Taxonomic placement of sterile morphotypes of endophytic fungi from *Pinus tabulaeformis* (*Pinaceae*) in northeast China based on rDNA sequences

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In a survey of the endophytic fungi from *Pinus tabulaeformis* in northeast China, approximately 11% of isolates did not produce spores, although various techniques were employed to promote sporulation. These sterile mycelia were grouped into 74 morphotypes based on similar cultural characters. Arrangement of isolates into morphotypes does not reflect species phylogeny, and therefore they were further identified based on phylogenetic analysis of the 5.8S gene and internal transcribed spacer (ITS1 and ITS2) regions, as well as sequence similarity comparison. Sequence analyses indicated that five morphotypes were *Basidiomycota*, while the other 69 morphotypes were *Ascomycota*. Further analyses resulted in two morphotypes being identified as *Fusarium sporotrichioides* and *Schizophyllum commune*. Twenty-two morphotypes were identified to generic level, seven to family (*Lophiostomataceae* and *Valsaceae*) level, and four to order (*Helotiales* and *Pezizales*) level. The 74 morphotypes were classified into 64 taxa, which indicates a high diversity of fungi on *Pinus*.

Key words: endophyte; molecular identification; mycelia sterilia; ribosomal RNA gene.

Introduction

Traditionally a culture-dependent process has been employed in endophytic studies (e.g. Petrini *et al*., 1982; Rodrigues, 1994; Fisher *et al*., 1995; Lodge *et al*., 1996; Brown *et al*., 1998; Taylor *et al*., 1999; Tomita, 2003; Kumar and Hyde, 2004; Suryanarayan and Thennarasan, 2004). Endophytic isolates can only be identified based on morphological characteristics if they sporulate on the media. Despite the development of various methods to promote sporulation, e.g. by growing them on modifications of artificial media and under various incubation conditions (Guo

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et al., 1998; Taylor *et al*., 1999), the number of isolates that do not sporulate ranges from 4.5 to 54% (Petrini *et al*., 1982, Espinosa-Garcia and Langenheim, 1990; Johnson and Whiteney, 1992; Fisher *et al*., 1993; Guo *et al*., 2000; Photita *et al*., 2001; Cannon and Simmons, 2002; Kumaresan and Suryanarayanan, 2002). Since conventional classification of fungi relies heavily on reproductive structures, these non-sporulating mycelia sterilia cannot be provided with taxonomic names. In order to appreciate the considerable diversity of these mycelia sterilia in culture, they are generally categorised as 'morphotypes' based on similar cultural characters (Taylor *et al*., 1999; Fröhlich *et al*., 2000; Guo *et al*., 2000; Arnold *et al*., 2001; Cannon and Simmons, 2002). This approach has been shown to be justifiable and related to endophyte diversity (Lacap *et al*., 2003).

Arrangement of taxa into different morphotypes, however, does not reflect species phylogeny, because morphotypes are not real taxonomic entities. Isolates having similar cultural characters are grouped into the same morphotype, but it may be that isolates included in a morphotype comprise distantly related taxa, or isolates divided into different morphotypes belong to the same species (Lacap *et al*., 2003). Molecular methods are therefore required for the identification and understanding of the diversity of these endophytic mycelia sterilia. A complementary approach to fungal taxonomy or phylogeny is represented by DNA sequence analyses. Because the 5.8S gene is highly conserved, it has been used successfully to assess phylogenetic relationships at higher taxonomic levels. Non-coding rDNA spacer regions, such as the internal transcribed spacers (ITS1 and ITS2), benefit from a fast rate of evolution, resulting in greater sequence variation between closely related species. Thus, fungal ITS sequences generally provide greater taxonomic resolution than sequences generated from coding regions, and are now routinely used in phylogenetic studies, as well as in the detection and identification of fungi (Guo *et al*., 2000, 2001, 2003; Arnold *et al*., 2001; Okane *et al*., 2001; Baayen *et al*., 2002; Lord *et al*., 2002; Anderson *et al*., 2003; Jeewon *et al*., 2004).

This study reports fungal endophytic communities isolated from *Pinus tabulaeformis* in Liaoning of northeast China. Despite employing various artificial media and subjecting the cultures to different incubation regimes, a high number of mycelia sterilia was isolated. To gain a better insight on the diversity and taxonomy of these endophytic communities, sterile mycelia were grouped into morphotypes based on similar cultural characters. The taxonomic placement and phylogenetic relationships of these sterile mycelia have also been investigated through pairwise sequence alignment and parsimony analysis of 5.8S gene and ITS regions of rDNA.

Materials and methods

Site and sampling procedure

The Chinese oil pine *Pinus tabulaeformis* selected for this study is broadly distributed in temperate regions of China and is the dominant taxon of northern Chinese forests. The pine grows to 25 m high, with needles up to 15 cm long. The present study was carried out at two sites of Liaoning Province of northeast China, i.e. Fenghuangshan (alt. 350-500m, co-ordinates 123˚32'E/40˚2'N) located southeast of Liaoning Province and Lingyuan (alt. 300-400m, co-ordinates 118˚50'E/40˚25'N) located northwest of Liaoning Province. Thirty individual mature pines were randomly chosen for this study at each site. The pines were about 30 years old and 20 m apart. One branch containing three age-classes (1-, 2-, and 3-yr-old branches), were randomly collected from each plant in August 2002. These branches were immediately placed in plastic bags, labelled, and taken to the laboratory. Samples were stored in 4˚C and processed within 3 days of collection.

Isolation and culture of endophytic fungi

The sampling regime was designed to isolate as many endophyte species as possible from different tissues. Each selected branch was divided into three age classes, i.e. 1-, 2-, and 3-yr-old branches with needles. Needles were removed from each age class branch, and the branches were cut into 5 mm segments. Three branch segments and three needles were randomly selected from each age class. The needles were also cut into 5 mm segments, and the branch segments were separated into bark and xylem. Eight needle, eight bark, and eight xylem segments were randomly selected from each age class. A total of 60 trees from both Fenghuangshan and Lingyuan were selected and 4320 segments were cultured, including 1440 needles, 1440 bark and 1440 xylem segments (8 segments × 1 needle/bark/xylem × 3 age-classes × 60 trees).

Surface sterilisation was performed according to Guo *et al*. (2000). After surface sterilisation, sets of four segments were evenly placed in each 90 mm Petri dishes containing malt extract agar (MEA, 2%; Sigma, St Louis, MO) supplemented with Rose Bengal (30 mg l^{-1}) to slow down fungal growth. Streptomycin sulphate (50 mg l^{-1}, North China Medicine, Shijiazhuang, China) was added to suppress bacterial growth. Petri dishes were sealed, incubated for 2 months at 25˚C and examined periodically. When colonies developed, they were transferred to new Petri dishes with MEA. Subcultures were then incubated on different media which included potato dextrose agar (PDA, 2%, Difco, Detroit), corn meal agar (CMA, 2%, Difco) and tap water agar (TWA,

0.8%). In addition, sterile needle and branch segments of *P. tabulaeformis* were also included to promote sporulation (Taylor *et al*., 1999; Guo *et al*., 2000). Isolates were incubated at 25˚C, with cool white fluorescent light for a light regime of 12:12 hours light:dark to induce sporulation.

Sources of sterile strains

Subcultures were examined periodically and identified based on morphological characters. The remaining cultures that failed to sporulate were named sterile mycelia. These sterile mycelia were divided into different 'morphotypes' based on similarity of cultural characteristics on MEA, e.g. colony colour, texture, and growth rates (Guo *et al*., 1998, 2000; Taylor *et al*., 1999; Arnold *et al*., 2000; Fröhlich *et al*., 2000). The morphotypes were given the prefix EMS recovered from Fenghuangshan and WMS recovered from Lingyuan.

Voucher morphotype strains are maintained in the China General Microbiological Culture Collection Center (CGMCC).

DNA extraction, PCR amplification and DNA sequencing

A hyphal tip was obtained from each fresh culture using a dissecting microscope and incubated on MEA in the dark at 25˚C for 3-30 days. Genomic DNA was extracted from fresh cultures following the protocol of Guo *et al*. (2000).

