DGGE coupled with ribosomal DNA gene phylogenies reveal uncharacterized fungal phylotypes

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Most fungal diversity studies have previously been based on morphological examination and cultivation methods. In this study we use a molecular method based on DGGE coupled with sequence analysis of 18S rRNA gene to assess fungal diversity on leaves of *Magnolia liliifera*. To achieve this, we extracted total genomic DNA and used fungal specific primers (NS1 and GCFung) to obtain fungal sequences. PCR-DGGE analysis recovered 14 operational taxonomic units (OTU) from different parts of the studied leaves. Phylogenetically, 8 OTUs belonged to the order *Pleosporales* and other bitunicate ascomycetes; 2 and 3 were related to the Xylariaceae, (*Xylariales*) and *Hypocreales*, respectively; 1 OTU was phylogenetically affiliated with the *Rhytismatales*. While this molecular approach identified taxa that were not recovered from morphological or cultural studies, it did not detect other taxa that were predominantly isolated using traditional methods. The three different parts of one leaf tested (petioles and midribs, leaf blade lower and upper parts) yielded different fungal taxa that possible indicate tissue-recurrence. The findings are compared with previous studies on the same host where endophytes were investigated using traditional culturing techniques.

Key words: DGGE, endophytes, fungal diversity, leaf fungi, phylogeny, rDNA, unculturable fungi

Introduction

Plant associated fungi are highly diverse, with saprobes, endophytes and pathogens occurring in all plant species examined, e.g *Proteaceae* (Lee *et al*., 2005), *Magnolia liliifera* (Promputtha *et al*., 2005a), grasses (Bacon and White, 1994), palms (Fröhlich *et al*., 2000; Rodrigues, 1996; Taylor *et al*., 1999), banana (Brown *et al*., 1998; Photita *et al*., 2004a,b) and mangroves (Suryanarayanan and Kumaresan, 2000). Fungal endophytes live inside plants for all or part of their life cycle without causing any disease symptoms or tissue

damage (Wilson, 1995). The endophytes may also become saprobes when the leaves senesce (Boddy and Griffith, 1989; Petrini, 1991; Photita *et al*., 2004a,b). De Bary (1866) was the first to observe endophytes and ever since then they have been widely studied (Petrini, 1991; Photita *et al*., 2004a,b; Suryanarayanan and Thennarasan, 2004; Vettraino *et al*., 2005). Endophytic fungi play important roles in plant life, forming symbiotic associations, facilitating nutrient cycling between plant and fungus, enhancing plant growth, increasing resistance and producing toxins that protect plants from animal and insect herbivores (Bultman and Murphy, 2000; Clay, 1987).

Endophytic fungi have previously been identified based on morphological characters from sporulating isolates on artificial media (Guo *et al*., 1998, 2003; Taylor *et al*., 1999). The fungi that do not sporulate on media have been termed mycelia sterilia and often been grouped as morphospecies (Guo *et al*., 2000, 2003; Promputtha *et al*., 2005a). Methods to promote sporulation in mycelia sterilia have been developed (Fröhlich *et al*., 2000; Guo *et al*., 1998; Taylor *et al*., 1999) and proportions of non-sporulating endophytes range from 11-54% (Fisher *et al*., 1994, Fröhlich *et al*., 2000; Guo *et al*., 2000; Kumar *et al*., 2004). To resolve the problem of identifying non-sporulating isolates, DNA sequence-based methodologies have been successfully used for the phylogenetic placement and classification of morphospecies obtained as endophytes (Guo *et al*., 2000, 2001, 2003; Promputtha *et al*., 2005a; Wang *et al*., 2005).

Despite the advances in identifying endophytes by enhanced cultural techniques and molecular identification of morphospecies, studies are still flawed by the fact that fast-growing fungi will be isolated preferentially while unculturable fungi and slow-growing fungi will escape detection. Culture-independent methods for screening fungal diversity from natural samples are therefore necessary (Guo *et al*., 2001; Kemp, 1994). With this in mind, Guo *et al*. (2001) developed a technique using direct amplification of rDNA gene extracted from frond tissue of *Livistona chinensis* followed by cloning, sequencing and phylogenetic analysis to identify endophytic fungi. They successfully recovered some endophytic fungi that had not previously been isolated from cultural studies. However, only 6 phylotypes were recovered: one plant, one basidiomycete and four ascomycetes. The most common endophytic fungi occurring on *Livistona chinensis*, such as *Guignardia*, *Pseudospiropes* and *Xylaria* species (Guo *et al*., 2000) however, were not encountered.

Advances in technology now provide additional molecular tools to evaluate diversity, and to study ecology and phylogeny (Countway *et al*., 2005; De Hoog *et al*., 2005; Green *et al*., 2004; Iotti *et al*., 2005; Jeewon *et al*., 2004; Le Bourhis *et al*., 2005; Lim *et al*., 2005). Denaturing gradient gel

electrophoresis (DGGE) is a technique that has effectively been used to estimate the diversity of prokaryotes and eukaryotes in natural samples (Anderson *et al*., 2004; Countway *et al*., 2005; Dar *et al*., 2005; Díez *et al*., 2001;Jeewon and Hyde, 2006).

