

## Molecular phylogeny of *Magnaporthaceae* (Sordariomycetes) with a new species *Ophioceras chiangdaoense* from *Dracaena loureiroi* in Thailand

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The phylogenetic relationship of the family *Magnaporthaceae* and allied genera were investigated using individual and combined analyses of 18S and 28S nuclear ribosomal DNA (rDNA) gene fragments. New DNA sequences representing six genera from *Magnaporthaceae* were analyzed phylogenetically based on Bayesian, likelihood and parsimony methods. *Magnaporthaceae*, encompassing genera such as *Buergenerula*, *Gaeumannomyces*, *Magnaporthe*, *Ophioceras*, *Pseudohalonestria* and anamorphic species including *Mycocleptodiscus* and *Pyricularia*, constitute a strongly supported monophyletic group. Results also indicate that *Ophioceras* is probably monophyletic. *Magnaporthe salvinii* is closely related to *Gaeumannomyces*, while *M. grisea* clusters with the anamorphic genus *Pyricularia*. The placement of *Mycocleptodiscus coloratus* and *Ceratosphaeria lampadophora* within the *Magnaporthaceae* is confirmed. Molecular data also provided further evidence to support the association of several anamorphic genera with the ascomycetous *Magnaporthaceae*. The phylogenetic significance of several morphologies currently used in the classification of members among *Magnaporthaceae* is discussed. Phylogenies also indicate that *Magnaporthaceae* bears close phylogenetic affinities to the order *Diaporthales* and *Ophiostomatales*. However, there appears to be sufficient evidence to establish a new order, *Magnaporthales* to accommodate the *Magnaporthaceae*. A new species (*Ophioceras chiangdaoense* sp. nov.) that was isolated from the leaves of *Dracaena loureiroi* is described and its phylogenetic relationship to is evaluated using the rDNA sequences.

**Key words:** rDNA, 28S rDNA, *Magnaporthaceae*, molecular systematics, phylogeny

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### Introduction

*Magnaporthaceae* is a small family of unitunicate, perithecial fungi in Sordariomycetes/Pezizomycotina/Ascomycota (Cannon and Kirk, 2007). Its taxonomic boundaries are not well defined, and the number of genera that should be accommodated within the family is still unclear. The *Dictionary of the Fungi* (Kirk *et al.*, 2001) accepted 9 genera and 26 species within the family, while the most recent dictionary (Kirk *et al.*, 2008) accepted 13 genera and 93 species. *Clavati-*

*sporella* was recently included, while *Junci-gena* (previously *Magnaporthaceae*) has been transferred to Hypocreomycetidae (Lumbsch and Huhndorf, 2007, Table 1). *Gaeumannomyces* and *Magnaporthe* species and their anamorphs constitute the most important members of this family, as they are serious pathogens of economically important plants worldwide (Yaegashi, 1977; Freeman and Ward, 2004). *Gaeumannomyces graminis* (Sacc.) Arx & D.L. Olivier causes take all, one of the most important root diseases of wheat (Freeman and Ward, 2004), while

*Magnaporthe grisea* (T.T. Hebert) M.E. Barr (anamorph: *Pyricularia oryzae*) is the causal agent of rice blast (Yaegashi, 1977). Other *Magnaporthe* / *Pyricularia* species cause blast disease of banana, maize, millet, pearl millet and a number of other grass species (Yaegashi and Hebert, 1976; Yaegashi, 1977; Landschoot and Jackson, 1989; Berbee, 2001). *Buergenerula* species have also been found to be associated with a distinctive leaf spot of *Carex* species (McKenzie, 1991). A serious disease, stem canker, caused by *Ophioceras* sp. in Malaysia, can be fatal to Rambutan (*Nephelium lappaceum*) trees if not controlled at the outset (Morton, 1987). The family *Magnaporthaceae* was established by Cannon (1994) to include a group of fungi similar to *Magnaporthe*. Morphological characters pertaining to members of this family include a lack of stromata, and black ascomata immersed mostly in decaying plant tissues, often with long hairy necks. Interascal tissue comprises thin-walled tapering paraphyses and asci that are often cylindrical, persistent, fairly thick-walled, lacking separable layers and with a large apical pore often surrounded by a ring that stains blue in iodine (J+). However, the type species *M. salvinii* (Catt.) R.A. Krause & R.K. Webster has a refractive ring that is not J+ (Hanlin, 1998). Ascospores are septate, often filiform, and surrounded by a sheath (Cannon, 1994; Kirk *et al.*, 2001). Members of this group share many similarities, most notably in teleomorph morphology and pathogenic potential, although their anamorphs are variable (Cannon, 1994). The importance of these morphological characters in delineation of genera within this family has not been clarified using phylogenetic methods.

The ordinal placement of the *Magnaporthaceae* and the genera within the family has long been problematic due to the lack of convincing morphological and inconclusive molecular data. Closely related families are difficult to identify and, therefore, the current phylogenetic position of *Magnaporthaceae* is uncertain within the class Sordariomycetidae (Kirk *et al.*, 2001; Lumbsch and Huhndorf, 2007). Some magnaporthaceous genera have been placed in, or are possibly closely related to various orders (e.g. *Diaporthales*, *Ophio-*

*stomatales*, *Phyllachorales* and *Sordariales*) (Barr, 1976a,b; Huang, 1976; Conway and Barr, 1977; Jensen, 1985; Cannon, 1994; Berbee, 2001; Zhang and Blackwell, 2001; Castlebury *et al.*, 2002; Wanderlei-Silva *et al.*, 2003).

The objectives of the present study were to use 18S and 28S rDNA sequence analyses to 1) confirm the ordinal placement of *Magnaporthaceae*; 2) assess genera that should be included in the family, and 3) establish which characters are important in placing taxa within the *Magnaporthaceae*. A new species of *Ophioceras* is also described and its phylogenetic relationship to other magnaporthaceous members is evaluated using the 18S and 28S rDNA sequences.

## Materials and methods

### *Collection, identification and DNA extraction of new fungi*

Decaying leaves of *Dracaena loureiroi* were collected during the rainy season (June–September) and cool dry season (November–February) from Chiang Dao National Park, Chiang Mai, Thailand (see Thongkantha *et al.*, 2008). Fungi were examined after incubation in a moist chamber for a minimum of 7 days. Ascoma contents were mounted in sterile water and viewed using an Olympus light microscope. Asci apical rings were tested for an amyloid reaction with Melzer's reagent. Attempts to isolate a new fungus in pure culture were unsuccessful therefore DNA was extracted directly from dried specimen using a modified Chelex method (Walsh *et al.*, 1991; Hirata and Takamatsu, 1996; Bahl *et al.*, 2005; Vijaykrishna *et al.*, 2006a). Ascospores were suspended in 300 µl of 5% Chelex solution (Bio-Rad, Richmond, California) and vortexed thoroughly for 1 min. Tubes were then incubated at 100°C for 10 min and vortexed for another 1 min to allow maximum disruption. After incubation for an additional 5 min at 100°C the contents were centrifuged for 2 min at a speed of 13000 g and the supernatant was transferred to a new tube. The sample was further incubated at the same temperature, vortexed and centrifuged and the supernatant was used for PCR.

