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Niche sharing reflects a poorly understood biodiversity phenomenon

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Key words

Eucalyptus
ITS
Mycosphaerella
systematics
Teratosphaeria

Abstract *Eucalyptus* spp. are susceptible to a large number of foliar pathogens, some of which can cause serious defoliation and die-back. In this study, a single leaf spot on a *Eucalyptus* leaf collected in Madagascar revealed an unusual association of microfungi with disease symptoms. Initial observations indicated that the leaf spot was associated with *Mycosphaerella marksii*, a common pathogen of eucalypts. However, more intensive scrutiny showed the presence of several other microfungi co-occurring in this, and other leaf spots on the leaf. A total of 41 single conidial propagules were subsequently obtained from a single lesion for morphological study and DNA sequence comparisons. Based on these data, 11 members of the *Capnodiales*, including one species of *Pestalotiopsis* (*Xylariales*), were observed. Of the capnodialean taxa, nine could be cultivated, which revealed one known species, *M. marksii*, two taxa in the *Cladosporium cladosporioides* species complex that were not treated here, and six new species, including *Passalora intermedia*, *Pseudocercospora madagascariensis*, *Teratosphaeria hortaea*, *Toxicocladosporium chlamydosporum*, *T. rubrigenum* and *T. veloxum*. Results of this study highlight a remarkable fungal biodiversity that can occur within a very specific niche. Furthermore, the results emphasise the importance of verifying the identity of fungal isolates in culture, as many taxa, especially those of the *Capnodiales*, frequently co-occur in the same niche, lesion or leaf spot.

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INTRODUCTION

The genus *Mycosphaerella* s.l. with its associated anamorph genera includes more than 10 000 names (Crous et al. 2000, 2001, 2004a, b, 2006a, b, e, 2007a–c, Crous & Braun 2003, Aptroot 2006, Arzanlou et al. 2007). Not surprisingly, this remarkably large genus, has recently been shown to be polyphyletic (Hunter et al. 2006, Crous et al. 2006b, d, 2007a), including *Davidiella* species with *Cladosporium* anamorphs (*Davidiellaceae*) (Braun et al. 2003, Crous et al. 2007b, Schubert et al. 2007b, Zalar et al. 2007, Dugan et al. 2008), *Schizothyrium* species with *Zygophiala* anamorphs (*Schizothyriaceae*) (Batzer et al. 2008), *Teratosphaeria* species with more than 12 anamorph genera (*Teratosphaeriaceae*) (Arzanlou et al. 2007, 2008, Crous et al. 2007a, 2008a, b, Cheewangkoon et al. 2008, Ruibal et al. 2008), and *Mycosphaerella* species with more than 20 anamorph genera (*Mycosphaerellaceae*) (Crous & Braun 2003). All of these families reside in the *Capnodiales* of the *Dothideomycetes* (Schoch et al. 2006).

Species in the *Mycosphaerella* complex have, in the past, been distinguished based on their host association (Crous & Braun 2003, Aptroot 2006) and morphology. Studies of these fungi in culture and the introduction of DNA-based techniques has, however, provided new evidence that host specificity does not apply to several taxa. The fact that species of *Mycosphaerella* can be isolated as endophytes (Crous & Wingfield 1996, Crous 1998, Ganley et al. 2004, Verkley et al. 2004) might explain why several species have in recent years been isolated from the same leaf spots (Crous 1998, Crous et al. 2004a, b, 2006e, 2007c, 2008a, b, Burgess et al. 2007). Furthermore, a substantial body of evidence has begun to emerge suggesting that some of these species may move from one host to another in the process of locating their preferred hosts on which they cause disease. This phenomenon, which has been referred to as the 'pogo stick hypothesis' (Crous & Groenewald 2005), has been shown for several species (Table 1). Although species of *Cladosporium* (*Davidiellaceae*) are generally accepted as having wide host ranges, recent DNA sequence-based studies have shown that many common species actually represent species complexes. Some of these taxa also appear to have a more defined host range than was originally accepted for them (Schubert et al. 2007a, b, Dugan et al. 2008).

Various species of *Mycosphaerella* and *Teratosphaeria*, including some species of *Cladosporium* that have commonly been treated as host-specific necrotrophic pathogens, appear to also exhibit a facultative saprobic behaviour on non-hosts. This suggests that the definitions of necrotroph and saprobe for this group of fungi are incompletely applied for the *Capnodiales*. This is especially true where species have apparently retained the ability to also grow on dead tissue when they lose the connection to their known susceptible host.

During the course of a study to describe novel species of *Capnodiales* from *Eucalyptus* leaves collected in Madagascar, a new species of *Pseudocercospora* was encountered. Upon closer examination, however, ramichloridium-like and stenella-like species were observed on the same lesion. This raised the question as to how many species might be present in a single leaf spot, which was further considered using studies of the fungi in culture as well as DNA sequence comparisons.

MATERIALS AND METHODS

Isolates

A single lesion on the leaf of a *Eucalyptus camaldulensis* tree growing near Morondavo was chosen for study. The leaf was

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Table 1 List of GenBank accession and culture collection numbers for fungal species isolated from a single lesion.

Species	Strain no.[1]	GenBank Accession number	
		ITS	LSU[2]
Cladosporium sp. 1	A35; CPC 15742	FJ790248	FJ790289
Cladosporium sp. 2	A40; CPC 15746	FJ790249	FJ790290
Mycosphaerella marksii	CPC 14655	FJ790250	FJ790291
Passalora intermedia	A11; CPC 15719	FJ790251	–
	A12; CPC 15720	FJ790252	–
	A13; CPC 15934	FJ790253	–
	A17; CPC 15724	FJ790254	FJ790292
	A21; CPC 15728	FJ790255	FJ790293
	A23; CPC 15730	FJ790256	FJ790294
	A24; CPC 15731	FJ790257	–
	A25; CPC 15732	FJ790258	–
	A26; CPC 15733	FJ790259	FJ790295
	A27; CPC 15734	FJ790260	–
	A30; CPC 15737	FJ790261	FJ790296
	A31; CPC 15738	FJ790262	–
	A32; CPC 15739	FJ790263	–
	A33; CPC 15740	FJ790264	–
	A36; CPC 15743	FJ790265	–
	A37; CPC 15744	FJ790266	–
	A39; CPC 15745; CBS 124154	FJ790267	FJ790297
Pseudocercospora madagascariensis	CPC 14621; CBS 124155	FJ790268	FJ790298
Teratosphaeria hortaea	A3; CPC 15711	FJ790269	–
	A4; CPC 15712	FJ790270	–
	A5; CPC 15713	FJ790271	–
	A6; CPC 15714	FJ790272	–
	A7; CPC 15715	FJ790273	–
	A8; CPC 15716; CBS 124156	FJ790274	–
	A9; CPC 15717	FJ790275	–
	A10; CPC 15718	FJ790276	FJ790299
	A14; CPC 15721	FJ790277	–
	A15; CPC 15722	FJ790278	–
	A16; CPC 15723	FJ790279	FJ790300
	A20; CPC 15727	FJ790280	–
	A22; CPC 15729	FJ790281	–
	A34; CPC 15741	FJ790282	–
Toxicocladosporium chlamydosporum	A1; CPC 15709; CBS 124157	FJ790283	FJ790301
	A2; CPC 15710	FJ790284	FJ790302
Toxicocladosporium rubrigenum	A18; CPC 15725	FJ790285	FJ790303
	A19; CPC 15726	FJ790286	FJ790304
	A28; CPC 15735; CBS 124158	FJ790287	FJ790305
Toxicocladosporium veloxum	A29; CPC 15736; CBS 124159	FJ790288	FJ790306

[1] A: Temporary laboratory identifier; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS.
[2] ITS: Internal transcribed spacers 1 and 2 together with 5.8S nrDNA; LSU: 28S nrDNA.

randomly selected from mature, green foliage on an apparently healthy tree, and kept together with other, similar leaves in a paper bag at room temperature under dry conditions. The chosen lesion was ± 5 × 5 mm in size, and extended through the leaf lamina. No fungal growth was observed on the surrounding, green leaf tissue, and the leaf was not incubated before isolation of microfungi. Initial examination under a stereo microscope (80× magnification) revealed only three species to be present, namely an ascomycete, a coelomycete and a hyphomycete. Microscopic mounts examined under higher magnification (1 000× magnification) revealed a mixture of several hyphomycetes to be present. Fungal conidia were subsequently removed by scraping the surface area of the lesion with a sterile scalpel blade, and making dilution plates of spores in sterile water on Petri dishes containing 2 % malt extract agar (MEA; Oxoid, Hampshire, England). Ascomata were removed from the lesion by means of a scalpel, squashed in a drop of sterile water, and streaked onto MEA plates. Forty-one single conidial and 10 single ascospore isolates were chosen for further study and DNA sequence comparisons. Colonies were subcultured onto 2 % potato-dextrose agar (PDA), synthetic nutrient-poor agar (SNA), MEA, and oatmeal agar (OA) (Gams et al. 2007), and incubated under continuous near-ultraviolet light at 25 °C to promote sporulation. All cultures obtained in this study are maintained in the culture collection of the CBS (Table 1). Nomenclatural novelties, descriptions and trace files of the ITS DNA barcodes were deposited in MycoBank (www.MycoBank.org).

