

Analysis of Endophytic Community Structure in Seeds of *Pholidota chinensis* Lindl. Based on High-throughput Sequencing

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Abstract [**Objectives**] This study was conducted to reveal the composition and structure of the endophytic microbial community in seeds of *Pholidota chinensis* Lindl., so as to provide an important basis for research on seed endophytes and resource development of *P. chinensis*. [**Methods**] The 16S rDNA V3-V4 region and the ITS1-ITS2 region of endophytes from *P. chinensis* seeds were amplified, followed by the construction of MiSeq libraries and sequencing on the Illumina MiSeq platform. Subsequently, OTU clustering and taxonomic analysis were performed. [**Results**] A total of 28 OTUs (operational taxonomic units) from the amplified 16S rDNA sequences of endophytic bacteria could be annotated. They were classified into Cyanobacteria, Proteobacteria, Bacteroidetes, Acidobacteria, and unclassified bacteria, with Cyanobacteria as the dominant phylum and *Streptophyta* as the dominant genus. The Shannon diversity index, Simpson index, and Chao index of the endophytic bacterial community were 0.66, 0.65, and 35.50, respectively. The endophytic fungal sequences were grouped into 315 OTUs, belonging to Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, Mortierellomycota, Mucoromycota, Rozellomycota, and unclassified fungi, with genera including *Penidiella*, *Botryosphaeria*, *Cladosporium*, and *Candida*. Among them, Ascomycota was the dominant phylum, and *Penidiella* was the dominant genus, in which *Penidiella aggregata* was the dominant species. The Shannon diversity index, Simpson index, and Chao1 index were 2.63, 0.23 and 402.85, respectively. [**Conclusions**] The endophytic bacteria and endophytic fungi in the seeds of *P. chinensis* have certain biodiversity.

Key words *Pholidota chinensis* Lindl.; Seed; High-throughput sequencing; Endophytic fungi; Endophytic bacteria; Diversity

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Chinese *Pholidota* Herb refers to the pseudobulb or whole plant of *Pholidota chinensis* Lindl. (Orchidaceae), also known as "Shi Ganlan" or "Shi Yurou". It is mainly distributed in the tropical rainforests of Southwest China and Southeast Asia^[1] and serves as a commonly used Chinese herbal medicine in Guangxi, China^[2]. As a key microbial symbiotic system during the growth and development of *P. chinensis*, the endophytic microbial community within its seeds is closely associated with various physiological processes, including the healthy growth of the plant, substance metabolism, and the synthesis of medicinal components, thereby holding significant academic value.

P. chinensis is an orchid species. It produces a large number of tiny seeds without endosperm, most of which are underdeveloped. Under natural conditions, these seeds require symbiotic fungi to germinate and develop into seedlings^[3]. Plant seeds harbor abundant endophytic microorganisms, which can be classified into endophytic bacteria, endophytic fungi, and endophytic actinomycetes^[4]. Plant endophytes are microorganisms that spend all or part of their life cycle within healthy plant tissues without causing apparent disease symptoms in the host plant^[5–7]. Under natural conditions, orchid seeds require fungal hyphae to germinate, and most processes such as normal growth, flowering, and fruiting of orchids involve fungal symbiosis^[8]. Since 1993, when foreign researchers isolated an endophytic fungus capable of producing the anticancer active ingredient taxol from the *Taxus brevifolia*, research on plant endophytes has gradually gained increasing attention^[9]. Plant endophytes can promote plant growth and development, regulate nutrient absorption, and have become a research hotspot in fields such as agricultural production, plant protection, natural active product research, medicinal resource development, and environmental protection^[10–13]. In recent years, plant seed endophytes have been confirmed to promote plant growth, development, and reproduction, enhance host plant stress resistance and disease and pest resistance, and transmit plant genetic information. They are closely associated with plant health^[4,14]. In various tissues and organs of common medicinal plants such as *Panax ginseng*, *Zanthoxylum nitidum*, and *Agastache rugosa*, a large number of endophytes have been isolated. It has been found that these