**Table 1.** Members of the *Magnaporthaceae* and their anamorphs.

Kirk <i>et al.</i> (2001)	Anamorph	Lumbsch and Huhndorf (2007)
<i>Buergenerula</i> Syd.	<i>Passalora</i> -like, <i>Nakataea</i> -like	<i>Buergenerula</i> Syd.
<i>Clasterosphaeria</i> Sivan.	<i>Clasterosporium</i>	<i>Clasterosphaeria</i> Sivan. <i>Clavatisporella</i> K.D. Hyde
<i>Gaeumannomyces</i> Arx & D.L. Olivier	<i>Pyricularia</i>	<i>Gaeumannomyces</i> Arx & D.L. Olivier
<i>Herbampulla</i> Scheuer & Nograsek	unknown	? <i>Herbampulla</i> Scheuer & Nograsek
<i>Juncigena</i> Kohlm., Volkm.-Kohlm. & O.E. Erikss.	<i>Cirrenalia</i>	
<i>Magnaporthe</i> R.A. Krause & R.K. Webster	<i>Pyricularia</i> , <i>Nakataea</i> , <i>Phialophora</i> , <i>Sclerotium</i>	<i>Magnaporthe</i> R.A. Krause & R.K. Webster
<i>Omnidemptus</i> P.F. Cannon & Alcorn	<i>Mycoleptodiscus</i>	<i>Omnidemptus</i> P.F. Cannon & Alcorn
<i>Ophioceras</i> Sacc.	unknown	<i>Ophioceras</i> Sacc.
<i>Pseudohalonectria</i> Minoura & T. Muroi	unknown	<i>Pseudohalonectria</i> Minoura & T. Muroi

### **Fungal cultures and DNA extraction**

In addition to the new species, 21 living cultures of different magnaporthaceous strains were obtained from American Type Culture Collection (ATCC), BIOTEC, Thailand (BCC), Centraalbureau voor Schimmelcultures (CBS), and Hong Kong University Culture Collection (HKUCC). Collections and cultures of anamorph / teleo-morph genera of *Magnaporthaceae*, *Buergenerula*, *Gaeumannomyces* (*Pyricularia*), *Magnaporthe* (*Pyricularia*, *Pyriculariopsis*), *Ophioceras* and *Pseudohalonectria* were subcultured onto potato dextrose agar (PDA) or 2% malt extract agar (MA) 5-10 days prior to DNA extraction.

A DNA extraction protocol as outlined by Cai *et al.* (2006) and Zhang *et al.* (2008) was used to extract DNA. Actively growing mycelia were scraped off cultures on agar plates. The mycelium was ground with 200 mg of sterilized quartz sand and 600  $\mu$ l of 2 $\times$  CTAB extraction buffer (2% w/v CTAB, 100 mM Tris-HCl, 1.4M NaCl, 20 mM EDTA, pH 8) in a 1.5 ml centrifuge tube. Contents were then incubated at 60°C in a water bath for 30 min with gentle swirling. The solution was then extracted 2-3  $\times$  with an equal volume of phenol: chloroform (1:1) at 13000 rpm for 30 min until no interface was visible. The supernatant phase containing the DNA was precipitated by addition of 2.5 volumes of absolute ethanol and kept at -20°C overnight. The DNA pellet was washed (70% ethanol) 2 times, dried (under vacuum), and resuspended

in TE buffer (1 mM EDTA, 10mM Tris-HCl, pH 8) and mixed together with RNase A (1 mg/ml<sup>-1</sup>).

### **PCR amplification and sequencing of 28S and 18S rDNA**

Approximately 900 nucleotides at the 5' end of the nuclear large ribosomal subunit gene (28S rDNA) were amplified by primer pairs LROR (5'-ACCCGCTGAACTTAAGC-3') and LRO5 (5'-TCCTGAGGG AACTTC G-3') (Vilgalys and Hester, 1990). A portion of the nuclear small ribosomal subunit gene (18S rDNA) was partially amplified using primers NS1 (5'-GTAGTCATATGCTTGTC TC-3') and NS4 (5'-CTTCCGTCAATTCCTT TAAG-3') (White *et al.*, 1990). Three  $\mu$ l of genomic DNA (extracted from mycelia) or 5  $\mu$ l (extracted from spores by using Chelex method) was used in a standard 50  $\mu$ l PCR mixture (25mM MgCl<sub>2</sub>, 10 Mg-free buffer, 2.5  $\mu$ M dNTPs, 1.5  $\mu$ M primers, and 1.5 unit of *Taq* DNA Polymerase) under the following thermal conditions: 94°C for 3 min, 94°C for 50 s, 30 cycles of 94°C for 50 s, 50°C for 1 min, and 72°C for 1.5 min, with a final extension step of 72°C for 10 min. Amplicons were checked on 1% agarose gels stained with ethidium bromide under UV light. Negative control reactions omitting DNA were included in all sets of amplifications to monitor for potential contamination by exogenous DNA. PCR products were purified using GFX<sup>TM</sup> PCR DNA and Gel Band Purification

Kit (Amersham Biosciences, Catalog no. 27-9602-01) following manufacturer's protocol. The amplified 18S and 28S rDNA fragments were directly sequenced. Sequencing reactions were performed and sequences determined automatically in an Applied Biosystem 3730 Genetic Analyzer/ Sequencer (Genome Research Center, The University of Hong Kong) using PCR primers mentioned above.

### **Phylogenetic analyses**

A total of 32 strains from the *Magnaporthaceae*, representing five teleomorphic genera (including the new species) and two anamorphic genera, were analysed along with reference fungal sequences, from different fungal families, that were downloaded from GenBank. Table 2 lists all *Magnaporthaceae* members that were included for analyses. Nucleotide sequences of the 28S and 18S rDNA were initially aligned using BioEdit (Hall, 1999) and Clustal X 1.83 (Chenna *et al.*, 2003) with default parameter settings. The alignments were then manually optimized using Se-al v2.0a11 (Rambaut, 1996). All ambiguously aligned portions of the alignment were removed for further phylogenetic analyses. Maximum parsimony (MP) analysis was conducted using PAUP\*4.0b10 (Swofford, 2002). A symmetric step matrix was generated, using the program STMatrix v2.1 (<http://www.lutzonilab.net/downloads/index.shtml>), where the relative frequencies of nucleotide substitutions are converted into costs of changes (Miadlikowska *et al.*, 2002). Gaps were treated as fifth state. Tree searches were carried out using the heuristic method with a random stepwise addition and tree bisection and reconstruction branch-swapping algorithm.

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MrModeltest v2.2 (Nylander, 2004) was used to determine the best-fit DNA substitution and gamma rate heterogeneity for each dataset. The best-fit model was used in maximum likelihood (ML) and Bayesian analyses with gaps treated as missing. ML analysis was carried out using the program Garli v 0.951 (Zwickl, 2006). Bayesian analysis was carried out, using the program MrBayes v3.13 (Huelssenbeck and Ronquist, 2001), for two replicates of 1 million generations, with 6 chains sampling every 100 generations. Branch support was estimated by performing 1000 MP bootstrap replicates (Felsenstein, 1985), 100 ML bootstrap replicates, and Bayesian posterior probabilities was calculated from the consensus of 18,000 trees after excluding the first 2000 trees as burnin.

Combinability of the 18S and 28S rDNA was assessed by detecting any conflicts that exist between the highly supported branches from trees obtained in different datasets. Confident branch support is defined as bootstrap values  $\geq 70\%$  and Bayesian posterior probabilities  $\geq 0.95$  (Alfaro *et al.*, 2003).

## **Results**

### **Taxonomy**

*Ophioceras chiangdaoense* Thongk.,  
Lumyong & K.D. Hyde, sp. nov.

(Figs 1-10)

MycoBank: MB 512870

Ascomata 200-310  $\times$  170-310  $\mu\text{m}$ , globosa vel subglobosa, paraphysata. Asci 85-125  $\times$  11-17  $\mu\text{m}$  ( $\bar{x}$  = 100  $\times$  14, n = 30), octospori, cylindrici, apedicellati. Ascospores 54-75.5  $\times$  4-5.5  $\mu\text{m}$  ( $\bar{x}$  = 60.2  $\times$  4.3, n = 30), multiseriatae, filiformes, fusoideae, 3-septatae, hyalinae.

