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Diversity, biocontrol, and growth promotion potential of culturable endophytic fungi isolated from root, shoot, and leaf of wild *Phoebe bournei* (Hemsl.) Yang across the seasons

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Draft

Abstract

Endophytic fungi were isolated from *Phoebe bournei* and their diversity and antimicrobial and plant growth-promoting activities were investigated. Of the 389 isolated endophytic fungi, 88.90% belonged to phylum Ascomycota and 11.10% to phylum Basidiomycota. The isolates were grouped into four taxonomic classes, 11 orders, 30 genera, and 45 species based on internal transcribed spacer sequencing and morphologic analysis. The host showed a strong affinity for the genera *Diaporthe* and *Phyllosticta*. The diversity of the fungi was highest in autumn, followed by spring and summer, and was lowest in winter. The fungi exhibited notable tissue specificity in *P. bournei*, and the species richness and diversity were highest in the root across all seasons. Five isolates showed antimicrobial activity against eight plant pathogens, and reduced the incidence of leaf spot disease in *P. bournei*. Additionally, 9 biocontrol isolates showed plant growth-promoting activity, with five significantly promoting *P. bournei* seedling growth. This is the first report on the endophytic fungi of *P. bournei* and their potential applicability to plant disease control and growth promotion.

Keywords: *Phoebe bournei*; Endophytic fungus; Fungal diversity; Antifungal activity; Plant growth promotion

Introduction

Endophytic fungi inhabit plants at different points in their life cycle without causing any disease symptoms (Aly et al. 2010). In natural ecosystems, plants live symbiotically with endophytes, which have a major effect on the species diversity and structure of plant communities (Márquez et al. 2010). Several hundred endophytic fungi have been identified in plant species worldwide (Strobel and Daisy 2003); however, in natural ecosystems, there are many that remain unknown.

The diversity and abundance of endophytic fungi suggest that they play a key role in plant growth and survival (Qiang et al. 2019). In mutualistic interactions, endophytic fungi enhance the host plant's response to abiotic and biotic stresses, stimulate plant growth, and suppress diseases, and potentially serve as biocontrol agents through the secretion of metabolites that inhibit growth and spore germination in microbial pathogens (Higginbotham et al. 2013; Tonial et al. 2016). Additionally, endophytes promote plant growth by releasing iron carriers, volatile compounds, and plant hormones (Huang et al. 2014) such as indole acetic acid (IAA) (Michele et al. 2013).

Phoebe bournei (Hemsl.) Yang, also known as jinsinan, is an evergreen tree in the *Lauraceae* family that is mainly cultivated in the south of China including in the cities of Fujian, Zhejiang, Guangxi, and Guizhou (Li et al. 2018). *P. bournei* is a durable hardwood, and has a distinct fragrance and attractive golden tint and texture,

making it a good material for constructing high-grade buildings and fine furniture. However, the natural forest populations of *P. bournei* have sharply declined over the past decades due to overexploitation. Thus, *P. bournei* is listed as a second-class national protected plant in China (Jia et al. 2014; Li et al. 2015). Endophytes of *Lauraceae* have been previously described (Wang et al. 2013; Zhang et al. 2018); however, to date the endophytic fungi associated with *P. bournei* have not been investigated.

We speculated that there are beneficial endophytic fungi in *P. bournei* that can promote plant growth and improve plant stress resistance. The aims of this study were as follows: 1) to isolate and identify endophytic fungi from different issue of *P. bournei* across different seasons; 2) to evaluate the diversity and abundance of endophytic fungi in *P. bournei*; and 3) to test the biocontrol and growth-promoting potential of endophytic fungi in *P. bournei*.

Materials and methods

Sample collection and isolation of endophytic fungi

Representative samples from the four seasons of spring, summer, autumn, and winter were collected on March 10, June 10, September 10, and December 10, respectively, of 2018 from four different sites in Guizhou Province, China including Luo si ke Nature Reserve (107°3'78.82" E, 26°24'84.67" N) , Leigong Mountain Nature Reserv (108°20'9.104"E, 26°37'46.495"N), Congjiang County (108°43'7.494"E, 25°59'40.455"N), and Taijiang County (106°65'6.568"E, 26°45'42.222"N). Ten different *P. bournei* plants (height 15–20 m and diameter at breast height 0.8–1.5 m) were selected from each site, and three tissues from each plant were sampled (root, shoot, and leaf), with four replications of each tissue taken from all sides of each plant. In total, 160 healthy leaf and 160 healthy shoot samples of equal length (25–30 cm) and diameter (1.0–1.5 cm) were collected with tree pruners. The shoots and leaves were cleaned with distilled water and cut using sterilised scissors. A stainless steel corer (9 cm in diameter) was used to obtain soil samples from all sides of the plant at a depth of 0.3–0.5 m, from which 160 root samples (0.5–1.0 cm in diameter) were collected. All samples were placed in sterile zippered pouches and immediately stored at 4°C until further use. Endophytic fungi were isolated within 24 h. Samples from each season were treated in the same manner.