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endophytes play important roles in promoting the growth of medicinal plants, enhancing stress tolerance, and participating in the synthesis of medicinal components^[15–18]. However, regarding the study of seed endophytes in *P. chinensis*, although there have been some preliminary reports on the isolation and identification of endophytes from the plant^[19], the composition and structure of its seed endophytic communities have not yet been systematically investigated. Current research has mostly focused on basic biological aspects such as morphological characteristics, germination conditions^[20–22], chemical composition, and pharmacological activities^[20–22], while studies on seed endophytic communities have not been reported.

Therefore, this study focuses on the seeds of *P. chinensis* and employs highthroughput sequencing technology to investigate the endophytic community structure and species diversity within its seeds, aiming to provide a reference for exploring the growth-related symbiotic microbes of *P. chinensis*. Furthermore, due to the longterm coexistence with their host plants, plant endophytes have acquired the ability to produce active components that are identical or similar in structure to those of the host plant^[23]. Therefore, this study also lays a certain theoretical foundation for the development of medicinal resources of *P. chinensis* and the utilization of endophytic resources.

Materials and Methods

Materials

The capsules of *P. chinensis* were collected from Baishengtun, Chetian Village, Zhongliang Township, Jinxiu Yao Autonomous County, Laibin City, Guangxi.

Methods

Total DNA extraction of seed endophytes The capsules of *P. chinensis* were cleaned with detergent solution, rinsed under running water for 2 h, and dried. They were then soaked in 75% ethanol for 3 min, rinsed three times with sterile water, and immersed in 5% sodium hypochlorite solution for 10 min. After immersion, they were rinsed three times with sterile water, and dried. Next, the capsules were opened, and the seeds were ground into a fine powder using liquid nitrogen. Total DNA of

seed endophytes was extracted following the instructions of the Omega E. Z. N. A™. Mag-Bind Soil DNA Kit. The integrity of the extracted genomic DNA was examined by agarose gel electrophoresis, and the DNA concentration was quantitatively detected using the Qubit system.

PCR amplification Using the total genomic DNA of seed endophytes as the template, endophytic fungi and endophytic bacteria were amplified with primers ITS1-ITS2 (ITS1F: CTTGGTCATTAGAGGAAGTAA, ITS2R: GCTGCGTTCATCGATGC) and V3-V4 (341F: CCTACGGGNGGCWGCAG, 805R: GACTACHVGGTATCTAATCC), respectively. A MiSeq library was constructed. The library size was verified by 2% agarose gel electrophoresis, and the library concentration was determined using a Qubit 3.0 fluorometer.

Highthroughput sequencing and diversity analysis Based on nextgeneration sequencing, amplicon sequences of the microbial community in the samples were obtained using the Illumina MiSeq platform. OTU clustering (ASV denoising) and taxonomic analysis were performed. On the basis of OTU clustering (ASV denoising) results, multiple diversity indices were analyzed, and the sequencing depth was detected. Based on the taxonomic information, statistical analysis of community structure was carried out at various taxonomic levels. On the basis of the above analyses, a series of indepth statistical and visual analyses, including alpha diversity analysis, beta diversity analysis, and species composition analysis, could be performed on the community composition and phylogenetic information of multiple samples.

Results and Analysis

OTU analysis of endophytes in *P. chinensis* seeds

A total of 80 655 valid sequences were obtained from the sequencing of endophytic fungi in *P. chinensis* seeds. Among them, 77 211 were used for subsequent OTU clustering and annotation, and were classified into 315 OTUs (Table 1).

A total of 48 808 valid sequences were obtained from the sequencing of endophytic bacteria in *P. chinensis* seeds. Among them, 43 655 were used for subsequent OTU clustering and annotation, and were classified into 28 OTUs (Table 1).

