Pseudocercospora flavomarginata sp. nov., from Eucalyptus leaves in Thailand

Abstract: Mycosphaerella represents one of the largest ascomycete genera accommodating more than 3000 names. Approximately 60 Mycosphaerella species have been linked to leaf diseases on Eucalyptus species, collectively known as Mycosphaerella Leaf Disease (MLD). Many hyphomycete and coelomycete anamorph genera are linked to Mycosphaerella and several species of the hyphomycete genus Pseudocercospora are associated with MLD symptoms on various Eucalyptus species. Eucalyptus trees in Vietnam and Thailand, particularly those of E. camaldulensis and hybrids of this species, commonly have a leaf spot disease caused by a species of Pseudocercospora. Lesions associated with this disease are very characteristic, with chlorotic margins and masses of brown conidiophores occurring predominantly on the abaxial lesion surface. The aim of this study was to characterise the Pseudocercospora species associated with this disease. This was achieved through studying the morphology of the fungus and via DNA sequence analysis from four nuclear gene regions. Results showed that the fungus represents an undescribed species of Pseudocercospora, that is formally described here as Pseudocercospora flavomarginata.

INTRODUCTION

Species of *Eucalyptus* are currently some of the most popular tree species for commercial forestry, with plantations expanding more rapidly than for any other tree species (Turnbull 2000). This is largely due to their favourable wood qualities, relatively rapid growth and their adaptability to a very wide range of different environments (Turnbull 2000). *Eucalyptus* spp. are thus propagated in commercially productive plantations in many tropical and sub-tropical countries, where timber is used primarily for the paper and pulp industry. In south-east Asia, the forestry industry is expanding rapidly, and it has been estimated that there are approximately 2 million ha of *Eucalyptus* plantations in the area (Old *et al.* 2003).


The teleomorph state, represented by abundant small pseudothecia, is most commonly associated with MLD on *Eucalyptus* leaves (Crous 1998). *Mycosphaerella* is, however, linked to approximately 23 anamorph genera including both coelomycetes and hyphomycetes (Crous *et al.* 2000, Crous & Braun 2003, Kirschner *et al.* 2004, Schubert & Braun 2005). Recent surveys of *Eucalyptus* foliage in many parts of the world have led to a significant increase in the number of *Mycosphaerella* spp. found on these trees (Crous *et al.* 2004, 2006).

The anamorphic genus *Pseudocercospora* Speg. is large and morphologically diverse (Crous *et al.* 2000). Species of *Pseudocercospora* occur on many plant hosts where they cause leaf spots (Crous & Braun 1996, Crous *et al.* 1997, Crous & Braun 2001, Braun & Dick 2002). These fungi typically have dematiaceous conidiophores and scolecosporous conidia with inconspicuous conidial scars and conidiogenous cells that proliferate sympodially and percurrently (Crous & Wingfield 1997, Crous *et al.* 2000, Crous & Braun 2003).

A very distinct leaf spot disease especially on *Eucalyptus camaldulensis* and hybrids of this and other species is well known in Thailand and Vietnam (Old *et al.* 2003). The symptoms of this disease are very obvious and not easily confused with other leaf spots. Based on the occurrence of abundant conidiophores that are found on the lesion, it is well recognised that the disease is caused by a species of *Pseudocercospora* (Old *et al.* 2003). The
fungus has, however, never been critically compared with other *Mycosphaerella* spp. occurring on *Eucalyptus*, nor has it been formally named. The aim of this study was, therefore, to characterise the fungus and to provide a name for it. This was achieved through critical study of its morphological characteristics by comparison to other *Pseudocercospora* spp. known to occur on *Eucalyptus* (Crous 1998, Braun & Dick 2002) and via comparisons of DNA sequences for the Large Subunit (LSU) and Internal Transcribed Spacer (ITS) region of the rRNA operon, the Actin (ACT) and Translation Elongation Factor 1-alpha (EF-1α) gene regions.

**MATERIALS AND METHODS**

**Sample collection and fungal isolations**

Leaf spots on *Eucalyptus camaldulensis* and hybrids of this species were collected from trees growing in plantations in various parts of Thailand. Diseased leaves showing the typical lesions with very distinct chlorotic margins and bearing conidiophores of a *Pseudocercospora* sp. were collected for subsequent laboratory study.

