



<b>Title</b>	<b>The family Pleosporaceae: intergeneric relationships and phylogenetic perspectives based on sequence analyses of partial 28S rDNA</b>
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## The family Pleosporaceae: intergeneric relationships and phylogenetic perspectives based on sequence analyses of partial 28S rDNA

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**Abstract:** The Pleosporaceae is an important loculoascomycete family. There has been disagreement, however, regarding the taxonomic placement of many genera within this family. This study investigates phylogenetic relationships among the genera *Cochliobolus*, *Kirschsteiniothelia*, *Leptosphaerulina*, *Macroventuria*, *Pleospora*, *Pyrenophora*, and *Wettsteinina*. Partial 28S rDNA sequences from taxa within these genera were analyzed with maximum parsimony, likelihood and Bayesian methods. *Cochliobolus* can be segregated broadly into two groups as previously proposed. *Pleospora* is polyphyletic in its current sense. Taxa with *Stemphylium* anamorphs are closely related to *Cochliobolus* and fit within the Pleosporaceae, whereas the affinities of *Pleospora herbarum* and *P. ambigua* are still ambiguous. *Pyrenophora* constitutes a monophyletic group within the Pleosporaceae, whereas *Leptosphaerulina* and *Macroventuria* appear to share phylogenetic affinities with the Leptosphaeriaceae and Phaeosphaeriaceae. Phylogenies indicate that *Wettsteinina* should be excluded from the Pleosporaceae. Similar findings are reported for *Kirschstei-*

*nothelia*, which is probably polyphyletic. Anamorphic characters appear to be significant (especially in *Cochliobolus*) while ascospore morphologies, such as shape and color and substrate occurrence are poor indicators of phylogenetic relationships among these loculoascomycetes.

**Key words:** anamorphs, ascospore morphology, Loculoascomycetes, phylogeny, *Pleospora*, polyphyletic, ribosomal DNA

### INTRODUCTION

The largest family within the Pleosporales, Pleosporaceae, comprises 17 genera and 111 species (Kirk et al 2001). Species are parasites or saprobes on wood and dead herbaceous stems or leaves (Sivanesan 1984). The classification in the Pleosporaceae has been based primarily on the *Pleospora* type of centrum development (Dong et al 1998) and asci that are interspersed with pseudoparaphyses in the ascostroma. These pseudoparaphyses originate above the hymenial layer and grow downward among the asci to fuse at the base of the locule (Wehmeyer 1975). Ascospores are usually brown and phragmosporous or dictyosporous (Dong et al 1998, Kirk et al 2001). Many pleosporaceous taxa are important plant pathogens. For instance *Cochliobolus heterostrophus* causes southern corn leaf blight and *Pyrenophora graminea* is the causal agent of barley leaf stripe (Agrios 2005). Their anamorphs are usually hyphomycetes and also have been reported to cause plant disease in cereals (e.g. *Bipolaris maydis*, *Exserohilum turcicum* and *Helminthosporium oryzae*) (Farr et al 1989, Berbee 1996, Krupinsky et al 2004, Agrios 2005).

The Pleosporaceae historically was placed in the Sphaeriales (e.g. Winter 1887, Ellis and Everhart 1892, Lindau 1897) based on immersed perithecia containing paraphyses (Wehmeyer 1975). The family then was transferred to the Pseudosphaeriaceae, which was later raised to ordinal rank as the Pseudosphaeriales (Theissen and Sydow 1917, Wehmeyer 1975). Luttrell (1955) treated the name Pseudosphaeriales as a synonym of the Dothideales and suggested that pseudoparaphyses were important in

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their taxonomy. He considered the Pseudosphaeriales as synonym of the Pleosporales and assigned the Pleosporaceae, Venturiaceae and Lophiostomataceae, to the Pleosporales. Such a scheme largely was accepted by other mycologists (e.g. Wehmeyer 1975). Wehmeyer (1975) pointed out that the family Pleosporaceae has never been clearly delimited following Luttrell's concept and as a result taxa with ascostromata of many different types, which previously were placed in other families, were placed arbitrarily in the Pleosporaceae.

The family Pleosporaceae is a heterogeneous group of bitunicate ascomycetes with genera primarily included based on ascospore characteristics, including shape, color, septation, pigmentation and presence or absence of mucilaginous sheaths (Luttrell 1955, 1973; Wehmeyer 1961, 1975; Eriksson 1981; Sivanesan 1984; Barr 1987b; Abler 2003). Other families, such as the Leptosphaeriaceae, Melanommataceae, Phaeosphaeriaceae and Sporormiaceae, however, also possess morphological characters similar to those of the Pleosporaceae, and this has resulted in considerable ambiguity and confusion in intergeneric and familial classification (Luttrell 1955, 1973; Wehmeyer 1961, 1975; von Arx and Müller 1975; Sivanesan 1984; Barr 1987a, b; Eriksson and Hawksworth 1986, 1991). Most genera in these families have morphological characters that overlap in many respects and the taxonomic organization among them has been modified on several occasions (TABLE I). Barr (1987b) redefined the Pleosporaceae to include *Clathrospora* (= *Comoclathris*), *Kirschsteiniotelia*, *Lewia* and *Pleospora* and grouped *Cochliobolus*, *Pyrenophora* and *Setosphaeria* into the family Pyrenophoraceae. Berbee (1996) disagreed, suggesting that all those genera belong to the Pleosporaceae.

Given the considerable taxonomic confusion, this study, based on phylogenetic analyses of rDNA sequence data, was undertaken to (i) verify the familial placement of these genera and assess whether they represent natural groups, (ii) determine which morphological characters are phylogenetically significant and therefore are useful for generic delineation and (iii) assess whether phylogenies based on molecular characters are concordant with any of the traditional morphology-based classification schemes.

#### MATERIALS AND METHODS

**DNA extraction.**—Twenty isolates of Pleosporaceae (*Cochliobolus* [two species], *Kirschsteiniotelia* [one species], *Leptosphaerulina* [four species], *Macroventuria* [two species], *Pleospora* [four species], *Pyrenophora* [four species] and *Wettsteinina* [three species]) and six species of allied genera (*Karstenula rhodostoma*, *Leptosphaeria maculans*,

*Lophiostoma caulium*, *Massarina ramunculicola*, *Phaeosphaeria vegans* and *Venturia carpophila*) were selected for this study. Species names and accession numbers of the isolates in this study are listed (TABLE II). For each isolate, pure cultures were plated on potato dextrose agar and incubated at 25 C 10–20 d before DNA extraction. Genomic DNA was extracted from fresh fungal mycelia following a protocol as outlined by Jeewon et al (2002, 2003, 2004) and Cai et al (2005). Briefly, mycelia were scraped off from the surface of the plate. The mycelia were ground with 200 mg of sterilized quartz sand and 600 µL of 2× CTAB extraction buffer (2% w/v CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8) in a 1.5 mL Eppendorf tube. The contents were incubated at 60 C in a water bath 40 min with gentle swirling every 10 min. The solution was extracted three times with an equal volume of phenol:chloroform (1:1) at 13 000 g 30 min until no interface was visible. The upper aqueous phase containing the DNA was precipitated by addition of 2.5 volumes of absolute ethanol and kept at –20 C overnight. The precipitated DNA was washed two times with 70% ethanol, dried under vacuum and suspended in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and treated with RNase (1 mg/mL) before DNA amplification.

