. Characteristics of Rhizosphere Soil Fungal Communities of *Cypripedium macranthos* Sw. at Different Latitudes in Heilongjiang Province. *Diversity* **2025**, *17*, 577. https://doi.org/10.3390/d17080577

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1. Introduction

The soil surrounding the root system is influenced by root growth, forming a unique zone where plant roots interact with soil microorganisms. The diversity of soil microorganisms associated with plant roots is immense, with approximately tens of thousands of species. The microbial communities between plant roots are highly diverse and have an extremely complex composition, often called the plant's second genome, and they are crucial for plant health. Research indicates that plants can actively shape the structure of their rhizosphere microbial communities. Meanwhile, the dynamics of rhizosphere soil microorganisms profoundly influence the bioavailability, absorption, and transformation processes of soil nutrients. Rhizosphere soil microorganisms inhabit the rhizosphere soil, primarily comprising bacteria and fungi [1–3]. The sustainability of forest ecosystems relies heavily on soil fungal communities to fulfil essential ecological functions [4]. Soil fungal

communities influence functions such as soil nutrient cycling [5], plant immunity enhancement by beneficial fungi [6], and decomposition of dead leaves [7,8]. Meanwhile, fungi are closely related to plant development and can help host plants to absorb nutrients better, improve their resistance, and influence the bacterial community in the soil [9]. Some fungi can also establish a close symbiosis with the root system, which promotes rapid growth and improves the overall resistance of the plant to external stresses [10]. Soil fungi form unique communities and distribution patterns in different ecosystems and play a vital role in forming soil structure and texture, enhancing fertility, and balancing soil microbiota [11].

Orchidaceae is one of the largest families of flowering plants, mostly perennial herbs. *Cypripedium* is an important genus in the subfamily of Orchidaceae. Its flowers are peculiar, colourful, and showy, one of the groups of alpine orchids with the highest ornamental value [12]. *C. macranthos* is a perennial herb with both decorative and medicinal value in the genus *Cypripedium*, family Orchidaceae. The roots and rhizomes are used as medicine, known as *Cypripedium macranthum* Swartz, which can diuretically reduce oedema, dispel wind and activate blood circulation, and have significant curative effects on general oedema and rheumatism pain [13,14]. Currently, research on *C. macranthos* has been carried out in various fields such as population dynamics studies [15], studies on biological characteristics [16,17], genetic diversity [18–20], seed germination [21–23], pollination [24], and chloroplast genome [25]. In recent years, due to commercial over-excavation and the destruction of the living environment, the wild population of *C. macranthos* has been rapidly shrinking, and *C. macranthos* has been in an endangered state; it is now a nationally protected class II plant [26]. *C. macranthos* is also a typical mycorrhizal fungal plant, and its seed germination, plant growth, and development are closely related to mycorrhizal fungi [27,28].

Orchid symbiotic fungi are considered to come directly from the rhizosphere soil and are closely related to fungal species in rhizosphere soil. Currently, research on *C. macranthos* focuses on its endophytic fungal composition, and most scholars have used traditional methods to isolate and cultivate endophytic fungi. And traditional tissue isolation methods have been used to study the endophytic fungal composition of *C. macranthos* roots, stems, and leaves [29,30]. In addition, the fungal composition of rhizosphere soil and non-rhizosphere soils has also been studied using high-throughput sequencing [31]. Fewer analyses have been reported from the perspective of fungal communities' characteristics of rhizosphere soils at different latitudes of *C. macranthos;* the traditional method of isolating and purifying fungi from culture media could not comprehensively reflect the fungal communities' composition. To address the above issues, we utilised high-throughput sequencing technology to study fungal composition in the rhizosphere soil of *C. macranthos* at different latitudes, providing a theoretical basis for restoring wild populations of *C. macranthos* and the sustainable use of its resources. In this study, we used high-throughput sequencing technology to characterise the rhizosphere soil fungal communities of *C. macranthos* at different latitudes in Heilongjiang Province, providing a theoretical basis for future isolation and screening of symbiotic mycorrhizal fungi of *C. macranthos* and carrying out conservation studies of *C. macranthos.* In particular, we aimed to (1) assess whether there are differences in the diversity of rhizosphere soil fungal communities of *C. macranthos* at different latitudes; (2) assess whether there are differences in the physicochemical properties of rhizosphere soils of *C. macranthos* at different latitudes; and (3) analyse the correlation between the physicochemical properties of soils and rhizosphere soil fungal communities of *C. macranthos,* and probe the key drivers for the composition of the rhizosphere soil fungal communities of *C. macranthos*.

2. Materials and Methods

2.1. Study Area and Sampling Locations

The study area is in Heilongjiang Province, the northernmost province of China. The geographical extent of Heilongjiang Province is 121°11′∼135°05′ E, 43°26′∼53°33′ N, with a forest cover of 47.3%, a forested land area of 26.17 million hm^2, of which 21.5 million hm^2 is forested, and a forest reserve of 22.4 billion m^3, which is rich in natural resources [32]. Heilongjiang Province has a cold-temperate and temperate continental monsoon climate, with long, cold winters, warm, humid summers, and various landform types. The average annual precipitation is 400–700 mm, the total yearly water resources amount to 77.58 billion m^3, and the annual water production modulus is 166,000 m^3. The average yearly temperature ranges from −4.00 to 7.93 °C. The main landform types are mountains, hills, and plains. The terrain is generally high in the north–south and low in the east–west direction, with the Daxing'anling Mountain Range in the northwest; the Xiaoxing'anling Mountain Range, Zhangguangcailing, Laoyaoling, Taipingling, and Wundashan in the central and southeastern parts of the region, respectively; the Three Rivers Plain in the northeast; and the northern half of the Songnen Plain in the southwest. The vegetation in the Daxing'anling region is primarily coniferous forest. The Songnen Plain and the Sanjiang Alluvial Low Plain areas are covered by grasslands and meadows, swamps, cultivated vegetation, and mixed coniferous and broad-leaved forests. Broad-leaved forests and thickets are primarily found in the areas of the Xiaoxing'anling Mountains, Zhangguangcailing Mountains, and Laoyaoling Mountains [33–35]. As shown in Figure 1, the sampling sites for this study were Heilongjiang Shengshan National Nature Reserve, Heilongjiang Langxiang National Nature Reserve, and Heilongjiang Xiaobeihu National Nature Reserve.

