J Ind Microbiol Biotechnol (2011) 38:1267–1278
DOI 10.1007/s10295-010-0905-4

ORIGINAL PAPER

# Isolation and characterization of endophytic huperzine A-producing fungi from *Huperzia serrata*

**Ya Wang · Qing Gui Zeng · Zhi Bin Zhang · Ri Ming Yan · Ling Yun Wang · Du Zhu**

Received: 26 August 2010 / Accepted: 8 November 2010 / Published online: 24 November 2010


**Abstract** *Huperzia serrata* is a producer of huperzine A (HupA), a cholinesterase inhibitor (ChEI). Over 120 endophytic fungi were recovered from this plant and screened for Hup-A and nine were found. These nine represented seven different fungal genera with the most significant producer being *Shiraia* sp. A total of 127 endophytic fungi isolates obtained from the root, stem, and leaf segments of *H. serrata* were grouped into 19 genera based on their morphological traits and sequence analysis of the internal transcribed spacers (ITS1-5.8S-ITS2), indicating endophytic fungi in *H. serrata* are diverse and abundant. *Aspergillus*, *Podospora*, *Penicillium*, *Colletotrichum*, and *Acremonium* were the frequent genera, whereas the remaining genera were infrequent groups. Overall, 39 endophytic fungi isolates showed acetylcholinesterase (AChE) inhibition in vitro. Nine endophytic fungi isolates from seven distinct genera were capable of producing HupA verified by thin-layer chromatography and reverse-phase high-performance liquid chromatography (RP-HPLC). Among the HupA-producing fungi, the yield of HupA produced by the *Shiraia* sp. Slf14 was 327.8 μg/l in potato dextrose broth, and the fungal HupA was further validated by mass spectrometry (ESI-MS). The present study demonstrated that *H. serrata* was a fascinating fungal reservoir for producing HupA and other ChEIs.

**Keywords** Endophytic fungi · ITS sequence · Phylogenetic analysis · Huperzine A · *Huperzia serrata*

Y. Wang · D. Zhu (✉)
Key Laboratory for Research on Active Ingredients in Natural Medicine of Jiangxi Province, Yichun University, Xuefu Road 576, 336000 Yichun, China
e-mail: zhudu12@163.com

Y. Wang · Q. G. Zeng · Z. B. Zhang · R. M. Yan · L. Y. Wang · D. Zhu
Key Laboratory of Protection and Utilization of Subtropic Plant Resources of Jiangxi Province, Jiangxi Normal University, Ziyang Road 99, 330022 Nanchang, China

## Introduction

*Huperzia serrata* (Thunb. ex Murray) Trev., the traditional Chinese medicine *Qian Ceng Ta*, a member of the Huperziaceae, grows at an altitude of 300–2,700 m in damp forests and rock crevices in China [45]. The whole plant has been used for over 1,000 years in China for the treatment of a number of ailments, including contusions, strains, swellings, schizophrenia, myasthenia gravis, and now organophosphate poisoning [4, 21]. Huperzine A (HupA), a lycopodium alkaloid isolated originally from *H. serrata*, has attracted intense attention since its marked anticholinesterase activity was discovered by Chinese scientists [19, 20]. HupA has been marketed in China as a new drug for Alzheimer's disease (AD) treatment and currently used in the USA as a supplement for preventing further memory degeneration [47]. ZT-1, the semi-synthetic derivative of HupA, is being developed as anti-AD new drug candidate both in China and in Europe [26].

Most of the HupA currently used in herbal supplements and medicines is sourced from Chinese club moss *H. serrata* and some other species in the Huperziaceae. However, *H. serrata* actually has very low HupA content (ca. 0.007%), very limited distribution, and extremely slow growth. Germinated spores require at least 15 years to develop into mature sporophytes that can be harvested for HupA collection [22, 25]. Other species in the Huperziaceae are even more difficult to obtain and are much rarer in nature than

*H. serrata*, making them even less desirable candidates as natural sources for HupA. Meanwhile, no successful commercial cultivation has been reported for *H. serrata* or other species in the Huperziaceae [23]. Thus, increasing efforts have been made to develop alternative means of HupA production, such as using complete chemical synthesis and Huperziaceae plant cell culture. Otherwise, wild populations of the plant may soon become extinct if current over-collection practices are not curtailed [22, 24–26]. However, thus far, the total chemical synthesis of HupA in an industrially feasible manner is not available, and the in vitro culture of lycopods has only met limited success [23].

Endophytic fungi are fungi that live for all, or at least a significant part, of their life cycle internally (intercellularly or intracellularly) and asymptomatically in plant parts [27, 28]. They ubiquitously inhabit most plant species, and have been isolated from a variety of plants [34]. Some endophytic fungi have been found to produce similar chemical compounds to those produced by its host [1, 33]. Others have been shown to be a potential source of natural products such as antibiotics, antiviral compounds, and anticancer agents [36], more than half of which were previously unknown [32]. Endophytic fungi have been recognized as a repository of novel compounds of immense value in agriculture, industry, and medicine [16, 35, 41]. Thus, if a microbial source of HupA is available, then fermentation processes using HupA-producing endophytic fungi may be an alternative way to produce HupA, and it would eliminate the need to harvest and extract the slow-growing and relatively rare Huperziaceae plant for this drug. So far, several endophytic HupA-producing fungi associated with Huperziaceae plants were isolated and identified [15, 18, 49], which demonstrated that fermentation processes using HupA-producing microorganisms may be a promising approach for producing HupA. As the initial step in the search of fungi capable of producing HupA and other novel natural compounds, the objectives of this study were as follows: (1) to identify the endophytic fungal isolates of *H. serrata* collected from the natural populations at Lushan Botanical Garden (Jiangxi Province, China) using partial sequences of the internal transcribed spacers (ITS1-5.8S-ITS2), and (2) to investigate the diversity of fungi associated with leaves, stems, and roots from *H. serrata*, and (3) to screen the endophytic HupA-producing fungi residing in the tissues of *H. serrata*.

## Materials and methods

### Materials

Ten healthy wild-plant materials of *H. serrata* were collected from the natural populations at Lushan Botanical Garden in Jiangxi Province, central China, in May 2007. The plants were over 10 years old and yet reached a height of less than 10 cm. All samples were immediately brought to the laboratory in an icebox, and the tissues were screened for endophytic fungi within 24 h.

Solvents used for chromatography were of high-performance liquid chromatography (HPLC) grade, while solvents used for extraction were of American Chemical Society grade. Authentic HupA (≥98% purity) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products of China. Acetylcholinesterase (AChE), acetylthiocholine iodide, and dithiobis nitrobenzoic acid (DTNB) were purchased from Sigma. All other chemicals were from China Medicine Shanghai Chemical Reagent Co. Ltd. PCR primers were synthesized by Shanghai Sangon Biologic Engineering Technology and Service Co. Ltd.

### Isolation and preliminary identification of endophytic fungi

To eliminate epiphytic microorganisms, all the *H. serrata* samples were thoroughly washed in running tap water, and then sterilized by washing in 75% ethanol for 2 min, 0.1% mercuric chloride (v/v) for 10 min, respectively. Afterwards, the samples were rinsed 4–5 times in sterile distilled water and then the roots and stems of the samples were cut into 0.5-cm lengths, leaves were cut into 0.5-$cm^2$ sections, and transferred to Petri dishes (9 cm in diameter) containing potato dextrose agar (PDA) medium (amended with 60 μg/ml streptomycin and 100 μg/ml ampicillin). The Petri dishes were incubated at 28°C in darkness and monitored every day to check the growth of endophytic fungal hyphae emerging from segments. Individual hyphal tips of the various fungi were removed from the agar plates, placed on new PDA medium, and incubated at 28°C for at least 10 days. Each fungal culture was checked for purity and transferred to another PDA plate by the hyphal tip method [37]. To ensure that the surface sterilization had removed all hyphae and chlamydospores externally adhering to the segments, they were placed in PDA agar plates and incubated at 28°C in darkness. Only segments that were negative in this test were used for isolation of endophytes. The fungal isolates were numbered and stored on the surface of the PDA plate at 4°C or as spores and mycelia in 15% (v/v) glycerol at −70°C.

