British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/mycres

Phylogenetic reassessment supports accommodation of *Phaeophleospora* and *Colletogloeopsis* from eucalypts in *Kirramyces*

Vera ANDJIC[a], *Paul A. BARBER*[a], *Angus J. CARNEGIE*[b], *Giles St J. HARDY*[a], *Michael J. WINGFIELD*[c], *Treena I. BURGESS*[a,*]

[a]*School of Biological Sciences and Biotechnology, Murdoch University, Murdoch 6150, Australia*
[b]*Forest Resources Research, NSW Department of Primary Industries, PO Box 100, Beecroft, NSW 2119, Australia*
[c]*Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa*

ARTICLE INFO

Article history:
Received 29 September 2006
Accepted 15 May 2007
Published online 26 July 2007
Corresponding Editor:
David L. Hawksworth

Keywords:
DNA sequence
Eucalyptus
Herbarium specimens
Mycosphaerella
Revision
Taxonomy

ABSTRACT

Species of *Phaeophleospora* are anamorphs of *Mycosphaerella* and they include some of the most serious foliar pathogens of *Eucalyptus* spp. grown in plantations worldwide. Pathogens assigned to this genus and occurring on *Eucalyptus* spp. were previously treated in *Kirramyces* and they are also phylogenetically closely related to other anamorphs of *Mycosphaerella* residing in the genus *Colletogloeopsis*. The primary aim of this study was to consider the appropriate taxonomic placement of these species. To achieve this goal, morphological characteristics and DNA sequence data from the ITS and translation EF1-α gene regions were used to compare the type species *P. eugeniae*, *Phaeophleospora* spp. and *Colletogloeopsis* spp. occurring on eucalypts, using ex-type cultures and herbarium specimens. Phylogenetic data and morphological comparisons supported the separation of *P. eugeniae* from *Phaeophleospora* species occurring on eucalypts. The name *Phaeophleospora* is retained for *P. eugeniae* and the name *Kirramyces* is resurrected for the species occurring on eucalypts (genera *Eucalyptus*, *Corymbia*, and *Angophora*). Sequence data from the type specimens of two previously described species of *Kirramyces*, *K. lilianiae* and *K. delegatensis*, show they reside in a clade with other *Kirramyces* spp. Morphological and DNA sequence comparisons also showed that there is considerable overlap between species of *Phaeophleospora* and *Colletogloeopsis* from eucalypts. Based on these findings, *Colletogloeopsis* is reduced to synonymy with the older *Kirramyces* and the description of *Kirramyces* is emended to include species with aseptate, as well as multiseptate, conidia produced in acervuli or pycnidia. Two new species of *Kirramyces*, *K. angophorae* and *K. corymbiae*, are also described.

Introduction

Phaeophleospora spp. are anamorphs of *Mycosphaerella* that cause leaf and shoot blight diseases on many plants, including members of the families *Myrtaceae*, *Proteaceae*, *Malvaceae*, *Elaeocarpaceae*, and *Sapotaceae*. The genus *Phaeophleospora* was introduced to accommodate the dark form of *Phleospora* (''*Phloeospora*'') by Rangel (1916). However, most taxonomists regarded the monotypic genus *Phaeophleospora* as a *nomen dubium* because the mode of conidiogenesis and the form of

* Corresponding author.
E-mail address: tburgess@murdoch.edu.au
0953-7562/$ – see front matter © 2007 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.mycres.2007.07.003

the conidia were not documented in the type description (Sutton 1977). Up until 1997, *Phaeophleospora* included only two species: *P. eugeniae* occurring on *Eugenia uniflora* and *P. elaeocarpi* occurring on *Elaeocarpus* spp. (Bond 1947).

Confusion regarding the taxonomic placement of *Phaeophleospora* first emerged when a suitable name was sought for a *Pseudocercospora* sp. causing widespread damage on *Eucalyptus* in South Africa. Type specimens of *Cercospora epicoccoides* and *C. eucalypti* were examined and it was concluded that neither of these species were correctly placed in the genus *Cercospora* (Crous *et al.* 1989). Thus, *C. epicoccoides* was shown to reside in *Phaeoseptoria* where it was known as *P. eucalypti*. Walker *et al.* (1992) later compared *P. eucalypti* with the type specimen of the genus *Phaeoseptoria*, *P. papayae,* and noted that *P. eucalypti* differed from the type specimen in having large, brown, cylindrical, rough-walled, percurrently proliferating conidiogenous cells, that were not present in the type species. *P. papayae* had also previously been redescribed by Morgan-Jones (1974) and both he and Walker *et al.* (1992) found large, brown, cylindrical, rough-walled, percurrently proliferating conidiogenous cells were not present.

In a search for a suitable genus for *P. eucalypti,* several genera were considered (Walker *et al.* 1992). These included *Scoleciasis*, *Sonderhenia,* and *Stagonospora*, but none were suitable because they were all characterised by smooth-walled conidiogenous cells and distoseptate conidia (*Sonderhenia*) or smooth-walled, hyaline conidia (*Stagonospora*). *P. eucalypti* was therefore removed from *Phaeoseptoria* and the new genus *Kirramyces* was introduced for species with pycnidial conidiomata, brown, euseptate, cylindrical to narrowly obclavate rough-walled conidia and brown roughened annellidic conidiogenous cells (Walker *et al.* 1992). The genus included three taxa, *K. epicoccoides, K. lilianiae*, characterised by brown rough-walled conidia, and *K. eucalypti* with pale yellowish-brown, finely roughened conidia. Walker *et al.* (1992) recognized the similarity in conidial size and shape between *K. eucalypti* and the *Stagonospora delegatensis* anamorph of *Mycosphaerella delegatensis*, but they noted that *S. delegatensis* differed from *Kirramyces* spp. in having paler, slightly less tapered and smooth conidia. These features also indicated that it was poorly accommodated in *Stagonospora*, but due to the lack of a suitable number of collections, the taxonomic position of this fungus was not resolved. Sankaran *et al.* (1995) later reduced *S. delegatensis* to synonymy with *K. eucalypti* without providing an explanation for their decision.

Crous *et al.* (1997) redescribed *Phaeophleospora eugeniae* based on the collection and designation of a neotype. In their study, it was concluded that *P. eugeniae* resembled species residing in *Kirramyces,* and *Kirramyces* was reduced to synonymy under the older name *Phaeophleospora*. Differences were noted between *Phaeophleospora* and *Kirramyces*, particularly in the gradient of pigmentation and number of septa in the conidia, but they did not consider these sufficiently important to justify separation at the generic level Crous *et al.* (1997). Other species of *Kirramyces* that had previously been described by Walker *et al.* (1992), as well as *K. proteae*, *K. hebes*, *K. phormii* and *K. destructans,* were re-allocated to *Phaeophleospora* (Crous *et al.* 1997).

Maxwell *et al.* (2003) selected *Phaeophleospora* for the anamorph of *M. ambiphylla* because the conidia of the fungus were produced in pycnidia. However, these authors noted that aseptate conidia had not previously been described for species residing in *Phaeophleospora*. Amongst *Mycosphaerella* anamorphs, aseptate conidia are more typical of *Colletogloeopsis* spp. with acervular conidiomata; however, the anamorph of *M. ambiphylla* produced conidia in pycnidia rather than acervuli. More recently, Crous *et al.* (2004) described the anamorph of *M. toledana* in the genus *Phaeophleospora* because the conidiomata were pycnidial rather than acervular. This species, like the anamorph of *M. ambiphylla,* produces aseptate conidia.

In a taxonomic re-evaluation of *Coniothyrium zuluensis*, Cortinas *et al.* (2006a) showed that this pathogen, which produces conidia in pycnidia, always clusters in the same clade as *Colletogloeopsis nubilosum* and *C. molleriana* whose conidia are produced in acervuli. Based on phylogenetic data, Cortinas *et al.* (2006a) emended the description of *Colletogloeopsis* to accommodate *Coniothyrium*-like anamorphs residing in *Mycosphaerella*, and included pycnidial, as well as acervular, conidiomata in this description. The authors could not place this fungus in the genus *Phaeophleospora*, as the type species *P. eugeniae* was found to be phylogenetically distant from *C. zuluensis* (Cortinas *et al.* 2006a). Likewise, Andjic *et al.* (2007), who investigated the phylogenetic relationship of *Phaeophleospora* species from eucalypts, found that these species were phylogenetically distant from the ex-type culture of *P. eugeniae* but close to *C. zuluense* and *M. nubilosa*. As a result, *Phaeophleospora* was no longer suitable to accommodate *Colletogloeopsis*-like species with pycnidial conidiomata as had previously been true for the anamorphs of *M. ambiphylla* (Maxwell *et al.* 2003) and *M. toledana* (Crous *et al.* 2004).

Species of *Phaeophleospora s. lat.* and *Colletogloeopsis* are common and important pathogens of eucalypts. New species that might reside in either of these genera are collected regularly. For practical taxonomic reasons and for the establishment of appropriate quarantine regulations, these fungi require appropriate names. This study emerged from the collection of apparently new species in these two genera, and the assignment of these new species to the appropriate genus necessitated a detailed phylogenetic and morphological study of related fungi. These included type specimens representing *Phaeophleospora, Kirramyces,* and *Colletogloeopsis*. The correct taxonomic placement of these genera was thus reassessed; suitable synonymies and combinations are proposed in *Kirramyces* and new species are described. *Phaeophleospora* remains as the monotypic genus for *P. eugeniae*.

Materials and methods

Isolates

Isolates were obtained by collecting conidia exuding from single pycnidia or acervuli using the tip of a sterile needle. These were transferred onto 2 % malt extract agar (MEA) containing streptomycin 150 $\mu g\,ml^{-1}$ (Sigma-Aldrich, Sydney, Australia) in a single spot and allowing it to hydrate for 5 min. Under a dissecting microscope, spores were streaked using a sterile needle and single spores immediately transferred to MEA plates. Cultures were grown at 25 °C for 2 weeks and then

transferred to fresh MEA plates. Cultures were maintained on 2 % MEA in tubes at 20 °C. Herbarium specimens were obtained for *Phaeophleospora lilianiae* (DAR 3833), *P. delegatensis* (DAR 45718b), species for which an ex-type culture or sequence data do not exist. Herbarium material used for measurements included: *P. destructans* (PREM 59259, PREM 59261), *P. epicoccoides* (PREM 59258, PREM 59260, MURU 422, MURU 423), and *P. eucalypti* (MURU 424, MURU 425). The cultures used in this study are maintained in the culture collections of Murdoch University (MUCC), the Forestry and Agricultural Biotechnology Institute, University of Pretoria (CMW), the New South Wales, Plant Pathology Herbarium (DAR), and State Forests of New South Wales (NSWF), Australia.