DNA phylogeny

Fungal colonies were established on agar plates and genomic DNA was isolated using a commercial DNA isolation kit (E.Z.N.A. Forensic DNA Isolation Kit, Omega Bio-Tek). The primers V9G (de Hoog & Gerrits van den Ende 1998) and LR5 (Vilgalys & Hester 1990) were used to amplify part (ITS) 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 first 900 bases at the 5' end of the 28S rRNA gene (LSU). The primer ITS4 (White et al. 1990) and LR0R (Rehner & Samuels 1994) were used as internal sequence primers to ensure high quality overlapping sequences were obtained. The PCR conditions, sequence alignment and subsequent phylogenetic analysis with gaps treated as missing data followed the methods of Crous et al. (2006c). Sequence data were deposited in GenBank (Table 1) and the alignment and trees in TreeBASE (http://www.treebase.org).

Taxonomy

Wherever possible, 30 measurements (1 000× magnification) were made of structures mounted in lactic acid, with the extremes of spore measurements given in parentheses. Colony

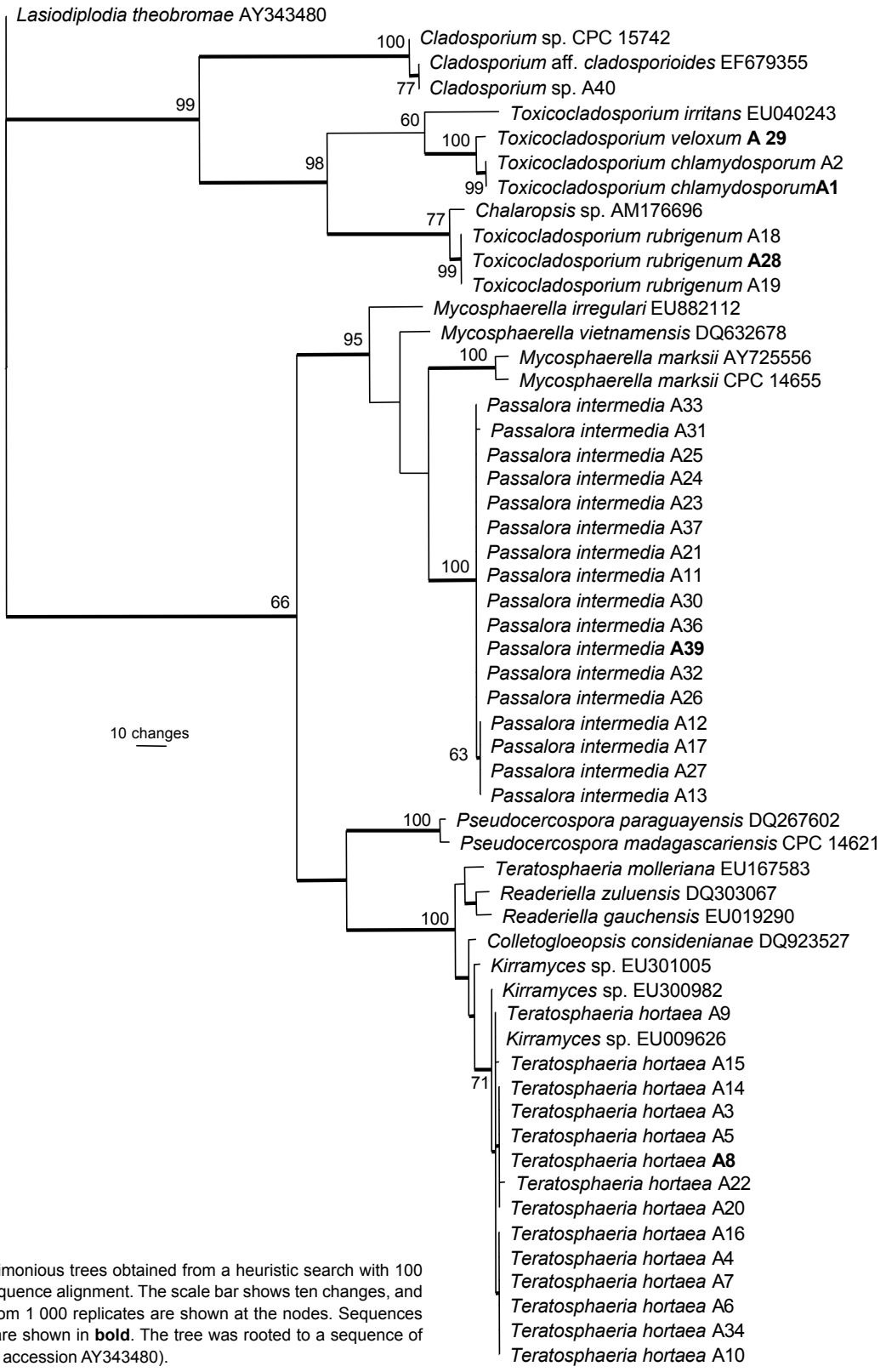

Fig. 1 One of 16 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the ITS sequence alignment. The scale bar shows ten changes, and bootstrap support values (> 59 %) from 1 000 replicates are shown at the nodes. Sequences of ex-type cultures for new species are shown in **bold**. The tree was rooted to a sequence of *Lasiodiplodia theobromae* (GenBank accession AY343480).

colours (surface and reverse) were assessed after 2–8 wk on MEA, OA and PDA at 25 °C in the dark, using the colour charts of Rayner (1970).

RESULTS

DNA phylogeny

Amplicons of ± 1 700 bases were obtained for the isolates listed in Table 1. The ITS and LSU sequences were used to obtain additional sequences from GenBank, which were added to the respective alignments. The manually adjusted ITS alignment contained 56 sequences (including the outgroup sequence) and 530 characters including alignment gaps (matrix available in TreeBASE). Of these, 181 were parsimony informative, 48 were variable and parsimony uninformative and 301 were constant.

Neighbour-joining analyses using three substitution models on the ITS sequence alignment yielded trees with identical topologies and differed from the tree shown in Fig. 1 with regard to the placement of the *Pseudocercospora* clade (data not shown). The parsimony analysis yielded 16 equally most parsimonious trees (TL = 504 steps, CI = 0.696, RI = 0.934,