All fungal isolates were tentatively grouped and dereplicated by observing their morphological and cultural characteristics, including the characteristics of colonies on plates and slants, the presence of aerial mycelia and substrate mycelia, spore mass color, distinctive reverse colony color, diffusible pigment, and sporophore and spore chain morphology. Some isolates sporulated readily on PDA

media after 1 week of inoculation in darkness at 28°C. Many colonies that were similar in color, shape, and size were observed for hyphal length and structure with light microscopes, which allowed them to be segregated into distinct isolates. The remaining sterile fungal isolates were subjected to a molecular method of identification. Based on the preliminary grouping, 35 morphotypes were selected for further molecular research.

### DNA extraction, PCR amplification, and sequencing

Mycelium of different endophytic fungi was ground with a sterile mortar in liquid nitrogen. The genomic DNA was extracted by the CTAB method [48]. Primers ITS1 (5′-TC CGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTC CGCTTATTGATATGC-3′) were used for amplification of the fungal ribosomal DNA (rDNA) internal transcribed spacer (ITS) regions 1 and 2 of the isolates [44]. The PCR mixture (20 μl) consisted of 100 ng genomic DNA, 2 μl of 10× PCR buffer, 1.5 μM $MgCl_2$, 0.5 μM each primer, 200 μM of each deoxyribonucleotide triphosphate, 1 unit Taq polymerase and autoclaved double-distilled water. PCR were performed with a pre-heating at 94°C for 5 min, followed by 35 cycles of 1 min at 94, 55°C for 40 s, 72°C for 1 min, and a final extension at 72°C for 10 min. Five microliters of PCR products were visualized by electrophoresis on a 1.5% (wt/v) agarose gel in 1× TBE buffer (40 mM Tris, 1 mM EDTA, pH 8.0) and then sequenced. Sequencing of the PCR products was performed by the service of Shanghai Shengon Company Ltd (Shanghai, China). The fragment of ITS region was sequenced in both directions using an ABI 377 automated DNA sequencer.

### Phylogenetic analysis of endophytic fungi

The ITS sequences of the endophytic fungi were compared to the data available in NCBI using BLAST searches to estimate the phylogenetic relationships of the endophytic fungi. The resulting sequences were aligned with the ClustalX software [43] with gaps treated as missing data. The phylogenetic tree was performed using the neighbor-joining method [29] and the Kimura two-parameter distance calculation in mega software version 3 [16]. The bootstrap was 1,000 replications to assess the reliable level to the nods of the tree.

### Fermentation and preparation of endophytic fungi extracts

The endophytic fungi isolates were inoculated, respectively, into 500-ml Erlenmeyer flasks containing 150 ml of the PDB and cultured at 120 rpm at 28°C for 14 days in a rotary shaker. The mycelial pellets were harvested by centrifugation at 12,000 × *g* for 10 min and dried at 45°C overnight, and then the dried mycelia were powdered. Each sample of raw material (1.0 g) was extracted with 75% ethanol (50 ml) for 30 min, repeated three times, and the extracts were filtered. The extraction was carried out in an ultrasonic bath at 40°C. The combined filtrates were evaporated under reduced pressure. Next, the dry residues were dissolved in 50 ml of 2.5% hydrochloric acid and purified by shaking twice with chloroform and then with ethylic ether. The water phases were rendered basic with 25% ammonia solution (pH 9), salted out with sodium chloride, and then exhaustively extracted with chloroform. In the last stage, the combined chloroform extracts were evaporated to dryness. The dry residues were dissolved in 10 ml of methanol (HPLC purity grade). The methanolic extracts were filtered through a 0.45-μm filter prior to chromatographic separation and spectroscopic analyses [40].

### Determination of AchE inhibitory activity of endophytic fungi extracts in vitro

In vitro AChE inhibition activity of the endophytic fungi extracts was compared to authentic HupA. It was carried out as the method of Atta-ur-Rahman et al. [2]. Briefly, a pre-incubation volume of 250 μl phosphate buffer (200 mM, pH 7.7) contained 15 μl fungal extract/15 μl authentic HupA, 80 μl DTNB (3.96 mg DTNB and 1.5 mg sodium bicarbonate dissolved in 10 ml phosphate buffer pH 7.7), and 10 μl AChE (0.22 U/ml). The mixture was incubated for 5 min at 25°C. Following preincubation, 15 μl of the substrate acetylthiocholine iodide (10.85 mg in 5 ml phosphate buffer) was added and incubated again for 5 min. The color developed was measured in a microwell plate reader at 412 nm (Bio-Rad, Hercules, CA). Percent inhibition was calculated using the formula: (control absorbance − sample absorbance)/control absorbance × 100.

### Screening of huperzine A-producing fungi

The HupA-producing endophytic fungi were screened by thin-layer chromatography (TLC). TLC analysis of the methanolic extract of endophytic fungi as well as the methanolic solution of HupA standard was developed in a solvent system (chloroform: acetone: isopropanol at 4:4:2 v/v/v) by spotting on the start line of a 0.25 mm (10 × 20 cm) silica gel plate. HupA was detected using a spray reagent consisting of 0.3% potassium permanganate (w/v), which appeared as yellowish spots. The HupA spot was identified by comigration with authentic HupA [49]. The area of the plate containing putative HupA was carefully removed by scraping off the silica and eluting with methanol after chromatography. After purification,

the fungal HupA was used for ESI-MS spectroscopic analysis.

High-performance liquid chromatography (HPLC) was performed using an RP-$C_{18}$ column (5 μm, 4.6 × 150 mm) (Agilent Technologies, Palo Alto, CA). A 20-μl amount of each methanolic extract was injected. The mobile phase was methanol: water (85: 15, v/v) at a flow rate of 1.0 ml $min^{-1}$. The effluent was monitored at 310 nm. Quantification was achieved by using the standard curve generated from the HupA standard over a concentration range of 0.01–0.09 mg $l^{-1}$ at which the peak area and height showed linear relationships with the absorbance ($r^2 = 0.9942$). The fungal HupA was further identified by mass spectroscopy analysis using the electrospray technique with a Waters ZQ 4000/2695 LC-ESI-MS trap. The sample purified by TLC was dissolved in 100% HPLC-grade methanol and was injected with a spray flow of 2 μl $min^{-1}$ and a spray voltage of 3.0 kV using the loop injection method.

## Statistical analysis and calculation formula

The colonization frequency (CF) of each endophyte was calculated according to the method of Hata & Futai [12], $CF = N_{COL}/N_t$ where $N_{COL}$ is the number of segments colonized by each endophytic fungi; $N_t$ is the total number of segments. The Isolation rate (IR) was calculated as follows [7]: IR = total number of isolates yielded in a given trial/total number of samples in a trial. Jaccard similarity coefficients were calculated for comparing the endophyte assemblages using the following formula: Similarity coefficient $= C/(A + B - C)$, where $A$ and $B$ are the total number of morphological species isolated from any three parts of host and $C$ the number of morphological species found in common. The results were expressed as percentages. The formula to calculate Margalef's richness index $(R1) = (S - 1)/\ln(n)$. Where $S$ is the number of species in the assemblage and $n$ is the number of isolates. ln denotes natural logarithm [39].