Primers ITS5 and ITS4 (White *et al*., 1990) were used to amplify the ITS regions. The DNA fragment was amplified in an automated thermal cycler (PTC-100^{TM}; MJ Research, Watertown, MA). Amplification was performed in a 50-μl reaction volume which contained PCR buffer (10 mM KCL, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCL, pH 8.8, 0.1% Triton X-100), 1.5 mM $MgCL_2$, 200 μM of each deoxyribonucleotide triphosphate, 15 pmol of each primer, *ca*. 100 ng template DNA, and 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI). The thermal cycling program was as follows: 3 minutes initial denaturation at 95˚C, followed by 35 cycles of 40 seconds denaturation at 94˚C, 50 seconds primer annealing at 52˚C, 1 minutes extension at 72˚C, and a final 10 minutes extension at 72˚C. A negative control using water instead of template DNA was included in the amplification process. Four microlitres of PCR products from each PCR reaction were examined by electrophoresis at 75 V for 2 h in a 0.8% (w/v) agarose gel in 1× TAE buffer (40 mM Tris, 1 mM EDTA, pH 8.0) and visualized with UV light after staining with ethidium bromide (0.5 μg ml^{-1}).

PCR products were purified using minicolumns (Wizard® PCR Preps DNA Purification System, Promega) according to the manufacturer's protocol. Purified PCR products were directly sequenced in the ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, CA). Primers ITS5 and ITS4 were used in the sequencing reactions.

Sequence data analysis

Each morphotype sequence was used as query sequence to search for similar sequences from GenBank and EMBL using BLAST program. The most similar reference sequences with query sequences were obtained and used for subsequent phylogenetic analyses with standard reference sequences. Reference taxa were included in all of the phylogenetic analyses to provide a phylogenetically established frame-work into which the test morphotype strains could be incorporated. These taxa were selected on the basis of, primarily, their established taxonomic disposition and, secondarily, for some, on their high nucleotide similarity with the sterile morphotype strains. These 5.8S gene and ITS (both ITS1 and ITS2) sequences were aligned using Clustal X program (Thompson *et al*., 1997) and the result were adjusted manually where necessary to maximize alignment. The alignment data were subsequently used for maximum parsimony analysis in which searches for most parsimonious trees were conducted with the heuristic search algorithms with tree-bisection-reconnection (TBR) branch swapping in PAUP 4.0b1a (Swofford, 1988). For each search, 1000 replicates of random stepwise sequence addition were performed. Maxtrees set to 10000, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. A preliminary analysis showed no major topographical differences between trees obtained with gaps treated either as fifth character state or as missing data (results not shown). Character states were all treated as unordered and of equal weight. Statistical support for the internal branches was estimated by bootstrap analysis with 1000 replications.

Results

The general origin of the morphotypes

A total of 205 sterile mycelia isolated from *P. tabulaeformis* were grouped into 74 morphotypes in this study, which are *Ascomycota* (69 morphotypes) and *Basidiomycota* (morphotypes EMS23, WMS2, WMS18, WMS21 and WMS25) as indicated by phylogenetic analysis of the 5.8S gene. We used reference taxa, from the *Ascomycota*, *Basidiomycota* and

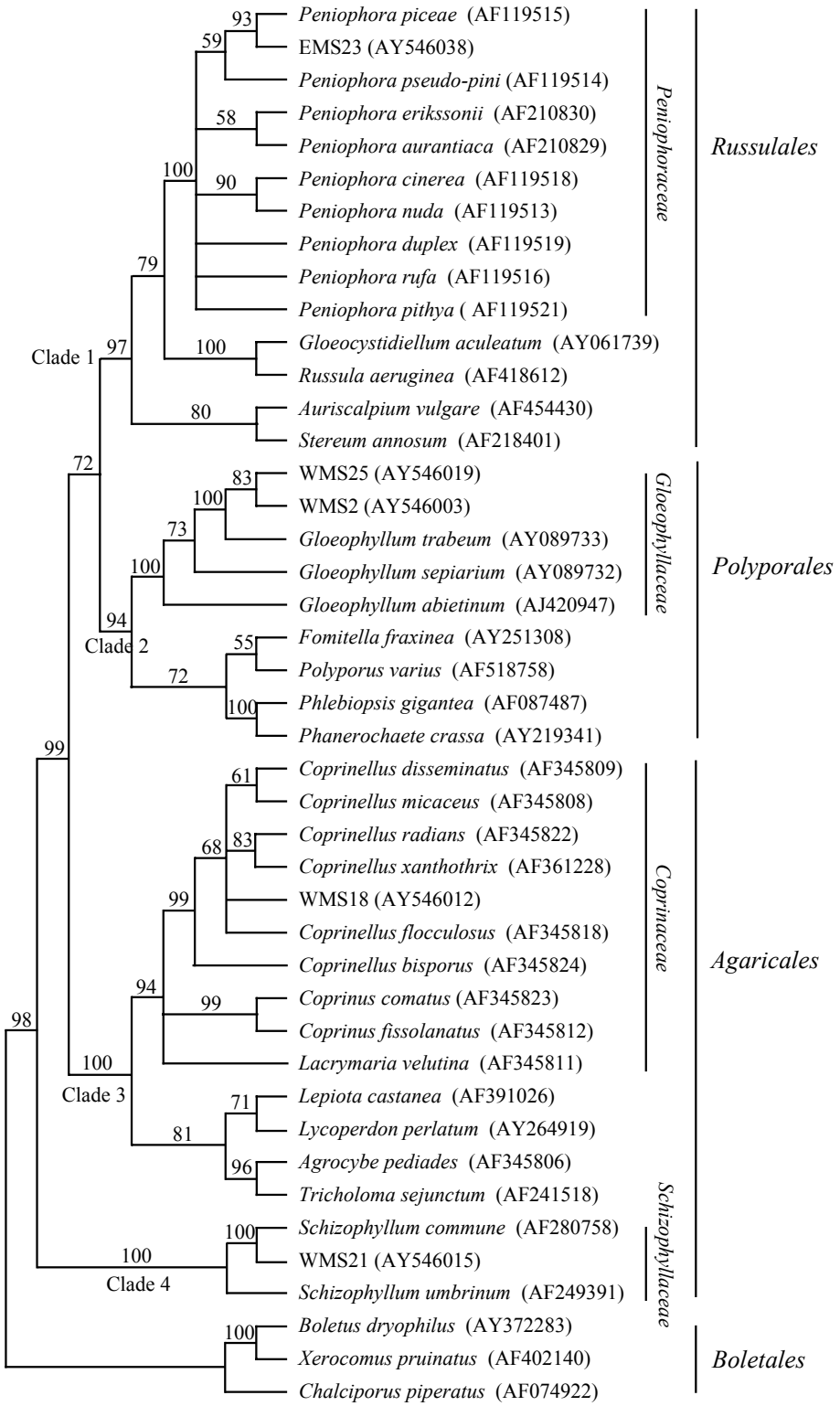
Zygomycota, as well as searching for similar 5.8S gene and ITS sequences using the BLAST program in GenBank and EMBL (data not shown).

Classification of basidiomycetous morphotypes

Based on the results of search for similar 5.8S gene and ITS sequences, the alignment of the sequences of the 43 taxa comprised 798 base sites. The heuristic research recovered two equally parsimonious trees of 2728 steps, and the strict consensus tree is shown in Fig. 1. In this tree there were four clades, of which morphotype EMS23 and 13 reference taxa of five families in the *Russulales* formed the first clade (i.e. clade 1) with 97% bootstrap support. Within the clade 1, morphotype EMS23 and nine *Peniophora* species are closely related to each other and this relationship is supported by 100% bootstrap support. Two morphotypes (WMS2 and WMS25) and seven reference taxa of five families in the *Polyporales* formed the second clade (i.e. clade 2) with 94% bootstrap support. Within the clade 2, morphotypes WMS2 and WMS25 and three *Gloeophyllum* species are closely related to each other and this relationship is supported by 100% bootstrap support. Morphotype WMS18 and 13 taxa of the *Coprinaceae* formed the third clade (i.e. clade 3) with 100% bootstrap support. Within clade 3, morphotype WMS18 and six *Coprinellus* species are closely related to each other and this relationship is supported by 99% bootstrap support. Morphotype WMS21 and two *Schizophyllum* species of the *Schizophyllaceae* in the *Agaricales* formed the fourth clade (i.e. clade 4) with 100% bootstrap support. Within the clade 4, morphotype WMS21 formed a terminal clade with *S. commune* with 100% bootstrap support.

The results of the 5.8S gene and ITS sequence similarity comparisons showed that strain EMS23 had a high nucleotide similarity with *P. piceae* (91.7%). The 5.8S gene and ITS sequence between morphotypes WMS25 and WMS2 is identical, and the morphotypes have a high sequence similarity (97.8%) with *G. trabeum*. Morphotype WMS18 had high nucleotide similarities with *Coprinellus* species (83.2-94%). Morphotype WMS21 had an identical sequence as *S. commune*.