DGGE has been successfully applied to document fungal communities (May *et al*., 2001; Nikolcheva *et al*., 2003, 2005; Vainio *et al*., 2000, 2005). Vainio and Hantula (2000) studied wood-inhabiting fungi and found that phylotypes isolated from environmental samples were comparable to fungi isolated from the same substrate at varying depths. Vainio *et al*. (2005) used DGGE to test the effect of sample treatment using a commercial formulation of *Phlebiopsis gigantea* on fungal communities of treated samples. Two different amplification products were observed on average from a single piece of sample (approximately 500 mm^3). The conclusion was that treatment of environmental samples did not reduce the overall fungal diversity within the treated plots. May *et al*. (2001) accessed fungal communities associated with whole plant corn silage. Results indicated that one inoculum dramatically influenced the fungal community. This method has, however, not yet been used in studies on fungal communities in living leaves, although Nikolcheva *et al*. (2003) investigated fungi on decaying leaves from freshwater from different hosts (alder, beech, linden, oak and red maple) and found that the highest diversity occurred one week after leaves were submerged. In another study, Nikolcheva *et al*. (2005) studied fungal communities occurring at the initial stage of leaf decaying in three plant species (linden, maple and oak) and found up to 7 operational taxonomic units (OTUs) on the second day, which was the highest biodiversity during the decay process.

In the present study we used DGGE to establish the fungal communities on living leaves of *Magnolia liliifera* collected from Doi Suthep Pui National Park, Chiang Mai, in Thailand. We chose this host because several studies had been published on endophytic and saprobic fungal communities on this host at this location (Promputtha *et al*. 2004, 2005a,b) that would permit comparison. The present work aims 1) to characterise the fungal communities based on DGGE coupled with phylogenetic analysis, 2) to reveal fungi that possibly are not recovered through cultural and microscopy techniques.

Methods

DNA extraction

Living, healthy leaves of *Magnolia liliifera* were collected at Doi Suthep-Pui National Park (1146 m alt, 18º48.402' North, 98º54.617' East), Chiang Mai, Thailand. Three leaves were used for DNA isolation. The leaves were divided into three parts (petioles and midribs, lower and upper parts of leaf blades) in order to assess whether fungal distribution from different leaf parts is different. Each part was separately ground into powder state with liquid nitrogen. DNA isolations were carried out 3 times from each part of leaves, using a modified CTAB procedure as outlined by Jeewon *et al*. (2002, 2004; Cai *et al*., 2005). Fifty milligrams of leaf powder was placed in a sterile 1.5 ml tube. 650 µl of pre-heated 2 × CTAB was then added, followed by 1 hour of incubation at 65°C. The same volume of pre-heated (65°C) phenol:chloroform : isoamylalcohol (25:24:1) was added (hot phenol extraction) into the sample tube and mixed gently. The contents were centrifuged at 14000 rpm for 30 min and the upper phase was transferred to another fresh tube. This step was repeated 3 times or until no interphase could be seen. DNA was precipitated from the supernatant (500 µl) by 2 volume of absolute ethanol (4°C) and kept at -20°C overnight. Then, samples were centrifuged at 4°C, 14000 rpm for 30 min. DNA pellets were washed in ice-cold 70% ethanol 2 times with centrifugation at 1000 rpm at 4°C in between and then dried in a vacuum. DNA samples were diluted in 75 µl of TE buffer containing RNase (10 $mg.\mu l^{-1}$) and incubated at 37°C for 2 hours and examined on 1% agarose gel containing ethidium bromide.

Polymerase Chain Reaction (PCR)

A partial sequence of 18S rDNA gene (about 320 bp) was amplified using primer pair NS1 (5'-GTAGTCATATGCTTGTCTC-3') (White *et al*., 1990) and GCfung (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCATTCCCCGTTACCCGTTG-3') as described by May *et al.* (2001). Each PCR reaction contained 5 µl of 10 × PCR buffer, 2 µl of $MgCl_2$ (25 mM), 4 µl of dNTP (2.5 µM) mixture, 1 µl of BSA (1 µg/µl), 0.3 µM of each primer, 0.8 units *Taq* Polymerase, and 10 ng template DNA. The optimized PCR thermal cycles for the primer pair NS1 and GCfung was as follows: initial denaturation at 95°C for 4 min and 35 cycles of 95°C for 1 min, annealing at 50°C for 1 min and 10 sec, extension at 72°C for 2 min, then followed by a last extension at 72°C for 8 min.

