

Fungi associated with black mould on baobab trees in southern Africa

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Abstract There have been numerous reports in the scientific and popular literature suggesting that African baobab (*Adansonia digitata*) trees are dying, with symptoms including a black mould on their bark. The aim of this study was to determine the identity of the fungi causing this black mould and to consider whether they might be affecting the health of trees. The fungi were identified by sequencing directly from mycelium on the infected tissue as well as from cultures on agar. Sequence data for the ITS region of the rDNA resulted in the identification of four fungi including *Aureobasidium pullulans*, *Toxicocladosporium irritans* and a new species of *Rachicladosporium* described here as *Rachicladosporium africanum*. A single isolate of an unknown *Cladosporium* sp. was also found. These fungi, referred to here as black

mould, are not true sooty mould fungi and they were shown to penetrate below the bark of infected tissue, causing a distinct host reaction. Although infections can lead to dieback of small twigs on severely infected branches, the mould was not found to kill trees.

Keywords *Adansonia* · *Aureobasidium* · *Rachicladosporium* · Sooty mould

Introduction

There have been various reports of baobab (*Adansonia digitata*) trees covered with black mould on their branches and stems and in some cases it has been suggested that this might be linked to death of these iconic trees (Guy 1971; Pearce et al. 1994; Wickens and Lowe 2008). This condition has commonly been referred to as “sooty mould” in the literature (Pearce et al. 1994). The infection appears to begin as orange-brown spots, mostly on the undersides of branches. The spots subsequently turn black and can coalesce to form larger patches (Pearce et al. 1994). Fallen twigs covered with the black fungus have commonly been found on the ground below severely affected trees in Zimbabwe (Pearce et al. 1994) and were presumed to have died due to the black mould infection. The fungus collected between 1989 and 1991 from the black stems of trees in Zimbabwe was identified as an *Antennulariella* sp. (Pearce et al. 1994) and it was concluded

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that this condition was mainly due to long-term environmental stresses and that the sooty mould was a secondary infection.

Sharp (1993) reported instances of “sooty mould” in Malawi, South Africa, Zambia and Zimbabwe. These reports, including observations made over an approximately 10-year-period, were closely associated with more than a decade of drought (Sharp 1993). In 1991, an article in *New Scientist* (Anonymous 1991) reported that an unknown black fungus was colonising the branches and trunks of apparently healthy baobab trees in South Africa and Zimbabwe. It was speculated that the fungi started growing on the trees after rain had ended the drought of the previous decade.

The term “sooty mould” refers to fungi that grow on the exudates of insects living on plants, but are also able to grow without these exudates (Chomnunti et al. 2014; Hughes 1976). These fungi do not penetrate through the epidermis of the host plants. In extreme cases, the extensive growth of the fungi on leaves can reduce the photosynthetic ability of the plants, but the fungi do not have a direct interaction with the plant cells that would lead to a physiological response from the plants (Chomnunti et al. 2014; Hughes 1976).

The taxonomy of the sooty moulds is complicated by the fact that they occur in complexes of up to eight different species in one sample (Hughes 1976). Although most sooty mould fungi belong to the family Capnodiaceae, members of the Antennulariellaceae, Cladosporiaceae, Coccodiniaceae, and Metacapnodiaceae (all Capnodiales) also include sooty mould fungi. Furthermore, not all fungi involved in sooty mould complexes reside in this order. For example, *Aureobasidium pullulans* (Dothideales) often forms part of sooty mould complexes (Chomnunti et al. 2014; Hughes 1976; Mirzwa-Mróz and Winska-Krysiak 2011).

Black fungal growth on the stems and branches of baobab trees was the most prevalent symptom found during a recent survey of baobab health in southern Africa. Trees at about 70 % of the sites surveyed had some level of black mould and at most of these sites, this was restricted to isolated patches on the main branches and stem (Fig. 1a). However, in some cases, the trees were extensively covered in black fungal growth, from the main stem to the tips of the branches (Fig. 1b). The aim of this study was to identify the