*Etymology*: In reference to Chiang Dao National Park, where the specimens were collected.

*Holotype*: CMU 26633, Thailand.

*Ascomata* 200-310  $\times$  170-310  $\mu\text{m}$ , globose to subglobose, immersed, dark brown

**Table 2.** Magnaporthaceous taxa and their GenBank accession number used in this study.

Taxon	GenBank accession No.		Source <sup>#</sup>
	18S rDNA	28S rDNA	
<i>Buergenerula spartinae</i>	DQ341471*	DQ341492*	ATCC22848
<i>Gaeumannomyces amomi</i>	DQ341472*	DQ341493*	CMUZE002
<i>G. cylindrosporus</i>	DQ341473*	DQ341494*	CBS610.75
<i>G. graminis</i> var. <i>avenae</i>	DQ341474*	DQ341495*	CBS870.73
<i>G. graminis</i> var. <i>graminis</i>	AF050488	AF362557	Chen <i>et al.</i> , 1999; Castlebury <i>et al.</i> , 2002
<i>G. graminis</i> var. <i>graminis</i>		DQ341496*	CBS352.93
<i>G. graminis</i> var. <i>tritici</i>	DQ341475*	DQ341497*	CBS541.86
<i>G. oryzinus</i>	DQ341476*	-	CBS235.32
<i>Magnaporthe grisea</i>	AB026819	AF362554	Sone <i>et al.</i> , 2000; Castlebury <i>et al.</i> , 2002
<i>M. salvinii</i>	DQ341477*	DQ341498*	CBS 243.76
<i>Mycoleptodiscus coloratus</i>	-	DQ341499*	CBS 720.95
<i>Ophioceras arcuatissporum</i>	AF050472	-	Chen <i>et al.</i> , 1999
<i>O. Chiangdaoense</i> sp. nov.	EU571271	EU571272	CMU26633
<i>O. commune</i>	AF050469	-	Chen <i>et al.</i> , 1999
<i>O. commune</i>	DQ341478*	DQ341500*	HKUCC9106
<i>O. commune</i>	DQ341479*	DQ341501*	CMUVJ10
<i>O. commune</i> (previously <i>O. dolichostomum</i> )	DQ341480*	DQ341502*	BCC3328
<i>O. dolichostomum</i>	DQ341482*	DQ341504*	CMURp50
<i>O. dolichostomum</i>	DQ341483*	DQ341505*	CMUVJ1
<i>O. dolichostomum</i>	DQ341485*	DQ341507*	HKUCC10113
<i>O. dolichostomum</i>	DQ341486*	DQ341508*	HKUCC3936
<i>O. fusiforme</i>	AF050473	-	Chen <i>et al.</i> , 1999
<i>O. hongkongense</i>	DQ341487*	DQ341509*	HKUCC3624
<i>O. leptosporum</i>	AF050474	-	Chen <i>et al.</i> , 1999
<i>O. leptosporum</i>	DQ341488*	DQ341510*	CBS168.96
<i>O. tenuisporum</i>	AF050475	AY346295	Chen <i>et al.</i> , 1999
<i>O. venezuelense</i>	AF050476	-	Chen <i>et al.</i> , 1999
<i>Pseudohalonectria falcata</i>	AF050477	-	Chen <i>et al.</i> , 1999
<i>P. lignicola</i>	AF050478	AY346299	Chen <i>et al.</i> , 1999
<i>P. suthepensis</i>	DQ341490*	DQ341513*	PDD76762
<i>Pyricularia borealis</i>	DQ341489*	DQ341511*	CBS 461.65
<i>P. higginsii</i>	-	DQ341512*	CBS 665.79

\* Sequences generated in this study.

# Culture collection numbers for newly generated sequences and original citations for GenBank sequences are given.

to black, solitary to gregarious. Neck composed of *textura intricata* with hyphae arranged in rows and fanning out obliquely, 93-273 × 52-68 µm, central, cylindrical, pale brown to black and hyaline at the apex. Peridium thin, comprising a few layers of dark brown to black-walled compressed cells, composed of large cells of *textura angularis*. Paraphyses hyaline, 13-17 µm at the widest point, filamentous, smooth, septate, obtuse. Asci 85-125 × 11-17 µm ( $\bar{x}$  = 100 × 14, n = 30), 8-spored, cylindrical, apedicellate, apex rounded or truncate, with a J- subapical ring. Ascospores 54-75.5 × 4-5.5 µm ( $\bar{x}$  = 60.2 × 4.3, n = 30), multiseriate, filiform, fusoid at both ends and typically 3-septate, both ends are slightly broader immediately above and

below each septum, hyaline, straight to slightly curved, smooth, thin-wall, light yellowish-brown in mass.

*Habitat/Known distribution:* Known to inhabit decaying leaves of *Dracaena lourei-roi*, Thailand.

*Specimens examined:* THAILAND: Chiang Mai, Chiang Dao National Park, in rainforest, on dead leaves of *Dracaena lourei-roi*, 12 July 2002, *S. Thongkantha S011-1*, CMU 26633 (**holotype**); *S011-2*, CMU 26634; 1 November 2005, *S. Thongkantha S011-3*, CMU 26887; *S011-4*, CMU 26888.

### 18S rDNA phylogeny

The 18S rDNA that consisted of 65 sequences resulted in a 1044 bp dataset of which 665 characters were constant and 119 were parsimony-informative. Maximum par-

simony analyses resulted in 84 equally most parsimonious trees (tree score, 1871.74). The maximum likelihood tree (-ln Likelihood, 6790.68) based on the best-fit model of evolution (GTR+I+G) is shown in Fig. 11. A strict consensus tree of the 84 most parsimonious trees (data not shown) was largely similar to the tree generated by the likelihood methods. The monophyletic clades that are shown in Fig. 11 (i.e. *Magnaporthaceae*, *Diaporthales*, *Ophiostomatales*, *Sordariales*, *Xylariomycetidae* and *Hypocreomycetidae*) also formed monophyletic clades in the strict consensus parsimony tree. However the interrelationship between these clades differed between the trees. In particular, the *Diaporthales* and *Ophiostomatales* formed a monophyletic sister clade to the *Magnaporthaceae*, however in the strict consensus parsimony tree, the *Ophiostomatales* was more closely related to the *Magnaporthaceae* and the *Diaporthales* was basal to this *Magnaporthaceae*-*Ophiostomatales* grouping. Nevertheless, the *Diaporthales* and *Ophiostomatales* relationship was supported by a low (65%) parsimony bootstrap value, supporting the maximum likelihood tree. These results indicate a close relationship of the *Diaporthales* and the *Ophiostomatales* to the *Magnaporthaceae*, however their interrelationship could not be confirmed using this gene segment.