**DNA amplification and sequencing of 28S rDNA.**—Approximately 900 nucleotides at the 5' end of the 28S rDNA region were amplified by primer pairs LROR/LRO5 (Vilgalys and Hester 1990). PCR was carried out in 50 µL reaction volume containing 31.7 µL sterile water, 5 µL of 10× Mg free PCR buffer, 3 µL of 25 mM MgCl<sub>2</sub>, 4 µL of 2.5 mM deoxyribonucleotide triphosphate (dNTPs), 1.5 µL of each 10 µM primers (LROR and LRO5), 3 µL of DNA template, 0.3 µL of 2.5 units of Taq DNA polymerase (Promega, Madison, Wisconsin). Typical amplification parameters were: initial denaturation of 95 C for 3 min; 35 cycles of denaturation at 95 C 1 min, annealing at 52 C 50 s and extension at 72 C 1 min and final extension of 72 C 10 min. Double-stranded DNA products were purified with GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, catalogue No. 27-9602-01) following manufacturer's protocol. 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.**—Fifty-four taxa from different fungal families were aligned initially with the computer program Bioedit (Hall 1999) and Clustal X (Thompson et al 1997) with default parameter settings, and alignments were manually edited by inserting gaps for optimization using Se-Al (Rambaut 1996). Phylogenetic analyses of LSU rDNA were performed with maximum parsimony employing a heuristic search (1000 random replicates) in PAUP\* v 4.0b10 (Swofford 2002). Ambiguously aligned regions also were excluded from the phylogenetic analyses.

TABLE I. Classification of Pleosporaceae based on published morphological studies

	Wehmeyer 1961	Wehmeyer 1975	Sivanesan 1984	Barr 1987	Eriksson and Hawksworth 1998	Kirk et al 2001	Eriksson 2005	This study
<i>Clathrospora</i>	<i>Allonecta</i>	<i>Asteromassarica</i>	<i>Clathrospora</i>	<i>Clathrospora</i>	<i>Cochliobolus</i>	<i>Cochliobolus</i>	<i>Cochliobolus</i>	<i>Cochliobolus</i>
<i>Platyspora</i>	<i>Catharinia</i>	<i>Cochliobolus</i>	<i>Kirschsteiniiothelia</i>	<i>Kirschsteiniiothelia</i>	<i>Extrawetsteinina</i>	<i>Extrawetsteinina</i>	<i>Decorospora</i>	<i>Decorospora</i> *
<i>Pleospora</i>	<i>Clathrospora</i>	<i>Cucurbitoidis</i>	<i>Levia</i>	<i>Levia</i>	<i>Falciformispora</i>	<i>Falciformispora</i>	<i>Extrawetsteinina</i>	<i>Extrawetsteinina</i> *
<i>Pyrenophora</i>	<i>Cochliobolus</i>	<i>Cucurbitaria</i>	<i>Pleospora</i>	<i>Pleospora</i>	<i>Kirschsteiniiothelia</i>	<i>Kirschsteiniiothelia</i>	<i>Falciformispora</i>	<i>Falciformispora</i> *
	<i>Cucurbitaria</i>	<i>Didymella</i>			<i>Kriegeriella</i>	<i>Kriegeriella</i>	<i>Kirschsteiniiothelia</i>	<i>Kriegeriella</i> *
	<i>Didymopilella</i>	<i>Eudartluca</i>			<i>Leptosphaerulina</i>	<i>Leptosphaerulina</i>	<i>Kriegeriella</i>	<i>Levia</i> *
	<i>Eudartluca</i>	<i>Gammamyces</i>			<i>Levia</i>	<i>Levia</i>	<i>Leptosphaerulina</i>	<i>Macrospora</i> *
	<i>Fenestella</i>	<i>Herpotrichia</i>			<i>Macrospora</i>	<i>Macrospora</i>	<i>Levia</i>	<i>Monacostroma</i> *
	<i>Gibberidea</i>	<i>Leptosphaeria</i>			<i>Macroventuria</i>	<i>Macroventuria</i>	<i>Macrospora</i>	<i>Platysporoides</i> *
	<i>Gilletiella</i>	<i>Massarina</i>			<i>Monacostroma</i>	<i>Platysporoides</i>	<i>Macroventuria</i>	<i>Pleospora</i>
	<i>Herpotrichia</i>	<i>Microthelia</i>			<i>Platysporoides</i>	<i>Pleospora</i>	<i>Monacostroma</i>	<i>Pseudouconia</i> *
	<i>Herpotrichiella</i>	<i>Ophiobolus</i>			<i>Pleospora</i>	<i>Pseudouconia</i>	<i>Platysporoides</i>	<i>Pyrenophora</i>
	<i>Leptosphaeria</i>	<i>Othia</i>			<i>Pseudouconia</i>	<i>Pyrenophora</i>	<i>Pleospora</i>	<i>Setosphaeria</i>
	<i>Letendrea</i>	<i>Paraphaeosphaeria</i>			<i>Pyrenophora</i>	<i>Setosphaeria</i>	<i>Pseudouconia</i>	<i>Zeuctomorpha</i> *
	<i>Licopolia</i>	<i>Pleomassarica</i>			<i>Setosphaeria</i>	<i>Teratosphaeria</i>	<i>Pyrenophora</i>	
	<i>Melanomma</i>	<i>Pleospora</i>			<i>Wetsteinina</i>	<i>Tremateia</i>	<i>Setosphaeria</i>	
	<i>Melanopsamma</i>	<i>Pyrenophora</i>			<i>Zeuctomorpha</i>	<i>Wetsteinina</i>	<i>Wetsteinina</i>	
	<i>Metasphaeria</i>	<i>Rhytidiella</i>						
	<i>Mycromicrothelia</i>	<i>Setosphaeria</i>						
	<i>Ophiobolus</i>	<i>Splanchnonema</i>						
	<i>Othia</i>	<i>Zeuctomorpha</i>						
	<i>Paralimnices</i>							
	<i>Parodiella</i>							
	<i>Platyspora</i>							
	<i>Pleomassarica</i>							
	<i>Pleospora</i>							
	<i>Pteridiospora</i>							
	<i>Pyrenophora</i>							
	<i>Teichospora</i>							
	<i>Teratosphaeria</i>							
	<i>Thyridaria</i>							
	<i>Thyridium</i>							
	<i>Tomasella</i>							
	<i>Trematosphaeria</i>							
	<i>Trichometasphaeria</i>							
	<i>Zignoella</i>							

\* Not examined.