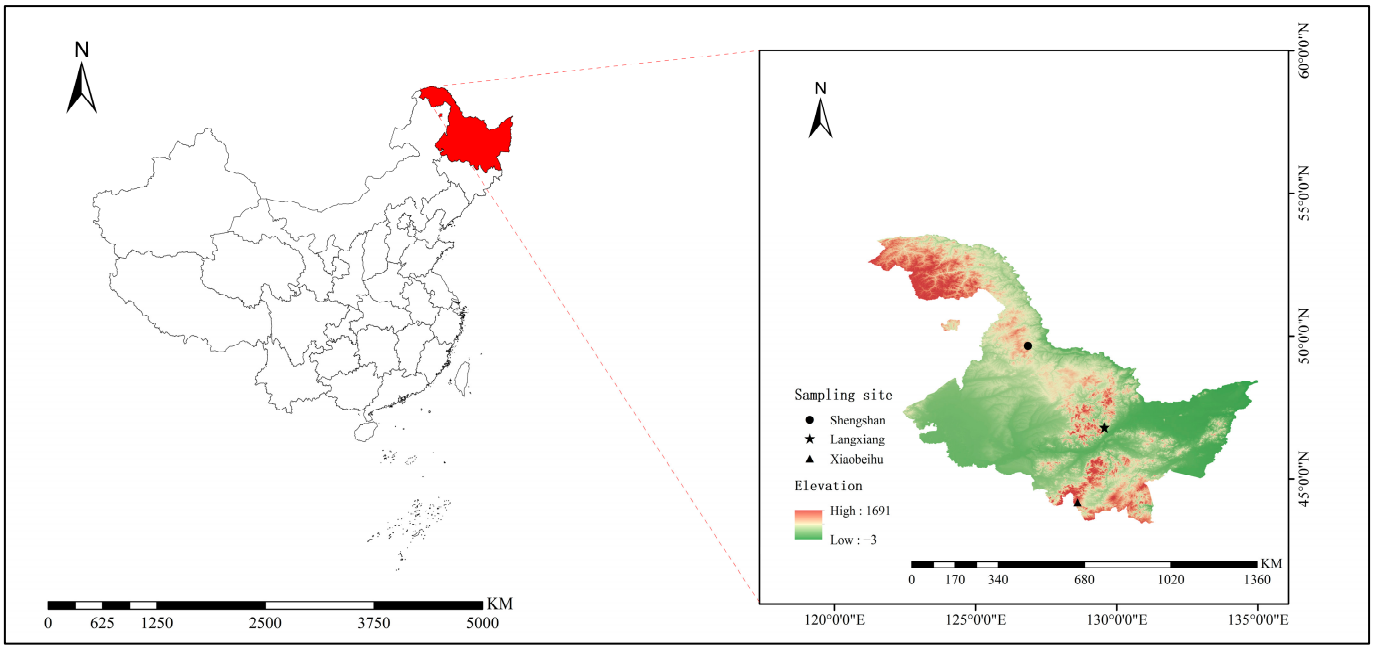


Figure 1. Map of China and the study area and sampling sites for this study.

2.2. Sample Collection

Samples were collected between May and June 2024 in the Shengshan National Nature Reserve, Langxiang National Nature Reserve, and Xiaobei Lake National Nature Reserve in Heilongjiang Province. Six populations were randomly selected at each site, and three plants were collected from each population to obtain the soil attached to their roots. The three root soil samples collected from each population were combined into one sample. To protect *C. macranthos*, this study considered soil from the 0–1 cm surface layer and the

5–15 cm soil layer around the orchid's root system as rhizosphere soil. After collecting the soil, the plants were buried in their original soil for protection. The rhizosphere soil samples of Heilongjiang Shengshan National Nature Reserve were numbered as A_1–6, those of Heilongjiang Langxiang National Nature Reserve were numbered as B_1–6, and those of Heilongjiang Xiaobeihu National Nature Reserve were numbered as C_1–6. All the samples were put into a sterile bag, placed in a thermostatic bag with ice packs, brought back to the laboratory, and stored in an ultra-low-temperature refrigerator (−80 °C) for preservation until later use.

2.3. Analysis of Soil Chemical Properties

In this study, the following methods were used to determine the physicochemical properties of the soil between the roots of *C. macranthos*: soil moisture content was determined by the drying method, soil pH was determined by the potentiometric method, soil carbon and nitrogen content were determined by the Dumas combustion method, soil ammonium nitrogen and nitrate nitrogen content were determined by a continuous analyser, soil phosphorus and available phosphorus content were determined by the colorimetric method, soil potassium content was determined by the sodium hydroxide fusion method, and soil fast-acting potassium content was determined by the ammonium acetate leaching and flame photometer method.

