Diversity and Antimicrobial Activity of Culturable Endophytic Fungi Isolated from Moso Bamboo Seeds

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Abstract

Bamboos, regarded as therapeutic agents in ethnomedicine, have been used to inhibit inflammation and enhance natural immunity for a long time in Asia, and there are many bamboo associated fungi with medical and edible value. In the present study, a total of 350 fungal strains were isolated from the uncommon moso bamboo (Phyllostachys edulis) seeds for the first time. The molecular diversity of these endophytic fungi was investigated and bioactive compounds were screened for the first time. All the fungal endophytes were categorized into 69 morphotypes according to culturable characteristics and their internal transcriber spacer (ITS) regions were analyzed by BLAST search with the NCBI database. The fungal isolates showed high diversity and were divided in Ascomycota (98.0%) and Basidiomycota (2.0%), including at least 19 genera in nine orders. Four particular genera were considered to be newly recorded bambusicolous fungi, including Leptosphaerulina, Simplicillium, Sebacina and an unknown genus in Basidiomycetes. Furthermore, inhibitory effects against clinical pathogens and phytopathogens were screened preliminarily and strains B09 (Gladosporium sp.), B34 (Curvularia sp.), B35 (undeﬁned genus 1), B38 (Penicillium sp.) and zzz816 (Shiraia sp.) displayed broad-spectrum activity against clinical bacteria and yeasts by the agar diffusion method. The crude extracts of isolates B09, B34, B35, B38 and zzz816 under submerged fermentation, also demonstrated various levels of bioactivities against bambusicolous pathogenic fungi. This study is the first report on the antimicrobial activity of endophytic fungi associated with moso bamboo seeds, and the results show that they could be exploited as a potential source of bioactive compounds and plant defense activators. In addition, it is the first time that strains of Shiraia sp. have been isolated and cultured from moso bamboo seeds, and one of them (zzz816) could produce hypocrellin A at high yield, which is significantly different from the other strains published.

Introduction

Bamboos are well-known for their therapeutical effects and potential health benefits. They are used as bioactive agents for a variety of applications, including bamboo charcoal (hintoncha), bamboo vinegar, bamboo juice, bamboo beer, bamboo salt, and tender shoot used in Chinese cuisine. There are also many traditional drugs associated with bamboos for treating fever and detoxification which have been used in Indian Ayurvedic medicine and Chinese herbal medicine since ancient times.

Moso bamboo (Phyllostachys edulis (Carr.) H. De Lahaie), a member of Bambusoideae (Poaceae), is one typical vegetative, monopodial bamboo species, and native to the subtropics of China. Because of giant size, high production, various uses and wide distribution, it has long been considered as the most important economic bamboo species in China. However, there is a considerable ecological problem in moso bamboo, as their flowers appear only once every 60–120 years, followed by the death of the flowered culms [1]. Sexual propagation plays a vital part in the sustainable production of moso bamboo, but the seeds are uncommon and have a low germination rate [2,3]. In particular, seed germination of moso bamboo is often associated with high fungal contamination [4] and some fungal endophytes have serious negative effects on the seed survival in tissue culture [5]. It has been demonstrated that bamboo seeds colonized by field and storage fungi, could be a source of potential pathogens, which might pose problems in nurseries [6,7].

Endophytic fungi colonize almost all plants and have been isolated from all plant parts such as roots, stems, leaves, barks, floral organs and even seeds [8–11]. The relationships between the endophytic fungi and their hosts may be saprophytic, pathogenic or even mutualistic [12,13]. Diverse endophytes have been investigated in seeds of several hosts, of which most (>90%) belong to Dothideomycetes and Sordariomycetes [8,14]. The seed-associated fungal endophytes were usually implicated in assisting seeds in germination of seed pods but only for a short time due to weather conditions and posing problems in nurseries [3,15]. Fungal endophytes in the tissues of bamboos have also been identified as species from Dothideomycetes and Sordariomycetes, and their molecular diversity has been analyzed based on internal

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transcriber spacer (ITS) region sequences of the ribosomal DNA [16,17]. However, the seed-associated endophytes from moso bamboo have not yet been investigated.

Some bamberculous fungi also have medicinal effects, similar to their host’s or even more effective. *Polyporus nyssae Cooke. et Mass.*, *Ganoderma lucidum* (Batsch) G. F. Atk. and *Dictyophora indusiata* (Vent.) Desv. are all well-known edible macrofungi [18], and have been used as ‘herbal’ treatments for various human diseases in China for over 1000 years [19–21]. Cytochalasin C and hypocrellins from *Engleromyces sinensis* have been used as ‘herbal’ treatments for various human diseases in China for over 1000 years [19–21]. Cytochalasin C and hypocrellins from *Engleromyces sinensis* (Vent.) Desv. are all well-known edible macrofungi [18], and have been used as ‘herbal’ treatments for various human diseases in China for over 1000 years [19–21].

