ITS rDNA phylogeny of selected *Mycosphaerella* species and their anamorphs occurring on *Myrtaceae*

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Species of *Mycosphaerella* and their anamorphs are commonly found on the leaves of *Myrtaceae*, many of which are defoliated by these pathogens. The taxonomy of these fungi has been based on minute morphological differences, and virtually nothing is known regarding their relatedness to each other. In this study, we present a phylogeny of 30 species of *Mycosphaerella* or their anamorphs from myrtaceous hosts, based on sequence data from the ITS regions of the ribosomal RNA operon. Fifteen of the species were also analysed for the 5′ end of the large subunit (28S), which produced a phylogeny similar to that obtained for the ITS data set. The *Mycosphaerella* species included in this analysis are all regarded as representatives of section *Plaga*, and appear to represent a monophyletic assemblage. *Mycosphaerella lateralis* was the only species shown to have a wide host range. In general, species clustered together based on their anamorph genera. Species with *Colletogloeopsis* and *Stenella* anamorphs always grouped in two respective clusters. However, species with *Mycovellosiella*, *Phaeophleospora*, *Pseudocercospora* and *Uwebraunia* anamorphs occurred separately, suggesting that they have evolved more than once within *Mycosphaerella*. Based on the ITS data set, all morphospecies were also shown to be phylogenetic species, although too few isolates were available to address questions relating to intraspecies variation. Nevertheless, ITS sequence data proved sufficient to distinguish morphologically similar taxa that have hitherto only been distinguished based on ascospore germination patterns and anamorph characteristics. Sequence data presented in this study should facilitate the identification of *Mycosphaerella* species occurring on *Myrtaceae* in the future.

INTRODUCTION

Many species of *Mycosphaerella* occur on the leaves of *Eucalyptus* (Crous 1998) and other myrtaceous hosts (Crous 1999). Several of these species are recognised as serious leaf and shoot pathogens. For example, *M. cryptica* causes leaf blight on *Eucalyptus* in Australia, and *M. juvenis* is a serious leaf pathogen on *E. nitens* and *E. globulus* in Southern Africa (Lundquist & Purnell 1987, Crous & Wingfield 1996).

Species of *Mycosphaerella* are distinguished on perithecium, ascus and ascospore morphology, as well as on host symptoms (Barr 1972). In the 1980s, a series of papers by Park & Keane (1982a, b, 1984) highlighted the fact that some of these species also have different modes of ascospore germination. The taxonomic value of this characteristic was expanded by Crous (1998) who identified 14 patterns of ascospore germination for taxa on *Eucalyptus*. Crous (1998) also introduced cultural characteristics and the morphology of asexual structures produced in culture as valuable taxonomic characters. However, even when all these characters are considered, it is often difficult to identify most species of *Mycosphaerella* on *Myrtaceae*. This problem arises from not all strains having the ability to produce anamorphs in culture, and little being known of their geographical distribution and host ranges.

Very few *Mycosphaerella* species occurring on *Eucalyptus* are known to occur in Australasia where this host genus is native. This led Crous (1999) to speculate that these fungi could have originated on other species of *Myrtaceae* and adapted to infect *Eucalyptus* where this tree is grown as an exotic. However, when the morphology of *Mycosphaerella* species from *Eucalyptus* was compared with that of species on other *Myrtaceae*, no evidence of host swapping was found (Crous 1999). If species of *Mycosphaerella* are really as host specific as reported in the literature (Chupp 1954, Corlett 1991), this would suggest that most of these fungi occur in Australasia, but have yet to be reported.

Although considerable progress has been made in identifying *Mycosphaerella* species from *Myrtaceae*, many problems belie this process. While taxonomic characters for this purpose have been considerably expanded, they remain relatively complex and equivocal. Characteristics of these fungi in culture are influenced by small differences in media. Ascospore germination patterns are also affected by culture conditions. Anamorph characteristics are in many cases difficult to interpret and synanamorphs are found in some species (Crous

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Table 1. Isolates included in the sequence analysis.

