Ribosomal DNA fingerprinting in the identification of non sporulating endophytes from *Magnolia liliifera* (*Magnoliaceae*)

Itthayakorn Promputtha[1], Rajesh Jeewon[3], Saisamorn Lumyong[1], Eric H.C. McKenzie[2] and Kevin D. Hyde[3*]

[1]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand 50200
[2]Landcare Research, Private Bag 92170, Auckland, New Zealand
[3]Centre for Research in Fungal Diversity, Department of Ecology & Biodiversity, The University of Hong Kong, Pokfulam Road, Hong Kong

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The sporulating endophytic fungi isolated from *Magnolia liliifera* were identified to genus or species level using morphological characters. Non-sporulating isolates are generally termed as 'mycelia sterilia' and are grouped as 'morphospecies' based on similar cultural characters. Mycelia sterilia were grouped to 31 morphospecies and were further identified based on ribosomal DNA (rDNA) sequence analysis. The 5.8S gene, ITS1 and ITS2 regions of rDNA from all morphospecies were amplified and sequenced. Phylogenetic analysis indicated that MS88 were related to the genus *Massarina* (*Lophiostomataceae*), MS9, MS11 and MS47 to *Xylaria* (*Xylariaceae*), MS19 to *Glomerella* (*Phyllachoraceae*), MS25 to *Hypoxylon* (*Xylariaceae*), MS27 to *Bionectria* (*Bionectriaceae*) and the remaining 24 morphospecies were related to *Phomopsis* and *Diaporthe* (anamorphic *Phomopsis*).

Key words: *Bionectria*, *Diaporthe*, *Glomerella, Hypoxylon*, mycelia sterilia, morphospecies, mycelia sterilia, *Phomopsis*, *Xylaria*.

Introduction

Fungal endophytes are fungi that live within plant tissues without causing obvious symptoms of tissue damage either intercellularly or intracellularly (Wilson, 1995; Schulz and Boyle, 2005). Endophytic fungi have been found in every plant species that have been studied including terrestrial plants such as grasses (Bacon and White, 1994; Groppe *et al*., 1999; Saikkonen *et al*., 2000), palms (Rodrigues, 1994, 1996; Fröhlich and Hyde, 1999; Taylor *et al*., 1999; Fröhlich *et al*., 2000), banana (Brown *et al*., 1998; Photita *et al*., 2004), and mangroves (Suryanarayanan *et al*., 1998; Kumaresan *et al*., 2002) and

*Corresponding author: K.D. Hyde; e-mail: kdhyde@hkucc.hku.hk

halophytes (Petrini and Fisher, 1986; Suryanarayanan and Kumaresan, 2000). Endophytic fungi have been isolated from several plant tissues including bark, flower, leaves, petioles, root, seed and twigs (Stone *et al*., 2000; Rolston *et al*., 1986; Suryanarayanan and Vijaykrishna, 2001; Kumar and Hyde, 2004). Several reports have been published on their biology (Jennings and Lysek, 1996; Clay, 1998; Brem and Leuchtmann, 2001; Arnold *et al*., 2003), evolution (Tsai *et al*., 1994; Carrol, 1998; Saikkonen *et al*., 2004), occurrence (Gamboa and Bayman, 2001; Romero *et al*., 2001; Kumar *et al*., 2004; Suryanarayanan and Thennarasan, 2004), taxonomy (Petrini, 1986; Guo *et al*., 2000, 2001, 2003; Jeewon *et al*., 2004) and biotechnological application (Tomita, 2003).

Identification of fungal endophytes has been based on isolation procedures, sterilisation techniques, cultural conditions and sporulation of isolates (Taylor *et al*., 1999; Guo *et al*., 1998). Several endophytes, however, cannot be identified due to the lack of sporulation. Several methods have been developed to promote of sporulation, e.g. exposure at 8ºC under fluorescent or near-ultraviolet light with a 12 hours dark-light cycle, or incubation at 4ºC in the darkness may induce sporulation in some fungi. Transferring non-sporulating isolates to natural media such as corn meal agar, V8 agar, potato carrot agar, or on chemically defined media such as Czapek's agar, peptone dextrose agar, may also induce sporulation. Sometimes it is necessary to grow the fungus on host substrate to induce sporulation (Guo *et al*., 1998; Taylor *et al*., 1999; Fröhlich *et al*., 2000). Yet, variable proportions of non-sporulating endophytes have been reported in studies ranging from 11% of total isolates from *Trachycarpus fortunei* in China (Taylor *et al*., 1999), 13% of isolates from two *Licuala* species in Australia and Brunei (Frohlich *et al*., 2000), 15% of isolates from ever green shrubs in western Oregon (Petrini *et al*., 1982), 16.5% of isolates from fronds of *Livistona chinensis* in Hong Kong (Guo *et al*., 2000), 23.6% of isolates from *Tripterygium wilfordii* in Guangdong, China (Kumar and Hyde, 2004), 27% of isolates from leaves of *Sequoia sempervirens* in central California (Espinosa-Garcia and Langenheim, 1990), and 54% of isolates obtained from twigs of *Quercus ilex* in Switzerland (Fisher *et al*., 1994).

Non-sporulating isolates are generally termed 'mycelia sterilia' and grouped as 'morphospecies' based on similarity in cultural characteristics such as, colony surface texture, hyphal pigments, exudates, margin shapes and growth rates (Bills, 1996; Umali *et al*., 1999; Fröhlich *et al*., 2000; Lacap *et al*., 2003). Grouping mycelia sterilia into morphospecies, however, may not reflect species phylogeny, because morphospecies are not real taxonomic entities (Guo *et al*., 2003). The taxonomic correctness of grouping into morphospecies based on cultural characteristics is questionable (Arnold *et al*., 2000; Guo *et*

al., 2003). Guo *et al*. (2003) pointed out that morphologically similar sterile mycelia can be unrelated taxa. Furthermore, interpretation of morphospecies as separate taxonomic entities may have implications on fungal diversity measurement and fungal species composition of a particular plant species (Rollo *et al*., 1995; Zhang *et al*., 1997; Guo *et al*., 2000, 2001, 2003; Lacap *et al*., 2003). To resolve this problem, alternative techniques have been developed to identify these fungi to family or genus level and also to assess the validity of the current morphospecies concept (Lacap *et al*., 2003; Guo *et al*., 2003).

Molecular techniques have been used successfully for phylogenetic placement and segregation of endophytic mycelia sterilia. Guo *et al*. (2000) identified 19 endophytic morphospecies from *Livistona chinensis* using cultural morphology, growth rates and rDNA sequence analysis. Based on phylogenetic analysis of the ITS and 5.8S regions, they identified all morphospecies to family and genus. In addition, Guo *et al*. (2003) identified 18 morphotypes from *Pinus tabulaeformis* to various taxonomic levels based on nrDNA sequence analysis and genetic variation in *Alternaria alternata* by RAMS (Guo *et al*., 2004). Lacap *et al*. (2003) compared nucleotide ITS and 5.8S rDNA sequence similarities and identified 6 morphotypes to genus.

