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Diversity Estimation and Antimicrobial Activity of Culturable Endophytic Fungi from *Litsea cubeba* (Lour.) Pers. in China

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Received: 6 December 2018; Accepted: 31 December 2018; Published: 6 January 2019

Abstract: Endophytes are important components of forest ecosystems, and have potential use in the development of medical drugs and the conservation of wild medicinal plants. This study aimed to examine the diversity and antimicrobial activities of endophytic fungi from a medicinal plant, *Litsea cubeba* (Lour.) Pers. The results showed that a total of 970 isolates were obtained from root, stem, leaf, and fruit segments of *L. cubeba*. All the fungal endophytes belonged to the phylum Ascomycota and could be classified into three taxonomic classes, nine orders, twelve families, and seventeen genera. SF15 (*Colletotrichum boninense*) was the dominant species in *L. cubeba*. Leaves harbored a greater number of fungal endophytes but lower diversity, while roots harbored the maximum species diversity of endophytic fungi. For the antimicrobial activities, seventeen isolates could inhibit the growth of plant pathogenic fungi, while the extracts of six endophytes showed antimicrobial activity to all the tested pathogenic fungi. Among these endophytes, SF22 (*Chaetomium globosum*) and SF14 (*Penicillium minioluteum*) were particularly effective in inhibiting seven plant pathogenic fungi growths and could be further explored for their potential use in biotechnology, medicine, and agriculture.

Keywords: endophytes; medicinal plants; pathogen; molecular identification; plant-microbe interaction

1. Introduction

The demand for new and useful compounds for disease prevention and control is ever growing [1]. Antibiotic resistance, the increasing incidence of fungal diseases, and the development of superbugs cause biodiversity loss and constantly bring challenges to the field of medicine [2,3]. Thus, there is an urgent need to find new antibiotics that are more effective, have lower toxicity, and a smaller environmental impact.

Forest ecosystems cover an area of approximately 38 million square kilometers and contain substantial resources [4,5]. Endophytes are an important component of the forest ecosystem, which inhabit the internal tissues of plants, have no detrimental effects on plants, and can sometimes improve plant growth performance [6,7]. Most of the natural compounds produced by endophytes have exhibited antimicrobial activity and, in many cases, these are related to the protection of the host from phytopathogenic microorganisms [8]. The endophyte *Beauveria bassiana* has been able to inhibit fungal pathogens by the production of bioactive metabolites [9]. The endophytic

fungus *Gliocladium catenulatum* can reduce the incidence of witches' broom disease in cacao by up to 70% [10]. Furthermore, some endophytic fungi can produce the same chemical compounds as the host, such as the paclitaxel producing fungus *Taxomyces andreanae* from *Taxus brevifolia* [11,12], and the podophyllotoxin generating fungus *Fusarium oxysporum* from *Juniperus recurva* [13]. There have been over 8600 discovered bioactive metabolites of fungal origin [14]. It is estimated that there are approximately 1 million fungal species of endophytic fungi in nature [15], whereas only a small percentage of endophytes have been discovered [16]. The enormous biodiversity and abundant fungal endophytes that occur in plant tissues show the potential role of endophytes in the production of novel natural antimicrobial compounds.

Litsea cubeba (Lour.) Pers. (Lauraceae) is a native woody species in China, Indonesia, and other countries in Southeast Asia [17]. It is a valuable traditional Chinese medicinal plant that has been used to treat rheumatic diseases, stomach aches, and common cold for thousands of years [18,19]. The active components of *L. cubeba* were reported to be antibacterial [20], anticancer [21], and anti-inflammatory [19]. Intercropping of *L. cubeba* and *Camellia oleifera* Abel. can reduce the incidence of *C. oleifera* disease, suggesting the role of *L. cubeba* in protecting economic plants from diseases. *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. [22], *Fusarium andiyazi* Marasas, Rheeder, Lampr., K.A. Zeller & J.F. Leslie [23], *Alternaria alternata* (Fr.) Keissl. [24], *Phomopsis* sp. [25], *Ceratosphaeria phyllostachydis* Zhang [26], *Rhizoctonia solani* Kühn [27], and *Phytophthora capsici* Leonian [28] cause diseases in main economic crops in South China, leading to a heavy decline in crop yield and quality. Currently, the associated microflora of medicinal plants is being paid increased amounts of attention for the exploitation of antimicrobial drugs [29]. However, to our knowledge, there are no reports on the biodiversity and bioactivity of endophytic fungi in *L. cubeba*. This study aimed to investigate the diversity and antimicrobial activities of endophytic fungi of *L. cubeba*, and, further, to screen them as potential biocontrol agents against seven plant pathogens.