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE gels were prepared with the aid of a Bio-Rad model 475 Gradient Delivery System. The gels contained 7% (wt/vol) of acrylamide (acrolamide/bisacrolamide 37.5:1) and a range of denaturant concentration from 10% to 55% (formamide and urea). DNA concentration of PCR products was estimated by spectrophotometer and 2 μg products were loaded on DGGE gels. The gels were run at 150 V for 7 hours in 1 × TAE buffer (pH 8.0) at 60°C. DGGE gels were stained with ethidium bromide in 1x TAE for 20 min and then destained in 1 × TAE for 15 min. The gel photographs were viewed by the computer program Gel Doc. DNA bands on the DGGE gels were excised under UV trans-illumination using sterile scalpels and then soaked in 30 μl of sterile double-distilled water at 4°C overnight. 0.7 μl of DNA solution was used for re-amplification, using the primer pair described above without GC clamp. Re-amplification was done with the following thermal program: 95°C for 3 min, followed by 36 cycles of 94°C for 1 min, 40 sec at 50°C, 30 sec at 72°C and 8 min of a last extension at 72°C. DNA was then purified by using purification kits (Amersham Biosciences GFX^{TM} PCR DNA and Gel Band Purification Kit). Purified DNA was sequenced, using the NS1 primer, in an automated sequencer (Applied Biosystem 3730 DNA Analyzer) at the Genome Research Centre, The University of Hong Kong.

Phylogenetic analysis

When DNA sequences were obtained from NS1 primer, a blast search was performed in GenBank to find possible sister groups (Table 1). Phylogenetic analyses were conducted in PAUP* 4.0b10 (Swofford, 2004) and multiple alignments were done in Bioedit (Hall, 1999) and Clustal X (Thompson *et al*., 1997). Parsimony analyses included representatives of members from other orders (sequences available from GenBank) to resolve phylogenetic relationships and to root cladograms. Trees were inferred using the heuristic search option with 1000 random addition sequence additions. Gaps were treated as missing data and characters were unordered and weighted equally and differentially. Clade stability was assessed in a bootstrap analysis with 1000 replicates, random sequence additions with maxtrees set to 2000 and other default parameters as implemented in PAUP*. Futher details are outlined in Jeewon *et al*. (2002, 2004).

Bayesian analyses were performed using the MrBayes3.0B4 program (Huelsenbeck and Ronquist, 2001). The Markov Chain-Monte Carlo (MCMC)

method was used to approximate the posterior probabilities of each branch, counting the occurrence of each branch in trees that were visited in the MCMC analysis progress.

Table 1. List of species used in the study and their GenBank accession numbers

Species	Accession number	Species	Accession number
Aureobasidium pullulans	AY137506	*Chaetomium globosum*	AB048285
Botryosphaeria tsugae	AF271127	*Clypeosphaeria uniseptata*	AY083812
Cladosporium cladosporioides	AF548071	*Daldinia* sp.	AY315425
Mycosphaerella latebrosa	AY251114	*Discostroma tosta*	AY083814
Didymella cucurbitacearum	AY293779	*Graphostroma platystoma*	AY083808
Dothidea ribesia	AY016343	*Haematon haematococca*	AY489697
Elytroderma deformans	AF203455	*Hypocrea rufa*	AY489694
Lophodermium pinastri	AF106014	*Hyponectria buxi*	AF130976
Massariosphaeria phaeospora	AF164368	*Hypoxylon fragiforme*	AB014046
Mycocalicium albonigrum	L37736	*Leuconectria clusiae*	AY489700
Passalora fulva	AY251109	*Monographella nivalis*	AF064049
Phaeococcomyces nigricans	AY843273	*Neocosmospora vasinfecta*	U44117
Rhizoscyphus ericae	AY524847	*Neurospora crassa*	AY046271
Sarcinomyces petricola	Y18702	*Seimatoantlerium* sp.	AF346555
Septoria rosae	AY251113	*Sordaria fimicola*	X69851
Sphaerulina oryzina	AY251103	*Thielavia terrestris*	U43969
Trimmatostroma macowanii	AY251118	*Viridispora diparietispora*	AY489703
Tryblidiopsis pinastri	AF106013	**Outgroup**	
Westerdykella cylindrical	AY016355	*Didymella cucurbitacearum*	AY293779
Mycosphaerella sp.	AY251116	*Pleospora* sp.	AY392129
Outgroup			
Cosmospora coccinea	AY489702		
Leuconectria clusiae	AY489700		

Results

Among the three leaves used for DNA extraction, two of them did not give any PCR product using the NS1 and FungGC primer pair. The DGGE profiles of the third leaf yielded 14 different bands from the different parts (Fig. 1). Three bands were from the lower parts, five bands from the upper parts of the leaf blades, and six from petioles and midribs. All fourteen bands were excised for sequencing analysis. Although some bands (eg. ML1 and MU1; ML2 and MR2) shared similar electrophoretic mobility the sequence analyses revealed that the fungal taxa were not identical or phylogenetically related.

