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ORIGINAL PAPER

Endophytic fungi assemblages from 10 *Dendrobium* medicinal plants (Orchidaceae)

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Abstract *Dendrobium* is the largest genus of tropical epiphytic orchid, some of which are traditional Chinese medicinal plants. The therapeutic components varied significantly among species. Endophytic microbes (fungi) hidden in medicinal plants may play an important effect on the overall quality of herb. Investigation of fungal composition in host plants is the first step toward elucidating the relationship endophyte-therapeutic content of herbal medicine. In this study, 401 culturable fungal endophytes were isolated and identified from 10 species of medicinal *Dendrobium* based on morphological and molecular techniques. The results showed that endophytic fungi from *Dendrobium* plants exhibited high biodiversity (37 genera, about 80 species). *Acremonium, Alternaria, Ampelomyces, Bionectria, Cladosporium, Colletotrichum, Fusarium, Verticillium* and *Xylaria* were the dominant fungal endophytes. Tropical epiphytic orchids appear to vary in degree of host specificity in their endophytic fungi.

Keywords Orchidaceae · Endophyte · Taxonomy · Medicinal plant

With about 1200 species worldwide, *Dendrobium* is known as one of the largest genera of Orchidaceae, mainly distributed in the South, East and Southeast Asia, such as China, Japan and Philippines (Lavarack et al. 2000). Some members of this genus have special pharmacologic effect on gastritis infection, cancer and aging, thus making them to be a precious treasure of Chinese traditional herbal drugs (Li et al. 2009). However, different species in this genus has shown dramatic differences in quality and quantity of chemical composition. *D. candidum* is being recognized as the most excellent in medicinal efficacy (Pharmacopoeia Committee of the Republic of China 2005). Effective components of a medicinal plant may be affected by many factors, such as plant germplasm, geological distribution, ecological environments and human elements (harvesting and processing) (Li et al. 2008). As far as we know, little work has been done concerning the relationships between the therapeutic components of the medicinal plants and endophytic microorganisms.

Orchids are unique among plants in their modes of nutrition (myco-heterotrophy) involving direct and often obligate relationships with fungi (Leake 1994). As to our knowledge, fungal endophytes have profound effects on plant ecology, fitness, evolution, even plant communities' structure and diversity (Brundrett 2006; Rodriguez et al. 2008). Moreover, chemistry analysis revealed that some endophytic fungi isolated from *Dendrobium* were of excellent bioactivity (Chen et al. 2000; Chen and Guo 2005). The aims of the present study are (1) to investigate species diversity of culturable endophytic fungi in 10 different *Dendrobium* species occurring in the southwestern China using traditional morphological and molecular methods, (2) elucidate distribution for endophytic fungi in host plant and (3) address the mutually or differently fungal species among these medicinal plants. This work will contribute to further evaluate fungal role in herbs and find some new natural products with bioactivity from endophytic fungi.

Juan Chen and Ke-Xing Hu are contributed equally to this work.

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Materials and methods

Plant materials

In 2004–2007, ten *Dendrobium* species plants were sampled from Yunnan and Guizhou province, China. Local sites and species were listed in Table 1. Plants were identified according to Chen et al. (1999).

Fungal isolation and cultivation

Endophytes were isolated from plants (root, stem and leaf) of each species. Surface sterilization and isolation of endophyte followed a modified procedure as described by Bayman et al. (1997). In brief, surface-sterilized in a sequence 75% ethanol for 30 s, 3% NaClO 1 min, 75% ethanol for 30 s and rinsed in sterile distilled water three times. Six 2–5 mm pieces were cut from each root. Eight 3 mm pieces were cut from each leaf from distal, central and proximal parts. Ten 3 mm pieces were cut from each stem. Four pieces of each root, leaf and stem were plated on isolation medium. Media used were malt extract agar (MEA) with 50 μg mL^{-1} streptomycin and 50 μg mL^{-1} chloramphenicol. Most of the endophyte isolates began to grow within the first 10 day of plating. The cultures were kept for one month or until it became clear that no more fungal isolates would appear.

Fungal colonies were transferred to Potato Dextrose Agar (PDA) for purity and identification. The pure entophytic fungal stains were photographed and preserved in Laboratory of Mycology, Biotechnology Center, Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences (CAMS). Strains were therefore selected for subculture from each primary inoculation plate on the basis of recognizably distinct morphology. The selected colonies were then subcultured to PDA plates, 25°C under dark conditions.