DNA extraction and PCR from cultures

Isolates were grown on 2 % MEA at 20 °C for four weeks and the mycelium harvested and placed in 1.5 ml sterile Eppendorf® tubes. Harvested mycelium was frozen in liquid nitrogen, ground to a fine powder, and genomic DNA extracted using a hexadecyl trimethyl ammonium bromide (CTAB) modified protocol of Graham *et al.* (1994). Six hundred microlitres of extraction buffer [2 % CTAB; 100 mm Tris–HCl (pH 8), 1.4 m NaCl 2 % PVP-40 100 $\mu g\,ml^{-1}$ proteinase K, 100 $\mu g\,ml^{-1}$ RNAse A] was added per 60 mg freeze-dried mycelium and incubated at 55 °C for 20 min. After incubation, the tubes were centrifuged for 2 min at 11 000 ***g***, and the supernatant transferred to a new tube and extracted with equal volume of chloroform isoamyl alcohol 24:1 (IAC), centrifuged for 20 s at 11 000 ***g***, the upper aqueous phase transferred to a new tube and 0.1 volumes of 7.5 m ammonium acetate and two volumes of 100 % added to precipitate the DNA. The tubes were inverted a few times, incubated at −20 °C for 60 min, centrifuged for 1 min at 11 000 ***g***, the supernatant discarded and the DNA pellet washed with 1 ml 70 % ice-cold ethanol and re-centrifuged at 1000 ***g*** for 1 min. The ethanol was decanted and the DNA allowed to air dry for 15 min. The DNA was resuspended in 30 µl ultra-pure PCR grade water. ITS1, ITS2 and 5.8S regions of the rDNA operon were amplified using primers ITS-1F (5′ CTT GGT CAT TTA GAG GAA GTA A 3′) (Gardes & Bruns 1993), ITS-4 (5′TCC TCC GCT TAT TGA TAT GC 3′) (White *et al.* 1990), and part of the translation elongation factor 1-α region was amplified using primers EF1-728F (5′ CAT CGA GAA GTT CGA GAA GG 3′) and EF1-986R (5′ TAC TTG AAG GAA CCC TTA CC 3′) (Carbone & Kohn 1999).

PCR was performed using GeneAmp PCR System 2700 Thermal Sequencer (Applied Biosystems, Foster City, CA). Each 25 µl reaction mixture contained 1 × PCR polymerisation buffer (67 mm Tris–HCl, 16.6 mm ammonium sulphate, 0.45 % Triton X-100, 0.2 $mg\,ml^{-1}$ gelatine, 0.2 mM of each dNTPs; Fisher Biotech, Perth, Australia), 25 mM $MgCl_2$ (Fisher Biotech), 0.6 pmol each primer (GeneWorks, Adelaide, Australia), approximately 5 ng DNA and 1 unit Taq DNA polymerase (Fisher Biotech). During the PCR reaction, the DNA first was denatured at 94 °C for 2 min, followed by 35 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 45 s) and elongation (72 °C for 1 min) and ended with a final elongation step at 72 °C for 5 min. To detect possible contamination in the amplification reaction, a negative control that contained all reaction components except the fungal template DNA, was used with every reaction. The PCR products were visualised on 1 % agarose gel containing ethidium bromide using an uv transluminator and purified with Ultrabind® DNA purification kit (MO BIO Laboratories, Solana Beach, CA) following the manufacturer's instructions.

DNA extraction and amplification from herbarium specimens

In the cases where ex-type cultures were not available for species required to define genera, DNA extractions were made directly from herbarium specimens representing the types. Several individual pycnidia or acervuli were carefully removed from the herbarium specimens and transferred to a 1.5 ml tube and ground with liquid nitrogen to a fine powder. DNA was extracted with CTAB extraction buffer as described previously (Wittzell 1999).

Initially the DNA from the herbarium specimens was amplified using ITS primers ITS-1F and ITS-4, followed by nested PCR using primers ITS-1 (5′ GTA TCG ATG AAG AAC GCA GC 3′) and ITS2 (GCTCGGTTCTTCATCGATGC) (White *et al.* 1990) primers with 1:5 dilution of initial PCR product as template. PCR mixtures and running conditions were as described above.

Some isolates showed false-positive amplification when subjected to nested PCR, thus direct PCR was used and the running conditions were changed. The magnesium concentration was increased to 4 mm and 0.8 % bovine serum albumin was added to each reaction. The DNA was denatured at 94 °C for 7 min, followed by 40 cycles of denaturation (94° C for 2 min), annealing (45° C for 1 min) and elongation (72° C for 2 min) and ended with a final elongation step at 72° C for 10 min.

Phylogenetic analyses

In order to compare species of *Phaeophleospora*, sequences in addition to those derived in this study were obtained from GenBank (Table 1). Sequence data were assembled using Sequence Navigator version 1.01 (Perkin Elmer, Melbourne, Australia) and aligned in Clustal X (Thompson *et al.* 1997). Manual adjustments were made visually by inserting gaps where necessary. All sequences derived in this study were deposited in GenBank and accession numbers are listed in Table 1.

Analyses were performed on the combined dataset of complete ITS and EF-1α sequences, after a partition homogeneity test (PHT) had been performed in PAUP version 4.0b10 (Swofford 2003) to determine whether sequence data from the two gene regions were statistically congruent (Farris *et al.* 1995; Huelsenbeck *et al.* 1996). Parsimony analysis with heuristic search was performed using PAUP with random stepwise addition in 100 replicates with the tree bisection–reconnection branch-swapping option and the steepest-descent option off. All ambiguous and parsimony-uninformative characters were excluded; gaps were treated as a fifth character. MaxTrees were unlimited, branches of zero length were collapsed, and all multiple equally parsimonious trees saved. Estimated levels of homoplasy and phylogenetic signal; tree length (TL), consistency index (CI) and retention index (RI) were determined (Hillis & Huelsenbeck 1992). Characters were unweighted and

Table 1 – Isolates considered in the phylogenetic study

Culture no.[a]	Teleomorph	Anamorph	Host	Location	Isolator	GenBank accession no. for the ITS sequence	GenBank accession no. for the EF-1α sequence
CMW 7127		*Phaeophleospora destructans*	*Eucalyptus grandis*	Sumatra, Indonesia	M.J. Wingfield	DQ632698	EF011658
CMW 17919		*P. destructans*	*E. urophylla*	China	T.I. Burgess	DQ632701	DQ632729
CMW 17917		*P. eucalypti*	*E. grandis x E. teretacornis*	NSW, Australia	A.J. Carnegie	DQ632711	DQ632725
CBS 113992, CMW 11687		*P. eucalypti*	*E. nitens*	New Zealand	M. Dick	DQ240001	DQ235115
CMW 22484	*Mycosphaerella. suttonii*	*P. epicoccoides*	*Eucalyptus* sp.	China	T.I. Burgess	DQ632705	DQ632714
MUCC 426	*M. suttonii*	*P. epicoccoides*	*E. globulus*	WA, Australia	S. Jackson	DQ632704	DQ632715
CMW 5348, STE-U 1346	*M. suttonii*	*P. epicoccoides*	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	AF309621	DQ240170
CBS 113313, CMW14457	*M. toledana*	*P. toledana*	*E. globulus*	Spain	P.W. Crous	AY725581	DQ235120
CMW 5351		*P. eugeniae*	*Eugenia uniflora*	Brazil	M.J. Wingfield	DQ632710	EF011663
CMW 11560	*M. nubilosa*		*E. globulus*	Tasmania	A. Milgate	DQ658232	DQ240176
CBS 114708, CMW9003	*M. nubilosa*		*E. nitens*	South Africa	G. C. Hunter	AF449099	DQ235112
CBS 110975, CMW 3279	*M. cryptica*	*Colletogloeopsis nubilosum*	*E. globulus*	Australia	A.J. Carnegie	AY309623	DQ235119
CBS 117262, CMW 7449		*C. zuluensis*	*E. grandis*	South Africa	L. Van Zyl	DQ240021	DQ240155
CBS 113399, CMW 13328		*C. zuluensis*	*E. grandis*	South Africa	L. Van Zyl	DQ240018	DQ240172
CBS 110499, CMW14180	*M. molleriana*	*C. molleriana.*	*E. globulus*	Western Australia	A. Maxwell	AY150675	DQ240169
CBS 111164, CMW4940	*M. molleriana*	*C. molleriana*	*Eucalyptus* sp.	Portugal	S. McCrae	AF309620	DQ235104
CBS 117924, CMW 11588	*M. molleriana*	*C. molleriana*	*E. globulus*	Tasmania		DQ239968	DQ240167
CBS 116154, CMW 4945	*M. africana*		*Eucalyptus*	South Africa	P.W. Crous	AF309602	DQ235099
CBS 116155, CMW 3026	*M. africana*		*Eucalyptus*	South Africa	P.W. Crous	DQ267577	DQ235098
CBS 111011, CMW 5147	*M. keniensis*		*Eucalyptus*	Kenya	T.A Couthino	DQ246259	DQ235100
CMW 4934	*M. ellipsoidea*	*Uwebraunia ellipsoidea*	*Eucalyptus*	South Africa	M.J. Wingfield	DQ246253	DQ235129
CMW 5166	*M. ellipsoidea*	*U. ellipsoidea*	*Eucalyptus*	South Africa	M.J. Wingfield	DQ246254	DQ235127
CBS 110969, CMW 4944	*M. colombiensis*		*E. urophylla*	Colombia	M.J. Wingfield	DQ204744	DQ211660
CBS 110967, CMW 11255	*M. colombiensis*		*E. urophylla*	Colombia	M.J. Wingfield	DQ204745	DQ211661
CMW 7773	*Neofusicoccum ribis*		*Ribes sp.*	New York, USA	B. Slippers	AY236936	AY236878
CBS 120495, DAR 77445		*Kirramyces corymbiae*	*Corymbia maculata*	NSW, Australia	A.J.Carnegie	EF011657	EF011661
CBS 120496, DAR 77446		*K. corymbiae*	*C. maculata*	NSW, Australia	A.J.Carnegie	EF011656	EF011662
CBS 120493, DAR 77452		*K. angophorae*	*Angophora floribunda*	NSW, Australia	A.J.Carnegie	EF011653	EF011660
CBS 120494, DAR 77451		*K. angophorae*	*A. floribunda*	NSW, Australia	A.J.Carnegie	EF011652	EF011659

a Designation of isolates and culture collections: CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW, Tree Pathology Co-operative Program Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; STE-U, Stellenboch University, South Africa; MUCC, Murdoch University culture collection, Australia; DAR, New South Wales, Plant Pathology Herbarium, Australia.

unordered branch and branch node support was determined using 1 K BS replicates with equal probability (Felsenstein 1985). Trees were rooted to *Neofusicoccum ribis*, which was treated as the outgroup taxon.

