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Isolation and characterization of endophytic taxol-producing fungi from *Taxus chinensis*

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Abstract This study investigated the endophytic fungi diversity of *Taxus chinensis* and screened the taxol-producing fungi in the host. A total of 115 endophytic fungi isolates obtained from bark segments of *T. chinensis* were grouped into 23 genera based on the morphological traits and sequence analysis of the internal transcribed spacers (ITS1-5.8S-ITS2), indicating endophytic fungi in *T. chinensis* are diverse and abundant. *Diaporthe*, *Phomopsis* (anamorph of *Diaporthe*), *Acremonium*, and *Pezicula* were the dominant genera, whereas the remaining genera were infrequent groups. The 13 representative species of the distinct genera were capable of producing taxol verified by reverse-phase high performance liquid chromatography (HPLC). Among the taxol-producing fungi, the yield of taxol produced by the *Metarhizium anisopliae*, H-27 was 846.1 μg l^{-1} in reformative potato dextrose liquid medium, and the fungal taxol was further validated by mass spectrometry (MS). The taxol-producing fungi (92.3%) were infrequent communities, suggesting that infrequent fungi associated with *T. chinensis* might be a fascinating reservoir of taxol-generating fungi.

Keywords Endophytic fungi · ITS sequence · Phylogenetic diversity · Taxol · *Taxus chinensis*

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Introduction

Endophytic fungi are microorganisms that reside in living plant tissues, apparently without inflicting negative effects [2]. Endophytes are presumably ubiquitous in the plant kingdom, some of which can improve the ecological adaptability of hosts [12, 15, 16]. Moreover, certain endophytic fungi are capable of synthesizing the medicinal products produced in plants [23]. At present, much research has focused on isolation of endophytic fungi from pharmaceutical plants, such as *Camptotheca acuminate* [11], pine [7], and *Taxus* plants [4, 9, 26], discovering a vast number of undescribed endophytic fungi species, some of which have potential to be used in the production of medicine. Therefore, investigations of endophytic fungi are crucial for conservation and utilization of fungal resources in plants.

Taxomyces andreanae, an endophytic fungus obtained from the Pacific Yew, producing taxol was discovered by Stierle et al. [19], uncovering a novel strategy to produce taxol to meet the growing demand in clinics. In the past decade, extensive efforts to isolate endophytic fungi in *Taxus* plants in different geographical settings have led to the discovery of some taxol-producing fungi with taxol yields ranging from 24 ng l^{-1} to 187.6 μg l^{-1} [6, 19]. Although the amount of taxol found in most *Taxus*-associated fungi is small when compared with that of the trees, the short generation time and high growth rate of fungi make it worthwhile to continue our systemic investigations of these species. However, to date, there are rare reports of endophytic fungi living in *Taxus chinensis*.

In the mountainous regions of QinBa (China), located in the joint belt of warm temperate and northern subtropical climate, there are virgin forests of *T. chinensis* trees. Whether the *T. chinensis* plants in the mentioned regions secrete abundant endophytic fungi is unknown. An answer

to this question will not only increase our knowledge about the diversity of endophytic fungi in *T. chinensis*, but also highlight some taxol-producing fungi. In this paper, we surveyed the endophytic fungi diversity of *T. chinensis* and screened the endophytic taxol-producing fungi residing in the tissues of *T. chinensis* in this area.

Materials and methods

Plant materials and surface sterilization

Healthy stems were collected from *T. chinensis* grown at the forest site (33.34°N, 107.59°W), located in the mountainous region of Qinba (China), in December 2007. The samples were surface-sterilized by washing in 70% ethanol (v/v) for 1 min and 0.1% mercuric chloride (v/v) for 8 min, respectively. The branches were rinsed six times in sterile distilled water, then employed to isolate endophytic fungi.