During the study of endophytes on the leaves of *Magnolia liliifera,* a commonly found tree in Doi Suthep-Pui National Park, Thailand, we encountered numerous sterile mycelia, which was grouped in to 31 morphospecies. To determine the phylogenetic position of mycelia sterilia we used the 5.8S gene and ITS regions. Because the 5.8S gene is highly conserved, it is phylogenetically applicable to assess relationships at higher taxonomic level, whereas the highly variable ITS regions are used for analysis of lower taxonomic level.

The objectives of this study were:

1. To identify non sporulating endophytes and classify them to familial and generic level.
2. To determine their phylogenetic relationships with related species.

Materials and methods

Plant species and site

Magnolia liliifera (*Magnoliaceae*) selected for this study is an evergreen tree which grows up to 15 m in height, having an irregular crown and a smooth pale brown bark. Leaves are 20-50 × 7-15 cm, dark green with 9-15 pairs of widely spaced side veins. Petioles are 5-7 cm and swollen at base (Gardner *et al*., 2000). This tree is distributed in few areas of tropical regions, India,

Myanmar, Nepal and Thailand. In Thailand, this tree is locally common in Doi Suthep-Pui National Park (Gardner *et al*., 2000). In this study the endophytes associated with *Magnolia liliifera* in Doi Suthep-Pui National Park, Chiang Mai Province, Thailand are characterised.

Isolation and culture of endophytic fungi

To investigate the endophytes living within leaf veins, interveins, petioles and twigs of *Magnolia liliifera*, leaf and twig samples were collected from ten trees. Two healthy mature leaves and two healthy twigs were randomly removed from each tree and placed individually into plastic bags. Samples were returned to laboratory and immediately washed in running tap water for 15 minutes. Ten disks of 5 mm diam were cut from leaf tissue containing veins, ten disks were cut from intervein leaf tissue and ten segments of 5 mm long were cut from the petiole of each leaf,. Ten segments of 5 mm long were cut from each twig. A total of 20 vein disks, 20 intervein disks, 20 petiole segments and 20 twig segments were subjected to the surface sterilisation procedure. The surface sterilisation technique chosen follows Taylor *et al*. (1999), but was modified based on the thickness of plant tissue following pilot experiments. Vein disks and intervein disks were surface sterilised by soaking in 95% ethanol for 1 minute, then immersed in a solution of sodium hypochlorite (3%) for 1 minute and finally immersed in 95% ethanol for 30 seconds. Petiole segments and twig segments were surface sterilised by soaking in 95% ethanol for 1 minute, then immersed in 3% sodium hypochlorite solution for 3 minutes and finally immersed in 95% ethanol for 30 seconds. Samples were washed in sterile distilled water and dried on sterile tissue paper. Ten of each tissue part of each tree were transferred to Rose Bengal agar, containing malt extract Difco (20 g per litre), rose bengal (0.033 g per litre), oxoid agar (15 g per liter) and chloramphenicol (50 mg per litre) (Bussaban *et al*., 2001). Labelled plates were incubated at 20°C under a cycle of 12 hours light/dark for 1-3 weeks (Gamboa and Bayman, 2001).

When fungal colonies developed from the disks/segments, the hyphal tips of the fungal colonies were transferred onto Potato Dextrose Agar (PDA) plates and corn meal agar (CMA, 2% Difco) slants containing a 1 cm^2 strip of autoclaved host leaf tissue. The tissue strip was included to encourage sporulation (Guo *et al*., 1998). Isolates were then incubated at 28-30°C under a cycle of 12:12 hours light:dark for 2 months.

Sources of cultures and identification

Cultures were examined periodically and identified when isolates sporulated. The remaining isolates which failed to were treated as mycelia sterilia. All mycelia sterilia were separated into morphospecies differentiated by culture characteristics such as colony surface, texture, hyphal pigmentation and growth rate on PDA (Guo *et al*., 1998, Umali *et al*., 1999, Bussaban *et al*., 2001, Suryanarayanan *et al*., 2003). The mycelia sterilia were divided into 31 morphospecies and were identified following DNA sequence analysis. ITS sequence data was generated from these morphotypes to enable their identification.

DNA extraction

Fungal cultures were grown on PDA plates for 5-20 days and total DNA was extracted from fresh mycelium using a protocol of Jeewon *et al*. (2003, 2004) and Lacap *et al*. (2003). Mycelium were directly scraped off from culture plates and transferred into 1.5 µl centrifuge tube. Mycelium was mixed with 0.2 g of sterile white quartz sand and 600 µl of preheated (60 °C) 2X CTAB buffer [2% v/w CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8.0]. Mycelium was ground with a plastic pestle for 5-10 min and incubated at 60 °C for 40 min with occasional gentle swirling every 10 min. Then 600 µl of phenol:chloroform (1:1) was added into each tube and gently mixed. The mixture was centrifuge at 13000 rpm for 30 min and the aqueous extraction layer was transferred into a new 1.5 ml tube. Phenol:chloroform (1:1) extraction was repeated 2-3 times until no interface was visible. Two volumes of 100% cold ethanol was added and the tube was inverted gently and stored overnight at -20 °C to precipitate DNA. Contents were then centrifuged at 11000 rpm for 30 min at 4 °C. The DNA pellet obtained was washed with 70% cold ethanol twice and dried using SpeedVac®. The pellet was suspended in 100 µl of TE buffer [10 mM Tris-HCl, 1 mM EDTA, pH 8] containing 20 µg/ml RNase. DNA samples were checked for purity by electrophoresis in 1% (w/v) agarose stained with ethidium bromide (10 mg/ml).

PCR amplification

Primer pair ITS4 and ITS5 (White *et al*., 1990) were used to amplify the 5.8S gene and flanking ITS1 and ITS2 regions. Amplification was performed in a 50 µl reaction volume containing 5 µl of 10X Mg free PCR buffer, 3 µl of 25 mM $MgCl_2$, 4 µl of 2.5 mM deoxyribonucleotide triphosphate (dNTPs), 1.5

µl of 10 µM primers (ITS4 and ITS5), 3 µl of DNA template, 0.3 µl of 2.5 units of *Taq* DNA polymerase. The thermal cycle consisted of 3 minutes initial denaturation at 95 °C, followed by 30 cycles of 1 minute denaturation at 95 °C, 50 seconds primer annealing at 52 °C, 1 minute extension at 72 °C, and a final 10 minutes extension at 72 °C. The PCR products were examined by electrophoresis in 1% (w/v) agarose gel with ethidium bromide (10 mg/ml) and check for size and purity.

