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# Antimicrobial activity of endophytic fungi isolated from *Dendrobium* species in Southwestern China

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[**Abstract**] **Objective**: To isolate and characterize endophytic fungi from seven *Dendrobium* species, and detect their antimicrobial activities. **Method**: Fungal endophytes were isolated by strictly sterile sample preparation and fungal identification methods were based on their ITS ribosomal DNA (ITS rDNA gene) sequences. The agar well diffusion method was then employed to evaluate the antimicrobial activity against six pathogenic organisms and the phylogenetic tree of active isolates was constructed by the MEGA. **Result**: Ninety-eight endophytic fungi obtained from seven *Dendrobium* spp., and among them twenty-four isolates, representing 11 genera and 14 species, displayed anti-microbial activities. The phylogenetic assay based on ITS-rDNA showed that 24 active isolates were sorted to 7 taxonomic orders: Hypocreales, Sordariales, Capnodiales, Eurotiales, Botryosphaeriales, Xylariales and Mucorales. The results of antimicrobial activity assay revealed that 1.02%, 10.2%, 18.4%, 1.02%, 1.02% and 10.2% of fermentation broths of 98 isolates displayed significant antimicrobial activities against *E. coli*, *B. subtilis*, *S. aureus*, *C. albicans*, *C. neoformans* and *A. fumigatus*, respectively. Four strains DL-R-3, DL-S-6, DG-R-10 and DN-S-1 displayed strong and broad antimicrobial spectrum. **Conclusion**: Endophytic fungi associated with *Dendrobium* species have fungal diversity, and possess diverse antimicrobial activity.

[**Key words**] antimicrobial activity; agar diffusion method; endophytic fungi; ITS analyses; tropical rainforest plants; *Dendrobium* plant

There is a general call for new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective, possess low toxicity, and have a minor environmental impact[1]. Where we can find the relevant drug resources? Recent studies have reported hundreds of natural products including substances of alkaloids, terpenoids, flavonoids, steroids, etc. from endophytes[2]. Endophytes are viewed as an outstanding source of bioactive natural products due to their abundance and their occupation of literally millions of unique biological niches (higher plants) growing in so many unusual environments[3].

The other thing that we had to notice was that plant scientists have begun to realize that all plants in natural ecosystems appear to be symbiotic with fungal endophytes[4]. In view of the unique colonization in certain hosts, it has been estimated that there might be as many as 1 million different endophyte species; however, only a handful of them have been described[5]. This increases the opportunity of finding and targeting new natural products from interesting endophytes among myriads of plants in different niches and ecosystems[2].

As one of the renowned traditional medicines, the native *Dendrobium* plants are distributed in tropical and subtropical rainforest areas where they have been used as medicine by indigenous (Dai ethnic) people for thousands of years in southwestern China. *Dendrobium* spp. belong to the family Orchidaceae and were precious herbal plants in Chinese traditional medicine as a therapeutic for nourishing the stomach, promoting secretion of saliva, and reducing fever[6]. Recently, a number of compounds from *Dendrobium* spp. were

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found to have beneficial activities such as antioxidant, immune stimulating and antitumor activity[7-10]. With the diminishing area of rainforest, *Dendrobium* plants and their endophytic fungi were rapidly disappearing. So, the study of endophytic fungi of *Dendrobium* materials has become extremely necessary.

The aim of the present research study was to screen for antimicrobial activity of endophytic fungi isolated from seven *Dendrobium* plants, *D. longicornu*, *D. minutiflorum*, *D. gratiosissimum*, *D. compactum*, *D. aphyllum*, *D. huoshanense* and *D. nobile*, found in the rainforest of Yunnan province in China. Those endophytic fungi with antimicrobial activity were identified and their phylogenetic affiliation was analyzed based on the internal transcribed spacer (ITS) sequence. This was an important study in discovering novel metabolites and exploring the relationship between active endophytic fungi and *Dendrobium* plants.

## 1 Materials and methods

**1.1** Collection of plant material Healthy and asymptomatic plant materials comprising stems and roots of seven *Dendrobium* spp. (*D. longicornu*, *D. minutiflorum*, *D. gratiosissimum*, *D. compactum*, *D. aphyllum*, *D. huoshanense*, *D. nobile*) were collected from different sites of the tropical rainforests in the Yunnan province and Chongqing municipality of China in January 2009 and were identified by Prof. Shunxing Guo. The fresh plant samples were brought to the laboratory in an icebox and treated within eight hours. A voucher specimen (Den7-200901) was deposited in the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College.

