# Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants

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Endophytic fungi residing in medicinal plants have not been systematically characterized. In this study, we classified 1160 fungal isolates from 29 medicinal plant species using traditional morphological methods. The colonization rate, isolation rate, and relative frequency of these endophytes were investigated. The relationship between the composition of endophytic fungi and the chemical constituents of host plants was also explored for the first time. The results showed that endophytic fungi from these medicinal plants exhibited high biodiversity, host-recurrence, tissue-specificity, and spatial heterogeneity. Taxa of *Alternaria, Colletotrichum, Phoma, Phomopsis, Xylariales*, and mycelia sterilia were the dominant fungal endophytes. Some phenolic compounds were found to more likely coexist with certain endophytic fungi in the same plants. Our systematic investigation reveals that traditional medicinal plants are a rich and reliable source of novel endophytic fungi. This study was the first step towards understanding host-endophyte relationships based on the plant chemistry.

**Key words:** biodiversity, endophytic fungi, host-endophyte relationships, host-preference, medicinal plants, plant chemistry, spatial heterogeneity, tissue-specificity

## Introduction

Endophytic fungi are a group of fungi that colonize living, internal tissues of plants without causing any immediate, overt negative effects (Hirsch and Braun, 1992). Many recent studies have revealed the ubiquity of these fungi, with an estimate of at least 1 million species of endophytic fungi residing in plants (Dreyfuss and Chapela, 1994) and even lichens (Li *et al.*, 2007). Endophytic fungi represent an important and quantifiable component of fungal biodiversity, and are known to affect plant community diversity and structure (Sanders, 2004; Gonthier *et al.*, 2006; Krings *et al.*, 2007). To date, only about 80,000–100,000 fungal species have been described (Hawksworth and Rossman, 1987; Kirk *et al.*, 2001), out of a conservative estimate of 1.5 million (Hawksworth, 1991). Recent studies of endophytic fungi from tropical and temperate forests support the high estimates of species

diversity (e.g., Kumar and Hyde, 2004; Santamaria and Bayman, 2005; Santamaria and Diez, 2005; Sánchez Márquez *et al.*, 2007). These estimates do not include several additional sources of fungal diversity (Ganley and Newcomb , 2006).

A variety of relationships exist between fungal endophytes and their host plants, ranging from mutualistic or symbiotic to antagonistic or slightly pathogenic (Schulz and Boyle, 2005; Arnold, 2007). Because of what appears to be their contribution to the host plant, the endophytes may produce a plethora of substances of potential use to modern medicine, agriculture, and industry, such as novel antibiotics, antimycotics, immuno-suppressants, and anticancer compounds (Strobel and Daisy, 2003; Mitchell *et al.*, 2008). There is great potential of finding new drugs from endophytes for treating new diseases in humans and animals (Kumar *et al.*, 2005). In addition, the studies of endophytic

fungi and their relationships with host plants will shed light on the ecology and evolution of both the endophytes and their hosts: the evolution of endophyte-plant symbioses; the ecological factors that influence the direction and strength of the endophyte-host plant interaction (Saikkonen *et al.*, 1998, 2004).

The relationships of endophytes with single or multiple plant hosts can be described in terms of host-specificity, host-recurrence, host selectivity, or host-preference (Zhou and Hyde, 2001; Cohen, 2006). Host-specificity is the relationship in which a fungus is restricted to a single host or a group of related species, but does not occur in other unrelated plants in the same habitat (Holliday, 1998). The frequent or predominant occurrence of an endophytic fungus on a particular host or a range of plant hosts is often defined as host-recurrence, but the fungus can also occur infrequently on other host plants in the same habitat (Zhou and Hyde, 2001). A single endophytic fungal species may form relationships with two related plant species but demonstrate a preference for one particular host, and this phenomenon is categorized as host-selectivity (Cohen, 2004, 2006). The term 'host-preference', however, is more frequently used by mycologists to indicate a common occurrence or uniqueness of the occurrence of a fungus on a particular host, and the term is also used to indicate the differences in fungal community compositions and isolation frequencies from different host plants (Suryanarayanan and Kumaresan, 2000; Bettucci *et al.*, 2004). The differences in endophyte assemblages from different hosts might be related to the chemical differences of the hosts (Paulus *et al.*, 2006).

Endophytic fungi frequently demonstrate single host-specificity at the plant species level but this specificity could be influenced by environmental conditions (Cohen, 2004). Some researchers use 'partial heterogeneity' or 'geographic variation' to indicate the endophytic fungal segregation impacted by environmental differences (Yahr *et al.*, 2006). Endophytes are also able to colonize multiple host species of the same plant family within the same habitat, and the distribution of some endophytes can be similar in closely related plant species. For example, most endophytic fungi belonging to *Balansieae* and originally isolated from the grass family *Poaceae* were also detected in its closely related families *Cyperaceae* and *Juncaceae* (Marks *et al.*, 1991). On the other hand, differences in endophytic fungal assemblages have been found in different tissues of the same plant species, or even in different tissues of a single plant, which is a reflection of tissue specificity (Collado *et al.*, 2001; Fröhlich *et al.*, 2001; Ganley and Newcombe, 2006).

Since natural products are likely adapted to a specific function in nature, the search for novel secondary metabolites should concentrate on organisms that inhabit novel biotopes (Schulz *et al.*, 2002)., Schulz *et al.* (2002) isolated about 6500 endophytic fungi from herbaceous plants and trees over a course of 12 years, screened them for biologically active compounds, and found a correlation between biological activity and biotope, e.g. a higher proportion of the fungal endophytes, in contrast to the soil isolates, inhibited at least one of the test organisms for anti-algal and herbicidal activities. Medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of pharmaceutical importance (Strobel *et al.*, 2004; Wiyakrutta *et al.*, 2004; Kumar *et al.*, 2005; Tejesvi *et al.*, 2007). The various natural products produced by endophytic fungi possess unique structures and great bioactivities, representing a huge reservoir which offers an enormous potential for exploitation for medicinal, agricultural and industrial uses (Tan and Zou, 2001; Zhang *et al.*, 2006).