2. Materials and Methods

2.1. Collection of Samples and Isolation of Endophytic Fungi

The leaves, branches, roots, and fruits of *Litsea cubeba* were collected from a planting base in Lichuan county of Jiangxi Province, China, in May 2016. The leaves and fruits samples were cut into small pieces of about 0.5 × 0.5 cm using a sterile knife, and the branch and root samples were cut into small segments 1 cm in length. These fragments were surface sterilized with 70% (v/v) ethanol for 3 min, 3% (v/v) NaClO for 3–5 min, and then rinsed with sterile water four times. Excess moisture was blotted by sterile filter papers [30]. Then, they were cultured on potato dextrose agar (PDA) medium supplemented with streptomycin (50 U/mL) and penicillin (30 U/mL) at 25 °C under dark conditions for 7–15 days. Pure fungal cultures were obtained by picking hyphal tips of the developing fungal colonies. The acquired isolates were preserved on PDA slants and deposited at 4 °C for identification.

2.2. Genomic DNA Extraction, PCR Amplification and Molecular Identification

The isolates were first identified based on the morphological characteristics of the colony culture and spores. Fungal genomic DNA was extracted from the mycelia using an Ezup Column Fungi Genomic DNA Purification Kit (Sangon Biotech, Inc., Shanghai, China) according to the manufacturer's protocol. The internal transcribed spacer (ITS) regions were amplified using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [31]. The reaction mixtures (50 µL) contained 25 µL 2 × Taq PCR Master mixture (Sangon Biotech, Inc., Shanghai, China), 2 µL of ITS4, 2 µL of ITS5, 2 µL of Template DNA, and 19 µL of ddH_2O. The reaction conditions were 94 °C for 5 min, 30 cycles at 94 °C for 50 s, 52 °C for 50 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min. The PCR products were examined by electrophoresis in 1% (w/v) agarose gels and then purified using the Agarose Gel DNA Extraction Kit (Takara, Japan) and sequenced.

The resultant sequences were compared with previously deposited sequences in the GenBank, NCBI (http://www.ncbi.nlm.nih.gov) using a basic local alignment search tool (BLAST). Sequence alignment and phylogenetic analysis were conducted using MEGA version 7 [32]. Phylogenetic trees were constructed using a neighbor-joining method. The ITS gene sequences of the potential novel isolates were deposited in GenBank under the accession numbers MF962537–MF962573.

2.3. Estimation and Quantification of Fungal Diversity

Fungal diversity and richness in different plant tissues were measured and quantified using various indices, including the colonization rate (*CR*), isolation rate (*IR*), and Shannon-Wiener (*H'*), Simpson's (D_s) diversity index and evenness index (*E*). The calculations were as follows.

$$CR = Nf/Nt \times 100, \quad (1)$$

$$IR = Ng/Nt \times 100, \quad (2)$$

$$H' = -\sum Pi \times \mathrm{Ln}(Pi), \quad (3)$$

$$D_s = 1 - \Sigma Pi^2, \quad (4)$$

$$E = H'/\mathrm{Ln}(S), \quad (5)$$

where *Nf* was the number of fragments with fungal growth, *Nt* was the total number of fragments, and *Ng* was the number of isolates of a given type isolated [33]. $Pi = n_i/N$, is the relative abundance of the endophytic fungal species, n_i is the number of isolates of one species, and *N* is the total species number of isolates [34,35]. *S* was the total number of the taxa (ITS genotype) present within each sample [16].

2.4. Antimicrobial Activity of Endophytic Fungi

The indicator strains include the following plant pathologens: the fungi *Colletotrichum gloeosporioides*, *F. andiyazi*, *A. alternata*, *Phomopsis* sp., *Ceratosphaeria phyllostachydis*, *R. solani*, and the Chromista *Phytophthora capsici*, provided by the Plant Pathology Laboratory, College of Forestry, Jiangxi Agricultural University, China.

A dual culture technique was applied to examine the antimicrobial activity of endophytic fungi from *L. cubeba* against fungal pathogens [36]. The mycelial discs (6 mm in diameter) of actively growing endophytes were placed at the periphery of the PDA plate. The mycelial discs of the pathogen were placed on the other side of the PDA plate, 4 cm away from the endophyte disc. The plate with only the pathogen was used as a control. Each treatment replicated 3 times. The dual culture plates were incubated for 3–8 days at 25 °C. The inhibition rate against pathogens was calculated according to the formula below.

$$\text{Inhibition rate (\%)} = (R_1 - R_2)/(R_1 - 0.6) \times 100, \quad (6)$$

where R_1 is the colony diameter of the control, R_2 is the colony diameter under experimental treatments, and 0.6 mm represents the mycelial discs.

