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Eyespot of cereals revisited: ITS phylogeny reveals new species relationships

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Abstract

Four species so far classified in *Pseudocercosporella* or *Ramulispora* (hyphomycetes) are associated with eyespot disease symptoms of cereals. Two of these have been linked to teleomorphs that were described in *Tapesia*. Sequence data derived from the Internal Transcribed Spacer region (ITS1, 5.8S and ITS2) of the rDNA operon showed, however, that the eyespot fungi associated with *Tapesia* are not congeneric with *Ramulispora sorghi*, the type of *Ramulispora*. The genus name *Tapesia* is now rejected in favour of the conserved name *Mollisia*, which appears to comprise heterogeneous fungi. *Tapesia yallundae* is not closely related to the type of *Mollisia*, *M. cinerea*, but clusters separately, being more closely allied to species with *Cadophora* anamorphs. A new holomorph genus, *Oculimacula,* is therefore proposed for teleomorphs of the eyespot fungi, while the anamorphs are accommodated in *Helgardia* gen. nov.

Introduction

Eyespot disease of cereals is widespread throughout the temperate regions of the world, and causes a damaging stem-base infection of these hosts (Fitt et al., 1990). Severe eyespot lesions girdle the stem and soften the stem-base, resulting in lodging and heavy crop losses (Scott and Hollins, 1974). Four cercosporoid species are known to be associated with eyespot disease of cereals (Nirenberg, 1981; Robbertse et al., 1995), while a sexual state is known for two of these species (Robbertse et al., 1995). The cercosporoid species associated with eyespot disease are rather unusual in resembling leaf spot pathogens of *Pseudocercosporella* Deighton.

The eyespot fungus was originally described as *Cercosporella herpotrichoides* Fron (Fron, 1912). Deighton (1973) established the new genus *Pseudocercosporella* for anamorphs of *Mycosphaerella* Johanson that were *Cercosporella*-like, but had unthickened and inconspicuous conidial scars. He included *C. herpotrichoides* in this genus. Nirenberg (1981) found that the best-known eyespot fungus on wheat, *Pseudocercosporella herpotrichoides*, includes two varieties, *P. herpotrichoides* (Fron) Deighton var. *herpotrichoides* and var. *acuformis* Nirenberg. These varieties were initially thought to correlate with two pathotypes, respectively known as the wheat-type (W-type) and the rye-type (R-type) (Priestley et al., 1992), though an examination of more strains found this to not always be the case (Lucas et al., 2000). In her treatment of this complex, Nirenberg (1981) followed Deighton (1973), and chose *Pseudocercosporella* in which to place *C. herpotrichoides* together with the new variety, as well as two new species which she described from eyespot lesions on cereals in Germany, namely *P. anguioides* Nirenberg and *P. aestiva* Nirenberg.

Nirenberg's treatment received wide recognition and was the first to highlight the fact that several taxa are involved in this disease complex. Von Arx

(1983), however, recognized that the eyespot fungi are unrelated to the *Mycosphaerella* anamorphs included in *Pseudocercosporella*. He observed them to have a mode of conidiogenesis similar to that of *Ramulispora sorghi* (Ellis & Everh.) Olive & Lefebvre, the type of *Ramulispora* Miura. He also found that conidia in all these species developed lateral branches. Robbertse et al. (1995) later demonstrated that the lateral conidial branches were, in most cases, the result of microcyclic conidiation, which is not uncommon among the cercosporoid taxa (Fernandez et al., 1991).

Von Arx (1983) expanded the genus *Ramulispora* to include those species that are indeed *Pseudocercosporella*-like, with or without lateral branches in the conidia that are formed in slimy masses, and parasitize the culm base of gramineous hosts. He transferred *P. herpotrichoides* to *Ramulispora* and indicated that the other species treated by Nirenberg (1981) also had to be allocated in this genus. This recommendation was followed by Boerema et al. (1992), in their treatment of the two varieties of *R. herpotrichoides*. In a later revision of this species complex, Robbertse et al. (1995) found that the two varieties shared a very low percentage RAPD similarity, exhibited differences in spore and colony morphology, infection pathway, fungicide sensitivity, virulence to specific hosts (Scott and Hollins, 1980) and distinct mating populations (Daniels et al., 1991; Dyer et al., 1994; Robbertse et al., 1994). These taxa were therefore recognized as separate species of *Ramulispora* (Robbertse et al., 1995), a genus known to represent pathogens of gramineous plants (Von Arx, 1983; Braun, 1995).

The discovery that the teleomorphs of the eyespot pathogens were actually discomycetes belonging to the genus *Tapesia* (Pers.) Fuckel (Wallwork and Spooner, 1988; Boerema et al., 1992) seemed to support the position taken by Von Arx (1983), namely to remove these pathogens from the *Mycosphaerella* anamorphs in *Pseudocercosporella*. *Tapesia* resides well outside *Mycosphaerella* (Stewart et al., 1999) in the *Helotiales*. But *Tapesia* is now recognized to be congeneric with species of the younger but better-known genus *Mollisia* (Fr.) P. Karst. (Dennis, 1968; Baral, 1985), and the name was therefore rejected in favour of the conserved name *Mollisia* (Hawksworth and David, 1989). Species of *Tapesia* thus require transfer to the recognized generic name *Mollisia*.

Ramulispora is typified by *R. sorghi*, a pathogen that causes prominent leaf spots on sorghum called sooty stripe, due to the abundant production of microsclerotia on the leaf surface (Olive et al., 1946; Braun, 1995). The latter pathogen was recently encountered on sorghum in the KwaZulu-Natal Province of South Africa, where it was associated with a severe outbreak of sooty leaf stripe (Mchau et al., 1996). In an attempt to clarify the taxonomic position of *R. sorghi*, as well as the eyespot pathogens of cereals, the present study was undertaken to infer a phylogeny for these fungi in comparison with other members representing their respective anamorph (*Ramulispora*) and teleomorph (*Tapesia*) genera. This was achieved by sequencing the Internal Transcribed Spacer region (ITS1, 5.8S and ITS2) of the rDNA operon, and comparing sequence data from the eyespot and *Ramulispora* isolates with those of known *Mycosphaerella* species (Crous et al., 2001).

Materials and methods

Isolates and DNA amplification

Isolates studied were obtained from the culture collections of the Centraalbureau voor Schimmelcultures (CBS), and the Department of Plant Pathology at the University of Stellenbosch (STE-U) (Table 1). Single-conidium subcultures were grown on malt extract agar (Biolab, Midrand, Johannesburg) (MEA) plates for 7 days. The isolation protocol of Crous et al. (2000) was used to isolate genomic DNA from fungal mycelia grown on MEA plates. The primers ITS1 (5′TTT CCG TAG GTG AAC CTG C3′) and ITS4 (5′TCC TCC GCT TAT TGA TAT GC′) (White et al., 1990) were used to amplify part of the nuclear rRNA operon using polymerase chain reaction (PCR). The amplified region included the 3′ end of the 18S (small subunit) rRNA gene, the first ITS (ITS1), the 5.8S rRNA gene, the second ITS (ITS2) region and the 5′ end of the 26S (large subunit) of the rRNA gene. The reaction mixture contained 5 µl of diluted sample, 1 × buffer, 8 mM $MgCl_2$, 500 µM of each of the dNTPs, 2.5 U (Bioline) Taq polymerase and 10 pM of each primer and made up to a total volume of 25 µl with sterile water. The cycling conditions comprised denaturing at 96 °C for 5 min, followed by 30 cycles of denaturation at 96 °C (30 s), annealing 55 °C (30 s) and elongation at 72 °C (90 s). A final elongation step at 72 °C for 7 min was included. PCR products were separated by electrophoresis at 75 V for 1 h in a 0.8% (w/v) agarose gel in 0.5 × TAE buffer (0.4 M Tris, 0.05 M NaAc and 0.01 M EDTA, pH 7.85) and visualized under UV light

Table 1. Strains sequenced in the present study

Teleomorph	Anamorph	Accession no.	Collector	Substrate	Origin	GenBank no. (ITS)
M. cinerea	Unknown	STE-U 5092 = CBS 412.81	O. Petrini	*Juniperus communis*	Switzerland	AY259135
M. dextrinospora	Unknown	STE-U 5093 = CBS 401.78	R.P. Korf	Decaying wood	Spain	AY259134
M. fusca	*T. fusca*	CBS 234.71	B. Aebi	*Fagus sylvatica*	Switzerland	AY259138
M. fusca	*T. fusca*	CBS 486.48	Unknown	*Azalea* sp.	Netherlands	AY259137
M. melaleuca	Unknown	STE-U 5094 = CBS 89.84	H. Butin	*Picea abies* needle	Germany	AY259136
Mycosphaerella capsellae	*P. capsellae*	CBS 112032, 112033	R. Evans	*Pisum sativum*	UK	AY259139, AY259140
Unknown	*R. sorghi*	STE-U 905 = CBS 110578	D. Nowell	*Sorghum bicolor*	South Africa	AY259131
Unknown	*R. sorghi*	STE-U 906 = CBS 110579	D. Nowell	*Sorghum bicolor*	South Africa	AY259132
Unknown	*R. sorghi*	STE-U 908 = CBS 110580	D. Nowell	*Sorghum bicolor*	South Africa	AY259133

using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, UK) following ethidium bromide staining.

Polymerase chain reaction products were purified using a NucleoSpin Extract 2 in 1 Purification Kit (Macherey-Nagel GmbH, Germany). The cycle sequencing reaction with 20–40 ng of purified PCR products and 10 pmol primer in a total volume of 10 µl was carried out with an ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA, USA) containing AmpliTaq DNA Polymerase. The reaction was set up as denaturing at 94 °C for 5 min, followed by 25 cycles of 96 °C for 10 s, 55 °C for 10 s and 60 °C for 4 min, with a final incubation of 30 s at 60 °C. The resulting fragments were analysed on an ABI Prism 3100 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut).