As part of our ongoing efforts towards finding novel anti-oxidant and anti-microbial agents, and other bioactive chemicals from natural resources, we had recently investigated the secondary metabolites of endophytes isolated from 29 traditional Chinese medicinal plants in Hong Kong, and found some endophytic fungal isolates exhibited strong anti-oxidant capacity and anti-microbial activity (Kumar and Hyde, 2004; Kumar *et al.*, 2005; Huang *et al.*, 2007a,b). It is important and necessary to identify and classify these potential bioactive fungi for a better understanding of the characteristics of the relevant endophytic communities.

The aim of this study was to investigate qualitatively and quantitatively the biodiversity of endophytic fungi from 29 traditional

Chinese medicinal plant species occurring in Hong Kong. Although there are limitations in classifying endophytes using the traditional morphological methodology, at present there are no workable alternatives (Duong *et al*., 2006; Hyde and Soytong, 2007) since molecular phylogenetic identification is still not applicable to all fungal taxa on a large scale. Host-recurrence, tissue-specificity, and spatial heterogeneity in endophyte distributions were analyzed based on both fungal community compositions and isolation frequencies. Furthermore, we explored and discussed the relationship between the compositions of endophytic fungal assemblages and the chemical constituents of their plant hosts for the first time.

## Materials and methods

### *Plant material*

Twenty-nine traditional Chinese medicinal plant species from seven families (*Apocynaceae, Asclepiadaceae, Asteraceae, Polygonaceae, Lamiaceae, Rubiaceae*, and *Solanaceae*) were collected from Kadoorie Farm and Botanic Garden (KFBG) and four more locations in the wild: Tai Po (TP), Hong Kong Island (HKI), Ma On Shan (MOS), and Ham Tin Wan (HTW). All the species were first identified morphologically in the field and subsequently verified by DNA sequencing analysis. Digital photos were taken for all the species studied.

### *Isolation of endophytic fungi*

A total of 20 samples of plant parts (leaf, stem, flower/inflorescence, fruit, or roots) from each species were first washed with running water. The leaves, flowers/inflorescences or fruits were cut into segments (5 × 5 mm), and stems or roots were cut into small pieces (10 mm in length). Surface sterilization and isolation of endophytic fungi followed a modified procedure as described by Schulz *et al*. (1993), and the details of the procedure were also given in our previous study (Huang *et al*., 2007b). Antibiotics penicillin G and streptomycin (Sigma, St. Louis, MO, USA) were added to the cultures to suppress the growth of bacteria. The pure endophytic fungal strains were photographed and preserved in the laboratory of School of Biological Sciences, the University of Hong Kong.

### *Identification of endophytic fungal isolates*

The morphological identification of endophytic fungal strains is based on the morphology of the fungal culture colony or hyphae, the characteristics of the spores, and reproductive structures if these features were discernible (Wei, 1979; Carmichael *et al*., 1980; Barnett and Hunter, 1998), and the identities of some major groups were subsequently verified with molecular methods following the procedures of Promputtha *et al*. (2007). For inducing sporulation, each of the isolated fungal strains was separately inoculated on PDA (potato dextrose agar), PCA (potato carrot agar), and WA (water agar) in Petri dishes. Measurements of all fungal characters were made in water mounts, and the slides were subsequently mounted in lactophenol and sealed with nail vanish. All experiments and observations were repeated at least twice. Those cultures that failed to sporulate were grouped as mycelia sterilia, and divided into different morphospecies according to their cultural characteristics. Some fungal isolates could not be identified to the species or genus level. This is a common problem concerning the identification of endophytes (Gamboa and Bayman, 2001; Promputtha *et al*., 2005).

### *Plant chemistry analysis*

Total phenolic content (TPC) was estimated for each of the 29 plant species using the Folin-Ciocalteu colorimetric method in a previous study (Huang *et al*., 2007b). Total flavonoid content (TFC) was determined in this study using a modified colorimetric method of Chun *et al*. (2003). All of the tests were performed in triplicate. The chemical constituents of host plants were analyzed in detail using several chromatographic and spectroscopic techniques: reversed phase-high performance liquid chromatography (RP-HPLC), liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI MS), and gas chromatography-mass spectrometry (GC-MS) as described previously (Huang *et al*., 2007b).

## Data analysis

All the TPC and TFC results were calculated as mean ± SD (standard deviation). Correlation coefficients ($r$) and coefficients of determination ($R^2$) were calculated using Microsoft Office Excel 2003. Colonization rate (CR) was calculated as the total number of segments/pieces colonized by endophytic fungi divided by the total number of segments/pieces incubated for that plant sample, and expressed as percentage. Isolation rate (IR) was calculated as the number of isolates obtained from segments/pieces, divided by the total number of segments/pieces, but not expressed as percentage. Isolation rate is a measure of fungal richness in a given sample of plant tissue, i.e. the incidence of multiple infections per segment/piece of fronds. 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 also expressed as percentage (Photita et al., 2001).

To investigate the relationship between endophytic fungal composition and plant chemistry, random coefficient models were used for reference and statistical design was based on the principle of probability (Longford, 1993; Davis and Lawrence, 1977). For each medicinal plant, one matrix was established as $A_k = [a_{ij}]_{m \times n}$ ($k$ refers to the plant species, varying from 1 to 29). The element $a_{ij}$ in row $i$ and column $j$ of matrix A were obtained by $a_{ij} = a_i \times a_j$ ($a_i$ refers to the presence-absence of one given compound in the given plant, $i$ varies from 1 to $m$, $m$ is the total number of detected compounds; $a_j$ refers to presence-absence of one given fungal taxon in the given plant, $i$ varies from 1 to $n$, $n$ is the total number of found fungal taxa; '1' represents presence, and '0' represents absence). All the 29 matrices were added up to get matrix B ($B = [b_{ij}]_{m \times n} = \sum A_k$, $b_{ij}$ refers to the number of plants with both the given compound and fungal taxon). Another matrix C $= [c_{ij}]_{m \times n}$ was established to compare the relative abundance of the given compound and fungal taxon coexisting in the plants. The element in matrix C was obtained by $c_{ij} = (b_{ij}/p_j) \times (b_{ij}/q_j)$, where $p_j$ is the total number of plants with the given compound, and $q_j$ is the total number of plants with the given fungal taxa. In the equation, $b_{ij}/p_j$ referred to the frequency of plants with the given compound colonized by the given fungal taxon, and $b_{ij}/q_j$ referred to the frequency of plants with the given fungal taxon possessing the given compound.