Accession No.	Teleomorph (*Mycosphaerella*)	Anamorph	Origin
STE-U 1124	*M. syzgii*	*Cercostigmina punctata*	*Syzygium*, South Africa
STE-U 784	*M. molleriana*	*Colletogloeopsis molleriana*	*Eucalyptus*, California, USA
STE-U 1214*	*M. molleriana*	*Colletogloeopsis molleriana*	*Eucalyptus*, Portugal
STE-U 355	*M. cryptica*	*Colletogloeopsis nubilosum*	*Eucalyptus*, Chile
STE-U 936*	*M. cryptica*	*Colletogloeopsis nubilosum*	*Eucalyptus*, Australia
STE-U 825	*M. lateralis*	*Dissoconium dekkeri*	*Eucalyptus*, South Africa
STE-U 1233*	*M. lateralis*	*Dissoconium dekkeri*	*Eucalyptus*, Zambia
STE-U 1457*	*Mycosphaerella* state unknown	*Mycovellosiella eucalypti*	*Eucalyptus*, Brazil
STE-U 1555	*M. tasmaniensis*	*Mycovellosiella tasmaniensis*	*Eucalyptus*, Australia
STE-U 1366	*Mycosphaerella* state unknown	*Phaeophleospora destructans*	*Eucalyptus*, Indonesia
STE-U 1454	*Mycosphaerella* state unknown	*Phaeophleospora eugeniae*	*Eugenia*, Brazil
STE-U 1346*	*M. suttoniae*	*Phaeophleospora epicoccoides*	*Eucalyptus*, Indonesia
STE-U 1266	*Mycosphaerella* state unknown	*Pseudocercospora basiramifera*	*Eucalyptus*, Thailand
STE-U 1106	*M. colombiensis*	*Pseudocercospora colombiensis*	*Eucalyptus*, Colombia
STE-U 801	*M. crystallina*	*Pseudocercospora crystallina*	*Eucalyptus*, South Africa
STE-U 16, 17	*Mycosphaerella* state unknown	*Pseudocercospora eucalyptorum*	*Eucalyptus*, South Africa
STE-U 760	*M. heimii*	*Pseudocercospora heimii*	*Eucalyptus*, Madagascar
STE-U 1312*	*M. heimioides*	*Pseudocercospora heimioides*	*Eucalyptus*, Indonesia
STE-U 1360, 1362*	*M. irregulariramosa*	*Pseudocercospora irregulariramosa*	*Eucalyptus*, South Africa
STE-U 1264*	*Mycosphaerella* state unknown	*Pseudocercospora natalensis*	*Eucalyptus*, South Africa
STE-U 1458*	*Mycosphaerella* state unknown	*Pseudocercospora paraguayensis*	*Eucalyptus*, Brazil
STE-U 1269	*Mycosphaerella* state unknown	*Pseudocercospora robusta*	*Eucalyptus*, Malaysia
STE-U 204	*Mycosphaerella* state unknown	*Pseudocercospora syzgiicola*	*Syzygium*, South Africa
STE-U 2768 2769	*M. walkeri*	*Sonderhenia eucalypticola*	*Eucalyptus*, Uruguay
STE-U 348	*M. marasasii*	*Stenella marasasii*	*Syzygium*, South Africa
STE-U 353*	*M. parkii*	*Stenella parkii*	*Eucalyptus*, Brazil
STE-U 794*	*M. africana*	Unknown	*Eucalyptus*, South Africa
STE-U 1109	*M. fluexuosa*	Unknown	*Eucalyptus*, Colombia
STE-U 1084*	*M. keniensis*	Unknown	*Eucalyptus*, Kenya
STE-U 935, 982*	*M. marksii*	Unknown	*Eucalyptus*, Australia
STE-U 937	*M. nubilosa*	Unknown	*Eucalyptus*, Australia
STE-U 1224*, 1225	*M. ellipsoidea*	*Uwebraunia ellipsoidea*	*Eucalyptus*, South Africa
STE-U 1004, 1005*	*M. juvenis*	*Uwebraunia juvenis*	*Eucalyptus*, South Africa

STE-U = Culture collection of the Department of Plant Pathology, University of Stellenbosch.
* Isolates also sequenced for the 5′ end of the 28S.

