Diversity and bioactive potential of culturable fungal endophytes of *Dysosma versipellis*; a rare medicinal plant endemic to China

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The plant *Dysosma versipellis* is known for its antimicrobial and anticancer properties but is a rare and vulnerable perennial herb that is endemic to China. In this study, 224 isolates were isolated from various tissues of *D. versipellis*, and were classified into 53 different morphotypes according to culture characteristics and were identified by sequence analyses of the internal transcribed spacer (ITS) region of the rRNA gene. Although nine strains were not assignable at the phylum level, 44 belonged to at least 29 genera of 15 orders of Ascomycota (93%), Basidiomycota (6%), and Zygomycota (1%). Subsequent assays revealed antimicrobial activities of 19% of endophytic extracts against at least one pathogenic bacterium or fungus. Antimicrobial activity was also determined using the agar diffusion method and was most prominent in extracts from four isolates. Moreover, high performance liquid chromatography (HPLC) and ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry analyses (UPLC–QTOF MS) showed the presence of podophyllotoxin in two *Fusarium* strains, with the highest yield of 277 μg/g in *Fusarium* sp. (WB5121). Taken together, the present data suggest that various endophytic fungi of *D. versipellis* could be exploited as sources of novel natural antimicrobial or anticancer agents.

Resistance to antibiotics and drugs in pathogenic bacteria and fungi and overuse of antibiotics are the major challenges for researchers all over the world¹. Thus, safer and novel antimicrobial drugs are eagerly awaited⁴, and natural secondary metabolites from endophytic fungi are increasingly considered due to their diverse structural classes and various bioactivities. These include antifungal⁵, antibacterial⁶, anticancer, anti-HIV⁷, and other promising bioactivities⁸. In addition, endophytic fungi are nontoxic and, thus, provide a promising source of novel drugs⁸.

Endophytic fungi inhabit living plant tissues without causing apparent disease or injury to the host⁹ and are ubiquitous in vascular plant species⁹,¹¹. Currently, less than 10% of the approximately one million known terrestrial endophytes have been investigated¹². However, several rare medicinal plants produce important bioactive compounds to survive in unique environments and may host novel and diverse fungal endophytes⁶,¹³, and these have rarely been isolated and characterized.

*Dysosma versipellis* (Hance) M. Cheng ex Ying (Fig. 1a) is commonly referred to as podophyllum, hemipilia, fatsia, or octagonal lotus, and is a rare and vulnerable perennial herb of the Berberidaceae family¹⁴. This plant species is endemic to China and is mainly distributed in high altitudes ranging from 200–2400 m above sea level in disjunct stands of warm-temperate, deciduous, montane forests (Fig. 1b) across central and eastern China¹⁵. *Dysosma* species including *D. aurantiocaulis*, *D. difformis*, *D. majorensis*, *D. pleiantha*, *D. tsayuensis*, *D. veitchii*, and *D. versipellis* have been identified in previous studies and six of these are endemic to China¹⁶. As a traditional Chinese medicine, extracts from the rhizomes of this plant has been used as antibacterial treatments for syphilis and an antidote for snake bites¹⁷. In recent decades, *D. versipellis* has attracted increasing pharmaceutical attention due to the discovery of podophyllotoxin (PTOX), which is a pivotal lignan and is used as a natural source of various anticancer PTOX derivatives¹⁸. Recent studies show antiviral and anti-inflammatory properties of the flavonoids quercitrin and kaempferol from this plant¹⁹. However, due to overexploitation and slow growth, all *Dysosma* species have been

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under the threat of extinction. Therefore, to protect these valuable medicinal plants and maintain the supply of PTOX for anticancer drugs, alternative sources are eagerly sought. Among these, endophytic fungi have the potential to produce PTOX for the production of podophyllotoxin. However, to date, only a few PTOX-producing fungi associated with Berberidaceae plants have been reported. In the present study, we investigated the diversity of culturable fungal endophytes of *D. versipellis* and screened the endophytic fungi for antimicrobial activities and PTOX-producing fungal isolates using HPLC and UPLC–QTOF MS analyses.

**Results and Discussion**

**Isolation, sequencing data, and diversity of culturable endophytic fungi.** In this study, a total of 224 fungal colonies (isolation rate, 41.2%) were isolated from 544 tissue segments of *D. versipellis* plants and included 62 (32.6%), 104 (42.3%), 33 (73.3%), and 25 (39.7%) strains from root, rhizome, stem, and leaf tissue segments, respectively (Table 1). The 224 isolates were assigned to 53 representative morphotypes (19, 22, 6, and 6 strains from roots, rhizomes, stems, and leaves, respectively) according to culture characteristics on potato dextrose agar (PDA; Fig. 1c), and all culturable morphotypes were identified according to ITS rDNA sequence analyses. Subsequently, 44 isolates were categorized at the genus level based on sequence similarity analyses, and the other nine isolates remained unidentified due to low sequence homology in the GenBank database. According to diversity and sequence data of 53 isolates recovered from *D. versipellis* tissues.

| Tissues | Segments examined | Segments infected | Total isolates | Endophytic species | Total CR% | Total IR% | Shannon $H'$ |
|---------|-------------------|-------------------|---------------|-------------------|-----------|-----------|--------------|
| Root    | 190               | 58                | 62            | 19                | 30.50%    | 32.60%    | 2.433        |
| Rhizome | 246               | 97                | 104           | 22                | 39.40%    | 42.30%    | 2.728        |
| Stem    | 45                | 31                | 33            | 6                 | 68.00%    | 73.30%    | 1.330        |
| Leaf    | 63                | 23                | 25            | 6                 | 36.50%    | 39.70%    | 1.242        |
| Total   | 544               | 209               | 224           | 53                |           |           |              |

Table 1. Endophytic isolates from *D. versipellis* tissues.