Morphology-based identification

After isolation, cultures were identified to genus or species level by microscopic and culture characters. Identification was based on the published descriptions (Nelson et al. 1983; Barnett and Hunter 1998; Ellis 1976). The cultures of mycelia sterilia (failed to sporulate) were selected for molecular identification.

Sequence-based identification

122 pure cultures were selected for DNA extraction, amplification, and sequencing. Universal fungal primers ITS1 and ITS4 (White et al. 1990) were used to amplify ribosomal internal transcribed spacer (ITS). PCR was carried out as follows: the reaction mixture in a total volume of 50 μL contained 5 μL 10 × buffer (with Mg^{2+}), 4 μL (10 μM) dNTPs, 4 μL (5 μM) each primer, 1.5 μL (3 U) Taq DNA polymerase, 29.5 μL H_2O and 2 μL genomic DNA. Samples were incubated in a thermal cycler at 95°C for 3 min, followed by 35 cycles of 94°C for 1 min, 53°C for 50 s, 72°C for 1 min; and finally 72°C for 7 min. Single-band PCR products were purified using Watson's PCR purification kit (Watson, China). Sequencing was performed with BigDye Terminator sequencing kit and an ABI 3730 automated sequencer (Applied Biosystems, USA).

Table 1 Details of plant species and sampling sites employed in the study

Plant name	Plant code	Site of collection	Habitat
Dendrobium candidum Wall.ex Lindl.	DC	Tropical Botanical Garden, Xishuangbanna, Yunnan, China. (21.41° N, 101.25° E, 580 m asl.)	Epiphytic
D. nobile Lindl.	DN	Puwen, Xishuangbanna, Yunnan, China. (22.55° N, 101.38° E, 858 m asl.)	Epiphytic
D. falconeri Hk.	DF	Tropical Botanical Garden, Xishuangbanna, Yunnan, China. (21.41° N, 101.25° E, 580 m asl.)	Epiphytic
D. loddigesii Rolfe.	DL	Xingyi, Guizhou, China (24.61° N, 104.55° E, 1,020 m asl.)	Epiphytic
D. primulinum Lindl.	DP	Dadugang, Xishuangbanna, Yunnan, China (100.91° E, 22.50° N, 1,200 m asl.)	Epiphytic
D. gratiosissimum Rchb. f.	DG	Dadugang, Xishuangbanna, Yunnan, China (100.91° E, 22.50° N, 1,200 m asl.)	Epiphytic
D. christyanum Rchb.f.	DCH	Tropical Botanical Garden, Xishuangbanna, Yunnan, China (21.41° N, 101.25°E, 580 m asl.)	Epiphytic
D. hancockii Rolfe.	DH	Tropical Botanical Garden, Xishuangbanna, Yunnan, China (21.41° N, 101.25° E, 580 m asl.)	Epiphytic
D. pendulum Roxb.	DPE	Puwen, Xishuangbanna, Yunnan, China (22.55° N, 101.38° E, 858 m asl.)	Epiphytic
D. moniliforme (L.)Sw.	DM	Puwen, Xishuangbanna, Yunnan, China (22.55° N, 101.38° E, 858 m asl.)	Epiphytic

Sequence-based identifications and phylogenetic analysis based on blast searches of ITS sequence data in the NCBI GenBank database. The species were accepted when identity between our sequence and that of the database was greater than 99%; only the genus was accepted when identity to a database match about 95%. And when the similarity was <95%, the isolates were considered unidentified (Sánchez et al. 2008).