The aims of the present study were firstly to produce a sequence-based estimate of the diversity of cultivable endophytes in moso bamboo seeds and their isolation frequencies. In addition, the bioactivities of these fungi against pathogenic microorganisms were investigated and the effective metabolites from these endophytes were examined.

**Materials and Methods**

In our study, the materials are only referred to the collection of moso bamboo seeds and no specific permissions are needed for the process. There are three noticeable reasons for this case:

1. Moso bamboo would die after flowering, so the traditional method was to collect seeds from the flowered plants;
2. Moso bamboo is a typical vegetative bamboo species, and because of high production, various purposes and wide distribution, it has long been considered as the most important economic bamboo species in China. Moso bamboo is not endangered or protected species in our country.
3. There are large-area artificial forests of stock plant transplantation for moso bamboo in China, so the seed collection didn’t need specific permissions.

**Collection of Seeds and Isolation of Fungal Endophytes**

Fresh and healthy seeds were collected from moso bamboo in one plantation (110°17′~110°47′ E, 25°04′~25°48′ N) in Guilin City in the Guangxi Zhuang Autonomous Region in China. More than 100 seeds were randomly selected for fungal isolation. There are three notable reasons for this case:

1. Moso bamboo would die after flowering, so the traditional method was to collect seeds from the flowered plants;
2. Moso bamboo is a typical vegetative bamboo species, and because of high production, various purposes and wide distribution, it has long been considered as the most important economic bamboo species in China. Moso bamboo is not endangered or protected species in our country.
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**DNA Extraction, Amplification, Sequencing and Molecular Identification**

Fungal mycelia from subcultured colonies were scraped from the surface of the agar and frozen at −20°C for one night for the extraction of DNA. Extractions were performed using E.Z.N.A.™ Fungal DNA Mini Kit (Omega Biotech, Norcross, United States) and the target regions of ITS rDNA were amplified by ITS1-F/ITS4 [27]. The PCR mixture (25 μL, total volume) contained 0.5 μL template, 0.5 μL of each primer (25 μM each), 12.5 μL 2× MasterMix (including 10× buffer, dNTPs and Taq polymerase) and ddH2O (Tiangen, Beijing, China). Thirty-five cycles consisting of denaturation at 94°C (30 s), annealing at 50°C (45 s) and extension at 72°C (60 s) were run and the final extension step at 72°C for 7 min was performed using Techne TC-512 (Keison Products, Beijing, China). Finally, the purified amplicons were sequenced by Invitrogen Biotechnology Co. Ltd. (Beijing, China). To identify the isolates, sequences were subjected to a BLAST search with the NCBI database (http://www.ncbi.nlm.nih.gov/). Only matches of sequences published in journals were used. Priority was given to sequences derived from authoritative materials, such as ex-type or ex-epitype cultures. The sequences of the present study were also deposited at GenBank.

**Fungal Culture and Crude Preparation**

Endophytic fungi isolates were cultured in PDA media. The fresh mycelia of different endophytic fungi were grown on plates at 25°C for more than 7 d. Five plugs (6 mm in diameter) of growing culture plus the adhering mycelium were subsequently added to 250 ml Erlenmeyer flasks containing 100 ml of Potato Dextrose Broth media (PDB, containing (g/L): potato 200 and dextrose 20; pH 6.0). All liquid cultures were kept at 25°C for 7–10 d with shaking (150 rpm). The fermentation of each fungus was filtered to separate the filtrates from the mycelia. The mycelia and filtrates were separately extracted with ethyl acetate (EtOAc) in order to obtain mycelial and filtrated extracts [28].

**Agar Diffusion Assay**

The endophytic fungi were screened using the agar diffusion method, as a rapid and qualitative selection of the bioactive microorganisms. Endophytic fungi were cultured on PDA media at 25°C over 7 d. Agar plugs (6 mm in diameter) of growing culture plus the adhering mycelium were subsequently added to Luria Broth Agar media (LBA, containing (g/L): tryptone 10, yeast extract 5, NaCl 10 and agar 20; pH 6.0) and PDA media, supplemented with 0.5% olive oil previously spread with bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Listeria monocytogenes* and *Salmonella bacteria*) and yeasts (*Rhodotorula rubra*, *Saccharomyces cerevisiae* and *Candida albicans*). The cultures of bacteria and yeasts were also deposited at CFCC. Plates were incubated at 37°C for 24 h for the bacteria and 28°C for 2–7 d for the yeasts. The inhibition zones around the agar plugs were measured to record the antimicrobial activity of fungal isolates [29].

