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Cryptic speciation and host specificity among *Mycosphaerella* spp. occurring on Australian *Acacia* species grown as exotics in the tropics

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Abstract: Species of *Mycosphaerella* and their anamorphs represent serious pathogens of two phyllodenous species of *Acacia*, *A. mangium* and *A. crassicarpa*. In recent years, these fungi have been collected during surveys in South America and South-East Asia, where these trees are widely planted as exotics. In this study, the *Mycosphaerella* spp. and their anamorphs were identified based on morphological and cultural characteristics. Identifications were confirmed using comparisons of DNA sequences for the internal transcribed spacers (ITS1 & ITS2), the 5.8S rRNA gene, elongation factor 1-α, histone 3, actin and calmodulin gene regions. The data revealed six new taxa, of which three are named in this study, along with their anamorphs. *Cercospora acaciae-mangii*, which is morphologically part of the *C. apii sensu lato* species complex, is distinguished based on its distinct phylogeny. *Mycosphaerella acaciigena*, collected in Venezuela, is distinguished from *M. konae* and *M. heimii*, and described as new. *Mycosphaerella thailandica*, a new species occurring on *Acacia* and *Musa*, is shown to be a sibling species to *M. colombiensis*, a foliar pathogen of *Eucalyptus*. *Mycosphaerella citri*, an important leaf and fruit pathogen of *Citrus* (*Rutaceae*), is shown to also occur on *Musa* (*Musaceae*) and *Acacia* (*Leguminosae*).

Taxonomic novelties: *Cercospora acaciae-mangii* Crous, Pongpanich & M.J. Wingf. sp. nov., *Mycosphaerella acaciigena* Crous & M.J. Wingf. sp. nov. (anamorph *Pseudocercospora acaciigena* Crous & M.J. Wingf. sp. nov.), *Mycosphaerella thailandica* Crous, Himaman & M.J. Wingf. sp. nov. (anamorph *Pseudocercospora thailandica* Crous, Himaman & M.J. Wingf. sp. nov.).
Key words: *Acacia*, *Ascomycetes*, *Cercospora*, *Mycosphaerella*, *Pseudocercospora*, *Stenella*, systematics.

INTRODUCTION

Plantations of exotic tree species in the tropics and Southern Hemisphere sustain important industries producing solid wood products and pulp. In many situations, they provide an alternative to logging of native forest trees and they contribute substantially to the economies of many developing countries. The most extensively planted trees in these plantations are species of *Pinus* L., *Eucalyptus* L'Herit. and *Acacia* L. Australian *Acacia* species have been planted as exotics in the tropics and Southern Hemisphere for many years. Until relatively recently, however, these have been less extensively planted than *Pinus* or *Eucalyptus* spp. In areas with temperate climates, *Acacia* spp. with pinnate leaves such as *Acacia mearnsii* De Wild. and *A. dealbata* Link are planted, although on a limited scale. More recently, phyllodenous *Acacia* spp. such as *Acacia mangium* Willd., *A. crassicarpa* A. Cunn. ex Benth. and *A. auriculiformis* A. Cunn. ex Benth. have been planted extensively in plantations in the tropics (Old *et al*. 2000).

The success of exotic plantation forestry can, to some extent, be attributed to the separation of trees from their natural enemies (Wingfield *et al*. 2001). In terms of *Acacia* spp., virtually nothing is known regarding the diseases that affect these trees, particularly where they are planted as exotics. A preliminary synthesis of the diseases of phyllodenous *Acacia* spp. was made by Old *et al*. (2000), and from this study it was clear that many pathogens were poorly defined and required rigorous taxonomic study.

Leaf and shoot pathogens belonging to the genus *Mycosphaerella* Johanson, have had a very distinct impact on plantations in the tropics and Southern Hemisphere. The pine pathogen *Dothistroma septosporum* (Dorog.) M. Morelet (teleomorph *M. pini* E. Rostrup) that has devastated plantings of *P. radiata* D. Don in many Southern Hemisphere countries is one example (Stone *et al*. 2003). Likewise, species of *Mycosphaerella* have had a very marked impact on *Eucalyptus* species planted in this area. For example, Mycosphaerella leaf blight resulted in the abandonment of *E. globulus* Labill. as a plantation species in South Africa (Purnell & Lundquist 1986), and this and other species in the genus continue to seriously threaten *Eucalyptus* plantings (Crous 1998).

Species of *Mycosphaerella* and its anamorphs have been recorded on phyllodenous *Acacia* spp. grown in the tropics (Old *et al*. 1996). These fungi have tentatively been recognised as members of two anamorph genera of *Mycosphaerella*, namely *Cercospora* Fresen. and *Pseudocercospora* Speg. (Old *et al*. 1996, Cannon *et al*. 1997). However, no intensive taxonomic studies have been conducted on these fungi, and the names used are tentative. Although the disease is known to occur widely on species of *Acacia* (Fig. 1), the correct identity of the causal organisms remains unresolved. This again has negative implications for disease management and quarantine programmes, which are aimed at restricting the movement of pathogens between countries.

This present study results from a collection of *Mycosphaerella* species and their anamorphs on two phyllodenous species of *Acacia*, *A. mangium* and *A. crassicarpa*, which are widely planted as exotics in the tropics and the Southern Hemisphere. These fungi have been collected in surveys in South America and South-East Asia during the course of the past four years. Their identification will hopefully contribute to a better understanding of their biology and the diseases that they cause. Identification of species included both morphological and cultural characteristics. More importantly for this group of fungi, however, identifications were also confirmed using comparisions of DNA sequences for the internal transcribed spacer (ITS1 & ITS2) and the 5.8S regions of the ribosomal RNA operon, as well as the elongation factor 1-α, histone, actin and calmodulin gene regions.

Fig. 1A–D. Typical Mycosphaerella leaf blotch symptoms on *Acacia mangium* leaves collected in Thailand.

MATERIALS AND METHODS

Isolates
Symptomatic leaves with leaf spots or blight were chosen for isolations. Excised lesions were placed in distilled water for approximately 2 h, after which they were placed on double-sided tape and fastened to the insides of Petri dish lids, suspended over 2 % malt extract agar (MEA) (Biolab, Midrand, South Africa). Germinating ascospores were examined after 24 h, and single-ascospore and conidial cultures established as explained by Crous (1998). Colonies were subcultured onto oatmeal agar (OA) (Gams *et al*. 1998) and incubated at 25 °C under continuous near-ultraviolet light, to promote sporulation.

DNA phylogeny
Genomic DNA was isolated from fungal mycelium grown on malt extract agar plates following the protocol of Lee & Taylor (1990). The primers ITS1 and ITS4 (White *et al*. 1990) were used to amplify part (ITS) of the nuclear rRNA operon spanning the 3' end of the 18S rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region and the 5' end of the 28S rRNA gene. Part of the elongation factor 1-α gene (EF) was amplified with primers EF1-728F and EF1-986R, part of the actin gene (ACT) with primers ACT-512F and ACT-783R and part of the calmodulin gene (CAL) with primers CAL-228F and CAL-737R (Carbone & Kohn 1999). Part of the histone H3 gene (HIS) was amplified with primers H3-1a and H3-1b (Glass & Donaldson 1995). PCR conditions and protocols, as well as alignment of the subsequent data and DNA phylogeny were treated and generated as explained in Crous *et al*. (2004b) elsewhere in this volume. Sequence data were deposited in GenBank (Table 1) and the alignments in TreeBASE (study accession number S1178). Uniquely fixed characters were identified by manual comparison of the aligned *Cercospora* sequences and unique character positions were calculated using the sequences of *C. apii* CPC 5087 and *C. acaciae-mangii* CPC 10526 as references. The nucleotides shown in the description represent the *C. acaciae-mangii* allele.