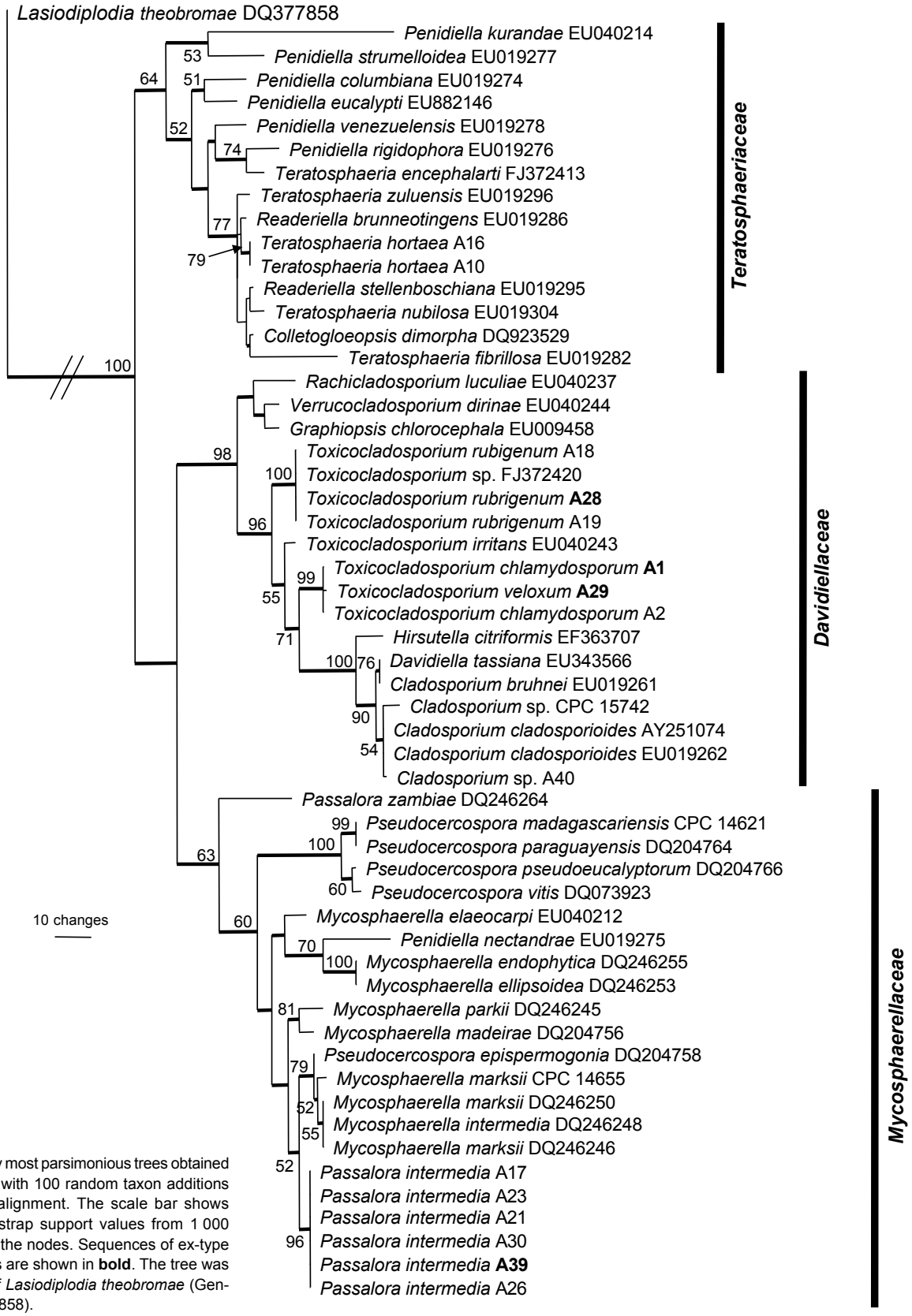

Fig. 2 One of 22 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the LSU sequence alignment. The scale bar shows ten changes, and bootstrap support values from 1 000 replicates are shown at the nodes. Sequences of ex-type cultures for new species are shown in **bold**. The tree was rooted to a sequence of *Lasiodiplodia theobromae* (GenBank accession DQ377858).

RC = 0.651), one of which is presented (Fig. 1). The manually adjusted LSU alignment contained 56 sequences (including the outgroup sequence) and 787 characters including alignment gaps (matrix available in TreeBASE). Of these, 148 were parsimony informative, 83 were variable and parsimony uninformative and 556 were constant.

Neighbour-joining analyses using three substitution models on the LSU sequence alignment, yielded trees with identical topologies and differed from the tree shown in Fig. 2 with regard to the placement of some species within the families and an unresolved ordering of the families (data not shown). The parsimony analysis yielded 22 equally most parsimonious trees (TL = 544 steps, CI = 0.574, RI = 0.848, RC = 0.486), one of which is presented (Fig. 2). The phylogenetic results obtained are discussed where applicable in the descriptive notes below.

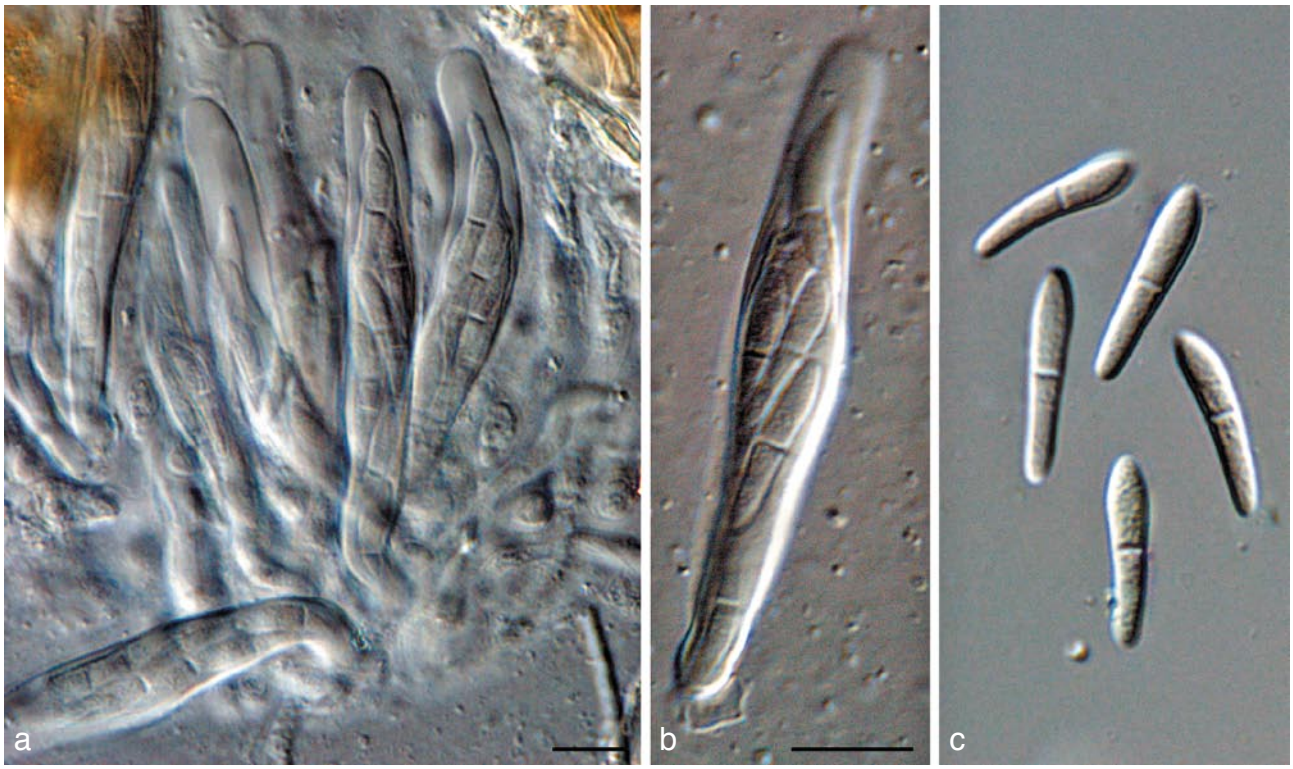

Fig. 3 *Mycosphaerella marksii* (CPC 14655). a. Asci in squashed ascoma; b. single ascus; c. ascospores with typical asymmetrical apical cells. — Scale bars = 10 µm.