2.4. Soil DNA Extraction, Amplicon Generation, and Sequencing

Microbial DNA was extracted from the rhizosphere soil of *C. macranthos* samples using the E.Z.N.A.® Soil (DNA Kit (Omega Bio-tek, Norcross, GA, USA)) according to the manufacturer's protocols. The fungal ITS gene was amplified by PCR (95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min) using primers ITS1F 5′-barcode-CTTGGTCATTTAGAGGAAGTAA-3′ and ITS2R 5′-GCTGCGTTCTTCATCGATGC-3′, where barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate with a 20 μL mixture containing 4 μL of 5× FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions. Purified PCR products were quantified by Qubit® 3.0 (Life Technologies, Carlsbad, CA, USA), and every twenty-four amplicons with different barcodes were mixed equally. The DNA was fragmented using an ultrasonic instrument. A DNA library was obtained by unwinding the DNA duplex product from PCR amplification and disrupting the uncircularised DNA molecules. The libraries were finally sequenced on an Illumina MGI-G99 platform (Shanghai BIOZERON Biotech. Co., Ltd., Shanghai, China) with the PE300 mode according to the standard protocols.

2.5. Sequencing Data Processing

High-throughput sequencing of the ITS region of the rhizosphere soil of *C. macranthos* was carried out by Shanghai Ling'en Biotechnology Co., Ltd. (Shanghai, China), to analyse the structure of the fungal communities. All bioinformatics analyses were performed using a combination of UPARSE (version 10) and R software (version 4.4.3), relying on the Synergis cloud platform (http://www.cloud.bioicroclass.com/CloudPlatform, accessed on 23 March 2025) for efficient data processing and analysis. Fastp (0.23.1) [36] software was used for quality control of raw sequencing sequences, and FLASH (v1.2.7) [37] software was used for splicing of raw sequencing data to remove low-quality reads, junctions, and chimeric sequences through a strict quality control process, and ultimately to obtain high-quality pure reads suitable for downstream analysis. Operational taxonomic unit (OTU)

clustering was performed using UPARSE software [38], and OTU clustering of sequences was performed based on 97% similarity. α-Diversity indices (including Chao1, Shannon, Simpson, and Good-coverage) were performed by IBM SPSS Statistics (21.0.0.0) software for data analysis. β-Diversity analysis was performed using the R language vegan package (version 2.6-10) to assess differences in community structure between samples. Principal coordinate analysis (PCoA) based on the Bray–Curtis distance matrix was used to visualise patterns of sample clustering. The Adonis function was used to test for the significance of community differences between treatments. To explore the correlation between microbial community structure and environmental factors, one-way ANOVA was performed using IBM SPSS Statistics (21.0.0.0) and presented by heatmap plotting with Origin2024. The overlap and uniqueness of OTUs between treatments were resolved by constructing a Wayne's diagram with the Venn Diagram plug-in.

2.6. Statistical Analysis

Experimental data entry, preliminary collation, and basic calculations were performed using Microsoft Office Excel 2019 software. The data were grouped and organised according to the research design to ensure that the data structure met the requirements for subsequent statistical analysis. Follow-up statistical analyses were performed using IBM SPSS Statistics (21.0.0.0) software, which was used to assess the significant differences between different treatment groups and explore the correlations between variables.

Data were analysed using IBM SPSS Statistics (21.0.0.0) software. We used one-way ANOVA (one-way ANOVA) for the overall comparison of means between groups, with the significance level of the ANOVA pre-set at $p < 0.05$. The normality of the data was assessed using the Shapiro-Wilk test. For data that deviated slightly from normality, we relied primarily on the results of the ANOVA, given its robustness. If the data deviated significantly from normal distribution or if the assumption of chi-squaredness of variance was violated considerably (Levene's test $p < 0.05$), the non-parametric Kruskal–Wallis H-test was considered an alternative or supplement. When the results of one-way ANOVA (or Kruskal–Wallis H-test) showed an overall significant difference ($p < 0.05$), Dunn's multiple comparisons test was selected for two-way comparisons between the groups. This method can accurately locate specific inter-group differences and effectively control the overall error rate.

3. Results

3.1. Quality Analysis of Rhizosphere Soil Fungi Sequencing of C. macranthos

Through high-throughput sequencing of 18 root zone soil samples from *C. macranthos*, 655,861 high-quality sequences with a length of approximately 230–270 bp were obtained. Subsequently, these high-quality sequences were clustered into OTUs based on a 97% similarity level, and after smoothing, 4228 OTUs were ultimately retained. As shown in Figure 2, the total number of OTUs across all samples was 68, accounting for less than 1.61% of the total OTU count, indicating that the fungal communities of *C. macranthos* exhibited a certain degree of environmental specificity across different study areas. The sample from Yichun (B_4) had the highest number of OTUs at 1121, while the sample from Shengshan (A_5) had the lowest number of OTUs at 535. At the group level, Yichun (B) had the highest number of OTUs at 3007, while Shengshan (A) had the lowest number of OTUs at 2674.

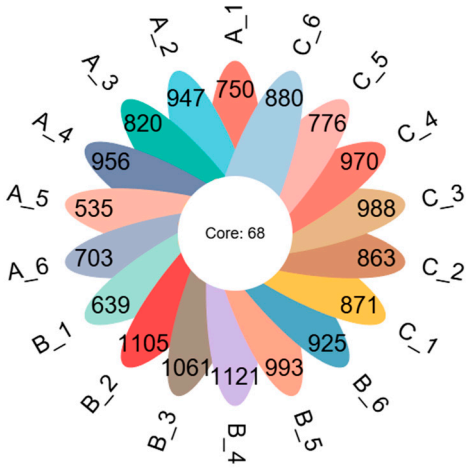


Figure 2. Petal diagram of the number of fungal OTUs contained in different samples.