Taxonomy
Fungal structures were mounted in lactic acid. The extremes of spore measurements (30 observations) are given in parentheses. Colony colours (surface and reverse) were rated after 1–2 mo on OA at 25 °C in the dark, using the colour charts of Rayner (1970). All cultures obtained in this study are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands (Table 1).

RESULTS

DNA Phylogeny
For each of the five loci sequenced, approximately 500, 320, 230, 320 and 395 bases were determined for ITS, EF, ACT, CAL, and HIS, respectively. A partition homogeneity test using the sequence data showed that only some loci could be combined ($p > 0.05$) in a phylogenetic analysis and these were ITS / ACT ($p = 0.131$), ACT / CAL ($p = 0.698$), ACT / HIS ($p = 0.186$), CAL / HIS ($p = 0.430$) and ACT / CAL / HIS ($p = 0.145$). Therefore, the ITS dataset, which contains additional sequences obtained from GenBank and for which sequence data for the other loci were not available, and the EF dataset were analysed separately and the ACT, CAL and HIS datasets were combined into a single analysis.

The manually adjusted alignment of the ITS sequences contains 44 taxa (including the two outgroups) and 521 characters including alignment gaps (TreeBASE study accession number S1178). Of these characters, 195 are parsimony-informative, 11 are variable and parsimony-uninformative, and 315 are constant. Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with similar topology and bootstrap values. Parsimony analysis of the alignment yielded 96 most parsimonious trees (TL = 350 steps; CI = 0.794; RI = 0.944; RC = 0.750), one of which is shown in Fig. 2. The neighbour-joining and parsimony analyses supported the same main clades (data not shown). Several well-supported clades are seen in the tree, the first of which (100 % bootstrap support) contains sequences of *Cercospora apii* and *C. beticola* (63 % bootstrap support) and four isolates of *C. acaciae-mangii* (64 % bootstrap support). Two *Mycosphaerella* species (CPC 10520 and 10521) cluster with *Pseudocercospora basiramifera* and *Ps. paraguayensis* (100 % bootstrap support). Three sequences of *M. fijiensis* (100 % bootstrap support) form a sister clade to the *Pseudocercospora* isolates (94 % bootstrap support). The sequences of the two *Passalora* sp. isolates (100 % bootstrap support) cluster with *Passalora loranthi* with a bootstrap support value of 100 %. Another well-supported clade (87 % bootstrap support) contains three *Mycosphaerella* species as well as *M. mangium*, *M. heimii* and *M. konae*.

Table 1. Isolates of *Mycosphaerella* spp. and their anamorphs included for sequence analysis.

Species	Accession number[1]	Host	Country	Collector	GenBank numbers (ITS, EF 1-α, ACT, CAL, HIS)
Cladosporium cladosporioides	ATCC 66669 / CPC 5100	Creosote-treated southern pine pole	U.S.A.	–	AY251070, AY752164, AY752192, AY752223, AY752254
Cladosporium herbarum	ATCC 66670 / CPC 5101	CCA-treated Douglas-fir pole	U.S.A.	–	AY251078, AY752165, AY752193, AY752224, AY752255
Cercospora apii	CBS 536.71 / CPC 5087	*Apium graveolens*	Romania	–	AY752133, AY752166, AY752194, AY752225, AY752256
	CPC 5123	*A. graveolens*	New Zealand	C.F. Hill	AY752134, AY752167, AY752195, AY752226, AY752257
Cercospora beticola	CBS 116.47 / CPC 5074	*Beta vulgaris*	Netherlands	–	AY752135, AY752168, AY752196, AY752227, AY752258
	CBS 122.31 / CPC 5072	*Beta vulgaris*	Germany	–	AY752136, AY752169, AY752197, AY752228, AY752259
	CPC 5125	*Beta vulgaris*	New Zealand	C.F. Hill	AY752137, AY752170, AY752198, AY752229, AY752260
	CPC 5128	*Beta vulgaris*	New Zealand	C.F. Hill	AY752138, AY752171, AY752199, AY752230, AY752261
Cercospora acaciae-mangii	CPC 10550	*Acacia mangium*	Thailand	–	AY752139, AY752172, AY752200, AY752231, AY752262
	CPC 10551	*A. mangium*	Thailand	–	AY752140, AY752173, AY752201, AY752232, AY752263
	CPC 10552	*A. mangium*	Thailand	–	–, AY752174, AY752202, AY752233, AY752264
	CPC 10553	*A. mangium*	Thailand	–	–, AY752175, AY752203, AY752234, AY752265
	CPC 10526	*A. mangium*	Thailand	M.J. Wingfield	AY752141, AY752176, AY752204, AY752235, AY752266
	CPC 10527	*A. mangium*	Thailand	M.J. Wingfield	AY752142, AY752177, AY752205, AY752236, AY752267
Mycosphaerella acaciigena	CPC 3837	*Acacia* sp.	Venezuela	M.J. Wingfield	AY752143 (ITS only)
Mycosphaerella citri	X126	*Citrus* sp.	Florida	–	AY752144, AY752178, AY752206, AY752237, AY752268
	CPC 10522	*A. mangium*	Thailand	M.J. Wingfield	AY752145, AY752179, AY752207, AY752238, AY752269
	X115 / rCRB2 / CBS 116426	*Musa* sp.	Florida	J. Cavaletto	AY752146, AY752180, AY752208, AY752239, AY752270
Mycosphaerella colombiensis	CBS 110967 / CPC 1104	*Eucalyptus urophylla*	Colombia	M.J. Wingfield	AY752147, AY752181, AY752209, AY752240, AY752271
	CBS 110968 / CPC 1105	*E. urophylla*	Colombia	M.J. Wingfield	AY752148, AY752182, AY752210, AY752241, AY752272
	CBS 110969 / CPC 1106	*E. urophylla*	Colombia	M.J. Wingfield	AY752149, AY752183, AY752211, AY752242, AY752273
Mycosphearella fijiensis	X300	*Musa* sp.	Tonga	F. Sumich	AY752150 (ITS only)
Mycosphaerella konae	CPC 2123	*Leucadendron* sp.	Hawaii	P.W. Crous	AY260086, AY752184, AY752212, AY752243, AY752274
	CPC 2125	*Leucadendron* sp.	Hawaii	P.W. Crous	AY260085, AY752185, AY752213, AY752244, AY752275
Mycosphaerella sp.	CPC 10516	*A. mangium*	Thailand	M.J. Wingfield	AY752151, AY752186, AY752214, AY752245, AY752276
	CPC 10518	*A. mangium*	Thailand	M.J. Wingfield	AY752152, AY752187, AY752215, AY752246, AY752277
	CPC 10520	*Acacia aulacocarpa*	Thailand	M.J. Wingfield	AY752153 (ITS only)
	CPC 10521	*A. aulacocarpa*	Thailand	M.J. Wingfield	AY752154 (ITS only)
	CPC 10524	*A. mangium*	Thailand	M.J. Wingfield	AY752155, AY752188, AY752216, AY752247, AY752278
Mycosphaerella thailandica	CPC 10547	*A. mangium*	Thailand	–	AY752156, –, AY752217, AY752248, AY752279
	CPC 10548	*A. mangium*	Thailand	–	AY752157, –, AY752218, AY752249, AY752280
	CPC 10549	*A. mangium*	Thailand	–	AY752158, –, AY752219, AY752250, AY752281
	CPC 10621	*A. mangium*	Thailand	–	AY752159, AY752189, AY752220, AY752251, AY752282
	X51	*Musa* sp.	Windward Isles	E. Reid	AY752160, AY752190, AY752221, AY752252, AY752283
	X58	*Musa* sp.	Windward Isles	E. Reid	AY752161, AY752191, AY752222, AY752253, AY752284
Passalora sp.	CPC 11147	*Acacia crassicarpa*	Indonesia	M.J. Wingfield	AY752162 (ITS only)
	CPC 11150	*A. crassicarpa*	Indonesia	M.J. Wingfield	AY752163 (ITS only)