Within the *Magnaporthaceae*, the interrelationship between the two trees (viz, strict consensus parsimony tree and likelihood tree) were similar (Fig. 11). *Ophioceras* species formed a strongly supported monophyletic group, also confirming the phylogenetic position of *O. chiangdaoense* within the genus. *Gaeumannomyces graminis* var. *avenae* was basal to the *Ophioceras* clade (100%). Sister to this clade consisted of the remaining *Gaeumannomyces* species, *Magnaporthe salvinii*, *Buergenerula spartinae* and *Ophioceras arcuatissporum*. The three species of *Pseudohalonectria* that were analysed were basal within the *Magnaporthaceae*. These results show that the morphological characters that were used to define these genera

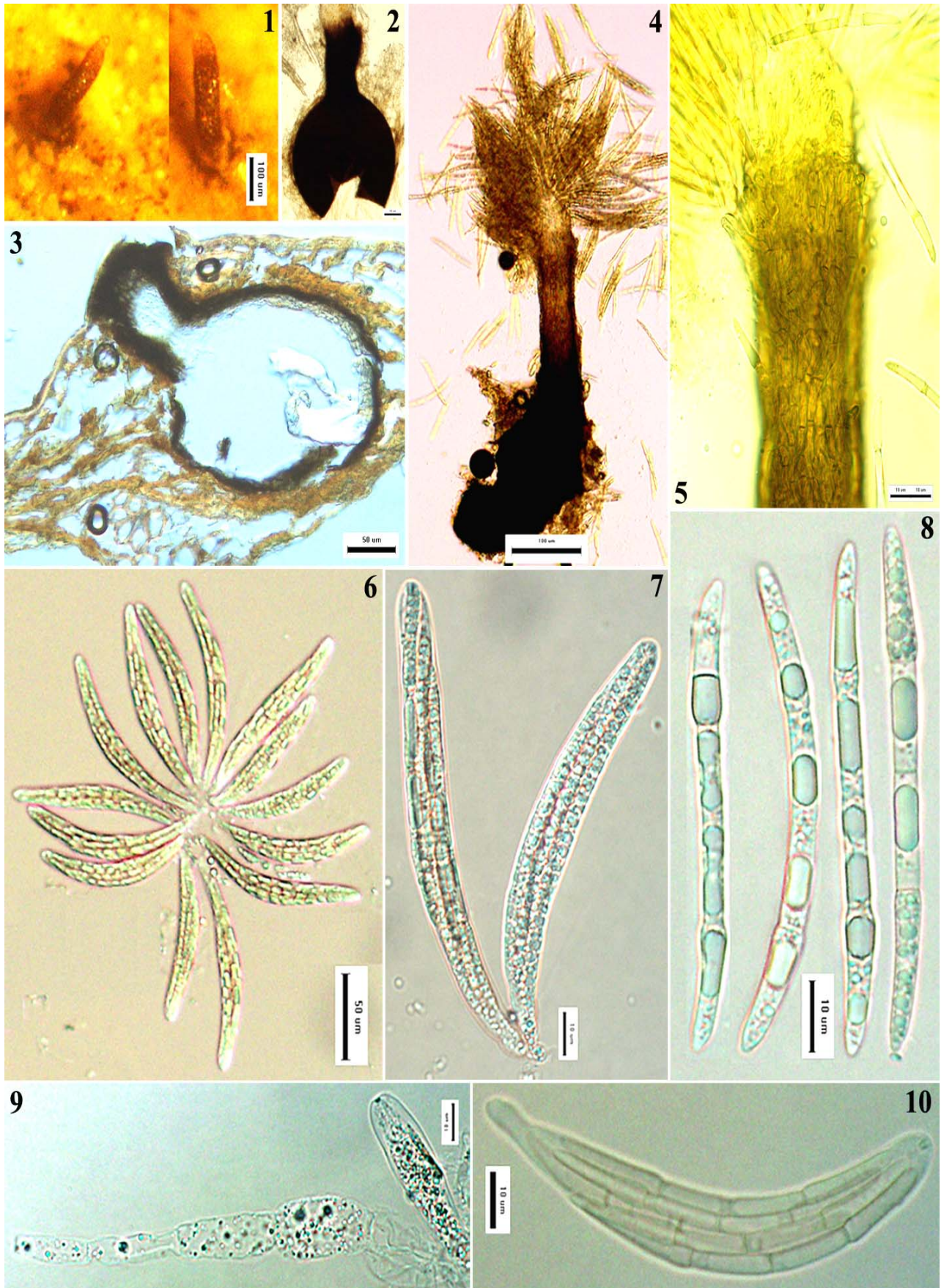
(*Gaeumannomyces*, *Magnaporthe*, *Buergenerula*) need to be clarified.

### 28S rDNA phylogeny

The 28S rDNA dataset consisted of 74 sequences and resulted in a 918-nt dataset. After the removal of 69 ambiguously aligned regions, 379 were constant, 146 parsimony-uninformative and 330 were parsimony-informative. Maximum parsimony analyses resulted in a single most parsimonious tree (tree score, 4566.85). The best-fit model of evolution for this dataset was GTR+I+G. The maximum likelihood tree (-ln Likelihood, 10926.95) generated based on the best-fit model is shown in Fig. 12. The phylogenetic relationships generated by the maximum parsimony analyses was similar to the maximum likelihood tree. The established orders (e.g. *Ophiostomatales*, *Hypocreales*) were monophyletic and highly supported by the 28S rDNA analyses, being consistent with the 18S rDNA analyses. However, due to the low support for the backbone of the 28S rDNA trees, the interrelationships between the clades could not be clarified. In the 28S rDNA surprisingly, the *Sordariales*-*Chaetosphaeriales* group was sister to the *Magnaporthaceae*, however, with low bootstrap support, indicating that the 28S rDNA might not be appropriate in clarifying the interrelationship of the families and orders within the Sordariomycetidae.

Within the *Magnaporthaceae*, again the *Ophioceras* species were highly supported, however, the other relationships within this family was not consistent with the 28S rDNA phylogeny. Strikingly, *Gaeumannomyces graminis* var. *avenae* was not closely related to the *Ophioceras* clade, but was most closely related to the other *Gaeumannomyces* species. Also, except the *Ophioceras* clade and the outgroup relationship of *Mycoleptodiscus* sp., the other relationships were not supported.

An analysis was also carried out using a concatenated dataset (18S rDNA and 28S rDNA genes) (data not shown). The phylogenetic tree produced by any method resulted in a topology similar to the one generated by the 18S rDNA gene (see Fig. 11).



**Figs 1-10.** *Ophioceras chiangdaoense* (from holotype). 1. Appearance of ascomata on the host surface. 2, 4. Ascomata. 3. Section of ascoma. 5. Neck. 6-7, 10. Asci. 8. Ascospores. 9. Paraphysis. Scale bars: 1, 4 = 100 μm; 2-3, 6 = 50 μm; 5 = 20 μm; 7-10 = 10 μm.

## Discussion

### *A new species of Ophioceras*

A new taxon collected from dried leaves of *Dracaena loureiroi* could be recognised as a species of *Ophioceras* based on morphology and phylogenetic analysis of 18S and 28S rDNA sequence data. *Ophioceras chiangdaoense* sp. nov. is in the same clade as *O. leptosporum* and they are a sister clade to the polyphyletic clade of *O. dolichostomum*, *O. commune*, *O. fusiforme*, *O. hongkongense*, *O. tenuisporum* and *O. venezuelense* (Figs 11-12). The shape and size of ascomata and ascospores of the new taxon are also most similar to *O. leptosporum* (Table 3).

The new taxon is characterized by absence of stroma on the host tissue, the dark colour, shape and size of ascomata with a long neck and filiform ascospores. These characters agree well with the morphological taxonomic concept of *Ophioceras* (Teng, 1934; Conway and Barr, 1977; Shearer *et al.*, 1999; Tsui *et al.*, 2001). The ascospores of *O. chiangdaoense* are relatively short and wide, and truncate at both ends. Other species of *Ophioceras* with short ascospores are *O. commune*, *O. fusiforme*, *O. leptosporum*, *O. palmae*, *O. parasiticum* and *O. tenuisporum*, but all of them have narrower ascospores (Table 3). *Ophioceras chiangdaoense* differs from previously described species in ascospore shape. The ascospores of *O. chiangdaoense* and *O. tenuisporum* are similar in length and are 4-celled but in *O. chiangdaoense* and *O. tenuisporum* are similar in length and are 4-celled but in *O. chiangdaoense* ascospores are broader ( $66\text{-}94 \times 1\text{-}1.5 \mu\text{m}$  vs.  $54\text{-}75 \times 3.9\text{-}5.4 \mu\text{m}$ ) (Shearer *et al.*, 1999).