As shown in the fungal dilution curve (Figure 3), when the sequencing volume reached 25,000 sequences, the growth rate of OTU numbers in each sample gradually decreased, although there was still a slight increase in OTU numbers. Combined with the Shannon–Wiener curve analysis, when the sequencing volume increased to 20,000 sequences, the Shannon–Wiener index curve tended to level off and no longer changed significantly with an increase in sequencing volume. This result indicates that the current sequencing depth of fungal ITS is sufficient to comprehensively reflect each sample's species composition and taxonomic information.

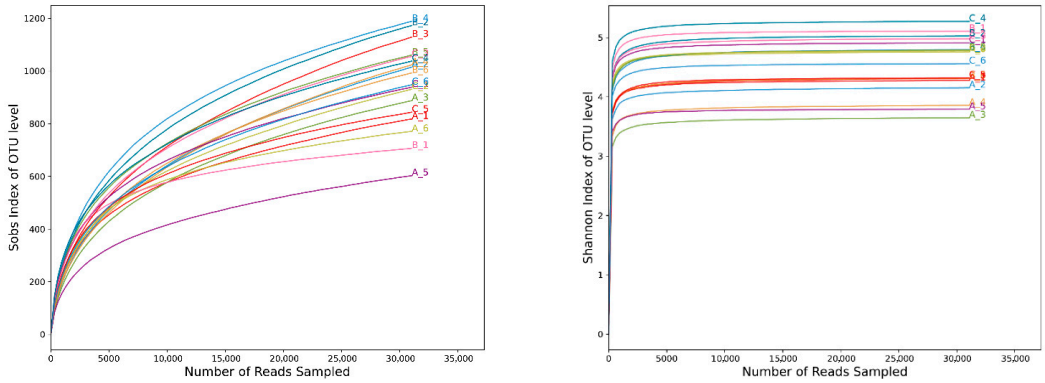


Figure 3. Dilution curve of rhizosphere soil samples and Shannon–Wiener curve of rhizosphere soil samples. Note: A1_6: Shengshan; B1_6: Langxiang; C1_6: Xiaobeihu.

3.2. Rhizosphere Soil Fungal Community Diversity of C. macranthos at Different Latitudes

Alpha-diversity indices of rhizosphere soil bacteria at different latitudes of *C. macranthos* are shown in Table 1. The results showed that the Chao1 index of the three sites ranged from 1215.90 to 1439.27, with the largest Chao1 index being B and the smallest A. However, there was no significant difference between the three different latitudes. The Shannon diversity index ranged from 4.08 to 4.80, with the largest Shannon diversity index being C and the smallest being A. There was a significant difference between A and B, and between C and A at the three different latitudes. There were substantial differences between A, B, and C, and no significant differences between B and C. The Simpson diversity index ranges from 0.92 to 0.97, where the largest Simpson diversity index is C and the smallest is A. There is a significant difference between A and C among the three different latitudes. In addition, the coverage reached 99% in all groups of samples, indicating that the probability of the species in the samples being measured was high and the likelihood of not being measured was low, which proved that the results of this sequencing could represent the real

situation of the microorganisms in the samples. In conclusion, a significant size relationship existed between the rhizosphere soil fungal diversity indices of *C. macranthos* at different latitudes, which indicated that there was spatial differentiation in soil fungal diversity.

Table 1. Fungal communities in rhizosphere soil samples of *C. macranthos* at different latitudes: alpha diversity.

Sampling Site	Chao1	Shannon	Simpson	goods_coverage
A	1215.90 ± 251.45 ^{a}	4.08 ± 0.40 ^{b}	0.92 ± 0.04 ^{b}	0.99
B	1439.27 ± 312.24 ^{a}	4.72 ± 0.34 ^{a}	0.96 ± 0.02 ^{ab}	0.99
C	1367.88 ± 124.99 ^{a}	4.80 ± 0.33 ^{a}	0.97 ± 0.03 ^{a}	0.99

Note: The higher the Chao1 in the alpha-diversity index, the greater the species richness; the higher the Shannon index, the higher the species diversity; the smaller the Simpson index, the higher the species diversity of the community. The numbers after "±" in the table are standard deviations; different letters in the same column indicate that the corresponding values are significantly different ($p < 0.05$).

This study analyses the alpha diversity of soil fungi in the rhizosphere of *C. macranthos* at different latitudinal gradients in Heilongjiang Province. The results showed that both the Shannon index and the Simpson index showed a decreasing trend with increasing latitude (from 44°–49°) (Figure 4). This clear negative correlation strongly supports the classical Latitudinal Diversity Gradient (LDG) theory [39], suggesting that the construction of rhizosphere soil fungal communities in *C. macranthos* follows the same broad eco-geographical pattern of decreasing biodiversity from low to high latitudes. This pattern may be mainly attributed to the systematic changes in environmental factors that occur with increasing latitude, and the resulting changes in the soil microenvironment that may be triggered. These changes may enhance environmental filtering effects at higher latitudes, limiting the abundance of suitable ecological niches or increasing survival pressures, leading to a decline in the diversity of rhizosphere soil fungal communities. The research results highlight that latitude may be a geographical driving factor for the symbiotic fungal communities of *C. macranthos* and suggest that root zone soil fungal communities in high-latitude regions may have relatively weak buffering capacity due to lower diversity. This is of great significance for understanding the adaptability of rare plants such as large trees and the stability of root zone soil mutualistic systems in the context of climate change. However, due to logistical constraints, sampling was limited to three representative latitudes (44°–49° N); therefore, the observed linear trends may not fully reflect patterns across the entire latitudinal gradient. Future studies with expanded sampling ranges are needed to validate broader patterns.

