

**CHAPTER 3**  
**SPECIES OF *MYCOSPHAERELLA* AND RELATED ANAMORPHS ON**  
***EUCALYPTUS* LEAVES FROM THAILAND**

**3.1 Introduction**

Species of *Eucalyptus* (Myrtaceae) are hosts to a wide range of fungal pathogens (Sankaran *et al.*, 1995a; Crous *et al.*, 2004c, 2006f, 2007h; Summerell *et al.*, 2006). *Eucalyptus* spp. are commonly planted as exotics in commercial plantations for fuel wood, timber, and the paper and pulp industries in various tropical and subtropical regions (Ball, 1995). Eucalypts have been cultivated extensively because of their fast growth rates and high adaptability to different soil types and climates (Turnbull, 2000). In 1995, the total *Eucalyptus* plantation area in South-East Asia already exceeded 2 million ha (Old *et al.*, 2003) and this number has continually increased over the years. In Thailand alone, a total of 443 000 ha *Eucalyptus* plantations was established by 2005 (Barney, 2005). In spite of these huge areas already afforested to *Eucalyptus*, this crop is still increasingly being planted in Thailand and other Asian countries to meet rising global timber demand.

Plant pathogenic microfungi associated with *Eucalyptus* spp. can substantially decrease timber yield (Park *et al.*, 2000; Old *et al.*, 2003). In particular, species of *Mycosphaerella* (Mycosphaerellaceae) and *Teratosphaeria* (Teratosphaeriaceae) have proven to be serious pathogens of *Eucalyptus*, causing severe leaf spot formation, defoliation, shoot die-back and stem cankers (Crous, 1998b; Maxwell *et al.*, 2003; Crous *et al.*, 2004d, 2006g, d, 2007f; Hunter *et al.*, 2004, 2006b; Jackson *et al.*, 2005a; Cortinas *et al.*, 2006b; Carnegie, 2007). Nonetheless, information about the diversity of *Mycosphaerella* and related anamorph species on *Eucalyptus* from Thailand is generally lacking, and presently only five species have been reported, namely *Pseudocercospora basiramifera* and *Ps. flavomarginata* (Crous, 1998b; Hunter *et al.*, 2006b),

*Mycosphaerella heimii*, *M. konae* and *M. thailandica* (Crous *et al.*, 2007h). Species of this pathogen complex reported from *Eucalyptus* in Asia include *Dissoconium aciculare*, *Kirramyces destructans*, *K. eucalypti*, *M. crystallina*, *M. eucalyptorum*, *M. gracilis*, *M. heimioides*, *M. marksii*, *M. obscuris*, *M. parkii*, *M. robusta*, *M. stramenticola*, *M. sumatrensis*, *M. verrocosiafricana*, *M. vietnamensis*, *M. yunannensis*, *Ps. deglupta*, *Ps. eucalyptorum*, *Ps. fatouae*, *Ps. paraguayensis*, *Ps. robusta*, *Septoria eucalyptorum*, *S. xenoparkii*, *Teratosphaeria fimbriata*, *T. gamsii*, *T. suberosa* and *T. suttonii* (Crous and Alfenas, 1995; Crous and Wingfield, 1997; Hunter *et al.*, 2004, 2006b, Crous, 1998b; Crous and Braun, 2003; Crous *et al.*, 2004c, 2006g, 2007f; Burgess *et al.*, 2007). Many *Eucalyptus* leaf pathogens originally described as related species of *Mycosphaerella* (i.e. *M. cryptica*, *M. gamsii*, *M. pseudocryptica*, *M. pseudosuberosa* and *M. suttonii*) have been re-classified into the genus *Teratosphaeria* (Teratosphaeriaceae) after substantial taxonomic revisions based on novel morphological characters integrated with their

DNA phylogeny obtained by using the 28S nrRNA gene (Crous *et al.*, 2007f, 2008). DNA sequencing of the ITS nrDNA gene has in the past proven highly effective to distinguish among species of *Mycosphaerella* (Crous *et al.*, 2000a, 2006d, b, 2007h; Cortinas *et al.*, 2006). The main objective of the present study was to identify species of *Mycosphaerella* and related anamorphs associated with *Eucalyptus* leaves collected from plantations in Thailand, and to resolve their taxonomy and DNA phylogeny

### 3.2 Materials and methods

#### 3.2.1 Isolates

Symptomatic *Eucalyptus* leaves were collected at various locations in Thailand (Table 3.1). Lesions with ascomata were removed, soaked in distilled water for 2 h, and then placed in the bottom of the Petri dish lids, with the top half of the dish containing 2 % malt extract agar (MEA; Oxoid, Gams *et al.*, 2007). Dishes were incubated at room temperature in the dark. After 24 h ascospore germination patterns from discharged ascospores on MEA were examined under a compound microscope. Single ascospore cultures were established on fresh MEA dishes as described by Crous (1998b).

Symptomatic leaves were also incubated in moist chambers (Petri dishes containing moist filter paper). Leaves were inspected daily for microfungi, and single conidial colonies of hyphomycetes and coelomycetes established on MEA (Crous, 2002). Cultures were plated onto fresh MEA, oatmeal agar (OA; Gams *et al.*, 2007) and pine needle agar (Slippers *et al.*, 2006) and subsequently incubated at 25 °C in dark, to promote sporulation. Reference strains are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands and BCC, BIOTEC, Thailand (Table 3.1).

### 3.2.2 DNA isolation, amplification and analyses

Genomic DNA was extracted from mycelia of fungal colonies cultivated on MEA using the UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA). The Primers V9G (de Hoog and Gerrits van den Ende, 1998) and LR5 (Vilgalys and Hester, 1990) were used to amplify part of the nuclear rDNA operon spanning the 3' end of the 18S rRNA gene (SSU), the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region (ITS2) and the 5' end of the 28S rRNA gene (LSU). The primers ITS4 (White *et al.*, 1990) and LR0R (Rehner and Samuels, 1994) were used as internal sequence primers to ensure good quality sequences over the entire length of the amplicon. To help resolve species of *Pseudocercospora*, the ITS region was supplemented with sequences of the translation elongation factor 1- $\alpha$  gene (EF-1 $\alpha$ ) using the primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) and the actin gene (ACT) using the primers ACT-512F and ACT-783R (Carbone and Kohn, 1999). The PCR amplifications were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) in a total volume of 12.5  $\mu$ L solution containing 10–20 ng of template DNA, 1  $\times$  PCR buffer, 2.5 mM MgCl<sub>2</sub>, 15 pmol for each primer, 60  $\mu$ M of each dNTP and 0.75 U *Taq* DNA polymerase (Bioline GmbH, Luckenwalde, Germany). PCR amplification conditions were set as follows: an initial denaturation temperature of 94 °C for 5 min, followed by 40 cycles of denaturation