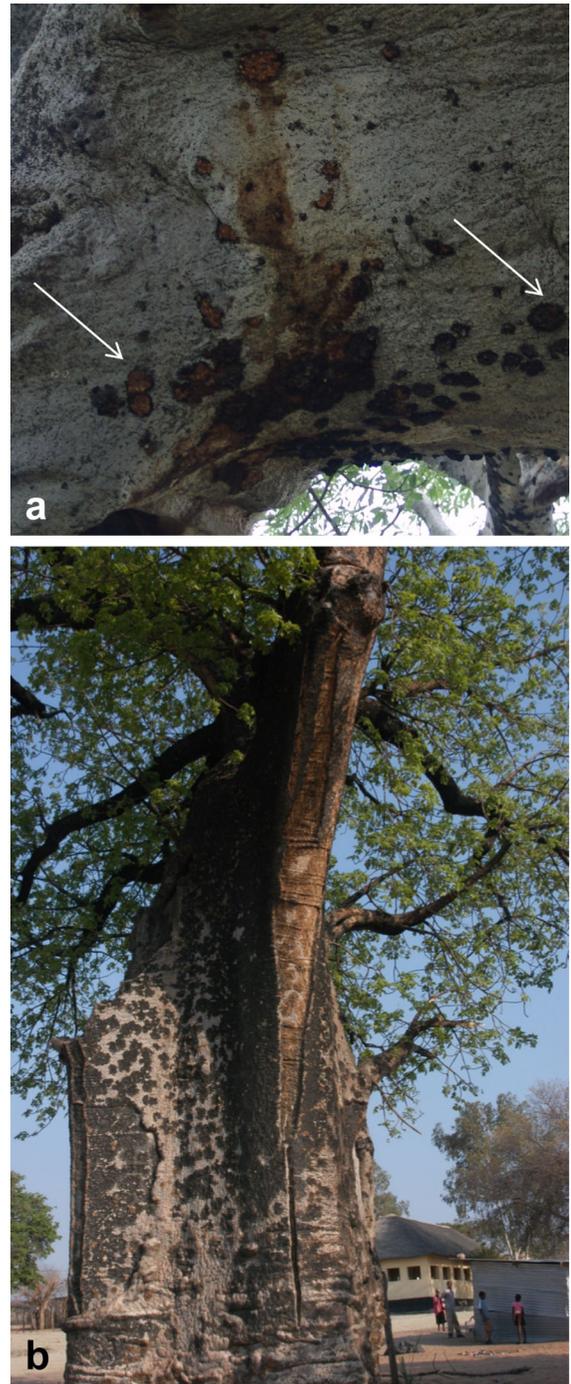


Fig. 1 **a** Baobab branch with patches of black mould starting to grow (arrows), **b** baobab tree covered in black mould from main stem to top branches

fungi causing the black mouldy growth on baobab stems and twigs and to study the extent to which they infect plant tissue.

Materials and methods

Isolation and DNA extraction

Branch and bark tissue was collected from four trees infested with black mould in the Venda area of the Limpopo Province of South Africa. Fungal isolations were made onto 2 % malt extract agar (MEA: 20 g malt extract Biolab, Merck, Midrand, South Africa; 15 g agar Biolab, Merck; 1000 mL dH₂O) amended with streptomycin. Cultures were purified by transferring hyphal tips to clean plates of 2 % MEA. Small pieces of fungal tissue were also removed from the bark and placed in Eppendorf tubes for direct DNA extraction. The Eppendorf tubes were placed in a microwave oven for 1 min at 100 % power after which 5 µL of SABAX water was added and mixed. The tubes were then centrifuged at 13,000 rpm for 1 min and the supernatant was used directly in PCR reactions. For DNA sequence-based identification of isolated fungi, cultures were grown at 25 °C and mycelium was scraped from the surface and freeze dried. DNA extractions were done using the method described by Möller et al. (1992).

PCR amplification, sequencing and analyses

PCR amplification of the ITS1 and ITS2 regions, and spanning the 5.8S gene, of the ribosomal DNA was done with primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). PCR reaction mixtures consisted of 1.5 U MyTaqTM DNA Polymerase (Bio-line, London, UK), 5 µL MyTaq PCR reaction buffer and 0.2 mM of each primer (made up to total volume of 25 µL with SABAX water). PCR conditions were 2 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C and 1 min at 72 °C, and finally one cycle of 8 min at 72 °C. PCR products were visualised with GelRed (Biotium, Hayward, California, USA) on 1 % agarose gels and PCR products were purified with the Zymo research DNA clean & concentratorTM- 5 kit (California, USA).

PCR fragments for each gene region were sequenced using the forward and reverse primers mentioned above. The ABI Prism[®] Big DyeTM Terminator 3.0 Ready Reaction Cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA) was used for the sequencing PCR. Sequences were determined with an ABI PRISMTM 3100 Genetic Analyzer

(Applied Biosystems). DNA sequences of opposite strands were edited and consensus sequences obtained using CLC Main workbench v6.1 (CLC Bio, www.clcbio.com).

BLAST searches were conducted on the NCBI (<http://www.ncbi.nlm.nih.gov>) database with the consensus sequences and closely related sequences downloaded for subsequent data analyses. Datasets were aligned in MEGA5 using the Muscle algorithm and manually adjusted where necessary. jModeltest v2.1.3 with the Akaike Information Criterion (AIC) (Darriba et al. 2012; Guindon and Gascuel 2003) was used to determine the best substitution model for each dataset and Maximum Likelihood (ML) analyses were conducted with PhyML v3.0 (Guindon and Gascuel 2003). Consensus trees were generated with the *consense* option in PHYLIP v3.6 (Felsenstein 2005). Sequences of representative isolates were deposited in GenBank.

Microscopic characterization of infection

Freehand sections of the branches covered with black mould were made to observe the interface between plant and fungal material and to determine whether the fungus could penetrate the plant tissue. The sections were examined using a Zeiss Axioscop 2 Plus compound microscope and images were captured using a Zeiss Axiocam MRc digital camera using Axiovision v4.8.3 (Carl Zeiss Ltd., Germany) software.