[1]CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; C.P.C.: Culture collection of Pedro Crous, housed at CBS; ATCC: American Type Culture Collection, Virginia, U.S.A; [2]Ex-type cultures.[3]ITS: internal transcribed spacer region, EF 1-α: elongation factor 1-alpha, ACT: actin, CAL: calmodulin, HIS: histone 3-a.

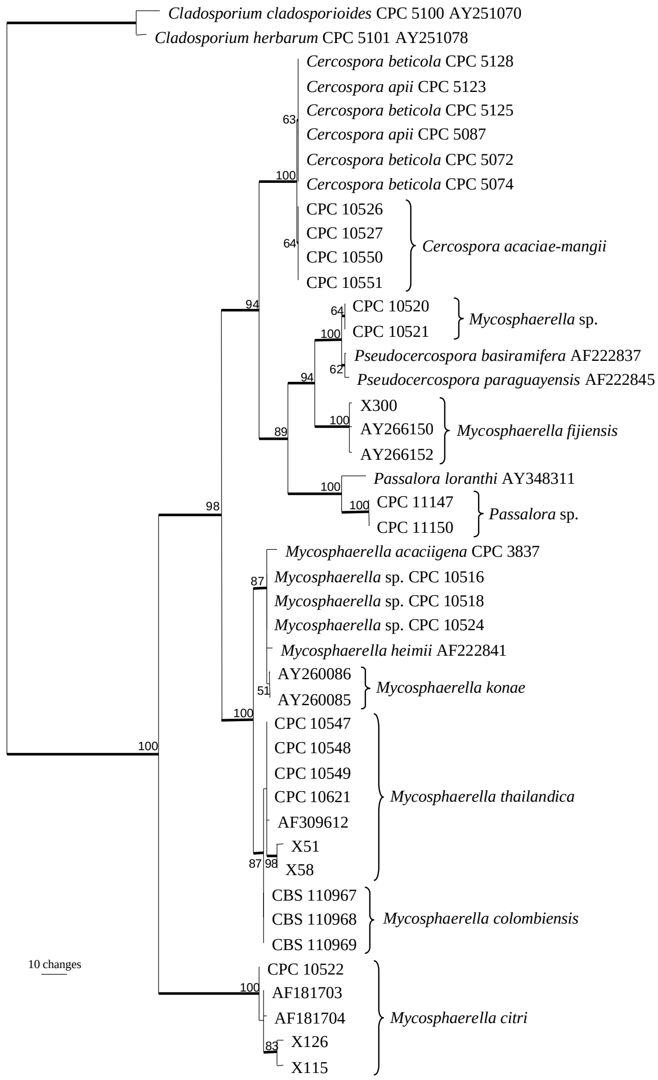


Fig. 2. One of 96 most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the ITS sequence alignment. The scale bar shows 10 changes; bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches. The tree was rooted to two *Cladosporium* species.

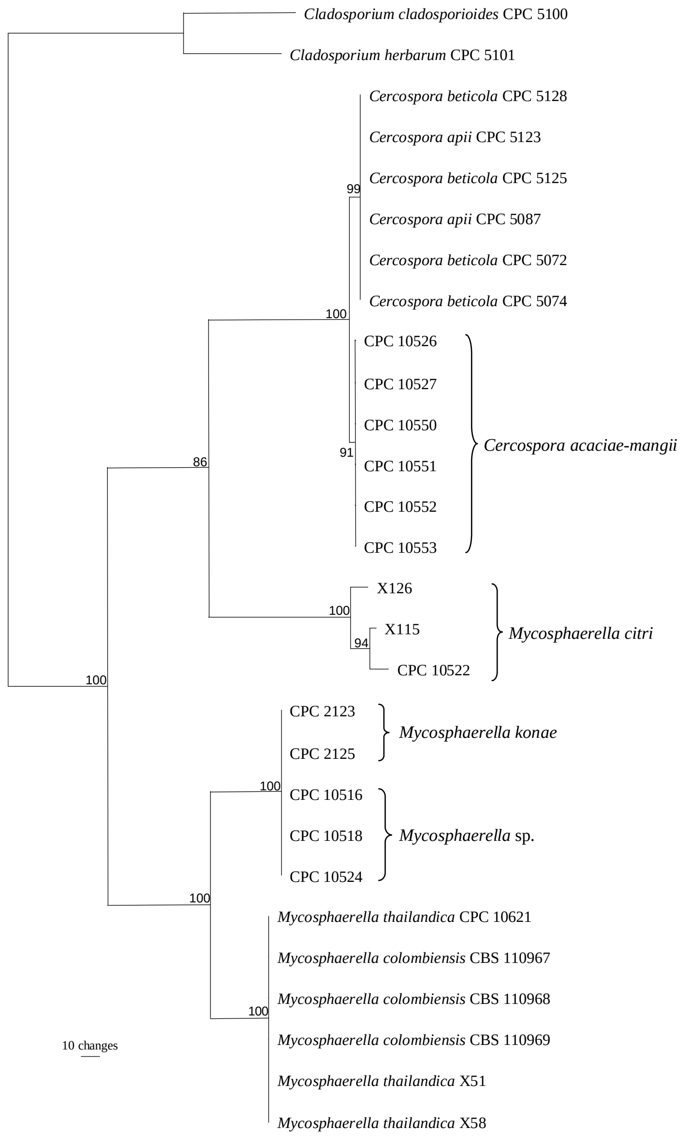


Fig. 3. Single most parsimonious tree obtained from a heuristic search with 100 random taxon additions of the EF 1-α sequence alignment. The scale bar shows 10 changes; bootstrap support values from 1000 replicates are shown at the nodes. The tree was rooted to two *Cladosporium* species.

The sequences of *M. thailandica* and *M. colombiensis* all cluster in the same clade (87 % bootstrap support), with only isolates X51 and X58 forming a distinct group (98 % bootstrap support). Five sequences of *M. citri*, two of which were obtained from GenBank, also formed a well-supported (100 % bootstrap support) clade.

