CHAPTER 5

MOLECULAR PHYLOGENY OF MAGNAPORTHACEAE (SORDARIOMYCETES): IMPLICATION ON TAXONOMY AND PHYLOGENETIC PLACEMENT

5.1 INTRODUCTION

The Magnaporthaceae is a small family of unitunicate, perithecial ascomycetes. Its taxonomic boundaries are however ill defined, and the number of genera that should be accommodated within the family is still unclear. While the *Dictionary of the Fungi* (Kirk *et al*., 2001) accepts 9 genera and 26 species, the latest ascomycete classification proposed by Eriksson (2005) incorporates 10 genera (Table 5.1). *Gaeumannomyces* and *Magnaporthe* species and their anamorphs constitute the most important members of this family, as they are serious pathogens of economic plants worldwide (Yaegashi, 1977; Freeman and Ward, 2004). *Gaeumannomyces graminis* (Sacc.) Arx & D.L. Olivier causes one of the most important root diseases of wheat (Berbee, 2001; Freeman and Ward, 2004) while *Pyricularia oryzae* Cavara is the causal agent of rice blast (Yaegashi, 1977). Other *Magnaporthe/Pyricularia* species cause blast disease of banana, maize, millet, pearl and a number of other grass species (Yaegashi and Hebert, 1976; Yaegashi, 1977; Landschoot and Jackson, 1989; Berbee, 2001). *Buergenerula* species (also Magnaporthaceae) have also been found to be associated with a distinctive leaf spot of *Carex* species (McKenzie, 1991b). A serious disease, stem canker, caused by *Ophioceras* sp. in Malaya, can be fatal to rambutan (*Nephelium lappaceum* L.) 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 lack of a stromata, 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, without separable layers and with a large apical pore often surrounded by a massive J+ apical ring. However, the type species *M. salvinii* (Catt.) R.A. Krause & R.K. Webster has a refractive ring that does not stain blue in iodine (Hanlin, 1998). Ascospores are septate, often filiform and with a sheath (Cannon, 1994; Kirk *et al.*, 2001).

Kirk *et al.* (2001) and Eriksson (2005) accepted nine and ten genera in the Magnaporthaceae respectively (Table 1). This group of fungi has many similarities, most notably in the teleomorph form and pathogenic effects, although their anamorphs are variable (Cannon, 1994). 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; Eriksson, 2005). Some magnaporthaceous genera have been placed or are possibly closely related to various orders (e.g Diaporthales, Ophiostomathales, Phyllachorales and Sordariales) [Barr, 1976a, b; Huang, 1976; Conway and Barr, 1977; Jensen, 1985; Cannon, 1994; Berbee, 2001; Castlebury *et al.*, 2002; Zhang and Blackwell, 2001; Wanderlei-Silva *et al.*, 2003].

The objectives of the present study was 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.

Table 5.1 Members of the Magnaporthaceae and their anamorphs.

Kirk *et al.* (2001)		Eriksson (2005)
Teleomorph	Anamorph	
Buergenerula Syd.	*Passalora*-like, *Nakataea*-like	*Buergenerula* Syd.
Clasterosphaeria Sivan	*Clasterosporium*	*Clasterosphaeria* Sivan.
Gaeumannomyces Arx & D.L. Olivier	*Pyricularia*	*Clavatisporella* K.D. Hyde
Herbampulla Scheuer & Nograsek	unknown	*Gaeumannomyces* Arx & D.L. Olivier
Juncigena Kohlm., Volkm.-Kohlm. & O.E. Erikss	*Cirrenalia*	?*Herbampulla* Scheuer & Nograsek
Magnaporthe R.A. Krause & P.K. Webster	*Pyricularia*, *Nakataea*, *Phialophora*, *Sclerotium*	*Juncigena* Kohlm., Volkm.-Kohlm. & O.E. Erikss.
Omnidemptus P.F. Cannon & Alcorn	*Mycoleptodiscus*	*Magnaporthe* R.A. Krause & R.K. Webster
Ophioceras Sacc.	unknown	*Omnidemptus* P.F. Cannon & Alcorn
Pseudohalonectria Minoura & T. Muroi	unknown	*Ophioceras* Sacc.
		Pseudohalonectria Minoura & T. Muroi

5.2 MATERIALS AND METHODS

5.2.1 Fungal cultures and DNA extraction

Living cultures were obtained from the American Type Culture Collection (ATCC), the BIOTEC, Thailand (BCC), the Centraalbureau voor Schimmelcultures (CBS), and Hong Kong University Culture Collection (HKUCC). Collections and cultures of anamorph/teleomorph genera of Magnaporthaceae fungi, *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 total of 29 taxa from the Magnaporthaceae, representing 5 teleomorphic genera, and an additional 2 anamorphic genera were included in the study. Magnaporthaceous strains and GenBank sequences used in the study are listed in Table 2. DNA extraction protocol as outlined by Jeewon *et al.* (2002, 2004) and Cai *et al.* (2006) was used. Briefly, actively growing mycelia were scraped off cultures on agar plates. The mycelium was ground with 200 mg of sterilized quartz sand and 600 µl of 2×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 two or three 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 (1 mg/ml).

5.2.2 PCR amplification and sequencing of 28S and 18S rDNA

Approximately 900 nucleotides at the 5′ end of 28S rDNA region were amplified by primer pairs LROR (5'-ACCCGCTGAACTTAAGC-3') and LRO5 (5'-TCCTGAGGGAAACTTCG-3') (Vilgalys and Hester 1990) (Figure 5.1). The 18S rDNA was partially amplified using primers NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3') (White *et al.*, 1990) (Figure 5.2). Three µl of genomic DNA was used in a standard 50 µl PCR mixture (25mM $MgCl_2$, 10 Mg-free buffer, 2.5 µM dNTPs, 1.5 µM primers, and 1.5 unit of *Taq* Polymerase) under the following thermal conditions: 94 C for 3 min, 94 C for 50s, 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 visualized on 1% agarose gel electrophoresis (stained with ethidium bromide) under UV light to check for size and purity. 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™ 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 the PCR primers mentioned above.

