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A phylogenetic analysis of *Mycosphaerellaceae* leaf spot pathogens of *Proteaceae*

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Many plant pathogenic foliicolous fungi recorded on *Proteaceae* hosts in South Africa are reminiscent of members of the *Mycosphaerellaceae* and their anamorphs. However, these fungi are often unusual and specific to the *Proteaceae*, and have proved difficult to classify in the past. To address this issue, a phylogenetic analysis of the ITS-1, 5.8S and ITS-2 DNA sequence data was performed to determine relationships between members of the *Mycosphaerellaceae* and some taxa apparently unique to the *Proteaceae*. Results from this study have confirmed *Batcheloromyces* to be affiliated to the *Mycosphaerellaceae*. Within *Mycosphaerella*, *Batcheloromyces* was also shown to be distinct from *Stigmina*, which clustered separately from *Pseudocercospora*. The separation between *Mycosphaerella* and *Teratosphaeria* (anamorph *Trimmatostroma*) was shown to be artificial, which was further supported by several *Trimmatostroma* species clustering within *Mycosphaerella*. From these data it is concluded that *Teratosphaeria* should be reduced to synonymy under *Mycosphaerella*, and that *Trimmatostroma* represents yet another additional anamorph of *Mycosphaerella*.

INTRODUCTION

Genera and species of *Proteaceae* are known to host a diverse and unusual range of plant pathogenic foliicolous fungi. *Proteaceae* in some regions have been studied better than those in others, and it has been noted that different taxa occur on native *Proteaceae* members in southern Africa, South America and Australia. The best studied region is southern Africa, particularly the *Proteaceae* in the mediterranean Cape Floral Kingdom (Taylor *et al*. 2001), but also *Proteaceae* in the summer rainfall areas of eastern South Africa, Malawi and Kenya (P. F. Cannon, CABI, pers. comm.). Approximately 65 fungal species have been recorded which are specific to *Proteaceae* in Africa (Taylor *et al*. 2001, Taylor, unpubl.) [mainly in the tribe *Proteeae* (subfamily *Proteoideae*), but also some members in the tribe *Conospermeae* and *Macadamieae* (McCarthy 1995)]. An estimated 50 fungal species have been recorded on Australian *Proteaceae* (Taylor, unpubl.) where members of all seven subfamilies are represented (McCarthy 1995). The greatest diversity of *Proteaceae* fungi probably exists in Australia, for it is here that *Proteaceae* diversity is highest, while the associated fungi are poorly studied (Crous *et al*. 2001c). *Proteaceae* in South America, where all the species are members of subfamily *Grevilleoideae* (McCarthy 1995), are also poorly studied and few fungal species have been recorded.

The foliicolous pathogens on southern African *Proteaceae* have long been problematic in terms of their taxonomic placement. The genus *Teratosphaeria* was originally described from *Protea grandiflora* (now known as *P. nitida*) with the type, *T. fibrillosa*, commonly associated with leaves of this host. Even within the *Proteaceae*, it is seldom recorded on alternative hosts. Other species in this genus include three members associated with *Proteaceae* in South Africa, *Teratosphaeria proteae-arboreae*, *T. maculiformis* and *T. microspora*. In addition, there are two other species, *Teratosphaeria concentrica* (on *Rubiaceae*) and *Teratosphaeria dispersa* (on *Euphorbiaceae*), which occur in Java and Chile, respectively. Müller & Oehrens (1982) stated that the arrangement of the ascomata is characteristic for species of *Teratosphaeria*, and noted that they also possessed periphysate ostioles. Characteristics of the genus include globose, often large ascomata in an extensive stroma, bitunicate asci which produce ascospores which are initially hyaline, but become pale brown (with maturity or discharge) (Müller & Oehrens 1982). The similarity of this genus with *Mycosphaerella*

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has been recognised previously, and the taxon has been placed in *Mycosphaerella*, as *M. proteae* (Müller & von Arx 1962), a synonym of *T. maculiformis* (Taylor & Crous 1998).

Several of the acknowledged anamorphs of *Mycosphaerella* are commonly encountered on *Proteaceae* hosts. These include species of *Cercostigmina*, *Pseudocercospora*, *Septoria* and *Stenella*. *Batcheloromyces* represents a rather unusual genus of hyphomycetes unique to *Proteaceae*. Previous studies have argued, however, that it has affinities to *Stigmina* (Sutton & Pascoe 1989), which is an anamorph of *Mycosphaerella* (Crous *et al*. 2001b).