Sequences were then aligned to other sequences obtained from the GenBank database with Clustal X 1.83 (Thompson et al. 1997). Phylogenetic analysis was performed with Mega 4.0 (Tamura et al. 2007) using Kimura

Table 2 Amount and distribution of endophytic fungi isolated from 10 *Dendrobium* medicinal plants based on morphological and molecular analysis

Fungal taxonomic	DC	DN	DF	DL	DPR	DG	DCH	DH	DPE	DM	Total
Acremonium	7	3	10	12	3		3	3	1	1	43
Alternaria (Anamorphic *Lewia*)	11	6	1	4			1	1			24
Ampelomyces	2	1	1	3			1				8
Arthrinium	1										1
Aureobasidium	2										2
Bionectria	2	1		6						1	10
Candida	1										1
Cercophora	1			1							2
Chaetomella				1	1					1	3
Chaetomium	3										3
Chaetophoma	1										1
Cladosporium		1		1		1		1	3		7
Colletotrichum		3	1	4	4						12
Davidiella				1							1
Fusarium (Anamorphic *Gibberella*)	21	20	9	22	5	12	1	7	9	8	114
Fusicoccum		2									2
Glomerularia	4									1	5
Hyalodendron	2										2
Lasiodiplodia				1							1
Nemania							1				1
Nigrospora	3										3
Paraconiothyrium		1		1							2
Periconiella	3										3
Pestalotia							1				1
Pestalotiopsis			1								1
Pezicula		1									1
Pleospora		1									1
Pleurophragmium									2		2
Schizophyllum				1						1	2
Sclerotium	1										1
Sirodesmium				3							3
Streptomyces	1										1
Thielavia				1				1			2
Trichoderma (Anamorphic *Hypocrea*)				1			1			1	3
Verticillium		7	4	5					1	3	20
Xylaria		5		2	1	1					9
Unidentified	16	22	13	33	5	2	10	1	1	0	102
Total	82	74	40	103	19	16	19	14	17	17	401
Shannon's diversity index	1.76	1.07	1.28	0.62	0.92	1.09	0.62	0.93	1.40	1.44	–

2-parameter model with a transition to transversion ration. Phylogenetic trees were built using the neighbour-joining (NJ) methods (Kumar et al. 2004). Bootstrap tests were performed using 500 replicates.

Data analysis

Relative frequency (RF) of isolation, used to represent fungal density, was calculated as the number of isolates of one species divided by the total number of isolates, and was expressed as percentage. Diversity of cultured endophytes was measured by the Shannon diversity index (H') according to the formula

$$H' = -\sum_{i=1}^{k} (Pi \times lnPi)$$

where k is the total number of fungal species, and Pi is the proportion of individuals that species i contributes to the total (Pielou 1975).

Results

Morphological identification of cultured endophytes

Fungal endophytes were abundant and diverse in healthy plant tissues of *Dendrobium*. In total, 401 culturable endophytes were recovered from 10 species of medicinal plants. Among them, 45.9% were classified into 19 genera, including 61 distinct species on the basis of culture morphology and mitosporic features, and 54.1% were mycelia sterilia, including 21 distinct morphtypes. In the six majority endophytic fungal groups, mycelia sterilia had the highest relative frequency (53.9%) in the 10 species of *Dendrobium* plants, followed by *Fusarium* (21.7%), *Acremonium* (8.22%), *Verticillium* (3.49%), *Alternaria* (2.99%) and *Cladosporium* (1.0%) (Table 2).

Phylogenetic affinities of cultured mycelia sterilia

Among 217 mycelia sterilia, 122 strains, representing 21 morphotypes, were selected to sequence the internal transcribed spacer region (ITS) of nuclear ribosomal DNA. Among them, 4 isolates belong to the Basidiomycota and 99 strains belong to Ascomycota. Together the basidiomycetous endophytes showed highest affinity to two different families when subjected to BLAST searches: Schizophyllaceae (for 2 strains, 99% identical to the *Schizophyllum* in the Genbank database) and Tricholomataceae (for other 2 strains) (Fig. 1).

Within the Ascomycota, 99 mycelia sterila endophytes in this study were distributed the three classes (Sordariomycetes, Dothideomycetes and Leotiomycetes), eight orders and 13 families (Figs. 2, 3). 32 endophytes that represented nine or ten genus were placed within various lineages of Dothideomycetes and one endophytic fungus belongs to Helotiales (Leotiomycetes) (Fig. 2). 13 cultured endophytes belong to *Alternaria*, and 2 isolations (1018 and 1070) are associated with the genus *Stemphylium*, and 2 isolatins (843 and 1006) belong to *Botryosphaeria*. Eight endophytic fungi resemble *Ampelomyces*.