Phylogenetic analysis

The nucleotide sequences of the rDNA gene generated in this study were added to the outgroup, *Botryosphaeria dothidea* (Moug.) Ces. & De Not. (AF027741) and other sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/) and TreeBASE (http://www.treebase.org/), which were assembled using Sequence Alignment Editor v2.0 (Rambaut, 2002). The sequences were aligned using CLUSTAL W software (Thompson et al., 1994). Adjustments for improvement were made by eye where necessary. Phylogenetic analyses were undertaken using PAUP Version 4.0b10 (Swofford, 2000). Alignment gaps were treated as missing characters and all characters were unordered and of equal weight. Heuristic searches were conducted using 1000 replicates of random addition sequences and tree bisection and reconstruction (TBR) as the branch-swapping algorithm to find maximum parsimony trees. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees was evaluated by 1000 bootstrap replications (Hillis and Bull, 1993). 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 1.6.6 (Page, 1996) and decay indices were calculated with AutoDecay Version 4.0.2 (Eriksson, 1998).

Results

Phylogenetic analysis

Approximately 520–560 bases were determined for each isolate, of which approximately 450–490 bases per sequence (spanning ITS1, 5.8S rRNA gene, ITS2 and the first part of the small subunit gene) were added to the alignment. The manually adjusted alignments of the nucleotide sequences contained 601 characters including alignment gaps (data not shown). Of the aligned nucleotide sites for the data set, 245 characters were parsimony-informative, 61 variable characters were parsimony-uninformative and 295 were constant. Sequences were deposited in GenBank (Table 1), and the alignment in TreeBASE (SN 1392).

Aligned sequences of 39 isolates and an outgroup were subjected to maximum parsimony analysis using the heuristic search option with 1000 random taxon-additions in PAUP (Swofford, 2000). The 14th most parsimonious tree obtained from the heuristic search was evaluated with 1000 bootstrap replications. The

three *R. sorghi* isolates (STE-U 905, 906 and 908) grouped in a strongly supported clade (100%), sharing 55% support with a subclade containing *P. capsellae* (Ellis & Everh.) Deighton (*M. capsellae* A.J. Inman & Sivan.) within *Mycosphaerella* (Figure 1). Species of *Mollisia* and *Tapesia* grouped in a large clade (100% bootstrap support), consisting of three well-defined subclades outside of the *Mycosphaerellaceae*

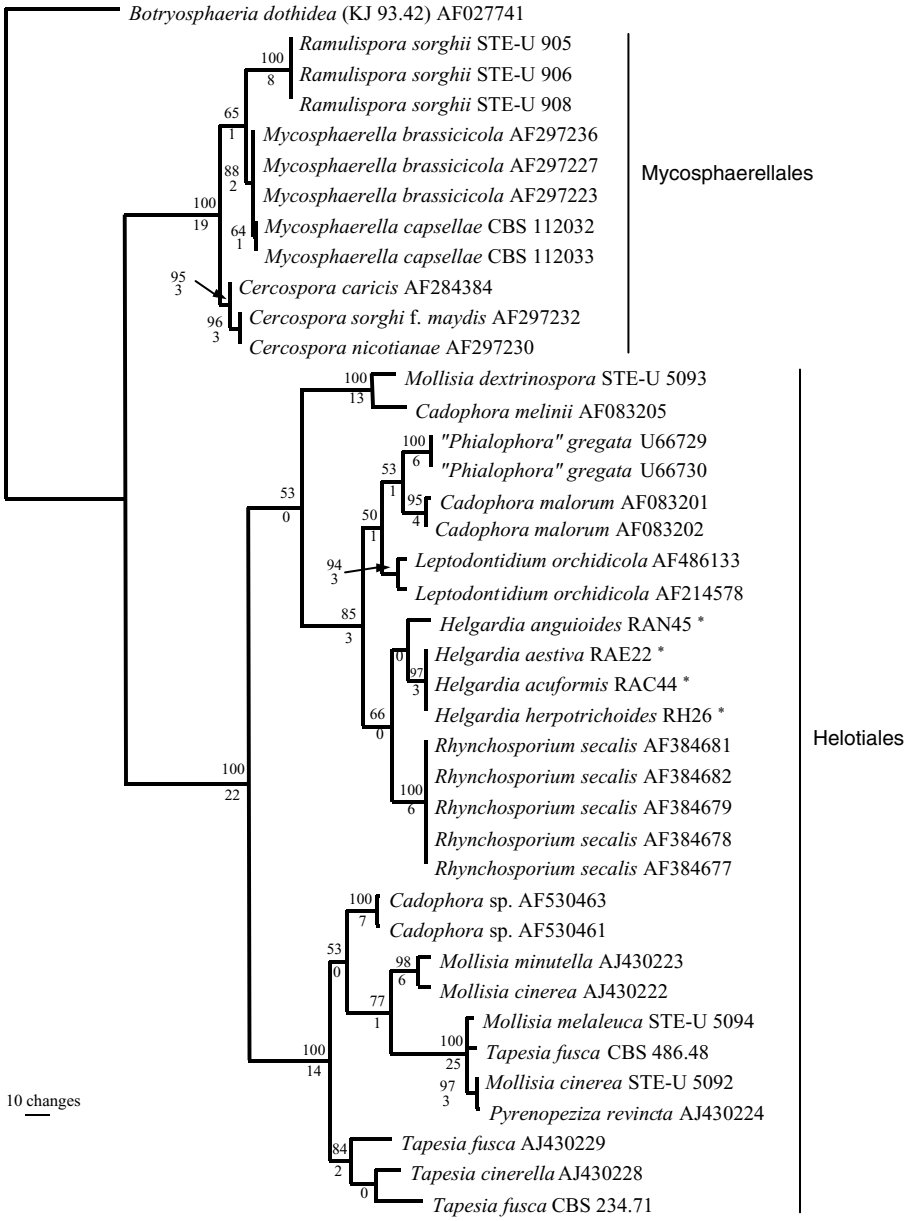


Figure 1. One of 14 most parsimonious trees (length = 606 steps, CI = 0.738, RI = 0.919, RC = 0.678) obtained from a heuristic search with 1000 random taxon-additions using a 601 bp alignment of ITS1, the 5.8S rRNA gene and ITS2. Bootstrap support values from 1000 replicates are shown above and decay values below the nodes. *B. dothidea* was used as outgroup (*Sequences from TreeBASE matrix M691).

(*Mycosphaerellales*), comprising species of *Mollisia*, *Tapesia* and *Pyrenopeziza* Fuckel of the *Dermateaceae* (*Helotiales*). *Mollisia dextrinospora* Korf and *Cadophora melinii* Nannf. clustered apart from the main clade. *M. cinerea* (Batsch) P. Karst. and *M. melaleuca* (Fr.) Sacc. grouped in a clade (100% bootstrap support) together with *M. minutella* (Sacc.) Rehm, *Pyrenopeziza revincta* (P. Karst.) Gremmen, *Tapesia fusca*, *T. cinerella* Rehm and *Cadophora* sp. The eyespot '*Ramulispora*' spp. clustered in a clade containing *Phialophora* Medlar (or rather *Cadophora* Lagerb. & Melin *sensu* Gams, 2000), *M. dextrinospora* Korf (STE-U 5093), *Leptodontidium* de Hoog, and *Rhynchosporium* Heinsen ex A.B. Frank isolates (97% bootstrap support). Within this clade, the four species of '*Ramulispora*' together with *Rhynchosporium secalis* (Oudem.) Davis formed a subclade with 88% bootstrap support.

Taxonomy

The four species associated with cereal eyespot are obviously not congeneric with *R. sorghi*. For the teleomorphs of these cereal pathogens, the genus *Tapesia* is not available being a rejected name in favour of the conserved name *Mollisia* (Hawksworth and David, 1989), with which it is considered as being synonymous. Furthermore, *Mollisia* also appears to be morphologically and ecologically heterogeneous, and is linked to several different anamorph genera.

Species of *Mollisia* in a broad sense, including the eyespot pathogens, grouped in a large clade containing two well-defined subclades. The first subclade includes the type of *Mollisia*, *M. cinerea* (CBS 412.81, STE-U 5092), with a phialidic anamorph suggestive of a moderately branched *Cystodendron* Bubák, and *Pyrenopeziza revincta*. Species of *Pyrenopeziza* have in the past been linked to *Cystodendron*/*Cadophora*-like anamorphs (Hütter, 1958). *T. fusca* (Pers.) Fuckel, the type of *Tapesia*, has also been linked to a *Cystodendron* anamorph (Aebi, 1972), and is thus distinct from the eyespot pathogens. Isolates identified as *T. fusca*, clustered with *M. cinerea*, apart from the eyespot pathogens.

Species of the second subclade have *Cadophora* (incl. several taxa presently still in *Phialophora*), *Leptodontidium* and *Rhynchosporium* anamorphs. The *Ramulispora*-like anamorphs of the eyespot pathogens of cereals are quite distinct from all these anamorphs of the Dermateaceae, though phylogenetically appear closely related to *Rhynchosporium* (Figure 1). *Ramulispora*, as typified by *R. sorghi*, is a member of the Mycosphaerellaceae. Therefore, it cannot be congeneric with a fungus having a Helotialean teleomorph (viz. the eyespot complex). The latter fungi do therefore not belong in *Ramulispora*, but require a new anamorph genus. *Mollisia*, as typified by *M. cinerea*, occurs in a separate cluster to the eyespot fungi, and has a different anamorph. Likewise, *Tapesia*, typified by *T. fusca*, has a different anamorph, and clusters with *Mollisia*, separate from the eyespot fungi. A new teleomorph genus thus needs to be described for the eyespot fungi.