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**Figure 1.** Habitat of *D. versipellis* and its endophytic fungi. Adult plants of *D. versipellis* (Bar = 20 mm) (a) growing among hillside shrubs (b) and representative fungal morphotypes isolated from *D. versipellis* growing on potato dextrose agar (PDA) for 2 weeks at 26°C (c).
| Fungal isolate | Accession number | Closest relatives in NCBI | ITS identity (%) | Tissue | IR % | Phylum; Class; Order | Classification |
|----------------|-----------------|---------------------------|------------------|--------|------|----------------------|----------------|
| WB5101         | KY940469        | *Acremonium nepalense* CBS 115254 (DQ825972)43 44 | 99               | Leaf   | 1.12 | Ascomycota; Sordariomycetes; Glomerellales | *Acremonium sp.* |
| WB5102         | KY940470        | *Alternaria alternata* CBS 112018 (AY673074)45 | 99               | Root   | 0.53 | Ascomycota; Dothideomycetes; Pleosporales | *Alternaria sp.* |
| WB5103         | KY940471        | *Arthrinium arundinis* CBS 114516 (KF144884)46 | 99               | Root   | 0.53 | Ascomycota; Sordariomycetes; Xylariales | *Arthrinium sp.* |
| WB5104         | KY940472        | *Ascomyctea* P7 (AY265338)47 | 80               | Rhizome | 0.41 | Ascomycota | Ascomycota |
| WB5105         | KY940473        | *Ascomyctea* (JX270545)48 | 84               | Root   | 0.53 | Ascomycota | Ascomycota |
| WB5106         | KY940474        | *Cladosporium* uredinicola SACCR 040661 (AY251071)49 | 99               | Rhizome | 0.41 | Ascomycota; Dothideomycetes; Capnodiales | *Cladosporium sp.* |
| WB5107         | KY940475        | *Colletotrichum exsolubilis* CCMCC 3.15131 (JX625182)50 | 99               | Leaf   | 1.12 | Ascomycota; Sordariomycetes; Glomerellales | *Colletotrichum sp.* |
| WB5108         | KY940476        | *Colletotrichum gigasporus* P1982 (KT269249)51 | 99               | Stem   | 2.22 | Ascomycota | *Colletotrichum sp.* |
| WB5109         | KY940477        | *Colletotrichum* gloeosporioides CBS 119204 (JX010150)52 | 99               | Leaf   | 19.04 | Ascomycota | *Colletotrichum sp.* |
| WB5110         | KY940478        | *Colletotrichum* karstii CCMCC 3.15123 (JX625163)46 | 99               | Leaf   | 1.12 | Ascomycota | *Colletotrichum sp.* |
| WB5111         | KY940479        | *Colletotrichum* siamense GM29 (KC512127)53 | 100              | Stem   | 2.22 | Ascomycota | *Colletotrichum sp.* |
| WB5113         | KY940481        | *Cylindrocarpon* lioidendra CBS 117640 (DQ178166)54 | 99               | Rhizome | 0.41 | Ascomycota; Hypocreales | *Cylindrocarpon sp.* |
| WB5114         | KY940482        | *Cylindrocarpon* pasicteptatum Cy196 (JF353505)55 | 99               | Root   | 0.53 | Ascomycota | *Cylindrocarpon sp.* |
| WB5115         | KY940483        | *Dactylonectria* alacerealis CBS 129087 (NK_121496)56 | 99               | Rhizome | 0.41 | Ascomycota; Sordariomycetes; Hypocreales | *Dactylonectria sp.* |
| WB5116         | KY940484        | *Diaporthe* perjuncta CBS 109745 (KC343172)57 | 96               | Rhizome | 0.41 | Ascomycota; Sordariomycetes; Diaporthales | *Diaporthe sp.* |
| WB5117         | KY940485        | *Diaporthe* sp. HKB37 (DQ902525)58 | 96               | Rhizome | 0.41 | Ascomycota | *Diaporthe sp.* |
| WB5118         | KY940486        | *Exophiala* sp. AS29-1 (AB752282)59 | 99               | Rhizome | 3.65 | Ascomycota; Eurotiales; Chaetothyriales | *Exophiala sp.* |
| WB5119         | KY940487        | *Fusarium* nematophilum BBA 70838 (HQ897786)60 | 99               | Rhizome | 2.43 | Ascomycota; Sordariomycetes; Diaporthales | *Fusarium sp.* |
| WB5120         | KY940488        | *Fusarium* oxysporum ERP-10 (JN222394)61 | 99               | Root   | 0.53 | Ascomycota; Sordariomycetes; Hypocreales | *Fusarium sp.* |
| WB5121         | KY940489        | *Fusarium* solani ATCC 56480 (FJ345335)62 | 100              | Root   | 0.53 | Ascomycota | *Fusarium sp.* |
| WB5122         | KY940490        | *Hypoxylon* fragiforme 22 (JN198512)63 | 99               | Rhizome | 0.41 | Ascomycota; Sordariomycetes; Xylariales | *Hypoxylon sp.* |
| WB5123         | KY940491        | *Ilyonectria* coprosmae CBS 119606 (JF735260)64 | 96               | Root   | 10.53 | Ascomycota; Sordariomycetes; Hypocreales | *Ilyonectria sp.* |
| WB5124         | KY940492        | *Ilyonectria* macrodyma K6 (IF807395)65 | 99               | Rhizome | 1.62 | Ascomycota | *Ilyonectria sp.* |
| WB5125         | KY940493        | *Ilyonectria* robusta CBS 117815 (JF735266)66 | 96               | Rhizome | 5.69 | Ascomycota | *Ilyonectria sp.* |
| WB5126         | KY940494        | *Ilyonectria* torresiensis CBS 112598 (FJ735351)67 | 99               | Rhizome | 1.62 | Ascomycota | *Ilyonectria sp.* |
| WB5127         | KY940495        | *Leotiomycetes* AK1466 (HQ759764)68 | 89               | Root   | 0.53 | Ascomycota; Sordariomycetes; Hypocreales | *Leotiomycetes* |
| WB5128         | KY940496        | *Minimelanolocus aquaticus* 15-0414 (KR215607)69 | 97               | Rhizome | 2.83 | Ascomycota; Eurotiales; Chaetothyriales | *Minimelanolocus sp.* |
| WB5129         | KY940497        | *Mucor* sp. CY118 (HQ607969)70 | 95               | Root   | 0.53 | Zygomycota; Zygomycetes; Mucorales | *Mucor sp.* |
| WB5130         | KY940499        | *Ochrroconis* cf. constricta CBS 124172 (GQ426096)71 | 99               | Leaf   | 1.12 | Ascomycota; Dothideomycetes; Venturiales | *Ochrroconis sp.* |
| WB5131         | KY940500        | *Ophioceras* sp. P2224 (KU747946)72 | 94               | Stem   | 2.22 | Ascomycota; Sordariomycetes; Magnaporthales | *Ophioceras sp.* |
| WB5132         | KY940487        | *Ophiostomalata* F1732 (KU747803)73 | 97               | Stem   | 2.22 | Ascomycota; Sordariomycetes; Ophiostomalatales | *Ophiostomalatales* |
| WB5133         | KY940501        | *Microsphaeropsis* sp. SAA1ACS (KY305064)74 | 99               | Root   | 0.53 | Ascomycota; Dothideomycetes; Pleosporales | *Microsphaeropsis sp.* |
| WB5134         | KY940502        | *Pestalotiopsis* oryzae CBS 115122 (KM199324)75 | 99               | Root   | 0.53 | Ascomycota; Sordariomycetes; Xylariales | *Pestalotiopsis sp.* |
| WB5135         | KY940503        | *Phialophora* musae BAN-C4 (IN123359)76 | 99               | Root   | 0.53 | Ascomycota; Eurotiales; Chaetothyriales | *Phialophora sp.* |
| WB5136         | KY940505        | *Phoma* putaminum CBS 372.91 (GU237843)77 | 99               | Root   | 0.53 | Ascomycota; Dothideomycetes; Pleosporales | *Phoma sp.* |
| WB5137         | KY940506        | *Phoma* salignella CIB 122.93 (GU237762)78 | 99               | Root   | 0.53 | Ascomycota | *Phoma sp.* |