Morphology

Colonies were established on Petri dishes containing 2 % MEA and oatmeal agar (OA; 20 g oats, boiled and filtered, with 20 g agar added and made up to 1000 mL with dH₂O), and incubated at 25 °C under continuous near-ultraviolet light to promote sporulation. Morphological observations were made with a Zeiss Axioscop 2 Plus compound microscope using differential interference contrast (DIC) illumination and images captured using the same camera and software mentioned above. Colony characters and pigment production were noted after 2 week of growth on MEA and OA incubated at 25 °C. Colony colours (surface and reverse) were rated according to the colour charts of Rayner (1970). Morphological descriptions were based on cultures sporulating on OA and taxonomic

novelties as well as metadata were deposited in MycoBank (www.MycoBank.org).

Results

Species identification

Numerous cultures (Table 1) with dark-coloured mycelium were obtained from the black mould-infested tissue. These were categorised in cladosporium-like and aureobasidium-like groups based on culture morphology.

DNA sequencing revealed that most of the aureobasidium-like isolates were *A. pullulans*, while one isolate grouped with an undescribed species in this genus (Fig. 2). The cladosporium-like isolates grouped in three different clades (Fig. 3). The largest of these groups clustered most closely to *Rachicladosporium*

americanum, but formed a distinct clade with high bootstrap support (95 %). The second group of isolates clustered with *Toxicocladosporium irritans* (84 % bootstrap support). A single isolate grouped with *Cladosporium cladosporioides*, *C. tenuissimum* and *Cladosporium oxysporum*. Because only a single isolate of this fungus was recovered, it was not subjected to further study.

Characterization of infection

Orange-coloured lesions were observed on branches with fresh black mould infection (Fig. 4a). Blackened galls could also be seen on branches with older infections (Fig. 4b). Some of the smaller branches with heavy infection and many galls were dead.

Where tree-fungus interactions were considered, sections through healthy and black mould infected branches were compared. Healthy branches had

Table 1 Isolates obtained from black mould on baobab trees in South Africa

Name	CMW no. ^a	Other no. ^{b,c}	Herbarium ^d	Collected by	Isolated by	ITS
<i>Aureobasidium pullulans</i>		CPC 21225		EM Cruywagen	PW Crous	KP662097
		CPC 21233		EM Cruywagen	PW Crous	KP662099
		CPC 21210		EM Cruywagen	PW Crous	KP662100
		CPC 21205		EM Cruywagen	PW Crous	KP662101
		SM1.4c_SA		EM Cruywagen	EM Cruywagen	KP662103
		SM1.5c_SA		EM Cruywagen	EM Cruywagen	KP662104
		SM1.6c_SA		EM Cruywagen	EM Cruywagen	KP662105
		SM2.3c_SA		EM Cruywagen	EM Cruywagen	KP662106
		SM3.1c_SA		EM Cruywagen	EM Cruywagen	KP662107
<i>Aureobasidium</i> sp.		CPC 21235		EM Cruywagen	PW Crous	KP662098
<i>Rachicladosporium africanum</i>	CMW 39098	CPC 21201		EM Cruywagen	PW Crous	KP662108
	CMW 39097	CPC 21214		EM Cruywagen	PW Crous	KP662109
	CMW 39099	SM1.1		EM Cruywagen	EM Cruywagen	KP662110
	CMW 39100	CBS 139400	PREM 61153	EM Cruywagen	EM Cruywagen	KP662111
		SM1.2_SA		EM Cruywagen	EM Cruywagen	KP662112
<i>Toxicocladosporium irritans</i>		SM1.3_SA		EM Cruywagen	EM Cruywagen	KP662113
		SM3_SA		EM Cruywagen	EM Cruywagen	KP662115
	CMW 39101	CPC 21221		EM Cruywagen	PW Crous	KP662116
<i>Cladosporium</i> sp.	CMW 39102	CPC 21231		EM Cruywagen	PW Crous	KP662117
		CPC 21209		EM Cruywagen	PW Crous	KP662118