The endophytes with high antimicrobial activity were selected and investigated for the in vitro antimicrobial activity of their extracts. Each of the endophytes were separately cultured on 200 mL PDA liquid medium at 25 °C, by shaking at 150 rpm for 8–12 days. The culture broth was collected by filtration and extracted with an equal amount of ethyl acetate three times. The organic phase was evaporated to dryness using a rotary evaporator. The dry extract was dissolved in 3 mL of methanol and formulated into 15 μg/mL of mycelia broth.

In vitro antimicrobial tests were conducted by testing the growth rate of the pathology fungi. The mycelial discs (6 mm in diameter) of the pathogen were placed in the center of the PDA plate containing 1.5 mL mycelia broth. The PDA plate without mycelia broth (containing only 1.5 mL methanol) was used as the control. The tested plates were cultured at 25 °C for 3–7 days. The formula for calculating the inhibition rate is the same as Formula (6).

2.5. Statistical Analyses

Statistical tests were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Turkey's multiple range test was used to pairwise multiple comparisons between treatments.

3. Results

3.1. Identification and Composition of Endophyte Assemblage

A total of 970 isolates were obtained from root, stem, leaf, and fruit segments of *L. cubeba* (Table 1). The maximum number of isolates was obtained from the leaves (438 isolates), followed by stems (241 isolates), fruits (149 isolates), and roots (142 isolates). Molecular identification of the isolates was conducted based on a comparative analysis of ITS gene sequences and their similarity to reference sequences (Figure 1). The results showed that the isolated endophytic fungi could be allocated to 36 operational taxonomic units (OTUs). All of them belonged to the Ascomycota phylum and were classified into three taxonomic classes (Eurotiomycetes, Dothideomycetes, and Sordariomycetes), nine orders (Eurotiales, Botryosphaeriales, Pleosporales, Hypocreales, Chaetosphaeriales, Sordariales, Diaporthales, Xylariales, and an unassigned order), twelve families and seventeen genera. Twenty-three fungal morphotypic groups were taxonomically assigned to species, and the other 13 were classified at the genus level (Table 1). SF15 (*Colletotrichum boninense*) accounted for 39.79% of the total isolates and was the dominant species in the whole fungal endophytic community, followed by SF4 (*Botryosphaeria dothidea*) (6.60%).

3.2. Diversity Estimation of Endophytic Fungi

The biodiversity of endophytic fungi in *L. cubeba* was quantitatively investigated in terms of the colonization rate (*CR*), isolation rate (*IR*), Shannon-Wiener (H'), and Simpson's (D_s) diversity index and evenness index (E) (Table 2). The total H' and D_s were 2.52 and 0.82, respectively. The highest biodiversity of endophytic fungi was observed in roots (H' = 2.74, D_s = 0.90), followed by stems (H' = 2.56, D_s = 0.90), fruits (H' = 1.99, D_s = 0.76), and leaves (H' = 1.43, D_s = 0.56). The leaf samples had the highest endophytic fungi colonization rate but the lowest species evenness (E = 0.51) compared to the other plant parts.

3.3. In Vitro Antimicrobial Activity of Endophytic Fungi

The results of dual culture experiments showed that 17 isolates inhibited the growth of pathogenic fungi, which was manifested by the occurrence of the inhibition zone or mycelial atrophy of pathogens (Table 3). Among them, 10 isolates exhibited antibiotic effects on all the tested pathogenic microbes. SF22 (*Chaetomium globosum*) showed the strong activity against *Ceratosphaeria phyllostachydis*, *Phomopsis* sp., and *Alternaria alternata*, with inhibition rates of 78.43, 73.20, and 70.23%, respectively.

The results of the antimicrobial test on the fermentation products support that the fermentation products of SF14, SF22, SF23, SF27, SF29 and SF32 showed antimicrobial activity against all the tested pathogen fungi (Table 4). The antimicrobial activity of the fermentation products was stronger than the endophytic fungi. The inhibition rate of SF22 (*Chaetomium globosum*) extracts against *Ceratosphaeria phyllostachydis* was 93.24%. The inhibition rate of SF14 (*Penicillium minioluteum*) extracts against *Phomopsis* sp. was 87.87%. The inhibition rates of the fermentation products of these two isolates against the other six pathogens were over 60%.

Table 1. Identification, abundance, and percentage recovery of endophytic fungi isolated from different tissues of *Litsea cubeba* (Lour.) Pers.