Fig. 1. Strict consensus tree of two equally parsimonious trees generated from the 5.8S gene and ITS (ITS1/2) sequences of 43 taxa showing the relationships of five morphotypes with reference taxa. The tree rooted with *Boletus dryophilus*, *Chalciporus piperatus* and *Xerocomus pruinatus* (TL=2728, CI=0.4622, HI=0.5378, RI=0.7261, RC=0.3357). Bootstrap values greater than or equal to 50% (1000 replicates) are shown at branches.


Peniophora piceae (AF119515)
EMS23 (AY546038)
Peniophora pseudo-pini (AF119514)
Peniophora erikssonii (AF210830)
Peniophora aurantiaca (AF210829)
Peniophora cinerea (AF119518)
Peniophora nuda (AF119513)
Peniophora duplex (AF119519)
Peniophora rufa (AF119516)
Peniophora pithya (AF119521)
Gloeocystidiellum aculeatum (AY061739)
Russula aeruginea (AF418612)
Auriscalpium vulgare (AF454430)
Stereum annosum (AF218401)
WMS25 (AY546019)
WMS2 (AY546003)
Gloeophyllum trabeum (AY089733)
Gloeophyllum sepiarium (AY089732)
Gloeophyllum abietinum (AJ420947)
Fomitella fraxinea (AY251308)
Polyporus varius (AF518758)
Phlebiopsis gigantea (AF087487)
Phanerochaete crassa (AY219341)
Coprinellus disseminatus (AF345809)
Coprinellus micaceus (AF345808)
Coprinellus radians (AF345822)
Coprinellus xanthothrix (AF361228)
WMS18 (AY546012)
Coprinellus flocculosus (AF345818)
Coprinellus bisporus (AF345824)
Coprinus comatus (AF345823)
Coprinus fissolanatus (AF345812)
Lacrymaria velutina (AF345811)
Lepiota castanea (AF391026)
Lycoperdon perlatum (AY264919)
Agrocybe pediades (AF345806)
Tricholoma sejunctum (AF241518)
Schizophyllum commune (AF280758)
WMS21 (AY546015)
Schizophyllum umbrinum (AF249391)
Boletus dryophilus (AY372283)
Xerocomus pruinatus (AF402140)
Chalciporus piperatus (AF074922)
Peniophoraceae
Gloeophyllaceae
Coprinaceae
Schizophyllaceae
Russulales
Polyporales
Agaricales
Boletales
Clade 1
Clade 2
Clade 3
Clade 4
93
59
58
100
90
79
97
100
80
72
83
100
73
100
94
55
72
100
99
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68
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81
96
100
100
98
100

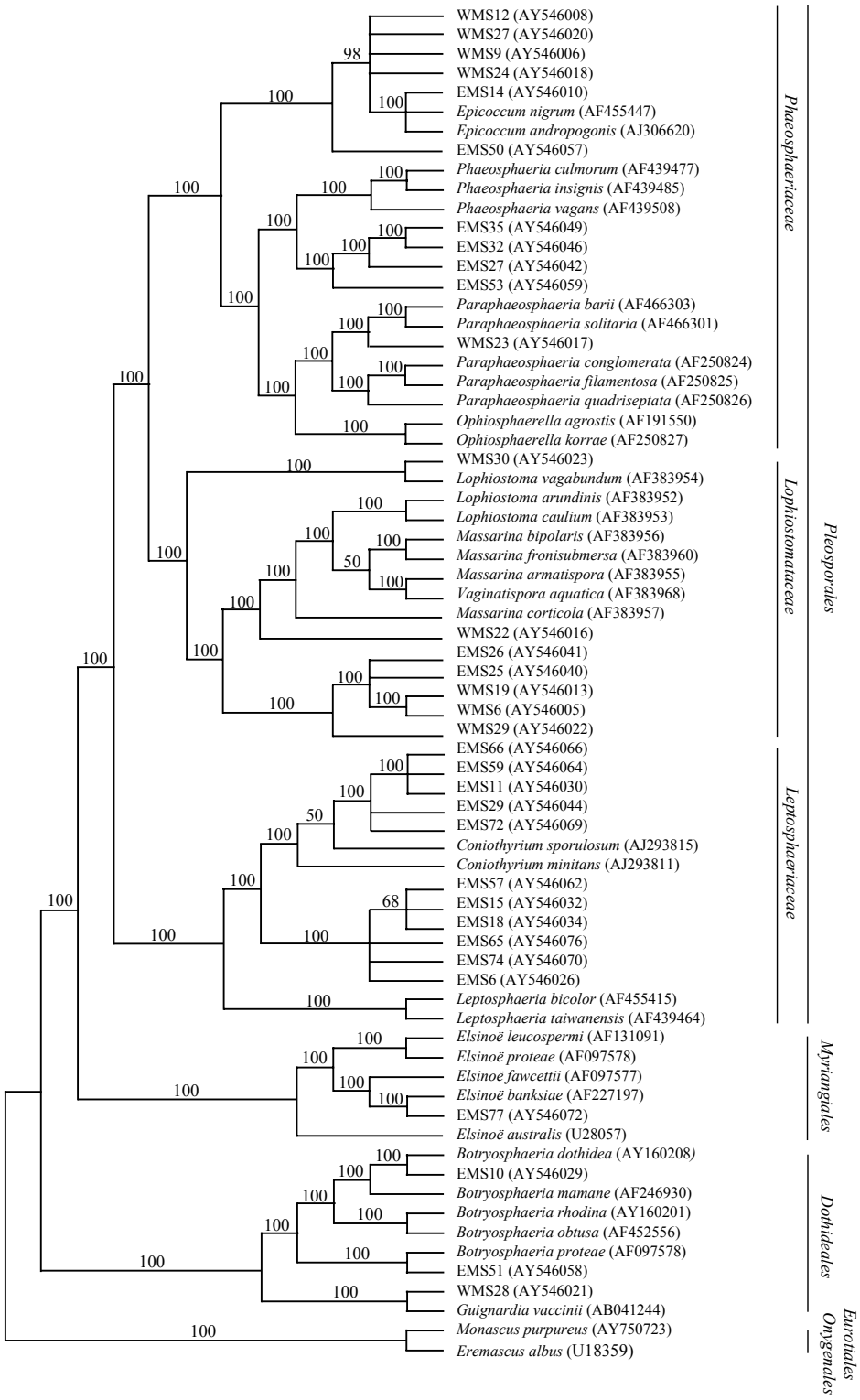
***Classification of ascomycetous morphotypes* - Dothideomycetidae**

Based on the results of search for similar 5.8S gene and ITS sequences, 35 reference sequences of the *Leptosphaeriaceae*, *Lophiostomataceae* and *Phaeosphaeriaceae* (*Pleosporales*), the *Elsinoaceae* (*Myriangiales*) and the *Botryosphaeriaceae* (*Dothieales*) were obtained from GenBank and EMBL for phylogenetic analysis. *Eremascus albus* (*Eremascaceae*) and *Monascus purpureus* (*Monascaceae*) of the *Eurotiomycetidae* were used as the outgroup.

The alignment of the 5.8S gene and ITS sequences of the 37 reference taxa and 33 morphotypes resulted in a data matrix of 677 base sites. The strict consensus tree is shown in Fig. 2. In this tree there were three clades, of which 29 morphotypes and 24 reference taxa of the *Leptosphaeriaceae*, *Lophiostomataceae* and *Phaeosphaeriaceae* in the *Pleosporales* formed the first clade with 100% bootstrap support. Within this clade, 11 morphotypes and 12 reference taxa of the *Phaeosphaeriaceae* formed a subclade with 100% bootstrap support. Furthermore, six morphotypes (EMS14, EMS50, WMS9, WMS12, WMS24 and WMS27) and two *Epicoccum* species are closely related to each other and this relationship is supported by 100% bootstrap support. Four morphotypes (EMS27, EMS32, EMS35 and EMS53) and three *Phaeosphaeria* species are closely related to each other and this relationship is supported by 100% bootstrap support. Morphotype WMS23 and five *Paraphaeosphaeria* species are closely related to each other and this relationship is supported by 100% bootstrap support. Seven morphotypes and eight reference taxa of the *Lophiostomataceae* formed the second subclade with 100% bootstrap support, furthermore, morphotype WMS30 and *Lophiostoma vagabundum* are closely related to each other and this relationship is supported by 100% bootstrap support, the other six morphotypes (EMS25, EMS26, WMS6, WMS19, WMS22 and WMS29) did not cluster together with any reference taxa. Eleven morphotypes and four reference species formed the third subclade, and they are closely related to each other and this relationship is supported by 100% bootstrap support. Both them belong to *Leptosphaeria* and its anamorph *Coniothyrium* of the *Leptosphaeriaceae*.