Oculimacula *Crous & W. Gams, gen. nov.*
Apothecia sessilia, gregaria, 0.5–2.5 mm diam., circularia vel lobata, subiculo hypharum plus minusve brunnearum persistentium insidentia, texto superficiali hypharum pallide brunnearum, angustarum substrato affixa. Discus levis, griseus, marginem *versus* pallide griseus, maturus emarginatus, applanatus ad convexus. Receptaculum pallide brunneum ad griseo-brunneum, crateriforme. Asci 8-spori, unitunicati, clavati vel subcylindrici vel fusoidei, breviter stipitati, poro apicali iodi ope caerulescente. Ascosporae biseriatae ad multiseriatae, hyalinae, leves, unicellulares, fusoideae vel subcylindricae-fusoideae vel clavatae, utrinque rotundatae, plerumque rectae. Paraphyses filiformes, sursum obtusatae, ascis longitudine similes. Excipulum medullare ex hyphis multiseptatis, hyalinis compositum, excipulum ectale e cellulis tenuitunicatis, fuscis, angularibus, marginem versus magis elongatis, constans.

Anamorphe: Helgardia Crous & W. Gams.

Type: AUSTRALIA. Yallunda Flat, on wheat stubble, 18 Nov. 1986, *H. Wallwork* and *B. Spooner*, K (holotype), ADW (isotype), of *Oculimacula yallundae* (Wallwork & Spooner) Crous & W. Gams.

Etymology: Oculimacula = Latin for eyespot, named after the characteristic lesions induced on stems of cereals.

Apothecia sessile, gregarious, 0.5–2.5 mm diam., circular to lobate, situated on a subiculum consisting of white to dark brown persistent hyphae, attached to the substrate via a superficial mat of pale brown, thin

hyphae. *Disk* smooth, grey with a pale grey margin, becoming emarginate and flattened to convex at maturity. *Receptacle* pale brown to grey-brown, cup-shaped. *Asci* 8-spored, unitunicate, clavate to subcylindrical or fusoid, with a short stalk, and an apical pore staining blue in Melzer's reagent. *Ascospores* bi- to multiseriate, hyaline, smooth, aseptate, fusoid to subcylindrical-fusoid or clavate with rounded ends, mostly straight. *Paraphyses* filiform with obtuse ends, similar in length to the asci. *Medullary excipulum* consisting of multiseptate, hyaline hyphae. *Ectal excipulum* consisting of thin-walled, dark brown, angular cells, becoming more elongated towards the margin. Anamorph *Helgardia* Crous & W. Gams.

Helgardia *Crous & W. Gams, gen. nov.*
Conidiophora fasciculata vel solitaria in hyphis superficialibus, vel e stromate pallide brunneo oriunda, subcylindrica vel geniculato-sinuosa, raro ramosa, hyalina ad pallide olivacea, levia, seu tantum e cellulis conidiogenis constantia seu uno vel duobus septis divisa, paulo distincta; cellulae conidiogenae integratae, ad apicem dense sympodialiter elongascentes; loci conidiogeni haud inspissati, inconspicui nec fuscescentes. Conidia solitaria, hyalina, levia, in acervis mucidis aggregata, acicularia-filiformia, recta vel curvata, uni- vel multiseptata, saepe conidia secundaria statim proferentia.

Type: FRANCE, holotype of *Helgardia herpotrichoides* (could not be traced in herb. PC); SOUTH AFRICA. Western Cape Province, Moorreesburg, on wheat stubble, 1991, *F. Bester*, CBS 110665 (Dried culture in herb. CBS designated here as *neotype*) of *Helgardia* (isolate genetically identical and sexually compatible with European isolates).

Etymology: *Helgardia*, named after the German mycologist and phytopathologist, Dr. Helgard I. Nirenberg, who first recognized the distinctiveness of these anamorphs on cereals.

Conidiophores fasciculate or solitary on the superficial mycelium, or arising from pale brown stromata, subcylindrical to geniculate-sinuous, rarely branching, hyaline to pale olivaceous, smooth, consisting of conidiogenous cells only, or slightly differentiated with up to 2 septa, conidiogenous cells integrated, proliferating sympodially at the apex, with inconspicuous, dense geniculations; loci unthickened, inconspicuous, not darkened. Conidia solitary, hyaline, smooth, arranged in slimy packets, acicular-filiform, straight to curved, one- to multiseptate, forming smaller, secondary conidia via microcyclic conidiation.

Oculimacula yallundae *(Wallwork & Spooner) Crous & W. Gams, comb. nov. Figures 2–6*

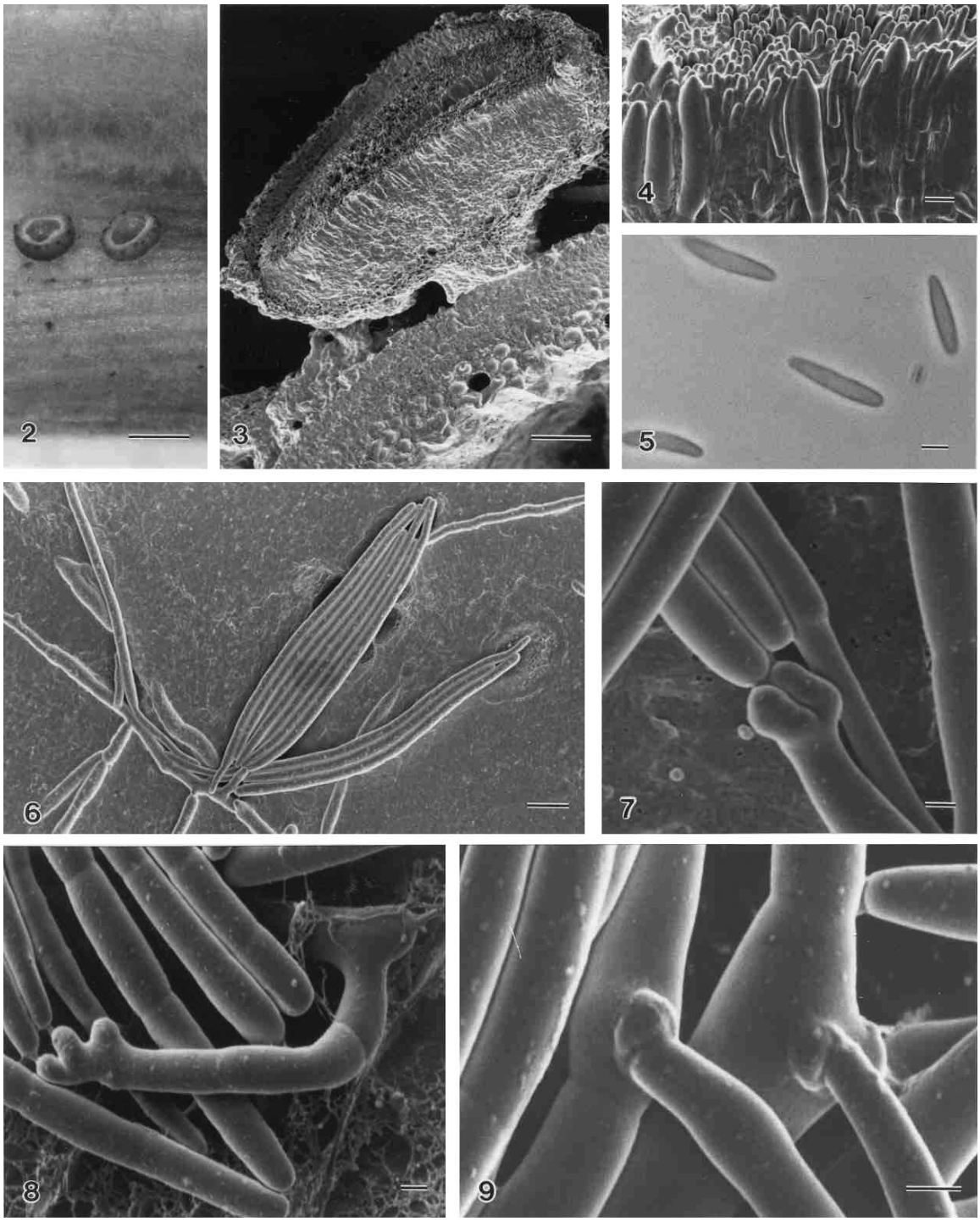
≡ *Tapesia yallundae* Wallwork & Spooner, Trans. Brit. Mycol. Soc. 71: 703 (1988). Anamorph: *Helgardia herpotrichoides* (Fron) Crous & W. Gams, comb. nov.
≡ *Cercosporella herpotrichoides* Fron, Ann. Sci. Agron. Franç. Étrangère, Sér. 4, 1: 11 (1912).
≡ *Pseudocercosporella herpotrichoides* (Fron) Deighton, Mycol. Pap. 133: 46 (1973).
≡ *Ramulispora herpotrichoides* (Fron) Arx, Proc. K. Ned. Akad. Wet. C 86(1): 36 (1983).

Oculimacula acuformis *(Boerema, R. Pieters & Hamers) Crous & W. Gams, comb. nov. Figure 7*

≡ *Tapesia yallundae Wallwork & Spooner var. acuformis Boerema, R. Pieters & Hamers, Neth. J. Pl. Pathol. 98(Suppl 1): 22 (1992).*
≡ *Tapesia acuformis (Boerema, R. Pieters & Hamers Crous, S. Afr. J. Bot. 61: 46 (1995)*. Anamorph: *Helgardia acuformis* (Nirenberg) Crous & W. Gams, comb. nov.
≡ *Pseudocercosporella herpotrichoides* var. *acuformis* Nirenberg, Z. Pflanzenkr. Pflanzensch. 88: 244 (1981).
≡ *Ramulispora herpotrichoides* var. *acuformis* (Nirenberg) Boerema, R. Pieters & Hamers, Neth. J. Pl. Pathol. 98(Suppl 1): 22 (1992) (combination also made by U. Braun, Nova Hedwigia 56: 423, 1993).
≡ *Ramulispora acuformis* (Nirenberg) Crous, S. Afr. J. Bot. 61: 46 (1995).