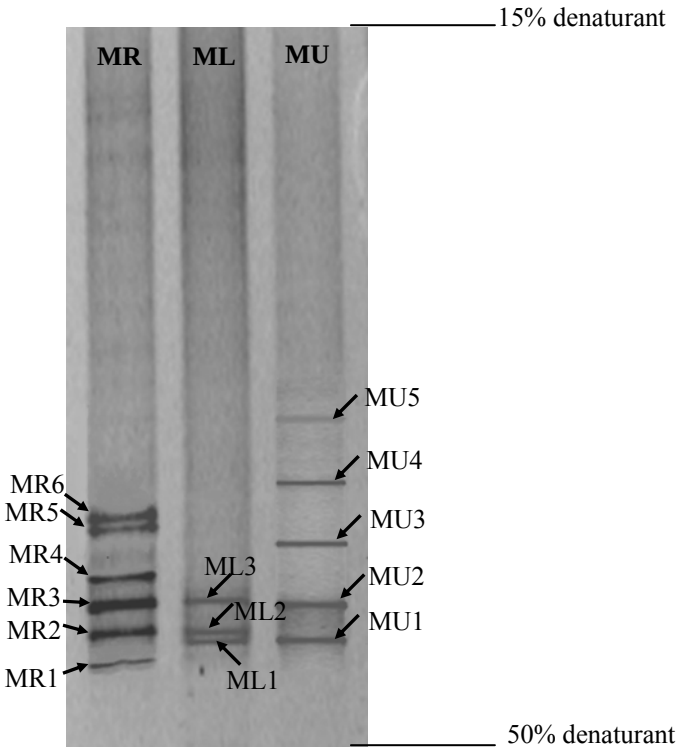

Fig. 1. Denaturant gradient gel electrophoresis profiles of 18S rDNA sequences amplified from DNA extracted directly from living leaf samples of *Magnolia liliifera*. Lane 1: Total genomic DNA extracted from leaf midrib and petiole (MR); Lane 2: Total genomic DNA extracted from upper parts of leaf blade (MU); Lane 3: Total genomic DNA extracted from lower parts of leaf blade (ML).

Table 2 shows the most similar taxa to the different OTUs following blast search results in GenBank. Blast search showed that ML1 and MR1 have high sequence similarity to *Trimmatostroma macowanii*; ML2 was similar to *Mycosphaerella* sp.; MU3 and MU4 were similar to *Botryosphaeria* species; MR2 and MR3 were similar to *Cordyceps* and uncultured *Hypocreales*; MR4 was similar to *Phaeococcomyces nigricans*; MR5 and MR6 were similar to *Hypoxylon fragiforme*; MU1 was similar to *Didymella curcubitacearum*; MU2 was similar to Fungal sp. (AY843229); and MU3 was similar to *Elytroderma deformans*.

The Maximum Parsimony (MP) and Bayesian analysis of the unitunicate ascomycete dataset comprised 25 taxa with *Pleospora* sp. and *Didymella cucurbitacearum* as outgroups with 5 OTUs from DGGE (MR2, MR3, MR5, MR6 and MU5) resulted in one MP tree (Fig. 2). MR5 and MR6

belong to the *Xylariaceae* with 55% bootstrap and 0.91 Baysian posterior probabilities. Phylogenetic results showed that MR2, MR3 and MU5 were hypocrealean taxa with of 92% bootstrap support and 1.00 posterior probability (Fig. 2). However, further phylogenetic placement of these OTUs could not be resolved.

Table 2. Percentage sequence similarities of uncultured phylotypes from *Magnolia liliifera* leaf colonizers to other sequences in GenBank.

OTU	Species	Similarity (%)	GenBank No	Family	Order
ML1	*Trimmatostroma macowanii*	299/300 (99)	AY251118	*Mycosphaerellaceae*	*Mycosphaerellales*
	Coccodinium bartschii	299/300 (99)	U77668	Icertae sedis	Icertae sedis
ML2	*Mycosphaerella* sp.	290/296 (97)	AY251116	*Mycosphaerellaceae*	*Mycosphaerellales*
	Mycosphaerella latebrosa	290/296 (97)	AY251114	*Mycosphaerellaceae*	*Mycosphaerellales*
ML3	*Botryosphaeria tsugae*	288/291 (98)	AF271127	*Botryosphaeriaceae*	*Dothideales*
	Trimmatostroma macowanii	285/288 (98)	AY251118	*Mycosphaerellaceae*	*Mycosphaerellales*
MU1	*Didymella cucurbitacearum*	313/316 (99)	AY293779	Icertae sedis	Icertae sedis
MU2	Fungal sp.	308/311 (99)	AY843229	Icertae sedis	Icertae sedis
	Fusicoccum dimidiatum	312/316 (98)	AF258605	*Botryosphaeriaceae*	*Dothideales*
MU3	*Elytroderma deformans*	294/295 (99)	AF203455	*Rhytismataceae*	*Rhytismatales*
MU4	*Botryosphaeria ribis*	304/309 (98)	AF271129	*Botryosphaeriaceae*	*Dothideales*
MU5	*Cordyceps sinensis*	294/295 (99)	AB187268	*Clavicipitaceae*	*Hypocreales*
MR1	*Trimmatostroma macowanii*	298/299 (99)	AY251118	*Mycosphaerellaceae*	*Mycosphaerellales*
MR2	*Cordyceps sinensis*	287/289 (99)	AB187268	*Clavicipitaceae*	*Hypocreales*
MR3	Uncultured Hypocreales	234/234 (100)	AY275188	Icertae sedis	Icertae sedis
MR4	*Phaeococcomyces nigricans*	244/245 (99)	AY843273	*Herpotrichiellaceae*	*Chaetothyriales*
	Hymenoscyphus ericae	290/309 (93)	AY524847	*Helotiaceae*	*Helotiales*
MR5	*Hypoxylon fragiforme*	288/289 (99)	AY083810	*Xylariaceae*	*Xylariales*
MR6	*Hypoxylon fragiforme*	288/289 (99)	AY083810	*Xylariaceae*	*Xylariales*

