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

**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, Translation 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.

Published as: Hunter GC, Wingfield BD, Crous PW, Wingfield MJ (2006). A multi-gene phylogeny for species of *Mycosphaerella* occurring on *Eucalyptus* leaves. *Studies in Mycology* **55**: 147–161.
INTRODUCTION

Species of Eucalyptus are native to Australia with isolated pockets of native Eucalyptus forests also occurring in Papua New Guinea, Indonesia 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 3000 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, Carnegie & Ades 2003). 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 Translation 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 this investigation where already in existence and 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 % (wt/v) malt extract agar (MEA) (Biolab, South Africa), at 25 °C 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 (98 % 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 (wt/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 MgCl₂, 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′) (White et al. 1990, Vilgalys & Hester 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 datasets 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 *Mycosphaerella ohnowa* Crous & M.J. Wingf. (CBS 112973) and *Mycosphaerella mexicana* Crous (CBS 110502) was excluded from the analyses. In the LSU dataset, 1075 characters were constant while 77 characters were parsimony-uninformative and 179 characters were parsimony-informative. Parsimony analysis of the LSU dataset 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* H.J. Swart & J. Walker (*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* (Kobayashi) Crous, *Ps. basiramifera* Crous] *Passalora* [Pass. eucalypti (Crous & Alfenas) Crous & U. Braun, Pass. zambiae Crous & T. Coutinho], and *Dissoconium* de Hoog, Oorschot & Hijwegen (*M. lateralis* Crous & M.J. Wingf., *M. communis* Crous & J.P. Mansilla).

Internal Transcribed Spacer (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.

A heuristic search of the ITS dataset 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 Deighton, Stenella Syd., 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).

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. ellipsoides* 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 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 dataset:** 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 0.05 is inordinately conservative (Cunningham 1997, Darlu & Lecointre 2002, Dettman et al. 2003). Thus, the LSU, ITS and EF-1α DNA sequence datasets were combined. The ACT dataset was omitted from the combined dataset due to the lack of resolution among species of *Mycosphaerella*. Therefore, the combined LSU, ITS and EF-1α dataset 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 dataset, 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. 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, 2006, 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 data from the LSU, ITS and 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 conidiomatal forms under differing environmental conditions (Crous et al. 2000, Cortinas et al. 2006). 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. vespa 9.5–16.5 × 2.5–4 μm and those of M. ambiphylla are (12–)14–15(–22) × (3.5–)4.5–5(–6) 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 synonymised 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 Pflanzenfamilie, 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 dataset 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 datasets 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 %). Furthermore, there is concordance across all gene regions for the node from which all four species branch, however, there is discord at nodes internal to that node. 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 datasets, *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 *Mycosphaerella parkii* Crous, M.J. Wingfield, F.A. Ferreira & Alfenas (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*. Both *M. marksii* and *M. intermedia* ascospores germinate in a typical Type B ascospore germination pattern with germ tubes growing parallel to the long axis of the ascospore with no distortion, darkening or constriction of the ascospore occurring (Carnegie & Keane 1994, Crous 1998, Dick & Dobbie 2001). Furthermore, overlap is seen in the ascospore dimensions of *M. marksii* and *M. intermedia* with those of *M. marksii* being 12.5–22.5(17.9) × 2.5–5.0(3.1) µm and those of *M. intermedia* 12–16 × 2–4 µm (Carnegie & Keane 1994, Dick & Dobbie 2001). Leaf lesions of these two species are also similar with those of *M. marksii* being grey on the adaxial leaf surface and yellow to red-brown on the abaxial leaf surface and surrounded by a red-brown margin while lesions of *M. intermedia* are pale on the abaxial surface and rust-brown with a slightly raised dark-brown margin surrounded by a red-purple zone on the adaxial leaf surface (Carnegie & Keane 1994, Dick & Dobbie 2001). Due to the phylogenetic and morphological similarity, we 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* Crous & M.J. Wingf., *M. aurantia* A. Maxwell and *M. keniensis* Crous & T. Coutinho 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. pseudoendophytica Crous & G.C. Hunter are the only Mycosphaerella spp. occurring on Eucalyptus that are known to have Pseudocercosporella anamorphs (Crous 1998, Crous et al. 2006).
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 datasets 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). To study species complexes, variable gene regions must be studied and the generation of greater numbers of datasets 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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## Table 1. Isolates of *Mycosphaerella* used in this study for DNA sequencing and phylogenetic analysis.