Helgardia anguioides *(Nirenberg) Crous & W. Gams, comb. nov. Figure 8*

≡ *Pseudocercosporella anguioides* Nirenberg, Z. Pflanzenkr. Pflanzensch. 88: 244 (1981).
≡ *Ramulispora herpotrichoides* var. *anguioides* (Nirenberg) U. Braun, Nova Hedwigia 56: 423 (1993).



Figures 2–9. Apothecia of *Oculimacula*, with *Helgardia* anamorphs. (2) Apothecia of *O. yallundae* on wheat stubble. (3) Vertical section through an apothecium of *O. yallundae*. (4) Section through an apothecium of *O. yallundae,* showing ascal layer. (5) Ascospores of *O. yallundae*. (6) Conidia and conidiogenous cells of *H. herpotrichoides*. (7) Conidial hila and conidiogenous cell of *H. acuformis*. (8) Conidial hila and conidiogenous cell of *H. anguioides*. (9) Conidia of *H. aestiva* giving rise to secondary conidia via microcyclic conidiation. Bars = 2 mm, 100, 5, 2, 10 μm in (a)–(e), and 1 μm in (f)–(h).

Helgardia aestiva *(Nirenberg) Crous & W. Gams, comb. nov. Figure 9*

≡ *Pseudocercosporella aestiva* Nirenberg, Z. Pflanzenkr. Pflanzensch. 88: 246 (1981).
≡ *Ramulispora aestiva* (Nirenberg) E.L. Stewart & Crous, Mycol. Res. 103: 1497 (1999).

Discussion

A recent reclassification of the eyespot pathogens in *Ramulispora* seemed to correct the inadequacy of their placement in *Pseudocercosporella*, which comprises anamorphs of *Mycosphaerella*. The present study has revealed that these assumptions about the phylogenetic position and affinity of the genus *Ramulispora* were incorrect, as was the placement of the sexual state of the eyespot fungi in the genus *Tapesia*. To address this issue, a new teleomorph genus, *Oculimacula*, with its associated anamorph genus *Helgardia*, are proposed. Although it can be argued that a teleomorph genus alone would suffice for these organisms, two related species, namely *H. anguioides* and *H. aestiva*, have not yet been linked to teleomorphs, and thus they require anamorph names for the present. Our data suggest, however, that their teleomorphs, if found, would reside in *Oculimacula*.

The genus *Mollisia* is known to have anamorphs that reside in the *Phialophora* complex, particularly *Cadophora* (Gams, 2000). As shown in the present study, and reported elsewhere (Webster et al., 1993; Nauta and Spooner, 2000), *Mollisia* is heterogeneous. The eyespot taxa reside in one clade together with some species of *Mollisia* that have *Cadophora* or *Cystodendron* or other anamorphs such as *Leptodontidium* and *Rhynchosporium*. The type species of *Mollisia*, *M. cinerea*, has an inconspicuously phialidic, unnamed anamorph, which is distinct from *Cadophora*. The molecular divergence also suggests that *Mollisia* species with *Cadophora* anamorphs will require a new teleomorph genus, while *Tapesia* might possibly be available for species with *Cystodendron* anamorphs (Aebi, 1972). The eyespot pathogens are sufficiently distinct ecologically and in their anamorphs from these two groups to warrant the introduction of a new holomorph. However, the ascomata offer relatively few criteria for this distinction.

The presence or absence of a subiculum has in the past been regarded as significant to separate genera such as *Tapesia* from *Mollisia* (Boudier, 1885; Saccardo, 1889; Rehm, 1891). In later years, less weight was placed in this feature, which appeared insignificant at the generic level (Dennis, 1968; Aebi, 1972; Baral, 1985; 1994), and hence Aebi (1972) reduced *Mollisia* (1871) to synonymy under *Tapesia* (1870). The genus *Mollisia* encompasses more than 100 species, and is better known than *Tapesia* (20 spp.) (Hawksworth and David, 1989). Therefore, Hawksworth and David (1989) proposed conservation of *Mollisia* over *Tapesia*, a proposal that was accepted by the Committee for Fungi and Lichens (Gams, 1992), and the conservation is now listed in the Code. *Mollisia*, however, consists of several different groups that can be distinguished primarily on the basis of their anamorph associations.

Deighton (1973) introduced the genus *Pseudocercosporella* to accommodate taxa with unthickened, not darkened or refractive conidial hila that were formerly placed in *Cercosporella* Sacc. He did not, however, consider the morphological similarity of *Pseudocercosporella* with *Ramulispora*, and hence placed *C. herpotrichoides* in *Pseudocercosporella*. Braun (1995) stated that if *R. sorghi*, the type of *Ramulispora*, had a teleomorph other than *Tapesia*, a new anamorph genus would have to be introduced to accommodate *R. herpotrichoides* and related taxa. We have shown here that *R. sorghi* (and hence *Ramulispora*) represents an anamorph of *Mycosphaerella*, as does *Pseudocercosporella*. *Ramulispora* is distinct from *Helgardia* in that *R. sorghi* induces characteristically sooty leaf spots, which is due to the abundant sclerotia that form on the leaf surface. The latter are, however, not produced in culture. Colonies of *R. sorghi* grow more slowly than those of *Helgardia*. They are compact, grey to black, and sporulate by forming masses of pink, slimy conidia. Slimy conidial masses are known to also occur in *Pseudocercosporella* and *Helgardia*.

A further issue not addressed in the present paper concerns the distinction between and priority of the genera *Pseudocercosporella* (1973) and *Ramulispora* (1920). Although Deighton (1973) did not compare these two genera when he introduced *Pseudocercosporella*, Von Arx (1983) chose to retain *Ramulispora* for taxa occurring on gramineous hosts. Morphologically, these two genera are similar, and also cluster closely together (Figure 1). With *Ramulispora* being the older name, the International Code for Botanical Nomenclature determines that all names in *Pseudocercosporella* actually would have to

be transferred to *Ramulispora*. To reach a final conclusion, however, more species of *Pseudocercosporella* and *Ramulispora* need to be compared in a larger morphological and molecular study. If these two genera were indeed shown to be synonymous, it is evident that the name *Pseudocercosporella* would deserve conservation over the lesser-known *Ramulispora*. A further 13 species of *Ramulispora* are known (www.speciesfungorum.org), but without cultures and molecular analyses, their correct phylogenetic affinities remain unclear. *Pseudocercosporella* has recently been monographed (Braun, 1995). It contains more than 100 species that are well known to plant pathologists and mycologists, and the genus should thus be retained. The erection of new generic names for the eyespot pathogens of cereals was necessary, however, as neither *Pseudocercosporella* nor *Ramulispora* is available for the anamorphs, nor are *Tapesia* or *Mollisia* for the teleomorphs.

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Species of *Cercospora* associated with grey leaf spot of maize

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Abstract: Grey leaf spot is a serious yield-reducing disease of maize (*Zea mays*) in many parts of the world where this crop is cultivated. The causal organism associated with the disease is *Cercospora zeae-maydis*. Two potential sibling species have been recognized as Groups I and II. The DNA sequences for the internal transcribed spacers (ITS1 & ITS2), the 5.8S rRNA gene, elongation factor 1-α, histone H3, actin and calmodulin gene regions suggest that Groups I and II are two distinct species. Furthermore, *Cercospora zeae-maydis* (Group I) can be distinguished from *C. zeina* sp. nov. (Group II) by its faster growth rate on artificial media, the ability to produce cercosporin, longer conidiophores, and broadly fusiform conidia. A PCR-based test that distinguishes the two species was developed using species-specific primers designed from the histone H3 gene.

Taxonomic novelties: *Cercospora zeina* Crous & U. Braun sp. nov.
Key words: Ascomycetes, *Cercospora zeae-maydis*, *Cercospora zeina*, grey leaf spot, maize, *Mycosphaerella*, systematics.

INTRODUCTION

Grey leaf spot of maize is a serious foliar disease of *Zea mays* in many countries where it is cultivated, especially in the eastern U.S.A. and Africa (Ward *et al*. 1999, Crous & Braun 2003). Since it was recognized as a "disease on the move" by Latterell & Rossi (1983), grey leaf spot has become increasingly important and is currently seen as one of the most serious yield-limiting diseases of maize (Nutter & Jenco 1992, Ward & Nowell 1998). The causal agent of grey leaf spot is generally regarded as *Cercospora zeae-maydis* Tehon & E.Y. Daniels, though *C. sorghi* Ellis & Everh. has also been reported from maize (Crous & Braun 2003). Chupp (1954) referred to a *C. sorghi* var. *maydis* Ellis & Everh., which is morphologically similar to *C. sorghi*, but suspected to represent a distinct species due to its lack of pathogenicity to sorghum. In recent years, it has become accepted that more than one species of *Cercospora* is associated with grey leaf spot of maize, namely *C. zeae-maydis* Group I, which is dominant in the U.S.A. and occurs elsewhere in the world, and *C. zeae-maydis* Group II, which is genetically and phenotypically distinct and occurs in the U.S.A., Africa and possibly elsewhere (Wang *et al*. 1998, Dunkle & Levy 2000, Goodwin *et al*. 2001).

The aim of the current study was to characterise the *Cercospora* species associated with grey leaf spot symptoms occurring on maize in South Africa. To achieve this goal isolates were subjected to DNA sequence analysis of several loci, namely the internal transcribed spacers (ITS1 & ITS2), the 5.8S rRNA gene, the elongation factor 1-α, histone 3, actin and calmodulin gene regions. Furthermore, South African isolates were morphologically compared to those isolates from the U.S.A., and the type specimen of *C. zeae-maydis*.