Isolation of endophytic fungi and colonization frequency

The barks of the sterile stems were cut into pieces of 1 cm^2, then plated on potato dextrose agar (PDA) for incubation at 28°C for 2–15 days to allow the growth of endophytic fungi, and checked regularly. Pure isolates were obtained by picking fungal tips. The colonization frequency (CF%) of each endophyte was calculated using the method as described by Fisher and Petrini [5].

DNA extraction, PCR amplification, and sequencing

A 0.5-g mycelia of different endophytic fungi was, respectively, ground with a sterile mortar in liquid nitrogen. DNA was extracted by the CTAB method [27].

The fungal ITS fragments were amplified using the universal primers ITS1 and ITS4 [22]. The PCR reaction mixtures (20 µl) were composed of 1 µl genomic DNA (100 ng), 2 µl 10 × PCR reaction buffer, 2 µM $MgCl_2$, 0.5 µl 10 µM forward and reverse primers, 0.5 µl 2.5 µM each of deoxyribonucleotide triphosphate, 0.2 µl 5 U of *Ex* Taq DNA polymerase (TaKaRa, DaLian), and 13.3 µl PCR quality water. The PCR reaction programs were pre-heating at 94°C for 5 min, 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. The PCR products were separated on 1.5% (wt/vol) agarose gel and purified using a DNA Gel Exaction Kit (cat. no. BS353). The resulting DNA was sequenced directly using the same primers.

Molecular phylogenetics

The ITS sequences of the endophytic fungi were compared with the data available in NCBI using BLAST searches to estimate the phylogenetic relationships of the endophytic fungi. The resulting sequences were aligned with the Clustal X software [25], with gaps treated as missing data. The phylogenetic tree was performed using the neighbor-joining method [18] and the Kimura two-parameter distance calculation in mega software version 3 [10]. The bootstrap was 1,000 replications to assess the reliable level to the nods of the tree.

Screening of taxol-producing fungi

The fungal endophytes were inoculated, respectively, into 500-ml Erlenmeyer flasks containing 300 ml of the liquid medium (potato 200 g l^{-1}, sugar 40 g l^{-1}, peptone 0.5 g l^{-1}, yeast extracts 0.8 g l^{-1}, $(NH_4)_2SO_4$ 3 g l^{-1}, KH_2PO_4 2 g l^{-1}, $MgSO_4{\cdot}7H_2O$ 0.5 g l^{-1}, and phenylalanine 0.01 g l^{-1}) and cultured at 160 rpm at 28°C for 14 days in a rotary shaker. The mycelial pellets were harvested by filtration and freezed at −20°C for 5 h, then thoroughly crushed in a mortar. The fermentation broths and ground mycelia were subjected to ultrasound-assisted extraction three times with ethyl acetate at room temperature for 5 h. All extracts were combined and condensed in a rotating evaporator under reduced pressure. The residues were redissolved with 10 ml of 100% methanol (v/v).

Fungal extracts were analyzed by reverse-phase HPLC using an RP-C18 column (4.6 × 150 mm, 5 µm) with UV absorbance at 228 nm. Each sample extract (20 µl) was injected on the RP-C18 column. The separation was performed at a flow rate of 1 ml min^{-1} with methanol–water (65:35, v/v) as the mobile phase. Standard curves were generated using a serial dilution of authentic taxol (0.01–0.08 mg ml^{-1}). There was a linear relationship between the concentration of standard taxol and area of absorbed peak ($r = 0.9992$). Taxol content analysis was performed according to the formula: $M = M_0 \times V_1 \times 10^6/V_2$. M (µg l^{-1})—taxol content in fermentation cultures; M_0 (mg ml^{-1})—taxol content in methanol solution (per ml); V_1 (ml)—volume of methanol used in redissolving of residues; V_2 (ml)—volume of fermentation broths for extraction.

Taxol was identified by MS analysis using the Electro Spray Ionization (ESI) technique with an Agilent 1100 LC/MSD trap. The nebulizer gas flow rate of the sample was 2 µl min^{-1}, and the capillary voltage was 2.2 kV.