DNA sequencing

PCR products were purified using minicolumns, purification resin and buffer according to the manufacturer's protocol (Wizard PCR Preps DNA Purification System, Promega, Madison, WI). The purified PCR products were directly sequenced in an automated sequencer at Genome Research Centre, Faculty of Medicine, The University of Hong Kong. Primer pair ITS4 and ITS5 (White *et al*., 1990) were used in the sequencing reaction.

Sequence data analysis

Sequences were aligned using ClustalX (1.83) (Thompson *et al*., 1997) and BioEdit (Hall, 1999) program. Alignments were manually edited where necessary. ITS sequences were used as probes in NCBI blast search database in order to retrieve similar sequences for inclusion in the phylogenetic analyses. Sequences obtained were split into different datasets in order to access phylogenetic relationships at the familial and species level. Phylogenetic analyses for maximum parsimony (MP) were performed by using PAUP 4.0 beta10 (Swofford, 2002). Parsimony trees were obtained using heuristic searches only because of the large data set. To increase the probability of finding all most-parsimonious trees, searches were implemented using the random sequence addition option and the tree bisection-reconnection (TBR) branch-swapping algorithm. Each search was repeated ten times from different random starting points using the stepwise addition option. Single-position gaps were treated as missing data. For phylogenetic analysis each homologous sequence position was treated as a discrete character with four possible unordered states (A, G, C or T) and equally weighted parsimony (with a transition transversion ratio of 1:1) were included in the parsimony analysis. A series of minor analyses under different conditions (different Transition Transversion ratios and treating gaps as missing or fifth state) was carried out to test the phylogenetic relationships among the taxa and to determine the most reliable parameters giving the best trees for subsequent analyses. Branch

support of the trees resulting from maximum parsimony (MP) was assessed by bootstrapping (Felsenstein, 1985; Sanderson, 1989). The bootstrap analysis was performed with 1000 replications using the heuristic search option as described above to estimate the reliability of inferred monophyletic groups. Consistency Index (CI), Retention Index (RI), Rescaled Consistency Index (RC) and Homoplasy Index (HI) were calculated for all parsimony trees. To access the taxonomic placement of these morphotypes at the ordinal and familial level only, 5.8S gene sequences were analised. In addition, ITS1, ITS2 (including 5.8S) for all morphotypes were also analysed phylogenetically to determine the phylogenetic affinities of these morphotypes at the generic and species level.

Results and discussion

Taxonomic placement of the 31 morphospecies

Identification of bitunicate mycelia sterilia MS88 to family and genus level

The search for similar ITS regions sequence in GenBank showed that MS88 had the high sequence similarities with species of *Leptosphaeriaceae*, *Lophiostomataceae*, and *Phaeosphaeriaceae* (*Pleosporales*) (data not shown). Using only sequences from the conserved 5.8S region, the phylogenies of MS88 were investigated to clarify its familial placement. *Creosphaeria sassafras* and *Daldinia grandis* (*Xylariaceae*) were used as outgroups. The maximum-parsimony tree was generated from a maximum parsimony analysis of 26 taxa is shown in Fig. 1. The tree had a length (TL) of 93, a consistency index (CI) of 0.731, a homoplasy index (HI) of 0.269, a retention index (RI) of 0.846 and a rescaled consistency index (RC) of 0.618. MS88 clustered with species of *Lophiostomataceae* (*Dothideales*). MS88 could be further identified to lower taxonomic level.

Maximum-parsimony analyses of aligned ITS1 & 2 and 5.8S sequences of 23 taxa were performed. The tree obtained with bootstrap support is shown in Fig. 2. [tree length (TL) of 2497, a consistency index (CI) of 0.558, a homoplasy index (HI) of 0.442, a retention index (RI) of 0.551 and a rescaled consistency index (RC) of 0.307]. MS88 clustered with *Massarina walkeri* with bootstrap support of 100%.

Sequence analysis and percentage of nucleotide similarities indicated that MS88 belong to the genus *Massarina* (*Lophiostomataceae*). The current taxonomic status of *Massarina walkeri* needs further investigation as it is not found to be phylogenetically related to other *Massarina* species (Liew *et al*., 2002; Aptroot, 1998).

Identification of unitunicate mycelia sterilia MS9, MS11, MS19, MS25, MS27 and MS47

Blast search results showed that MS9, MS11, MS19, MS25, MS27 and MS47 are unitunicate ascomycetes. MS9, MS11, MS25 and MS47 had high sequence similarities (86-95%) with species of *Xylariaceae* (*Xylariales*), MS27 had relatively higher sequence similarities (98%) with species of the *Bionectriaceae* (*Hypocreales*) than other sequences, and MS19 had high sequence similarities (96-100%) with species of the *Phyllachoraceae* (*Phyllachorales*). Subsequently, the 5.8S sequence of MS9, MS11, MS19, MS25, MS27, MS47 and their relative sequences from GenBank were aligned. Sequences from the 5.8S region were aligned with other members of the Sordariomycetes. A total of 31 taxa were analysed and designated outgroups were *Guignardia vaccinii* (*Mycosphaerellales*) and *Botryosphaeria parva* (*Dothideales*).

Maximum parsimony analysis revealed that MS9, MS11, MS25 and MS47 are species of *Xylariaceae* (Fig. 3). MS25 clusters with *Hypoxylon perforatum* with 57% bootstrap support while MS27 formed a clade with *Bionectria ochroleuca* (*Bionectriaceae*) with a strong bootstrap support of 100%. MS19 clustered with *Glomerella* and *Colletotrichum* species (*Phyllachoraceae*) with a 94% bootstrap support.

Further analyses based on ITS and 5.8S gene sequences showed that MS9, MS11 and MS47 are *Xylaria* species (Fig. 4). All these mycelia sterilia are more related to *X. acuta* and *X. longipes*. *Xylaria* is widespread genus and common endophytic fungi in plants (Bayman *et al*., 1998; Fröhlich *et al*., 2000; Rogers, 2000).

MS25 belongs to family *Xylariaceae* (*Xylariales*) and further analyses showed that MS25 belongs to the genus *Hypoxylon*. The parsimonious tree had a tree length (TL) of 1258, a consistency index (CI) of 0.438, a homoplasy index (HI) of 0.517, a retention index (RI) of 0.504 and a rescaled consistency index (RC) of 0.244 (Fig. 5). MS25 clusters with *Hypoxylon* species and formed subclade with *Hypoxylon annulatum* and *H. fendleri* with a strong bootstrap support of 91%. *Hypoxylon* is the largest genera within *Xylariaceae* and have been found on dead wood and some species living as endophytes in living plants (Petrini and Petrini, 1985).

