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

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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*.

Key words: *Ascomycete*, *Eucalyptus* leaf disease, *Pseudocercospora*

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

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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 genus *Mycosphaerella* is one of the largest genera of *Ascomycetes*, accommodating more than 3000 names (Corlett, 1991, 1995; Crous, 1998; Aptroot, 2006). Approximately 60 *Mycosphaerella* species have been associated with diseases on *Eucalyptus* leaves, collectively known as Mycosphaerella Leaf Disease (MLD) (Crous, 1998; Maxwell *et al*., 2003; Crous *et al*., 2004, 2006; Hunter *et al*., 2004, 2006). Symptoms of MLD include leaf spots, defoliation, twig cankers and in severe cases stunting of tree growth (Park and Keane, 1982; Carnegie and Keane, 1994; Crous, 1998).

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 and Braun, 2003; Kirschner *et al*., 2004; Schubert and 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* is large and morphologically diverse (Crous *et al*., 2000). Species of *Pseudocercospora* occur on many plant hosts where they cause leaf spots (Crous and Braun, 1996; Crous *et al*., 1997; Crous and Braun, 2001; Braun and Dick, 2002). These fungi typically have dematiaceous conidiophores and scolecosporous conidia with inconspicuous conidial scars and conidiogenous cells that proliferate sympodially and percurrently (Crous and Wingfield, 1997; Crous *et al*., 2000; Crous and 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 and 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, Johannesburg, 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 days 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 month 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 hours, 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 (100% EtOH). Isolated DNA was cleaned by the addition of