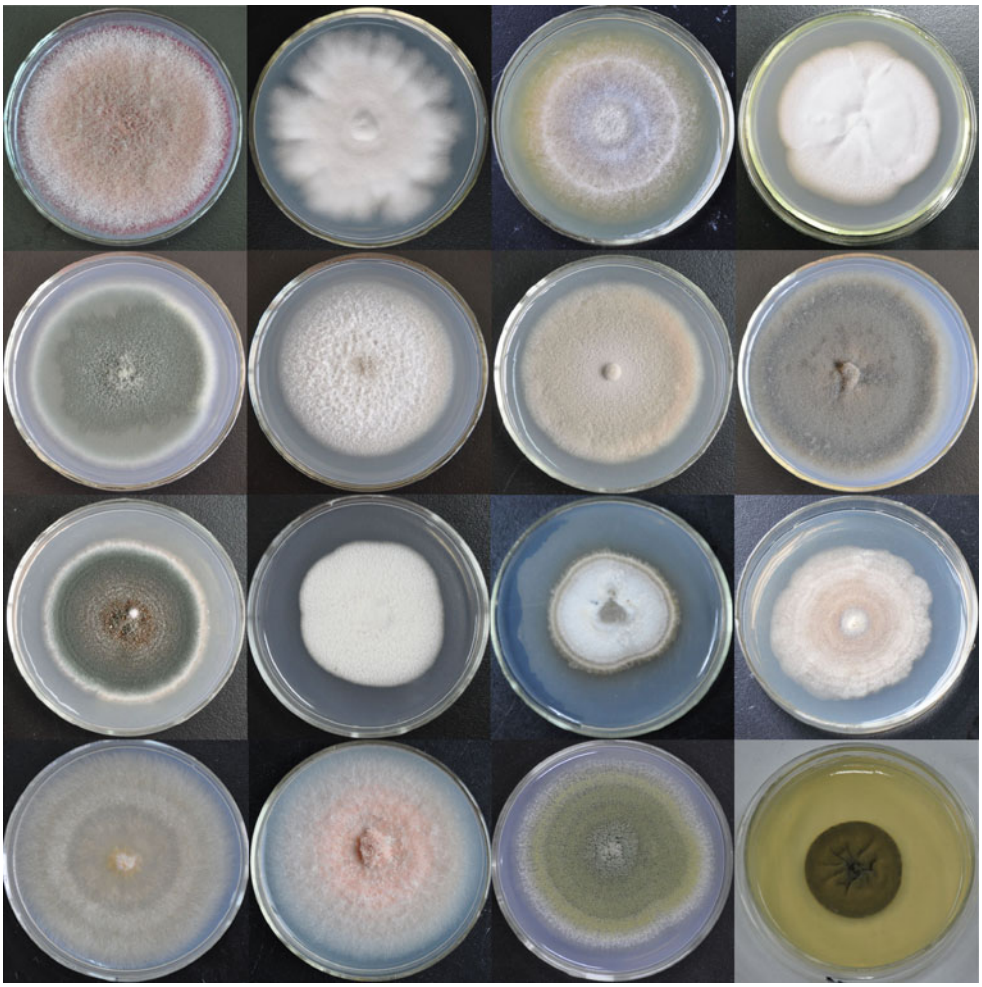

**Fig. 1** Colony morphology of some endophytic fungi isolates recovered from *H. serrata* tissues

## Results

### Isolation and characterization of culturable endophytic fungi community associated with *H. serrata*

The surface disinfection treatments used for leaf, stem and root segments were efficient eliminating epiphytic fungi because the segments imprint yielded no fungi. A total of 127 endophytic fungi isolates were isolated and purified from the 246 tissue segments of *H. serrata* grown in the Lushan Mountain, China. A proportion of isolates were identified to genus and/or species level based on the morphology of conidia, colonies, and unique phenotypic characters (Fig. 1). To confirm the reliability of morphological identification, all 35 morphotypes were subjected to molecular identification based on ITS sequence analysis. In total 30 distinctive genotypes were grouped into 19 fungal genera and 13 fungal species (Fig. 2; Table 1), which corresponded well with morphological differences between these fungal cultures. In addition, the stems of *H. serrata* were colonized by a great number of endophytes relative to leaves and roots, also the endophyte assemblage of stems was more species rich, as indicated by R1 (Table 2). It seems likely that stem tissues may provide better niche or more entry for endophytes colonization and penetration, which we need toinvestigate more extensively.

Tissue specificity of endophytic fungi was also observed in the present study. For example, *Penicillium janthinellum*, *Aspergillus flavus*, and *Acremonium* sp. colonized stems exclusively, but *Paraconiothyrium brasiliense* only colonized leaves (Fig. 2). The Jaccard similarity coefficients also indicated that each plant part hosts different groups of fungi, suggesting heterogeneity of the endophyte assemblage (Table 3). Analysis of distribution frequencies of the 127 endophytic fungi revealed that the fungal communities in the host contained a few frequent genera and many infrequent groups (Table 4). *Aspergillus*, *Podospora*, *Penicillium*, *Colletotrichum*, and *Acremonium* were the frequent genera, accounting for colonization frequencies ranging from 10.5 to 18.7%. Among the rare morphotypes, *Coniothyrium*, *Chaunopycnis*, *Cladosporium*, *Botrytis*, *Leptosphaeria*, *Capronia*, *Paraphaeosphaeria*, *Mortierella*, *Alternaria*, and *Shiraia* were the infrequent genera, accounting for colonization frequencies ranging from 1.2 to 4.9%, whereas others only showed that of 0.4%.

### Molecular phylogenetics

The ITS neighbor-joining tree of the endophytic fungi is shown in Fig. 3. The 25 morphospecies (SF3, SF4, SF6, LF6, SF7, Slf14, LF15, LF17, RF22, SF25, LF30, LF33, LF40, LF46, SF58, SF64, LF70, LF73, SF96, LF116, SF123, SF136, LF146, SF153, and RF161) sharing sequence max identity of ≥98% with available data in NCBI (Table 4) were grouped into 12 genera of *Penicillium*, *Aspergillus*, *Cladosporium*, *Mortierella*, *Coniothyrium*, *Paraphaeosphaeria*, *Leptosphaeria*, *Shiraia*, *Alternaria*, *Podospora*,

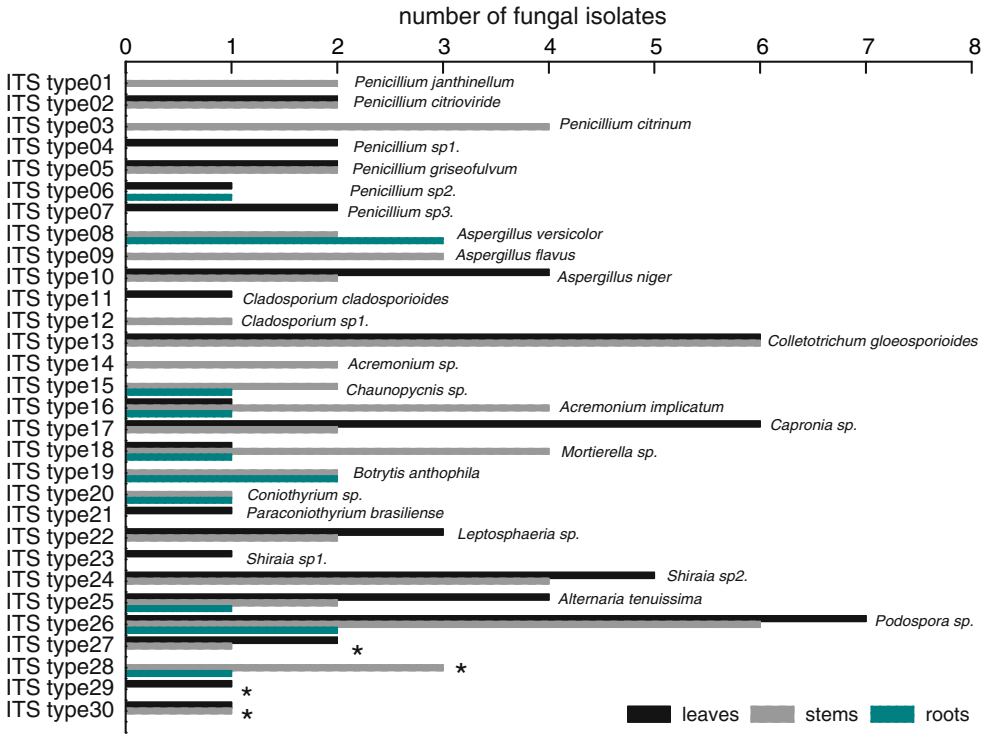


**Fig. 2** The frequency of 30 different ITS-based genotypes determined from total endophytic fungi. *Denotes undescribed fungal species. Genus and/or species names of identified fungi are indicated above the corresponding column

