Mycosphaerella spp. on *Eucalyptus* in Asia; new species; new hosts and new records

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Burgess, T.I., Barber, P.A., Sufaati, S., Xu, D., Hardy, G.E. StJ. and Dell, B. (2007) *Mycosphaerella* spp. on *Eucalyptus* in Asia; new species, new hosts and new records. Fungal Diversity 24: 135-157.

Leaf spots caused by *Mycosphaerella* spp. are of increasing concern in eucalypt plantations around the world, but few species have been reported infecting eucalypts in Asia. From leaf material collected in China, Vietnam and Indonesia, we describe three new *Mycosphaerella* spp.; *M. vietnamiensis*, *M. obscuris* and *M. yunnanensis*. *Mycosphaerella crystallina*, previously known only from South Africa, and *M. stramenticola*, previously reported from *Eucalyptus* leaf litter in Brazil, are new records. *Mycosphaerella citri*, an important pathogen of citrus, was found infecting *Eucalyptus*. *Mycosphaerella marksii* and *M. suttoniae*, which have been previously reported in Asia, were regularly encountered. *Pseudocercospora* spp. were also common throughout the region. This study highlights the potential biodiversity of *Mycosphaerella* spp. on eucalypts in South-East Asia.

Key words: exotic plantations, host jumps, ITS, phylogenetics, tropical forestry

Introduction

Eucalypts were introduced from Australia to the rest of the world in the 19th century. In the last 40 years there has been a rapid expansion in areas under afforestation, particularly in the tropics and the Southern Hemisphere (Eldridge *et al*., 1994) to meet the world's demand for paper and pulp. In South-East Asia and south China there are over two million ha of plantations, predominantly *Eucalyptus grandis*, *E. urophylla* and their hybrids (Cotterill and Brolin, 1997; Martin, 2003; Qi, 2003).

The initial success of eucalypts as exotics has been due to the absence of pests and pathogens naturally associated with them in their regions of origin

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(Barber, 2004; Burgess and Wingfield, 2002a; Wingfield, 1999). However, as the planted areas have increased and more plantations have been established on less suitable sites, disease problems have increased (Burgess and Wingfield, 2002a; Day, 1950; Wingfield, 1999; Wingfield *et al*., 2001; Summerell *et al*., 2006). Pathogens on exotic eucalypts are predominantly those introduced with germplasm, either on seed or chaff or as endophytes of vegetative material (Burgess and Wingfield, 2002b; Burley, 1987). However, exotic eucalypts are often planted in close association with native plant species and the genetic uniformity of clonal eucalypt plantations places a very high selection pressure on indigenous pathogens to jump hosts and infect eucalypts (Burgess and Wingfield, 2002; Slippers *et al*., 2005). A classic example of this is the movement of the rust *Puccinia psidii*, from guava to eucalypts in South America (Coutinho *et al*., 1998).

Mycosphaerella spp. and their anamorphs have become one of the major disease causing agents affecting exotic eucalypt plantations throughout the world (Crous, 1998) with over 80 species associated with Mycosphaerella leaf blotch disease of eucalypts. Many of these species (e.g. *M. suttonii*) have broad host and geographic ranges, while others such as *M. molleriana*, only known on *E. globulus*, appear to be more specific (Carnegie *et al*., 1994; Crous, 1998; Park and Keane, 1984, 1982b; Park *et al*., 2000). *Mycosphaerella* spp. found on eucalypts appear to be eucalypt-specific and are thought to be moved about via seed. However, many *Mycosphaerella* spp. known to infect eucalypts have not been found in Australia (Crous, 1998; Crous *et al*., 2004a, 2006) and may have emerged on native plants and adapted to eucalypts outside Australia (Crous, 1998; Crous *et al*., 2001, 2004a, 2006).

Few *Mycosphaerella* spp. have been documented from eucalypts in Asia. In his detailed book on *Mycosphaerella* spp. and their anamorphs associated with leaf spot diseases of eucalypts, Crous (1998) reported 11 species with known distribution in Asia; *M. gracilis*, *M. heimioides*, *M. marksii*, *M. parkii*, *M. suberosa*, *M. suttoniae*, *Phaeophleospora destructans*, *Phaeoramularia eucalyptorum*, *Pseudocercospora basiramifera*, *Ps. deglupta* and *Ps. robusta*. It is now known that *Colletogloeopsis zuluensis* (formerly *Coniothryium zuluense*) is an anamorph of an unknown *Mycosphaerella* sp. (Cortinas *et al*., 2006) and this taxon has been reported from throughout Asia (Old *et al*., 2003). Recently, 21 new *Mycosphaerella* spp. have been described from *Eucalyptus*, including five with an Asian distribution; *M. eucalyptorum*, *M. gamsii*, *M. gracilis, M. sumatrensis and M. verrucosiafricana* (Crous *et al*., 2006)

During 2003 and 2004, a number of surveys of eucalypt diseases were conducted in various Asian countries. This study presents a collection of

Table 1. Isolates considered in the phylogenetic study. Those in bold were isolated during this study.

Culture no.[1]	teleomorph	anamorph	Host	Location	Collector	GenBank Accession no.
CPC 10522	*M. citri*	*Stenella citri-grisea*	*Acacia mangium*	Thailand	M.J. Wingfield	AY752145
CBS 116426	*M. citri*	*S. citri-grisea*	*Musa* sp.	Florida	J. Cavaletto	AY752146
CMW 22517	***M. citri***	***S. citri-grisea***	***E. camaldulensis* hybrid**	**South East Vietnam**	**T.I. Burgess**	**DQ632684**
CMW 22518	***M. citri***	***S. citri-grisea***	***E. camaldulensis* hybrid**	**South East Vietnam**	**T.I. Burgess**	**DQ632685**
CBS 110969	*M. colombiensis*	*Ps. colombiensis*	*Eucalyptus*	Colombia	M.J. Wingfield	AY752149
CMW 11255	*M. colombiensis*	*Ps. colombiensis*	*Eucalyptus*	Colombia	M.J. Wingfield	DQ240011
CBS 110968	*M. colombiensis*	*Ps. colombiensis*	*Eucalyptus*	Colombia	M.J. Wingfield	AY752148
ATCC 262271	*M. cruenta*	*P. cruenta*	Cowpea	Puerto Rico		AY266153
MURU 118	*M. cryptica*	*Colletogloeopsis nubilosum*	*E. globulus*	Western Australia	A. Maxwell	AY509753
MURU 115	*M. cryptica*	*Co. nubilosum*	*E. globulus*	Western Australia	A. Maxwell	AY509754
CMW 3042	*M. crystallina*	*Ps. crystallina*	*Eucalyptus*	South Africa		DQ239997
CBS 681.95, STE-U-802	*M. crystallina*	*Ps. crystallina*	*Eucalyptus*	South Africa		AY490757
CMW 22534	***M. crystallina***		***E. urophylla***	**China**	**T.I. Burgess**	**DQ632681**
CBS 118496	*M. eucalyptorum*		*Eucalyptus*	Indonesia	M.J. Wingfield	DQ302954
ATCC 22117	*M. fijiensis*	*Paracercospora fijiensis*	*Musa sp.*	Hawaii	D.S. Merideth	AF297234
CBS 110743	*M. flexuosa*	*Stenella* sp.	*E. globulus*	Colombia	M.J. Wingfield	DQ302955
CBS 111055	*M. flexuosa*	*Stenella* sp.	*E. grandis*	Colombia	M.J. Wingfield	DQ302956
CMW 9094	*M. fori*	*Pseudocercospora* sp.	*Eucalyptus*	South Africa	G. Hunter	AF468868
CMW 9095	*M. fori*	*Pseudocercospora* sp.	*Eucalyptus*	South Africa	G. Hunter	AF468869
CBS 118495	*M. gamsii*		*Eucalyptus*	India	W. Gams	DQ302959
CBS 111189	*M. gracilis*	*Ps. gracilis*	*E. urophylla*	Indonesia	M.J. Wingfield	DQ302960
CMW 10189	*M. grandis*		*Eucalyptus*	Ethiopia	A. Gezahgne	AY244407
L/1/2/1	*M. grandis*				A. Milgate	AY045513
CMW 4942	*M. heimii*	*Ps. heimii*	*Eucalyptus*	Madagascar		AF309606
CMW 3046	*M. heimioides*	*Ps. heimioides*	*Eucalyptus*	Indonesia		AF309609
CMW 5825	*M. irregulariramosa*	*Ps. irregulariramosa*	*Eucalyptus*	South Africa		AF468878
CBS 112224	*M. jonkershoekensis*		*Protea lepidocarpodendron*	Australia	P.W. Crous	DQ302968
MURU 257	*M. lateralis*	*Dissoconium dekkeri*	*E. globulus*	Western Australia	A. Maxwell	AY509762
CPC 3746	*M. maideirae*		*E. grandis*	Maideira, Portugal	S. Denman	DQ302979
CBS 112895	*M. maideirae*		*E. grandis*	Maideira, Portugal	S. Denman	AY725553
CMW 9091	*M. marksii*		*Eucalyptus*	South Africa	G. Hunter	AF468871
CMW 9092	*M. marksii*		*Eucalyptus*	South Africa	G. Hunter	AF468872