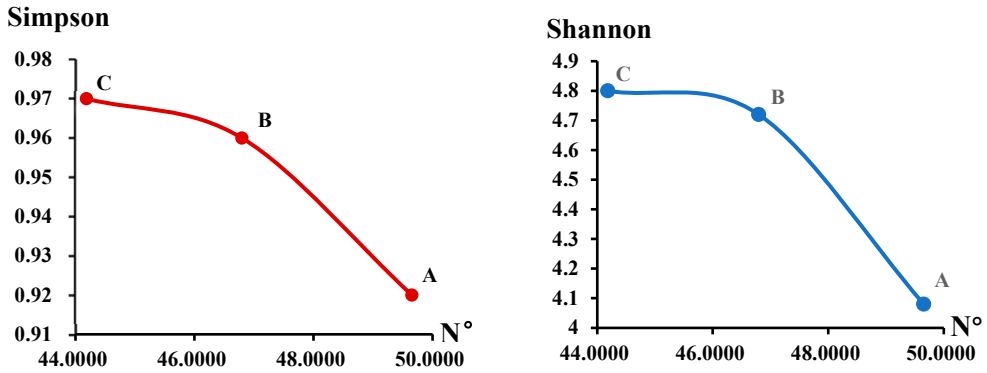


Figure 4. Alpha diversity of rhizosphere soil fungal communities of *C. macranthos* at different latitudes as a function of the latitudinal gradient. Note: A: Shengshan, 49° N; B: Langxiang, 46° N; C: Xiaobeihu, 44° N.

3.3. Community Composition of Rhizosphere Soil Fungi of C. macranthos

OTUs generated based on 97% similarity clustering were annotated at the phylum, class, order, family, genus, and species levels using the Unite database for the fungal community in the rhizosphere soil of the large-flowered orchid. The classification statistics of the annotation results are summarised in Table 2. In the combined analysis of all samples across the entire study area, a total of 8 fungal phyla, 48 classes, 152 orders, 376 families, 1008 genera, and 2039 species were identified, indicating that the rhizosphere soil fungal community exhibits high taxonomic diversity, with particularly notable richness at the genus and species levels. There were differences in the richness of taxonomic units among different sites: the phylum level was the most stable (all sites detected 8 phyla); at the class, order, family, genus, and species levels, Site B had the highest richness (35 classes, 101 orders, 225 families, 465 genera, and 751 species). The average richness across all sites was approximately 8 phyla, 34 classes, 103 orders, 215 families, 425 genera, and 676 species.

Table 2. Statistics of soil fungi in the rhizosphere soil zone of *C. macranthos* at different latitudes.

Taxonomy	Number of Microbial Species in Each Sampling Site			Total
	A	B	C	
Phylum	8	8	8	8
Class	34	35	34	48
Order	96	101	111	152
Family	198	225	223	376
Genus	395	465	415	1008
Species	625	751	652	2039

Note: A: Shengshan; B: Langxiang; C: Xiaobeihu.

Analysis of Rhizosphere Soil Fungal Composition of *C. macranthos* at Different Taxonomic Levels

At the phylum level, eight fungal phyla were detected in the rhizosphere soil samples of *C. macranthos* (Figure 5) by sequencing Ascomycota, Basidiomycota, Chytridiomycota, Cryptomycota, Microsporidia, Mucoromycota, Olpidiomycota, and Zoopagomycota. Among them, Ascomycota, Basidiomycota, and Mucoromycota accounted for more than 90% of the species OTU abundance in all three sites, making them the dominant phyla. At the family level, a total of 376 fungal families were detected in the rhizosphere soil samples of *C. macranthos*, among which the top 10 dominant families were Mortierellaceae, Sebacinaceae, Cortinariaceae, Russulaceae, Nectriaceae, Pyronemataceae, Thelephoraceae, Archaeorhizomycetaceae, Hydnaceae, and Unclassified. At the genus level, by sequencing in the rhizosphere soil samples of *C. macranthos*, 1008 fungal genera were detected, among which the top 10 dominant genera were *Sebacina*, *Cortinarius*, *Mortierella*, *Russula*, *Linnemannia*, *Tomentella*, *Hypotarzetta*, *Fusarium*, *Archaeorhizomyces*, and *Clavulina*. Among them, Sebacinaceae, Nectriaceae, and *Sebacina* were similar in percentage at different latitudes, and according to a previous study, Sebacinaceae, Nectriaceae, and *Sebacina* were presumed to be the specific fungi for this species. The differences in the other fungi are presumed to be associated with the different latitudinal environments.

3.4. Differences in Rhizosphere Soil Community Structure of C. macranthos at Different Latitudes

To analyse the overall differences (β diversity) in the structure of soil fungal communities in the rhizosphere soil of *C. macranthos* at different latitudes, principal coordinate analysis (PCoA) was conducted based on the Bray–Curtis distance matrix (Figure 6). As shown in the figure, the rhizosphere soil fungal community samples of *C. macranthos* from different latitudinal sampling sites formed a significant spatial separation in two dimensions, each clustering into independent clusters. This intuitive spatial distribution

pattern indicates that the latitudinal gradient is the driving factor for the composition of the fungal community in the rhizosphere soil of *C. macranthos*. Principal coordinate axis 1 (PCoA1) explained 13.83% of the inter-sample variability in fungal community composition, and principal coordinate axis 2 (PCoA2) explained 10.98% of the inter-sample variability in fungal community composition, which together contributed 24.81% of the total variation. To quantify the statistical contribution and significance of the latitude factor to the observed differences in fungal community structure, we performed an Adonis analysis (PERMANOVA). The results of the Adonis test (labelled in Figure 6: $R^2 = 0.225$, Pr = 0.001) showed that the amount of explanation (R^2) was 0.225, indicating that latitudinal grouping significantly explained the differences in fungal community composition between samples. All differences in fungal communities between samples were highly significant($p < 0.01$).