Endophytic Fungal Taxon	Isolate Codes	Accession Numbers	The Closet Genbank Taxa	Similarity (%)	Numbers of Isolates from Plant Tissues				Total Abundance (Percentage Recovery)
					Roots	Stems	Leaves	Fruits	
Aspergillus fumigatus 1	SF25	MF962555	*Aspergillus fumigatus* (KP131566.1)	99	6	0	0	0	0.62
Aspergillus fumigatus 2	SF48	MF962572	*Aspergillus fumigatus* (EU833205.1)	99	1	0	0	0	0.10
Botryosphaeria dothidea	SF4	MF962539	*Botryosphaeria dothidea* (FJ478129.1)	99	1	46	11	6	6.60
Clonostachys sp.	SF31	MF962561	*Clonostachys* sp. (LC133855.1)	99	5	2	3	0	1.03
Calonectria curvispora	SF39	MF962566	*Calonectria curvispora* (GQ280568.1)	99	25	2	0	0	2.78
Chaetomium globosum	SF22	MF962552	*Chaetomium globosum* (KM268652.1)	99	1	0	1	0	0.21
Colletotrichum boninense	SF15	MF962548	*Colletotrichum boninense* (MF076585.1)	99	0	1	3	0	0.41
Colletotrichum gloeosporioides 1	SF40	MF962567	*Colletotrichum gloeosporioides* (EU552111.1)	99	2	32	284	68	39.79
Colletotrichum gloeosporioides 2	SF3	MF962538	*Colletotrichum gloeosporioides* (KU534983.1)	99	4	0	41	4	5.06
Diaporthe phaseolorum 1	SF8	MF962542	*Diaporthe phaseolorum* (KX866868.1)	99	7	37	18	4	6.80
Diaporthe phaseolorum 2	SF45	MF962570	*Diaporthe phaseolorum* (AF001018.2)	99	0	0	0	9	0.93
Diaporthe eres	SF30	MF962560	*Diaporthe eres* (KX866867.1)	97	0	11	0	0	1.13
Diaporthe sp.	SF34	MF962563	*Diaporthe* sp. (EF42278.1)	97	0	7	0	0	0.72
Fusarium graminearum	SF35	MF962564	*Fusarium graminearum* (KF624778.1)	99	5	4	11	10	3.09
Nigrospora sphaerica	SF13	MF962546	*Nigrospora sphaerica* (KM510416.1)	100	0	0	1	6	0.72
Nemania diffusa	SF10	MF962543	*Nemania diffusa* (KP133219.1)	99	3	4	0	0	0.72
Phomopsis sp.1	SF5	MF962540	*Phomopsis* sp. (KP184328.1)	99	0	8	29	0	3.82
Phomopsis sp.2	SF7	MF962541	*Phomopsis* sp. (JX436795.1)	98	14	12	10	13	5.05
Phomopsis sp.3	SF21	MF962551	*Phomopsis* sp. (AB505410.1)	97	3	3	0	4	1.03
Phomopsis sp.4	SF38	MF962565	*Phomopsis* sp. (HQ832822.1)	99	1	22	0	0	2.37
Phomopsis sp.5	SF44	MF962569	*Phomopsis* sp. (HM595506.1)	99	0	0	0	9	0.93
Phomopsis fukushii	SF11	MF962544	*Phomopsis fukushii* (KT951302.1)	97	1	0	2	1	0.41
Phyllosticta capitalensis	SF1	MF962537	*Phyllosticta capitalensis* (KR056285.1)	100	1	15	9	9	3.51
Pestalotiopsis sp.1	SF24	MF962554	*Pestalotiopsis* sp. (HQ607806.1)	99	0	9	0	0	0.93
Pestalotiopsis sp.2	SF46	MF962571	*Pestalotiopsis* sp. (HE608797.1)	99	2	0	0	0	0.21
Pestalotiopsis sp.3	SF49	MF962573	*Pestalotiopsis* sp. (EF423541.1)	100	1	0	0	5	0.62
Pestalotiopsis disseminata	SF28	MF962558	*Pestalotiopsis disseminate* (JQ323000.1)	99	3	13	0	1	0.62
Pestalotiopsis vismiae	SF12	MF962545	*Pestalotiopsis vismiae* (KM015217.1)	99	2	1	3	0	1.75
Penicillium rubens	SF18	MF962550	*Penicillium rubens* (LT558865.1)	100	0	1	0	0	0.10
Penicillium janthinellum	SF27	MF962557	*Penicillium janthinellum* (KM268648.1)	99	29	0	0	0	2.99
Penicillium citrinum	SF32	MF962562	*Penicillium citrinum* (LT558897.1)	100	5	0	0	0	0.52
Penicillium minioluteum	SF14	MF962547	*Penicillium minioluteum* (L14505.1)	99	3	0	0	0	0.31
Phoma sp.	SF26	MF962556	*Phoma* sp. (HQ631000.1)	99	5	9	11	0	2.58
Thozetella sp.	SF16	MF962549	*Thozetella* sp. (KU059840.1)	96	1	1	0	0	0.21
Talaromyces sp.	SF23	MF962553	*Talaromyces* sp. (KU556510.1)	99	3	0	0	0	0.31
Talaromyces amestolkiae	SF29	MF962559	*Talaromyces amestolkiae* (LT558956.1)	99	8	1	1	0	1.03
Total					142	241	438	149	100.00

Table 2. The Index of endophytic fungi flora diversity of *Litsea cubeba* (Lour.) Pers.