Table 1 continued. Isolates considered in the phylogenetic study. Those in bold were isolated during this study.

Culture no.[1]	teleomorph	anamorph	Host	Location	Collector	GenBank Accession no.
CMW 22520	***M. marksii***		***Eucalyptus***	**Irian Jaya, Indonesia**	**S. Sufaati/P. Barber**	**DQ632674**
CMW 22529	***M. marksii***		***Eucalyptus***	**Yunnan, China**	**B. Dell/P. Barber**	**DQ632671**
CMW 22530	***M. marksii***		***Eucalyptus***	**Yunnan, China**	**B. Dell/P. Barber**	**DQ632672**
CMW 22531	***M. marksii***		***Eucalyptus***	**Yunnan, China**	**B. Dell/P. Barber**	**DQ632673**
AF309591	*M. marasasii*	*S. marasasii*	*Syzigium*	South Africa		AF309591
MURU215	*M. mexicana*		*E. globulus*	Western Australia	A. Maxwell	AY509768
MURU 051	*M. nubilosa*		*E. globulus*	Western Australia	A. Maxwell	AY509777
MURU 057	*M. nubilosa*		*E. globulus*	Western Australia	A. Maxwell	AY509778
CMW 23439	***M. obscuris***	***Pseudocercospora* sp.**	***Eucalyptus***	**Irian Jaya, Indonesia**	**S. Sufaati/P. Barber**	**DQ632677**
CBS 119973, CMW 23440	***M. obscuris***	***Pseudocercospora* sp.**	***E. pellita***	**South East Vietnam**	**T.I. Burgess**	**DQ632676**
CBS 110949	*M. ohnowa*		*E. grandis*	South Africa	M.J. Wingfield	AY725575
CBS 112896 CMW 4937	*M. ohnowa*		*Eucalyptus*	South Africa		AF309604
CMW 14775, STE-U 353	*M. parkii*	*S. parkii*	*Eucalyptus*	Brazil		AF309590
MURU 248	*M. parva*		*E. globulus*	Western Australia	A. Maxwell	AY509779
MURU 249	*M. parva*		*E. globulus*	Western Australia	A. Maxwell	AY509780
CPC 10983	*M. perpendicularis*		*Eucalyptus*	Colombia	M.J. Wingfield	DQ303006
CBS 114782	*M. pseudoafricana*		*E. globulus*	Zambia	T.A. Coutinho	DQ303008
CPC 12085	*M. pseudosuberosa*	*Trimmatostroma* sp.	*Eucalyptus*	Uruguay	M.J. Wingfield	DQ303011
CBS 115608	*M. secundaria*		*E. grandis*	Brazil	A.C. Alfenas	DQ303018
CBS 111002	*M. secundaria*		*E. grandis*	Colombia	M.J. Wingfield	DQ303017
NZs	*M. suberosa*				A. Milgate	AY045503
Ke/2/1	*M. suttoniae*	*Phaeophleospora epicoccoides*				AY045519
CMW 5348, STE-U 1346	*M. suttoniae*	*P. epicoccoides*	*Eucalyptus*	Indonesia		AF309621
CMW 22484	***M. suttonii***	***P. epicoccoides***	***E. urophylla***	**Guandong, China**	**T.I. Burgess**	**DQ632705**
CMW 22485	***M. suttonii***	***P. epicoccoides***	***E. urophylla***	**Guandong, China**	**T.I. Burgess**	**DQ632683**
CBS 118506	*M. stramenticola*		*Eucalyptus*	Brazil	A.C. Alfenas	DQ303043
CBS 119972, CMW 23438	***M. stramenticola***		***E. camaldulensis* hybrid**	**South East Vietnam**	**T.I. Burgess**	**DQ632668**
CMW 22514	***M. stramenticola***		***E. grandis***	**Lake Toba, Indonesia**	**P.A. Barber**	**DQ632669**
CPC 12147	*'M. stramenticola'*		*A. mangium*	Thaoland	W. Himaman	DQ303041
CPC 11171	*M. sumatrensis*		*Eucalyptus*	Indonesia	M.J. Wingfield	DQ303048
CPC 11178	*M. sumatrensis*		*Eucalyptus*	Indonesia	M.J. Wingfield	DQ303050
CPC 10547	*M. thailandica*	*Ps. thailandica*	*A. mangium*	Thailand		AY752156

Table 1 continued. Isolates considered in the phylogenetic study. Those in bold were isolated during this study.