Bayesian analysis was conducted on the same aligned and combined dataset as that used in the distance analysis. First, MrModeltest v2.2 (Nylander 2004) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with MrBayes v3.1 (Ronquist & Heuelsenbeck 2003) applying a general time reversible (GTR) substitution model with gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites. Two independent runs of MCMC using four chains were run over 10 M generations. Trees were saved each 10 K generations, resulting in 10 001 trees. Burn-in was set at 500 001 generations (i.e. 51 trees), well after the likelihood values converged to stationery, leaving 9950 trees from which the consensus trees and PPs were calculated.

Morphological comparisons

In order to re-assess the taxonomical status of *Phaeophleospora* spp. associated with eucalypts and to determine the correct generic placement for apparently undescribed species obtained from *Corymbia* spp. and *Angophora floribunda*, representative isolates of the unknown species and representative isolates of *P. destructans*, *P. epicoccoides* and *P. eucalypti* were compared *in vivo* and *in vitro* with type specimens and with previous observations from published literature. The only available isolate of *P. eugeniae* (CMW5351) was sterile in culture and it could not be used for morphological comparisons. Ex-holotype cultures of *P. elaeocarpi*,

P. delegatensis and *P. lilianiae* do not exist. The herbarium specimens were examined for *P. epicoccoides* (DAR 6338), *P. eucalypti* (*Septoria normae*) (DAR 65274), *P. lilianiae* (DAR 3832, DAR 3833), *P. delegatensis* (DAR 45718b), *P. eugeniae* (IMI 372655), *Colletogloeopsis nubilosum* (PDD37677) and *C. molleriana* (PREM 54395). Where morphological characteristics of species could not be determined from culture or herbarium specimens, data from published literature were included. Available isolates were characterised using cultural characteristics useful for *Phaeophleospora* species separation, such as conidial pigmentation, number of septa, and conidial size (Crous *et al.* 1997).

Four replicates of each isolate used in comparisons were prepared using 55 mm diam Petri plates and 2 % MEA. After 30 d, cultures were photographed and squash mounts of fruiting structures were prepared on slides in lactoglycerol and observed under an Olympus BH2 light microscope. Each isolate was assessed for conidial size, shape, pigmentation, and number of septa. Unknown species were also assessed for growth rate after one month growing at 20 °C in the dark. The growth rate was determined by measuring perpendicular colony diameters. Wherever possible, 30 measurements of all taxonomically relevant structures were recorded for each species and the extremes have been presented in parentheses. Colony colour for the unknown species was described using notations in the Munsell®Soil Color Charts (Gretag Macbeth, New Windsor, New York, revised 2000). Measurements of conidial size were obtained using the image analysis software Olysia BioReport 3.2 soft imaging system. Data analyses were performed using descriptive statistics in Microsoft Excel. The drawings were prepared using a drawing tube attached to a BH2 Olympus Microscope. These drawings were then scanned on a flatbed scanner at 300 dpi, imported into the software program Macromedia Freehand version 10, and traced into a vector file. This file was then imported into Adobe Photoshop version, airbrushed, and stippled using the Andromeda Series 3 Screen Filter (Barber, unpubl. technique).

Results

Phylogenetic analyses

After repeated attempts to amplify the whole ITS region of herbarium specimens of *Phaeophleospora delegatensis* and *P. lilianiae*, sequence data for the ITS 1 region were obtained. For both species, BLASTn searches on GenBank returned the closest match as *Mycosphaerella molleriana*. A phylogenetic analysis based solely on the ITS1 region placed *P. delegatensis* (EF011654) and *P. lilianiae* (EF011655) in a strongly supported clade with all other *Phaeophleospora* spp. and *Colletogloeopsis* spp. from eucalypts, including newly described species *Colletogloeopsis stellenboschiana* (CBS116428) and *C. gauchensis* (CMW17328, CMW17330) and undescribed *Colletogloeopsis* spp. (CBS111149, CBS110906, CBS116427, CPC18, CBS113621), and far from the type species of *Phaeophleospora, P. eugeniae* (data not shown; TreeBASE SN3058).

The multiple gene genealogies for ITS and EF-1α sequence data compared 28 isolates representing *Phaeophleospora* spp. (including *P. eugeniae*) and *Colletogloeopsis* spp. from *Eucalyptus* and two unknown taxa isolated in this study from *Corymbia* and *Angophora*. The aligned dataset for the combined gene regions consisted of 967 characters of which 428 were parsimony informative and used in the analyses. The partition homogeneity test showed a significant ($P = 0.001$) difference between the data from different gene regions (sum of lengths of original partition was 1406 range for 1 K randomisations was 1409–1428). However, the differences in the topology between the trees was not in the *Phaeophleospora/Colletogloeopsis* clade (data not shown, TreeBASE SN3058), which is the focus of this study, and thus data were combined as suggested previously (Hognabba & Wedin 2003). The combined dataset contained no significant ($P < 0.01$ gl = −090) phylogenetic signal compared with 1000 random trees. Initial heuristic searches of unweighted characters in PAUP resulted in a single most parsimonious trees of 898 steps (CI = 0.63, RI = 0.81).

Phylogeny generated from the combined ITS and EF-1α data (Fig 1, TreeBASE, SN3058) indicates that *Phaeophleospora* and *Colletogloeopsis* species from eucalypts, including isolates of two unknown taxa, resided in a strongly supported clade, clearly separate from *P. eugeniae*. Furthermore, species of *Phaeophleospora* (long 0-multiseptate conidia) and *Colletogloeopsis* (short 0-1 septate conidia) were intermixed within the clade with highly supported BS values. Both Bayesian analysis and parsimony analysis place all *Phaeophleospora* spp. and *Colletogloeopsis* spp. together in a strongly supported clade.

Morphological comparisons

Re-examination of the type specimen of *Phaeophleospora eugeniae* in this study has shown conidia of *P. eugeniae* to differ in conidial pigmentation, length, width, and number of septa from other *Phaeophleospora* species. While conidia of *P. eugeniae* show variation in pigmentation along the conidial length, ranging from light brown cells near the base to subhyaline cells at the apex, the conidia of other *Phaeophleospora* species are uniformly pigmented. Also, conidia of *P. eugeniae* are much longer, broader, and have a greater number of septa when compared with conidia of other *Phaeophleospora* spp. (Table 2).

Morphological observations from herbarium specimens agreed well with the published descriptions, although there were minor exceptions (Table 2). Observation of the type specimen of *P. eugeniae* in the present study showed conidia were slightly shorter (100–115 μm) and had less septa (18–20 septa) than those described previously in the literature (110–120 μm, 16–30 septa). Specimens of *P. destructans* and *P. eucalypti* showed high levels of variability in conidial length, depending on the origin of the specimen. For example, specimens of *P. destructans* from China had shorter conidia (38–47 μm) than those from Indonesia (49–55 μm). The specimen of *P. eucalypti* from Queensland had slightly longer conidia (42–47 μm) than *P. eucalypti* from New South Wales (38–46 μm). Specimens of *P. destructans* had shorter conidia (38–47 μm) than previously recorded (50–65 μm) by Wingfield *et al.* (1996). *Phaeophleospora epicoccoides*, *P. eucalypti* and *P. destructans* produced shorter conidia *in vitro* than *in vivo*.