Sequences of ITS regions showed that MS19 had high similarities with *Glomerella* and its anamorph *Colletotrichum* species (96-100%) and results indicated that MS19 was related to *Phyllachoraceae*. The heuristic search under the maximum parsimony criterion yielded a tree (Fig. 6) with tree length (TL) of 608, a consistency index (CI) of 0.811, a homoplasy index (HI) of 0.189, a retention index (RI) of 0.772 and a rescaled consistency index (RC) of

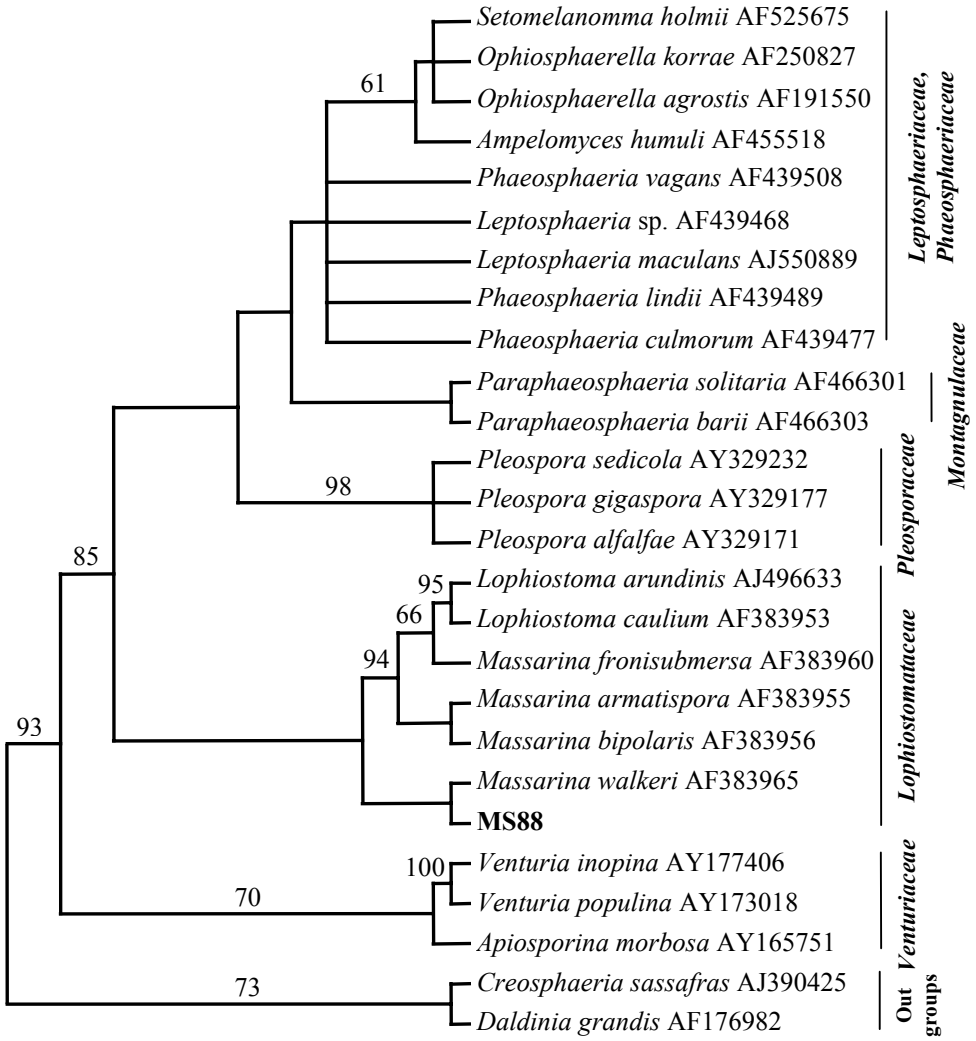

Fig. 1. Maximum-parsimony tree generated from the 5.8S sequences of 26 taxa showing the relationships of MS88 with reference taxa. The tree was rooted with *Creosphaeria sassafras* and *Daldinia grandis* (tree length = 93, CI = 0.731, HI = 0.269, RI = 0.846, RC = 0.618). Bootstrap values higher than or equal to 50% (1000 replicates) are shown at each branches.

0.626. MS19 clusters with *Glomerella cingulata* and *G. acutata* with a strong bootstrap support of 92%. The results indicated that MS19 is a *Glomerella* or *Colletotrichum* species.

Identification of the other 24 morphospecies using ITS and 5.8S sequences

Blast search results indicated that 24 morphospecies (MS1, MS2, MS3, MS4, MS5, MS6, MS7, MS8, MS10, MS12, MS13, MS14, MS15, MS16, MS17, MS18, MS20, MS21, MS22, MS23, MS24, MS26, MS28 and MS109),