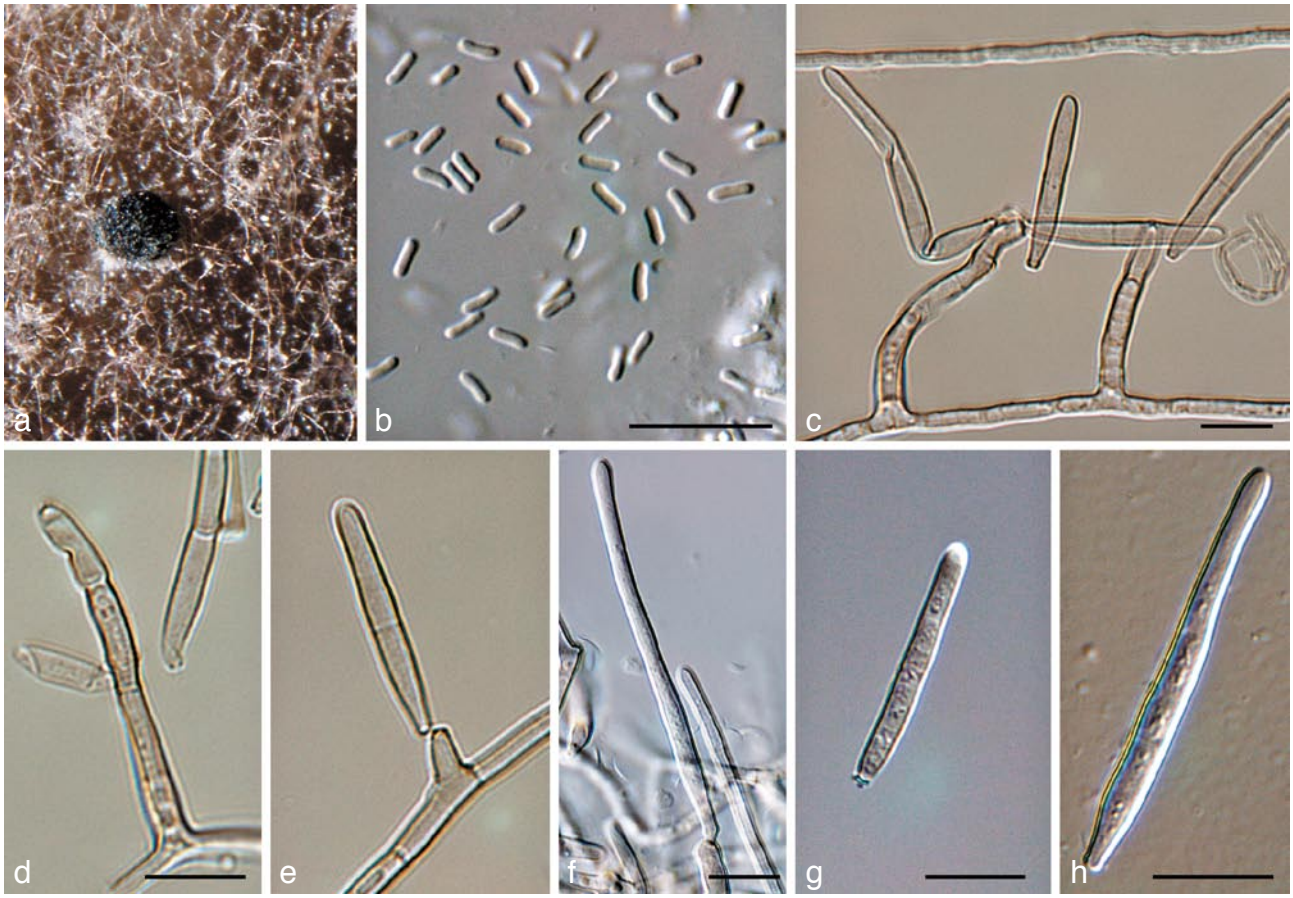

Fig. 4 *Passalora intermedia* (CPC 15745). a. Spermatogonium forming on OA; b. spermatia; c–f. conidiophores giving rise to conidia; g, h. conidia. — Scale bars = 10 µm.

Taxonomy

Several species of capnodialean fungi were isolated from the single *Eucalyptus* leaf lesion on which the present study was based. These are treated below.

Mycosphaerella marksii Carnegie & Keane, Mycol. Res. 98: 414. 1994 — Fig. 3

Description — Carnegie & Keane (1994), Crous (1998).

Specimen examined. Madagascar, Morondavo, on leaves of *Eucalyptus camaldulensis*, Aug. 2007, *M.J. Wingfield*, cultures CPC 14655 = CBS 124153, CPC14656, 14657.

Notes — *Mycosphaerella marksii* is a well-known species pathogenic to *Eucalyptus* (Carnegie & Keane 1994, Crous 1998, Crous et al. 2006e), but also occurring on several other hosts (Arzanlou et al. 2008). Isolates are morphologically variable, and recently Cheewangkoon et al. (2008) delineated *M. pseudomarksii* from this complex, which is presently known only to occur on eucalypts in Thailand.

Passalora intermedia Crous & M.J. Wingf., *sp. nov.* — MycoBank MB509535; Fig. 4

Conidiophoris solitariis, modice brunneis, laevibus, 0–3-septatis, ad 70 µm longis et 4 µm latis. Cellulis conidiogenis terminalibus vel lateralibus, pallide ad modice brunneis, laevibus, 15–20 × 3–3.5 µm; locis fuscatis et inspissatis, 1–1.5 µm latis. Conidiis solitariis, pallide brunneis, laevibus, guttulatis, subcylindricis vel anguste obclavatis, apice subobtuso, basi oblong, obconice subtruncata, 1–8-septatis, (35–)50–75(–100) × (2.5–)3 µm; hilis inspissatis et fuscatis, 1–1.5 µm latis.

Etymology. Name reflects the morphological variability of this species, which is somewhat intermediate between *Pseudocercospora* and *Passalora*.

Conidiophores solitary, medium brown, smooth, 0–3-septate, up to 70 µm tall and 4 µm wide. *Conidiogenous cells* terminal and lateral, pale to medium brown, smooth, 15–20 × 3–3.5 µm; loci darkened and thickened, 1–1.5 µm wide. *Conidia* solitary, pale brown, smooth, guttulate, subcylindrical, becoming narrowly obclavate; apex subobtuse, base long obconically subtruncate, 1–8-septate, (35–)50–75(–100) × (2.5–)3 µm; hila thickened and darkened, 1–1.5 µm wide.

Mycelium pale to medium brown, consisting of septate, branched, smooth, 2–3 µm wide hyphae. *Conidiophores* solitary, arising from superficial mycelium, medium brown, smooth, 0–3-septate, subcylindrical, straight to variously curved or geniculate-sinuous, unbranched or branched, up to 70 µm tall and 4 µm wide. *Conidiogenous cells* terminal and lateral, pale to medium brown, smooth, 15–20 × 3–3.5 µm, with 1–3 thickened and darkened loci, 1–1.5 µm wide, proliferating sympodially. *Conidia* solitary, pale brown, smooth, guttulate, subcylindrical when small, becoming narrowly obclavate when larger, apex subobtuse, base long obconically subtruncate, straight to slightly curved, 1–8-septate, (35–)50–75(–100) × (2.5–)3 µm; hila thickened and darkened, 1–1.5 µm wide; microcyclic conidiation observed in culture. *Spermatogonia* forming on OA. *Spermatia* cylindrical with obtuse ends, smooth, hyaline, 3–5 × 1 µm.

Cultural characteristics — *Colonies* on MEA erumpent, spreading with moderate aerial mycelium and smooth, lobate margins; surface isabelline, margin sepia; reverse sepia to brown-vinaceous; reaching 25 mm diam after 1 mo; on OA spreading with sparse to moderate aerial mycelium, margins feathery; surface olivaceous-grey, with patches of pale olivaceous-grey; colonies reaching 20 mm after 1 mo at 25 °C.

Specimen examined. Madagascar, Morondavo, on leaf of *Eucalyptus camaldulensis*, Aug. 2007, *M.J. Wingfield*, CBS H-20197 holotype, cultures ex-type A39 = CPC 15745 = CBS 124154.

Notes — *Passalora intermedia* has conidial hila that are somewhat thickened and darkened, but not prominently refractive, thus appearing intermediate between *Pseudocercospora* and *Passalora*, though it clusters apart from the *Pseudocercospora* clade. Morphologically, *P. intermedia* is distinct from the *Passalora* species currently known from eucalypts by having longer conidia (Crous 1998, Crous & Braun 2003), and phylogenetically it does not correspond to any taxon presently known from this host.

Pseudocercospora madagascariensis Crous & M.J. Wingf., *sp. nov.* — MycoBank MB509536; Fig. 5

Pseudocercosporae paraguayensis similis, sed conidiis brevioribus, (15–)30–45(–60) × 2(–2.5) µm.

Etymology. Name reflects the Island of Madagascar and the origin of the fungus.

Leaf spots amphigenous, subcircular to circular, 1–2 mm diam, medium brown with sporulation within and adjacent to lesion (endophyte?); also occurring with a species of *Ramichloridium* and *Stenella* on the same spots. *Mycelium* internal and external, pale to medium brown, consisting of septate, branched, smooth hyphae, 1–2.5 µm wide. *Caespituli* fasciculate, amphigenous, medium brown on leaves, up to 50 µm wide and 30 µm high. *Conidiophores* arising singly from superficial mycelium, or aggregated in dense fascicles arising from the upper cells of a brown stroma, up to 30 µm wide and 20 µm high; conidiophores pale to medium brown, smooth, 0–1-septate, subcylindrical, straight to variously curved or geniculate-sinuous, unbranched or branched above, 15–20 × 2–3.5 µm. *Conidiogenous cells* terminal, pale brown, smooth, tapering to flat-tipped apical loci, proliferating sympodially, 10–15 × 2–2.5 µm. *Conidia* solitary, pale brown, smooth, subcylindrical to narrowly obclavate, apex subobtuse, base long obconically subtruncate to truncate, straight to curved, 1–3(–4)-septate, (15–)30–45(–60) × 2(–2.5) µm; hila and scars inconspicuous.

Cultural characteristics — *Colonies* on MEA flat, spreading with moderate aerial mycelium and smooth, lobate margins;

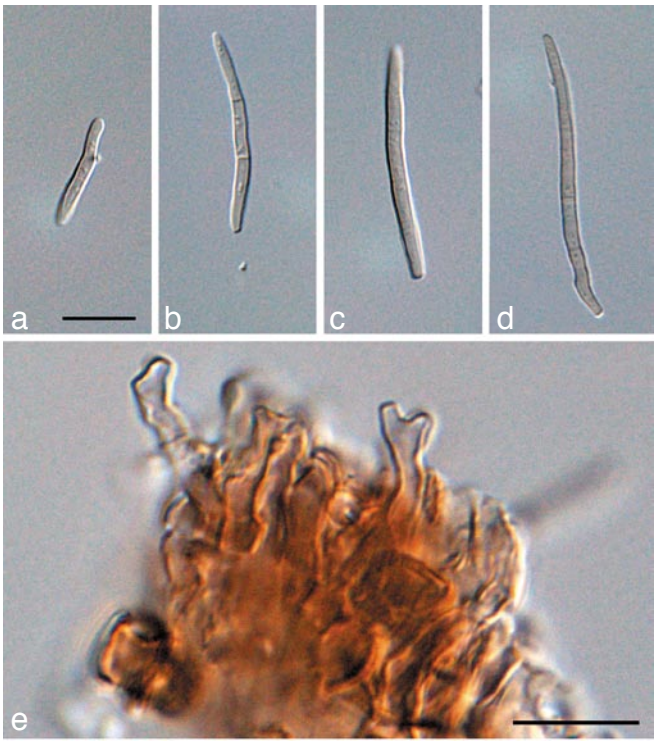

Fig. 5 *Pseudocercospora madagascariensis* (CPC 14621). a–d. Conidia; e. fasciculate conidiophores. — Scale bars = 10 µm.

pale olivaceous-grey with patches of white or olivaceous-grey; reverse iron-grey; reaching 35 mm diam after 1 mo; on OA flat, spreading with moderate aerial mycelium, margins smooth, regular, pale olivaceous-grey, reaching 45 mm after 1 mo.