Among the Sordariomycetes (Fig. 3), 124 endophytic fungi formed five clades. The largest clade, comprising 32 endophytes, was recovered from *Dendrobium* surveyed. The clade of endophyte contains several well supported branches on the ITS tree and represent 4 genus: *Fusarium* (anamorphic *Gibberella*), *Bionectria, Neonectria* and *Trichoderma* (anamorphic *Hypocrea*) within Hypocreales. An additional group of 25 endophytes, which had highest ITS BLAST affinity for *Xylaria*, *Arthrinium* and

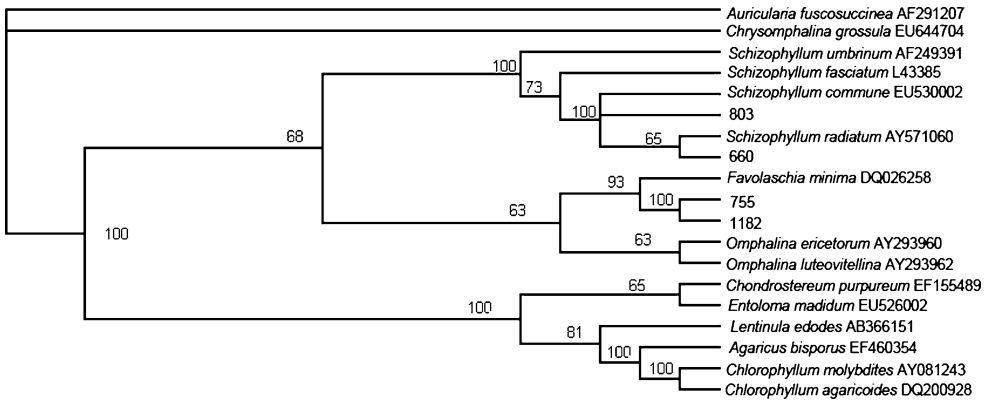

Fig. 1 Neighbour-Joining tree generated in Mega from the alignment of ITS sequences of endophytic fungi obtained in culture from 10 species of *Dendrobium* medicinal plants, and 4 endophytes of Basidiomycota, using the Kimura two parameter models with complete deletion gap handling and 500-replication bootstrapping. Nodes with bootstrap values inferior to 50% were eliminated. Bootstrap values are indicated along to relevant nodes