Leaf lesions were examined under a dissection microscope for the presence of *Pseudocercospora* conidiophores. A sterile inoculation needle was used to scrape conidia from the lesions and these were spread onto 2 % MEA agar plates (wt/v) (malt extract agar) (Biolab, South Africa) and incubated at 25 °C. Subsequently, single germinating conidia were lifted from the plates and transferred to fresh 2 % MEA agar plates. Agar plates were incubated at 25 °C in the dark for 21 d to allow for culture growth. Isolates of *Mycosphaerella* species occurring on *Eucalyptus* and known to have *Pseudocercospora* anamorphs were also included in this study for comparative purposes (Table 1). These isolates were all grown on 2 % MEA agar plates for approximately 1 mo to ensure sufficient mycelial growth. All cultures used during this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) of the University of Pretoria, Pretoria, South Africa. Representative cultures have also been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (Table 1).

**DNA extraction**

Mycelia were scraped from actively growing cultures, freeze dried for 24 h, and then ground into a fine powder using liquid nitrogen. DNA was isolated according to the method of Hunter
et al. (2004). A 1 : 1 phenol : chloroform extraction was used. DNA was precipitated by the addition of absolute ethanol (98 % EtOH). Isolated DNA was cleaned by the addition of 70 % ethanol (70 % EtOH) and dried under vacuum. Sterile water was used to resuspend the isolated DNA. RNaseA (10 µg/µL) was added to the resuspended DNA and incubated at 37 °C for approximately 2 h to digest any residual RNA. Isolated DNA was visualised in a 1 % agarose gel (wt/v) (Roche Diagnostics, Germany) stained with ethidium bromide.

**PCR amplification and purification**

DNA (ca. 20 ng) isolated from the unknown *Pseudocercospora* sp. and representative *Mycosphaerella* isolates that were used for comparative purposes, was used as a template for amplification with the polymerase chain reaction (PCR). For the purposes of this study, four nuclear gene regions were chosen for amplification and subsequent DNA sequencing. These included the internal transcribed spacer (ITS) region and the large subunit (LSU) of the rRNA operon, a portion of the translation elongation factor 1-alpha gene (EF-1α) and a portion of the actin (ACT) gene regions.

All PCR reaction mixtures for the four gene regions were performed in a total volume of 25 µL containing 10 × PCR Buffer (5 mM Tris-HCl, 0.75 mM MgCl₂, 25 mM KCl, pH 8.3) (Roche Diagnostics, South Africa), 2.5 mM of each dNTP (dATP, dTTP, dCTP, dGTP) (Roche Diagnostics, South Africa), 0.2 µM of forward and reverse primers (Inqaba Biotech, South Africa), 1.25 U Taq DNA Polymerase (Roche Diagnostics, South Africa) and DNA (20 ng/µL). Sterilised distilled water was added to achieve a final volume of 25 µL.

The ITS-1, ITS-2 and the 5.8 S gene regions of the internal transcribed spacer (ITS) region of the rRNA operon were amplified using primers ITS-1 (5’–TCC GTA GGT GAA CCT GCG G–3’) and LR-1 (5’–GGT TGG TTT CTT TTC CT–3’) (Vilgalys & Hester 1990, White et al. 1990). PCR reaction conditions for the ITS followed those of Crous et al. (2004) and Hunter et al. (2004). A portion of the large subunit (containing domain D1–D3) of the nuclear rRNA operon was amplified using primers LROR (5’–ACC CGC TGA GGT GAA CCT GCG G–3’) and LR7 (5’–TAC TAC CAC CAA GAT CT–3’) (Vilgalys & Hester 1990). PCR reaction conditions were as follows: an initial denaturation temperature of 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 62 °C for 30 s, primer extension at 72 °C for 1 min and a final elongation step at 72 °C for 7 min.
A portion of the translation elongation factor 1-alpha gene (EF-1α) was amplified using the 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). Reaction conditions were as follows: an initial denaturation temperature of 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s and primer extension at 72 °C for 30 s. The reaction was completed with a final extension at 72 °C for 7 min.