**Table 3.1** Isolates of *Mycosphaerella* or its anamorphs used for DNA analysis and morphological studies.

Species	Accession number <sup>1</sup>	Host	Location	GeneBank number (ITS, LSU, EF,ACT)
<i>Mycosphaerella heimii</i>	CPC15428	<i>Eucalyptus</i> sp.	Burirum, Thailand	EU882123, --
	CPC15439	<i>Eucalyptus</i> sp.	Burirum, Thailand	EU882122, EU882141, --
	CPC15430	<i>Eucalyptus</i> sp.	Vientein, Laos	EU882121, EU882140, --
<i>M. irregulari</i>	CPC15408, CBS123242	<i>Eucalyptus</i> sp.	Udonthani, Thailand	EU882110, --
	CPC15431	<i>Eucalyptus</i> sp.	Udonthani, Thailand	EU882111, --
	CPC 15432	<i>Eucalyptus</i> sp.	Udonthani, Thailand	EU882112, --
<i>M. quasiparkii</i>	CPC15409, CBS123243	<i>Eucalyptus</i> sp.	Burirum, Thailand	EU882125, EU882143, --
	CPC15433	<i>Eucalyptus</i> sp.	Burirum, Thailand	EU882126, --
	CPC15434	<i>Eucalyptus</i> sp.	Burirum, Thailand	EU882127, --
<i>M. pseudomarkii</i>	CPC15410, CBS123241	<i>Eucalyptus</i> sp.	Chiang Mai, Thailand	EU882113, EU882137, --
	CPC15435	<i>Eucalyptus</i> sp.	Chiang Mai, Thailand	EU882114, --
	CPC15436	<i>Eucalyptus</i> sp.	Chiang Mai, Thailand	EU882115, --
<i>M. thailandica</i>	CPC15437	<i>Eucalyptus</i> sp.	Vientein, Laos	EU882120, --
	CPC15438	<i>Eucalyptus</i> sp.	Vientein, Laos	EU882119, --
	CPC15439	<i>E. camaldulensis</i>	Maharakam, Thailand	EU882118, EU882139, --
<i>M. vietnamensis</i>	CPC15440	<i>E. camaldulensis</i>	Ubonratchathani, Thailand	EU882117, EU882138, --
	CPC15441	<i>Eucalyptus</i> sp.	Burirum, Thailand	EU882116, --
	CPC15442	<i>Eucalyptus</i> sp.	Vientein, Laos	EU882104, --
	CPC15443	<i>Eucalyptus</i> sp.	Burirum, Thailand	EU882107, EU882135, --
	CPC15444	<i>Eucalyptus</i> sp.	Vientein, Laos	EU882106, EU882134, --
CPC15445	<i>E. camaldulensis</i>	Udonthani, Thailand	EU882105, --	

**Table 3.1** Isolates of *Mycosphaerella* or its anamorphs used for DNA analysis and morphological studies. (continued)

Species	Accession number <sup>1</sup>	Host	Location	GeneBank number (ITS, LSU, EF,ACT)
<i>Mycosphaerella</i> sp.	CPC15446	<i>E. camaldulensis</i>	Burirum, Thailand	EU882108, EU882136, --
	CPC15447	<i>E. camaldulensis</i>	Burirum, Thailand	EU882109, --, --
	CPC15448	<i>E. camaldulensis</i>	Ubonratchathani, Thailand	EU882124, EU882142, --
<i>Penidiella eucalypti</i>	CPC15411, CBS123246	<i>Eucalyptus</i> sp.	Mahasarakam, Thailand	EU882131, EU882145, --
	CBS123245	<i>E. camaldulensis</i>	Burirum, Thailand	EU882132, --, --
	CPC15449	<i>E. camaldulensis</i>	Burirum, Thailand	EU882133, EU882146, --
<i>Pseudocercospora chiangmeiensis</i>	CPC15412, CBS123244	<i>E. camaldulensis</i>	Chiang Mai, Thailand	EU882128, EU882144, EU882147, EU882150
	CPC15450	<i>E. camaldulensis</i>	Chiang Mai, Thailand	EU882129, --, --
	CPC15451	<i>E. camaldulensis</i>	Chiang Mai, Thailand	EU882130, --, --

<sup>1</sup> CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; CPC: Culture collection of Ratchadawan Cheewangkoon, housed at Chiang Mai University, Thailand.



temperature of 94 °C for 45 s, primer annealing at 48 °C for 30 s, primer extension at 72°C for 90 s and a final extension step at 72 °C for 7 min. The primer annealing temperature for EF-1 $\alpha$  and ACT was at 55 °C. The resulting fragments were sequenced using the PCR primers together with a BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, CA) and analysed on an ABI Prism 3100 DNA Sequencer (Perkin-Elmer, Norwalk, CN). The generated sequences were compared with other fungal DNA sequences from NCBI's GenBank sequence database using a blast search; sequences with high similarity were added to the alignments. The additional GenBank sequences were manually aligned using Sequence Alignment Editor v. 2.0a11 (Rambaut, 2002). The phylogenetic analyses of the aligned sequence data were performed using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford, 2003) and consisted of neighbour-joining analyses with the uncorrected ('p'), the Kimura 2-parameter and the HKY85 substitution models. Alignment gaps were treated as missing data and all characters were unordered and of equal weight. Any ties were broken randomly when encountered. For parsimony analyses, alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed using the heuristic search option with 100 random simple taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1,000 bootstrap replications (Hillis and Bull, 1993). Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were calculated and the resulting trees were printed with TreeView v. 1.6.6 (Page, 1996). New sequences were lodged in GenBank and the alignments and phylogenetic trees in TreeBASE ([www.treebase.org](http://www.treebase.org)).

### 3.2.3 Morphology

Preparations from cultured fungal colonies were mounted on glass slides with clear lactic acid for microscopic examination. Sections of ascomata were made by hand for examination purposes. Measurements of all taxonomically relevant parameters were made at 1,000  $\times$  magnification with 30 measurements per structure

where possible. Colony colours on MEA (surface and reverse) were determined using the colour charts of Rayner (1970) after 15 d at 25 °C in the dark.

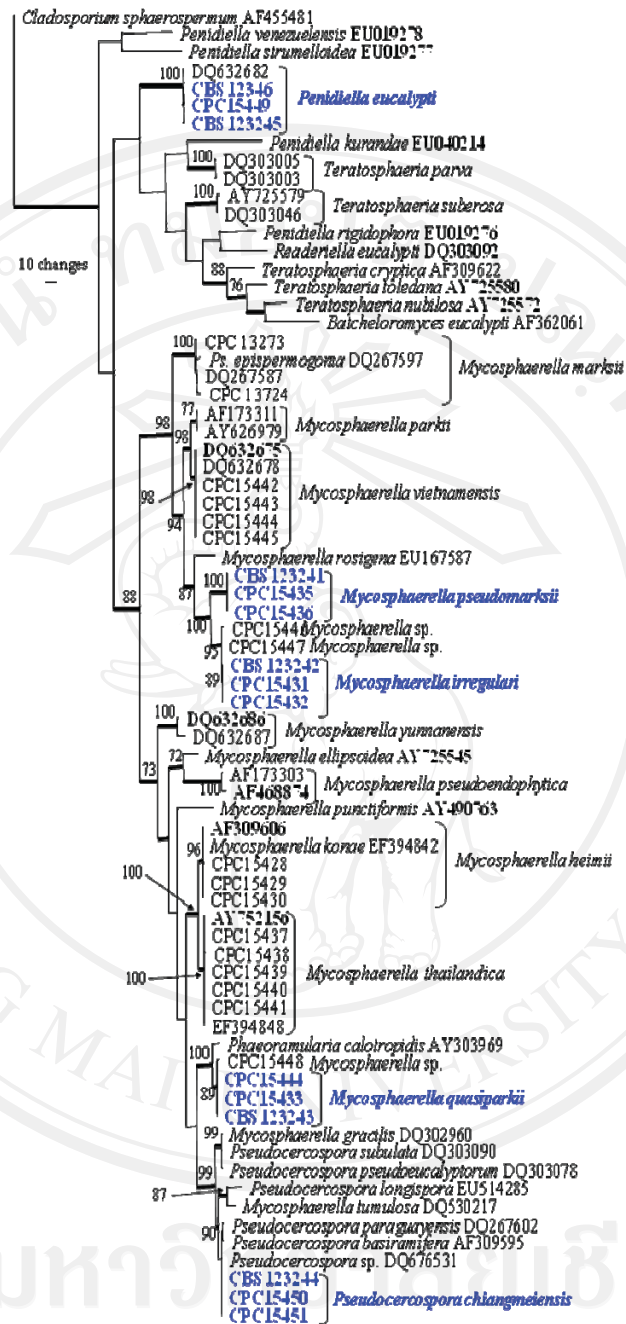
### 3.3 Results

#### 3.3.1 Phylogenetic analysis

Approximately 1,700 bases, spanning the ITS and LSU regions, were obtained for isolates listed in Table 1. These two regions were analysed separately; ITS to determine species level relationships and LSU for the generic placement. Approximately 300 and 220 bases were determined for EF-1 $\alpha$  and ACT, respectively, and these were concatenated with the corresponding ITS sequences for a combined analysis of the *Pseudocercospora* clade.