**1.2** Isolation, identification and phylogenetic analysis of endophytic fungi Healthy plant samples were cleaned in running tap water to remove all soil, and finally washed with double distilled water to minimize the microbial contamination from sample surface. The surface sterilization was done adopting the methodology by Wang et al.[11] The cleaned samples were cut into about 1 cm long and then surface sterilized by immersion in 70% ethanol for 1 min, 5% sodium hypochlorite solution for 5 min, and sterile distilled water twice. The surface-sterilized stems and roots were cut into small pieces using a sterile blade and placed on plates with PDA (potato-dextrose-agar) medium for incubation at 25 ℃. Actively growing mycelia immerging from plant tissues were sub-cultured on PDA petri plates for identification and fermentation. Similar procedure, but without surface sterilization, was used as negative control to check for contamination. All the isolated endophytic fungi have been deposited in the culture collection in the Laboratory of Microbiology, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College.

Endophytic fungi that showed antimicrobial activity were identified based on their ITS ribosomal DNA (ITS rRNA gene) sequences. The total genomic DNA was extracted from fungal mycelia grown with PDA using the CTAB (Cetyl trimetyl ammonium bromide) method[12]. Primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the ITS regions from the DNA extract. The PCR reaction was performed with the following cycles: ①94 ℃ for 3 min; ②30 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s and 72 ℃ for 1 min; ③72 ℃ for 10 min[13]. The PCR products were purified using microcon columns (Millipore, USA), and sequenced using ABI Prism 310 genetic analyzer (ABI, USA) as per the manufacturer′s instructions. A BLAST search was used to search for closest matched sequences in the GenBank database (http://www.ncbi.nlm.nih.gov)[14].

The active fungal sequences and other relatived sequences were multiply aligned using BioEdit 7.0.5[12] and the alignments were adjusted manually, where necessary, to maximize alignment. The ITS sequences of active endophytic fungi obtained in this study were deposited in GenBank (Table 1).

The sequences were aligned using the CLUSTAL X program[15], and molecular evolutionary analyses were conducted using MEGA version 4.0[16]. The Kimura two-parameter model[17] was used to estimate evolutionary distance. The phylogenetic tree was constructed using the neighbor-joining (NJ)

Table 1 Closest relatives of *Dendrobium* plants endophytic fungi isolates based on BLAST analyses

| Strain | GenBank accession number | Host plants | Closest related species | Similarity/% |
|---|---|---|---|---|
| L-R[a]-3 | GU441576 | *Dendrobium longicornu* | *Cladosporium cladosporioides* | 100 |
| L-R-4 | GU441577 | *D. longicornu* | *Trichoderma viride* | 100 |
| DL-R-5 | GU441578 | *D. longicornu* | *Penicillium brevicompactum* | 100 |
| DL-R-7 | GU441579 | *D. longicornu* | *Hypocrea viridescens* | 95 |
| DL-R-14 | GU441580 | *D. longicornu* | *P. swiecickii* | 100 |
| DL-S[b]-1 | GU441581 | *D. longicornu* | *Trichoderma viride* | 100 |
| DL-S-6 | GU441582 | *D. longicornu* | *Chaetomium globosum* | 100 |
| DM-R-1 | GU441583 | *D. minutiflorum* | *Fusarium tricinctum* | 100 |
| DM-R-2 | GU441584 | *D. minutiflorum* | *F. tricinctum* | 100 |
| DM-R-3 | GU441585 | *D. minutiflorum* | *F. tricinctum* | 100 |
| DM-R-7 | GU441586 | *D. minutiflorum* | *Pestalotiopsis microspora* | 100 |
| DM-S-2 | GU441587 | *D. minutiflorum* | *P. clavispora* | 100 |
| DM-S-3 | GU441588 | *D. minutiflorum* | *F. proliferatum* | 100 |
| DG-R-6 | GU441589 | *D. gratiosissimum* | *P. microspora* | 100 |
| DG-R-7 | GU441590 | *D. gratiosissimum* | *P. microspora* | 100 |
| DG-R-10 | GU441591 | *D. gratiosissimum* | *Gibberella moniliformis* | 100 |
| DG-S-5 | GU441592 | *D. gratiosissimum* | *F. proliferatum* | 100 |
| DC-R-1 | GU441593 | *D. compactum* | *Botryosphaeria mamane* | 95 |
| DC-R-7 | GU441594 | *D. compactum* | *F. proliferatum* | 100 |
| DA-S-3 | GU441595 | *D. aphyllum* | *F. proliferatum* | 100 |
| DH-R-7 | GU441596 | *D. huoshanense* | *F. tricinctum* | 100 |
| DN-S-1 | GU441597 | *D. nobile* | *P. microspora* | 100 |
| DN-S-2 | GU441598 | *D. nobile* | *Mucor racemosus* | 100 |
| DN-S-5 | GU441599 | *D. nobile* | *Fusicoccum arbuti* | 100 |