Parsimony analysis of bitunicate ascomycetes showed that ML2 was related to *Passalora fulva* and clustered with taxa of the family *Mycosphaerellaceae* (*Mycosphaerella latebrosa*, *Mycosphaerella* sp., *Sphaerulina oryzina*, *Septoria rosae*) with 0.74 posterior probabilities (Fig. 3). ML1 and MR1 were sister taxa to *Cladosporium cladosporoides*

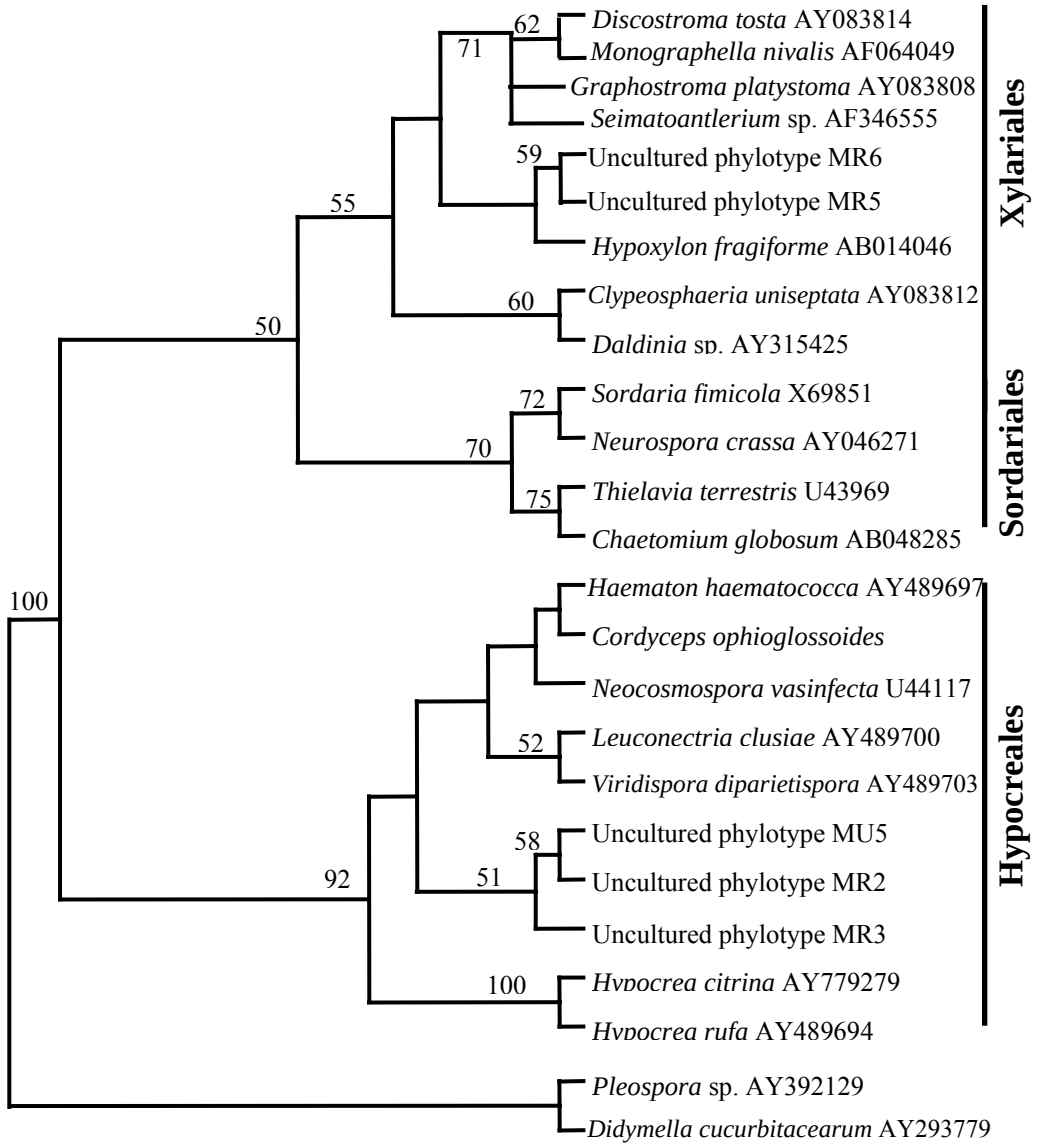

Fig. 2. Maximum-parsimony tree generated from partial 18S rDNA sequences of 25 taxa showing the relationships of MR2, MR3, MR5, MR6 and MU5 with reference taxa. The tree was rooted with *Pleospora* sp. and *Didymella cucurbitacearum* (Tree length = 125, CI = 0.688, RI = 0.851, RC = 0.586, and HI = 0.312). Bootstrap values ≥ 50% (2000 replicates) are shown on the branches.