Parts	No. of Tissue	No. of Fungi	No. of Strains	No. of Genus	*CR* %	*IR* %	*H′*	D_s	*E*
Root	450	142	142	27	32.22	31.56	2.74	0.90	0.83
Stem	450	232	241	21	51.56	53.56	2.56	0.90	0.84
Leaf	450	413	438	16	91.78	97.33	1.43	0.56	0.51
Fruit	450	139	149	14	30.89	33.11	1.99	0.76	0.75
Total	1800	926	970	36	51.44	53.89	2.52	0.82	0.70

Abbreviations: No.: number; *CR*: colonization rate; *IR*: isolation rate; *H′*: Shannon-Wiener diversity index; D_s: Simpson's diversity index; *E*: evenness index.

Table 3. Antimicrobial activities of endophytic fungi from *Litsea cubeba* (Lour.) Pers.

No.	Endophytic fungi	Inhibition Ratio of Pathogen Mycelium Growth (%)						
		①	②	③	④	⑤	⑥	⑦
SF11	*Phomopsis fukushii*	$46.15 \pm 0.33^{W-}$	$55.56 \pm 0.53^{W-}$	$45.91 \pm 0.71^{W+}$	$29.52 \pm 0.32^{W+}$	$46.56 \pm 0.52^{W-}$	$55.35 \pm 1.61^{W+}$	—
SF14	*Penicillium minioluteum*	$39.23 \pm 0.27^{W+}$	$58.82 \pm 0.28^{W+}$	$52.20 \pm 0.21^{W+}$	$60.95 \pm 0.41^{W-}$	$52.67 \pm 0.47^{W+}$	$53.46 \pm 0.31^{W+}$	$55.49 \pm 0.37^{W+}$
SF22	*Chaetomium globosum*	$58.61 \pm 0.44^{W+}$	$73.43 \pm 0.43^{W+}$	$53.12 \pm 0.14^{W+}$	$70.23 \pm 0.30^{W+}$	$73.20 \pm 0.24^{W+}$	$78.43 \pm 0.49^{W+}$	$56.60 \pm 0.35^{W+}$
SF23	*Talaromyces* sp.	$42.31 \pm 0.27^{W+}$	$64.05 \pm 0.18^{W+}$	$49.06 \pm 0.21^{W+}$	$42.86 \pm 0.24^{W+}$	$55.73 \pm 0.27^{W+}$	$52.83 \pm 0.28^{W+}$	$52.44 \pm 0.34^{W-}$
SF24	*Pestalotiopsis* sp.1	$46.92 \pm 0.25^{W+}$	$52.94 \pm 0.31^{W-}$	$56.60 \pm 0.29^{W+}$	$68.57 \pm 0.43^{W-}$	$57.25 \pm 0.14^{W+}$	$50.31 \pm 0.28^{W+}$	$39.63 \pm 0.39^{W-}$
SF27	*Penicillium janthinellum*	$38.46 \pm 0.41^{W+}$	$39.22 \pm 0.20^{W+}$	$47.17 \pm 0.41^{W-}$	$31.43 \pm 0.40^{W-}$	$53.44 \pm 0.32^{W+}$	$40.88 \pm 0.37^{W+}$	$35.37 \pm 0.21^{W+}$
SF28	*Pestalotiopsis disseminata*	$50.00 \pm 0.23^{W+}$	$58.17 \pm 0.21^{W+}$	$57.23 \pm 0.27^{W+}$	$39.05 \pm 0.21^{W-}$	$45.04 \pm 0.21^{W-}$	$48.43 \pm 0.31^{W-}$	$25.61 \pm 0.37^{W-}$
SF29	*Talaromyces amestolkiae*	$60.77 \pm 0.43^{W-}$	$78.43 \pm 0.18^{W-}$	$60.38 \pm 0.37^{W-}$	$59.05 \pm 0.19^{W+}$	$73.28 \pm 0.27^{W-}$	$64.78 \pm 0.42^{W+}$	$51.22 \pm 0.43^{W-}$
SF31	*Clonostachys sp.*	$33.08 \pm 0.38^{W+}$	$47.06 \pm 0.35^{W+}$	$48.43 \pm 0.41^{W+}$	$28.57 \pm 0.33^{W-}$	$32.06 \pm 0.25^{W-}$	—	—
SF32	*Penicillium citrinum*	$50.77 \pm 0.45^{W+}$	$62.75 \pm 0.29^{W+}$	$61.01 \pm 0.32^{W+}$	$39.05 \pm 0.21^{W+}$	—	—	$80.49 \pm 0.27^{W-}$
SF35	*Fusarium graminearum*	$36.92 \pm 0.43^{W+}$	$49.67 \pm 0.33^{W+}$	$53.46 \pm 0.31^{W+}$	$37.14 \pm 0.26^{W+}$	$49.62 \pm 0.28^{W+}$	$40.88 \pm 0.36^{W-}$	$32.93 \pm 0.17^{W-}$
SF39	*Calonectria curvispora*	$45.38 \pm 0.25^{W+}$	—	—	—	$70.13 \pm 0.19^{W+}$	$59.12 \pm 0.18^{W-}$	—
SF44	*Phomopsis* sp.5	$50.77 \pm 0.40^{W+}$	$71.42 \pm 0.25^{W+}$	$57.86 \pm 0.32^{W-}$	$40.95 \pm 0.44^{W-}$	$49.62 \pm 0.36^{W+}$	$55.35 \pm 0.23^{W+}$	—
SF49	*Pestalotiopsis* sp.3	$46.92 \pm 0.24^{W+}$	$70.21 \pm 0.35^{W+}$	$56.60 \pm 0.35^{W+}$	$52.38 \pm 0.33^{W-}$	$51.91 \pm 0.39^{W+}$	$57.23 \pm 0.31^{W+}$	—