Culture no.[1]	teleomorph	anamorph	Host	Location	Collector	GenBank Accession no.
X51	*M. thailandica*	*Ps. thailandica*	*Musa* sp.	Windward Isles	E. Reid	AY752160
CPC 10621	*M. thailandica*	*Ps. thailandica*	*A. mangium*	Thailand		AY752159
CPC 10549	*M. thailandica*	*Ps. thailandica*	*A. mangium*	Thailand		AY752158
CPC 11167	*M. verrucosiafricana*		*Eucalyptus*	Indonesia	M.J. Wingfield	DQ303056
CPC 11170	*M. verrucosiafricana*		*Eucalyptus*	Indonesia	M.J. Wingfield	DQ303058
CBS 119974, CMW 23441	***M. vietnamensis***		***E. grandis* hybrid**	**South East Vietnam**	**T.I. Burgess**	**DQ632675**
CMW 23442	***M. vietnamensis***		***E. camaldulen*sis hybrid**	**South East Vietnam**	**T.I. Burgess**	**DQ632678**
STE-U-2769	*M. walkeri*	*Sonderhenia eucalypticola*				AF309616
CBS 119975, CMW 23443	***M. yunnanensis***		***Eucalyptus***	**Yunnan, China**	**B. Dell/P. Barber**	**DQ632686**
CBS 119976, CMW 23444	***M. yunnanensis***		***Eucalyptus***	**Yunnan, China**	**B. Dell/P. Barber**	**DQ632687**
CMW 23445	***M. yunnanensis***		***Eucalyptus***	**Yunnan, China**	**B. Dell/P. Barber**	**DQ632688**
CBS 208.94	*Mycosphaerella* sp.		*E. grandis*	Indonesia	A.C. Alfenas	DQ303029
CBS 209.94	*Mycosphaerella* sp.		*E. grandis*	Indonesia	A.C. Alfenas	DQ303030
CBS 110678	*Mycosphaerella* sp.		*E. globulus*	Brazil	F.A. Ferreira	DQ303031
CBS 111036	*Mycosphaerella* sp.		*E. grandis*	Colombia	M.J. Wingfield	DQ303036
CPC 10521	*Mycosphaerella* sp.		*A, aulacocarpa*	Thailand	M.J. Wingfield	AY752154
CMW 22515	***Mycosphaerella* sp.**		***E. camaldulensis* hybrid**	**South East Vietnam**	**T.I. Burgess**	**DQ632682**
CMW 22516	***Mycosphaerella* sp.**		***E. camaldulensis* hybrid**	**South East Vietnam**	**T.I. Burgess**	**DQ632670**
CMW 22519	***Mycosphaerella* sp.**		***Eucalyptus***	**Irian Jaya, Indonesia**	**S. Sufaati/P. Barber**	**DQ632689**
CMW 22532	***Mycosphaerella* sp.**		***E. camaldulensis* hybrid**	**South East Vietnam**	**T.I. Burgess**	**DQ632679**
CMW 22533	***Mycosphaerella* sp.**		***Eucalyptus***	**Irian Jaya, Indonesia**	**S. Sufaati/P. Barber**	**DQ632680**
STE-U 1458		*Ps. paraguayensis*	*Eucalyptus*	Brazil	M.J. Wingfield	AF309596
		Ps. paraguayensis	*Eucalyptus*	Brazil	M.J. Wingfield	AF222845
STE-U 1266		*Ps. basiramifera*	*Eucalyptus*	Thailand	M.J. Wingfield	AF309595
		Ps. basiramifera	*Eucalyptus*	Thailand	M.J. Wingfield	AF222837
CPC 11648		*Ps. fatouae*	*Fatoua villosa*	Korea	H.D. Shin	DQ303077
CBS 114243		*Ps. pseudoeucalyptorum*	*E. nitens*	New Zealand	W. Gams	AF725527
STE-U 16		*Ps. eucalyptorum*	*Eucalyptus*	South Africa		AF309598
CBS 111072		*Pseudocercospora* sp.	*E. pellita*	Thailand	M.J. Wingfield	DQ303082
CPC 11654		*Pseudocercospora* sp.	*Morus bombycis*	Korea	H.D. Shin	DQ303086
CPC 11680		*Pseudocercospora* sp.	*Ampelopsis brevipenduncula*	Korea	H.D. Shin	DQ303088
CMW 22521		***Pseudocercospora* sp.**	***E. camaldulensis***	**Thailand**	**K. Ramawong**	**DQ632690**

Table 1 continued. Isolates considered in the phylogenetic study. Those in bold were isolated during this study.

Culture no.[1]	teleomorph	anamorph	Host	Location	Collector	GenBank Accession no.
CMW 22522		***Pseudocercospora* sp.**	***E. camaldulensis* hybrid**	**South East Vietnam**	**T.I. Burgess**	**DQ632691**
CMW 22523		***Pseudocercospora* sp.**	***Eucalyptus***	**Irian Jaya, Indonesia**	**S. Sufaati/P. Barber**	**DQ632693**
CMW 22524		***Pseudocercospora* sp.**	***Eucalyptus***	**Irian Jaya, Indonesia**	**S. Sufaati/P. Barber**	**DQ632694**
CMW 22525		***Pseudocercospora* sp.**	***Eucalyptus***	**Irian Jaya, Indonesia**	**S. Sufaati/P. Barber**	**DQ632692**
CMW 22526		***Pseudocercospora* sp.**	***Eucalyptus***	**Yunnan, China**	**B. Dell/P. Barber**	**DQ632695**
CMW 22527		***Pseudocercospora* sp.**	***Eucalyptus***	**Yunnan, China**	**B. Dell/P. Barber**	**DQ632696**
CMW 22528		***Pseudocercospora* sp.**	***Eucalyptus***	**Yunnan, China**	**B. Dell/P. Barber**	**DQ632697**
CBS 110999		*S. pseudoparkii*	*E. grandis*	Colombia	M.J. Wingfield	DQ303023
CBS 110988		*S. pseudoparkii*	*E. grandis*	Colombia	M.J. Wingfield	DQ303021
CBS 111088		*S. xenoparkii*	*Eucalyptus*	Indonesia	M.J. Wingfield	DQ303026
CBS 111085		*S. xenoparkii*	*Eucalyptus*	Indonesia	M.J. Wingfield	DQ303028
CPC 11671		*Stenella* sp.	*Lonicera japonica*	Korea	H.D. Shin	DQ303097

[1]Designation of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; STE-U = Stellenbosch University, South Africa; MURU = Murdoch University, Perth, Western Australia.

isolates from China, Indonesia and Vietnam representing new species and new records of either host or location.

Materials and Methods

Isolates

Mycosphaerella spp. were isolated in the following manner. Ascospores of *Mycosphaerella* were isolated using the methods Crous (1998), by shooting the spores onto 2% Malt Extract Agar (MEA). Plates were examined 24-48 hours later for the presence of germinating ascospores using an Olympus VT II dissector microscope. Herbaria specimens were heated at 70ºC and submitted to MURU (Murdoch University, Western Australia). Ex-type cultures were submitted to Centraalbureau voor Schimmelcultures (CBS), other cultures used in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (CMW), University of Pretoria.

Molecular phylogenetic characterisation

For each isolate, approximately 50 mg of fungal mycelia was scraped from the surface of 21-day-old cultures, ground using a glass rod, suspended in 200 µl of DNA extraction buffer (200 mM Tris-HCL pH 8.0, 150 mM NaCl, 25 mM EDTA, 0.5% SDS) and incubated for 1 hr at 70°C. DNA was purified using the Ultrabind® DNA purification kit according to the manufactures instructions (MO BIO Laboratories, Solana Beach, CA). A part of the internal transcribed spacer (ITS) region of the ribosomal DNA operon was amplified using the primers ITS-1F (5’ CTT GGT CAT TTA GAG GAA GTA A) (Gardes and Bruns, 1993) and ITS4 (5’ TCC TCC GCT TAT TGA TAT GC) (White *et al*., 1990). The PCR reaction mixture (25 µl), PCR conditions and visualisation of products were as described previously (Burgess et al., 2001) except that 1 U of Taq polymerase (Biotech International, Needville, TX) was used in each reaction. PCR products were cleaned and sequenced as described previously (Taylor et al., 2005).

In order to compare the *Mycosphaerella* isolates used in this study with other *Mycosphaerella* spp., ITS rDNA sequences obtained from GenBank, including all species known from Asia and isolates used in recent studies (Crous *et al*., 2001, 2004a, 2004b, 2006, Hunter *et al*., 2004b, Maxwell *et al*., 2003), were used in the phylogenetic analysis (Table 1). Trees were rooted using *Neofusicoccum ribis*. Sequence data were analysed using Sequence Navigator version 1.0.1™ (Perkin Elmer Corp. Foster City, CA) and manually aligned by inserting gaps. Gaps were treated as a fifth character, all ambiguous characters and parsimony uninformative characters were excluded prior to analysis. The most parsimonious trees were obtained in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford, 2003) by using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option off. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (Hillis and Huelsenbeck, 1992). Characters were unweighted and unordered. Branch and branch node supports were determined using 1000 bootstrap replicates (Felsenstein, 1985).