Morphotype EMS77 and five *Elsinoë* species of the *Elsinoaceae* (*Myriangiales*) formed the second clade with 100% bootstrap support, furthermore morphotype EMS77 and five *Elsinoë* species are closely related to

Fig. 2. Strict consensus tree of 23755 equally parsimonious trees generated from the 5.8S gene and ITS (ITS1/2) sequences of 70 taxa showing the relationships of 33 morphotypes with reference taxa. The tree rooted with *Eremascus albus* and *Monascus purpureus* (TL=2426, CI=0.4163, HI=0.5837, RI=0.8005, RC=0.3333). Bootstrap values greater than or equal to 50% (1000 replicates) are shown at branches.


WMS12 (AY546008)
WMS27 (AY546020)
WMS9 (AY546006)
WMS24 (AY546018)
EMS14 (AY546010)
Epicoccum nigrum (AF455447)
Epicoccum andropogonis (AJ306620)
EMS50 (AY546057)
Phaeosphaeria culmorum (AF439477)
Phaeosphaeria insignis (AF439485)
Phaeosphaeria vagans (AF439508)
EMS35 (AY546049)
EMS32 (AY546046)
EMS27 (AY546042)
EMS53 (AY546059)
Paraphaeosphaeria barii (AF466303)
Paraphaeosphaeria solitaria (AF466301)
WMS23 (AY546017)
Paraphaeosphaeria conglomerata (AF250824)
Paraphaeosphaeria filamentosa (AF250825)
Paraphaeosphaeria quadriseptata (AF250826)
Ophiosphaerella agrostis (AF191550)
Ophiosphaerella korrae (AF250827)
WMS30 (AY546023)
Lophiostoma vagabundum (AF383954)
Lophiostoma arundinis (AF383952)
Lophiostoma caulium (AF383953)
Massarina bipolaris (AF383956)
Massarina fronisubmersa (AF383960)
Massarina armatispora (AF383955)
Vaginatispora aquatica (AF383968)
Massarina corticola (AF383957)
WMS22 (AY546016)
EMS26 (AY546041)
EMS25 (AY546040)
WMS19 (AY546013)
WMS6 (AY546005)
WMS29 (AY546022)
EMS66 (AY546066)
EMS59 (AY546064)
EMS11 (AY546030)
EMS29 (AY546044)
EMS72 (AY546069)
Coniothyrium sporulosum (AJ293815)
Coniothyrium minitans (AJ293811)
EMS57 (AY546062)
EMS15 (AY546032)
EMS18 (AY546034)
EMS65 (AY546076)
EMS74 (AY546070)
EMS6 (AY546026)
Leptosphaeria bicolor (AF455415)
Leptosphaeria taiwanensis (AF439464)
Elsinoë leucospermi (AF131091)
Elsinoë proteae (AF097578)
Elsinoë fawcettii (AF097577)
Elsinoë banksiae (AF227197)
EMS77 (AY546072)
Elsinoë australis (U28057)
Botryosphaeria dothidea (AY160208)
EMS10 (AY546029)
Botryosphaeria mamane (AF246930)
Botryosphaeria rhodina (AY160201)
Botryosphaeria obtusa (AF452556)
Botryosphaeria proteae (AF097578)
EMS51 (AY546058)
WMS28 (AY546021)
Guignardia vaccinii (AB041244)
Monascus purpureus (AY750723)
Eremascus albus (U18359)
Phaeosphaeriaceae
Lophiostomataceae
Leptosphaeriaceae
Pleosporales
Myriangiales
Dothideales
Eurotiales
Onygenales

each other and this relationship is supported by 100% bootstrap support. Morphotypes EMS10, EMS51 and WMS28 and six reference species of the *Botryosphaeriaceae* (*Dothideales*) formed the third clade with 100% bootstrap support. Within this clade, two morphotypes (EMS10 and EMS51) and five *Botryosphaeria* species are closely related to each other and this relationship is supported by 100% bootstrap support. Morphotype WMS28 and *Guignardia vaccinii* are closely related to each other and this relationship is supported by 100% bootstrap support.

The results of the 5.8S gene and ITS sequence similarity comparisons showed that morphotype EMS14 had relatively high nucleotide similarity (99%) with two *Epicoccum* species. Morphotypes WMS9, WMS12, WMS24 and WMS27 had identical sequences, and these four morphotypes and morphotype EMS50 had high nucleotide similarities (95.6-96.7%) with two *Epicoccum* species. There were high nucleotide similarities (98.2-98.8%) among morphotypes EMS27, EMS32, EMS35 and EMS53, and these four morphotypes had high nucleotide similarity (89.7-92.3%) with three *Phaeosphaeria* species. Morphotype WMS23 had high nucleotide similarities (83.4-91.3%) with five *Paraphaeosphaeria* species.

Morphotype WMS30 had a high nucleotide similarity (90.3%) with *Lophiostoma vagabundum*. There were identical sequences between morphotypes WMS6 and WMS19, and morphotypes EMS25 and EMS26, respectively. Furthermore, there were nine (98.3% nucleotide similarity) nucleotide differences between morphotypes WMS6 (WMS19) and EMS25 (EMS26). There were nine base pair differences (98.3% nucleotide similarity) between morphotypes WMS29 and EMS25 (EMS26).

Morphotypes EMS11, EMS59 and EMS66 had identical sequences. There were two nucleotide differences between morphotypes EMS29 and EMS72, between morphotypes EMS11 (EMS59 and EMS66) and EMS29, and between morphotypes EMS11 (EMS59 and EMS66) and EMS72. There were high nucleotide similarities (88.6-94.5%) between these five morphotypes and two *Coniothyrium* species. There were relatively high sequence similarities (> 99.6%) among the six morphotypes (EMS6, EMS15, EMS18, EMS57, EMS65 and EMS74), furthermore, there was one base difference (99.9% nucleotide similarity) among morphotypes EMS15, EMS18 and EMS57. Morphotypes EMS6 and EMS65 had identical sequences, and there were two nucleotide differences between morphotypes EMS6 (EMS65) and EMS74.

Morphotype EMS77 had high nucleotide similarities with five *Elsinoë* species (80.1-95.3%). There was a high nucleotide similarity between morphotype EMS10 and *B. dothidea* (96%) and between morphotype EMS51

and *B. proteae* (88.2%). Morphotype WMS28 had a high nucleotide similarity with *G. vaccinii* (81.3%).

***Classification of ascomycetous morphotypes* – Helotiales**

The alignment of the 5.8S gene and ITS sequences of the 39 taxa comprised 546 base sites. The heuristic research recovered 12 equally parsimonious trees of 1261 steps, and the strict consensus tree is shown in Fig. 3. In this tree the morphotypes EMS38 and EMS55 formed a clade with all 34 species of the *Helotiales* with 100% bootstrap support. Morphotype EMS55 is closely related to *Pyrenopeziza revincta* of the *Dermateaceae* and this relationship is supported by 100% bootstrap support. Morphotype EMS38 was placed between *Rutstroemiaceae* and *Hemiphacidiaceae* and did not cluster together with any reference taxa. The 5.8S gene and ITS sequences of morphotype EMS55 had a high similarity with *P. revincta* (93%).

***Classification of ascomycetous morphotypes* – Pezizales**

The alignment of the 5.8S gene and ITS sequences of the 26 taxa comprised 925 base sites. The heuristic research recovered two equally parsimonious trees of 3579 steps, and the strict consensus tree is shown in Fig. 4. In this tree the five morphotypes and all reference taxa of the nine families in *Pezizales* formed a clade with 80% bootstrap support. Within the clade, morphotypes WMS5 and WMS10 formed a subclade with four reference species of the *Sarcosomataceae* with 65% bootstrap support, furthermore, morphotype WMS5 and *Sarcosoma latahense* are closely related to each other and this relationship is supported by 98% bootstrap support. Morphotype WMS10 and *Strumella coryneoidea* are closely related to each other and this relationship is supported by 75% bootstrap support. The other three morphotypes WMS13, WMS17 and WMS20 did not cluster together with any reference taxa.

