# A multi-gene phylogeny for species of *Mycosphaerella* occurring on *Eucalyptus* leaves

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**Abstract:** Species of the ascomycete genus *Mycosphaerella* are regarded as some of the most destructive leaf pathogens of a large number of economically important crop plants. Amongst these, approximately 60 *Mycosphaerella* spp. have been identified from various *Eucalyptus* spp. where they cause leaf diseases collectively known as Mycosphaerella Leaf Disease (MLD). Species concepts for this group of fungi remain confused, and hence their species identification is notoriously difficult. Thus, the introduction of DNA sequence comparisons has become the definitive characteristic used to distinguish species of *Mycosphaerella*. Sequences of the Internal Transcribed Spacer (ITS) region of the ribosomal RNA operon have most commonly been used to consider species boundaries in *Mycosphaerella*. However, sequences for this gene region do not always provide sufficient resolution for cryptic taxa. The aim of this study was, therefore, to use DNA sequences for three loci, ITS, Elongation factor 1-alpha (EF-1α) and Actin (ACT) to reconsider species boundaries for *Mycosphaerella* spp. from *Eucalyptus*. A further aim was to study the anamorph concepts and resolve the deeper nodes of *Mycosphaerella*, for which part of the Large Subunit (LSU) of the nuclear rRNA operon was sequenced. The ITS and EF-1α gene regions were found to be useful, but the ACT gene region did not provide species-level resolution in *Mycosphaerella*. A phylogeny of the combined DNA datasets showed that species of *Mycosphaerella* from *Eucalyptus* cluster in two distinct groups, which might ultimately represent discrete genera.

**Key words:** Actin, *Ascomycetes*, Translation Elongation factor 1-alpha, Multi-gene phylogeny, *Mycosphaerella*, Mycosphaerella Leaf Disease, ribosomal RNA operon.

## INTRODUCTION

Species of *Eucalyptus* are native to Australia with isolated pockets of native *Eucalyptus* forests also occurring in Papua New Guinea and the Philippines (Turnbull 2000). Many *Eucalyptus* spp. have been removed from these centres of origin to new environments where they are typically propagated in plantations for the production of paper, pulp and other wood products (Wingfield 1999, Turnbull 2000, Wingfield *et al*. 2001). In these non-native environments, *Eucalyptus* trees are susceptible to many pests and diseases including those known in their areas of origin and others that have undergone host shifts (Wingfield 2003, Slippers *et al*. 2005). These pests and diseases cause significant annual losses to *Eucalyptus* plantations resulting in decreased revenue for commercial forestry companies.

*Mycosphaerella* Johanson is one of the largest genera of the ascomycetes, accommodating more than 2000 species. Approximately 60 *Mycosphaerella* spp. have been associated with leaf diseases of many *Eucalyptus* spp., and these are collectively referred to as Mycosphaerella Leaf Disease (MLD) (Crous 1998, Maxwell *et al*. 2003, Crous *et al*. 2004a). The disease is particularly prevalent on the juvenile leaves and shoots of *Eucalyptus* trees, where infection results in premature defoliation, twig cankers and stunting of tree growth (Lundquist & Purnell 1987, Crous 1998, Park *et al*. 2000). However, several *Mycosphaerella* spp. can also infect adult *Eucalyptus* foliage, and this has been attributed to their ability to produce a proto-appressorium that enables direct cuticle penetration (Ganapathi 1979, Park & Keane 1982b). In some

situations, trees may thus be subjected to infection by a suite of different *Mycosphaerella* spp.

Identification of *Mycosphaerella* spp. based on morphology is known to be difficult. This is because these fungi tend to produce very small fruiting structures with highly conserved morphology, and they are host-specific pathogens that grow poorly in culture. Traditionally, morphological characters of the teleomorph and anamorph have been used in species delimitation (Crous 1998). Park & Keane (1982a) introduced ascospore germination patterns as an additional characteristic to identify *Mycosphaerella* spp., and Crous (1998) subsequently identified 14 different ascospore germination patterns for the *Mycosphaerella* spp. occurring on *Eucalyptus*. Crous (1998) and Crous *et al*. (2000) also introduced features of these fungi growing in culture and especially anamorph morphology as important and useful characteristics on which to base species delimitation. DNA-based methods such as RAPDs and species-specific primers have also been employed to distinguish between *Mycosphaerella* species occurring on *Eucalyptus* (Carnegie *et al*. 2001, Maxwell *et al*. 2005).

Comparisons of DNA sequence data have emerged as the most reliable technique to identify *Mycosphaerella* spp. The majority of studies employing DNA sequence data for species identification have relied on sequence data from the Internal Transcribed Spacer (ITS) region of the ribosomal RNA operon (Crous *et al*. 1999, 2001, 2004a, b, Hunter *et al*. 2004a, b). Although comparisons of gene sequences for this region have been useful, the resolution provided by this region is not uniformly adequate to discriminate between individuals

of a species complex or to effectively detect cryptic species (Crous *et al*. 2004b). Thus, recent studies have shown the importance of employing Multi-Locus Sequence Typing (MLST) to effectively identify cryptic fungal species and to study species concepts (Taylor & Fischer 2003).

A single morphological species does not always reflect a single phylogenetic unit (Taylor *et al*. 2000). Within *Mycosphaerella*, teleomorph morphology is conserved and the anamorph morphology provides additional characteristics to discriminate between taxa (Crous *et al*. 2000). Yet the collective teleomorph and anamorph morphology is often not congruent with phylogenetic data. Thus, recent phylogenetic studies have led to the recognition of several species complexes within *Mycosphaerella* (Crous *et al*. 2001, 2004b, Braun *et al*. 2003). Most of these studies have been based on comparisons of sequences for the ITS regions of the ribosomal DNA operon. Given the important data that have emerged from them, it is well recognised that greater phylogenetic resolution will be required for future taxonomic studies on *Mycosphaerella* species.

The aim of this study was to use MLST to consider species and anamorph concepts in *Mycosphaerella* spp. occurring on *Eucalyptus*. This was achieved by sequencing four nuclear gene regions, namely part of the Large Subunit (D1−D3 of LSU) and ITS region of the nuclear rRNA operon, and a portion of the Actin (ACT) and Elongation Factor 1-alpha (EF-1α) gene regions.

## MATERIALS AND METHODS

### *Mycosphaerella* isolates
For this study, an attempt was made to obtain cultures of as many *Mycosphaerella* spp. known to infect *Eucalyptus* leaves as possible. All cultures used in the investigation are housed in culture collections of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (Table 1). All cultures were grown on 2 % (w/v) malt extract agar (MEA) (Biolab, South Africa), at 25 ℃ for approximately 2–3 mo to obtain sufficient mycelial growth for DNA extraction.

### DNA isolation
Mycelium from actively growing cultures was scraped from the surface of cultures, freeze-dried for 24 h and then ground to a fine powder using liquid nitrogen. DNA was isolated using the phenol : chloroform (1: 1) extraction protocol as described in Hunter *et al*. (2004a, b). DNA was precipitated by the addition of absolute ethanol (100 % EtOH). Isolated DNA was cleaned by washing with 70 % Ethanol (70 % EtOH) and dried under vacuum. SABAX water was used to resuspend the isolated DNA. RNaseA (10 µg/µL) was added to the resuspended DNA and incubated at 37 °C for approximately 2 h to digest any residual RNA. Isolated DNA was visualised in a 1 % agarose gel (w/v) (Roche Diagnostics, Mannheim), stained with ethidium

bromide and visualised under ultra-violet light.

### PCR amplification and purification
DNA (*ca*. 20 ng) isolated from the *Mycosphaerella* spp. used in this study was used as a template for amplification using the Polymerase Chain Reaction (PCR). All PCR reactions were mixed in a total volume of 25 µL containing 10× PCR Buffer (5 mM Tris-HCl, 0.75 mM $MgCl_2$, 25 mM KCl, pH 8.3) (Roche Diagnostics, South Africa), 2.5 mM of each dNTP (dATP, dTTP, dCTP, dGTP) (Roche Diagnostics, South Africa), 0.2 µM of forward and reverse primers (Inqaba Biotech, South Africa) and 1.25 U Taq DNA Polymerase (Roche Diagnostics, South Africa) and DNA (20 ng/µL). Sterilised distilled water was added to obtain a final volume of 25 µL.

The ITS-1, ITS-2 and the 5.8 S gene regions of the ITS region of the rRNA operon were amplified using primers ITS-1 (5`− TCC GTA GGT GAA CCT GCG G –3`) and LR-1 (5`- GGT TGG TTT CTT TTC CT – 3`) (Vilgalys & Hester 1990, White *et al*. 1990). Reaction conditions for the ITS gene regions followed those of Crous *et al*. (2004a) and Hunter *et al*. (2004a, b).

A portion of the LSU (including domains D1−D3) of the rRNA operon was amplified using primers LR0R (5'-ACC CGC TGA ACT TAA GC-3') (Moncalvo *et al*. 1995) and LR7 (5'-TAC TAC CAC CAA GAT CT-3') (Vilgalys & Hester 1990). PCR cycling conditions were as follows: an initial denaturation step of 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 62 °C for 30 s, primer extension at 72°C for 1 min and a final elongation step at 72 °C for 7 min.

A portion of the EF-1α was amplified using the primers EF1-728F (5'-CAT CGA GAA GTT CGA GAA GG-3') and EF1-986R (5'-TAC TTG AAG GAA CCC TTA CC-3') (Carbone & Kohn 1999). Reaction conditions were: an initial denaturation step of 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s and primer extension at 72 °C for 30 s. The reaction was completed with a final extension at 72 °C for 7 min.

A portion of the ACT gene was amplified using the primers ACT-512F (5'-ATG TGC AAG GCC GGT TTC GC-3') and ACT-783R (5'-TAC GAG TCC TTC TGG CCC AT-3') (Carbone & Kohn 1999). PCR reaction conditions were: an initial denaturation step at 96 °C for 2 min, followed by 10 cycles of denaturation at 94 °C for 30 s, primer annealing at 61 °C for 45 s and extension at 72 °C for 45 s. This was followed by 25 cycles of denaturation at 94 °C for 30 s, primer annealing at 61 °C and elongation at 72 °C for 45 s with an increase of 5 s per cycle. The reaction was completed with a final elongation step at 72 °C for 7 min.

All PCR products were visualised in 1.5 % agarose gels (wt/v) stained with ethidium bromide and viewed under ultra-violet light. Sizes of PCR amplicons were estimated by comparison against a 100 bp molecular weight marker (O' RangeRuler™ 100 bp DNA ladder) (Fermentas Life Sciences, U.S.A.). Prior to DNA sequencing, PCR products were purified through Centri-sep spin columns (Princeton Separations, Adelphia, NJ) containing Sephadex G-50 (Sigma Aldrich, St. Louis, MO) as outlined by the manufacturer.

## DNA sequencing and phylogenetic analysis

Purified PCR products were used as template DNA for sequencing reactions on an ABI PRISM™ 3100 Automated DNA sequencer (Applied Biosystems, Foster City, CA). The ABI Prism Big Dye Terminator Cycle sequencing reaction kit v. 3.1 (Applied Biosystems, Foster City, CA) was used for sequencing reactions following the manufacturer's instructions. Most sequencing reactions were performed with the same primers used for PCR reactions. Exceptions were in the case of the ITS region where two internal primers ITS-2 (5'-GCT GCG TTC TTC ATC GAT GC-3') and ITS-3 (5'-GCA TCG ATG AAG AAC GCA GC-3') (White *et al*. 1990) were included for the sequencing reactions. Similarly, for the LSU region two internal primers LR3R (5'-GTC TTG AAA CAC GGA CC-3') and LR-16 (5'-TTC CAC CCA AAC ACT CG-3') were used for the sequencing reactions.

All resulting sequences were analysed with Sequence Navigator v. 1.0.1 (Applied Biosystems, Foster City, CA). Sequence alignments were done using MAFFT (Multiple alignment program for amino acid or nucleotide sequences) v. 5.667 (Katoh *et al*. 2005) and manually adjusted where necessary. Phylogenetic analyses and most parsimonious trees were generated in PAUP v. 4.0b10 (Swofford 2002) by heuristic searches with starting trees obtained through stepwise addition with simple addition sequence and with the MULPAR function enabled. Tree Bisection Reconnection (TBR) was employed as the swapping algorithm. All gaps were coded as missing data and characters were assigned equal weight. Branch support for nodes was obtained by performing 1000 bootstrap replicates of the aligned sequences. For parsimony analyses, measures that were calculated include tree length (TL), retention index (RI), consistency index (CI), rescaled consistency index (RC) and homoplasy index (HI). *Botryosphaeria ribis* Grossenb. & Duggar was used as the outgroup to root all trees.

A Partition Homogeneity Test (Farris *et al*. 1994), of all possible combinations, consisting of 1000 replicates on all informative characters was conducted in PAUP to determine if the LSU, ITS and EF-1α data sets were combinable. All sequences of *Mycosphaerella* spp. used in this study have been deposited in GenBank (Table. 1). Sequence alignments and trees of the LSU, ITS, EF-1α and ACT have been deposited in TreeBASE (accession numbers: LSU = SN2535, ITS = SN2534, EF-1α = SN2536, ACT = SN2537).

Parsimony and distance analyses of combined DNA sequence alignments were conducted in PAUP. Parsimony analyses of all DNA sequence alignments were identical to those described earlier. For distance analyses, Modeltest v. 3.04 (Posada & Crandall 1998) was used to determine the best evolutionary model to fit the combined DNA sequence alignment. A neighbour-joining analysis with an evolutionary model was conducted in PAUP. Here, the distance measure was a general time-reversible (GTR) and the proportion of sites assumed to be invariable (I) was 0.4919, identical sites were removed proportionally to base frequencies estimated from all sites, rates of variable sites assumed to follow a gamma distribution (G) with shape parameter of 0.6198. Ties (if encountered) were broken randomly.

## RESULTS

### DNA sequencing and phylogenetic analysis

*Large Subunit (LSU) phylogeny*: The LSU alignment had a total length of 1714 characters. An indel of 383 bp present in *M. ohnowa* Crous & M.J. Wingf. (CBS 112973) and *Mycosphaerella mexicana* Crous (CBS 110502) was excluded from the analyses. In the LSU data set, 1075 characters were constant while 77 characters were parsimony-uninformative and 179 characters were parsimony-informative. Parsimony analysis of the LSU data set resulted in the retention of thirty most parsimonious trees (TL = 663, CI = 0.519, RI = 0.878, RC = 0.456). One of these trees (Fig. 1) could be resolved into two clades (Clades 1−2). Clade 1, supported with a bootstrap value of 70 %, included *Mycosphaerella* isolates characterised by *Phaeophleospora* Rangel (*M. ambiphylla* A. Maxwell, *M. suttoniae* Crous & M.J. Wingf.), *Colletogloeopsis* Crous & M.J. Wingf. [*M. molleriana* (Thüm.) Lindau, *M. vespa* Carnegie & Keane, *M. cryptica* (Cooke) Hansf.], *Uwebraunia* Crous & M.J. Wingf. [*M. nubilosa* (Cooke) Hansf.], *M. ohnowa*, *Readeriella* Syd. & P. Syd. (*M. readeriellophora* Crous & J.P. Mansilla), and *Passalora* Fr. (*M. tasmaniensis* Crous & M.J. Wingf.) anamorphs.