1998). It is, therefore, uncertain whether many of the morphological species of *Mycosphaerella* on *Myrtaceae* represent phylogenetic species.

In recent years, several molecular methods have facilitated the process of identifying *Mycosphaerella*'s. Random amplified polymorphic DNAs (RAPDs) were employed by Johansen *et al*. (1994) to distinguish *Mycosphaerella* species on *Musa*, while McDonald & Martinez (1990) used restriction fragment length polymorphism (RFLPs) to study the population structure of *Mycosphaerella graminicola* in wheat fields. Stewart *et al*. (1999) utilised sequence data from ITS1 and ITS2 regions of the rRNA operon to separate species occurring on *Musa* and several other diverse hosts. Using similar sequence data, Crous *et al*. (1999) were able to separate several species occurring on *Eucalyptus*. These authors also found that *M. lateralis*, a species formerly thought to occur only on *Eucalyptus*, has a wide host range.

One of the major impediments to the taxonomy of *Mycosphaerella*, lies in that they are usually identified from host tissue. Cultures of these fungi have seldom been made, and where they are available they tend to grow extremely slowly. They are also not easily maintained in culture collections. In recent years, we have assembled a collection of isolates of *Mycosphaerella* from *Myrtaceae* in order to undertake the present study. Our aim here is to test whether morphological species, many of which are separated on minute details, are also phylogenetically distinct. In doing so, our objective has also been to make available a set of sequence data for as many species as possible. Our overall intention has been that this will facilitate identification of these fungi on *Myrtaceae* in the future.

MATERIALS AND METHODS

The morphology of the species used in this investigation has been examined in previous studies (Crous 1998, 1999). Data on the isolates included in the present study are presented in Table 1; some of these also formed the basis of a previous study (Crous *et al*. 1999). Genomic DNA was isolated from fungal mycelium collected directly from MEA plates using the isolation protocol of Raeder & Broda (1985). DNA quantification was by UV spectroscopy, using a Beckman Du Series 7500 Spectrophotometer.

Two DNA regions were amplified, the ITS1 and 2 regions including the 5.8S gene of the ribosomal RNA operon. The primer pair ITS1 and ITS4 (White *et al*. 1990) were used for this amplification. The 5′ end of the large subunit (LSU) was also sequenced in this study. Based on their morphology and