## Results and discussion

### Biodiversity of endophytic fungi

The 1160 endophytic fungal strains isolated from the 29 traditional Chinese medicinal plants were classified into 31 taxonomic groups, including a total of 73 distinct morphospecies (Table 1). Mycelia sterilia consists of various morphological fungal types, but not forming true spores. This group of fungi is considerably prevalent in endophyte studies (Lacap et al., 2003). In the 13 frequently encountered endophytic fungal groups, mycelia sterilia had the highest relative frequency (27.16%) in the 29 medicinal plants. *Colletotrichum* which are frequently identified as endophytes (Photitia et al., 2005; Devarajan and Suryanarayanan, 2006) was the second most frequent endophytic group, followed by *Phomopsis*, *Alternaria*, *Phoma*, and *Xylariales*, with relative frequencies of 19.31%, 11.03%, 9.05%, 7.07%, and 5.25%, respectively (Fig. 1). These were the dominant genera or order of endophytic fungi found in this study, similar to the findings reported previously for many tropical endophytic fungi (Corrado and Rodrigues, 2004; Krohn et al., 2007). The 18 infrequent endophytic fungal species or genera included *Aureobasidium pullulans*, *Botryosphaeria*, *Chaetomella*, *Chaetomium*, *Cladosporium*, Coelomycetes, *Drechslera*-like, *Ellisembia*, *Epheliş Flagellospora*, *Helminthosporium*, *Pestalotiopsis*, *Physalospora*, *Pyrenochaeta*, *Pyriculariopsis*, *Rhizosphaera*, *Spiropes*, and *Verticillium*. Among them, *Spiropes*, *Ellisembia*, and *Helminthosporium* are reported here for the first time as endophytic fungi.

### Host recurrence of endophytic fungi

All the 29 medicinal plants were found to harbor various endophytic fungi. The colonization rate and the isolation rate of endophytic fungi from these plants ranged from 36.7% to 100%, and 0.45 to 1.75, respectively. A

**Table 1.** Number and taxonomic identification of endophytic fungi isolated from 29 Chinese medicinal plants.

| Plant host / Fungal taxon [a] | Al | Asc | As | Co | Fu | Gl | Hy | MS | Ph | Pho | Phy | To | Xy | Others | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Apocynaceae | | | | | | | | | | | | | | | |
| *Allamanda cathartica (Aca)* | | | | 6 | | | | 23 | 1 | 5 | | | | | 35 |
| *Alstonia scholaris (Asc)* | 2 | 5 | 4 | 12 | | | | 9 | | | | | | 1 | 33 |
| *Alyxia sinensis (As)* | | | | 9 | | 5 | | 13 | 2 | | | | 2 | | 31 |
| *Catharanthus roseus (Cr)* | 7 | | | 6 | | | 4 | 19 | 2 | 4 | | 3 | | | 45 |
| *Cerbera manghas (Cm)* | 9 | | 1 | 15 | 4 | | | 1 | 9 | 3 | | 2 | | | 44 |
| *Melodinus suaveolens (Ms)* | 7 | | | 8 | | | | 19 | | 8 | | | | | 42 |
| *Nerium oleander (No)* | | 1 | | 1 | | | 5 | 21 | 4 | | | 7 | | 3 | 42 |
| *Plumeria rubra (Pr)* | 8 | 1 | 1 | 2 | | | 1 | 12 | | 4 | 1 | | 1 | 4 | 35 |
| *Strophanthus divaricatus (Sd)* | 1 | | | 3 | | | | 31 | 1 | 1 | 8 | | 24 | 1 | 70 |
| *Tabernaemontana divaricata (Td)* | | | | 26 | | 8 | | 12 | 14 | 19 | | | 1 | 3 | 83 |
| *Thevetia peruviana (Tp)* | | 6 | 1 | 9 | 1 | | | 4 | 6 | 4 | | | | 5 | 36 |
| *Trachelospermum jasminoides (Tj)* | | | 1 | 1 | | 5 | 1 | 14 | 9 | 10 | 1 | | 1 | | 43 |
| Asclepiadaceae | | | | | | | | | | | | | | | |
| *Asclepias curassavica (Ac)* | 9 | | 1 | 2 | 1 | | 5 | | 2 | | | | 3 | 4 | 27 |
| *Dischidia chinensis (Dc)* | 13 | 2 | | | | | | 19 | | | | | | | 34 |
| *Graphistemma pictum (Gp)* | 1 | | | 15 | | | 4 | 4 | | 9 | | | | | 33 |
| *Gymnema sylvestre (Gs)* | 4 | | | | 4 | | | 11 | 5 | 7 | | | 2 | 5 | 38 |
| *Hoya carnosa (Hc)* | | | | 4 | 1 | 6 | | 7 | | 2 | | | 9 | | 29 |
| *Toxocarpus wightianus (Tw)* | | | | 11 | | | | | 18 | 4 | 3 | 3 | | 4 | 43 |
| *Tylophora ovata (To)* | | | | 15 | 1 | 14 | | 4 | 1 | | | | 6 | 2 | 43 |
| Asteraceae | | | | | | | | | | | | | | | |
| *Artemisia capillaris (Acap)* | 27 | 1 | | | | | | 7 | | 1 | | | 1 | 5 | 42 |
| *Artemisia indica (Ai)* | | 13 | 2 | | | | | 15 | | 5 | | | | 4 | 39 |
| *Artemisia lactiflora (Al)* | | 2 | | 20 | | | | 3 | | 2 | | | | | 27 |
| Lamiaceae | | | | | | | | | | | | | | | |
| *Scutellaria indica (Si)* | | | | 19 | | | | 1 | 6 | | | | | 7 | 33 |

**Table 1 (continued).** Number and taxonomic identification of endophytic fungi isolated from 29 Chinese medicinal plants.