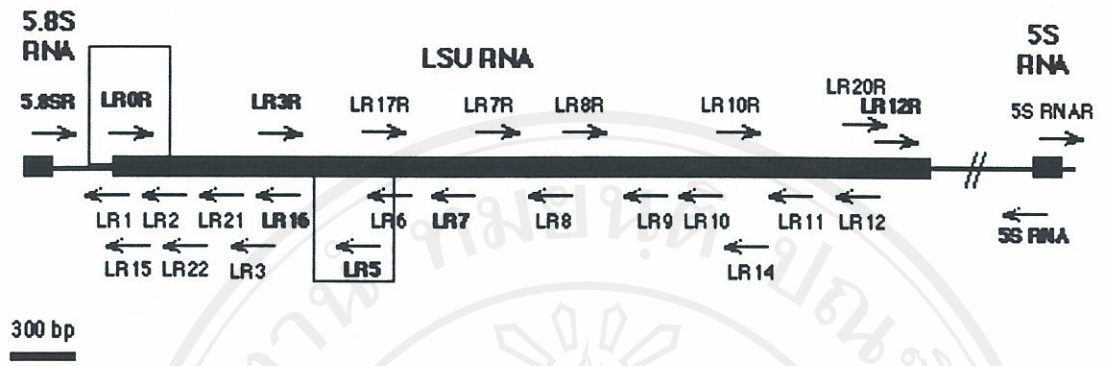

Figure 5.1 Primers for amplify and sequencing partial 28S rDNA. Note LROR and LR were used in this study.

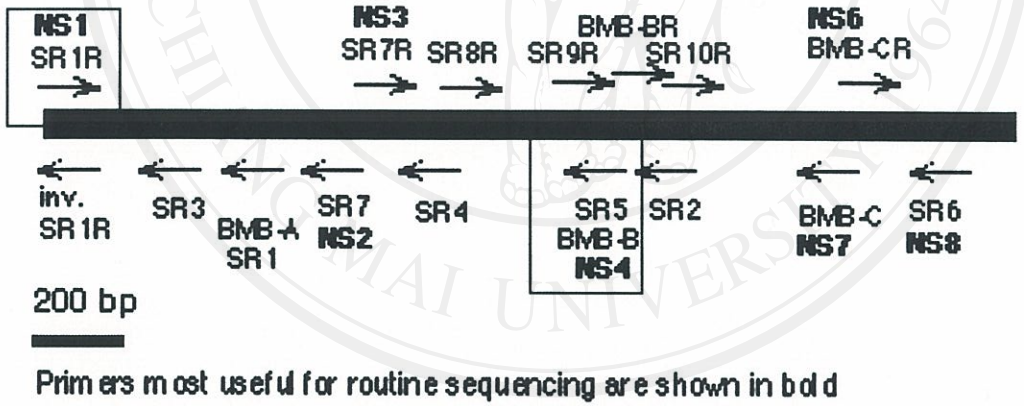

Figure 5.1 Primers for amplify and sequencing partial 18S rDNA. Note NS1 and NS4 were used in this study.

Table 5.2 Magnaporthaceous taxa and their GenBank accession number used in this study.

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

* Sequences determined for this study.

5.2.3 Phylogenetic analyses

Nucleotide sequences of the 28S and 18S rDNA from different fungal families were initially aligned with the Clustal X 1.83 (Chenna *et al.*, 2003) with default parameter settings and Bioedit (Hall, 1999). The alignments were then manually edited to optimize alignment using Se-al v2.0a11 (Rambaut, 1996). Four datasets, which differed in taxon sampling, were analysed.

Dataset I, contained 28S rDNA sequences of Magnaporthaceae taxa and some Magnaporthaceae-like taxa (*Ceratosphaeria*, *Linocarpon*), was used to examine phylogenetic relationships among long neck perithecia with filiform or fusiform ascospore pyrenomycetes and their anamorphic taxa. Dataset II, consisting of partial 18S rDNA sequences and 5 taxa from the Magnaporthaceae, was analysed with other ascomycota taxa. Dataset III, a smaller dataset, derived from Dataset II was analysed to resolve the phylogenetic relationships of Magnaporthaceae species and it affinities to similar families/orders. Dataset IV is a combination of 18S-28S rDNA sequences and analysed separately to see if there is any incongruence between the phylogenies generated from individual datasets.

Two additional 18S-28S rDNA datasets were also analysed for the combined dataset. The method of assessing combinability of these two datasets in this study is by simply comparing highly supported clade among trees generated from different data sets to detect conflict (Miller and Huhndorf, 2005). High support typically refers to bootstrap support values ≥70% and Bayesian posterior probabilities ≥95% (Alfaro *et al.*, 2003).

All sequence datasets were analysed individually. Each datasets was subjected to 3 methods of phylogenetic analysis: bayesian, maximum likelihood (ML) and maximum parsimony (MP). The bayesian analysis was conducted using the computer program MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). The MP and ML analyses were carried out using PAUP*4.0b10 (Swofford, 2002).

MP1 and MP2 analyses were maximum parsimony treated gap as missing and fifth state respectively. MP3 analysis was maximum parsimony carried out on data matrices containing only variable characters. Symmetric step matrices were created for unambiguous portions of 18S and 28S rDNA alignments using the STMatrix 2.1 program (written by S. Zoller and available upon request from S.Z. or F.L.), as

outlined in Miadlikowska *et al.* (2002). The 18S and 28S rDNA each were subjected to a specific symmetric step matrix. Gaps were used as a fifth character states for unambiguous portions of the alignment. All ambiguously aligned regions were excluded. However, these regions were recoded with the INAASE program (Lutzoni *et al.*, 2000) and then re-integrated into the dataset for MP analysis.

Maximum parsimony was based on heuristic seach option with the random addition sequence and TBR branch swaping.). Relative support for branches was estimated with 1000 bootstrap replications (Felsenstein, 1985).

MrModeltest 2.2 (Nylander, 2004) was used to determine the best-fit model of evolution for each datasets. Best model selected was GTR+I+G. Bayesian analyses employed a Markov Chain Monte Carlo (MCMC) method. MCMC chains were run for 1,000,000 generation with trees sampled every 100th generation resulting in 10,000 total trees. The MCMC chains always achieved stationary phase after the first 2000 trees, so the burnin of 200,000 generations was discarded. Bayesian posterior probabilities (BPT) were determined from a consensus tree generated from the remaining 8,000 trees (Alfaro *et al.*, 2003).