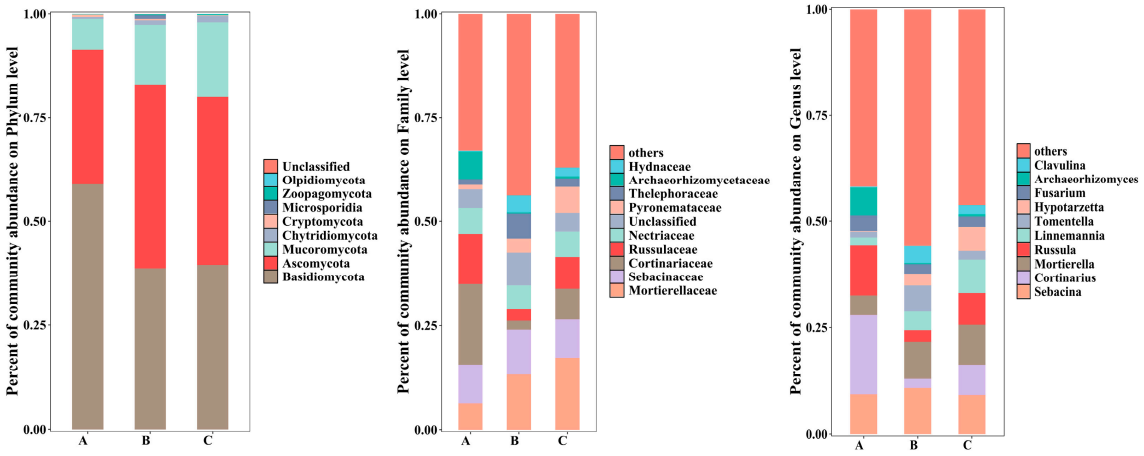


Figure 5. Soil fungal community composition of rhizosphere soils of *C. macranthos* at different latitudes at the phylum, family, and genus taxonomic levels. Note: A: Shengshan; B: Langxiang; C: Xiaobeihu.

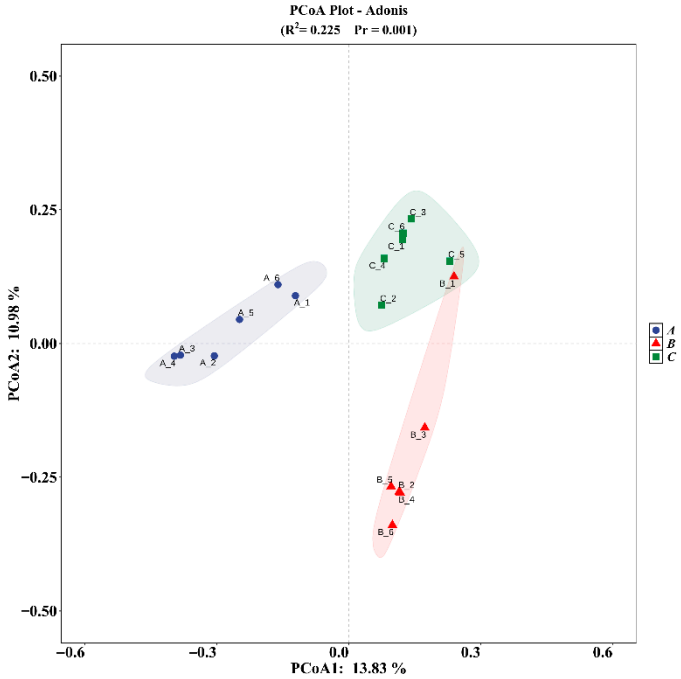


Figure 6. PCoA analysis of rhizosphere soil fungi of *C. macranthos* at different latitudes. Note: A: Shengshan; B: Langxiang; C: Xiaobeihu.

3.5. Soil Properties and Their Effects on the Rhizosphere Soil Fungal Communities of C. macranthos

3.5.1. Soil Properties

To investigate whether there were differences in the rhizosphere soil properties of *C. macranthos* at different latitudes, we analysed the experimental data on soil physicochemical properties at three latitudes using one-way analysis of variance (ANOVA) combined with Duncan's multiple comparison test. The results showed that soil pH (PH), total nitrogen (TN), total phosphorus (TP), total potassium (TK), ammonium nitrogen (AN), nitrate nitrogen (NN), available phosphorus (AP), and quick-acting potassium (AK) ranged from 6.31 to 6.84, 0.81 to 1.11 g/kg, 1.41 to 1.73 g/kg, 24.14 to 28.85 g/kg, 6.27 to 18.08 mg/kg, 0.34 to 0.88 mg/kg, 12.38 to 15.92 mg/kg, and 43.71 to 118.16 mg/kg (Table 3). TN, TP, and AP showed no statistically significant difference among them. Meanwhile, pH, TK, AN, NN, and AK were the most important factors in terms of spatial variability. This indicates significant differences ($p < 0.05$) and spatial heterogeneity in soil chemical properties at different latitudes.

Table 3. Rhizosphere soil properties of *C. macranthos* at different latitudes.