**Disk Diffusion Assay**

The antifungal activities of fungal extracts were tested in a number of pathogenic fungi: *Carvalunana ergostriata*, *Pleospora herbarum*, *Arthrinium saccharum*, *Arthrinium phaeospermum* and *Phoma herbarum*. These cultures of fungi were all deposited at CFCC. The bioactive extracts of mycelia and filtrates were assessed for antimicrobial activity by the disc diffusion method at a concentration of 100 μg/disk. Antimicrobial activity against pathogenic fungi was estimated by the size (diameter in mm) of growth inhibition zones. Each inhibition assay was repeated three times, and analysis of variance was conducted by SPSS 18.0 for Windows (SPSS Inc., Chicago, USA).

**Results**

**Isolates, Sequence Data and Diversity**

A total of 350 fungal isolates were designated into 69 morphotypes based on cultural characteristics. Sequences of ITS
region were generated for the isolates from each morphotype (69 in total). ITS sequences were compared with those deposited in GenBank using a BLAST search (http://www.ncbi.nlm.nih.gov/), and directly with sets of authentic sequences from published studies of taxa (Table S1). The results show that all 350 isolates represented at least 19 genera (Table 1). The majority of ITS sequences from the fungal isolates did not show complete sequence identity with sequences present in GenBank, ranging from 0.2% to greater than 10% sequence variation. All the ITS sequences have been deposited in GenBank, and the accession numbers are HQ654261 and HQ696018–85 corresponding to individual isolates (Table S1).

For the further taxonomic analysis, the ITS sequences of the 69 representative isolates were aligned with reference sequences from GenBank based on sequence similarity, or because they were potentially related taxa. The sequence alignments indicated that the isolates belonged to the phyla Ascomycota and Basidiomycota, corresponding to nine orders (Table 1). In the Basidiomycota, isolate zzz919 was close to *Sebacina endomycorrhiza* (Sebacinaeae, Sebacinales, Agaricomycetes) and isolate zzz1735 was placed in the Basidiozymetes without any similar defined sequence at the generic level. Within the Ascomycota (Table 1), three classes (Dothideomycetes, Eurotiales and Sordariomycetes) were included and at least seven orders (Capnodiales, Dothideales, Pleosporales, Eurotiales, Hypocreales, Phyllachorales and Xylariales) were detected from the fungal isolates. Ten strains (B01, B05, B06, B08, B09, B10, B11, B25, zzz409 and zzz1737) belonged to the genus *Cladosporium* in the Capnodiales, and only one (B23) to *Aurobasidium* in the Dothideales. Twenty-three isolates belonging to the Pleosporales were placed respectively in the genus *Alternaria* (isolates zzz407 and zzz1740), *Curvularia* (isolate B34), *Leptosphaeria* (isolates zzz511), *Shiraia* (16 isolates, including zzz1225, zzz1228, zzz612, zzz818, zzz1227 and zzz1739) and undefined genus 1 (three isolates, including B35, zzz1429 and zzz1623).

One isolates (zzz714) was affiliated to the Dothideomycetes, but the published NCBI reference sequence was not identified at order level. Three isolates (B19, B32 and B34) were species of *Penicillium* (Eurotiales, Eurotiomycetes) with well supported sequence alignment. Sordariomycetes contained three orders (Hypocreales, Phyllachorales and Xylariales), seven genera (*Fusarium*, *Simplicillium*, *Colletotrichum*, *Arthrinium*, *Monographella*, *Pestalotiopsis* and *Xylaria*) and 29 culturable strains. Seven of them (zzz101, zzz305a, zzz612, zzz818, zzz1227 and zzz1739) represented taxa from the dominant genus *Fusarium* (Hypocreales). Another isolate (B26) was close to *Simplicillium* (Hypocreales) with high similarity. Phyllachorales contained 12 isolates (B12, B21, B24, B31, B37, zzz303, zzz305, zzz920, zzz1420, zzz1633, zzz1738 and zzz1943) with high similarity, all of which were close to several species of *Colletotrichum* In Xylariales, isolates B16, B20, zzz304, zzz1022, zzz1530 and zzz130 were analogous with *Arthrinium* species, and isolates B13, zzz2045 and zzz1741 were assigned to *Monographella*, *Pestalotiopsis* and *Xylaria*, with high identities, respectively. Three hundred and forty-three isolates belonged to the Ascomycota (90.0% frequency) and only seven to the Basidiomycota (2.0% frequency), representing at least 19 genera in nine orders (Table 1). Pleosporales was the most frequent order.

