A phylogenetic analysis of Mycosphaerellaceae leaf spot pathogens of Proteaceae

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Received 30 September 2002; accepted 16 April 2003.

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 Proteaceae (subfamily Proteoideae), but also some members in the tribe Conospermaeae and Macadamiae (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

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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* spp. of *Proteaceae* hosts. These include species of *Cercostigma, Pseudocercospora, Septoria* and *Stenella. Batheloromyces* 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 the ABI Prism 377 DNA Sequencer at 25°C (MEA, Biolab, Midrand, South Africa), and incubated for 2 wk-old colonies. Fungal mycelia were collected directly from MEA plates (Raeder & Broda 1985). DNA quantification was done by UV spectroscopy using the ABI Prism 377 DNA Sequencer at 25°C (MEA, Biolab, Midrand, South Africa), and incubated for 2 wk-old colonies. Fungal mycelia were collected directly from MEA plates (Raeder & Broda 1985). DNA quantification was done by UV spectroscopy using the ABI Prism 377 DNA Sequencer at 25°C (MEA, Biolab, Midrand, South Africa), and incubated for 2 wk-old colonies. Fungal mycelia were collected directly from MEA plates (Raeder & Broda 1985). DNA quantification was done by UV spectroscopy using the ABI Prism 377 DNA Sequencer at 25°C (MEA, Biolab, Midrand, South Africa), and incubated for 2 wk-old colonies. Fungal mycelia were collected directly from MEA plates (Raeder & Broda 1985). DNA quantification was done by UV spectroscopy using the ABI Prism 377 DNA Sequencer at 25°C (MEA, Biolab, Midrand, South Africa), and incubated for 2 wk-old colonies.

**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, *Sonnerentia, 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*), and clade 8 (*Stenella*).

**DISCUSSION**

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

Table 1. Fungal isolates included for ITS sequence analysis.

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Anamorph</th>
<th>Teleomorph</th>
<th>Collection details</th>
<th>GenBank no. (ITS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STE-U 1387, CBS 110892</td>
<td>Batcheloromyces leucadendri</td>
<td>–</td>
<td>Leucadendron sp., Stellenbosch, RSA, L. Swart</td>
<td>AY260100</td>
</tr>
<tr>
<td>STE-U 1388, CBS 11577</td>
<td>Batcheloromyces leucadendri</td>
<td>–</td>
<td>Leucadendron laevoeham, Stellenbosch, RSA, L. Swart</td>
<td>AY260101</td>
</tr>
<tr>
<td>STE-U 1518, CBS 110896</td>
<td>Batcheloromyces proteae</td>
<td>–</td>
<td>Protea cynaroides, Stellenbosch, RSA, L. Swart</td>
<td>AY260099</td>
</tr>
<tr>
<td>STE-U 1820 – STE-U 1321, CBS 110891</td>
<td>Cercostigmnia protearum var. leucadendri</td>
<td>–</td>
<td>Leucospermum sp., Stellenbosch, RSA, P. W. Crous</td>
<td>AY260089</td>
</tr>
<tr>
<td>STE-U 2123, CBS 110895</td>
<td>Mycosphaerella bellula</td>
<td>–</td>
<td>Leucospermum sp., Montagu, RSA, G. Matthews</td>
<td>AY260091</td>
</tr>
<tr>
<td>STE-U 2155, CBS 110899</td>
<td>Mycosphaerella holualoana</td>
<td>–</td>
<td>Leucospermum sp., Kona, Hawaii, P. W. Crous</td>
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</tr>
<tr>
<td>STE-U 2126, CBS 110698</td>
<td>Mycosphaerella holualoana</td>
<td>–</td>
<td>Leucospermum sp., Holualoa, Hawaii, P. W. Crous &amp; M. E. Palm</td>
<td>AY260084</td>
</tr>
<tr>
<td>STE-U 2179, CBS 110697</td>
<td>Stenella sp.</td>
<td>Mycosphaerella wainemanu</td>
<td>Leucospermum Hybrid 24, Waimea, Hawaii, P. W. Crous &amp; M. E. Palm</td>
<td>AY260083</td>
</tr>
<tr>
<td>STE-U 1470</td>
<td>Septoria protearum</td>
<td>–</td>
<td>Protea cynaroides, Pretoria</td>
<td>AY260081</td>
</tr>
<tr>
<td>STE-U 5212, CBS 110700</td>
<td>Septoria protearum</td>
<td>–</td>
<td>Protea sp., Tenerife, Spain, S. Denman</td>
<td>AY260082</td>
</tr>
<tr>
<td>STE-U 4299, IMI 136770, CBS 110755</td>
<td>Stigmatina platani</td>
<td>–</td>
<td>Platanus orientalis, India</td>
<td>AY260090</td>
</tr>
<tr>
<td>STE-U 1876</td>
<td>–</td>
<td>Mycosphaerella fibrillosa</td>
<td>Protea nitida, Cederberg, RSA, J. E. Taylor</td>
<td>AY260094</td>
</tr>
<tr>
<td>STE-U 1872, CBS 110756</td>
<td>Trimmatostroma macowanii</td>
<td>–</td>
<td>Protea nitida, Cederberg, RSA, J. E. Taylor</td>
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</tr>
<tr>
<td>STE-U 1488, CBS 111029</td>
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<td>Protea sp., Hermanus, RSA, P. W. Crous</td>
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<tr>
<td>STE-U 1832, CBS 110890</td>
<td>Trimmatostroma micropora</td>
<td>Mycosphaerella micropora</td>
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<tr>
<td>STE-U 1848, CBS 111031</td>
<td>Trimmatostroma micropora</td>
<td>Mycosphaerella micropora</td>
<td>Protea cynaroides, Somerset, West, RSA, J. E. Taylor</td>
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<td>STE-U 1958, CBS 110130</td>
<td>Trimmatostroma elginensis</td>
<td>–</td>
<td>Protea grandiceps, Elgin, RSA, J. E. Taylor &amp; S. Denman</td>
<td>AY260093</td>
</tr>
</tbody>
</table>
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 telemorphs 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 telemorph morphology does not fit comfortably in any of the sections proposed for Mycosphaerella (Crous et al. 2000). Being prolific 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.


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


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


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


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 congerenic 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 eusepate, 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 conidioidal 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.

ACKNOWLEDGEMENTS

The authors are grateful to Kang Ji-Chuan for technical advice. J.T. would like to thank the South African National Research Foundation for post-doctoral support, enabling this study to take place.

REFERENCES


Corresponding Editor: D. L. Hawksworth

Mycosphaerella spp. of Proteaceae