Whole rDNA analysis reveals novel and endophytic fungi in *Bletilla ochracea* (*Orchidaceae*)

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Endophytes within leaf and root tissues of *Bletilla ochracea* (*Orchidaceae*) were investigated using DGGE and random cloning analysis. Eighteen operational taxonomic units (OTUs) of endophytic fungi from leaves and ten taxa from roots were revealed. Two dominant ascomycete OTUs were *Mycosphaerella* species (41%) (*Mycosphaerellaceae*). An unknown Ascomycete sp. 2 (13.6%) and an *Alternaria* sp. (9%) were also common. One *Sebacina* sp. (*Sebacinaceae*, Basidiomycota) (46%), two *Fusarium* species (30.7%) and a *Nectria* sp. (13.4%) (*Nectriaceae*) were common in the orchid roots. The diversity within leaves (H′, 2.354) was higher than that within roots (H′, 1.560). Fungal communities within leaf and root tissues were significantly different.

Key words: DGGE, fungal diversity, endophytes, ITS, *Orchidaceae*, phylogenetic analysis

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Introduction

The term ‘endophyte’ is commonly defined as all organisms, including bacteria (Kobayashi and Palumbo, 2000), fungi (Stone *et al*., 2000), algae (Peters, 1991), and insects (Feller, 1995), that grow inside living plant tissues without causing disease symptoms (Petrini, 1991; Mostert *et al*., 2000; Stone *et al*., 2000; Sanchez-Márquez *et al*., 2007). Endophytic fungi can be latent pathogens (Brown *et al*., 1998; Jumpponen, 2001; Photita *et al*., 2004), mutualists, for example mycorrhizal fungi (Sieber, 2002), and/or saprobes (Gardes, 2002; Promputtha *et al*., 2007), but should be detected within the tissues of healthy host plants (Mostert *et al*., 2000; Schulz and Boyle, 2005). Fungal endophytes play important roles in ecosystem processes such as decomposition and nutrient cycling, and have beneficial symbiotic relationships with roots of many plants (Christensen, 1989).

The *Orchidaceae* is one of the largest plant families, including almost 10% of all flowering plant species (Jones, 2006). They are fasci-nating plants for researchers and have beautiful flowers and special mycorrhizal symbiosis (Griesbach, 2002; Zettler *et al*., 2004). Orchids are usually divided into two groups, the epiphytic orchids and the terrestrial orchids based on their photosynthetic ability (Bidar-tondo, 2005; Zettler *et al*., 2004). Most studies of orchid fungal associations have focused on terrestrial photosynthetic orchids (Otero *et al*., 2002; McCormick *et al*., 2004, 2006; Shef-ferson *et al*., 2005; Irwin *et al*., 2007). Studies have often shown associations between specific mycorrhizal fungi and Orchid species (Zettler *et al*., 2004; Otero *et al*., 2007; Shefferson *et al*., 2008;). Orchid mycorrhizae have often been characterized as belonging to several anamorphic genera: *Epulorhiza*, *Ceratorhiza*, and *Moniliopsis* (Warcup, 1981a; Moore, 1988; Ma *et al*., 2003; Pereira *et al*.,

2003, 2005), other studies have revealed teleomorph genera (*Ceratobasidium*, *Oliveonia*, *Sebacina*, *Thana-tephorus and Tulasnella*) as well as several genera of Basidiomycota (Warcup and Talbot, 1966, 1971; Currah *et al*., 1997; Taylor *et al*., 2003; Zettler *et al*., 2004).

Apart from mycorrhizal fungi within Orchid roots, many of the endophytic fungi are not mycorrhizal, and studies on these endophytic fungi are lacking (*e.g*. see Rasmussen, 2002; Dearnaley, 2007), especially in leaf tissues. Based on knowledge of endophytes in other plants (Guo *et al*., 2000, 2001, 2003; Schulz and Boyle, 2005; Li *et al*., 2007), it is likely that all orchids contain a large community of fungal endophytes which are an important component of fungal biodiversity. To understand potential symbiosis with distinctive endophytes and thus to elucidate adaptive significance of the Orchid plant, it is essential to gain insight on fungal endophytes and their genetic diversity.

Traditional approaches for revealing fungal endophytes involve isolation procedures, sterilization techniques, cultural conditions and sporulation of isolates (Taylor *et al*., 1999; Guo *et al*., 1998; Koide *et al*., 2005; Ganley and Newcombe, 2006; Hyde and Soytong, 2007). Endophyte isolations commonly result in a considerable number of sterile mycelia (*sensu* Lacap *et al*., 2003), and these fungi can not be identified due to lack morphological characters. Molecular techniques have been successfully employed in phylogenetic analysis for the identification of morphospecies by applying rDNA sequences (Guo *et al*., 2000, 2001, 2003; Promputtha *et al*., 2005, 2007; Wang *et al*., 2005). The problem with these methods is that many endophytes do not grow out on the artificial media and are not isolated (Hyde and Soytong, 2007). Allen *et al*. (2003) concluded that unculturable *Sebacina*-like basidiomycete endophytes were present in the *Gaultheria shallon* (*Ericaceae*) roots and represented a significant component of the root endophyte communities, but that they were absent from cultured endophytes.

DNA-based techniques have the advantage of allowing direct identification of dominant fungi within plant tissues and are not limited by culturability or affected to contaminants (Duong *et al*., 2006). PCR with fungus-specific primers from the genomic DNA extracted directly from natural samples, coupled with separation methods such as random cloning, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) analysis and amplified ribosomal DNA restriction analysis (ARDRA) can reveal hidden taxa (Anderson and Cairney, 2004; Duong *et al*., 2006; Seena *et al*., 2008) and have been applied in mycorrihiza (Bougoure and Cairney, 2005), endophyte and saprobe studies (Duong *et al*., 2006).

In the present study, we used combined ITS-PCR, random cloning, DGGE and phylogenetic analysis to investigate the fungal communities within roots and leaves of the terrestrial Orchid *Bletilla ochracea* in southwest China. The main purpose of this study was to compare endophytic diversity between roots and leaves of the same plants, and to establish whether there was community consistency within different organs of a single host. This study is a preliminary step towards determining relationships between orchids and their endophytes, and towards a more comprehensive knowledge of orchid endophytes in nature.

Materials and methods

Sampling sites and treatments

Bletilla ochracea, a species of orchid, were collected from their native habitat from a mountain near Guiyang City in Guizhou Province, China (26°30′24.1″N, 106°27′43.3″E) in August 2006. The altitude was *ca*. 1310m above sea level, mean annual temperature 15.3ºC, and mean annual precipitation 1100-1200 mm. These samples were taken to the laboratory together with the soil, and replanted for the further experiments. The sample plants were treated as follows to remove the microorganism on the plant surface. Healthy leaves and roots were cut from experimental plants, and debris or soil on the surface was removed by careful rinsing under gently running tap water. Roots were examined at 5-10 mm intervals using a microscope, and those with the hyphal pelotons or coils within the cortical cells were selected for further DNA

extraction. Adult leaves were cut into six 2-4 cm diameter discs. Root pieces and leaf discs were surface-sterilized in a sequence of 75% ethanol for 1 minute, 0.1% $HgCl_2$ for 3.5 minutes, and finally rinsed in five changes of sterile distilled water. Genomic DNA was extracted from the sample at once, or place in sterile paper bag, and stored at -70°C until further analysis.

DNA extraction

Total DNA was extracted from samples using a modified protocol of CTAB (Doyle and Doyle, 1987; Guo *et al*., 2001). Approximately 500 mg of root or 1g of leaf tissues were placed in a mortar with liquid nitrogen and ground into fine powder for 5-10 minutes. The powder was immediately placed into 1.5 mL Eppendorf microcentrifuge tubes, and the protocol of DNA extraction was as described previously (Doyle and Doyle, 1987; Guo *et al*., 2001). The DNA pellet was washed with 70% ethanol twice or more and allowed to air dry, and then resuspended in 200-500 µl TE buffer (10mM Tris–HCl, 1mM EDTA, pH 8.0) and measured by a fluorometer (Beckman, DU-800) with approximately 100 ng/µl of DNA. The total DNA samples were stored at -20°C for PCR amplification.

ITS amplification, and cloning

The fungal ITS regions, including the intervening 5.8S rDNA and flanking ITS1 and ITS2, were amplified with universal primers of ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al*., 1990), and with the fungal specific primers ITS1F (5′-CTTGGTCATTTA GAGGAAGTAA-3′) (Gardes and Bruns, 1993) and ITS4 directly from the total DNA of samples. The reactions of ITS1-ITS4 primer pair were carried out as same as described in Yang and Liu (2005). The protocol of ITS1F-ITS4 primer amplification was as follows: 3 minutes initial denaturation at 95°C, followed by 35 cycles of 50 seconds denaturation at 94°C, 50 seconds primer annealing at 56°C, 1 minute extension at 72°C, and a final 10 minutes extension at 72°C. PCR products were electrophoresed in 1.2% (w/v) agarose gels, stained with ethidium bromide and checked for size and purity under UV light.

Primary PCR products with multiple bands were excised together from the agarose gels with a sterile scalpel, and directly purified with PCR Product Purification Kit (Tiangen, China) according to the manufacturer's protocol. Purified PCR products of multiple bands were cloned into pMD18-T vector (Takara) with an overnight ligation reaction at 16°C, and transformed into the cells of *E. coli*, DH5α (Tiangen) by the protocol provided by the manufacturer. Recombinants were identified by blue-white screening, and as many as possible clones on Luria-Bertani medium were confirmed and selected by PCR with primers ITS1 and ITS4 for further DGGE analysis. Recombinant colonies with inserts were incubated overnight at 37°C with shaking at 200 rpm in 3-5 mL of Luria-Bertani broth (Difco, Detroit, Mich.) added to 100 ng/mL of ampicillin. The cultured clones above supplemented with 16% glycerol were stored at -70°C for further sequencing.

ITS amplification for DGGE

Preparation for DGGE: ITS amplification (ITS1-ITS4) of each recombinant was carried out directly from single clone of DH5α colony. ITS amplification of ITS1F-ITS4 primer clones was also performed with the same procedure of ITS1-ITS4 and with the same primers of ITS1 and ITS4, because the site of ITS1 was located at the inner region of ITS1F. Secondly, the additional 40 bp GC-rich sequences (Sheffield *et al*., 1989) were introduced into the fungal ITS sequences by the PCR. This GC clamp stabilized the melting behavior of the DNA fragment, and made it suitable for analysis by DGGE. These PCR products were subjected to DGGE to examine the variation of different fungal taxa and within a single species.