5.3 RESULTS

5.3.1 Phylogenies based on 28S rDNA dataset (dataset I)

Results obtained from this dataset are summarized in Table 3. The transitions and transversions were weighted for MP3 analysis in the original rate substitution stepmatrix and are as follows: A↔C=2.22, A↔G=1.96, A↔T=2.19, C↔G=2.07, C↔T=1.73 and G↔T=2.12. One of the best bayesian trees based on KH test generated from this dataset with bootstrap support above and bayesian posterior probabilities below the branches for the major orders is shown in Figure 5.3. All members of the Magnaporthaceae grouped together in a well supported clade (with 99% BPT support). This group was a sister to members of the Boliniales, Chaetosphaeriales, Trichosphaeriales and Sordariales but there were no statistical support for this relationship. The family Magnaporthaceae separates into 3 clades. They include Clade A (*Buergenerula, Gaeumannomyces, Magnaporthe* and *Pyricularia* species) supported by 78% BT and 94% BPT. Clade B consists of

Ophioceras species. All species of *Ophioceras* (except *O. tenuisporum*) forms a strong monophyletic clade with 99 % BT and 100% BPT and having *Mycoleptodiscus coloratus* as sister taxon. Clade C, with 89% BPT, is characterized by members of *Pseudohalonectria* as well as *Ceratosphaeria lampadophora* and *Ophioceras tenuisporum* as sister taxa.

5.3.2 Phylogenies based on 18S rDNA dataset (dataset II)

This dataset consisted of 103 taxa from major fungal groups including 5 taxa from the Magnaporthaceae. Results from this dataset provided little resolution at the backbone of the tree or for most of the major clades. The affinities of the Magnaporthaceae with other recognized orders could not be confidently assessed and therefore the results are not shown. When the number of sister taxa was reduced in the dataset (not ingroups), results showed that there is a strong phylogenetic relationship between Magnaporthaceae and Diaporthales and Ophiostomatales. It should be mentioned however, that the Magnaporthaceae was monophyletic with high statistical support and the relationships among the different genera were similar to those phylogenies derived from the 28S rDNA dataset.

5.3.3 Phylogenies based on 18S rDNA dataset (dataset III)

This dataset consisted of 65 taxa and results from MP1, MP2, MP3, bayesian and ML analyses are summarized in Table 5.3. The transitions and transversions were weighted for MP3 analysis in the original rate substitution stepmatrix and are as follows: A↔C=1.93, A↔G=1.78, A↔T=1.89, C↔G=1.96, C↔T=1.60 and G↔T=2.76.

Given that heuristics search found a large number of trees, I limit bootstrap analyses to maxtrees=1000 instead of 5000 as in other analyses. One of the best bayesian trees generated from this dataset with bootstrap support above and BPT below the branches are given on Figure 5.4. Phylogenies generated are generally similar in topology to those of from 28S rDNA dataset. A total of 27 Magnaporthaceae taxa forms a strongly support (93% BT and 100% BPT) monophyletic group with the Diaporthales and Ophiostomatales as sister orders.

Table 5.3 Results of the phylogenetic analysis of 18S and 28S rDNA.

	28S rDNA-73 taxa					18S rDNA-65 taxa					Combine 18S-28S rDNA-51 taxa				
	MP1	MP2	MP3	Bayesian Fig.1	ML	MP1	MP2	MP3	Bayesian Fig.2	ML	MP1	MP2	MP3	Bayesian Fig.3	ML
No. of trees generated	259	14	7	10, 001	1	35,909	35,909	35,909	10, 001	1	1	2	12	10, 001	2
Total characters	918	918	925	918	918	1044	1044	1044	1044	1044	1951	1951	1961	1951	1850
Excluded characters	63	63	63	63	63	0	0	0	0	0	101	101	101	101	101
Parsimony-informative	292	317	324	-	-	244	254	254	-	-	441	487	496	-	-
No. of addition random sequence replicate in heuristic search	1,000	1,000	1,000	-	-	1	1	1	-	-	10	10	10	-	-
Tree length	2038	2245	2441	2049	2051	955	996	996	955	959	2050	2310	2558	2054	2056
Consistency index (CI)	0.334	0.362	0.391	0.332	0.332	0.515	0.525	0.525	0.515	0.513	0.438	0.474	0.503	0.437	0.436
Retention index (RI)	0.637	0.635	0.634	0.634	0.633	0.787	0.785	0.785	0.787	0.785	0.700	0.694	0.691	0.699	0.698
Rescaled consistency index (RC)	0.213	0.230	0.248	0.211	0.210	0.406	0.412	0.412	0.406	0.403	0.306	0.328	0.348	0.305	0.305
Homoplasy index (HI)	0.666	0.638	0.609	0.667	0.668	0.485	0.475	0.475	0.485	0.487	0.562	0.526	0.497	0.563	0.564
No. of bootstrap replicate	1,000	1,000	1,000	-	-	1,000	1,000	1,000	-	-	1,000	1,000	1,000	-	-
No. of addition random sequence replicate in bootstrap heuristic search	1	1	1	-	-	1	1	1	-	-	1	1	1	-	-

MP1=maximum parsimony, gapmode were treated as missing

MP2= maximum parsimony, gapmode were treated as newstate

MP3= maximum parsimony, gapmode were treated as newstate with a symmetric step matrix generated with the program STMatrix v.2.2

