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 ºC 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.

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).

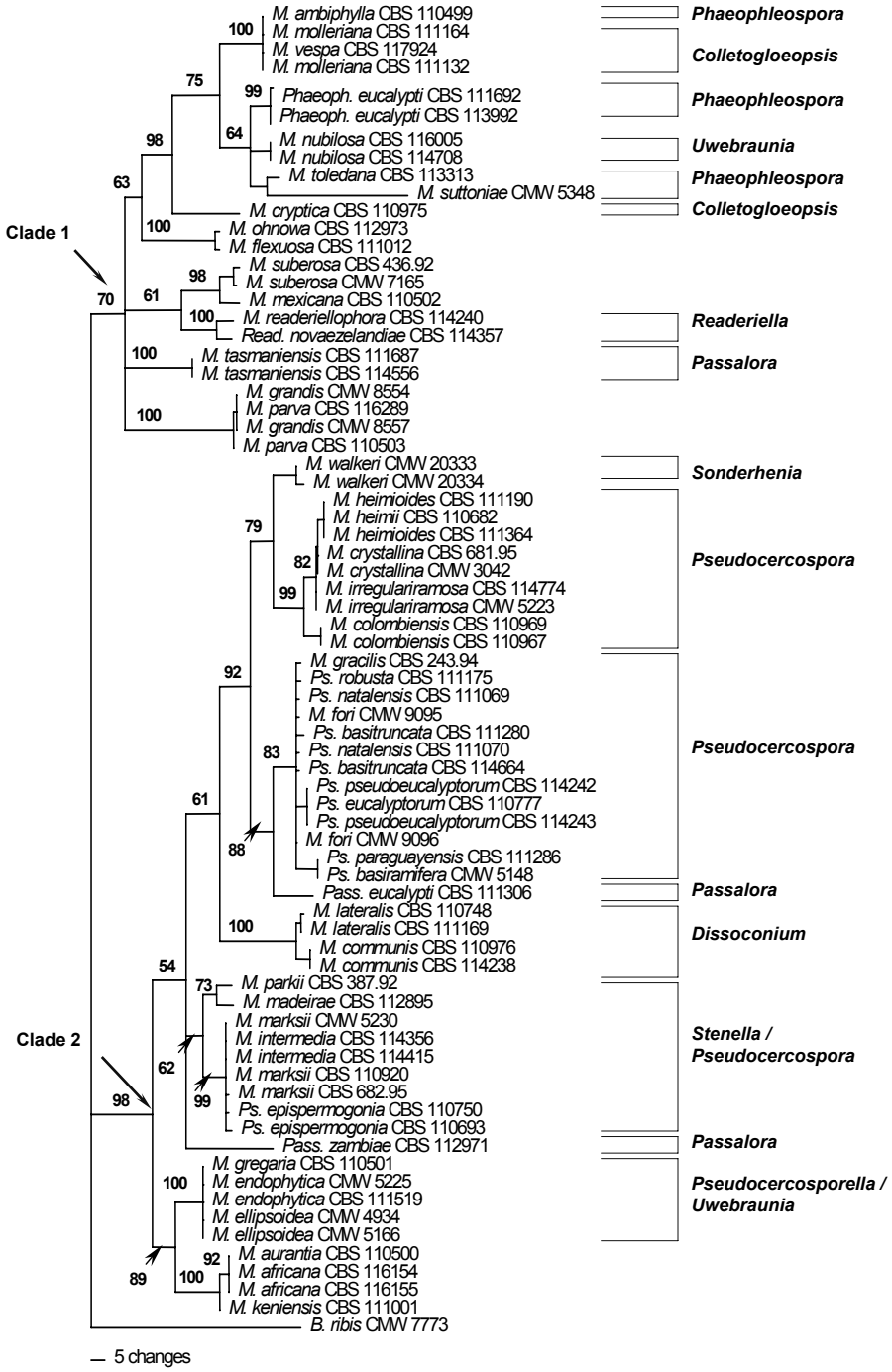

Fig. 1. Phylogram obtained from the Large Subunit (LSU) rDNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves showing two well-supported main clades (Clades 1−2). Tree length = 663, CI = 0.519, RI = 0.878, RC = 0.456. Bootstrap values based on 1000 replicates are indicated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