All tissue samples were washed under running tap water, distilled water, and sterile water in that order. The samples were cut into small pieces (0.5×0.5 cm for leaves and 0.5 cm in length for shoots and roots) and sterilised as previously described (Adams et al. 2021; Schulz et al. 1993). The samples were immersed in 75% (v/v) ethanol for 40 s, rinsed three times with sterile water, then soaked in 3% (v/v) NaClO (roots for 3 min,

shoots for 2 min, and leaves for 1 min) and rinsed three to five times with sterile water for 2 min each time. The sterile water used for the last rinse was inoculated onto a potato dextrose agar (PDA) as the blank control. Eight segments were placed in a Petri dish (90 mm in diameter) containing culture medium (PDA medium: potato 200 g, glucose 20 g, agar 20 g, distilled water 1 l, natural pH; rose bengal agar [RBA] medium: peptone 5 g, glucose 10 g, potassium dihydrogen phosphate 1 g, magnesium sulfate [$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$] 0.5 g, agar 20 g, 1/3000 Bengal red solution 100 ml, distilled water 1 l; Czapek's medium: sodium nitrate 3 g, dipotassium phosphate 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, potassium chloride 0.5 g, ferrous sulfate 0.01 g, sucrose 30 g, agar 20 g, distilled water 1 l; and potato sucrose agar [PSA] medium: potato 200 g, sucrose 20 g, agar 20 g, distilled water 1 l, natural pH) supplemented with 50 $\mu\text{g/ml}$ chloramphenicol. All samples were incubated in the dark at 28°C for at least 5–10 days. Pure fungal cultures were obtained by picking colonies from the advancing edge using forceps and transferring these to fresh PDA plates. Pure isolates were preserved on PDA slants and stored at 4°C for further identification.

Identification of endophytic fungi

The fungal isolates were identified by their morphologic features and by sequencing the internal transcribed spacer (ITS) region. Morphologic characterisation of isolates grown on PDA in the dark for 10–14 days was based on macroscopic features such as surface colony texture, odour, and colour (Kim et al. 2011). The size and shape of conidia and phialides were measured under a light microscope (model CX21; Olympus, Tokyo, Japan). Fungal genomic DNA was extracted from the mycelia using the DNA Plant Mini Kit (Shenggong Biotech, Shanghai, China) according to the manufacturer's instructions. The ITS region was amplified using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). PCR was carried out in a volume of 25 μl containing 23 μl Golden Medal MIX (Taq DNA polymerase, dNTPs, buffer systems, and dyes that have been optimised; Thermo Fisher Scientific, Waltham, MA, USA), 1 μl of each primer (1 $\mu\text{mol/l}$), and 1 μl template DNA (1 ng/ μl). The reaction conditions were as follows: initial incubation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 40 s; and incubation at 79°C for 1 s for plate reading. The PCR products were sequenced by Beijing Sunbiotech (Beijing, China). After manual editing, an initial search was carried out using BLAST to identify the closest matches. The sequences obtained in the current study were then aligned using ClustalW with sequences of type materials and those with high similarity identified in the BLAST search. The aligned sequences were then manually realigned and a phylogenetic tree was constructed with the neighbour-joining method using MEGA v7.0 software. A

maximum likelihood analysis was performed to determine the phylogenetic relationships among isolates. A bootstrap analysis with 1000 replicates was performed (Kumar et al. 2016). The sequences have been deposited in GenBank under accession numbers.

In vitro inhibition test by dual culture

In order to assess the biocontrol potential of endophytic fungi against common leaf and root diseases such as anthracnose, leaf spot, and root rot, pathogenic strains known to cause leaf or root disease in other plants (Ye et al. 2018; Luo and Yu 2020; Iliger et al. 2020; Li et al. 2021) were tested including *Colletotrichum nymphaeae*, *Fusarium petroliphilum*, *Colletotrichum acutatum*, *Penicillium chermesinum*, *Fusarium oxysporum*, *Fusarium solani*, *Pestalotiopsis microspora*, and *Colletotrichum gloeosporioides*, which were provided by the plant pathology laboratory of the College of Forestry, Guizhou University, China. *P. microspora* and *C. gloeosporioides* are pathogenic fungi that cause leaf spot in *P. bournei* (Chen et al. 2018; Yao and Yu 2021). The antimicrobial activity of *P. bournei* endophytic fungi against pathogens was evaluated in a dual culture experiment. Mycelial discs (8 mm in diameter) of an endophytic fungus and pathogen were placed on each side of a PDA plate along the diameter separated by a distance of 4 cm. In order to exclude the effects of the medium pH, the PDA medium was buffered with 0.2 mol/l phosphate buffer (pH 7). As a control, a mycelial disc (8 mm in diameter) of pathogenic fungus was placed on each side of a PDA plate along the diameter. The plates were incubated in the dark at 25°C for 3–8 days. Three independent replicates were prepared. The growth inhibition rate was calculated with the following formula: inhibition rate = $(R_c - R_p)/R_c \times 100\%$, where R_c and R_p are the radial growth of the pathogen on the control and dual culture plates, respectively. The results were classified as follows: $\geq 75\%$, strong inhibition; 50%–75%, medium inhibition; 10%–25%, low inhibition; and $\leq 10\%$, no inhibition.

Suppression of leaf spot disease in vitro

Five endophytic fungus isolates (3-hcg-5, 3-ye-3, 3-ccg-1, 1-cg-18, and 3-ccg-2) that inhibited the growth of *P. microspora* were selected and their ability to control leaf spot disease in *P. bournei* seedlings was assessed. Detached healthy leaves were stabbed with a sterile needle and a PDA plug of *P. microspora* (5 mm in diameter) was placed on the wound. Five endophytic fungi were separately cultured on PDA plates at 28°C for 7 days; inoculation suspensions of each fungus were prepared by flooding the agar surface with sterilised distilled water to obtain a spore suspension (1×10^6 CFU/ml). The suspensions were separately sprayed daily on the detached

leaves inoculated with *P. microspora*. Unsprayed leaves inoculated with *P. microspora* and leaves inoculated with sterile agar plugs only served as a positive and negative control, respectively. The treated leaves were sealed in a Petri dish with moist filter paper and cultured at 25°C for 10 days in an incubator with lighting, after which they were photographed and % inhibition of the pathogen was calculated with the following formula: % inhibition = $(R_1 - R_2)/R_1 \times 100$, where R_1 and R_2 are the diameter of the spot in the control and in the presence of endophytic fungus, respectively. Three independent biological replicates were prepared for each treatment (30 healthy leaves per treatment).