A portion of the actin (ACT) gene was amplified using the primers ACT-512F (5’−ATG TGC AAG GCC GGT TTC GC−3’) and ACT-783R (5’−TAC GAG TCC TTC TGG CCC AT−3’) (Carbone & Kohn 1999). PCR reaction conditions were as follows: an initial denaturation step at 96 °C for 2 min, followed by 10 cycles of denaturation at 94 °C for 30 s, primer annealing at 61 °C for 45 s and extension at 72 °C for 45 s. This was followed by 25 cycles of denaturation at 94 °C for 30 s, primer annealing at 61 °C for 45 s and elongation at 72 °C for 45 s with an increase of 5 s per cycle for elongation. The reaction was completed with a final elongation step at 72 °C for 7 min.

All PCR products were visualised in 1.5 % agarose gels (wt/v) stained with ethidium bromide, under ultra-violet light. Sizes of PCR amplicons were estimated by comparison against a 100 bp molecular weight marker (O’ RangeRuler™ 100bp DNA ladder) (Fermentas Life Sciences, U.S.A.). For further DNA sequencing, PCR products were purified through Centri-sep spin columns (Princeton separations, Adelphia, NJ) containing Sephadex G-50 (Sigma Aldrich, St. Louis, MO) as outlined by the manufacturer.

**DNA sequencing and phylogenetic analysis**

The purified PCR products were used as template DNA for cycle sequencing reactions using the ABI Prism Big Dye Terminator Cycle sequencing reaction kit v. 3.1 (Applied Biosystems, Foster City, CA) following the manufacturers instructions. The same primers as used for the PCR reactions were also used for sequencing reactions. However, additional internal primers were used for both the ITS and LSU regions. These were ITS-2 (5’−GCT GCG TTC TTC ATC GAT GC−3’) and ITS-3 (5’−GCA TCG ATG AAG AAC GCA GC−3’) (White et al. 1990) in the case of the ITS and LR3R (5’−GTC TTG AAA CAC GGA CC−3’) and LR-16 (5’−TTC CAC CCA AAC ACT CG−3’) in the case of LSU. The precipitated sequencing reactions were then run on an ABI PRISM™ 3100 Automated DNA sequencer (Applied Biosystems, Foster City, CA).
All resulting sequences were analysed with Sequence Navigator v. 1.0.1 (Applied Biosystems, Foster City, CA). Sequence alignments were done using MAFFT v. 5.667 (Katoh et al. 2005) incorporating the E-INS-i alignment strategy. A partition homogeneity test (Farris et al. 1994), on all possible combinations, of 1000 replicates on all informative characters was conducted in PAUP v. 4.10b (Swofford 2002) to determine if the DNA datasets from the four gene regions were combinable.

For phylogenetic analyses, both parsimony and distance analyses were conducted. For parsimony analysis, most parsimonious trees were generated by heuristic searches with starting trees obtained through stepwise addition with the MULPAR function enabled. Tree bisection reconnection (TBR) was employed as the branch-swapping algorithm. All gaps were coded as missing data and characters were assigned equal weight. Statistic support for nodes was obtained by performing 1000 bootstrap replicates.

Modeltest v. 3.04 (Posada & Crandal 1998) was used to determine the most appropriate nucleotide substitution model to be applied to the combined DNA sequence alignment and GTR + I + G was chosen from the Akaike Information Criterion (AIC) (base frequencies: $\pi_A = 0.2387$, $\pi_C = 0.2588$, $\pi_G = 0.2780$, $\pi_T = 0.2245$; substitution rates: $A/C = 1.4579$, $A/G = 1.8851$, $A/T = 1.7352$, $C/G = 1.2094$, $C/T = 4.2937$, $G/T = 1.0000$; proportion of invariable sites (I) = 0.5562; gamma shape distribution parameter = 0.5825). Following this, a neighbour-joining (NJ) analysis using the GTR + I + G substitution model was conducted in PAUP. Here, identical sites were removed proportionally to base frequencies estimated from all sites, rates of invariable sites assumed to follow a gamma distribution and ties were broken if encountered. Mycosphaerella lateralis Crous & M.J. Wingf. (anamorph: Dissoconium dekkeri de Hoog & Hijwegen) was used as an outgroup to root all trees.