Morphology

Leaves were examined for the presence of lesions and associated fungal structures using an Olympus VT II dissector microscope in AQIS-approved laboratories. Sporocarps were mounted in lactoglycerol and examined with an Olympus BH2 compound microscope. Size ranges of spores were derived from at least 50 individual ascospores and the extremes given in parentheses. Sections of sporocarps were prepared using a sharp razor blade and mounted in lactoglycerol and measured.

Pieces of agar with ascospores at various stages of germination were then removed using a scalpel, mounted on a slide in lactoglycerol and squashed gently. Microscopic examination and drawing of germination patterns was carried out using an Olympus BH2 compound microscope and drawing tube. Anamorphs observed in culture were mounted on a slide in lactoglycerol and measured and drawn using the method described above. Culture growth and colour after 3 weeks on MEA was determined with the aid of colour charts (Munsell® Soil Color Charts, Gretag Macbeth, New Windsor, New York,)

Results

Phylogenetic analysis

Initially, a blast search was conducted on GenBank to compare the sequence of the unknown *Mycosphaerella* isolates with existing sequence data. Some isolates had high homology with known species whilst others had poor homology to any *Mycosphaerella* species currently on GenBank. The sequences were then aligned with matching or closely related *Mycosphaerella*

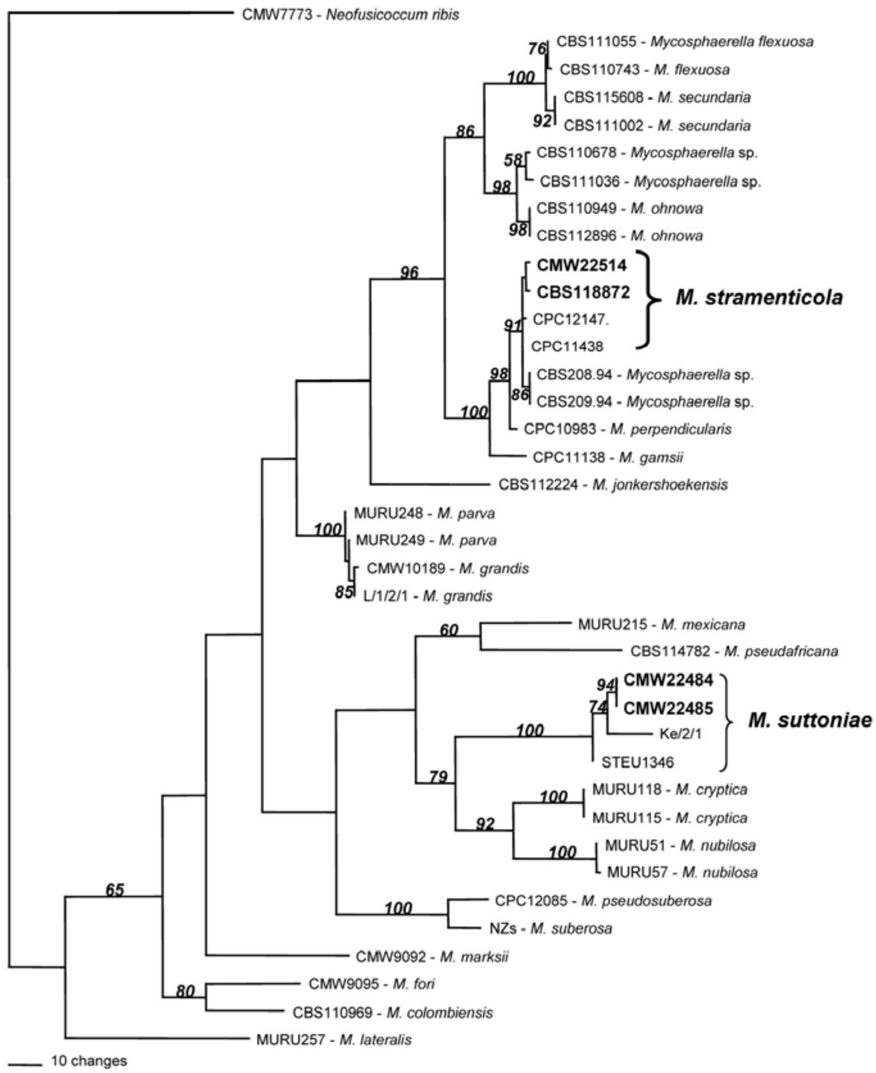

Fig. 1. A phylogram of one of the five most-parsimonious trees obtained from the ITS sequence data of the *Mycosphaerella* isolates. Isolates obtained from *Eucalyptus* spp. in Asia are in bold. Bootstrap support is given above the branches. The tree is rooted to *N. ribis*.

spp. The aligned dataset was very large and was divided into three datasets each containing genetically similar species and representative species from the other alignments. The first alignment of 38 taxa consisted of 625 characters of which 230 were parsimony informative. The dataset contained significant phylogenetic signal compared to 1000 random trees ($P<0.001$, $g1 = -0.56$) and

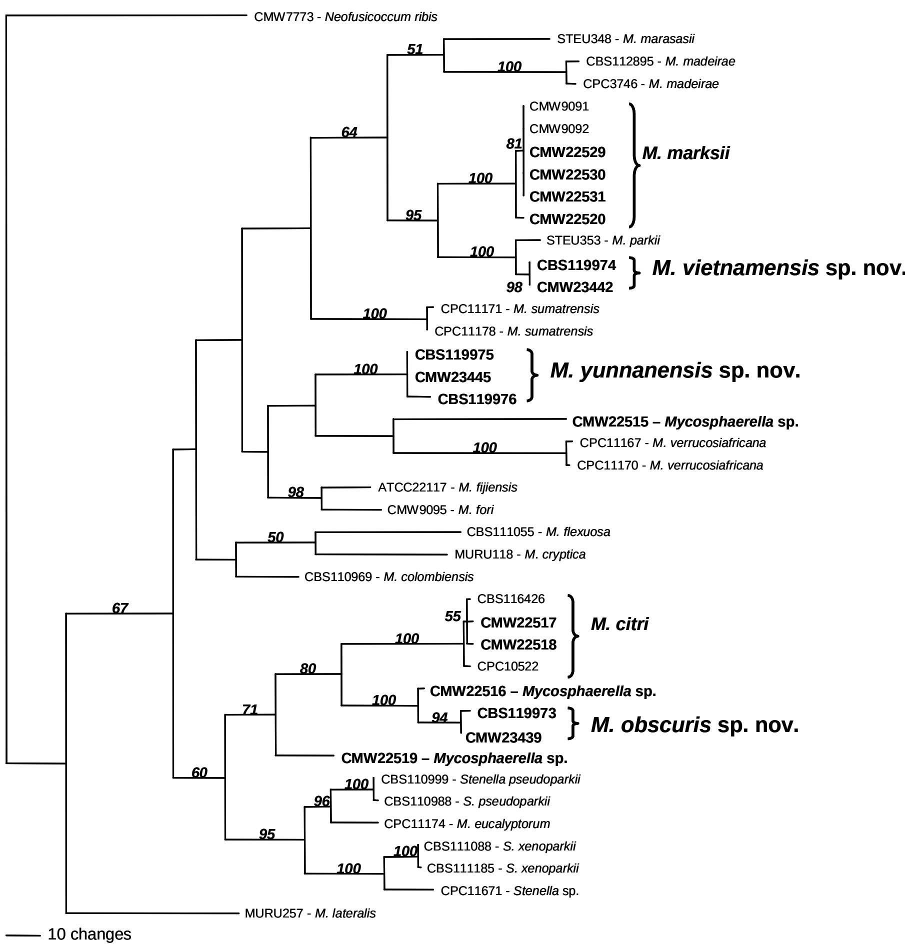

Fig. 2. A phylogram of one of the 24 most-parsimonious trees obtained from the ITS sequence data of the *Mycosphaerella* isolates. Isolates obtained from *Eucalyptus* spp. in Asia are in bold. Bootstrap support is given above the branches. The tree is rooted to *N. ribis*.

heuristic searches in PAUP resulted in five most parsimonious trees of 868 steps (CI = 0.54, RI = 0.79) (Figure 1, TreeBASE = SN2980). Four isolates obtained in this study corresponded to two known species; *M. suttonii* and the newly described *M. stramenticola*.