### Table 1. Number of endophytic fungi isolated from moso bamboo seeds and the frequency of colonization (FC%).

| Genus (when stated in GenBank) | Phylum; Subclass; Order; | Strain | Isolates number | FC% |
|--------------------------------|--------------------------|--------|-----------------|-----|
| **Cladosporium**               | Ascomycota; Dothideomycetes; Capnodiales | B01, B05, B06, B08, B09, B10, B11, B25, zzz409, zzz1737 | 84 | 24.00 |
| **Aureobasidium**              | Ascomycota; Dothideomycetes; Dothideales | B23 | 2 | 0.57 |
| **Alternaria**                 | Ascomycota; Dothideomycetes; Pleosporales | zzz407, zzz1740 | 12 | 3.43 |
| **Curvularia**                 | Ascomycota; Dothideomycetes; Pleosporales | B34 | 2 | 0.57 |
| **Leptosphaeria**              | Ascomycota; Dothideomycetes; Pleosporales | zzz511 | 6 | 1.71 |
| **Phoma**                      | Ascomycota; Dothideomycetes; Pleosporales | B29, zzz202 | 18 | 5.14 |
| **Shiraia**                    | Ascomycota; Dothideomycetes; Pleosporales | B02, B17, B18, B22, B27, B33, zzz510, zzz613, zzz815, zzz816, zzz1021, zzz1023, zzz1225, zzz1226 | 66 | 18.90 |
| undefined genus 1              | Ascomycota; Dothideomycetes; Pleosporales | B35, zzz1429, zzz1632 | 10 | 2.86 |
| undefined genus 2              | Ascomycota; Dothideomycetes; Pleosporales | zzz714 | 1 | 0.29 |
| **Penicillium**                | Ascomycota; Eurotiomycetes; Eurotiales | B19, B32, B38 | 6 | 1.71 |
| **Fusarium**                   | Ascomycota; Sordariomycetes; Hypocreales | zzz101, zzz305a, zzz612, zzz818, zzz1124, zzz1327, zzz1739 | 48 | 13.70 |
| **Simplicillium**              | Ascomycota; Sordariomycetes; Hypocreales | B26 | 2 | 0.57 |
| **Colletotrichum**             | Ascomycota; Sordariomycetes; Phyllachorales | B12, B21, B24, B31, B37, zzz303, zzz305, zzz920, zzz1428, zzz1633, zzz1738, zzz1943 | 54 | 15.43 |
| **Arthrinium**                 | Ascomycota; Sordariomycetes; Xylariales | B16, B28, zzz304, zzz1022, zzz1320, zzz1842 | 24 | 6.85 |
| **Monographella**              | Ascomycota; Sordariomycetes; Xylariales | B13 | 4 | 1.14 |
| **Pestalotiopsis**             | Ascomycota; Sordariomycetes; Xylariales | zzz2045 | 2 | 0.57 |
| **Xylaria**                    | Ascomycota; Sordariomycetes; Xylariales | zzz1741 | 2 | 0.57 |
| **Sebacina**                   | Basidiomycota; Agaricomycetes; Sebacinales | zzz919 | 6 | 1.71 |
| undefined genus 3              | Basidiomycota; Basidiomycetes | zzz1735 | 1 | 0.29 |