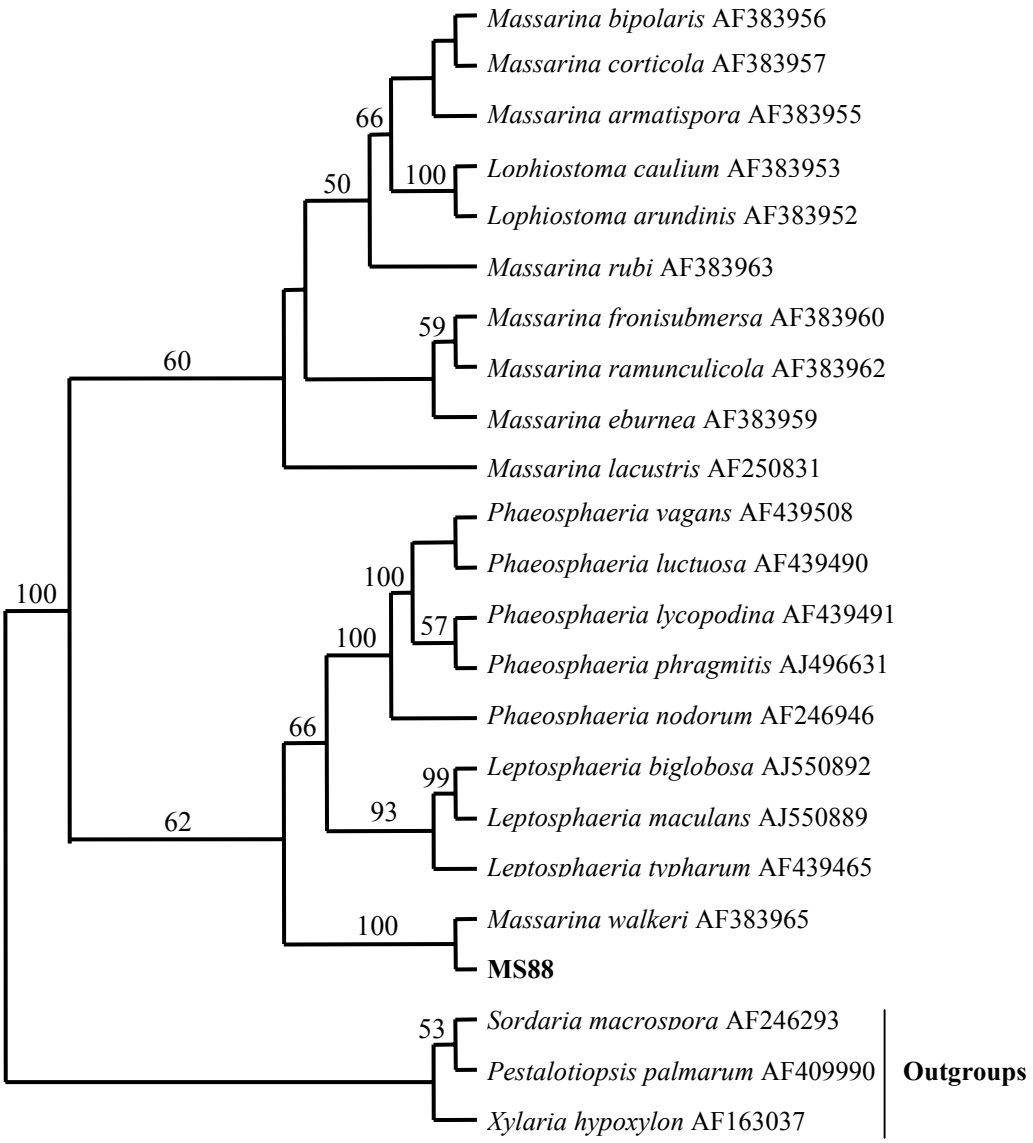

Fig. 2. Maximum-parsimony tree generated from ITS1, 5.8S and ITS2 sequences of 23 taxa showing the relationships of MS88 with reference taxa. The tree was rooted with *Sordaria macrospora*, *Pestalotiopsis palmarum* and *Xylaria hypoxylon* (tree length = 2497, CI = 0.558, RI = 0.551, RC = 0.307, HI = 0.442). Bootstrap values higher than or equal to 50% (1000 replicates) are shown at branches.

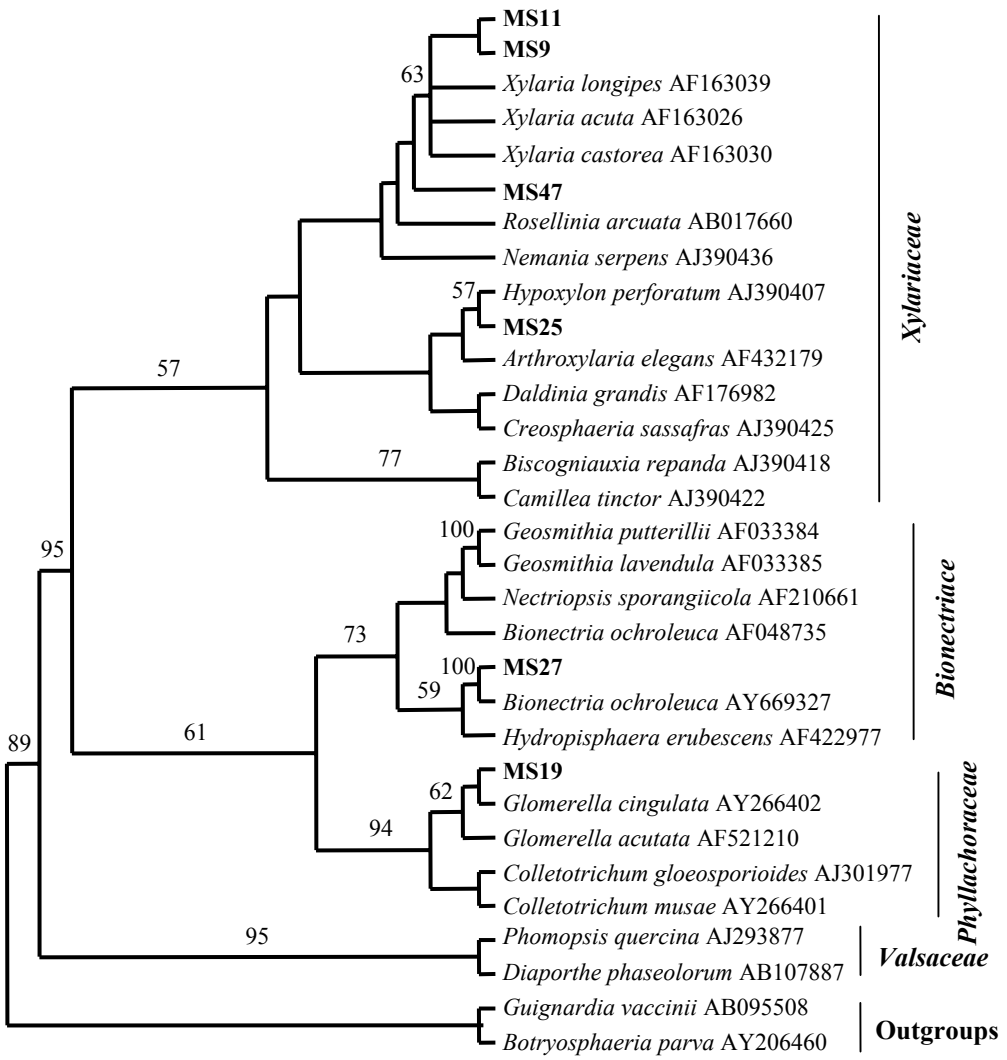

Fig. 3. Maximum parsimony tree analysis based on 5.8S gene sequences of 31 taxa, rooted with *Guignardia vaccinii* and *Botryosphaeria parva* (tree length = 322, CI = 0.573, HI = 0.427, RI = 0.739, RC = 0.423. This tree showing the relationship of MS9, MS11, MS19, MS25, MS27 and MS47 with reference taxa. Bootstrap values higher than or equal to 50% (1000 replicates) are shown at branches.

were highly similar to species of the *Diaporthe* (*Valsaceae*, *Diaporthales*) and its anamorph *Phomopsis* (86-98%).

Phylogenetic analyses indicated that all 24 morphospecies are closely related to *Diaporthe* and its anamorph *Phomopsis* with very strong bootstrap support of 100% (Fig. 7). MS28, MS24, MS15, MS20, MS109, MS13 and