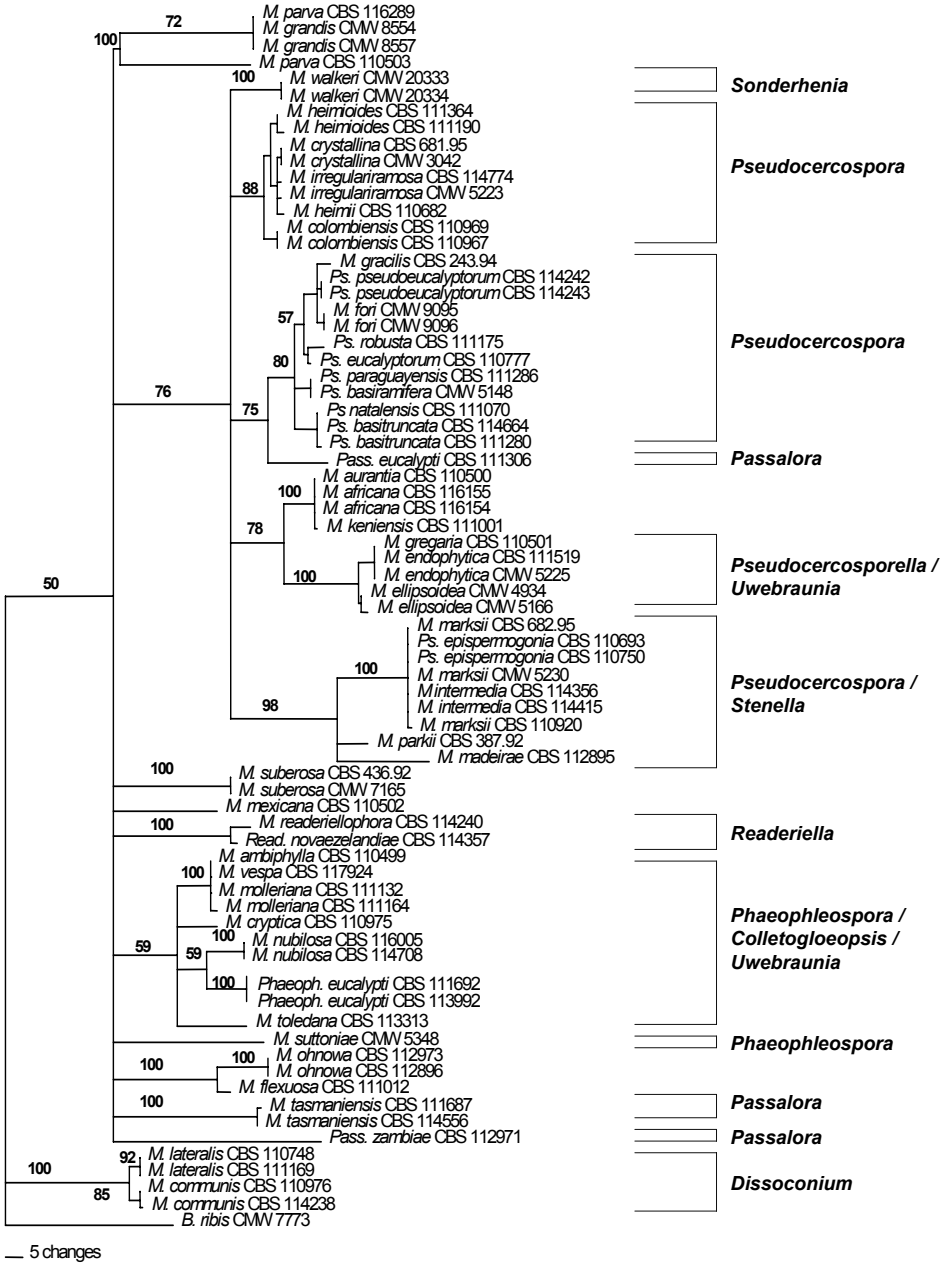

Fig. 2. Phylogram obtained from the Internal Transcribed Spacer (ITS) DNA sequence alignment of *Mycosphaerella* spp. occurring on Eucalyptus leaves indicating two monophyletic clades (Clades 1−2). Tree length = 871, CI = 0.358, RI = 0.782, RC = 0.280.