The second major clade (Clade 2) resolved in the LSU tree was well-supported with a bootstrap value of 98 %. *Mycosphaerella* species in this clade also grouped strongly following their anamorph associations. Here *Mycosphaerella* isolates could be resolved into several sub-clades also characterised by their anamorph associations. These were *Sonderhenia* (*M. walkeri* R.F. Park & Keane.), *Pseudocercospora* Speg. [*M. heimioides* Crous & M.J. Wingf., *M. heimii* Crous, *M. crystallina* Crous & M.J. Wingf., *M. irregulariramosa* Crous & M.J. Wingf., *M. colombiensis* Crous & M.J. Wingf., *M. gracilis* Crous & Alfenas, *Pseudocercospora robusta* Crous & M.J. Wingf., *Ps. natalensis* Crous & T. Coutinho, *M. fori* G.C. Hunter, Crous & M.J. Wingf., *Ps. basitruncata* Crous, *Ps. pseudoeucalyptorum* Crous, *Ps. eucalyptorum* Crous, M.J. Wingf., Marasas & B. Sutton., *Ps. paraguayensis* (Koboyashi) Crous, *Ps. basiramifera* Crous] *Passalora* [*Pass. eucalypti* (Crous & Alfenas) Crous & U. Braun, *Pass. zambiae* Crous & T. Coutinho], and *Dissoconium* (*M. lateralis* Crous & M.J. Wingf., *M. communis* Crous & J.P. Mansilla).

*Internal Transcribed Spacer Region (ITS) phylogeny*: The ITS sequence alignment consisted of a total of 793 characters. Of these 499 characters were constant, 62 characters were variable and parsimony-uninformative and 232 characters were parsimony-informative. A 185 bp indel was observed in isolates of *M. gregaria* Carnegie & Keane (CBS 110501), *M. endophytica* Crous & H. Smith (CBS 111519) and *M. endophytica* (CMW 5225) and was excluded in the phylogenetic analysis.

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**Table 1.** Isolates of *Mycosphaerella* used in this study for DNA sequencing and phylogenetic analysis.

| Teleomorph | Anamorph | Isolate No.[a] | | | Host | Country | Collector | GenBank Accession No. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CMW | CBS | STEU | | | | LSU | ITS | ACT | EF-1a |
| *M. africana* | Unknown | 3026 | 116155 | 795 | *E. viminalis* | South Africa | P.W. Crous | DQ246258 | DQ267577 | DQ147608 | DQ235098 |
| | | 4945 | 116154 | 794 | *E. viminalis* | South Africa | P.W. Crous | DQ246257 | AF309602 | DQ147609 | DQ235099 |
| *M. ambiphylla* | *Phaeophleospora* sp. | 14180 | 110499 | N/A | *E. globulus* | Australia | A. Maxwell | DQ246219 | AY725530 | DQ147669 | DQ235103 |
| *M. aurantia* | Unknown | 14460 | 110500 | N/A | *E. globulus* | Australia | A. Maxwell | DQ246256 | AY725531 | DQ147610 | DQ235097 |
| *M. colombiensis* | *Pseudocercospora colombiensis* | 4944 | 110969 | 1106 | *E. urophylla* | Colombia | M.J. Wingfield | DQ204744 | AY752149 | DQ147639 | DQ211660 |
| | | 11255 | 110967 | 1104 | *E. urophylla* | Colombia | M.J. Wingfield | DQ204745 | AY752147 | DQ147640 | DQ211661 |
| *M. communis* | *Dissoconium commune* | 14672 | 114238 | 10440 | *E. globulus* | Spain | J.P. Mansilla | DQ246262 | AY725541 | DQ147655 | DQ235141 |
| | | 14673 | 110976 | 849 | *E. cladocalyx* | South Africa | P.W. Crous | DQ246261 | AY725537 | DQ147654 | DQ235140 |
| *M. cryptica* | *Colletogloeopsis nubilosum* | 3279 | 110975 | 936 | *E. globulus* | Australia | A.J. Carnegie | DQ246222 | AF309623 | DQ147674 | DQ235119 |
| | | 2732 | N/A | 355 | *Eucalyptus* sp. | Chile | M.J. Wingfield | N/A | AF309622 | N/A | N/A |
| *M. crystallina* | *Pseudocercospora crystallina* | 3042 | N/A | 800 | *E. bicostata* | South Africa | M.J. Wingfield | DQ204746 | DQ267578 | DQ147637 | DQ211662 |
| | | 3033 | 681.95 | 802 | *E. bicostata* | South Africa | M.J. Wingfield | DQ204747 | AY490757 | DQ147636 | DQ211663 |
| *M. ellipsoidea* | *Uwebraunia ellipsoidea* | 4934 | N/A | 1224 | *Eucalyptus* sp. | South Africa | Unknown | DQ246253 | AF309592 | DQ147647 | DQ235129 |
| | | 5166 | N/A | 1225 | *Eucalyptus* sp. | South Africa | Unknown | DQ246254 | AF309593 | DQ147648 | DQ235127 |
| *M. endophytica* | *Pseudocercosporella endophytica* | 14912 | 111519 | 1191 | *Eucalyptus* sp. | South Africa | P.W. Crous | DQ246255 | DQ267579 | DQ147646 | DQ235131 |
| | | 5225 | N/A | 1192 | *Eucalyptus* sp. | South Africa | P.W. Crous | DQ246252 | DQ267580 | DQ147649 | DQ235128 |
| *M. flexuosa* | Unknown | 5224 | 111012 | 1109 | *E. globulus* | Colombia | M.J. Wingfield | DQ246232 | AF309603 | DQ147653 | DQ235126 |
| *M. fori* | *Pseudocercospora* sp. | 9095 | N/A | N/A | *E. grandis* | South Africa | G.C. Hunter | DQ204748 | AF468869 | DQ147618 | DQ211664 |
| | | 9096 | N/A | N/A | *E. grandis* | South Africa | G.C. Hunter | DQ204749 | DQ267581 | DQ147619 | DQ211665 |
| *M. gracilis* | *Pseudocercospora gracilis* | 14455 | 243.94 | 730 | *E. urophylla* | Indonesia | A.C. Alfenas | DQ204750 | DQ267582 | DQ147616 | DQ211666 |
| *M. grandis* | Unknown | 8557 | N/A | N/A | *E. globulus* | Chile | A.Rotella | DQ246241 | DQ267583 | DQ147644 | DQ235108 |
| | | 8554 | N/A | N/A | *E. globulus* | Chile | M.J. Wingfield | DQ246240 | DQ267584 | DQ147643 | DQ235107 |
| *M. gregaria* | Unknown | 14462 | 110501 | N/A | *E. globulus* | Australia | A. Maxwell | DQ246251 | DQ267585 | DQ147650 | DQ235130 |
| *M. heimii* | *Pseudocercospora heimii* | 4942 | 110682 | 760 | *Eucalyptus* sp. | Madagascar | P.W. Crous | DQ204751 | AF309606 | DQ147638 | DQ211667 |
| *M. heimioides* | *Pseudocercospora heimioides* | 14776 | 111364 | N/A | *Eucalyptus* sp. | Indonesia | M.J. Wingfield | DQ204752 | DQ267586 | DQ147632 | DQ211668 |
| | | 3046 | 111190 | 1312 | *Eucalyptus* sp. | Indonesia | M.J. Wingfield | DQ204753 | AF309609 | DQ147633 | DQ211669 |
| *M. intermedia* | Unknown | 7163 | 114356 | 10902 | *E. saligna* | New Zealand | K. Dobbie | DQ246247 | AY725546 | N/A | N/A |
| | | 7164 | 114415 | 10922 | *E. saligna* | New Zealand | K. Dobbie | DQ246248 | AY725547 | DQ147627 | DQ235132 |
| *M. irregulariramosa* | *Pseudocercospora irregulariramosa* | 4943 | 114774 | 1360 | *E. saligna* | South Africa | M.J. Wingfield | DQ204754 | AF309607 | DQ147634 | DQ211670 |
| | | 5223 | N/A | 1362 | *E. saligna* | South Africa | M.J. Wingfield | DQ204755 | AF309608 | DQ147635 | DQ211671 |
| *M. ohnowa* | Unknown | 4937 | 112896 | 1004 | *E. grandis* | South Africa | M.J. Wingfield | N/A | AF309604 | DQ147662 | DQ235125 |
| | | 4936 | 112973 | 1005 | *E. grandis* | South Africa | M.J. Wingfield | DQ246231 | AF309605 | DQ147661 | DQ235124 |
| *M. keniensis* | Unknown | 5147 | 111001 | 1084 | *E. grandis* | Kenya | T. Coutinho | DQ246259 | AF309601 | DQ147611 | DQ235100 |

**Table 1.** (Continued).

| Teleomorph | Anamorph | Isolate No.[a] | | | Host | Country | Collector | GenBank Accession No. | | | |
| | | CMW | CBS | STEU | | | | LSU | ITS | ACT | EF-1a |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *M. lateralis* | *Dissoconium dekkeri* | 14906 | 110748 | 825 | *E. grandis × E. saligna* | South Africa | G. Kemp | DQ204768 | AF173315 | DQ147651 | DQ211684 |
| | | 5164 | 111169 | 1232 | *E. globulus* | Zambia | T. Coutinho | DQ246260 | AY25550 | DQ147652 | DQ235139 |
| *M. madeirae* | *Pseudocercospora* sp. | 14458 | 112895 | 3745 | *E. globulus* | Madeira | S. Denman | DQ204756 | AY725553 | DQ147641 | DQ211672 |
| *M. marksii* | Unknown | 14781 | 682.95 | 842 | *E. grandis* | South Africa | G. Kemp | DQ246249 | DQ267587 | DQ147624 | DQ235133 |
| | | 5150 | 110920 | 935 | *E. botryoides* | Australia | A.J. Carnegie | DQ246250 | AF309588 | DQ147625 | DQ235134 |
| | | 5230 | N/A | 782 | *E. botryoides* | Australia | A.J. Carnegie | DQ246246 | DQ267588 | DQ147626 | DQ235135 |
| *M. mexicana* | Unknown | 14461 | 110502 | N/A | *E. globulus* | Australia | A. Maxwell | DQ246237 | AY725558 | DQ147660 | DQ235123 |
| *M. readeriellophora* | *Readeriella readeriellophora* | 14233 | 114240 | 10375 | *E. globulus* | Spain | J.P. Mansilla | DQ246238 | AY725577 | DQ147658 | DQ235117 |
| *M. molleriana* | *Colletogloeopsis molleriana* | 4940 | 111164 | 1214 | *E. globulus* | Portugal | S. McCrae | DQ246220 | AF309620 | DQ147671 | DQ235104 |
| | | 2734 | 111132 | 784 | *E. globulus* | U. S. A. | M.J. Wingfield | DQ246223 | AF309619 | DQ147670 | DQ235105 |
| *M. nubilosa* | *Uwebraunia juvenis* | 3282 | 116005 | 937 | *E. globulus* | Australia | A.J. Carnegie | DQ246228 | AF309618 | DQ147666 | DQ235111 |
| | | 9003 | 114708 | N/A | *E. nitens* | South Africa | G.C. Hunter | DQ246229 | AF449099 | DQ147667 | DQ235112 |
| *M. parkii* | *Stenella parkii* | 14775 | 387.92 | 353 | *E. grandis* | Brazil | M.J. Wingfield | DQ246245 | AY626979 | DQ147612 | DQ235137 |
| *M. parva* | Unknown | 14459 | 110503 | N/A | *E. globulus* | Australia | A. Maxwell | DQ246243 | AY626980 | DQ147645 | DQ235110 |
| | | 14917 | 116289 | 10935 | *Eucalyptus* sp. | South Africa | P.W. Crous | DQ246242 | AY725576 | DQ147642 | DQ235109 |
| *M. suberosa* | Unknown | 5226 | 436.92 | 515 | *E. dunnii* | Brazil | M.J. Wingfield | DQ246235 | AY626985 | DQ147656 | DQ235101 |
| | | 7165 | N/A | N/A | *E. muelleriana* | New Zealand | Unknown | DQ246236 | DQ267589 | DQ147657 | DQ235102 |
| *M. suttonii* | *Phaeophleospora epicoccoides* | 5348 | N/A | 1346 | *Eucalyptus* sp. | Indonesia | M.J. Wingfield | DQ246227 | AF309621 | DQ147673 | DQ235116 |
| *M. vespa* | *Colletogloeopsis* sp. | 11558 | 117924 | N/A | *E. globulus* | Tasmania | Unknown | DQ246221 | DQ267590 | DQ147668 | DQ235106 |
| *M. tasmaniensis* | *Passalora tasmaniensis* | 14780 | 111687 | 1555 | *E. nitens* | Tasmania | M.J. Wingfield | DQ246233 | DQ267591 | DQ147676 | DQ235121 |
| | | 14663 | 114556 | N/A | *E. nitens* | Tasmania | M.J. Wingfield | DQ246234 | DQ267592 | DQ147677 | DQ235122 |
| *M. toledana* | *Phaeophleospora toledana* | 14457 | 113313 | N/A | *Eucalyptus* sp. | Spain | P.W. Crous | DQ246230 | AY725580 | DQ147672 | DQ235120 |
| *M. walkerii* | *Sonderhenia eucalypticola* | 20333 | N/A | N/A | *E. globulus* | Chile | M.J. Wingfield | DQ267574 | DQ267593 | DQ147630 | DQ235095 |
| | | 20334 | N/A | N/A | *E. globulus* | Chile | M.J. Wingfield | DQ267575 | DQ267594 | DQ147631 | DQ235096 |
| Unknown | *Passalora eucalypti* | 14907 | 111306 | 1457 | *E. saligna* | Brazil | P.W. Crous | DQ246244 | AF309617 | DQ147678 | DQ235138 |
| Unknown | *Passalora zambiae* | 14782 | 112971 | 1227 | *E. globulus* | Zambia | T. Coutinho | DQ246264 | AF725523 | DQ147675 | DQ235136 |
| Unknown | *Pseudocercospora epispermogonia* | 14778 | 110750 | 822 | *E. grandis × E. saligna* | South Africa | G. Kemp | DQ204757 | DQ267596 | DQ147629 | DQ211673 |
| | | 14786 | 110693 | 823 | *E. grandis × E. saligna* | South Africa | G. Kemp | DQ204758 | DQ267597 | DQ147628 | DQ211674 |
| Unknown | *Phaeophleospora eucalypti* | 11687 | 113992 | N/A | *E. nitens* | New Zealand | M. Dick | DQ246225 | DQ267598 | DQ147664 | DQ235115 |

**Table 1.** (Continued).

| Teleomorph | Anamorph | Isolate No.[a] | | | Host | Country | Collector | GenBank Accession No. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CMW | CBS | STEU | | | | LSU | ITS | ACT | EF-1a |
| | | 14910 | 111692 | 1582 | *Eucalyptus* sp. | New Zealand | M.J. Wingfield | DQ246224 | DQ267599 | DQ147663 | DQ235114 |
| Unknown | *Pseudocercospora basitruncata* | 14914 | 114664 | 1202 | *E. grandis* | Colombia | M.J. Wingfield | DQ204759 | DQ267600 | DQ147622 | DQ211675 |
| | | 14785 | 111280 | 1203 | *E. grandis* | Colombia | M.J. Wingfield | DQ204760 | DQ267601 | DQ147621 | DQ211676 |
| Unknown | *Pseudocercospora basiramifera* | 5148 | N/A | N/A | *E. pellita* | Thailand | M.J. Wingfield | DQ204761 | AF309595 | DQ147607 | DQ211677 |
| Unknown | *Pseudocercospora eucalyptorum* | 5228 | 110777 | 16 | *E. nitens* | South Africa | P.W. Crous | DQ204762 | AF309598 | DQ147614 | DQ211678 |
| Unknown | *Pseudocercospora natalensis* | 14777 | 111069 | 1263 | *E. nitens* | South Africa | T. Coutinho | DQ267576 | N/A | DQ147620 | N/A |
| | | 14784 | 111070 | 1264 | *E. nitens* | South Africa | T. Coutinho | DQ204763 | AF309594 | DQ147623 | DQ211679 |
| Unknown | *Pseudocercospora paraguayensis* | 14779 | 111286 | 1459 | *E. nitens* | Brazil | P.W. Crous | DQ204764 | DQ267602 | DQ147606 | DQ211680 |
| Unknown | *Pseudocercospora pseudoeucalyptorum* | 14908 | 114242 | 10390 | *E. globulus* | Spain | J.P. Mansilla | DQ204765 | AY725526 | DQ147613 | DQ211681 |
| | | 14911 | 114243 | 10500 | *E. nitens* | New Zealand | W. Gams | DQ204766 | AY725527 | DQ147615 | DQ211682 |
| Unknown | *Pseudocercospora robusta* | 5151 | 111175 | 1269 | *E. robusta* | Malaysia | M.J. Wingfield | DQ204767 | AF309597 | DQ147617 | DQ211683 |
| Unknown | *Readeriella novaezelandiae* | 14913 | 114357 | 10895 | *E. botryoides* | New Zealand | M.A. Dick | DQ246239 | DQ267603 | DQ147659 | DQ235118 |
| *Botryosphaeria ribis* | *Fusicoccum ribis* | 7773 | N/A | N/A | *Ribus* sp. | U. S. A. | G. Hudler. | DQ246263 | DQ267604 | DQ267605 | DQ235142 |

[a]CMW: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
STEU: Culture collection of Stellenbosch University, South Africa. Isolate numbers from Crous (1998).
N/A: Not available

A heuristic search of the ITS data set resulted in the retention of four most parsimonious trees (TL = 871, RI = 0.782, CI = 0.358, RC = 0.280). One of these phylogenetic trees (Fig. 2) generated by parsimony analysis of the ITS alignment could be resolved into two monophyletic clades (Clades 1−2). Clade 1 was only weakly supported with a bootstrap value of 50 % after 1000 bootstrap replicates. Clade 1 could be further resolved into several smaller sub-clades where isolates grouped strongly based on their anamorph affiliations. These included *Sonderhenia*, *Pseudocercospora*, *Passalora*, *Uwebraunia*/*Pseudocercosporella*, *Stenella*, *Readeriella*, *Phaeophleospora* and *Colletogloeopsis*. The second monophyletic clade (Clade 2) grouped sister to the first larger monophyletic clade and

contained isolates of *M. lateralis* and *M. communis* (*Dissoconium* anamorphs). This clade was well-supported with a bootstrap value of 100 % after 1000 bootstrap replicates.