To resolve the speculation of the apparent affinities of genera such as *Teratosphaeria* and *Batcheloromyces* to *Mycosphaerella*, their phylogeny was determined using parsimony analysis of the rDNA (ITS-1, 5.8S and ITS-2) gene. These results are considered in association with ecological and pathological data of the fungal taxa concerned.

MATERIALS AND METHODS

Isolates and morphology

Cultures that were derived from single conidia or ascospores were obtained from foliicolous fruiting structures on various *Proteaceae* hosts. Conidia and ascospores were cultured on 2% malt extract agar (MEA, Biolab, Midrand, South Africa), and incubated at 25 °C under near-ultraviolet light using the methods of Crous (1998). Subcultures are maintained in the Culture Collection of the Centraalbureau voor Schimmelcultures (CBS). Accession numbers and other data pertaining to the various isolates examined are listed in Table 1.

DNA extraction and amplification

Total DNA of single spore isolates was extracted from 2-wk-old colonies. Fungal mycelia were collected directly from MEA plates (Raeder & Broda 1985). DNA quantification was done by UV spectroscopy using a Beckman Du Series 7500 Spectrophotometer. Template DNA was amplified in a 50 µl PCR reaction by using reagents and primers ITS-1 and ITS-4 described in White *et al*. (1990). The reaction was set up as follows: initial denaturation at 96 ° 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, Middlesex). A negative control using water instead of template DNA was set up for each experiment. The PCR products were separated on a 0.8% (w/v) agarose (Promega, Madison, Wisconsin) gel stained with ethidium bromide and visualised under UV illumination. PCR products were purified by using a QIAquick PCR Purification Kit (Qiagen GmbH, Germany) and sequenced with the PCR primers using the ABI Prism 377 DNA Sequencer (Perkin Elmer, Norwalk, CN), with an ABI PRISM™ Dye Terminator Cycle sequencing Ready Reaction Kit (Perkin Elmer, Warrington). Both kits were used as recommended by the manufacturers.

Phylogenetic analysis

The nucleotide sequences of the 5.8S rRNA gene and the flanking internal transcribed spacers (ITS-1 and ITS-2) were assembled using Sequence Navigator™ version 1.0.1. (Perkin Elmer, Applied Biosystems, Foster City, CA). Alignments of the sequence files were conducted using the CLUSTAL W software (Thompson *et al*. 1994). Adjustments were made by eye where necessary. Alignment gaps were coded as a fifth character in the analysis. Phylogenetic analysis of aligned DNA sequences was performed using PAUP Version 4.0b10 (Swofford 2000). The most parsimonious trees were inferred from the original sequence data set using the heuristic search option with simple and 1000 randomizations of sequence input orders. The clade stability was evaluated by 1000 bootstrap replications. Other measures including tree length, consistency index, retention index and rescaled consistency index (CI, RI and RC) were also calculated. Resulting trees were printed with TreeView Version 1.6.5 (Page 1996) and decay indices were calculated with AutoDecay Version 4.0.2 (Eriksson 1998). Sequence data of the various species were deposited in GenBank (Table 1), and the alignment in TreeBase (submission no. SN1398). *Mycocentrospora acerina* (TreeBase matrix M691, Stewart *et al*. 1999) was used as outgroup.

RESULTS

The alignment of the sequences of the 5.8S ribosomal RNA gene and the flanking internal transcribed spacers (ITS-1 and ITS-2) had a total consensus length of 519 bp, including the alignment gaps. Of the aligned sites, 173 (33.33%) were constant, 140 (26.97%) variable characters were parsimony-uninformative and 206 (39.69%) were informative for use in parsimony. The ITS-1 and ITS-2 showed sequence variation.

The phylogenetic tree (Fig. 1) divided the isolates into eight clades. Although some clades contained more than one anamorph genus (clade 1, *Sonderhenia*, *Septoria*, *Cercospora*, and clade 6, *Stenella* and *Uwebraunia*), most clades represented species of only one anamorph genus, such as clade 2 (*Pseudocercospora*, except for one species of *Stenella*), clade 3 (*Pseudocercospora*), clade 4 (*Stigmina*), clade 5 (*Trimmatostroma*, except for one species of *Colletogloeopsis*), clade 7 (*Batcheloromyces*), clade 8 (*Stenella*).

DISCUSSION

The present study has provided valuable information to further assist in the circumscription and delimitation

Table 1. Fungal isolates included for ITS sequence analysis.