The manually adjusted EF sequence alignment (TreeBASE study accession number S1178) contains 28 taxa (including the two outgroups) and 300 characters including alignment gaps; of these characters 233 are parsimony-informative, 24 are variable and parsimony-uninformative, and 43 are constant. Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with similar topology (data not shown). Between the neighbour-joining and parsimony analyses, the trees supported the same main clades (data not shown). Parsimony analysis of the alignment yielded a single most parsimonious tree (TL = 611 steps; CI = 0.876; RI = 0.966; RC = 0.846), which is shown in Fig. 3.

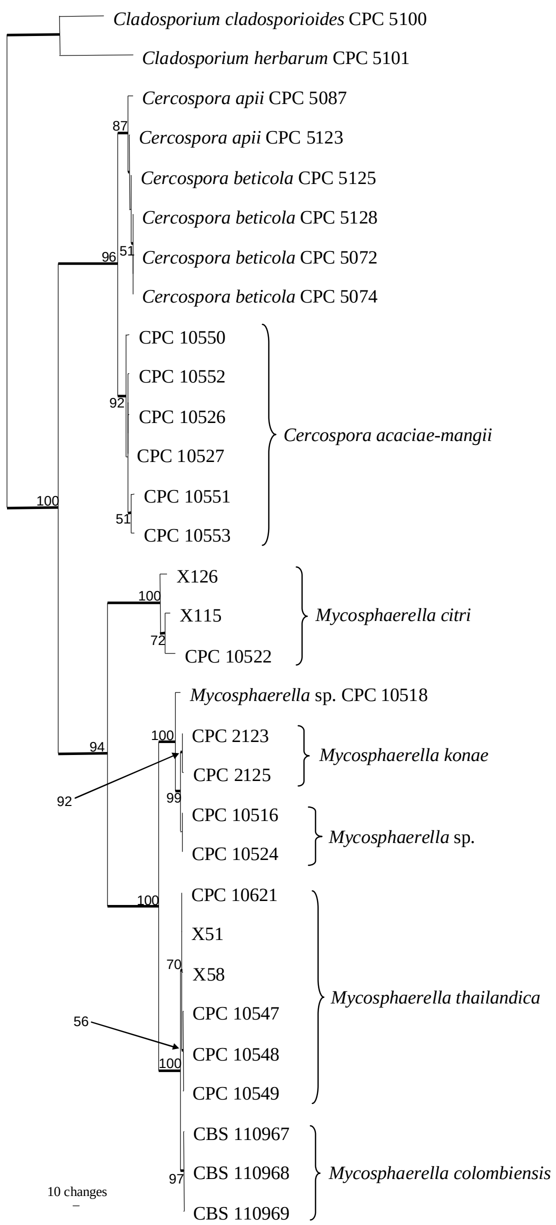


Fig. 4. One of 18 most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the combined ACT, CAL and HIS sequence alignment. The scale bar shows 10 changes and bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches. The tree was rooted to two *Cladosporium* species

Several well-supported clades are seen in the tree, one of which (100 % bootstrap support) contains sequences of *C. apii* and *C. beticola* (99 %) and isolates of *C. acaciae-mangii* (91 %). The *M. citri* clade (100 %) contains three isolates, two of which are more closely related, grouping with a bootstrap support value of 94 %. The three *Mycosphaerella* spp. form a well-supported clade (100 %) together with the *M. konae* isolates. Three sequences of each of *M. thailandica* and *M. colombiensis* all cluster in the same clade (100 %). The EF dataset failed to separate *M. konae* and the *Mycosphaerella* sp., as well as *M. thailandica* and *M. colombiensis*.

For ACT, CAL and HIS, respectively 209, 312 and 388 bases (including alignment gaps) were included in the manually adjusted alignment consisting of all three loci for 44 taxa (including the two outgroups). The combined data set (TreeBASE study accession number S1178) used for phylogenetic analysis contains a total of 909 characters, of which 306 are parsimony-informative, 54 were variable and parsimony-uninformative, and 549 were constant. The topology of the trees generated with neighbour-joining analysis using the three substitution models were identical (data not shown). Parsimony analysis of the combined data yielded 18 most parsimonious trees, one of which is shown in Fig. 4. Between the neighbour-joining and parsimony analyses, the trees differed only in the placement of the *M. citri* clade (data not shown). Distance analysis grouped the *M. citri* clade with the *Cercospora* clade (bootstrap support value of approximately 70 % irrespective of which substitution model is used), whereas it groups (94 %) with the clades containing the other *Mycosphaerella* species when a parsimony analysis is performed. As with the ITS and EF trees, a clear separation is found between the clade containing *C. apii*/*C. beticola* isolates (87 %) and *C. acaciae-mangii* (92 %). The *M. citri* clade (100 %) contains three isolates, two of which once again are more closely related, and is supported by a lower bootstrap support value of 72 %. The clade containing the three *Mycosphaerella* sp. and two *M. konae* isolates is also well-supported (100 %), with *M. konae* clustering with a bootstrap support of 92 % and the isolate CPC 10518 sitting outside of the cluster (99 %) formed by the rest of the isolates in this clade. Another well-supported clade (70 %) in this tree contains the *M. thailandica* (70 %) and *M. colombiensis* (97 %) isolates.

Taxonomy

Cercospora acaciae-mangii Crous, Pongpanich & M.J. Wingf., **sp. nov.** MycoBank MB500118.
Teleomorph: *Mycosphaerella* sp. Fig. 5.

Etymology: Named after its host *Acacia mangium*.

Maculae amphigenae, medio-brunneae, inter marginem et costam, margine atro-brunneo, leviter elevato cinctae. Stromata nulla vel bene evoluta, brunnea, ad 30 µm diam. Conidiophora medio-brunnea, levia, longa, fasciculata (3–20), recta vel apice geniculato-sinuoso. Cellulae conidiogenae integratae, terminales vel intercalares, ad 100 µm longae, sympodiales; cicatrices conidiales incrassatae, fuscatae, refractivae, ad 3 µm latae. Conidia solitaria, hyalina, levia, aciculares, 50–350 × 3.5–5 µm, pluriseptata, basi (in hilo) incassata, fuscata, refractiva. *Cercosporae apii* similis, sed hospite *Acacia* et nonnullis nucleotideis differens: elongation factor 1-alpha (EF) in positionibus 42 (T), 47 (C), 144 (C), 198 (G), 217 (A), 224 (A), 235 (A), 245 (C), 257 (G); actinum (ACT) in positionibus 70 (T), 172 (A), 175 (A); calmodulinum (CAL) in positionibus 37

(C), 81 (A), 109 (C), 114 (C), 117 (A), 148 (A), 149 (T), 189 (T), 270 (G), 279 (T); histonum H3 (HIS) in positionibus 112 (A), 114 (T), nucleotide deleto inter positiones 122 et 123, 135 (G), 148 (C), 151 (T), 381 (C).

Leaf spots amphigenous, covering up to half of the leaf lamina from the margin to the mid rib; infections intermixed with that of *M. thailandica*; lesions medium brown, surrounded by a raised, dark brown border. *Stromata* lacking to well developed, brown, up to 30 µm diam, giving rise to conidiophores. *Conidiophores* medium brown, smooth, long, flexuous, in fascicles that vary in number from 3–20, straight, or with upper part geniculate-sinuous. *Conidiogenous cells* integrated, terminal or intercalary, up to 100 µm long, proliferating sympodially, loci thickened, darkened, refractive, up to 3 µm wide. *Conidia* solitary, hyaline, smooth, acicular, 50–350 × 3.5–5 µm, multiseptate, with a thickened, darkened, refractive scar. Morphologically indistinguishable from *C. apii s. l.* (Crous & Braun 2003).