The second alignment of 41 taxa consisted of 625 characters of which 241 were parsimony informative. The data set contained significant phylogenetic signal compared to 1000 random trees ($P<0.001$, $g1 = -0.64$) and heuristic searches in PAUP resulted in 24 most parsimonious trees of 1099 steps (CI = 0.48, RI = 0.74) (Figure 2, TreeBASE = SN2980). This alignment contained 16 isolates obtained in this study, corresponding to two known

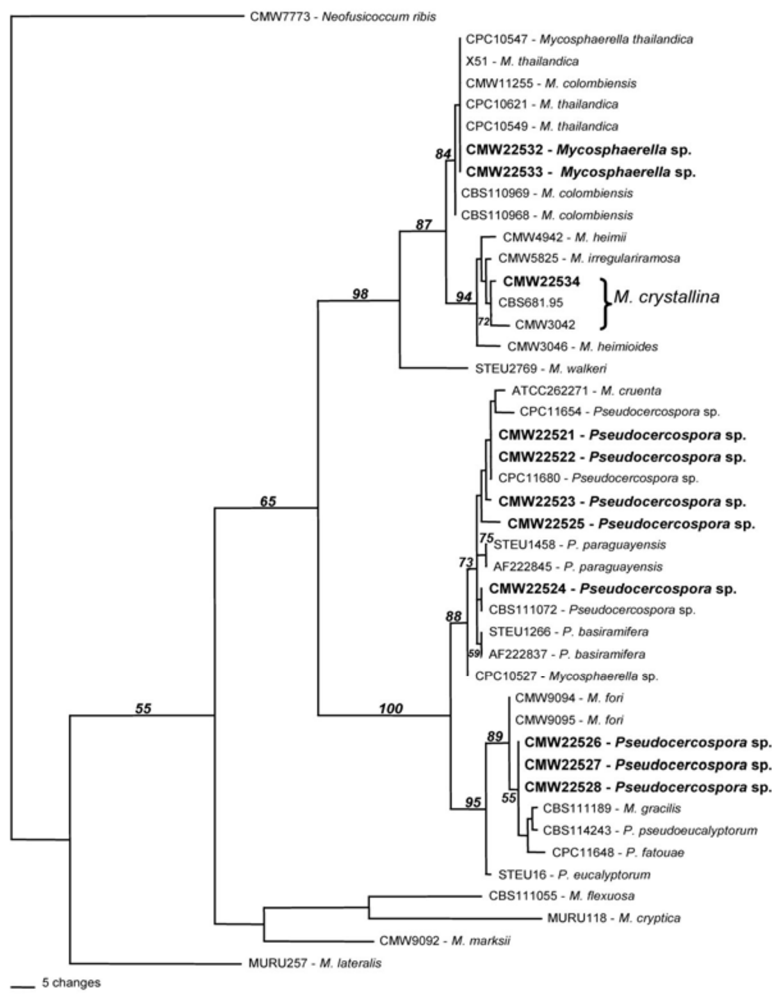

Fig. 3. A phylogram of one of the 384 most-parsimonious trees obtained from the ITS sequence data of the *Mycosphaerella* isolates, predominantly with *Pseudocercospora* anamorphs. Isolates obtained from *Eucalyptus* spp. in Asia are in bold. Bootstrap support is given above the branches. The tree is rooted to *N. ribis*.

species (*M. marksii* and *M. citri*) and six undescribed species. For three of the apparent new species only a single isolate was obtained, these have not been described (Figure 2). Three other species were identified, each in a terminal clade with high bootstrap support and these will be described below in the morphology section. The third alignment of 44 taxa, predominantly with *Pseudocercospora* anamorphs, consisted of 625 characters of which 144 were parsimony informative. The data set contained significant phylogenetic signal compared to 1000 random trees (P<0.001, g1 = -0.40) and heuristic searches in PAUP resulted in 384 most parsimonious trees of 427 steps (CI = 0.62, RI = 0.87) (Figure 3, TreeBASE = SN2980). The resultant tree consisted of two main clades. In the first clade, one isolate was identified as *M. crystallina*.

In addition, there were two isolates with ITS sequence identical to *M. colombiensis* and *M. thailandica*, two species that cannot be separated based on ITS alone. Based on their origin, they most likely belong to *M. thailandica*. In the second clade there were 8 isolates with *Pseudocercospora* anamorphs whose identity could also not be confirmed with ITS alone. Two isolates, CMW22521 and CMW22522 from *E. camaldulensis* in Thailand and Vietnam respectively, have identical ITS sequence as an undescribed *Pseudocercospora* sp. isolated from *Ampelopsis brevipeduncula* in Korea (CPC11680). Isolate CMW22524 from a eucalypt in Irian Jaya, Indonesia also had identical sequence to an isolate from *E. pellita* in Thailand (CPC111072).

Taxonomy

Mycosphaerella obscuris Barber & T.I. Burgess, **sp. nov.** (Fig. 4)
Mycobank 500739

Etymology: Named after the lack of distinguishing morphological features of the species.

Pseudothecia amphigena, solitaria, nigra, immersa, substomalia, ad 95 µm diam. *Asci* aparaphysati, fasciculati, bitunicati, subsessiles, obovoidei, recti vel incurvi, 8-sporati, 26–32 × 7–10 µm. *Ascosporae* bi- vel triseriatae, superpositae, hyalinae, guttulatae, parietibus crassibus, rectae vel subfalcatae, fusoideo-ellipsoideae, apicibus subobtusis, ad medium uniseptatae, ad medium cellulae apicalis latissimae, ad septum subconstrictae, ad fines ambas (sed ad finem basalem manifeste) angustatae, 9–11 × 2–3 µm. *Mycelium* ramosum, septatum, pallide brunneum, 2–3.5 µm latum. *Conidiorum* cellulae discretae vel absentes, pallide brunneae, terminales vel non terminales, laevigatae, subcylindricae, rectae, proliferationibus 1 vel 2 percurrentibus, 1–2 × 2–2.5 µm. *Conidia* pallide brunnea, verruculosa, septis 1–3, raro 0 vel 4, recta, falcata vel sigmoidea, immatura obpyriformia, matura ellipsoidea ad anguste obclavata vel subcylindrica, apice subobtuso, ad medium vel ad basim latissima, ad basim truncatum angusta, (9) 20–28 (–38) × (4–) 5–6 (8) µm.