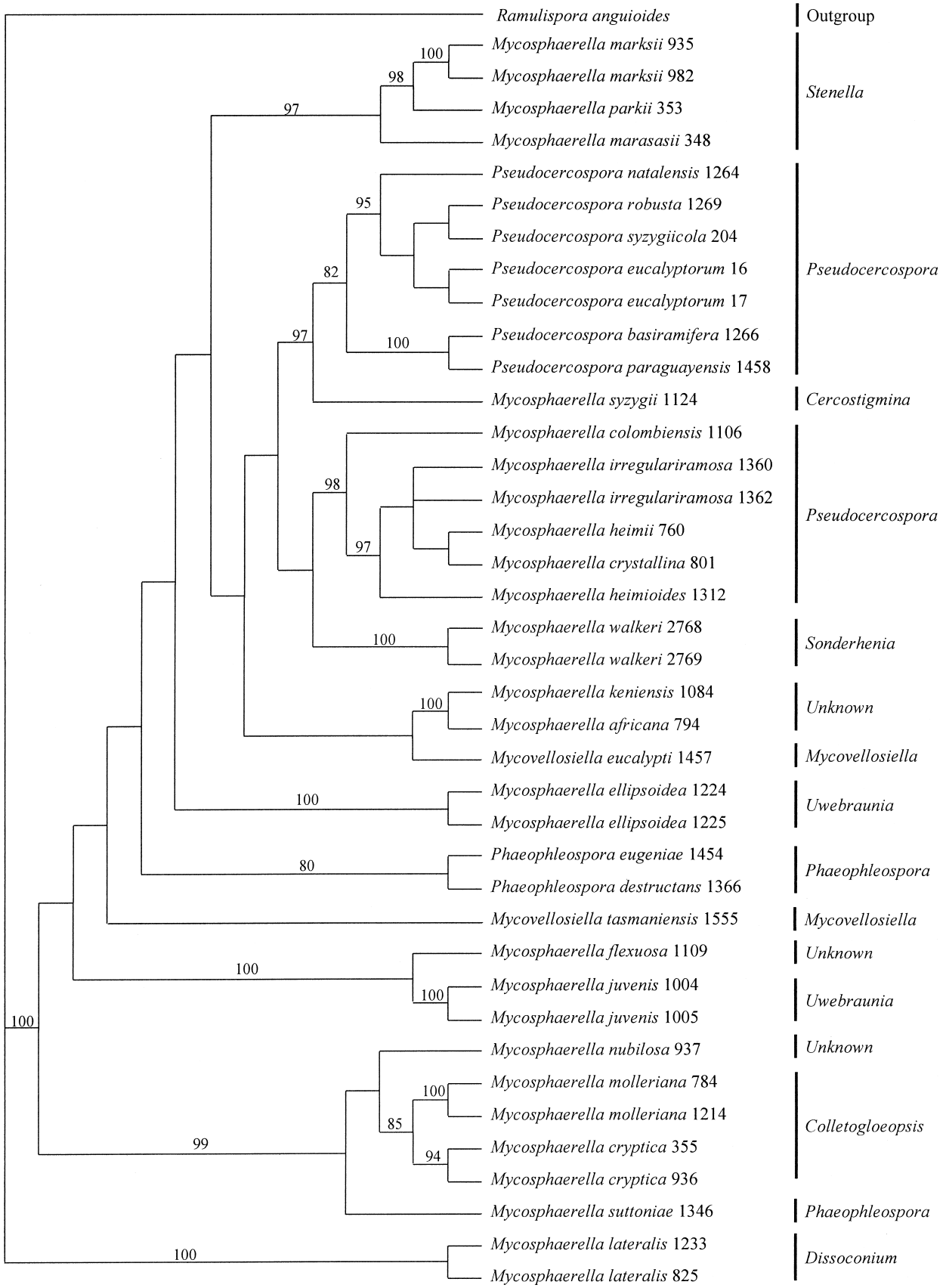

Fig. 1. Cladogram of ITS phylogeny of *Mycosphaerella* species on *Myrtaceae*. One of 20 most parsimonious trees (length = 1411, CI = 0.556, RI = 0.719, HI = 0.444) inferred using heuristic and branch swapping options of PAUP Version 4.0b1. Bootstrap support of 1000 replications is listed above the branches.

the ITS data set, specific isolates were selected (Table 1) for the LSU analysis. Primer pair ITS1 and LR6 were used for the LSU amplification and sequencing.