Continued
Table 2. Culturable endophytic fungi from D. versipellis and corresponding isolation rates (IR%).

| Fungal isolate | Accession number | Closest relatives in NCBI | ITS identity (%) | Tissue | IR % | Phylum; Class; Order | Classification |
|----------------|------------------|---------------------------|------------------|--------|------|----------------------|----------------|
| WB5139         | KY940507         | Phyllosticta sp. MUC03547 (AB454364) | 99               | Rhizome | 0.41 | Basidiomycota; Agaricomycetes; Agaricales | Phyllosticta sp. |
| WB5140         | KY940508         | Paathyrella candolleana P73 (AM712281) | 99               | Rhizome | 0.41 | Basidiomycota; Agaricomycetes; Agaricales | Paathyrella sp. |
| WB5141         | KY940509         | Pseudocercospora humidula CPC11358 (GU124676) | 99               | Stem    | 26.7 | Ascomycota; Dothideomycetes; Capnodiaceae | Pseudocercospora sp. |
| WB5112         | KY940480         | Pyrenochaeta sp. P2916 (KT270113) | 98               | Root    | 1.05 | Ascomycota; Dothideomycetes; Pleosporales | Pyrenochaeta sp. |
| WB5142         | KY940510         | Pyrenochaeta sp. CBS 135108 (KF251149) | 97               | Leaf    | 1.12 | Ascomycota; Dothideomycetes; Pleosporales | Pyrenochaeta sp. |
| WB5143         | KY940467         | Ramichloridium sp. NC1_3.3F1a (FJ425199) | 96               | Stem    | 2.22 | Ascomycota; Dothideomycetes; Capnodiaceae | Ramichloridium sp. |
| WB5144         | KY940511         | Rhexocercosporidium sp. Dfel14 (EU543257) | 99               | Rhizome | 0.41 | Ascomycota; Leotiomyces; Helotiales | Rhexocercosporidium sp. |
| WB5145         | KY940512         | Rhizoctonia sp. Rh183 (JF519833) | 99               | Rhizome | 0.81 | Basidiomycota; Agaricomyotina incertae sedis | Rhizoctonia sp. |
| WB5146         | KY940513         | Rhizoctonia sp. R14 (AY927321) | 95               | Root    | 0.53 | | Rhizoctonia sp. |
| WB5147         | KY940514         | Sordariomyces RfEF169 (JN859389) | 95               | Root    | 2.11 | Ascomycota; Sordariomyces; Sordariales | Sordariales |
| WB5148         | KY940515         | Sordariomyces AK0924 (JQ793094) | 88               | Rhizome | 0.81 | Ascomycota; Sordariomyces | Sordariomyces |
| WB5151         | KY940517         | Virgaria nigra NRBC 9453 (AB670716) | 99               | Rhizome | 0.41 | Ascomycota; mitosporic Phycomycetes | Virgaria sp. |
| WB5152         | KY940518         | Volutella consors CBS 139.79 (KM231768) | 98               | Rhizome | 0.81 | Ascomycota; Sordariomyces; Hypocreales | Volutella sp. |
| WB5153         | KY940519         | Xenacremumum falcatus CBS 400.85 (KM231832) | 99               | Rhizome | 0.41 | Ascomycota; Sordariomyces; Hypocreales | Xenacremumum sp. |
| WB5150         | KY940516         | Xylariales Ws6110H (GQ924056) | 95               | Rhizome | 2.44 | Ascomycota; Sordariomyces; Xylariales | Xylariales |

In further analyses, 49 representative morphotypes belonged to four classes of the Ascomycota phylum, including Dothideomycetes, Eurotiomycetes, Leotiomycetes, and Sordariomycetes. Most of the isolates (n = 28) from D. versipellis belonged to Sordariomycetes class in this study. This class was represented by seven orders: Glomerellales (7 isolates), Hypocreales (13 isolates), Diaporthales (2 isolates), Xylariales (4 isolates), Magnaporthales (1 isolate), Ophiostomales (1 isolate), Sordariales (1 isolate); and 13 genera: Acremonium, Arthrinium, Colletotrichum, Cylindrocarpon, Dactylonectria, Diaporthe, Fusarium, Ilyonectria, Pestalotiopsis, Pestalotiopsis, Phialophora, Sporothrix, Xylaria. Three isolates were assigned to Eurotiomycetes class and Chaetothyriales order, representing the genera Exophiala, Minimelanolocus and Phialophora. Finally, two isolates were assigned to Leotiomycetes class. One (WB5144) was classified as Rhexocercosporidium genus of the Helotiales order. No sequence similarity with any reference species was detected in GenBank database.