Specimen examined. Madagascar, Morondavo, on leaf of *Eucalyptus camaldulensis*, Aug. 2007, *M.J. Wingfield*, CBS H-20192 holotype, cultures ex-type CPC 14621 = CBS 124155, CPC 14622.

Notes — Phylogenetically, *P. madagascariensis* is closely related to *M. irregulari* for which no anamorph is known (Cheewangkoon et al. 2008) and *M. vietnamensis* that has a *Pseudocercospora* anamorph (Burgess et al. 2007). Morphologically, it is distinct from *M. vietnamensis*, having narrower conidia, and from the taxa in the *P. paraguayensis* species complex (Crous 1998) due to its shorter conidia.

Teratosphaeria hortaea Crous & M.J. Wingf., *sp. nov*. — MycoBank MB509537; Fig. 6

Cellulis conidiogenis in hyphis usque ad 5 µm longis, locis solitariis, phialidicis, proliferantibus per spissescentem loci, vel cellulis conidiogenis subcylindraceis-ampulliformibus, sympodialiter proliferantibus, 3–5 × 3–4 µm. Conidiis ellipsoideis, pallide ad modice brunneis, apice obtuse rotundato, basi subtruncata, (4–)5–6(–7) × (2–)2.5(–3) µm.

Etymology. Name reflects the morphological similarity of this species to the hyphomycete genus *Hortaea*.

On SNA. *Mycelium* consisting of branched, septate, smooth to finely verruculose, medium brown, 2–3 µm wide hyphae. *Conidiogenous cells* randomly distributed on hyphal cells, with cells becoming septate, up to 5 µm long, and giving rise to a single conidiogenous locus, which can be phialidic (exophiala-like), inconspicuous, with a minute non-flaring collarette, apex 1–1.5 µm wide, giving rise to single conidia (percurrent proliferation not seen, and appears to be via periclinal thickening of the locus); alternatively conidiogenous cells develop on hyphal cells, as subcylindrical to ampulliform, brown, erect cells, that give rise to conidia via sympodial proliferation, 3–5 × 3–4 µm. *Conidia* ellipsoid, pale to medium brown, apex obtusely rounded, widest in middle, tapering towards a subtruncate base, 1 µm wide, (4–)5–6(–7) × (2–)2.5(–3) µm. On MEA conidia become 1-septate, and frequently undergo microcyclic conidiation (percurrently), and in general are darker brown, up to 15 µm long, 5 µm wide, with minute marginal frill, and subtruncate to truncate base.

Cultural characteristics — *Colonies* on MEA erumpent, spreading, lacking aerial mycelium; surface black, appearing crumpled, slimy, with feathery margin; reverse black; reaching 20 mm diam after 1 mo. On OA spreading, with sparse aerial mycelium, and even catenulate margin; surface olivaceous-grey; colonies reaching 25 mm diam after 1 mo at 25 °C. Colonies fertile, with typical black yeast-like growth.

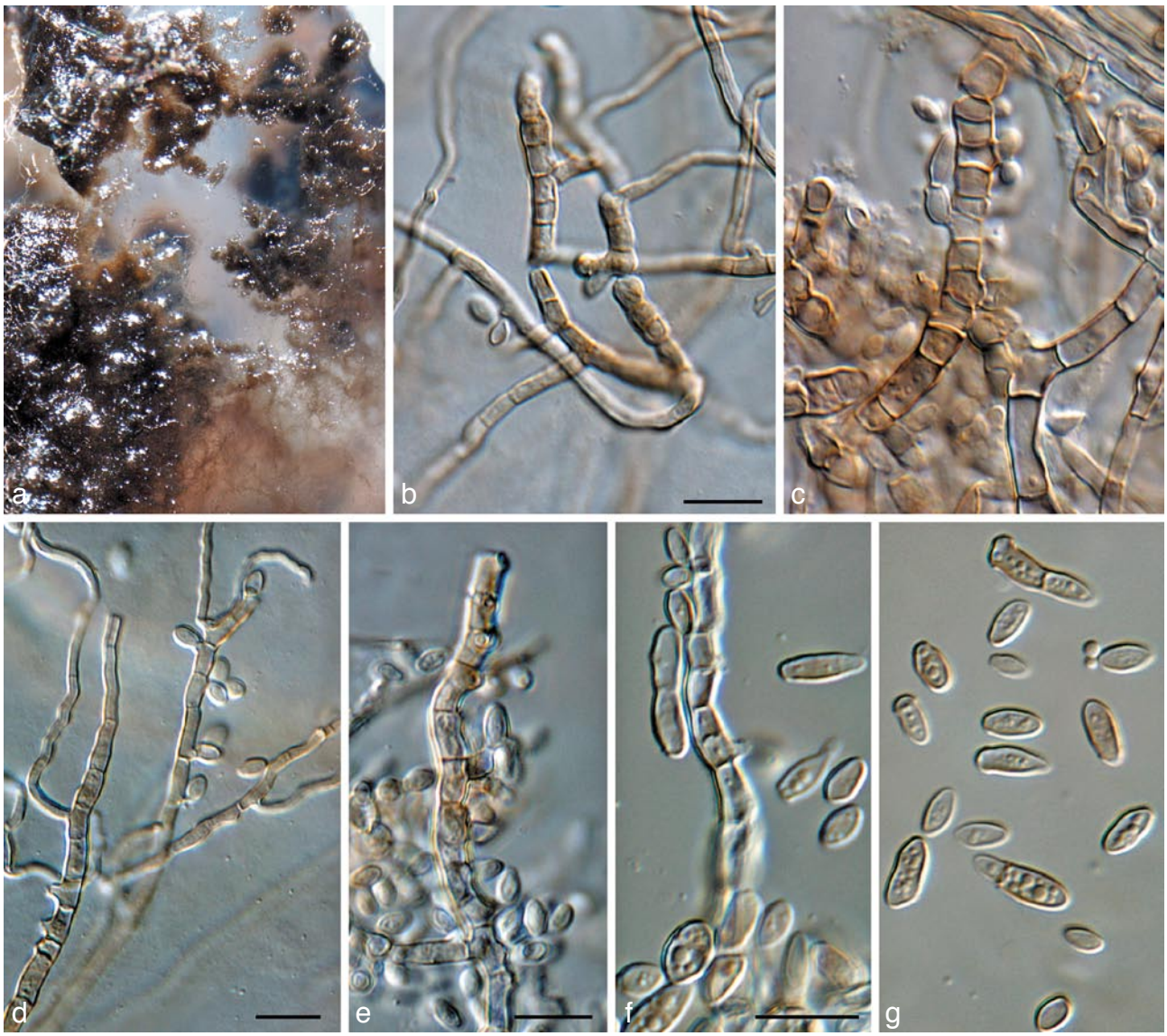

Fig. 6 *Teratosphaeria hortaea* (CPC 15716). a. Colonies on SNA; b–f. hyphae with conidiogenous cells that give rise to conidia via sympodial or percurrent proliferation, in some cases appearing phialidic with periclinal thickening; g. conidia. — Scale bars = 10 µm.

Specimen examined. Madagascar, Morondavo, on leaf of *Eucalyptus camaldulensis*, Aug. 2007, *M.J. Wingfield*, CBS H-20194 holotype, cultures ex-type A8 = CPC 15716 = CBS 124156.

Notes — *Teratosphaeria hortaea* is unusual in that it clusters among *Colletogloeopsis*/*Kirramyces* coelomycetes (Crous et al. 2009), but represents a hyphomycete. Furthermore, although species of *Colletogloeopsis* have been observed to have conidiogenous cells that proliferate percurrently or sympodially, *T. hortaea* appears to have sympodial proliferation, and phialides that proliferate percurrently, or are reminiscent of *Hortaea* or *Rhizosphaera*. Although these genera belong to the *Dothideomycetes*, they do not cluster among anamorphs of *Teratosphaeria*, suggesting that if the fungus were to be defined based on its anamorph state, a new genus would have to be proposed to accommodate it.