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

Isolate No.			Teleomorph	Anamorph	Origin	GenBank Accession No.			
CMW	CPC	CBS	(*Mycosphaerella*)			LSU	ITS	ACT	EF-1α
4944	1106	110969	*M. colombiensis*	*P. colombiensis*	*E. urophylla,* Colombia	DQ204744	AY752149	DQ147639	DQ211660
11255	1104	110967	*M. colombiensis*	*P. colombiensis*	*E. urophylla,* Columbia	DQ204745	AY752147	DQ147640	DQ211661
3042	800	N/A	*M. crystallina*	*P. crystallina*	*E. bicostata,* South Africa	DQ204746	DQ267578	DQ147637	DQ211662
3033	802	681.95	*M. crystallina*	*P. crystallina*	*E. bicostata,* South Africa	DQ204747	AY490757	DQ147636	DQ211663
9095	N/A	N/A	*M. fori*	*Pseudocercospora* sp.	*E. grandis,* South Africa	DQ204748	AF468869	DQ147618	DQ211664
9096	N/A	N/A	*M. fori*	*Pseudocercospora* sp.	*E. grandis,* South Africa	DQ204749	DQ267581	DQ147619	DQ211665
14455	730	243.94	*M. gracilis*	*P. gracilis*	*E. urophylla,* Indonesia	DQ204750	DQ267582	DQ147616	DQ211666
4942	760	110682	*M. heimii*	*P. heimii*	*Eucalyptus* sp., Madagascar	DQ204751	AF309606	DQ147638	DQ211667
14776	N/A	111364	*M. heimioides*	*P. heimioides*	*Eucalyptus* sp., Indonesia	DQ204752	DQ267586	DQ147632	DQ211668
3046	1312	111190	*M. heimioides*	*P. heimioides*	*Eucalyptus* sp., Indonesia	DQ204753	AF309609	DQ147633	DQ211669
4943	1360	114774	*M. irregulariramosa*	*P. irregulariramosa*	*E. saligna,* South Africa	DQ204754	AF309607	DQ147634	DQ211670
5223	1362	N/A	*M. irregulariramosa*	*P. irregulariramosa*	*E. saligna,* South Africa	DQ204755	AF309608	DQ147635	DQ211671
14458	3745	112895	*M. madeirae*	*Pseudocercospora* sp.	*E. globulus,* Madeira	DQ204756	AY725553	DQ147641	DQ211672
14778	822	110750	*M. marksii*	*P. epispermogonia*	*E grandis×saligna,* South Africa	DQ204757	DQ267596	DQ147629	DQ211673
14786	823	110693	*M. marksii*	*P. epispermogonia*	*E grandis×saligna,* South Africa	DQ204758	DQ267597	DQ147628	DQ211674
14914	1202	114664	Unknown	*P. basitruncata*	*E. grandis,* Colombia	DQ204759	DQ267600	DQ147622	DQ211675
14785	1203	111280	Unknown	*P. basitruncata*	*E. grandis,* Colombia	DQ204760	DQ267601	DQ147621	DQ211676
5148	N/A	N/A	Unknown	*P. basiramifera*	*E. pellita,* Thailand	DQ204761	AF309595	DQ147607	DQ211677
5228	16	110777	Unknown	*P. eucalyptorum*	*E. nitens,* South Africa	DQ204762	AF309598	DQ147614	DQ211678
14784	1264	111070	Unknown	*P. natalensis*	*E. nitens,* South Africa	DQ204763	AF309594	DQ147623	DQ211679
14779	1459	111286	Unknown	*P. paraguayensis*	*E. nitens,* Brazil	DQ204764	DQ267602	DQ147606	DQ211680
14908	10390	114242	Unknown	*P. pseudoeucalyptorum*	*E. globulus,* Spain	DQ204765	AY725526	DQ147613	DQ211681
14911	10500	114243	Unknown	*P. pseudoeucalyptorum*	*E. nitens,* New Zealand	DQ204766	AY725527	DQ147615	DQ211682
5151	1269	111175	Unknown	*P. robusta*	*E. robusta,* Malaysia	DQ204767	AF309597	DQ147617	DQ211683
13586	N/A	118841	Unknown	*P. flavomarginata*	*E. camaldulensis,* Thailand	DQ153306	DQ155657	DQ166513	DQ156548
13594	N/A	118824	Unknown	*P. flavomarginata*	*E. camaldulensis,* Thailand	DQ153308	DQ155658	DQ166514	DQ156549
13590	N/A	118823	Unknown	*P. flavomarginata*	*E. camaldulensis,* Thailand	DQ153307	DQ155659	DQ166515	DQ156550
14906	825	110748	*M. lateralis*	*Dissoconium dekkeri*	*E. grandis×saligna,* South Africa	DQ204768	AF173315	DQ147651	DQ211684

CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
CMW: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.
CPC: Working collection of Pedro Crous, formerly in STE-U, now housed at CBS. Isolate numbers from Crous (1998).
N/A: Not available.

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 hours to digest any residual RNA. Isolated DNA was visualised in a 1% agarose gel (wt/v) (Roche Diagnostics, Mannheim, 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 MgCl$_2$, 25 mM KCl, pH 8.3) (Roche Diagnostics, Johannesburg, South Africa), 2.5 mM of each dNTP (dATP, dTTP, dCTP, dGTP) (Roche Diagnostics, Johannesburg, South Africa), 0.2 µM of forward and reverse primers (Inqaba Biotech, Pretoria, South Africa), 1.25 U Taq DNA Polymerase (Roche Diagnostics, Johannesburg, 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 and 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 ACT TAA GC-3') (Moncalvo, Wang and Hseu, 1995) and LR7 (5'-TAC TAC CAC CAA GAT CT-3') (Vilgalys and Hester, 1990). PCR reaction conditions were as follows: an initial denaturation temperature of 96°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 62°C for 30 seconds, primer extension at 72°C for 1 minute and a final elongation step at 72°C for 7 minutes.

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

and Kohn, 1999). Reaction conditions were as follows: an initial denaturation temperature of 96°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 56°C for 30 seconds and primer extension at 72°C for 30 seconds. The reaction was completed with a final extension at 72°C for 7 minutes.