Note: [a] root; [b] stem.

algorithm[18] and maximum-parsimony (MP) analyses, with bootstrap values calculated from 1 000 replicate runs, using the software routines included in the MEGA software [19].

**1.3** Fungal culture and the fermentation broth preparation Each of the isolated fungi was grown on PDA at 25 ℃ for 7 d. Five pieces (5 mm in diameter) of the grown culture cut from the plate were inoculated into 1 000 mL Erlenmeyer flask containing 250 mL of liquid PD medium, respectively. All flasks were incubated for 10 d at 25 ℃ on a rotary shaker at 180 r · $min^{-1}$. After suspension culture, the culture broth was separated from the mycelia by filtration in vacuum. Crude fermentation broth was blended thoroughly and centrifuged at 4 000 r · $min^{-1}$ for 5 min. Liquid supernatant was extracted with an equal volume of ethyl acetate thrice. The organic solvent extract was then evaporated under reduced pressure to yield an ethyl acetate extract. Then the ethanol extracts were used for antimicrobial screening.

**1.4** Antimicrobial activity assay The agar well diffusion method was used to evaluate the antimicrobial activity[20]. Six pathogenic micro-organisms, all obtained from the Chinese Academy of Medical Sciences, were used as indicator organisms: three pathogenic fungi (*Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*) were used to determine the antifungal activity, and two gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and one gram-negative (*Escherichia coli*) bacteria were used as antibacterial activity. The bacteria and spores of indicator organisms were diluted with melted beef extract peptone (BEP) medium and Sabouraud's agar (SA) medium into $1 \times 10^6$/mL and $5 \times 10^5$/mL, respectively, and poured onto the 9 cm diameter petri plate which had already contained about 8 mL of solidified BEP or SA medium. After solidification, four circular equidistant well (7.8 mm in diameter) were made in the BEP or SA layer using sterile cork borers. The ethyl acetate extracts were dissolved in dimethyl sulfoxide (DMSO, 2%) to a final concentration of 1 g · $L^{-1}$. Then 100 μL of each extract solution was added into the wells. DMSO

(2%) was used as a control. After incubation at 37 ℃ for 24 h for bacteria and, 25 ℃ for 48 h for fungi, the diameter of the zone of inhibition (in cm) were observed, measured and recorded. All tests were performed in triplicate.

## 2 Results

**2.1** Endophytic fungi and their phylogenetic analysis

Ninety-eight morphologically distinct endophytic fungi were isolated from the stems and roots of seven *Dendrobium* plants. Among them, there were 22 strains from *D. longicornu*, 14 from *D. minutiflorum*, 21 from *D. gratiosissimum*, 13 from *D. compactum*, 3 from *D. aphyllum*, 19 from *D. huoshanense* and 6 from *D. nobile* (Fig. 1). Twenty-four antimicrobially active strains isolated from *Dendrobium* spp. were identified by their ITS ribosomal DNA and they were distributed to 11 genera (Table 1).

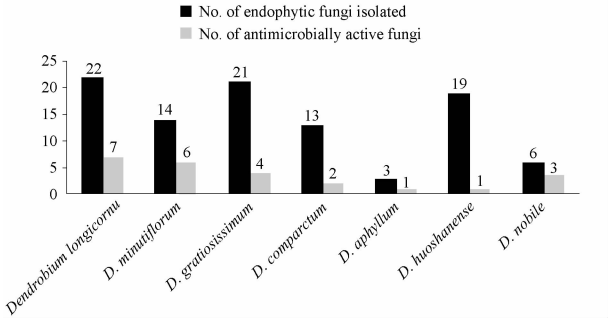



Fig. 1 Number of endophytic fungi and the antimicrobially active fungi isolated from each *Dendrobium species*