The primers of PCR for DGGE analysis were forward primer ITS1 and the reverse primer ITS4-GC clamp (<u>CGCCCGCCGCGCG CGGCGGGCGGGGCGGGGCACGGG</u>TCCT CCGCTTATTGATATG) [the GC clamp sequence is underlined], and reaction conditions were as following protocol: 3 minutes at 94°C (1 cycle); 40 seconds at 94°C, 40 seconds at 57°C, and 60 seconds at 72°C (34 cycles); and finally 10 minutes at 72°C (1 cycle). PCR products were analyzed by 1.2% agarose gel electrophoresis. Amplifications

were performed on a My Cycler Thermo-cycler (Bio-Rad).

DGGE analysis and sequencing

DGGE was performed by a DCode Universal Mutation Detection System instrument and a gradient former model 475 according to the manufacturer's instructions (Bio-Rad). Electrophoresis was performed with 1 mm thick 7.5% polyacrylamide gels with a ratio of acrilamide to bisacrilamide of 37.5:1, and a vertical denaturing gradient of urea and formamide from 30% to 50%. The running buffer was 1 × TAE (40mM Tris, 40 mM acetic acid, 1mM EDTA, pH 7.4). Approximately 50ng of PCR products for DGGE were mixed with the same volume of loading buffer dye (2% bromophenol blue, 2% xylene cyanol, 100% glycerol) and added to individual wells. Gels were run at a constant temperature of 58°C for 14-16 hours at 80V, stained with ethidium bromide (50ug/ml) by gently shaking for 15 minutes and visualized under UV illumination. Gel images were stored by using the Bio Imaging Systems (Syngene).

The ITS clones from leaf and root tissues were analysed by DGGE, and grouped into different OTUs. One clone of the every same OTU was sequenced by using the vector M13 primers with an ABI automated Sequencer (ABI 3730) (Perkin Elmer). These determined ITS sequences were submitted to phylogenetic analysis.

Phylogenetic analysis

ITS sequences were initially aligned by using the program package Clustal X 1.81 (Thompson *et al*., 1997) under the default settings (multiple alignment parameters: gap opening 10.00 and gap extension 0.20) and was followed by manual adjustments by using BioEdit version 5.0.6 (Hall, North Carolina State University, Raleigh, NC).

All analyses were conducted in PAUP 4.0b 10 (Swofford, 1998). Topology was determined by maximum parsimony (MP) analysis and neighbour-joining (NJ) analysis for the ITS sequences. Robustness of clades was estimated by bootstrap analysis (Felsenstein, 1985) with 1000 replications. Maximum parsimony (MP) analyses were performed with heuristic searches consisting of 1000 random sequence addition replicates with tree bisection-reconnection (TBR) branch swapping. All characters were equally weighted and unordered, and gaps were treated as missing data. Neighbour-joining method by genetic distance analysis among different sequences was also used to generate trees with qualitatively identical results. The phylogenic tree was edited by Treeview (Page, 1996).

To establish the general placement of the clone sequences, known taxa sequences of Eumycota and Plantae for comparison were obtained through a BLAST search from GenBank for phylogenetic analysis (Table 1). Because the ITS regions were highly variable in nucleotides and in length, the alignment of these regions among distantly taxa was not reliable. The ITS regions were therefore excluded from the data set for this analysis, and only the less variable 5.8S gene sequences among distant groups were used in the initial phylogenetic analysis. To further identify these sequences to as low taxonomic level as possible, both the 5.8S gene and the ITS regions were used in the subsequent analysis, which contained more closely related taxa.

Fungal diversity analysis

The Shannon-Weiner diversity index (H′) was employed to evaluate and compare the diversity of fungal communities between different tissues of *Bletilla ochracea* plant, and H′ was calculated according to the formula

$$H' = -\sum_{i=1}^{k} pi \times \ln pi$$

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

Results

PCR amplification and ITS cloning

Total DNA extracted from living surface-sterilized leaves and roots of *Bletilla ochracea* contained genomic DNA of endophytic fungi. ITS sequences including the 5.8S region of fungi and plant were amplified from total DNA with universal primers of ITS1 and ITS4, and the fungal specific primers ITS1F and ITS4

Table 1. Sequences and sources used to construct phylogenetic trees.

Taxon	GenBank accession No.	Taxon	GenBank accession No.
Ascomycota		*Fusarium napiforme*	X94175
Alternaria alternata	AB369904	*Fusarium oxysporum*	EU285552
Alternaria alternata	AF455539	*Fusarium oxysporum*	EU326216
Alternaria mali	EF136372	*Fusarium oxysporum f. cubense*	EF590328
Alternaria tenuissima	AY154710	*Fusarium proliferatum*	EF577235
Alternaria tenuissima	EU315000	*Fusarium proliferatum*	EU151490
Capnobotryella sp.	AM746201	*Fusarium solani*	AM412643
Cercospora beticola	AF297222	*Fusarium tricinctum*	AY188923
Cercospora kikuchii	AY633838	*Gibberella avenacea*	AY147282
Cercospora nicotianae	AF297230	*Gibberella fujikuroi*	EU326193
Cladophialophora boppii	AB109182	*Gibberella moniliformis*	EU151483
Cladophialophora carrionii	AB109171	*Gibberella moniliformis*	EU364867
Cladophialophora carrionii	AB109178	*Gibberella avenacea*	AY147282
Cladophialophora chaetospira	EU035404	*Glomerella graminicola*	EF187914
Cladophialophora emmonsii	AB109184	*Glomerella tucumanensis*	AY944752
Cladophialophora potulentorum	EU035410	*Heteroconium eucalypti*	DQ885893
Cladosporium bruhnei	EF679352	*Hortaea werneckii*	AY213656
Cladosporium chlorocephalum	AF393686	*Leptosphaeria senegalensis*	DQ836777
Cladosporium cladosporioides	DQ810182	*Leptosphaeria tompkinsii*	DQ836790
Cladosporium cladosporioides	EF136373	*Leptosphaerulina americana*	AY278318
Cladosporium funiculosum	AY362000	*Leptosphaerulina trifolii*	AY131203
Cladosporium macrocarpum	EF679380	*Lewia infectoria*	EU301053
Cladosporium variabile	EF679403	*Monochaetia camelliae*	AF377286
Cladosporium vignae	AY361998	*Monochaetia karstenii*	AF405300
Colletotrichum acutatum	AJ301987	*Mycosphaerella areola*	DQ459084
Colletotrichum capsici	EF458673	*Mycosphaerella brassicicola*	AF297236
Colletotrichum caudatum	AB042305	*Mycosphaerella cryptica*	AY509753
Colletotrichum coccodes	AB233340	*Mycosphaerella eumusae*	AY923758
Colletotrichum dematium	AB046607	*Mycosphaerella fijiensis*	AY923765
Colletotrichum destructivum	AF451908	*Mycosphaerella grandis*	AY045514
Colletotrichum higginsianum	AB042303	*Mycosphaerella mexicana*	AY509769
Colletotrichum lupini	AJ301975	*Mycosphaerella musicola*	AY646504
Colletotrichum orbiculare	AB269939	*Mycosphaerella musicola*	AY646472
Colletotrichum truncatum	AJ301937	*Mycosphaerella nubilosa*	AY509775
Colletotrichum truncatum	AJ301976	*Mycosphaerella parva*	AY509781
Cylindrocarpon liriodendri	DQ178165	*Mycosphaerella suberosa*	AY045504
Davidiella dianthi	AF393698	*Nectria haematococca*	DQ535186
Davidiella tassiana	AY361985	*Nectria lugdunensis*	DQ247780
Dichocladosporium chlorocephalum	EU009458	*Neocosmospora ornamentata*	AF178413
Discostroma tricellulare	EU030327	*Neonectria macrodidyma*	DQ069037
Exophiala attenuata	EF025392	*Neonectria radicicola*	EF495233
Exophiala dermatitidis	AB087205	*Neonectria radicicola*	AJ875336
Exophiala dermatitidis	DQ826738	*Neonectria ramulariae*	DQ779782
Exophiala jeanselmei	DQ836795	*Neonectria ramulariae*	AJ279446
Exophiala oligosperma	DQ836797	*Neophaeosphaeria conglomerata*	AF250824
Exophiala pisciphila	DQ826739	*Neophaeosphaeria filamentosa*	AF250820
Exophiala placitae	EU040215	*Pestalotiopsis cocculi*	EF055192
Exophiala salmonis	AM176667	*Pestalotiopsis disseminata*	AB251918
Exophiala salmonis	AY213652	*Pestalotiopsis funereoides*	AY838893
Exophiala spinifera	EU257701	*Pestalotiopsis karstenii*	AY681473
Exserohilum rostratum	EF222027	*Pestalotiopsis lespedezae*	EF055203
Fungal endophyte isolate	DQ979639	*Pestalotiopsis microspora*	DQ001002
Fungal endophyte isolate	EF419910	*Pestalotiopsis neglecta*	EF055210
Fungal endophyte isolate	EF419938	*Pestalotiopsis neglecta*	EU342212
Fungal endophyte isolate	EF419954	*Pestalotiopsis vismiae*	EF055221
Fungal endophyte isolate	EF419966	*Phaeosphaeria avenaria*	EF452729

Table 1 (continued). Sequences and sources used to construct phylogenetic trees.

Taxon	GenBank accession No.	Taxon	GenBank accession No.
Fungal endophyte sp.	EF495231	*Sebacina incrustans*	AF490395
Phaeosphaeria halima	AF422971	*Sebacina* sp.	AF440664
Phaeosphaeria halima	AF422991	*Serendipita vermifera*	DQ520096
Phaeosphaeria nodorum	AF181708	*Sebacina vermifera*	DQ983814
Phaeosphaeria phragmitis	AJ496631	*Tremellales* sp.	EF060917
Phaeosphaeria pontiformis	AJ496632	*Tulasnella albida*	AY373294
Phaeococcomyces catenatus	AF050277	*Tulasnella calospora*	DQ388045
Phaeococcomyces chersonesos	AJ507323	*Tulasnella calospora*	EF393622
Rhynchosporium secalis	AF384682	*Tulasnella danica*	AY373297
Strelitziana africana	DQ885895	*Tulasnella eichleriana*	AY373292
Truncatella angustata	EU342216	*Tulasnella pruinosa*	DQ457642
Xanthoria elegans	AF278756	*Tulasnella tomaculum*	AY373296
Uncultured endophytic fungus	EF504333	*Tulasnella violea*	AY373293
Uncultured endophytic fungus	EF504576	Uncultured endophytic fungus	EF504366
Uncultured endophytic fungus	EF505438	Uncultured mycorrhiza	AY634132
Uncultured endophytic fungus	EF505583	Uncultured *Sebacinales*	EF127237
Basidiomycota		Plantae	
Cryptococcus anemochorus	DQ830986	*Bletilla striata*	AF273334
Cryptococcus arboriformis	AB260936	*Bletilla striata*	AF461466
Cryptococcus bhutanensis	EU266557	*Bletilla striata*	EU100762
Cryptococcus cellulolyticus	AF444442	*Coelogyne cristata*	AF302742
Cryptococcus diffluens	AF444374	*Coelogyne dayana*	AF281126
Cryptococcus dimennae	EU266559	*Coelogyne harana*	AF302749
Cryptococcus flavescens	AM176643	*Coelogyne plicatissima*	AF281125
Cryptococcus laurentii	AF410468	*Coelogyne rhabdobulbon*	AF281127
Cryptococcus rajasthanensis	AM262981	*Coelogyne veitchii*	AF302759
Dioszegia aurantiaca	EU266500	*Coelogyne virescens*	AF281122
Dioszegia crocea	AJ581078	*Diuris punctata*	DQ904024
Dioszegia fristingensis	EU070925	*Michelia chapensis*	DQ234270
Dioszegia hungarica	EU252552	*Phalaenopsis amabilis*	AY391519
Dioszegia takashimae	DQ003332	*Pleione albiflora*	AY101967
Epulorhiza sp.	EF393629	*Pleione bulbocodioides*	EU100770
Epulorhiza sp.	AJ31344	*Pleione chunii*	AY008471
Kwoniella mangroviensis	EF215528	*Pleione formosana*	EU100756
Piriformospora indica	AF019636	*Pleione grandiflora*	AF461476
Sebacina aff. epigaea	AF490393	*Pleione hookeriana*	AF461469
Sebacina allantoidea	AF490396	*Pleione pleionoides*	AF461480
Sebacina epigaea	AF490397	*Pleione x confusa*	AF461479
Pestalotiopsis olivacea	EF055215		