Holotype: **Thailand**, Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, 28 May 2003, K. Pongpanich, **holotype** herb. CBS 9874; culture ex-type CBS 116365 = CPC 10526.

Host: *Acacia mangium.*

Cultures: Colonies irregular, fast growing, covering the dish after 1 mo; aerial mycelium fluffy to woolly, surface white to pale olivaceous-grey (21””’d), with patches of grey-olivaceous (21””b) sporulation; reverse iron-grey (25””’k).

Distribution: Thailand.

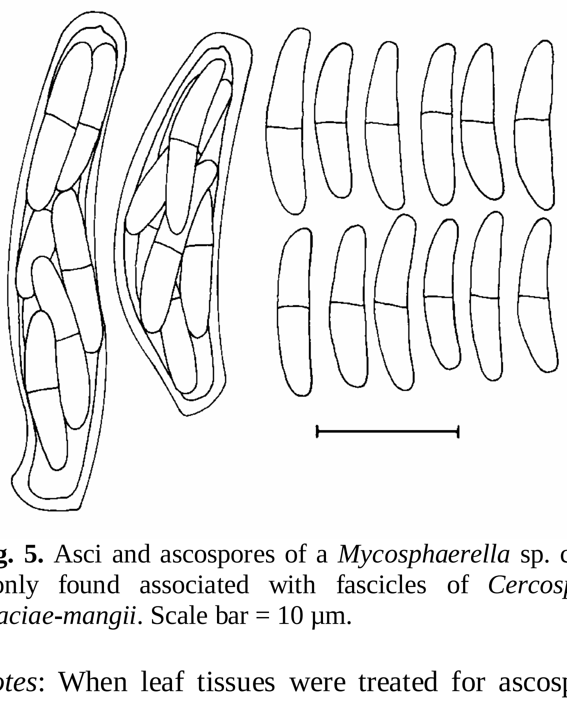


Fig. 5. Asci and ascospores of a *Mycosphaerella* sp. commonly found associated with fascicles of *Cercospora acaciae-mangii*. Scale bar = 10 µm.

Notes: When leaf tissues were treated for ascospore discharge, several ascospores of a *Mycosphaerella* sp. were obtained that gave rise to a *Cercospora* anamorph. Upon germination, however, these ascospores could not with certainty be traced back to the *Mycosphaerella* state, as they were only harvested after 48 h, and had hence started to distort. The formal naming of the *Mycosphaerella* teleomorph thus awaits further collections of fresh material. A probable candidate which occurred on the lesions from which the cultures were derived has the following morphology: *Ascomata* pseudothecial, amphigenous, erumpent, black, aggregated in moderately dense clusters, globose, up to 90 µm diam; apical ostiole 5–10 µm diam; wall of 2–3 layers of medium brown *textura angularis*. *Asci* fasciculate, bitunicate, subsessile, obovoid to narrowly ellipsoid or subcylindrical, straight or slightly incurved, 8-spored, 30–40 × 7–9 µm. *Ascospores* tri- to multiseriate, overlapping, hyaline, non-guttulate, thin-walled, curved, fusoid-ellipsoidal with obtuse ends, medianly 1-septate, widest at the median, unconstricted septum, tapering towards both ends, (10–)12–13(–15) × (2–)2.5–3 µm *in vivo*.

The *Cercospora* anamorph closely matched others within the *C. apii s. l.* complex (Crous & Braun 2003), but could be separated phylogenetically, and is thus described as *C. acaciae-mangii*.

Additional specimens and cultures examined: **Thailand**, Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, 28 May 2003, K. Pongpanich, herb. CBS 9876, CPC 10550, 10526–10528 (single-ascospore isolates), CPC 10551–10553 (single-conidial isolates of *C. acaciae-mangii*).

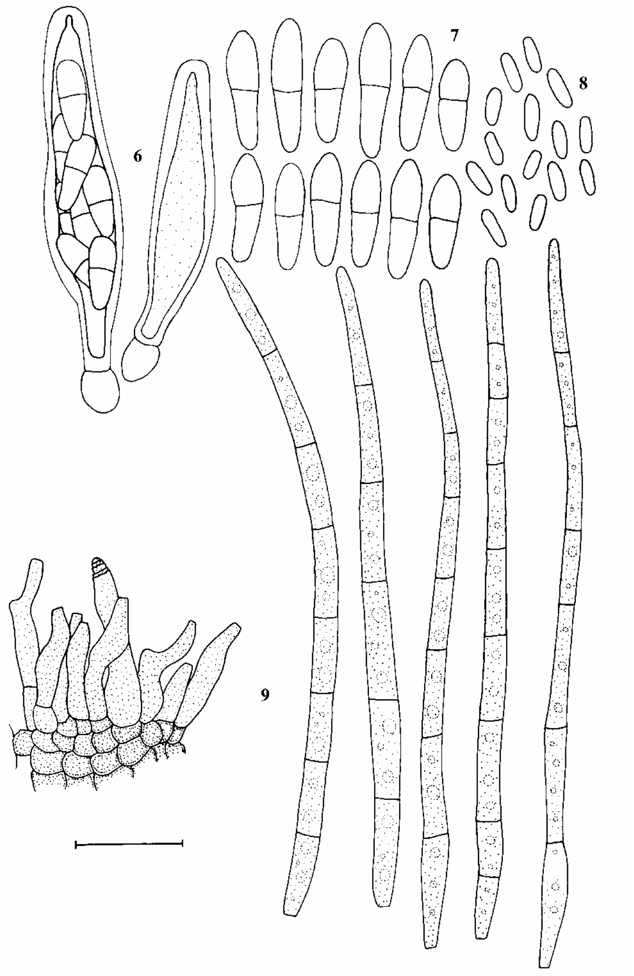
Mycosphaerella acaciigena Crous & M.J. Wingf., **sp. nov.** MycoBank MB500119. Figs 6–9.

Anamorph: *Pseudocercospora acaciigena* Crous & M.J. Wingf., sp. nov.

Etymology: Named after the host genus, *Acacia*.

Mycosphaerella heimii similis sed ascosporis ad septum modice constrictis differens.

Leaf spots amphigenous, elongated along the length of the leaf, not confined to the margins, variable in width, up to 2 cm diam, medium brown, surrounded by a raised, dark brown border. *Ascomata* pseudothecial, amphigenous, erumpent, black, aggregated in clusters of up to 100, forming black spots up to 1 mm diam on the lesions; ascomata globose, up to 80 µm diam; apical ostiole 5–10 µm diam; wall of 2–3 layers of medium brown *textura angularis*. *Asci* fasciculate, bitunicate, subsessile, obovoid to narrowly ellipsoid, straight or slightly incurved, 8-spored, 25–40 × 8–11 µm. *Ascospores* tri- to multiseriate, overlapping, hyaline, non-guttulate, thin-walled, straight, fusoid-ellipsoidal with obtuse ends, medianly 1-septate, widest in the middle of the apical cell, slightly constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (8–)9–10(–11) × (2.5–)3 µm *in vivo*. *Spermogonia* intermixed with and similar to the ascomata in general morphology. *Spermatia* rod-shaped, hyaline, 3–6 × 1 µm *in vivo*.