| Plant host / Fungal taxon [a] | Al | Asc | As | Co | Fu | Gl | Hy | MS | Ph | Pho | Phy | To | Xy | Others | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Polygonaceae | | | | | | | | | | | | | | | |
| *Polygonum capitatum (Pca)* | 10 | | | 12 | | | | 22 | | 4 | | | | | 48 |
| *Polygonum chinense (Pc)* | | | 3 | | 2 | | 9 | 3 | 2 | 3 | | 1 | 8 | 10 | 41 |
| *Polygonum cuspidatum (Pcu)* | | 7 | | | | 1 | | 22 | | 16 | | | | | 46 |
| *Polygonum multiflorum (Pm)* | | 1 | 2 | | | | 14 | 8 | | 4 | | 1 | 3 | 2 | 35 |
| Rubiaceae | | | | | | | | | | | | | | | |
| *Pavetta hongkongensis (Ph)* | 1 | | | 12 | | | | 6 | | 4 | | | | | 23 |
| Solanaceae | | | | | | | | | | | | | | | |
| *Cestrum nocturnum (Cno)* | 6 | | | 14 | | | | 5 | | 9 | | | | 6 | 40 |
| **Total** | 105 | 39 | 14 | 224 | 14 | 39 | 43 | 315 | 82 | 128 | 13 | 17 | 61 | 66 | 1160 |

[a] The morphologically identified taxon; Al: *Alternaria* spp. (4 morphospecies); Asc: Ascomycete spp. (6 morphospecies); As: *Aspergillus* spp. (3 morphospecies); Co: *Colletotrichum* spp. (6 morphospecies); Fu: *Fusarium* spp. (3 morphospecies); Gl: *Gliocladium* sp.; Hy: Hyphomycete spp. (6 morphospecies); MS: Mycelia sterilia spp. (13 morphospecies); Ph: *Phoma* spp. (4 morphospecies); Pho: *Phomopsis* spp. (4 morphospecies); Phy: *Phyllosticta* sp.; To: Torula sp.; Xy: *Xylariales* sp.; Others (the total number of isolates was no more than 10): 5 isolates of *Aureobasidium pullulans* (AcapL1, AscS2, PcS11, PmL12, and PrL5); one isolate of *Botryosphaeria* sp. from *P. chinense*; 5 isolates of *Chaetomella* sp. (one from *T. ovata*, and 4 from *T. peruviana*); one isolate of *Chaetomium* sp. from *N. oleander*; 8 isolates of *Cladosporium* sp. (PcL7, PmL15, PrS4, TpF1, and each two from *A. curassavica* and *N. oleander*); 10 isolates of Coelomycetes spp. (3 morphospecies; AcS8, CnoS5, PcL11, 4 from *A. indica*, and 3 from *T. divaricata*); one isolate of *Drechslera*-like sp. from *A. capillaries*; 5 isolates of *Ellisembia* sp. from *G. sylvestre*; 2 isolates of *Ephelis* sp. from *A. capillaries*; 6 isolates of *Flagellospora* sp. (AcS1, PrL2, and 4 from *P. chinense*); one isolate of *Helminthosporium* sp. from *S. divaricatus*; 2 isolates of *Pestalotiopsis* sp. (AcapF6 and PcL10); 2 isolates of *Physalospora* sp. from *C. nocturnum*; 2 isolates of *Pyrenochaeta* sp. (PcS23 and PrS3); one isolate of *Pyriculariopsis* sp. from *T. ovata*; 3 isolates of *Rhizosphaera* sp. from *C. nocturnum*; 7 isolates of *Spiropes* sp. from *S. indica*; and 4 isolates of *Verticillium* sp. from *T. wightianus*.
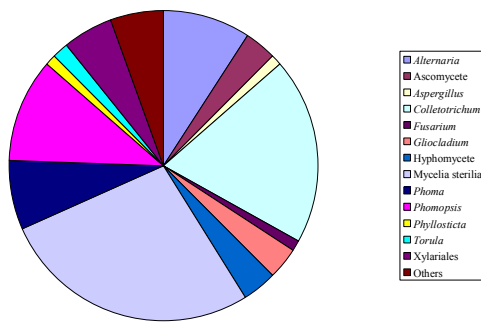
**Fig. 1.** Relative frequencies of different endophytic taxa isolated from 29 traditional Chinese medicinal plants.
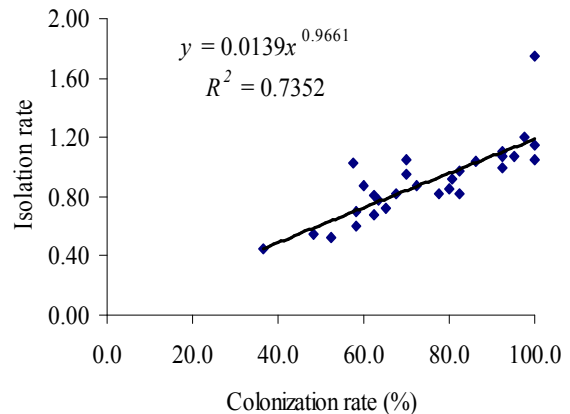