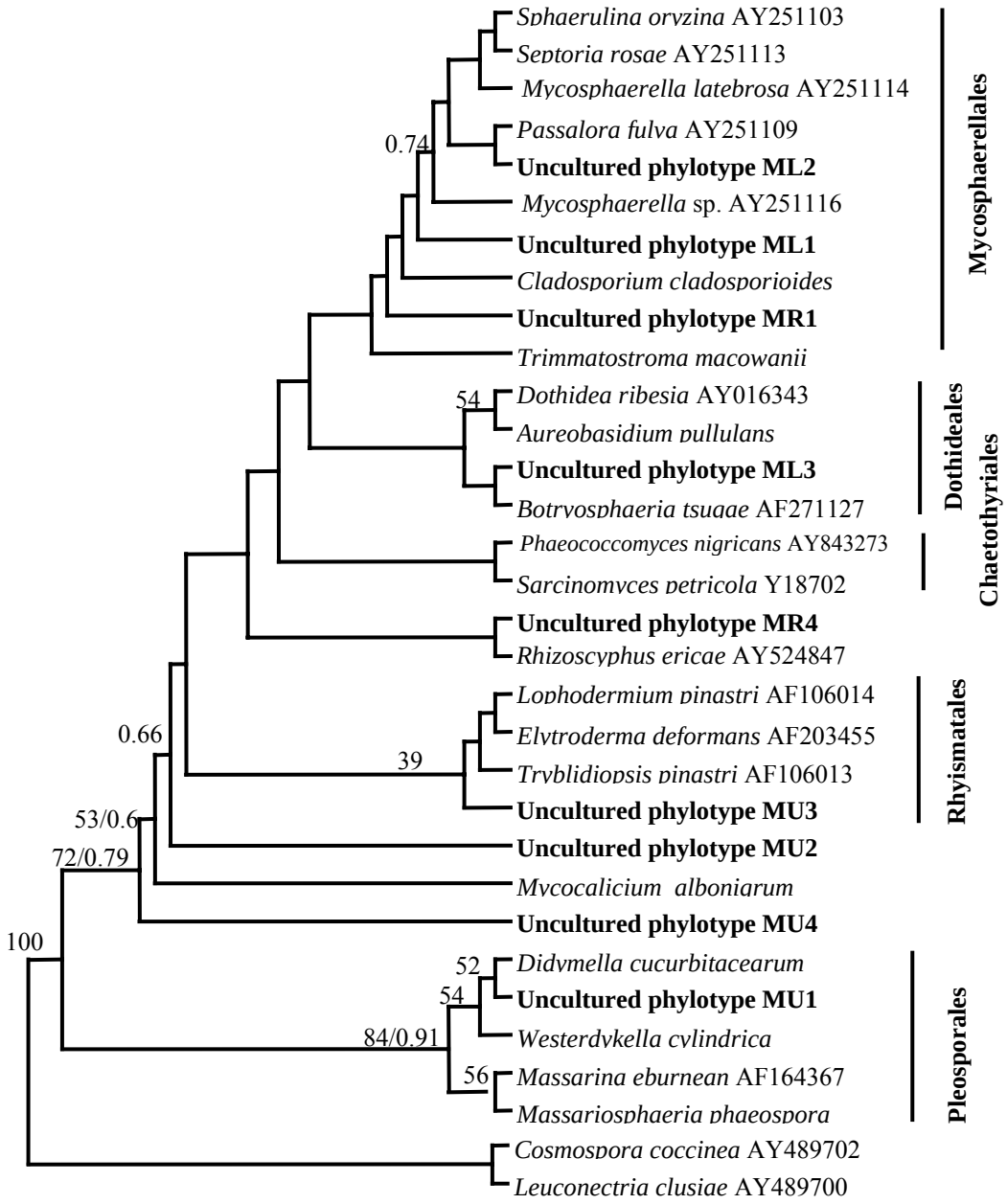

Fig. 3. Maximum-parsimony tree generated from partial 18S sequences of 32 taxa showing the relationships of ML1, ML2, ML3, MR1, MR4, MU1, MU2, MU3 and MU4 with reference taxa. The tree was rooted with *Cosmospora* and *Leuconectria clusiae* (tree length = 117, CI = 0.709, HI = 0.291, RI = 0.806, RC = 0.572). The numbers above branches were bootstrap and posterior probability values.