MATERIALS AND METHODS

Isolates

Single-conidial isolates were obtained from symptomatic maize leaves, and cultured as explained in Crous (1998). Cultural characteristics and morphology of *Cercospora* isolates (Table 1) were determined on plates containing 2% malt extract agar (MEA) (20 g/L), 2% potato-dextrose agar (PDA), oatmeal agar (OA), and carnation leaf agar (CLA) [1% water agar (10 g/L) with autoclaved carnation leaves placed onto the medium] (Gams *et al*. 1998). Plates were incubated at 25 °C under continuous near-UV light, to promote sporulation.

DNA phylogeny

Isolates of *C. zeae-maydis*, *C. beticola, C. apii*, and an unidentified *Cercospora* sp. (Table 1) were used for phylogenetic analysis. The protocol of Lee & Taylor (1990) was used to isolate genomic DNA from fungal mycelium of monoconidial cultures grown on MEA in Petri dishes. The primers ITS1 and ITS4 (White *et al*. 1990) were used to amplify part (ITS) of the nuclear rRNA operon spanning the 3' end of the 18S rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region and the 5' end of the 28S rRNA gene. To obtain additional sequence information, four other loci were also sequenced. Part of the elongation factor 1-α gene (EF) was amplified with primers EF1-728F and EF1-986R, part of the actin gene (ACT) with primers ACT-512F and ACT-783R,

Table 1. *Cercospora* isolates used for sequence analysis.

Species	Accession number[1]	Host	Country	Collector	GenBank numbers[2] (ITS, EF ACT, CAL, HIS)
Cercospora apii	CBS 114418; CPC 10924	*Apium graveolens*	Italy	Meutri	AY840517, AY840484, AY840448, AY840415, AY840382
	CBS 116455; CPC 11556*	*A. graveolens*	Germany	K. Schrameyer	AY840519, AY840486, AY840450, AY840417, AY840384
	CBS 116504; CPC 11579	*A. graveolens*	Germany	K. Schrameyer	AY840520, AY840487, AY840451, AY840418, AY840385
	CBS 119.25; CPC 5086	*A. graveolens*	—	L. J. Klotz	AY840512, AY840479, AY840443, AY840410, AY840377
	CBS 121.31; CPC 5073	*Beta vulgaris*	Austria	—	AY840513, AY840480, AY840444, AY840411, AY840378
	CBS 127.31; CPC 5119	*B. vulgaris*	Hungary	—	AY840514, AY840481, AY840445, AY840412, AY840379
Cercospora beticola	CBS 116456; CPC 11557*	*B. vulgaris*	Italy	V. Rossi	AY840527, AY840494, AY840458, AY840425, AY840392
	CBS 116501; CPC 11576	*B. vulgaris*	Iran	A. A. Ravanlou	AY840528, AY840495, AY840459, AY840426, AY840393
	CBS 116502; CPC 11577	*B. vulgaris*	Germany	S. Mittler	AY840529, AY840496, AY840460, AY840427, AY840394
	CBS 116.47; CPC 5074	*B. vulgaris*	Netherlands	G. E. Bunschoten	AY752135, AY752168, AY752196, AY752227, AY752258
	CBS 124.31; CPC 5070	*B. vulgaris*	Romania	—	AY840523, AY840490, AY840454, AY840421, AY840388
	CBS 126.31; CPC 5064	*B. vulgaris*	Germany	—	AY840525, AY840492, AY840456, AY840423, AY840390
	CPC 5125	*B. vulgaris*	New Zealand	C. F. Hill	AY752137, AY752170, AY752198, AY752229, AY752260
	CPC 5128	*B. vulgaris*	New Zealand	C. F. Hill	AY752138, AY752171, AY752199, AY752230, AY752261
	CPC 10168	*B. vulgaris*	New Zealand	C. F. Hill	AY840533, AY840500, AY840464, AY840431, AY840398
Cercospora canescens	ATCC 32779	*Vigna radiata*	Taiwan	—	AY266164, —, —, —, —
Cercospora sorghi	—	*Sorghum bicolor*	U.S.A., Texas	—	AF291707, —, —, —, —
Cercospora sorghi var. *maydis*	—	*Zea mays*	U.S.A., North Carolina	—	AF297233, —, —, —, —
	—	*Z. mays*	Kenya	—	AF297232, —, —, —, —
Cercospora sp.	CPC 12062	*Z. mays*	South Africa, KwaZulu-Natal	P. Caldwell	DQ185071, DQ185083, DQ185095, DQ185107, DQ185119
Cercospora zeae-maydis	—	*Z. mays*	U.S.A., Indiana	—	AF291709, —, —, —, —
	CBS 117755 = A358	*Z. mays*	U.S.A., Indiana	B. Fleener	DQ185072, DQ185084, DQ185096, DQ185108, DQ185120
	CBS 117756 = A359	*Z. mays*	U.S.A., Delaware	B. Fleener	DQ185073, DQ185085, DQ185097, DQ185109, DQ185121
	CBS 117757* = A360	*Z. mays*	U.S.A., Wisconsin	B. Fleener	DQ185074, DQ185086, DQ185098, DQ185110, DQ185122
	CBS 117758 = A361	*Z. mays*	U.S.A., Iowa	B. Fleener	DQ185075, DQ185087, DQ185099, DQ185111, DQ185123
	CBS 117759 = A362	*Z. mays*	U.S.A., Tennessee	B. Fleener	DQ185076, DQ185088, DQ185100, DQ185112, DQ185124
	CBS 117760 = A363	*Z. mays*	U.S.A., Pennsylvania	B. Fleener	DQ185077, DQ185089, DQ185101, DQ185113, DQ185125
	CBS 117761 = A364	*Z. mays*	U.S.A., Indiana	B. Fleener	DQ185078, DQ185090, DQ185102, DQ185114, DQ185126
	CBS 117762 = A365	*Z. mays*	U.S.A., Missouri	B. Fleener	DQ185079, DQ185091, DQ185103, DQ185115, DQ185127
	CBS 117763 = A367	*Z. mays*	U.S.A., Iowa	B. Fleener	DQ185080, DQ185092, DQ185104, DQ185116, DQ185128
Cercospora zeina	CBS 118820 = CPC 11995*	*Z. mays*	South Africa, KwaZulu-Natal	P. Caldwell	DQ185081, DQ185093, DQ185105, DQ185117, DQ185129
	CPC 11998	*Z. mays*	South Africa, KwaZulu-Natal	P. Caldwell	DQ185082, DQ185094, DQ185106, DQ185118, DQ185130
	—	*Z. mays*	U.S.A., North Carolina	—	AF291710, —, —, —, —

[1]CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS.

[2]ITS: internal transcribed spacer region, EF: partial elongation factor 1-alpha gene, ACT: partial actin gene, CAL: partial calmodulin gene, HIS: partial histone H3 gene.

*Ex-type cultures.

and part of the calmodulin gene (CAL) with primers CAL-228F and CAL-737R (Carbone & Kohn 1999). Part of the histone H3 gene (HIS) was amplified with primers CylH3F and CylH3R (Crous *et al*. 2004a). Sequencing was done with the same PCR primers. The PCR conditions, sequence alignment and subsequent phylogenetic analysis followed the methods of Crous *et al.* (2004b). The new sequences were added to a subset of the alignment (TreeBASE matrix M2038) of Crous *et al*. (2004b) and additional sequences were obtained from GenBank. Sequence data were deposited in GenBank and alignments in TreeBASE (S1509, M2712).

Development of a species-specific diagnostic test

The histone H3 gene was found to be most effective in separating the three species described in the present study. Therefore, this area was targeted for the development of a species-specific diagnostic test. Primers CylH3F and CylH3R were used as external primers and their amplification product functions as a positive control. Three species-specific primers were designed for *C. zeae-maydis*, *C. zeina* sp. nov. and an undescribed *Cercospora* species, respectively: CzeaeHIST (5'-TCGACTCGTCTTTCACTTG-3'), CzeinaHIST (5'-TCGAGTGGCCCTCACCGT-3') and CmaizeHIST (5'-TCGAGTCACTTCGACTTCC-3'); all of them species-specific. These internal, species-specific primers, together with the external primers, were used in separate PCR reactions in a total volume of 12.5 µl, containing 1 µl of diluted genomic DNA, 1× PCR buffer, 2 mM $MgCl_2$, 48 µM of each of the dNTPs, 0.7 pmol CylH3F, 3 pmol of CylH3R, 4 pmol of the specific internal primer and 0.7 units (Bioline) *Taq* polymerase. The amplification reactions were done on a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, Connecticut). The initial denaturation step was done at 94 °C for 5 min, followed by 15 cycles of denaturation at 94 °C (20 s), annealing at 58 °C (30 s) and elongation at 72 °C (40 s) as well as 25 cycles of denaturation at 94 °C (20 s), annealing at 55 °C (30 s) and elongation at 72 °C (40 s). A final elongation step at 72 °C (5 min) was included to ensure that full length products are obtained. The PCR products were separated on a 1 % agarose gel and visualized under UV-light after ethidium bromide staining.