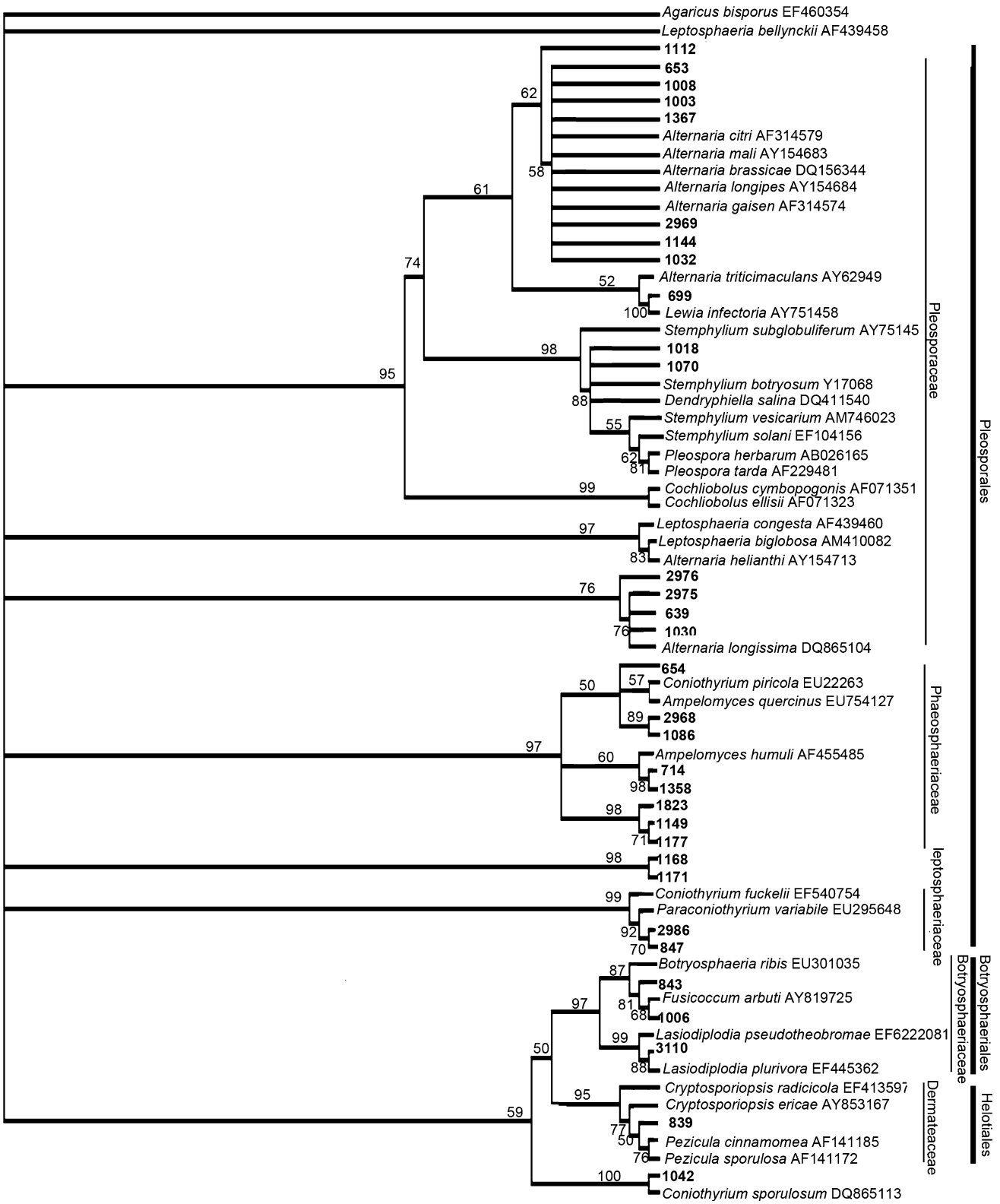

Fig. 2 Phylogenetic relationships among 32 endophytic fungi from *Dendrobium* medicinal plants (*in bold*) derived from Dothideomycetes and Leotiomycetes (Ascomycota). Neighbour-Joining tree generated in Mega from the alignment of ITS sequences. Bootstrap values (≥50%) are indicated along to relevant nodes