Results

Isolation, colonization frequency of culturable endophytic fungi in *T. chinensis*

The 115 endophytic fungal strains were isolated from the bark tissues of *T. chinensis* grown in the Qinba mountains,

Table 1 Analysis of the endophytic fungi obtained from *T. chinensis* in the mountainous region of Qinba (China)

Fungal isolate[a]	CF%[b]	ITS identity (%)	Accession number	Closest relatives in NCBI	Taxon[c]	Fungal taxol[d]
H-1	0.9	99	FJ375136	*Diaporthe eres*	*Diaporthe eres*	–
H-3	0.9	100	FJ375139	*Alternaria alternata*	*Alternaria* sp.	–
H-4	0.9	99	FFJ375140	*Fusarium solani*	*Fusarium solani*	+
H-5	0.9	100	FJ375146	*Rhizopus oryzae*	*Rhizopus oryzae*	–
H-7	0.9	99	FJ375145	*Paraconiothyrium brasiliense*	*Paraconiothyrium brasiliense*	+
H-8	3.4	86.87	FJ375144	*Pyrenochaeta romeroi*	Unidentified	–
H-9	0.9	97	FJ375143	*Trichoderma saturnisporum*	*Trichoderma* sp.	+
H-12	10.3	99	FJ375142	*Diaporthe phaseolorum*	*Phomopsis* sp.	+
H-13	0.9	99	FJ375141	*Aspergillus versicolor*	*Aspergillus* sp.	+
H-14	0.9	99	FJ375137	*Paraconiothyrium brasiliense*	*Paraconiothyrium brasiliense*	–
H-15	22.2	≥99	FJ375138	*Acremonium alternatum*	*Acremonium alternatum*	–
H-17	0.9	100	FJ375154	*Aspergillus versicolor*	*Aspergillus* sp.	–
H-18	0.9	99	FJ375153	*Trichoderma gamsii*	*Trichoderma* sp.	–
H-19	2.6	99	FJ375152	*Xylaria venosula*	*Xylaria* sp.	+
H-20	0.9	99	FJ375151	*Mycorrhizal basidiomycete*	*Mycorrhizal basidiomycete*	–
H-21	0.9	100	FJ375150	*Cladosporium tenuissimum*	*Cladosporium tenuissimum*	+
H-22	0.9	100	FJ375149	*Sordaria macrospora*	*Sordaria* sp.	+
H-23	0.9	99	FJ375148	*Neonectria radicicola*	*Neonectria radicicola*	–
H-25	15.4	98	FJ375147	*Pezicula sporulosa*	*Pezicula* sp.	–
H-26	0.9	97	FJ375162	*Epacris microphylla*	*Epacris* sp.	–
H-27	0.9	≥99	FJ375161	*Metarhizium anisopliae*	*Metarhizium anisopliae*	+
H-28	0.9	100	FJ375160	*Cryptococcus flavescens*	*Cryptococcus flavescens*	–
H-29	0.9	99	FJ375159	*Hypocrea lixii*	*Hypocrea lixii*	–
H-30	0.9	84	FJ375158	*Ascomycete* sp.	*Fusarium* sp.	–
H-31	0.9	86	FJ375157	*Preussia flanaganii*	Unidentified	–
H-32	18.8	99	FJ375156	*Diaporthe phaseolorum*	*Diaporthe* sp.	–
H-33	4.3	98	FJ375155	*Pezicula sporulosa*	*Pezicula* sp.	+
H-34	1.7	100	FJ375167	*Botryosphaeria obtuse*	*Botryosphaeria obtuse*	–
H-37	0.9	100	FJ462758	*Coniothyrium diplodiella*	*Coniothyrium diplodiella*	+
H-38	0.9	88	FJ375165	*Ceratobasidium* sp.	Unidentified	+
H-39	0.9	97	FJ375164	*Epacris microphylla*	*Epacris* sp.	+

–, No taxol was detected in metabolites of endophytic fungi; +, taxol was detected in metabolites of endophytic fungi

[a] Isolates with prefix H- were cultivated from *T. chinensis*

[b] CF%, colonization frequency

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

[d] HPLC validation of fungal taxol was based on three replicate tests

China, and assigned to 31 morphotypes based on the morphological characteristics.