Toxicocladosporium chlamydosporum Crous & M.J. Wingf., *sp. nov*. — MycoBank MB509538; Fig. 7

Toxicocladosporio irritanti simile, sed ramoconidiis majoribus, (15–)16–17(–18)× (2.5–)3–4 µm, et conidiis intercalaribus longioribus et angustioribus, (8–)9–10(–11) × 3(–3.5) µm.

Etymology. Name reflects the conspicuous chlamydospores formed in culture.

On SNA. *Mycelium* consisting of branched, septate, smooth, brown, 2–3 µm wide hyphae, containing swollen, globose, dark brown chlamydospore-like cells up to 12 µm diam. *Conidiophores* dimorphic. *Macronematous conidiophores* solitary, erect, arising from superficial mycelium, penicillate, subcylindrical, straight to once geniculate-sinuous, medium brown, smooth, 20–45 µm long, 3–4 µm wide at base, which is not

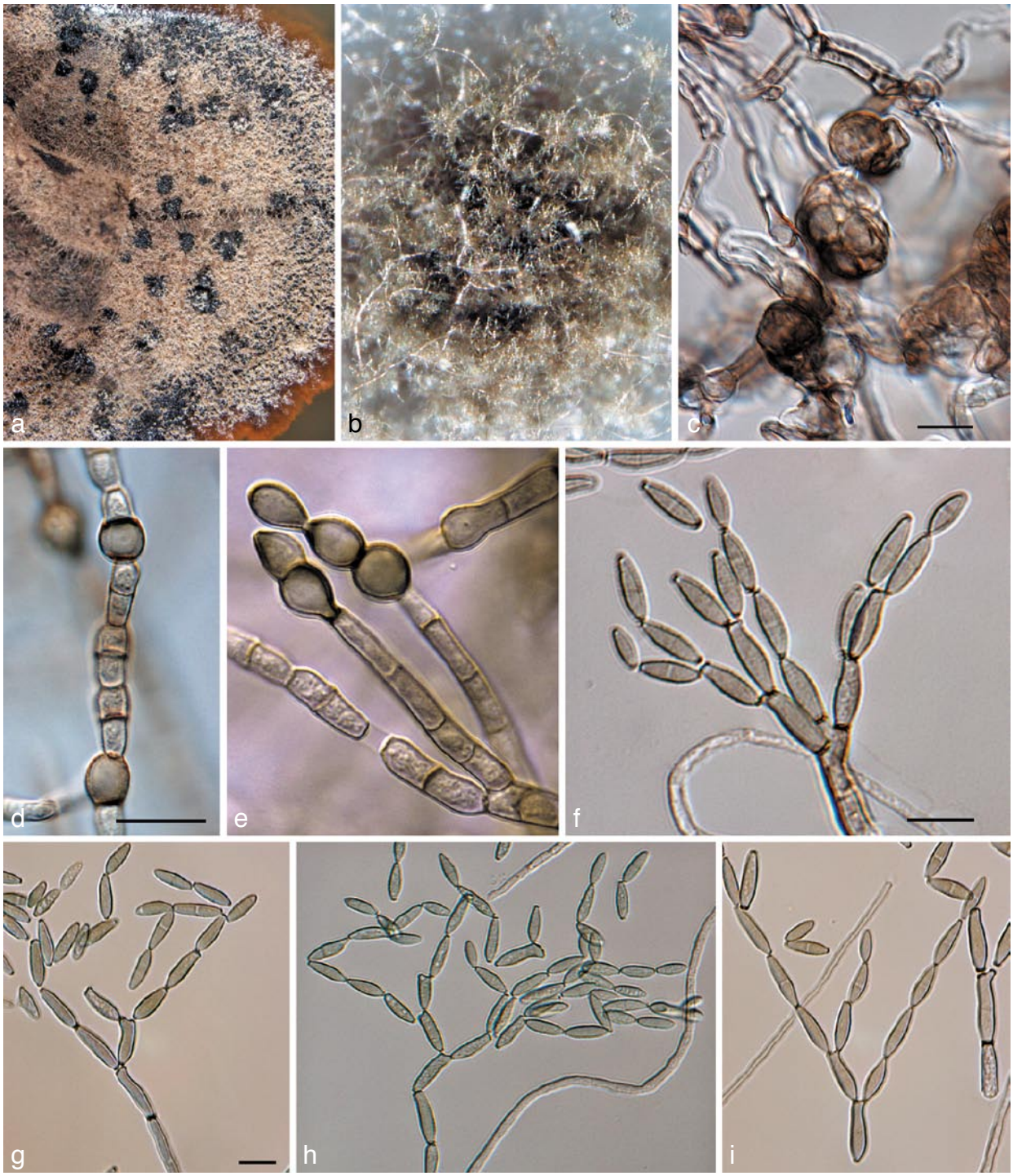

Fig. 7 *Toxicocladosporium chlamydosporum* (CPC 15709). a. Colony forming black sclerotial structures on MEA; b. sporulation on OA; c–e. microsclerotia and chlamydospore-like cells formed in culture; f–i. conidiophores with penicillate heads of branched conidial chains. — Scale bars = 10 µm.

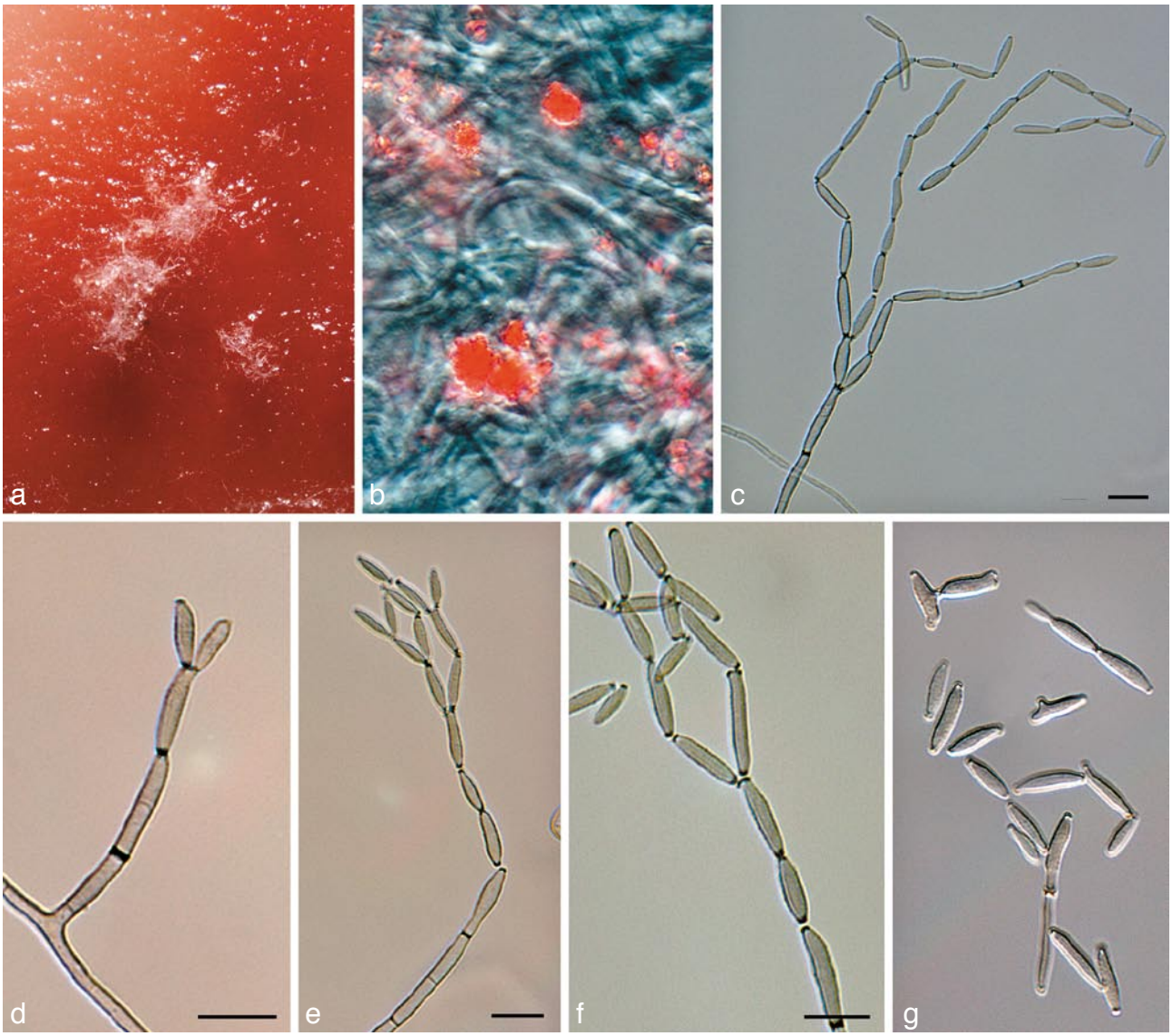

Fig. 8 *Toxicocladosporium rubrigenum* (CPC 15728). a. Typical red colony on OA; b. red crystals formed in colonies on SNA; c. macroconidiophore with chains of branched conidia; d–f. microconidiophores with catenulate conidia; g. conidia. — Scale bars = 10 µm.