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 and Kohn, 1999). PCR reaction conditions were as follows: an initial denaturation step at 96°C for 2 minutes, followed by 10 cycles of denaturation at 94°C for 30 seconds, primer annealing at 61°C for 45 seconds and extension at 72°C for 45 seconds. This was followed by 25 cycles of denaturation at 94°C for 30 seconds, primer annealing at 61°C for 45 seconds and elongation at 72°C for 45 seconds with an increase of five seconds per cycle for elongation. The reaction was completed with a final elongation step at 72°C for 7 minutes.

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[TM] 100bp DNA ladder) (Fermentas Life Sciences, Hanover, U.S.A.). For further DNA sequencing, PCR products were purified through centri-sep spin columns (Princeton separations, Adelphia, U.S.A.) containing sephadex G-50 (Sigma Aldrich, St. Louis, U.S.A.) 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, U.S.A.) 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[TM] 3100 Automated DNA sequencer (Applied Biosystems, Foster City, U.S.A.).

All resulting sequences were analysed with Sequence Navigator v. 1.0.1 (Applied Biosystems, Foster City, U.S.A.). Sequence alignments were done

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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 and 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 and 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 90mm 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 days for one month. Three plates were used per isolate at each temperature, and the experiment was repeated once. Colony colours and morphologies were described after one month using the 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 600bp. 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. 4). 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 data set 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. 4).

Clade I could be further resolved into four well-supported sub-clades (sub-clades 1–4) (Fig. 4). 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., *Mycosphaerella gracilis* Crous & Alfenas, *Pseudocercospora eucalyptorum* Crous, M.J. Wingf., Marasas & B. Sutton, *Pseudocercospora*

pseudoeucalyptorum Crous (Bootstrap = 98%). Clade 3 contained isolates of *Pseudocercospora basitruncata* Crous and *Pseudocercospora natalensis* Crous & T. Coutinho (Bootstrap = 100%) and Clade 4 contained isolates of *Mycosphaerella colombiensis* Crous & M.J. Wingf., *Mycosphaerella crystallina* Crous & M.J. Wingf., *Mycosphaerella irregulariramosa* Crous & M.J. Wingf., *Mycosphaerella heimii* Crous and *Mycosphaerella 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. 5). 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. 4). 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. 1). These are very distinct angular 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 month. 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. 2, 3)

MycoBank number: 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: Chang Gao Province near Pratchinburi: 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, Chang Gao Province near Pratchinburi on leaves of *Eucalyptus camaldulensis*, 2004, M.J. Wingfield (CMW 17703, PREM 58953); Thailand, Chang Gao Province near Pratchinburi on leaves of *Eucalyptus camaldulensis*, 2004 M.J. Wingfield (CMW 17707, PREM 58954); Thailand, Chang Gao Province near Pratchinburi on leaves of *Eucalyptus camaldulensis*, 2004 M.J. Wingfield (CMW17708, PREM 58955).