The results of the 5.8S gene and ITS sequence similarity comparisons showed that five morphotypes had very low sequence similarities with any reference taxa (< 60%), but there were high sequence similarities among morphotypes WMS13, WMS17 and WMS20 (95.1-97.5%).

***Classification of ascomycetous morphotypes* – Rhytismataceae**

Based on the results of search for similar reference sequences, the alignment of the 5.8S gene and ITS sequences of the 43 taxa comprised 510 base sites. The heuristic research recovered 100 equally parsimonious trees of

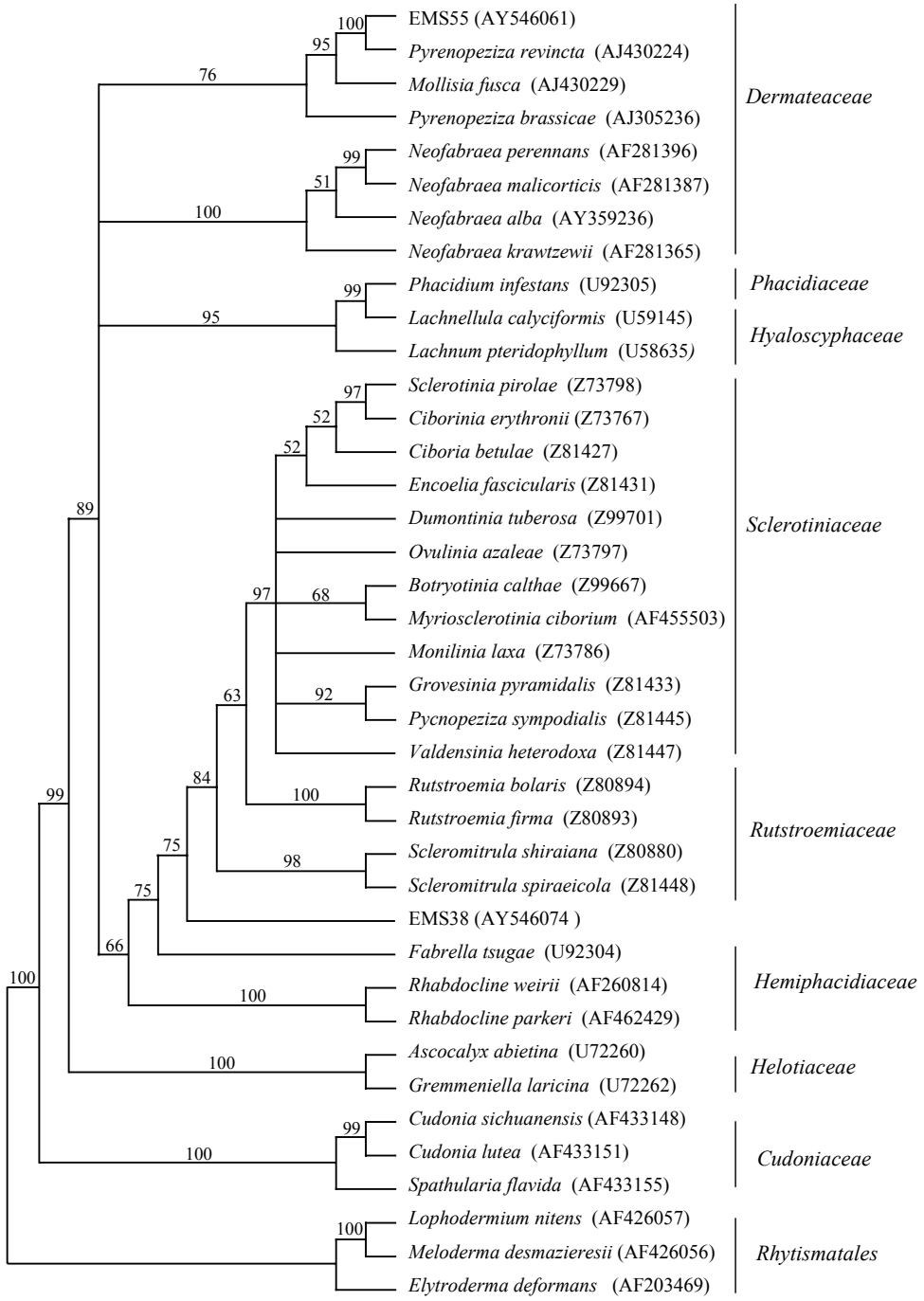


Fig. 3. Strict consensus tree of 12 equally parsimonious trees generated from the 5.8S gene and ITS (ITS1/2) sequences of 39 taxa showing the relationships of two morphotypes with reference taxa. The tree rooted with *Elytroderma deformans*, *Meloderma desmazieresii* and *Lophodermium nitens* (TL=1261, HI=0.5274, CI=0.4726, RI=0.6872, RC=0.3248). Bootstrap values greater than or equal to 50% (1000 replicates) are shown at branches.

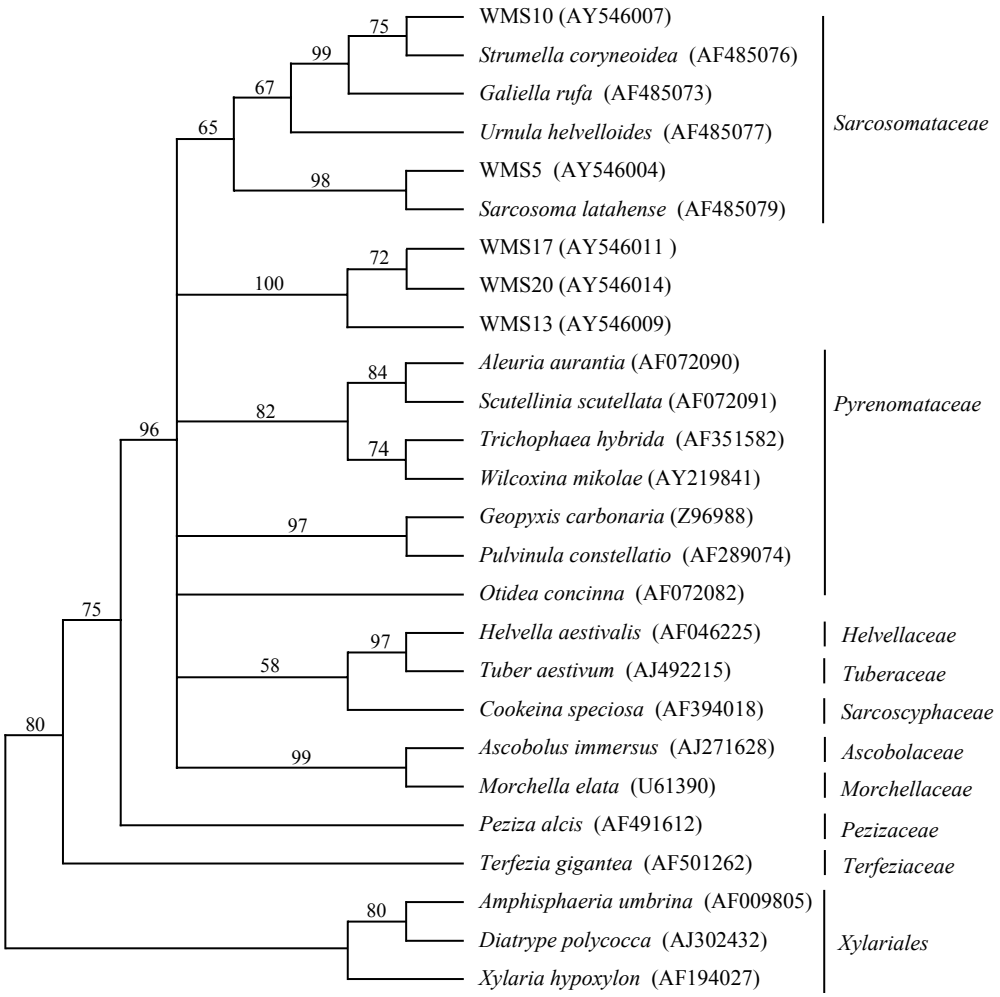


Fig. 4. Strict consensus tree of two equally parsimonious trees generated from the 5.8S gene and ITS (ITS1/2) sequences of 26 taxa showing the relationships of five morphotypes with reference taxa. The tree rooted with *Amphisphaeria umbrina*, *Diatrype polycocca* and *Xylaria hypoxylon* (TL=3579, CI=0.4767, HI=0.5233, RI=0.3931, RC=0.1874). Bootstrap values greater than or equal to 50% (1000 replicates) are shown at branches.