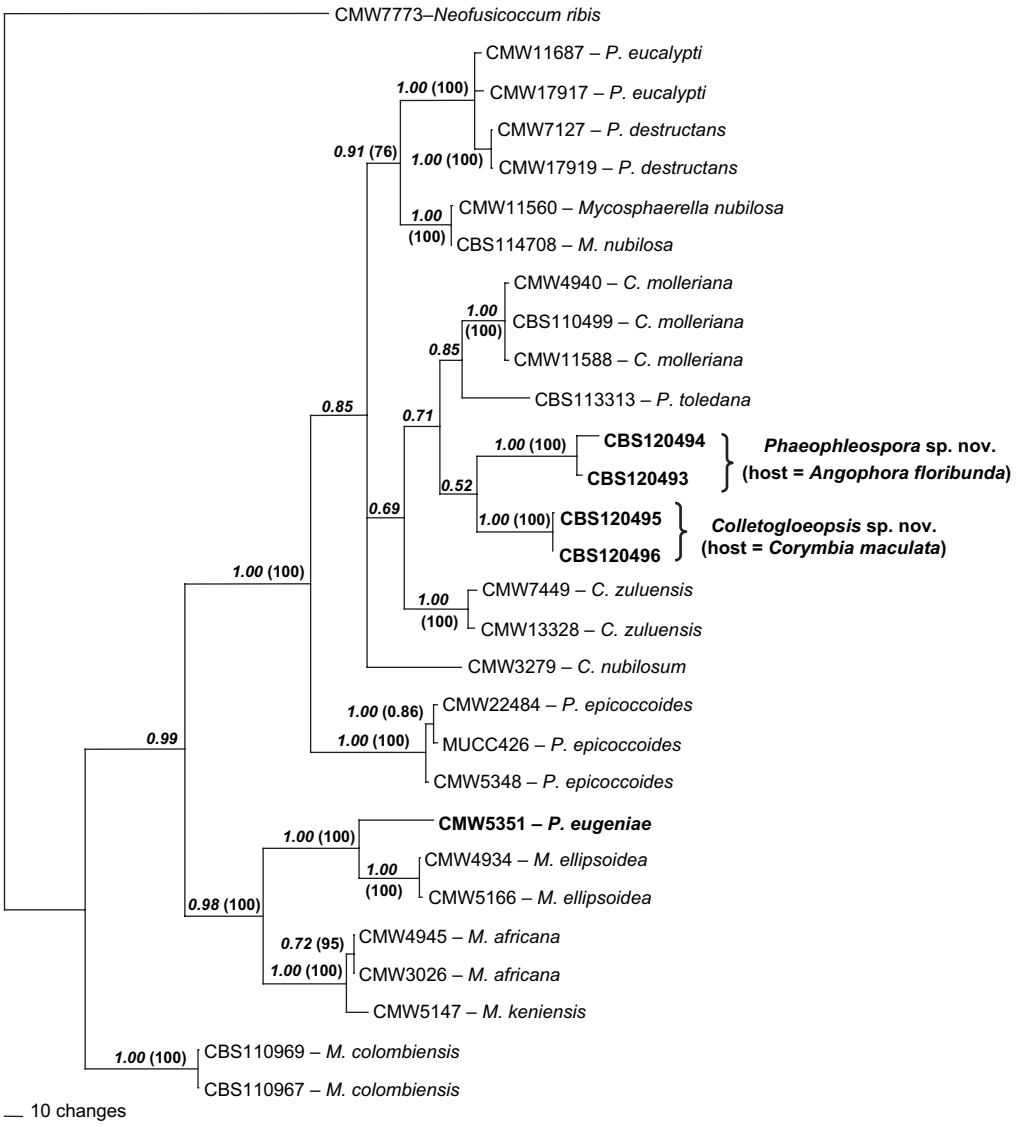


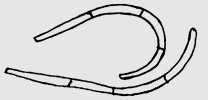
Fig 1 – Consensus phylogram of 9500 trees resulting from Bayesian analysis of the combined ITS and EF-1a sequence data for isolates of *Phaeophleospora* and *Colletogloeopsis*. PPs of the branch nodes are indicated in italics and BS values resulting from parsimony analysis are indicated in brackets. The tree is rooted to *Neofusicoccum ribis*.

Taxonomy

Based on phylogenetic analyses and morphological observations, it is clear that *Phaeophleospora eugeniae* is not related to other species of *Phaeophleospora* that are found on eucalypts. Although it has conidia that are peripherally similar to other species of *Phaeophleospora*, they are much longer, broader, and have more septa. Re-examination of type specimens in this study has shown that pigmentation is uniform along the length of conidia in all species of *Phaeophleospora* occurring on eucalypts. This is different to *P. eugeniae*, where there is distinct gradation in conidial pigmentation from light brown basal cells to subhyaline apical cells. Moreover, phylogenetic analysis has shown that taxa of *Colletogloeopsis* from eucalypts are congeneric with taxa of *Phaeophleospora* from eucalypts. A genus is thus needed to accommodate species of *Phaeophleospora* other than *P. eugeniae*. The most appropriate repository for these species is *Kirramyces*, which we resurrect, with an emended description for species of *Phaeophleospora* occurring on eucalypts.

This study has shown that anamorphs of *Mycosphaerella* from eucalypt leaves and stems, currently residing in *Colletogloeopsis*, occur in a single monophyletic assemblage together with species of *Kirramyces*. However, these fungi all have single-celled conidia that are morphologically very different to the multiseptate conidia of *Kirramyces* spp. On the other hand, one of the unknown species emerging from this study, residing in the same phylogenetic group, and for which a name is needed, has either aseptate or up to three septate conidia. This implies that there is an obvious gradation from single-celled to multi-septate conidia in the

Table 2 – Morphological features of conidia of *Phaeophleospora*, *Colletogloeopsis*, and *Kirramyces* species from eucalypts recorded in published literature and in the present study

Fungus	Specimen number	Pigmentation	Conidial length (*in vivo*) μm	Conidial length (*in vitro*) μm	Conidial width (*in vivo*) μm	Conidial width (*in vitro*) μm	Number of septa	Schematic drawings of conidia
Phaeophleospora eugeniae (Crous *et al.* 1997)	IMI 372655	Sub-hyaline to medium brown	110–120	n/a	7–8	n/a	16–30	
Present study	IMI 372655	Versicoloured	100–115	n/a	4–5	n/a	18–20	
P. epicoccoides (Walker *et al.* 1992)	K 39488	Medium brown	32–50.5	n/a	5–6	n/a	1–4	
(Crous & Wingfield 1997)	PREM 54963	Medium brown	45–55	40–55	3.5–4	3.5–5	1–7	
Present study	MURU 422	Medium brown	44–50	n/a	3.5–4	n/a	n/a	
	MURU 423	Medium brown	45–48	n/a	3.5–4	n/a	n/a	
	PREM 59260	Medium brown	45–53	36–45	3–4	3–4.5	3–6	
	PREM 59258	Medium brown	41–49	n/a	3–4	n/a	n/a	
	DAR 6338	Medium brown	34–48	n/a	3.5–5	n/a	n/a	
P. eucalypti (Walker *et al.* 1992)	K(M) 39487	Pale brown	35–50	n/a	3–4	n/a	0–2	
Septoria normae (Heather 1961)	DAR 65742	Hyaline, yellow to light brown	24–57		3–3.5		1–2	
Present study	MURU 425	Pale brown	42–47	25–36	2–3	2–3.5	0–3	
	MURU 424	Sub-hyaline	38–46	22–28	2–3	2–3	0–3	
	DAR 65742	Sub-hyaline	35–46	n/a	2–3	n/a	1–2	
P. destructans (Wingfield *et al.* (1996))	PREM54416	Pale brown	50–65	n/a	2.5–3	n/a	1–3	
Present study	PREM 59261	Pale brown	38–47	35–40	2–2.5	2–3	1–3	
	PREM 59259	Pale brown	49–55	33–40	2–2.5	2–2.5	1–3	
Kirramyces corymbiae Present study	DAR 77445	Pale brown	17–23	16.5–22	3.5–5	2.5–3.5	0	
K. angophorae Present study	DAR 77452	Sub-hyaline to pale brown	9–15	10.5–22.5	2.5–4	3–4.5	0–3	
P. lilianiae (Walker *et al* 1992)	DAR 3833	Medium brown	35–50	n/a	4–6	n/a	1–3	
Present study	DAR 3832	Medium brown	40–48	n/a	5–6	n/a	1–3	
	DAR 3833	Medium brown	35–43	n/a	5–7	n/a	1–3	
P. delegatensis (Park & Keane 1984)	DAR 45718b	Hyaline	21–51	n/a	3–5	n/a	1	
P. toledana (Crous *et al.* 2004)	CBS 59896	Medium brown	10–12	n/a	3–3.5	n/a	0	
Colletogloeopsis nubilosum (Crous & Wingfield 1997)	PDD 37677	Medium brown	10–15	n/a	4–5	n/a	0	

C. molleriana (Crous & Wingfield 1997)	PREM 54395	Medium brown	9–12	n/a	3–3.5	n/a	0	
C. zuluensis (Cortinas *et al.* 2006a)	IMI 370886	Sub-hyaline to pigmented	4.5–5	n/a	2–2.5	n/a	0–1	
C. gauchensis(Cortinas *et al.* 2006b)	CBS 19722	Medium brown	5–6	n/a	2.5	n/a	0	
C. stellenboschiana (Crous *et al.* 2006)	CBS H-19688	Medium brown	7–9	n/a	3.5	n/a	0	

In vivo, herbarium specimens; *in vitro*, isolates from culture; n/a, not applicable (the isolates did not produce conidia in culture or were not available). Schematic drawings are to scale; relative to *Phaeophleospora eugeniae* with a length of 110 µm.

monophyletic lineage that includes species of *Colletogloeopsis* and those species of *Phaeophleospora* known from eucalypts, now shown to be more appropriately accommodated in *Kirramyces*.

Kirramyces, as emended to include *Phaeophleospora* and *Colletogloeopsis* species known from eucalypts, produces fruiting bodies that are pycnidial, acervular, or both, conidiogenous cells that proliferate percurrently and/or sympodially, and conidia that are rough-walled, or in the case of *Colletogloeopsis* smooth-walled, pigmented, subhyaline to medium brown, with none or up to seven septa (Table 3). Thus, the generic description of *Kirramyces* is emended to accommodate additional species with black, erumpent acervuli, cylindrical to subcylindrical, subhyaline conidiogenous cells and aseptate, fusoid and ellipsoidal, smooth conidia. *Phaeophleospora* is distinguished from *Kirramyces* by the patterns of pigmentation, length of the conidia, and number of septa.

An emended description for *Kirramyces* is as follows:

Kirramyces J. Walker, B. Sutton & Pascoe, *Mycol. Res.* **96**:919 (1992).

Mycelium immersed. *Conidiomata* pycnidioid to acervuloid, immersed to erumpent, brown to black, solitary, unilocular; wall 2–5 cells thick, of brown textura angularis or textura epidermoidea; ostiole central, circular, not papillate. *Conidiogenous cells* discrete or produced on superficial hyphae (when cultivated), ampulliform, doliiform to lageniform or short cylindrical to sub-cylindrical, subhyaline to brown, verruculose, with 1-several percurrent or sympodial proliferations, formed from the inner cells of the pycnidial wall. *Conidia* holoblastic, pigmented, aseptate or euseptate, fusoid to cylindrical to long obclavate, ellipsoidal tapered to obtuse apices, bases truncate to subtruncate with a marginal frill, smooth to verruculose.

Kirramyces epicoccoides (Cooke & Massee) J. Walker, B. Sutton & Pascoe, *Mycol. Res.* **96**:919 (1992).

Basionym: *Cercospora epicoccoides* Cooke & Massee, *Grevillea* **19**: 91 (1891).

Synonyms: *Hendersonia grandispora* McAlpine, *Proc. Linn. Soc. N.S.W.* **28**: 99 (1903).

Phaeoseptoria eucalypti Hansf., *Proc. Linn. Soc. N.S.W.* **82**: 225 (1957).