**Fig. 2.** The relationship between colonization rate and isolation rate of endophytic fungi from 29 medicinal plants.

$$y = 0.0139x^{0.9661}$$
$$R^2 = 0.7352$$
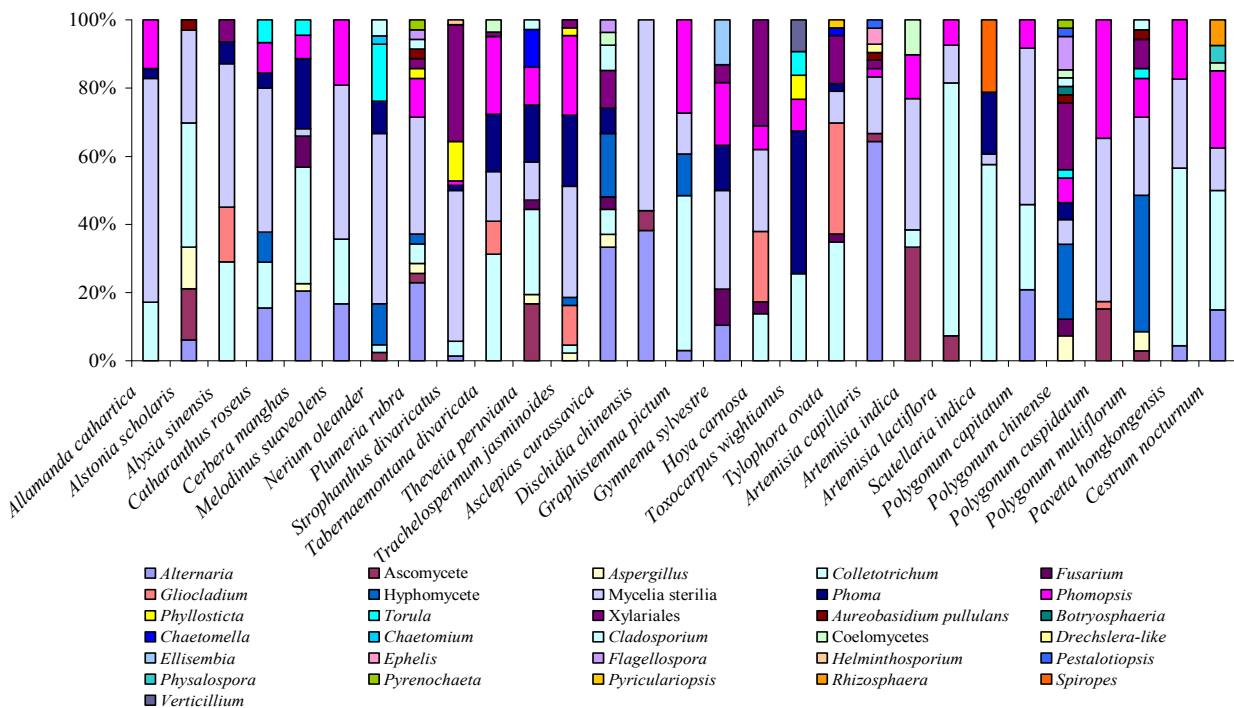


**Fig. 3.** Relative frequencies of different endophytic fungal taxa isolated from each of the 29 medicinal plants.

positive correlation was detected between their colonization and isolation rates (Fig. 2). In addition, different endophytic fungal taxa showed different relative frequencies in different plants (Fig. 3). Among the 29 medicinal plants, *Polygonum chinense* yielded the greatest fungal diversity, with 15 different taxa being isolated from its leaves and stems. Thirteen and ten different fungal taxa were isolated from *Plumeria rubra* and *Asclepias curassavica*, respectively. *Dischidia chinensis* yielded the lowest fungal diversity, with only

three taxa of endophytic fungi isolated. Interestingly, the highest number of fungal isolates (83) was obtained from the leaves and stems of *Tabernaemontana divaricata*, but they belong to only seven different taxa. Among these fungal taxa, *Colletotrichum* was the most frequent (31.3%), followed by *Phomopsis* (22.9%), *Phoma* (16.9%), mycelia sterilia (14.5%).

The common endophytic fungi had a wide distribution in the plant hosts and hence a high isolate abundance. For example,

mycelia sterilia were found in all the plant samples except *A. curassavica* and *Toxocarpus wightianus*. Twenty-three of the 29 host plants were colonized by *Colletotrichum* species. *Colletotrichum* had a relatively high occurrence in 13 of the host plants (RF > 20%), especially in *Artemisia lactiflora* (RF = 74.1%). *Phomopsis* species were found in 22 of the host plants, with a major occurrence in five of them. *Alternaria* species were found in 14 of the host plants, with a major occurrence in seven, especially in *Artemisia capillaris* (RF = 64.3%). *Phoma* species were obtained from 15 of the plant hosts, with a major occurrence in three. *Xylariales* species were found in 12 of the plant hosts, with major occurrences in *Strophanthus divaricatus* and *Hoya carnosa* (RF = 34.3 and 31%, respectively).

*Pestalotiopsis* is a common endophytic genus that has been reported in many previous studies (Li and Strobel, 2001; Hu *et al.*, 2006; Tejesvi *et al.*, 2007), but only two *Pestalotiopsis* fungal isolates were detected in two of the host plants (*A. capillaris* and *P. chinense*) in the present study. Moreover, the five isolates of *Aureobasidium pullulans* obtained in this study came from five different host plants. Some of the endophytic fungi were found in only one host species. For example, the only isolate of *Chaetomium* came from the stem of *Nerium oleander*, and five isolates of *Ellisembia* all came from the stem of *Gymnema sylvestre*. Although only seven isolates of *Spiropes* were obtained in this study, they all came from the leaves of *Scutellaria indica* with a RF value of 21.2% in the host species.

### Tissue specificity of endophytic fungi

The distribution of endophytic fungi in leaves and stems were investigated for all the medicinal plants, but their occurrence in fruits, roots, or flowers/inflorescences was investigated for only some of the host species (e.g., fruits of *Thevetia peruviana*, roots of *Tylophora ovata*, and flowers/inflorescences of *Catharanthus roseus*, *Pavetta hongkongensis*, *T. peruviana*, *A. curassavica*, and *A. capillaris*). The composition and abundance of the endophytes varied according to the host tissues tested. The leaves, stems, and fruits

harbored more endophytic fungi than the flowers or roots, whose CR and IR values were 74.1, 76.9, 83.3, 33.8 and 42.9%, 0.89, 0.94, 0.92, 0.41 and 0.57, respectively. A total of 549 fungal isolates were obtained from the leaves of these 29 medicinal plants, while 568 isolates from the stems, 28 isolates from the flowers/inflorescences, 11 isolates from the fruits, and 4 isolates from the roots, respectively (Table 2).

The 13 most frequent fungal taxa had a nearly ubiquitous presence in the leaves and the stems of the 29 host plants, only *Fusarium* could not be found in the leaves and *Phyllosticta* not found in the stems. For the other 18 infrequent endophytic fungal taxa, they appeared to be more abundant in the stems (35 isolates of 13 taxa) than in the leaves (27 isolates of 8 taxa). Some endophytic fungi more frequently colonized the leaves, and others the stems. For example, more endophytic isolates of *Colletotrichum*, mycelia sterilia, or *Alternaria* were obtained from the leaves than from the stems, whereas more isolates of *Phoma*, *Phomopsis*, or *Xylariales* were detected in the stems than in the leaves. Endophytic strains of *Phyllosticta*, *Physalospora* an d *Spiropes* were only isolated from the leaves. In contrast, isolates of *Chaetomium*, *Drechslera*-like, *Ellisembia*, *Epheliş Helminthosporium*, *Pyrenochaeta*, and *Rhizosphaera* were detected only in the stems. The one isolate of *Pyriculariopsis* was found only in the root of *Tylophora ovata*.