1064 steps, and the strict consensus tree is shown in Fig. 5. In this tree, 17 morphotypes formed a clade with 23 reference taxa of the *Rhytismataceae* (*Rhytismatales*) with 94% bootstrap support. The 17 morphotypes and *Lophodermium* species are closely related to each other and this relationship is supported by high bootstrap support.

The results of the 5.8S gene and ITS sequence similarity comparisons showed that morphotype EMS45 had high nucleotide similarity (93.4%) with *L. macci*. The other 16 morphotypes had high nucleotide similarities (90.2-

94%) with *L. australe*, *L. conigenum* and *L. indianum*, and there were relatively high sequence similarities (97.2-99.1%) among the 16 morphotypes, particularly there were high sequence similarities between morphotypes EMS39 and EMS54 (99.1%) and between morphotypes EMS4 and EMS63 (99.1%).

***Classification of ascomycetous morphotypes* – Amphisphaeriaceae, Nectriaceae, Valsaceae *and* Xylariaceae**

Based on the results of search for similar 5.8S gene and ITS sequences, 12 morphotypes and 41 reference taxa were included in the phylogenetic analysis. The strict consensus tree is shown in Fig. 6. In this tree seven morphotypes and ten reference taxa of the *Valsaceae* formed a clade with 100% bootstrap support. Within this clade four morphotypes (EMS21, EMS33, EMS34 and EMS43) and *Amphiporthe leiphaemia* are closely related to each other and this relationship is supported by 100% bootstrap support. Morphotypes EMS17 and EMS19 and *Diaporthe* (anamorph *Phomopsis*) are closely related to each other and this relationship is supported by 100% bootstrap support. Morphotypes EMS9 did not cluster together with any reference taxa. The results of the 5.8S gene and ITS sequence similarity comparison showed that morphotypes EMS21, EMS33, EMS34 and EMS43 had relatively high nucleotide similarities (97.5-98.8%) with *A. leiphaemia*, and there were relatively high nucleotide similarities (98.5-99.8%) among the four morphotypes, of which there were two base differences between morphotypes EMS33 and EMS43 (99.8%), and there were three base differences between morphotypes EMS21 and EMS33 (99.6%) and five base differences between morphotypes EMS21 and EMS43 (99.3%). Morphotypes EMS17 and EMS19 had high nucleotide similarities with *Diaporthe* (anamorph *Phomopsis*) species (83.6-94%), and there were only four nucleotide differences between morphotypes EMS17 and EMS19 (99.4%).

Two morphotypes (EMS13 and EMS40) and 11 reference taxa of the *Nectriaceae* formed the second clade with 100% bootstrap support. Within this clade, the two morphotypes and *Gibberella* (anamorph *Fusarium*) are closely related to each other and this relationship is supported by 100% bootstrap support. There were relatively high sequence similarities between morphotype EMS40 and *F. sporotrichioides* (99%) and between morphotype EMS13 and *G. avenacea* (98%).

Two morphotypes (EMS5 and EMS68) and seven reference taxa of the *Amphisphaeriaceae* formed the third clade, within this clade, morphotype EMS68 and two *Discostroma* are closely related to each other and this relationship is supported by 100% bootstrap support. Morphotype EMS5 and

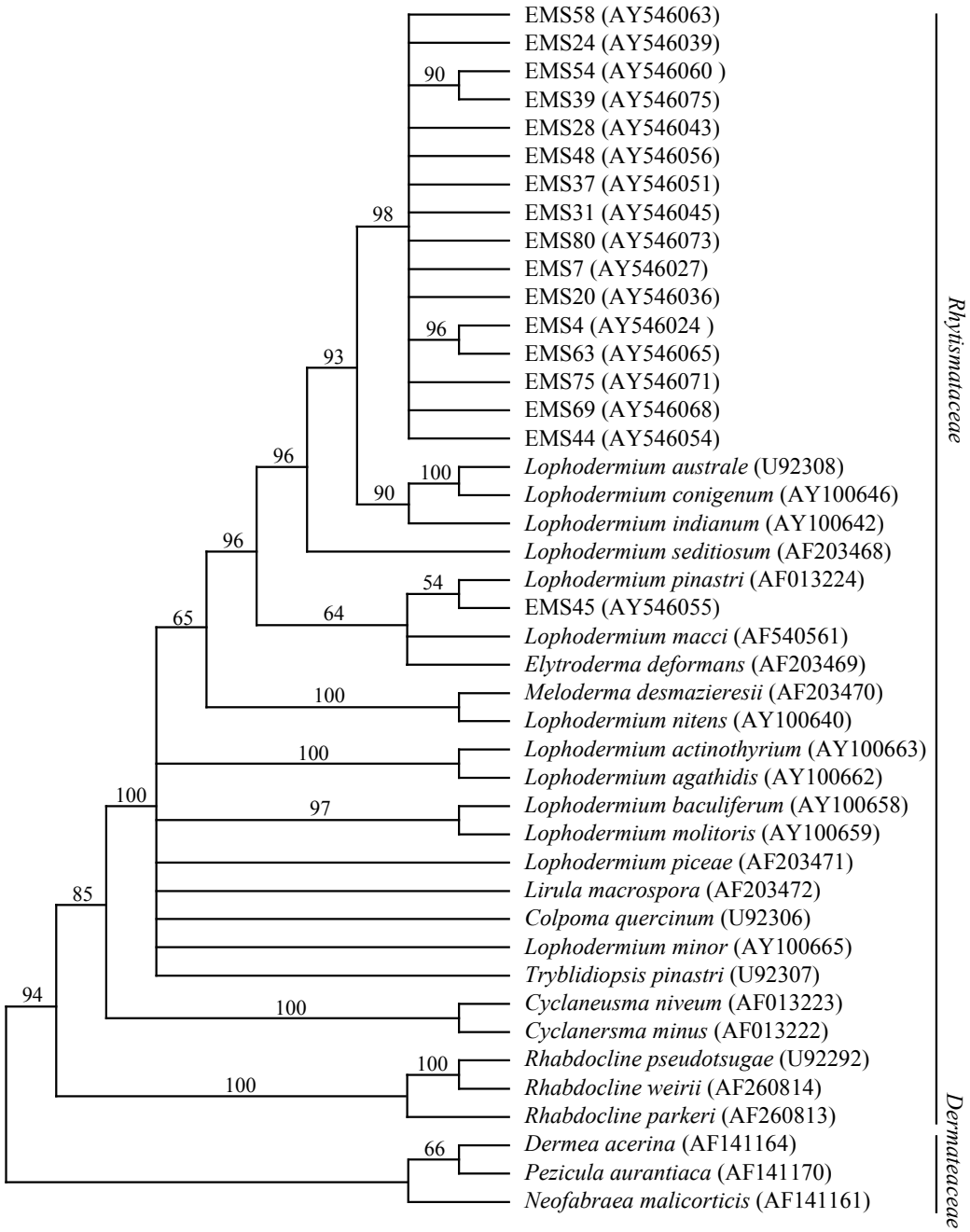


Fig. 5. Strict consensus tree of 100 equally parsimonious trees generated from the 5.8S gene and ITS (ITS1/2) sequences of 43 taxa showing the relationships of 17 morphotypes with reference taxa. The tree rooted with *Dermea acerina*, *Neofabraea malicorticis* and *Pezicula aurantiaca* (TL=1064, CI=0.4671, HI=0.5329, RI=0.6621, RC=0.3093). Bootstrap values greater than or equal to 50% (1000 replicates) are shown at branches.