(Fig. 1a). The PCR products contained dif ferent ITS sequences of fungal taxa.PCR products with multiple bands were cloned into *E. coli*, DH5α. recombinants and a single ITS sequence of the fungus or plant was obtained (Fig. 1b). Random ITS clones were selected for further DGGE analysis (Fig. 1c). We obtained 203 clones and 211 clones from leaf tissues and root tissues respectively.

DGGE analysis

PCR products of ITS clones with additional 40-bp GC-rich sequences were subjected to DGGE to elucidate the diversity of fungal interspecies as well as intraspecies. All ITS clones of endophytic fungi from the leaf tissues grouped into 18 different ITS clone sequences (Fig. 2a) and 10 from the root tissues (Fig. 2b) with a total of 203 clones (leaf tissues) and 211 clones (root tissues) based on DGGE and sequence analysis. In addition, we also obtained 26 ITS clones and 25 ITS clones of *Bletilla ochracea* respectively from the leaf and root tissues. All ITS clones amplified from leaf and root tissues migrated in the range of 35% to 40% denaturant concentration (Fig. 2). In cases where ITS sequences had similar lengths, but large differences in G+C

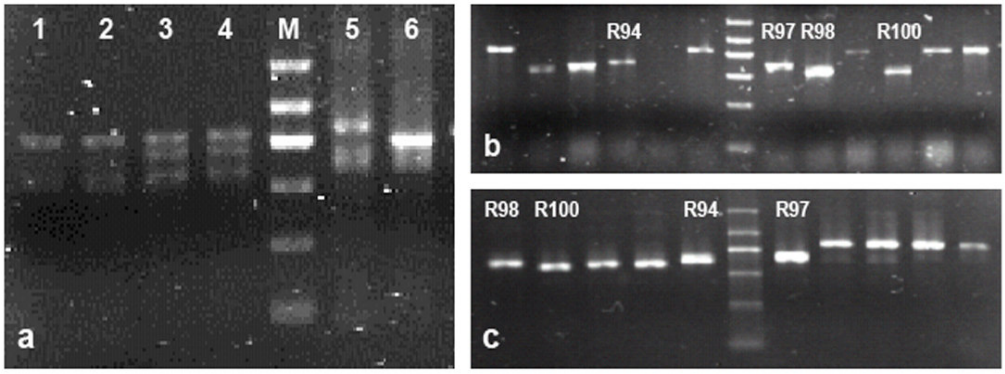

Fig. 1. Agarose electrophoresis profiles of ITS sequences amplified from genomic DNA extracted directly from leaves and roots of *Bletilla ochracea* (**a**), and ITS clones amplified by ITS1-ITS4 primers (**b**) from root tissues, and the same clones amplified by ITS1-ITS4-GC clamp primers for DGGE analysis (c). Lanes, **a**: 1, 2, ITS sequences of ITS1-ITS4 primers PCR from leaf tissues; 3, 4, ITS sequences of ITS1-ITS4 primers PCR from root tissues; 5, ITS sequences of ITS1F-ITS4 primers PCR from leaf tissues; 6, ITS sequences of ITS1F-ITS4 primers PCR from root tissues; Marker of **a, b, c**: 1200 bp, 900 bp, 700 bp, 500 bp, 300 bp, 100bp.

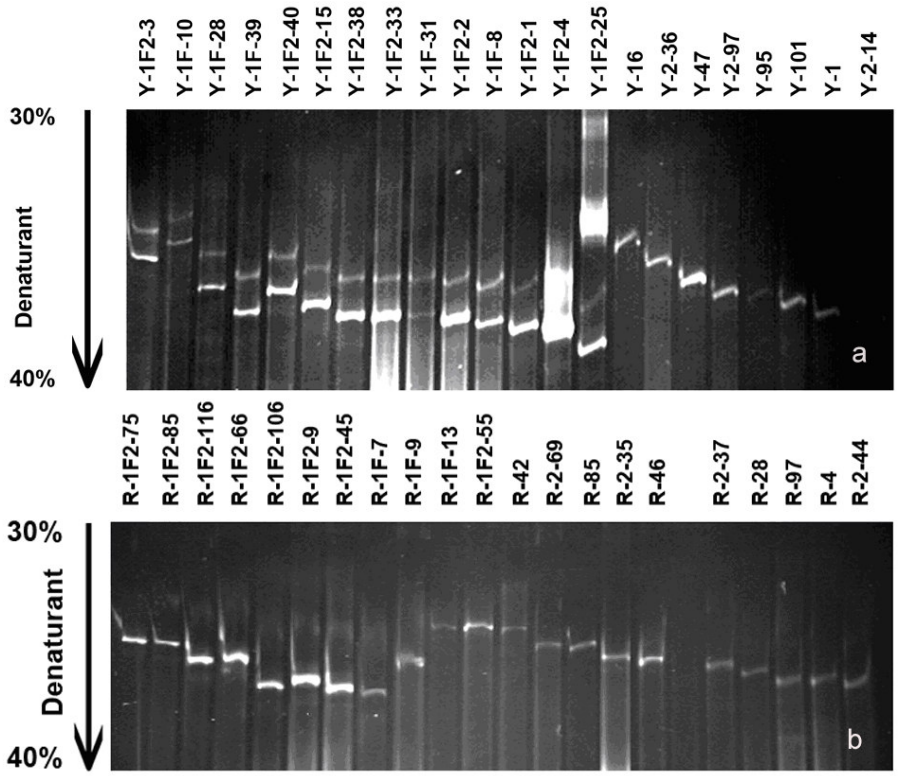

Fig. 2. DGGE profiles of ITS sequences including the 5.8S region of representative clones of different groups from leaf tissues (a) and root tissues (b) of *Bletilla ochracea*.

percentages, similar migration behaviors occurred on the agarose gel. However, these sequences can be easily distinguished from each other by DGGE. In this study, two ITS clones of Y-1F-8 and Y-1F-39 from leaf tissues and the same length, but they were different withonly 79.3% similarity (identities = 444/560). clones of Y-1F-8 and Y-1F-39 from leaf tissues ad the same length, but they were different with only 79.3% similarity (identities = 444/560). They could be separated by DGGE (Fig. 2a), however the

two ITS sequences had the same position on the agarose gel (data not shown).

Taxonomic placement of endophytic fungi from leaf tissues by sequence analysis

DGGE, sequence and phylogenetic analysis revealed 18 OTUs of endophytic fungi and one kind of plant clone from the leaf tissues.

Phylogenetic analysis of 5.8S gene sequences. In order to establish the general taxonomic placement, neighbor-joining phylogenetic trees of the 65 aligned 5.8S gene sequences (42 references and 23 clones) (Fig. 3) was constructed with 1000 bootstrap replications, and *Michelia chapensis*, a plant in the family of *Magnoliaceae*, was used as outgroup. The data resulted in two main clades