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be effective against Curvularia eragrostidis. In the disk diffusion test, none of the crude extracts were found to display the same trend (Table 3), but ethyl acetate extracts of the variations in the calculation of DGI of five endophytic fungi displayed the most marked activity against bambusicolous pathogens. Comparing crude extracts of mycelia and filtrates, the more important purpose of the present study was to investigate the antimicrobial activity of the culturable fungi associated with bamboo seeds and screen the bioactive strains which might have applied potentials. There were 69 representative endophytes from moso bamboo seeds, which were screened by agar diffusion assay, to confirm if they demonstrated antimicrobial activities against clinical pathogens. The tested micro-organisms included model bacteria (S. aureus, B. subtilis, L. monocytogenes and Salmonella sp.) and yeasts (C. albicans, R. rubra and S. cerevisiae). The preliminary evaluation demonstrated that the various fungal isolates displayed different antimicrobial effects (Table 2). Endophytic fungi strain B09 inhibited the growth of two human pathogenic bacteria S. aureus and B. subtilis and also displayed good activity against C. albicans. Strain B34 had effect on four clinical microorganisms (B. subtilis, L. monocytogenes, Salmonella sp. and C. albicans) and B35 was also active against two bacterial species (S. aureus and B. subtilis) and two fungal species (C. albicans and R. rubra). Strain B38 displayed the widest spectrum of anti-microorganism (six species – S. aureus, B. subtilis, L. monocytogenes, Salmonella sp., C. albicans and R. rubra) and had the strongest activity against three of them (S. aureus, B. subtilis and C. albicans), as well as strain zzz816. However isolate zzz816 showed higher activity against R. rubra in the antifungal assay and less inhibitory effect on L. monocytogenes and Salmonella sp. in the antibacterial test. There was no fungal endophyte with distinct bioactivity against S. cerevisiae.

Of 69 representative isolates, five strains (B09, B34, B35, B38 and zzz816) had inhibitory effects on at least four of the pathogenic micro-organisms tested, and these were selected to continue in the bioactive compounds screening. To test the bioactivity of the endophytic isolates against plant pathogenic fungi, five pathogenic bambusicolous fungi (Curvularia eragrostidis, Phleospora herbarum, Arthrinium sacchari, Arthrinium phaeospermum and Phoma herbarum) were selected for the further antagonism test [4]. Bioactivity of extracts from endophytic fungi were estimated from the size (diameter in mm) of growth inhibition zones (DGI), which is an indication of the efficacy of antifungal activity, and the effect of ethyl acetate extracts was tested at 100 μg/ml against pathogens. Comparing crude extracts of mycelia and filtrates, the variations in the calculation of DGI of five endophytic fungi displayed the same trend (Table 3), but ethyl acetate extracts of mycelia showed higher growth inhibition than the related filtrates. In the disk diffusion test, none of the crude extracts were found to be effective against Curvularia eragrostidis and Phoma herbarum, and strain B35 didn’t exhibit antifungal activity distinctly against any of the plant pathogens, either from mycelia or filtrates. Ethyl acetate extracts of B09 were found to be the most effective agents, against the widespread plant pathogenic fungus Phleospora herbarum, both from mycelia and filtrates. With bioactivity against Arthrinium sacchari, DGI of extracts from zzz816 were significantly higher than the others under the same conditions. From the calculation of DGI, the mycelial and filtrated extracts of B38 and zzz816 had the most marked activity against Arthrinium phaeospermum.

Strain B38 and zzz816 displayed the same broad-spectrum of bioactivity against bambusicolous pathogens, but extracts of B09 inhibited the growth of Phleospora herbarum more significantly, and

| Isolate No. | Staphylococcus aureus | Bacillus subtilis | Listeria monocytogenes | Salmonella bacteria | Candida albicans | Rhodotorula rubra | Saccharomyces cerevisiae |
|-------------|----------------------|-----------------|-----------------------|-------------------|-----------------|-------------------|--------------------------|
| B09         | ++                   | ++              | ++                    | ++                | ++              | ++                | ++                       |
| B34         | ++                   | ++              | ++                    | +++               | +++             | +                 | +                        |
| B35         | ++                   | ++              | +                     | ++                | +               | +                 | +                        |
| B38         | +++                  | +++             | +                     | +++               | +++             | +                 | +                        |
| zzz816      | ++                   | ++              | +                     | ++                | ++              | +                 | +                        |

Table 2. Antimicrobial activity of fungal isolates from moso bamboo seeds against human pathogens.

![Image]