**Table 1** Occurrence of identified species of endophytic fungi obtained from *H. serrata* at Lushan Botanical Garden (China)

| Fungal group | Closest related species (GenBank accession no.) | Relative frequency[a] |
|---|---|---|
| *Penicillium griseofulvum* | *Penicillium griseofulvum* (EU780695) | 3.15 |
| *Penicillium citrioviride* | *Penicillium citrioviride* (GU388431) | 3.15 |
| *Penicillium citrinum* | *Penicillium* sp. FF47 (FJ379822) | 3.15 |
| *Penicillium janthinellum* | *Penicillium janthinellum* (EF550979) | 1.57 |
| *Penicillium* sp1. | *Penicillium* sp. S17e3 (EU142860) | 1.57 |
| *Penicillium* sp2. | *Penicillium* sp. IBT 12396 (AJ005493) | 1.57 |
| *Penicillium* sp3. | *Penicillium* sp. E75-3-2 (EU142851) | 1.57 |
| *Aspergillus versicolor* | *Aspergillus versicolor* (EU042148) | 3.94 |
| *Aspergillus flavus* | *Aspergillus flavus* (DQ467970) | 2.36 |
| *Aspergillus niger* | *Aspergillus niger* (FJ629339) | 4.72 |
| *Cladosporium cladosporioides* | *Cladosporium* sp. HKB6 (EF029809) | 0.79 |
| *Cladosporium* sp1. | *Cladosporium* sp. QLF108 (FJ025146) | 0.79 |
| *Colletotrichum gloeosporioides* | *Colletotrichum gloeosporioides* (EU326190) | 9.45 |
| *Acremonium* sp. | *Acremonium* sp. KMU 4528 (AB158289) | 1.57 |
| *Chaunopycnis* sp. | *Chaunopycnis alba* (AF389191) | 2.36 |
| *Acremonium implicatum* | *Acremonium implicatum* (AF368810) | 4.72 |
| *Capronia* sp. | *Capronia pilosella* (DQ826737) | 6.30 |
| *Mortierella* sp. | *Mortierella* sp. dd08037 (FJ810151) | 4.72 |
| *Botrytis anthophila* | *Botrytis anthophila* (AJ716305) | 3.15 |
| *Coniothyrium* sp. | *Coniothyrium* sp. RM3-4 (DQ993627) | 1.57 |
| *Paraconiothyrium brasiliense* | *Paraconiothyrium brasiliense* (EU295632) | 0.79 |
| *Leptosphaeria* sp. | *Leptosphaeria* sp. South-west0034 (FJ537121) | 3.94 |
| *Shiraia* sp1. | *Shiraia bambusicola* (AB354989) | 0.79 |
| *Shiraia* sp2. | *Shiraia* sp. SUPER-H168 (EU267793) | 7.09 |
| *Alternaria tenuissima* | *Alternaria tenuissima* (AB369462) | 5.51 |
| *Podospora* sp. | *Podospora* sp. LZ612 (DQ810183) | 11.81 |

[a] Relative frequency means isolate frequency of a given endophyte divided by the sum of isolate frequencies of all endophytes × 100

**Table 2** Colonization, isolation, species richness, and multiple infection rates of fungal endophytes of each healthy tissue of *H. serrata*

| Parameter | Leaves | Stems | Roots | Total |
|---|---|---|---|---|
| No. of samples | 82 | 82 | 82 | 246 |
| No. of isolates recovered | 53 | 60 | 14 | 127 |
| No. of samples with isolates | 37 | 46 | 15 | 98 |
| Colonization frequency (CF) | 0.45 | 0.56 | 0.18 | 0.40 |
| Isolation rate (IR) | 0.65 | 0.73 | 0.17 | 0.52 |
| Margalef's richness index (R1) | 4.79 | 5.37 | 3.41 | – |

**Table 3** Jaccard similarity coefficients for the endophytic fungi isolated from tissues

| Tissues | Roots | Stems | Leaves |
|---|---|---|---|
| Roots | 1.00 | 0.38 | 0.20 |
| Stems | | 1.00 | 0.43 |
| Leaves | | | 1.00 |

*Colletotrichum*, and *Acremonium*. Among these endophytic fungi, the strains SF3, SF4, SF6, LF6, SF7, Slf14, LF17, RF22, SF25, LF30, LF33, LF40, LF46, SF58, LF73, SF96, LF116, SF123, SF136, and RF161 were located with high bootstrap support (≥98%) in their own cluster, whereas the strains LF70, SF153, LF15, SF64, and LF146 formed their own cluster with a bootstrap value from 65 to 96%. The five strains RF53, SF113, RF54, SF50, and LF86 shared sequence max identities from 92 to 97% with *Botrytis anthophila* (100% bootstrap), *Chaunopycnis alba* (88% bootstrap), *Aspergillus versicolor* (100% bootstrap), and *Capronia pilosella* (77% bootstrap), respectively. However, the three strains LF5, LF41, and SF142 were clustered to *Leptosphaeria microscopica* (52% bootstrap), *Penicillium* sp. (99% bootstrap), and *Penicillium* sp. (67% bootstrap), respectively, but sequence identities with the available references in NCBI were low (90–97%). In addition, the strains