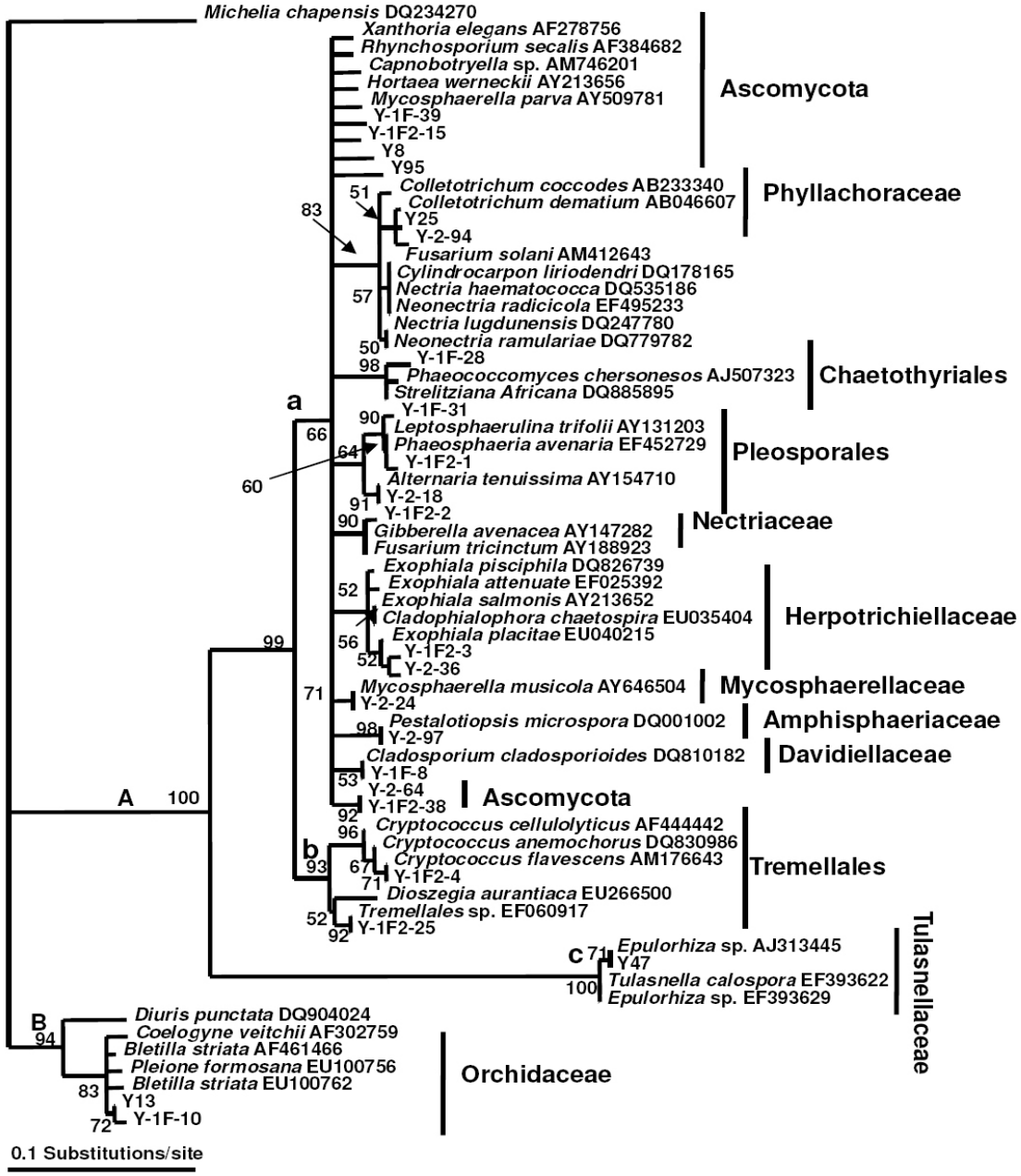

Fig. 3. Neighbor-joining phylogenetic tree showing the relationship between endophytic fungi and orchid plant from leaf tissues of *Bletilla ochracea* and related fungi and plants based on the sequences of 5.8S of rDNA. The tree was rooted with *Michelia chapensis* (DQ234270). Bootstrap values > 50% (1000 replicates) are shown at the branches.

the family of *Magnoliaceae*, was used as outgroup. The data resulted in two main clades (A and B), representing the fungal group and orchid plant group. Clade A contained three subclades (subclades Aa, Ab and Ac, with 66%, 93% and 100% bootstrap support values (BSV) respectively). These comprised the main fungal groups of Ascomycota and Basidiomycota. Subclade Aa of Clade A was a large group of Ascomycota including 20 clones. The highly conserved 5.8S gene could be used for identifying distant groups to family or higher

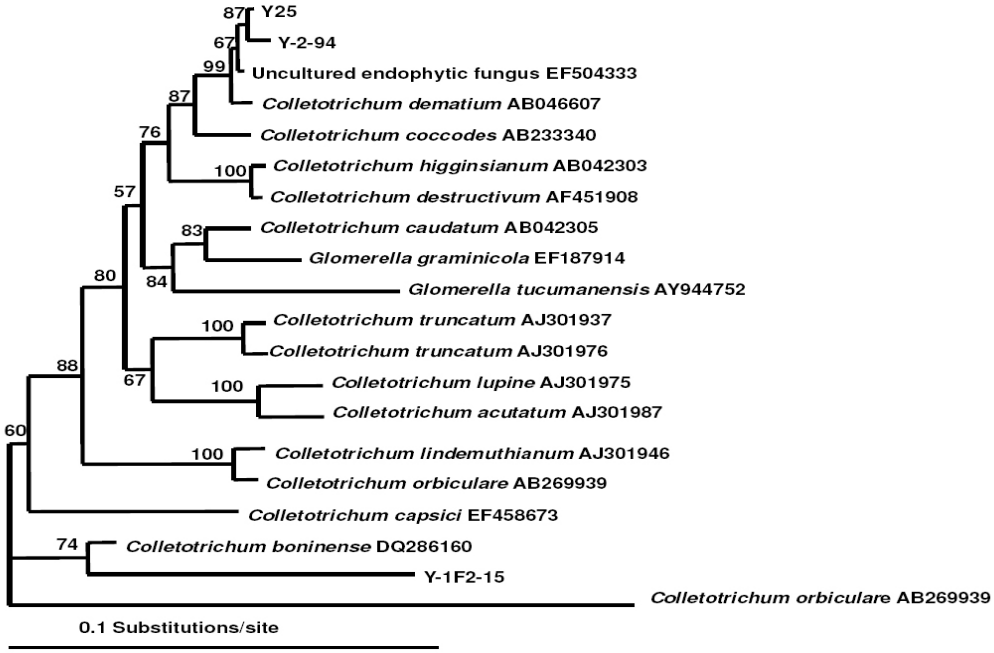

Fig. 4. Neighbor-joining phylogenetic tree showing the relationship between endophytic fungi and related fungi based on the sequences of 5.8S gene and ITS regions of nuclear rDNA. The tree was rooted with *Plectosphaerella cucumerina*. Bootstrap values > 50% (1000 replicates) are shown at the branches.

level. Among these clones, Y25 and Y-2-94, Y-1F2-2, Y-1F2-3 and Y-2-36, Y-2-24, Y-2-97, and Y-1F-8 clustered with *Phyllachoraceae*, *Nectriaceae*, *Herpotri-chiellaceae*, *Mycosphaerellaceae*, *Amphisphae-riaceae* and *Davidiellaceae*. Y-1F-28, Y-1F-31 and Y-1F2-1 clustered with *Chaetothyriales* and *Pleosporales*. However, Y8, Y95, Y-1F-39, Y-1F2-15, Y-2-64, Y-1F2-38 could only be placed at the higher taxonomic level of Asco-

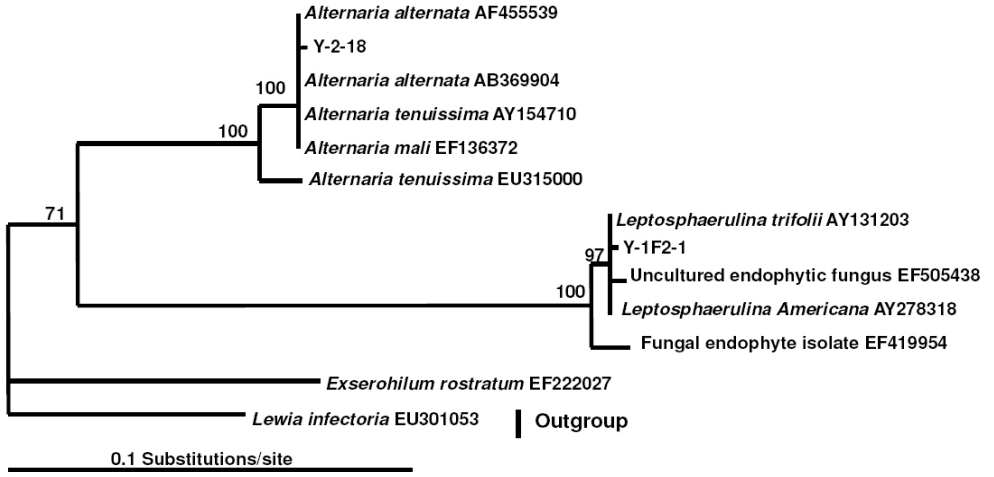

Fig. 5. Neighbor-joining phylogenetic tree showing the relationship between endophytic fungi and related fungi based on the sequences of ITS1-5.8S-ITS2 of rDNA. Bootstrap values > 50% (1000 replicates) are shown at the branches.

mycota because of lack of close phylogenetic taxa in this NJ tree. Subclade Ab including Y-1F2-4 and Y-1F2-25 was a main clade of Clade A, and consisted of species of *Tremellales* (93% BSV) of Basidiomycota. Y47 clustered with the species of *Tulasnellaceae* (Subclade Ac, 100% BSV) belonging to *Cantharellales*, a different order to the *Tremellales*. Subclade Ab was more closely related to subclade c than subclade Ac. Y13 and Y-1F-10 belonged to *Orchidaceae* in Clade B, and hence not within the scope of this study.

Phylogenetic analysis of ITS regions. The ITS regions (ITS1-5.8S-ITS2) were used to further identify these fungi.

Phyllachorales. Y25 and Y-2-94, Y-1F2-15 were identified as *Phyllachoraceae* and other Ascomycota in 5.8S NJ tree (Fig. 3), and the NJ tree based on ITS regions of the 20 aligned sequences indicated that Y25 and Y-2-94 belonged to *Colletotrichum* (*Phyllachoraceae*) (Fig. 4). Because the ITS regions of Y25 and Y-2-94 were the same size with 99.3% similarity (identities = 573/577), Y25 and Y-2-94 were only slightly variable within 1 OTU. The clone of Y-1F2-15 could be a species of *Colletotrichum* as it clustered with *C. boninense* (DQ286160) with 74% BSV, but

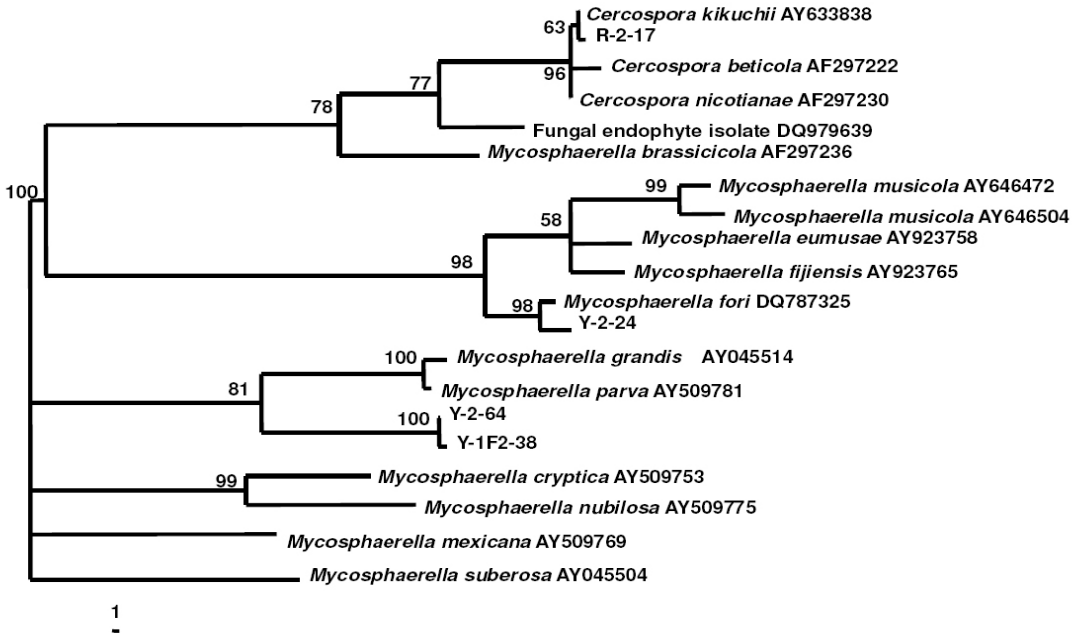

Fig. 6. Maximum-parsimony tree showing the relationship between endophytic fungi and related fungi based on the sequences of ITS1-5.8S-ITS2 of rDNA. The tree was rooted with *Mycosphaerella suberosa* (TL=375, CI=0.6800, HI=0.3200, RI=0.7853, RC=0.5340). Bootstrap values > 50% (1000 replicates) are shown at the branches.

interestingly it had a distant phylogenetic relationship with Y25 and Y-2-94.