Accession no.[a]	Anamorph	Teleomorph	Collection details	GenBank no. (ITS)
STE-U 1837, CBS 110892	*Batcheloromyces leucadendri*	–	*Leucadendron* sp., Stellenbosch, RSA, *L. Swart*	AY260100
STE-U 1838, CBS 11577	*Batcheloromyces leucadendri*	–	*Leucadendron laureolum*, Stellenbosch, RSA, *L. Swart*	AY260101
STE-U 1518, CBS 110696	*Batcheloromyces proteae*	–	*Protea cynaroides*, Stellenbosch, RSA, *L. Swart*	AY260099
STE-U 1869	*Cercostigmina protearum* var. *leucadendri*	–	*Leucadendron* sp., Stellenbosch, RSA, *P. W. Crous*	AY260089
STE-U 1321, CBS 110891	–	*Mycosphaerella bellula*	*Leucospermum* sp., Stellenbosch, RSA, *P. W. Crous*	AY260092
STE-U 2120	–	*Mycosphaerella bellula*	*Protea repens*, Montagu, RSA, *G. Matthews*	AY260091
STE-U 2161, CBS 110695	–	*Mycosphaerella holualoana*	*Leucospermum* sp., Kona, Hawaii, *P. W. Crous*	AY260088
STE-U 2155, CBS 110699	–	*Mycosphaerella holualoana*	*Leucospermum* sp., Holualoa, Hawaii, *P. W. Crous & M. E. Palm*	AY260084
STE-U 2126, CBS 110698	–	*Mycosphaerella holualoana*	*Leucospermum* sp., Holualoa, Hawaii, *P. W. Crous & M. E. Palm*	AY260087
STE-U 2125, CBS 111028	*Pseudocercospora* sp.	*Mycosphaerella konae*	*Leucadendron* 'Safari Sunset', Holualoa, Hawaii, *P. W. Crous & M. E. Palm*	AY260085
STE-U 2123, CBS 11261	*Pseudocercospora* sp.	*Mycosphaerella konae*	*Leucadendron* 'Safari Sunset', Holualoa, Hawaii, *P. W. Crous & M. E. Palm*	AY260086
STE-U 2179, CBS 110697	*Stenella* sp.	*Mycosphaerella waimeana*	*Leucospermum* Hybrid 24, Waimea, Hawaii, *P. W. Crous & M. E. Palm*	AY260083
STE-U 1470	*Septoria protearum*	–	*Protea cynaroides*, Pretoria, RSA, *L. Swart*	AY260081
STE-U 5212, CBS 110700	*Septoria protearum*	–	*Protea* sp., Tenerife, Spain, *S. Denman*	AY260082
STE-U 4299, IMI 136770, CBS 110755	*Stigmina platani*	–	*Platanus orientalis*, India	AY260090
STE-U 1876	–	*Mycosphaerella fibrillosa*	*Protea nitida*, Cederberg, RSA, *J. E. Taylor*	AY260094
STE-U 1872, CBS 110756	*Trimmatostroma macowanii*	–	*Protea nitida*, Cederberg, RSA, *J. E. Taylor*	AY260095
STE-U 1488, CBS 111029	*Trimmatostroma macowanii*	–	*Protea* sp., Hermanus, RSA, *P. W. Crous*	AY260096
STE-U 1832, CBS 110890	*Trimmatostroma microspora*	*Mycosphaerella microspora*	*Protea cynaroides*, Stellenbosch, RSA, *L. Swart*	AY260097
STE-U 1848, CBS 111031	*Trimmatostroma microspora*	*Mycosphaerella microspora*	*Protea cynaroides*, Somerset West, RSA, *J. E. Taylor*	AY260098
STE-U 1958, CBS 111030	*Trimmatostroma elginensis*	–	*Protea grandiceps*, Elgin, RSA, *J. E. Taylor & S. Denman*	AY260093

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of the genus *Mycosphaerella*. Although *Mycosphaerella* is presently one of the largest genera of ascomycetes, several previous studies have delineated it from other genera (Crous *et al*. 2000, 2001b). This has also led to a re-evaluation of anamorph concepts, and a reduction in the number of anamorph genera linked to *Mycosphaerella*. The present study has done the opposite, however, namely to again extend the number of anamorph genera linked to *Mycosphaerella*.