Template DNA was amplified in a 50 µl PCR reaction using the reagents described in Crous *et al*. (1999). The reaction conditions were as follows: initial denaturation at 96 °C for 2 min, followed by 40 cycles of denaturation at 94 ° for 30 s, annealing at 53° for 30 s, extension at 75 ° for 2 min, and final extension at 75° for 7 min in a Hybaid Omnigene Temperature Cycler (Hybaid, Middx, UK). A negative control using water instead of template DNA was set up for each experiment. PCR products were accessed using agarose gel electrophoresis. The agarose gels were stained with ethidium bromide and visualised under UV illumination. PCR products were purified by using a QIAquick PCR Purification Kit (Qiagen, Germany). The ABI Prism 377 DNA Sequencer (Perkin–Elmer, Norwalk, CON) was used to sequence the amplified fragments. An ABI PRISM™ Dye Terminator Cycle sequencing Ready Reaction Kit (Perkin–Elmer, Warrington) was used according to the manufacturer's recommendations and the resultant reaction run on the DNA sequencer. The DNA sequences were aligned using Clustal and Sequence Navigator™ version 1.0.1 (Perkin–Elmer, Applied Biosystems, Foster City, CA). Further adjustments were made by eye where deemed necessary. Gaps generated in the alignment process were treated as missing data in the analysis. Phylogenetic analysis of aligned DNA sequences was performed using PAUP Version 4.0b1 (Swofford 1998). The most parsimonious trees were produced from the sequence data using the heuristic search. Other measures including tree length, consistency index, retention index and rescaled consistency index (CI, RI and RC) were also determined. All the sequence data generated in this study has been deposited in GenBank (ITS: AF309588-AF309625; LSU: AF309573-AF309587). *Ramulispora anguioides* (Crous *et al*. 1999) and a *Cladosporium* sp. (AF050266) were chosen as outgroups for the ITS and LSU data sets, respectively.

RESULTS

The ITS data set produced 20 most parsimonious trees with a length of 1411 steps (CI = 0.556, RI = 0.719, HI = 0.444) using the heuristic search option with 1000 randomizations of sequence input orders. Of the 657 characters analysed, 338 were parsimony informative. Subsequently, 1000 bootstrap replicates with 1000 random addition sequences were applied. The majority consensus tree (Fig. 1) showed the same topology of the major clades to the most parsimonious tree. A similar clustering was also observed in the neighbor-joining tree (g1 statistic = −0.842, g2 = 1.410). The LSU data set produced 1 most parsimonious tree (Fig. 2) with a tree length of 669 steps (CI = 0.740, RI = 0.694, HI = 0.260) using the heuristic search option with 1000 randomizations of sequence input orders. Of the 886 characters analysed, 102 were parsimony informative. Subsequently, 1000 bootstrap replicates with 1000 random addition sequences were applied (g1 statistic = −1.376, g2 = 2.888). The partition homogeneity test ($P = 0.01000$) indicated that the data sets could not be combined.

The ITS data set resolved a major *Mycosphaerella* clade, as well as a smaller clade with *Dissoconium* anamorphs (Fig. 1). The major clade consisted of several subclades that correlated with the different anamorph genes. Most genera were clearly defined, except *Mycovellosiella*, *Phaeophleospora*, *Pseudocercospora* and *Uwebraunia* that evidently evolved more than once within *Mycosphaerella*. The LSU data set also supported one major and one smaller clade. In contrast to the ITS data set, the separation of *Dissoconium* anamorphs from the major *Mycosphaerella* clade was not supported. Anamorph forms such as *Pseudocercospora* and *Uwebraunia* were also shown to have evolved more than once using the LSU. In general the two data sets agreed, supporting the monophyly of this set of *Mycosphaerella* species.

DISCUSSION

This study has shown that *Mycosphaerella* species on *Myrtaceae* represent a monophyletic assemblage. This emerged from the ITS sequence as well as the LSU data sets. Using a smaller set of isolates, Crous *et al*. (1999) also concluded that the *Mycosphaerella* species from *Myrtaceae* were largely monophyletic. The results of the present study provide further evidence to support this. The *Mycosphaerella* species included in this analysis are all regarded as representatives of section *Plaga* (Barr 1972, Crous *et al*. 2000). Species placed in this section are regarded as plant pathogens, not saprobes, and are characterised by obovoid to ellipsoidal or cylindrical asci, and small to medium sized fusiform to obovoid ascospores with rounded ends. Saprobes with wide host ranges have been placed in other sections of *Mycosphaerella* (Crous *et al*. 2000).