### Spatial heterogeneity of endophytic fungi

The 29 medicinal plant species were collected from five different field sites in Hong Kong. The host plants and number of plant samples differed among the sites, but the overall colonization and isolation rates of endophytic fungi from different sites did not differ greatly, with CR values ranging from 58.3% to 83.3%, IR values ranging from 0.69 to 0.9. These findings were consistent with previous report (Photita *et al.*, 2001).

The taxa abundance and isolation frequency of endophytic fungi from each location are given in Table 3. Twenty-four fungal taxa were obtained from site KFBG, 11 from TP, 14 from HKI, 10 from MOS, and 9 from HTW. The KFBG site had the

**Table 2.** Number of isolates of different endophytic fungal taxa in different plant tissues.

| Tissue | Al | Asc | As | Co | Fu | Gl | Hy | MS | Ph | Pho | Phy | To | Xy | Others | Total |
|--------|----|-----|----|----|----|----|----|----|----|-----|-----|----|----|--------|-------|
| Leaf | 54 | 15 | 7 | 153 | | 16 | 22 | 188 | 2 | 21 | 13 | 14 | 17 | 27 | 549 |
| Stem | 35 | 24 | 6 | 68 | 13 | 23 | 19 | 117 | 79 | 102 | | 3 | 44 | 35 | 568 |
| Flower | 16 | | | | | | 2 | 6 | | 2 | | | | 2 | 28 |
| Fruit | | | 1 | 3 | | | | 4 | | 3 | | | | | 11 |
| Root | | | | | 1 | | | | 1 | | | | | 2 | 4 |
| Total | 105 | 39 | 14 | 224 | 14 | 39 | 43 | 315 | 82 | 128 | 13 | 17 | 61 | 66 | 1160 |

**Table 3.** Relative isolation frequencies of different endophytic fungal taxa from five different sites.

| Location [a] | Al | Asc | As | Co | Fu | Gl | Hy | MS | Ph | Pho | Phy | To | Xy | Others [b] |
|----------|----|-----|----|----|----|----|----|----|----|-----|-----|----|----|---------|
| KFBG | 7.4 | 3.6 | 1.4 | 11.0 | 1.1 | 2.2 | 6.2 | 31.5 | 6.3 | 13.2 | 1.6 | 2.2 | 6.5 | 5.7 |
| TP | 13.6 | 6.4 | 3.6 | 20.9 | | | | 25.5 | 16.4 | 3.6 | 2.7 | 2.7 | | 4.5 |
| HKI | 5.2 | 0.7 | | 33.8 | 2.0 | 8.2 | 1.3 | 24.6 | 3.9 | 11.5 | | | 6.2 | 2.6 |
| MOS | | 8.7 | 1.4 | 40.6 | 1.4 | | | 7.2 | 17.4 | 5.8 | | | | 17.4 |
| HTW | 64.3 | 2.4 | | | | | | 16.7 | | 2.4 | | | 2.4 | 11.9 |

[a] KFBG: Kadoorie Farm and Botanic Garden; TP: Tai Po; HKI: Hong Kong Island; MOS: Ma On Shan; HTW: Ham Tin Wan.
[b] Others referred to 18 infrequent endophytic fungal taxa (KFBG: 11 taxa; TP: 2 taxa; HKI: 4 taxa; MOS: 3 taxa; HTW: 4 taxa).

broadest distribution of endophytic taxa, which was apparently related to the largest number of hostplants sampled from the site. However, three medicinal plants (*P. chinense*, *P. rubra*, and *A. curassavica*) with the broadest distribution of endophytic taxa were all collected from KFBG. In addition, more endophytic fungal isolates and taxa were obtained from KFBG than HKI in the same host plant T. divaricata, which was collected from the two different locations for comparative analysis of endophyte distribution. Ascomycete sp., mycelia sterilia, and Phomopsis were found in all the five collection sites. *Colletotrichum* and *Phoma* were found at four of the five sites (except for HTW). *Colletotrichum* were the dominant endophytes in all these sites, and *Phoma* had frequent occurrence at sites TP and MOS (RF > 10%). *Alternaria* were also found in four sites (except for MOS), and it was the most common endophyte at HTW (RF = 64.3%). Site-specific fungal taxa included the following: *Gliocladium*, Coelomycetes, *Botryosphaeria*, *Chaetomium*, Coelomycetes, *Flagellospora*, *Helminthosporium*, and *Rhizosphaera* were found only at site KFBG,

*Verticillium* only at Tai Po, *Xylariales*, *Ellisembia*, and *Pyriculariopsis* only at HKI, *Ephelis* and *Drechslera*-like endophytes only at HTW, and Spiropes only at MOS (RF = 10.1%).