Table 1 Annotation results of endophytes in *P. chinensis* seed

Group	Total sequences	Valid sequences	Sequences used for OTU clustering	OTUs
Endophytic fungi	80 655	80 588	77 211	315
Endophytic bacteria	49 360	48 808	43 655	28

Species distribution of endophytes in *P. chinensis* seeds

Statistical analysis methods were applied to observe the community structure of the samples at different taxonomic levels. Usually, species with a relative abundance of less than a certain threshold (1%) in all samples are classified as "others", while the remaining species are analyzed as dominant species.

The endophytic fungi of *P. chinensis* were classified into Ascomycota, Basidiomycota, Mucoromycota, and others, with their relative abundances being 92.44%, 4.25%, 2.68%, and

0.64%, respectively (Fig. 1).

The phylum Ascomycota was further classified into 5 classes, 8 orders, 10 families, and 12 genera. The class Saccharomycetes accounted for 8.04%, comprising one OTU belonging to the order Saccharomycetales, the family Saccharomycetales_fam_Incertae_sedis, and the genus *Candida*. The class Leotiomycetes accounted for 2.38%, comprising one OTU belonging to the order Helotiales, the family Dermateaceae, and the genus *Pyrenopeziza*. The class Eurotiomycetes comprised two OTUs, including Eurotiales

(2.77%) with the family Aspergillaceae and genus *Aspergillus*, and Phaeomoniellales (4.34%) with the family Phaeomoniellaceae. The class Sordariomycetes comprised two OTUs, including Ophiostomatales (1.42%) with the family Ophiostomataceae and genus *Ophiostoma*, and Glomerellales (3.65%) with the family Glomerellaceae and genus *Colletotrichum*. The class Dothideomycetes accounted for 66.11% and included two orders, three families, and comprised four OTUs: Capnodiales (57.86%) with the family Teratosphaeriaceae and genus *Penidiella* (2 OTUs); Cladosporiaceae (4.67%) with the genus *Cladosporium* (1 OTU); and Botryosphaeriales (6.87%) with the family Botryosphaeriaceae and genus *Botryosphaeria* (1 OTU) (Fig. 2).

The phylum Basidiomycota included one class, Tremellomycetes (4.25%), which comprised one OTU belonging to the order Tremellales, family Tremellaceae, and the genus *Cryptococcus*.

The phylum Mucoromycota included one class, Mucoromycetes (2.68%), comprising one OTU belonging to the order Mucorales, the family Lichtheimiaceae, and the genus *Rhizomucor*.

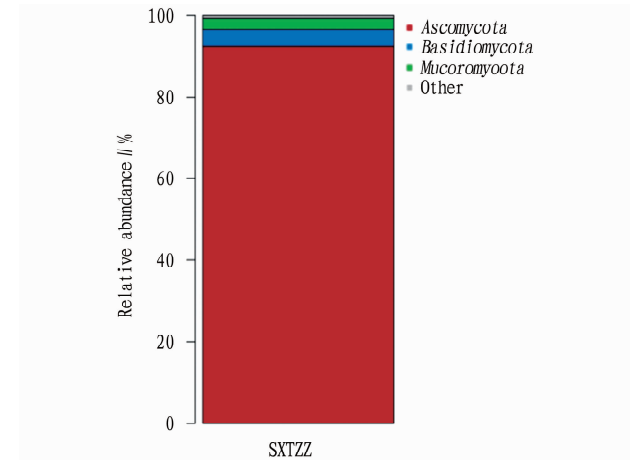


Fig. 1 Phylum-level abundance distribution of endophytic fungi in *P. chinensis* seeds