**Table 4** Analysis of the endophytic fungi obtained from *H. serrata* at Lushan Botanical Garden (China)

| Strains | CF (%)[a] | GenBank accession no.[b] | Closest relatives in NCBI | Taxon[c] | Max identity (%) | AchE inhibitory activity (%)[d] | Fungal HupA[e] |
|---|---|---|---|---|---|---|---|
| LF146 | 2.8 | GU985229 | *Penicillium griseofulvum* | *Penicillium griseofulvum* | 99 | 64.4 ± 0.7 | + |
| SF64 | 2.8 | GU985208 | *Penicillium griseofulvum* | *Penicillium griseofulvum* | 98 | 52.5 ± 1.2 | − |
| LF73 | 2.4 | GU985204 | *Penicillium* sp. | *Penicillium citrinum* | 98 | 15.4 ± 0.8 | − |
| SF142 | 2.0 | GU985213 | *Penicillium* sp. | *Penicillium* sp1. | 97 | 67.8 ± 0.3 | + |
| SF136 | 2.8 | GU985212 | *Penicillium citrioviride* | *Penicillium citrioviride* | 98 | 51.8 ± 1.6 | − |
| SF58 | 2.0 | GU985207 | *Penicillium janthinellum* | *Penicillium janthinellum* | 98 | 53.3 ± 1.9 | − |
| LF116 | 0.8 | GU985227 | *Penicillium* sp. | *Penicillium* sp2. | 98 | 47.2 ± 0.4 | − |
| LF41 | 0.4 | GU951765 | *Penicillium* sp. | Unidentified | 95 | 18.9 ± 2.1 | − |
| LF46 | 0.4 | GU951767 | *Penicillium* sp. | *Penicillium* sp3. | 98 | 31.6 ± 1.1 | − |
| SF50 | 2.0 | GU985217 | *Aspergillus versicolor* | *Aspergillus versicolor* | 97 | 46.2 ± 1.8 | − |
| RF161 | 3.3 | GU985232 | *Aspergillus versicolor* | *Aspergillus versicolor* | 99 | 56.9 ± 0.4 | − |
| LF40 | 2.4 | GU951764 | *Aspergillus flavus* | *Aspergillus flavus* | 99 | 90.2 ± 1.5 | + |
| SF6 | 2.8 | GU951769 | *Aspergillus niger* | *Aspergillus niger* | 98 | 16.1 ± 2.3 | − |
| LF70 | 1.2 | GU599165 | *Cladosporium* sp. | *Cladosporium cladosporioides* | 99 | 86.5 ± 1.3 | + |
| SF153 | 1.2 | GU985231 | *Cladosporium* sp. | *Cladosporium* sp1. | 99 | 64.8 ± 2.3 | − |
| RF83 | 0.4 | GU985223 | *Mycoleptodiscus terrestris* | Unidentified | 89 | 75.9 ± 0.7 | + |
| SF61 | 0.4 | GU985221 | *Mycoleptodiscus terrestris* | Unidentified | 89 | 43.9 ± 0.6 | − |
| LF86 | 2.8 | GU985224 | *Capronia pilosella* | *Capronia* sp. | 97 | 36.1 ± 1.3 | − |
| RF22 | 2.8 | GU985216 | *Mortierella* sp. | *Mortierella* sp. | 100 | 24.7 ± 1.7 | − |
| LF23 | 0.4 | NS | | *Shiraia* sp1. | | 39.8 ± 0.3 | − |
| RF53 | 1.2 | GU985219 | *Botrytis anthophila* | *Botrytis anthophila* | 92 | 19.4 ± 0.5 | − |
| SF123 | 1.2 | GU985228 | *Coniothyrium* sp. | *Coniothyrium* sp. | 99 | 27.3 ± 1.7 | − |
| LF6 | 1.2 | GU985234 | *Paraconiothyrium brasiliense* | *Paraconiothyrium brasiliense* | 99 | 24.5 ± 1.3 | − |
| SF96 | 1.6 | GU985209 | *Leptosphaeria* sp. | *Leptosphaeria* sp. | 99 | 45.2 ± 2.5 | − |
| LF5 | 0.4 | GU985233 | *Leptosphaeria microscopica* | Unidentified | 90 | 78.3 ± 1.7 | + |
| LF51 | 0.4 | NS | | *Leptosphaeria* sp. | | 49.7 ± 0.4 | − |
| LF15 | 4.1 | GU951760 | *Shiraia bambusicola* | *Shiraia* sp1. | 100 | 69.7 ± 2.9 | + |
| Slf14 | 0.4 | GQ355934 | *Shiraia* sp. | *Shiraia* sp2. | 99 | 96.5 ± 3.2 | + |
| LF33 | 2.0 | GU951763 | *Alternaria tenuissima* | *Alternaria tenuissima* | 100 | 9.8 ± 2.2 | − |
| SF36 | 0.8 | NS | | *Alternaria tenuissima* | | 35.5 ± 0.6 | − |
| SF7 | 13.8 | GU951770 | *Podospora* sp. | *Podospora* sp. | 100 | 5.9 ± 0.8 | − |
| LF56 | 0.4 | NS | | *Podospora* sp. | | 23.8 ± 1.2 | − |
| SF3 | 15.9 | GU951768 | *Colletotrichum gloeosporioides* | *Colletotrichum gloeosporioides* | 99 | 54.6 ± 2.1 | − |
| LF17 | 0.8 | GU951761 | *Colletotrichum* sp. | *Colletotrichum* sp. | 99 | 51.6 ± 1.6 | − |
| RF54 | 1.2 | GU985220 | *Chaunopycnis alba* | *Chaunopycnis* sp. | 95 | 60.2 ± 0.4 | − |
| LF30 | 7.3 | GU951762 | *Acremonium implicatum* | *Acremonium implicatum* | 99 | 64.3 ± 3.1 | + |
| SF4 | 6.1 | GU985205 | *Acremonium* sp. | *Acremonium* sp. | 99 | 46.9 ± 0.6 | − |
| SF25 | 4.9 | GU985206 | *Acremonium* sp. | *Acremonium* sp. | 99 | 43.3 ± 2.3 | − |
| SF127 | 0.4 | GU985211 | *Acremonium* sp. | *Acremonium* sp. | 99 | 37.4 ± 0.7 | − |

− No HupA was detected in metabolites of endophytic fungi, + HupA was detected in metabolites of endophytic fungi

[a] *CF* colonization frequency

[b] *ns* not sequenced

[c] Identification based on morphological traits and ITS sequence analysis

[d] The inhibition effects of fungal extracts on AChE activity in vitro based on three replicate tests. The inhibition effects of authentic HupA was 93.2 ± 0.3, values are mean ± SE of three replications

[e] TLC, HPLC validation of fungal HupA was based on three replicate tests

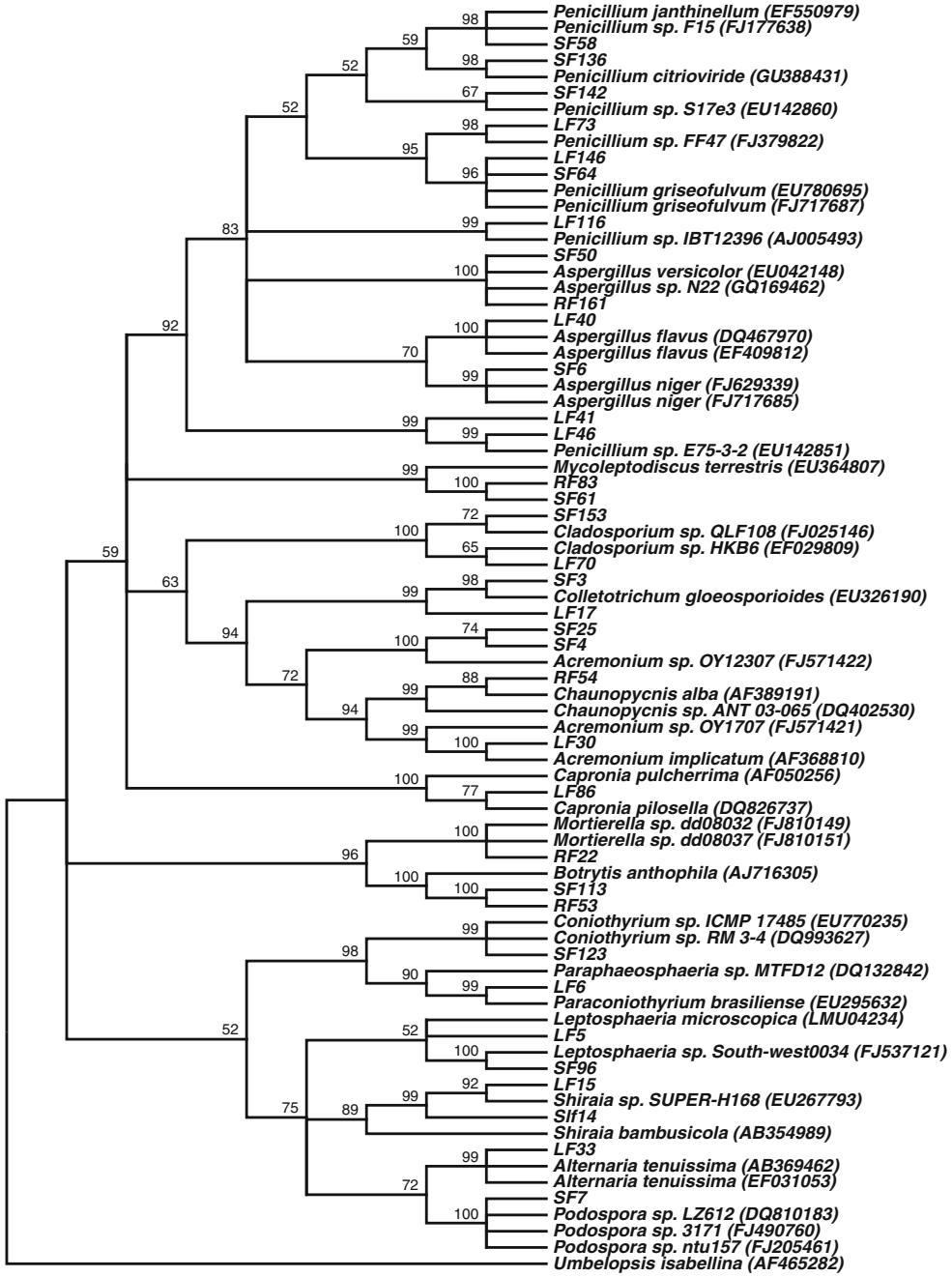


**Fig. 3** Neighbor-joining tree of the ITS sequences of the endophytic fungi associated with *H. serrata*. *Numbers at nodes* are bootstrap scores (above 50%) obtained from 1,000 replications. *Umbelopsis isabellina* is used as an outgroup

RF83 and SF61 were all placed within the clade *Mycoleptodiscus terrestris* with 99% support, respectively, but the sequence identities with the available references in NCBI were very low (89%). These fungi might represent novel species or even new genera.