The taxonomy of *Mycosphaerella* species is typically based on characters associated with teleomorph structures on host tissue. Only in recent years has some focus been given to characteristics of these fungi in culture. *Mycosphaerella*'s are isolated with difficulty and because cultures grow slowly, they are prone to contamination and poor revival after storage. It is primarily for this reason that few cultures of these fungi are available for study. This is a major impediment to resolving taxonomic questions relating to this important group of fungi. In the present study, the majority of species considered were from *Eucalyptus*, with a few species from other *Myrtaceae*. It is thus difficult to draw strong inferences concerning the relatedness of the species on *Eucalyptus* and other *Myrtaceae*. However, some patterns did emerge and these present challenging hypotheses for future study.

Our results show that species with *Pseudocercospora* anamorphs clustered in two discrete clades. The first included *P. natalensis*, *P. robusta*, *P. eucalyptorum*, *P. basiramifera* and *P. paraguayensis* from *Eucalyptus*, and *P. syzgiicola* from *Syzygium*. In these species, a range of morphological characters can be found. Species included have smooth or finely verruculose conidia that are olivaceous to medium brown, thin- to thick-walled, straight to flexuous, moderate (3–6) to multi-septate (−11), with hila that are either truncate, or obconically truncate to subtruncate, and with or without lateral branches. Fascicles of conidiophores also vary from loose to dense, and occur on either one or both sides of the leaf.

The second clade represents *Mycosphaerella*'s with *Pseudocercospora* anamorphs that all originate from *Eucalyptus*. It