The genus *Teratosphaeria* is similar in morphology to *Mycosphaerella*, but has been separated on the basis of its ascomatal arrangement and periphysate ostioles (Müller & Oehrens 1982). Placement of this genus has proved difficult. Currently *Teratosphaeria* is placed in the *Pleosporaceae* (*Pleosporales*) (Kirk *et al*. 2001). However, in the past the genus has been placed in the *Clypeosphaeriaceae*, *Montagnellaceae*, *Pleosporaceae*, *Stigmateaceae* (Syn. *Venturiaceae*) and *Phaeosphaeriaceae* (Müller & Oehrens 1982). Of the current species recognised in *Teratosphaeria*, *T. maculiformis* has caused the most confusion. This species was introduced as *Didymella maculiformis*, but was removed to *Mycosphaerella* as *M. proteae*, and was subsequently placed in *Teratosphaeria* as *T. maculiformis*, due to its prominently developed periphysoid ostioles (Taylor & Crous 1998). The first anamorph genus associated

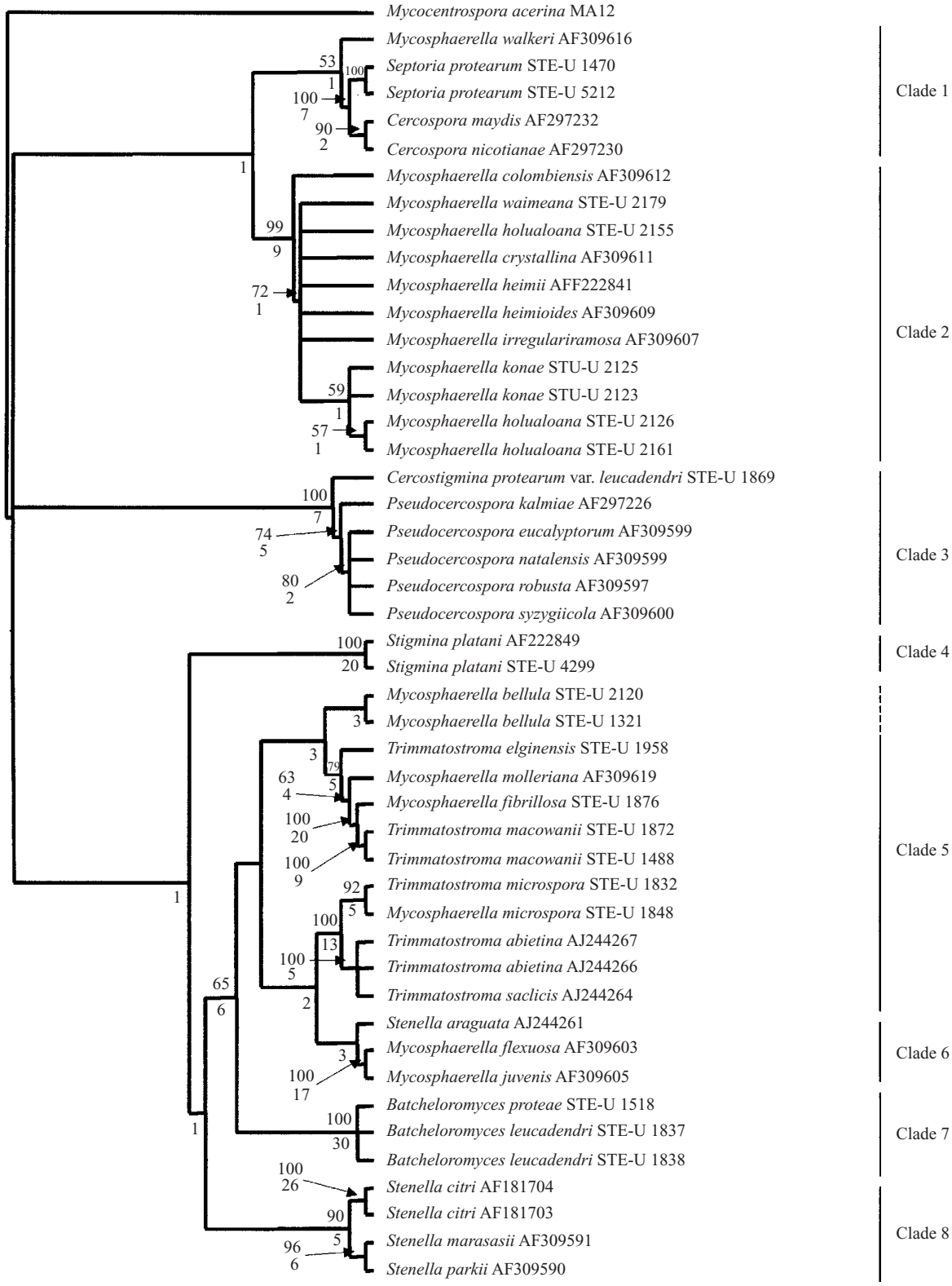

Fig. 1. Strict consensus of 40 most parsimonious heuristic trees (TL = 1172 steps, CI = 0.560, RI = 0.753, RC = 0.421, HI = 0.440). Bootstrap support values from 1000 replicates and decay values are shown at the nodes, above and below the branches, respectively. *Mycocentrospora acerina* was included as outgroup.