TABLE II. Fungal species and GenBank accession number for taxa used in phylogenetic analyses

Species	Isolates/sources	GenBank Accession number	Family*
Ingroup			
<i>Bimuria novae-zelandiae</i>	CBS107.79	AY016356	Melanommataceae
<i>Bipolaris papendorffii</i>	9084c	AF163980	Anamorphic <i>Cochliobolus</i> , Pleosporaceae
<i>Cochliobolus cynodontis</i>	BRIP16821	AF163982	Pleosporaceae
<i>Cochliobolus hawaiiensis</i>	BRIP15933	AF163979	Pleosporaceae
<i>Cochliobolus heterostrophus</i>	CBS134.39	AY544645	Pleosporaceae
<i>Cochliobolus lunatus</i>	95/1937b	AF163988	Pleosporaceae
<i>Cochliobolus nodulosus</i>	ICMP10306	AY849940	Pleosporaceae
<i>Cochliobolus pallescens</i>	ICMP11023	AY849941	Pleosporaceae
<i>Curvularia oryzae</i>	MRL1089	AF163991	Anamorphic <i>Cochliobolus</i> , Pleosporaceae
<i>Curvularia brachyspora</i>	ATCC58872	AF279380	Anamorphic <i>Cochliobolus</i> , Pleosporaceae
<i>Curvularia heteropogonicola</i>	IMI268958	AF163986	Anamorphic <i>Cochliobolus</i> , Pleosporaceae
<i>Dothidea ribesia</i>	CBS195.58	AY016360	Dothideaceae
<i>Dothidea sambuci</i>	CBS198.58	AF382387	Dothideaceae
<i>Karstenula rhodostoma</i>	CBS690.94	AY787933	Melanommataceae
<i>Kirschsteiniothelia elaterascus</i>	HKUCC7769	AY787934	Pleosporaceae
<i>Leptosphaeria doliolum</i>	ATCC32813	U43473	Leptosphaeriaceae
<i>Leptosphaeria maculans</i>	ICMP13554	AY849946	Leptosphaeriaceae
<i>Leptosphaerulina argentiniensis</i>	CBS569.94	AY849947	Pleosporaceae
<i>Leptosphaerulina australis</i>	ICMP10312	AY849948	Pleosporaceae
<i>Leptosphaerulina briosiana</i>	CBS441.74	AY849949	Pleosporaceae
<i>Leptosphaerulina trifolii</i>	CBS235.58	AY849950	Pleosporaceae
<i>Letendraea helminthicola</i>	CBS884.85	AY016362	Tubeufiaceae
<i>Lophiostoma caulium</i>	CBS 623.86	DQ528763	Lophiostomataceae
<i>Macroventuria anomochaeta</i>	CBS525.71	AY787936	Pleosporaceae
<i>Macroventuria wentii</i>	CBS526.71	AY849952	Pleosporaceae
<i>Massarina ramunculicola</i>	HKUCC7649	DQ528762	Lophiostomataceae
<i>Microxyphium citri</i>	CBS451.66	AY004337	Anamorphic Ascomycetes
<i>Myriangiium duriaei</i>	CBS260.36	AY016365	Myriangiaceae
<i>Phaeosphaeria avenaria</i>	AFTOL-ID280	AY544684	Phaeosphaeriaceae
<i>Phaeosphaeria vagans</i>	CBS604.86	AY849953	Phaeosphaeriaceae
<i>Pleomassaria siparia</i>	CBS279.74	AY004341	Pleomassariaceae
<i>Pleospora ambigua</i>	CBS366.52	AY787937	Pleosporaceae
<i>Pleospora halophila</i>	CBS410.73	AY849955	Pleosporaceae
<i>Pleospora herbarum</i> var. <i>herbarum</i>	CBS191.86	AF382386	Pleosporaceae
<i>Pleospora sedicola</i>	CBS109843	AY849958	Pleosporaceae
<i>Pleospora tomatonis</i>	CBS109844	AY849959	Pleosporaceae
<i>Preussia terricola</i>	AFTOL-ID282	AY544686	Sporormiaceae
<i>Pyrenophora dictyoides</i>	ICMP14523	AY849960	Pleosporaceae
<i>Pyrenophora seminiperda</i>	ICMP6169	AY849961	Pleosporaceae
<i>Pyrenophora tetrarhenae</i>	ICMP6139	AY849962	Pleosporaceae
<i>Pyrenophora trichostoma</i>	CBS392.54	AY849963	Pleosporaceae
<i>Pyrenophora triticirepentis</i>	AFTOL-ID173	AY544672	Pleosporaceae
<i>Setomelanomma holmii</i>	CBS110217	AF525678	Dothideales Incertae sedis
<i>Setosphaeria monoceras</i>	CBS154.26	AY016368	Pleosporaceae
<i>Stylodothis puccinioides</i>	CBS193.58	AY004342	Dothideaceae
<i>Trematosphaeria heterospora</i>	CBS644.86	AY016369	Melanommataceae
<i>Venturia carpophila</i>	ICMP5402	AY849967	Venturiaceae
<i>Venturia hanliniana</i>	ATCC96019	AF050290	Venturiaceae
<i>Westerdykella cylindrica</i>	CBS454.72	AY004343	Sporormiaceae
<i>Wettsteinina dryadis</i>	CBS448.54	AY849968	Pleosporaceae
<i>Wettsteinina macrotheca</i>	CBS647.86	AY849969	Pleosporaceae
<i>Wettsteinina pachyasca</i>	CBS646.86	AY849970	Pleosporaceae
Outgroup			
<i>Glyphium elatum</i>	CBS268.34	AF346420	Mytiliniaceae

\* Familial classification follows Kirk et al (2001).