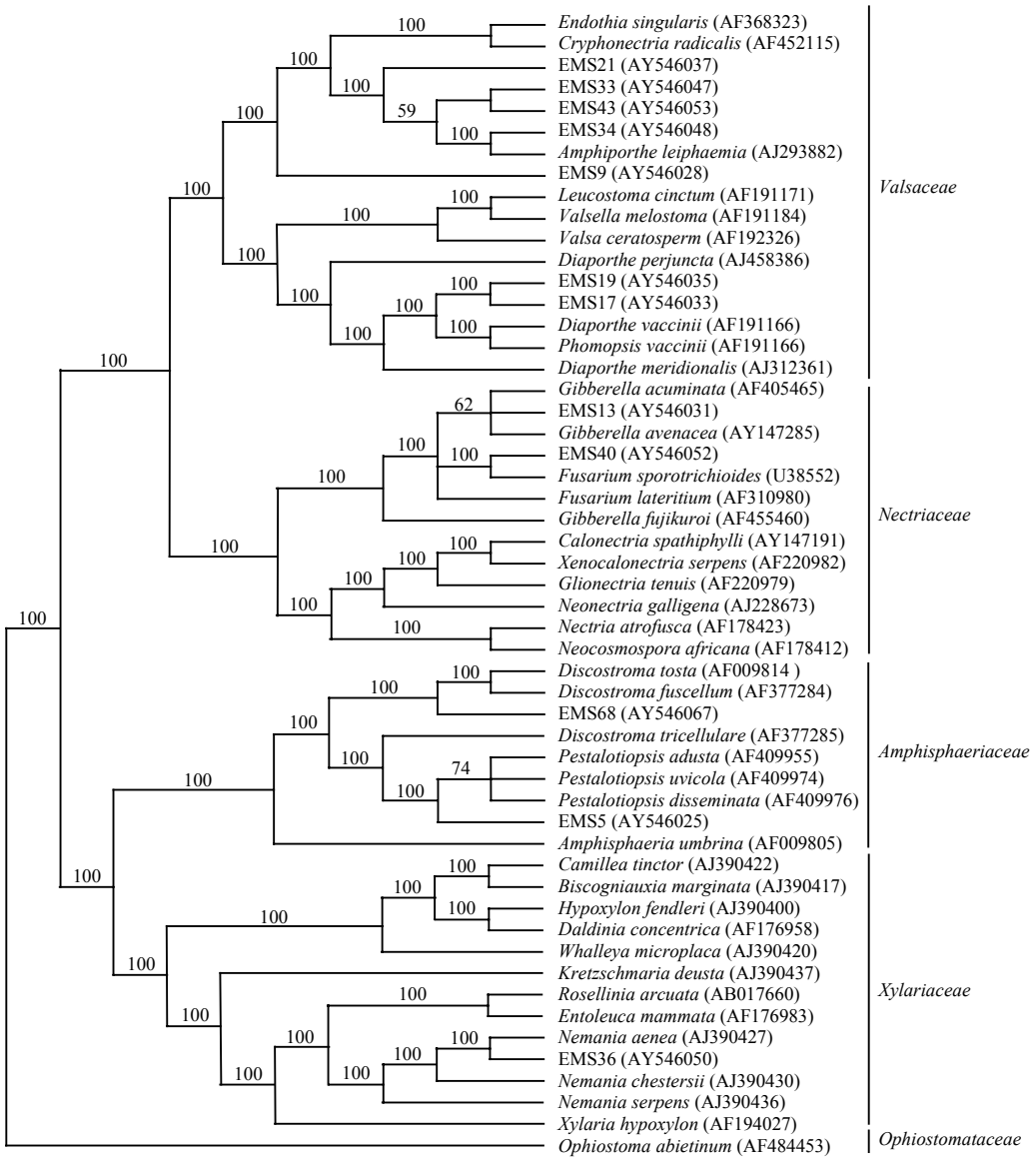


Fig. 6. Strict consensus tree of 256 equally parsimonious trees generated from the 5.8S gene and ITS (ITS1/2) sequences of 53 taxa showing the relationships of 12 morphotypes with reference taxa. The tree rooted with *Ophiostoma abietinum* (TL=1903, CI=0.4556, HI=0.5444, RI=0.7609, RC=0.3467). Bootstrap values greater than or equal to 50% (1000 replicates) are shown at branches.

three *Pestalotiopsis* species are closely related to each other and this relationship is supported by 100% bootstrap support. Morphotype EMS5 had relatively high nucleotide similarities (97.2-97.7%) with *Pestalotiopsis* species. Morphotype EMS68 had high sequence similarities (92-96.3%) with two *Discostroma* species.

Morphotype EMS36 formed the fourth clade with 12 reference species of the *Xylariaceae* with 100% bootstrap support. Within this clade, morphotype EMS36 and three *Nemania* species are closely related to each other and this relationship is supported by 100% bootstrap support. Morphotype EMS36 had high nucleotide similarities (90.8-96.8%) with three *Nemania* species.

Discussion

The main objective of this study was to establish the taxonomic placement of 74 morphotypes isolated from *P. tabulaeformis* within the fungal kingdom. The 74 morphotypes were *Ascomycota* (69) and *Basidiomycota* (5) based on the results of phylogenetic analysis of the 5.8S gene and ITS regions.

Phylogenetic position of morphotypes related to Basidiomycetes

Based on the phylogenetic analysis and sequence similarity comparison of the 5.8S gene and ITS regions, morphotype EMS23 is congeneric with *Peniophora*. Morphotypes WMS25 and WMS2 are conspecific due to having identical 5.8S gene and ITS sequences and are identified as *Gloeophyllum*. Morphotype WMS18 is inferred to belong to *Coprinellus*. Morphotype WMS21 is identified as *Schizophyllum commune*, because of identical 5.8S gene and ITS sequences.

Basidiomycetous endophytes have been reported in several previous studies (Chapela and Boddy, 1988; Griffith and Boddy, 1990; Bills and Polishook, 1992; Bettucci and Saravay, 1993; Rodrigues, 1994; Brown *et al*., 1998), while they are generally rarely isolated. The results of the present study indicated that basidiomycetous strains, as endophytic fungi, can be successfully isolated from *P. tabulaeformis*, although we cannot identify them using traditional methods due to their non-sporulation in artificial media. Similarly, Guo *et al*. (2001) have been successful in detecting and identifying a basidiomycetous endophyte directly from frond tissue of *Livistona chinensis* based on rDNA sequence analysis, while basidiomycetous species were not found in their study of endophytes from the same host with traditional culturing methods (Guo *et al*., 2000).

Phylogenetic position of morphotypes related to Dothideomycetidae

Based on the phylogenetic analysis and sequence similarity comparison of the 5.8S gene and ITS regions, morphotypes EMS10 and EMS51 belong to the genus *Botryosphaeria*. *Botryosphaeria* and its anamorph *Diplodia*, *Dothiorella*, *Fusicoccum*, *Lasiodiplodia* and *Sphaeropsis* are widely distribute

in woody plants, furthermore, some species are important pathogens. The occurrence of the ascomycete *B. quercuum* has been associated with cankers on oak (Sivanesan, 1984; Morehart, 1994), while it has been reported to be a colonizer of the wood and bark of dead branches of beech on the ground (Ellis and Ellis, 1987). *Botryosphaeria* and its anamorphs have been reported as endophytes in previous studies (Barengo *et al*., 2000; Danti *et al*., 2002).

Morphotype WMS28 is congeneric to *Guignardia*. *Guignardia* and its anamorph *Phyllosticta* are widespread in world. *Guignardia citricarpa* is known to cause black spot of citrus (McOnie, 1967). Carroll *et al*. (1977) showed that *P. concentrica*, the conidial state of *G. philoprina* represented the single widespread endophyte with a frequency of occurrence in petiole and needle often reaching 100% in *Taxus baccata*. *Guignardia* and its anamorph *Phyllosticta* have been isolated as endophytes from various plants (Carroll and Carroll, 1978; Petrini *et al*., 1982; Taylor *et al*., 1999; Guo *et al*., 2000; Cannon and Simmons, 2002).

Species of *Pleosporales* have been reported as endophytes in previous studies (Carroll *et al*., 1977; Petrini *et al*., 1982; Bertoni and Cabral, 1988; Collado *et al*., 1999; Fisher and Petrini, 1990; Lodge *et al*., 1996; Tomita, 2003). *Coniothyrium* and *Epicoccum* species are ubiquitous endophytes among different hosts from tropical and temperate regions (Carroll *et al*., 1977; Bertoni and Cabral, 1988; Fisher and Petrini, 1990; Lodge *et al*., 1996; Barengo *et al*., 2000). Morphotype EMS77 is identified in the genus *Elsinoë*. *Elsinoë* species have not previously been reported as endophytes.

Phylogenetic position of morphotypes related to Helotiales *and* Pezizales

Morphotype WMS10 has a relatively close relationship with *Strumella*, morphotype WMS5 belongs to *Sarcosoma*, and morphotypes WMS17, WMS20 and WMS13 are placed in the *Pezizales*, because these three morphotypes do not cluster together with any reference taxa.

Strumella species are widespread, and *S. coryneoidea* is known to cause canker of oak and sometimes of other trees. There are no reports of *Strumella* and *Sarcosoma* species presence as asymptomatic colonizers of living tissues in previous studies. This is firstly reported of *Strumella* and *Sarcosoma* species occurring as endophytes in *Pinus*.