^a CMW Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa

^b CPC Culture collection of Pedro Crous, housed at CBS

^c CBS Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

^d PREM National Collection of Fungi, Pretoria, South Africa

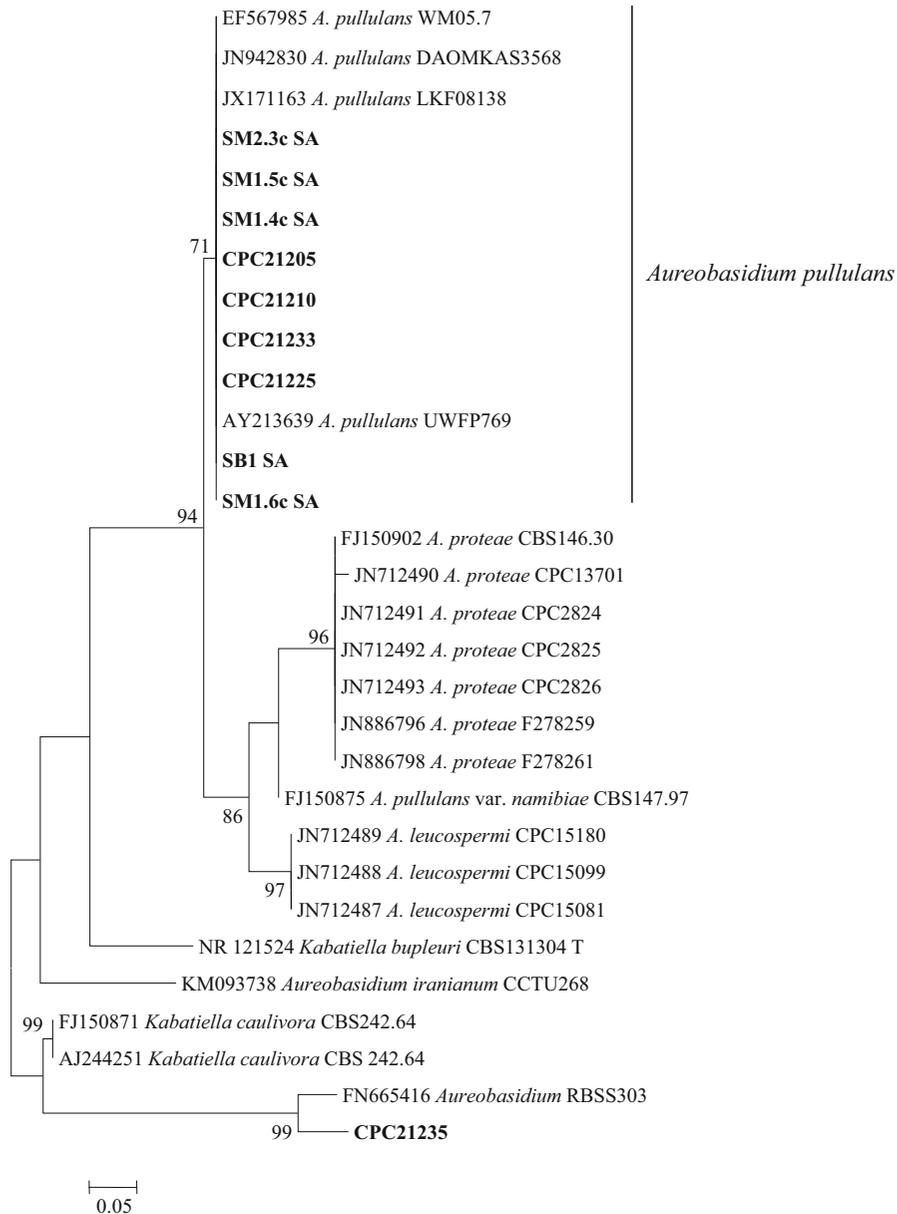


Fig. 2 Midpoint rooted ML tree of aureobasidium-like isolates based on ITS sequence data with isolate numbers of sequences obtained in this study printed in *bold* type. Sequence with SB number was obtained by direct sequencing from plant material

cream-coloured xylem tissue (Fig. 4c) while sections through infected branch tissue showed brown discoloration and wood malformation (Fig. 4d). A single thin layer of brown bark (Fig. 4e) was present in healthy branches while newly infected branches showed evidence of new bark tissue being produced to exclude the fungus (Fig. 4f). Successive layers of bark with fungal material between them were found where sections were made through the black galls (Fig. 4g),

apparently representing a strong host response to infection. Fungal structures penetrating the wood tissues were also observed (Fig. 4h).

Taxonomy

Based on differences in morphology and ITS sequences, the *Rachicladosporium* isolates from