Leaf spots distinct, scattered over leaves, amphigenous, circular to angular, 3−20 mm diam (Fig. 1), pale to dark brown becoming 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. 2, 3). *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. 2, 3). *Spermagonium* present in
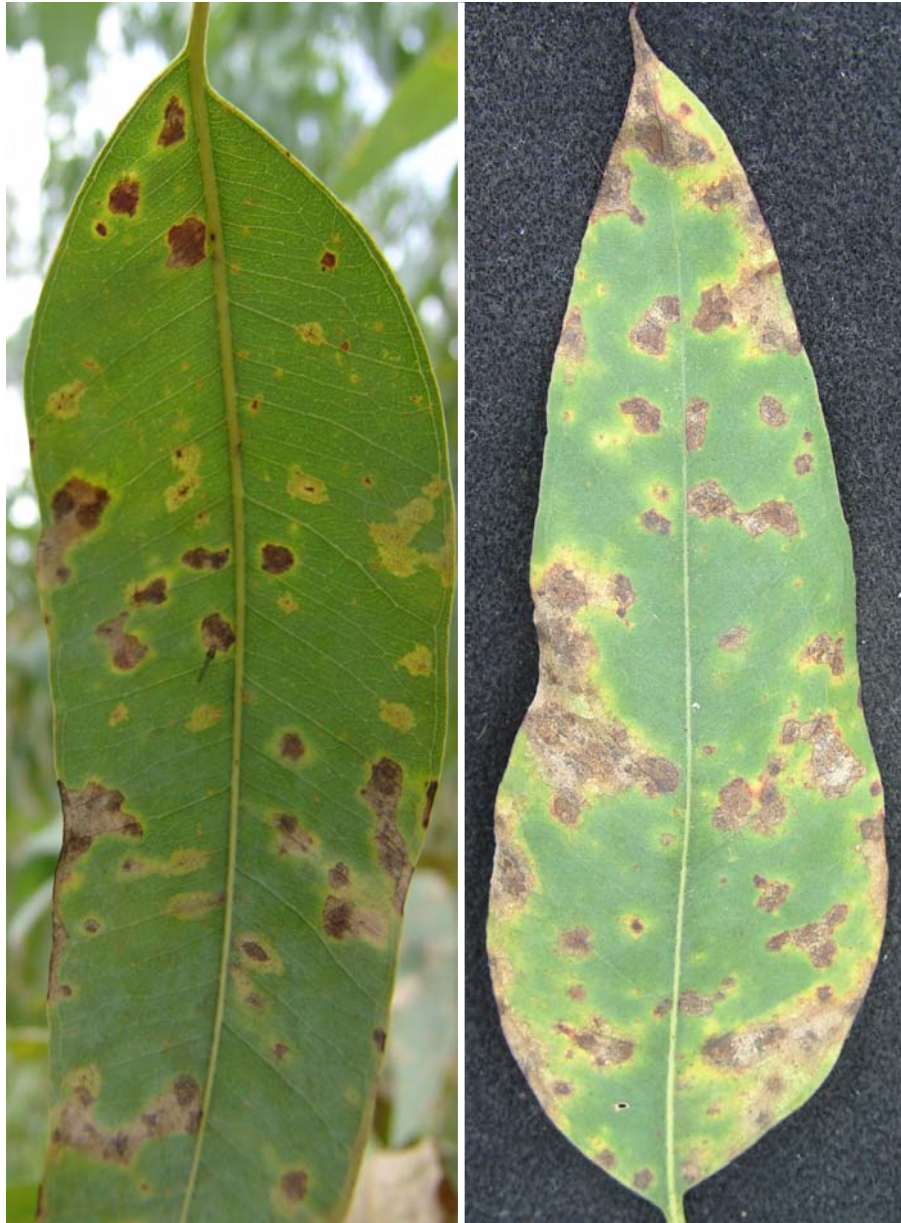
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Fig. 1. Symptoms of *Pseudocercospora flavomarginata* on leaves of *Eucalyptus