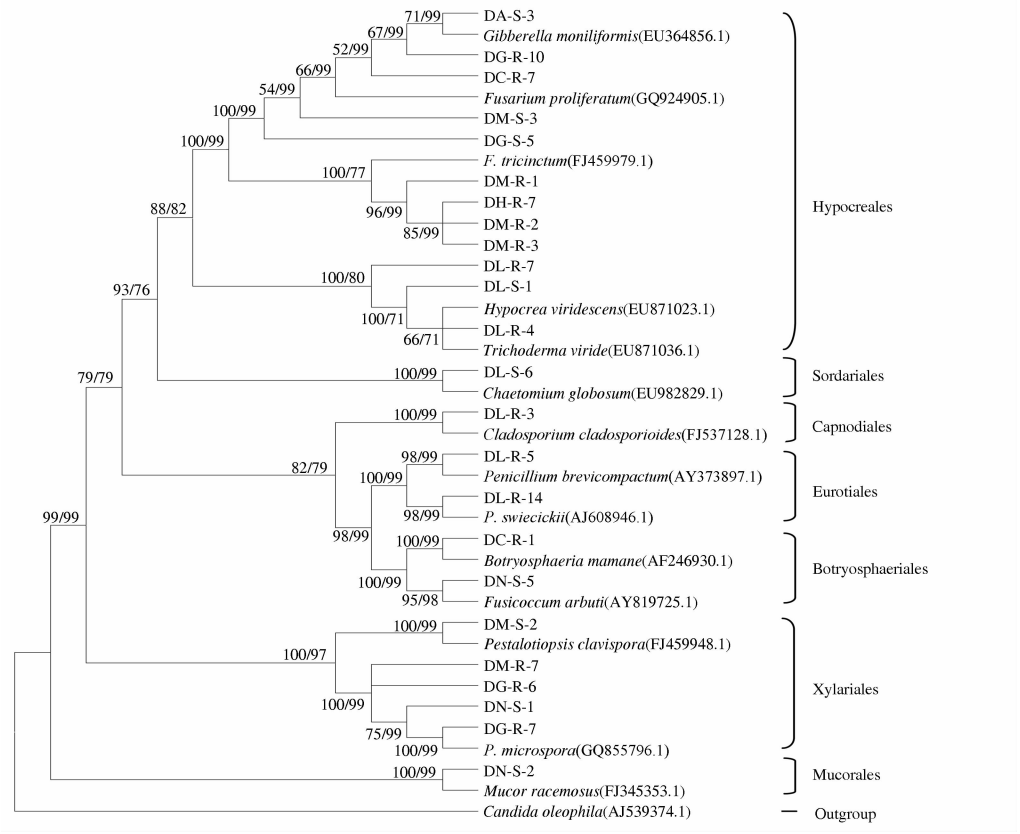
The phylogenetic assay of antimicrobial active fungi based on ITS-rDNA (Fig. 2) showed that 24 strains could be sorted to 7 different clades, corresponding to 7 taxonomic orders (Hypocreales, Sordariales, Capnodiales, Eurotiales, Botryosphaeriales, Xylariales, Mucorales). The Hypocreales group, the biggest group, was made up 12 strains. The 9 strains DA-S-3, DG-R-10, DC-R-7, DM-S-3, DG-S-5, DM-R-1, DH-R-7, DM-R-2 and DM-R-3, were closely related to some species of *Fusarium* and its teleomorph *Gibberella*. The strains DL-R-4 and DL-S-1 were closely related to *Trichoderma viride* and the strain DL-R-7 was closely related to *Hypocrea viridescens*.

The Sordariales clade was monophyletic group with DL-S-6 closely related to *Chaetomium globosum*, and formed a cluster supported by 100%/99% (NJ/MP) bootstrap. The order Capnodiales was represented by the strain DL-R-3, which was genotypically closely related, and formed a cluster supported by a 100% bootstrap value with *Cladosporium cladosporioides* (100% BLAST similarity). The order Eurotiales was represented by the strain DL-R-5 and DL-R-14, which were closely related to *Penicillium brevicompactum* by 98%/99% bootstrap value and *Penicillium swiecickii* by 98%/98% bootstrap value (100% BLAST similarity). The Botryosphaeriales group was made up of DC-R-1 and DN-S-5, closely related to *Botryosphaeria mamane* and *Fusicoccum arbuti*, respectively.