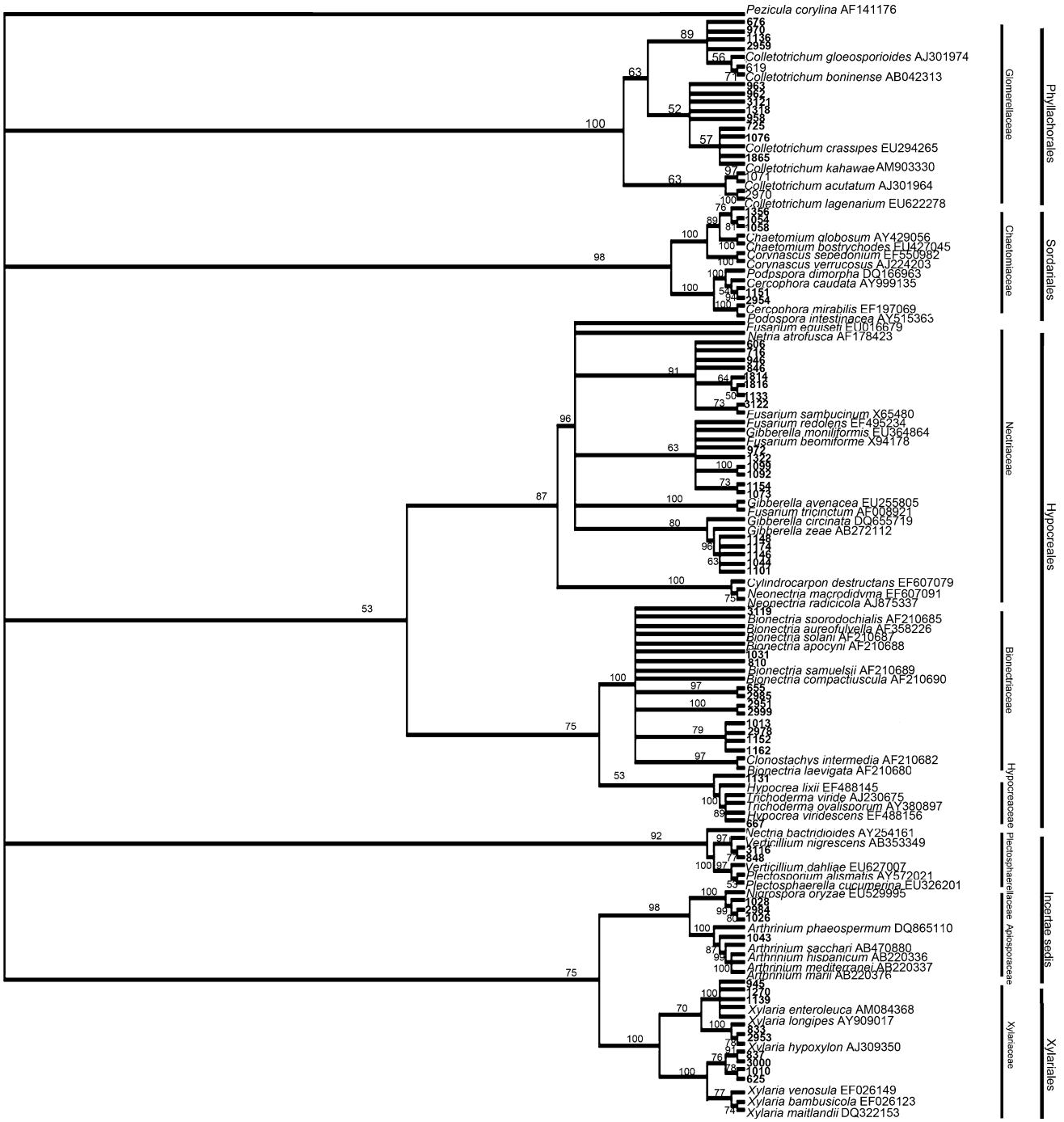

Fig. 3 Phylogenetic relationships among 67 isolates of endophytic fungi belong to Sordariomycetes (Ascomycota) from 10 species of *Dendrobium* plants (*in bold*). Neighbour-Joining tree generated in Mega from the alignment of ITS sequences. Bootstrap values (≥50%) are indicated along to relevant nodes

Nigrospora forms a second well supported clade. Twelve endophyte isolates representing single genus (*Colletotrichum*) were placed within the Phyllachorales. Five endophytes (1356, 1054, 1058, 1151 and 2954) were placed within Sordriales and had the highest ITS BLAST affinity for *Chaetomium, Cercophora* and *Podospora*. Finally, endophyte 3116 and 848 formed a group with taxa of *Verticillium*, anamorphic Plectosphaerellaceae and were placed with Incertae sedis of Sordariomycetes. Amount and distribution of endophytic fungi isolated from 10

Dendrobium medicinal plants based on morphological and molecular analysis were listed in Table 2.

Distribution of endophytic fungi in different *Dendrobium* host plants and organ

Excluding unidentified isolates (102 stains), 299 endophytic fungi from 10 *Dendrobium* medicinal plants belong to 37 genera based on combined morphological and molecular identification (Table 2). The Shannon's diversity index indices of endophytic fungi in 10 medicinal plants were significantly greater in *D. candidum* (1.76) and lowest in *D. loddigesii* and *D.christyanum* (0.62). Different endophytic fungi showed different relative frequencies in different plants.

Except for *D. primulinum, D. gratiosissimum, D. hancockii* and *D. moniliforme*, other plant species had exclusive endophytic fungal colonization (Table 2). *Arthrinium*, *Aureobasidium*, *Candida*, *Chaetomium, Chaetophoma*, *Glomerularia*, *Hyalodendron*, *Nigrospora*, *Periconiella*, *Streptomyces* and *Sclerotium* exclusive only in *D. candidum*. *Davidiella* and *Lasiodiplodia* only in *D. loddigesii. Pleurophragmium* in *D. pendulum. Pestalotia* and *Nemania* in *D. christyanum*. *Pestalotiopsis* in *D. falconeri. Pleospora, Nectria, Fusicoccum* and *Pezicula* exclusive in *D. nobile.* The genus *Fusarium* was generalists common to all *Dendrobium* plants analysised.