Ten isolates were assigned to Dothideomycetes class, comprising three orders: Pleosporales (6 isolates), Capnodiaceae (3 isolates) and Ventariales (1 isolate) and eight genera (Alternaria, Cladosporium, Ochroconis, Microsphaeropsis, Phoma, Pseudocercospora, Pyrenochaeta and Ramichloridium). Three times were assigned to Eurotiomycetes class and Chaetothyriales order, representing the genera Exophiala, Minimelanolocus and Phialophora. Exophiala nigra was exclusively detected in roots. Another 19 isolates only colonized rhizomes, and Acremonium and Ochroconis were exclusively present in leaves. Pseudocercospora and Ramichloridium only colonized stems. Based on these varying spatial distributions of endophyte communities in D. versipellis, we suggested that these microorganisms have adapted to distinct tissue microenvironments, resulting in clear tissue specificity among endophytic fungi in D. versipellis, as indicated in a previous study of Indian medicinal plants.34,25 Additionally, the isolates WB5143 (Ramichloridium sp., Fig. 1c), WB5104 (Ascomycota) and WB5136 (Cadophora sp.) have darkly pigmented and septate hyphae of thick walls. These are referred as dark septate fungi (DSF) and were isolated from roots. Junpapon & Trappe suggested that DSF frequently colonize roots of mycorrhizal or nonmycorrhizal plants and play unique roles in terrestrial ecosystems35. However, in contrast with the common root tissue habitat of DSE, Ramichloridium sp. (WB5143) was isolated from stems of plants.

Antimicrobial activity of ethanolic fraction of culture supernatants of endophytic fungal species.

In this study, antimicrobial-producing fungi belonged to the genera Fusarium, Cladosporium, Ilyonectria,
at 26 °C.

**Table 3.** Antibacterial and antifungal activities of endophytic fungi from *D. versipellis* against five pathogens.

| Isolate No | Taxa (accession number) | Inhibition zone in diameter on Petri plates (mm) | S. aureus | E. coli | B. subtilis | A. fumigatus | C. tropicalis |
|------------|-------------------------|-----------------------------------------------|----------|---------|------------|-------------|--------------|
| WB5106     | Cladosporium sp. (KY940474) | 10.9 ± 0.3                                     | 10.8 ± 0.5 | 11.0 ± 0.3 | —          | 19.1 ± 0.7   |
| WB5121     | Fusarium sp. (KY940489)    | 18.7 ± 0.9                                     | 21.3 ± 0.7 | 10.0 ± 0.1 | 7.3 ± 0.3  | —            |
| WB5127     | Ilyonectria sp. (KY940494) | —                                               | —         | 7.5 ± 0.4 | —          | 21.0 ± 0.3   |
| WB5134     | Microsphaeropsis sp. (KY940501) | 7.3 ± 0.5                                     | 9.7 ± 0.2 | 8.0 ± 0.5 | —          | —            |
| WB5136     | Cadophora sp. (KY940503)   | 15.0 ± 0.4                                     | 14.0 ± 0.3 | —         | —          | 8.0 ± 0.5    |
| WB5138     | Phoma sp. (KY940506)       | 10.2 ± 0.5                                     | 10.3 ± 0.2 | 15.5 ± 0.3 | —          | —            |
| WB5145     | Rhizoctonia sp. (KY940512) | 10.9 ± 0.2                                     | 17.8 ± 0.2 | —         | —          | —            |
| WB5147     | Sordariales (KY940514)     | 9.6 ± 0.3                                      | 10.8 ± 0.4 | —         | —          | 13.7 ± 0.2   |
| WB5148     | Sordariomyces (KY940515)   | 25.0 ± 0.5                                     | —         | 10.0 ± 0.4 | 7.0 ± 0.5  | 18.0 ± 0.3   |
| WB5151     | Virgaria sp. (KY940517)    | 9.6 ± 0.3                                      | —         | —         | —          | —            |
| Positive control-1 | Ampicillin | 17.0 ± 0.3                                      | 18.6 ± 0.2 | 21.5 ± 0.3 | —          | —            |
| Positive control-2 | Fluconazole | —       | —       | —       | 25.0 ± 0.3 | 18.1 ± 0.2   |
| Negative control | 10% DMSO | —       | —       | —       | —          | —            |

**Microsphaeropsis, Cadophora, Phoma, Rhizoctonia, Virgaria.** In addition, the ethanolic extracts of two unidentiﬁed isolates also inhibited the microbial growth (Table 3).

Endophytic strains of *Fusarium* are well-known producers of various metabolites screened in the host plants; the commercially important drug precursor PTOX was originally found in the endangered genus *Dysoxylum*, but is also produced by the endophytic *F. oxysporum* from *Juniperus recurva* plants. Other natural agents include taxol which was originally found in *Taxus* plants and was produced by endophytic *F. proliferatum* from *Taxus x media*. Additionally, 2-methylbutyraldehyde-substituted α-pyrones, beauvericin, and subglutinol A and B are dominant antimicrobial compounds that are produced by endophytic *Fusarium* spp. isolated from medicinal plants. Most members of the genus *Cladosporium* also produce antimicrobial compounds, and *C. uredinicola* from *Tinospora cordifolia* was found to possess anti-insect properties, potentially protecting plants against insect pests. In the present study, *Cladosporium* sp. (WB5106) exhibited high antimicrobial activity against *S. aureus*, *E. coli*, *B. subtilis*, and *C. tropicalis*, but did not show any activity against *A. fumigatus*.

Interestingly, all of the present endophytic fungal strains that produce antimicrobial compounds were isolated from roots or rhizomes of *D. versipellis*. Similar studies had also showed medicinal plants with antifungal, antibacterial, anticancer, and antioxidant activities may provide more feasible opportunities to isolate and culture endophytic fungal producers. However, further studies are required to characterize dynamic changes of endophytic communities and uncultured fungi and to confirm fungal tissue specificity in *D. versipellis*.