camaldulensis*. *P. flavomarginata* produces necrotic lesions surrounded by characteristic chlorotic tissue. Lesions bear clusters of dark brown conidiophores on the abaxial and adaxial lesion surfaces.
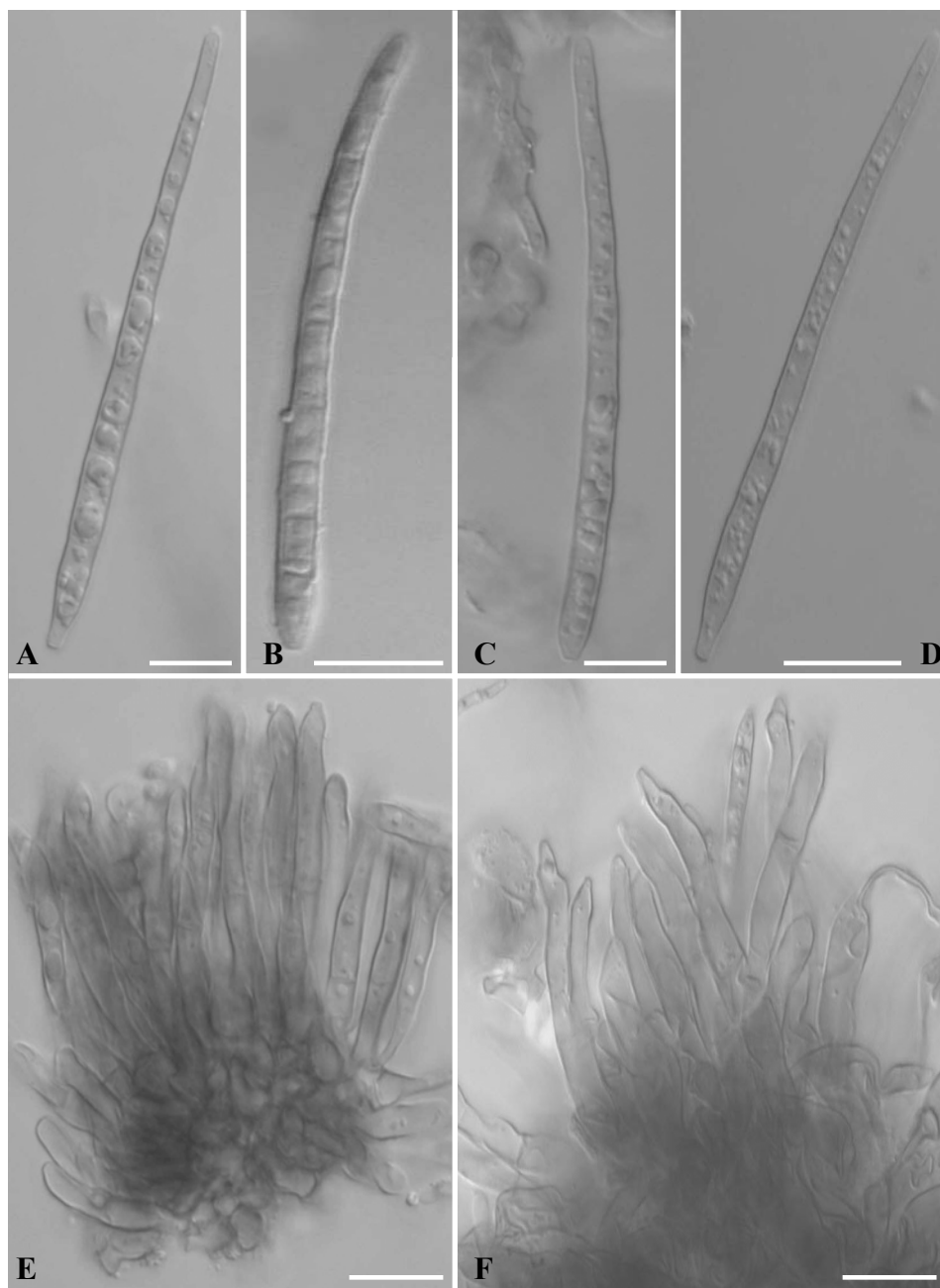
Fig. 2. Morphological characteristics of *Pseudocercospora flavomarginata* (PREM 58952, MycoBank 500513). **A–D.** Straight or slightly curved, guttulate, acicular to obclavate conidia with obtuse apex and rounded to long obconic-truncate base. **E–F.