Analysis of distribution frequencies of the 115 morphotypes revealed that the fungal communities in the host contained a few frequent genera and many infrequent groups (Table 1). *Phomopsis* (anamorph of *Diaporthe*), *Diaporthe*, *Pezicula*, and *Acremonium* were the dominant genera, accounting for colonization frequencies ranging from 10.3 to 22.2%. Among the rare morphotypes, *Botryosphaeria obtuse* H-34, *Xylaria* sp. H-19, the fungus H-8 (unidentified), and *Pezicula* sp. H-33 represented isolation frequencies of 1.7, 2.6, 3.4, and 4.3, respectively, whereas others only showed that of 0.9%.

Molecular phylogenetics

The ITS neighbor-joining tree of the endophytic fungi is shown in Fig. 1. The 24 morphospecies (H-1, H-3, H-4, H-5, H-7, H-12, H-13, H-14, H-15, H-17, H-18, H-19, H-20, H-21, H-22, H-23, H-25, H-27, H-28, H-29, H-32, H-33, H-34, and H-37) sharing sequence similarities of ≥98% with available data in NCBI (Table 1) were grouped into 17

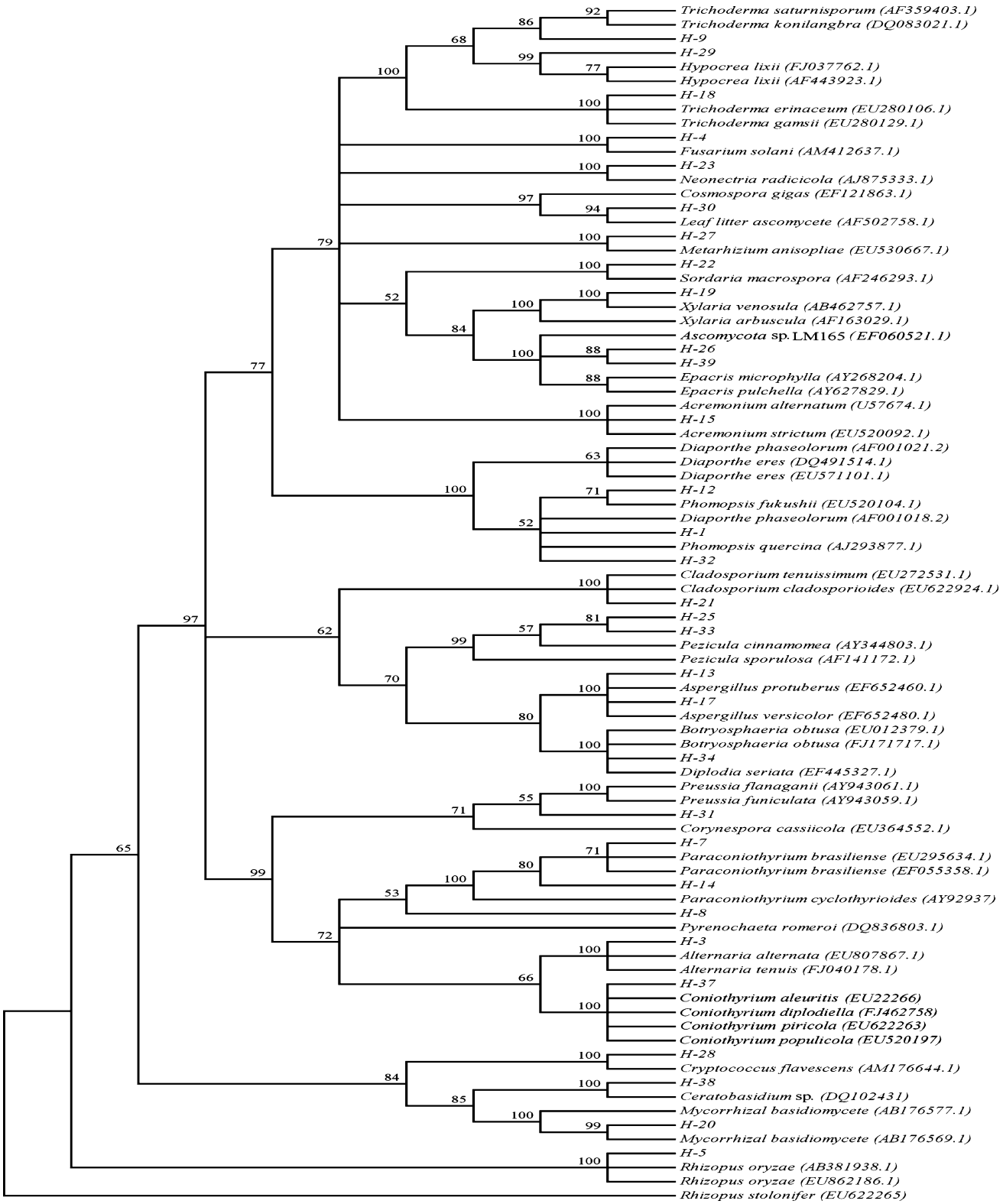

Fig. 1 Neighbor-joining tree of the ITS sequences of the endophytic fungi associated with *T. chinensis*. Numbers at nodes are bootstrap scores (above 50%) obtained from 1,000 replications. *Rhizopus stolonifer* is used as an outgroup

genera of *Diaporthe*, *Alternaria*, *Paraconiothyrium*, *Aspergillus*, *Acremonium*, *Trichoderma*, *Xylaria*, *Mycorrhizal*, *Cladosporium*, *Sordaria*, *Neonectria*, *Metarhizium*, *Cryptococcus*, *Hypocrea*, *Botryosphaeria*, *Pezicula*, and *Coniothyrium*. Among these endophytic fungi, the strains (H-3, H-4, H-5, H-13, H-15, H-17, H-18, H-19, H-20, H-21, H-23,