Pleosporales. Y-2-18 and Y-1F2-1 were placed in genus *Alternaria* and *Leptosphaerulina* within *Pleosporaceae* (*Pleosporales*), because they clustered with the species of these genus with the strong BSV (100% and 97%) (Fig. 5). In Fig. 9, Y-1F-31 clustered with 6 species of genus *Phaeosphaeria* within *Phaeosphaeriaceae* (*Pleosporales*) and other 5 references, and was identified to genus *Phaeosphaeria*.

Capnodiales. Further analysis of the taxonomic levels of Y-2-24, Y-1F2-38 and Y-2-64 based on the maximum-parsimony tree was shown in Fig. 6, Y-2-24 was in a subclade clustered with *Mycosphaerella*, belonged to the family *Mycosphaerellaceae* (*Capnodiales*), species with a 98% BSV, and was formed a terminal cluster with *M. fori* with a 98% BSV and 98.5% sequence similarity (identities =530/538). Y-1F2-38 and Y-2-64 clustered in the other subclade with *Mycosphaerella* species with a 81% BSV, but had distant relationship with Y-2-24. Because Y-1F2-38 and Y-2-64 had the same size of ITS regions with 99.8% similarity (identities = 555/556), they both were just one single OTU. Y-1F-8 had been identified to *Davidiellaceae* (*Capnodiales*) according to 5.8S sequence, and

combined with sequences of ITS regions (Fig. 8) to further reveal that Y-1F-8 was a species of *Cladosporium* (*Davidiellaceae*), and is very close relationship with *C. cladosporioides* (DQ810182) of highly ITS similarity of 100% (identities = 551/551).

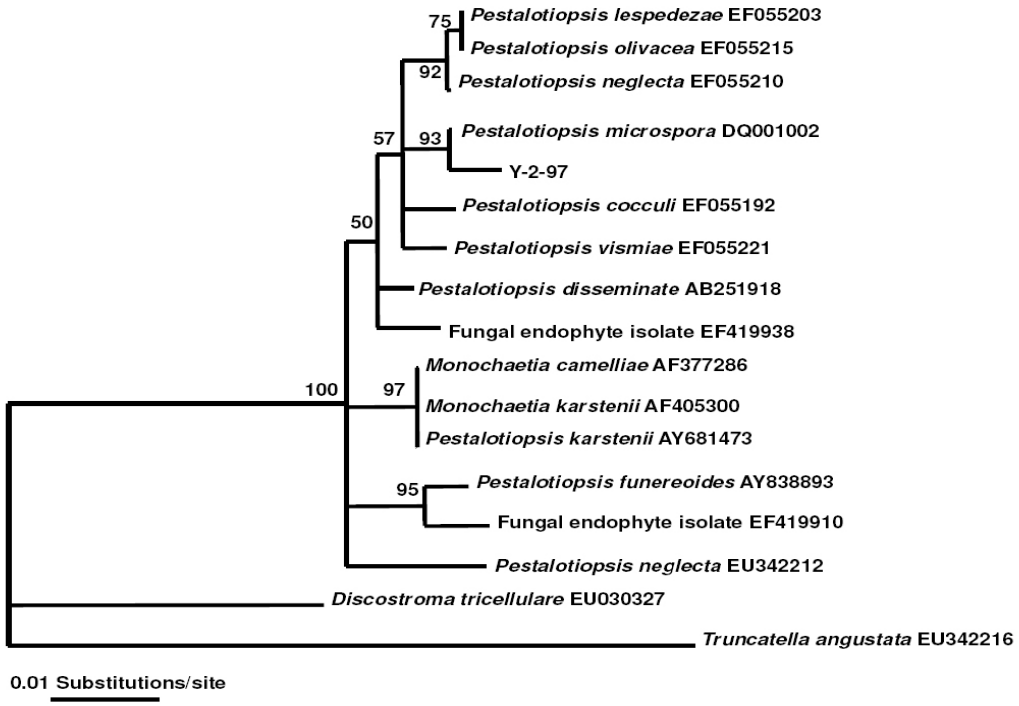

Fig. 7. Neighbor-joining phylogenetic tree showing the relationship between endophytic fungi and related fungi based on the sequences of ITS1-5.8S-ITS4. The tree was rooted with *Truncatella angustata*. Bootstrap values > 50% (1000 replicates) are shown at the branches.

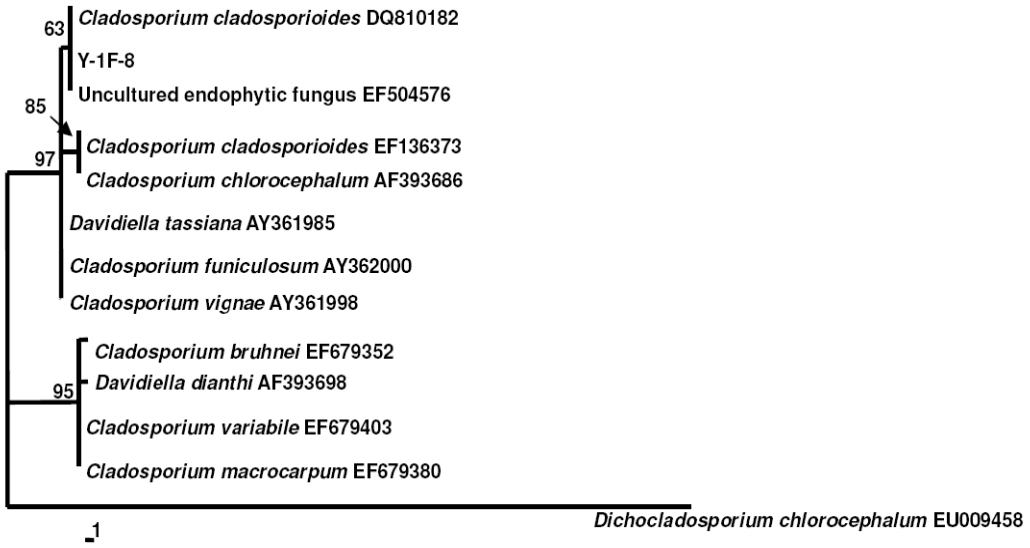

Fig. 8. Maximum-parsimony tree showing the relationship between endophytic fungi and references based on the sequences of ITS1-5.8S-ITS2 of nuclear rDNA. The tree was rooted with *Dichocladosporium chlorocephalum* (TL=96, CI=1.0000, HI=0.0000, RI=1.0000, RC=1.0000). Bootstrap values > 50% (1000 replicates) are shown at the branches.

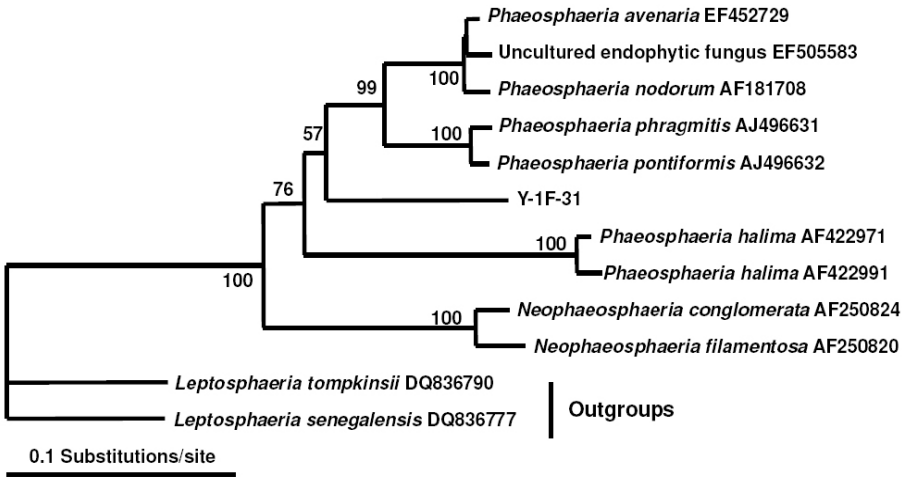

Fig. 9. Neighbor-joining phylogenetic tree showing the relationship between endophytic fungi and related fungi based on the sequences of ITS1-5.8S-ITS2 of rDNA. Bootstrap values > 50% (1000 replicates) are shown at branches.

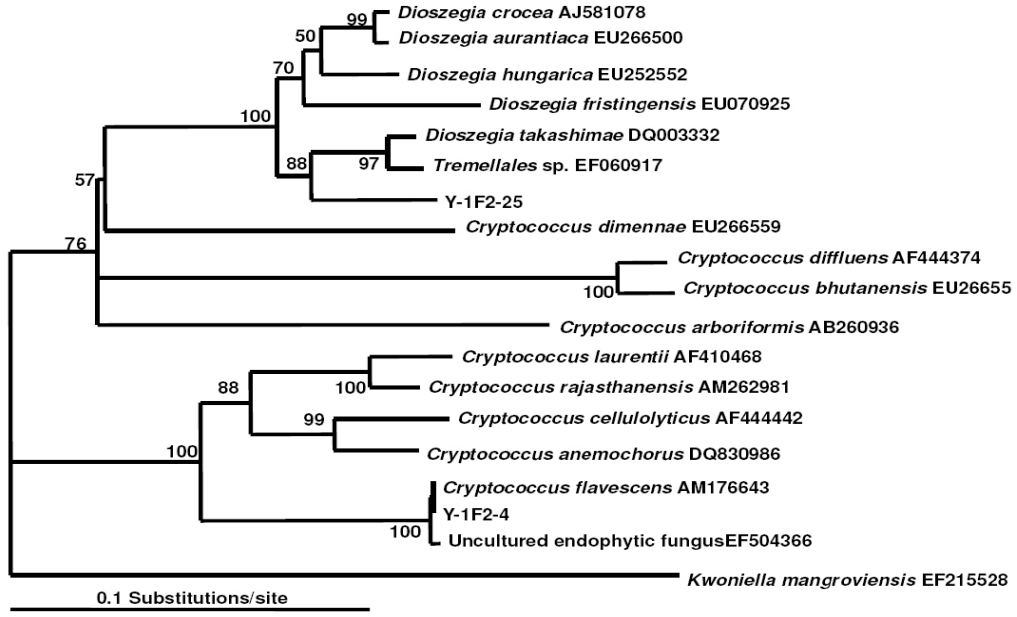

Fig. 10. Neighbor-joining phylogenetic tree showing the relationship between endophytic fungi and related fungi based on the sequences of ITS1-5.8S-ITS2 of rDNA. The tree was rooted with *Kwoniella mangroviensis*. Bootstrap values > 50% (1000 replicates) are shown at the branches.