Phaeoseptoria luzonensis Tak. Kobay., *Trans. Mycol. Soc. Japan* **19**: 377 (1978).

Phaeophleospora epicoccoides (Cooke & Massee) Crous, F.A. Ferreira & B. Sutton, *S. Afr. J. Bot.* **63**: 113 (1997).

Teleomorph: *Mycosphaerella suttonii* Crous & M.J. Wingf., *Can. J. Bot.* **75**: 783 (1997).

The following additional species are thus accepted in *Kirramyces*:

Kirramyces delegatensis (R.F. Park & Keane) Andjic, **comb. nov.**

MycoBank no.: MB511196

Basionym: *Stagonospora delegatensis* R.F. Park & Keane, *Trans. Br. mycol. Soc.*, **83**: 95 (1984).

Table 3 – Comparison of morphological characters defining *Phaeophleospora*, *Kirramyces* and *Colletogloeopsis*

Morphological Characters	*Phaeophleospora* (Crous *et al.* 1997)	*Colletogloeopsis* (Crous & Wingfield 1997) (Cortinas *et al.* 2006a)	*Kirramyces* (present study)
Conidiomata	Pycnidial	Pycnidial, acervular	Pycnidial, acervular
Conidiogenous cell	Pigmented, cylindrical to ampulliform, proliferation percurrent	Subhyaline to pigmented, doliiform to subcylindrical or somewhat irregular, proliferation percurrent and sympodial	Sub-hyaline to pigmented, ampulliform, doliiform to lageniform, or short cylindrical to subcylindrical, somewhat irregular proliferation percurrent and sympodial
Conidia	Pigmented, basal cell light-brown, apical cell pale brown, euseptate, vermiform, long, subcylindrical to obclavate, smooth to rough walled	Pigmented, aseptate rarely 1-septate, subcylindrical, fusoid to ellipsoidal, smooth to verruculose	Pigmented, 0–7, cylindrical-subcylindrical, fusoid to ellipsoidal, rough to smooth walled

Phaeophleospora delegatensis (R.F. Park & Keane) Crous, *Mycol. Mem.* **21**: 51 (1998).
Teleomorph: *Mycosphaerella delegatensis* (R.F. Park & Keane) Crous, *Trans. Br. mycol. Soc.* **83**: 95 (1984).

Kirramyces destructans M.J. Wingf. & Crous, *S. Afr. J. Bot.* **62**: 325 (1996).
Synonym: *Phaeophleospora destructans* (M.J. Wingf. & Crous) Crous, F.A. Ferreira & B. Sutton, *S. Afr. J. Bot.* **63**: 113 (1997).
Teleomorph: not seen but presumed to be a *Mycosphaerella* sp. based on phylogenetic analysis.

Kirramyces eucalypti (Cooke & Massee) J. Walker, B. Sutton & Pascoe, *Mycol. Res.* **96:** 920 (1992).
Basionym: *Cercospora eucalypti* Cooke & Massee, *in* Cooke, *Grevillea* **18**: 7 (1889).
Synonyms: *Septoria pulcherrima* Gadgil & M.A. Dick, *N. Z. J. Bot.* **21**: 49 (1983).
Pseudocercospora eucalypti (Cooke & Massee) Y.L. Guo & X.J. Liu, *Mycosystema* **2**: 234 (1989).
Stagonospora pulcherrima (Gadgil & M.A. Dick) H.J. Swart, *Trans. Br. mycol. Soc.* **90**: 285 (1988).
Phaeophleospora eucalypti (Cooke & Massee) Crous, F.A. Ferreira & B. Sutton, *S. Afr. J. Bot* **63**: 113 (1997).
Teleomorph: not seen but presumed to be a *Mycosphaerella* sp. based on phylogenetic analysis.

Kirramyces lilianiae J. Walker, B. Sutton & Pascoe. *Mycol. Res.* **96**: 921 (1992).
Synonym: *Phaeophleospora lilianiae* (J. Walker, B. Sutton & Pascoe) Crous, F.A. Ferreira & B. Sutton, *S. Afr. J. Bot.* **63**: 115 (1997).
Teleomorph: not seen but presumed to be a *Mycosphaerella* sp. based on phylogenetic analysis.

Kirramyces toledana (Crous & G. Bills), Andjic, **comb. nov.**
Mycobank no. MB511197
Synonym: *Phaeophleospora toledana* Crous & Bills, *Stud. Mycol.* **50**: 208 (2004).
Teleomorph: Mycosphaerella toledana Crous & Bills

Colletogloeopsis spp. from eucalypts are synonymised with *Kirramyces* spp. and new combinations are proposed as follows:

Kirramyces gauchensis (M.N. Cortinas, Crous & M.J. Wingf.) Andjic, M.N. Cortinas & M.J. Wingf. **comb. nov.**
Mycobank no. MB571199
Basionym: *Colletogloeopsis gauchensis* M.N. Cortinas, Crous & M.J. Wingf., *Stud. Mycol.* **55:** 143 (2006).
Teleomorph: not seen but presumed to be a *Mycosphaerella* sp. based on phylogenetic analysis.

Kirramyces molleriana (Crous & M.J. Wingf.), Andjic, & M.J. Wingf. **comb. nov.**
Mycobank no. MB11199
Basionym: *Colletogloeopsis molleriana* Crous & M.J. Wingf, *Can. J. Bot.* **75:** 670 (1997).
Teleomorph: Mycosphaerella molleriana (Thüm.) Lindau, *Nat. Pflanzenfam. (Leipzig)* **1:** 424 (1897).

Kirramyces nubilosum (Ga, nap. & Corbin), Andjic, **comb. nov.**
Mycobank no. MB511200
Basionym: *Colletogloeum nubilosum* Ganap. & Corbin, *Trans. Br. mycol. Soc.* **72:** 237 (1979).
Synonym: *Colletogloeopsis nubilosum* (Ganap. & Corbin) Crous & M.J. Wingf., *Can. J. Bot.* **75:** 668 (1997).
Teleomorph: *Mycosphaerella cryptica* (Cooke) Hansf., *Proc. Linn. Soc. N.S.W.* **81**: 35 (1956).

Kirramyces stellenbochiana (Crous) Andjic, **comb. nov.**
Mycobank no. MB511201
Basionym: *Colletogloeopsis stellenboschiana* Crous, *Stud. Mycol.* **55**: 110 (2006).

Kirramyces sp.
Teleomorph: *Mycosphaerella pseudocryptica* Crous, *Stud. Mycol.* **55**:116 (2006), anamorph as *Colletogloeopsis*

Kirramyces zuluensis (M.J. Wingf., Crous & T.A. Cout.) Andjic & M.J. Wingf., **comb. nov.**
Mycobank no. MB511202

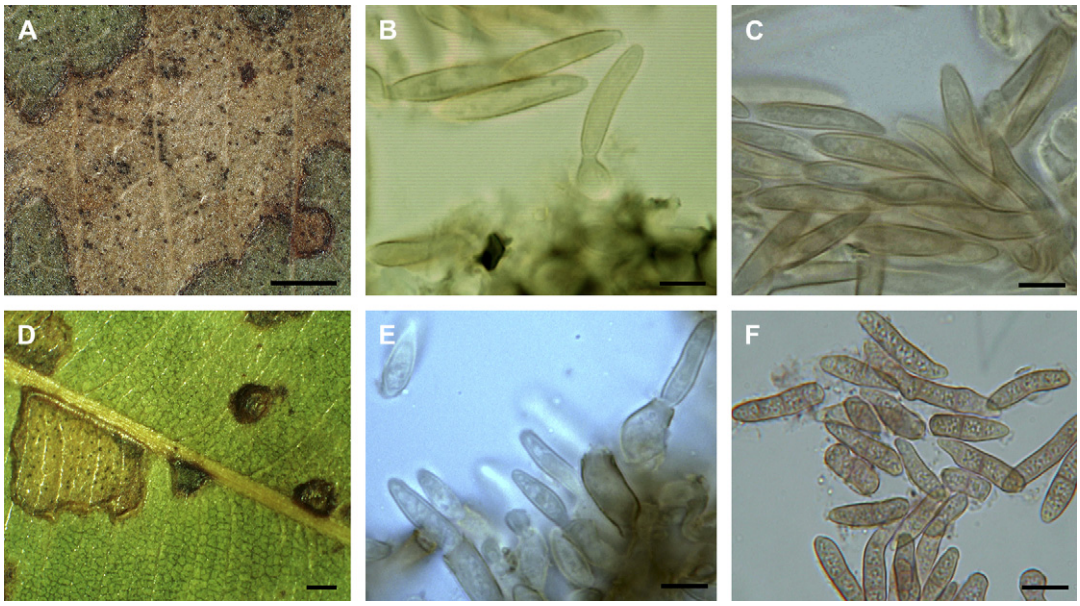


Fig 2 – *Kirramyces corymbiae* (A). Leaf lesion on *Corymbia maculata* showing small black pycnidia (B). Conidium attached to a conidiogenous cell, (C). Aseptate conidia; *Kirramyces angophorae* (D). Leaf lesion on *Angophora floribunda* showing small black pycnidia (E). Conidia attached to conidiogenous cells, (F). Aseptate to multi-septate conidia. Bars = (A, D) 10 mm, (B–C, E–F) 10 μm. Squash mounts prepared from *in vivo* material.

Basionym: *Coniothyrium zuluense* M.J. Wingf., Crous. & T .A. Cout., *Mycopathologia* **136:** 142 (1997).

Synonym: *Colletogloeopsis zuluensis* (M.J. Wingf., Crous & T.A. Cout.) M.N. Cortinas, M.J. Wingf. & Crous, *Mycol. Res.* **110**: 233 (2006).

Teleomorph: Not seen but presumed to be a *Mycosphaerella* sp. based on phylogenetic analysis.

Based on phylogenetic analysis and morphological observations it is clear that the new species isolated from *Angophora* and *Corymbia* should reside in the genus *Kirramyces* and they are described below.

Kirramyces corymbiae Carnegie, Andjic & P.A. Barber, **sp. nov.** Figs 2, 3A–C

MycoBank no.: MB510110

Etym.: Named after the host on which this fungus is found.

Teleomorph: Not seen but presumed to be a *Mycosphaerella* sp. based on phylogenetic analysis.