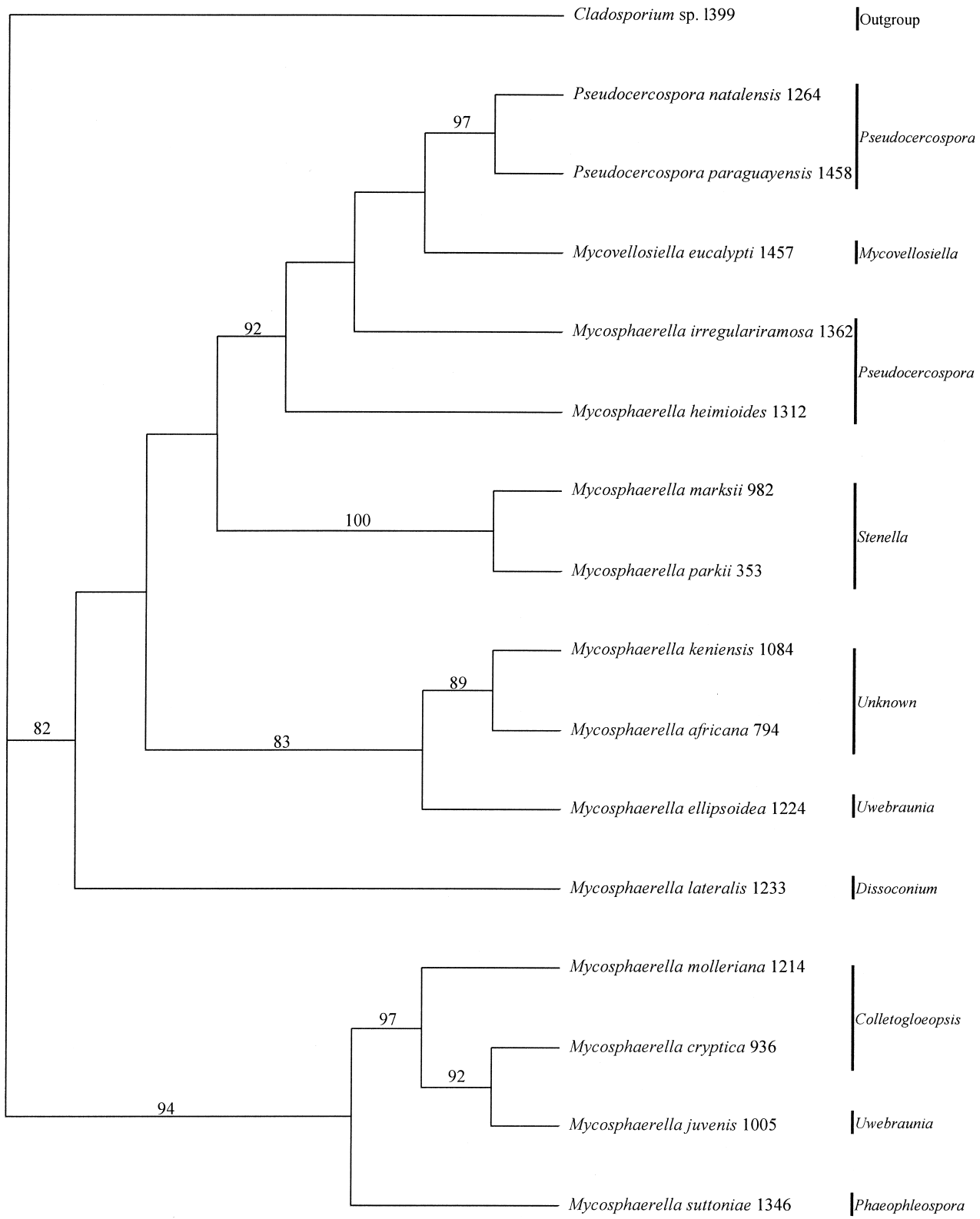

Fig. 2. Cladogram of LSU phylogeny of *Mycosphaerella* species on *Myrtaceae*. The most parsimonious tree (length = 669, CI = 0.740, RI = 0.694, HI = 0.260) inferred using heuristic and branch swapping options of PAUP Version 4.0b1. Bootstrap support of 1000 replications is listed above the branches.

includes *M. heimii*, *M. crystallina*, *M. irregulariramosa*, *M. heimioides* and *M. colombiensis*, the first four species being very closely related. Morphologically, the first four species, which we consider to represent the *M. heimii* complex, are very similar in conidial pigmentation and shape. Species in this clade have pale brown, smooth to finely verruculose, obclavate to subcylindrical conidia, and small, dense fascicles with conidiophores that are frequently reduced to conidiogenous cells. They are also similar in culture, producing red crystals in MEA and water agar. Although their ascospores show some

variation in germination patterns, the ascospore morphology is similar in all three taxa. *M. colombiensis* also occurs in this clade, but clusters separately from the *M. heimii* complex, and is morphologically distinct. Isolates of *M. colombiensis* do not produce red crystals in culture, and generally have shorter and wider conidia than those found in the *M. heimii* complex (Crous 1998).

The genus *Cercostigmina* was erected for pseudocercosporalike species with sporodochial conidiomata and integrated conidiogenous cells that proliferate precurrently rather than sympodially, and produce euseptate, verruculose, acicular-subcylindric, or narrowly obclavate conidia (Braun 1993). Uncertainty existed, however, regarding the separation of this genus from *Pseudocercospora*, as many intermediate forms exist. The present isolate (STE-U 1124) belongs to *Cercostigmina s. str*. Other than in its mode of conidiogenesis, *C. punctata* is morphologically quite distinct from the *Pseudocercospora* species compared in this study; it has relatively dense sporodochial conidiomata and verruculose conidia. In the ITS phylogeny presented here (Fig. 1), it clustered between the two *Pseudocercospora* clades, suggesting that although it is a distinct species, the genus *Cercostigmina* should once again be reduced to synonymy with *Pseudocercospora*.

Species with *Phaeophleospora* anamorphs emerged in two separate clades. *Ph. eugeniae* from *Eugenia* grouped with *Ph. destructans* from *Eucalyptus*, while *Ph. suttoniae* from *Eucalyptus* grouped separately. This suggests that the very distinct *Phaeophleospora* anamorph evolved more than once in *Mycosphaerella*. This study also presents the first molecular evidence that *Ph. eugeniae* is congeneric with other species of *Mycosphaerella*, and that it is an older name for *Kirramyces* (Walker *et al*. 1992), as proposed by Crous *et al*. (1997). It further suggests that teleomorphs of other *Phaeophleospora* species will also belong to *Mycosphaerella*.