**Screening of PTOX-producing fungi.** Crude extracts of endophytic fungi were screened for fungal PTOX using HPLC and UPLC–QTOF MS analyses. In these analyses, PTOX from *Fusarium* sp. WB5121 and WB5122 had retention times that corresponded with the standard PTOX (Fig. 2) and corresponding yields were 277 and 1.25 µg/g (wet weight of crude extracts), respectively, after culture in 200 mL of potato dextrose broth (PDB) at 26 °C ± 2 °C with shaking at 125 rpm for 10 days. Associated MS spectra showed the same peak MH+ at m/z 459.12 for standard and fungal PTOX from *Fusarium* sp. WB5121, and that of the fungal PTOX from *Fusarium* sp. WB5121 yielded a peak MH+ at m/z 459.13 (Fig. 3), indicating the presence of endogenous PTOX in isolates of *Fusarium* sp. WB5121 and WB5122 strains.

In conclusion, *D. versipellis* harbors a rich and diverse range of endophytic fungi and provides a fungal resource for the study of PTOX and other unique secondary metabolites. Among the present endophytic fungi, 18.9% and 3.7% of strains produced antimicrobial and anticancer metabolites, respectively. Hence, future studies of metabolic pathways, mutual relationships, and fungal species identification are warranted.

**Materials and Methods**

**Collection of plant material.** The wild plant samples of *D. versipellis* were collected from Yongfu county, Guangxi province of China (109°36′E; 24°37′N). Samples were placed in polyethylene bags, labeled, transported to the laboratory, and refrigerated at 4 °C, as described previously. Plant specimens were identified by Dr. Tan and were preserved in the herbarium of the Guangxi Botanical Garden of Medicinal Plants.

**Fungal isolation and cultivation.** Endophytic fungi were isolated from stems, leaves, and roots of plants. Procedures for surface sterilization of plant tissues and isolation and cultivation of fungi are described by Tan et al. Briefly, stems, leaves, and roots were separated from plants, were washed thoroughly in running tap water, and were surface-sterilized in a sequence of 70% ethanol (v/v) for 30 s and sodium hypochlorite solution (2.5%, v/v) for 5 min. All tissues were then rinsed three times with sterile distilled water and were surface-dried with sterile filter paper. Subsequently, 0.5 × 0.5-cm pieces were excised using a sterile blade and were placed on PDA containing 50-µg/mL oxytetracycline and 50-µg/mL streptomycin. Nine segments were plated per Petri dish (90-mm diameter). Petri dishes were then wrapped in parafilm and were incubated at 25 °C in the dark for more than one week. Samples were checked daily and colonies were routinely isolated, purified, and maintained in PDA for
identification and antimicrobial assays. Pure endophytic fungi were finally photographed and preserved in the laboratory of Mycology, Guangxi Botanical Garden of Medicinal Plants.

**DNA extraction, PCR amplification, sequencing, and molecular identification.** To produce fungal mycelia, all strains were grown on PDA plates at 25 °C for 10 days. Mycelia were scraped using sterile pipette tips and were then freeze-dried, and DNA from endophytic fungi were then extracted using E.Z.N.A.TM Fungal DNA Mini Kits (Omega Bio-tek, Norcross, USA) according to the manufacturers’ instructions for use as templates in polymerase chain reactions (PCR). The primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were constructed for molecular phylogenetic studies and were used to amplify ribosomal internal transcribed spacers (ITS)39. The PCR mixture (50 µL) contained 25 µL of Taq PCR Master Mix (Qiagen, Beijing), 2 µL of each primer at 5 µM, 19 µL of H2O, and 2 µL of genomic DNA. PCR were performed using a thermal cycler (BioRAD) with an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min, and then a final extension step at 72 °C for 7 min. Subsequently, 5-µL PCR products were analyzed electrophoretically in 1% (w/v) agarose gels stained with ethidium bromide. After visual inspection under UV light, 45-µL aliquots of PCR products were purified and sequenced at the Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. Sequences were then compared with ITS sequences from reliable isolates listed in the NCBI database (http://www.ncbi.nlm.nih.gov). Only sequence matches with high similarity to those published in previous studies were included in analyses. All identified isolates were categorized at genus or family levels according to the ownership criterion as follows: species of the same genera have sequence similarity (SS) of >95% and those of the same families had SS of <95%40.

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**Figure 2.** Representative base peak ion chromatograms of *Fusarium* sp. (WB5122) extract (a) and standard podophyllotoxin (PTOX) samples (b) from UHPLC-QTOF-MS/MS analyses performed in negative ionmode.

**Figure 3.** MS spectra of PTOX; standard podophyllotoxin (a); fungal PTOX isolated from *Fusarium* sp. WB5122 (b); the arrow indicates the molecular ion of PTOX at m/z 459.12 (MH+).
The sequences obtained in this study were previously submitted to the GenBank database with accession numbers from KY940469 to KY940519.

**Crude extract preparation of fungal fermentation broth.** Fifty-three strains were precultured on PDA (potato extract, 200 g/L; dextrose, 20 g/L) for 7 days, and five plugs (6 mm of diameter) of each fungus were then pre-inoculated into 500-mL Erlenmeyer flask containing 200-mL PDB containing 200 g/L potato extract and 20 g/L dextrose. All cultures were incubated on a rotary shaker (125 rpm) at 26 °C ± 2 °C in the dark for 10 days. Cultures were then filtered to collect fermentation broth and wet mycelia were discarded. Fermentation broth was extracted with four volumes of ethanol for one day and filtrates were further concentrated in vacuo to remove organic solvent. Concentrates were then volatilized in a water bath at 60 °C and dried residues and were finally stored at −20 °C. Crude extracts were diluted with 10% dimethyl sulfoxide (DMSO) to 10 mg/mL and were sterilized by filtration using a Millipore filter (0.22 μm) prior to antimicrobial assays.