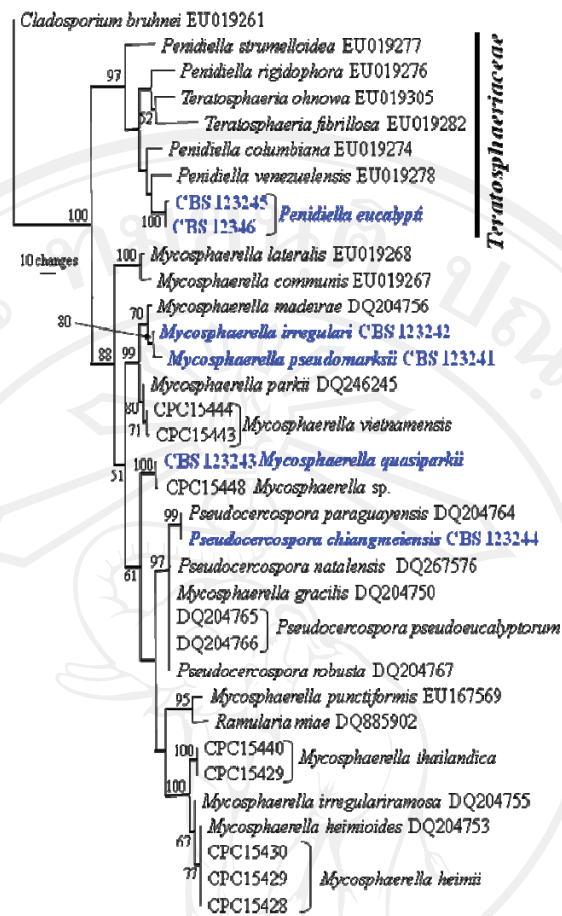
The manually adjusted ITS alignment contained 73 taxa (including the outgroup sequence) and, of the 533 characters used in the phylogenetic analysis, 228 were parsimony-informative, 59 were variable and parsimony-uninformative and 246 were constant. Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with similar topology and bootstrap values; 1,100 equally most parsimonious trees were obtained from the heuristic search, one of which is shown in Fig. 1 (TL = 1386, CI = 0.444, RI = 0.797, RC = 0.354). The phylogenetic tree derived from the ITS region (Fig. 3.1) showed that some of the isolates belong to known species, whereas others appeared to be new to science.

The manually adjusted LSU alignment contained 35 taxa (including the outgroup sequence) and, of the 797 characters used in the phylogenetic analysis, 121 were parsimony-informative, 54 were variable and parsimony-uninformative and 622 were constant. Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with similar topology and bootstrap values. Two equally most parsimonious trees were obtained from the heuristic search, one of which is shown in Fig. 3.2 (TL = 407, CI = 0.570, RI = 0.805, RC = 0.459). The phylogenetic tree of the LSU region (Fig. 3.2) showed two distinct groups of fungal isolates; the first was the



**Fig. 3.1** One of 1,100 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the ITS sequence alignment using PAUP v. 4.0b10. The scale bar shows 10 changes and bootstrap support values higher than 70 % from 1,000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches and extype sequences are printed in bold face. The tree was rooted to *Cladosporium sphaerospermum* (GenBank accession AF455481). Teratosphaeriaceae clade (97 % bootstrap support), whereas the second was the Mycosphaerellaceae clade (88 % bootstrap support).





**Fig. 3.2** One of two equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the LSU sequence alignment using PAUP v. 4.0b10. The scale bar shows 10 changes and bootstrap support values from 1 000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches and ex-type sequences are printed in **bold** face. The tree was rooted to *Cladosporium bruhnei* (GenBank accession EU019261).

The manually adjusted combined (ITS, EF-1 $\alpha$  and ACT) alignment contained 16 taxa (including the outgroup sequence) and, of the 1 012 characters used in the phylogenetic analysis, 339 were parsimony-informative, 139 were variable and parsimony-uninformative and 534 were constant. Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with similar topology and bootstrap values. A single most parsimonious tree was obtained from the heuristic search and is shown in Fig. 3.3 (TL = 876, CI = 0.822, RI = 0.852, RC = 0.700). The two trees differed only in their placement of *Ps. basiramifera*; in the one tree it is the

closest sister of *Ps. chiangmaiensis* and in the other it is the sister of *Ps. flavomarginata*.



**Fig. 3.3** Single most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the combined ITS, EF and ACT sequence alignment using PAUP v. 4.0b10. The scale bar shows 100 changes and bootstrap support values from 1 000 replicates are shown at the nodes. The tree was rooted to *Teratosphaeria cryptica* (GenBank accessions DQ239971, DQ235119 and DQ147674, respectively). *Ps.* = *Pseudocercospora*.

### 3.3.2 Taxonomy

Several taxonomic novelties, namely three *Mycosphaerella*, one *Pseudocercospora* and one *Penidiella* species, were found. These species do not match any species presently described from these genera, or any linked to sequences available in GenBank, and are thus described as new below.

***Mycosphaerella irregulari*** Cheewangkoon, K.D. Hyde & Crous, *sp. nov.* — MycoBank MB507001; Fig. 3.4, 3.5

*Anamorph.* Unknown.

*Mycosphaerellae tasmaniensis* similis, sed ascosporis cum tubis germinalibus irregulariter latis.

*Etymology.* Named after the irregular width of its ascospore germ tubes.

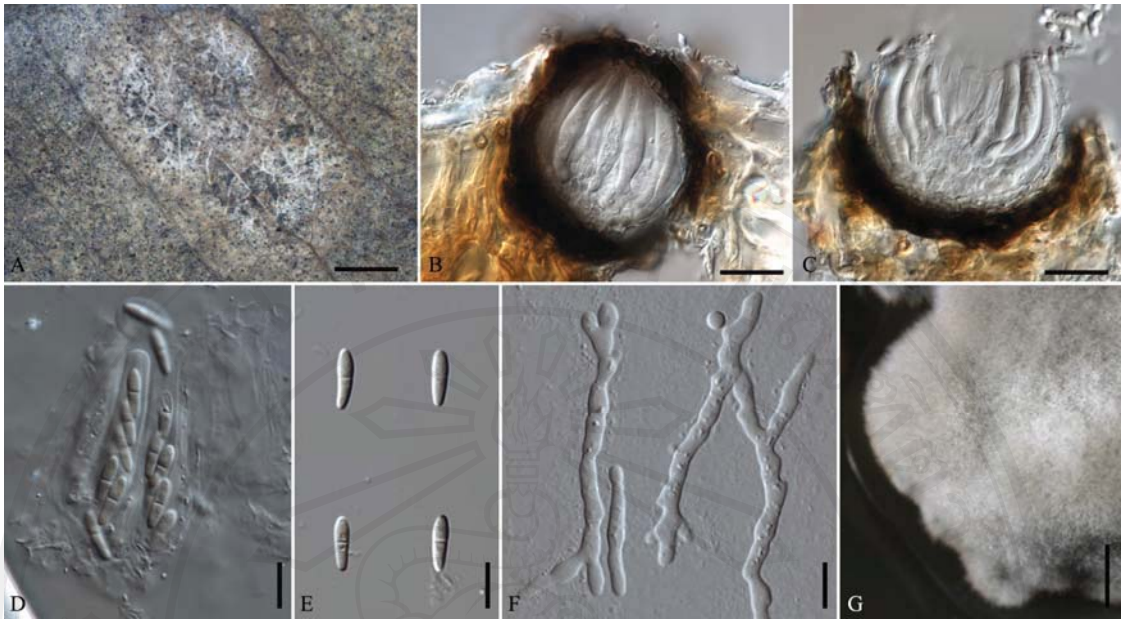
*Leaf spots* amphigenous, subcircular to oval, pale brown with grey centres, 5–12 mm diam, surrounded by a thin, medium brown margin. *Mycelium* external, smooth, septate, branch, medium brown, (2–)2.5–3(–4)  $\mu\text{m}$  wide hyphae. *Ascomata* epiphyllous, black, subepidermal to erumpent, ovoid to subglobose, 45–83  $\times$  45–78  $\mu\text{m}$ ; apical ostiole 20–25  $\mu\text{m}$  wide; wall thick (6.5–)7–10(–12)  $\mu\text{m}$ , consisting of 2–3 layers of medium brown *textura angularis*. *Asci* paraphysate, fasciculate, sessile, subcylindrical to narrowly obovoid, straight to slightly curved, 8-spored, (25–)35–40(–45)  $\times$  (6–)7–8(–10)  $\mu\text{m}$ . *Ascospores* bi- to tri-seriate, overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, fusoid-ellipsoidal with obtuse ends, widest just above the septum, or in the middle of the apical cell, medianly 1-septate or slightly longer in the basal cell, slightly constricted at septum, tapering toward both ends, but with more prominent taper towards lower end, at times with a mucous-like coating, (8–)9–11(–13)  $\times$  2.5–3(–3.5)  $\mu\text{m}$ .