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

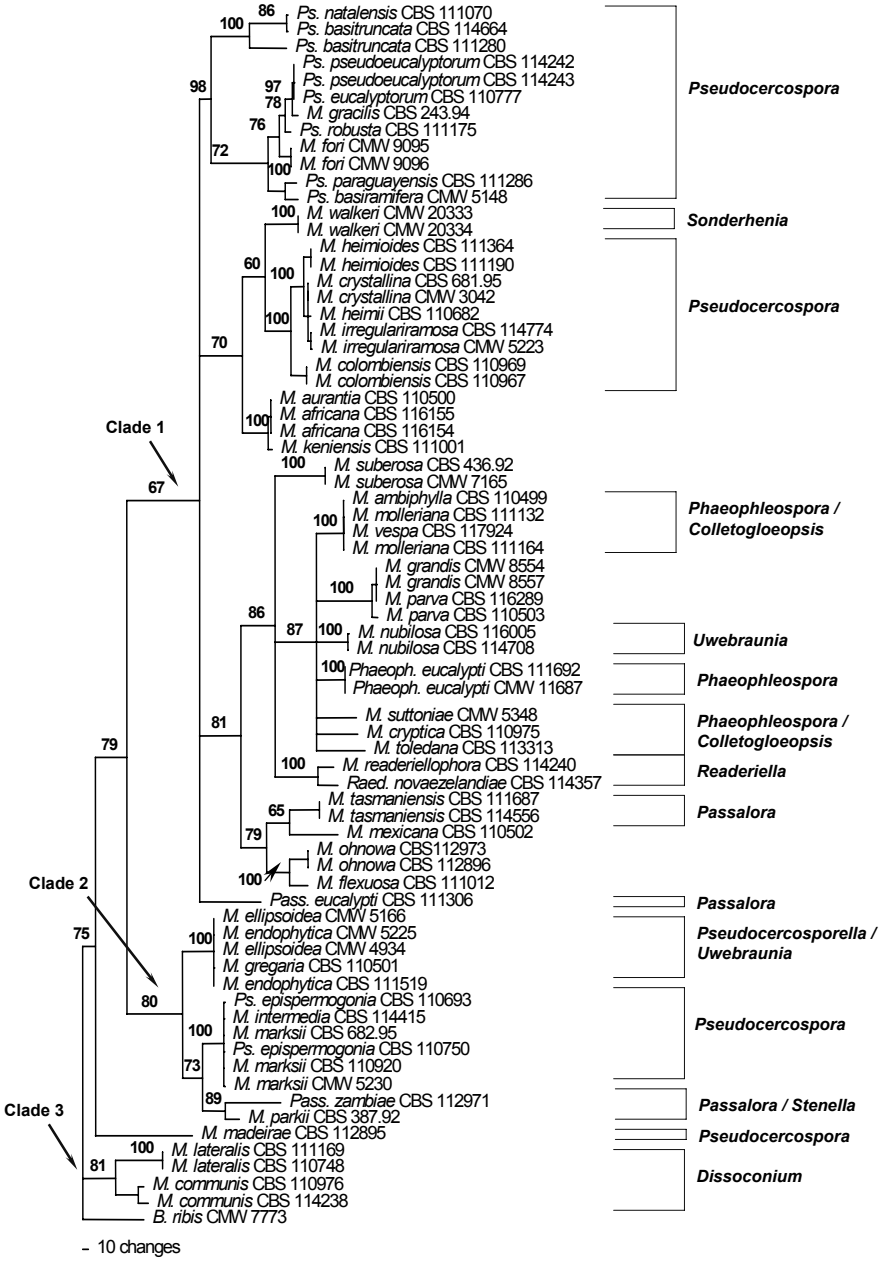

Fig. 3. Phylogram obtained from the Elongation factor 1-alpha (EF-1α) DNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves showing three main clades. Tree length = 3194, CI = 0.345, RI = 0.777, RC = 0.268.