AchE inhibitory activity of endophytic fungi extracts

A total of 127 endophytic fungi extracts were tested for their in vitro AChE-inhibitory activity, 39 of which showed AChE inhibition using HupA as a positive control (Table 4). Out of the 39 endophytic fungi extracts tested, 28.2% showed potent (>60% inhibition) AChE inhibition, 46.2% showed moderate (30–60% inhibition) inhibition, and 25.6% showed low activity (<30% inhibition). Among these endophytic fungi, the five strains LF5, Slf14, LF40, LF70, and RF83 showed a more potent effect in AChE inhibition, with a percent inhibition from 75.9 to 96.5%. Especially the extract of strain Slf14 obviously exhibited stronger inhibition activity (96.5%) than authentic HupA (93.2%).

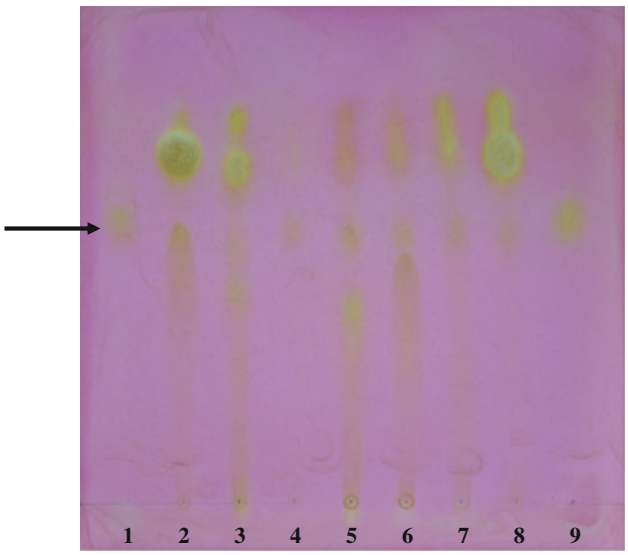


**Fig. 4** Thin-layer chromatography analysis of authentic HupA (*lanes 1*, *9*), and fungal LF5 (*lane 2*), RF83 (*lane 3*), Slf14 (*lane 4*), LF70 (*lane 5*), LF40 (*lane 6*), LF146 (*lane 7*), LF30 (*lane 8*) HupA formation in potato dextrose broth on silica gel. *Arrow* indicates the presence of HupA

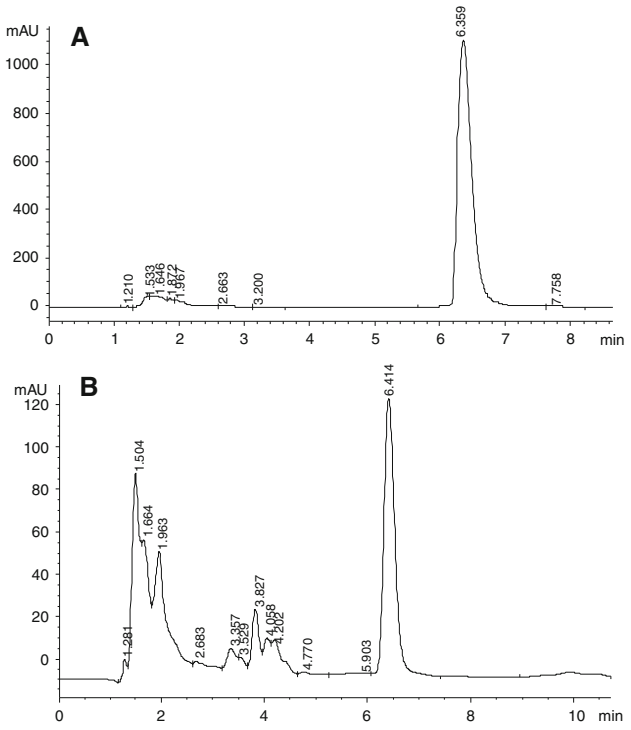


**Fig. 5** High-performance liquid chromatogram of standard huperzine A (**a**) and fungal huperzine A (**b**); the mobile phase was methanol/water (85:15), flow rate at 1.0 ml/min; Retention time of standard huperzine A: 6.359 min; retention time of fungal huperzine A: 6.414 min

### Screening of HupA-producing fungi

The extracts of the 39 endophytic fungi cultures were examined for the presence of HupA by TLC analysis. Nine (belonging to seven different genera) of them showed positive results for HupA production in PDB (Fig. 4). One of the fungal compounds exhibited the same $R_f$ values (0.74) as authentic HupA.

Results of HPLC analysis also confirmed the presence of HupA. Under the same HPLC conditions, we screened the extracts of the nine representative species to detect fungal HupA. The results showed the peak positions and peak shapes of the nine representative species from the different genera were identical or very close to that of the chemical reference (retention time: 6.321–6.557 min), demonstrating the nine distinct fungi produced HupA (Table 4). Among these HupA-producing fungi, the *Sharaia* sp. Slf14, had the highest HPLC peak area, and the HupA yield of the fungus was 327.8 μg/l or 142.6 μg/gdcw (HupA per dry wt of mycelium) when it was cultured in 100 ml PDB at 28°C with 120 rpm shaking for 14 days (Fig. 5). The MS confirmation of the fungal HupA (*Sharaia* sp. Slf14) is shown in Fig. 6. The authentic HupA yielded $MH^+$ at *m/z* 243.32 while the fungal HupA yielded a peak $MH^+$ at *m/z* 243.34. On the basis of LC-ESI-MS assays, the fungus, *Sharaia* sp. Slf14, did produce HupA.

## Discussion

Endophytic fungi obtained from the tissues (leaves, stems, and roots) of *H. serrata* from the natural populations at Lushan Botanical Garden represented a phylogenetically diverse array of fungal taxa. There were at least 15 fungal genera including five frequent genera and ten infrequent genera (Fig. 1; Table 4), confirming that a few species are frequent colonizers, and yet a majority of the groups are infrequent inhibitors in grasses and other plants in temperate regions and sub-tropical regions [10, 14, 17, 30, 31]. *Aspergillus*, *Podospora*, *Penicillium*, *Colletotrichum* and *Acremonium* were frequent colonizers in our study, whereas they are not cosmopolitan species within *H. serrata* growing other regions, showing dominant genera residing in the plant growing in different places are distinct. Gong et al. [8] isolated 180 fungi strains from *H. serrata* in Anhui Province and identified them as belonging to 13 genera based on morphological characteristics; *Phoma* and *Phacodium* were frequent colonizers. Except for *Aspergillus*, *Penicillium*, *Alternaria*, and *Colletotrichum* belonging to the identified genera in our study, other strains were designated to different genera. Chen et al. [3] isolated 52 fungi strains from *H. serrata* in Hainan Province and identified them as belonging to 11 genera based on rDNA ITS sequences analysis; *Colletotrichum* and *Hypoxylon* were frequent colonizers. Only *Colletotrichum* was recovered in our study. To our knowledge, *Podospora*, *Coniothyrium*, *Paraphaeosphaeria*, *Leptosphaeria*, *Mortierella*, *Capronia*,