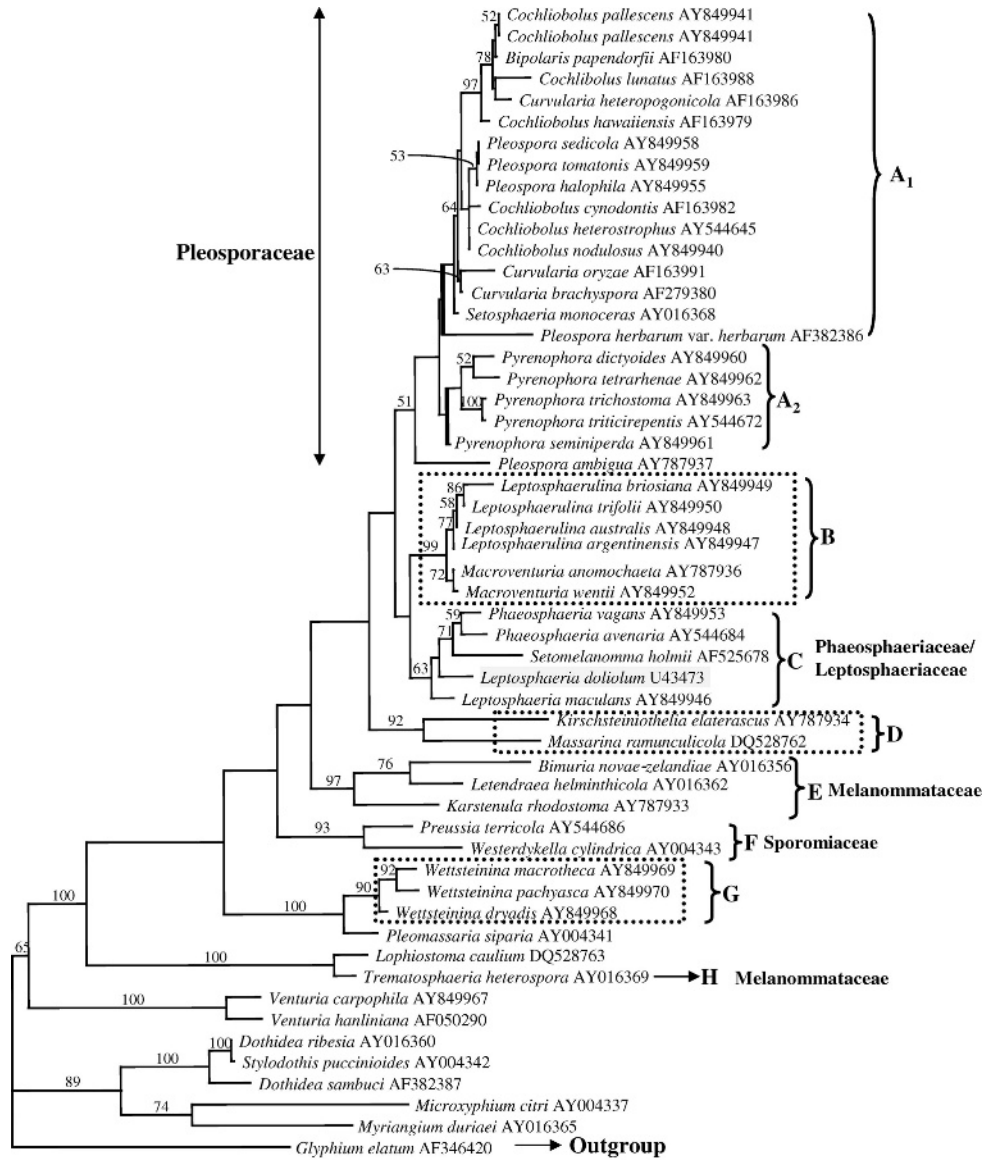
Maximum parsimony analyses were carried out treating gaps as missing and as fifth character (newstate) with the exclusion of ambiguously aligned portions. Parsimony analyses also were carried out including the ambiguously aligned portions as recoded characters using INAASE (Lutzoni et al 2000), while the gaps were treated as fifth character. Heuristic search option with the tree-bisection-reconnection (TBR) branch swapping, MULTREES options (saving all optimal trees) effective and random sequence additions set to 1000 was employed to generate parsimony trees. Reliability for each branch of the best tree was assessed by the bootstrap method (Felsenstein 1985) with 1000 replicates (one random addition of sequences per bootstrap replicate). The model of substitution used for Bayesian and ML was chosen with Mrmodeltest 2.2 (Nylander 2004). Independent Bayesian phylogenetic analysis was performed in MrBayes 3.0 (Huelsenbeck and Ronquist 2001) using a uniform SYM+I+G model, as selected by hLRT in Mrmodeltest 2.2([SYM+I+G] lset nst = 6 rates = invgamma; prset statefreqpr = fixed (equal)). The Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling approach was used to calculate posterior probabilities. Four simultaneous Markov chains, three heated and one cold, were run under a general time reversible (GTR) model of sequence evolution and gamma approximation for rate variation among sites. Chains were analyzed with random starting trees for  $10^6$  generations. Trees collected before the stable likelihood value point were discarded as "burn-in" (Huelsenbeck and Ronquist 2001). The remaining trees were used to build a majority rule consensus tree where the percentage of the remained best trees supporting a branch represents the Bayesian posterior probabilities. For maximum likelihood (ML) analyses, tree was inferred with PAUP\* using the heuristic search option starting with a parsimony tree with random sequence addition. The Kishino-Hasegawa and Templeton tests were performed to determine whether the trees inferred under different optimality criteria were significantly different.

## RESULTS

The dataset consisted of 54 taxa, each with 886 aligned nucleotide characters, of which 204 (23%) were parsimony informative. Designated outgroup was *Glyphium elatum*. A total of 60 characters, which were ambiguously aligned, were excluded in the analyses. This data matrix has been deposited in TreeBase. A heuristic search with random addition of taxa (1000 replicates) and treating gaps as missing and as fifth character (newstate) generated 18 and 60 equally parsimonious trees respectively. All trees were similar in topology and not significantly different. Recoding ambiguously aligned characters (60 in total) in INAASE as four characters and treating gaps as missing data yielded four most parsimonious trees (not statistically different), one of which is shown (FIG. 1). A similar approach to gap treatment was undertaken when dataset was subjected to ML and

Bayesian analyses. Maximum likelihood analyses (under the SYM+I+G evolutionary model as estimated by MrModeltest 2.2) yielded a single tree of log likelihood  $-5160.83120$ . Estimated nucleotide frequencies were: A = 0.25707, C = 0.20575, G = 0.30922 and T = 0.22796, shape parameter (alpha) was 0.6066 (total length = 714, CI = 0.520, RI = 0.738, RC = 0.383, HI = 0.480). Phylogenies obtained from ML analyses were essentially similar to those obtained from MP analyses (results not shown). Bayesian analyses resulted in a tree (total length = 717, CI = 0.517, RI = 0.736, RC = 0.381, HI = 0.483) with identical topologies obtained from other optimality criterion, however support as measured by Bayesian posterior probabilities option was higher and provided significant (>95%) support for most of the clades as defined below (FIG. 2). Given that the relationships among ingroups were identical in all analyses, we selected the MP tree (FIG. 1, with bootstrap support based on 1000 replicates) to explain systematic relationships pertaining to members of the Pleosporaceae.

The maximum parsimony tree generated based on sequence analysis of the 28S rDNA dataset produced nine monophyletic clades (FIG. 1). Clades A<sub>1</sub> and A<sub>2</sub> are members of the Pleosporaceae and did not receive bootstrap support (FIG. 1) but moderate Bayesian support (FIG. 2). Clade A<sub>1</sub> comprises 16 species from different pleosporaceae genera while Clade A<sub>2</sub>, characterized only by *Pyrenophora* species is monophyletic and supported by high Bayesian support (93%). Four species of *Leptosphaerulina* and two species of *Macroventuria* clustered together within Clade B and form a well supported monophyletic group with 99% bootstrap support (100% Bayesian support, FIG. 2). However the position of this clade is not consistent. Phylogenies from Bayesian analyses place this monophyletic group basal to other members of the Phaeosphaeriaceae and Leptosphaeriaceae (Clade C), whereas MP analyses place it basal to Clade A (Pleosporaceae). Similar results were obtained from members of the Phaeosphaeriaceae and Leptosphaeriaceae (Clade C) which are basal to Clade B (MP analyses) or basal to Clade A (Bayesian analyses). The latter grouping was moderately supported whereas the node supporting clades B and C did not receive bootstrap support (FIG. 1). Clade D, characterized by *Kirschsteiniothelia elaterascus* (Pleosporaceae) and *Massarina ramunculicola* (Massarinaaceae), is monophyletic and strongly supported in all analyses. Clade E, with 97% bootstrap support, includes *Bimuria novae-zealandiae* and *Karstenula rhodostoma* (Melanommataceae) and *Letendraea helminthicola* (Tubeufiaceae). *Preussia terricola* and *Westerdykella cylindrica* clustered together in Clade F (Sporomiaceae). Clade G, another strongly supported