Figs 6–9. *Mycosphaerella acaciigena* and its *Pseudocercospora* anamorph. 6. Asci. 7. Ascospores. 8. Spermatia. 9. Conidiophores and conidia. Scale bar = 10 µm.

Pseudocercospora acaciigena Crous & M.J. Wingf., **sp. nov.** MycoBank MB500120.

Differt a *P. thailandica* conidiis longioribus, ad 15-septatis; a *P. acaciae-confusae*, *P. hyaloconidiophora* et *P. acaciae* conidiis obclavatis, pallide brunneis, 2–2.5(–3) µm latis.

Conidiomata amphigenous, pale brown, up to 80 µm diam; stromata well developed, brown, up to 60 µm wide and 30 µm high. *Mycelium* predominantly internal, consisting of smooth, branched, septate, pale brown, 3–4 µm wide hyphae. *Conidiophores* aggregated in dense fascicles arising from the upper cells of the stroma; conidiophores pale brown, smooth, unbranched or branched, 0–3-septate, subcylindrical, straight to geniculate-sinuous, 15–30 × 3–5 µm. *Conidiogenous cells* terminal, pale brown, smooth, subcylindrical, tapering to flat tipped apical loci, proliferating sympodially, or several times percurrently, 15–20 × 3–4 µm; conidial scars inconspicuous. *Conidia* solitary, pale brown, smooth, guttulate, narrowly obclavate, apex subobtuse, base long obconically subtruncate, straight to curved, 3–15-septate, (40–)50–75(–80) × 2–2.5(–3) µm *in vivo*; hila inconspicuous.

Holotype: **Venezuela**, Acarigua, on leaves of *A. mangium*, May 2000, M.J. Wingfield, herb. CBS 9873, **holotype** of *M. acaciigena* and *P. acaciigena*; cultures ex-type CBS 115432, 112515, 112516 = CPC 3836–3838.

Cultures: Colonies on OA with thin yellow-brown line of pigment diffusing into the agar; margin thin, smooth, slimy, white (1–2 mm wide); surface pale olivaceous-grey (21''''d), with sparse aerial mycelium. On MEA margin smooth, regular, aerial mycelium sparse; surface colour variable, predominantly pale olivaceous-grey (23''''d), with patches of smoke-grey (19''''d) and olivaceous-grey (21''''i); reverse olivaceous-grey (21''''i).

Host: *Acacia mangium* (*Leguminosae*).

Distribution: Venezuela.

Notes: The dense black clusters of raised ascomata on both sides of the leaf lamina is a very characteristic feature of this species. The holotype specimen of *M. acaciigena* is also colonized by a species of *Cercospora*. The latter appears to be distinct from the *C. apii s. l.* complex, as conidia tend to have more rounded bases, and be more subcylindrical in shape and shorter than the typical conidia of *C. apii*, which have more truncate bases, and are longer and acicular in shape. A few conidia of a *Stenella* sp. were also found to be present, though fructification was sparse. As no cultures of the latter two fungi were obtained, they are not treated further and await additional collections.

Mycosphaerella citri Whiteside, Phytopathology 62: 263. 1972. Fig. 10.

Anamorph: *Stenella citri-grisea* (F.E. Fisher) Sivan., In: Sivanesan, Bitunicate ascomycetes and their anamorphs: 226. 1984.

≡ *Cercospora citri-grisea* F.E. Fisher, Phytopathology 51: 300. 1961.

Leaf spots amphigenous, covering up to half of the leaf lamina from the margin to the mid rib; infections intermixed with that of *M. thailandica* and *C. acaciae-mangii*; lesions medium brown, surrounded by a raised, dark brown border. *Mycelium* consisting of verruculose, branched, septate, red-brown to medium brown hyphae, 2–3 µm wide. *Conidiophores* arising singly from superficial mycelium, red-brown to medium brown, verruculose, subcylindrical to irregular, 1–3-septate, straight to variously curved, 5–20 × 2.5–4 µm. *Conidiogenous cells* terminal, verruculose, medium brown, unbranched, tapering to rounded apices with flat, thickened, darkened, refractive loci, proliferating sympodially, 5–10 × 2.5–4 µm. *Conidia* solitary, medium brown to red-brown, verruculose, narrowly obclavate, apex subobtuse, base long obconically subtruncate, straight to curved, (0–)3–5(–10)-septate, (10–)35–65(–120) × (2–)2.5(–3) µm *in vivo* (description based on *Acacia* isolate CPC 10522 = CBS 116366).

Cultures: Colonies with smooth, regular margins, moderately fast growing, covering the dish after 2 mo; aerial mycelium moderate, surface olivaceous-grey (21 """'i), reverse greenish black (33 """'k); cultures fertile.

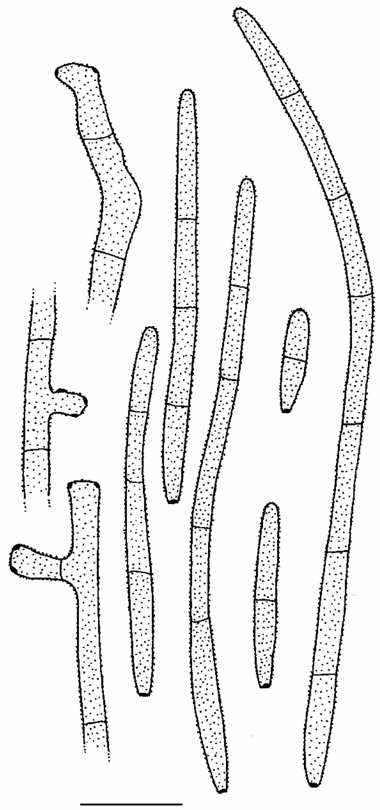


Fig. 10. Conidiophores and conidia of *Stenella citri-grisea* formed *in vitro* from isolate CBS 116366. Scale bar = 10 µm.

Hosts: *Acacia mangium*, *Musa* sp., and species of *Aeglopsis* Swingle, *Citrus*, *Fortunella* Swingle, *Murraya* L., *Poncirus* Rafin. (Rutaceae) (Pretorius *et al*. 2003).

Distribution: Thailand (*Acacia*), on Rutaceae in Brazil, Costa Rica, Cuba, Dominican Republic, El-Salvador, Gabon, Haiti, Hong Kong, Japan, Puerto Rico, Surinam, Taiwan, Thailand, USA (FL, HI, TX), Venezuela, Virgin Islands (Pretorius *et al*. 2003).

Notes: In culture, conidia of CBS 116366 closely resembled the morphology of isolates described from *Citrus* (Fisher 1961, Sivanesan 1984).

Culture examined: **Thailand**, Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, 28 May 2003, K. Pongpanich, CBS 116366 = CPC 10522 (single-ascospore isolate).

Mycosphaerella thailandica Crous, Himaman & M.J. Wingf., **sp. nov.** MycoBank MB500121. Figs 11–15.

Anamorph: *Pseudocercospora thailandica* Crous, Himaman & M.J. Wingf., sp. nov.

Etymology: Named after its country of origin, Thailand.