**Morphological studies**

Conidia and conidiophores of the undescribed Pseudocercospora sp. were mounted in lactic acid or bromophenol blue on microscope slides. Leaf tissue was mounted in Jung Tissue Freezing Medium (Leica Microsystems AG, Wetzlar, Germany) and sections (10 µm) were cut using a Leica CM 100 Freezing microtome (Leica Microsystems AG, Wetzlar, Germany). Both cross sections and structures taken from the surface of lesions were examined under a Zeiss Axioskop light microscope (Carl Zeiss, Jena, Germany) using differential interference contrast. Fifty measurements of all taxonomically relevant structures were made at × 1000 magnification. Morphological characteristics of the unidentified Pseudocercospora sp. from
Thailand were compared with other *Pseudocercospora* spp. known to occur on *Eucalyptus* (Crous 1998, Braun & Dick 2002). Representative herbarium specimens of the Thailand *Pseudocercospora* sp. have been deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

Growth characteristics of the undescribed *Pseudocercospora* sp. on agar medium were also defined. Plugs (5 mm diam) of agar were cut from the actively growing margins of pure cultures of ex-type isolates and transferred to the centres of 90 mm 2 % MEA plates. Agar plates were incubated in the dark at temperatures between 0 °C and 35 °C at five degree intervals. Colony diameters were determined every 7 d for one mo. Three plates were used per isolate at each temperature, and the experiment was repeated once. Colony colours and morphologies were described after one mo using the using colour charts of Rayner (1970).

**RESULTS**

**PCR Amplification and analysis of sequence data**

Amplification of the LSU, ITS, EF-1α and ACT gene regions for all the isolates used in this study resulted in amplification products of approximately 600 bp for ITS, 300 bp for EF-1α, 250 bp for ACT and 1500 bp for LSU.

Results of the partition homogeneity test using 1000 replicates resulted in a P-value > 0.001 for all possible combinations of the LSU, ITS, EF-1α and ACT DNA sequence alignments. Therefore, DNA sequence alignments of the ITS, EF-1α, ACT and LSU gene regions were combined. For parsimony analysis, heuristic searches of the combined DNA sequence alignment resulted in the retention of six most parsimonious trees, one of which is shown here (Fig. 1). The tree had a length of 1417 steps (CI = 0.709, RI = 0.8961, HI = 0.291). A total of 2665 characters were evaluated of which 1998 were constant, 153 were parsimony uninformative and 514 were parsimony informative. Bootstrap searches of the combined dataset produced a tree with the same topology of the most parsimonious tree. Isolates of *Mycosphaerella* spp. and isolates of the unknown *Pseudocercospora* sp. could be resolved into two well-supported monophyletic clades (Clades I–II) with bootstrap values of 83 % and 96 % respectively (Fig. 1).

Clade I could be further resolved into four well-supported sub-clades (sub-clades 1–4) (Fig. 1). Clade 1 included isolates of *P. flavomarginata*, *P. paraguayensis* (Kobayashi) Crous and *P. basiramifera* Crous (Bootstrap = 100 %), Clade 2 contained isolates of
Mycosphaerella fori G.C. Hunter, Crous & M.J. Wingf., M. gracilis Crous & Alfenas, P. eucalyptorum Crous, M.J. Wingf., Marasas & B. Sutton, P. pseudoeucalyptorum Crous (Bootstrap = 98 %). Clade 3 contained isolates of P. basitruncata Crous and P. natalensis Crous & T. Coutinho (Bootstrap = 100 %) and clade 4 contained isolates of M. colombiensis Crous & M.J. Wingf., M. crystallina Crous & M.J. Wingf., M. irregulariramosa Crous & M.J. Wingf., M. heimii Crous and M. heimioides Crous & M.J. Wingf. (Bootstrap = 100 %).

For distance analysis, neighbour-joining analyses yielded a phylogenetic tree with the same topology as the most parsimonious trees generated by parsimony analyses (Fig. 2). Here isolates of Mycosphaerella and Pseudocercospora were resolved into two well-supported monophyletic clades containing the same isolates as those in the parsimony analysis (Fig. 1). Furthermore, for the distance analysis, isolates of P. flavomarginata grouped into a well-supported clade of their own, sister to P. paraguayensis and P. basiramifera.