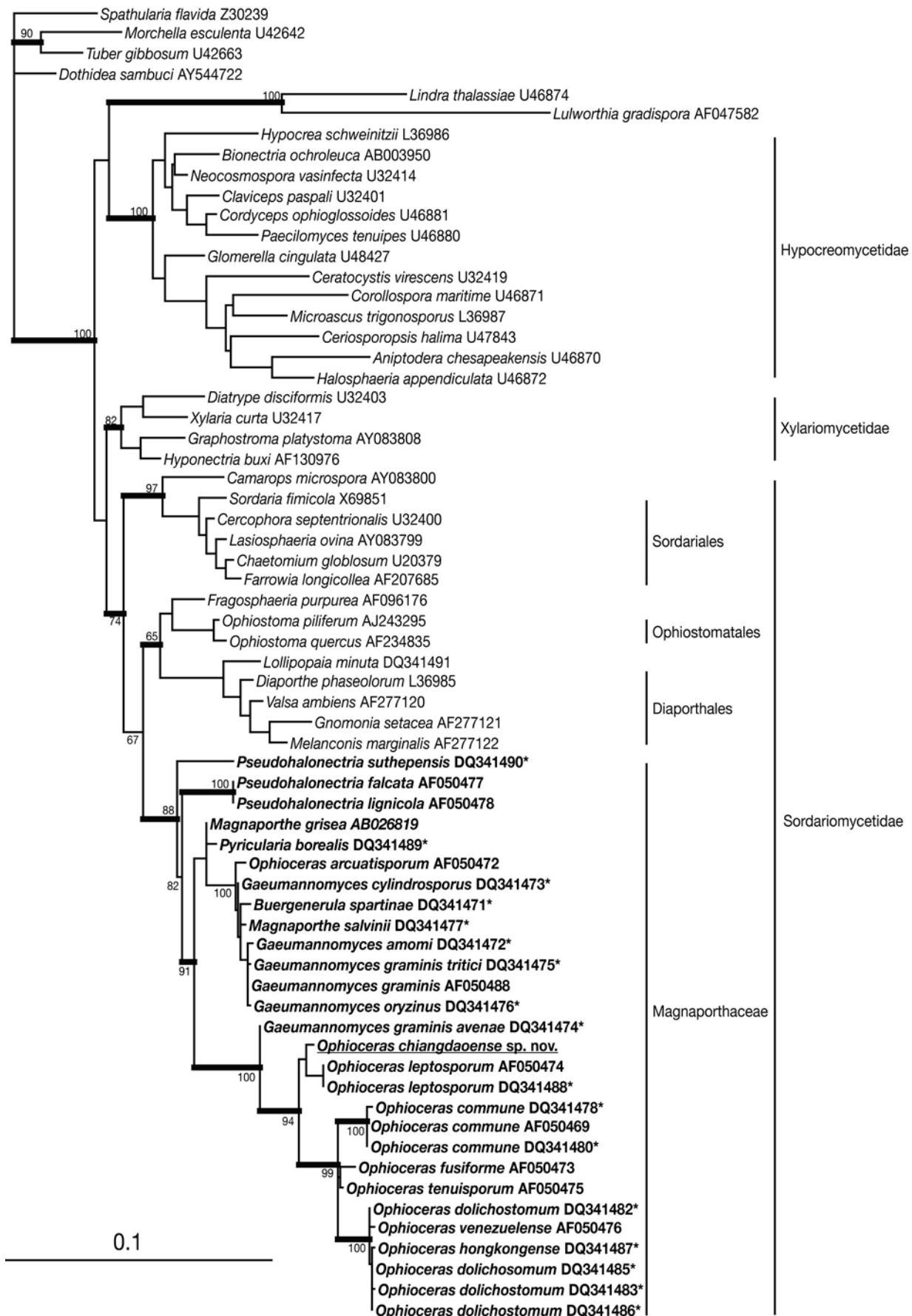
### *Phylogeny of Magnaporthaceae*

Based on rDNA sequence analysis, we recognize the family *Magnaporthaceae* as monophyletic. Phylogenies also show that *Magnaporthaceae* are closely related to the *Diaporthales* and *Ophiostomatales*, but whether they belong to any of those orders is contentious.

### *Ordinal classification of Magnaporthaceae and relationships to other orders*

When the type genus *Magnaporthe* (*M. salvinii*) was originally described by Krause and Webster (1972), it was considered to belong to the *Diaporthales* (*Diaporthaceae*). However, Arx and Müller (1975) placed it in the *Pleosporaceae* based on developmental and morphological characters (thick-walled asci), and the type of anamorph. *Magnaporthe salvinii* and *M. grisea* (T.T. Hebert) M.E. Barr lack a stroma, but were still accommodated in the *Diaporthales* by Monod (1983) and Alexopoulos *et al.* (1996). The order *Diaporthales* is characterized by perithecial ascocarps produced in a stroma of fungal and substrate origin, or directly from somatic hyphae on the substrate. The *Diaporthales* contains important plant pathogens and saprobes with many taxa having coelomycetous anamorphs (Alexopoulos *et al.*, 1996), quite unlike the hyphomycetous anamorphs associated with *Magnaporthe* species (e.g., *Pyricularia*; Cannon, 1994). Winka and Eriksson (2000) found that *Valsa leucostoma* and *Cryphonectria cubensis* (*Diaporthales*) appeared basal to the other pyrenomycetes in subclass Hypocreomycetidae, Sordariomycetidae and Xylariomycetidae in an analysis of long sequences of 18S rDNA dataset (1728 bp). But in the shorter dataset (998 bp) analysis it was shown that *Diaporthales* clade is a sister group to *Papulosaceae*, *Ophiostomatales*, *Sordariales* and *Phyllachorales*. The results of the short dataset resembled our study with the exception of *Papulosaceae* and *Phyllachorales* (Fig. 11). *Gaeumannomyces* and *Magnaporthe* are major plant pathogens (Yaegashi, 1977; Freeman and Ward, 2004), while *Ophioceras* and *Pseudohalonestria* species are mostly saprobes (Luo *et al.*, 2004). These genera differ from *Diaporthe* and *Gnomonia* (*Diaporthales*) in having multiseptate, filiform ascospores and distinct, often long, ascomatal necks. Filiform ascospores and long necked ascomata are also found in the diaporthalean genus *Lollipopaia* (Inderbitzin and Berbee, 2001). *Ophioceras* has been placed in *Gnomoniaceae* (*Diaporthales*), a family lacking true stromatic development (Wehmeyer, 1975). Species of *Ophioceras* and *Pseudohalonestria* have features common to both the *Sordariales* and *Diaporthales*, but are thought to belong to the





**Fig. 11.** Maximum likelihood tree of 64 fungal species based on the 18S rDNA. Numbers on the branches indicate maximum parsimony bootstrap values ( $\geq 50$ ). Thickened branches indicate  $\geq 95$  Bayesian posterior probabilities. The tree is rooted to *Spathularia flavida* (*Rhytismatales*). Scale bar, 0.01 substitutions per site.