Five strains were previously identified as belonging to the genus *Pestalotiopsis* within the order Xylariales, and two are included here (Fig. 2). Strain DM-S-2 was closely related and formed a cluster supported by 100%/99% bootstrap value, with *P. clavispora* (100% BLAST similarity). Four strains DM-R-7, DG-R-6, DN-S-1 and DG-R-7 were grouped with *P. microspora* (100% BLAST similarity and 100%/99% bootstrap value).

The last order Mucorales was represented by the strain DN-S-2, which was genotypically closely related, and formed a cluster supported by a 100%/99% bootstrap value with *Mucor racemosus* (100% BLAST similarity), which belonged to Zygomycota fungus.

**2.2** Antimicrobial activity The antibacterial and antifungal activity of fermentation broths of 98 endophytic fungi were evaluated by agar well diffusion. Twenty-four strains (24.5%) displayed antimicrobial activity against at least one pathogenic microorganism (Table 2). Among them, 7 strains were from *D. longicornu*, 6 from *D. minutiflorum*, 4 from *D. gratiosissimum*, 2 from *D. compactum*, 1 from *D. aphyllum*, 1 from *D. huoshanense* and 3 from *D. nobile* (Fig. 1). It was observed that 18 strains (18.4%) displayed antagonistic activity against *S. aureus*, 10 strains (10.2%) against *B. subtilis* and *A. fumigatus*, and only 1 strain (1.02%) displayed antagonistic activity against *E. coli*, *C. albicans* and *C. neoformans*.




Numbers above or below branches indicate bootstrap values of NJ and MP analyses ( >50%, right) from 1 000 bootstrap replicates.

Fig. 2 Neighbour-joining (NJ) phylogenetic tree based on ITS-rDNA sequences of endophytic fungi associated with *Dendrobium* spp.

Table 2 Antimicrobial activities in fermentation broths of 24 antimicrobially active fungi from *Dendrobium* spp. cm

| Strain | Antimirobial activity ($\bar{x} \pm s, n = 3$) | | | | | |
|---|---|---|---|---|---|---|
| | *Escherichia coli* | *Bacillus subtili* | *Staphylococcus aureus* | *Candida albicans* | *Cryptococcus neoformans* | *Aspergillus fumigates* |
| DL-R-3 | – | 1. 195 ± 0. 011 | 1. 683 ± 0. 017 | 1. 749 ± 0. 058 | – | 1. 531 ± 0. 010 |
| DL-R-4 | – | 1. 029 ± 0. 035 | 0. 937 ± 0. 023 | – | – | 1. 479 ± 0. 018 |
| DL-R-5 | – | 0. 983 ± 0. 013 | 1. 364 ± 0. 006 | – | – | – |
| DL-R-7 | – | – | – | – | 1. 607 ± 0. 006 | 0. 875 ± 0. 011 |
| DL-R-14 | – | 0. 973 ± 0. 002 | 1. 009 ± 0. 010 | – | – | – |
| DL-S-1 | – | – | 1. 250 ± 0. 002 | – | – | 1. 187 ± 0. 007 |
| DL-S-6 | – | 1. 800 ± 0. 011 | 1. 634 ± 0. 007 | – | – | – |
| DM-R-1 | – | 1. 078 ± 0. 002 | 1. 271 ± 0. 011 | – | – | – |
| DM-R-2 | – | – | 0. 896 ± 0. 005 | – | – | 1. 524 ± 0. 006 |
| DM-R-3 | – | – | 1. 135 ± 0. 001 | – | – | – |
| DM-R-7 | – | – | – | – | – | 1. 657 ± 0. 008 |
| DM-S-2 | – | – | 1. 217 ± 0. 006 | – | – | – |
| DM-S-3 | – | – | 0. 969 ± 0. 006 | – | – | – |
| DG-R-6 | – | – | 1. 085 ± 0. 015 | – | – | – |
| DG-R-7 | – | – | 0. 885 ± 0. 005 | – | – | – |
| DG-R-10 | – | – | 0. 927 ± 0. 007 | – | – | 1. 774 ± 0. 008 |
| DG-S-5 | – | – | – | – | – | 1. 107 ± 0. 008 |
| DC-R-1 | – | 1. 543 ± 0. 008 | 1. 393 ± 0. 013 | – | – | – |
| DC-R-7 | – | 1. 518 ± 0. 008 | – | – | – | 1. 691 ± 0. 010 |
| DA-S-3 | – | – | 0. 927 ± 0. 007 | – | – | – |
| DH-R-7 | – | – | 1. 100 ± 0. 001 | – | – | – |
| DN-S-1 | – | 1. 802 ± 0. 007 | 1. 477 ± 0. 004 | – | – | 1. 590 ± 0. 005 |
| DN-S-2 | 1. 209 ± 0. 004 | – | – | – | – | – |
| DN-S-5 | – | 1. 181 ± 0. 002 | – | – | – | – |