**Antimicrobial activity.** Five pathogens, including the fungi A. fumigatus and C. albicans and bacteria E. coli, B. subtilis, and S. aureus, were used to test antimicrobial activities of 53 crude fungal EtOH extracts, and inhibitory effects were assayed using the agar diffusion method with 10-mg/mL extracts at 100 μg/disk. Ampicillin sodium (100 μg/disk) and fluconazole (25 μg/disk) were used as positive antimicrobial controls and 10% DMSO was used as a negative control. Antimicrobial activities were determined according to diameters of inhibition zones (IZ) and experiments were repeated three times.

**Determination of PTOX-producing fungi.** PTOX-producing endophytic fungi were screened using HPLC22,23 analyses and the agent was identified using UPLC–QTOF MS. In these experiments, crude extracts of fungal isolates were dissolved in 1 mL of 80% methanol (v/v) and were filtered through 22-μm syringe filters prior to HPLC analyses (Agilent 1260, USA), which were performed using a Zorbax SB-C18 column (5 μm, 4.6 mm × 250 mm; Agilent, USA). Gradient elution was then performed with acetonitrile/H2O binary solvent-delivery gradient elution at a flow rate of 1.0 mL/min as follows: 0–20 min, 20% acetonitrile; 20–25 min, 60% acetonitrile; 25–30 min, acetonitrile; volume fraction. Analytes were detected at 207 nm and injection volumes for all fungal methanol extracts and PTOX standard were 20 and 5 μL, respectively. PTOX standard was purchased from Sigma-Aldrich Corporation (St. Louis, Missouri, USA).

Fungal PTOX was further identified using a UPLC–QTOF MS system (Waters, USA) as described previously. Briefly, chromatographic separation was performed with an Acquity UPLC HSS T3 C18 column (1.8 μm, 2.1 mm × 100 mm) with an injection volume of 0.3 μL and a binary gradient elution mixture comprising water with 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B) as follows: 0–3.5 min, 10–35%; 3.5–5.5 min, 35–40%; 5.5–6.5 min, 40–60%; 6.5–8.0 min, 60–90%; 8.1–10 min, 100% B. The mobile phase was applied at a flow rate of 0.5 mL/min and the temperature of the column oven was set to 35 °C. The MS was operated in negative ion mode and was set to total ion chromatogram mode with the following mass conditions: capillary voltage = 2500 V, cone voltage = 40 V, low collision energy = 6 V, source temperature = 100 °C, desolvation temperature = 400 °C, and desolvation gas flow = 800 L/h. Data acquisition and processing were conducted using MassLynx version 4.1 (Waters, Manchester, UK).

**Statistical analyses.** Colonization rates (CR%) of fungal strains isolated from D. versipellis were calculated as follows: CR% = (Nec/Nos) × 100, where Nec represents the number of segments infected by fungi and the Nos represents the total number of segments investigated. Isolation rates (IR%) of the strains were calculated as follows: IR% = (Ni/Ns) × 100, where Ni represents the number of segments from which fungal species were isolated and Ns is the total number of segments incubated. The diversity of fungal species from D. versipellis was evaluated using the Shannon–Weiner Index (H′) with the following formulas:

\[
H' = - \sum (p_i \times \ln p_i) = \frac{\ln n}{n}
\]

where \(n\) represents the numbers of individuals and \(N\) represents the total number of individuals. All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

**References**
1. Aksoy, D. Y. & Unal, S. New antimicrobial agents for the treatment of Gram-positive bacterial infections. *Clin Microbiol Infect* **14**, 411–420 (2008).
2. Katoch, M. et al. Diversity, phylogeny, anticanic and antimicrobial potential of fungal endophytes associated with Monarda citriodora. *L. BMC Microbiology* **17**, 44 (2017).
3. Silva-Hughes, S. A. F. et al. Diversity and anti-fungal activity of the endophytic fungi associated with the native medicinal cactus Opuntia humifusa (Cactaceae) from the United States. *Microbiological Research* **175**, 67–77 (2015).
4. Salam, N. et al. Endophytic actinobacteria associated with Dracaena cochinchinensis Lour.: isolation, diversity, and their cytotoxic activities. *BioMed Research International* **10.1155/2017/1308563** (2017).
5. Zhang, D. W. et al. A novel assay for screening inhibitors targeting HIV-1 integrase dimerization based on Ni-NTA magnetic agarose beads. *Scientific Reports* **6**, 25375 (2016).
6. Cui, J. L. et al. Diversity and antioxidant activity of culturable endophytic fungi from alpine plants of Rhodiola cremlata, R. angusta, and R. schauffenni. *PloS ONE* **10**(3), e0118204 (2015).
7. Strobel, G. A. Endophytes as sources of bioactive products. *Microbes infect.* **5**, 535–544 (2003).
8. Pupo, M. T. et al. Microbial natural products: a promising source of bioactive compounds. (In: Taft CA, editor Modern Biotechnology in Medicinal Chemistry and Industry. Kerala: Research Signpost, p 51–78, 2006).
9. Petri, O. et al. Ecology, metabolite production, and substrate utilization in endophytic fungi. *Nat Toxins* **1**, 185–196 (1992).
10. Arnold, A. E., Maynard, Z. & Gilbert, G. S. Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. *Mycol. Res.* **105**, 1502–1507 (2001).
11. Hawkesworth, D. L. The variety of fungal- algal symbioses, their evolutionary significance, and the nature of lichens. *Bot. J. Linn. Soc.* **96**, 1–20 (1988).
12. Ganley, R. J., Brunsonfeld, S. J. & Newcombe, G. A community of unknown, endophytic fungi in western white pine. PNAS 101, 10107–10112 (2004).

13. Pupo, M. T. et al. Microbial natural products: a promising source of bioactive compounds. (In: Taft CA, editor. Modern Biotechnology in Medicinal Chemistry and Industry. Kerala: Research Signpost, p 51-78, 2006).

14. Wang, S. & Xie, Y. China species red list (Vol.1). (Beijing: Higher Education Press, p 324, 2004).

15. Ying, T. S., Zhang, Y. L. & Balfour, D. E. The endemic genera of seed plants of China. (Beijing: Science Press, 1993).

16. Goni, R. et al. A Frontiers Board of Flora of China: Flora of China (Vol. 29). (Beijing: Science Press, 2001).

17. Jiangsu New Medical College. Dictionary of Chinese Traditional Medicine. (Shanghai: Shanghai Science and Technology Press, 1986).