Location	pH	Total Nitrogen TN (g/kg)	Total Phosphorus TP (g/kg)	Total Potassium TK (g/kg)	Ammonium Nitrogen AN (mg/kg)	Nitrate Nitrogen NN (mg/kg)	Available Phosphorus AP (mg/kg)	Quick-Acting Potassium AK (mg/kg)
A	6.3 ± 0.3^{b}	0.9 ± 0.3^{a}	1.5 ± 0.5^{a}	27.2 ± 1.6^{a}	18.1 ± 4.7^{a}	0.3 ± 0.2^{c}	15.9 ± 6.2^{a}	118.2 ± 25.2^{a}
B	6.8 ± 0.4^{a}	1.1 ± 0.4^{a}	1.7 ± 0.7^{a}	24.1 ± 1.2^{b}	9.2 ± 2.2^{b}	0.9 ± 0.2^{a}	12.4 ± 8.2^{a}	43.7 ± 19.1^{b}
C	6.5 ± 0.1^{ab}	0.8 ± 0.1^{a}	1.4 ± 0.4^{a}	28.9 ± 1.8^{a}	6.3 ± 2.2^{b}	0.6 ± 0.1^{b}	13.6 ± 5.2^{a}	99.1 ± 40.1^{a}

Note: Numbers after "±" are standard deviations; different letters in the same column indicate significant differences ($p < 0.05$) in the corresponding values.

3.5.2. Correlation Analysis of Soil Properties and Rhizosphere Soil Fungal Communities of *C. macranthos*

This study conducted Spearman correlation analysis to investigate the environmental factors influencing the variation in the fungal community structure (family level) of the rhizosphere soil of *C. macranthos*, as well as the response patterns of specific fungal groups to changes in the soil environment. The results showed the following (Figure 7): Mortierellaceae was significantly positively correlated with NN and extremely significantly negatively correlated with AN; Cortinariaceae was extremely considerably negatively correlated with pH and NN, and significantly positively correlated with AN and AK; Russulaceae was extremely considerably negatively correlated with pH and TN, significantly negatively correlated with NN, and significantly positively correlated with TK; Pyronemataceae was significantly negatively correlated with AN and significantly positively correlated with NN; Thelephoraceae showed a significant negative correlation with TN, while Archaeorhizomycetaceae showed a significant negative correlation with TP and a significant positive correlation with AN and AK.

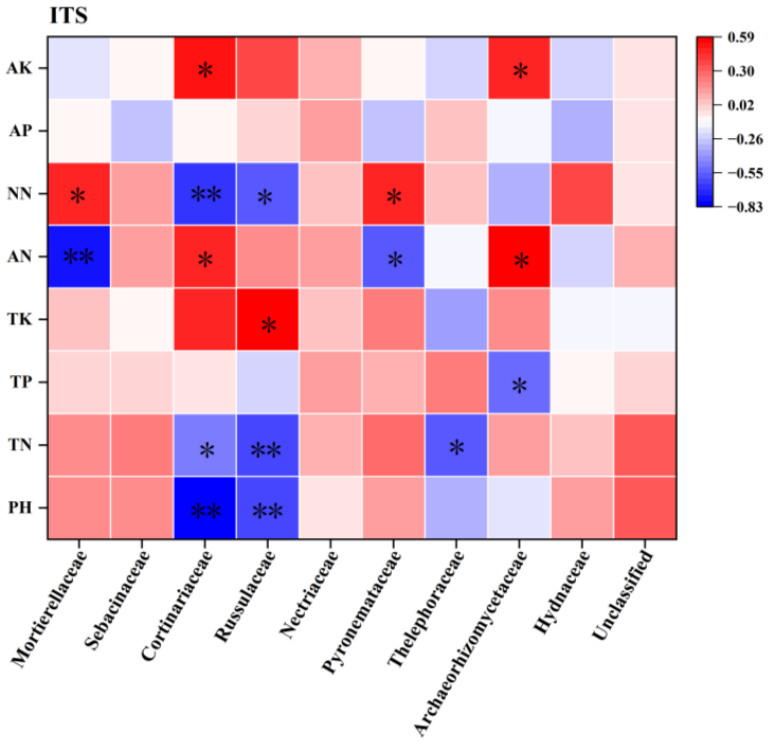


Figure 7. Heat map of correlation between horizontal abundance of soil fungal families and environmental factors in the rhizosphere soil of *C. macranthos* at different latitudes. Note: ITS (Internal Transcribed Spacer); Environmental factors: pH (soil pH value); TN (total nitrogen, g/kg); TP (total phosphorus, g/kg); TK (total potassium, g/kg); AN (ammonium nitrogen, mg/kg); NN (nitrate nitrogen, mg/kg); AP (available phosphorus, mg/kg); AK (quick-acting potassium, mg/kg). "*" indicates a significant correlation at $p < 0.05$, and "**" indicates a highly significant correlation at $p < 0.01$.

4. Discussion

Soil fungi are key to ecosystem stability and forest productivity, influencing soil nutrient dynamics and plant health [40]. *C. macranthos* has medicinal value and is an endangered orchid, so it is crucial to investigate the composition and diversity of its rhizosphere soil fungal communities for the conservation of *C. macranthos*. In this study, we used high-throughput sequencing technology to compare and analyse the diversity of fungal communities in the rhizosphere soils of *C. macranthos* at different latitudes in Heilongjiang Province. We investigated the characteristics of the rhizosphere soils of *C. macranthos* at varying latitudes in Heilongjiang Province. The results of this study lay a theoretical foundation for the study of mycorrhizal fungi of *C. macranthos*. The key findings showed that the alpha diversity of rhizosphere soil fungal communities of *C. macranthos* at different latitudes differed significantly (Table 1), and Shannon's diversity index and Simpson's diversity index decreased with an increase in latitudinal gradient (Figure 4), which suggests that the diversity of rhizosphere soil fungi in *C. macranthos* conforms to the pattern of the biotic latitudinal gradient changes, which is consistent with the general latitudinal gradient pattern theory proposed by Hillebrand [41] in "On the generality of the latitudinal diversity gradient". PCoA analyses of the rhizosphere soil fungi of *C. macranthos* at different latitudes and Adonis test results of $R^2 = 0.225$, $p < 0.01$, indicated that there was a highly significant difference in the composition of the rhizosphere soil fungal communities of *C. macranthos* at different latitudes (Figure 6).