*Translation Elongation factor 1-alpha (EF -1α) phylogeny*: The EF-1α alignment contained 373 characters. Of these, 41 characters were constant, 23 characters were variable and parsimony-uninformative and 309 characters were parsimony-informative. Heuristic searches resulted in the retention of six most parsimonious trees (TL = 3194, RI = 0.777, CI = 0.345, RC = 0.268), one of which is shown (Fig. 3). Species of *Mycosphaerella* could be resolved into three clades (Clades 1−3).
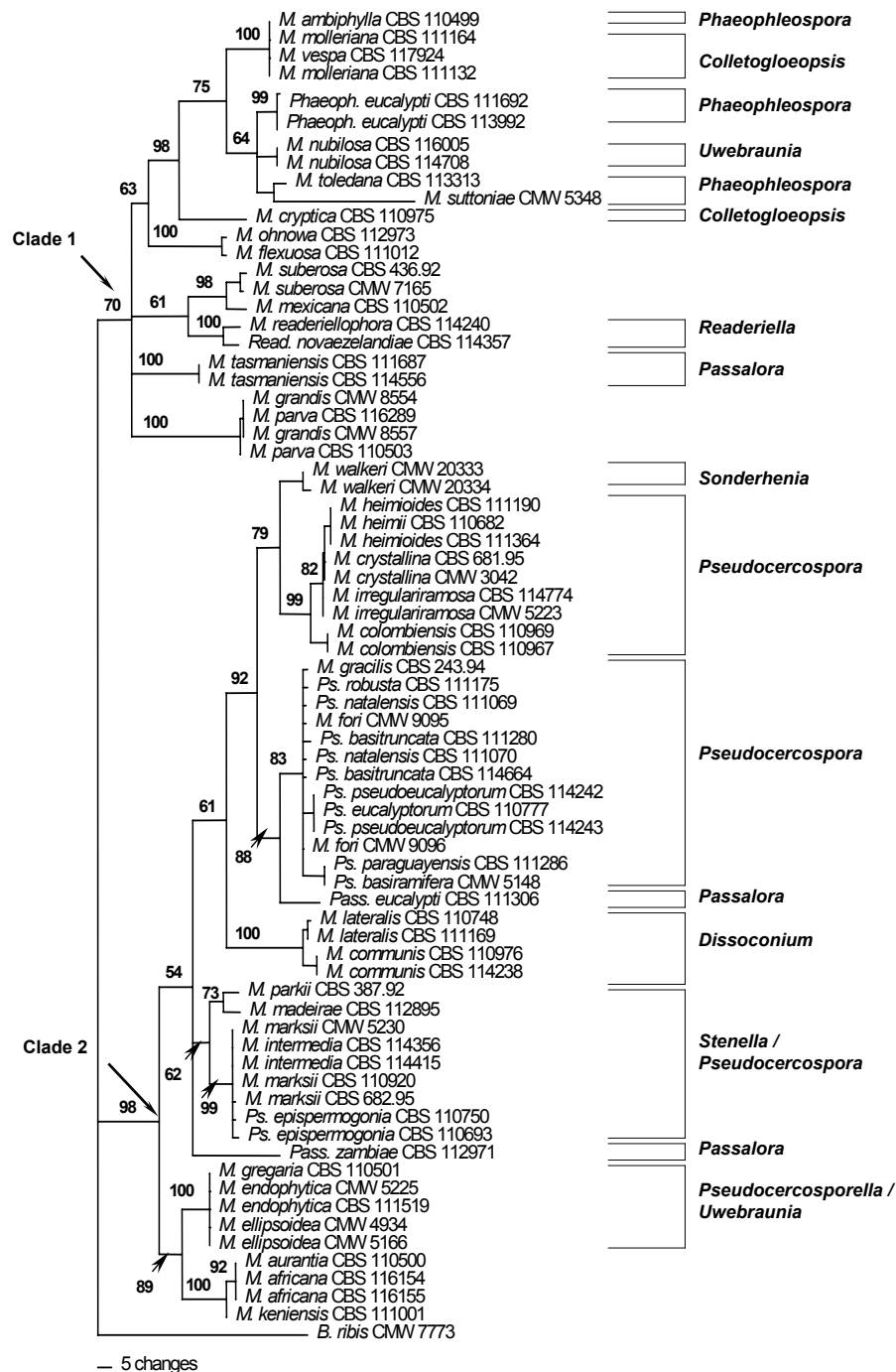


**Fig. 1.** Phylogram obtained from the Large Subunit (LSU) rDNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves showing two well-supported main clades (Clades 1−2). Tree length = 663, CI = 0.519, RI = 0.878, RC = 0.456. Bootstrap values based on 1000 replicates are indicated above branches. Anamorph affinities are indicated next to the vertical lines.

Clade 1 was weakly supported with a bootstrap value of 67 %. This clade contained *Mycosphaerella* isolates represented by *Pseudocercospora*, *Sonderhenia*, *Phaeophleospora*, *Colletogloeopsis*, *Uwebraunia*, *Readeriella* and *Passalora* anamorphs. Clade 2 was sister to Clade 1 and had a higher bootstrap support of 80 %. Within this clade, *Mycosphaerella* isolates could be separated into three sub-clades that were well-supported. These three sub-clades contained species of *Mycosphaerella* that produced *Pseudocercosporella*, *Uwebraunia*, *Pseudocercospora*, *Passalora* and *Stenella* anamorphs. Clade 3 with bootstrap support of 80 % included isolates of *M. lateralis* and *M. communis* and was basal to Clades 1 and 2.

*Actin (ACT) phylogeny*: The aligned ACT sequence dataset contained a total of 294 characters. Of these, 135 characters were constant, 30 characters were variable and parsimony-uninformative and 129 characters were parsimony-informative. Heuristic

searches of the aligned ACT dataset resulted in the retention of six most parsimonious trees (TL = 1007, RI = 0.682, CI = 0.235, RC = 0.160). One of these trees, shown in Fig. 4, was very poorly resolved and all deeper nodes were present in a basal polytomy. However, certain smaller clades were resolved and these included a clade including *M. fori*, *M. gracilis*, *Ps. eucalyptorum*, *Ps. pseudoeucalyptorum*, *Ps. robusta*, *Ps. basitruncata*, *Ps. natalensis*, *Ps. basiramifera* and *Ps. paraguayensis*. This clade was supported with a bootstrap value of only 67 %. Another clade supported with a bootstrap value of 100 % contained isolates of *M. ellipsoidea* Crous & M.J. Wingf., *M. endophytica* and *M. gregaria*. Isolates of *M. ambiphylla*, *M. molleriana* and *M. vespa* also clustered together with 100 % bootstrap support. Isolates of *M. intermedia* M.A. Dick & Dobbie, *M. marksii* Carnegie & Keane and *Pseudocercospora epispermogonia* Crous & M. J. Wingf. grouped together in a clade that was supported with a bootstrap value of
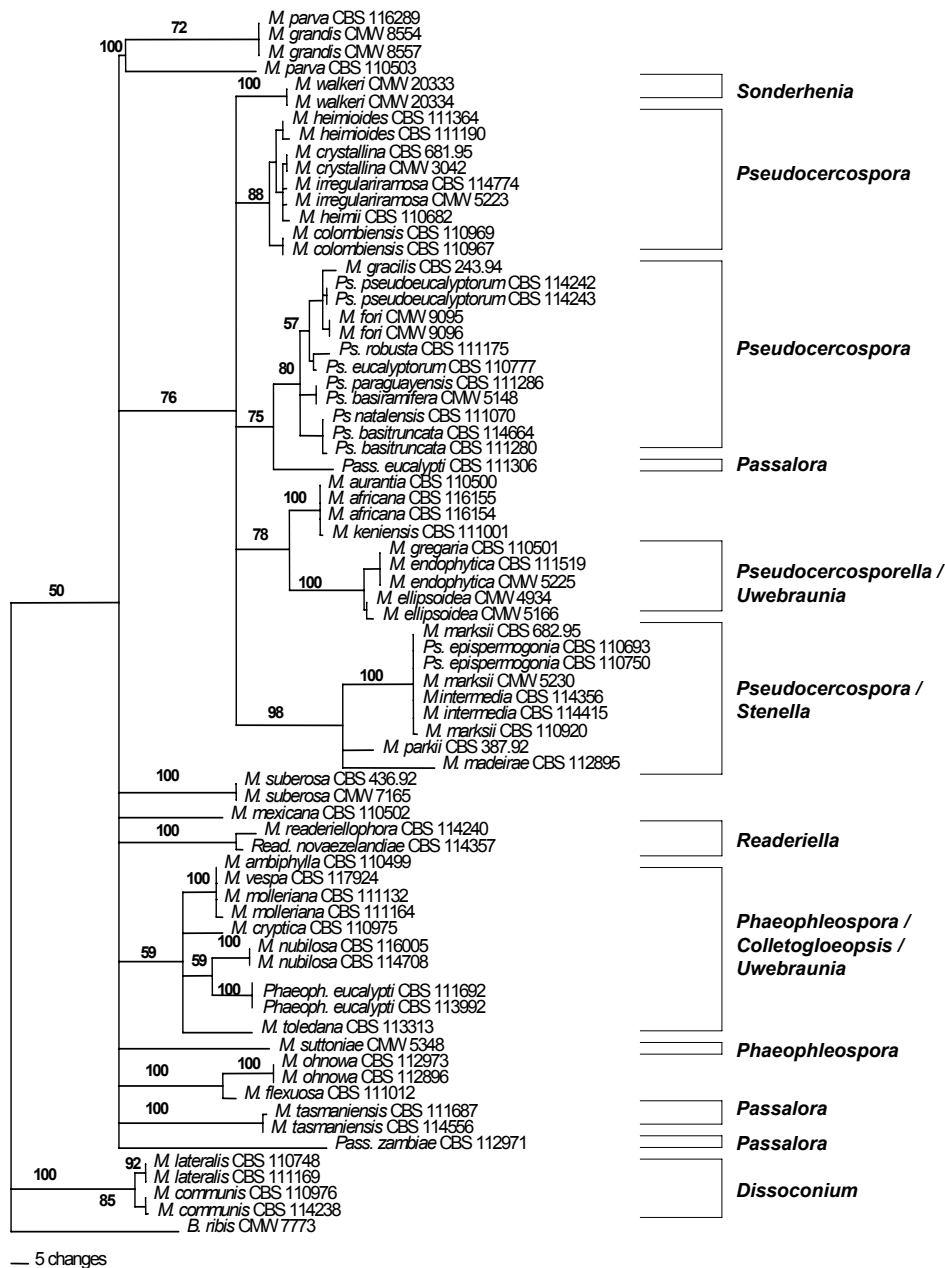


**Fig. 2.** Phylogram obtained from the Internal Transcribed Spacer (ITS) DNA sequence alignment of *Mycosphaerella* spp. occurring on Eucalyptus leaves indicating two monophyletic clades (Clades 1−2). Tree length = 871, CI = 0.358, RI = 0.782, RC = 0.280.

84 %. Isolates of *M. flexuosa* Crous & M.J. Wingf., *M. lateralis* and *M. communis* were also accommodated in a well-supported clade with a bootstrap value of 99 %. Isolates of *M. grandis* Carnegie & Keane and *M. parva* R.F. Park & Keane were also resolved into a clade with a bootstrap value of 99 %.