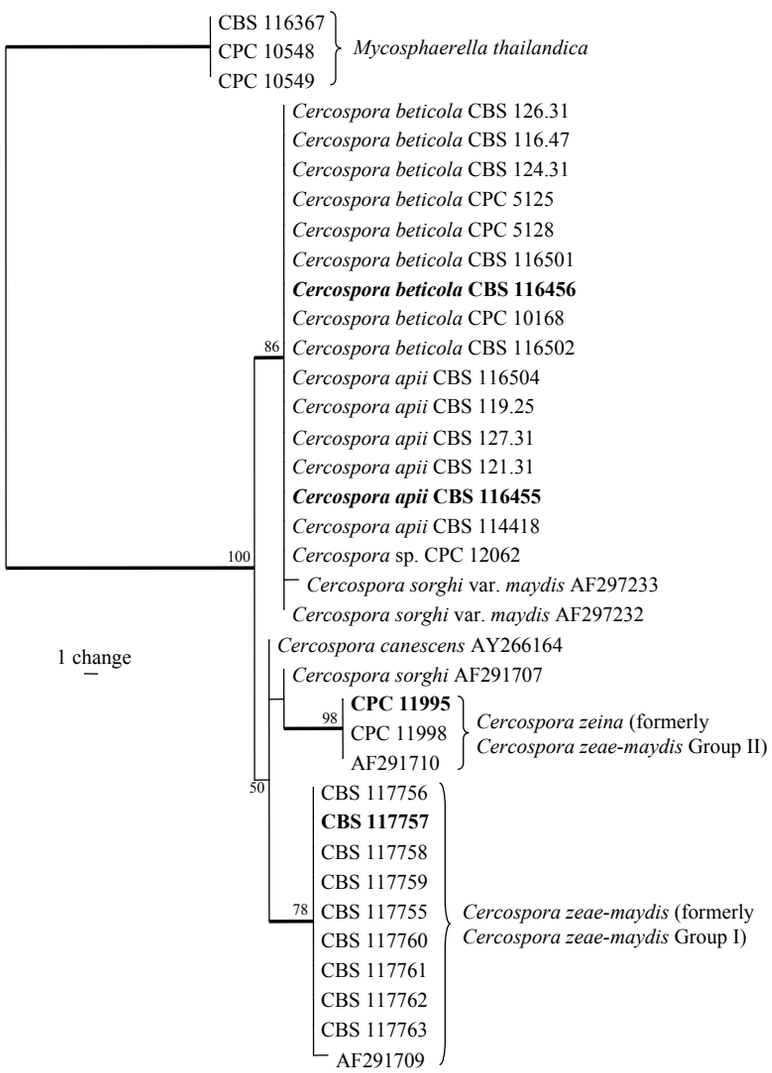

Fig. 1. One of six most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the ITS sequence alignment. The scale bar shows a single change, and bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches, and ex-type strains are shown in bold print. The tree was rooted to three *Mycosphaerella thailandica* strains.

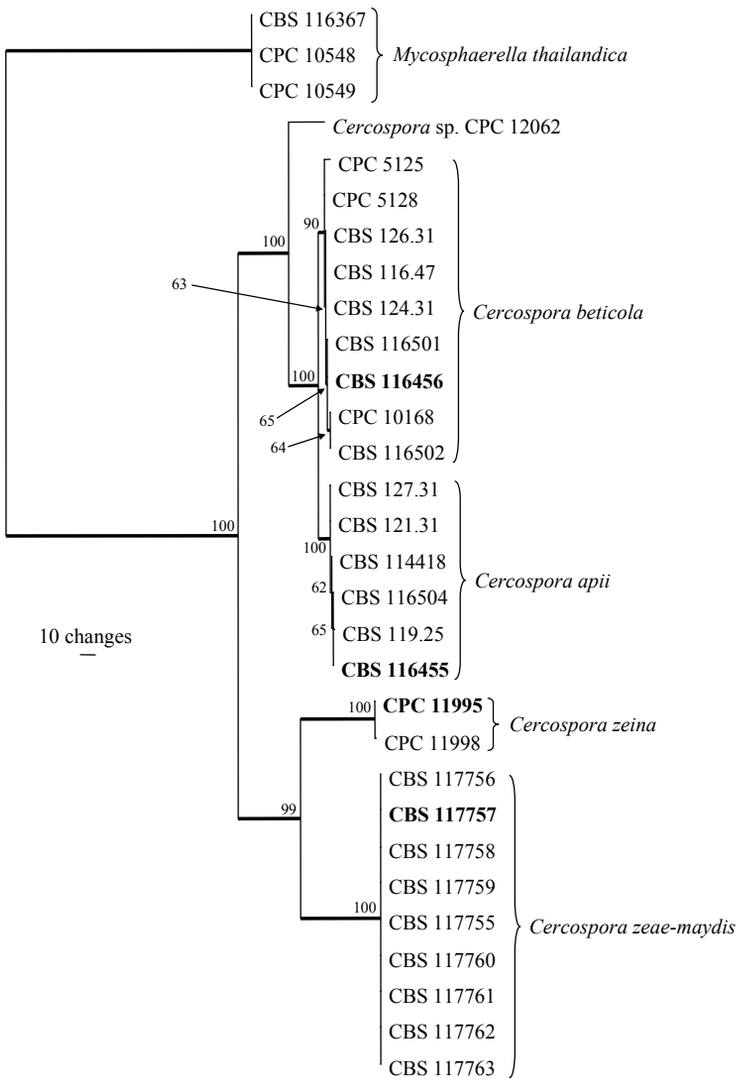

Fig. 2. One of two most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the combined ITS, elongation factor 1-alpha, actin, calmodulin and histone H3 sequence alignment. The scale bar shows 10 changes, and bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches, and type strains are shown in bold print. The tree was rooted to three *Mycosphaerella thailandica* strains.

Taxonomy

Morphological examinations were made from cultures sporulating on CLA, as well as on host material. Structures were mounted in lactic acid, and 30 measurements at × 1000 magnification were made of each structure. The 95 % confidence levels were determined and the extremes of spore measurements given in parentheses. Colony colours were noted after 3 wk growth on MEA, PDA and OA at 25 °C in the dark, using the colour charts of Rayner (1970). All cultures studied are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands. Type specimens were deposited at the National Collection of Fungi in Pretoria (PREM), South Africa (Table 1).

RESULTS

DNA phylogeny

Approximately 500, 310, 230, 320 and 400 bases were determined for ITS, EF, ACT, CAL, and HIS loci, respectively, of the isolates listed in Table 1. Because sequences for the last four loci were not available for other isolates, a separate tree that included more isolates was generated using only ITS sequences (Fig. 1). A partition homogeneity test showed that all loci could be combined (p = 0.747) into a single analysis (Fig. 2).

The ITS data matrix contained 36 taxa (including the three outgroup isolates) and 487 characters including alignment gaps. Of these characters, 40 were parsimony-informative, one was variable and parsimony-uninformative, and 446 are constant. Neighbour-joining analysis using three substitution models (uncorrected “p”, Jukes-Cantor and HKY85) on the sequence data yielded trees with similar topology and bootstrap values. Parsimony analysis of the alignment yielded six most parsimonious trees (TL = 44 steps; CI = 0.955; RI = 0.986; RC = 0.942), one of which is shown in Fig. 1. Three distinct clades were obtained. The first clade (86 % bootstrap support) contained *C. apii* and *C. beticola* together with two isolates of *C. sorgh* var. *maydis* and an undescribed *Cercospora* sp. (CPC 12062) from *Zea mays* in South Africa. The second clade (98 % bootstrap support) contained three

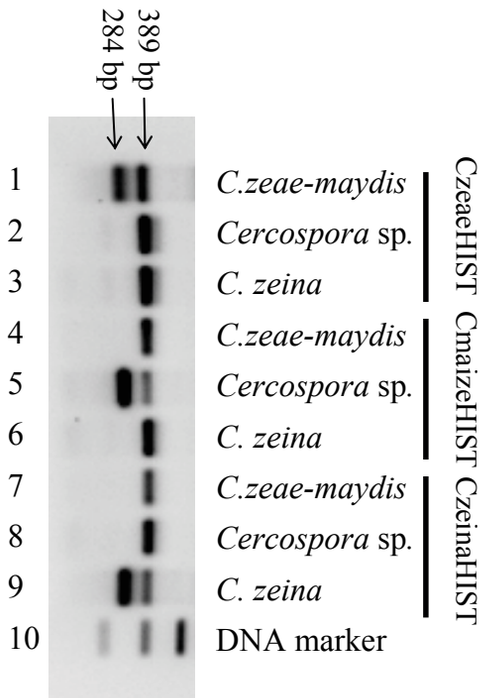

Fig. 3. Identification of *C. zeae-maydis*, an unidentified *Cercospora* sp. and *C. zeina* using the species-specific primers. Lane 10 contains the DNA marker. The 389 bp fragment, which acts as the positive control, is present in all PCR amplifications (lanes 1–9). The species-specific fragment (284 bp) is observed when the amplification reaction contains C. zeae-maydis DNA and primer CzeaeHIST (lane 1, strain CBS 117757), *Cercospora* sp. DNA and primer CmaizeHIST (lane 5, strain CPC 12062) or C. zeina DNA and primer CzeinaHIST (lane 9, strain CPC 11995).

isolates of the new species (*C. zeina*, formerly *C. zeae-maydis* Group II). The isolates of *C. sorghi* var. *sorghi* and *C. canescens* had ITS sequences similar to those of *C. zeae-maydis* Group II (= *C. zeina*), but there was no bootstrap support for this branch. The third clade (78 % bootstrap support) contained isolates of *C. zeae-maydis* (formerly *C. zeae-maydis* Group I). The neighbour-joining and parsimony analyses provided trees with similar topologies (data not shown).

The combined data matrix contained 30 taxa (including the three outgroup taxa) and 1643 characters including alignment gaps. Of these characters, 406 were parsimony-informative, 10 were variable and parsimony-uninformative, and 1227 were constant. Parsimony analysis of the alignment yielded two most parsimonious trees (TL = 519 steps; CI = 0.948; RI = 0.986; RC = 0.935), one of which is shown in Fig. 2. Three distinct clades were obtained, the first (100 % bootstrap support) containing clades with *C. beticola* (90 % bootstrap support) and *C. apii* (100 % bootstrap support) with *Cercospora* sp. CPC 12062 as a sister taxon (100 % bootstrap support). Similar to the ITS tree, the *C. zeina* and *C. zeae-maydis* isolates formed distinct and well-supported clades (each with a bootstrap support value of 100 %). Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with similar topology and bootstrap values to that obtained using parsimony (data not shown).