Detecting Antimicrobial Activities of the Culturable Strains

Of 69 representative isolates, five strains (B09, B34, B35, B38 and zzz816) had inhibitory effects on at least four of the pathogenic micro-organisms tested, and these were selected to continue in the bioactive compounds screening. To test the bioactivity of the endophytic isolates against plant pathogenic fungi, five pathogenic bambusicolous fungi (Curvularia eragrostidis, Phleospora herbarum, Arthrinium sacchari, Arthrinium phaeospermum and Phoma herbarum) were selected for the further antagonism test [4]. Bioactivity of extracts from endophytic fungi were estimated from the size (diameter in mm) of growth inhibition zones (DGI), which is an indication of the efficacy of antifungal activity, and the effect of ethyl acetate extracts was tested at 100 μg/ml against pathogens. Comparing crude extracts of mycelia and filtrates, the variations in the calculation of DGI of five endophytic fungi displayed the same trend (Table 3), but ethyl acetate extracts of mycelia showed higher growth inhibition than the related filtrates. In the disk diffusion test, none of the crude extracts were found to be effective against Curvularia eragrostidis and Phoma herbarum, and strain B35 didn’t exhibit antifungal activity distinctly against any of the plant pathogens, either from mycelia or filtrates. Ethyl acetate extracts of B09 were found to be the most effective agents, against the widespread plant pathogenic fungus Phleospora herbarum, both from mycelia and filtrates. With bioactivity against Arthrinium sacchari, DGI of extracts from zzz816 were significantly higher than the others under the same conditions. From the calculation of DGI, the mycelial and filtrated extracts of B38 and zzz816 had the most marked activity against Arthrinium phaeospermum.

Strain B38 and zzz816 displayed the same broad-spectrum of bioactivity against bambusicolous pathogens, but extracts of B09 inhibited the growth of Phleospora herbarum more significantly, and

| Isolate No. | Staphylococcus aureus | Bacillus subtilis | Listeria monocytogenes | Salmonella bacteria | Candida albicans | Rhodotorula rubra | Saccharomyces cerevisiae |
|-------------|----------------------|-----------------|-----------------------|-------------------|-----------------|-------------------|--------------------------|
| B09         | ++                   | ++              | ++                    | ++                | ++              | ++                | ++                       |
| B34         | ++                   | ++              | ++                    | +++               | +++             | +                 | +                        |
| B35         | ++                   | ++              | +                     | ++                | +               | +                 | +                        |
| B38         | +++                  | +++             | +                     | +++               | +++             | +                 | +                        |
| zzz816      | ++                   | ++              | +                     | ++                | ++              | +                 | +                        |
others more weakly. Extracts of B34 had low activity against plant pathogens, with no measureable effect from B35 either.

Discussion

The analysis of ITS region revealed that a remarkable diversity of fungal endopytes from moso bamboo seeds was mainly distributed in Dothideomycetes and Sordariomycetes. Many species in the two subclasses have been described from bamboos, including saprophytes, pathogens and endophytes [4,16,30,31]. Previously, a total of 65 fungi belonging to 37 genera have been reported on stored seeds of different species of bamboos [4]. In this study, we obtained at least 10 genera in Dothideomycetes, seven genera in Sordariomycetes, one in Eurotiomycetes and two in Agaricomycetes as endophytes from moso bamboo seeds, and some of them were reported from bamboo seeds or moso bamboo for the first time.

At least 12 genera in the present study have been reported as pathogens and endophytes, and in order of frequency, they were Cladosporium (24.0%), Shiraia (18.90%), Colletotrichum (15.43%), Fusarium (13.70%), Arthrinium (6.85%), Phoma (5.14%), Alternaria (3.43%), Penicillium (1.71%), Aureobasidium (0.57%), Curvularia (0.57%) and Xylaria (0.57%) (Table 1).

Four of them, Fusarium, Arthrinium, Alternaria and Aureobasidium, have been reported as pathogens of moso bamboo [32–34]. Other than Aureobasidium, they have previously been reported on bamboo seeds [4,35] and several species, including F. pallidoroseum, are seed-borne and capable of causing infection of emerging seedlings [4]. Isolate B23 was highly similar to Aureobasidium pullulans by ITS sequence (only 2% difference). This is a common fungus on all plant material and A. pullulans has been reported on moso bamboo in China in one previous study [34].

Another three genera (Cladosporium, Phoma and Curvularia) have been reported as pathogens of some bamboo species excluding moso bamboo, and they were also associated with seeds of some bamboo species [4]. These genera were reported from moso bamboo for the first time as far as the authors are aware.

Fusarium sp. has been documented to infect seeds of Bambusa nutans in India and Thailand [4,35]. Six isolates obtained during the study by Mohanan [4] and Shukla et al. [35] shared high