*Ascospore germination* — Germinating from both ends, remaining hyaline; germ tubes grow parallel to the long axis of the spore, with lateral branches parallel or perpendicular to the long axis of the spore; germination tubes irregular in width, constrict at the median septum of the spore, becoming (11–)13–15(–16.5)  $\times$  4–5(–5.5)  $\mu\text{m}$ , slightly distorting (Type I; Crous 1998b).

*Cultural characteristics* — Colonies reach 15 mm diam on MEA after 15 d at 25 °C in the dark; circular, convex, with a slightly undulate, smooth margin, and medium aerial mycelium; pale greenish grey to pale olivaceous-grey (surface); olivaceous-black (reverse).

*Specimen examined*. Thailand, Udonthani, on living leaves with leaf spot symptom of *Eucalyptus* sp., July 2007, R. Cheewangkoon, holotype CBS H-20135, cultures ex-type CBS 123242 = CPC 15408, CPC 15431, CPC 15432.

*Notes* — The ascospore morphology of *M. irregulari* is similar to that of *M. tasmaniensis* (Crous *et al.*, 1998b), *M. flexuosa* (Crous, 1998b), *M. ellipsoidea* (Crous and Wingfield, 1996) and *M. heimii* (Crous and Swart, 1995). However, *M. irregulari* can be distinguished from these species by its irregular germ tubes and germination pattern. Phylogenetically, it is also not closely related to any of the species cited above, but clusters near to *M. pseudomarksii* (100 % bootstrap, Fig. 1), which has a distinct morphology.



**Fig. 3.4** *Mycosphaerella irregulari*. a. Leaf spot; b, c. sections through ascomata; d. asci; e. ascospores; f. germinating ascospores; g. colony on MEA. — Scale bars: a = 1 mm; b, c = 20  $\mu\text{m}$ ; d–f = 10  $\mu\text{m}$ .



**Fig. 3.5** *Mycosphaerella irregulari*. a. Asci; b. germinating ascospores; c. ascospores. — Scale bar = 10  $\mu\text{m}$ .

*Mycosphaerella pseudomarksii* Cheewangkoon, K.D. Hyde & Crous, *sp. nov.*

— MycoBank MB507003; Fig. 6, 7

*Mycosphaerellae marksii* similis, sed ascosporis majoribus, (12–)14–17 (–18.5)  $\times$  (2.5–)3(–3.5)  $\mu\text{m}$ .



*Etymology.* Named after *Mycosphaerella marksii* to which it is morphologically similar.

*Ascomata* amphigenous in apparently healthy tissue (endophyte?), occurring on greenish brown part of the leaf after incubation in moist chambers for 2 d, black, subepidermal to erumpent, globose to subglobose,  $42\text{--}60 \times 45\text{--}80 \mu\text{m}$ ; apical ostiole  $20\text{--}35 \mu\text{m}$  wide; wall consisting of 2–3 layers of medium brown *textura angularis*. *Asci* paraphysate, fasciculate, bitunicate, sessile, subcylindrical to narrowly ovoid, slightly curved, 8-spored,  $(40\text{--})42\text{--}45(\text{--}48) \times (6.5\text{--})7\text{--}8(\text{--}8.5) \mu\text{m}$ . *Ascospores* bi- to tri-seriate overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, fusoid with obtuse ends, widest in the middle of the asymmetrical apical cell, medianly 1-septate or with slightly longer basal cell; tapering toward both ends, but with more prominent taper towards lower end,  $(12\text{--})14\text{--}17(\text{--}18.5) \times (2.5\text{--})3(\text{--}3.5) \mu\text{m}$ . *Spermatogonia* well developed, amphigenous, dark brown, subepidermal to erumpent, globose to subglobose, up to  $90 \mu\text{m}$  diam. *Spermatia* hyaline, smooth, rod-shaped, with obtuse ends,  $(3.5\text{--})4\text{--}5(\text{--}5.5) \times 1.5\text{--}2 \mu\text{m}$ .

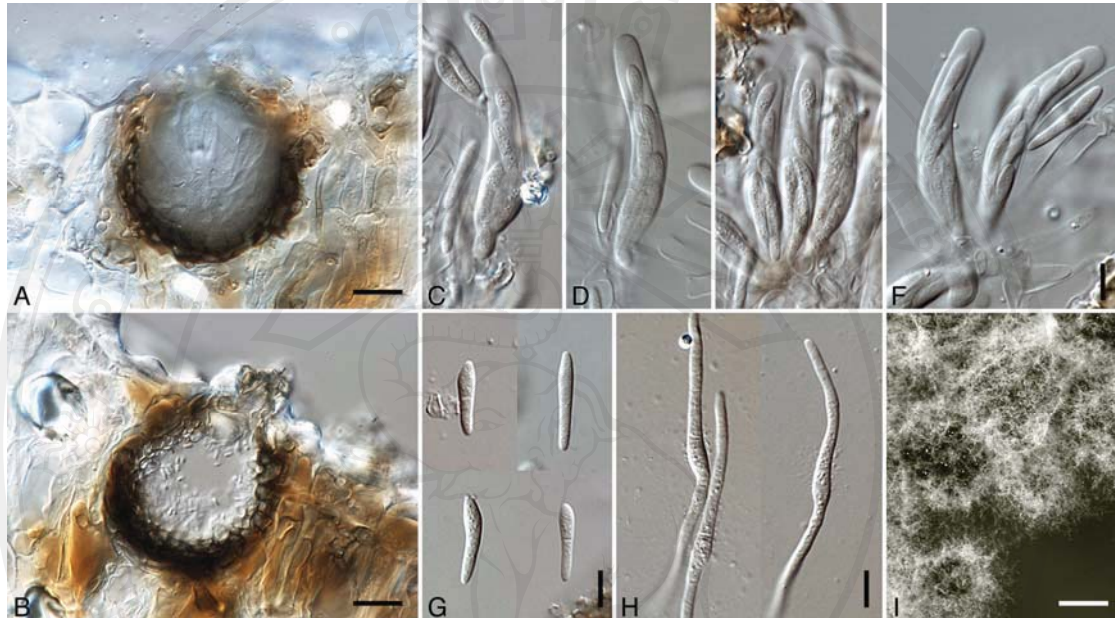
*Ascospore germination* — Germinating from both ends, with a thin, mucous-like coat visible surrounding ascospores on agar; germ tube growing parallel to the long axis of the spore, regular in width, remaining hyaline, not distorting or becoming constricted at septum (Type B; Crous, 1998b). *Cultural characteristics* — Colonies reach 15 mm diam on MEA after 15 d at 25 °C in the dark; subcircular, convex, with even margin, slightly folded, with sparse aerial mycelium; pale olivaceous-grey to olivaceous-grey (surface); olivaceous-black (reverse).

*Specimen examined.* Thailand, Chiang Mai, Mae Tang, on living leaves with leaf spot symptom of *Eucalyptus* sp., June 2007, R. Cheewangkoon, holotype CBS H-20134, cultures ex-type CBS 123241 = CPC 15410, CPC 15435, CPC 15436.