H-27, H-28, H-29, H-34, and H-37) were located with a high bootstrap support (≥99%) in their own cluster, whereas the strains (H-1, H-7, H-9, H-12, H-14, H-32, and H-33) formed their own cluster with a bootstrap value from 52 to 86%.

The three strains (H-9, H-26, and H-39) shared sequence similarities of 97% with *Trichoderma saturnisporum* (86% bootstrap) and *Epacris microphylla* (100% bootstrap), respectively. However, the three strains (H-30, H-31, and H-38) were clustered to a leaf litter *Ascomycete* (GenBank description) with a bootstrap value of 94%, *Preussia flanaganii* (55% bootstrap), and *Ceratobasidium* sp. (100% bootstrap), respectively, but sequence identities with the available references in NCBI were very low (84–88%). In addition, the strain (H-8) was not similar to any references with a bootstrap value of 53%. These fungi might represent novel species or even new genera.

Screening of taxol-producing fungi

Under the same HPLC conditions, we screened the extracts of the 31 representative species to detect fungal taxol. The results showed the peak positions and peak shapes of the 13 representative species from the different genera were identical or very close to that of the chemical reference (retention time = 7.659 ± 0.2 min), demonstrating the 13 distinct fungi produced taxol (Table 1). Among these taxol-producing fungi, the *M. anisopliae*, H-27, had the highest HPLC peak area, and the taxol yield of the fungus was 846.1 μg l^{-1} in the liquid medium (Fig. 2.)