(*Mycosphaerellaceae*) and *Trimmatostroma macowanii*. ML3 was related to species of *Dothideales* (*Aureobasidium pullulans, Botryosphaeria tsugae* and *Dothidea ribesia*). MR4 clustered with *Rhizoscyphus ericae* (*Helotiaceae*, *Helotiales*). MU3 was related to the group comprising *Elytroderma deformans, Lophodermium pinastri*, and *Tryblidiopsis pinastri* (*Rhytismataceae*, *Rhytismatales*). However, these relationships did not have any support (Fig. 3). MU1 belongs to the family *Pleosporaceae* and is related to *Didymella cucurbitacearum*, *Massarina eburnea, Massariosphaeria phaeospora*, and *Westerdykella cylindrica*) with relatively high support (84 bootstrap and 0.91 posterior probabilities) (Fig. 3). The phylogenetic placement of MU2 and MU4 could not be resolved using available DNA sequences.

Discussion

Previous studies on *Magnolia liliifera* have already shown that this host harbours numerous saprobic and endophytic fungal taxa (Promputtha *et al*., 2004, 2005a,b). There, is however, one major limitation of these previous studies as they targeted fungi that either produce fruiting-bodies (which can be identified upon microscopic examination) or those fungi that can be easily cultured on artificial media. It has already been shown in several other studies that direct morphological examination of fruiting structures on substrates or culture-dependent methods give bias estimates of fungal communities (Duong *et al*., 2004; Guo *et al*., 2001; Promputtha *et al*., 2004). This study targets fungal communities using DGGE with sequence analyses in an attempt to characterize unknown fungal taxa from *Magnolia liliifera*.

Studies on endophytes of Magnolia liliifera

We found fourteen operational taxonomic units (OTUs) on healthy leaf using PCR-DGGE coupled with sequence analyses. Phylogenetic analyses were useful to establish their taxonomic placement and systematic relationships with known fungi.

Fungal communities on *Magnolia liliifera* have been investigated by various methods. Promputtha *et al*. (2005a) identified 77 taxa of endophytic fungi from leaves of *Magnolia liliifera* of which 46 were sporulating and belonged to 8 genera (*Colletotrichum* (10), *Corynespora* (2), *Curvularia* (2), *Fusarium* (5), *Guignardia* (2), *Phomopsis* (11), *Trichoderma* (1), and *Xylaria* (13); 31 were morphospecies. In order to identify those morphospecies at a lower taxonomic level, rDNA gene sequence analyses were performed. The authors successfully identified the morphospecies into 6 ascomycete genera

(*Bionectria* - one morphospecies, *Diaporthe* - 24 morphospecies, *Glomerella* - one morphospecies, *Hypoxylon* - one morphospecies, *Massarina* - one morphospecies, and *Xylaria* - 3 morphospecies). Most of sporulating and non-sporulating fungi were common endophytic fungi that were also found in many other studies (Kumar *et al*., 2004; Suryanarayanan *et al*., 2005).

In the present study, 14 OTUs were successfully recovered from one leaf sample only using DGGE, revealing some typical endophytic genera (e.g. xylariaceous, and hypocrealean genera) as well as taxa that appear unable to grow on artificial media. Twelve OTUs were distributed amongst 6 different orders of ascomycetes (*Dothideales*, *Hypocreales*, *Mycosphaerellales*, *Pleosporales*, *Rhytismatales* and *Xylariales*) and 2 bitunicate fungi (MU2 and MU4), whose phylogenetic placement could not be resolved. This is not surprising as O'Brien *et al*. (2005) sequenced all kinds of organisms (soil and litter microorganisms from a mixed deciduous forest in the Southeastern United States) using a universal primer and identified them using the available data from GenBank. They found that 12% of the sequences could not be identified even to phylum level. Most endophytic isolates recovered using traditional methodologies and rDNA gene sequence analyses from *Magnolia liliifera* resulted in mostly unitunicate ascomycetes (Promputtha *et al*., 2004, 2005a). Among the 77 taxa previously identified from leaves of *Magnolia liliifera*, only one species of *Massarina*, *Corynespora*, *Curvularia* (*Pleosporales*) and *Guignardia* (*Botryosphaeriales*) were isolated. In contrast, most OTUs identified using DGGE in this study are bitunicate ascomycetes. MU1 belongs to the *Pleosporales* and is sister taxon to *Didymella*, a genus without proper familial placement (Fig. 3). Surprisingly no endophytic or saprobic fungi had previously been isolated from *Magnolia liliifera* that were related or similar to the *Mycosphaerellales* and *Rhytismatales*. Therefore, the OTUs (ML1, ML2, MR1 and MU3) recovered from DGGE sequence analyses in this study indicate that these taxa are possibly unculturable or slow growing and have gone undetected in previous studies. In contrast, however, diaporthalean taxa and many others commonly isolated as endophytes (especially 24 *Phomopsis* species of 31 morphospecies from *Magnolia liliifera*) using artificial media were not identified using DGGE.