Table 3. Antifungal activity of ethyl acetate extracts of the mycelia and filtrates of endophytic fungi from moso bamboo seeds tested by disk diffusion assay.a

| Isolate No. | Curvularia eragrostidis | Pleospora herbarum | Arthrinium Sacchari | Arthrinium Phaeospermum | Phoma herbarum |
|-------------|------------------------|-------------------|---------------------|------------------------|----------------|
| DMSO        | -                      | -                 | -                   | -                      | -              |
| B09M        | -                      | 13.72±0.41*       | 10.38±0.59*         | 9.74±0.31*             | -              |
| B09F        | -                      | 9.85±0.13*        | 7.99±0.23*          | 7.93±0.25*             | -              |
| B34M        | -                      | 7.86±0.23*        | 8.07±0.18*          | 8.06±0.32*             | -              |
| B34F        | -                      | -                 | -                   | -                      | -              |
| B35M        | -                      | -                 | -                   | -                      | -              |
| B35F        | -                      | -                 | -                   | -                      | -              |
| B38M        | -                      | 11.90±0.07*       | 14.07±0.14*         | 13.83±0.07*            | -              |
| B38F        | -                      | 7.35±0.51*        | 8.42±0.45*          | 9.80±0.20*             | -              |
| zzz816M     | -                      | 11.80±0.29*       | 10.99±0.42*         | 14.09±0.26*            | -              |
| zzz816F     | -                      | 8.22±0.16*        | 9.52±0.22*          | 10.39±0.13*            | -              |

*aDiameter of growth inhibition in mm.
*bmm±SD: millimeter ± standard deviation.
*cEthyl acetate extracts of the mycelia.
*dEthyl acetate extracts of the filtrates. Statistical analysis of the data was performed with SSPS 18.0 using Student-Newman-Keuls test for determining significant difference (α=0.05).
*"low activity, "moderate activity, "high activity.

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Figure 1. Colony morphology of isolates of Shiraia sp. on PDA media. A. Upper colour of colony from isolate 816 (Endophytic fungi from moso bamboo seeds); C. Reverse colour of colony from isolate 816; B. Upper colour of colony from isolate of fruit body of S. bambusicola; D. Reverse colour of colony from isolate of fruit body. doi:10.1371/journal.pone.0095838.g001
similarly with three species of Penicillium (3% difference in ITS sequences) in the present study (Table S1 and Table 1).

Species of the other three genera, _Shiraia, Colletotrichum_ and _Xyaria_, are common pathogens of bamboos, but have not yet been reported on moso bamboo seeds [33,36,37]. A total 122 isolates (34.00%) from moso bamboo seeds in our study were close to some species of these genera. One exception was isolate zzz1740 as the generic position was undefined in the present study, with a 5% sequence variation. Isolate B17 was obviously close to _Shiraia_ sp. by sequence alignment, but was proved to be far from _S. bambusicola_ and other _Shiraia-like_ fungi with 5% base pair differences based on ITS [38].

Five taxa isolated with low frequency in our study had not yet been reported as pathogens of bamboos. _Pestalotiopsis_ (0.57%) have been reported as endophytes [16], _Leptosphaerulina_ (1.71%), _Simplicillium_ (0.57%), _Scehacina_ (1.71%) and an undetermined genus (2.86%) were new bambusicolous fungi which have previously been reported as pathogens of other plants [39–41]. Actually, the similarity of the ITS sequences was a little low, especially as isolate zzz919. This isolate had 18% difference of ITS sequence with _Scehacina endomycorrhiza_ (HQ696070). To determine these unknown taxa, such as zzz714 (0.29%) and zzz1735 (0.29%), further studies of other conservative genes and morphology are needed.

In tropical humid areas such as the Guangxi Zhuang Autonomous Region, bamboo seeds have been reported to be particularly vulnerable to several field and storage fungi, and many of these fungi are potential pathogens [6,7]. Many of the seed-associated endophytes might affect the viability of seeds and pose problems in nurseries [4]. To date, far more than 1100 species of bambusicolous fungi are known, with only a few previously known from bamboo seeds, including endophytic fungi [4,30,35,42]. The sampling of the present study focused on the diversity of endophytes from moso bamboo seeds in China. The results showed that at least 19 genera of endophytes were identified. It was difficult to define all taxa at species level. Some of the taxa were identified according to the accepted generic variation at species level after comparing these to the taxa by ITS sequences in published references. Several taxa could be identified only to the family, order or subclass level [9]. Furthermore, the endophytic diversity in this study presumably only accounts for a fraction of the total diversity within this one plantation of moso bamboo.