## 3 Discussion

Endophytic fungi, a group of microbes remaining relatively unstudied, represent an important genetic resource for exploiting new natural products, and had been a target of micro-ecological and bioactive screening studies. In the present study, twenty-four strains with antimicrobial activity were obtained from 98 endophytic fungi of seven *Dendrobium* plants and they were distributed to 11 genera and 14 species, according to the results of molecular identification, showed high diversity. Though differing in fungal species composition and structure, culturable communities of these bioactive endophytic fungi from seven *Dendrobium* spp. displayed similar phylogenetic affiliation at the phyla level. They were mainly from Deuteromycota and Ascomycota, and only one strain (DN-S-2) belonging to *Mucor racemosus* was from Zygomycota. Moreover, the number of species from the two genera *Fusarium* (including its teleomorph *Gibberella*) and *Pestalotiopsis* was 17. This accounted for 70.8% of 24 active isolates. Therefore, the fungal diversity within any particular host may be very high[21]. No two isolates may be identical, even from the same species or host[12,22]. But the endophytes from any particular host usually include one to several taxa that are adapted to that host[23].

In the current study, 24 endophytic fungi displayed different antimicrobial ability against Gram-negatives, Gram-positives and fungi (Table 2). Most of strains showed certain selectivity to pathogens, even those from the same species. None of the fungal broths inhibited the Gram-negative bacterium *Escherichia coli* except DNL-S-2 showing low activity against it. Four strains DL-R-3, DL-S-6, DG-R-10 and DN-S-1 possessed comparatively strong and broad antimicrobial spectrum. DL-R-3 was the only strain of the 98 endophytic fungi that displayed strong anti-fungal activity against *Candida albicans*. DL-S-6 and DN-S-1 displayed anti-Gram-positive activity against *Bacillus subtilis*. DG-R-10 had the best effect on the fungi *Aspergillus fumigatus*. These four isolates could be good candidates for further studies of their anti-Gram-positive and anti-fungi activities. The other seven strains (DL-R-4, DL-R-7, DL-S-1, DM-R-2, DM-R-7, DC-R-7 and DC-R-1) also had strong effect on *Bacillus subtilis*, *Staphylococcus aureus*, *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*. All these results demonstrate that the endophytic fungi from *Dendrobium* plants are promising sources in the search for antimicrobial metabolites.

In summary, the current study of endophytic fungi of *Dendrobium* plants can contribute to our understanding of the community structure and diversity of associated organisms and facilitate the further study of active strains and their metabolites.

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# 中国西南 7 种石斛植物活性内生真菌及其遗传关系

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[**摘要**] **目的**:对中国西南地区 7 种石斛植物的内生真菌进行抗菌活性筛选,考察活性菌株之间的遗传关系。**方法**:采用组织块法分离真菌,琼脂扩散法进行抑菌活性筛选,通过 rDNA 的 ITS 序列为基础鉴定活性真菌,用邻接法(NJ)和最大简约法(MP)进行遗传关系考察。**结果**:从 7 种石斛属植物分离到 98 株真菌,它们对 *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Candida albicans*, *Cryptococcus neoformans* 和 *Aspergillus fumigatus* 的抑制率分别是 1.02%, 102%, 184%, 1.02%, 1.02%, 10.2%,其中 DL-3, DL-18, DL-21, DM-2, DG-10 和 DN-1 显示出了对 *B. subtilis*, *S. aureus* 和 *A. fumigatus* 较强的生长抑制性。24 株活性真菌隶属于 7 个目, 11 个属, 14 个种, NJ 和 MP 系统树阐明活性菌之间的亲缘关系。**结论**:本研究对于考察珍惜濒危石斛属植物的内生真菌的生物活性以及它们之间的遗传关系具有重要的生态价值,为石斛根茎部活性真菌的研究提供参考。

[**关键词**] 抑菌活性; 琼脂扩散法; 内生真菌; ITS 序列分析; 热带雨林植物; 石斛

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