Screening of endophytic fungi for plant growth-promoting traits

A total of 27 isolates showing antifungal activity against at least one of the tested pathogens were selected for the evaluation of plant growth-promoting activity. There were three replicates in each experiment.

1) IAA production

Using the Salkowski colorimetric method (Glickmann and Dessaux 1995), the selected isolates were cultured for 1 week and then inoculated into Jin's culture medium (peptone 10 g, MgSO₄ 1.5 g, K₂HPO₄ 1.5 g, H₂O 1 l, glycerin 10 ml, pH 7.2) for liquid fermentation. One week later, the supernatant of each strain was collected and 100 µl was added to the same volume of ferric chloride colorimetric solution (0.5 mol/l FeCl₃ 1 ml, 98% H₂SO₄ 30 ml, H₂O 50 ml). The colour change was observed after 0.5 h of incubation in the dark. A sample containing 100 µl IAA standard solution (50 µg/ml) and the same volume of ferric chloride colorimetric solution was used for colour comparison. If the colour of the strain sample was the same as that of the standard solution, the result was positive, indicating that the strain could secrete IAA (with a darker colour reflecting more IAA secretion); otherwise, the result was recorded as negative.

2) Phosphate solubilisation

Phosphate solubilisation by the isolated endophytic fungi was assessed using Pikovskaya's agar medium (0.5 g [NH₄]₂SO₄, 20 mg NaCl, 20 mg KCl, 0.1 g MgSO₄·7H₂O, 3 mg FeSO₄·7H₂O, 3 mg MnSO₄·7H₂O, 0.5 g yeast extract, 10 g glucose, and 15 g agar in 1 l distilled water) supplemented with 0.3% insoluble tricalcium phosphate (Ca₃[PO₄]). An agar plug of the isolated endophytic fungi (5 mm in diameter) was inoculated onto the medium, followed by incubation at 28°C for 24–72 h. A clear zone around colonies indicated phosphate solubilisation (positive result) (Kumar et al. 2018); otherwise, the result was recorded as negative.

3) Potassium solubilisation

Isolated endophytic fungi (as an agar plug 5 mm in diameter) were inoculated onto one side of a PDA plate, and 1 g sterilised potassium feldspar powder was placed on the opposite side, followed by incubation at 28°C for 24–72 h. Chemotaxis of mycelia (hyphae) towards the potassium-containing mineral powder was observed under a stereomicroscope as an indicator of potassium solubilisation (positive result); otherwise, the result was recorded as negative.

Evaluation of the effect of plant growth-promoting strains on *P. bournei* seedling growth

Five endophytic fungus isolates (4-hby-3, 3-hcg-5, 1-cg-2, 1-cg-18, and 4-cg-4) were randomly selected for evaluation of plant growth-promoting traits. A 10-ml sample of the endophytic fungal suspension (1×10^6 CFU/ml) was inoculated into soil with *P. bournei* seedlings. Seedlings treated with 10 ml sterilised distilled water served as a control. After treatment, seedlings were grown at $25^\circ\text{C} \pm 2^\circ\text{C}$ on a 12:12-h light/dark cycle. At 90 days after inoculation, they were photographed and height, chlorophyll content, dry weight, fresh weight, root length, and root activity were measured. Chlorophyll was extracted from leaves with 80% acetone and quantified as previously described (Porra et al. 1989). Root activity was also measured as previously described (Yin et al. 2014).

Statistical analysis

Fungal diversity and richness in different plant tissues were measured and quantified using various indicators including isolation rate (*IR*), relative frequency (*IF*), the Shannon–Wiener (*H'*) index, Simpson’s diversity index (*D*), Pielou’s evenness index (*E*), Margalef’s index (*M*), and Sorenson’s similarity coefficient (*C_s*), which were calculated as follows:

$$IR = Ng/Nt \times 100\% \tag{1}$$

$$IF = Ng/Nf \times 100\% \tag{2}$$

$$H' = -\sum_1^k P_i \times \ln P_i \tag{3}$$

$$D = 1 - \sum P_i^2 \tag{4}$$

$$M = (S - 1)/\log_2(N) \tag{5}$$

$$E = H/\ln(S) \tag{6}$$

$$Cs = 2j/(a + b) \quad (7)$$

where N_g is the number of endophytic fungal strains in the tissue sample, N_t is the total number of samples, and N_f is the total number of isolated strains; k is the total number of endophytic fungal species in a plant or tissue; P_i is the percentage of a specific endophytic fungus strain in the total number of isolated strains; S is the number of species; N is the total number of strains; H' is the diversity index; and j is the number of endophytic fungal species in two tissues, a is the number of endophytic fungal species in one tissue, and b is the number of endophytic fungal species in the other tissue.