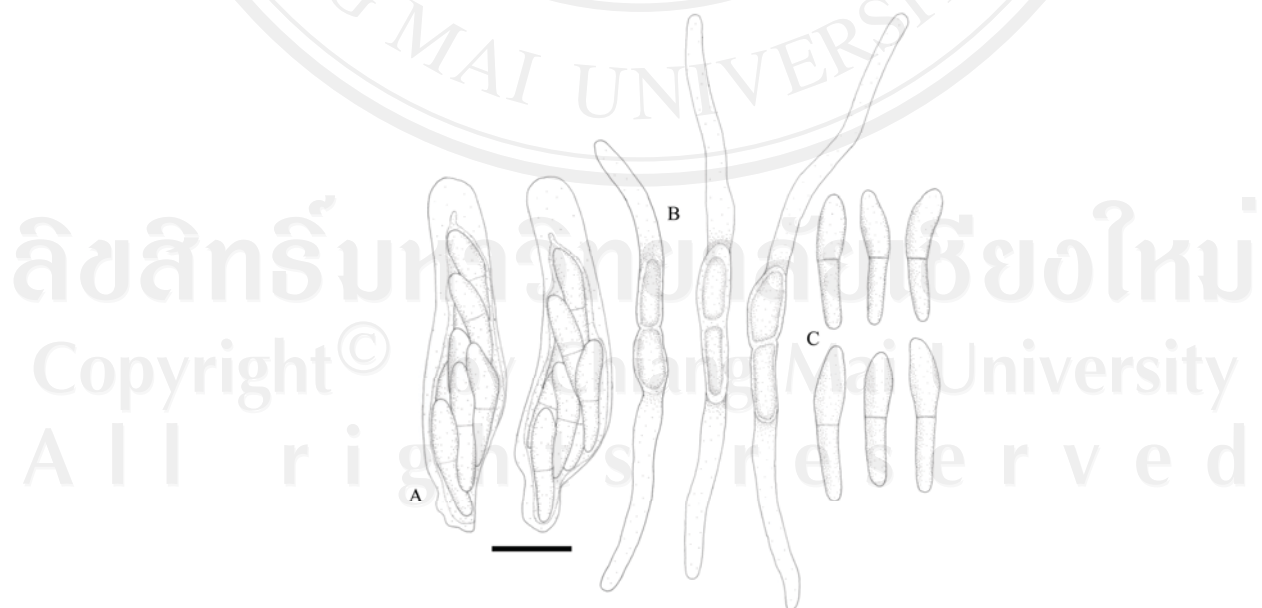
*Notes* — *Mycosphaerella pseudomarksii* was most similar to *M. marksii* (Carnegie and Keane, 1994) based on its asymmetrical apical ascospore cells and ascospore germination patterns. However, germinating ascospores of *M. pseudomarksii* have a visible mucilaginous sheath, which was not observed in germinating ascospores of *M. marksii*. Furthermore, ascospores of *M. pseudomarksii* were slightly longer and wider  $(12\text{--})14\text{--}17(\text{--}18.5) \times (2.5\text{--})3(\text{--}3.5) \mu\text{m}$  than those of *M. marksii*  $(11\text{--})12\text{--}14(\text{--}16) \times 2\text{--}2.5(\text{--}3) \mu\text{m}$ . Crous and Wingfield (1996) observed



considerable variation in ascospore dimensions of several collections of *M. marksii* ( $12.5\text{--}22.5 \times 2.5\text{--}5 \mu\text{m}$ ) commenting that this may represent a species complex. Phylogenetically the two species are also distinct (Fig. 3.1)



**Fig. 3.6** *Mycosphaerella pseudomarksii*. a. Vertical section through an ascoma; b. vertical section through a spermatogonium; c–f. asci; g. ascospores; h. germinating ascospores; i. colonies on MEA. — Scale bars: a, b = 10  $\mu\text{m}$ ; c–h = 10  $\mu\text{m}$ .



**Fig. 3.7** *Mycosphaerella pseudomarksii*. a. Asci; b. germinating ascospores; c. ascospores. — Scale bar = 10  $\mu\text{m}$ .

*Mycosphaerella quasiparkii* Cheewangkoon, K.D. Hyde & Crous, *sp. nov.* —  
Mycobank MB507002; Fig. 3.8, 3.9

*Anamorph.* Unknown.

*Mycosphaerellae parkii* similis, sed ascosporis ellipsoideis et coloniis bubalinis in agaro MEA.

*Etymology.* Named after its similarity to *Mycosphaerella parkii*.

*Leaf spots* amphigenous, round to irregular, separate, becoming confluent, 5–15 mm diam, medium brown on adaxial surface, pale brown on abaxial surface, surrounded by a raised border, which is dark brown on the adaxial surface and paler brown on the abaxial surface. *Ascomata* epiphyllous, black, subepidermal to erumpent, subglobose, 40–60 × 40–55 µm; apical ostiole 10–15 µm wide; wall consisting of 3–4 layers of medium to dark brown *textura angularis*. *Asci* paraphysate, fasciculate, bitunicate, sessile, broadly ellipsoid to obclavate, straight to slightly curved, 8-spored, (30–)45–50(–57) × (7–)8.5–9(–9.5) µm. *Ascospores* bi- to multiseriate, overlapping, hyaline, guttulate, thin-walled, straight to slightly curved, ellipsoidal to obovoid with obtuse ends, widest in the middle of the apical cell, medianly 1-septate, not constricted at the septum, tapering toward both ends, with a thin mucilaginous sheath, (9–)10–11(–12.5) × (2.5–)3–3.5(–4.5) µm.

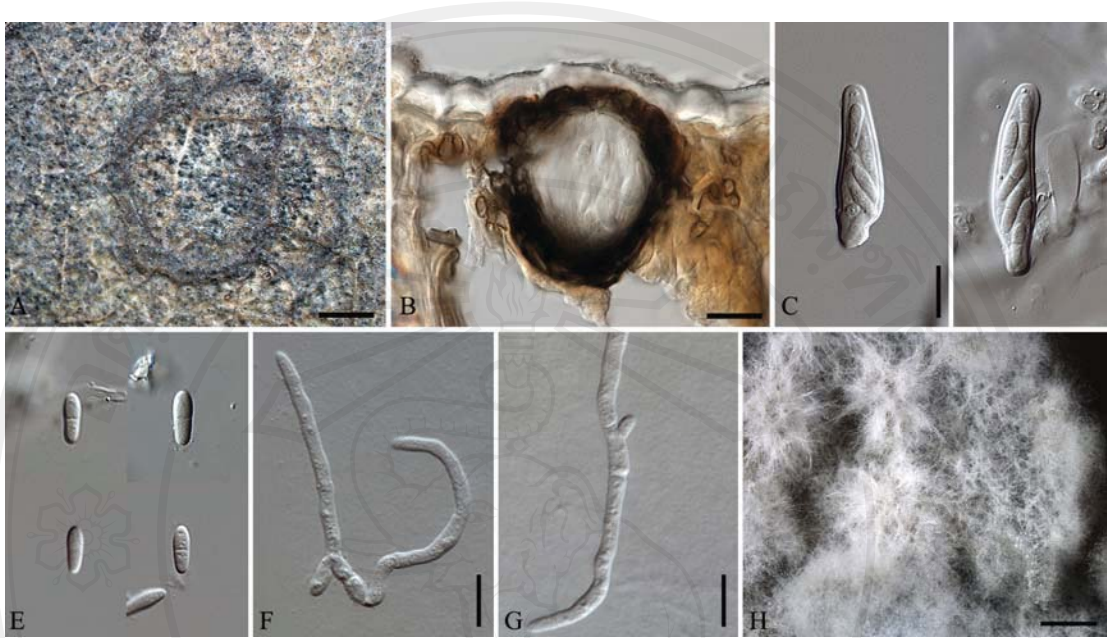
*Ascospore germination* — Germinating with more than one germ tube per cell. Initial germ tubes originating from polar ends, growing parallel to the long axis of the spore, with perpendicular germ tubes developing later; ascospores remain hyaline, but become constricted at the median septum, and distorting, (2.8–)3.5–4(–4.5) µm wide (Type D; Crous, 1998b).

*Cultural characteristics* — Colonies reach 27 mm diam on MEA after 15 d at 25 °C in the dark; circular, low convex, with entire edge and sparse aerial mycelium; buff (surface); vinaceous-buff (reverse).