The composition and abundance of the endophytes are varied according to the host tissue tested (Fig. 4). Nine frequently encountered endophytic fungal groups were found in root, stem and leave. *Fusarium,* the common encounter fungal genera, was found in 68.2% of root, 20.6% of stem and 11.2% of leaves. *Colletotrichum* was absent in roots but abundant in the stems and leaves. For another 21 infrequent endophytic fungal taxa, they appeared more frequently colonized the root than stem or leaves.

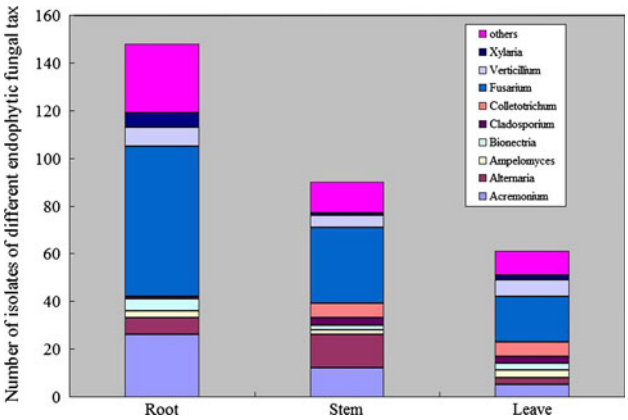

Fig. 4 Number of isolates of different endophytic fungal taxa in different plant tissues

Discussion

All the 10 species of *Dendrobium* plants were found to harbor various endophytic fungi. The difference of species composition of endophytes assemblages were observed in 10 species different tropical *Dendrobium* medicinal plants in this study. The endophyte taxa, *Fusarium*, *Verticillium*, *Acremonium*, *Ampelomyces*, *Alternaria*, *Colletotrichum* and *Cladosporium* were found in most *Dendrobium* plants analyzed. Furthermore, exclusive endophyte was found in six plants (*D. candidum, D. nobile, D. falconeri, D. loddigesii, D. christyanum, D. pendulum*), although the number of each fungal is small. For example, eleven fungal genera exclusively colonized in *D. candidum*. Two fungal genera were only found in *D. loddigesii* and a single genus was only observed in *D. pendulum* and *D. falconeri.* This result could indicate that host specificity might exist to some extent. Stone et al. (2000) have stated that each rare fungus species was represented only once or twice in different plants, but the number of the rare species isolated is usually proportional to intensity of sampling.

Additionally, Xylariales, Botryosphaeriales, Hypocreales, Pleosporales and Capnodiales, which known from other tropic epiphytic orchid, also were recovered from *Dendrobium*, and 11 genera, including *Aureobasidium, Chaetophoma, Periconiella, Nigrospora, Hyalodendron, Sirodesmium, Glomerularia, Chaetomella*, *Thielavia* and *Pleurophragmium,* are firstly reported as endophyte from *Dendrobium* plants.

Molecular taxonomic techniques demonstrated that mycorrhizal fungi are important for adult, green, epiphytic plants (Suárez et al. 2006) and the main mycobionts found in epiphytic orchids are similar to terrestrial photosynthetic species and include *Ceratobasidium*, *Tulasnella* and Sebacinales species (Pereira et al. 2005; Suárez et al. 2008). In this study, we did not observe mycorrhizal fungi from root of adult plants of *Dendrobium*. The possible reason, on one hand, is that green and photosynthetic epiphytic orchids for mycorrhizal fungi are facultative in mature stage, and on the other hand, is that endophytic fungi of samples were not been exhausted or is that the isolation methods (plating fragments of surface sterilized) is not much more effective to isolate mycorrhizal fungi than using single pelotons from the root cell.

In brief, endophytic fungi in *Dendrobium* medicinal plants are very abundant and fungal distribution exhibited host specificity and insignificant tissue specificity. Among 10 species medicinal plants of *Dendrobium* studied, fungal diversity and specificity in *D. candidum* were the highest relativity. *D. candidum* is one of the most valuable materials of traditional Chinese medicine among this genus because of their high quality polysaccharides and alkaloids (Pharmacopoeia Committee of the Republic of China

2005). Whether these distinct fungi are associated with quality and quantitative of different *Dendrobium* plants need further verification.

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Conflict of interest None.

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