*Phylogeny of combined data set:* A partition homogeneity test of the combined LSU, ITS and EF-1α alignment conducted in PAUP resulted in a P-value of 0.001 for all possible combinations of the LSU, ITS and EF-1α DNA alignments. This value is less than the conventionally accepted P-value of P > 0.05 required to combine data. However, several studies have accepted a P-value of 0.001 or greater and have further stated that the conventional P-value of 0.005 is inordinately conservative (Cunningham 1997, Darlu & Lecointre 2002, Dettman *et al.* 2003). Thus, the LSU, ITS and EF-1α DNA sequence data sets were combined. The ACT dataset was omitted from the combined data set due to

the lack of resolution among species of *Mycosphaerella*. Therefore, the combined LSU, ITS and EF-1α data set had a total length of 2880 characters. Of these, 1459 were constant, 150 were variable and parsimony-uninformative and 701 characters were parsimony-informative. An indel of 382 bp was excluded for *M. ohnowa* CBS 112973 and *M. mexicana* CBS 110502 and another indel of 186 bp was excluded for *M. gregaria* CBS 110501 and *M. endophytica* CMW 5225 and CBS 111519. A parsimony analysis resulted in the retention of ten most parsimonious trees (TL = 1677, CI = 0.384, RI = 0.817, RC = 0.314, HI = 0.616). One of these trees (Fig. 5) exhibited a similar topology to that obtained from the LSU alignment. From the analysis of the combined data set, isolates of *Mycosphaerella* could again be resolved into two clades (Clades 1−2) (Fig. 5). Clade 1 was poorly supported with a bootstrap value of only 66 % and the same isolates were contained in this clade as in the LSU Clade 1
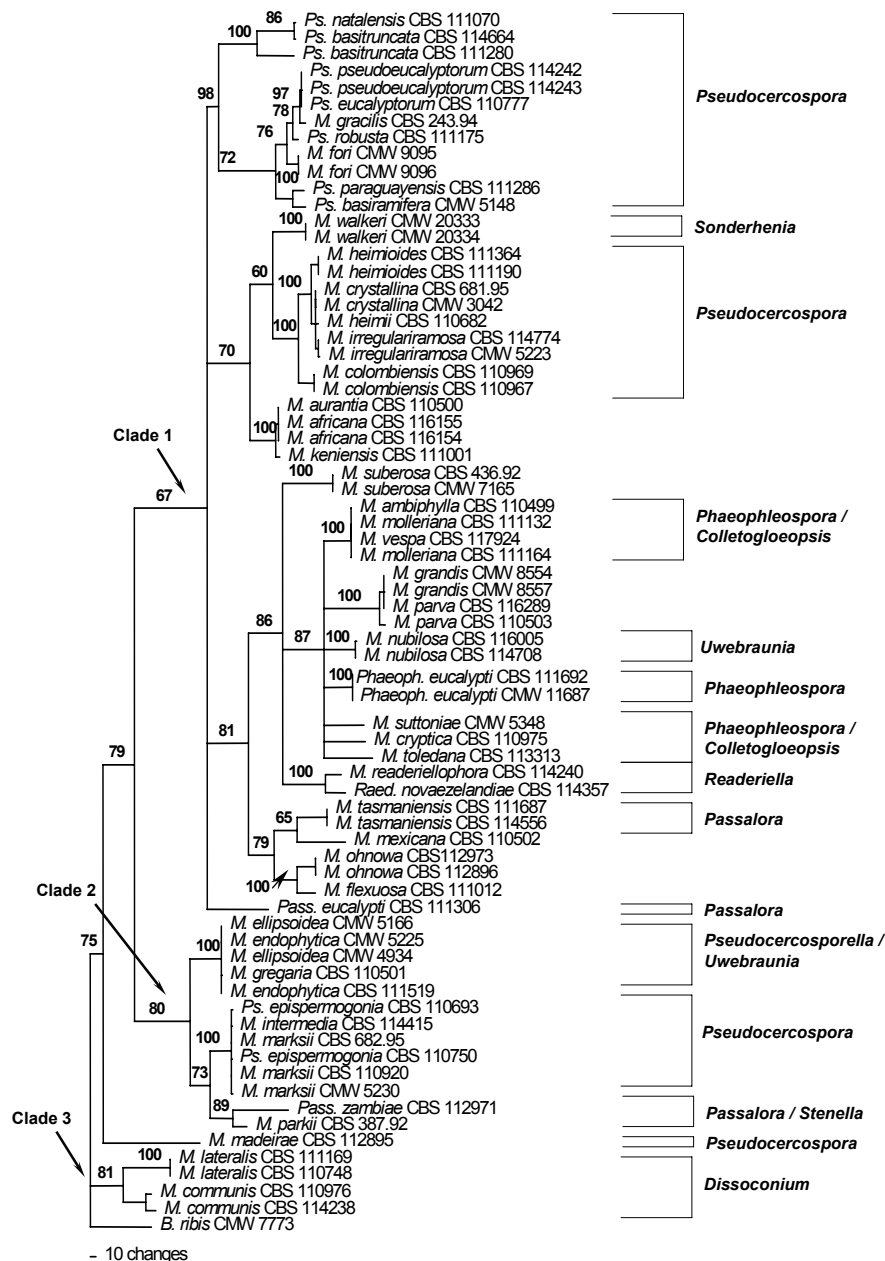


**Fig. 3.** Phylogram obtained from the Elongation factor 1-alpha (EF-1α) DNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves showing three main clades.  Tree length = 3194, CI = 0.345, RI = 0.777, RC = 0.268.

(Fig. 1). Clade 2 of the combined phylogenetic tree was well-supported with a bootstrap value of 81 %. This clade could be further resolved into several smaller well-supported sub-clades containing *Mycosphaerella* isolates that grouped according to their anamorph associations (Fig. 5). Neighbour-joining analysis yielded a phylogenetic tree with the same topology as the most parsimonious trees (data not shown). Here, all *Mycosphaerella* spp. could be resolved into two main clades (Clade 1−2), similar to those of the parsimony analysis (Fig. 5). *Mycosphaerella* spp. could be further sub-divided into several sub-clades corresponding to their anamorph associations, similar to those observed for the parsimony analysis.

## DISCUSSION

Results of this study represent the first attempt to employ DNA sequence data from a relatively large number of nuclear gene regions in order to consider the phylogenetic relationships for *Mycosphaerella* spp. occurring on *Eucalyptus* leaves. Other similar studies have relied entirely on sequence data of the ITS region (Crous *et al.* 1999, 2001, 2004a, and 2006 – this volume, Hunter *et al.* 2004b). Although the ITS region offers sufficient resolution to distinguish most taxa, it has not been adequate to separate cryptic taxa in *Mycosphaerella* (Crous *et al.* 2004b). Results of the present study showed that combined DNA sequence
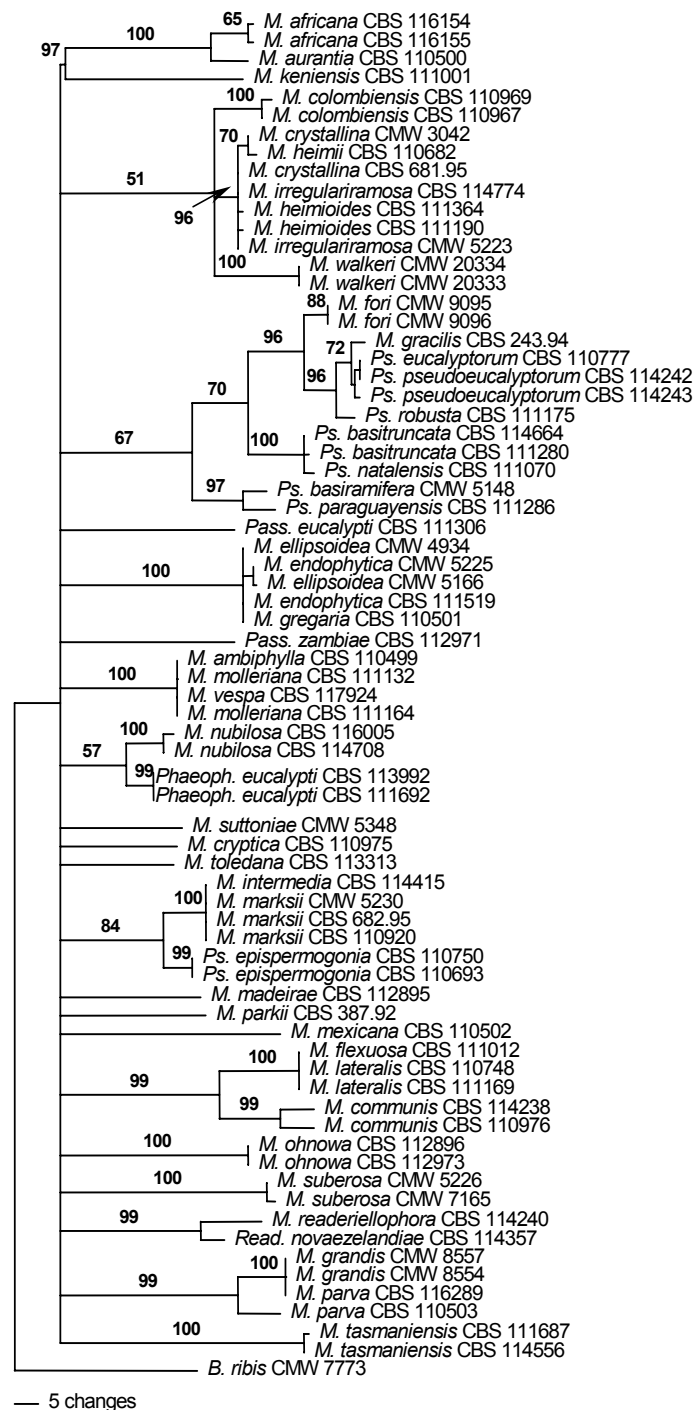


**Fig. 4.** Phylogram obtained from the Actin (ACT) DNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves. Tree length = 1007, CI = 0.235, RI = 0.682, RC = 0.160.

data from the LSU, ITS, EF-1α gene regions offer increased genetic resolution to study species concepts in *Mycosphaerella*. However, genes such as the ACT, did not support data emerging from the other loci sequenced, and indicated variation within some clades that were well supported by sequences of other loci and morphological characteristics. These observations led us to exclude ACT data from the final analyses. A similar finding has also emerged from other studies including greater numbers of *Mycosphaerella* species (Crous & Groenewald, unpubl. data).

*Mycosphaerella ambiphylla*, *M. molleriana* and *M. vespa* grouped together in a well-supported clade in the phylogeny emerging from the combined alignment. This

was also true for the ITS, EF-1α and ACT phylogenies where these isolates grouped in a distinct clade with a 100 % bootstrap support. *Mycosphaerella molleriana* and *M. vespa* both have *Colletogloeopsis* anamorphs, however, *M. ambiphylla* produces a *Phaeophleospora* anamorph (Crous & Wingfield 1997a, Maxwell *et al*. 2003). Interestingly, the *Phaeophleospora* anamorph of *M. ambiphylla* was differentiated from *Colletogloeopsis* only by the fact that conidia are produced in a pycnidium as opposed to an acervulus (Maxwell *et al*. 2003). Application of conidiomatal structure to differentiate anamorphs of *Mycosphaerella* has previously been viewed with circumspection especially because *Mycosphaerella* anamorphs can produce different
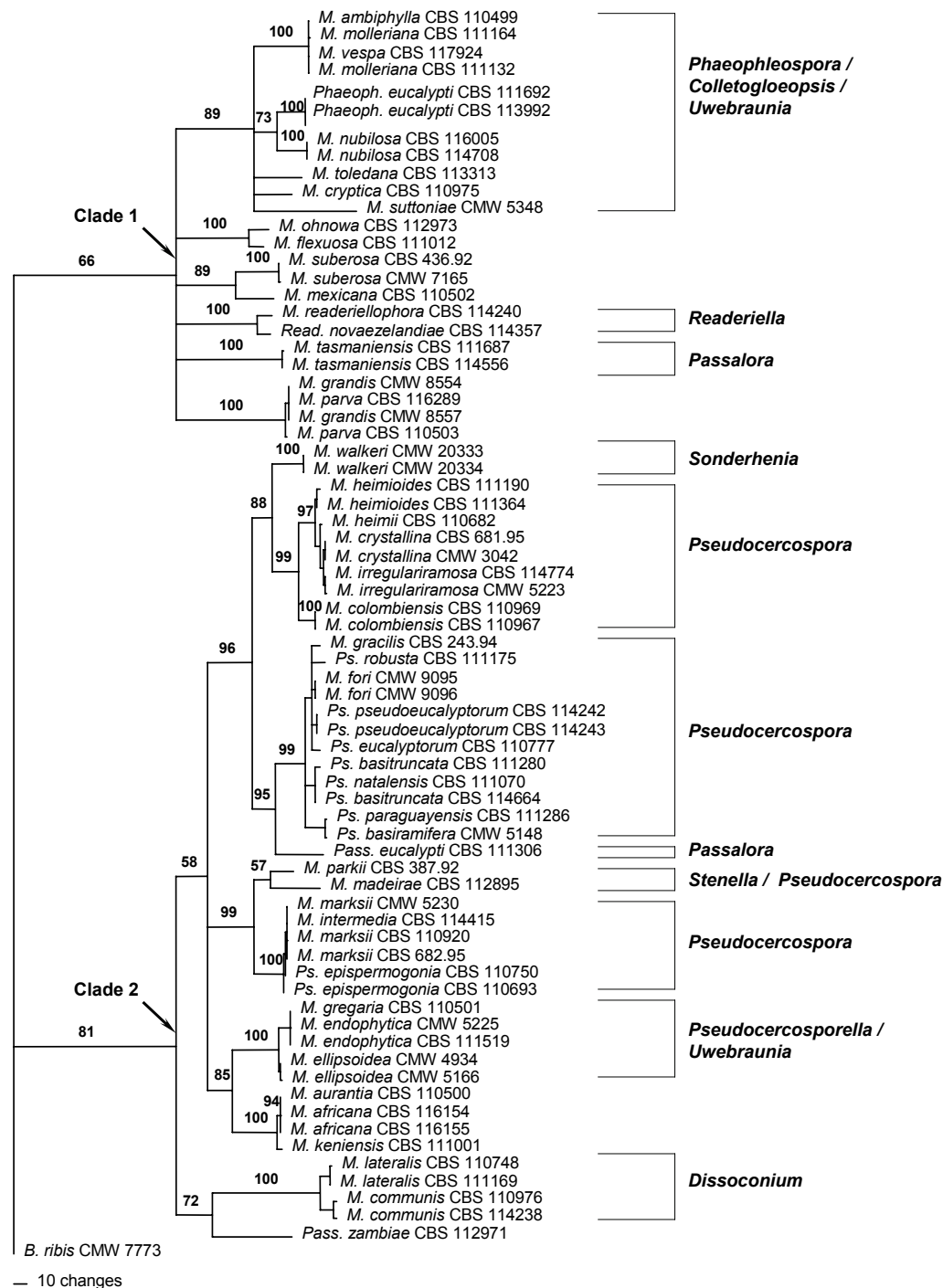


**Fig. 5.** Phylogram obtained from the combined LSU, ITS and EF-1α DNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves showing two main clades. Tree length = 1677, CI = 0.384, RI = 0.817, RC = 0.314.

conidiomatal forms under differing environmental conditions (Crous *et al.* 2000, Cortinas *et al.* 2006 – this volume). Therefore, the placement of the *M. ambiphylla* anamorph in *Phaeophleospora* is questioned and it should be re-evaluated in terms of its morphological similarities to *Colletogloeopsis*.

Ascospore germination patterns of *M. ambiphylla*, *M. molleriana* and *M. vespa* are all similar, with germ tubes that grow parallel to the long axis of the spore and ascospores with a slight constriction at the median septum, typical of a type C ascospore germination pattern (Crous 1998, Carnegie & Keane 1998, Maxwell *et al.* 2003). Furthermore, overlap is seen in ascospore dimensions of the three species where those of *M. molleriana* are (11−)12−14(−17) × (2.5−)3.5−4(−4.5) µm, those of *M. ambiphylla* are (12−)14−15(−22) ×(3.5−)4.5−5(−6) µm and those of *M. vespa* 9.5−16.5 × 2.5−4 µm (Crous 1998, Carnegie & Keane 1998, Maxwell *et al.* 2003). Leaf lesions of the three species are also similar, pale brown to dark red-brown with lesions of *M. vespa* and *M. ambiphylla* often producing a red margin that was, however, not observed in *M. molleriana* (Crous 1998, Carnegie & Keane 1998, Maxwell *et al.* 2003). Morphological features of *M. ambiphylla*, *M. molleriana* and *M. vespa* are also very similar. This is supported in the DNA phylogeny of the present study where these three species appear to represent a single taxon and therefore suggest that *M. ambiphylla*, *M. molleriana* and *M. vespa* should be synonomised under *M. molleriana*, which is the oldest epithet. We therefore reduce *M. ambiphylla* and *M. vespa* to synonymy with *M. molleriana* as follows:

**Mycosphaerella molleriana** (Thüm.) Lindau, Natürliche Pfanzenfamilie, 1: 424. 1897.

≡ *Sphaerella molleriana* Thüm., Revista Inst. Sci. Lit. Coimbra 28: 31. 1881.

= *Mycosphaerella vespa* Carnegie & Keane, Mycol. Res. 102: 1275. 1998.

= *Mycosphaerella ambiphylla* A. Maxwell, Mycol. Res. 107: 354. 2003.

*Anamorph*: *Colletogloeopsis molleriana* Crous & M.J. Wingf., Canad. J. Bot. 75: 670. 1997.