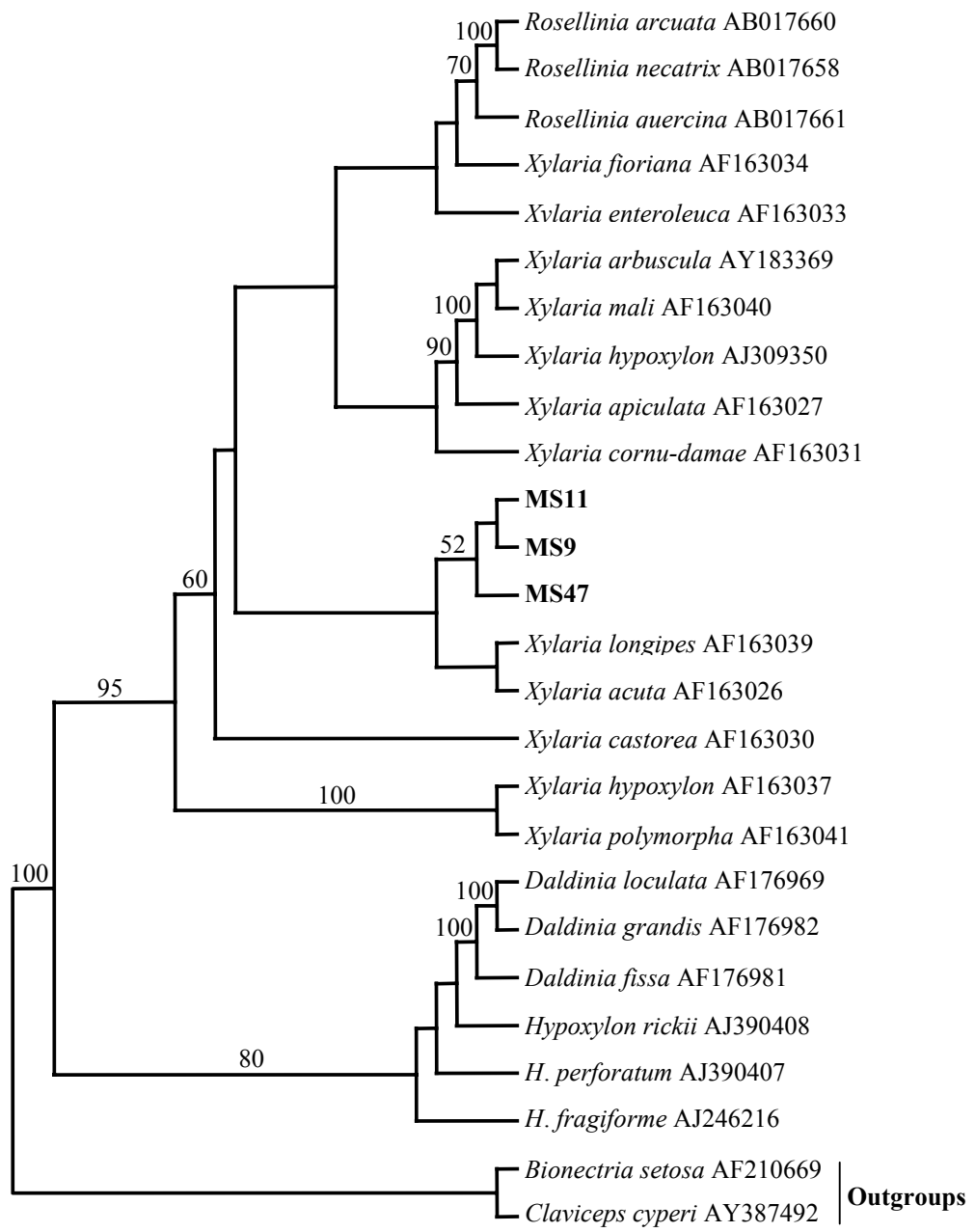

Fig. 4. Maximum parsimony tree generated from ITS1, 5.8S and ITS2 sequences of 26 taxa showing the relationships of MS9, MS11 and MS47 with referent taxa. The tree is rooted with *Bionectria setosa* and *Claviceps cyperi*. (TL = 933.5, CI = 0.563, HI = 0.437, RI = 0.596, RC = 0.336). Bootstrap values higher than or equal to 50% (1000 replicates) are shown at branches.

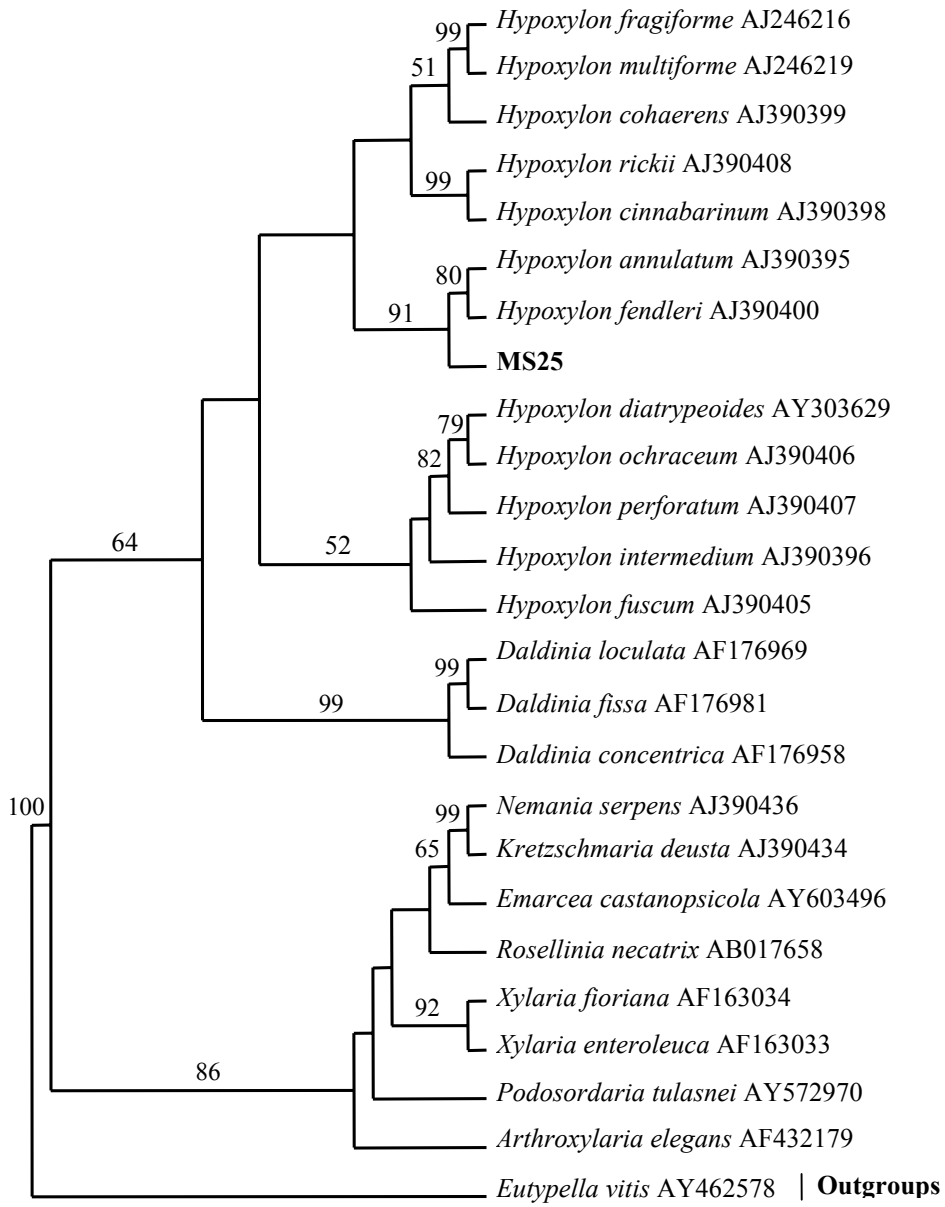

Fig. 5. Maximum parsimony tree generated from whole ITS1-5.8S-ITS2 sequence of 25 taxa showing the relationships of MS25 with reference taxa. The tree is rooted with *Eutypella vitis*. (TL = 1258, CI = 0.438, HI = 0.517, RI = 0.504, RC = 0.244). Bootstrap values higher than or equal to 50% (1000 replicates) are shown at branches.