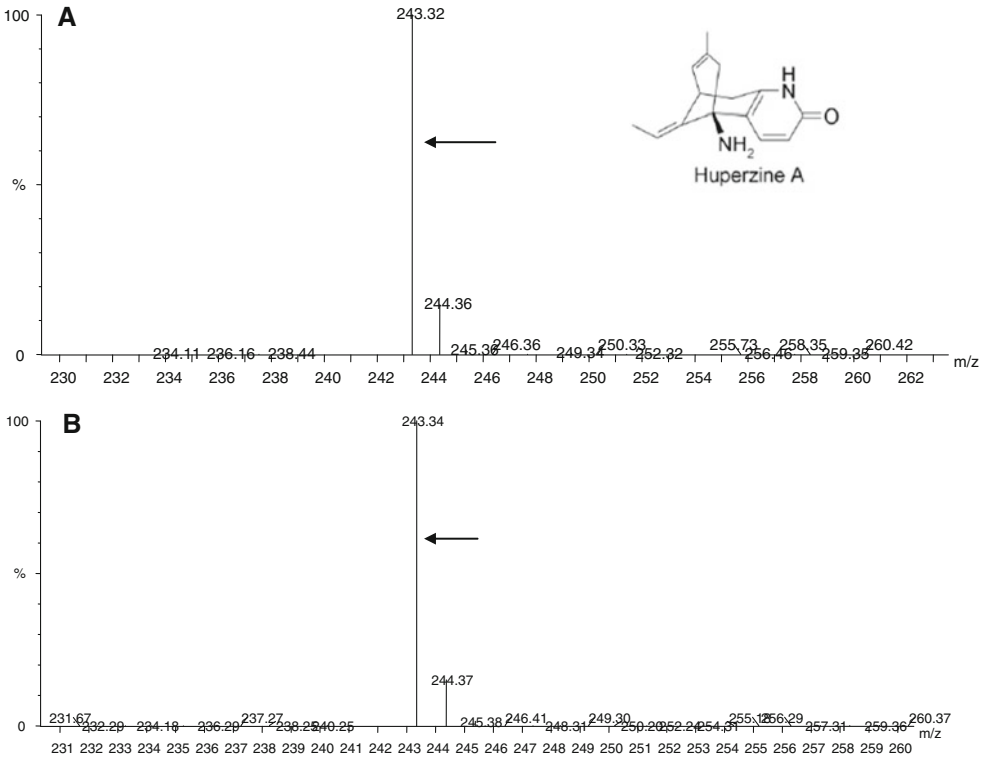


**Fig. 6** Electrospray mass spectra of huperzine A. **a** Standard huperzine A; **b** fungal huperzine A isolated from *Sharaia* sp., Slf14. *Arrow* indicates the molecular ion of HupA at *m/z* 243 ($M + H^+$)

and *Chaunopycnis* are never reported as endophytic fungi from *H. serrata*, and *Capronia* has been identified as endophytes by the culturable approach for the first time. As the present molecular database is not large enough to contain all fungal rDNA sequence information, some fungi are difficult to identify especially the nonsporulating ones. Some strains recovered here, such as RF83, SF61, and LF5 shared sequence max identities with known fungi (89–90%), suggesting that they could be undescribed taxa. The quantitative difference of endophytic fungi and distinctive fungal community within *H. serrata* is probably due to the climatic and regional distinction [13, 35]. Because of the limitations of traditional techniques, the present study probably did not detect all endophytes within the *H. serrata* tissues [11]. The detection of endophytes relies on the ability of the fungi to grow out from living tissues onto the agar. Had other molecular approaches (e.g., denaturing gradient gel electrophoresis, terminal restriction fragment length polymorphism) [5, 6, 42] coupled with phylogeny analysis, more taxa, including those slow growing and unculturable ones, would have been revealed.

A total of 39 endophytic fungi isolates, accounting for 30.7% of the total isolates, showed AChE inhibition in vitro (Table 4). Then the fungal extracts with AChE inhibition were analyzed by TLC, RP-HPLC, and LC-ESI-MS, nine (belonging to seven different genera) of the 39 endophytic fungi were verified for produced HupA (Table 4). Meanwhile, it was most interesting to observe that two endophytic fungi (SF153 and RF54) showed potent AChE inhibition (>60%) in spite of the absence of producing HupA. This implies that other AChE inhibitors (not HupA) may exist in strains SF153 and RF54. Future studies should be conducted to validate this. Previous studies demonstrated that the content of HupA in the leaf is richer than that in the stem and root of *H. serrata* [9, 38]. Interestingly, in the present study, most of HupA-producing fungi (77.8%, seven of the nine) were isolated from leaf tissues, only two from stem and root tissues. Maybe it further supports the theory of Young et al. [46] that during the course of evolution, the symbiotic endophytes developed machinery to biosynthesize and tolerate high levels of secondary metabolites in order to better compete and survive in association with the medicinal plant tissues.

Quantitative HPLC analysis showed that the HupA content of *Sharaia* sp. Slf14 (327.8 μg/l or 142.6 μg/gdcw) was higher than that of reported fungi *Acremonium* sp.2F09P03B (8.32 μg/l) [18], *Cladosporium cladosporioides* LF70 (56.84 μg/l) [49], *Blastomyces* sp. HA15, and *Botrytis* sp. HA23 (20–30 μg/gdcw) [15], indicating its potent potential for HupA commercial production. To meet the commercial need of HupA, we need to carry out further work to improve the HupA yield of the *Sharaia* sp. Slf14 by genetic engineering. Meanwhile, the discovery of HupA-producing endophytic fungi associated with Huperziaceae plants is valuable for both basic research and in industrial applications. So far, a biosynthetic pathway of

HupA in members of the Huperziaceae has been speculated by briefly [23], and the genes related to HupA biosynthesis from *H. serrata* have not been cloned. Meanwhile, no genes or biosynthetic pathways have yet been identified in any fungi; the molecular basis of HupA synthesis in fungi is still totally unknown. Therefore, genomic and metabolomic studies of these HupA-producing endophytic organism and the plant harboring it would be interesting to reveal the secondary metabolite pathway(s) used by the plant and its endophytic counterpart for generation of the important drug compounds. Not only that, since alkaloids are believed to act as defense compounds in the plants producing them, the production of the same by the endophytic population of those plants possibly demonstrates a probable symbiotic relationship between them in vivo.

**Acknowledgments** This work was supported by National Natural Science Foundation of China (Grant No. 21066014), Jiangxi Natural Science Foundation of China (Grant No. 2008GQN0075), Jiangxi Province key technology projects of China (Grant No. 2010BSA19200) and graduate student innovation fund of Jiangxi normal university (Grant No. YJS2010068). We thank Prof. Lehua Zhang from Lushan botanical garden, China for helping with the collection and identification of the plant material. We also wish to thank the anonymous reviewers for constructive comments.