| Teleomorph       | Anamorph                  | Isolate No. | Host          | Country   | Collector   | GenBank Accession No. |
|------------------|---------------------------|-------------|---------------|-----------|-------------|-----------------------|
|                  |                           | CMW         | CBS           | STEU      | LSU         | ITS                   | ACT         | EF-1α       |
| *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. ambiphilla*  | *Phaeophleospora* sp.     | 14180       | 110499        | N/A       | *E. globulus* | South Africa          | 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   | AY907057   | 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   |
| Teleomorph         | Anamorph                | Isolate No. | Host          | Country  | Collector | LSU     | ITS     | ACT     | EF-1α    |
|--------------------|-------------------------|-------------|---------------|----------|-----------|---------|---------|---------|----------|
| M. gracilis       | Pseudocercospora gracilis | 14455       | E. urophylla   | Indonesia| A.C. Alfenas | DQ204750| DQ267582| DQ147616| DQ211666 |
| M. grandis        | Unknown                 | 8557        | E. globulus   | Chile    | A. Rotella | DQ246241| DQ267583| DQ147644| DQ235108 |
| M. gregaria       | Unknown                 | 8554        | E. globulus   | Chile    | M.J. Wingfield | DQ246240| DQ267584| DQ147643| DQ235107 |
| M. heimii         | Pseudocercospora heimii | 14462       | E. globulus   | Australia| A. Maxwell | DQ246251| DQ267585| DQ147650| DQ235130 |
| M. heimioides     | Pseudocercospora heimioides | 14776      | Eucalyptus sp. | Indonesia| M.J. Wingfield | DQ204752| DQ267586| DQ147632| DQ211668 |
| M. intermedia     | Unknown                 | 7163        | E. saligna    | New Zealand| K. Dobbie | DQ246247| AY725546| N/A     | N/A      |
| M. irregularirimosa | Pseudocercospora irregularirimosa | 4943        | E. saligna    | South Africa| M.J. Wingfield | DQ204754| AF309607| DQ147634| DQ211670 |
| M. ohnowa         | Unknown                 | 5223        | E. saligna    | South Africa| M.J. Wingfield | DQ204755| AF309608| DQ147635| DQ211671 |
| M. keniensis      | Unknown                 | 4937        | E. grandis    | South Africa| M.J. Wingfield | N/A     | AF309604| DQ147662| DQ235125 |
| M. lateralis      | Uwebranxia lateralis   | 5147        | E. grandis    | Kenya     | T. Coutinho | DQ246259| AF309601| DQ147611| DQ235100 |
| M. madeirae       | Pseudocercospora sp     | 14457       | E. globulus   | Zambia    | T. Coutinho | DQ246260| AY25550 | DQ147652| DQ235139 |
| M. marksii        | Unknown                 | 14781       | E. grandis    | South Africa| G. Kemp   | DQ246249| DQ267587| DQ147624| DQ235133 |
|                   |                         | 5150        | E. botryoides | Australia | A.J. Carnegie | DQ246250| AF309588| DQ147625| DQ235134 |
| Teleomorph            | Anamorph                  | Isolate No. | Host        | Country | Collector | GenBank Accession No. |
|----------------------|---------------------------|-------------|-------------|---------|-----------|-----------------------|
|                      |                           |             |             |         |           |                       |
| **CMW**              | **CBS**                   | **STEU**    |             |         |           |                       |
| M. mexicana          | Unknown                   | 14461       | N/A 110502  | E. globulus | A. Maxwell | DQ246237 AY725558 DQ147660 DQ235123 |
| M. readeriellophora  | Readeriella readeriellophora | 14233     | 114240 10375 | E. globulus | J.P. Mansilla | DQ246238 AY725577 DQ147658 DQ235117 |
| M. molleriana        | Colletogloeopsis molleriana | 4940       | 111164 1214 | E. globulus | S. McCrae | DQ246220 AF309620 DQ147671 DQ235104 |
| M. nubilosa          | Uwebraunia juvenis        | 3282        | 116005 937  | E. globulus | A. J. Carnegie | DQ246228 AF309618 DQ147666 DQ235111 |
| M. parkii            | Stenella parkii           | 14775       | 387.92 353  | E. grandis | M.J. Wingfield | DQ246245 AY626979 DQ147612 DQ235137 |
| M. parva             | Unknown                   | 14459       | 110503  N/A | E. globulus | A. Maxwell | DQ246243 AY626980 DQ147645 DQ235110 |
| M. suberosa          | Unknown                   | 5226        | 436.92 515  | E. dunnii | M.J. Wingfield | DQ246235 AY626985 DQ147656 DQ235101 |
| M. suttoniae         | Phaeopheospora epicoccoides | 5348       | 1346 Eucalyptus sp. | Indonesia | M.J. Wingfield | DQ246227 AF309621 DQ147673 DQ235116 |
| M. vespa             | Colletogloeopsis sp.      | 11558       | 117924  N/A | E. globulus | Unknown | DQ246221 DQ267590 DQ147668 DQ235106 |
| M. tasmaniensis      | Passalora tasmaniensis    | 14780       | 111687 1555 | E. nitsa | M.J. Wingfield | DQ246233 DQ267591 DQ147676 DQ235121 |
| M. toledana          | Phaeopheospora toledana    | 14457       | 113313  N/A | E. nitsa | unknown | DQ246234 DQ267592 DQ147677 DQ235122 |
| M. walkerii          | Sonderhenia eucalyptica    | 20333       | 111306 1457 | E. saligna | M.J. Wingfield | DQ267574 DQ267593 DQ147630 DQ235095 |
| Unknown              | Passalora eucalypti       | 14907       | 111306 1457 | E. globulus | P.W. Crous | DQ246244 AF309617 DQ147678 DQ235138 |
| Unknown              | Passalora zambiae          | 14782       | 112971 1227 | E. globulus | T. Coutinho | DQ246264 AF725523 DQ147675 DQ235136 |