Amphisphaeriaceae. Y-2-97 belonged to the family *Amphisphaeriaceae* (*Xylariales*) (Fig. 3), and further analyses showed that Y-2-97 belonged to the genus *Pestalotiopsis* (Fig. 7). Y-2-97 was closely related to *P. microspora* with a 93% BSV and 99.5% similarity (identities = 603/606).

Nectriaceae. Y-1F2-2 together with some clones from root tissues were further identified to the species of *Gibberella* and its anamorph of *Fusarium* of *Nectriaceae* (*Hypocreales*) (Fig. 13), and Y-1F2-2 clustered with *G. avenacea* with 99% BSV and 99.8% similarity (identities = 560/561).

Tulasnellaceae. Y47 belonged to *Tulasnellaceae* (*Cantharellales*) of Basidiomycota (Fig. 3), and further identifications based on ITS regions showed that Y47 was species of genus *Epulorhiza* (Fig. 11).

Tremellales. Y-1F2-25 and Y-1F2-4 belonged to *Tremellales* of Basidiomycota(Fig. 3), and further identified to the species of

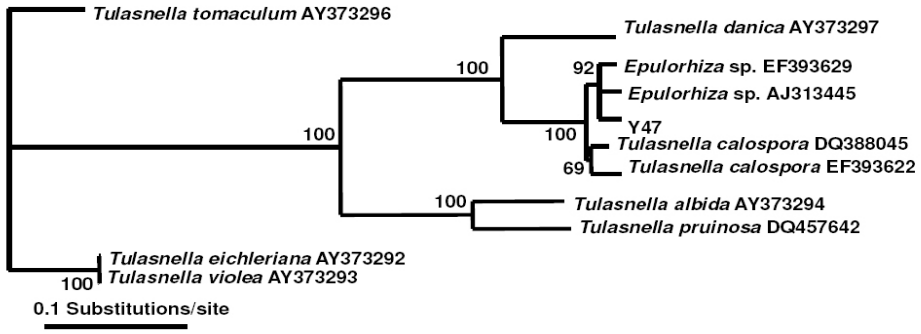

Fig. 11. Neighbor-joining phylogenetic tree showing the relationship between endophytic fungi and related fungi based on the sequences of ITS1-5.8S-ITS2 of rDNA. The tree was rooted with *Tulasnella tomaculum*. Bootstrap values > 50% (1000 replicates) are shown at the branches.

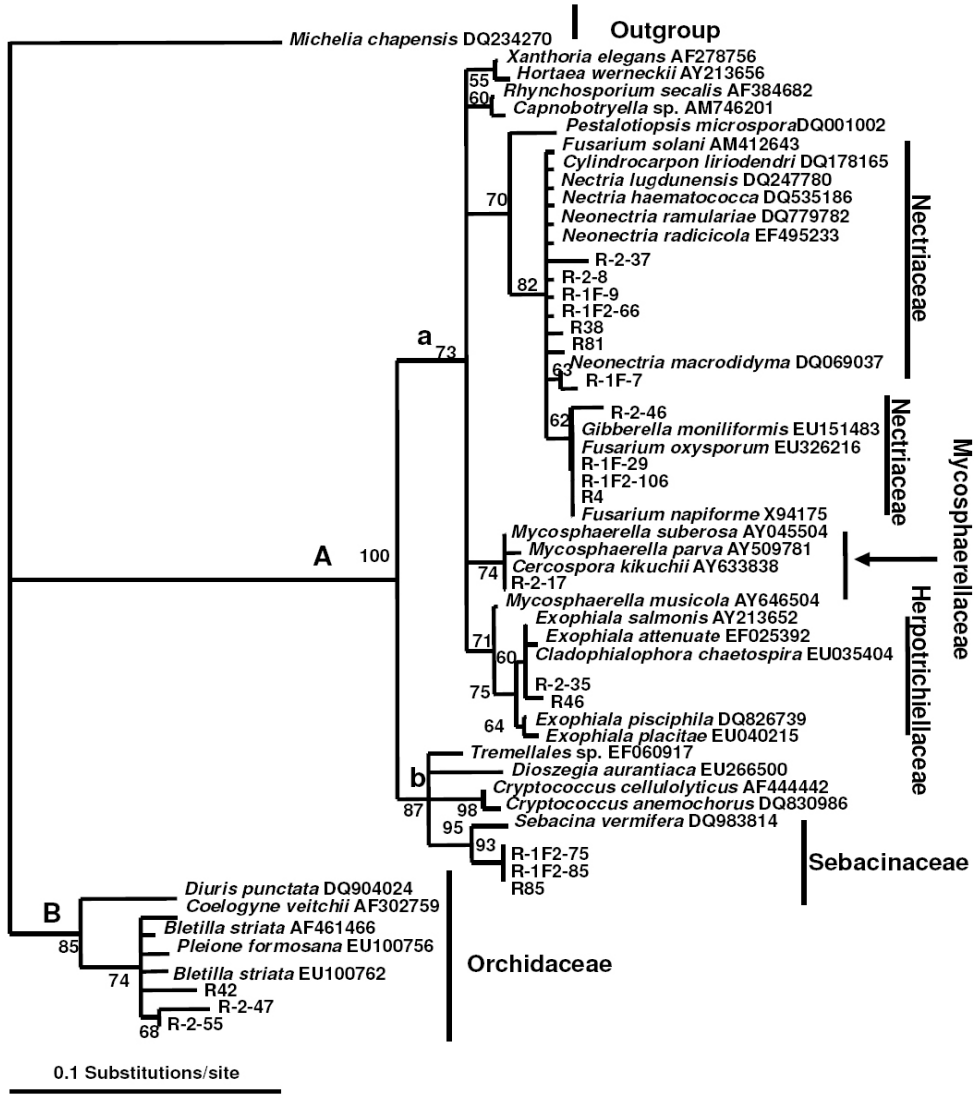

Fig. 12. Neighbor-joining phylogenetic tree showing the relationship between endophytic fungi from root tissues of *Bletilla ochracea*, orchid plant and references based on the sequences of 5.8S of rDNA. Bootstrap values > 50% (1000 replicates) are showed at the branches. Accession numbers of GenBank nucleotide database are given for all sequences.

genus *Dioszegia* and *Cryptococcus*, respectively from the NJ tree (Fig.10).Y-1F2-25 had a distant phylogenetic relationship with other species of *Dioszegia*, Y-1F2-4, however, had a very strong BSV (100%) and 100% sequence similarity (identities = 529/529) in its subclade with *Cryptococcus flavescens* (Fig. 10).

Chaetothyriales. Y-1F2-3 and Y-2-36 were placed at family *Herpotrichiellaceae* (*Chaetothyriales*) (Fig. 14), although Y-1F2-3 and Y-2-36 were grouped with species of

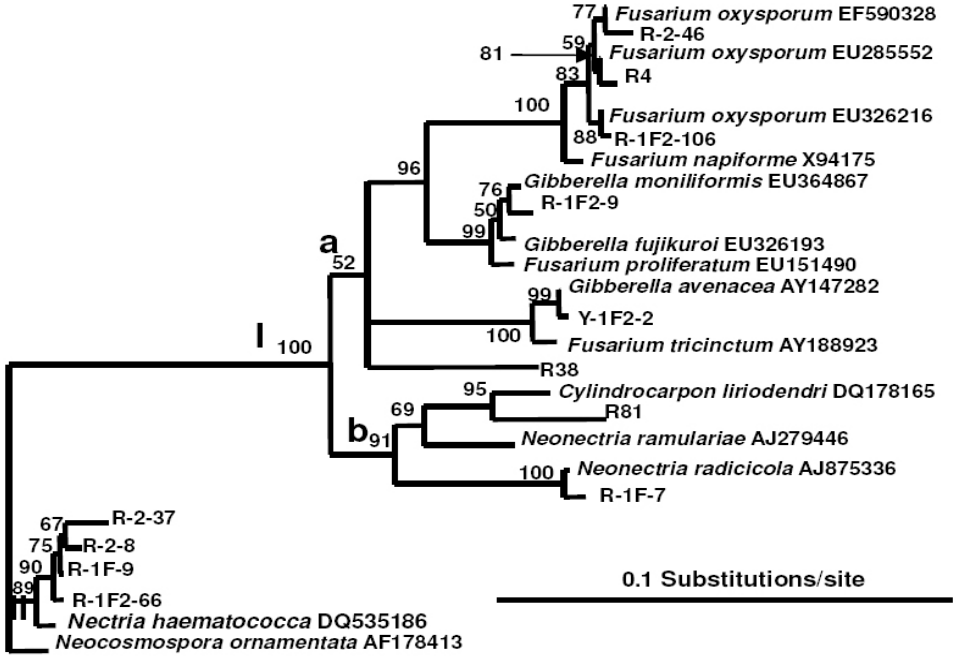

Fig. 13. Neighbor-joining phylogenetic tree showing the relationship between endophytic fungi and related fungi based on ITS1, 5.8S and ITS2 sequences. The tree was rooted with *Neocosmospora ornamentata*. Bootstrap values > 50% (1000 replicates) are shown at the branches.

Phaeococcomyces with 92% BSV, they could not form a terminal cluster with them. And because Y-1F2-3 and Y-2-36 had higher sequence similarity of 99.4% (identities = 681/685), we regarded Y25 and Y-2-94 as one OTU.

Based on phylogenetic analysis of 5.8S in Fig. 3, Y-1F-28, Y-1F-39, Y8 and Y95 were identified to the order or higher levels. And because there are not enough molecular data in GenBank to construct phylogenetic trees, these clones could not be identified to a lower taxonomic level.

As a result, we combined the 5.8S gene and the ITS regions to identify the fungal ITS clones from the leaf tissues of *Bletilla ochracea* by phylogenetic analysis to 18 taxa of genus or higher level listed in Table 2.

Taxonomic placement of endophytic fungi from root tissues by sequence analysis

On the basis of DGGE and phylogenetic analysis of ITS regions, 10 OTUs of endophytic fungi and one kind of plant clone sequence from the root tissues were grouped.

Phylogenetic analysis of sequences based on 5.8S gene. Phylogeny generated from 5.8S gene sequences resulted in two main clades of A and B with 100% and 85% BSV (Fig. 12), respectively, representing fungal group and plant group of orchid. Clade A includes two subclades (Aa and Ab, 73% and 87% BSV) of large fungal groups of Ascomycota and Basidiomycota. Subclade Aa was a group of Ascomycota including 14 clones.