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FIG. 1. The maximum parsimony tree generated based on sequence analysis of the 28S rDNA dataset (Total length = 884, CI = 0.581, RI = 0.742, RC = 0.432, HI = 0.419). Designated outgroup is *Glyphium elatum*. Bootstrap support values above 50% shown at nodes are based on 1000 replicates. Clades A–H represent members from different genera forming distinct monophyletic groups.

monophyletic group, consists of three species of *Wettsteinina* (Pleosporaceae) and basal to them is *Pleomassaria siparia* (Pleomassariaceae). *Lophiostoma caulium* (Lophiostomataceae) and *Trematosphaeria heterospora* (Melanommataceae) nest together in Clade H with a 100% bootstrap and Bayesian support.

#### DISCUSSION

The family Pleosporaceae (Nitschke 1869) is the largest within the Pleosporales. The taxonomy of this

family traditionally has been based on few characters. Various classification schemes have been proposed to arrange taxa in this family, but to date there is considerable taxonomic confusion. This is the first phylogenetic study encompassing a broader taxon sampling from members of the Pleosporaceae. Results obtained generally are congruent with previously described phylogenies and morphology-based classification schemes. Based on current data the monophyly of Pleosporaceae is supported. However there are discrepancies in the taxonomy of several

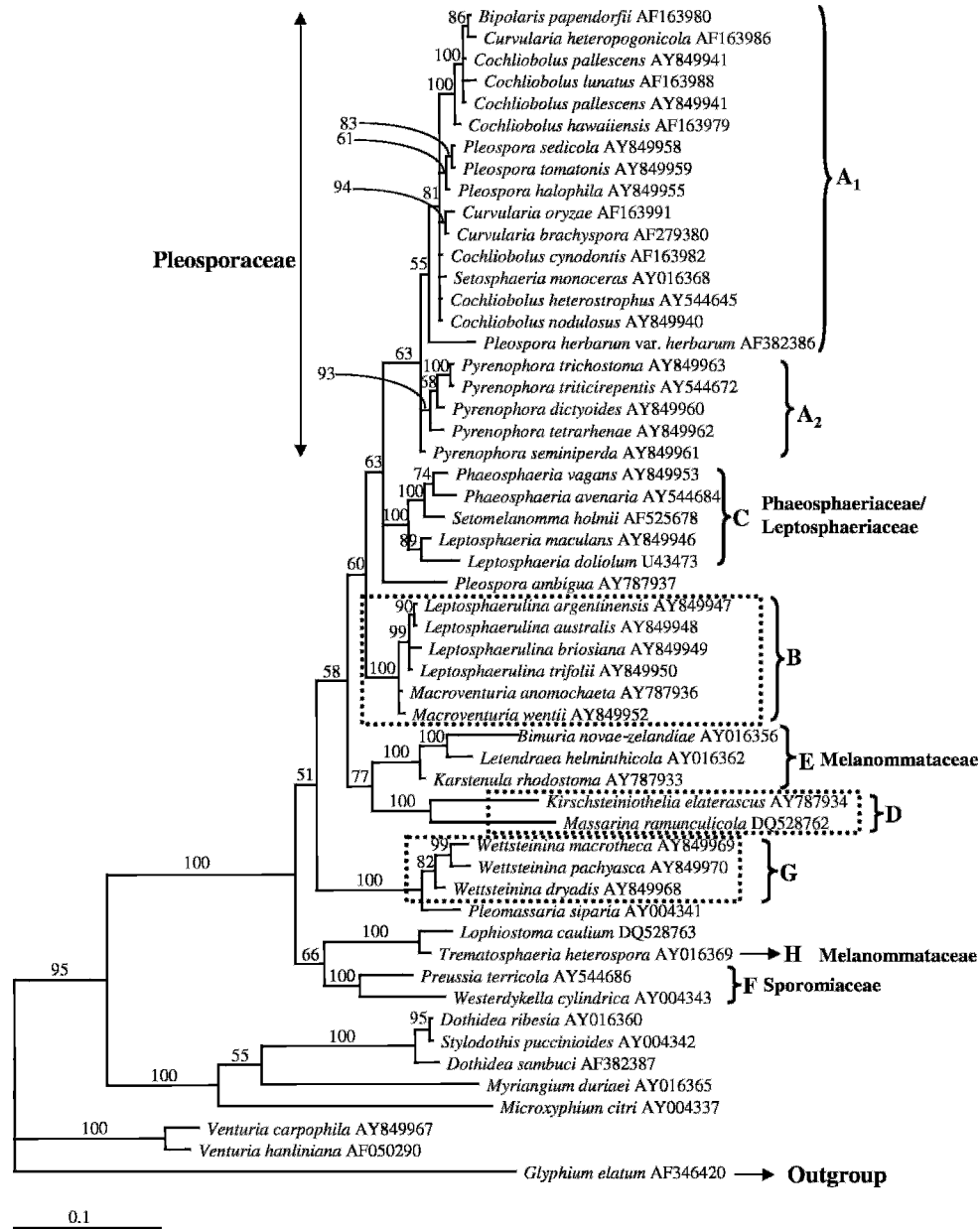


FIG. 2. Phylogeny of the *Pleosporaceae* and allied genera within Pleosporales estimated under Bayesian analysis (Total length = 717, CI = 0.517, RI = 0.736, RC = 0.381, HI = 0.483). Outgroup is *Glyphium elatum*. Bayesian support is shown above the branches.

pleosporaceous genera, and based on results obtained from rDNA phylogenies and existing morphological data we provide evidence for some reclassification.