Differences in the Shannon–Wiener and Simpson’s diversity indices across samples were evaluated by one-way analysis of variance (ANOVA). The % inhibition of eight phytopathogens by endophytic fungi was assessed by ANOVA as a measure of the latter’s antifungal properties. IAA secretion by endophytic fungi was analysed by one-way ANOVA.

Results

Identification and composition of endophytic fungi of *Pheobe bournei*

Using molecular and morphological methods, a total of 389 endophytic fungal strains (in total of 45 species) were isolated and purified from root, shoot and leaf segments of *P. bournei* in different seasons. Among the different tissues, the maximum number of isolates was obtained from the root (186 isolates, 36 species), followed by the shoot (128 isolates, 27 species) and then the leaf (75 isolates, 20 species). The maximum number of isolates was obtained in autumn (156 isolates, 32 species), followed by spring (98 isolates, 29 species), summer (78 isolates, 18 species), and winter (57 isolates, 13 species). At the phylum level, most isolates belonged to Ascomycota (88.90%), with very few belonging to Basidiomycota (11.10%). The 389 endophytic fungi strains were identified to belong to 45 fungal species (Table. S1). Morphologic features of the 45 species were shown in Figure S1. The 45 fungal species grouped into 4 taxonomic classes (Sordariomycetes, Eurotiomycetes, Dothideomycetes and Agaricomycetes), 11 orders (Capnodiales, Xylariales, Glomerellales, Hypocreales, Trichosphaeriales, Diaporthales, Eurotiales, Botryosphaeriales, Pleosporales, Polyporales and Agaricales), and 30 genera (Table. S1 and Figure 1), which were obtained from different *P. bournei* tissues across different seasons. All sequences of 45 fungal species have been submitted to the GenBank database, and the accession numbers were listed in Table. S1.

The relative frequency of each genus out of all the isolates led to the following classification: dominant genus $\geq 10\%$, common genus $5\%–10\%$ and rare genus $\leq 5\%$. Among the endophytic fungi of *P. bournei*, the two dominant genera were *Diaporthe* (IR = 13.6%; IF = 17.48%) and *Phyllosticta* (IR = 8%; IF = 10.28%); and three common genera were *Phomopsis* (IR = 6.6%; IF = 8.48%), *Xylaria* (IR = 4%; IF = 5.14%) and *Botryosphaeria* (IR = 4%; IF = 5.14%). The isolation rate and relative frequency of the other genera were $\leq 5\%$. There were big differences in abundance between dominant genera, common genera and rare genera (Table S1).

Relative abundance analyses and diversity estimation of endophytic fungi of *P. bournei*

At the genus level, the relative abundance of endophytic fungi associated with different *P. bournei* tissues in different seasons was analysed, as shown in Figure 2. Out of the 30 genera isolated, *Diaporthe* (13.6%), *Phyllosticta* (8%) and *Phomopsis* (6.6%) displayed the highest richness. The relative abundance of endophytic fungi was highest in autumn (25 genera) and spring (23 genera), followed by summer (15 genera) and winter (10 genera). Across the different tissues, the relative abundance of endophytic fungi was highest in the root (26 genera), followed by the shoot (23 genera) and the leaf (18 genera). The comparison of the endophytic fungi associated with *P. bournei* in the different seasons showed that the genera *Diaporthe*, *Trichoderma* and *Phoma* were present in all seasons (Figure 2). However, the genus *Aspergillus* was isolated only in spring, the genus *Cylindrocladiella* was isolated only in summer, the genera *Nemania*, *Pestalotiopsis* and *Fusicoccum* were isolated only in autumn and no endemic genera were found in winter (Figure 2A–D). In addition, the analysis of endophytic fungi in different tissues showed that the most abundant genera isolated from the leaf were *Xylaria*, *Nectria*, *Haematonectria* and *Botryosphaeria* (Figure 2G). Four endemic genera *Arthrimum*, *Irpex*, *Montagnula* and *Phoma* were isolated from the root (Figure 2E). It was also found that certain endophytic fungi were isolated only in certain tissues, or in certain seasons. For example, *Colletotrichum gloeosporioides* 3-cby-1 was only found in the shoot (Figure 2F), and *Xylaria arbuscula* 1-ye-5 was only observed in autumn (Figure 2C). This indicates that some endophytic fungi of *P. bournei* have specific host selectivity.

The diversity indices of endophytic fungus species associated with *P. bournei* are summarised in Table S2. The highest biodiversity of endophytic fungi was observed in autumn, with H' , M , and D reaching maximum values of 4.36, 3.42, and 0.73, respectively. Among the different plant tissues, the highest biodiversity of endophytic fungi was in the root, with H' , M , and D reaching maximum values of 1.35, 7.02, and 0.79, respectively. Thus, there was considerable diversity in the number and population distribution of endophytic fungi in different seasons and plant parts of *P. bournei*. The highest Pielou's evenness index value of endophytic fungi ($E=2.13$)

was in the spring, despite the relatively lower species richness and diversity values (H' , D , and M) compared to autumn. Among the different tissues, endophytic fungi in the leaf showed the highest Pielou's evenness index ($E=0.56$); H' , D , and M values were also lower in the leaf than in the shoot and root. Across the different seasons, the Sorenson's similarity index of endophytic fungi associated with *P. bournei* was the highest in the spring and autumn ($Cs=0.55$), followed by the winter and summer ($Cs=0.48$). Thus, the root and shoot had the highest similarity ($Cs=0.53$), followed by the shoot and leaf ($Cs=0.37$).