swollen, and lacks rhizoids, up to 4-septate. *Micronematous conidiophores* erect, subcylindrical, up to 15 µm tall and 5 µm wide, 0–1-septate, medium brown. *Conidiogenous cells* terminal, integrated, subcylindrical, medium brown, 10–25 × 3–4 µm, smooth; loci flat tipped, thickened, darkened, 1–2 µm wide. *Conidia* in branched chains, brown, smooth to finely verruculose, ellipsoid to cylindrical-oblong. *Ramoconidia* rarely observed, 0–1-septate, fusoid-ellipsoidal to subcylindrical, (15–)16–17(–18) × (2.5–)3–4 µm. *Secondary ramoconidia* 0–1-septate, fusoid-ellipsoidal, (9–)10–14(–16) × (2.5–)3–4 µm. *Intercalary conidia* 0–1-septate, fusoid-ellipsoidal, (8–)9–10(–11) × 3(–3.5) µm. *Terminal conidia* aseptate, fusoid-ellipsoidal, 6–7(–9) × 2.5(–3) µm (conidia dark brown and verruculose on MEA).

Cultural characteristics — *Colonies* on MEA erumpent, spreading, with sparse aerial mycelium; surface irregular and sectored, with feathery margin, centre fuscous-black, outer region greyish sepia; reverse dark mouse grey; reaching 15 mm diam after 1 mo. Huge black sclerotial bodies are observed on MEA, consisting of an agglomeration of chlamydospore-like cells; they remain sterile, and eventually resemble hollow fruiting bodies, though they lack an ostiole or defines wall. On OA spreading, with sparse aerial mycelium, and even catenulate margin; surface iron-grey with patches of pale olivaceous-grey; colonies reaching 15 mm diam after 1 mo at 25 °C. Colonies fertile.

Specimen examined. Madagascar, Morondavo, on leaf of *Eucalyptus camaldulensis*, Aug. 2007, *M.J. Wingfield*, CBS H-20193 holotype, cultures ex-type A1 = CPC 15709 = CBS 124157.

Notes — The genus *Toxicocladosporium* is presently known from a single species, *T. irritans*, isolated from mouldy paint in Suriname (Crous et al. 2007a). *Toxicocladosporium irritans* also has dimorphic conidiophores, and conidial loci and hila that are thickened and darkened. *Toxicocladosporium chlamydosporum* is distinct from *T. irritans* having larger ramoconidia, and longer, narrower intercalary conidia, and by the fact that it forms chlamydospores and sclerotial bodies in culture.

Toxicocladosporium rubrigenum Crous & M.J. Wingf., *sp. nov*. — MycoBank MB509539; Fig. 8

Toxicocladosporio irritanti simile, sed conidiophoris penicillatibus, dense ramosis, et coloniis in OA cum pigmento conspicue rubro.

Etymology. Name reflects a red pigment produced in oatmeal agar colonies.

On SNA. *Mycelium* consisting of branched, septate, smooth, hyaline to pale brown, 1.5–2 µm wide hyphae. *Conidiophores*

dimorphic. *Macronematous conidiophores* solitary, erect, arising from superficial mycelium, terminally densely penicillate, subcylindrical, straight to curved, medium brown, smooth, up to 100 µm long, 2–4 µm wide at base, which is not swollen, and lacks rhizoids, up to 8-septate. *Micronematous conidiophores* erect, subcylindrical, up to 30 µm tall and 2–3 µm wide, 0–1-septate, medium brown. *Conidiogenous cells* predominantly terminal, integrated, subcylindrical, medium brown, 15–20 × 2.5–3 µm, smooth; loci flat tipped, thickened, darkened, 0.5–1 µm wide. *Conidia* in densely branched chains, medium brown, smooth, ellipsoid to cylindrical-oblong, aseptate; hila darkened, thickened, 0.5–1 µm wide. *Ramoconidia* (13–)14–15(–16) × 2.5–3(–3.5) µm. *Secondary ramoconidia* (9–)10–12(–14) × 2.5–3(–3.5) µm. *Intercalary conidia* 7–8(–9) × 2(–2.5) µm. *Terminal conidia* (4–)6–7 × 2(–2.5) µm.

Cultural characteristics — *Colonies* on MEA erumpent, spreading, with sparse aerial mycelium; surface sectored, with feathery margin, centre pale olivaceous-grey, outer region olivaceous-grey; reverse fuscous-black to greyish sepia; reaching 20 mm diam after 1 mo. On OA spreading, with sparse aerial mycelium, and even catenulate margin; surface red, with patches of vinaceous; colonies reaching 25 mm diam after 1 mo at 25 °C. Colonies fertile.

Specimen examined. Madagascar, Morondavo, on leaf of *Eucalyptus camaldulensis*, Aug. 2007, *M.J. Wingfield*, CBS H-20195 holotype, cultures ex-type A28 = CPC 15735 = CBS 124158.

Notes — *Toxicocladosporium rubrigenum* produces densely branched penicillate conidiophores, and colonies that form a prominent red pigment on OA, which are characteristics distinct from other species in the genus.

Toxicocladosporium veloxum Crous & M.J. Wingf., *sp. nov*. — MycoBank MB509540; Fig. 9

Toxicocladosporio chlamydosporo simile, sed chlamydosporis nullis, coloniis in vitro celeriter crescentibus, et conidiis atriore brunneis et majoribus, (8–)9–10 × 2(–2.5) µm.

Etymology. Named after its rapid growth in culture.

On SNA. *Mycelium* consisting of branched, septate, smooth to verruculose, hyaline to medium brown, 2.5–3 µm wide hyphae. *Conidiophores* solitary, erect, arising from superficial mycelium, straight to once geniculate-sinuous, medium to dark brown, smooth to finely verruculose, 30–60 × 3–5 µm, 1–4-septate, forming a loose penicillate head. *Conidiogenous cells* terminal, integrated, subcylindrical, straight, 10–25 × 3–4 µm, medium brown, smooth to finely verruculose; loci terminal and lateral, thickened, darkened, at times subdenticulate, 0.5–1 µm wide. *Conidia* in branched chains, brown, smooth to finely verruculose, ellipsoid to cylindrical-oblong. *Ramoconidia* rarely observed, 0–1-septate, fusoid-ellipsoidal to subcylindrical, (15–)16–17(–18) × (2.5–)3–4 µm. *Secondary*