Conidiomata pycnidialia amphigena, subepidermalia, solitaria ad raro aggregata, atra, globosa, uniloculata, ad 90 μm diam; paries ex 2 vel 3 stratis texturae angularis constans. *Cellulae conidiogenae* discretae, subhyalinae ad pallide brunneae, doliiformes, 6–13 μm. *Conidia* holoblastica, fusiformia, recta ad plerumque curvata, raro sigmoidea, apice subobtuso, basi truncata, non prominente guttulata, pallide brunneae, aseptata, (14–)17–23(–24) × 3.5–5 μm.

Typus: **Australia**: *New South Wales*: Mandalong (native forest), on leaves of *C. maculata*, 15 Jan. 2003, *A.J. Carnegie* (DAR 77445 — *holotypus*; culture ex-type DAR 77445).

Leaf spots amphigenous, sub-circular to irregular, single to confluent, 1–10 mm diam., yellow-brown with thin green-brown to red-purple margin. *Conidiomata* pycnidial, amphigenous, sub-epidermal, single to occasionally aggregated, black, globose, unilocular, to 90 μm diam; wall of 2–3 layers of *textura angularis*. *Conidiogenous cells* discrete, subhyaline to light brown, doliiform, 6–13 μm. *Conidia* holoblastic, fusiform, straight to mostly curved, occasionally sigmoidal, apex sub-obtuse, base truncate, not prominently guttulate, pale brown, aseptate, (14–)17–23(–24) × 3.5–5 μm.

Conidial germination on MEA after 24 h: Conidia becoming 1–2-septate, germ tubes growing at an acute angle from both ends of the conidia, each germ tube less than 10 μm long at 24 h.

Cultures: Colonies slow-growing, 9–14 mm diameter on MEA after one month at 25° C in the dark, margin white 5Y 8/1, top dark grey 5Y 3/1, bottom light pink 5YR 8/3, colony sectored. *Conidia*: fusiform, pale brown, straight to mostly curved, aseptate (8.5–)10.5–22.5(–25) × (1.5–)3–4.5(–5)(mean = 16.5 × 3.5 μm).

Hosts: C. variegata, *C. maculata*, and *C. henryi*.

Geographical distribution: Native forests and plantations in NSW Australia, very common and occasionally damaging.

Additional specimens examined: **Australia:** *NSW:* Mallanganee, Richmond Range State Forest, on *C. variegata*, 22 June 2002, *A.J. Carnegie* (DAR 77447): Kempsey, adj. Bains Dairy Plantation, on *C. maculata*, 29 May 2003, *A. J. Carnegie* (DAR 77448); Kiwarak State Forest, on *C. maculate,* 31 May 2003, *A.J. Carnegie* (DAR 77449); Dilkoon, Zuill Plantation, on *C. variegata*, 24 Aug. 2003, *A.J. Carnegie* (DAR 77450); Baryugil, Ibbot Plantation, on *C. maculata, A.J. Carnegie* (DAR 77446).

Kirramyces angophorae Andjic, Carnegie & P.A. Barber, **sp. nov.** Figs 2D–F, 4

MycoBank no.: MB510110

Etym.: Named after the host on which this species was found.

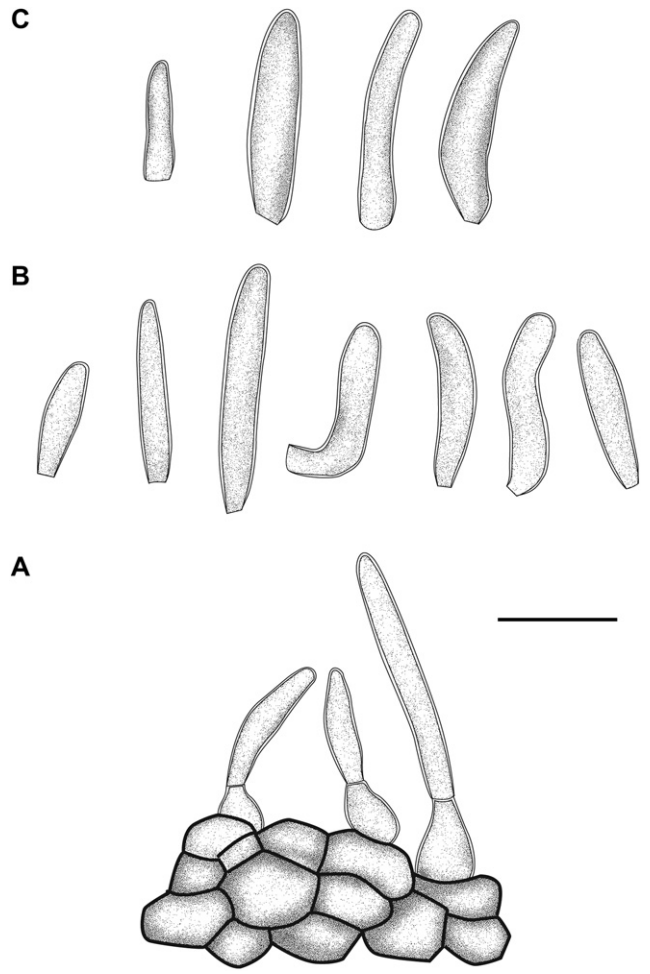


Fig 3 – *Kirramyces corymbiae*. (A–B) Conidiogenous cells and conidia produced *in vivo*. (C) Conidia produced on MEA. Bar = 10 μm.

Teleomorph: not seen but presumed to be a *Mycosphaerella* sp. based on phylogenetic analysis.

Conidiomata pycnidialia amphigena, plerumque, hypophyllosa, solitaria, atrobrunnea ad atra, uniloculata, ad 92 μm diam. parietibus 3 – stratis texturae angularis. *Cellulae conidiogenae* cellulis superis stromatum orientes, doliiformes ad subcylindraceae vel ampuliformes, aseptatae ad 1-septatae, 6.5–12 × 2.5–4 μm, parietibus crassis, subhyalinae ad pallide brunneae, verruculosae, enteroblasticae prolificantes, 1-3 percurrenter, raro sympodialiter. *Conidia* solitaria, aseptata ad 1-3 septata, subhyalina et pallide brunneae, verrucolosa, fusiformia, subcylindraceae ad ellipsoidea, recta ad parvum curvata, basis truncata, fimbriata, margine imbricato, apice subobtuso ad obtuso, (4.5–)9–15(–19) × (1.5– 2.5–4 (–4.5) (mean = 12 × 3.5 μm).

Typus: **Australia**: *NSW,* Greenwich, Lane Cove Bushland, on leaves of *Angophora floribunda,* 27 Feb. 2005, *A.J. Carnegie MURU 426* (DAR 77452 - holotypus; culture ex-type DAR 77452).

Leaf spots amphigenous, circular to irregular, 2–8 mm diam., single to confluent, red–brown with prominent purple border. *Conidiomata* pycnidial, amphigenous, predominantly hypophyllous, solitary, dark brown to black, unilocular, up to 92 μm diam; wall of three layers of *textura angularis*. *Conidiogenous cells* arising from upper cells of the stroma, doliiform to subcylindrical or ampulliform, aseptate to 1- septate, 6.5–12 × 2.5–4 μm, thick-walled, subhyaline to pale brown, verruculose, proliferating enteroblastically, 1-3 times percurrently, occasionally sympodially. *Conidia* single, aseptate to 1-3 euseptate, subhyaline to pale brown, verruculose, fusoid, subcylindrical to ellipsoidal, straight to slightly curved; base truncate with a marginal frill, apex sub-obtuse to obtuse, (4.5–)9–15(–19) × (1.5–)2.5–4(–4.5) (mean = 12 × 3.5 μm).

Cultures: Colonies 28 × 22 mm after one month at 25 °C in the dark, produce red pigmentation (10R 5/6) in agar, upper surface of culture olive 5Y 5/3, reverse dark olive 5Y 3/2. *Conidiogenous cells* 5.5–1.5 × 2.5–6 μm. *Conidia* (8.5–)10.5–22.5 (–25.5) (mean = 16.5 μm) × (1.78–)3–4.5(5–) (mean = 3.5 μm) 0–3 euseptate, lateral branches present as secondary conidia, mycelium in culture producing a synanamorph resembling

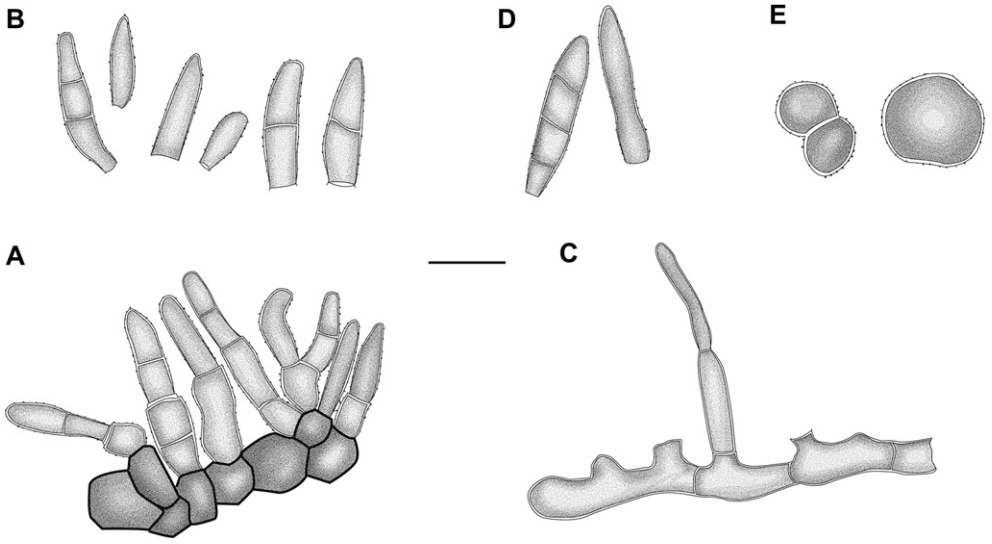


Fig 4 – *Kirramyces angophorae*. (A–B) Conidiogenous cells and conidia produced *in vivo*; (C–D) conidiogenous cells and conidia produced on MEA; (E) mycelium in culture producing chlamydospore-like synanamorph. Bar = 10 μm.

chlamydospores. *Chlamydospores* (9.5–)10–13(–13.5) × (7.5–)9–11.5(–14) (mean = 11.5 × 10.5 μm), dark brown, rounded, thick-walled.