The analysis of fungal community composition showed that at the phylum classification level, the dominant fungal groups in the rhizosphere soils of *C. macranthos* at different latitudes were Ascomycota, Basidiomycota, and Mucoromycota, which accounted for an

absolute dominance of up to 90% of the species OTU abundance in all three sites. This is similar to the results obtained in the study of inter- and non-rhizosphere soil fungal dominant groups of *C. macranthos* by Fu Yajuan et al. [13]. Among the top 10 dominant mycorrhizal groups at the family and genus levels, the top three families were Mortierellaceae, Sebacinaceae, and Cortinariaceae, while the three most abundant genera were *Sebacina*, *Cortinarius*, and *Mortierella*. This is similar to the results obtained by Fan Jie et al. [31] in the analysis of intra- and rhizosphere soil fungal community composition of *C. macranthos* and by Liu Huanchu et al. [42] in their study of mycorrhizal diversity and community composition in co-occurring *Cypripedium* species. The results of family abundance showed that *S. grandiflora* and *C. macranthos* are the most common species in the world, but *S. grandiflora* is not the only one. The results of family-level abundance showed that Sebacinaceae, Nectriaceae, and *Sebacina* had similar percentages at different latitudes (Figure 5), suggesting that they are the specific dominant fungi of *C. macranthos*, while the differences in abundance of the other fungal families are supposed to be related to different latitudinal environments.

Despite the significant findings of this study, several limitations still need to be pointed out. Follow-up studies should integrate more diverse data and employ complementary statistical methods to understand these potential factors better. Since the composition of mycorrhizal fungal communities in orchids is influenced by various factors, the present study investigated the diversity and community structure of rhizosphere soil mycorrhizal fungi of *C. macranthos* plant populations at different latitudes in Heilongjiang Province. Although molecular biology techniques can rapidly obtain sequence information of mycorrhizal fungi and carry out the analysis of mycorrhizal communities, the isolation and culture techniques used to obtain mycorrhizal fungal strains are still valuable for the in-depth analysis of the mechanism of interactions between orchids and mycorrhizal fungi, as well as for the bioconservation of orchids. Therefore, it is necessary to strengthen the isolation and culture of mycorrhizal fungi of *Cypripedium* in the future to promote the deepening of the research on the symbiotic mechanism of mycorrhizal fungi of *Cypripedium*.

5. Conclusions

This study systematically revealed the characteristics of fungal community structure in the rhizosphere soil of *C. macranthos* at different latitudes.

Based on the α diversity (Shannon and Simpson indices) of fungi in the rhizosphere soil of *C. macranthos*, there was a significant decreasing trend with increasing latitude, which was highly consistent with the traditional latitudinal diversity gradient (LDG) theory. This finding provides new empirical support for the latitudinal distribution patterns of rhizosphere microorganisms in *C. macranthos*, further deepening our understanding of the environmental adaptability of the microbial symbiotic system of this endangered species. β-Diversity analysis (PCoA and Adonis test) further confirmed that fungal community composition varies significantly across latitudes (Adonis test $R^2 = 0.225$, $p < 0.01$), indicating that latitude may be a key geographical factor shaping the rhizosphere fungal communities of the *C. macranthos.* Combined with synchronous analysis of soil physicochemical properties, the study identified pH, total potassium (TK), ammonium nitrogen (AN), nitrate nitrogen (NN), and available potassium (AK) as core driving factors, revealing the direct influence of latitudinal heterogeneity in soil chemical environments on fungal community assembly. Notably, Spearman correlation analysis showed that soil nitrogen forms (e.g., the ratio of ammonium nitrogen to nitrate nitrogen) were significantly associated with the abundance of specific fungal families (e.g., Mortierellaceae, Cortinariaceae), suggesting that fungi's differentiated utilisation strategies for soil nitrogen environments may be the core mechanism for their adaptation to latitude gradients.

This study not only elucidates the latitudinal gradient characteristics of the rhizosphere fungal communities of *C. macranthos* at the molecular level and their driving mechanisms but also provides a scientific basis for endangered Orchidaceae species conservation: on the one hand, it clarifies that the selection of symbiotic mycorrhizal fungi should prioritise potential functional groups from low-latitude regions; on the other hand, it emphasises the critical role of maintaining soil chemical environment stability (especially nitrogen form balance) in the recovery of the *C. macranthos* population. These findings lay an important foundation for developing conservation strategies for the microbial symbiotic system of *C. macranthos* and the optimisation of artificial propagation techniques.

Author Contributions: Methodology, L.M.; Investigation, J.Q., S.D. and J.L.; Writing—original draft, J.Q.; Supervision, S.D., M.L. and L.M. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the following projects: Heilongjiang Provincial Survey of National Key Protected Wild Plants (No. ZQTYB240100013), the Special Survey Project of Orchidaceae Plants in Heilongjiang Langxiang National Nature Reserve (2024-206), and the Key Plant Survey in the Hanma National Nature Reserve of the Greater Khingan Range, Inner Mongolia (No. HFW240100014).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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