Note: Data presented are the means ± SD (n = 3). ① *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.; ② *Phytophthora capsici* Leonian; ③ *Fusarium andiyazi* Marasas, Rheeder, Lampr., K.A. Zeller & J.F. Leslie; ④ *Alternaria alternata* (Fr.) Keissl.; ⑤ *Phomopsis* sp.; ⑥ *Ceratosphaeria phyllostachydis* Zhang; ⑦ *Rhizoctonia solani* Kühn; $^{-}$: No inhibition zone; $^{+}$: Inhibition zone; w: Pathogen hyphae shrink; –: No inhibition.

Table 4. Antimicrobial activity of the metabolites of endophytic fungi from *Litsea cubeba* (Lour.) Pers.

No.	Endophytic fungi	Inhibition Ratio of Pathogen Mycelium Growth (%)						
		①	②	③	④	⑤	⑥	⑦
SF14	*Penicillium minioluteum*	76.32 ± 1.35^{aB}	75.21 ± 1.63^{bBC}	69.00 ± 2.40^{aC}	61.08 ± 1.85^{bD}	87.87 ± 1.97^{aA}	84.73 ± 4.03^{aA}	60.01 ± 3.03^{bD}
SF22	*Chaetomium globosum*	66.18 ± 3.98^{bC}	91.73 ± 1.67^{aA}	66.67 ± 3.39^{aC}	77.60 ± 2.72^{aB}	80.00 ± 6.25^{abB}	93.24 ± 2.10^{aA}	61.00 ± 3.82^{bC}
SF23	*Talaromyces* sp.	79.34 ± 3.22^{aAB}	55.10 ± 0.85^{dD}	37.33 ± 1.31^{bF}	45.21 ± 3.25^{cE}	80.63 ± 3.55^{abA}	65.00 ± 2.19^{bC}	73.60 ± 1.36^{aB}
SF27	*Penicillium janthinellum*	80.66 ± 2.80^{aA}	71.07 ± 3.87^{bcAB}	29.87 ± 6.01^{bcE}	42.16 ± 3.55^{cDE}	74.80 ± 4.02^{bAB}	56.08 ± 8.85^{bcCD}	64.78 ± 3.76^{bBC}
SF29	*Talaromyces amestolkiae*	27.37 ± 4.30^{dCD}	64.19 ± 6.58^{cdA}	25.33 ± 6.73^{cD}	46.73 ± 6.43^{cB}	50.65 ± 4.27^{cAB}	45.00 ± 5.53^{cdB}	42.14 ± 3.30^{cBC}
SF32	*Penicillium citrinum*	50.34 ± 3.70^{cB}	60.24 ± 5.04^{dA}	61.23 ± 2.53^{aA}	42.23 ± 3.57^{cBC}	32.76 ± 1.55^{dD}	33.78 ± 2.70^{dCD}	45.12 ± 2.91^{cB}