**Morphology**

One of the most obvious distinguishing features of the Pseudocercospora sp. on E. camaldulensis found in Thailand and Vietnam is the symptoms associated with the taxon (Fig. 3). These are very distinct angular chlorotic spots that later become necrotic lesions on the leaves, typically surrounded by halos of chlorotic tissue. Lesions typically bear dense clusters of dark brown conidiophores on the abaxial surface but these are occasionally also found on the adaxial surfaces of lesions. Lesions can be very common, mostly on leaves on the lower branches, and susceptible Eucalyptus clones can be seriously defoliated.

Cultures of the Pseudocercospora sp. grew relatively rapidly on 2 % MEA. Results from growth comparisons at different temperatures showed that the optimal temperature for growth was 25 °C. At 25 °C, cultures had a growth rate of 29 mm in one mo on MEA. Minimum and maximum temperatures were between 5–10 °C and 30–35 °C, respectively. Cultures were pale olivaceous-grey on the surface, and greenish-black in reverse. Cultures exhibited irregular margins and produced profuse aerial mycelium while colony borders were generally darker (greenish-black) than the colony centre that tended to become paler as the culture aged.

**Taxonomy**

DNA sequence comparison of the Pseudocercospora sp. considered in the study has shown that the species is different from all other Mycosphaerella spp. considered during this study. The symptoms associated with this fungus are also very obvious and unique. In addition, the
morphology of the fungus is unlike that of any other Pseudocercospora sp. known on Eucalyptus. We therefore describe it as a new species as follows:

**Pseudocercospora flavomarginata** G.C. Hunter, Crous & M.J. Wingf., sp. nov.  (Figs. 4, 5)  
MycoBank No.: MB500513

**Etymology:** Named for the characteristic chlorotic borders surrounding the angular necrotic lesions on *Eucalyptus camaldulensis* leaves.

**Conidiophorae** dense fasciculatae, brunneae, apicem versus pallidiores, non ramosae, parietibus crassis, laeves, 0–4-septatae, subcylindricae, rectae ver curvatae, e cellulis stromatis bene evoluti exorientes, (18–)32–36(–53) × (2–)3–4(–5) µm. *Conidia* solitaria, recta vel subfalcata, pallide brunnea, laeves, parietibus crassis, guttulata, acicularia vel obclavata, apice obtusa, basi rotundata vel longe obconico-truncata, 2–7-septata, (28–)46–54(–90) × (2–)3(–4) µm. *Teleomorpha* ignota.

**Holotype:** Thailand: Chachoengsao Province near Pratchin Buri: on leaves of *Eucalyptus camaldulensis* 2004, M.J. Wingfield (PREM 58952– holotypus; cultura viva ex-types CBS 118841, 118823, 118824).

**Additional material examined (paratypes):** Thailand, Chachoengsao Province near Pratchin Buri on leaves of *Eucalyptus camaldulensis*, 2004, M.J. Wingfield (CMW 17703, PREM 58953); Thailand, Chachoengsao Province near Pratchin Buri on leaves of *Eucalyptus camaldulensis*, 2004 M.J. Wingfield (CMW 17707, PREM 58954); Thailand, Chachoengsao Province near Pratchin Buri on leaves of *Eucalyptus camaldulensis*, 2004 M.J. Wingfield (CMW17708, PREM 58955).

**Leaf spots** appear as chlorotic spots, distinct, scattered over leaves, amphigenous, circular to angular, 3–20 mm diam (Fig. 3), pale to dark brown becoming necrotic and darker with age, definite chlorotic margin on abaxial and adaxial leaf surfaces but more obvious on adaxial leaf surfaces. *Mycelium* internal and external, pale brown, septate, branched, thick-walled, smooth to finely verruculose, 2–4 µm diam. *Caespituli* amphigenous, predominantly epiphyllous, evenly distributed over lesion, brown to black on leaves, 74 µm wide and 90 µm high (Figs. 4, 5). *Conidiophores* fasciculate, grouped in dense fascicles, conidiophores brown becoming paler towards apex, unbranched, thick-walled, smooth, 0–4-septate, subcylindrical, straight to curved, arising from cells of a well developed stroma (18–)32–36(–53) × (2–)3–4(–5) µm. *Stromata* well-developed, prominent, immersed becoming erumpent, brown, 56 µm wide and 47 µm high. *Conidiogenous cells* terminal, smooth, thick-walled, pale brown, unbranched, tapering to a rounded apex, proliferating sympodially or 1–2 times percurrently,
(6–)14–17(−25) × (2–)3(−5) μm. Conidial scars, unthickened and not darkened. Conidia solitary, straight or slightly curved, pale brown, smooth, thick-walled, guttulate, acicular to obclavate, apex obtuse, base rounded to long obconic-truncate, 2–7-septate, (28–)46–54(−90) × (2–)3(−4) μm. Hilum unthickened, not darkened (Figs. 4, 5). Spermagonium present in lesions, well developed, prominent, immersed becoming erumpent 64 μm wide and 57 μm high. Spermatia not observed. Teleomorph unknown.