ML= maximum linklihood, gapmode were treated as missing

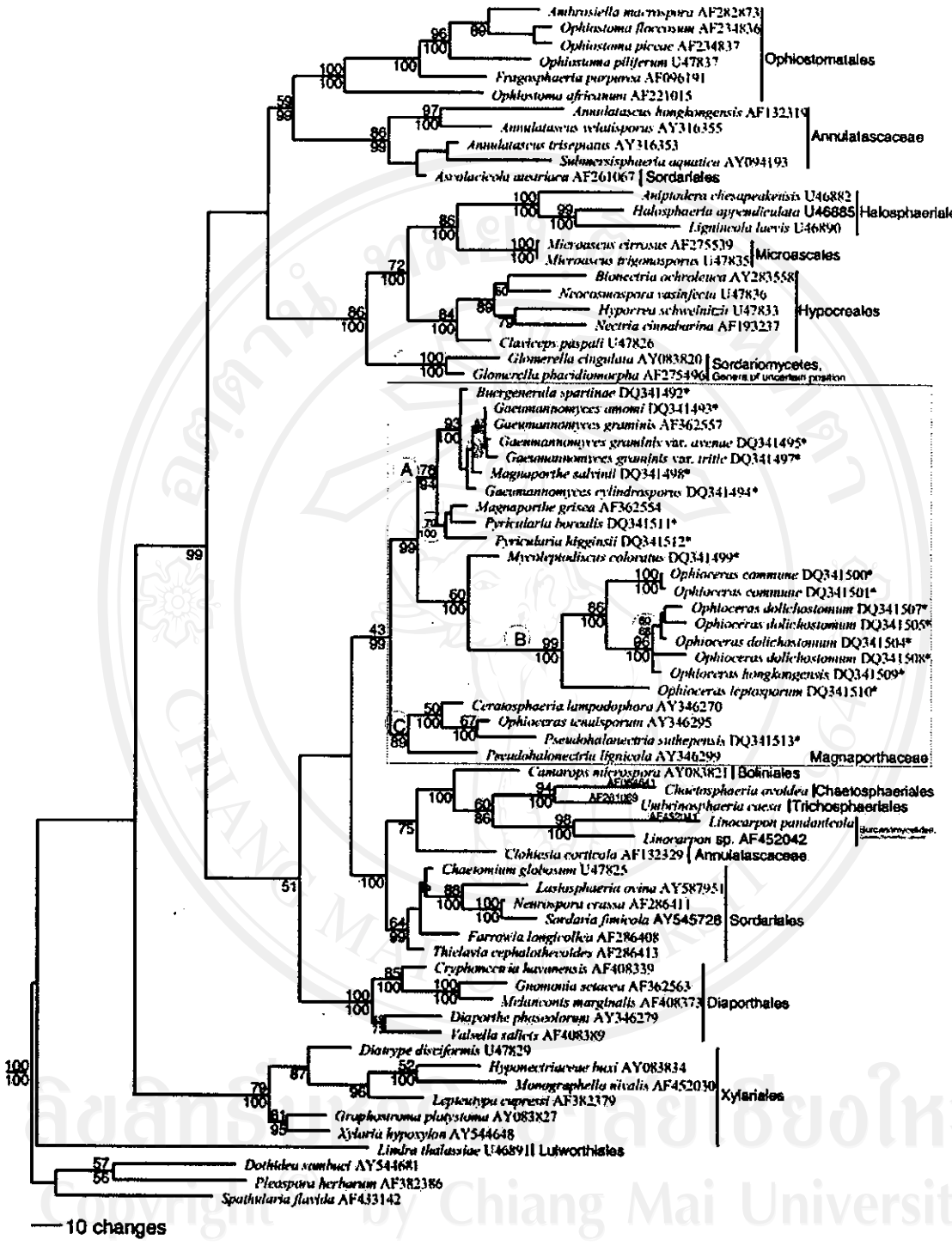

Figure 5.3 One of the best bayesian trees resulting from analysis of alignment I (28S rDNA-73 taxa) for the major order of the Ascomycetes. The numbers above the branches indicate MP1 bootstrap support proportions from 1000 replicates and numbers below the branches indicate pooled posterior probabilities, and *=the current study taxa.

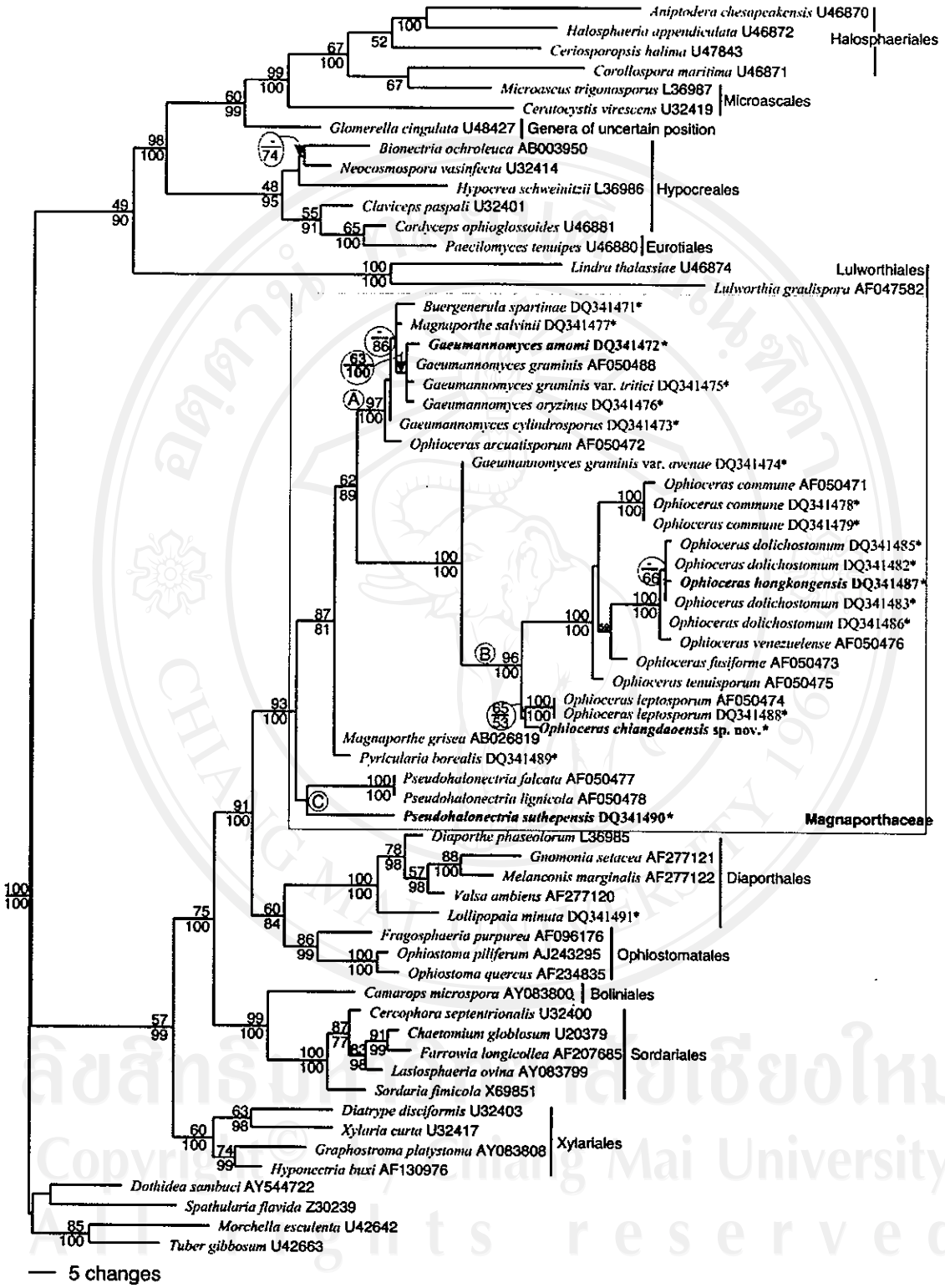

Figure 5.4 One of the best bayesian trees resulting from analysis of alignment III (18S rDNA-65 taxa). The numbers above the branches indicate MP1 bootstrap support proportions from 1000 replicates and numbers below the branches indicate pooled posterior probabilities, and *=the current study taxa.