**Table 3.** A comparison of *Ophioceras chiangdaoense* with some other previously described species.

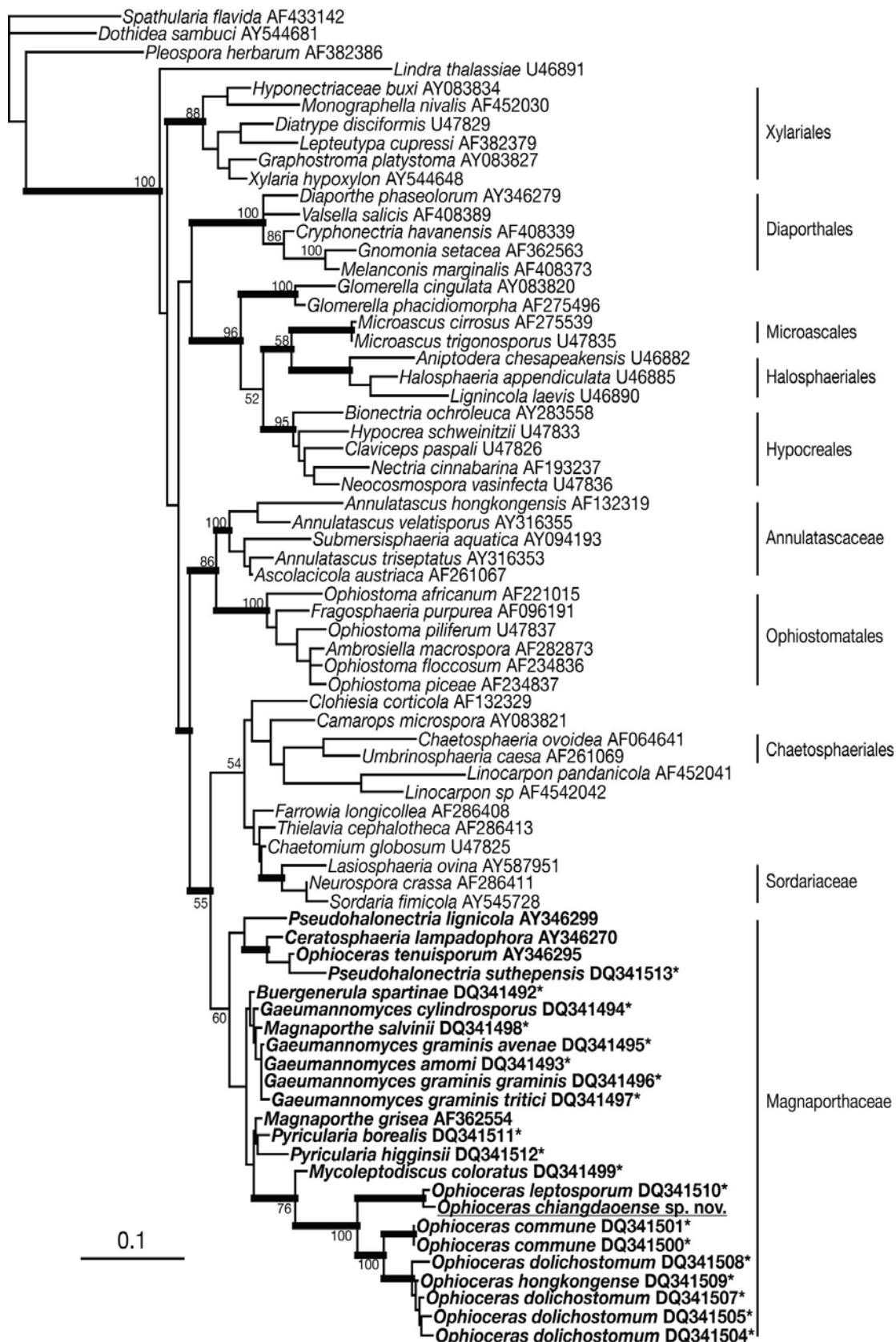
<i>Ophioceras</i> species (References)	Ascomata (µm)	Asci (µm)	Ascospores (µm)	Ascospore septation
<i>O. arcuatisporum</i> (Shearer <i>et al.</i> , 1999)	313-324 × 252-340	276-307 × 15-20	170-239 × 4-7	5-12
<b><i>O. chiangdaoense</i> sp. nov. (this study)</b>	<b>200-310 × 170-310</b>	<b>85-125 × 11-17</b>	<b>54-75 × 3.9-5.4</b>	<b>3</b>
<i>O. commune</i> (Shearer <i>et al.</i> , 1999)	150-350 × 260-400	64-118 × 4-12	50-110 × 2	3-7
<i>O. dolichostomum</i> (Conway & Barr, 1977)	500 diam.	100-130 × 8-12	94-110 × 2-3	3-7
<i>O. fusiforme</i> (Shearer <i>et al.</i> , 1999)	360-500 × 330-450	70-112 × 6-12	64-104 × 1.5-3	3-5
<i>O. guttulatum</i> (Tsui <i>et al.</i> , 2001)	400-600 × 1200-1800	130-160 × 14-17	100-128 × 4-5	3-5
<i>O. hongkongense</i> (Tsui <i>et al.</i> , 2001)	500-640 × 700-800	100-125 × 12-14	72-101 × 3.5-4.5	3-6
<i>O. leptosporum</i> (Shearer <i>et al.</i> , 1999)	250-300 diam.	70-95 × 5-6	70-80 × 1-1.5	3-7
<i>O. palmae</i> (Tsui <i>et al.</i> , 2001)	164-320 × 244-288	76-96 × 10-14	79-90 × 3-4	5
<i>O. parasiticum</i> (Teng, 1934)	600-800 diam	100-140 × 9-11	48-70 × 2.5-3.3	3-9
<i>O. tenuisporum</i> (Shearer <i>et al.</i> , 1999)	240-625 × 260-775	82-114 × 4-6	66-94 × 1-1.5	3
<i>O. venezuelense</i> (Shearer <i>et al.</i> , 1999)	730-890 × 745-868	148-180 × 11-18	130-158 × 2-4	5

*Sordariales* because all species possess peridial tissue of *textura angularis* (Conway and Barr, 1977; Shearer, 1989). Recent classification schemes, however, have shown that *Magnaporthaceae* may be misplaced in the *Diaporthales*, *Ophiostomatales* or *Sordariales* (Chen *et al.*, 1999; Zhang and Blackwell, 2001; Castlebury *et al.*, 2002; Wanderlei-Silva *et al.*, 2003; Réblová and Seifert, 2004; Tang *et al.*, 2007). These previous investigations, and 18S-28S rDNA sequence data in this study suggest that magnaporthaceous taxa are closely related and comprise a single order.

The *Hyponectriaceae* and *Magnaporthaceae* were assumed by Cannon (1994) to provide a possible link between the *Diaporthales* and *Phyllachorales* and he suggested assigning a new order to them. Both families have, however, been accommodated in *Phyllachorales* by Barr (1976b). Results here reveal that there is no close phylogenetic affiliation of *Magnaporthaceae* with any other members of the *Xylariales* or *Diaporthales*. The latter appears to be more closely related to other ascomycete orders rather than the family *Magnaporthaceae*.

Another family with morphological similarities to the *Magnaporthaceae* is the *Annulatasceae* (Krause and Webster, 1972; Ho and Hyde, 2000; Lee *et al.*, 2004). type species of *Magnaporthe* and *Annulatasceus* have cylindrical asci with large apical rings and other similar characters (Krause and Webster 1972; Ho *et al.*, 2000). The representatives of *Annulatasceae* used in this study do not cluster near to the *Magnaporthaceae* (Fig. 12) and the family appears to be related to the order *Ophiostomatales*. Similar results were found by recent studies (Réblová and Winka, 2001; Campbell and Shearer, 2004; Huhndorf *et al.*, 2004; Vijaykrishna *et al.*, 2006a; Tang *et al.*, 2007)

Phylogenies based on both sequence datasets suggest that *Magnaporthaceae* is not well-accommodated in any known fungal order. Its phylogenetic placement, monophyly and level of diversity support the erection of a new order to accommodate it. Such a taxonomic arrangement has already been suggested by Cannon (1994), who erected the *Magnaporthaceae* in the order *Diaporthales*. We therefore introduce a new order here.