Among these clones, R-2-8, R-2-37, R-1F-9, R-1F2-66, R38, R81, R-1F-7, R4, R-2-46, R-1F2-106, R-1F2-9 clustered with the family *Nectriaceae*, and R-2-17, R-2-35, R46 were placed at the family *Mycosphaerellaceae* and *Herpotrichiellaceae*, respectively. Subclade Ab including R85, R-1F2-75 and R-1F2-

85 was consisted of species in *Tremellales* of Basidiomycota. R85, R-1F2-75 and R-1F2-85 were formed a terminal cluster with the *Sebacinaceae* (95% BSV). R42, R-2-47 and R-2-55 belonged to *Orchidaceae* plant in Clade B, and not within the scope of this study.

Phylogenetic analysis of sequences based on ITS regions. The diversity of these fungal clones was further investigated based on ITS regions. The phylogeny revealed two main clades (I, II) with 100% and 89% BSV (Fig. 13), containing sequences from *Neonectria* and its anamorph *Cylindrocarpon*, *Fusarium* and its telemorphs of *Gibberella* and *Nectria*, and fungal clones from root tissues.

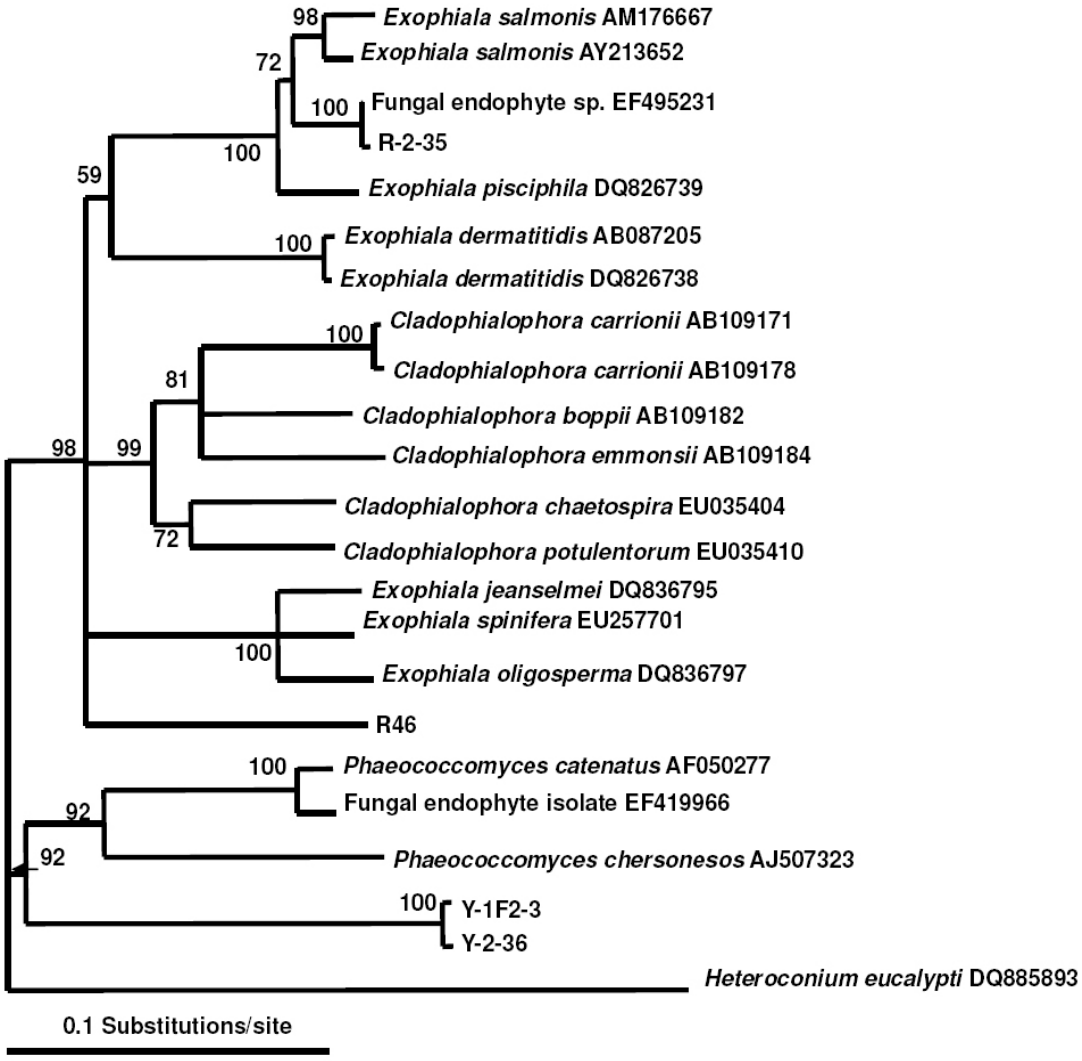

Fig. 14. Neighbor-joining phylogenetic tree showing the relationship between endophytic fungi and related fungi based on ITS1, 5.8S, ITS2 sequences. The tree was rooted with *Heteroconium eucalypti*. Bootstrap values > 50% (1000 replicates) are shown at the branches.

Clade I included subclade Ia consisting of fungal clones of R4, R-2-46, R-1F2-106, R-1F2-9, and R38 and subclade Ib consisting of R81 and R-1F-7. Fungal clones from subclade Ia grouped together with reference species of *Fusarium* and its telemorph *Gibberella* (*Nectriaceae*). Among these clones, R4, R-2-46 and R-1F2-106 showed 98.7-98.9% sequence similarity (identities = 537/544-538/544) among them, so we regarded them as one OUT. R-1F2-9 was placed in a terminal group with *Gibberella moniliformis* and showed high

sequence similarity (99.3%, identities = 554/558). R38 had a distant relationship with other clones because of forming a single cluster with no any references in this group. However, clones from subclade Ib, R81, clustered with *Cylindrocarpon liriodendri*, and R-1F-7, clustered with *Neonectria radicicola*, were supported with high bootstrap values (Fig. 13).

In clade II, fungal clones of R-2-37, R-2-8, R-1F-9 and R-1F2-66 were regarded as a single OTU of genus *Nectria* (*Nectriaceae*) since they all clustered with *Nectria haematococca* (89% BSV) and had 98.6-99.6% sequence similarity (identities = 564/572-570/572) among them (Fig. 13).

In the Fig. 14, R-2-35 and R46 clustered with 15 references of species of family *Herpotrichiellaceae*. R-2-35 was identified to genus *Exophiala* on the basis of 100% BSV with species of *Exophiala* in the subcluster. R46 was placed at family level of *Herpotrichiellaceae* because no references formed the terminal cluster with it, and had distant relationship with *Exophiala* and *Cladophialophora*. R-2-17 was further identified to genus *Cercospora*, anamorph of *Mycosphaerella* (*Mycosphaerellaceae*) based on phylogentic analysis in Fig. 6, and it was formed a subclade with *Cercospora kikuchii* (AY633838) with 99.8% similarity (identities = 535/536).

Further analysis of R85, R-1F2-75 and R-1F2-85 based on the maximum parsimony tree indicated they all had strong similarities to each other (99.5-99.7%) and all clustered with *Sebacina* species (Fig. 15). They were the same OUT of *Sebacina* (*Sebacinaceae*) in *Sebacinales* of Basidiomycota. However, interestingly, they were not closely claded with *Sebacina vermifera* which is the classic Orchid endophyte (Milligan and Williams, 1988).

As a result, we identified the fungal clones within the roots based on 5.8S gene and the ITS regions to 10 taxa of genus or family level listed in Table 3.

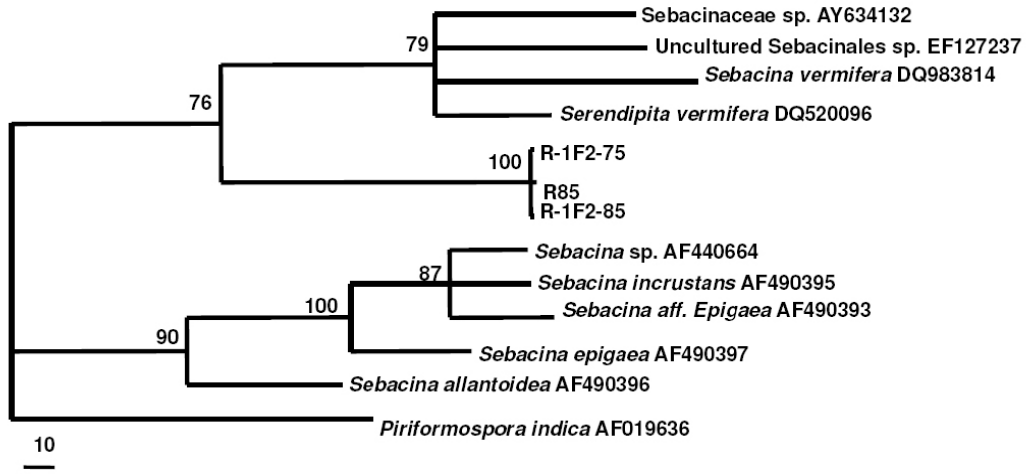

Fig. 15. Maximum-parsimony tree showing the relationship between endophytic fungi from roots and related fungi based on the sequences of ITS1-5.8S-ITS2. The tree was rooted with *Piriformospora indica* (TL=931, CI=0.6853, HI=0.3147, RI=0.6601, RC=0.4524). Bootstrap values > 50% (1000 replicates) are shown at the branches.

Fungal community composition and fungal diversity

All the ITS clones were grouped into 18 OTUs of endophytic fungi from leaf tissues (Table 2) and 10 OTUs from root tissues (Table 3) by DGGE and phylogentic analysis.

Within the leaves, the groups of endophytic fungi were consisted of Ascomycota (91% to total clones) and Basidiomycota (9%). Of all the 18 OTUs, 15 taxa belonged to Ascomycota, and the other 3 taxa of Basidiomycota. The 15 taxa of Ascomycota included 2 taxa of *Mycosphaerella* (41%) in *Mycosphaerellaceae*, 2 taxa of *Alternaria* (9%) and *Leptosphaerulina* (5.7%) in *Pleosporaceae*, 2 taxa of *Colletotrichum* (6.2%) in *Phyllachoraceae*, 1 taxon of *Gibberella* (4.5%) in *Nectriaceae*, 3 taxa of lower proportion (3.4%, sum to total) of *Cladosporium*, *Pestalotiopsis* and *Phaeosphaeria*, 2 taxa of

order *Chaetothyriales* (5.1%) and 3 taxa of not identifying to lower level in Ascomycota (15.8%). The 3 taxa of Basidiomycota consisted of 2 yeast taxa of *Dioszegia* and *Cryptococcus* of order *Tremellales* (7.9% to total clones) and 1 mycorrhizal fungus of *Epulorhiza* (1.1%). Of all the 18 taxa, 2 taxa of *Mycosphaerella* were the dominant species within the leaves and Ascomycota sp.2 and *Alternaria* sp. were also main groups.