**Phylogenetics of *Cochliobolus*, *Pleospora* and *Setosphaeria* (Clade A<sub>1</sub>).**—Several phylogenetic studies within the loculoascomycetes have included *Pleospora* as a typical representative of the Pleosporales, which is defined by the presence of cellular pseudoparaphyses (Berbee 1996, Dong et al 1998, Silva-Hanlin and Hanlin 1999, Lumbsch

2000, Lumbsch and Lindemuth 2001, Lumbsch et al 2001). *Cochliobolus*, *Pleospora* and *Setosphaeria* currently are accepted genera within the family Pleosporaceae (Sivanesan 1984, Eriksson and Hawksworth 1998, Kirk et al 2001, Eriksson 2005). Our results confirm that these genera are closely related and should be classified in one family, the Pleosporaceae. *Cochliobolus* has *Bipolaris* and *Curvularia* anamorphs, and results obtained from 28S rDNA analyses confirm a close phylogenetic connection between these asexual and sexual fungi. Phylogenies also corroborate find-



ings reported by Berbee et al (1999) that *Cochliobolus* species can be divided broadly into two groups. With the inclusion of different taxa in this study we found that *C. pallescens* (= *Curvularia pallescens*), *Bipolaris papendorfii*, *C. lunatus* (= *Curvularia lunatus*), *C. hawaiiensis* (= *Bipolaris hawaiiensis*) and *Curvularia heteropogonicola* represent a distinct monophyletic group (similar to Group 2 as defined by Berbee et al 1999). Although morphologically related *Cochliobolus* species do not constitute a monophyletic lineage. *Cochliobolus cynodontis*, *C. heterostrophus* and *C. nodulosus* form a sister group with three *Pleospora* species, which are known to produce *Stemphylium* anamorphs. Berbee et al (1999) also reported that *Curvularia heteropogonicola* is phylogenetically distinct from *Cochliobolus cynodontis*, *C. heterostrophus* and *C. nodulosus*. Two anamorphic species, *Curvularia oryzae* and *C. brachyspora*, however do not appear to belong to any of the two groups as defined by Berbee et al (1999). A similar phylogenetic phenomenon was reported for *Cochliobolus homomorphus* (Berbee et al 1999).

*Polyphyly of Pleospora (Clade A<sub>1</sub>)*.—The sister group relationship of *Pleospora herbarum* and *P. ambigua* is consistent in all analyses. Although morphologically uniform *Pleospora* does not appear to be monophyletic. Inclusion of *P. bjoerlingii*, *P. iqbalii* and *P. rubicunda* in our analyses showed that these species are unrelated even to any members of the Pleosporaceae (results not shown). They were found to be basal to *Westerdykella cylindrica* and *Preussia terricola*, which are members of the Sporormiaceae. Molecular data from other studies also have shown that *Pleospora* is a polyphyletic genus (Berbee et al 1996). All pleosporaceous taxa sampled in this study (Clade A) are characterized by septate pseudoparaphyses. There are morphologies, however, that are unique to each genus. *Pleospora* is distinct from *Cochliobolus* in having yellow-brown to dark brown ellipsoidal and dictyoseptate ascospores, whereas *Cochliobolus* has hyaline, filiform, multiseptate ascospores (Sivanesan 1984). An affinity between *Curvularia* and *Setosphaeria* can be explained by the fact that both have hyaline ascospores and a *Drechslera* anamorph (Sivanesan 1984). Their anamorphs also play an important role as plant pathogens in monocotyledonous plants (i.e. *Bipolaris hawaiiensis*, *B. maydis*, *Curvularia oryzae*, *Drechslera prolata* and *Exserohilum turcicum*) (Farr et al 1989, Luna et al 2002, Krupinsky et al 2004).

Our results are consistent with published concepts in that anamorphic characters might be useful in the

systematics of *Cochliobolus*, but whether this holds true for *Pleospora* remains uncertain. For instance *Pleospora herbarum* is known to possess *Stemphylium* anamorphs, yet it is not closely related to *Pleospora sedicola*, *P. tomatonis* and *P. halophila*, which possess similar *Stemphylium* anamorphs. *Pleospora ambigua* (of which no anamorph is known) clusters in the Pleosporaceae but not with other *Pleospora* species. It should be mentioned that some species of *Pleospora* also are known to possess *Phoma*, *Dendryphion* and *Diplodia*-like anamorphs (Sivanesan, 1984).

*Systematics of Pyrenophora (Clade A<sub>2</sub>)*.—This genus originally was placed in Pleosporaceae by Wehmeyer (1961) but later transferred to a new family, Pyrenophoraceae (Barr 1987b). Later it was included again in the Pleosporaceae (Eriksson 1984, Berbee 1996). *Pyrenophora* species are morphologically characterized by the absence of definite pseudoparaphyses, which are present in other pleosporaceous taxa. Our results indicate that *Pyrenophora* is monophyletic and phylogenetically distinct from other members of the Pleosporaceae but cluster within this family.

Sequence analyses also elucidate taxonomic relationships at the species level. *Pyrenophora trichostoma* and *P. tritici-repentis* cluster with high bootstrap support. Based on morphological similarity these two species have recently been synonymized (Ciuffetti and Tuori 1999). Molecular evidence provided here suggests that they are phylogenetically related but their sequences differ in several base pairs. Whether they should be treated as synonyms cannot be judged with this dataset alone.

Similar results were obtained with *P. dictyoides* and *P. tetraerhena* (albeit with low support), while *P. seminiperda* appears to be distinct from these other four species. *Pyrenophora dictyoides* and *P. tetraerhena* are characterized by sclerotia that form in culture and ascospores with one or two longitudinal septa in the median cell (Paul and Parbery 1968, Sivanesan 1984). *Pyrenophora trichostoma* and *P. tritici-repentis* possess ascospores with mucilaginous sheaths (Dennis 1978, Sivanesan, 1984). *Pyrenophora seminiperda* is unique in having multitransversely septate with two vertical septa laid at right angles and ascospores that are surrounded by a mucilaginous sheath when young (Sivanesan 1984). Although taxon sampling is sparse it can be argued that formation of sclerotia, shape of ascospores, septation and gelatinous sheath appear to be significant in delineating species. Based on phylogenetic analysis of ITS and the *gpd* data Zhang and Berbee (2001) showed that *Pyrenophora* is monophyletic. They also discussed relationships between most *Pyrenophora* and anamorphic *Drechslera* species

and found that phylogenies were consistent with the relationships proposed from prior morphological studies.

*Phylogeny of Leptosphaerulina and Macroventuria (Clade B).*—The genera *Leptosphaerulina* and *Macroventuria* currently are accommodated in the Pleosporaceae (Eriksson and Hawksworth 1998, Kirk et al 2001, Eriksson 2005). Our molecular analyses show that *Leptosphaerulina* and *Macroventuria* are phylogenetically related. This relationship respectively receives moderate bootstrap and high Bayesian support. However a classification within the Pleosporaceae is contentious from the standpoint of phenotypic characters and sequence data. Both genera consist of species that do not possess pseudoparaphyses. This character has been shown to be phylogenetically significant in segregating the bitunicate fungi into two major orders: Dothideales and Pleosporales (Luttrell 1973, Barr 1979, Silva-Hanlin and Hanlin 1999, Lumbsch and Lindemuth 2001). Assuming that this character is taxonomically important as has been postulated by various mycologists (Liu et al 1999, Silva-Hanlin and Hanlin 1999, Liew et al 2000) these two genera would have to be transferred into a different family whose allies include species characterized by the absence of pseudoparaphyses. Silva-Hanlin and Hanlin (1999) also found that although *Leptosphaerulina* belongs to the Pleosporales, but its affinities to other members of the Pleosporaceae are still unclear.