Antimicrobial activity of endophytic fungi from P. bournei

Dual culture experiments were performed to assess the antimicrobial activity of endophytic fungi based on the inhibition zone or mycelial atrophy in pathogens (Table 1). We identified 27 isolates that inhibited the growth of plant pathogenic fungi. Of these, five (*Trichoderma longibrachiatum* 1-cg-18, *Trichoderma harzianum* 3-hcg-5, *Trichoderma tomentosum* 3-ccg-1, *Cylindrocladiella pseudohawaiiensis* 3-ccg-2, and *Hypoxyton fragiforme* 3-ye-3) showed effects on all tested pathogenic fungi. In particular, isolate 1-cg-18 showed strong inhibitory activity against *C. nymphaeae* (80.43%), *F. petroliphilum* (75.82%), *F. solani* (75.20%), *C. acutatum* (68.84%), *Pestalotiopsis microspora* (60.23%), *F. oxysporum* (30.32%), *P. chermesinum* (24.23%), and *C. gloeosporioides* (22.87%). Additionally, isolate 1-xj-3 (*Phomopsis* sp.) showed inhibitory activity against five of the pathogenic fungi while 21 isolates showed inhibitory activity against one or two strains. There were 18 isolates that showed no inhibitory activity against the tested pathogenic fungi.

Five endophytic fungus isolates showed antimicrobial activity against *P. microspora*, which causes leaf spot on *P. bournei* (Table 1). In order to further assess the biocontrol effect of endophytic fungi on leaf spot disease in *P. bournei*, the five endophytic fungi isolates and *P. microspora* were both inoculated onto *P. bournei* leaves in vitro. Compared to the control (inoculated with *P. microspora* alone), the spots on *P. bournei* leaves were significantly reduced by inoculation with 3-hcg-5, 3-ye-3, 3-ccg-1, 1-cg-18, and 3-ccg-2 isolates (Figure 3A), with a significant increase in the inhibition rate (%) (Figure 3B). These results suggest that the five fungal endophyte isolates are potential biocontrol agents for leaf spot disease in *P. bournei*.

Plant growth-promoting traits of endophytic fungi

Fungal endophyte isolates exhibiting inhibitory activity were further studied for their plant growth-promoting traits including IAA production, phosphate and potassium solubilisation. Among the 27 isolates, 9 showed positive results for one or more of these traits, while isolates 1-cg-18 and 1-cg-13 were positive for all three traits

(Table 2).

Five of the 9 positive endophytic fungi isolates were randomly selected for growth parameter evaluation. Compared to the control (treated with water), *P. bournei* seedlings inoculated with biocontrol isolates (4-hby-3, 3-hcg-5, 1-cg-2, 1-cg-18, and 4-cg-4) showed obviously enhanced growth (Figure 4A), as evidenced by the significant increases in growth parameters including seedling height, fresh weight, dry weight, chlorophyll content, root length, and root activity (Figure 4B–G). These results indicate that the biocontrol isolates can potentially promote plant growth.

Discussion

Plant endophytic fungi form a hyper-diverse community and are the main constituents of the plant microbiome (Samaga and Rai 2016). Some endophytic fungi grow very slowly on artificial medium or lack a structure for sporulation, and some cannot be cultured on artificial medium. As such, it is difficult to identify endophytic fungi using classic morphology-based approaches. In this study, we used molecular biology combined with morphologic examination to identify endophytic fungi, constructed a phylogenetic tree based on rDNA sequences, and analysed the taxonomic status of endophytic fungi from the phylogenetic perspective. When performing the BLAST search in the NCBI GenBank sequence database, we applied the following criteria: when the similarity between a specific sequence and the reference sequence was <95%, it was considered as an unidentified strain; when it was >95%, it was identified to the genus level; and when it was >97%, it was identified to the species level. These thresholds have been widely used to identify endophytic fungi (Landeweert et al. 2003; Qu et al. 2012). In this study, the similarity of isolated endophytic fungi to species found in GenBank ranged from 98% to 100%. In order to confirm the results of molecular identification, we performed macroscopic and microscopic characterisation of the morphology using the Dictionary of Fungi as a reference (Kirk et al. 2008).

Previous studies have shown that the successful isolation of endophytic fungi depends on many factors such as method of plant material disinfection, disinfectant type and time, and type of culture medium (Chen et al. 2016). In this study, four popular media were used for cultures (Burge et al. 1977; Sharma and Pandey 2010). There was an obvious difference in the number of strains isolated with the different types of medium, with the most isolates obtained using PDA medium (201 isolates), followed by PSA (118 isolates), Czapek's (53 isolates), and RBA medium (17 isolates). The *Colletotrichum* and *Nigrospora* genera were isolated only in RBA medium, while *Fomitopsis* and *Trametes* were isolated only in CDA medium. Additionally, different culture conditions

such as photoperiod and temperature influence spore production of endophytic fungi; previous studies have shown that they could be isolated in the temperature range of 25°C–30°C (Adams et al. 2021; Wang et al. 2021; Zhang et al. 2021). In this study we selected a temperature of 28°C for endophytic fungus culture, which is favourable for fungal growth and spore germination (Zhang et al. 2021). More species can likely be obtained at different temperatures; future studies will examine the effect of different temperatures on the isolation of endophytic fungi.