The results of the sequence analysis show that morphotype EMS55 is identified as *Pyrenopeziza*, and morphotype EMS38 is placed in the *Helotiales*. *Pyrenopeziza* species have not so far been reported as endophytes.

Phylogenetic position of morphotypes related to Rhytismataceae

The results of the 5.8S gene and ITS sequence similarity comparisons and phylogenetic analysis showed that 17 morphotypes (EMS4, EMS7, EMS20, EMS24, EMS28, EMS31, EMS37, EMS39, EMS44, EMS45, EMS48, EMS54, EMS58, EMS63, EMS69, EMS75 and EMS80) are species of the *Lophodermium*, furthermore, morphotypes EMS4 and EMS63 and morphotypes EMS39 and EMS54 are conspecific, respectively, because they have a relatively high sequence similarity (99.1%).

Rhytismataceous species are often plant pathogens and have a worldwide distribution, particularly in the needles of conifers (Staley, 1964; Ziller, 1969; Hunt and Ziller, 1978). *Lophodermium pinastri* is known to cause needle cast and to occur in living needles in its spermogonial form (Gremmen, 1959; Minter, 1981; Teng, 1996). Kendrick and Burges (1962) reported that 40% needles of living *Pinus sylvestris* were infected by *L. pinastri* in the spring in Delaware Forest in Cheshire, the UK and there were no obvious symptoms at this stage. *L. pinastri* has also been isolated as an endophyte from the needles of *P. silvestris* in Switzerland (Carroll *et al*., 1977) and in France (Gourbière *et al*., 2001). *Lophodermium* species or their *Leptostroma* anamorphs have been isolated from apparently healthy green coniferous needles in previous studies (Carroll and Carroll, 1978; Sieber-Canavesi and Sieber, 1987; Petrini and Fisher, 1988; Petrini *et al*., 1989). Deckert *et al*. (2002) identified endophytic isolates from needles of *P. strobus* as *Lophodermium nitens* based on DNA analysis. In the present study, although *Leptostroma* species have not been isolated as endophytic fungi from *P. tabulaeformis* using conventional culturing techniques, there are 17 morphotype strains non-sporulated in cultures and are identified as *Lophodermium* based on rDNA sequence analysis.

Phylogenetic position of morphotypes related to Amphisphaeriaceae, Nectriaceae, Valsaceae *and* Xylariaceae

The results of the sequence analyses indicate that morphotype EMS5 belong to the concolourous group of the genus *Pestalotiopsis*, morphotype EMS68 has a relatively close relationship with *Discostroma*, and morphotype EMS36 is identified as a *Nemania* species.

Endophytic xylariaceous fungi have been isolated most frequently from plants in tropical regions (Dreyfuss and Petrini, 1984; Rodrigues and Samuels, 1990; Pereira *et al*., 1993; Rodrigues, 1994; Fisher *et al*., 1995; Lodge *et al*., 1996; Fröhlich *et al*., 2000; Guo *et al*., 2000; Photita *et al*., 2001).

Xylariaceous endophytes are also found in plants from temperate regions, although they are usually not common (e.g. Fisher and Petrini, 1990; Taylor *et al*., 1999). Xylariaceous endophytic fungi have often isolated from conifers. Carroll *et al*. (1977) isolated xylariaceous fungi from *Abies alba*, *Picea excelsa*, *Pinus silvestris*, *Pseudotsuga menziesii*, and *Taxus baccata* in Switzerland and *Pinus nigra* in France. Xylariaceous fungi were also obtained from the foliage of some *Cupressaceae*, such as *Chamaecyparis lawsoniana* (Petrini and Carroll, 1981) and from *Chamaecyparis thyoides* (Bills and Polishook, 1992).

Pestalotiopsis species are ubiquitous in distribution, occurring on a wide range of substrata, and have often been isolated as endophytes from various plants (Bertoni and Cabral, 1988; Lodge *et al*., 1996; Hata *et al*., 1998; Taylor *et al*., 1999; Photita *et al*., 2001; Cannon and Simmons, 2002; Guo, 2002; Wang and Guo, 2004; Wei and Xu, 2004). *Discostroma* and and *Nemania* species have not been reported as endophytes in previous endophyte studies.

Based on the phylogenetic analysis and sequence similarity comparisons of the 5.8S gene and ITS regions, morphotypes EMS13 and EMS40 are congeneric to *Gibberella* and its anamorph *Fusarium*, and morphotype EMS40 is identified as *F. sporotrichioides* due to relatively high sequence similarity (99%). *Gibberella* and its anamorph *Fusarium* occur on both herbaceous and woody plants as important pathogens that may cause enormous economic loss. *Fusarium* species have been often isolated as endophytes from various hosts, e.g. *F. mangiferae* and *F. sterilihyphosum* from mango (Britz *et al*., 2002), *F. solani* from temperate palms (Taylor *et al*., 1999), three *Fusarium* species (*F.* cf. *avenaceum*, *F. decemcellulare* and *F. solani*) from *Manilkara bidentata* (Lodge *et al*., 1996), and *F. lateritium* from *Salix fragilis* and *Quercus robur* (Petrini and Fisher, 1990).

The results of sequence analysis indicate that morphotypes EMS21, EMS33, EMS34 and EMS43 belong to the genus *Amphiporthe*, furthermore, morphotypes EMS33 and EMS43 are conspecific due to differences of only two nucleotides in the ITS region. Morphotypes EMS17 and EMS19 are identified as *Diaporthe* and its anamorph *Phomopsis*, and these two morphotypes are highly conspecific.

Phomopsis species are often plant pathogens and distributed worldwide. The connection of the teleomorph *Diaporthe* and anamorph *Phomopsis* is well established (Wehmeyer, 1993). *Phomopsis* species have often been isolated as endophytes from pines (Hata and Futai, 1996; Hata *et al*., 1998) and from other plants (Carroll and Petrini, 1983; Espinosa-Garcia and Langenheim, 1990; Rodrigues and Samuels, 1990; Lodge *et al*., 1996; Southcott and Johnson, 1997; Brown *et al*., 1998; Taylor *et al*., 1999; Fröhlich *et al*., 2000; Guo *et al*.,

2000). Ragazzi *et al*. (2003) reported that *Amphiporthe* species as endophytic fungi exist in oak species in Italy, and Kowalski and Kehr (1996) point out that *A. leiphaemia* was isolated as an endophyte from living branches in several European tree species.

Molecular phylogeny as a means to identify sterile endophytes

In a survey of the endophytic fungi from *Pinus tabulaeformis* at two different climatic sites of northeast China, approximately 11% isolates did not produce any spores, although various methods to promote sporulation in culture were employed. If the isolates do not produce any spores in culture they cannot be identified using morphological taxonomy, and therefore molecular techniques have been employed to identify such fungi (e.g. Arnold *et al*., 2000; Guo *et al*., 2000, 2001, 2003; Okane *et al*., 2001; Baayen *et al*., 2002). However, there are limitations in the identification of sterile mycelia by means of DNA sequence analyses (Guo *et al*., 2000, 2001). Although some morphotypes had relatively high similarities in ITS region sequences and clustered together with high bootstrap supports with reference taxa, there is still insufficient information at present to determine whether the terminal clades included one or more species in the phylogenetic analysis. For most of the taxa included in our analyses, the level of inter-specific and intra-specific variations is still unknown. Different levels of variations have been reported in the different taxa in previous studies (O'Donnell, 1992; Carbone and Kohn, 1993; Morales *et al*., 1993; Cunnington *et al*., 2004).

Another major limitation as demonstrated in our previous studies is the limited number of sequences in GenBank and EMBL (Guo *et al*., 2000, 2001). However, as more sequences become available this identity of more taxa can be revealed. The sterile mycelia morphotypes in our study that were given taxonomic placement above family levels could be further resolved once more references are available in these databases. Nevertheless, we have shown that despite the limitations, molecular identification based on nucleotide sequences is still appropriate to identify and classify endophytes to familial and general level.

In this study we found many fungi, i.e. *Schizophyllum commune*, *Coprinellus*, *Gloeophyllum* and *Peniophora* species of Basidiomycetes and *Discostroma*, *Elsinoë*, *Nemania*, *Pyrenopeziza*, *Sarcosoma* and *Strumella* species of Ascomycetes, which have not been observed as endophytes in previous studies. Our results indicate that molecular techniques are promising methods to find more new endophytic fungi.

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