Note: Data presented are the means ± SD ($n = 3$). Means followed by the same lowercase letters within a column and by the same uppercase letters within a row do not differ significantly at $p \leq 0.05$ according to Turkey's test. ① *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.; ② *Phytophthora capsici* Leonian; ③ *Fusarium andiyazi* Marasas, Rheeder, Lampr., K.A. Zeller & J.F. Leslie; ④ *Alternaria alternata* (Fr.) Keissl.; ⑤ *Phomopsis* sp.; ⑥ *Ceratosphaeria phyllostachydis* Zhang; ⑦ *Rhizoctonia solani* Kühn.

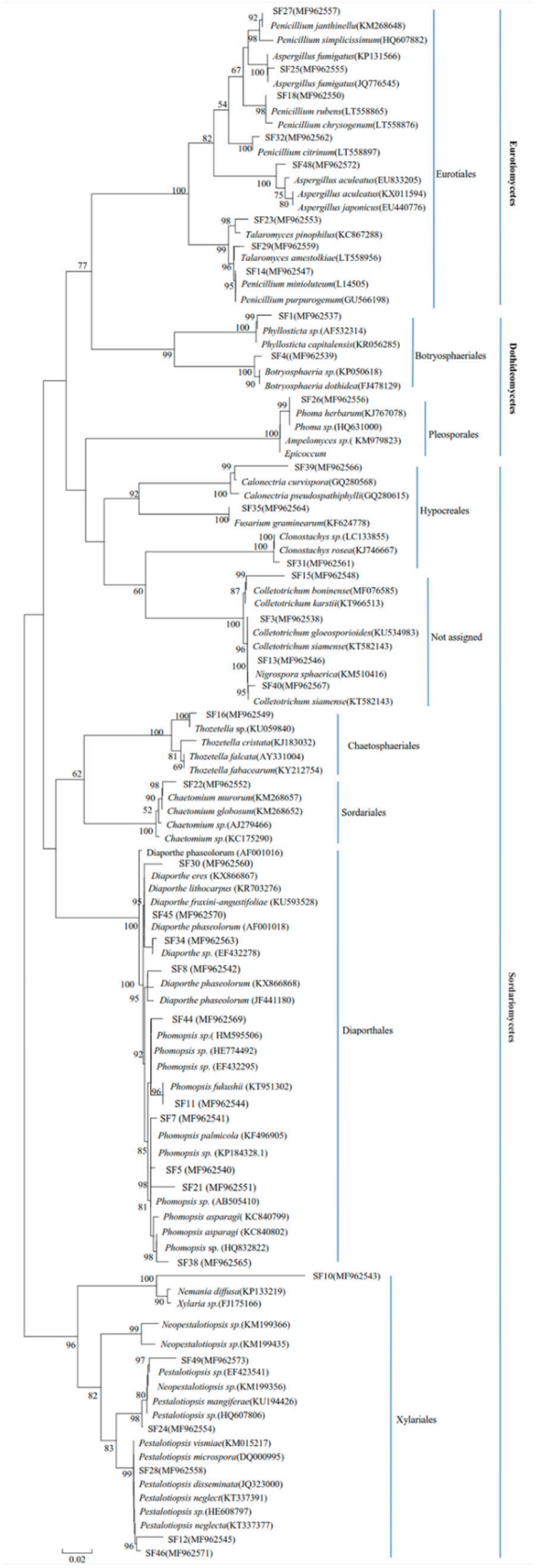

Figure 1. Neighbor-joining phylogenetic tree based on internal transcribed spacer (ITS)–rDNA gene sequences of endophytic fungi associated with *Litsea cubeba* (Lour.) Pers. Bootstrap percentages (>50) after 1000 replications are shown.

4. Discussion

Medicinal plants are legitimate targets to isolate endophytic fungi for their role in producing pharmacologically important secondary metabolites [37]. These fungal endophytes can be used to treat plant diseases. This is the first study that demonstrates the diversity, phylogeny, and bioactive potential of endophytic fungi associated with a medicinal plant, *L. cubeba*. In this study, all the fungal isolates were identified as Ascomycota, which is consistent with previous findings on *Ophiopogon japonicas* [38], *Calotropis procera* [39], and *Cannabis sativa* [35]. It is estimated that the phylum Ascomycota covers about 8% of the Earth's land and is among the most prevalent and diverse phyla of eukaryotes [37,40]. Endophytic fungi are ubiquitously distributed thoughout various classes of Ascomycota, including Eurotiomycetes, Dothideomycetes, Leotiomycetes, Pezizomycetes, and Sordariomycetes [6,41]. Katoch et al. [37] observed that the endophytic fungi in *Monarda citriodora*, a medicinal plant, were mainly distributed in the Sordariomycetes class, followed by Eurotiomycetes and Dothideomycetes. A similar presentation of classes was found in this study, indicating that endophytic fungi isolated in this study were cosmopolitan endophytes.