(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

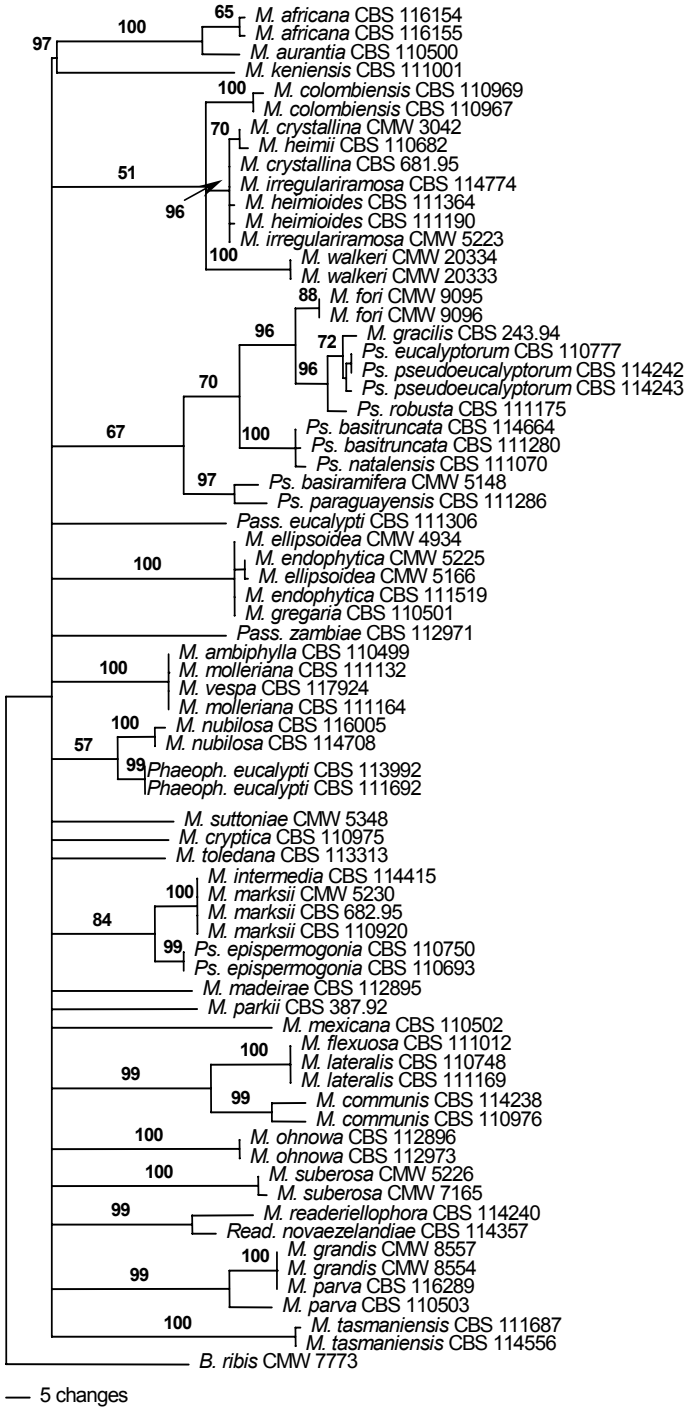

Fig. 4. Phylogram obtained from the Actin (ACT) DNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves. Tree length = 1007, CI = 0.235, RI = 0.682, RC = 0.160.