Development of a species-specific diagnostic test

Easy and rapid identification of *C. zeae-maydis*, *C. zeina* and the new *Cercospora* sp. is possible using three multiplex PCR amplifications. A 389 bp fragment, which serves as the positive control, is present for all three species, while the second 284 bp fragment is only observed for the *Cercospora* species recognised by the specific internal primer (Fig. 3). Primers CzeaeHIST, CzeinaHIST, and CmaizeHIST are therefore specific for *C. zeae-maydis, C. zeina* and the *Cercospora* sp., respectively, and can be used for their identification and detection.

Taxonomy

Cercospora zeae-maydis Tehon & E.Y. Daniels, Mycologia 17: 248. 1925. Fig. 4.

Leaf spots oblong, forming extended streaks or irregular, greyish to brownish spots, shape and size variable, often with a narrow brown border line or margin. *Caespituli* amphigenous, mostly hypophyllous, punctiform to subeffuse, brown. *Mycelium* internal. *Stromata* lacking or small, with a few swollen substomatal brown cells. *Conidiophores* in small to moderately large fascicles (3–14), emerging through the stomata, usually divergent, erect, straight, subcylindrical to flexuous, distinctly geniculate–sinuous, unbranched, 40–180 × 4–8 µm, obscurely (0–)1–8-septate, uniformly pale olivaceous to medium brown, thin-walled, smooth; conidiogenous cells integrated, terminal, occasionally intercalary, 10–40 µm long, conidiogenous loci conspicuously thickened and darkened, 2–3 µm wide. *Conidia* solitary, broadly obclavate–subcylindrical, 30–100 × 4–9 µm, 1–10-septate, hyaline, thin-walled, smooth, apex obtuse, base obconically truncate, hila somewhat thickened and darkened, 2–3 µm wide (based on type specimen).

Specimens examined: **U.S.A.**, Illinois, Alexander Co., McClure, on *Zea mays*, 29 Aug. 1924, P.A. Young (ILLS 4276) **holotype**, BPI 442569 **isotype**; Indiana, Princeton, 2003, B. Fleener, YA-03 = A358 = CBS 117755; Delaware, 1997, B. Fleener, DE-97 = A359 = CBS 117756; Wisconsin, Janesville, 2002, B. Fleener, **epitype designated here**, CBS H-17774, JV-WI-02 = A360 = CBS 117757, culture ex-type; Iowa, Johnston, 2004, B. Fleener, JH-IA-04 = A361 = CBS 117758; Tennessee, Union City, 1999, B. Fleener, UC-TN-99 = A362 = CBS 117759; Pennsylvania, New Holland, 1999, B. Fleener, NH-PA-99 = A363 = CBS 117760; Indiana, Princeton, 1999, B. Fleener, PR-IN-99 = A364 = CBS 117761; Missouri, Dexter, 2000, B. Fleener, DEXTER-MO-00 = A365 = CBS 117762; Iowa, Reinbeck, 1999, B. Fleener, RENBECK-IA-99 = A367 = CBS 117763.

Cultural characteristics: Colonies on PDA reaching 15–25 mm diam after 3 wk, and forming ample spermatogonia; colonies on MEA erumpent, with sparse aerial mycelium; margins smooth, but irregular; surface olivaceous-grey with irregular patches of white or smoke-grey; reverse iron-grey; colonies fertile. On OA colonies spreading with moderate aerial mycelium; margins smooth but irregular; surface red with patches of white and pale olivaceous-grey; fertile.

Substrate: *Zea mays.*

Distribution: Azerbaijan, Brazil, Cameroon, Canada, China, Colombia, Congo, Costa Rica, Ecuador,

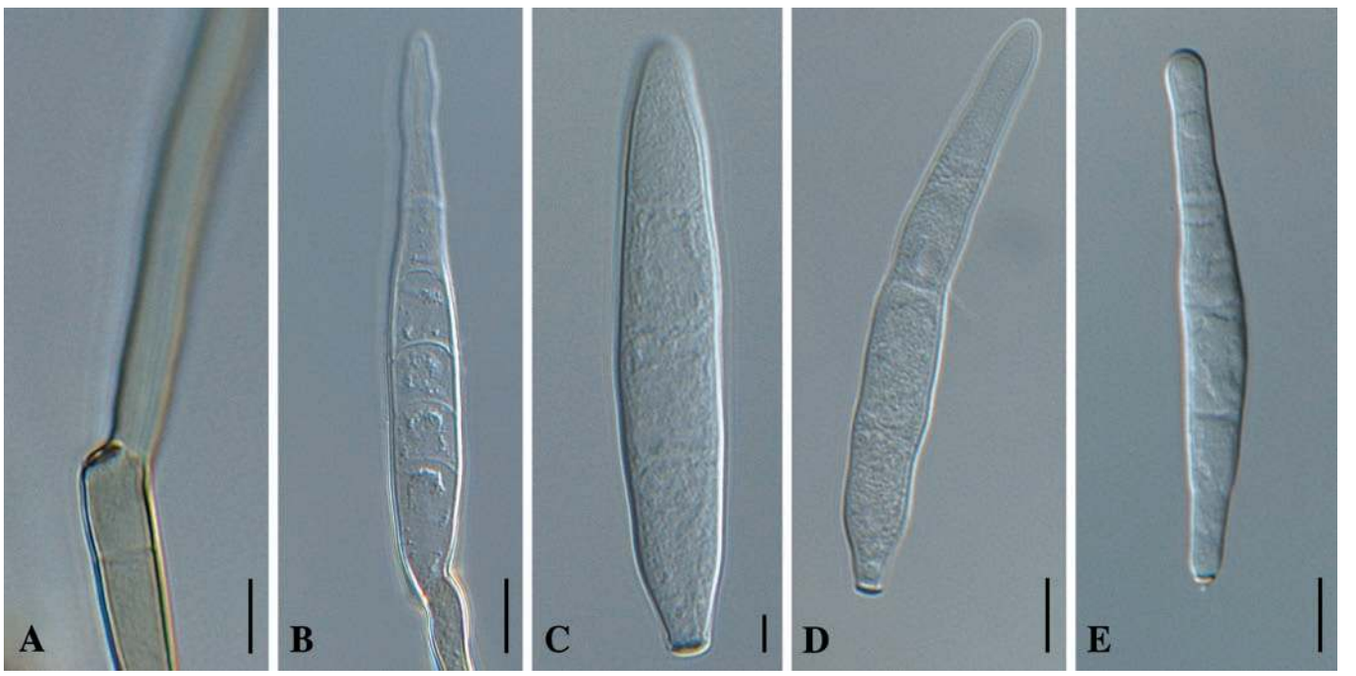

Fig. 4. *Cercospora zeae-maydis*. A. Conidiophore with darkened, refractive conidiogenous locus. B. Germinating conidium. C–E. Conidia in vitro. Bars = 10 µm.

Ethiopia, Georgia, Guatemala, Kenya, Malawi, Mexico, Mozambique, Nigeria, Panama, Peru, South Africa, Swaziland, Tanzania, Trinidad and Tobago, Uganda, USA (CO, DE, IA, IL, KS, KY, MD, MN, NC, OH, PA, SC, TN, VA, WI, WV), Venezuela, Zambia, Zimbabwe (Crous & Braun 2003).

Cercospora zeina Crous & U. Braun, **sp. nov.** MycoBank MB500863. Fig. 5.

Cercospora zeae-maydis affinis, a qua imprimis differt conidiophoris brevioribus (ad 100 µm longis), conidiis late fusiformibus, coloniis in cultura crescentibus tardioribus, sine pigmento rubro.

Leaf spots amphigenous, confined by leaf veins, 2–3 mm wide, variable in length from 5–40 mm; lesions becoming confluent, pale grey to pale brown; borders indistinct, chlorotic in younger leaf spots. *Caespituli* fasciculate, amphigenous, punctiform to subeffuse, grey to brown on leaves, up to 120 µm high and wide. *Mycelium* internal, consisting of pale brown, septate, branched, smooth hyphae, 3–4 µm wide. *Stromata* lacking or small, a few swollen substomatal cells, brown, up to 30 µm diam. *Conidiophores* aggregated (3–20) in loose to semi-dense fascicles arising from the upper cells of an inconspicuous brown stroma, emerging through stomata, usually divergent, erect, straight, subcylindrical to flexuous, distinctly geniculate–sinuous, unbranched or branched above, 40–100 × 5–7 µm, 1–5-septate, uniformly pale olivaceous to medium brown, thin-walled, smooth; conidiogenous cells integrated, terminal, 40–60 × 5–6 µm, with several conidiogenous loci that are conspicuously thickened, darkened and refractive, 2–3 µm wide. *Conidia* solitary, broadly fusiform, (40–)60–75(–100) × (6–)7–8(–9) µm, (1–)3–5(–10)-septate, hyaline, thin-walled, smooth, apex subobtuse, base subtruncate, hila somewhat thickened, darkened and refractive, 2–3 µm wide (based on type specimen).

Specimen examined: **South Africa**, KwaZulu-Natal, Pietermaritzburg, on *Zea mays*, 2005, P. Caldwell, CBS H-17775 **holotype**, CBS 118820 = CPC 11995, culture ex-type.

Cultural characteristics: Colonies on PDA reaching 10–15 mm diam after 3 wk, and forming spermatogonia; colonies on MEA erumpent, with sparse aerial mycelium; margins smooth, but irregular; surface olivaceous-grey with irregular patches of white or iron-grey; reverse iron-grey; colonies fertile. On OA colonies are spreading with moderate whitish aerial mycelium; margins smooth but irregular, olivaceous-grey; fertile.