The fungal endophytes discovered in *L. cubeba* in this study were not identical to those reported in other studies. Ho et al. (2012) [42] isolated endophytic fungi from twigs of seven medicinal herbs belonging to the Lauraceae family (including *L. cubeba*) and found that the endophytes from *L. cubeba* belonged to six genera (*Pestalotiopsis*, *Arthrinium*, *Diaporthe*, *Xylaria*, *Hypoxylon*, and *Pyrenochaeta*). Only two genera (*Pestalotiopsis* and *Diaporthe*) were consistent with the results of the present study. This may due to the differences in sites, seasons, and climates [6].

The variation in endophytic communities was also found in spatial distribution. The endophytic community in *L. cubeba* exhibited tissue specificity. A similar phenomenon was also observed in *Dendrobium officinale* [16], which may be caused by the different external environments or by the biological differences among tissues and organs [6]. Microorganisms in the environment usually show low diversity and low abundance compared with the soil [43]. The results of the present study support this point that roots harbor the maximum species diversity of endophytic fungi. Leaves harbor a greater number of fungal endophytes but with a lower diversity than other plant samples. This may be because the large surface area and the presence of stomata in leaves exposed to the external environment provide access for the entry of fungal mycelium, so that leaves may harbor a greater number of endophytic fungi [36]. However, the substantial organic compounds in leaves were largely inaccessible to foliar microorganisms, and microorganisms may present in the leaves in the form of co-metabolism, thus limiting the diversity of endophytic fungi in leaves [4,44,45].

Some fungal endophytes have been considered as beneficial mutualisms in protecting the host from pathogens [46]. In this study, the fungal endophytes were investigated for antifungal activity using a dual culture method. The results showed that 17 isolates inhibited the growth of plant pathogenic fungi. SF22 (*Chaetomium globosum*) showed strongest anti-pathogen activity. Previous studies demonstrated that some endophytic fungi could produce metabolites with antimicrobial function [6,37]. The endophytic extracts were screened for antifungal activity, and the results indicate that there were six endophytes exhibiting strong anti-pathogen activity. The extracts of SF22 (*C. globosum*) and SF14 (*Penicillium minioluteum*) were particularly effective in inhibiting pathogen growth. The dominant fungi, SF15 (*Colletotrichum boninense*), was less efficacious, though previous studies reported that *Colletotrichum* sp. showed a broad range of antifungal activity [47]. This phenomenon showed that there was no direct relationship between antifungal activity and fungal colonization rate [36]. *Chaetomium globosum* was reported to have disease control capacity by producing chaetoviridins and chaetoglobosin [48,49]. The application of the culture filtrates of *C. globosum* to maize showed efficacy in the inhibition of northern corn leaf blight [48]. *Penicillium* sp. was also reported to be efficacious against plant pathogenic fungi [50] and, interestingly, *P. minioluteum* attracted more attention for its beneficial effects on plant stress tolerance [51]. The growth inhibitory activity against plant pathogenic fungi by these endophytes indicates that endophytic fungi have the potential to be used as biocontrol agents in the future.

5. Conclusions

This study is the first to investigate the diversity of endophytic fungi in *L. cubeba*. The results demonstrated that *L. cubeba* harbors a rich fungal endophytic community with antimicrobial activities. SF22 (*C. globosum*) and SF14 (*P. minioluteum*) were found to have anti-pathogenic fungi properties and, thus, could be sources of novel natural antimicrobial compounds. Meanwhile, the results highlighted the potential use of endophytes in the development of drugs and the conservation of medicinal plants.

Author Contributions: L.Z. designed the study; D.Y. and Y.C. carried out the experiment and analyzed the data. F.W. wrote the first draft of the manuscript; F.W., D.Y., L.Z., Y.C., X.H., L.L. and J.L. contributed with suggestions and corrections, and approved the final manuscript. F.W. and D.Y. contributed equally to this work.

Funding: This work was supported by the National Natural Science Foundation of China [grant numbers 31660189, 31570594], and Hunan Provincial Natural Science Foundation of China (2018JJ2217, 2018JJ3281).

Acknowledgments: The authors thank Key Laboratory of State Forestry Administration on Forest Ecosystem Protection and Restoration of Poyang Lake Watershed (JXAU) for providing experimental equipment support.

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

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