**Fig. 12.** Maximum likelihood tree of 65 fungal species based on the 28S rDNA. Numbers on the branches indicate maximum parsimony bootstrap values ( $\geq 50\%$ ). Thickened branches indicate  $\geq 95$  Bayesian posterior probabilities. The tree is rooted to *Spathularia flavida* (Rhytismatales). Scale bar, 0.01 substitutions per site.

**Magnaporthales** Thongkr., Vijaykr. & K.D. Hyde, **ord. nov.**  
MycoBank: MB 512852.

*Ascomata* perithecia, cum collum breve vel longa, lutea, brunnea vel nigra, substrata colourata, immersa vel superficialia, paraphysata, paraphyses lates ad basim et gradatim angustati. *Asci* cylindrici, 8-spori, unitunicati, cum annuli caerulescenti, variabili. *Ascosporae* septatae, saepe filiformia, hyalinae, lutae vel brunnae.

*Apresoria* praesentia. *Anamorphosi* variabili sed cellulae conidiogenae semper pigmentatae. Sclerotia interdum praesentia.

*Ascomata* perithecia, with short or long necks, yellow, brown or black, often staining substrate, immersed or superficial, paraphysate, paraphyses wide at the base and tapering gradually. *Asci* formed from a crozier system, cylindrical, 8-spored, unitunicate, with a refractive apical, variable in size. *Ascospores* septate, often filiform, hyaline, yellow to brown.

*Apresoria* present. *Anamorphs* variable but conidiogenous cells always pigmented, belonging to the form genera *Clasterosporium*, *Dactylaria*, *Mycoleptodiscus Nakataea*, *Phialophora*, *Pseudotracylla* and *Pyricularia* Sclerotia sometimes formed.

*Families included: Magnaporthaceae.*

*Representative genera: Gaeumannomyces, Ophioceras, Magnaporthe, Pseudohalonestria.*

*Nutrition:* necrotrophic parasites or saprotrophs often on grasses, but also on submerged wood.

### ***Intergeneric relationships within***

#### ***Magnaporthaceae***

The inclusion of genera and species in the *Magnaporthaceae* (*Magnaporthales*) has predominantly been based on morphological characters and more recently on phylogeny (Table 1). Eriksson (1999) and Schoch *et al.* (2007) included *Clavatisporella* in the family. *Ophioceras* and *Pseudohalonestria* were also members of the *Magnaporthaceae* following phylogenetic support in an investigation by Chen *et al.* (1999) and Inderbitzin and Berbee (2001). *Juncigena* previously considered to belong to the *Magnaporthaceae* (Eriksson, 1999; Kirk *et al.*, 2001) has recently been placed in Hypocreomycetidae *incertae sedis*, supported by phylogeny data of Schoch *et al.* (2007).

In our dataset, *Magnaporthe salvinii*, the type species of *Magnaporthaceae*, is more closely related to *Buergenerula spartinae*, five species of *Gaeumannomyces* and *Ophioceras arcuatisporum* Shearer, J.L. Crane & W. Chen than to other species of *Ophioceras* or to *Pseudohalonestria* species (Fig. 11). This is supported by the fact that there are only minor differences in ascospore characters in all three genera (Barr, 1977). *Magnaporthe* is similar to *Buergenerula* in all characters except for ascospore septation. Most *Buergenerula*, *Magnaporthe* and *Gaeumannomyces* species are usually found as pathogens on monocotyledonous plants. Ascomatal and ascus features of these genera are similar to each other, in addition to the presence of appressoria (Cannon and Alcorn, 1994). In addition, anamorphic and pathogenicity characters of *Gaeumannomyces* share many similarities with *Magnaporthe* (Cannon, 1994). Despite the fact that *O. arcuatisporum* forms a sister relationship to the clade supporting *Buergenerula*, *Gaeumannomyces*, and *M. salvinii* (with high support) it was found to be closer to *G. cylindrosporus* than *G. graminis* based on 18S rDNA sequences analyses (Chen *et al.*, 1999). This taxon was isolated from submerged herbaceous debris but whether it is a parasite on its substrate is unknown (Shearer *et al.*, 1999). The authors commented only on the presence of paraphyses and periphyses to represent this species. *Gaeumannomyces cylindrosporus* is distinguished from *G. graminis* and its varieties by shorter, wider, fusoid ascospores, 40-70 × 3-5 µm with 3-5 septa (Walker, 1980). Likewise, *O. arcuatisporum* ascospores are much longer and broader (170-239 × 4-7 µm) than those of *G. cylindrosporus* as well as all of *G. graminis* varieties (70-130 × 2-3.5 µm). In this study we found that *M. grisea* groups together with *Pyricularia borealis* and not with *M. salvinii* as would be expected from a morphological perspective. *Magnaporthe grisea* has also been linked to *Gaeumannomyces* based on 18S rDNA sequence phylogenies (Bryan *et al.*, 1995) and molecular data herein are consistent with previous findings and shows that *M. grisea* and *M. salvinii* are phylogenetically distinct taxa.

The majority of taxa within *Magnaporthaceae* are members of the genera

*Ophioceras* (34 species + 2 varieties) and *Pseudohalonectria* (13 species). These two genera often occur in aquatic habitats on dead plant materials (Luo *et al.*, 2004; Tsui and Hyde, 2004; Shenoy *et al.*, 2005; Vijaykrishna *et al.*, 2006b; Vijaykrishna and Hyde, 2006). Based on morphological characters they are similar to each other (Shearer 1989; Hyde, 1992; Hyde *et al.*, 1999; Tsui *et al.*, 2001, 2004). Hanlin (1998) and Hyde *et al.*, (2000) pointed out that *Pseudohalonectria* differs from *Ophioceras* in having bright yellow, membranous ascomata. In both genera, asci become detached from the ascogenous hyphae and lie free in the ascomatal cavities. However, in *Pseudohalonectria* ascospores are discharged through the ascomatal beaks and accumulate in masses. By contrast, in *Ophioceras*, intact asci are forced up through the neck to the apex. The narrow canal of the beak allows the passage of only one ascus.

Phylogenetically significant characters that are useful for delineating *Gaeumannomyces*, *Ophioceras* and *Pseudohalonectria* have been questioned (Chen *et al.*, 1999). They found that *G. graminis* falls within the *Ophioceras/Pseudohalonectria* clade (6 and 2 different species, respectively). Our study based on both individual and combined datasets, with the inclusion of three additional taxa (*Buergenerula biseptata* (Rostr.) Syd. and some other species of *Gaeumannomyces* and *Ophioceras*), provides additional phylogenetic insights and resolution. *Ophioceras* seems to be more closely related to *Buergenerula*, *Gaeumannomyces* and *Magnaporthe* species than to *Pseudohalonectria* species with high support. Results here do not corroborate those of Chen *et al.*, (1999) who suggested that *Ophioceras* is phylogenetically closer to *Pseudohalonectria* than *Gaeumannomyces*.