## References

1. Aly AH, Debbab A, Kjer J, Proksch P (2010) Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal Divers. doi:10.1007/s13225-010-0034-4
2. Atta-ur-Rahman, Choudhary MI, Thomsen WJ (2001) Bioassay techniques for drug development. Harwood Academic Publishers, The Netherlands, pp 142–143
3. Chen XY, Qi YD, Wei JH, Zhang Z, Wang DL, Feng JD, Gan BC (2010) Molecular identification of endophytic fungi from medicinal plant *Huperzia serrata* based on rDNA ITS analysis. World J Microbiol Biotechnol. doi:10.1007/s11274-010-0480-x
4. College JNM (1985) The dictionary of traditional Chinese medicine. Shanghai Sci-Tech Press, Shanghai
5. Curlevski NJA, Chambers SM, Anderson IC, Cairney JWG (2009) Identical genotypes of an ericoid mycorrhiza-forming fungus occur in roots of *Epacris pulchella* (Ericaceae) and *Leptospermum polygalifolium* (Myrtaceae) in an Australian sclerophyll forest. FEMS Microbiol Ecol 67:411–420
6. Duong LM, Jeewon R, Lumyong S, Hyde KD (2006) DGGE coupled with ribosomal DNA phylogenies reveal uncharacterized fungal phylotypes on living leaves of *Magnolia liliifera*. Fungal Divers 23:121–138
7. Frohlich J, Hyde KD, Petrini O (2000) Endophytic fungi associated with *palms*. Mycol Res 104:1202–1212
8. Gong YX, Jiang TH, Chen FM, Zhang XP (2007) Isolation and screening of endophytic fungi from *Huperzia Serrata* and their antifungal activity. J Anhui Norm Univ 30:689–692
9. Gu YH, Wu QQ (2005) HPLC method for the determination of huperzine A in *Huperzia serrata*. Chin Pharmacol Bull 21:1017–1018
10. Guimaraes DO, Borges WS, Kawano CY, Ribeiro PH, Goldman GH, Nomizo A, Thiemann OH, Oliva G, Lopes NP, Pupo MT (2008) Biological activities from extracts of endophytic fungi isolated from *Viguiera arenaria* and *Tithonia diversifolia*. FEMS Immunol. Med Microbiol 52:134–144
11. Guo LD, Huang GR, Wang Y, He WH, Zheng WH, Hyde KD (2003) Molecular identification of white morphotype strains of endophytic fungi from *Pinus tabulaeformis*. Mycol Res 107:680–688
12. Hata K, Futai K (1995) Endophytic fungi associated healthy pine needles infested by the pine needle gall midge, *Thecodiplosis japonensis*. Can J Bot 73:384–390
13. Higgins KL, Arnold AE, Miadlikowska J, Sarvate SD, Lutzoni F (2007) Phylogenetic relationships, host affinity, and geographic structure of boreal and arctic endophytes from three major plant lineages. Mol Phylogenet Evol 42:543–555
14. Huang YJ, Wang JF, Li GL, Zheng ZH, Su WJ (2001) Antitumour and antifungal activities in endophytic fungi isolated from pharmaceutical plants *Taxus mairei*, *Cephalataxus fortune* and *Torreya grandis*. FEMS Immunol Med Microbiol 31:163–167
15. Ju Z, Wang J, Pan SL (2009) Isolation and preliminary identification of the endophytic fungi which produce Hupzine A from four species in Hupziaceae and determination of huperzine A by HPLC. Fudan Univ J Med Sci 36:445–449
16. Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163
17. Li HY, Qing C, Zhang YL, Zhao ZW (2005) Screening for endophytic fungi with antitumor and antifungal activities from Chinese medicinal plants. World J Microbiol Biotechnol 21:1515–1519
18. Li WK, Zhou JY, Lin ZW, Hu ZB (2007) Study on fermentation condition for production of huperzine A from endophytic fungus 2F09P03B of *Huperzia serrata*. Chin Med Biotechnol 2:254–259
19. Liu JS, Yu CM, Zhou YZ, Han YY, Wu FW, Qi BF, Zhu YL (1986) Study on the chemistry of huperzine-A and huperzine-B. Acta Chimi Sin 44:1035–1040
20. Liu JS, Zhu YL, Yu CM, Zhou YZ, Han YY, Wu FW, Qi BF (1986) The structures of huperzine A and B, two new alkaloids exhibiting marked anticholinesterase activity. Can J Chem 64:837–839
21. Ma XQ (1997) Chemical studies on natural resources of *Huperzia* and its related genera in China. PhD Thesis, Chinese Academy of Sciences, Shanghai
22. Ma XQ, Gang DR (2004) The Lycopodium alkaloids. Nat Prod Rep 21:752–772
23. Ma XQ, Gang DR (2008) In vitro production of huperzine A, a promising drug candidate for Alzheimer's disease. Phytochemistry 69:2022–2028
24. Ma X, Tan C, Zhu D, Gang DR (2005) Is there a better source of huperzine A than *Huperzia serrata*? Huperzine A content of Huperziaceae species in China. J Agric Food Chem 53:1393–1398
25. Ma X, Tan C, Zhu D, Gang DR (2006) A survey of potential huperzine A natural resources in China: the Huperziaceae. J Ethnopharmacol 104:54–67
26. Ma X, Tan C, Zhu D, Gang DR, Xiao P (2007) Huperzine A from *Huperzia speciesan* ethnopharmacological review. J Ethnopharmacol 113:15–34
27. Petrini O (1991) Fungal endophytes of tree leaves. In: Andrew JH, Hirano SS (eds) Microbial ecology of leaves. Springer, Berlin Heidelberg New York, pp 179–197
28. Saikkonen K, Faeth SH, Helander M, Sullivan TJ (1998) Fungal endophytes: a continuum of interactions with host plants. Annu Rev Ecol Syst 29:319–343
29. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

30. Sánchez Márquez S, Bills GF, Zabalgogeazcoa I (2007) The endophytic mycobiota of the grass *Dactylis glomerata*. Fungal Divers 27:171–195
31. Sánchez Márquez S, Bills GF, Zabalgogeazcoa I (2008) Diversity and structure of the fungal endophytic assemblages from two sympatric coastal grasses. Fungal Divers 33:87–100
32. Schulz B, Boyle C, Draeger S, Rommert AK, Krohn K (2002) Endophytic fungi: a source of novel biologically active secondary metabolites. Mycol Res 106:996–1004
33. Stierle A, Strobei G, Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of pacific yew. Science 260:214–216
34. Stone JK, Bacon CW, White JF Jr (2000) An overview of endophytic microbes: endophytism defined. In: Bacon CW, White JF (eds) Microbial endophytes. Marcel Dekker, New York, pp 3–29
35. Strobel G, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 67:491–502
36. Strobel G, Daisy B, Castillo U, Harper J (2004) Natural products from endophytic microorganisms. J Nat Prod 67:257–268
37. Strobel G, Yang XS, Sears J, Kramer R, Sidhu RS, Hess WM (1996) Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*. Microbiology 142:435–440
38. Sun YM, Yu HY, Yang YS, Yang JY, Zhang M (2002) Determination of hupzine A in *Huperzia serrata* by HPLC. Chin Tradit Herb Drugs 33:1078–1080
39. Suryanarayanan TS, Kumaresan V (2000) Endophytic fungi of some halophytes from an estuarine mangrove forest. Mycol Res 104:1465–1467
40. Szypula W, Pietrosiuk A, Suchocki P, Olszowska O, Furmanowa M, Kazimierska O (2005) Somatic embryogenesis and in vitro culture of *Huperzia selago* shoots as a potential source of huperzine A. Plant Sci 168:1443–1452
41. Tan R, Zou W (2001) Endophytes: a rich source of functional metabolites. Nat Prod Rep 18:448–459
42. Tao G, Liu ZY, Hyde KD, Lui XZ, Yu ZN (2008) Whole rDNA analysis reveals novel and endophytic fungi in *Bletilla ochracea* (Orchidaceae). Fungal Divers 33:101–122
43. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: Xexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882
44. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and application. Academic Press, San Diego, pp 315–322
45. Yang QP, Kou XL, Fugal KB, Mclaughlin JL (2003) Determination of Huperzine A in formulated products by reversed-phase-liquid chromatography using diode array and electrospray ionization mass spectrometric detection. Phytomedicine 10:200–205
46. Young DH, Michelotti EJ, Sivendell CS, Krauss NE (1992) Antifungal properties of taxol and various analogues. Experientia 48:882–885
47. Zangara A (2003) The psychopharmacology of Huperzine A: an alkaloid with cognitive enhancing and neuroprotective properties of interest in the treatment of Alzheimer's disease. Pharmacol Biochem Behav 75:675–686
48. Zhang D, Yang Y, Castlebury LA, Cerniglia CE (2006) A method for the large scale isolation of high transformation efficiency fungal genomic DNA. FEMS Microbiol Lett 145:261–265
49. Zhang ZB, Zeng QG, Yan RM, Wang Y, Zou ZR, Zhu D (2010) Endophytic fungus *Cladosporium cladosporioides* LF70 from *Huperzia serrata* produces Huperzine A. World J Microbiol Biotechnol. doi:10.1007/s11274-010-0476-6