Leaf spots on *E. pellita* amphigenous, subcircular to angular, vein-limited, up to 3 mm diam., grey to brown, surrounded by a thick, raised, brown border; on *Eucalyptus* sp. confined to leaf margins extending inward, irregular, up to 6 mm wide and 155 mm long, surrounded by a thin, raised concolorous border. *Pseudothecia* amphigenous, single, black, immersed, sub-stomatal, up to 95 µm diam.; apical ostiole 10-20 µm diam.; wall of 2-3 layers of medium brown textura angularis. *Asci* aparaphysate, fasciculate, bitunicate, subsessile, obovoid, straight or incurved, 8-spored, 26-32 × 7-10 µm. *Ascospores* bi- to tri-seriate, overlapping, hyaline, guttulate, thick-walled, straight to slightly curved, fusoid-ellipsoidal with subobtuse ends, medianly 1-septate, widest at the

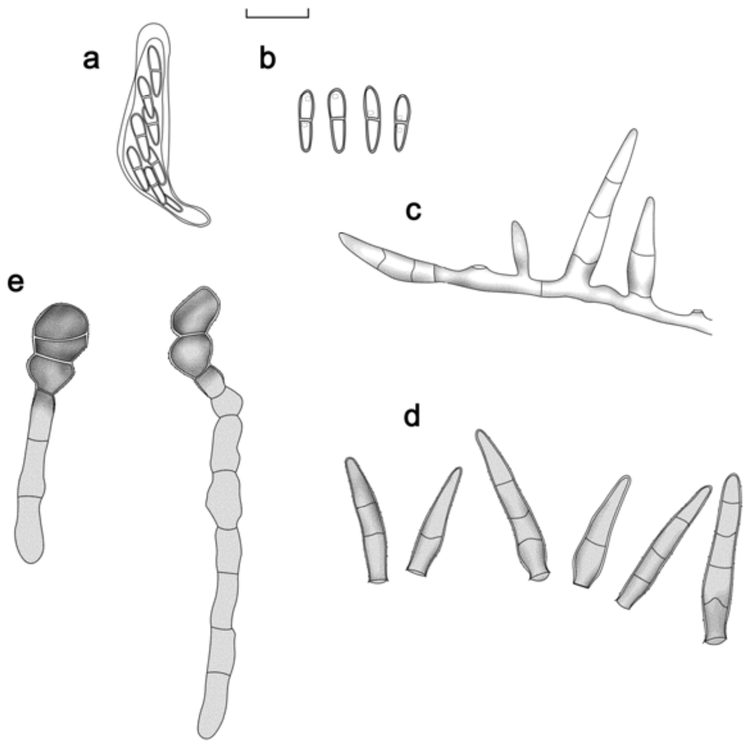

Fig. 4. *Mycosphaerella obscuris* (MURU 418) and its *Pseudocercospora* anamorph (CBS 119975). (a) Ascus. (b) Ascospores. (c) Conidiogenous cells and conidia. (d) Conidia. (e) Germinating conidia. Scale bar = 10 µm.

middle of the apical cell, slightly constricted at the septum, tapering towards both ends but more prominently towards lower end, 9-11 × 2-3 µm ($\bar{x}$ = 10 × 2.8 µm).

Mycelium branched, septate, pale brown, 2-3.5 µm wide. *Conidiogenous cells* absent or discrete, pale brown, terminal or not, smooth, sub-cylindrical, straight, 1-2 percurrent proliferations, 1-2 × 2-2.5 µm. *Conidia* pale brown, verruculose, with 1-3, rarely 0 or 4 septa, straight, curved or sigmoidal, obpyriform when immature becoming ellipsoidal to narrowly obclavate to sub-cylindrical at maturity, apex subobtuse, widest at the middle or towards the base, tapering to the truncate base, (9) 20-28 (38) × (4) 5-6 (8) ($\bar{x}$ = 25.3 × 5.6 µm). *Conidia* germinating *in vitro* from one end, conidium distorting and becoming more melanised upon germination with germ tubes parallel to the long axis of the spore.

Cultures 10-20 mm diam. after 3 wk on MEA, very dark greenish black, appressed colonies with moderate greenish grey aerial mycelium and smooth but irregular margins.

Anamorph: *Pseudocercospora* sp.

Habitat: On living leaves of *E. pellita* and *Eucalyptus* sp.

Known distribution: Vietnam and Indonesia.

Specimens examined. VIETNAM, BauBang Research Station from leaves of *E. pellita*, 07/07/2004, T.I. Burgess (MURU 418; **holotype**) (ex-holotype culture CBS 119973, CMW 23439); INDONESIA, Irian Jaya, Wasur National Park on leaves of Eucalyptus sp., 05/01/2004, S. Saafati (MURU 416) (culture CMW23440).

Notes: *Mycosphaerella obscuris* is morphologically similar to *M. africana* (Crous and Wingfield, 1996), *M. gregaria* (Carnegie *et al*., 1997), *M. vietnamensis* and *M. yunnanensis* (this paper) but can be distinguished from these species based upon the production of a *Pseudocercospora* anamorph *in vitro*. Conidia of this anamorph state became very melanised and distorted upon germination in culture.

Mycosphaerella vietnamensis Barber & T.I. Burgess **sp. nov.** (Fig. 5)
Mycobank MB500740

Etymology: Named after the country it was collected from.

Pseudothecia aggregata, nigra, immersa erumpescentia, substomalia, ad 80 µm diam. *Asci* aparaphysati, fasciculati, bitunicati, subsessiles, anguste ellipsoidei, recti vel subincurvi, 8-sporati, 32–50 × 9.5–12 µm. *Ascosporae* bi- vel triseriatae, superpositae, hyalinae, guttulatae, parietibus crassibus, rectae vel subfalcatae, fusoideo-ellipsoideae, apicibus obtusis, ad medium uniseptatae, in medio cellulae apicalis latissimae, ad septum subconstrictae, ad fines ambas (sed ad finem basalem manifeste) angustatae, 10–12 × 3–4 µm.

Leaf spots amphigenous, sub-circular to circular, vein-limited, 2-5 mm diam., medium brown, surrounded by a thin, raised, brown border and occasionally a spreading pink margin. *Pseudothecia* aggregated, black, immersed, becoming erumpent, up to 80 µm diam.; apical ostiole 10-15 µm diam; wall of 2-3 layers of medium brown *textura angularis*. *Asci* aparaphysate, fasciculate, bitunicate, subsessile, narrowly ellipsoid, straight or slightly incurved, 8-spored, 32-50 × 9.5-12 µm. *Ascospores* bi-seriate to tri-seriate, overlapping, hyaline, guttulate, thick-walled, straight to slightly curved, fusoid-ellipsoidal with obtuse ends, medianly 1-septate, widest at the middle of the apical cell, slightly constricted at the septum, tapering towards both ends but more prominently towards lower end, 10-12 × 3-4 µm.

Mycelium internal and external, consisting of smooth, branched, septate, pale to medium brown, 3-6 µm wide hyphae. *Conidiomata* fasciculate, hypophyllous, medium brown, up to 90 µm wide and 70 µm high. *Conidiophores* aggregated in dense fascicles arising from the upper cells of a brown stroma up to 80 µm wide and 90 µm high; conidiophores pale to medium brown, smooth, unbranched or branched, 1-3-septate, subcylindrical, straight to variously curved, 17-38 × 3-5.5 µm. *Conidiogenous cells* terminal, unbranched, subcylindrical, pale brown, smooth, proliferating sympodially, tapering to a truncate or bluntly rounded apex, 12.5-23.5 × 3.5-5.5 µm. Conidia solitary, pale brown, smooth to verruculose, narrowly obclavate to subcylindrical, apex obtuse, base obconic-truncate, 2-5 septate, 33.5-60 × 3-3.5 µm ($\overline{x}$ = 46.4 × 3.3 µm), hila inconspicuous.