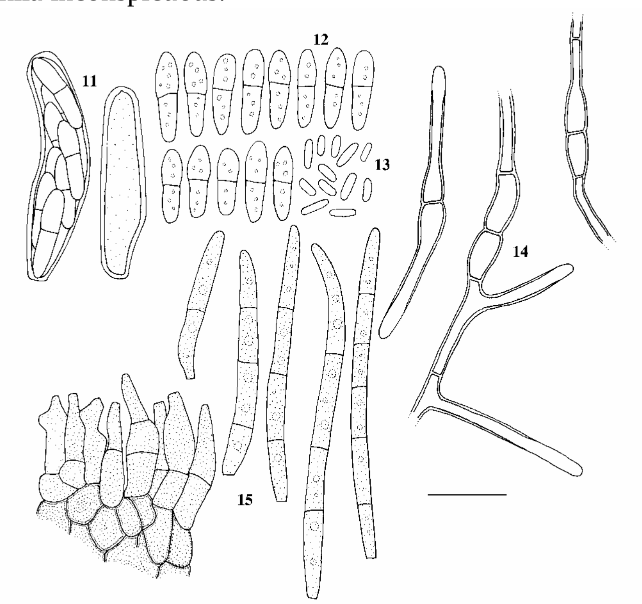
Mycosphaerellae colombiensi similis, sed ascosporis ad septum modice constrictis differens; ascosporae modo C germinantes.

Leaf spots amphigenous, irregular blotches covering large parts of the leaf lamina; associated symptoms include tip blight, or lesions all along the margin of the leaf, frequently extending to the middle of the leaf lamina; lesions medium brown, surrounded by a raised, dark brown border. *Ascomata* pseudothecial, amphigenous, subepidermal, becoming erumpent, black, globose, up to 80 µm diam; apical ostiole 5–10 µm diam; wall of 2–3 layers of medium brown *textura angularis*. *Asci* fasciculate, bitunicate, subsessile, obovoid to narrowly ellipsoid, straight or slightly incurved, 8-spored, 30–40 × 6–8 µm. *Ascospores* tri- to multiseriate, overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, fusoid-ellipsoidal with obtuse ends, medianly 1-septate, widest in middle of the apical cell, slightly constricted at the septum, tapering towards both ends, but more prominently towards the lower end, (9–)10–11(–12) × (2–)2.5–3 µm *in vivo*. Spermogonia intermixed with and similar to the ascomata in general morphology. Spermatia rod-shaped, hyaline, 3–5 × 1 µm *in vivo*.

Pseudocercospora thailandica Crous, Himaman & M.J. Wingf., **sp. nov.** MycoBank MB500122.

Differt a *P. acaciigena* conidiis brevioribus, ad 6-septatis; a *P. acaciae-confusae*, *P. hyaloconidiophora* et *P. acaciae* conidiis obclavatis-subcylindraceis, pallide brunneis, 2-2.5(–3) µm latis.

Conidiomata amphigenous, pale brown, up to 60 µm diam; stromata well developed, brown, up to 25 µm wide and 30 µm high. *Mycelium* predominantly internal, consisting of smooth, branched, septate, medium brown, 3–4 µm wide hyphae. *Conidiophores* aggregated in dense fascicles arising from the upper cells of the stroma; conidiophores pale brown, smooth, unbranched, 0–2-septate, subcylindrical, straight to variously curved, 10–20 × 5–6 µm. *Conidiogenous cells* terminal, pale brown, smooth, subcylindrical, tapering to flat tipped apical loci, proliferating sympodially, 10–15 × 3–5 µm; conidial scars inconspicuous. *Conidia* solitary, pale brown, smooth, guttulate, narrowly obclavate to subcylindrical, apex subobtuse, base long obconically subtruncate, straight to curved, 3–6-septate, (25–)30–45(–60) × 2–2.5(–3) µm *in vivo*; hila inconspicuous.



Figs 11–15. *Mycosphaerella thailandica* and its *Pseudocercospora* anamorph. 11. Asci. 12. Ascospores. 13. Spermatia. 14. Germinating ascospores on MEA. 15. Conidiophores and conidia. Scale bar = 10 µm.

Holotype: **Thailand**, Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, 28 May 2003, K. Pongpanich, herb. CBS 9875, **holotype** of both *M. thailandica* and *P. thailandica*, cultures ex-type CBS 116367 = CPC 10547–10549.

Ascospore germination on MEA after 24 h: Germinating with germ tubes parallel to the long axis of the ascospore, constricted at the original septum, ascospores becoming 2.5–3 µm wide, developing several lateral branches.

Cultures: Colonies slightly erumpent, having smooth, regular margins, fast growing, covering the dish after 60 d; aerial mycelium fluffy, surface grey-olivaceous (21 ""b), reverse olivaceous-black (25 ""k); cultures sterile.

Host: *Acacia mangium.*

Distribution: Thailand.
Notes: Morphologically *M. acaciigena* is similar to *M. thailandica*, except that the *Pseudocercospora* conidia of *M. acaciigena* tend to be longer, and ascomata of *M. acaciigena* are arranged in dense, superficial clusters, which differ from what was observed on the type of *M. thailandica*. However, additional specimens studied from Thailand (herb. CBS 9879, Mar. 2003) also tend to have ascomata arranged in clusters, though not as pronounced as observed for *M. acaciigena*. This could indicate that the clustering is a result of the host tissue, or that *M. acaciigena* also occurs in Thailand. Further collections and cultures would be required, however, to resolve this issue.

Mycosphaerella thailandica is morphologically similar to *M. colombiensis* Crous & M.J. Wingf., which is a pathogen of *Eucalyptus* (Crous 1998). Although the latter two species can be distinguished based on ascospore morphology and germination patterns.

Additional specimens and cultures of unidentified spp. examined: **Thailand**, Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, May 2002, W. Himaman, herb. CBS 9878; Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, Mar. 2003, K. Pongpanich, herb. CBS 9879; Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, 2003, K. Pongpanich, ascospore cultures CPC 10516–10525, 10621–10625.

Cultures of unidentified Mycosphaerella spp. examined: **Thailand**, Chachoengsao Province, Sanamchaikhet, on leaves of *A. mangium*, 28 May 2003, K. Pongpanich, CPC 10516, 10518, 10524 (single-ascospore isolates of *Mycosphaerella* sp. in the *M. konae* clade); CPC 10520, 10521 (single-ascospore isolates of *Mycosphaerella* sp. in the *M. basiramifera* clade).

DISCUSSION

Results of this study have clearly emphasised the paucity of knowledge regarding the taxonomy of leaf pathogens of *Acacia* spp. that are of considerable economic importance to the forestry industry. In the review of diseases of *Acacia* spp. grown in plantations in the tropics, Old *et al*. (2000) noted that two species, tentatively identified as species of *Cercospora* and *Pseudocercospora*, occur on *A. mangium*, *A. auriculiformis* and *A. crassicarpa*. In this study we have described three species of *Mycosphaerella*, and one that is currently known only from its anamorph. We have also identified at least three other, as yet undescribed species from these trees in various tropical countries. Several of these fungi are peripherally similar to each other and this probably explains why they have not previously been recognised.