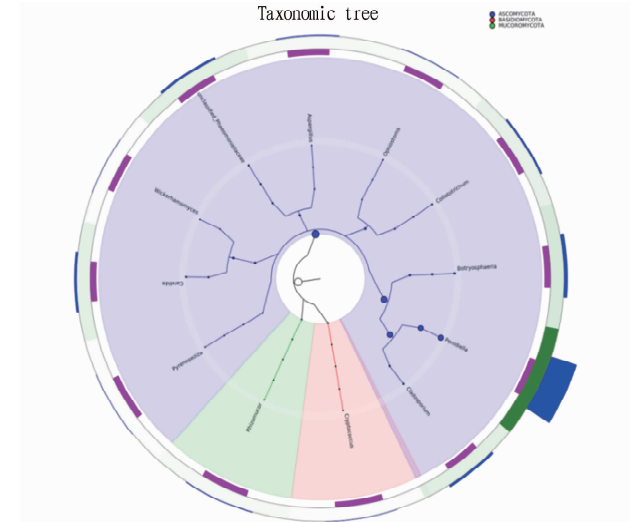


Fig. 2 Visualization of taxonomic and phylogenetic information of endophytic fungi in *P. chinensis* seeds

The endophytic bacteria in *P. chinensis* seeds were classified into Cyanobacteria_Chloroplast (87.08% relative abundance), unclassified_Bacteria (12.62%), and others (0.3%) (Fig. 3).

The phylum Cyanobacteria_Chloroplast included one class, Chloroplast (87.08%), one order, Chloroplast, one family, Chloroplast, one genus, Streptophyta, comprising two OTUs. Unclassified_Bacteria accounted for 12.62% and comprised one OTU (Fig. 4).

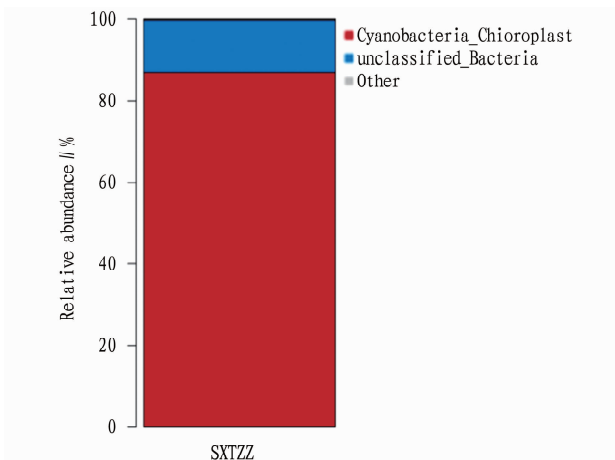


Fig. 3 Phylum-level abundance distribution of endophytic bacteria in *P. chinensis* seeds

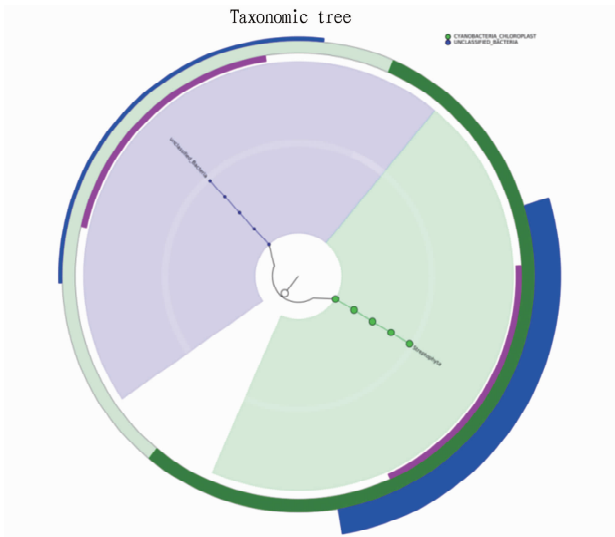


Fig. 4 Visualization of taxonomic and phylogenetic information of endophytic bacteria in *P. chinensis* seeds

Species diversity analysis

Alpha diversity analysis of the endophytes in *P. chinensis* seeds was performed to reflect the richness and diversity of the microbial community. For endophytic fungi, the Shannon index, the Simpson index, the Chao index and the Ace index were 2.63, 0.23, 402.85, and 402.85, respectively, and the library coverage for each sample was nearly 1.00. For endophytic bacteria, the Shannon index, the Simpson index, the Chao index and the Ace index were 0.66, 0.65, 35.50, and 38.94, respectively, and the library coverage for each sample was nearly 1.00 (Table 2).