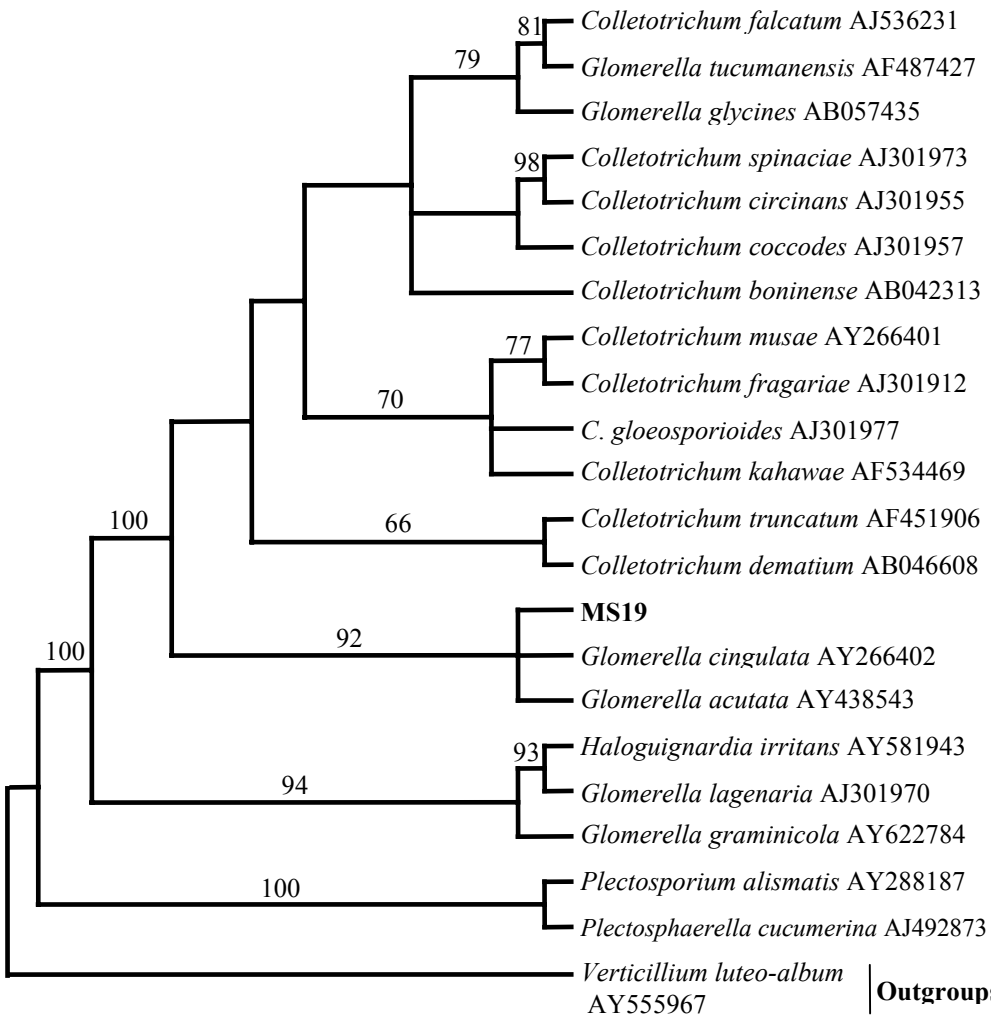

Fig. 6. Maximum parsimony tree generated from whole ITS1-5.8S-ITS2 sequence from 22 taxa showing the relationships of MS19 with reference taxa. The tree was rooted with *Verticillium luteo-album* (TL = 608, CI = 0.811, HI = 0.189, RI = 0.772, RC = 0.626). Bootstrap values higher than or equal to 50% (1000 replicates) are shown at branches.

MS10 appear to be more related to *Phomopsis eucommicola*. Furthermore, MS28, MS24, MS15, MS20, MS109 and MS10 clusters with each other with very strong bootstrap support while MS13 formed a terminal cluster with *Phomopsis eucommicola* but this relationship was not supported. MS2, MS5, MS3 and MS7 cluster with *Phomopsis amygdali* with a strong bootstrap support of 100%. MS1, MS21, MS14, MS22, MS23, MS8 and MS6 interspersed with four *Phomopsis* (*P. eucommii*, *P. lagerstroemiae*, *P. asparagi* and *P. saccharata*) and *Diaporthe meridionalis*. Within this clade MS1, MS21