ITS AND EF-1 $\alpha$  phylogenies revealed that *Leptosphaerulina briosiana* is closely related to *L. trifolii*, while *L. argentinensis* is related to *L. australis* (Abler 2003). At the species level, 28S rDNA phylogenies here corroborate with those of Abler (2003). *Leptosphaerulina argentinensis* and *L. australis* possess 5-septate ascospores and differ from *L. briosiana* and *L. trifolii* that have 3–4-septate ascospores. Irwin and Davis (1985) delineated *Leptosphaerulina* based on ascospore shape and size but most importantly on the number of transverse septa. The phylogenetic segregation of *Leptosphaerulina* species sampled in this study into two groups corresponds to the number of septa. Borm et al (2002) also have shown that *Leptosphaerulina chartatum* is related to *Pleospora rudis* and *Pseudotrichia aurata* based on 18S rDNA sequence analysis. Silva-Hanlin and Hanlin (1999) also found that *L. chartatum* and *L. crassiasca* were related to other pleosporaceous taxa, although the presence of pseudoparaphyses does not appear to be common in this genus. Morphologically *Leptosphaerulina* can sometimes be mistaken for *Pleospora* because the ascospores often become brown after

discharge. *Pleospora*, however, differs in having larger ascomata and ascospores that turn brown before discharge (Wu and Hanlin 1992). Based on 18S rDNA phylogenies, Silva-Hanlin and Hanlin (1999) found that *Leptosphaerulina*, although characterized by the absence of pseudoparaphyses, is related to *Didymella bryoniae* and *Didymella* ('*Mycosphaerella*') *citrullina* (both referred to the Dothideomycetes incertae sedis) and *Phaeosphaeria microscopica* (Leptosphaeriaceae). It also has been found that *Botryosphaeria ribis* and *B. dothidea* (placed in the Dothideales by Sivanesan 1984), which possess pseudoparaphyses, are phylogenetically distinct from other Pleosporales members (Silva-Hanlin and Hanlin 1999). It is highly plausible that the presence of pseudoparaphyses is not a definite characteristic of the Pleosporales as previously argued.

Similar disagreements are reported for the classification of *Macroventuria*. There are only two species (*M. wentii* and *M. anomochaeta*) and they initially were classified in the Venturiaceae (van der Aa 1971), mainly based on perithecia with well developed setae and ascospore shape. This genus has been referred to the Pseudosphaeriaceae by Barr (1982) and recently to the Pleosporaceae (Eriksson and Hawksworth 1998). *Macroventuria* is similar ecologically and morphologically to other members of the Pleosporaceae. The saprobic mode of life and morphology suggest a primitive organization, like that of *Wettsteinina* and *Pyrenophora* (Müller and von Arx 1950). *Macroventuria*, although superficially similar to *Leptosphaerulina*, differs in having ascospores that are almost hyaline, matures quickly in pure culture and have ellipsoid ascospores (van der Aa 1971). Ecologically, *Leptosphaerulina* species are mostly isolated as pathogens from alfalfa and turf grass (Sundheim and Wilcoxson 1965, Abler 2003) whereas *Macroventuria* species have a saprobic mode of life.

The familial placement based on sequence analyses of these two genera at present is unresolved. Barr (1982) maintained *Leptosphaerulina* and *Macroventuria* in the Pseudosphaeriaceae and Eriksson and Hawksworth (1998) accommodated them in the Pleosporaceae, but results show that placement in either of these families is doubtful. This is because (i) phylogenetically they are more closely related to other members of the Leptosphaeriaceae and Phaeosphaeriaceae and (ii) Pseudosphaeriaceae have been treated as a synonym of Pleosporaceae (Shoemaker and Babcock 1987). Although results do not appear to be consistent with any previous classification schemes, there is sufficient evidence to indicate that *Macroventuria* should not be included in the Venturiaceae (*sensu* van der Aa 1971). This is because *Venturia* is phylogenetically distant from most of the

members of Pleosporales. The traditional placement of *Macroventuria* in Venturiaceae indicates that morphology of the asci and ascospores and perithecia with well developed setae have been given too much importance.

*Position of Wettsteinina (Clade G).*—*Wettsteinina* commonly occurs as pathogens or saprobes on plant leaves and stems (Shoemaker and Babcock 1987) and no molecular studies have addressed phylogenetic relationships of *Wettsteinina* species at the familial level. *Wettsteinina* initially was referred to Pseudosphaeriaceae (Wehmeyer 1961), and other authors even considered it as a synonym of *Pseudosphaeria* (family Pseudosphaeriaceae, order Pseudosphaeriales). Molecular data here show that *Wettsteinina* is monophyletic but phylogenetically distinct from other members of the Pleosporaceae. Instead it forms a strongly supported sister group relationship with *Pleomassaria* (Pleomassariaceae). Luttrell (1955) pointed out that *Wettsteinina* morphologically is consistent with Luttrell's Dothidea-type of centrum. *Wettsteinina* has 1- to 7-transversely septate ascospores and resembles *Didymella*, *Leptosphaeria* and *Massarina*. However *Wettsteinina* lacks true pseudoparaphyses that are typical of other pleosporaceous members with the exception of *Leptosphaerulina* (Wehmeyer 1961). Phylogenies generated here indicate that the inclusion of *Wettsteinina* in the Pleosporaceae is doubtful. With its apparent lack of pseudoparaphyses and phylogenetic distance from other pleosporaceous taxa, we consider that *Wettsteinina* should be excluded from the Pleosporaceae. Placement within the Pleomassariaceae merits consideration because *Pleomassaria siparia* forms a well supported sister group relationship with *Wettsteinina* species. However to gain further insight into the phylogeny of *Wettsteinina*, a broader taxon sampling incorporating more taxa from Pleomassariaceae is necessary.

*Polyphyly of Kirschsteiniothelia (Clade D).*—Although *Kirschsteiniothelia* currently is accepted in the Pleosporaceae (Eriksson and Hawksworth 1998, Kirk et al 2001, Eriksson 2005), few phylogenetic studies have included *Kirschsteiniothelia* species. *Massarina ramunculicola* formerly was placed in the family Massarinaceae. In contrast *Kirschsteiniothelia elaterascus* has been referred to the Pleosporaceae. Hawksworth (1985) monographed *Kirschsteiniothelia* and placed it in Pleosporaceae, but he pointed out the substrate recurrence and the formation of refractive regions in the ascospores possibly reflect distosepta, which are atypical of the Pleosporaceae. Barr (1993a, b)

argued that *Kirschsteiniothelia* belongs to the Pleomassariaceae. *Kirschsteiniothelia elaterascus* surprisingly does not seem to fit within the Pleomassariaceae or Pleosporaceae. Instead it clusters with *Massarina ramunculicola* in a single monophyletic clade with high support. A close phylogenetic connection between *Kirschsteiniothelia* and *Massarina* was unexpected from a taxonomic viewpoint. Boise (1985) published new combinations of taxa in the Pleomassariaceae and Massarinaceae, suggesting that fungi in these two families have similar characters and might be closely related. Assuming that Barr's treatment is correct (accept *Kirschsteiniothelia* in Pleomassariaceae), then *Kirschsteiniothelia* also can be closely related to taxa within the Massarinaceae. *Kirschsteiniothelia* and *Massarina* possess several characters that unite them. These include 1-celled and fusoid ascospores, cellular pseudoparaphyses and particularly their occurrence on woody litter. Occurrence on this type of woody substratum is atypical in the Pleosporaceae (Hawksworth (1985). *Massarina ramunculicola* has been shown to have close phylogenetic affinities to the other Massarinaceae (e.g. *Massarina arundinariae* and *M. phragmiticola*) (Vijaykrishna pers comm). These relationships however received low statistical support and whether *M. ramunculicola* and *Kirschsteiniothelia elaterascus* can be assigned to the Massarinaceae is contentious. *Kirschsteiniothelia aethiops* also was found to be related to *Glyphium elatum*, whose taxonomic placement within the Dothideomycetes is uncertain (results not shown). We also included *Kirschsteiniothelia maritima* in other analyses but found that it was not related to the other *Kirschsteiniothelia* species. It therefore is highly likely that this genus is truly polyphyletic, just as *Pleospora*.