with a species of *Teratosphaeria* was *Trimmatostroma*. Taylor & Crous (2000) recorded a fourth species of *Teratosphaeria* from *Proteaceae* which was notably similar to *T. maculiformis*, but which produced a *Trimmatostroma* anamorph in culture and on the host material. The first evidence that *Trimmatostroma* could be an anamorph of *Mycosphaerella* was provided from a molecular study by de Hoog *et al.* (1999). These findings are supported by those of the present study, where *Teratosphaeria microspora*, which has a

Trimmatostroma anamorph, clustered within *Mycosphaerella*. Furthermore, *Teratosphaeria fibrillosa*, the type of *Teratosphaeria*, also clustered closely with other well-known species of *Mycosphaerella* (clade 5). This finding suggests that *Teratosphaeria* (1912) and *Mycosphaerella* (1884) are synonymous.

Before the advent of molecular DNA techniques, studies of *Mycosphaerella* tended to combine morphological and ecological characteristics for species identification. Because *Mycosphaerella* teleomorphs tend to not vary dramatically in their morphology, special emphasis was placed on their anamorphs for identification, and to determine relatedness, especially by means of cultural comparisons (Crous *et al*. 2000). Furthermore, ecological information such as the host, the relationship with the host and the distribution of taxa were also taken into account. When all of the above information is reviewed for the species of *Teratosphaeria* from *Proteaceae*, there are some interesting observations. Firstly, the teleomorph morphology does not fit comfortably in any of the sections proposed for *Mycosphaerella* (Crous *et al*. 2000). Being foliicolous pathogens, species of *Teratosphaeria* would best be accommodated in section *Plaga*, though their asci and ascospores resemble those of section *Tassiana*, and none of these sections have ever been linked to *Trimmatostroma* anamorphs (Crous *et al*. 2000). It is clear, therefore, that the present finding will also require a redefinition of the sections proposed within *Mycosphaerella* (Crous *et al*. 2000). Furthermore, these data also provide further proof that species of *Trimmatostroma* should be regarded as anamorphs of *Mycosphaerella*. Of the five species of *Teratosphaeria* presently known, *T. dispersa* appears to have affinities elsewhere. Müller & Oehrens (1982) describe asci in young pseudothecia to be separated by filiform paraphysoids, which is atypical of the genus *Mycosphaerella*. The remaining four species, however, belong to *Mycosphaerella*.

Mycosphaerella concentrica (Racib.) Joanne E. Taylor & Crous, **comb. nov.**

Basionym: *Gibbelina concentrica* Racib., *Parasit. Alg. Pilze Java's* (Jakarta) **2**: 11 (1900).

Teratosphaeria concentrica (Racib.) E. Müll., *Beitr. Kryptogamenfl. Schweiz* **11** (2): 316 (1962).

Mycosphaerella fibrillosa (Syd. & P. Syd.) Joanne E. Taylor & Crous, **comb. nov.**

Basionym: *Teratosphaeria fibrillosa* Syd. & P. Syd., *Annls Mycol*. **10**: 40 (1912).

Mycosphaerella microspora (Joanne E. Taylor & Crous) Joanne E. Taylor & Crous, **comb. nov.**

Basionym: *Teratosphaeria microspora* Joanne E. Taylor & Crous, *Mycol. Res*. **104**: 631 (2000); as '*microsporum*'.

Anamorph: *Trimmatostroma microspora* Joanne E. Taylor & Crous, *Mycol. Res*. **104**: 631 (2000).

Mycosphaerella proteae (Syd.) Arx, *Beitr. Kryptogamenfl. Schweiz* **11** (2): 357 (1962).

Basionym: *Oligostroma proteae* Syd., *Annls Mycol*. **12**: 265 (1914).

Didymella maculiformis G. Winter, *Hedwigia* **23**: 169 (1884).

Oligostroma maculiformis (G. Winter) Doidge, *Bothalia* **1**: 31 (1921).