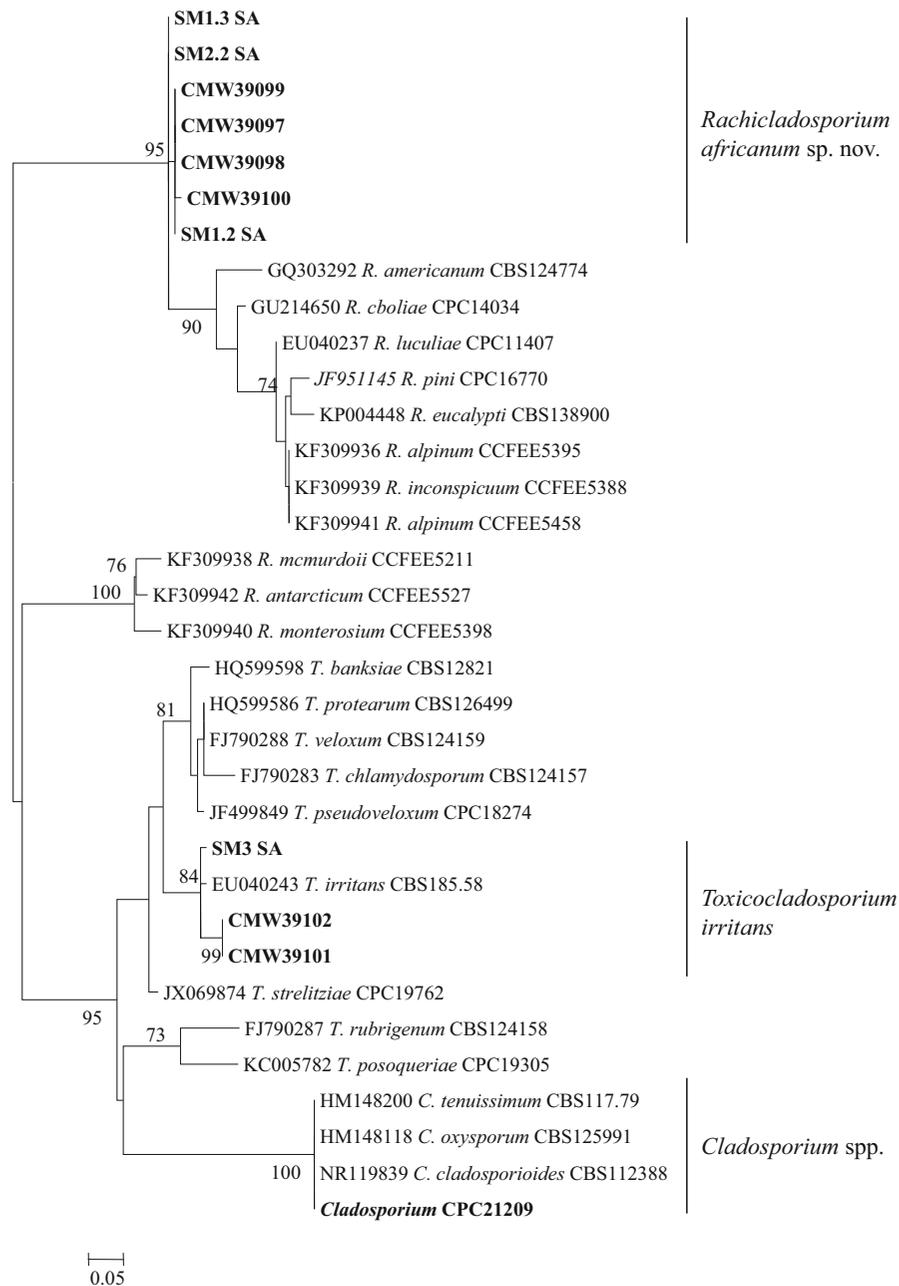


Fig. 3 Midpoint rooted ML tree of cladosporium-like isolates based on ITS sequence data with isolate numbers of sequences obtained in this study printed in *bold* type. Sequences with SM numbers were obtained by direct sequencing from plant material

baobabs in Africa represented a single taxon that could be differentiated from all other species in this genus. The fungus is thus described as follows:

Description of Rachicladosporium africanum Cruywagen, Crous, M.J. Wingf., sp. nov.—Mycobank MB811049; Fig. 5.

Etymology

The name reflects the continent of Africa where the fungus was collected.

On oatmeal agar. *Mycelium* hyaline to pale brown, smooth, septate, branched, 2–5 μm wide, sometimes