In their revision of the genus *Cercospora*, Crous & Braun (2003) regarded 281 names to be synonymous with the older *C. apii*, and treated these as part of the *C. apii s. l.* species complex. Currently there are no *Mycosphaerella* teleomorphs known within this complex. The collection of a *Mycosphaerella* sp. that gave rise to a *Cercospora* anamorph matching the description of *C. apii s. l.* in the present study is thus an exciting development. Isolates were obtained from single conidia, as well as single ascospores. Comparison of DNA sequence data for several genes (Figs 2–4) showed that these ascospore and conidial isolates cluster closely together within the *C. apii* clade, but that they represent a distinct lineage. We have described these as morphologically similar to *C. apii*, but representing a phylogenetically distinct species, named *C. acaciae-mangii*. These isolates will add a valuable indication of the variation that can be expected within the *C. apii s. l.* species complex. They will also promote our understanding of the species limits and genetic entities within this complex.

The *Pseudocercospora* anamorphs of *M. acaciigena* and *M. thailandica* are morphologically very similar, differing chiefly in conidial size and septation, and are quite distinct from *P. acaciae-confusae* (Sawada) Goh & W.H. Hsieh, which has pale yellowish brown, cylindrical conidia, and causes irregularly angular spots 0.5–2 mm diam (Hsieh & Goh 1990). *Pseudocercospora hyaloconidiophora* Goh & W.H. Hsieh is distinguished by having hyaline conidiophores and conidia (Hsieh & Goh 1990). Furthermore, *P. acaciae* Kamal & R.P. Singh is distinguished by its very long (up to 270 µm), thick-walled, smooth conidiophores, and obclavate conidia that are much wider than observed in the present collections (21.5–70 × 7–11 µm) (Kamal & Singh 1980).

Mycosphaerella acaciigena, which was collected in Venezuela, is morphologically similar, but phylogenetically distinct from the *M. heimii* Crous/*M. konae* Crous, Joanne E. Taylor & M.E. Palm species complex (Crous 1998, Crous *et al.* 2004a). Several other isolates obtained from Thailand (CPC 10516, 10518, 10524), could, however, represent one of the latter species, and this will be resolved once fertile collections have been obtained for morphological comparison. Isolates CPC 10520 and CPC 10521 appear to represent another, undescribed species closely related to *P. basiramifera* Crous/*P. paraguayensis* (Kobayashi) Crous (Fig. 2). The *Passalora* sp. (CPC 11147, 11150) from *A. crassicarpa* which clusters with *Cercospora loranthi* McAlpine (= *Passalora fide* V. Beilharz, in press), is clearly distinguishable based on morphological and phylogenetic differences. This species is treated elsewhere in this volume (Beilharz *et al.* 2004).

Mycosphaerella thailandica is morphologically very similar to *M. colombiensis*, which is a leaf pathogen of *Eucalyptus* in Colombia (Crous 1998). Morphologically, the two species can be distinguished by the constricted ascospores of *M. thailandica*, while those of *M. colombiensis* are not constricted. In the ITS dataset (Fig. 2), these species cluster together. However, in both the EF-1α, and combined actin, calmodulin & histone datasets (Figs 3, 4), it is clear that *M. thailandica* is a cryptic species closely related to, but distinct from *M. colombiensis*.

Mycosphaerella citri is an important foliar and fruit pathogen of *Citrus*, causing premature leaf drop, as well as reduced tree vigour, yield and fruit size (Mondal *et al.* 2003). In a recent phylogenetic study of the genus *Cercospora*, Goodwin *et al.* (2001) included one isolate from a *Musa* sp. (rCRB2 = CBS 116426), which, although identified as *M. fijiensis* M. Morelet, clustered with an isolate of *M. citri*. They subsequently concluded that the isolate was either misidentified or contaminated. The same isolate was obtained from Dr S.B. Goodwin for inclusion in the present study. We can now confirm that this isolate represents *M. citri*, and not *M. fijiensis*. Furthermore, an ex-ascospore isolate obtained from leaves of *Acacia mangium* in Thailand in the present study, also represented *M. citri*. As far as we are aware, this is the first record confirmed based on DNA sequence data, of a serious *Mycosphaerella* pathogen having alternative hosts. Species of *Acacia*, *Citrus*, and *Musa* are all native to parts of South-East Asia, and this might explain the host-sharing observed here. The fact that these trees are also widely planted as exotics in tropical and sub-tropical parts of the world, and that the important pathogen *M. citri* could infect three unrelated hosts, is cause for considerable concern. An examination of the various gene trees generated in the current study support the view of Pretorius *et al.* (2003) that *M. citri* is more variable than previously believed. Furthermore, our results show that speciation is occurring in *M. citri*. Although the isolates occurring on *Musa* and *Acacia* appear to fall within the morphological variation accepted for *M. citri*, this appears to be changing. We expect that in the future, this species will evolve into separate, cryptic species or lineages depending on its host.

Host sharing was also found in the *M. colombiensis/thailandica* complex, where *M. thailandica*, occurs on *Acacia* and *Musa*. However, in this case, lineages are more distinct than those in the *M. citri* complex, and the fungus on *Acacia* and *Musa* could thus be named as *M. thailandica*. In the *Cercospora apii s. l.* complex, *C. acaciae-mangii* represents an additional example of a morphologically similar species, which can be separated based on its host and phylogeny. Ironically, in all three examples where host sharing has been observed, isolates were obtained from ascospores, again suggesting that the presence of the teleomorph enhances speciation. Other taxa in the *C. apii s. l.* complex lack teleomorphs, and still cluster together in clades emerging from comparisons of the various gene regions sequenced, despite their different hosts.

An intriguing question relating to the fungi described in this study is where they might have originated. The host trees are native to tropical parts of Australia and Papua New Guinea, and it is logical to assume that the fungi have been introduced into plantation areas from one or more of these native tree populations. Alternatively, and as illustrated, they could have jumped from completely unrelated hosts. The two undescribed cercosporoid fungi reported by Old *et al*. (2000) were both found in Northern Australia (Old *et al*. 1996, Cannon *et al*. 1997), and match the description of the fungi described here. The remaining species might have evolved together with the *Acacia* spp. on which they occur. However, there is growing evidence to show that pathogens of *Eucalyptus* have adapted from native plants to infect these important plantation trees (Wingfield *et al*. 2001).

There are many native species of *Acacia* and trees of related genera in areas where Australian *Acacia* spp. are being propagated commercially. It seems likely that both fungi occurring on *Acacia* spp. in their native environment, and others that have more recently adapted to infect these trees as exotics will be encountered. The latter group of new pathogens could seriously threaten the trees in their native environment, if they were to be transferred back to these areas.

This situation would be similar to that found with Eucalyptus rust caused by *Puccinia psidii* G. Winter, which is native in Latin America on various *Myrtaceae*, and has adapted to infect *Eucalyptus* in that area (Coutinho *et al*. 1998). This rust fungus is presently considered to be one of the most serious threats to *Eucalyptus* in areas such as Australia where there are no rust pathogens of these trees.

Mycosphaerella spp. and their anamorphs include some of the most important leaf and shoot pathogens of forest plantation trees, fruit trees and shrubs (Old *et al*. 2000, Park *et al*. 2000, Stone *et al*. 2003, Crous *et al*. 2004a, b). In the case of *Eucalyptus*, plantations in the tropics and the Southern Hemisphere have been seriously damaged by these fungi (Crous 1998). We might thus expect the same situation for *Acacia* spp. in the future. It is thus imperative that these fungi are correctly characterised and named. Management strategies to reduce the impact of the diseases associated with these fungi will rest strongly on a clear understanding of the relative importance of the various species. Likewise, quarantine measures aimed at excluding these fungi from new areas will depend on our ability to identify them.

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