*Systematics of related families.*—The family Leptosphaeriaceae was introduced by Barr (1987) and comprises four genera and 119 species (Kirk et al 2001). Classification in the family has been based primarily on the type genus *Leptosphaeria*, which is morphologically similar to *Phaeosphaeria* (Kirk et al 2001). The familial position of these two genera within the loculoascomycetes has been debated by several mycologists (e.g. Sivanesan 1984, Barr 1987b, Hawksworth et al 1995, Morales et al 1995). Luttrell (1973), von Arx and Müller (1975) and Sivanesan (1984) placed *Leptosphaeria* in the Pleosporaceae, while Barr (1987a, b) and Eriksson and Hawksworth (1991) placed it in the Leptosphaeriaceae. Eriksson and Hawksworth (1986) considered that *Leptosphaeria* should be accommodated better in the Phaeosphaeriaceae.

The taxonomic delineation of *Phaeosphaeria* and allied genera such as *Leptosphaeria* is problematic (Hyde et al 2000). Re-examination and taxonomic reassessment of species previously placed in *Leptosphaeria* resulted in the disposition of many species into allied genera especially *Phaeosphaeria* (Hyde et al 2000). Many mycologists realized that there are problems in delimiting *Leptosphaeria* and *Phaeosphaeria* because they are similar in gross appearance.

Comparative analyses of the 28S rDNA of *Leptosphaeria* showed a close phylogenetic relatedness to other members of the Phaeosphaeriaceae, as well as a common evolutionary history pertaining to the Pleosporaceae. Although there is a minor topological incongruence between the parsimony and Bayesian phylogenies, this monophyletic group receives high statistical support and is consistent with existing classification of Phaeosphaeriaceae and Leptosphaeriaceae. Our results are congruent with those reported by Rossman et al (2002) who found that *Setomelanomma holmii* and *Leptosphaeria doliolum* are related phylogenetically and in addition both families share close evolutionary relationships to the Pleosporaceae. Despite major differences in opinions in the classification of *Leptosphaeria*, results show that they are more closely related to the Phaeosphaeriaceae as proposed by Eriksson and Hawksworth (1986). Whether Phaeosphaeriaceae is a synonym of Leptosphaeriaceae needs to be re-evaluated based on a larger taxon sampling and multigene sequence analyses. In other phylogenetic studies, incorporating more taxa from *Leptosphaeria*, *Phaeosphaeria*, and *Septoria nodorum* and *Ophiobolus herpotrichus*, we found that species from these four genera group into the combined Phaeosphaeriaceae and Leptosphaeriaceae family as a strongly supported monophyletic lineage (Kodsueb et al 2005a). Our results point out that Phaeosphaeriaceae and Leptosphaeriaceae are more closely related to the Pleosporaceae than other families. This has been shown by Rossman et al (2002) and Kodsueb et al (2005a).

Another taxonomic issue addressed in this study is the phylogenetic placement of *Bimuria*, *Karstenula* and *Trematosphaeria*, which all belong to the Melanommataceae (Kirk et al 2001). Of special interest was the sister group relationship of *Bimuria* and *Letendraea*. Hawksworth et al (1979) described detailed morphological affinities of *Bimuria* to *Pleospora* and *Montagnula*. It is highly probable that the family Melanommataceae is not strictly monophyletic. The Melanommatales were not monophyletic in an analysis based on a combination of 18S and a 28S rDNA sequence analyses (Lumbsch and Lindemuth 2001). Kodsueb et al (2005b) have shown that *Letendraea* does not belong in the Tubeufiaceae and

excluded this genus based on morphological and phylogenetic data. Results here also correspond to those of Lumbsch and Lindemuth (2001). Although *Preussia* and *Westerdykella* always group together, the position of the strongly supported monophyletic clade varies. MP analyses position these two genera basal to the core of the melanommataceous genera (*Bimuria* and *Karstenula*), whereas they cluster with other Melanommataceae and Lophiostomataceae species in the Bayesian analyses. In other sequence datasets it also was found that the addition of anamorphic Sporormiaceae (*Berkleasium micronesicum* and *B. nigroapicale*) resulted in a moderately supported clade characterizing the Sporormiaceae family, which was nested between the Melanommataceae and Pleomassariaceae (Jeewon pers comm).

#### CONCLUSIONS

Nine clades of pleosporalean fungi have been identified by our analysis. Although relationships among these clades sometimes are weakly supported and some may vary in detail, some tentative conclusions can be drawn. Several current taxonomic hypotheses are supported by our molecular data, and this makes it possible to propose some taxonomic hypotheses about the relationships among the Pleosporaceae. All taxa within Clade A are monophyletic and are housed within the Pleosporaceae, indicating a single origin of fungi characterized by pseudoparaphyses. However the Pleosporaceae do not appear to be monophyletic in its current circumscription because other members, such as *Leptosphaerulina*, *Macroventuria*, *Kirschsteiniiothelia* and *Wettsteinina*, appear to be more closely related to other families. *Pleospora* species bearing *Stemphylium* anamorphs are related to *Cochliobolus*. *Pyrenophora* is monophyletic and should be accommodated within Pleosporaceae. *Leptosphaerulina* and *Macroventuria* possibly need to be re-assigned to a different family. The phylogeny of *Wettsteinina* is largely in disagreement with morphology-based schemes and it should be excluded from the Pleosporaceae. *Kirschsteiniiothelia* is probably polyphyletic.

While the taxa presented in this analysis represent a broad range of bitunicate fungi, it is clear that additional taxa, especially from some of the more speciose genera including *Kirschsteiniiothelia*, *Pleospora* and *Wettsteinina*, will need to be sampled to resolve the phylogeny in more detail. The presence of the four clades of the Pleosporaceae phylogeny does provide a framework for future taxon selection to strengthen our understanding of Loculoascomycete evolution. Another major observation in this study was the lack of statistical support for any of the major

nodes within the Pleosporales. Neither 18S (other studies) nor 28S sequence analyses could offer significant phylogenetic signal to define proper familial boundaries. Addition of more taxa with broader taxon sampling from all families as well as from other dothideomycetes failed to resolve some of the major clades. Future molecular phylogenetic studies should include different genes as well.

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