***Relationship between endophytic fungal composition and plant chemistry***

Preliminary identification of chemical constituents in medicinal plants using RP-HPLC, LC-ESI MS, and GC-MS showed that major types of phenolic compounds were phenolic alkaloids, phenolic acids, flavonoids, tannins, phenolic terpenoids, quinones, stilbenes, and volatile and aliphatic compounds. Among them, flavonoids were the major type, and TFC ($y$) was positively related with TPC ($x$) ($y = 20.012x + 4.4176$, $r = 0.7817$). Interestingly, the lowest number of fungal taxa (3) were obtained from *D. chinensis*, which also had the lowest TFC and TPC values (8.72 mg rutin g$^{-1}$ and 2.8 mg gallic acid g$^{-1}$, respectively), while only four fungal taxa were found from each of the host plants with the highest TFC values (*Melodinus suaveolens*: 194.8 mg rutin g$^{-1}$ and
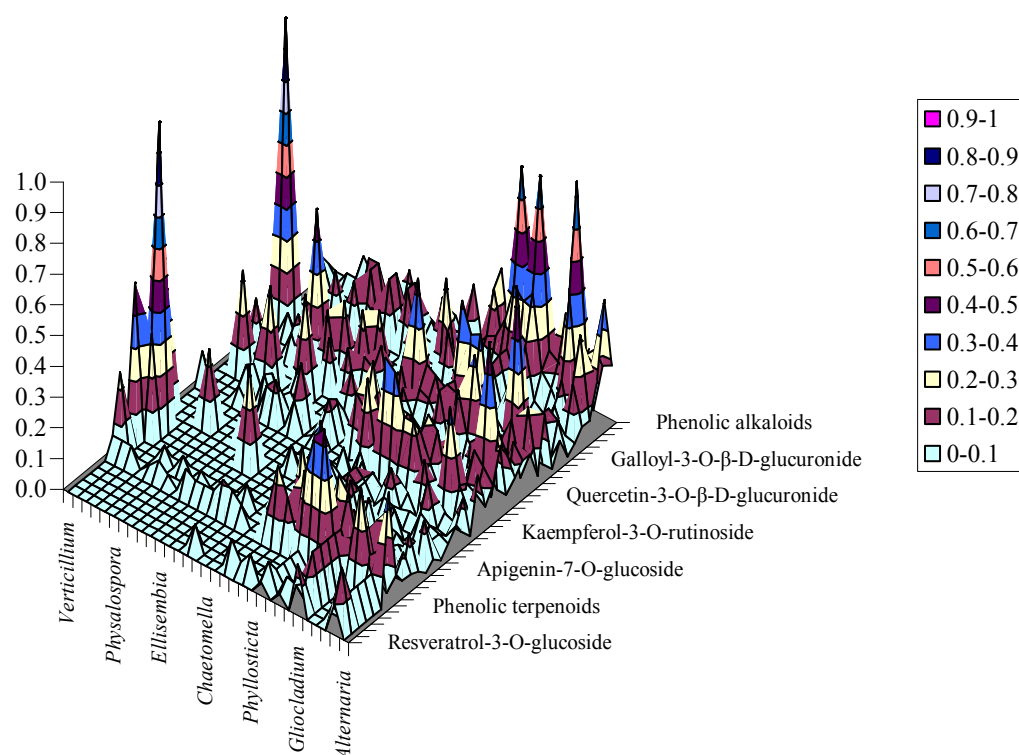
**Fig. 4.** The relative abundance of 37 phenolic compounds and 31 endophytic fungal taxa coexisting in 29 medicinal plants.

*S. indica*: 192.7 mg rutin g$^{-1}$) or the highest TPC values (*Polygonum capitatum*, *M. suaveolens*, *Polygonum cuspidatum*, and *S. indica*; 86.9, 70.1, 63.3, and 58.8 mg gallic acid g$^{-1}$, respectively). The plants with more fungal taxa had moderate TFC and TPC values. The relationship equation was established between the TFC value ($x$) and the number of endophytic isolates ($y$) ($y = -0.0018x^2 + 0.4047x + 28.958$ and $R^2 = 0.2792$).

Thirty-seven identified phenolic compounds were investigated for their relationship with a total of 31 endophytic fungal taxa using the established matrix based on random coefficient models and probability statistics. The element in the matrix C (37 × 31) represented the relative abundance of the given compound and fungal taxon coexisting in the plants, which varied from 0 to 1. '0' means the given compound could not coexist with the given fungal taxon, and '1' means

the plant with the given compound must be colonized by the given fungal taxon. Because $c_{ij}$ was the product of two probabilities ($b_{ij}/p_j \times b_{ij}/q_j$), the possibility of the given compound and fungal taxon coexisting in plants was relatively high when c > 0.25 (0.5 × 0.5). Among a total of 1147 elements, there were 51 elements with a value more than 0.25, and ten of them were even higher than 0.5 (Fig. 4). Some phenolic compounds could coexist with most of the fungal taxa, and others only correlated with several given fungal taxa. The most common endophytes more likely coexisted with the frequently detected compounds. For example, chlorogenic acid was detected in 21 medicinal plants. Twenty-nine fungal taxa were isolated from these plants. Among the fungal isolates, the most frequent taxa (mycelia sterilia, *Colletotrichum*, *Phomopsis*, *Alternaria*, *Phoma*, and *Xylariales*) all noticeably coexisted with chlorogenic acid (c = 0.705, 0.741, 0.701, 0.412,

0.317, and 0.254, respectively). Phenolic terpenoids were detected in 16 of the medicinal plants and 27 endophytic fungal taxa, with high occurrence in all the above six dominant fungal taxa (each c > 0.25). Each of the five flavonoids (rutin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, kaemp-ferol-3-*O*-rhamnoside, and kaempferol-3-*O*-rutinoside) was found to coexist with more than 20 fungal taxa. Among these flavonoids, rutin mostly co-occurred with *Colletotrichum* (c = 0.565). Tannins were discovered in 9 plants, coexisting with 21 fungal taxa. Among these fungal taxa, mycelia sterilia, *Phomopsis*, and *Torula* more likely coexisted with tannins (c = 0.263, 0.409, and 0.3, respectively). Only 3 plants were detected to contain gallic acid, however, 19 fungal taxa were obtained from these three plants, and gallic acid mostly co-occurred with *Botryosphaeria* (c = 0.33). Some infrequent endophytes were found in only one or two host plant species, but more likely coexisted with certain given com-pounds, such as *Pestalotiopsis* with quercetin-3-*O*-galactoside, and *Verticillium* with apiin/catechin (all c = 0.5). *Spiropes* coexisted with baicalin and an unknown flavonoid (both c = 1), but only in one plant (*S. indica*).