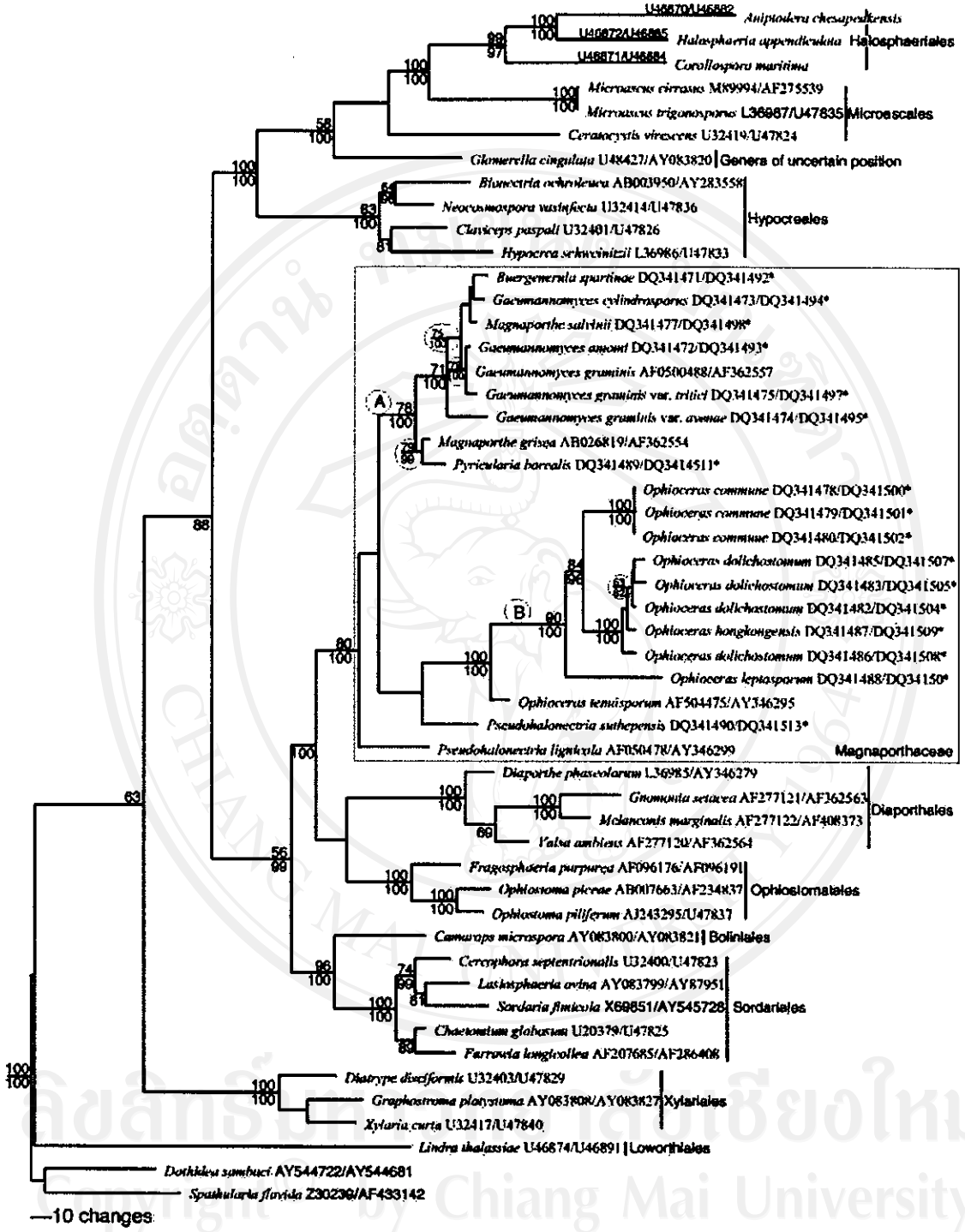

Figure 5.5 One of the best bayesian trees resulting from analysis of alignment IV (combine 18S and 28S rDNA-51 taxa). The numbers above the branches indicate MP1 bootstrap support proportions from 1000 replicates and numbers below the branches indicate pooled posterior probabilities.

5.3.4 Phylogenies based on the combined dataset (dataset IV)

A summary of the number of trees generated and indices are shown in Table 5.3. The transitions and transversions were weighted for MP3 analysis in the original rate substitution stepmatrix and are as follows: A↔C=2.38, A↔G=1.98, A↔T=2.16, C↔G=2.44, C↔T=1.57 and G↔T=2.76. MP1 analysis (treating gaps as missing data) generated a single tree which was slightly more resolved than those from MP2 (weighted parsimony) and MP3 analyses (Figure 5.3). Phylogenies generated are essentially similar to those derived of from individual datasets in that all Magnaporthaceae taxa (21 in all) form a monophyletic group with high support. However there are only 2 Clades (A & B) that correspond with phylogenies from other datasets. Two species of *Pseudohalonectria* are basal to the *Ophioceras* clade (Clade B) but they did not cluster together as expected. Their relationships are still unresolved because of sparse taxon sampling.

5.4 DISCUSSION

Based on rDNA sequence analysis, I recognize the family Magnaporthaceae as monophyletic. Phylogenies also show that they are closely related to other fungal orders such as Diaporthales and Ophiostomatales but whether they belong to any of those orders is contentious. In this study, I elaborate the possible phylogenetic relationships of Magnaporthaceae at the ordinal level, investigate the intergeneric clasification and provide insights regarding possible anamorphic teleomorphic connections.

5.4.1 Ordinal classification of Magnaporthaceae

Phylogenies based on both sequence datasets suggest that the Magnaporthaceae cannot be accommodated in any known fungal order. Instead, given the high statistical support I have for the monophyletic nature of this family, it is highly plausible that it should be established as a new order. Such a taxonomic arrangement has already been suggested by Cannon (1994), who erected the Magnaporthaceae to ordinal status. These results are therefore consistent with Cannon's (1994) scheme. However, I refrain from formerly introducing a new order and await a broader taxon sampling and phylogenies from other genes.