The MS confirmation of the fungal taxol (*M. anisopliae*, H-27) is shown in Fig. 3. The authentic taxol yielded MH+ at *m/z* 854.2 and MNa+ at *m/z* 876.3. The fungal taxol yielded a peak MH+ at *m/z* 852.8 and a characteristic fragment peak MNa+ at *m/z* 876.3. On the basis of HPLC-MS assays, the fungus, H-27, did generate taxol in vitro.

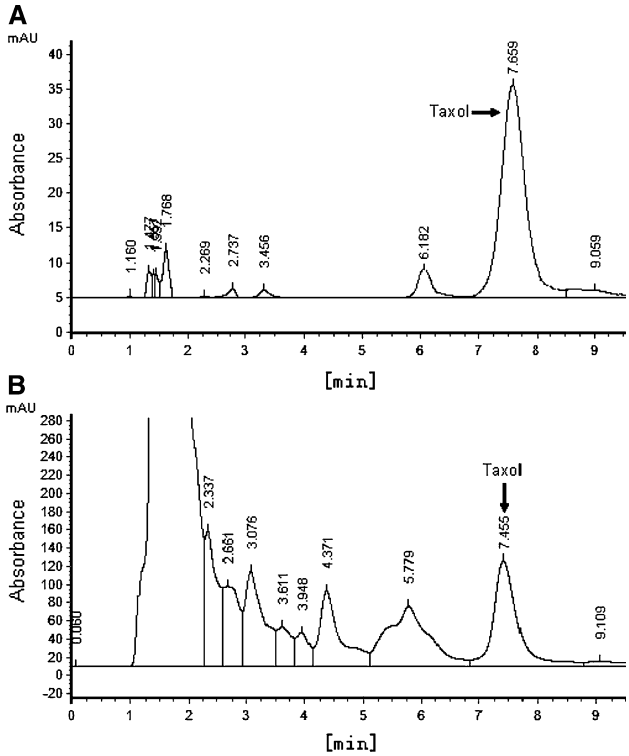

Fig. 2 Verification of taxol by HPLC chromatogram. **a** The standard curve of the authentic taxol. **b** Ethyl acetate extracts from the mycelial cultures of the *M. anisopliae*, H-27

Discussion

Endophytic fungi obtained from the stems of *T. chinensis* in the mountainous region of QinBa (China) represented a phylogenetically diverse array of fungal taxa, including 3 frequent genera and 20 rare genera (Table 1; Fig. 1), confirming that a few species are frequent colonizers, and yet a majority of the groups are rare inhibitors in woody plants in temperate regions and tropical regions [13, 24]. *Diaporthe*, *Phomopsis* (anamorph of *Diaporthe*), *Acremonium*, and *Pezicula* are frequent colonizers in *T. chinensis*, whereas they are not cosmopolitan species within other *Taxus* plants, such as *Taxus mairei* and *Taxus baccata* [3, 21], showing dominant genera residing in different yews are distinct.

Among the 20 infrequent genera, *Xylaria*, *Coniothyrium*, and *Botryosphaeria* have not been reported from *Taxus* trees; however, they have been obtained from other host plants [1, 14, 17]. *Fusarium* and *Alternaria* were the dominant genera acquired from *T. baccata* [3]. *Paraconiothyrium*, *Sordaria*, *Mycorrhizal*, *Cryptococcus*, *Metarhizium*, and *Epacris* were first discovered in *Taxus* plants. Some genera recovered here, such as *Trichoderma*, *Ascomycete*, *Preussia*, and *Ceratobasidium,* shared ITS similarities with known fungi (84–97%), suggesting they could be undescribed taxa. The distinctive fungal community within *T. chinensis* might be a result of host specificity and geographic settings [8, 20].