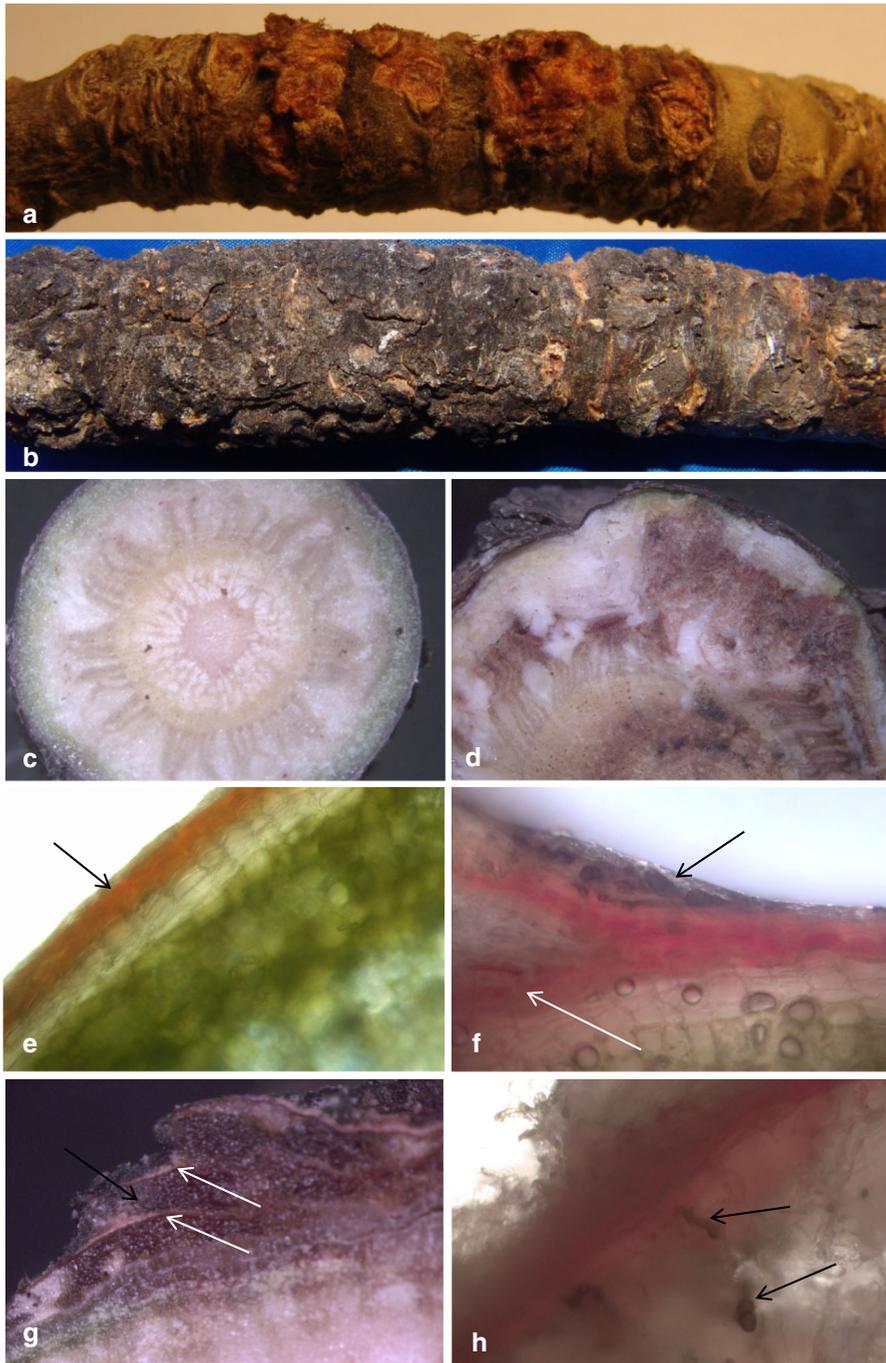


Fig. 4 **a** Baobab twig with reddish brown patches where fungus is starting to colonise, **b** twig with blackened appearance and galls forming due to black fungal colonisation, **c** section through healthy twig with cream coloured wood, **d** section through infected twig with brown internal discolouration and wood malformation, **e** section through healthy twig with single layer

of bark (*arrow*), **f** section through infected twig with fungal structures growing inside bark (*black arrow*) and new layer of bark forming (*white arrow*), **g** successive layers of bark (*white arrows*) with fungal and host material in between (*black arrow*), **h** fungal hyphae penetrating below bark into host cells (*arrow*)