Teratosphaeria maculiformis (G. Winter) Joanne E. Taylor & Crous, *IMI Descr. Fungi Bact*. **1346**: 1 (1998).

Euryachora maculiformis Nel, *Annls Univ. Stellenbosch, ser. A* **20** (2): 11 (1942).

Mycosphaerella proteae-arboreae (P. S. van Wyk, Marasas & Knox-Dav.) Joanne E. Taylor & Crous, **comb. nov.**

Basionym: *Teratosphaeria proteae-arboreae* P. S. van Wyk, Marasas & Knox-Dav., *J. S. Afr. Bot*. **41**: 232 (1975).

Several anamorphs of *Mycosphaerella* have been recorded from *Proteaceae*. The close association between *Cercospora* and *Septoria* (Crous *et al*. 2001b) is again seen in clade 1 of the present study. The decision to merge *Cercostigmina* with *Pseudocercospora* (Crous *et al*. 2001a) is also supported in the present study, where *Cercostigmina proteae* var. *leucadendri* clustered with species of *Pseudocercospora*. Furthermore, as found for the *Mycosphaerella* spp. occurring on *Myrtaceae*, species of *Pseudocercospora* occurring on *Proteaceae* have also evolved more than once in *Mycosphaerella* (Fig. 1).

The genus *Batcheloromyces* was introduced by Marasas *et al*. (1975) for dematiaceous hyphomycetes that cause leaf spots on members of the *Proteaceae*. Species of *Batcheloromyces* are characterised by forming numerous sporodochial conidiomata, and having conidiogenous cells with ragged, irregular annellations that produce verrucose, predominantly single-celled conidia that can also be arranged in false chains (Taylor *et al*. 1999). *Batcheloromyces* was treated by Sutton & Pascoe (1989), who concluded that it was congeneric with *Stigmina*. Taylor *et al*. (1999) argued that these two genera should be retained as separate entities. This was mainly based on the fact that species of *Stigmina* have distoseptate conidia that are borne singly. In contrast, those of *Batcheloromyces* are transversely euseptate, and can be borne in false chains. To add to the confusion, Crous *et al*. (2001b) concluded from a further molecular study that distoseptation and conidial catenulation were unreliable for distinguishing anamorph genera in *Mycosphaerella*, which once again raised questions about the tenability of separating *Batcheloromyces* and *Stigmina*. Furthermore, the single isolate of *Stigmina platani* investigated, also appeared closely related to species of *Pseudocercospora* (Crous *et al*. 2001b).

From the results of the present study (Fig. 1), it can be seen that *Stigmina platani* (type of *Stigmina*)

(clade 4) is clearly resolved from *Pseudocercospora* (clades 2, 3) and *Batcheloromyces* (clade 7). These results are in support of ecological and pathological data that suggest *Batcheloromyces* to be endemic to South African *Proteaceae*. Of further interest, is the fact that *Batcheloromyces* is here also shown to represent yet another anamorph genus within *Mycosphaerella*.

One issue not yet fully resolved entails to separation of genera in *Passalora* from *Pseudocercospora* and *Stenella* (Crous *et al*. 2001b). Although it appears that all three anamorph genera have evolved more than once within *Mycosphaerella*, and that these genera are best retained as separate entities, examples have been found of *Passalora* morpho-types clustering within *Pseudocercospora* (Crous *et al*. 2001b), or as seen in Fig. 1, *Mycosphaerella waimeana* (*Stenella* anamorph) clustering within *Pseudocercospora* (clade 2). The explanation offered by Crous *et al*. (2001b) was that the answer might lie in the degree of scar thickening (as has been the case with *Paracercospora* and *Pseudocercospora*), and that species in which the scars are slightly thickened, may be more likely to cluster with *Pseudocercospora* than *Passalora*. This definition was largely provided to try and explain the few odd species that clustered outside of their normal clades. As a consequence, however, it makes the normal taxonomic treatment of species in this complex extremely complicated. In Crous *et al*. (2001b), the position of *Stigmina* was also not resolved. However, the addition of one additional sequence, as done here, has clearly separated *Stigmina* (clade 4) from *Pseudocercospora* (clades 2, 3). Given the fact that many of these anamorph genera have evolved more than once within *Mycosphaerella*, it is quite possible, therefore, that as more species are added to the larger phylogeny, many of these odd isolates will eventually cluster in their own clades representative of species of *Passalora*, *Pseudocercospora* or *Stenella*.

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