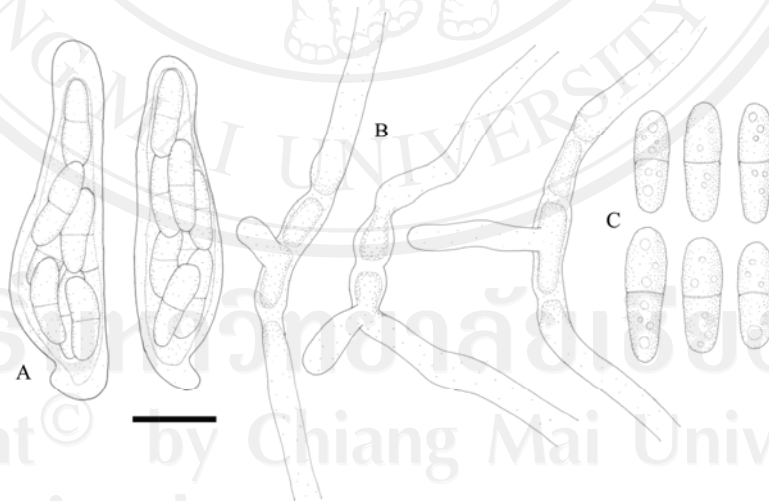
*Specimen examined.* Thailand, Burirum, on living leaves with leaf spot symptom of *Eucalyptus* sp., July 2007, P. Suwannawong, holotype CBS H-20132, cultures ex-type CBS 123243 = CPC 15409, CPC 15433, CPC 15434.

*Notes* — *Mycosphaerella quasiparkii* is morphologically similar to *M. parkii* (Crous *et al.*, 1993e, 2006g) with which it also shares the same ascospore germination pattern. However, *M. quasiparkii* has more ellipsoid ascospores and paler colonies on

MEA. Phylogenetically, *M. quasiparkii* clusters close to *Phaeoramularia calotropidis* (AY303969, Fig. 3.1).



**Fig. 3.8** *Mycosphaerella quasiparkii*. a. Leaf spot; b. section through an ascoma; c, d. asci; e. ascospores; f, g. germinating ascospores; h. colony on MEA. — Scale bars: a = 1 mm; b, = 20  $\mu\text{m}$ ; c–g = 10  $\mu\text{m}$ .



**Fig. 3.9** *Mycosphaerella quasiparkii*. a. Asci; b. germinating ascospores; c. ascospores. — Scale bar = 10  $\mu\text{m}$ .



*Penidiella eucalypti* Cheewangkoon, K.D. Hyde and Crous, sp. nov. —

Mycobank MB507004; Fig. 3.10, 3.11

Teleomorph. Unknown.

Differt a omnibus speciebus Penidiellae conidiis mucosis in vitro (MEA) formantibus.

Etymology. Named after its host genus, Eucalyptus.

Mycelium consisting of branched, septate, smooth to slightly verruculose, pale to medium brown, (2.5–)3–4(–5)  $\mu\text{m}$  wide hyphae. Conidiophores macro-nematous, occasionally micronematous, arising from superficial mycelium, solitary, erect, straight to slightly curved, branched laterally or not, medium to dark brown, slightly thick-walled, wall  $\leq 1$   $\mu\text{m}$  wide, smooth to finely verruculose, (30–) 150 – 200(–220)  $\times$  (4–)4.5–5.5(–6.5)  $\mu\text{m}$ , wider at the base, (5.5–)6–7(–8.5)  $\mu\text{m}$ , (0–)4–6(–8) septate. *Conidiogenous cells* terminal, cylindrical to subcylindrical, tapering to a flattened apical region, smooth to finely verruculose, medium brown, (10–)13–17(–25)  $\times$  (3–)4–5(–6)  $\mu\text{m}$ ; scars thickened, somewhat darkened, 2–2.5  $\mu\text{m}$  wide. *Ramoconidia*: primary ramoconidia subcylindrical or obovoid, (0–)1(–3)-septate, base truncate, with (1–)2–3(–4) apical hila, pale olivaceous to pale brown, smooth to finely verruculose, wall  $< 1$   $\mu\text{m}$  wide, (25–)35–40(–48)  $\times$  (3.5–)4–5(–6.5)  $\mu\text{m}$ ; scars thickened and slightly darkened, 2–2.5  $\mu\text{m}$  wide; giving rise to chains of up to 12 conidia; secondary ramoconidia obovoid, 0–1-septate, some constricted at septum, base truncate, with 2–3 (–4) apical hila, pale olivaceous, smooth, (13–)15–20(–28)  $\times$  (4.5–)5–6(–7.5)  $\mu\text{m}$ ; intercalary conidia obovoid, 0–1(–2)-septate, some constricted at septa, base truncate, with 2–3 apical hila, pale olivaceous, smooth, (10–)12–15(–18)  $\times$  (3.5–)4–5(–5.5)  $\mu\text{m}$ . *Conidia* in branched acropetal chains, broadly fusiform to obovoid, 0–1-septate, pale olivaceous, paler towards the apex, (9–)12–15(–19)  $\times$  (4–)5–6(–7)  $\mu\text{m}$ ; terminal conidia ovoid, 0-septate, pale olivaceous to hyaline, paler towards the apex, base truncate, conidia have a wing-like mucilaginous sheath in culture, extending up to 5  $\mu\text{m}$  wide on each side, tapering towards the polar ends.

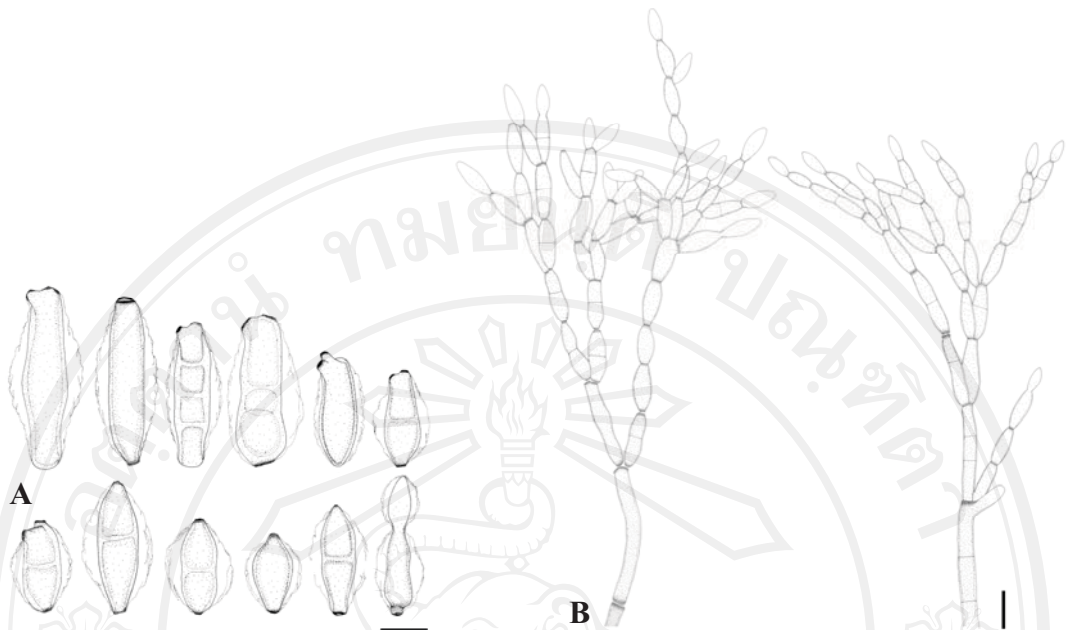
Cultural characteristics — Colonies on MEA reaching 20 mm diam after 15 d at 25 °C; margin feathery, colonies erumpent, spreading, with moderate aerial mycelium. Surface grey-olivaceous, reverse olivaceous-black.



**Fig. 10** *Penidiella eucalypti*. a. Colony on MEA; b–h. catenulate conidia; i. conidiogenous cell and primary ramoconidia; j. primary ramoconidium (left) and secondary ramoconidium (right); k. conidia with mucilaginous sheath. — Scale bars: b = 60  $\mu\text{m}$ ; c, d = 80  $\mu\text{m}$ ; e = 30  $\mu\text{m}$ ; f, g = 20  $\mu\text{m}$ ; h, i = 15  $\mu\text{m}$ ; j, k = 10  $\mu\text{m}$ .