** Fasciculate conidiophores that are unbranched, thick walled, subcylindrical, straight to curved. Bar = 10 μm.
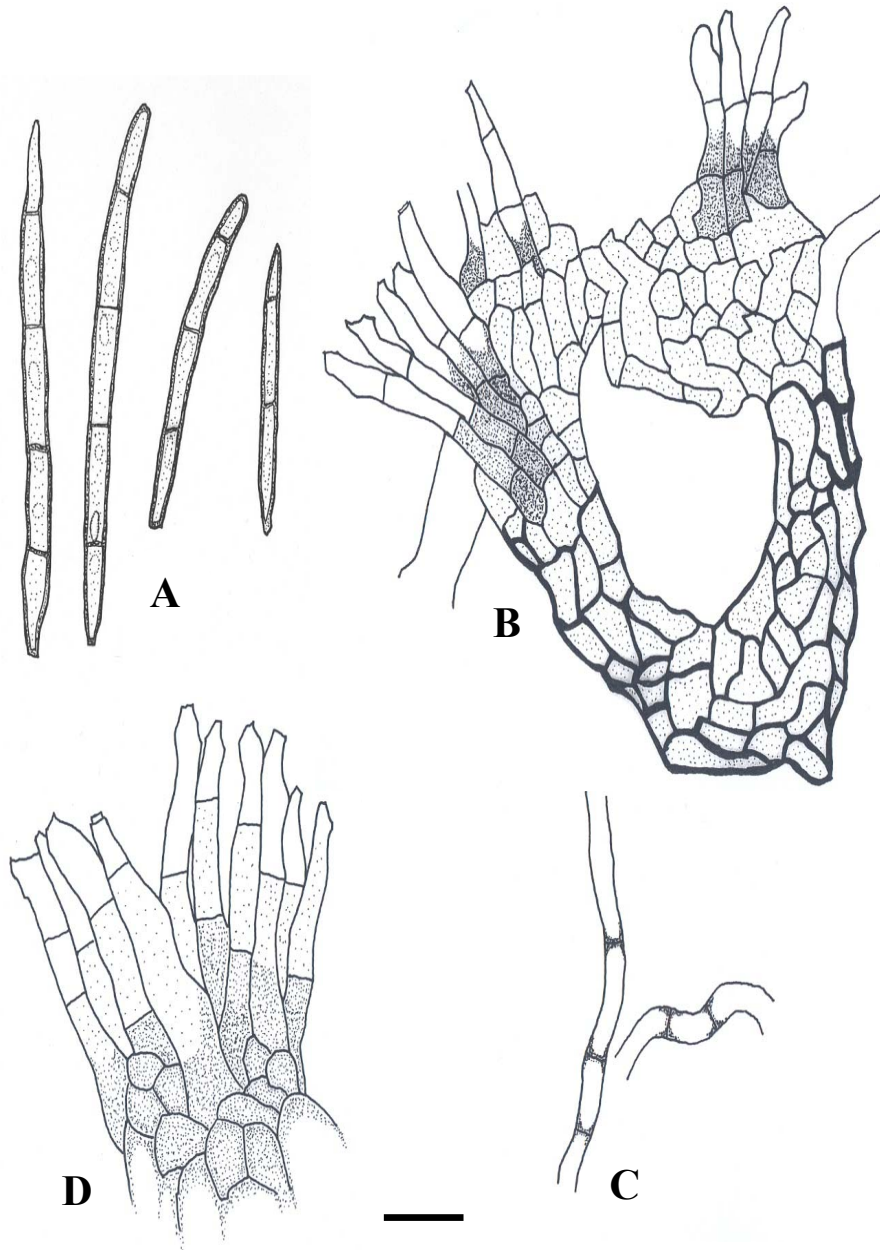
Fig. 3. Line drawings of *Pseudocercospora flavomarginata*. **A.** Conidia. **B.** Well-developed, immersed young spermagonium with developed conidiophores that become lighter towards the apex. **C.** Septate, branched, thick walled, smooth, external mycelium. **D.** Fascicle of conidiophores. Bar = 10 μm.