Endophytic fungi associated with *P. bournei* in our study mostly belonged to Ascomycota with a few belonging to Basidiomycota, consistent with earlier findings (González and Tello 2011; Yao et al. 2017). For example, it was found that endophytic fungi associated with *Acanthus ilicifolius* var. *xiamenensis* mostly belonged to phylum Ascomycota and Basidiomycota, with the former being more highly represented among isolates (Chi et al. 2019). Endophytic fungi are distributed in different classes of Ascomycota including Sordariomycetes, Eurotiomycetes, and Dothideomycetes (Jumpponen and Jones 2009; Porrasalfaro et al. 2011). In the present work, the five major classes of endophytic fungi found to be associated with *P. bournei* were Sordariomycetes, Eurotiomycetes, and Dothidiomycetes in phylum Ascomycota and Polyporales and Agaricales in phylum Basidiomycota (Table S1 and Figure 1). Differences among regions from which the plants were collected could explain the profile of isolated microbial communities (Dhayanithy et al. 2019). The most frequently detected isolates in *P. bournei* belonged to the *Diaporthe* genus, which was the main endophytic fungus group associated with the host and was present in nearly all host tissues. This finding was in accordance with investigations in other plants: for example, *Diaporthe* was the predominant genus of endophytic fungi associated with *Glycyrrhiza glabra* (Arora et al. 2019).

We isolated 45 species of endophytic fungi from *P. bournei* based on ITS sequencing and morphologic examination. Most of the species have also been isolated from other plants—e.g., *Colletotrichum siamense*, *Phomopsis asparagi*, and *Phyllosticta capitalensis* from *Bruguiera sexangula* (Lour.) Poir, which exhibit antimicrobial and antioxidant activities (Zhang et al. 2021). *C. gloeosporioides* isolated from pearl millet was shown to enhance plant growth (Nandhini et al. 2018). *Schizophyllum commune* and *Cladosporium tenuissimum* were isolated from the tropical mangrove *Rhizophora mucronata* (Tuan et al. 2018). *Botryosphaeria dothidea* and *Fusarium graminearum* were also isolated from the medicinal plant *Litsea cubeba* (Lour.) Pers (Wu et al. 2019); *B. dothidea* was the dominant species in the whole endophytic fungus community, and *F. graminearum* exerted antibiotic effects on all tested pathogenic microbes (Wu et al. 2019). *Aspergillus versicolor* and *T. longibrachiatum* were also found in *Panax notoginseng* and showed antimicrobial activities against host plant

pathogens responsible for root-rot disease.

Endophytic communities show variable distribution in host plant tissues. In *Holcus lanatus*, the root was enriched in the endophytic fungi colonization of *Hypericum mysorense* and *H. Japonicum* compared to the stem (Márquez et al. 2010). We observed clear differences in the distribution of endophytic fungi in *P. bournei*. The root had the most abundant and diverse endophytic fungus community followed by the shoot, while the rate of isolation from leaf was relatively low (Table S2). This is similar to findings in *Dendrobium officinale* (Jin et al. 2017). The lower abundance and diversity of endophytic fungi in the leaf may be due to the large surface area and presence of stomata that are exposed to the external environment, which is not conducive to endophyte growth (Gond et al. 2012). Some endophytic fungi such as *P. microspora*, *Daldinia fissa*, and *Ilyonectria radicola* were detected in the root, stem, and leaf of *P. bournei* whereas others were present only in specific tissues (Figure 2). Thus, endophytes have varying degrees of affinity for different plant tissues (Dhayanithy et al. 2019). The tissue specificity of endophytic fungi associated with *P. bournei* may be attributable to differences in the plant tissue microenvironment. Endophytes spend all or part of their life cycle colonising the healthy tissues of host plants either inter- or intracellularly (Zhao et al. 2010). The distribution of endophytic fungi in plants is affected by factors such as location, season, and water availability (Kim et al. 2013; Jin et al. 2017; Yeh and Kirschner 2019). The highest species diversity of endophytic fungi associated with *Juniperus rigida* and *Larix kaempferi* was in April and November, respectively (Kim et al. 2013). In general, the richness and abundance of endophytic fungi are higher in hotter and wetter seasons (Kim et al. 2013). In our study, the highest endophytic fungal diversity was observed in autumn, followed by spring, summer, and lastly, winter (Table S2). This may be because endophytic fungi and their hosts are affected by environmental factors such as temperature and rainfall that vary with the season. Autumn in Guizhou is warm and humid, which could have affected endophytic fungi dispersal.

Endophytic fungi can increase host resistance to biotic stress by enhancing the plant defence system (Zabalgogezcoa 2008). The inhibitory activity of endophytic fungi against pathogenic plant fungi highlights their potential as biocontrol agents. Eight endophytic fungi isolated from *Zanthoxylum bungeanum* suppressed the growth of pathogenic fungi (*Fusarium sambucinum* and *Pseudocercospora zanthoxyli*) on the plant (Li et al. 2016). Of the 81 strains of endophytic fungi that were isolated from *Camellia oleifera*, five showed an inhibitory effect on anthracnose-causing pathogens (Yu et al. 2018). We found that 27 endophytic fungus species isolated from *P. bournei*—which accounted for 60% of the total—showed antimicrobial activity against at least one of the eight plant pathogenic fungi tested, with five of these (*T. longibrachiatum* 1-cg-18, *T. harzianum* 3-hcg-5, *T.*

387 *tomentosum* 3-ccg-1, *C. pseudohawaiiensis* 3-ccg-2 and *H. fragiforme* 3-ye-3) showing broad-range inhibition of
388 all eight pathogens (Table 1). In particular, the endophytic fungi exhibited a potent inhibitory effect against the
389 *P. bournei* leaf spot pathogen *P. microspora*. Thus, these isolates can potentially be used for the biological
390 control of leaf spot disease in *P. bournei*.