*Specimens examined.* Thailand, Payakpoompisai, Mahasarakam, on leaves of *Eucalyptus camaldulensis*, July 2007, P. Suwannawong, holotype CBS H-20136, cultures ex-type CBS 123246 = CPC15411, AGI064.1, AGI064.2 (occurring on a lesion in association with *Harknessia* sp.); Satuk, Burirum, on leaves of *Eucalyptus camaldulensis*, July 2007, R. Cheewangkoon, cultures CBS 123245, CPC15449 (occurring on a lesion in association with several microfungi).





**Fig. 3.11** *Penidiella eucalypti*. a. Conidia; b. conidial branching system. — Scale bars = 10 µm.

Notes — *Penidiella eucalypti* is a typical species of *Penidiella* by having solitary conidiophores with a branching system consisting of ramoconidia that form secondary ramoconidia and conidia, with slightly thickened, darkened scars (Crous *et al.*, 2007f). Other than being phylogenetically distinct (Fig. 3.1, 3.2), *P. eucalypti* is distinct from most other species of *Penidiella* by having a prominent branching system which develops on a single terminal conidiogenous cell. Another character that has not previously been reported in the genus is the distinct mucilaginous sheath observed on conidia formed on MEA.

*Pseudocercospora chiangmaiensis* Cheewangkoon, K.D. Hyde and Crous, *sp.*

*nov.* — MycoBank MB507005; Fig. 3.12, 3.13

*Teleomorph.* Unknown.

*Pseudocercosporae basiramiferae* similis, sed cellulis conidiogenis terminalibus et intercalaribus et stromatibus bene evolutis.

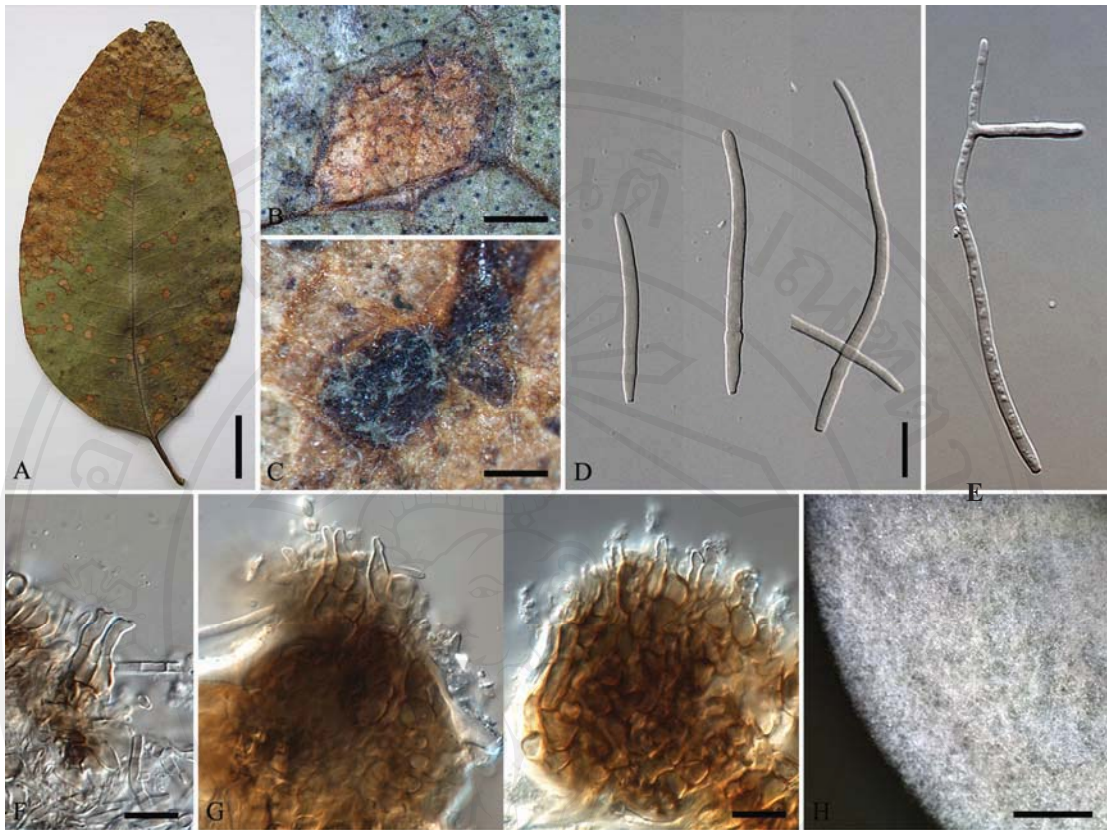
*Etymology.* Named after Chiang Mai, the province in Thailand from where it was collected.

*Leaf spots* amphigenous, subcircular to angular, 2–6 mm diam, pale to medium brown, surrounded by a slightly raised, dark-brown border, becoming confluent with age, leading to leaf blight from the leaf tip. *Mycelium* predominantly internal, consisting of smooth, septate, branched (1.5–)2–3(–4)  $\mu\text{m}$  wide hyphae. *Caespituli* amphigenous, more prominent on abaxial leaf surface, pale grey on leaves, often on dark brown to black and thickened leaf tissue, 25–50  $\times$  50–100  $\mu\text{m}$ . *Stromata* immersed, becoming erumpent, medium brown, 25–70  $\times$  30–70  $\mu\text{m}$  wide. *Conidiophores* reduced to conidiogenous cells or one supporting cell, occasionally arising from upper cells of stroma, subcylindrical, 1–3(–6)-septate with intercalary conidiogenous cells, (13–)20–25(–60)  $\times$  3–4(–4.5)  $\mu\text{m}$ , situated on the superficial part of the stroma. Conidiogenous cells terminal or intercalary, subcylindrical to obclavate, medium brown, becoming paler toward apex, straight to geniculate sinuous, tapering to a truncate or bluntly rounded apex, at times subdenticulate, smooth, medium to thick-walled, variable in length, (5–)10–11(–12)  $\times$  (2–)3(–5)  $\mu\text{m}$ , unbranched, proliferating sympodially; conidial scars thickened at the rim, not darkened, inconspicuous. Conidia solitary, subcylindrical to narrowly obclavate, tapering toward the subobtuse apex; base obconic-subtruncate, (2–)3–5(–10)-septate, straight to slightly curved, pale to medium brown, smooth, thin-walled, guttulate, (40–)50–60(–100)  $\times$  (2–)2.5–3(–3.5)  $\mu\text{m}$  (up to 140  $\mu\text{m}$  long in moist chambers); hilum thickened and somewhat darkened at the rim (paracercospora-like), not refractive, at times inconspicuous; microcyclic conidiation observed when incubated in a moist chamber.

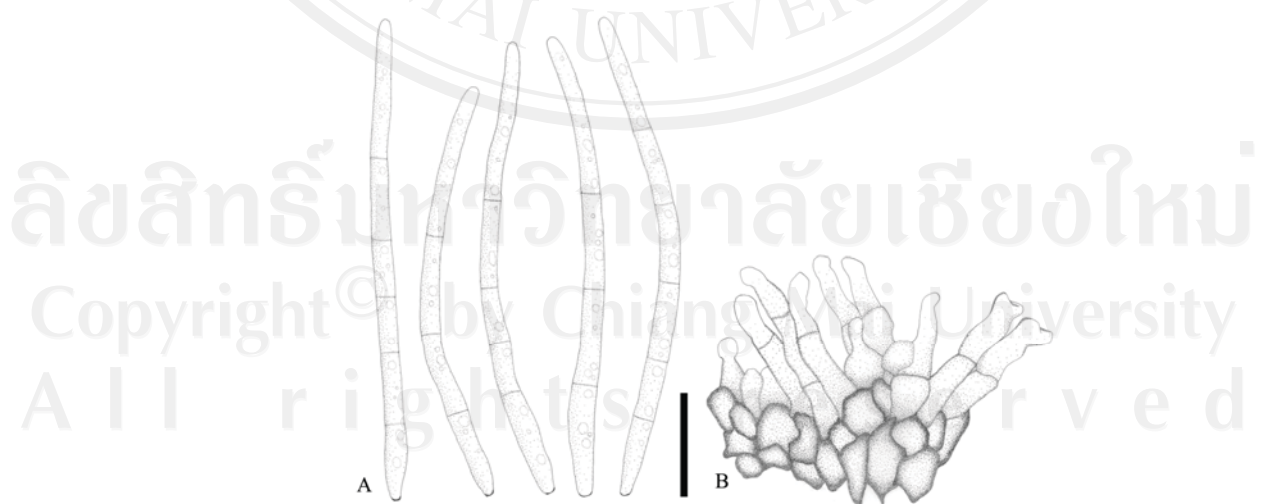
Cultural characteristics — Colonies reaching 18 mm diam on MEA after 15 d at 25 °C in the dark; colonies circular, convex, with entire margin and medium aerial mycelium; pale greenish grey (surface), fuscous-black (reverse).