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 month at 25°C in the dark. Colonies pale olivaceous-grey 21'''''b (surface) and greenish-black 33'''''i (reverse) (Rayner, 1970). Margins regular to irregular, aerial mycelium profuse. Border darker (greenish-black) than colony centres, 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* F. Muell. (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 and Alfenas, 1995; Crous and 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 and Alfenas, 1995; Crous and 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 numerously septate and longer than those of *P. paraguayensis*, conidia that are more numerously septate than those of *P. paraguayensis* and conidium development that is sympodial and percurrent, in contrast to the exclusively sympodal 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 data sets 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, *P. irregulariramosa*, *P. heimii* 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 Phillipines (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* an increase the known host range of this species.
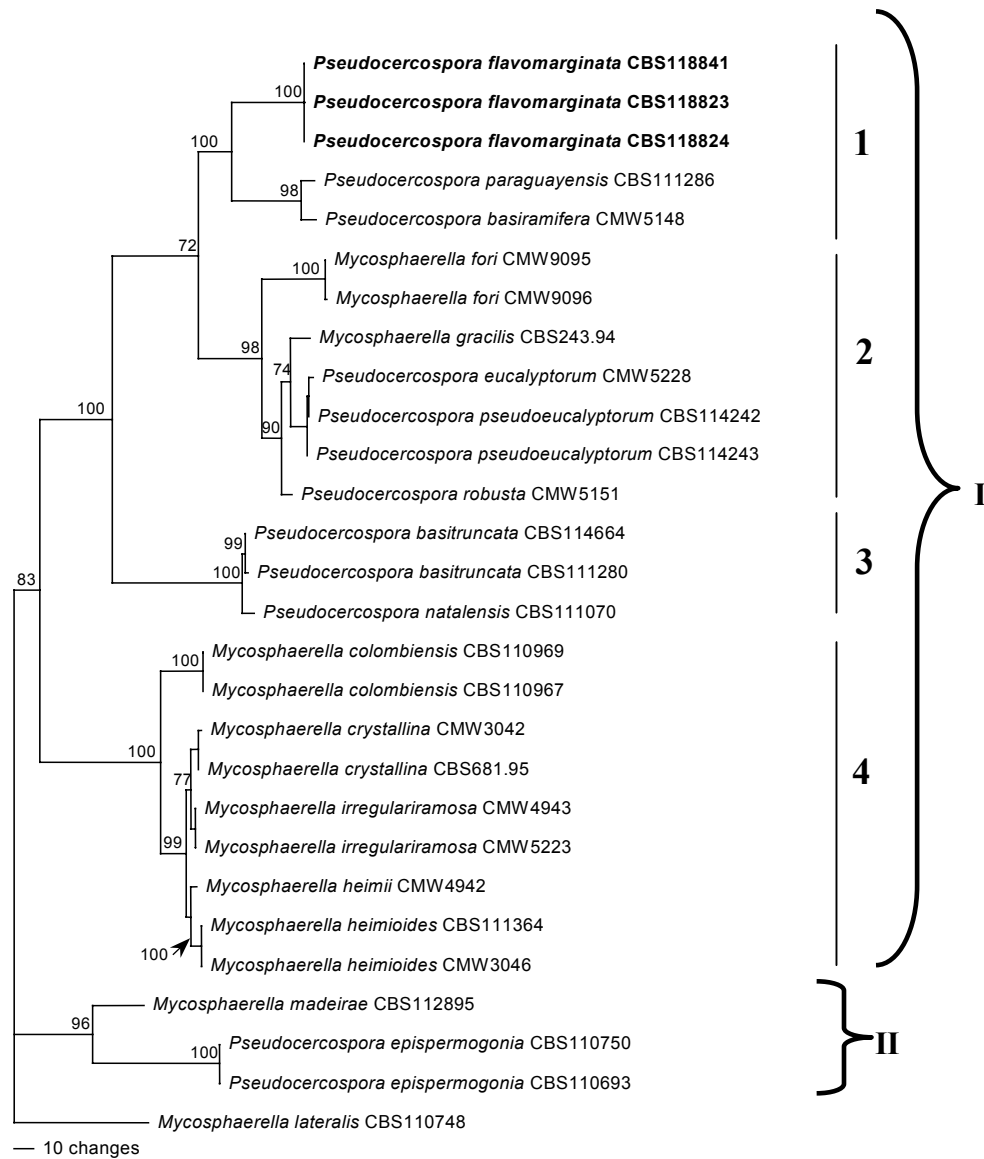
Fig. 4. Phylogram of *Mycosphaerella* and *Pseudocercospora* spp. occurring on *Eucalyptus* indicating the phylogenetic placement of *Pseudocercospora flavomarginata*. One of six most parsimonious trees generated from a heuristic search of combined DNA alignments of LSU, ITS, ACT and EF-1α data in PAUP v. 4.0b10 (Length= 1417, CI = 0.79, RI = 0.8961, HI = 0.291). Bootstrap values of 1000 replicates are indicated above branches.
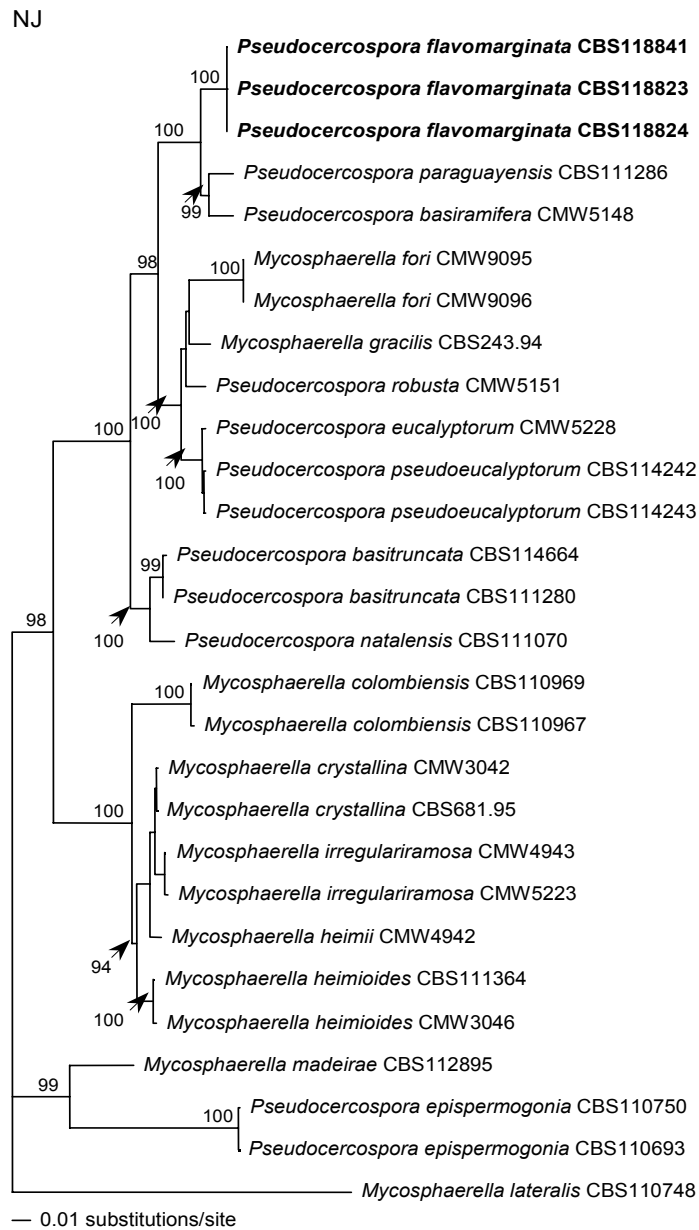
Fig. 5. Neighbour-joining (NJ) tree of *Mycosphaerella* and *Pseudocercospora* spp. obtained from distance analysis of combined LSU, ITS, ACT and EF-1α DNA sequence alignments using the GTR + I + G DNA substitution model. Bootstrap values after 1000 replicates are indicated above branches. The tree was rooted to *Mycosphaerella lateralis*.

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*.

Acknowledgements

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