Host: Angophora floribunda.

Geographical Distribution: Native forests in NSW, Australia.

Notes: Kirramyces angophorae can be distinguished from other *Kirramyces* spp. by producing a synanamorph with chlamydospore-like structures and a red pigment in culture. Unlike other *Kirramyces* spp., *K. angophorae* produces longer conidia in culture than on the host.

Key to Kirramyces *species occurring on eucalypts*

1 Conidia versicoloured, apex and basal cells lighter than the rest of conidial body, on average >100 μm long..... **Phaeophleospora**
Conidia uniformly pigmented, on average <70 μm long..... **Kirramyces**

2(1) Conidia aseptate to rarely 1-septate 3
Conidia 1 to multi-septate, rarely aseptate 10

3(2) Conidia aseptate, fusiform, straight to mostly curved, occasionally sigmoidal, apex sub-obtuse, base truncate, on average >17 μm in length..... **corymbiae**
Conidia on average < 15 μm in length 4

4(3) Conidia on average < 6 μm in length 5
Conidia on average > 6 μm in length 6

5(4) Conidia broadly ellipsoidal, finely verruculose, apex obtuse to sub-obtuse, base sub-truncate to bluntly rounded, 5–6 × 2.5 μm..... **gauchensis**
Conidia, fusoid to subcylindrical to ellipsoidal, smooth to verruculose, apex obtuse, base, subtruncate, 4.5–5 × 2–2.5 μm..... **zuluensis**

6(4) Conidia on average <10 μm in length..... **stellenboschiana**
Conidia on average >10 μm in length.....7

7(6) Conidia fusoid.....8
Conidia subcylindrical to ellipsoidal.....9

8(7) Conidia, finely verruculose, 12–14 × 4 μm; teleomorph *Mycospharella pseudocryptica* ..... **Kirramyces sp.**
Conidia, verruculose, apex acutely rounded, base truncate with a minute marginal frill, 10–12 × 3–3.5 μm; teleomorph *Mycosphaerella toledana* ..... **toledana**

9(7) Conidia, aseptate rarely becoming 1-septate in culture, 9–12 × 3–3.5 μm; teleomorph *Mycosphaerella molleriana*…**molleriana**
Conidia, aseptate, 10–15 × 4–5 μm; teleomorph *Mycospharella cryptica*..... **nubilosum**

10(2) Conidia on average <30 μm in length, ...**angophorae**
Conidia on average >30 μm in length.....11

11(10) Conidia medium brown, typically 3–5 septate 12
Conidia hyaline to sub-hyaline to pale brown, typically 1–3 septate 13

12(11) Conidia typically 3-5-septate, occasionally with up to 7 septa, subcylindrical to narrowly obclavate, apex sub-obtuse 45–55 × 3.5–4 μm; teleomorph *Mycosphaerella suttoniae*..... **epicoccoides**
Conidia typically 3-septate, cylindrical, apex obtuse, 40–48 × 5–6 μm, no known teleomorph ..... **lilianiae**

13(11) Conidia 1-septate, hyaline, cylindrical, straight or curved, smooth, thin walled, apex obtuse, base truncate 21–51 × 3–5 μm; teleomorph *Mycosphaerella delegatensis* ..... **delegatensis**
Conidia subhyaline to pale brown 14

14(13) Conidia typically 1-2-septate, less typically 0-3-septate, subcylindrical to narrowly obclavate, thick walled, finely verruculose, apex sub-obtuse, inconspicuous marginal frill present 35–50 × 3–4 μm, no known teleomorph.... **eucalypti**
Conidia 1-3-septate, cylindrical, verruculose, apex obtuse, marginal frill mostly absent 50–65 × 2.5 μm, no known teleomorph .....**destructans**

Additional specimens examined: **Australia**: *NSW*: Raymond Terrace, on *Angophora floribunda*, 29 Nov. 2005, *A.J. Carnegie* (DAR 77451).

Discussion

Mycosphaerella spp. and their anamorphs include some of the most important pathogens of eucalypts. Many of them have also been moved around the world through the establishment of plantations of these trees. In recent years, numerous new species of these fungi have been described (Crous *et al.* 2004, 2006), and based on the large number of eucalypt species, it seems likely that many more will be discovered in the future. Many of these fungi are morphologically similar or difficult to distinguish based on morphology and their contemporary taxonomy relies heavily on DNA sequence comparisons. Results of this study, using phylogenetic inference and morphological characteristics, have led to the discovery of two new species of these fungi. In order to accommodate these species, the need to modify the boundaries of the genera to which they belong became evident.

The present phylogenetic and morphological study has shown the type specimen of the genus *Phaeophleospora*, *P. eugeniae*, is well separated from *Phaeophleospora* spp. occurring on eucalypts. This phylogenetic separation logically led to the resurrection of the previous generic name, *Kirramyces*, for these species. Furthermore, phylogenetic analysis combined with the overlapping morphological characters of *Kirramyces* spp. and *Colletogloeopsis* spp. occurring on eucalypts, supporting the synonymy of these genera. Thus, anamorphs of *Mycosphaerella* residing in *Phaeophleospora* occurring on eucalypts, as well as species of *Colletogloeopsis*, have been transferred to the newly resurrected genus *Kirramyces*. Re-examination in the present study of the type specimens of both *Phaeophleospora* and *Kirramyces* has shown that variation in pigmentation of conidia is a useful morphological feature in distinguishing between these two genera.

The phylogenetic relationship between the genera *Phaeophleospora* (now *Kirramyces*) and *Colletogloeopsis* has been shown in previous studies (Andjic *et al.* 2007; Cortinas *et al.* 2006b; Crous *et al.* 2001, 2006; Hunter *et al.* 2006). The two genera have not previously been combined, mainly because *Phaeophleospora* produced pycnidioid conidiomata and 0-multiseptate conidia, whereas *Colletogloeopsis* produced acervular conidiomata and aseptate or rarely 1-septate conidia. The emendment of the description of *Colletogloeopsis* to accommodate species with pycnidia (Cortinas *et al.* 2006a) resulted in conidial size and septation being the only morphological characters separating the two genera. However, discovery of new species such as *K. angophorae*, with aseptate as well as multiseptate conidia, precludes retaining *Colletogloeopsis* for aseptate species in this group. Furthermore, these differences are not phylogenetically supported between species within the genus.

In the present study, it was possible to obtain the sequences for the ITS1 region from the type specimens of *K. lilianiae* and *K. delegatensis*. Walker *et al.* (1992) described *K. lilianiae* as morphologically very similar to *K. epicoccoides*. However, due to the lack of a suitable number of collections of *K. lilianiae*, comparison between the two species was not possible. *Stagonospora delegatensis*, first described by Park & Keane (1984) was later considered by Swart (1988) to be congeneric with *Septoria pulcherrima*. Walker *et al.* (1992) noted that *S. delegatensis* was similar to *K. eucalypti* and thus was a possible candidate to transfer to *Kirramyces*, but required further collections. It was later reduced to synonymy with *K. eucalypti* (Sankaran *et al.* 1995). Subsequently, Crous (1998) re-examined the type specimen of *S. delegatensis* and supported the placement of this fungus in the genus *Kirramyces*. However, based on conidial shape, they chose to retain the species as separate from *K. eucalypti* and transferred it to *Phaeophloeospora* as *P. delegatensis*. Results of the present study based on ITS1 sequence data have shown that *K. delegatensis* and *K. lilianiae* cluster together with other *Kirramyces* species occurring on eucalypts, therefore confirming its placement in *Kirramyces*.

Kirramyces was originally described for three species: *K. epicoccoides*, *K. eucalypti* and *K. lilianiae*. Based on the results of the present and previous studies, the genus now includes 14 species. These all reside in a well-resolved, monophyletic clade based on DNA sequence comparisons. They also have conidia ranging from those that are aseptate to multiseptate.

Mycosphaerella is a heterogenous genus that is linked closely to a large number of anamorphs that lack known teleomorphs (Crous & Braun 2003). Previous authors have debated whether anamorphic states should be used to separate genera, subgenera, or sections within *Mycosphaerella*. Sutton & Hennebert (1994) held the view that different conidiogenous events and conidiomatal types in anamorphs linked to *Mycosphaerella* may prove useful in grouping species at some subgeneric level. Based on phylogeny, this has not proved to be true, as many anamorphs of *Mycosphaerella* spp. are currently polyphyletic (Crous *et al.* 2006).

Hunter *et al.* (2006) suggested that anamorph relationships based on phylogenetic position within *Mycosphaerella* cannot be predicted. However, results of the present study have shown that all *Kirramyces* spp. from eucalypts (including several as yet undescribed species) reside in the same strongly supported clade. This is also true for species of *Pseudocercospora* (Crous & Braun 2003) and *Readeriella* (Crous *et al.* 2004, 2006; Hunter *et al.* 2006). Morphologically, *Readeriella* spp. are somewhat similar to species in the genus *Kirramyces,* but are distinctly different as *Readeriella* spp. have obvious phialidic conidiogenesis. This was a key feature used by Sutton (1980) to differentiate between the genera *Microsphaeropsis* and *Coniothyrium* occurring on eucalypts. A number of species collected and identified as *Microsphaeropsis* or *Kirramyces,* based on morphological characters, have subsequently been compared based on DNA sequence data and these have resolved taxonomic conflicts between *Readeriella* and *Kirramyces* (Andjic, unpubl. data). In the case of *Readeriella* and *Kirramyces*, the mode of conidiogenesis appears to be phylogenetically significant. We suspect that new collections and subsequent DNA sequence comparisons for previously described *Microsphaeropsis* spp. from eucalypts, including *M. conielloides*, *M. callista*, *M. globulosa,* and *M. olivaceae* will show that these fungi reside in the genus *Readeriella*.