*Specimen examined.* Thailand, Chiang Mai, Doi Lor, on leaves of *Eucalyptus camaldulensis*, June 2007, P. Suwannawong, holotype CBS H-20133, cultures ex-type CBS 123244 = CPC 15412, CPC 15450, CPC 15451.

Notes — Of the *Pseudocercospora* species known from *Eucalyptus* (Crous, 1998b; Braun and Dick, 2002; Crous *et al.*, 2004c, 2006g, 2007h; Hunter *et al.*, 2006b; Carnegie *et al.*, 2007), *Ps. chiangmaiensis* is morphologically most similar to *Ps. Basiramifera* (Crous, 1998b) in conidium morphology (dimensions, shape,



**Fig. 3.12** *Pseudocercospora chiangmaiensis*. a. Leaf spots; b, c. close up of leaf spot; d, e. conidia; f, g. conidiogenous cells; h. colony on MEA. — Scale bars: b = 1 mm; c = 300  $\mu$ m; d–g = 10  $\mu$ m.



**Fig. 3.13** *Pseudocercospora chiangmaiensis*. a. Conidia; b. conidiophores with conidiogenous cells. — Scale bar = 10  $\mu$ m.

microcyclic conidiation and scar thickening along the rim). *Pseudocercospora chiangmaiensis* is distinct from *Ps. basiramifera* based on its terminal and intercalary conidiogenous cells in vivo, and by its conidiophores occurring on well-developed stromata. Phylogenetically it is closely related to *Ps. basiramifera* (Fig. 3.3) but differs from it with 5 nucleotide positions on ITS, 34 on EF-1 $\alpha$  and 12 on ACT.

### 3.4 Discussion

Although numerous species of *Mycosphaerella* have been associated with *Eucalyptus* leaf diseases in tropical regions around the world (Crous, 1998b; Crous *et al.*, 2000, 2006g, 2007h, c, 2008), only a few of these species have been documented from Asia. The number of novel species found in the present study from sampled *Eucalyptus* leaves collected in Thailand, was thus not totally unexpected. The three new *Mycosphaerella* spp. (i.e. *M. irregulari*, *M. pseudomarksii* and *M. quasiparkii*) identified here were difficult to distinguish from other species of *Mycosphaerella* based on their ascospore morphology and germination patterns alone, which are the characters that have been commonly used in the past for taxonomic classification of *Mycosphaerella* spp. (Park and Keane, 1982; Crous, 1998b). Therefore, the DNA sequencing data generated here proved particularly helpful in distinguishing these species.

*Mycosphaerella pseudomarksii* has ascospores with asymmetrical apical cells similar to *M. marksii*, the only difference being ascospore dimensions and the presence of a mucilaginous ascospore sheath in *M. pseudomarksii*. Ascospores of *M. irregulari* are fusoid-ellipsoidal, thus being similar as those of *M. ellipsoidea*, *M. flexuosa*, *M. heimii* and *M. tasmaniensis*. Although these species have similar ascospore dimensions, *M. irregulari* is characterised by the distinctive irregular width of its ascospore germination tubes. *Mycosphaerella quasiparkii* has an ascospore morphology and germination pattern almost identical to *M. parkii*, but the presence of a thin mucous-like layer on ascospores of *M. quasiparkii* and its buff colonies, distinguish it from *M. parkii*, which lacks an ascospore sheath and has olivaceous-grey colonies. *Pseudocercospora chiangmaiensis*, which is also newly described from *Eucalyptus*, shares some morphological features (conidial dimensions, hilum thickening and microcyclic conidiation) with *Ps. basiramifera*. It is distinct, however,



by having terminal and intercalary conidiogenous cells in vivo and having conidiophores arising from a well-defined stroma. Phylogenetic analyses of the ITS and ACT genes showed limited differences (only 1 nucleotide) between *Ps. Chiangmaiensis* and *Ps. Assamensis*, which Arzanlou *et al.* (2008) recently described from banana. Morphologically, however, they differ in the basal conidial cell shape and marginal thickening along the hilum. These differences were supported by analyses based on the EF gene, which indicated these two species to differ by 38 nucleotides.

Other than new species of *Mycosphaerella* and *Pseudocercospora*, the present study also led to the discovery of a novel species of *Penidiella*. *Penidiella eucalypti* is distinct in that it has a distinctive branching system developed chiefly on a single conidiogenous locus, and conidia with a persistent, characteristic mucilaginous sheath in vitro. Results from the phylogenetic analyses also indicated that this species belongs to a clade represented by an undescribed *Teratosphaeria* sp. (DQ632682) which could represent its teleomorph.

Two strains that were phylogenetically closely related to *M. irregulari* (95 % bootstrap), namely CPC 15446 and CPC 15447, represent two undescribed species of *Mycosphaerella*. *Mycosphaerella* sp. CPC 15446 differs from *M. irregulari* by having ascospores with more obtuse apical cells and germination tubes that are regular in width. Although insufficient material was available of *Mycosphaerella* sp. CPC 15447, it occurred in lesions in association with *M. thailandica* and *M. heimii*. Another undescribed *Mycosphaerella* species, CPC 15448, was closely related to *M. quasiparkii* (89 % bootstrap). More collections are required to resolve their status. In the present study, three known species were also identified from the diseased *Eucalyptus* leaf samples, namely *M. heimii*, *M. thailandica* and *M. vietnamensis*. Although *M. thailandica* and *M. heimii* were previously reported on *Eucalyptus* in Thailand (Crous *et al.*, 2007h), *M. vietnamensis* represents a record for this country.

This study has again demonstrated that morphological characters and molecular techniques are complementary, and necessary, to uncover the diversity and geographical range of *Mycosphaerella* and *Teratosphaeria* species occurring on *Eucalyptus*. Although five new fungal species have been identified and one species represents a new record from diseased *Eucalyptus* leaves from Thailand, it is still



unknown whether these species are native or exotic. We expect that more unidentified disease-causing microfungi await discovery in Thailand, because the expanding area of *Eucalyptus* plantations allow fungal pathogens to cross geographical barriers to infect new hosts (i.e. from exotic *Eucalyptus* to other native trees) more easily, and also increase the chance of infection by native fungi to the exotic plantations (Slippers *et al.*, 2005c). Some examples of introduced pathogens from exotic *Eucalyptus* are *T. cryptica*, *T. nubilosa* (Park and Keane, 1982; Wingfield *et al.*, 1995), and *T. suttonii* (Chipompha, 1987; Crous *et al.*, 1989b, 1997a). These species were described from Australia where *Eucalyptus* is native, but were found later in other countries where this host has been planted as an exotic. In addition, the study of *Mycosphaearella* spp. on exotic *Acacia* in the tropics (Crous *et al.*, 2004d) again revealed examples of host sharing of *Mycosphaearella citri* on *Citrus*, *Acacia* and *Musa*.

More extensive research should thus be carried out to provide information concerning the fungal diversity of exotic *Eucalyptus* plantations in Thailand and other Asian countries to promote the understanding of the evolution of new pathogens and the movement of fungi between continents.