*Mycosphaerella flexuosa* has no known anamorph (Crous 1998). An isolate of this fungus included in the present study grouped together with isolates of *M. ohnowa* in the LSU, ITS, EF-1α and combined data set with high bootstrap support. This similarity was also observed in a recent study of *Mycosphaerella* spp. on *Eucalyptus* based on ITS sequence data (Crous *et al.* 2004a). *Mycosphaerella ohnowa* is also not known to produce an anamorph (Crous *et al.* 2004a). Although these two species are phylogenetically similar, they can be distinguished from one another based on different ascus and ascospore dimensions, ascospore germination patterns and cultural characteristics (Crous 1998, Crous *et al.* 2004a). Although morphologically distinct, it is interesting that these two taxa are phylogenetically so closely related and might suggest a recent speciation event.

Isolates of *M. grandis* and *M. parva* consistently grouped together in a separate clade in all of the DNA sequence data sets in this study. This has also been shown by Crous *et al.* (2004a), where isolates of these two species grouped together in a distinct clade based on ITS DNA sequences. *Mycosphaerella grandis* was originally described from *E. grandis* in Australia, and recognised as a distinct species of *Mycosphaerella* due to its lesion characteristics, and ascospore morphology (Carnegie & Keane 1994). However, Crous (1998) examined the type of *M. grandis* and *M. parva* and found the two species to be congeneric, and reduced them to synonymy under *M. parva*. Results from the present study support the synonymy.

*Mycosphaerella lateralis* and *M. communis*, both known to have *Dissoconium* anamorphs, showed various phylogenetic placements in this study. From the LSU phylogeny, *M. lateralis* and *M. communis* were situated within a large *Mycosphaerella* clade sister to a *Pseudocercospora* sub-clade. However, in the ITS and EF-1α phylogenies the *Dissoconium* clade was situated basal to the larger *Mycosphaerella* clade. This is consistent with findings of Crous *et al.* (1999, 2000) where the *Dissoconium* clade also resided outside the larger monophyletic *Mycosphaerella* clade. The LSU gene region is well-known to be conserved and to show less nucleotide differences than the ITS and EF-1α gene regions. Although the house-keeping genes investigated here lead to the conclusion that *Dissoconium* could be different from *Mycosphaerella s. str.*, this proved not to be the case when LSU data were considered. *Dissoconium* is morphologically identical to *Uwebraunia*, and the separation of these two genera no longer seems tenable. Only two species, *M. ellipsoidea* and *M. nubilosa,* have *Uwebraunia* anamorphs (Crous *et al.* 2004a). However, cultures of both species produced these anamorphs only upon initial isolation, and those that are currently available are sterile. In contrast, strains with *Dissoconium* anamorphs readily produce those in culture, and they usually sporulate profusely. It appears that the status of *Uwebraunia* will only be resolved once fresh, sporulating collections of either *M. ellipsoidea* or *M. nubilosa* can be obtained.

*Mycosphaerella* spp. with *Pseudocercospora* anamorphs grouped into three clades in all of the phylogenies generated in this study. Species in the *Pseudocercospora* clades have short branch lengths arising from a common internode, suggesting that they have speciated relatively recently from a common ancestor (Ávila *et al.* 2005) and, most likely have co-evolved with their *Eucalyptus* hosts as suggested by Crous *et al.* (2000). Ávila *et al.* (2005) suggested that *Pseudocercospora* may represent a monophyletic lineage. But, results of this and other studies (Ayala-Escobar *et al.* 2006) have shown that *Pseudocercospora* is paraphyletic in *Mycosphaerella* and has evolved more than once in the genus. The availability of new DNA datasets for several gene regions are likely to resolve cryptic species and species complexes within *Pseudocercospora,* as has already been shown for the *M. heimii* and the *P. eucalyptorum* species complexes (Crous *et al.* 2000, 2004a).

*Mycosphaerella heimioides*, *M. heimii*, *M. crystallina* and *M. irregulariramosa* are all morphologically similar

and are regarded as members of the *M. heimii* species complex (Crous & Wingfield 1997b, Crous *et al*. 2001). Previous studies based on ITS DNA sequence data have demonstrated the phylogenetic relatedness of these four species (Crous *et al*. 2001, Crous *et al*. 2004a). However, bootstrap support for their phylogenetic placement was low (Crous *et al*. 2004a). The phylogeny of combined DNA sequence data in this study showed that the four species in the *M. heimii* complex reside in a well-supported clade (bootstrap support 97 %). The short branch lengths indicate that the four species have also recently diverged from a common ancestor.

In the phylogeny based on the combined sequence data sets, *M. gracilis* grouped in a well-supported *Pseudocercospora* clade that also included isolates of *Ps. robusta*, *M. fori*, *Ps. pseudoeucalyptorum*, *Ps. eucalyptorum*, *Ps. basitruncata*, *Ps. natalensis*, *Ps. paraguayensis* and *Ps. basiramifera*. This is the first study in which DNA sequence data for *M. gracilis* have been incorporated into a phylogeny. In the ITS, EF-1α and ACT phylogenies, *M. gracilis* was phylogenetically most closely related to *Ps. pseudoeucalyptorum*. However, *M. gracilis* (anamorph: *Pseudocercospora gracilis* Crous & Alfenas) can be distinguished from *Ps. pseudoeucalyptorum* by its single conidiophores arising exclusively from secondary mycelium, which is different to *Ps. pseudoeucalyptorum* in which conidiophores arise from loose or dense fascicles of a stroma (Crous 1998, Crous *et al*. 2004a). Furthermore, conidia of *Ps. gracilis* are more septate, longer, and more uniformly cylindrical in shape than those of *Ps. pseudoeucalyptorum* (Crous 1998, Crous *et al*. 2004a). Results of the present study clearly emphasise the fact that species which are morphologically distinct, can be very closely related.

An interesting result emerging from the phylogenetic analyses in this study was the placement of *Pseudocercospora epispermogonia* in relation to *Mycosphaerella marksii* and *Mycosphaerella intermedia*. Sequences for all but the ACT gene region showed that these three taxa represent the same phylogenetic species. Although it has previously been suggested that *M. marksii* should have a *Stenella* anamorph because of its proximity to *M. parkii* (Crous *et al*. 2001), the current data suggest that this anamorph could be *Ps. epispermogonia*. Crous & Wingfield (1996) described *Ps. epispermogonia* from spermatogonia on lesions colonised by *M. marksii*, but failed to link the two states because single-ascospore cultures did not form an anamorph in culture. *Mycosphaerella intermedia* is morphologically similar to *M. marksii*, and probably represents the same taxon. We therefore reduce *M. intermedia* to synonymy with *M. marksii* as follows:

**Mycosphaerella marksii** Carnegie & Keane, Mycol. Res. 98: 413−416. 1994.

= *Mycosphaerella intermedia* M. A. Dick & Dobbie, New Zealand J. Bot. 39: 270. 2001.

Anamorph: *Pseudocercospora epispermogonia* Crous & M.J. Wingf., Mycologia 88: 456. 1996.

*Mycosphaerella africana*, *M. aurantia* and *M. keniensis* have no known anamorphs. Previous studies based on ITS sequence data have suggested that *M. africana* and *M. keniensis* grouped close to *Mycosphaerella* spp. with *Passalora* anamorphs. It has thus been assumed that *M. africana* and *M. keniensis* would have *Passalora* anamorphs if they were to be found (Crous *et al*. 2000). However, the phylogenies emerging from LSU, ITS and EF-1α sequences and the combined data for the three regions showed that *M. africana*, *M. keniensis* and *M. aurantia* consistently group separately from the *Passalora* anamorphs, close to a clade of isolates with *Uwebraunia* and *Pseudocercosporella* anamorphs. The association of these three taxa to *Passalora* is thus doubted. Furthermore, the clade containing *M. africana*, *M. aurantia* and *M. keniensis* is also well-supported and seems to represent a single evolving lineage.

Moreover, results of the present study show that *M. aurantia* and *M. africana* represent a single phylogenetic species. These two species consistently grouped together in all phylogenies with *M. keniensis* grouping as a sister. *Mycosphaerella aurantia* was described from leaves of *E. globulus* in south-western Australia and is known only from this location (Maxwell *et al*. 2003). Morphologically, *M. aurantia* produces asci and ascospores that are similar in size and morphology to *M. africana*. However, the ascospores of *M. aurantia* are not constricted at the median septum whereas those of *M. africana* had such constrictions, and ascospores of *M. aurantia* are longer (9−)11−12(−15) μm than those of *M. africana* (7−)8−10(−11) μm (Crous 1998, Maxwell *et al*. 2003). Furthermore, *M. aurantia* produces lateral hyaline germ tubes that grow parallel to the long axis of the ascospore and become slightly verrucose to produce lateral branches upon prolonged incubation (Maxwell *et al*. 2003). This is in contrast to ascospores of *M. africana* that germinate in an irregular fashion producing distinctly dark verrucose germ tubes from different positions of the distorted ascospore (Crous 1998). It is intriguing that these two species, which are morphologically quite distinct, would represent a single phylogenetic species. Additional isolates of these species are required to determine whether they represent two distinct taxa or are conspecific.

*Mycosphaerella gregaria* was described from leaves of *E. grandis* in Victoria, Australia (Carnegie & Keane 1997). No anamorph has been observed for this species (Carnegie & Keane 1997, Crous 1998). An isolate of *M. gregaria*, collected from *E. globulus* in Australia, consistently grouped in a clade with isolates of *M. endophytica* and *M. ellipsoidea*. *Mycosphaerella endophytica* and *M. ellipsoidea* are known to have *Pseudocercosporella* and *Uwebraunia* anamorphs, respectively (Crous 1998). Based on previous studies employing ITS sequence data, isolates of *M. endophytica* grouped sister to isolates of *M. aurantia*, *M. ellipsoidea* and *M. africana* (Crous *et al*. 2004a). However, based on sequence data from the four gene regions employed in this study, isolates of *M. endophytica* grouped in a distinct well-supported clade with *M. ellipsoidea*. This is interesting because *M. ellipsoidea* has an *Uwebraunia* anamorph (Crous & Wingfield 1996). *Mycosphaerella endophytica* and *M.*

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*pseudoendophytica* Crous & G. Hunter are the only *Mycosphaerella* spp. occurring on *Eucalyptus* that are known to have *Pseudocercosporella* anamorphs (Crous 1998, Crous *et al*. 2006 – this volume).

Phylogenies emerging from analyses of sequences for the four gene regions considered in this study suggest that *Mycosphaerella* constitutes heterogenous groups of which only a few are closely linked to certain anamorph genera. It is evident that for the larger part the evolution of the anamorph genera within *Mycosphaerella* has been polyphyletic, and not monophyletic as previously suggested. This can be seen by the multiple evolution of anamorph genera such as *Passalora, Pseudocercospora*, *Phaeophleospora* and *Stenella* within *Mycosphaerella* (Crous *et al*. 2006). It would thus not be advisable to predict anamorph relationships based on the phylogenetic position within *Mycosphaerella*. Not only has the same morphology evolved more than once in the group, but disjunct anamorph morphologies also frequently cluster together (Crous *et al*. 2000, 2004a, 2006). This makes the interpretation difficult, and predictions based on position in clades unreliable.

The production of four nucleotide sequence data sets for species of *Mycosphaerella* occurring on *Eucalyptus* leaves should serve as a framework for the more accurate taxonomic placement of these fungi in future. The importance of species complexes in *Mycosphaerella* has become more evident in this genus in recent years (Crous *et al*. 2004a, b, 2006 – this volume). To study species complexes, variable gene regions must be studied and the generation of greater numbers of data sets should allow for increased resolution at the species level. This in turn will aid in the resolution of species complexes and cryptic speciation. Studies of the deeper branches for groups in *Mycosphaerella* can in future utilise sequence data for the LSU region that have not previously been available. These should provide a more lucid indication and support for phenotypic characters that are phylogenetically informative.

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# A multigene phylogeny of the Dothideomycetes using four nuclear loci

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***Abstract:*** We present an expanded multigene phylogeny of the Dothideomycetes. The final data matrix consisted of four loci (nuc SSU rDNA, nuc LSU rDNA, *TEF1*, *RPB2*) for 96 taxa, representing five of the seven orders in the current classification of Dothideomycetes and several outgroup taxa representative of the major clades in the Pezizomycotina. The resulting phylogeny differentiated two main dothideomycete lineages comprising the pseudoparaphysate Pleosporales and aparaphysate Dothideales. We propose the subclasses Pleosporomycetidae (order Pleosporales) and Dothideomycetidae (orders Dothideales, Capnodiales and Myriangiales). Furthermore we provide strong molecular support for the placement of Mycosphaerellaceae and Piedraiaceae within the Capnodiales and introduce Davidiellaceae as a new family to accommodate species of *Davidiella* with *Cladosporium* anamorphs. Some taxa could not be placed with certainty (e.g. Hysteriales), but there was strong support for new groupings. The clade containing members of the genera *Botryosphaeria* and *Guignardia* resolved well but without support for any relationship to any other described orders and we hereby propose the new order Botryosphaeriales. These data also are consistent with the removal of Chaetothyriales and Coryneliales from the Dothideomycetes and strongly support their placement in the Eurotiomycetes.

***Key words:*** bitunicate asci, hamathecium, loculoascomycetes, pseudoparaphyses

## INTRODUCTION

Members of the Dothideomycetes often are found as pathogens, endophytes or epiphytes of living plants and also as saprobes degrading cellulose and other complex carbohydrates in dead or partially digested plant matter in leaf litter or dung. Combinations of these niches can be occupied by a single fungus as it passes through its life cycle; for example several fungi initiate their life cycles on living plants and switch to saprobic states when the plant dies or leaves are lost. The nutritional modes are not limited to associations with plants and several species are lichenized, while others occur as parasites on fungi or members of the kingdom animalia.

Although to a casual observer there is little to distinguish the flask-, spherical- or disk-shaped fruiting bodies of the Dothideomycetes from several other ascomycete groups, they share a distinctive pattern of development. The asci bearing the sexual spores develop in locules already formed lysigenously within vegetative hyphae. This, defined as ascolocular development, is in contrast to ascohymenial development found in the majority of other fungal classes. Ascohymenial development generates asci in a broad hymenium interspersed with apically free paraphyses and the reproductive structure is derived from cells after fertilization.

Building on earlier descriptions of ascolocular development Nannfeldt (1932) proposed the group ''Ascoloculares'' and in 1955 this was formally proposed as a class ''Loculoascomycetes'' by Luttrell (1955). The importance of ascus morphology and dehiscence, in addition to the presence of surrounding elements inside the ascostromata, was emphasized (Luttrell 1951). The bitunicate ascus remains a defining character in modern dothideomycete taxonomy. It consists of a thick extensible inner layer (endotunica) and a thin inextensible outer layer (ectotunica). Most species release their ascospores by the extension of the inner ascus wall and the rupture of the outer wall, similar to a jack-in-the-box (fissitunicate), but variations are numerous. Another character of note, the centrum, defined as the tissues and cells occupying the cavity of the sexual structure, was expanded by Luttrell when he described three different ascostromatal developmental types exemplified by the genera *Dothidea*, *Pleospora* and *Elsinoë* forming part of the currently accepted orders, Dothideales, Pleosporales and Myriangiales (see tolweb.org/Dothideomycetes for details). The ha-

mathecium (Eriksson 1981) (i.e. the sterile centrum tissues existing between the asci) is one of the most reliable characters used to delineate ordinal classifications within the Dothideomycetes. The presence of pseudoparaphyses (sterile cells extending down from the upper portion of the ascoma, initially attached at both ends, although the upper part may become free) is a notable character for the Pleosporales, together with mainly ostiolate flask-shaped pseudothecia. Conversely the absence of pseudoparaphyses and the presence of fascicles of asci are important in the Dothideales. The Myriangiales also do not have pseudoparaphyses but produce single globose asci in multiple locules. Several additional orders currently accepted are defined by combinations of centrum and ascomal characters. For a summary of different centrum types and features see Kirk et al (2001 p 224–225).