Substrate: *Zea mays.*

Distribution: South Africa, Uganda, U.S.A. (NC, NY, OH, VA), Zambia, Zimbabwe (Wang *et al*. 1998, Dunkle & Levy 2000).

Notes: *Cercospora zeae-maydis* has conidia of similar dimensions to those of *C. zeina*. However, *C. zeina* can be distinguished by having shorter conidiophores (up to 100 µm) and more broadly fusiform conidia, versus longer conidiophores (up to 180 µm) and broadly obclavate–subcylindrical conidia of *C. zeae-maydis*. Colonies of *C. zeina* grow more slowly in culture and lack the red pigment associated with cercosporin production, typical of *C. zeae-maydis* (Goodwin *et al*. 2001).

DISCUSSION

In a recent review of grey leaf spot of maize, Ward *et al*. (1999) discussed the complexities and importance of this disease in the U.S.A., as well as in Africa. Several papers have commented on the disease being associated with two or more species (Wang *et al*. 1998, Dunkle & Levy 2000, Goodwin *et al*. 2001). A review

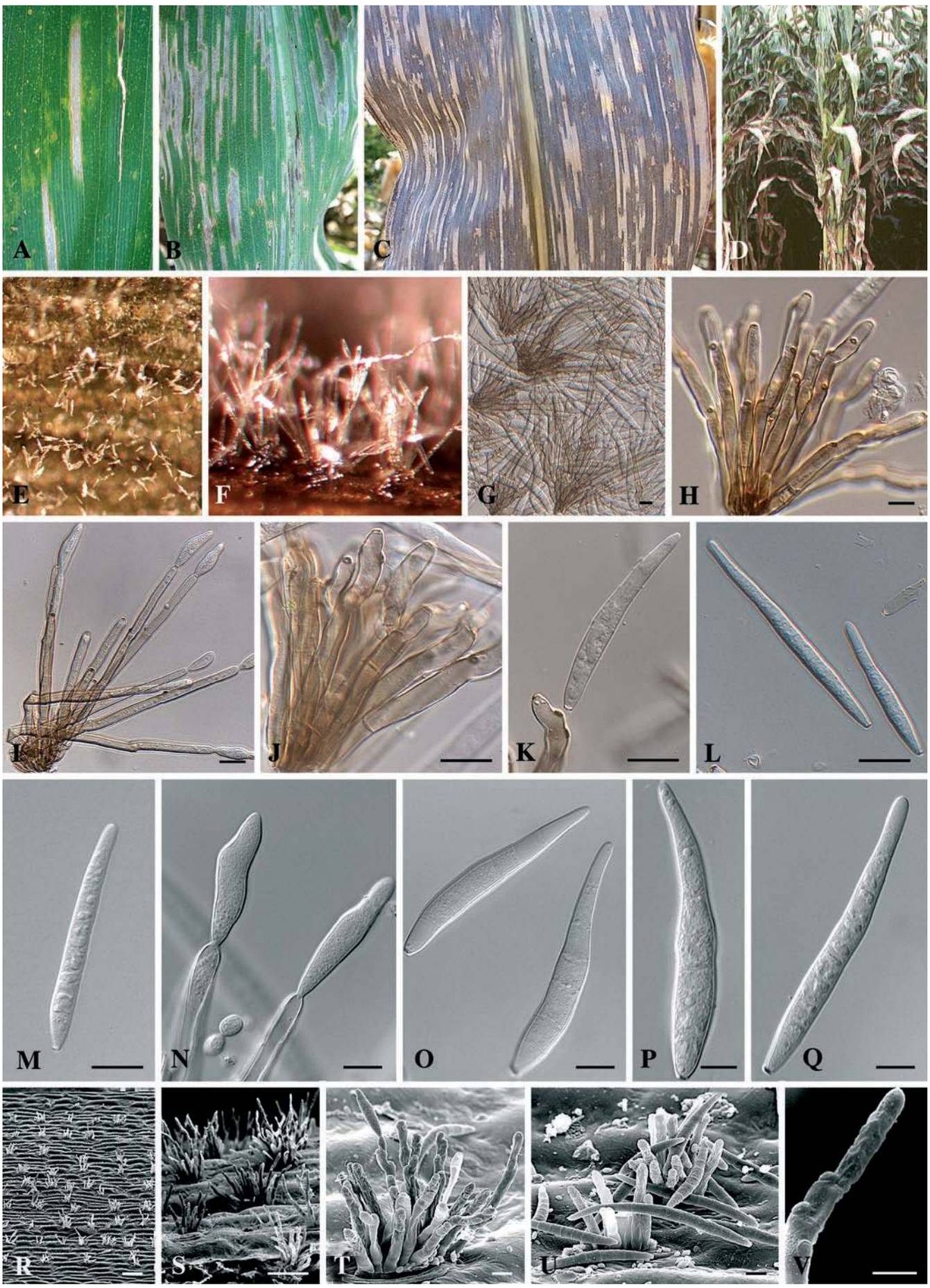

Fig. 5. *Cercospora zeina*. A–C. Close-up of grey leaf spot lesions on maize. D. Heavily infected plant. E–G. Conidiophores fascicles on leaf surface. H–J. Conidiophores. K, N. Conidiogenous cells giving rise to conidia. L–M, O–Q. Conidia. R–U. Scanning electron micrographs of conidiophores and conidia. V. Conidiogenous cell showing thickened loci. Scale bars: G–Q = 10 µm, R = 100 µm, S = 50 µm, T–U = 8 µm, V = 5 µm.

of the literature suggests that there are two possible species complexes associated with grey leaf spot, namely the *C. sorghi* complex (*C. sorghi* and *C. sorghi* var. *maydis*), and the *C. zeae-maydis* complex (Groups I and II).

The description of *C. zeina* has now resolved some of this taxonomic uncertainty, by demonstrating that Group II is, in fact, a distinct species (*C. zeina*) and that Group I, to which the name *C. zeae-maydis* applies, apparently does not occur in South Africa. Further collections from other African countries, as well as other locations in South Africa would be required, however, to determine if *C. zeae-maydis* is truly absent from the continent.

Grey leaf spot disease was first recorded from South Africa in 1988 (Ward *et al*. 1997). The possible source of inoculum was later postulated by Ward *et al*. (1999) to have been from infested maize residues imported from the U.S.A. However, as argued by Dunkle & Levy (2000), if this was indeed the case, such inoculum would have more likely contained *C. zeae-maydis*, which dominates over *C. zeina* throughout most of the maize-producing areas of the eastern and midwestern U.S.A. Given the distribution of *C. zeina* throughout Africa and the fact that there is more genetic diversity of the pathogen in Africa than in the U.S.A. (Dunkle & Levy 2000), it was thought to be more likely that *C. zeina* was introduced to the U.S.A. from Africa, than *vice versa*. Dunkle & Levy (2000) also considered a third possibility, namely that *C. zeina* was introduced to Africa and the U.S.A. on another host, as maize is not native to Africa. However, the most likely hypothesis may be that *C. zeina* is indeed native to Africa, but that it has jumped from another indigenous host (such as sorghum) onto maize. It is interesting to note that the ITS sequence of the *C. zeina* isolates was more similar to that of an isolate of *C. sorghii* var. *sorghi* than to that of the presumably American species *C. zeae-maydis*. Although they are morphologically distinct, further comparisons between *C. zeina* and *C. sorghi* are needed.

Although species of *Mycosphaerella* and their anamorphs are generally assumed to be host-specific (Corlett 1991, Crous & Braun 2003), some species have been observed to also have the ability to colonise hosts other than those on which they are assumed to be primary pathogens. This was recently observed for the greasy leaf-spot pathogen of *Citrus*, *Mycosphaerella citri* Whiteside, which was isolated from other hosts such as *Acacia* and *Musa* (Crous *et al*. 2004b). This finding subsequently led to the formulation of the pogo stick hypothesis (Crous & Groenewald 2005), where species of *Mycosphaerella* can jump to another host as a secondary colonizer, where they sporulate on lesions of the primary *Mycosphaerella* pathogen, producing enough inoculum to enable them to continue the search for their real host.

A further interesting finding was the isolation of a single, fast-growing isolate from grey leaf spot lesions caused by *C. zeina*. Although it was originally suspected that this isolate may represent *C. zeae-maydis* (fast growing and forming a red pigment in agar), this has proven to not be the case. Morphologically this isolate (CPC 12062) appeared more similar to isolates in the *Cercospora apii* complex (*C. apii* and *C. beticola*). Although only a few of the species in this complex are known from culture, CPC 12062 proved distinct based on DNA sequence data when compared to the more than 100 sequences currently available in our unpublished database. This isolate may represent an unrelated pathogen from another host that has "jumped" onto maize (Crous & Groenewald 2005). By using the PCR-based method described here as a diagnostic tool, it is relatively easy to identify the three *Cercospora* species on maize that are treated in this study.

Both *C. zeae-maydis* and *C. zeina* have the ability to form ample spermatogonia on host tissue as well as in culture. Although there has been an earlier report of a possible *Mycosphaerella* teleomorph (Latterell & Rossi 1977), this has remained unconfirmed. Wang *et al*. (1998) were unable to find evidence of the *MAT-2* mating type idiomorph in isolates of *Cercospora zeae-maydis*, and our current mating studies with isolates of *C. zeae-maydis* and *C. zeina* have also given negative results. Further population-level studies are thus needed to determine the level of variation present in populations, and whether sexual reproduction occurs within populations of these two fungi. Published results do not support the existence of cryptic sex, however, as Wang *et al*. (1998) reported the variation to be rather low in populations of both species.

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