The other purpose of the present study was to investigate the antimicrobial activity of the culturable fungi associated with bamboo seeds and screen the potentially useful bioactive strains. Of 69 fungal isolates, B09 ( _Cladosporium_ sp.), B34 ( _Curvularia_ sp.), B35 (undefined genus 1), B38 ( _Penicillium_ sp.) and zzz816 ( _Shiraia_ sp.) displayed broad-spectrum activities against human pathogenic bacteria ( _S. aureus, B. subtilis, L. monocytogenes_ and _Salmonella_ sp.) and clinical yeasts ( _R. rubra, S. cerevisiae_ and _C. albicans_ ) by the agar diffusion method. Furthermore, the crude extracts from five endophytic fungi also exhibited differences in the extent of antimicrobial activity against bambusicolous pathogenic fungi ( _A. Sacchari, A. Phaeospermum, C. eragrostidis, Pseudoperiza herbarum_ and _Phoma herbarum_ ). In particular, B09, B38 and zzz816 showed broad-spectrum and effective bioactivity in antagonistic tests, and these will be tested as potential biocontrol agents in further studies.

It is noticeable that isolate zzz816 was closely related to _Shiraia_ species, a known bambusicolous fungus in East and Southeast Asia. This fungus is mainly found on _Brachystachyum densiflorum_ and related species in China and _Bambusa_ species in Japan [36,43]. There is no information about _S. bambusicola_ in the Guangxi Zhuang Autonomous Region. Interestingly, fruiting bodies of this fungus frequently occur on _B. densiflorum_ in China, while they don’t usually appear on _P. edulis_ or other _Phyllostachys_ spp. and as endophytes on _Take_ and _Sasa_ species [16]. It is hypothesized that this fungus can live on various bamboos as asymptomatic endophytes without producing fruiting bodies, due to limiting conditions such as nutrition or host structure. The fruit body of _S. bambusicola_ has been used in traditional medicine in China, and its compounds have been found to be useful for antitumor activity and antiangiogenesis [36]. Hypocrellins, as the dominant effective compounds of _S. bambusicola_, have attracted a great deal of attention because of their light-induced antifungal, antiviral and antitumor activities. It is crucial to enhance the production of this compound for future research and therapeutic applications [44,45]. The paclitaxel (taxol), was well-known for the clinical application against different types of cancer, but the low extraction efficiency (0.0074%) has highly restricted the corresponding development [46]. To break the bottleneck of production, there were many endophytic fungi of _Pestalotiopsis_ isolated from yew trees, and some of them have been applied to improving taxol yield significantly [47]. Similarly, in contrast to the traditional resource of the fruiting body, one hypocrellin-producing strain zzz816 ( _S. bambusicola_ ) was isolated from the moso bamboo seeds, and it was significantly different from the original strains in the previous reports by the colony colour (Figure 1). In a preliminary test, zzz816 exhibited the highest content of hypocrellins among all the unmodified strains as far as is known and it is believed that the production efficiency of the active agent would be improved tremendously by breeding of novel industrial mutants and optimization of the fermentation process in the further research.

The species of _Cladosporium_ (B09), _Curvularia_ (B34) and _Penicillium_ (B38) have all been recorded as widespread strains among plants as endophytes, saprophytes or pathogens and there are many bioactive agents with antiviral, antifungal and antitumor activities from these corresponding species [48–53]. Potentially all three fungal isolates from moso bamboo seeds could be a source of original chemical products. Strain B35 was closely related to species of _Plesporales_ isolated from plants as endophytes, but the specific genus could not be identified by the ITS sequence alignment. Further studies of sequencing gene SSU and a rapid analytical method based on reverse-phase high-performance liquid chromatography were in progress to confirm the taxonomic status of the endophytic fungi and identify new and useful bioactive agents from these undiscovered species.

This is the first report analyzing the diversity of fungi from moso bamboo seeds, and the presented results could contribute to the understanding of the ecological role of bambusicolous fungi. Furthermore, screening of all the fungal isolates for biological activity demonstrated that five endophytic strains (B09, B34, B35, B38 and zzz816), had potential agricultural and pharmaceutical applications.

Strain zzz816 was isolated from moso bamboo seeds as fungal endophytes, and produced high-yield hypocrellins. Unlike hypocrellin biosynthesis strains generally originating from the fruiting body of _S. bambusicola_, this study suggests that it might be feasible to enhance the efficiency of industrial hypocrellin production using high-yield strains on the selection of novel plantations, and the future development of fermentation product yields would be improved furtherly at the base of strains breeding and process optimization.

**Supporting Information**

**Table S1** Taxon designation of fungal endophytes from moso bamboo seeds based on sequence data from the internal transcribed spacer regions of nuclear ribosomal DNA (ITS rDNA).

(DOC)
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Author Contributions

Conceived and designed the experiments: XYS YLC JGL CLH. Performed the experiments: XYS YLC JGC. Analyzed the data: XYS LF. Contributed reagents/materials/analysis tools: JGC JG. Wrote the paper: XYS YLC CLH.

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