When the genus *Kirramyces* was erected, it was speculated that there might be two groups in this genus (Walker *et al*. 1992). One group could be separated based on species with pale brown, smooth to finely verruculose conidia and pycnidial walls of *textura epidermoidea*. Species in the other group have darker brown, more distinctly verruculose conidia, and pycnidial walls of *textura angularis*. The unifying character is the brown, verruculose, percurrently proliferating conidiogenous cells and pigmented conidia. That *Ph. destructans* (pale brown, finely verruculose conidia and conidiogenous cells) clusters with *Ph. eugeniae* (medium to dark brown, verruculose conidia and conidiogenous cells), suggests that these groups should be retained as one genus.

Sonderhenia anamorphs of *Mycosphaerella* species are primarily distinguished from those of *Phaeophleospora* by having transversely distoseptate conidia. Although *M. walkeri* clustered separately from species of *Phaeophleospora*, species of the latter genus clustered in two clades. This indicates that more species will have to be considered to fully address the relationship between *Sonderhenia* and *Phaeophleospora*.

Mycosphaerella molleriana and *M. cryptica* have *Colletogloeopsis* anamorphs that produce acervular conidiomata. Both these species grouped in the same clade in the ITS as well as the LSU phylogeny. This clade, and others with coelomycete anamorphs such as *Sonderhenia* and *Phaeophleospora*, were dispersed amongst hyphomycete taxa with solitary conidiophores. In the LSU tree, *M. molleriana* and *M. cryptica* grouped with *M. juvenis* (*Uwebraunia* anamorph with solitary conidiophores) and *M. suttoniae* (*Phaeophleospora* anamorph with pycnidia). In the ITS phylogeny, species with *Colletogloeopsis* anamorphs clustered in a similar clade, while *M. ellipsoidea* grouped separately. Crous (1998) also reported on a species of *Phaeophleospora* with typical pycnidia but which also forms a *Cercostigmina* synanamorph with conidiophore fascicles in culture. The latter observations also suggest that conidiomatal structure has a relatively low phylogenetic value in the *Mycosphaerella* spp. from *Myrtaceae*. Those genera separated solely on conidiomatal structure (*Pseudocercosporella*, *Septoria* and *Phloeosporella*) clearly need to be re-evaluated (Crous *et al*. 2000).

Although no anamorph is known for *M. marksii*, it clustered with *M. parkii* (*Stenella* anamorph) in a previous study (Crous *et al*. 2000). Here, these two eucalypt leaf pathogens also clustered in the *Stenella* clade with *M. marasasii* from *Syzygium*. This suggests that if an anamorph exists for *M. marksii*, it would probably be a species of *Stenella*. Furthermore, the genera *Mycovellosiella* and *Stenella* both have conidia that can occur solitarily or in chains, are pigmented, and that have thickened, darkened, refractive conidial loci. A previous study (Crous *et al*. 2000) concluded that differences in conidial catenulation are insufficient to separate anamorphs of *Mycosphaerella*. These two genera are, therefore, primarily separated by the verrucose nature of the superficial mycelium in *Stenella*, in contrast to the smooth hyphae of *Mycovellosiella* spp. Although these differences might appear minimal, both analyses in this study (Figs 1–2) still support this separation.

We have shown that most species of *Mycosphaerella* from *Myrtaceae* can be distinguished on sequence data from the ITS and LSU regions of the rRNA operon. In general, the morphological characteristics used to distinguish these species appear to have been upheld, and although differences between species are often relatively small, these can continue to be used with some confidence. *Mycosphaerella* species are important pathogens of valuable crop plants such as *Eucalyptus*. For effective disease management, it is crucial to be able to identify these fungi accurately. Because many species are very similar to each other, mycologists and plant pathologists have found this difficult or even impossible. Sequence data emerging from this study should considerably simplify the identification of *Mycosphaerella* spp. in future. It should likewise promote a more comprehensive understanding of *Mycosphaerella* spp., particularly in the native range of *Eucalyptus*.

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