DGGE analysis coupled with phylogeny revealed that 5 phylotypes were unitunicate ascomycetes. Operational taxonomic units MR2, MR3 and MU5 belong to the order *Hypocreales*. In particular, they have high sequence similarity to those of *Cordyceps* species amongst many available small subunit sequences of other hypocrealean species in the GenBank (Table 2, Fig. 2). *Cordyceps* and its anamorphs are parasites of insects (Liang *et al*., 2005) and their occurrence as endophytes is surprising. No *Cordyceps* species was

isolated from *Magnolia liliifera*. Promputtha *et al*. (2005a) found other hypocrealean genera isolated from the host such as *Trichoderma* and *Fusarium*. Other fungal diversity studies based on DGGE and sequence analyses have also reported taxa with phylogenetic affinities to the *Hypocreales* (Bougoure and Cainey, 2005, Gomes *et al*., 2003). In our study, however, sequence analyses revealed that none of the OTUs recovered are *Trichoderma* or *Fusarium*.

OTUs MR5 and MR6 are closely related to *Hypoxylon* species. *Hypoxylon* comprises common endophytes and saprobes of various plant hosts (Petrini, 1991; Suryanarayanan *et al*., 2005). An endophytic species of this genus was also isolated from *Magnolia liliifera* (Promputtha *et al*., 2005a). *Xylaria* is another common endophyte that belongs to *Xylariaceae*, a family is known to harbor numerous endophytic species, and it was abundant in *Magnolia liliifera* (Promputtha *et al*., 2005a). Our molecular approach used here failed to recover any *Xylaria* species.

This study demonstrated that DGGE could be used to detect known and abundant fungi (*Xylariales*, *Hypocreales* and *Pleosporales*) as well as unknown endophytic fungi (*Mycosphaerellales*, *Dothideales*, *Helotiales* and *Rhytismatales*). On the other hand, taxa such as *Diaporthe* and *Xylaria* that are abundant in cultural studies and other commonly found endophytic taxa were not recovered through DGGE. There may be several reasons for this. It has been suggested that some endophytes are not abundant in leaves, while others occupy spaces as small as single cells (Ghimire and Hyde, 2004; Varma *et al*., 2004). In such cases there may not be enough fungal DNA to allow successful molecular detection. It might be plausible that these fungi present in a small amounts in the leaf tissues are not detected by DGGE, but being fast-growing fungi they are recovered abundantly on artificial media in traditional endophytic studies. The number of fungi identified by DGGE is small when compared to direct observation and traditional culturing of endophytes (Promputtha *et al*., 2005a). However, when comparing to other studies such as Nikocheva *et al*. (2003, 2005), the number of fungal taxa revealed in this study is relatively large. The number of endophytic fungi isolated from parts of leaves is hard to estimate as parts, rather than the whole leaf is used. Fungal endophytes show various patterns within the leaves; some are confined to single plant cells, others occur internally or externally as single hyphae or they may grow throughout the veins (Varma *et al*., 2004).

DGGE is a suitable method that can be applied in future studies to estimate fungal diversity, but it has several drawbacks. The primer pair NS1 and GCFung, as described by May *et al*. (2001) amplifies less than 400 nucleotides and it appears to be specific to ascomycetes. In addition, the region

amplified and sequenced (partial 18S rDNA gene) is rather conserved and therefore not appropriate to properly identify taxa at the genus or species level. Further studies should consider primers that are more universal (for fungi) and that give better phylogenetic resolution at generic or species level.

Fungi on different parts of leaves

Tissue specificity and recurrence in fungi have been studied at different host-taxonomic levels (Parungao *et al*., 2002; Paulus *et al*., 2006, Photita *et al*., 2001, Polishook *et al*., 1996; Varma *et al*., 2004) as reviewed by Zhou and Hyde (2001). They concluded that many host-specific endophytic fungi become saprobes at leaf senescence. However, it is hard to say whether a given fungus is host-specific or host-recurrent.

In this study we applied DGGE to establish whether the method could detect differences in fungal communities present in different *Magnolia liliifera* leaf parts. We found a different fungal spectrum in different leaf parts. Different numbers of unitunicate and bitunicate ascomycetes were found in midrib and leaf blades. Two of six sequence types (MR1 and MR4) from the midrib were bitunicate ascomycetes and one of 8 sequence types (MU5) from leaf blades was a unitunicate ascomycete.

Fungi have been shown to be tissue-recurrent in several studies (Photita *et al*., 2001, Promputtha *et al*., 2004; Van Ryckegem and Verbeken 2005a,b; Wong *et al*., 2001). Photita *et al*. (2001) studied fungal diversity on *Musa acuminata* in Hong Kong and found that distribution of fungal communities was different in leaf blades and petioles. Of 46 taxa from 2 study sites, only 11 taxa were present in both leaves and petioles. Six taxa were only identified from the petioles. Promputtha *et al*. (2004) documented the saprobic fungal communities on dead leaves of *Magnolia liliifera* with 5 taxa on midribs and petioles, and 33 taxa on leaf blades. The only one species found on both leaf blades and petiole was *Sporidesmium crassisporum*. The OTUs identified in this study illustrate differences in fungal communities between tissue types but given the sparse leaf samples studied, we refrain from making conclusive statements as to whether these taxa are tissue recurrent.

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