*LSU, ITS, ACT, EF-1α are GenBank Accession Numbers.*
| Teleomorph | Anamorph | Isolate No. | STEU | Host | Country | Collector | LSU     | ITS     | ACT     | EF-1α  |
|------------|----------|-------------|------|------|---------|-----------|---------|---------|---------|--------|
| 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 |
|            |          | 14910 | 111692 | 1582 | *Eucalyptus sp.* | New Zealand | M.J. Wingfield | DQ246224 | DQ267599 | DQ147663 | DQ235114 |
| Unknown    | *Pseudocercospora basistruncata* | 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 |
| Teleomorph       | Anamorph              | Isolate No. | Host     | Country | Collector | GenBank Accession No. |
|------------------|-----------------------|-------------|----------|---------|-----------|----------------------|
| Unknown          | Readeriella novaezelandiae | 14913       | 114357   | 10895   | E. botryoides | New Zealand          |
| Botryosphaeria ribis | Fusicoccum ribis       | 7773        | N/A      | N/A     | Ribus sp.  | U. S. 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
Figure 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.
Figure 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.
Dissoconium

Phaeophleospora / Colletogloeopsis / Uwebraunia

Pseudocercospora / Stenella

Readeriella

Pseudocercosporella / Uwebraunia

Pseudocercospora

Sonderhenia
Figure 3. Phylogram obtained from the Translation 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.
Figure 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.
**Figure 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.