Correspondingly, the 10 fungal taxa within roots contained 9 taxa of Ascomycota (54%) and 1 taxon of Basidiomycota (46%). The 9 taxa of Ascomycota consisted of 6 taxa of *Fusarium* (30.7%) and its telemorph *Gibberella* (4.3%), *Nectria* (13.4%), *Neonectria* (1.1%) and *Cylindrocarpon* (0.5%) in *Nectriaceae*, 2 taxa of family *Herpotrichiellaceae* (2.2%) and 1 taxon of *Cercospora* (1.6%) in *Mycosphaerellaceae*. Of the 9 taxa, 2 taxa of *Fusarium* were dominant species, and species of family *Nectriaceae* were the main groups within root tissues. The only 1 taxon of *Sebacina* in Basidiomycota from roots was also the dominant species with 46% proportion to total.

Table 2. Fungal diversity within leaf tissues.

OTU	Taxon	No. of clones	Proportion to total
Y-2-18	*Alternaria* sp.	16	9.04%
Y-1F-39	Ascomycete sp.1	3	1.70%
Y8	Ascomycete sp.2	24	13.56%
Y95	Ascomycete sp.3	1	0.57%
Y-1F-28	*Chaetothyriales* sp.	7	3.96%
Y-1F-8	*Cladosporium* sp.	2	1.13%
Y-1F2-15	*Colletotrichum* sp.1	7	3.96%
Y25,Y-2-94	*Colletotrichum* sp.2	4	2.26%
Y-1F2-4	*Cryptococcus* sp.	4	2.26%
Y-1F2-25	*Dioszegia* sp.	10	5.65%
Y47	*Epulorhiza* sp.	2	1.13%
Y-1F2-2	*Gibberella* sp.1	8	4.52%
Y-1F2-3,Y-2-36	*Herpotrichiellaceae* sp.1	2	1.13%
Y-1F2-1	*Leptosphaerulina* sp.	10	5.65%
Y-2-24	*Mycosphaerella* sp.1	50	28.25%
Y-1F2-38, Y-2-64	*Mycosphaerella* sp. 2	23	12.99%
Y-2-97	*Pestalotiopsis* sp.	2	1.13%
Y-1F-31	*Phaeosphaeria* sp.	2	1.13%

Table 3. Fungal diversity within root tissues.

OTU	Taxon	No. of clones	Proportion to total
R-2-17	*Cercospora* sp.	3	1.61%
R81	*Cylindrocarpon* sp.	1	0.54%
R-2-35	*Exophiala* sp.	2	1.08%
R-2-37, R-2-8,R-1F-9, R-1F2-66	*Nectria* sp.	25	13.44%
R38	*Fusarium* sp.1	19	10.22%
R4,R-2-46, R-1F2-106	*Fusarium* sp.2	38	20.43%
R-1F2-9	*Gibberella* sp.2	8	4.30%
R46	*Herpotrichiellaceae* sp.2	2	1.08%
R-1F-7	*Neonectria* sp.	2	1.08%
R-1F2-75,R-1F2-85, R85	*Sebacina* sp.	86	46.24%

A broad fungal spectrum above showed very high diversity within leaves and roots. The Shannon-Weiner diversity index (H′) of fungi within leaves and roots were 2.354 and 1.560, respectively. The results indicated that fungal diversity in leaf tissues were higher than that in root tissues, and also revealed that fungal communities within leaves and roots were significantly different to each other

Discussion

This is the first report on the fungal diversity and their phylogenetic relationships within leaves and roots of terrestrial orchids in China using combined molecular methods especially DGGE.

Comparison of fungal communities with previous studies.

The mycorrhizal fungi (*Sebacina* sp., *Epulorhiza* sp.), and some dominant species of Ascomycota, non-mycorrhizal fungi, *e.g*. *Mycosphaeella* (41%), *Alternaria* (9%) in leaves, *Fusarium* (30.7%) and its telemorph *Gibberella* (4.3%), *Nectria* (13.4%) in roots were detected in this study. Several studies had revealed diversity of endophytic fungal communities, including mycorrhizal fungi and non-mycorrhizal fungi in tropical orchid plants (Bayman *et al*., 1997; McCormick *et al*., 2004; Porras-Alfaro and Bayman, 2007; Richardson and Currah, 1995). These studies however revealed the presence of limited fungal communities within tropical orchids as they used traditional isolation methods only (see Hyde and Soytong, 2007; Shefferson *et al*., 2008). Bayman *et al*. (1997) isolated endophytic fungi within leaf and root tissues of epiphytic *Lepanthes* plants. Comparing with them, we observed a higher diversity and significant differences within leaf and root tissues of adult plants in our present study. To date, little is known of roles which these fungi play in distribution, population size, and genetic diversity of orchid plants (Bayman *et al*., 2008), especially of the genus *Bletilla*. and hence the studies of the only mycorrhizal fungi or special groups of endophytes for diversity maybe miss critical fungi for orchid establishment. So it is necessary to wholely investigate on fungal communities within plant.

Are fungi within Orchid tissues specialists or generalists.

Previous studies concerning host specificity of orchid mycorrhizae using *in vitro* and *in situ* approaches have often lead to conflicting results (Masuhara and Katsuya, 1994). However, this confusion may be because isolation techniques are inherently biased by choice of and response to growth medium (Allen *et al*., 2003; McCormick *et al*., 2004). Some studies have shown that mycorrhizal are often host specific in nonphotosynthetic and photosynthetic orchids (Otero *et al*., 2007; Shefferson *et al*., 2005; Taylor *et al.,* 2003). McCormick *et al*. (2004) found unrelated photosynthetic orchids to support a range of mycorrhizal fungi; some were specific to hosts while others were not. Otero *et al*. (2002, 2004) studied mycorrhizal associations of some tropical epiphytic orchids and found they comprised generalists. On the other hand, mycorrhizal fungi have been found in a wide variety of orchid species around the world (McCormick *et al*., 2000; Otero *et al*., 2002; Warcup, 1981). The mycorrhizae present may also change during the development of individual plants of some orchids. In *Gastrodia ellata*, *Mycena osmundicola* was mycorrhizal in the protocorm stage but was replaced by *Armillaria mellea* in subsequent stages (Xu and Mu, 1990). In the present study, we detected only 1 species of mycorrhizae (*Sebacina* sp.) within roots, and this was the dominant species as found within *Caladenia carnea* (Bougoure *et al*., 2005), hence *Sebacina* sp. seems to be a mycorrhizae which is specific to *Bletilla ochracea*. We also isolated many other dominant species of non-mycorrhizal fungi (shown in Table 2 and 3). These taxa cannot be ignored even though we do not know their roles in mycorrhizal ecology. So the facts above implied that fungal specificity to orchids may be narrow and temporary during their special life stages, and fungal diversity through their whole life cycles is universe and affected by the factors of field sites and environment, even different host plants.

Fungal diversities within leaves and roots.

It was surprising that in this study communities of mycorrhizal and non-mycorrhizal fungi of leaf and root tissues differed significantly. It was also surprising that we also found a mycorrhizal fungus (*Epulorhiza* sp.) within the leaves. The 10 OTUs extracted from roots consisted of of one OTU of *Sebacina* (*Sebacinales*, Basidio- mycota), but also six OTUs of *Nectriaceae* (*Hypocreales*) and 3 OTUs of 2 families of *Mycosphaerellaceae* and *Herpotrichiellaceae*. The fungal diversity within leaves (18 OTUs) were higher than within the roots, and the 18 OUTs were

distributed amongst 9 different orders (*Capnodiales* (3 OTUs), *Cantharellales*, *Chaethyriales*, *Chaetothyriales*, *Hypocreales*, *Phyllachorales*, *Pleosporales*, *Tremellales* and *Xylariales*) of Ascomycota, 3 OTUs of Ascomycete, whose phylogenetic placement could not be resolved and 3 OUTs of Basidiomycota. Bayman *et al.* (1997) used traditional isolation techniques and found that *Xylaria* spp. (*Xylariales*) and *Rhizoctonia*-like taxa (Basidiomycota) comprised the majority of endophytes within epiphytic orchids, and the fungal communities within the leaves and roots were surprising similar.

Comparisons of methodology with previous studies

DGGE has been used extensively for examination of fungal communities in different ecological systems such as grass, wheat, wood, soil (Smit *et al*., 1999; van Elsas *et al*., 2000; Vainio and Hantula, 2000) by using PCR amplification of the nuclear ribosomal RNA genes. In comparison with the more conserved coding regions of the rRNA genes, *e.g*. SSU rDNA and LSU rDNA, the variable ribosomal DNA (rDNA) internally transcribed spacer (ITS) regions generally provide greater taxonomic resolution (Anderson *et al*., 2003; Lord *et al*., 2002). Additionally, ITS data are considered useful for the relative ease with which ITS data can be recovered and the abundance of ITS data in GenBank (21,075 fungal ITS sequences before 2004, Lutzoni *et al*., 2004). There have been studies of endophytes using similar approaches. Guo *et al*. (2001) and Duong *et al*. (2006) studied endophyte disversity within plants using molecular approaches. Guo *et al*. (2001) developed a technique using direct amplification of ITS sequences extracted from frond tissues of *Livistona chinensis* followed by cloning, sequencing and phylogenetic analysis to identify endophytic fungi, however, they only obtained 6 phylotypes. The most common endophytic taxa occurring in *Livistona chinensis*, such as *Guignardia*, *Pseudospiropes* and *Xylaria* species (Guo *et al*., 2000), however, were not detected. Duong *et al*. (2006) used a molecular method based on DGGE coupled with sequence analysis of the 18S rRNA gene to assess fungal diversity within leaves of *Magnolia liliifera*, and recovered 14 OTUs distributed among 6 different orders and 2 unknown taxa. This method, however, failed to reveal any *Xylaria* species, the common endophytes from leaves of most plants (Arnold *et al*., 2003, 2007). These molecular methods can overcome the main limitations of previous studies of possibly unculturable or slow growing fungi on artificial media. Duong *et al*. (2006) could detect more abundant endophytes and higher diversities than that of Guo *et al*. (2001). In our study, we used random cloning, combining with DGGE and phylogenetic analysis to investigate the fungal communities within roots and leaves. In the step of random cloning, we can obtained plentiful ITS clones of endophytes, and used DGGE to group the different ITS sequences of different G+C% even if they are the same length, so we can obtain the more abundant fungal information (data shown in results) within the plant than that of previous studies (Guo *et al*., 2001; Duong *et al*., 2006).

This study has significance for orchid biology. The population size of *Bletilla ochracea* is decreasing and is at risk of extinction because of our human activities. It is possible that availability of endophytic fungi is one of the limiting factors for establishment of new plants and populations. So further research should elaborate on the possible connections between endophytes and plants within single organs, among organs of a single plant, and possibly among host species of different field sites.

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