Since the Magnaporthaceae is monophyletic but should have a relationship with other ascomycete order(s) I discuss its relationships with the Calosphaeriales, Chaetosphaeriales, Diaporthales, Ophiostomatales, Phyllachorales, Sordariales and Xylariales.

When the type genus *Magnaporthe* (*M. salvinii*) was originally described by Krause and Webster (1972), it was considered to belong to the Diaporthales (Diaporthaceae), but von 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 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), however they are quite unlike *Magnaporthe* species (Cannon, 1994).

Gaeumannomyces and *Magnaporthe* are major plant pathogens (Yaegashi, 1977; Freeman and Ward, 2004), while *Ophioceras* and *Pseudohalonectria* 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 minuta* Inderbitzin (Inderbitzin and Berbee 2001). *Ophioceras* has been placed in Gnomoniaceae, a family lacking true stromatic development in the Diaporthales (Wehmeyer, 1975). Species of *Ophioceras* and *Pseudohalonectria* have features common to both the Sordariales and Diaporthales, but thought to belong to the Sordariales because all species possess peridial tissue of *textura angularis* (Conway and Barr, 1977; Shearer, 1989). Recent classification schemes, however, have shown that the Magnaporthaceae does not properly belong to the Diaporthales, Ophiostomatales and Sordariales (Chen *et al.*, 1999; Zhang and Blackwell, 2001; Castlebury *et al.*, 2002; Wanderlei-Silva *et al.*, 2003; Réblová and Seifert, 2004). These previous investigations and 18S-28S rDNA sequence data in this study suggest that magnaporthaceous taxa are closely related and comprise a single order. In separate analyses (results not shown), the Diaporthales

was found to be less related to Magnaporthaceae than the Calosphaeriales, Chaetosphaeriales, Ophiostomatales and the *Pleurostoma-Phialophora*, *Togninia* species complex.

The Hyponectriaceae and Magnaporthaceae were assumed by Cannon (1994) to possess possible links between the Diaporthales and Phyllachorales and at that time he suggested to assign a new order to them. Both families have however, been accommodated in Phyllachorales by Barr (1976a). 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 will morphological similarities to the Magnaporthaceae is the Annulatascaceae (Krause and Webster, 1972; Ho and Hyde, 2000; Lee *et al.*, 2004). The type species of *Magnaporthe* and *Annulatascus* have cylindrical asci with large apical rings and other characters are also not distinctly different (Krausee and Webster, 1972; Ho *et al.*, 2000). The representative of Annulatascaceae used in this study does not cluster near to the Magnaporthaceae (Figure 5.3) and the families appear to be unrelated. Therefore, a separate order is deemed necessary.

5.4.2 Intergeneric relationships within Magnaporthaceae

Phylogenetic relationships of the family Magnaporthaceae was established to accommodate the genera *Buergenerula, Gaeumannomyces, Mycoleptodiscus* (anamorph of *Omnidemptus*), *Ophioceras* and *Pseudohalonectria* centred around *Magnaporthe* (*Pyricularia*). I was unable to include the rare isolates of *Clasterosphaeria*, *Clavatisporella, Herbampulla* and *Juncigena* in this study however, based on their morphology I confer that they should also probably be included in the Magnaporthaceae.

The inclusion of genera and species in the Magnaporthaceae has predominantly been based on morphological characters and more recently on phylogeny (Table 5.1 and 5.4). Eriksson (1999) included *Clavatisporella* K.D. Hyde in the family. *Ophioceras* and *Pseudohalonectria* were members of the Magnaporthaceae following phylogenetic support in an investigation by Chen *et al.* (1999) and Inderbitzin and Berbee (2001). In this dataset, *M. 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* and *Pseudohalonectria* species (Clade A, Figure 5.3-5.5). This is supported by the fact that there are only minor differences in ascospore characters of 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). Beside, the features of *Gaeumannomyces* anamorph taxa have many more links with those of *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). *Ophioceras arcuatisporum* was isolated from submerged herbaceous debris but whether it is a parasite on its substrates is not known (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 with 3-5 septa (Walker, 1980). Likewise, *O. arcuatisporum* ascospores are much longer and broader (170-239 × 4-7) than those of *G. cylindrosporus* as well as all of *G. graminis* varieties (70-130 × 2-3.5). In this study I found that *M. grisea* groups together with *Pyricularia borealis* and not with *M. salvinii*. *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 distant taxa.

At present, of the majority of taxa within Magnaporthaceae are *Ophioceras* (34 species + 2 variety) and *Pseudohalonectria* (12 species). These two genera often occur in aquatic habitats and dead plant materials (Luo *et al.*, 2004). Based on morphological characters they are similar to each other (Hyde *et al.*, 1999c; Shearer, 1989; Tsui *et al.*, 2001). Hanlin (1998) and Hyde *et al.* (2000) has pointed out that

Pseudohalonectria differs from *Ophioceras* in having bright yellow, membraneous 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 their beaks and accumulate in masses. In contrast, in *Ophioceras*, the whole asci are forced up through the neck to the apex. The narrow canal of the beak allows the passage of only one ascus (Hyde *et al.*, 2000).

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). My present study based on both individual and combined datasets, with the inclusion of 3 additional taxa (*B. biseptata* and some other species of *Gaeumannomyces* and *Ophioceras*), provided more phylogenetic insights and resolution. *Ophioceras* seems to be more related to *Buergenerula*, *Gaeumannomyces* and *Magnaporthe* species than to *Pseudohalonectria* species with high support.

Based on this study together with results from previous studies (Shearer, 1989; Chen *et al.*, 1999; Hyde *et al.*, 1999c; Hyde *et al.*, 2000; Tsui *et al.*, 2001), *Buergenerula*, *Magnaporthe*, *Gaeumannomyces*, *Omnidemptus*, *Ophioceras* and *Pseudohalonectria* can broadly be categorised into 3 major groups: a) hyperparasites with appressoria (*Buergenerula*, *Gaeumannomyces* and *Magnaporthe*); b) presence of dark brown to black ascomata with a single ascus discharged through the beak of mature ascomata (*Ophioceras*); c) presence of yellow to brown ascomata with mass ascospores at the mature beak tips (*Pseudohalonectria*). However, to have a clearer picture of the validity of other specific characters, a broader taxon sampling with the inclusion of rare species and sequence analyses of other genes are necessary. One oddity in our study is the phylogenetic relationships of *O. tenuisporum* and *O. leptosporum* despite the fact that they are characterized by narrow ascospores (Figure 5.3 clade C). *Ophioceras leptosporum* appears to be distinct to other *Ophioceras* species while *O. tenuisporum* clusters with *P. suthepensis* with moderate support. The affinities of *O. tenuisporum* (sequence from Chen *et al.*, 1999) to *P. suthepensis* (this study) maybe the result of an artifact in our dataset or contaminant. More *Pseudohalonectria* species should be included to resolve this.