Based on our studies, together with results from previous studies (Shearer, 1989; Chen *et al.*, 1999; Hyde *et al.*, 1999, 2000; Tsui *et al.*, 2001, 2004), *Buergenerula*, *Magnaporthe*, *Gaeumannomyces*, *Omnidemtus*, *Ophioceras* and *Pseudohalonectria* can broadly be categorized into three major groups: a) hyperparasites with apressoria (*Buergenerula*, *Gaeumannomyces* and *Magnaporthe*); b) presence of dark brown to black ascomata with a single ascus discharged through the beak of the

ascomata (*Ophioceras*); c) presence of yellow to brown ascomata with ascospores amassing at the ascomatal beaks (*Pseudohalonectria*).

#### **Placement of recently described taxa in Magnaporthaceae**

This study confirms the placement of three recently described taxa in their respective genera. *Gaeumannomyces amomi* was established by Bussaban *et al.* (2001) as an endophyte from wild ginger (*Amomum siamense*) in Thailand. In the phylogenies inferred based on combined and individual 18S and 28S rDNA datasets, it clusters with varieties of *G. graminis* as well as *G. oryzae* (Figs 11-12). Results reported here are consistent with those based on ITS sequence data as reported by Bussaban *et al.* (2005). *Ophioceras hongkongense* was described by Tsui *et al.* (2001), who noted that the ascospores are similar to those of *O. commune* and *O. fusiforme* in length and number of septa, but are wider. The inclusion of *O. hongkongense* in the genus is supported by molecular data (Figs 11-12). This species appears to be more related to the type species of *Ophioceras*, *O. dolichostomum* than *O. commune* and *O. fusiforme*. The ascomata, asci and ascospores in *O. hongkongense* are similar to those of *O. dolichostomum* in length but they are broader (3.5-4.5  $\mu\text{m}$  as compared to 2-3  $\mu\text{m}$ ). *Pseudohalonectria suthepensis* was isolated from dead leaves of *Magnolia liliifera* in Thailand by Promputtha *et al.* (2004). The ascospores and asci of this species are longer than those of most *Pseudohalonectria* species with the exception of *P. falcata* and *P. lutea*.

#### **Magnaporthaceae and their anamorphs**

DNA sequence analyses have been useful to verify and predict anamorph-teleomorph connections especially for those fungi that cannot be cultured or that fail to sporulate under artificial conditions (e.g. Crous *et al.*, 2007; Damm *et al.*, 2007; Phillips *et al.*, 2007; Shenoy *et al.*, 2007). *Pyricularia* has been linked to *Magnaporthe* based predominantly on physiological characters (e.g. Ellis, 1971, 1976; Matsuyama *et al.*, 1977; Walker, 1980) and more recently molecular information (e.g. Bryan *et al.*, 1995; Kato *et al.*, 2000; Bussaban *et al.*, 2001, 2005).

Krause and Webster (1972) established *Magnaporthe salvinii*, with *Nakataea sigmoidea* (Cavara) Hara anamorph and a sclerotial state of *Sclerotium oryzae* Catt. An isolate of this species was unavailable for this study. *Magnaporthe grisea* commonly known by its anamorph, *Pyricularia oryzae*, has a wide host range on grasses and is the causal agent of rice blast (Yaegashi, 1977). Our phylogenetic analyses also found that *P. borealis* is related to *M. grisea* (Figs 11-12), likewise *P. higginsii* grouped with *M. grisea* in the 28S rDNA analysis (Fig. 12). *Pyricularia zingiberis* has been reported to be the anamorph of *G. amomi* based on ITS sequence analysis (Bussaban *et al.*, 2005). They also reported that species with obpyriform conidia (*P. higginsii* and *P. junci-cola*, previously transferred to *Dactylaria*) represent a monophyletic lineage and grouped within the family *Magnaporthaceae* with high bootstrap support, and they suggested that both species should be maintained in *Pyricularia*. A connection between the phialidic anamorph *Harpophora graminicola* (Deacon) W. Gams (= *Phialophora radiculicola* Cain) and *Gaeumannomyces cylindrosporus* was supported by ITS sequence similarity (Walker, 1980; Bryan *et al.*, 1995). Further work based on other genes is needed to confirm other anamorphic counterparts (e.g. *Dactylaria*, *Nakataea*, *Phialophora*, *Pyriculariopsis*) and to establish whether they are related to the family *Magnaporthaceae*.

Some *Phialophora*-like species have been reported to be the anamorphs of some *Gaeumannomyces* species. For example, *Harpophora graminicola* (Deacon) W. Gams (= *G. cylindrosporus*) and *Phialophora* sp. (= *G. graminis* var. *graminis*), the take-all fungus, were investigated by Ward and Akrofi (1994), and Freeman and Ward (2004). Bryan *et al.* (1995) found that *G. graminis* var. *tritici* and *G. graminis* var. *avenae* are more closely related to each other than either is to *G. graminis* var. *graminis*. Similar results were obtained in our study (28S rDNA sequence data) except that our results do not totally agree with the conclusions of Bryan *et al.* (1995) based on the morphologies of the anamorphs. *Gaeumannomyces graminis* var. *tritici* and *G. graminis* var. *avenae* have *Phialophora*

anamorphs with simple hyphopodia, while the *G. graminis* var. *graminis* anamorph has lobed hyphopodia. Bussaban *et al.* (2001) also showed that *G. amomi* possesses distinctive irregular hyphopodia in culture. Phylogenies generated in this study show that *G. graminis* var. *tritici* is more closely related to *G. amomi*, than to *G. graminis* var. *avenae*, which is separated from these three species.

*Mycoleptodiscus affinis* is the anamorph of *Omnidemtus affinis* (Cannon and Alcorn, 1994). *Omnidemtus* is distinguished from *Magnaporthe* by its ascospores, which are similar in shape, but remain hyaline at maturity, and asci that have a J+ apical ring. *Mycoleptodiscus coloratus* groups in the *Magnaporthaceae* clade (Fig. 12) in 28S rDNA analysis. Our result confirmed that *Mycoleptodiscus* is an anamorphic *Magnaporthaceae*.

#### **Phylogenies of recently described taxa**

Some species have recently been described in *Ceratosphaeria* and *Lollipopaia* (Hyde *et al.*, 1997; Inderbitzin and Berbee, 2001). Based on current morphological classification, these genera have filiform ascospores with or without long necked ascomata that are similar to *Magnaporthaceae*, while some *Ceratosphaeria* species have also been transferred to the *Magnaporthaceae* (e.g. *M. grisea* was previously treated as *Ceratosphaeria grisea* (Barr, 1977)). Huhndorf *et al.* (2004) investigated the phylogeny of *Ceratosphaeria*, *Ophioceras* and *Pseudohalonectria*. They suggested that *Ceratosphaeria lampadophora* had affinities with *Ophioceras tenuisporum* and *Pseudohalonectria lignicola*. Hyde *et al.* (1997) noted that in *Ceratosphaeria lampadophora* ascomata are black, superficial and globose with a long neck. The ascomal wall had several different layers that resemble those of *P. eubenangeensis* (Hyde *et al.* 1999). Our results also support a close relationship between *Ceratosphaeria lampadophora* and *O. tenuisporum* and *P. suthensis*. It is highly possible that *Ceratosphaeria lampadophora* is an earlier name for *Ophioceras/Pseudohalonectria* species, but broader taxon sampling is required before any taxonomic changes are proposed. *Lollipopaia minuta* was described as a new genus from a tropical rain forest in Thailand by Inderbitzin and Berbee (2001).

The ascospores have long necks and are seated on a pseudoparenchymatous stroma. The type species is similar to *Ophioceras* and *Pseudohalonectria* in ascospore shape, ascus and ascospore morphology. However, phylogenetic analyses of the small subunit rDNA here confirmed the placement of *L. minuta* within the *Diaporthales* with high bootstrap support.

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