### Endophytic fungi in Traditional Chinese medicinal plants

Diverse endophytic fungi reside in plants, representing a rich resource of bioactive natural products with potential for exploitation in pharmaceutical and agri-cultural arenas (Schulz *et al*., 2002). However, it is thought that most of the endophytic fungal diversity remains to be discovered. All the medicinal plants in this study were found to harbor endophytic fungi, and the 1160 fungal isolates obtained from these plants belonged to 31 fungal groups at different taxonomic levels, ranging from species to order. In addition, 73 morpho-species were recognized. Mycelia sterilia was a large group of fungi that failed to sporulate, and was ubiquitous in all the plant endophytic isolation. Additionally, *Alternaria*, *Colle-totrichum*, *Phoma*, *Phomopsis*, and *Xylariales* were also predominant fungal taxa in the 29 host plants. Most of these taxa were

previously found to be common endophytes of tropical, subtropical or temperate plants (Pandey *et al*., 2003; Rodrigues and Menezes, 2005; Dai *et al*., 2006). The colonization and propagation of these endophytes may in some way offer significant benefits to their hosts by producing useful substances (Strobel *et al*., 2004). Indeed, some anti-oxidant and anti-microbial metabolites have already been found in endophytic fungal isolates (Kumar *et al*., 2005; Huang *et al*., 2007a,b).

Previous studies reported distinct endophyte community compositions in different host plants suggesting host preferences (Cannon and Simmons, 2002; Cohen, 2006). This study also found the significant differences in both presence /absence and relative abundance of fungal endophytes in the medicinal plants occurring in Hong Kong. The colonization rate and the isolation rate of endophytic fungi from these plants varied greatly. Some medicinal plants harbored more endophytic fungi than others. The number of fungal taxa colonizing these hosts ranged from three to 15, and many of the fungal taxa had different isolate frequent-cies in different hosts. Some of the common endophytes not only existed in more plant hosts but also had higher relative frequencies within each of the hosts. In contrast, some other endophytic fungi were detected in only one given plant host. These descriptions of host-preference were consistent with previous reports (Arnold *et al*., 2001; Bettucci *et al*., 2004).

The endophytic fungi in these 29 medicinal plants exhibited tissue-specificity. Some fungal endophytes were more likely found in the leaves while others in the stems. Some infrequent fungal endophytes were found in only one type of tissue (leaf /stem/root). The difference in endophyte assemblages from various tissues indicated that some fungal endophytes have an affinity for different tissue types and this might be a reflection of their capacity for utilizing or surviving within a specific substrate (different tissue texture and chemistry) (Rodrigues, 1994; Photita *et al*., 2001). Many previous reports also discovered tissue-specificity in

endophytic fungi (e.g., Taylor *et al.*, 2001; Ganley and Newcombe, 2006).

Spatial variability has not been thoroughly explored for endophytic fungi and may be difficult to discern because stratum, substrate, or host preferences confound spatial patterns (Arnold *et al.*, 2000). In this study, among-site differences were found in endophytic composition and abundance, but this spatial variation may contain the component of host-preferences as the collection sites also differ in plant composition. Some sites harbored more fungal endophytes than the others, and some endophytes were found in only one location. Spatial heterogeneity in the distribution of endophytes was also reported in previous studies (Arnold *et al.*, 2001; Gallery *et al.*, 2007). Such spatial heterogeneity may be partly due to differences in environmental conditions, including humidity, temperature, rainfall and potential inoculum sources (Photita *et al.*, 2001; Santamaria and Bayman, 2005).

The host plants in our study possessed different phenolic compounds (e.g., alkaloids, phenolic acids, flavonoids, tannins, terpenoids, quinones, stilbenes, volatile and aliphatic compounds), and these different hosts were colonized by various different endophytic fungi. Some correlation apparently exists between the endophytic fungal assemblages and the host chemistry. Unfortunately, it was impossible to identify or quantify all the chemical compounds nor all the endophytic fungi present in the plant hosts. In this study, only the total contents of phenolics and flavonoids of the 29 medicinal hosts were investigated and their major phenolic compounds and fungal endophytes identified. However, the results suggested that the total contents of phenolics and flavonoids of the host plants influence both the quantity of endophytic fungal taxa and the number of endophytic isolates. Moderate TPC and TFC appear to favor the growth of endophytic fungi. The plants with too low or too high TPC and TFC were colonized with fewer endophytic fungi. Based on the established matrix, some phenolic compounds were found to more likely coexist with some endophytic fungal taxa, such as chlorogenic acid with mycelia sterilia, *Colletotrichum*, and *Phomop-*

*sis*, rutin with *Colletotrichum*, and *Spiropes* with baicalin. Although some endophytic fungi may prefer the hosts with specific compounds, the presence of a given compound could not guarantee the presence of a given endophyte. The quantity of the specific compounds could also play a role in fungal colonization. Furthermore, the colonization of endophytes can induce the plants to produce certain compounds, and some of the special compounds with small quantity might be actually produced by the endophytes within the host plants. There exist other hypotheses that microbial symbionts could affect plant nutrition, defensive chemistry, and biodiversity (Rudgers *et al.*, 2007). The host-endophyte relationship is complex and involves many factors, but studying the presence-absence of a given endophyte in the presence of a given compound of the host plant is an important first step toward our understanding of this intricate relationship. Further studies are needed to reveal the interaction between the host plant and its endophytes.

In conclusion, this study investigated endophytic fungal diversity and host-endophyte relationship based on traditional metholody. The 1160 endophytic fungal isolates from 29 medicinal plants were identified and classified. *Alternaria*, *Colletotrichum*, *Phoma*, *Phomopsis*, *Xylariales*, and mycelia sterilia were the dominant fungal taxa. The evidence for host-preference, tissue-specificity, and spatial heterogeneity was found in the endophyte distribution based on fungal community compositions and isolation frequencies. Certain correlations exist between the endophytic fungal assemblages and the host plant chemistry.

Studying endophytes as we have done here is a method-dependent process (Guo *et al.*, 2001) and thus the fungi we isolated are dependent on our methodology. We were also unable to identify the mycelia sterilia. Future studies should use molecular sequence data to identify mycelia sterilia (e.g. Wang *et al.*, 2005; Promputtha *et al.*, 2005; Sánchez Márquez *et al.*, 2007). Total fungal communities should be detected by extracting the entire host DNA (for example from leaves) with various methods to sequence individual taxa. Potentially successful methods include

DNA cloning (Guo *et al*., 2001; Seena *et al*., 2008), DGGE (Duong *et al*., 2006) or T-RLFP (Nikolcheva *et al*., 2003; Nikolcheva and Bärlocher, 2005).

## Acknowledgements

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