The 13 representative species belonging to the different genera, accounting for 11.3% of the total isolates, were verified for producing taxol in vitro (Table 1), which was surprising. *Fusarium solani*, some species of *Aspergillus* and *Phomopsis* have been proven to be capable of producing taxol in vitro [3, 4]. *Paraconiothyrium brasiliense*, *Trichoderma* sp., *Xylaria* sp., *Cladosporium tenuissimum*, *Sordaria* sp., *Metarhizium anisopliae*, *Pezicula* sp., *Coniothyrium diplodiella*, *Epacris* sp., and one unidentified species (H-38) have not been obtained from other yews, demonstrating that *T. chinensis* harbored novel and highly diverse taxol-producing fungi. In addition, some taxol-producing fungi

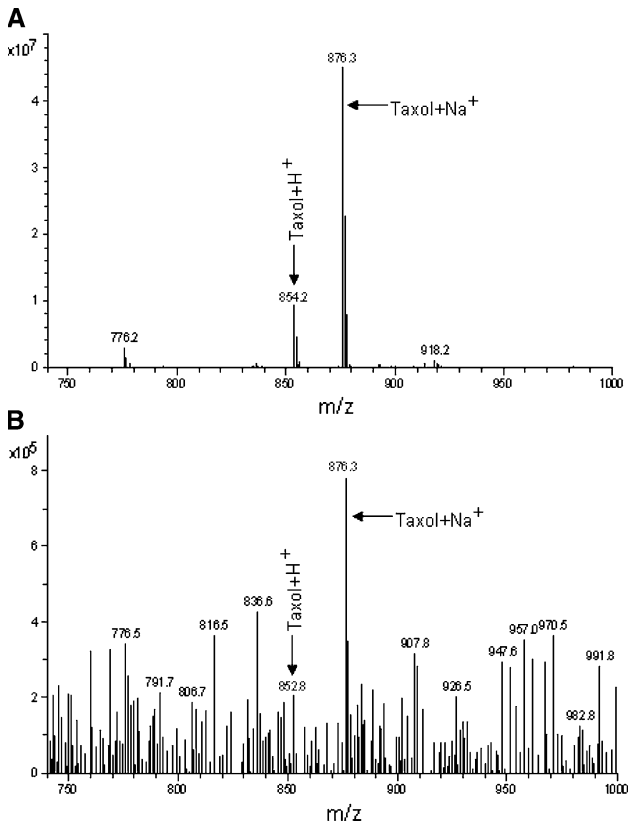

Fig. 3 Mass spectrometry analysis of the reference taxol (**a**) and the fungal taxol of the *M. anisopliae*, H-27 (**b**). The diagnostic mass spectral fragment ions are at *m/z* 854 (M + H)+ and 876 (M + Na)+

reported from other *Taxus* plants have not been acquired from *T. chinensis*, suggesting certain taxol-generating fungi seem to be host-specific. Interestingly, the taxol-producing fungi (92.3%) were recovered as infrequent genera, revealing that infrequent genera from *T. chinensis* grown in subtropic regions might be a huge source of taxol-producing fungi. The endophyte that possesses peculiar metabolitic functions could achieve extraordinary success in occupying a niche within plant tissues or even contribute to host defenses against the invading pathogens. From this point of view, infrequent fungal communities from *T. chinensis* producing taxol could be an evolutionary adaptation.

Quantitative HPLC analysis showed the taxol content of *M. anisopliae*, H-27, was higher than that of reported fungi, ranging from 24 ng l^{-1} to 187.6 μg l^{-1} [6, 19], indicating its potent potential for taxol commercial production. The high taxol yield suggests that in order to isolate taxol-producing fungal species, more consideration should be given to different hosts populating unique conditions.

To meet the commercial need of taxol, we need to carry out further work to improve the taxol yield of the *M. anisopliae*, H-27, by genetic engineering. Moreover, analysis of genes of these diverse fungi involved in taxol synthesis will provide significant insight into understanding of the coevolutionary mechanisms of the endophyte host.

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