Data emerging from this study provide clear evidence that at least some groups of anamorphs of *Mycosphaerella* spp.

reside in strongly monophyletic lineages. These are generally also consistent with their morphological features. These are interesting and important observations that most likely reflect ecological adaptation and evolutionary events. How these relate to a possible subdivision of *Mycosphaerella* based on phylogenetic inference is difficult to predict. Clearly, many anamorph genera are emerging in discrete clades that are very different to the one that accommodates *M. punctiformis*, the type species of the genus. There is good evidence that *Mycosphaerella* is polyphyletic and its subdivision into more natural subdivisions will emerge in time. We believe the anamorphs of this important genus encompass valuable ecological inference and should not be lost from future phylogenetic treatments of the group.

This study has clarified the generic placement of a large number of *Mycosphaerella* spp. or their anamorphs occurring on leaves, shoots, and stems of eucalypts. These also include some of the most important pathogens of *Eucalyptus* residing in *Mycosphaerella*. For example, the stem pathogens, *K. zuluensis* and *K. gauchensis* cause the disease known as *Coniothyrium* canker, which is one of the most important diseases of *Eucalyptus* spp. grown in plantations (van Zyl 1999; Wingfield; Crous & Couthinho 1997). Likewise, *K. destructans*, *K. eucalypti*, *K epicoccoides,* and *K. nubilosum* (anamorph of *M. cryptica*) represent four of the most important leaf pathogens of *Eucalyptus* spp. (Park *et al.* 2000; Barber 2004; Burgess *et al.* 2006; Carnegie 2007). Of these, *K. destructans* is particularly damaging because it infects both leaves and shoots of trees and it has caused substantial damage to plantations in southeast Asia. The majority of the most important *Mycosphaerella* spp. that infect eucalypts reside in the phylogenetic clade accommodating species of *Kirramyces*. The evolutionary significance of this relationship deserves further study.

Acknowledgements

This work was funded in part by the Australian Research Council DP0343600, 'Population genetics of fungal pathogens that threaten the biosecurity of Australia's eucalypts'. V.A. is a recipient of a Murdoch University Doctoral Research Scholarship. Pedro W. Crous is thanked for useful discussion on the correct generic name for this group. We acknowledge funding from various grants to the University of Pretoria linked to tree protection research and a collaborative research agreement linking the University of Pretoria and Murdoch University. Finally we thank Alex George for editing the Latin descriptions.

REFERENCES

Andjic V, GEStJ Hardy, Cortinas MN, Wingfield MJ, Burgess TI, 2007. Multiple gene genealogies reveal important relationships between *Phaeophleospora* spp. infecting *Eucalyptus* leaves. *FEMS Microbiology Letters* **268**: 22–33.

Barber PA, 2004. Forest pathology: the threat of disease to plantation forests in Indonesia. *Plant Pathology Journal* **3**: 97–104.

Bond TET, 1947. *Phaeophleospora elaeocarpi* on *Elaeocarpus amoenus*. *Ceylon Journal of Science, sect. A* **12**: 185.

Burgess TI, Andjic V, GEStJ Hardy, Dell B, Xu D, 2006. First report of *Phaeophleospora destructans* in China. *Journal of Tropical Forest Science* **18**: 144–146.

Carnegie AJ, 2007. Forest health condition in New South Wales, Australia, 1996–2005. II. Fungal damage recorded in eucalypt plantations during forest health surveys and their management. *Australasian Plant Pathology* **36**: 225–239.

Carbone I, Kohn LM, 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* **91**: 553–556.

Cortinas MN, Burgess TI, Dell B, Xu D, Wingfield MJ, Wingfield BD, 2006a. First record of *Colletogloeopsis zuluense* comb nov. causing a stem canker of *Eucalyptus* spp. in China. *Mycological Research* **110**: 229–236.

Cortinas MN, Crous PW, Wingfield BD, Wingfield MJ, 2006b. Multigene phylogenies and phenotypic characters distinguish two species within the *Colletogloeopsis zuluensis* complex associated with *Eucalyptus* stem cankers. *Studies in Mycology* **55**: 135–149.

Crous PW, Braun U (eds), 2003 Mycosphaerella *and its anamorphs 1. Names published in Cercospora and Passalora*. Centraalbureau voor Schimmelcultures, Utrecht.

Crous PW, 1998. *Mycosphaerella* spp and their anamorphs associated with leaf spot diseases of *Eucalyptus*. *Mycologia Memoir* **21**: 1–170.

Crous PW, Ferreira FA, Sutton BC, 1997. A comparison of the fungal genera *Phaeophleospora* and *Kirramyces* (coelomycetes). *South African Journal of Botany* **63**: 111–115.

Crous PW, Groenewald JZ, Mansilla JP, Hunter GC, Wingfield MJ, 2004. Phylogenetic reassessment of *Mycosphaerella* spp and their anamorphs occurring on *Eucalyptus*. *Studies in Mycology* **50**: 195–214.

Crous PW, Hong L, Wingfield BD, Wingfield MJ, 2001. ITS rDNA phylogeny of selected *Mycosphaerella* species and their anamorphs occurring on *Myrtaceae*. *Mycological Research* **105**: 425–431.

Crous PW, Wingfield MJ, 1997. *Colletogloeopsis* a new coelomycete genus to accommodate anamorphs of two species of *Mycosphaerella* on *Eucalyptus*. *Canadian Journal of Botany* **75**: 667–674.

Crous PW, Wingfield MJ, Mansilla JP, Alfenas AC, Groenewald JZ, 2006. Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus* II. *Studies in Mycology* **55**: 99–131.

Crous PW, Wingfield MJ, Marasas WFO, Sutton BC, 1989. *Pseudocercospora eucalyptorum* sp. nov. on *Eucalyptus* leaves. *Mycological Research* **93**: 394–398.

Farris JS, Kallersjo M, Kluge AG, Bult C, 1995. Testing significance of incongruence. *Cladistics* **10**: 315–319.

Felsenstein J, 1985. Confidence intervals on phylogenetics: an approach using bootstrap. *Evolution* **39**: 783–791.

Gardes M, Bruns T, 1993. ITS primers with enhanced specificity for basidiomycetes — application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113–118.

Graham GC, Meyers P, Henry RJ, 1994. A simplified method for preparation of fungal DNA for PCR and RAPID analysis. *Biotechniques* **16**: 48–50.

Heather WA, 1961. Studies on *Septoria* n. sp. causing a leaf blotch of *Eucalyptus dalrympleana Maid*. Sydney, Australia, MSc thesis, University of Sydney.

Hillis DM, Huelsenbeck JP, 1992. Signal noise and reliability in molecular phylogenetic analysis. *Journal of Heredity* **83**: 189–195.

Hognabba F, Wedin M, 2003. Molecular phylogeny of the *Sphaerophorus globusus* species complex. *Cladistics* **19**: 224–232.

Huelsenbeck JP, Bull JJ, Cunningham CV, 1996. Combining data in phylogenetic analysis. *Trends in Ecology and Evolution* **11**: 152–158.

Hunter GC, Wingfield BD, Crous PW, Wingfield MJ, 2006. A multi-gene phylogeny for species of *Mycosphaerella* occurring on *Eucalyptus* leaves. *Studies in Mycology* **55**: 147–161.

Maxwell A, Dell B, Neumeister-Kemp HG, Hardy GEStJ, 2003. *Mycosphaerella* species associated with *Eucalyptus* in south-western Australia: new species new records and a key. *Mycological Research* **107**: 351–359.

Morgan-Jones G, 1974. Icones generum Coelomycetum. Fascicle VII. *University of Waterloo Biology Series* **14**: 1–42.

Nylander JAA, 2004. *MrModeltest*. Version 2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Park RF, Keane PJ, 1984. Further *Mycosphaerella* species causing leaf diseases of *Eucalyptus*. *Transactions of the British Mycological Society* **83**: 93–105.

Park RF, Keane PJ, Wingfield MJ, Crous PW, 2000. Fungal diseases of eucalypt foliage. In: Keane PJ, Kile GA, Podger FD, Brown BN (eds), *Diseases and Pathogens of Eucalypts* CSIRO Publishing, Melbourne, pp. 153–240.

Rangel E, 1916. Fungos do Brasil novo ou mal conhecidos por Eugenia Rangel. *Archive Musium Nacional Rio de Janeiro* **18**: 157–164.

Ronquist F, Heuelsenbeck JP, 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Sankaran KC, Sutton BC, Minter DW, 1995. A checklist of fungi recorded on eucalypts. In: *Mycological Papers*, **vol. 170**. CAB International, Oxon.

Sutton BC, 1977. *Coelomycetes* VI. Nomenclature of generic names proposed for *Coelomycetes*. *Mycological Papers* **141**: 65.

Sutton BC, 1980. *The Coelomycetes: fungi imperfecti with pycnidia, acervuli and stromata*. Commonwealth Mycological Institute, Kew.

Sutton BC, Hennebert GL, 1994. Interconnections amongst anamorphs and their possible contribution to ascomycetes systematics. In: Hawksworth DL (ed), *Ascomycetes Systematics: problems and perspectives in the nineties*. Plenum Press, New York, pp. 77–98.

Swart HJ, 1988. Australian leaf-inhabiting fungi XXVI. Some noteworthy coelomycetes on *Eucalyptus*. *Transactions of the British Mycological Society* **90**: 279–291.

Swofford DL, 2003. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, MA.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**: 4876–4882.

van Zyl LM, 1999. *Factors associated with Coniothyrium Canker of Eucalyptus in South Africa*. PhD thesis, University of the Orange Free State.

Walker J, Sutton BC, Pascoe IG, 1992. *Phaeoseptoria eucalypti* and similar fungi on *Eucalyptus* with description of *Kirramyces* gen. nov. (*Coelomycetes*). *Mycological Research* **96**: 911–924.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innes MA, Gelfand DH, Sninsky JJ, White TJ (eds), *PCR Protocols: a guide to methods and applications* Academic Press, San Diego, pp. 315–322.

Wingfield MJ, Crous PW, Boden D, 1996. *Kirramyces destructans* sp. nov. a serious leaf pathogen of *Eucalyptus* in Indonesia. *South African Journal of Botany* **62**: 325–327.

Wingfield MJ, Crous PW, Couthinho TA, 1997. A serious new canker disease of *Eucalyptus* in South Africa caused by a new species of *Coniothyrium*. *Mycopathologia* **136**: 139–145.

Wittzell H, 1999. Chloroplast DNA variation and reticulate evolution in sexual and apomictic sections of dandelions. *Molecular Ecology* **8**: 2023–2035.