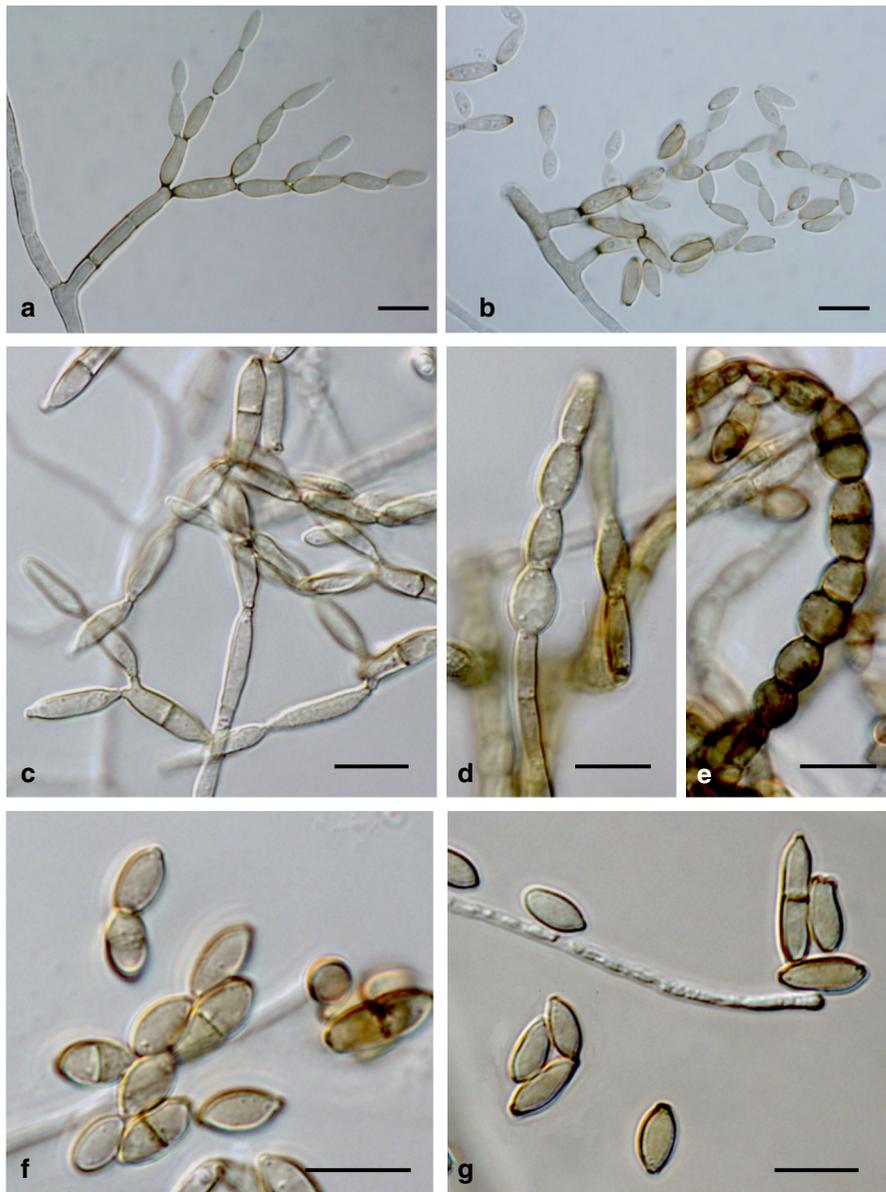


Fig. 5 *Rachicladosporium africanum* on oatmeal agar (type material). **a** macronematous conidiophore with apical conidiogenous cell, **b** micronematous conidiophores, **c** conidiophores

with conidial chains and ramoconidia, **d**, **e** chlamydospores, **f**, **g** conidia. Scale bar = 10 μm

constricted at septa and forming intercalary chlamydospores (Fig. 5d, e) that are brown, thick-walled and up to 8 μm diam. *Conidiophores* (Fig. 5a–c) dimorphic, macronematous, subcylindrical, straight when young, becoming flexuous, pale brown and verruculose, up to 180 μm tall and 3–5 μm diam, or micronematous, reduced to conidiogenous cells. *Conidiogenous cells* mostly terminal, sometimes

intercalary, cylindrical, 5–20 \times 3–4 μm . *Conidogenesis* holoblastic, sympodial with single or multiple (up to three) conidiogenous loci, 1.5–2 μm diam; ramoconidia subcylindrical, (9–)11–16(–17) \times (2–) 3–4 μm , 0–1-septate, sometimes slightly constricted at septum, smooth to verruculose, hila 1–2 μm diam, darkened, thickened and slightly refractive. *Conidia* (Fig. 5f, g) blastocatenate, ellipsoid to fusoid

(5–)6–11(–15) × (2–)3–4(–5) µm, 0–1-septate; hila darkened, thickened and slightly refractive, 0.5–1 µm diam.

Culture characteristics

Colonies on MEA reaching 17 mm diam after 10 days at 25 °C in the dark, elevated and folded at the centre while flat at the edge with a smooth margin. On oatmeal agar greenish olivaceous in the centre and grey-olivaceous at the margin; reverse grey-olivaceous.

HOLOTYPE: South Africa, *Limpopo*, Venda area, on African baobab tree (*Adansonia*), Jul. 2012, *E.M. Cruywagen* (PREM 61153, ex-type culture CMW 39100 = CBS 139400).

PARATYPE: South Africa, *Limpopo*, Sagole vil-lage, on baobab tree Jul. 2012, *E.M. Cruywagen* (CMW 39097 = CPC 21214).

Notes

This species is phylogenetically most closely related to *R. americanum* and *R. cboliae*, but *R. africanum* has smaller terminal conidia and ramoconidia than *R. americanum* (conidia 10–18 × 3–4 µm; ramoconidia 13–23 × 3–4 µm) (Cheewangkoon et al. 2009; Crous et al. 2009). Furthermore *R. africanum* also forms chlamydospores whereas these are absent in *R. americanum*. *Rachicladosporium cboliae*, sporulating on OA, also forms chlamydospores (up to 6 µm) but these are smaller than those of *R. africanum* as are the conidia (6–10 × 2–3 µm) and ramoconidia (7–12 × 3–4 µm) (Crous et al. 2009).

Discussion

Black mould on the surface of African baobab (*Adansonia digitata*) stems and branches has been linked to an apparent decline of these iconic trees in various parts of southern Africa (Alberts 2005; Pearce et al. 1994; Sharp 1993). This study represents a first attempt to characterise the fungi involved in the black mould complex on baobab trees in Africa using DNA-based techniques. The most commonly encountered species associated with this syndrome were *A. pullulans*, *T. irritans* and a novel species of *Rachicladosporium*. The new species is described in this study as *R.*

africanum sp. nov. Both methods used to isolate and identify the fungi, namely direct PCR and traditional culture-based isolation methods, revealed the same species composition associated with the black mould syndrome on baobabs. This suggests that other unculturable fungi are unlikely to be involved in the black mould problem.