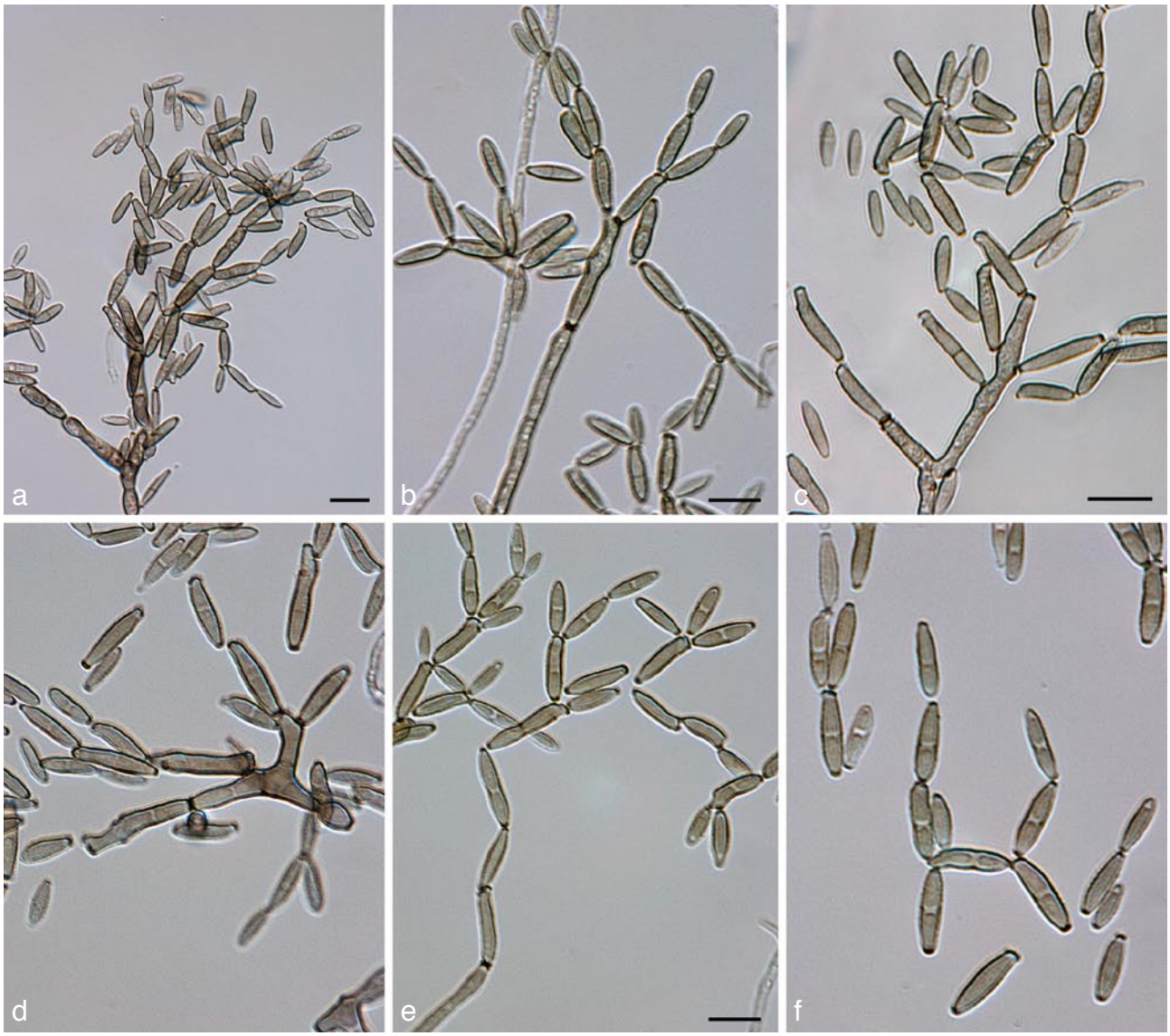

Fig. 9 *Toxicocladosporium veloxum* (CPC 15729). a–d. Conidiophores giving rise to branched conidial chains; e, f. ramo- and intermediate conidia. — Scale bars = 10 µm.

ramoconidia 0–1-septate, fusoid-ellipsoidal, (11–)12–14(–15) × (2.5–)3–4 µm. *Intercalary conidia* 0–1-septate, fusoid-ellipsoidal, (9–)10–11(–12) × 2.5(–3) µm. *Terminal conidia* aseptate, fusoid-ellipsoidal, (8–)9–10 × 2(–2.5) µm.

Cultural characteristics — *Colonies* on MEA erumpent, spreading with sparse aerial mycelium; surface folded, with feathery margin, centre pale olivaceous-grey, outer region olivaceous-grey; reverse iron-grey; reaching 25 mm diam after 1 mo. On OA spreading, flat, with sparse aerial mycelium, and even catenulate margin; surface iron-grey with patches of smoke-grey; colonies reaching 30 mm diam after 1 mo at 25 °C. Colonies fertile, lacking sclerotial bodies.

Specimen examined. Madagascar, Morondavo, on leaf of *Eucalyptus camaldulensis*, Aug. 2007, *M.J. Wingfield*, CBS H-20196 holotype, cultures ex-type A29 = CPC 15736 = CBS 124159.

Notes — Compared with *Toxicocladosporium chlamydosporum*, conidia of *T. veloxum* are darker brown and somewhat larger. Colonies also lack chlamydospores, grow faster in culture, and they are not as darkly pigmented as those in *T. chlamydosporum*.

DISCUSSION

Results of this study revealed a remarkable number of fungi, including an equally surprising number of new taxa, occurring within a single small lesion on a *Eucalyptus* leaf. The fact that members of the *Capnodiales* can co-occur on the same lesion is well known (Crous 1998, Burgess et al. 2007, Crous et al. 2007a, c, d, 2008a, b), although the mechanisms allowing them to occupy the same niche is not understood, and may be related to their ability to produce similar toxins (Harelimana et al. 1997, Yun et al. 1998). Species of these fungi occurring on a defined substrate could be opportunists not necessarily on their ideal host. This would not be unusual as these fungi have been collected from very diverse habitats including the surfaces of rocks (Ruibal et al. 2008). It is also entirely possible that one of the fungi on the lesion studied had a level of pathogenicity allowing the initial development of the spot and that the other fungi either invaded the dead tissue as saprobes, or they could have been endophytes in previously asymptomatic tissue (Crous & Wingfield 1996, Crous 1998, Ganley et al. 2004, Verkley et al. 2004).

The present study has revealed the presence of at least 11 species of capnodialean fungi in a single leaf spot. To the best of our knowledge, there are no prior studies reflecting this remarkable number of taxa in a single lesion on *Eucalyptus*. This phenomenon might be relatively common and this would imply that many species have been overlooked in studies concerning fungi associated with *Eucalyptus* leaf spots. These results would justify studies of the entire fungal community of single lesions on different *Eucalyptus* spp. from different parts of the world.

Although the ascomata of *M. marksii* were prominent in the lesion considered in this study, this species is unrelated to *Pseudocercospora madagascariensis*, which occurred intermingled with conidiophores of two species of *Cladosporium*, three species of *Toxicocladosporium*, one species of *Teratosphaeria*, and *Passalora*, respectively. Furthermore, attempts to culture a species of *Ramichloridium* and one species of *Stenella* were unsuccessful (Fig. 10), while an unidentified coelomycete and species of *Pestalotiopsis* were also found in the lesion. It is, therefore, possible that a more rigorous isolation technique such as extinction plating (Collado et al. 2007) might have yielded more taxa than the already large number that emerged from this study.

The two species of *Cladosporium* isolated belong to the *C. cladosporioides* species complex. Resolving species in the latter complex has proven to be more difficult than in *C. herbarum* (Schubert et al. 2007b) or *C. sphaerospermum* (Zalar et al. 2007, Dugan et al. 2008), which also included numerous undescribed taxa. A study is presently underway to elucidate this complex, and thus the *Eucalyptus* isolates must await further treatment.

This study does not represent the first species of fungi described from *Eucalyptus* leaves in Madagascar. A previous study of *Eucalyptus* leaf fungi by Crous & Swart (1995) revealed several capnodialean fungi including *T. suttonii* (*Kirramyces epicoccoides*), *Pseudocercospora eucalyptorum* and *Mycosphaerella heimii* (*Pseudocercospora heimii*). The present study arose from an observation that a single lesion on an *E. camaldulensis* leaf harboured an unusually large number of fungi. The results of this study and that of Crous & Swart (1995) suggest that many more species of fungi are likely to occur on *Eucalyptus* in Madagascar. Consequently, a systematic survey of the fungi including pathogens on these trees in that country is likely to be mycologically productive. Numerous native *Myrtaceae* also occur in Madagascar and it would be interesting to compare leaf fungi on these trees with those occurring on introduced *Eucalyptus* spp.

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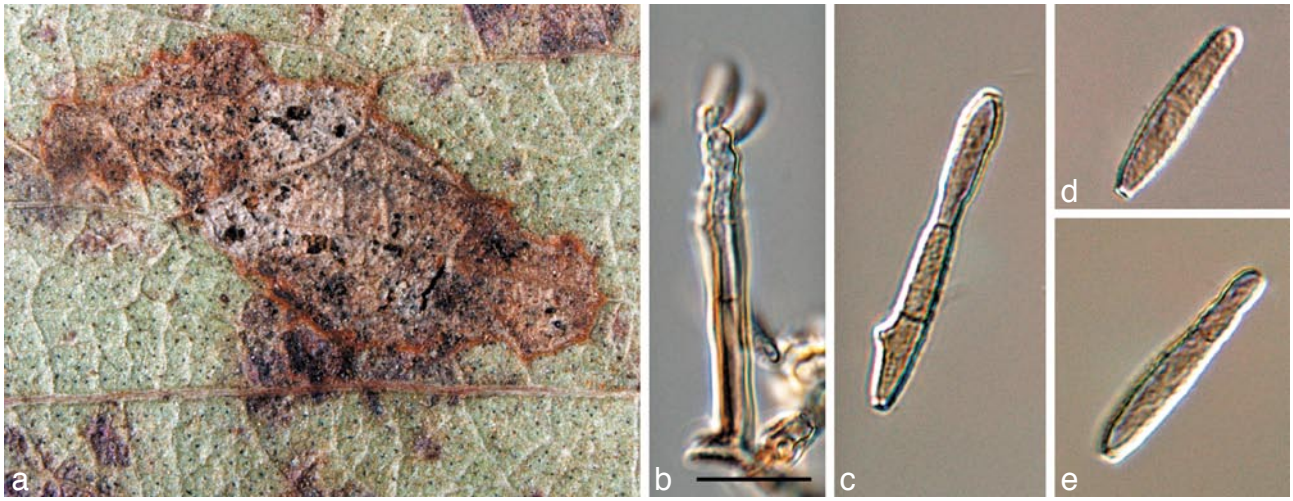

Fig. 10 a. Single leaf spot examined in this study; b, c. unidentified species of *Ramichloridium* and *Stenella*, respectively. — Scale bar = 10 µm.

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