18. Canol, C. et al. Molecules of interest: podophyllotoxin. Phytochemistry 54, 115–120 (2000).

19. Chen, R. D. et al. Flavonoid glycosides from callus cultures of Diosyos versipellis. China. Journal of Chinese Material Medic 41(1), 87–91 (2016).

20. Chaurasia, O. P. et al. Podophyllum L.: an endangered and anticancerous medicinal plant-an overview. Indian J. Tradit. Know. 11, 234–241 (2012).

21. Vasundhara, M. & Trappe, J. M. Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. New Phytologist 191, 139–164 (2012).

22. Mishra, A. et al. Season and tissue type affect fungal endophyte communities of the Indian medicinal plant Tinospora cordifolia more strongly than geographic location. Mycol. Prog. 11, 655–688 (2012).

23. Hata, K. & Futai, K. Endophytic fungi associated with healthy pine needles and needles infested by the pine needle gall midge. Mycol. Prog. 11, 1115–1121 (2012).

24. Tan, X. M. et al. In vitro seed germination and seedling growth of an endophytophilic orchid, Dendrobium officinale, endemic to China using mycorrhizal fungi (Tulasnella sp.). Scientia Horticulturae 165, 62–68 (2014).

25. Almeida, R. et al. Diversity of endophytic fungi in roots of nine属 orchid species (Orchidaceae) is broader than expected from adult plants (Orchidaceae), with descriptions of seven new speices. Studies in Mycology 73, 655–688 (2011).

26. Gomes, R. et al. Chemical constituents from endophytic fungus Diaporthe theobromae. Journal of Chinese Material Medic 41, 4134–4140. PMID: 12985237 (2003).

27. Feng, Y. & Wang, S. China species red list (Vol.1). (Beijing: Higher Education Press, p 324, 2004).

28. Mishra, A. et al. Diversity of aerobic Gram-positive rods in the clinical laboratory (an overview of the taxonomy, phylogeny, and typification of nectriaceous fungi in Cosmospora, Acremonium, Diaporthe, Musicillium, and Microtheca). Mycol. Prog. 68, 79–113 (2011).

29. Jumpponen, A. & Trappe, J. M. Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. New Phytologist 140, 295–310 (1998).

30. Tejevi, M. V. et al. Bioactivity and genetic diversity of endophytic fungi in Rhododendron tomentosum Harmaja. Fungal Diversity 47, 97–107 (2011).

31. Xiong, Z.Q. et al. Diversity of endophytic fungi and screening of fungal paclitaxel producer from Anglogiap yew, Taxus s media. BMC Microbiology 13, 71 (2013).

32. Lee, J. C. et al. Subglutinolins A and B: immunosuppressive compounds from the endophytic fungus Fusarium subglutinans. J. Org. Chem. 60, 7076–7077 (1995).

33. Almeida, R. et al. Endophytic fungi associated with healthy pine needles and needles infested by the pine needle gall midge. Thecodiplosis lapponica. Can. J. Bot. 73, 384–390 (1995).

34. Sun, Y. et al. Endophytic fungi associated with two Sauadera species growing in alkaline soil in China. Mycosphere 2, 239–248 (2011).

35. Grafenhan, T. et al. An overview of the taxonomy, phylogeny, and typification of nectriaceous fungi in Cosmospora, Acremonium, Fusarium, Stilbella, and Volutella. Stud. Mycol. 68, 79–113 (2011).

36. Bussaban, B. et al. Molecular and morphological characterization of Pyricularia oryzae and allied genera. Mycologia 97(5), 1002–1011 (2005).

37. Hata, K. et al. Phylogenetic re-evaluation of Arthrinum. JMA Fungus 4(1), 133–154 (2013).

38. Altindere, M. et al. Antimicrobial activity and biodiversity of endophytic fungi in Dendrobium devinnium and Dendrobium thysiforum from Vietnam. Curr. Microbiol. https://doi.org/10.1007/s00284-010-9848-2 (1997).

39. Fu, Y. J. et al. An analytical pipeline to compare and characterise the anthocyanin antioxidant activities of purple sweet potato cultivars. Food Chemistry 194, 46–54 (2016).

40. Hata, K. & Futai, K. Endophytic fungi associated with healthy pine needles and needles infested by the pine needle gall midge. Thecodiplosis lapponica. Can. J. Bot. 73, 384–390 (1995).

41. Sun, Y. et al. Endophytic fungi associated with two Sauadera species growing in alkaline soil in China. Mycosphere 2, 239–248 (2011).

42. Grafenhan, T. et al. An overview of the taxonomy, phylogeny, and typification of nectriaceous fungi in Cosmospora, Acremonium, Fusarium, Stilbella, and Volutella. Stud. Mycol. 68, 79–113 (2011).

43. Bussaban, B. et al. Molecular and morphological characterization of Pyricularia oryzae and allied genera. Mycologia 97(5), 1002–1011 (2005).

44. Cantrell, S. A. et al. Fungal communities of young and mature hypersaline microbial mats. Mycologia 105(4), 827–836 (2013).

45. Braun, U. et al. Phylogeny and taxonomy of Cladosporium–like hyphomycetes, including Davidiella gen. nov., the teleomorph of Cladosporium s.str. Mycol. Prog. 2(1), 3–18 (2003).

46. Taro, G. et al. Endophyte Colletotrichum species from Betelia ochracea (Orchidaceae), with descriptions of seven new species. Fungal Divers. 61(1), 139–164 (2013).

47. Glyenou, K. et al. The local environment determines the assembly of root endophytic fungi at a continental scale. Environ. Microbiol. https://doi.org/10.1111/1462-2920.13112 (2015).

48. Wei, B. S. et al. The Colletotrichum gloeosporioides species complex. Stud. Mycol. 73(1), 115–180 (2012).

49. Almeida, R. et al. Diversity and pathogenicity of Colletotrichum species isolated from sour sop in Colombia. Eur. J. Plant Pathol. 139(2), 319–332 (2014).

50. Halleen, F. et al. Neocentria lioriodendi sp. nov., the main causal agent of black foot disease of grapevines. Studies in Mycology 55, 227–234 (2006).