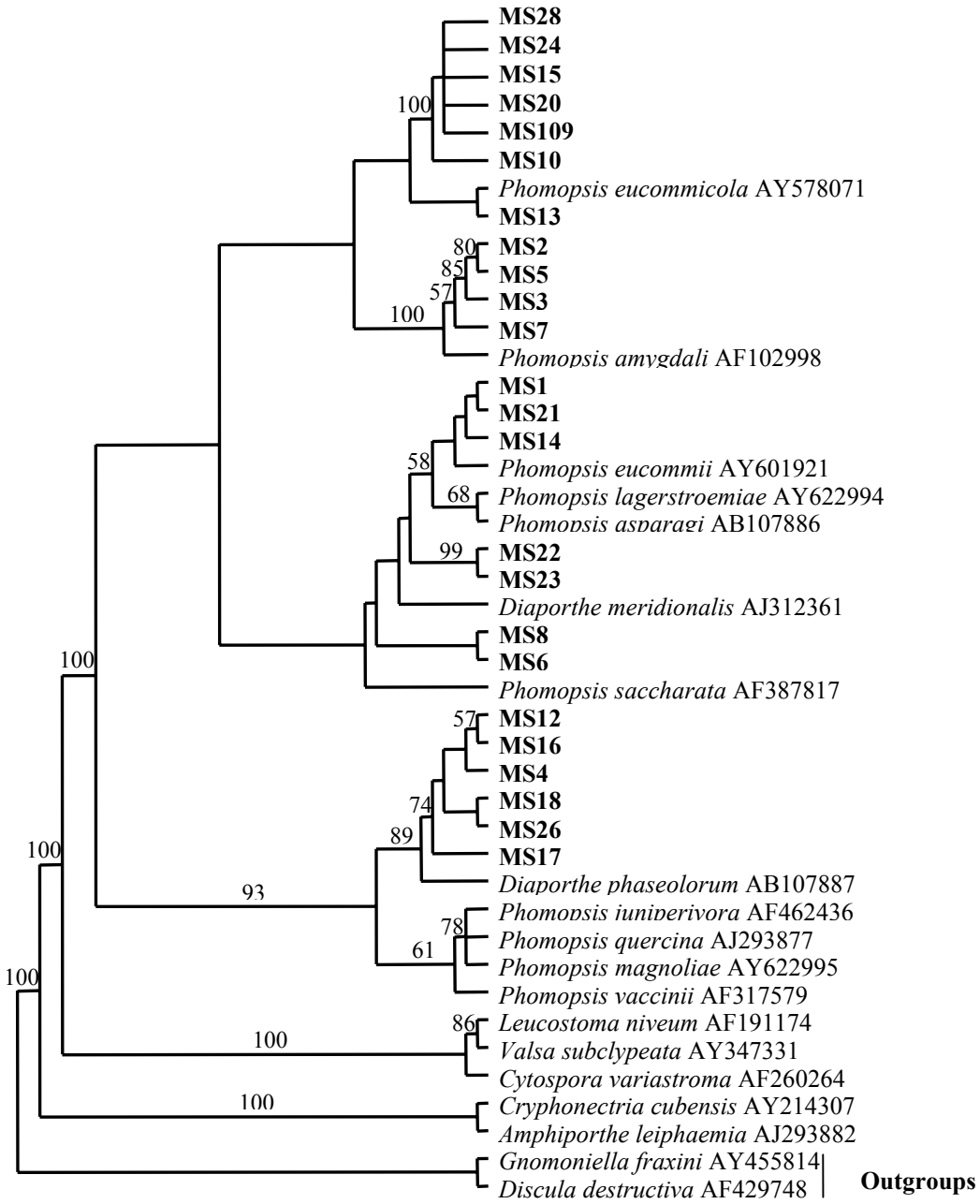

Fig. 7. Maximum parsimony tree generated from whole ITS1-5.8S-ITS2 sequence from 43 taxa rooted with *Gnomoniella fraxini* and *Discula destructiva* (TL = 608, CI = 0.811, HI = 0.189, RI = 0.772, RC = 0.626). This tree showing the relationships of the 24 morphospecies with reference taxa. Bootstrap values higher than or equal to 50% (1000 replicates) are shown at branches.

and MS14 appears to be more related to *Phomopsis eucommii* while MS22 and MS23 cluster with each other with high bootstrap support. MS12, MS16, MS4, MS18, MS26 and MS17 formed a strongly supported clade (89 %). It is highly probable that all these isolates are actually *Diaporthe* species.

Comparison of taxa identified by traditional and molecular techniques

Sequence analysis of the 5.8S gene and ITS regions of rDNA have been widely used in taxonomic placement of fungi at different levels in recent years. Guo *et al*. (2000) identified endophytes using 5.8S and ITS regions and showed that unidentified morphotypes from *Livistona chinensis* were mainly filamentous Ascomycota. Guo *et al*. (2001) detected and identified endophytic fungi from frond tissues of *Livistona chinensis* based on rDNA sequence and reported that one isolate belonged to the Basidiomycota and four isolates belonged to Ascomycota that could be identified to family and genus level (*Herpotrichiellaceae*, *Glomerella*, *Mycosphaerella*). Guo *et al*. (2000) identified 19 morphotypes from *Livistona chinensis* based on cultural morphology, growth rates and ribosomal DNA (rDNA) sequence analysis. In addition, Guo *et al*. (2003) identified 18 morphotype strains of mycelia sterilia from *Pinus tabulaeformis* to various taxonomic levels based on nuclear ribosomal DNA (nrDNA) sequence analysis. Lacap *et al*. (2003) compared nucleotide sequence similarities of ITS regions and 5.8S gene and identified 6 morphotypes of mycelia sterilia based on rDNA sequence analysis, all of which have been identified to genus level.

In this study we have used a similar approach to identify non-sporulating endophytes from *Magnolia liliifera*. Forty-six taxa in eight genera were identified following the identification of sporulating isolates. A further 31 morphotypes were recognised for the non-sporulating isolates resulting in 77 possible taxa. Molecular analysis showed these morphotypes belong in five families (six genera), with only *Bionectria* and *Hypoxylon* being recognised as additional genera with respect with previous studies. Most other non-sporulating isolates were placed in *Colletotrichum/Glomerella*, *Diaporthe/Phomopsis* or *Xylaria* and it is unclear whether these morphotypes were the same as the sporulating isolates where these genera were also common. Ten sporulating forms of *Colletotrichum* were identified and one non-sporulating form was identified by molecular analysis, but it is not possible to determine species diversity using the data available. Thirteen forms of *Xylaria* were identified based on colony characters and a further three forms clustered together in the sequence analysis. Eleven sporulating forms of *Diaporthe*/*Phomopsis* were recognised, while 24 forms were established by molecular analysis although many of these may be the same species. Molecular

finding indicate that these are at least four different strains or species of *Phomopsis* and two different species of *Diaporthe*.

This is now the fourth study that has employed molecular techniques to increase our knowledge of fungal diversity of endophytes. However, we feel that little additional information has been gathered considering the amount of effort and costs involved. The main problem is that we have used traditional techniques, i.e. surface sterilisation followed by transfer of mycelium growing from tissues placed on agar. Most previous endophytic studies have isolated relatively poorly diverse groups of taxa comprising mostly *Colletotrichum*, *Diaporthe/Phomopsis, Fusarium, Guignardia/Phyllosticta* and *Xylariaceae* species. All of these taxa grow relatively quickly in artificial media and therefore may not reflect the majority of endophytes within plants. Certain taxa that appear as saprobes within days of leaf fall (e.g. *Hyponectria* sp.) have never been isolated as endophytes, although it is highly likely that they are present in the leaves at senescence. We therefore suspect that the methods presently used to detect endophytes bias the result to those that readily grow in culture. By sequencing non-sporulating isolates we are only perpetuating this phenomena and that is why most of the morphotypes are identified in the above common endophytic genera.

Future studies on endophytes must take a completely novel approach. Our present endophyte methodology probably results in data that has low biological significance and presumably a small fraction of the total endophytic genotype diversity has been detected. Although the data provides pleasant malleable data, suitable for statistical evaluation, it may not have much relevance to the fungi living inside plants. Therefore until new methods are employed to establish endophyte diversity in living plants (e.g. DGGE) we see little value in work of this sort being carried out.

The ecological function of these endophytes are also not fully understood. In addition, it is unknown whether endophytic, parasitic and saprobe taxa possess different phylogenetic histories (Photita *et al*., 2004). Using rDNA sequence analyses, previous work and this study have focused primarily on identification of endophytes but information on molecular diversity in general and phylogenetic affinities are still lacking.

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