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

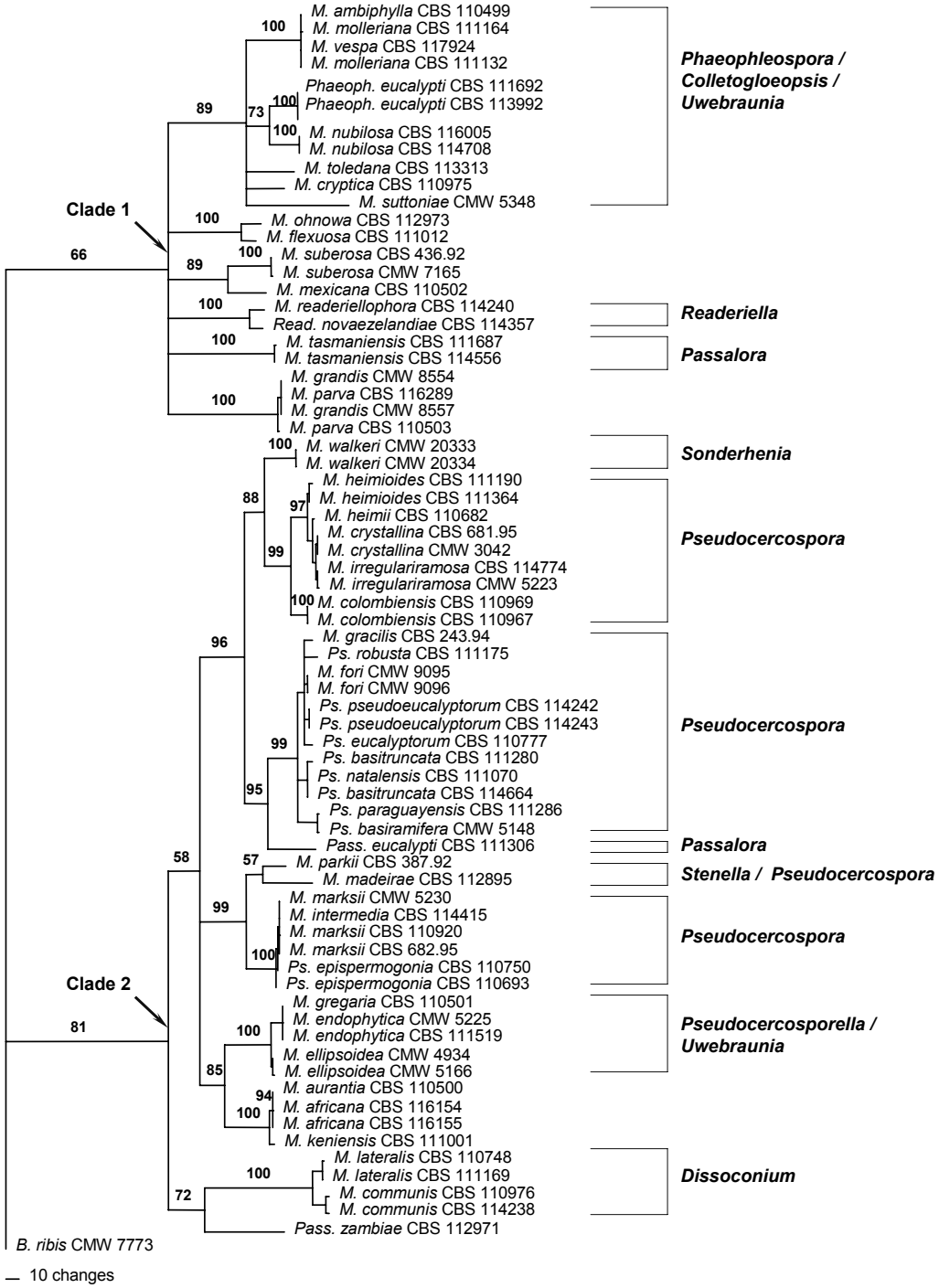

Fig. 5. Phylogram obtained from the combined LSU, ITS and EF-1α DNA sequence alignment of *Mycosphaerella* spp. occurring on *Eucalyptus* leaves showing two main clades. Tree length = 1677, CI = 0.384, RI = 0.817, RC = 0.314.

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.*

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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