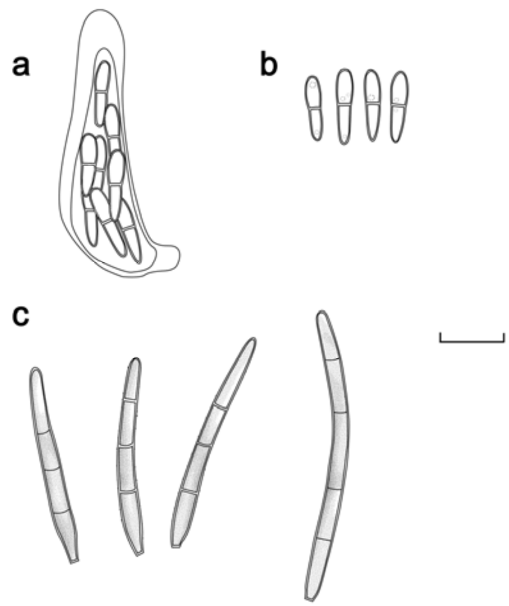

Fig. 5. *Mycosphaerella vietnamensis* (MURU 411) and its presumed *Pseudocercospora* anamorph. (a) Ascus. (b) Ascospores. (c) Conidia. Scale bar = 10 µm.

Cultures 35-35 mm diam. after 3 week on MEA, greenish grey aerial mycelium on surface, very dark greenish grey on reverse, feathery, even margins.

Anamorph: presumed *Pseudocercospora* sp.

Habitat: On living leaves of *E. grandis* and *E. camaldulensis*.

Known distribution: Vietnam.

Specimens examined. VIETNAM, South East Forestry Institute nursery from leaves of *E. grandis* hybrid, 06/07/2004 T.I. Burgess (MURU411 **holotype**) (ex-holotype culture CBS 119974, CMW 23441); SongMay Research Station from leaves of *E. camaldulensis* hybrid, 06/07/2004, T.I. Burgess (MURU 413) (culture, CMW23442)

Notes: *Mycosphaerella vietnamensis* is confined at present to eucalypts in Vietnam. It is morphologically similar to *M. africana* (Crous & Wingfield, 1996), *M. gregaria* (Carnegie *et al.*, 1997), *M. obscuris* and *M. yunnanensis* (this paper). It can be distinguished from *M. africana* and *M. obscuris* in having aggregated pseudothecia and from *M. obscuris* in lacking the production of a *Pseudocercospora* anamorph in vitro. A *Pseudocercospora* sp. occurred in close proximity on the same lesions from which *M. vietnamensis* was isolated but as it was not produced in culture the connection between these states could not be confirmed. This *Pseudocercospora* state differs to the anamorph of *M. obscuris* in having less roughened walls and longer and narrower conidia, but is morphologically similar to *P. epispermogoniana* Crous & M.J. Wingf. (conidia solitary, narrowly obclavate, 1-7-septate, hila inconspicuous).

Mycosphaerella yunnanensis Barber, Dell & T.I. Burgess, **sp. nov.** (Fig. 6)
Mycobank MB500741

Etymology: Named after the region in China from which it was collected.

Pseudothecia aggregata, nigra, immersia erumpescentia, ad 80 µm diam. *Asci* aparaphysati, fasciculati, bitunicati, subsessiles, ovoidei vel obclavati, incurvi, 8-sporati, 27–38 × 7.5–10.5 µm. *Ascosporae* bi- vel tri-seriatae, superpositae, hyalinae, eguttulatae, parietibus crassibus, rectae vel subfalcatae, fusoideo-ellipsoideae, apicibus obtusis, medio uniseptatae, in medio vel prope apicem cellulae apicalis latissimae, ad septum non- vel sub-constrictae, ad fines ambas (sed ad finem basalem manifeste) angustatae, (9.5–) 10–12.5 × (2–) 2.5–3 (–3.5) µm.

Leaf spots spreading inward from the leaf margins, irregular, up to 14 mm wide and 140 mm long, medium brown, surrounded by thick, dark brown border. *Pseudothecia* hypophyllous, single, black, immersed, up to 70 µm diam.; apical ostiole 10-15 µm diam.; wall of 2-3 layers of medium brown textura angularis. *Asci* aparaphysate, fasciculate, bitunicate, subsessile, ovoid to obclavate, incurved, 8-spored, 27-38 x 7.5-10.5 µm. *Ascospores* bi-seriate to tri-seriate, overlapping, hyaline, non-guttulate, thick-walled, straight to slightly curved, fusoid-ellipsoidal with obtuse ends, medianly or unequally 1-septate, widest at the middle or near the apex of the apical cell, not or slightly constricted at the septum, tapering towards both ends but more prominently towards lower end, (9.5)10-12.5 × (2)2.5-3 (3.5) µm ($\bar{x}$ = 11.2 × 2.7 µm).

Cultures 10-20 mm diam. after 3 wk on MEA, very dark greenish-black, appressed colonies with smooth, even margins.

Ascospore germination on 2% MEA up to 24 hours: Type I. Ascospores remaining hyaline but germ tubes becoming pale brown, germinating from both ends, with germ tubes either parallel to the long axis of the spore or somewhat irregular, constriction at the septum with slight distortion, lateral branching occurring either from the original ascospore or in close proximity to; ascospores becoming 4-5 µm diam.

Anamorph: not seen.

Habitat: On living leaves of *Eucalyptus urophylla*.

Known distribution: South-west China.

Specimens examined. CHINA, Yunnan: Lancang from leaves of *Eucalyptus urophylla* May 2005, B. Dell (MURU 407; **holotype**) (ex-holotype culture CBS 119975 [=CMW23443], CBS 119976 [=CMW 23444], CMW 23445).

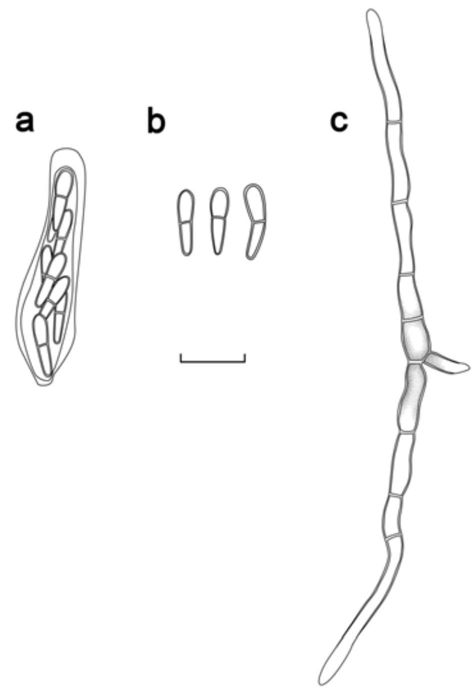

Fig. 6. *Mycosphaerella yunnanensis* (MURU 407). (a) Ascus. (b) Ascospores. (c) Germinating ascospore. Scale bar = 10 µm.

Notes: *Mycosphaerella yunnanensis* at present has a very restricted distribution being confined to *Eucalyptus urophylla* occurring at elevation of ca. 1,000 m in Yunnan Province of China. It was isolated from lesions colonised by *M. marksii* but the germinating ascospores of the two species were markedly different on agar. *Mycosphaerella yunnanensis* is most similar to *M. tasmaniensis* in its germination pattern; however, it differs from this species in ascospore morphology with thick walls and slight constrictions at the septa. An unidentified *Mycosphaerella* sp. was also isolated (CMW 22526, 22527 and 22528) from the same lesions and sequence data of the ITS region obtained. The germination pattern was indistinguishable from that of *M. yunnanensis*. This species aligned within the *Pseudocercospora* clade.

Discussion

Three new *Mycosphaerella* spp. have been described for eucalypts in Asia; *M. vietnamiensis, M. obscuris and M. yunnanensis*. *Mycosphaerella marksii* and *M. suttonii* were regularly encountered and these have been found in the region before (Crous, 1998). *Mycosphaerella crystallina*, with a previous known distribution of only South Africa, was found in China. *Mycosphaerella stramenticola*, recently described from leaf litter in Brazil was found in both Vietnam and Indonesia. *Pseudocercospora* spp. were common throughout the region but due to small morphological and phylogenetic differences between species these were not described here.