391 *Trichoderma* spp. are free-living fungi that are highly active in plant root and foliage and in the soil. Some
392 species are plant endophytic fungi that have potential applications as a biocontrol agent for plant diseases (Bailey
393 et al. 2008). For example, *Trichoderma citrinoviride* PG87 isolated from *Panax ginseng* was shown to exert
394 inhibitory activity against all host pathogens (Park et al. 2019), while *Hypoxylon* spp. can also inhibit pathogenic
395 fungi (Song et al. 2009; Tomscheck et al. 2010). *Cylindrocladiella* spp. are known saprobes although some
396 species are plant pathogens and there are reports of others that are plant endophytes. However, the biocontrol
397 potential of *Cylindrocladiella* spp. against plant pathogens has not been investigated (Mejía et al. 2008; Gond et
398 al. 2012).

399 Endophytic fungi inhabit the tissues of healthy plants at some point during their life cycle, without causing
400 pathologic stress to the host or obvious infection (Petrini 1991). According to this definition, endophytic fungi
401 can include pathogenic, saprophytic, and mycorrhizal fungi that do not harm the host at certain stages of their
402 life cycle; while this concept is controversial it is nonetheless widely recognized and accepted. Many
403 mycorrhizal fungi such as *Passalora*, *Uwebraunia*, *Houjia*, and *Pseudoveronaea* have been isolated from
404 *Lauraceae* plants that are exclusively pathogenic in other plant species (Zhou et al. 2021). Some pathogenic
405 *Fusarium* species were shown to act as mycorrhizal fungi in Orchidaceae (Jiang et al. 2019). Although the *P.*
406 *bournei* isolates included some pathogenic and saprophytic species, they did not cause obvious disease
407 symptoms during the period of sampling. In this study, some of the endophytic fungi of *P. bournei* were
408 themselves plant pathogens. For example, black rot of *Sedum morganianum* is caused by *Corynespora smithii*
409 (Schroers et al. 1999) and *Dalbergia odorifera* leaf blight is caused by *P. asparagi* (Samaga et al. 2014). Maize
410 seedling stalk rot mainly caused by *F. graminearum* is an important soil-borne disease that can result in
411 significant yield loss. Some pathogenic fungi present in healthy *P. bournei* do not cause disease, possibly
412 because the plants are in the latent stage of disease or because the fungi are only pathogenic in specific hosts
413 (Paul et al. 2013). Follow-up studies are required to determine whether these fungal species can cause disease in
414 *P. bournei*.

415 Endophytic fungi may directly or indirectly contribute to the fitness of host plants (Shah et al. 2018). It was
416 found that some endophytic fungal isolates promoted host growth via siderophore production, phosphate

solubilisation, and IAA and gibberellin secretion (Tsavkelova et al. 2006; Bilal et al. 2018). Other studies have demonstrated that IAA was produced by a plant's endophytic fungi and stimulated plant growth (Bilal et al. 2018; Qiang et al. 2019) and in one report, all 38 endophytic fungi associated with *G. glabra* were found to produce IAA (Arora et al. 2019). In our study, 9 endophytic fungi showed plant growth-promoting traits, accounting for 20% of all endophytic fungi isolated from *P. bournei*. *T. longibrachiatum* stimulated plant growth and enhanced plant tolerance to NaCl stress by producing IAA and 1-aminocyclopropane-1-carboxylate deaminase (Zhang et al. 2019). In the present study, the isolate 1-cg-18 (*T. longibrachiatum*) produced IAA and also demonstrated the ability to inhibit the growth of pathogenic fungi (Tables 1 and 2). The remaining three IAA-producing isolates—which belonged to the *Xylaria*, *Phomopsis*, and *Microdiplodia* genera—were reported to have antimicrobial activity (Shiono et al. 2015; Arora et al. 2019). Meanwhile, the biocontrol isolates (4-hby-3, 3-hcg-5, 1-cg-2, and 1-cg-18, 4-cg-4) clearly enhanced *P. bournei* seedling growth. Thus, endophytic fungi of *P. bournei* not only protect against fungal pathogens but also promote plant growth. Our findings provide insights into the functions of endophytic fungi in *P. bournei* that may have important implications for the protection of this endangered species.

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Figure captions

Figure 1. Phylogenetic tree constructed using the Neighbor-Joining method based on internal transcribed spacer (ITS)-rDNA gene sequences of endophytic fungi associated with *Pheobe bournei*. Eight blue labeled isolates were the important strains that showed antimicrobial activities or plant growth promoting activities in the following experiment. Bootstrap percentages (>50) determined using 1000 replications are shown.

Figure 2. Isolation rate (IR, %) of endophytic fungi at the genus level in spring (A), summer (B), autumn (C) and winter (D); and in the root (E), shoot (F), leaf (G) and total plant (H) of *Phoebe bournei*.

Figure 3. Biocontrol effect of biocontrol isolates on leaf spot disease of *P. bournei*. (A) Observation of biocontrol effect of different isolates on leaf spot *in vitro*; CK1 (positive control), inoculated with the pathogen *P. microspora* alone; CK2 (negative control), inoculated with sterile agar plugs alone. (B) Determination of the inhibition (%), different letters on the bars indicate significant differences ($P < 0.05$). 3-hcg-5, inoculated with *P. microspora* and 3-hcg-5 isolate; 3-ye-3, inoculated with *P. microspora* and 3-ye-3 isolate; 3-ccg-1, inoculated with *P. microspora* and 3-ccg-1 isolate; 1-cg-18, inoculated with *P. microspora* and 1-cg-18 isolate; 3-ccg-2, inoculated with *P. microspora* and 3-ccg-2 isolate.