Table 2 Diversity indices of endophytes in *P. chinensis* seeds

Group	Shannon	Simpson	Chao	Coverage	Ace
Endophytic fungi	2.63	0.23	402.85	1.00	402.85
Endophytic bacteria	0.66	0.65	35.50	1.00	38.94

Conclusion and Discussion

The composition of endophytes in *P. chinensis* seeds was investigated using Illumina high-throughput sequencing technology. It was found that the endophytic fungi of *P. chinensis* exhibit rich genetic diversity and belong to the phyla Ascomycota, Basidiomycota, and Mucoromycota. Among these, Ascomycota was the dominant fungal phylum in *P. chinensis* seeds, with *Penidiella* being the dominant genus. The diversity of endophytic bacteria in *P. chinensis* was relatively low, with Cyanobacteria_Chloroplast as the dominant phylum and *Streptophyta* as the dominant genus. This study also lays a certain theoretical foundation for the development of medicinal resources of *P. chinensis* and the utilization of endophytic resources.

According to the literature^[24–25], the endophytic fungi of the medicinal orchid *Dendrobium* mainly belong to three major phyla: Ascomycota, Basidiomycotina, and Deuteromycotina, and are distributed among genera such as *Neurospora*, *Sordaria*, *Chaetomium*, *Colletotrichum*, *Cosmospora*, and *Fusarium*. Both *P. chinensis* and *Dendrobium* belong to the Orchidaceae family, and their endophytic communities overlap at the phylum and genus levels, indicating a certain degree of homology. The diversity of endophytic bacteria in *P. chinensis* is relatively low, with Cyanobacteria_Chloroplast as the dominant phylum and *Streptophyta* as the dominant genus. According to the literature^[26], the endophytic bacteria in the seeds of *Dendrobium nobile* belong to the phyla Proteobacteria and Cyanobacteria. Both belong to the Orchidaceae family, and there is an overlap in endophytic bacteria between *P. chinensis* and *Dendrobium nobile*, indicating a certain degree of homology. Endophytic bacteria can enhance plant adaptation to stress, strengthen the ecological balance of the system, and thereby ensure the healthy growth of the host plant. The fruit of *P. chinensis* is a capsule, and the seeds are enclosed within the pericarp, isolated from the external environment, which may be one of the reasons for the relatively low diversity of its endophytic bacteria.

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assisting the Kidney water's luster, which is stored in the hair. In terms of the controlling cycle of the Five Elements, Sophorae Flavescens Radix uses Wood to suppress excessive hair oiliness (Earth), while Camelliae seed meal uses Earth to absorb greasiness and prevent the herbal liquid from becoming overly cloying and stagnant.

Regarding the use of guiding herbs, Rosmarini Folium directs the medicinal action specifically to the top of the head; Spirodelae Herba carries the medicinal effect upward; and Speranskiae Herba penetrates the hair follicles. For the harmonization of yin and yang, pungent-warm herbs such as Gleditsiae Fructus and Artemisiae Argyi Folium are combined with yin-nourishing, moistening herbs such as Ecliptae Herba and Ligustri Lucidi Fructus to prevent damage to yin. For the interdependence of ascending and descending, Rosmarini Folium and Spirodelae Herba guide the medicinal action upward, while Mori Cortex and Sophorae Flavescens Radix clear dampness and drain heat.

This study involves a preliminary evaluation of the effectiveness of a shampoo product, conducted in accordance with relevant ethical guidelines. All 30 volunteers who used the shampoo were fully informed about the study and signed informed consent forms. In the preliminary safety assessment, the product was found to pose no significant risk to humans. Therefore, prior ethical approval was not sought. Additionally, to verify the product's efficacy, skin tests were also performed on mice. All animal experimental procedures followed the principles of laboratory animal welfare and ethics, minimizing both discomfort and the number of animals used.

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