Cultures: Cultures 29 mm diam on MEA after 1 mo at 25 °C in the dark. Colonies pale olivaceous-grey 21’’’’’’’b (surface) and greenish-black 33’’’’’’’i (reverse). Margins regular to irregular, aerial mycelium profuse. Border darker (greenish-black) than colony centers, which become paler with age. Colony not sectored and folding absent.

Cardinal temperatures: Minimum 5–10 °C, optimum 25 °C, maximum 30–35 °C.

Hosts: Eucalyptus camaldulensis, E. camaldulensis hybrids.

Distribution: Thailand, Vietnam.

Notes: Pseudocercospora flavomarginata is morphologically and phylogenetically similar to Pseudocercospora paraguayensis. However, P. flavomarginata can be distinguished from P. paraguayensis by the very distinct chlorotic borders around the typically irregular lesions. Conidiophores are longer and have more septa than those of P. paraguayensis. Furthermore, conidium development is sympodial and percurrent in P. flavomarginata, but exclusively sympodial in P. paraguayensis.

DISCUSSION

Pseudocercospora flavomarginata is a fungal pathogen of E. camaldulensis that is very well-known in Thailand and Vietnam where it causes leaf spots typically on the lower leaves of young E. camaldulensis trees (Old et al. 2003). It is thus unfortunate that it has not previously been formally named. Perhaps this is to some extent due to the fact that there are various Pseudocercospora spp. on Eucalyptus leaves and DNA sequence data have not previously been available to reinforce the fact that it represents a novel taxon.

Although Pseudocercospora basiramifera is known to occur in Thailand on E. camaldulensis and E. pellita (Crous 1998), it was not encountered during the present study. Pseudocercospora flavomarginata can be distinguished from P. basiramifera by the presence of red lesion margins in P. basiramifera as opposed to the very characteristic chlorotic margins found around lesions of P. flavomarginata. Furthermore, P. flavomarginata has
smaller conidia that are 2−7-septate and (28−)46−54(−90) × (2−)3(−4) µm in contrast to conidia of P. basiramifera, which are 3−10-septate and (35−)50−70(−80) × 2(−3) µm (Crous 1998).

Various Pseudocercospora spp. other than P. flavomarginata are known to occur in south-east Asia on Eucalyptus. These include Pseudocercospora eucalyptorum, P. gracilis Crous & Alfenas, and P. heimioides Crous & M.J. Wingf. (Crous et al. 1989, Crous & Alfenas 1995, Crous & Wingfield 1997). Pseudocercospora flavomarginata is common in Thailand and Vietnam and can be distinguished from P. eucalyptorum, P. gracilis and P. heimioides by characteristic leaf lesions with prominent yellow borders. This in contrast to pale to grey-brown lesions with purple borders produced by P. eucalyptorum, pale brown lesions with red to brown borders formed by P. gracilis, and brown to absent leaf spots of P. heimioides (Crous et al. 1989, Crous & Alfenas 1995, Crous & Wingfield 1997, Crous 1998).

Phylogenetically, P. flavomarginata is closely related to P. paraguayensis, which is known from eucalypts in South America (Crous 1998). Pseudocercospora flavomarginata can be distinguished from P. paraguayensis by the prominent chlorotic borders around lesions, conidiophores that are more numerous septate and longer than those of P. paraguayensis, conidia that are more numerous septate than those of P. paraguayensis and conidium development that is sympodial and percurrent, in contrast to the exclusively sympodial development in P. paraguayensis.