5.4.3 Placement of recently described taxa and *Ophioceras chiangdaoensis* sp. nov. in Magnaporthaceae

This study confirms the placement of *Ophioceras chiangdaoensis* sp. nov. and three recently described taxa in their respective genera. *Gaeumannomyces amomi* was established by Bussaban *et al.* (2001b) as an endophyte from wild ginger (*Amomum siamense*) in Thailand. In the phylogenies of combine and individual 18S and 28S rDNA datasets, it clusters with varieties of *G. graminis* (Figures 5.3-5.5 Clade A,) as well as *G. oryzae* (Figure 5.4, Clade A). Results reported here are consistent with those based on ITS sequence data as reported by Bussaban *et al.* (2005). *Ophioceras chiangdaoensis* sp. nov. was described from *Dracaena lourieri* in the current study, which has short ascospores similar to those of *O. commune*, *O. fusiforme* and *O. leptosporum* but all of them have the narrower ascospores. 18S rDNA sequences analyses show that *Ophioceras chiangdaoensis* sp. nov. groups in a high supported clade and closely related to *O. leptosporum* (Figure 5.4, Clade B). *Ophioceras hongkongensis* was described by Tsui *et al.* (2001) who noted that the ascospores are similar to those of *O. commune* and *O. fusiformis* in length and number of septa, but are wider. The inclusion of *O. hongkongensis* in the genus is supported by molecular data (Figure 5.4, Clade B). This species appears to be more related to the type species of *Ophioceras*, *O. dolichostomum* than *O. commune* and *O. fusiformis*. The ascomata, asci and ascospores in *O. hongkongensis* are similar to those of *O. dolichostomum* in length but they are broader (3.5-4.5 µm as compared to 2–3 µm). *Pseudohalonectria suthepensis* was isolated from dead leaves of *Magnolia liliifera* in Thailand by Promputtha *et al.* (2004a). The ascospores and asci of this species are longer than those of most *Pseudohalonectria* species with the exception of *P. falcata* and *P. lutea*. 18S rDNA sequences analyses show that *P. suthepensis* groups in a well supported clade and sister to *P. falca and P. lignicola* complex (Figure 5.4, Clade C).

5.4.4 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 (Rossman *et al.*, 2001b). *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; Couch and Kohn, 2002; Bussaban *et al.*, 2001c; 2005).

Krause and Webster (1972) established *Magnaporthe salvinii*, with a *Nakataea sigmoidea* Hara anamorph and a sclerotial state of *Sclerotium oryzae* Catt.. This isolate 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 also related to *M. grisea* (Figure 5.3-5.5), likewise *P. higginsii* grouped with *M. grisea* in the 28S rDNA analysis (Figure 5.3). *Pyricularia zingiberis* has been reported to be the anamorph of *G. amomi* based on ITS sequences analysis (Bussaban *et al.*, 2005). They also reported that obpyriform conidia species (*P. higginsii* and *P. juncicola* 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 still be maintained in *Pyricularia*. A connection between the phialidic anamorph *Harpophora graminicola* (Deacon) W. Gams (=*Phialophora radicicola* Cain) and *G. 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 species of *Phialophora* have been reported to be the anamorphs of some *Gaeumannomyces* species. For example, *P. graminicola* (= *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 are obtained in our study (28S rDNA sequence data) except that we do not totally agree with the Bryan *et al.* (1995) conclusions based on the morphologies of the anamorphs. *Gaeumannomyces graminis* var. *tritici* and *G. avenae* have *Phialophora* anamorphs with simple hyphopodia, while the *G.* var. *graminis* anamorph has lobed hyphopodia. Bussaban *et al.* (2001c) 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 3 species.

Mycoleptodiscus affinis is the anamorph of *Omnidemptus affinis* (Cannon and Alcorn, 1994). *Omnidemptus* 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 (Figure 5.3, Clades A, B) in 28S rDNA analysis. Our result confirmed that *Mycoleptodiscus* is an anamorphic Magnaporthaceae.

5.4.5 Phylogenies of recently described taxa

Some species recently described are *Ceratosphaeria* and *Lollipopaia* (also new genus) (Hyde *et al.*, 1997b; Inderbitzin and Berbee, 2001). Based on current morphological classification, these genera have filiform ascospores with or without long necks ascomata that are similar to Magnaporthaceae, while some *Ceratosphaeria* species has 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.* (1997b) 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.*, 1999c). Our results also support a close relationship between *Ceratosphaeria lampadophora* and *O. tenuisporum* and *P. suthepensis*. It is highly possible that *Ceratosphaeria lampadophora* is an earlier name for *Ophioceras/Pseudohalonectria* species, however a greater sampling is required before any taxonomical changes are proposed. *Lollipopaia minuta* was described as a new genus from a tropical rain forest in Thailand by Inderbitzin and Berbee (2001). The ascomata have long necks and are seated on a pseudoparenchymatous stroma. The type species is similar to *Ophioceras* and *Pseudohalonectria* in ascomata 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.

Table 5.4 Magnaporthaceae species member accepted by using morphological taxonomy and molecular taxonomy base on 18S, 28S and ITS rDNA sequences analyses.