Rachicladosporium species have been isolated from leaf and twig litter in the USA (Cheewangkoon et al. 2009; Crous et al. 2009), leaf spots on *Luculia* sp. in New Zealand (Crous et al. 2007) and needles of *Pinus monophylla* in the Netherlands (Crous et al. 2011). More recently, *R. eucalypti*, the first species in the genus associated with sexual structures, was isolated from leaf spots on *Eucalyptus globulus* in Ethiopia (Crous et al. 2014). It is not clear whether any of these species are pathogenic to their hosts, but the genus is closely related to the Cladosporiaceae and the Capnodiaceae, both families that include known plant pathogens and sooty mould fungi (Crous et al. 2009). Other *Rachicladosporium* species have all been isolated from rocks and include *R. antarcticum* and *R. mcmurdoii* from Antarctica and *R. alpinum*, *R. inconspicuum*, *R. montessorium* and *R. paucitum* from Italy (Egidi et al. 2014).

The relationship of *Rachicladosporium* associated with the black mould on Baobab to rock inhabiting fungi (RIF) aligns with reports of several sooty mould groups that are also related to RIF, including groups in the Chaetothyriales (Gueidan et al. 2008) and Capnodiales (Ruibal et al. 2009). RIF are typically melanised, slow-growing organisms that have high tolerance for drought stress, radiation and low nutrients (Gueidan et al. 2008; Ruibal et al. 2009). It has been hypothesised that rock inhabiting fungi might have given rise to various plant and insect pathogens, as the inhospitable habitat may pre-dispose these fungi to easily adapt to new hosts and environments (Gueidan et al. 2008; 2011).

Crous et al. (2013) described a novel species of *Ochrocladosporium* (Pleosporales), *O. adansoniae*, from black mould symptoms on African baobabs in South Africa. The genus includes three species with the other two being *O. elatum* (isolated from wood) and *O. frigidarii* (isolated from a cooled room) (Crous et al. 2007). The previous isolation of *O. adansoniae* by Crous et al. (2013) was only obtained from a single tree from the same region as the present study. Interestingly, this species was not isolated in the

present study and this suggests that the fungi associated with the black mould syndrome represent a complex of fungi that are apparently not consistently present. Clearly much more intensive sampling is required to resolve the question of spatial and temporal variation in the species complex associated with black mould on Baobabs more fully.

T. irritans found in this study was first described from mould growing on paint in Suriname (Crous et al. 2007). It has subsequently been isolated from diverse substrates including ancient documents (Mesquita et al. 2009), patients with atopic dermatitis (Zhang et al. 2011) as well as a sub-surface ice cave in Antarctica (Connell and Staudigel 2013). These reports suggest that the fungus is able to colonise substrates that may be low in nutrients. It seems unlikely to be involved in a disease reaction on baobab as there is no evidence of this fungus infecting plants. It is probably associated only with superficial colonisation of plant tissue and not responsible for the growth inside the plant cells.

A. pullulans was the most commonly isolated fungus in this study. This yeast-like black fungus is often isolated from plant material and associated with sooty mould complexes (Hughes 1976; Mirzwa-Mróz and Winska-Krysiak 2011). This fungus can colonise almost any substrate and has even been found growing actively inside the Chernobyl containment structure where it is subjected to continuous high radiation (Zhdanova et al. 2000). Although this fungus can grow in areas of low water and nutrient availability (Yurlova et al. 1999; Zalar et al. 2008), it is likely growing only superficially on the black mouldy growths on the baobab trees.

Single isolates of unidentified *Cladosporium* and *Aureobasidium* species were collected from the black mould samples in this study. The unidentified *Aureobasidium* species grouped distant from the known species in this genus and might represent a novel species. It is apparent that there is a second black-yeast species involved in addition to *A. pullulans*. *Cladosporium* species are often involved in sooty mould complexes (Hughes 1976; Sherwood and Carroll 1974). This genus includes many plant pathogens and saprophytes (Bensch et al. 2012) and some of these species might contribute to the host response seen in baobab trees. The infrequent isolation of this fungus, however, suggests that it is not a major contributor to the observed disease symptoms.

Despite the fact that none of the commonly occurring fungi identified in this study are known plant pathogens, our observations showed that they were able to penetrate through the bark where they appear to cause the Baobab trees to produce a wound response. This is a major difference from sooty mould fungi that colonise only the surface of plants and grow on honeydew from insects (Chomnunti et al. 2014; Crous et al. 2009; Hughes 1976). Therefore, reference to the black mould on the stems and branches of baobab as “sooty mould” should be avoided. The infection was, however, still superficial and not of such a nature that we would expect it to be involved in the decline of the trees.

The results of this study suggest that the fungi associated with black mould syndrome on baobabs in southern Africa represent an assemblage of species. The composition of this assemblage is apparently also variable over time and space. This variability, along with the superficial nature of the infections, argues against these fungi being involved in the decline of these iconic trees. While it is a fact that the black mould is common on declining trees, this might simply be due to the fact that these trees are stressed and unable to resist the growth of what appear to be opportunistic colonists of their branches and stems.

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