Figure 4. The effect of different biocontrol isolates on *P. bournei* seedlings growth. (A) Analysis of seedlings growth on 90th day after five different biocontrol strains treatments. The photographs show representative seedlings. (B) Measurement of seedling height, (C) Measurement of fresh weight, (D) Measurement of dry weight, (E) Measurement of chlorophyll content, (F) Measurement of root length, (G) Measurement of root activity. Bars indicate the mean $\pm$ SE for each set of three independent experiments. Different letters above the bars indicate a significant difference at the 0.05 probability level according to the Duncan test.

Table 1 Antimicrobial activity of endophytic fungi from *Pheobe bournei* against the fungal pathogens *Colletotrichum nymphaeae*, *Fusarium petroliphilum*, *Colletotrichum acutatum*, *Penicillium chermesinum*, *Fusarium oxysporum*, *Fusarium solani*, *Pestalotiopsis microspora* and *Colletotrichum gloeosporioides*.

Isolate number	<i>Colletotrichum fragariae</i>	<i>Phytophthora capsici</i>	<i>Fusarium</i> sp.	<i>Penicillium</i>	<i>Colletotrichum capsici</i>	<i>Fusarium oxysporum</i>	<i>Pestalotiopsis microspora</i>	<i>Colletotrichum gloeosporioides</i>
3-bye-2	-	-	-	-	-	-	-	-
3-cxg-2	-	-	-	-	+	-	-	-
3-cye-2	-	-	+	-	-	-	-	-
4-cg-4	-	-	-	+	-	-	-	-
3-xj-4	-	-	-	-	-	-	-	-
1-ye-5	-	-	-	-	-	-	-	-
3-ye-3	+++	+	++	++	++	+	+	+
4-xg-3	-	-	-	-	-	-	-	-
1-cj-3	-	-	+	-	-	++	-	-
3-hxg-1	-	-	-	-	-	-	-	-
3-cby-1	-	-	-	-	-	-	-	-
1-cg-9	-	-	-	-	-	+	-	-
3-ccg-2	+	+	+	+	+	+++	+++	+++
3-hxg-6	-	-	-	-	-	-	-	-
4-cxg-3	-	-	-	-	-	-	-	-
4-cg-2	-	-	-	-	-	-	-	-
3-ccg-1	+	++	+++	+	+	+	+	++
3-hcg-5	+	+++	++	+	+	+	++	++
4-hxg-2	-	-	-	-	-	-	-	-
1-cg-18	+++	+++	++	+	++	++	+++	+
3-hby-20	-	-	-	-	-	++	-	-
3-xj-5	-	-	-	+	-	-	-	-
4-hby-4	-	-	-	-	-	-	-	-
2-dye-5	++	-	+	-	-	-	-	-
1-by4	-	-	-	-	-	-	-	-
1-cj-4	-	+	-	-	+	+	-	-
1-cg-1	-	-	-	-	-	-	-	-
1-xj-3	++	-	+	++	++	+++	-	-
3-cj-2	-	-	-	-	-	-	-	-
1-cg-13	-	-	+	-	-	-	-	-
1-cg-2	-	-	-	-	++	-	-	-
3-hcg-21	++	-	-	-	-	-	-	-
3-by-22	-	++	-	-	-	-	-	-
4-hye-7	-	-	-	-	-	++	-	-
4-hye-17	-	+	-	-	-	-	-	-
4-bye-2	-	-	-	-	-	-	-	-
4-bhye-5	+	-	-	-	-	++	-	-
3-hbye-3	-	+	-	++	-	-	-	-
4-hye-1	-	++	-	-	+	-	-	-
3-bye-10	+	-	-	-	-	+	-	-
3-hcg-3	-	-	-	-	-	-	-	-
3-cg-2	-	-	-	-	-	-	-	-
2-hye-3	-	-	-	++	-	-	-	-
4-hby-3	+	-	-	-	-	-	-	-

3-ccg-3

Note: +++ indicates strong inhibition ; ++ indicates medium inhibition; + indicates low inhibition ; — indicates no inhibition.

Table 2 Observation of plant growth-promotion traits of isolated fungal endophytes

Isolate number	Phosphate	Potassium dissociati	IAA production
3-cxg-2	—	—	—
3-cye-2	—	—	—
4-cg-4	+	—	+
3-ye-3	—	—	—
1-cj-3	—	—	—
1-cg-9	—	—	—
3-ccg-2	—	—	—
3-ccg-1	—	—	—
3-hcg-5	—	+	—
1-cg-18	+	+	+
3-hby-20	+	—	—
3-xj-5	+	—	—
2-dye-5	—	—	—
1-cj-4	—	+	+
1-xj-3	—	—	—
1-cg-13	+	+	—
1-cg-2	+	—	—
3-hcg-21	—	—	—
3-by-22	—	—	—
4-hye-7	—	—	—
4-hye-17	—	—	—
4-bhye-5	—	—	—
3-hbye-3	—	—	—
4-hye-1	—	—	—
3-bye-10	—	—	—
2-hye-3	—	—	—
4-hby-3	—	—	+

Note: IAA, indole acetic acid; +, positive; -, negative.

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