Species name	Morphological accepted	Phylogenetic relationship accepted
Buergenerula biseptata	Cannon (1994)	-
B. caricis	Cannon (1994)	-
B. spartinae	Cannon (1994)	This study
B. typhae	Cannon (1994)	-
B. zelandica	McKenzie (1991a)	-
Clasterosphaeria cyperi	Sivanesan (1984).	-
Gaeumannomyces amomi	Bussaban *et al.* (2001c)	Bussaban *et al.* (2005) and This study
G. cariceti	*Index of Fungi* 6: 1092	-
G. caricis	Walker (1980), Cannon (1994)	Bussaban *et al.* (2005)
G. cylindrosporus	Walker (1980)	Bussaban *et al.* (2005) and This study
G. graminis	Walker (1980), Cannon (1994)	This study
G. graminis var. *avenae*	Walker (1980)	Bryan *et al.* (1995) and this study
G. graminis var. *graminis*	Walker (1980)	Bryan *et al.* (1995) and this study
G. graminis var. *tritici*	Walker (1980)	Bryan *et al.* (1995) and this study
G. oryzinus	*Index of Fungi* 3: 80 (1961-70)	This study
Herbampulla crassirostris	Cannon (1994)	-
Juncigena adarca	Kohlmeyer (1997)	-
Magnaporthe grisea	Barr (1977), Cannon (1994)	Couch and Kohn (2002) and This study
M. oryzae	Index of Fungi 7: 368	-
M. poae	Cannon (1994)	-
M. rhizophila	Cannon (1994)	-
M. salvinii	Krause and Webster (1972); Cannon (1994)	This study
Omnidemptus affinis	Cannon and Alcorn (1994)	-
Ophioceras arcuatisporum	Shearer *et al.* (1999)	Chen *et al.* (1999) and this study
O. bambusae	Saccardo's Syll. fung. 22: 306	-
O. chiangdaoensis sp. nov.	This study	This study
O. commune	Shearer *et al.* (1999)	Chen *et al.* (1999) and this study
O. corni	Saccardo's Syll. fung. 20: 242; 22: 307	-
O. diaporthoides	Saccardo's Syll. fung. 9: 938 (1891); 12: 482; 15: 233; 20: 242	-
O. dolichostomum	Conway and Barr (1977), Hyde (1992b)	This study
O. friesii (Mont.) Sacc.	Saccardo's Syll. fung. 2: 359 (1883)	-
O. fusiforme	Shearer *et al.* (1999)	Chen *et al.* (1999) and this study
O. guttulatum	Tsui *et al.* (2001)	-

Table 5.4 (continued).

Species name	Morphological accepted	Phylogenetic relationship accepted
O. hongkongense	Tsui *et al.* (2001)	This study
O. hyptidis	*Saccardo's Syll. fung.* 11: 353; 12: 482; 17: 852	-
O. hystrix	*Saccardo's Syll. fung.* 2: 359 (1883)	-
O. indicus	*Kavaka* 15:7 (1989)	-
O. leptosporum	Walker (1980)	Chen *et al.* (1999) and this study
O. longisporum	*Saccardo's Syll. fung.* 2: 360 (1883); 12: 482.	-
O. marcrocarpum	*Saccardo's Syll. fung.* 2: 359; XII: 482.	-
O. majusculum	*Saccardo's Syll. fung.* 14: 616 (1897); 20: 242.	-
O. miyazakiense	*Index of Fungi* 7: 439	-
O. ohiense	*Saccardo's Syll. fung.* 11: 353; 12: 482	-
O. palmae	Tsui *et al.* (2001)	
O. parasiticum	Teng (1934)	
O. petrakii	Tilak, S.B. Kale & S.V.S. Kale (1969)	
O. sambuci	*Saccardo's Syll. fung.* 9: 938 (1981); 12: 482	
O. sorghi	*Index of Fungi* 2: 195	-
O. tambopataense	*Index of Fungi* 7: 439	-
O. tenuisporum	Shearer *et al.* (1999)	Chen *et al.* (1999) and this study
O. therryanum	*Saccardo's Syll. fung.* 2: 360 (1883); 12: 482; 20: 242	
O. tjibodense	*Saccardo's Syll. fung.* 14: 617; 15: 233	
O. venezuelense	Shearer *et al.* (1999)	Chen *et al.* (1999) and this study
O. zeae	Index of Fungi 2: 84 (1951-60)	-
Pseudohalonectria adversaria	Shearer (1989)	-
P. aomoriensis	Index of Fungi 7: 253	-
P. eubenangeensis	Hyde *et al.* (1999c)	-
Pseudohalonectria falcata	Shearer (1989)	Chen *et al.* (1999) and this study
Pseudohalonectria fuxianii	Cai *et al.* (2002)	-
Pseudohalonectria lignicola	Tsui *et al.* (2003)	Chen *et al.* (1999) and this study
Pseudohalonectria longirostrum	Shearer (1989)	-
Pseudohalonectria lutea	Shearer (1989)	-
Pseudohalonectria palmicola	Hyde *et al.* (1999c)	-
Pseudohalonectria phialidica	Shearer (1989)	-
Pseudohalonectria tayloriae	Index of Fungi 7: 577	-
Pseudohalonectria suthepensis	Promputtha *et al.* (2004a)	this study
Some Magnaporthaceous anamorphic taxa		
Cirrenaria adarca	Kohlmeyer (1997)	-
Clasterosporium anomalum	Sivanesan (1984).	-
C. caricinum	Saccardo's Syll. fung. 4: 386 (1886); 12: 111; 19: 304	-
C. cyperi	Sivanesan (1984).	-
C. fragellatum	Cannon (1994)	-
C. scleriae	Cannon (1994)	-

Table 5.4 (continued).

Species name	Morphological accepted	Phylogenetic relationship accepted
Harpophora graminicola	Gams (2000)	Bryan *et al.* (1995), Bussaban *et al.* (2005)
Mycoleptodiscus affinis	Cannon and Alcorn (1994)	-
M. atromaculans	Bills and Polishook (1992), Cannon (1994)	-
M. coloratus	Alcorn 1994	This study
M. disciformis	*Matsushima Mycological Memoirs* 7: 58 (1993)	-
M. geniculatus	Alcorn (1994)	-
M. indicus	Sutton (1973), Cannon (1994)	-
M. lateralis	Cannon (1994)	-
M. lunatus	Cannon and Alcorn (1994), Cannon (1994)	-
M. minimus	Cannon (1994)	-
M. sphaericus	Cannon (1994)	-
M. taiwanensis	Cannon (1994)	-
M. terrestris	Cannon (1994)	-
M. unilateralis	Cannon (1994)	-
M. variabilis	Alcorn (1994)	-
Pyricularia borealis	*Stud. Myco.* 26: 114 (1985)	this study
Pyricularia costina	Bussaban *et al.* (2003)	Bussaban *et al.* (2005)
Pyricularia higginsii	*Index of Fungi* 2 (1951-60): 219, *Mycologia* 46: 810 (1954)	Bussaban *et al.* (2005) and this study
Pyricularia juncicola	*Index of Fungi* 3 (1961-70): 515	Bussaban *et al.* (2005)
Pyricularia zingiberis	*Saccardo's Syll. fung.* 25: 723	Bussaban *et al.* (2005)