DNA sequence comparisons in this study resolved Pseudocercospora spp. into two well-supported monophyletic clades, supporting results of Crous et al. (2000), and the recent finding of Ayala-Escobar et al. (2006) that Pseudocercospora is paraphyletic within Mycosphaerella. While these groups are very clearly delineated, it is not possible to link them to any distinct morphological characteristics. However, larger sequence datasets that consider additional isolates of Cercospora spp. and specifically Pseudocercospora spp. might in future resolve specific lineages that could be linked to phylogenetically informative morphological characteristics.

Pseudocercospora crystallina Crous & M.J. Wingf., P. irregulariramosa Crous & M.J. Wingf., P. heimii Crous and P. heimioides are morphologically similar species that have been shown, based on ITS sequence data, to group in a single clade (Crous et al. 2000, 2001). These fungi are regarded as a species complex known as the Mycosphaerella heimii complex (Crous et al. 2000). Based on DNA sequence results from the four gene regions in this study, species within the M. heimii complex clustered together, supporting previous findings. Based on the distance of the M. heimii complex from its most recent ancestor, it is evident that the
divergence from this node is relatively recent. This suggests that speciation amongst members of the *M. heimii* complex has occurred recently.

*Eucalyptus* spp. are native to Australia, Papua New Guinea, Indonesia and the Philippines (Turnbull 2000). It is possible that *P. flavomarginata* may be present in Australia and other areas where *Eucalyptus* spp. are native. Further surveys of both natural and commercially propagated *Eucalyptus* spp. could result in collections of *P. flavomarginata* and increase the known host range of this species.

*Eucalyptus camaldulensis* is one of the species that is most commonly grown in south-east Asia. Considering that many commercially propagated eucalypts are clones or hybrids (Old *et al.* 2003), it will be important to consider the host distribution of *P. flavomarginata* in this area. It is also well-known that some clones can be particularly susceptible to pathogens while others, even those relatively closely related, can be resistant. Therefore, future surveys and pathogenicity studies would aid in selecting and propagating genotypes of *Eucalyptus* that are tolerant to pathogens such as *P. flavomarginata*. 
REFERENCES


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Table 1. Isolates of *Mycosphaerella* and *Pseudocercospora* used for DNA sequencing and phylogenetic analysis.

<table>
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<th>Anamorph</th>
<th>Origin</th>
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**CBS**: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

**CMW**: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.


**N/A**: Not available.
**Figure 1.** Phylogram of *Mycosphaerella* and *Pseudocercospora* spp. occurring on *Eucalyptus* indicating the phylogenetic placement of *Pseudocercospora flavomarginata*. One of six most parsimonious trees generated from a heuristic search of combined DNA alignments of LSU, ITS, ACT and EF-1α data in PAUP v. 4.0b10 (Length = 1417, CI = 0.79, RI = 0.8961, HI = 0.291). Bootstrap values of 1000 replicates are indicated above branches. The tree was rooted to *Mycosphaerella lateralis*.
Figure 2. Neighbour-joining (NJ) tree of *Mycosphaerella* and *Pseudocercospora* spp. obtained from distance analysis of combined LSU, ITS, ACT and EF-1α DNA sequence alignments using the GTR + I + G DNA substitution model. Bootstrap values after 1000 replicates are indicated above branches. The tree was rooted to *Mycosphaerella lateralis.*
Figure 3. Symptoms of *Pseudocercospora flavomarginata* on leaves of *Eucalyptus camaldulensis*. *Pseudocercospora flavomarginata* produces necrotic lesions surrounded by characteristic chlorotic tissue. Lesions bear clusters of dark brown conidiophores on the abaxial and adaxial lesion surfaces.
Figure 4. Morphological characteristics of *Pseudocercospora flavomarginata* (PREM 58952, MycoBank 500513). **A–D.** Straight or slightly curved, guttulate, acicular to obclavate conidia with obtuse apex and rounded to long obconic-truncate base. **E–F.** Fasciculate conidiophores that are unbranched, thick walled, subcylindrical, straight to curved. Bar = 10 µm.
Figure 5. Line drawings of *Pseudocercospora flavomarginata*. A. Conidia. B. Well-developed, immersed young spermagonium with developed conidiophores that become lighter towards the apex. C. Septate, branched, thick walled, smooth, external mycelium. D. Fascicle of conidiophores. Bar = 10 μm.