The different classification systems proposed thus far exhibited an emphasis on varying characters. For instance, the presence and morphology of characters in the hamathecium, together with ascostroma shape were used as the main characters to define ordinal groups by Luttrell (1955), while von Arx and Müller (1975) emphasized the form of the ascus and the specific opening of the ascoma. Although basing her classification on the work of Luttrell, Barr (1987) employed additional characters such as the morphology of pseudoparaphyses.

The best studied species in this group tend to be plant pathogens on important agricultural crops. Therefore a large body of work in dothideomycete taxonomy and systematics concerns descriptions of anamorphs, the predominant morphological state encountered on agricultural crops; in fact several families in this class (e.g. Pleosporaceae, Mycosphaerellaceae, Tubeufiaceae) include a high proportion of anamorphic species. These include both hyphomycetes and coelomycetes. Many of the hyphomycetes have sympodially proliferating conidiogenous cells. *Phoma*-like and other coelomycetes occur in several families (e.g. Leptosphaeriaceae, Lophiostomataceae); these have ostiolate pycnidia lined with phialidic, annellidic or holoblastic conidiogenous cells and produce small, aseptate conidia in slime. Other important species include the group now informally referred to as the "black yeasts" (some of which also belong to the Eurotiomycetes) characterized by the production of dark, slimy colonies and sporulation patterns that resemble the budding of true yeasts but actually are reduced versions of phialidic, annellidic or sympodially proliferating conidiogenous cells (de Hoog 1974). A selection of the variety of morphological structures exhibited by teleomorph and anamorph forms in the Dothideomycetes is shown (FIG. 1).

The refinement of character state homologies and the development of morphology-based classifications into a phylogenetic classification system are accelerating with the advent of molecular data. Initial analyses using DNA sequence data from the small subunit ribosomal RNA gene did not support the monophyly of the Loculoascomycetes (Spatafora et al 1995, Berbee 1996). A more recent phylogeny produced from protein gene coding data (Liu and Hall 2004) was inferred as supporting the taxonomic concepts for a monophyletic lineage for ascostromatic taxa, but the ontogenetic designations were considered oversimplified by some (Lumbsch et al 2005). Other studies combining data from protein-coding genes and the ribosomal operon have shown the paraphyly of ascostromatic, bitunicate lineages (Lutzoni et al 2004, Reeb et al 2004). An example is the group of fungi that recently were transferred to the Eurotiomycetes based on nuclear small subunit ribosomal sequences, the "black yeasts" of the Chaetothyriales (Winka et al 1998). Together with the Verrucariales and Pyrenulales these bitunicate taxa have been placed within a separate subclass, the Chaetothyriomycetidae (Miadlikowska and Lutzoni 2004), which is sister of the Eurotiomycetidae (Lutzoni et al 2004, Reeb et al 2004) in the class Eurotiomycetes (also see Geiser et al in this issue).

Several studies provide the groundwork for a phylogenetically based classification for the Dothideomycetes. Most have used nuclear small subunit ribosomal data, but nuclear large subunit ribosomal and mitochondrial small subunit sequences also were used (Lindemuth et al 2001, Lumbsch and Lindemuth 2001). This allowed for the reassessment of specific morphological characters proposed in earlier work. Specifically, poor support for phylogenetic groups based on the morphology of pseudoparaphyses was found while phylogenetic correlation of their presence or absence was well supported (Liew et al 2000, Lumbsch and Lindemuth 2001), although a single exception to this was noted (Silva-Hanlin and Hanlin 2000). In spite of these recent examples of interordinal, molecular-based phylogenetic studies, a large number of species within the ascostromatic Ascomycota remain listed as Dothideomycetes or Chaetothyriomycetes incertae sedis (Eriksson 2006). Furthermore the question of whether Dothideomycetes represents a natural group derived from a single ancestor is not settled and the need to investigate its relationships to a number of the bitunicate lichen species such as the currently separate class Arthoniomycetes remains essential. The main focus of this study however is to provide an extension of previous ribosomal DNA-based phylogenetic studies and combine a number of smaller phylogenetic analyses
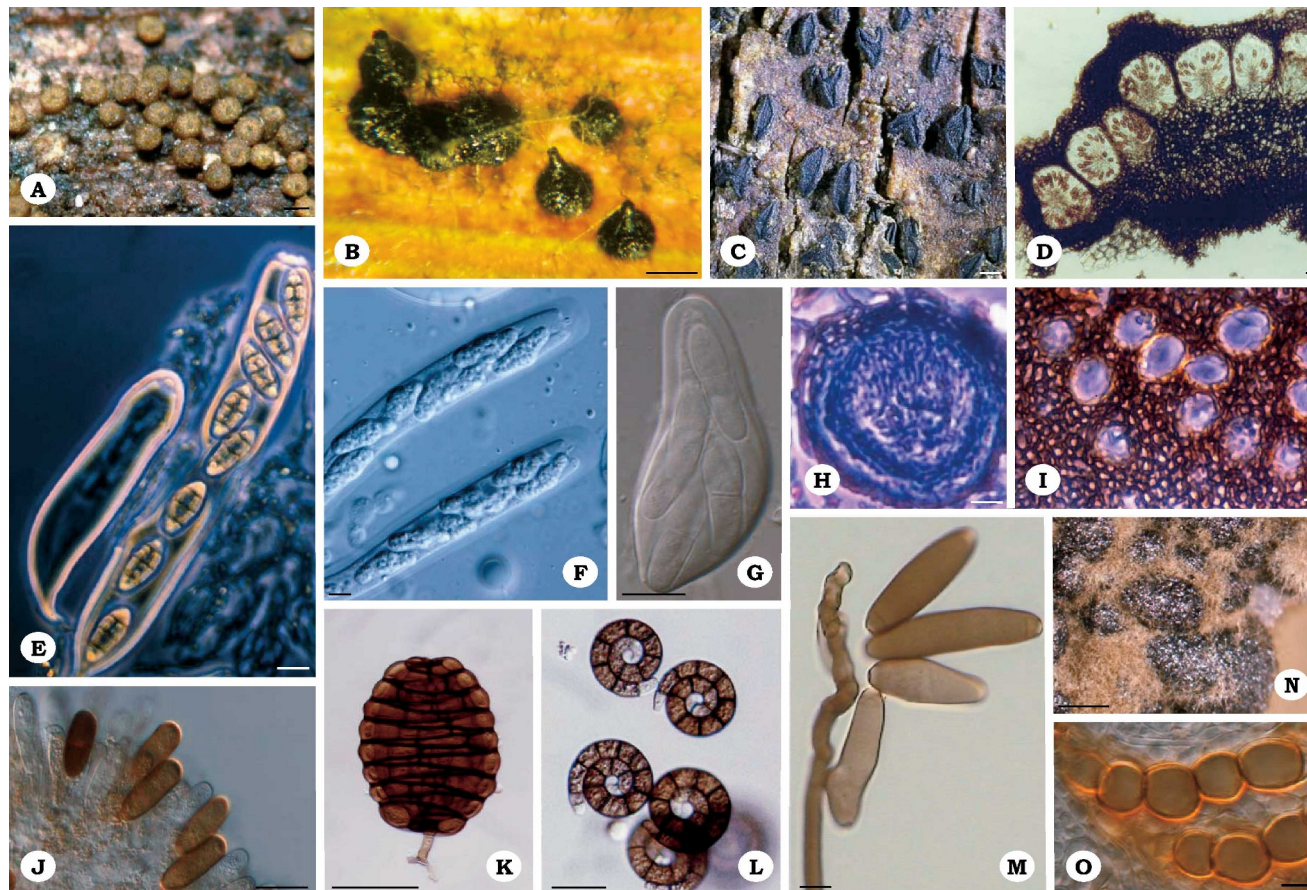
FIG. 1. A selection of dothideomycete morphological forms. Teleomorphs, ascostromata: A. Light-colored, flask-shaped pseudothecia of *Tubeufia cerea* (Tubeufiaceae) on wood. B. Dark pseudothecia of *Cochliobolus heterostrophus* (Pleosporales) on corn leaf. C. Hysterothecia of *Hysteropatella prostii* (Hysteriales), with slit-like openings. Teleomorphs, asci and locules: D. *Stylodothis puccinioides* (Dothideales), multiascus locules. E. *Pyrenophora brizae* (Pleosporales) bitunicate asci, one with broken ectotunica. F. *Guignardia magniferae* (Botryosphaeriales) asci with ascospores. G. Bitunicate ascus of *Davidiella tassiana* (Capnodiales). H. *Phaeosphaeria avenaria*, juvenile ascoma with pseudoparaphyses. I. *Myriangium duriaei* (Myriangiales), monascus locules in stroma. Anamorphs: J. Conidia borne in pycnidium of *Dothiorella* sp. (Botryosphaeriales). K, L. Helical conidia, in two different dimensions, of *Helicoon* and *Helicoma* spp. (Tubeufiaceae). M. Conidia and conidiophore of *Bipolaris sp.* (Pleosporales). N. Stroma of *Trimmatostroma abietis* (Capnodiales) bearing conidia in culture. O. Chlamydospores of *Trimmatostroma abietis* (Capnodiales). Scale bars are approximations obtained from published sources; the bar indicates 10 μm except in A, B, C and N where it indicates 200 μm. Photo credits, courtesy of: Jean-Paul Priou (A), B. Gillian Turgeon (B), Hans-Otto Baral (C), Robert A. Shoemaker (D, E, H, I), Gary Samuels (F), Pedro W. Crous (G, J, N, O), Clement K.M. Tsui (K, L), Keith A. Seifert (M).

within the framework of a multiple gene analysis showing intraordinal relationships in the Dothideomycetes.

## MATERIALS AND METHODS

*Sampling and alignments.*—Sequence data were obtained from GenBank and the Assembling the Fungal Tree of Life Project (AFTOL; http://ocid.nacse.org/research/aftol/). All strains and sequences used in this study are listed (SUPPLEMENTARY TABLE I). DNA alignments are available from the AFTOL Web site and TreeBASE (SN2913-11828). A number of sequences generated by the AFTOL project and available from the AFTOL Web site as well as from

GenBank were used. Newly generated DNA sequences were deposited at GenBank (TABLE I supplement). Genes used were nuclear small subunit ribosomal RNA gene DNA (nuc SSU), nuclear large subunit rDNA (nuc LSU), elongation factor la gene (*TEF1*), and the second largest subunits of RNA polymerase II gene (*RPB2*). Herbaria and culture collections where strains and specimens used in this study are deposited are listed (TABLE I supplement).

*Phylogenetic analysis.*—Maximum and weighted parsimony (MP and WP) analyses were performed on a combined dataset with a total of 117 taxa that included 96 Dothideomycetes. Nineteen taxa contained data for only three loci to maximize taxon sampling. The majority of the missing data were in the terminal branches of the tree, and care was

taken to include complete data sampling for taxa on branches underpinned by the more basal nodes. Two taxa with only ribosomal data (AFTOL ID 1856 *Phoma herbarum* and AFTOL ID 1864, *Didymella cucurbitacearum*) also were included to clarify the position of the clade surrounding *Phoma herbarum*. Removal of these taxa did not significantly affect support values in other parts of the tree. Likewise a comparison of a parsimony and Bayesian analysis with and without complete sets of characters yielded trees with congruent topologies. DNA sequences from a single strain (*Leptosphaeria maculans* DAOM 229267) inadvertently were included twice in the final analysis but were left in the final tree to ensure correct comparison across all approaches. We rooted the tree with three taxa from the class Pezizomycetes as outgroups (*Pyronema domesticum, Caloscypha fulgens Gyromitra californica*) (not shown in figure).

For the WP analyses the unambiguously aligned regions were subjected to symmetric step matrices for eight partitions (i.e. nuc SSU rDNA, nuc LSU rDNA and six codon positions of *TEF1* and *RPB2*) to incorporate the differences in substitution rates and patterns as described in Lutzoni et al (2004). MP and WP analyses were performed with only parsimony informative characters with these settings: 100 replicates of random sequence addition, TBR branch swapping and MULTREES in effect. Maximum likelihood was performed with PHYML (Guindon and Gascuel 2003) using a GTR+I+$\Gamma$ model of evolution. In all preceding cases nodal support was verified by nonparametric bootstrapping under the conditions mentioned, using 500 replicates.

Initial incongruence in the single gene trees for the taxa used was tested by examining single gene analyses with WP under the conditions previously mentioned for a set of taxa containing data for all four loci. A 70% majority rule consensus tree was compared in each case. Phylogenetic analysis using Bayesian inference of maximum likelihood was performed with a parallelized version of MrBayes v 3.1.2 across four processors (Altekar et al 2004). MrBayes was run with these parameters: a general time reversible model of DNA substitution (GTR) with gamma-distributed rate variation across sites (invariance, partitioning across genes and codons). A Markov chain Monte Carlo (MCMC) analysis with metropolis coupling was run starting from a random tree for $5 \times 10^6$ generations, sampling every 100th cycle. Four chains were run simultaneously with the initial 1000 cycles discarded as burn-in. Two additional runs with $5 \times 10^6$ generations were compared to confirm that stationarity in likelihood values was reached and compared. The phylogenies obtained in all cases were congruent. A 50% majority rule tree from a total of 45 000 trees obtained from a single run is presented (FIG. 2).

### RESULTS AND DISCUSSION

*Data analyses.*—The alignment for the phylogenetic analyses, after excluding introns and ambiguously aligned regions, consisted of 5098 base pairs, 1882 of which were parsimony informative. The reciprocal comparisons of 70% bootstrap trees from each gene with 61 core taxa did not reveal any incongruence (data not shown). Therefore all of 109 taxa in the current taxon sampling were used in the combined analyses. The heuristic search in MP and WP analyses yielded six MPTs with 20 917 steps (CI = 0.204, RI = 0.535) and three MPTs with 34 319.54 steps, respectively. In model-based methods, ML heuristic search analysis resulted in a tree of $-94\,457.67$ log likelihood and resulted after the GTR model was applied with a gamma value of 0.395 across four rate categories with a proportion of invariant sites equal to 0.287. The Bayesian analysis converged on the plateau of the log-likelihood on a mean value of $-93\,955$. The tree from Bayesian analyses is shown (FIG. 2) with all of the bootstrap proportions as well as the Bayesian posterior probabilities. Internodes were considered strongly supported if they received all of bootstrap proportions $\geq$ 70% and posterior probabilities $\geq$ 95% (Lutzoni et al 2004).

*Overview.*—The tree (FIG. 2) contains representatives of the major classes in the Ascomycota, as defined previously (Eriksson 2001). The supraclass relationships in our analysis indicated no support for a close relationship between the Dothideomycetes and Sordariomycetes, alluded to in an earlier study (Lutzoni et al 2004) and the sister relationships of the Sordariomycetes and Leotiomycetes are supported in agreement with recent data (Lumbsch et al 2005). A few taxon pairs containing isolates used in previous works have remarkably high similarity to each other over all four loci. Two examples noted in this analysis were incorrectly identified strains, namely ''*Clathrospora diplospora*'' CBS 174.52 = *Alternaria alternata* and ''*Epipolaeum longisetosum=Raciborskiomyces longisetosus*'' CBS 180.53 = *Cladosporium herbarum*.

*Non-Dothideomycete bitunicate groups.* Several lineages historically associated with the loculoascomycetes, such as the two species representing the Coryneliales, also were included. The placement of *Caliciopsis orientalis* together with *Caliciopsis pinea* (FIG. 2) indicates a close relationship with the Eurotiomycetidae (Geiser et al this issue). Other ordinal groups traditionally associated with the Dothideomycetes and now placed in the Eurotiomycetes were mentioned earlier. These groups share a number of centrum characters with members of the Dothideomycetes, such as the presence of periphysoids (Verrucariales, Chaetothyriales) and periphysate ostioles (Verrucariales, Chaetothyriales, Pyrenulales). The phylogeny (FIG. 2) confirms the separation of the Chaetothyriales and Verrucariales from the Dothideomycetes.