This study has highlighted the difficulties encountered when attempting to distinguish between *Mycosphaerella* spp. according to ascospore morphology. The new species described in this study (*M. obscuris*, *M. vietnamensis and M. yunnanensis*) overlap in their dimensions. Germination patterns of ascospores were first used as a distinguishing feature between eucalypts by Park and Keane (1982a) and later Crous (1998) categorised these germination patterns as more species were described. We were unable to obtain germination patterns of *M. obscuris* and *M. vietnamensis*, but fortunately these species were not associated with other *Mycosphaerella* spp. from individual lesions. It is not uncommon to isolate up to four *Mycosphaerella* spp. from a single lesion (Park and Keane, 1982a; Crous, 1998; Maxwell *et al*., 2003; Kuluratne *et al*., 2004). This was observed in the present study when isolating *M. yunnanensis* from lesions of *M. marksii* and another morphologically indistinct but phylogenetically distinct species. The germination pattern of *M. yunnanensis* was easily distinguishable from *M. marksii* but not the undescribed species. We chose not to describe this species as we had sequence data of fewer isolates and it resided deep in the *Pseudocercospora* clade. This clade is unresolved and further work is required to determine whether the undescribed species can be distinguished from *M. yunnanensis* according to morphology of the teleomorph or of the anamorph states, if they exist, as is the case with *M. swartii* and *M. walkerii* (Park and Keane, 1984; Crous, 1998).

The production of the anamorph state in culture distinguished *M. obscuris* from the other new species described in the present study. This anamorph state is typical of fungi previously allocated to the genus *Cercostigmina*, with conidiogenous cells that proliferate percurrently rather than sympodially, and produce euseptate, verruculose, acicular-subcylindric, or narrowly obclavate conidia (Braun, 1993). However, species in this genus have since been re-allocated to *Pseudocercospora* (Braun and Hill, 2002).

The *Pseudocercospora* sp. found in the present study on lesions associated with *M*. *vietnamensis* is morphologically similar to *P*. *epispermogoniana*. Hunter *et al*. (2006) recently linked this species to *M*. *marksii*, discounting the suggestion by Crous *et al*. (2001) that *M*. *marksii* should have a *Stenella* anamorph due to its proximity to *M*. *parkii* according to ITS sequence data.

An intriguing find was *M*. *citri* infecting *E*. *camaldulensis* in Vietnam. *Mycosphaerella citri* is a major pathogen of *Citrus* causing serious premature defoliation during the fall and winter on all varieties (Timmer *et al*., 2000). Only recently this pathogen has been found on *Acacia mangium* in Thailand and confirmed from bananas (Crous *et al*., 2004b). This was the first report of a major pathogen being able to infect hosts from unrelated genera and the observed phenomenon may be due to species of *Acacia*, *Citrus* and *Musa* all being native to parts of South-East Asia. Our study has added another host genus, *Eucalyptus*, whose natural distribution is not sympatric with species of *Citrus* and *Musa*. The presence of *M*. *citri* on eucalypts most likely represents a host-jump rather than co-evolution.

Mycosphaerella thailandica has recently been described as causing disease on *Acacia* and *Musa* in Thailand (Crous *et al*., 2004b). In their study, it was shown to be a sister species of *M*. *colombiensis*, a foliar pathogen of *Eucalyptus*, which cannot be separated based on ITS sequence data alone (Crous *et al*., 2004b). To date, *M*. *colombiensis* has only been found in Colombia, thus it is likely that the Asian isolates from this study are *M*. *thailandica* and as such would represent a new host species. However, this cannot be determined without sequencing more gene regions.

Mycosphaerella stramenticola has very recently been described from *Eucalyptus* leaf litter in Brazil (Crous *et al*., 2006). In this study we obtained two isolates from lesions on *Eucalyptus* leaves in Vietnam and Indonesia with identical ITS sequence data and overlapping morphological and culture characteristics. An isolate from *Acacia mangium* in Thailand also has identical ITS sequence. This species has a wider distribution than first reported and is associated with lesions on living foliage as well as a saprophyte on leaf litter.

Several *Pseudocercospora* isolates were recovered in this study. Over 20 species have been described from *Eucalyptus*, but morphological features used to distinguish between species are limited and there are only small differences in ITS sequence data (Crous *et al*., 2001, 2004a, b). To date, other gene regions have not been sequenced from the known species and for this reason we did not attempt to classify the apparently new species obtained in this study. Two *Pseudocercospora* isolates, one each from Thailand and Vietnam, were most closely related to *M*. *cruenta* which is a pathogen of cowpeas. We do not feel that this represents range expansion and a host jump, but rather a new, as yet, unidentified *Pseudocercospora* sp. Two other undescribed *Pseudocercospora* species isolated from *Eucalyptus* leaf lesions in South-east Asia have also been found on other hosts in Korea. This again emphasizes that either the ITS region is insufficient for the separation of *Pseudocercospora* spp. or as suggested by Crous *et al*. (2004b), many *Mycosphaerella* species have a broader host range than was first thought.

The *Mycosphaerella* species described in this study did not appear to be causing a significant level of damage to their hosts in plantations throughout the region. Lesions on young leaves were small and larger blights were only observed on older leaves. The association of the majority of these species alone on lesions suggests they may be primary pathogens rather than endophytes as is expected for numerous other *Mycosphaerella* spp. The exception to this is *M*. *yunnanensis* which was described from typical lesions of *M*. *marksii*.

The recent years have seen an exponential increase in the number of new *Mycosphaerella* spp. and their anamorphs described from *Eucalyptus*. This is due to their impact in an expanding world-wide forestry industry, but also because of the advent of molecular phylogenies which has enabled the separation of cryptic species (Crous *et al*., 2001, 2004b, 2006; Hunter *et al*., 2004a, 2006; Maxwell *et al*., 2005). Many of the new species have been described from eucalypt plantations in exotic environments and less than half these species have been found in Australia. However, eucalypt plantation forestry in Australia is relatively new and as more research is being conducted many of these *Mycosphaerella* spp., such as *M*. *heimii* and *M*. *fori*, are being found in Australia (Jackson *et al*., 2005; Whyte *et al*., 2005). Of the species described in exotic environments, many will have originated with their hosts whilst others seem to be moving onto eucalypts in their new environment (Burgess and Wingfield, 2002). A example of this is *Phaeophleospora destructans* which causes a serious leaf blight of young eucalypts in Asia (Burgess *et al*., 2006; Wingfield *et al*., 1996). Such pathogens pose a serious threat to the biosecurity of native *Eucalyptus* in Australia

In this study, besides the description of four new species, there were new host records for other known species such as *M*. *citri* and the known distribution of other species has been expanded. In addition, three phylogenetically distinct *Mycosphaerella* spp. were not described due to lack of material and three *Pseudosercospora* spp. were not described due to lack of distinguishing features. This study, whilst opportunistic rather than comprehensive has highlighted the potential biodiversity of *Mycosphaerella* spp. on eucalypts in Asia.

Acknowledgements

This work was funded by the Australian Research Council DP0343600, 'Population genetics of fungal pathogens that threaten the biosecurity of Australia's eucalypts'. Material collected under AQIS permit no 200214708. In country support was provided as follows: China - Research Institute of Tropical Forestry, Asian Paper and Pulp Ltd; Indonesia: - Toba Pulp; Vietnam - Forest Research Institute of Vietnam. We thank Alex George for the editing the Latin descriptions and Dianne White is thanked for her technical support.

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(Received 27 July 2006; accepted 12 October 2006)