51. Cabral, A. et al. Cylindrocarpon rot root: multi-genie analysis reveals novel species within the Ilyonectria radicicola species complex. Mycol. Prog. 11(3), 655–688 (2012).

52. Miao, C. P. et al. Rhizospheric fungi of Panax notoginseng: diversity and antagonism to host phytopathogens. J. Ginseng. Res. 40(2), 127–134 (2016).

53. Testelova, T. et al. Symbiotic germination capability of four Epipactic species (Orchidaceae) is broader than expected from adult ecology. Am. J. Bot. 99(6), 1020–1032 (2012).

54. Gomes, R. R. et al. Diaporthe: a genus of endophytic, saprobic and plant pathogenic fungi. Persoonia 31, 1–41 (2013).

55. Li, Q. & Wang, G. Diversity of fungal isolates from three Hawaiian marine sponges. Microbiol. Res. 164(2), 233–241 (2009).

56. Hirose, D. et al. Microfungi associated with withering willow wood in ground contact near Syowa Station, East Antarctica for 40 years. Polar Biol. 36, 919–923 (2013).
61. Zhao, J. et al. Endophytic fungi from pigeon pea (Cajanus cajan (L.) Millsp.) produce antioxidant Cajaninstilbene acid. J. Agric. Food Chem. 60(17), 4314–4319 (2012).
62. Khot, P. D. et al. Sequencing and analysis of fungal RNA operons for development of broad-range fungal PCR assays. Appl. Environ. Microbiol. 75(6), 1559–1565 (2009).
63. Wu, L. et al. Geographic and tissue influences on endophytic fungal communities of Taxus chinensis var. mairei in China. Curr. Microbiol. 66(1), 40–48 (2013).
64. Gao, Y. et al. Characterization of five fungal endophytes producing Cajaninstilbene acid isolated from pigeon pea (Cajanus cajan (L.) Millsp. PLoS ONE 6(11), E27589 (2011).
65. U’Ren, J. M. et al. Host and geographic structure of endophytic and endolichenic fungi at a continental scale. Am. J. Bot. 99(5), 898–914 (2012).
66. Liu, X. Y. et al. Backbone tree for Chaetothyriales with four new species of Minimelanolocus from aquatic habitats. Fungal Biol. 119(11), 1046–1062 (2015).
67. Rodrigues, A. et al. Ecology of microfungal communities in gardens of fungous-growing ants (Hymenoptera: Formicidae): a year-long survey of three species of attine ants in Central Texas. FEMS Microbiol. Ecol. 78(2), 244–255 (2011).
68. Lian, X. & de Hoog, G. S. Indoor wet cells harbour melanized agents of cutaneous infection. Med. Mycol. 48(4), 622–628 (2010).
69. Del Olmo-Ruiz, M. & Arnold, A. E. Community structure of fern-affiliated endophytes in three neotropical forests. J. Trop. Ecol. 33(1), 60–73 (2017).
70. Micalizzi, E. W. et al. Microbial inhibitors of the fungus Pseudogymnoascus destructans, the causal agent of white-nose syndrome in bats. PLoS one 12(6), E017790 (2017).
71. Maharachchikumbura, S. S. et al. Pestalotiopsis revisited. Stud. Mycol. 79, 121–186 (2014).
72. Ban, Y. et al. The response of dark septate endophytes (DSE) to heavy metals in pure culture. PLoS one 7(10), E47968 (2012).
73. Avekamp, M. M. et al. Highlights of the Didymellaceae: a polyphasic approach to characterise Phoma and related pleosporalean genera. Stud. Mycol. 65, 1–60 (2010).
74. Motohashi, K. et al. Phylogenetic analyses of Japanese species of Phyllosticta sensu stricto. Mycoscience 50, 291–302 (2009).
75. Vastiota, M. et al. Phylogenetic studies in Pithyrella focusing on sections Pennatea and Spalidaceae–new evidence for the paraphyly of the genus. Mycol. Res. 112 (PT 10), 1153–1164 (2008).
76. Crox, P. W. et al. Phylogenetic lineages in the Capnodiales. Stud. Mycol. 64, 17–4758 (2009).
77. Quadrelvieg, W. et al. Sizing up Septoria. Stud. Mycol. 75(1), 307–390 (2013).
78. Diaz Arias, M. M. et al. Diversity and biogeography of sooty blotch and flyspeck fungi on apple in the eastern and midwestern United States. Phytopathology 100(4), 345–355 (2010).
79. Ruiwen Zhang et al. Diosgenin production of Dioscorea zingiberensis cultures stimulated by its endophytic fungi. Journal of Biotechnology 1365, 151 (2008).
80. Kelderer, M. et al. Planting in the inter-row to overcome replant disease in apple orchards: a study on the effectiveness of the practice based on microbial indicators. Plant Soil 357(1–2), 381–393 (2012).
81. Manici, L. M. & Bonora, P. Molecular genetic variability of Italian binucleate Rhizoctonia spp. isolates from strawberry. Eur. J. Plant Pathol. 118(1), 31–42 (2007).
82. Knapp, D. G. The dark side is not fastidious–dark septate endophytic fungi of native and invasive plants of semiarid sandy areas. PLoS ONE 7(2), E32570 (2012).
83. Nonaka, K. et al. Virgaria boninensis, a new hyphomycete (Xylariaceae) from soils in the Bonin Islands, Japan. Mycoscience 54, 394–399 (2013).
84. Lombard, L. et al. Generic concepts in Nectriaceae. Stud. Mycol. 80, 189–245 (2015).
85. Herrera, J. et al. Shifting fungal endophyte communities colonize Bouteloua gracilis: effect of host tissue and geographical distribution. Mycologia 102(5), 1012–1026 (2010).

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Author Contributions
X.M.T. and Y.Q.Z. conceived and designed the experiments. X.M.T., L.L.H., H.Z.T. and Y.W. performed the experiments. X.M.T., X.L.Z., X.H.X. and L.Y.Y. analyzed the data. X.M.T. and Y.Q.Z. wrote the manuscript. All authors reviewed the manuscript.

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