*Dothideomycetes-Arthoniomycetes clade.* The relationship of the Dothideomycetes and Arthoniomycetes (node A) is well supported by Bayesian and

maximum likelihood but not parsimony, although in an analysis without third codon positions, support by MP bootstrap and WP bootstrap increased. The internal node supporting the monophyly of the Dothideomycetes (node B) also had higher support in maximum likelihood and the two parsimony processes when the more saturated third codon positions were omitted. In more complete analyses containing characters from the RPB1 locus, this node was moderately supported and the *Trypethelium* strain is shown inside the Dothideomycetes (see Spatafora et al this issue).

Although taxon sampling for the Arthoniomycetes is sparse in our dataset, these levels of support (FIG. 2) largely agree with other recent large analyses where the Dothideomycetes is resolved as monophyletic but with low statistical support (Lumbsch et al 2005). A possible sister relationship of Dothideomycete/Arthoniomycetes has been proposed (Barr 1987, Tehler 1990) and there is some phylogenetic support for this (Lumbsch et al 2005, Lutzoni et al 2004). Clear differences between the groups exist, such as the ascohymenial type development of the Arthoniomycetes apothecium (Henssen and Thor 1994). More thorough sampling of Arthoniomycetes will test the monophyly of its relationship with the Dothideomycetes. It is premature to comment on the ultimate monophyly of the Dothideomycetes, but it seems quite reasonable that increased sampling of taxa and genes could increase support for this node. As pointed out by Lumbsch et al (2005), most of the large scale interclass relationships have been in conflict in recent publications and taxon sampling should be an important consideration before making major classification changes.

*Dothideomycetes.* The addition of protein gene data illustrates that the lineages clustering around the core orders Pleosporales and Dothideales correlate with the presence or absence of pseudoparaphyses and other centrum characteristics. The node supporting the Dothideales, Capnodiales, Myriangiales and Mycosphaerellaceae (C) is strongly supported. This node was unaffected when third base codon positions were removed, but a small increase in parsimony bootstrap support was noted at node M, combining the Dothideales and Myriangiales, although ML bootstrap decreased. Saturation and the specific evolutionary model applied might have influenced this. Node C might indicate a single loss of pseudoparaphyses in all the terminal clades. However previous molecular phylogenies based on nuc SSU rDNA data have shown the presence of members of the aparaphysate genus *Leptosphaerulina* nested within the Pleosporales (Silva-Hanlin and Hanlin 2000), which could imply multiple, isolated losses of this character in other parts of the tree.
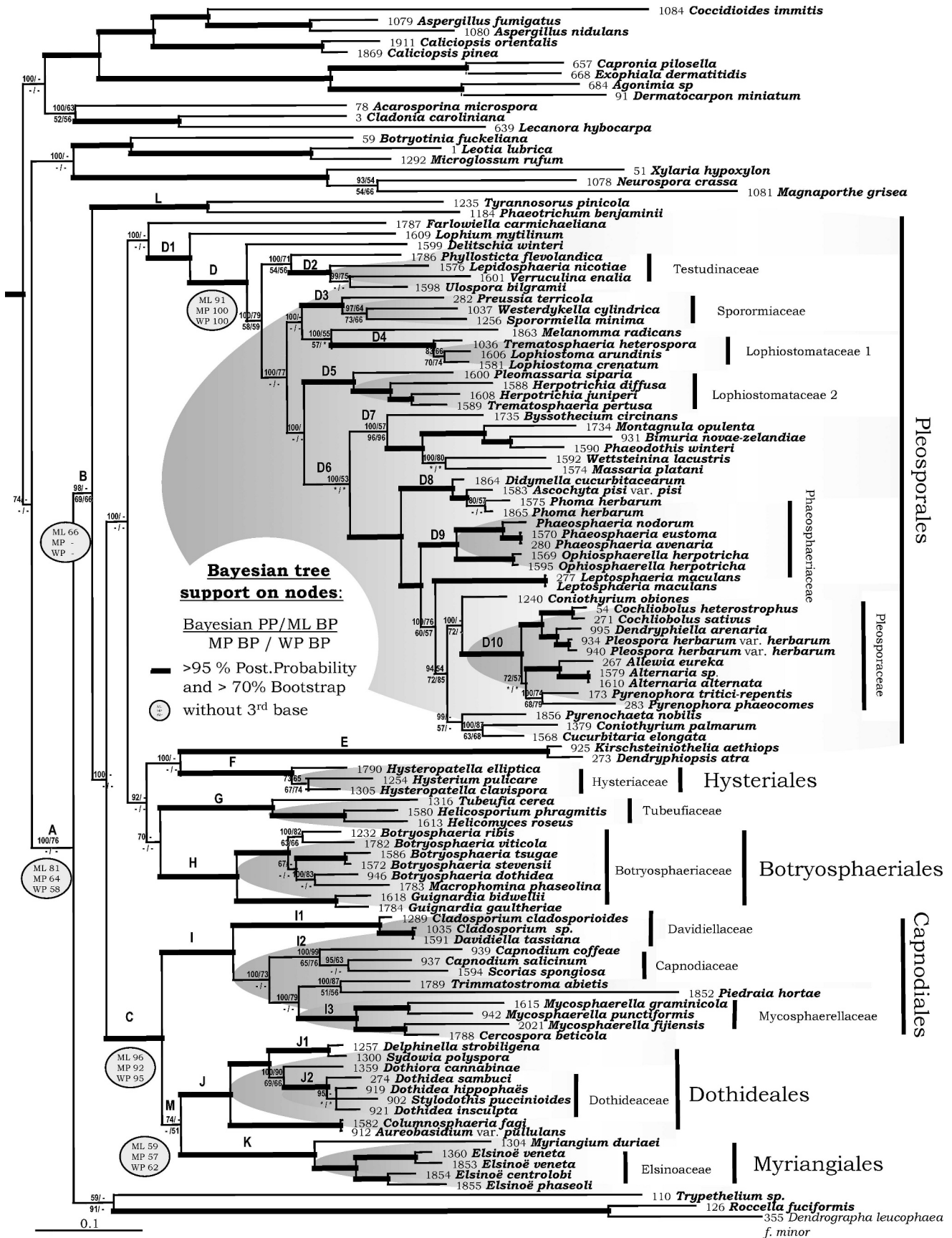
Anamorphs play an important role in the life cycles of many orders of Dothideomycetes. Many are coelomycetes, especially phialidic, *Phoma*-like anamorphs, which may be a plesiomorphic anamorph character in the class, perhaps serving some kind of spermatial function. In the Pleosporaceae and Mycosphaerellaceae hyphomycetes with sympodially proliferating conidiogenous cells with scars, and dry conidia, are particularly common and strictly anamorphic species may comprise the majority in these families. The Capnodiales, with their multitude of hyphomycete and coelomycete synanamorphs, and the helicoconidial anamorphs of the Tubeufiaceae, contain particularly distinctive anamorph groups. The anamorph genera of both hyphomycetes and coelomycetes, lacking teleomorph connections, continue to be examined for their phylogenetic relationships, many of them undoubtedly will be found to be associated with the Dothideomycetes. Several clades are well supported (FIG. 2) and will be discussed in more detail below.

*Aparaphysate Dothideomycetes.*—We hereby propose an emendation of the subclass Dothideomycetidae (*nom. nud.*) (Kirk et al 2001), which has been superceded by the Dothideomycetes O.E. Erikss. and Winka (2000). Dothideomycetidae *sensu* Lutzoni et al (2004) also was included in the Sordariomycetes as subclass Dothideomycetidae along with the subclass Sordariomycetidae (syn. Sordariomycetes *s. str.*) and Arthoniomycetidae (syn. Arthoniomycetes), although there was no strong statistical support for this broadened concept of Sordariomycetes. We validate and emend the concept of Dothideomycetidae *sensu* Kirk et al (2001) to include the bitunicate orders Dothideales, Capnodiales and Myriangiales, which lack paraphyses, pseudoparaphyses and paraphysoids. This emended subclass overlaps with the Loculoparenchymatomycetidae (Barr 1983) but differs by including the Myriangiales and excluding the Asterinales, now listed under its constituent families as Dothideomycetes et Chaetothyriomycetes incertae sedis by Eriksson (2006).

**Dothideomycetidae** P.M. Kirk, P.F. Cannon, J.C. David & J.A. Stalpers, ex Schoch, Spatafora, Crous et Shoemaker, **subclass nov.**
≡ *Dothideomycetidae* P.M. Kirk, P.F. Cannon & J.C. David & J.A. Stalpers, in Kirk et al, Dictionary of Fungi, 9th ed., p 165, 572. 2001 (nom. nud.).

Ascomata immersa vel erumpentia vel superficialia, minuta vel magnitudine media, separata vel in stromate basilari aggregata, unilocularia vel plurilocularia, ostiolata, nonnumquam periphysata. Pseudoparaphyses absentes, periphysoideae nonnumquam praesentes. Asci globosi vel ellipsoidei vel clavati vel

Bayesian tree support on nodes:

Bayesian PP/ML BP
MP BP / WP BP

■ >95 % Post.Probability and > 70% Bootstrap

(ML/MP/WP) without 3rd base

subcylindrici. Ascosporae hyalinae vel subhyalinae vel fuscae, unicellulares vel pluriseptatae vel muriformes. Anamorphoses seu coelomycetes seu hyphomycetes.

Ascomata immersed, erumpent or sometimes superficial, minute, small or medium-sized; separate or merged or grouped on basal stroma, uni- to multi-loculate apical pore mostly present, when present ostiolar canal at times periphysate, stromatic tissues may contain pseudoparenchymatous cells. Pseudo-paraphyses lacking, periphysoids may be present; Asci globose, subglobose, ovoid to ellipsoid, saccate, oblong, clavate or subcylindrical, Ascospores hyaline, subhyaline or dark brown, variable in shape and size, one celled or one to several septate or muriform.

Anamorphs coelomycetous and/or hyphomycetous.

*Type order.* Dothideales (1897) Lindau in Engler & Prantl, Nat. Pflanzenfam. 1(1):373. 1897.

*Represented orders.* Dothideales Lindau 1897, Capnodiales Woron. 1925, Myriangiales Starbäck 1899.

*Dothideales.* Species from this order generally have smaller ascomata and fewer asci than the pseudoparaphysate Pleosporales (node D) and traditionally have been segregated because of the absence of pseudoparaphyses in their pseudothecia. The species included in this order encompass saprotrophs, hemibiotrophs and biotrophs. It is represented by eight species in our analysis, including the recent epitype isolate of *Dothidea sambuci*, the type of the genus *Dothidea* (Shoemaker et al 2003). The family Dothideaceae includes biotrophs, necrotrophs and saprobes on plant tissue. *Stylodothis puccinoides* was redescribed as a separate species from *Dothidea* but remains closely associated with the genus in our phylogeny.

Three members of the Dothioraceae are polyphetic in the tree. The so-called black yeast anamorphs associated with Dothideomycetes tend to occur in this family, with *Aureobasidium pullulans* (probably an anamorph species complex based on the ITS sequences deposited in GenBank), and the micro-morphologically similar *Hormonema dematioides* (teleomorph *Sydowia polyspora*, perhaps also a complex of anamorph species) (de Hoog 1974). These species are found commonly on moist surfaces of plants and can convert from yeast to meristematic growth under nutritional stress. Some progress in the resolution of the nature of *Aureobasidium pullulans* has been made here with the linkage of *Columnosphaeria fagi* (H.J. Hudson) M.E. Barr to a ''neotype'' culture CBS 584.75 of *A. pullulans* var. *pullulans* (SUPPLEMENTARY TABLE I).

*Capnodiales.* The node I is well supported in this multigene analysis. This same node is present in a ribosomal rDNA phylogeny containing ''*Raciborskiomyces longisetosus*'' as erroneous name for a *Cladosporium* species with *Capnodium citri* (Lumbsch and Lindemuth 2001). Synapomorphies are limited in this expanded order and these taxa have not been grouped together before. The presence of short, periphyses-like cells in the ostiolar pore of some genera of the Capnodiales such as *Capnodium* also are reported from other families, including the Mycosphaerellaceae (von Arx and Müller 1975) and might be a synapomorphy uniting these taxa. We hereby propose an expansion of the current Capnodiales to include the Mycosphaereallaceae and Piedraiaceae. The constituent families are discussed below.

*Capnodiaceae.* An ascostromatal family without pseudoparaphyses, the Capnodiaceae are leaf epiphytes associated with the honeydew of insects. Also known as sooty molds, they tend to live in complex communities, with multiple species, and often multiple fungal parasites of those species, inhabiting a common, sooty mass. They are noted for the production of darkly pigmented hyphae, often of very characteristic morphology (Hughes 1976, Reynolds 1998). The members of this order have superficial ascostromata with ovoid asci in fascicles and hyaline to dark, one to multiseptate ascospores. The sooty molds are highly pleomorphic and often highly pleoanamorphic. The order includes many anamorphic species, all dematiaceous, including several conidiomatal, mycelial (often with dry-spored, blastic phragmo- or dictyoconidia) or presumably spermatial (usually phialidic) hyphomycete genera or pycnidial synanamorphs (Hughes 1976).

*Mycosphaerellaceae.* The *Mycosphaerellaceae* is characterized by small pseudothecial ascomata that are immersed in host tissue, single and superficial, or imbedded in a pseudoparenchymatal stroma, papil-

←

FIG. 2. Dothideomycete phylogeny. 50% majority rule consensus tree of 45 000 trees obtained by Bayesian inference and MCMCMC under GTR+I+Γ applied across seven partitions. Only orders and families with more than two members under the current classification of Eriksson (2005) are shown in shadow. Bar indicates the nucleotide substitutions per site. Nodes of interest are labeled alphabetically and support values are shown above and below. Bayesian PP = posterior probability, ML BP = maximum likelihood bootstrap, MP BP = maximum parsimony bootstrap, WP = weighted parsimony bootstrap. Gaps (–) show a collapsed node and asterisks show the presence of a differently resolved node under the specific statistical sampling method used.

late, ostiolate, lacking interascal tissue. Asci vary from ovoid to saccate to subcylindrical, usually stipitate, with or without an apical chamber, lacking any other apical mechanism. Ascospores are hyaline to slightly pigmented, 1-septate, but in some cases also 3-septate, and sometimes are enclosed in a sheath. *Mycosphaerella* has close to 30 anamorph genera associated with it, most of which have cicatrized, sympodially proliferating conidiogenous cells and single or acropetally catenate, dry conidia. The two clades delineated within *Mycosphaerella* here also were recognized in a separate study employing multiple genes to resolve relationships in *Mycosphaerella* (Hunter et al 2006). Node I1 contains the type of *Mycosphaerella*, *M. punctiformis*, and the bulk of *Mycosphaerella* species, while the second clade (above I4) appears to contain more extremotolerant species (Crous et al unpubl data).

*Mycosphaerella* is distinguished from *Davidiella* (*Cladosporium* anamorphs) by lacking irregular lumens or inclusions in its ascospores and not having anamorphs with protruberant, thickened, darkened, *Cladosporium*-like scars (Braun et al 2003, Aptroot 2006). As shown in this study *Davidiella* with its *Cladosporium* anamorphs (type species *Davidiella tassiana*, anamorph *Cladosporium herbarum*) clusters in a well supported clade apart from *Mycosphaerella* s.str. (Mycosphaerellaceae), and thus a new family is proposed for clade I1.

**Davidiellaceae** Schoch, Spatafora, Crous et Shoemaker, **fam. nov.**

Ascomata *Mycosphaerellae* similia, sed lumen ascosporarum forma variabile et anamorphe *Cladosporium*.

Ascomata immersed to erumpent, small or medium-sized; separate or aggregated, uniloculate, apical pore present, periphysate; wall of several layers of brown, thickened, pseudoparenchymatal cells. Pseudoparaphyses lacking. Asci bitunicate, 8-spored, obovoid to ellipsoid or subcylindrical, fasciculate, with or without apical chamber. Ascospores hyaline to pale brown, smooth to somewhat roughened, mucous sheath sometimes present, one-septate, thick-walled, with irregular lumens. Anamorphs are species of *Cladosporium*.

*Typus. Davidiella tassiana* (De Not.) Crous & U. Braun, Mycol. Prog. 2:8. 2003.

The position of a single representative of the Piedraiaceae, *Piedraia hortae*, is refined here as associated with the Capnodiales and allies but not the Myriangiales as reported earlier (Lindemuth et al 2001). This species was described with an ascus containing only one thin wall (Shoemaker and Egger 1982). The specialized parasites in this family are almost exclusively associated with human hair in tropical regions. It is shown with low parsimony bootstrap support (I3) with *Trimmatostroma abietis*, a meristematic anamorph species isolated from conifer needles and rock surfaces. This species was shown to be closely related to *Mycosphaerella* and its allies in a recently published molecular phylogeny (Selbman et al 2005).

*Myriangiales.* The Myriangiales are reported to be related to the Dothideales (node M), although without any significant bootstrap support for this placement. They generally have ascostromata without ostioles in monoascal locules. The species of the type genus, *Myriangium*, has globose asci scattered at many levels in an undifferentiated stromatic mass (Sivanesan 1984). The order includes saprobic, epiphytic or biotrophic organisms. The anamorphs of this order, when known, generally are acervular coelomycetes with polyphialidic conidiogenous cells, such as the *Sphaceloma* anamorphs of *Elsinoë* species (Kirk et al 2001).

*Paraphysate Dothideomycetes.*

We hereby propose a new subclass for the pseudoparaphysate taxa supported by node D1.

**Pleosporomycetidae** Schoch, Spatafora, Crous et Shoemaker, **subclass nov.**

Ascomata perithecialia vel hysterothecialia vel cleistothecialia, immersa vel erumpentia. Hamathecii pseudoparaphyses cellulares vel trabeculatae, maturae nonnumquam deliquescentes. Asci bitunicati, plerumque basilares, nonnumquam lateraliter extendentes, cylindrici vel clavati vel oblongi vel saccati. Ascosporae colore, forma septisque variabiles, plerumque heteropolares sed nonnumquam etiam symmetricae.

Ascomata perithecioid, hysterothecioid or cleistothecioid, conchate or dolabrate, immersed, erumpent or superficial; globose, sphaeroid, turbinate, ovoid, obpyriform, conoid, doliiform, dimidiate. Hamathecium of wide to narrow cellular or trabeculate pseudoparaphyses, deliquescing at maturity in some. Asci bitunicate, usually basal, at times extending laterally, cylindric, clavate, oblong or saccate. Ascospores variable in pigmentation, shape and septation, usually with bipolar asymmetry, but some symmetrical.

*Type order.* Pleosporales Luttrell ex M.E. Barr.

*Represented order.* Pleosporales Luttrell ex M.E. Barr.

*Pleosporales.* The Pleosporales is the largest order in the Dothideomycetes. It contains several well known plant pathogens such as *Cochliobolus heterostrophus*, the causative agent for southern blight on corn, *Leptosphaeria maculans*, causing black leg on rape seed and

*Phaeosphaeria nodorum* causing stagonospora blotch in cereals. In this analysis a strain of *Delitschia winteri* is placed above node D, supporting the rest of the Pleosporales according to Eriksson's broad concept (2001). *Delitschia* shares features common to several bitunicate species occurring on dung; they are darkly pigmented, usually strongly constricted ascospores with germ slits (Barr 2000). The family Delitschiaceae was described by Barr (2000) for species previously placed in the Sporormiaceae. The delineation is based on an ostiole containing periphyses and asci with wide outer ascus walls and an ocular chamber containing refractive rods. This placement was confirmed with nuc SSU rDNA sequence comparisons (Kruys 2005). A combined nuc SSU analysis of *Delitschia winteri* grouped it close to another species of the genus, *D. didyma* (AF242264), confirming the identification of the strain used (results not shown). Members of this family are hypersaprotrophic on old dung and exposed wood.

There was also strong support for the monophyly of Pleosporales, with *Lophium mytilinum* branching at its most basal node (D1). This species is found as a saprobe on wood and on cones of conifers and is listed incertae sedis as part of the Mytilinidiaceae (Eriksson 2006). The family contains species with characteristic conch shaped ascomata. Analyzing additional taxa from the Mytilinidiaceae and related groups also will be important to investigate ancestral character states for the Pleosporales but they should be placed as Pleosporomycetidae incertae sedis for now.

The morphology of ascospores has played an important role in delimiting families in the Pleosporales. However, as noted from some of the first molecular based phylogenies of the Dothideomycetes, several family relationships might be poorly supported (Lindemuth et al 2001). Perhaps the strains chosen are not good exemplars for their families or are derived from misidentified specimens. However it seems unlikely that this can account for all the relationships (FIG. 2) and a reassessment at this level of classification seems urgent. Here we will discuss only briefly a selection of highlighted families (FIG. 2).

The most basal node inside the Pleosporales (D2) supports two members of the Testudinaceae, provisionally included among Ascomycota incertae sedis by Eriksson (2006). Members of this family are mainly isolated from soil and produce reduced, cleistothecioid ascostromata. This clade unexpectedly contains the ostiolate marine species, *Verruculina enalia* (Didymosphaeriaceae) as also noted in an earlier phylogenetic analysis (Kruys 2005). The next well supported clade above node D3 supports the Spor-

ormiaceae. These fungi are found commonly on dung but some occur on other substrates (e.g. wood, soil and plant debris). A large number of species in this group have germ slits. This morphological variability was confirmed in a phylogenetic study using DNA sequences from multiple ribosomal loci (Kruys 2005).

The Lophiostomataceae and Melanommataceae are inferred as paraphyletic in the next set of clades (above D4 and D5), with one clade including two species of *Lophiostoma* (Lophiostomataceae 1). This clade also contains one species of *Trematosphaeria heterospora*, which was classified as *Lophiostoma heterosporum* (Barr 1992). The second clade (Lophiostomataceae 2) includes members of the Lophiostomataceae and Pleomassariaceae as well as Melanommataceae. Node D5 contains a diverse group of species isolated from diseased and decaying plants as well as soil (each currently classified under a different family). This overlapped with relationships reported before, using molecular-based phylogenies (Liew et al 2000, 2002), but like many of the other clades will require more intense sampling to address family and genus descriptions.

The more terminal branches in the Pleosporales (D6) include well studied families containing important plant pathogens, saprobes and animal pathogens with numerous anamorphs. *Didymella cucurbitacearum* forms a clade with the anamorphs *Ascochyta pisi* and *Phoma herbarum* (D8), parasites on agricultural crops. *Leptosphaeria* (Leptosphaeriaceae), shown on a single branch, is a large genus with pale to dark brown and septate ascospores. Members of this family have flask-shaped pseudothecia with narrow asci and a characteristic thin apex. Many species are associated with coelomycetous anamorphs. *Phoma* anamorphs are particularly common (Camara et al 2001, Verkley et al 2004). The Phaeosphaeriaceae (D9) are distinguished from the Leptosphaeriaceae by ascomal wall morphology and all have pycnidial coelomycetes, mostly classified in *Stagonospora*, characterized by holoblastic or sometimes annellidic conidiogenesis and the production of phragmoconidia. Unnamed pycnidial microconidial anamorphs also are reported in some species (Leuchtmann 1984). In a poorly supported clade a trio of species without any clear phylogenetic placement are noted. Two of these species are anamorphs, *Coniothyrium palmarum* and *Pyrenochaeta nobilis*, linked to the teleomorphs *Leptosphaeria* and *Herpotrichia*.

The next well supported node (D10) contains the Pleosporaceae, which have ascostromata that are mainly flask-shaped pseudothecia embedded in the substrate with 1-septate to muriform ascospores. In

addition to species found in marine environments and as parasites on animals a number of important grass and cereal crop parasite genera, *Cochliobolus*, *Pyrenophora* and *Lewia*, are included in this family. The sexual states are normally well linked with single anamorph genera. Important anamorph species include the well known genera *Alternaria* (with *Ulocladium* paraphyletic within it), *Stemphylium*, the so-called helminthosporia (*Bipolaris*, *Curvularia*, *Drechslera*, *Exserohilum*) and a few other genera such as *Dendryphion* and *Dendryphiopsis*.

*Dothideomycetes incertae sedis.*—A number of orders could not be placed in any of the two subclasses defined and will be discussed in more detail. Two orders, Jahnulales and Patellariales, currently listed by Eriksson (2006) are not included in this analysis but a separate comparison using deposited sequences from nuc SSU obtained from GenBank combined with our complete taxa revealed them to be separate from the groups referred to in this paper (data not shown).

Members of Hysteriales have been reported with pseudoparaphyses in apothecioid ascomata with elongated openings (von Arx and Müller 1975, Barr 1987, Luttrell 1974) and are often saprobes on wood or weak parasites of woody plants. Four members of the Hysteriales agreeing mainly with Luttrell's original definitions are included (FIG. 2) and it is clear that these are not a monophyletic group, a proposition also mentioned by Luttrell (1973). *Farlowiella carmichaeliana* could not be resolved with any certainty.

The phylogeny also supports a relationship between the dung fungus *Phaeotrichum benjaminii* and *Tyrannosorus pinicola* (FIG. 2). *Phaeotrichum* is characterized by dark-brown, septate spores and cleistothecioid ascostromata. *T. pinicola* produces ostiolate ascostromata with characteristic long, sharp spines and have been isolated from wood and plant material. The multiple germ slits that were described for *T. pinicola* may be linked to the terminal germ pores characteristic of *P. benjaminii*.

Node E supports *Kirschsteiniothelia aethiops* with its *Dendryphiopsis atra* anamorph. These two species also appear unrelated to other species in the genus (Shearer 1993) based on nuc SSU rDNA data and the genus is reportedly heterogenous (Hawksworth and Eriksson 2003). *K. aethiops* does not have close associations with the Pleosporaceae and should be placed in a separate family.

The Tubeufiaceae clade (above node G) contains species with a variety of nutritional modes. They often are reported as saprobes from terrestrial and freshwater environments, but some species are hyperparasites and others can parasitize insects. Teleomorphs consist of brightly colored ascostromata, with long,

hyaline, multiseptate ascospores (Rossman 1987). The best-known anamorphs of the Tubeufiaceae are helicosporous hyphomycetes and well known genera include *Helicodendron*, *Helicomyces* and *Helicoon*. Recent DNA sequence-based comparisons did not find strong correlation between these anamorph forms and phylogenetic groups. (Tsui et al 2006). Combining recent focused phylogenies into a large scale dataset is required before placement of this group in the current classification.

*Botryosphaeriaceae.* The position of the Botryosphaeriaceae (H) within the Dothideomycetes has been enigmatic. The taxonomy of this group of plant-associated fungi has relied mostly on anamorph descriptions; sequence data recently have linked several anamorph genera to the genus *Botryosphaeria* (Jacobs and Rehner 1998). Associated anamorphs were divided into two groups, those with thin-walled, hyaline conidia (*Fusicoccum*), and those with thick-walled, pigmented conidia (*Diplodia*) (Denman et al 2000). In a recent phylogenetic study employing LSU sequence data to resolve relationships among members of the Botryosphaeriaceae, Crous et al (2006) segregated *Botryosphaeria* into several genera, supported by morphological differences of their anamorphs. From the phylogenetic results obtained in this study, it is clear that the Botryosphaeriaceae deserves an order separate from the Pleosporales and Dothideales, which is introduced below.

**Botryosphaeriales** Schoch, Crous & Shoemaker, **ord. nov.**

> *Family. Botryosphaeriaceae* Theiss. & P. Syd., Ann. Mycol. 16:16 (1918).
>
> *Type. Botryosphaeria* Ces. & De Not., Comment Soc. crittog. Ital. 1:211 (1863)..
>
> *Type species. B. dothidea* (Moug. : Fr.) Ces. & De Not., Comment Soc. crittog. Ital. 1:212 (1863).

Ascomata unilocularia vel plurilocularia, pariete multistratoso fusco inclusa, singularia vel aggregata, raro in stromate submersa. Asci bitunicati, endotunica crassa, stipitati vel sessiles, clavati, camera apicali distincta, pseudoparaphysibus hyalinis, septatis, ramosis vel simplicibus intermixti. Ascosporae hyalinae vel pigmentatae, unicellulares vel septatae, ellipsoideae vel ovoideae, nonnumquam appendicibus vel tunica gelatinosis praeditae. Anamorphoses: conidiomata pycnidialia, unilocularia vel multilocularia, saepe in stromate submersa, cellulis conidiogenis phialidicis, conidia hyalina vel pigmentata, tenui- vel crassitunicata proferentibus, quae nonnumquam appendicibus vel tunica gelatinosis praedita sunt.

*Ascomata* uni- to multilocular with multilayered dark brown walls, occurring singularly or in clusters, frequently embedded in stromatic tissue. *Asci* bituni-

cate, with a thick endotunica, stalked or sessile, clavate, with a well developed apical chamber, intermixed with hyaline, septate pseudoparaphyses, branched or not. *Ascospores* hyaline to pigmented, septate or not, ellipsoid to ovoid, with or without mucoid appendages or sheath. *Anamorphs* have uni- to multilocular pycnidial conidiomata, frequently embedded in stromatic tissue, with hyaline, phialidic conidiogenous cells, and hyaline to pigmented, thin- to thick-walled conidia, which sometimes have mucoid appendages or sheaths.

*Conclusion.*—This multigene phylogeny contributes to the overall phylogenetic classification of the Dothideomycetes. We emend a previously proposed subclass, the Dothideomycetidae, and propose a new one, the Pleosporomycetidae, based on the presence or absence of pseudoparaphyses as defined by Barr (1987) based on Luttrell (1955). The orders according to Eriksson (2006) are largely upheld with the exception of the Hysteriales, but we also expand this classification with an additional order, the Botryosphaeriales, and redefine the Capnodiales to include the currently defined Mycosphaerellaceae and Piedraiaceae. A new family, the Davidiellaceae, is proposed to accommodate *Davidiella* species with *Cladosporium* anamorphs. Several clades did not correlate with familial relationships under Eriksson's classification (2006) and should be addressed in subsequent analyses. Similarly a number of small clades are incertae sedis and remain to be addressed in the future. The strains used in this study, although validated by morphological examinations in previous publications (e.g. Berbee 1996) as well as by comparisons with sequences from GenBank, should continue to be validated by more intensive taxon sampling in a number of clades. The value of additive sampling in this study, where two strains used in previous studies could be shown to be misidentified, supports this.

One large gap in this analysis is the absence of lichenized lineages. A single unidentified *Trypethelium* species was included, but numerous lichenized ascostromatic bitunicate species (such as those in the Pyrenulales) remain candidates for placement in the Dothideomycetes. In fact a study by Del Prado et al (2006) shows good support for a placement of the lichenized Trypetheliaceae within the Dothideomycetes. In addition, numerous lineages remain unresolved in this class. For example the current classification of Eriksson (2006) contains 23 families placed in orders but more than 40 families remain listed as Chaetothyriomycetes et Dothideomycetes incertae sedis. It appears likely that, in the process of combining the comprehensive body of work already done on the biology, ontogeny and morphology of these fungi within a molecular-based phylogenetic context, they will continue to surprise and challenge us well into the future.

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