Mycol Progress (2014) 13:219–240
DOI 10.1007/s11557-013-0907-5

ORIGINAL ARTICLE

Molecular phylogenetic analyses reveal three new *Ceratocystis* species and provide evidence for geographic differentiation of the genus in Africa

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Received: 11 March 2013 / Revised: 25 April 2013 / Accepted: 10 May 2013 / Published online: 1 June 2013

Abstract The emergence of wattle wilt disease on non-native *Acacia mearnsii* trees in Africa, caused by the indigenous fungus *Ceratocystis albifundus*, has highlighted a need to better understand the diversity, ecology and distribution of *Ceratocystis* species in natural African environments. In this study we applied phylogenetic inference to identify and characterize isolates of *Ceratocystis* collected in a natural savanna ecosystem in South Africa. Three new species were recognized and are described as *C. cryptoformis* sp. nov. in the *C. moniliformis* complex, as well as *C. thulamelensis* sp. nov. and *C. zambeziensis* sp. nov., both residing in the *C. fimbriata* complex. Incorporating the new species into global phylogenies of *Ceratocystis* provided insights into the patterns of evolution and biogeography of this group of fungi. Notably, the African continent was identified as an important centre of diversification of *Ceratocystis* spp., from which several lineages of these fungi were shown to have radiated.

Keywords Biogeographic lineages · Fungal plant pathogens · GCPSR · GMYC · Microascales · Savanna ecosystem · Species delimitation

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Introduction

It is well known that natural ecosystems harbor unknown pathogens and novel pathotypes threatening cultivated plant systems. These wild pathogens and their co-evolved hosts generally occur in dynamic equilibrium (Frank 1992; Thompson and Burdon 1992), maintaining low disease incidence in steady environmental conditions (Dinoor and Eshed 1984; Burgess and Wingfield 2002). Widespread epidemics in natural plant communities involving native pathogens arise as a consequence of a disruption of this equilibrium. This may happen shortly after evolutionary changes in pathogen populations that give rise to more aggressive pathotypes, or more commonly, in association with anthropogenic disturbances or dramatic changes in environmental conditions that affect host susceptibility (Castello et al. 1995; Burdon et al. 2006; Anderson et al. 2004; Dodds and Thrall 2009). In contrast, when wild pathogens are introduced into cultivated plant systems, they are more likely to initiate devastating disease outbreaks as is the case for several fungal diseases of agricultural and forestry importance (Stukenbrock and Mcdonald 2008).

A vivid illustration of the consequences associated with the adoption of wild plant pathogens in cultivated plant systems is found in the Wattle Wilt Disease (WWD) system affecting forest plantations based on Australian *Acacia* species in Africa. The WWD is caused by the fungus *Ceratocystis albifundus* M.J. Wingf., De Beer & M.J. Morris (Wingfield et al. 1996). The disease was first discovered in South Africa on *A. mearnsii* De Wild. (Morris et

al. 1993). It is now known to be distributed, at least, across southern and eastern Africa (Roux et al. 2001b, 2005; Heath et al. 2009) and may affect other non-native *Acacia* species (Morris et al. 1993). Interestingly, *C. albifundus* has been found colonizing wounds on several native African trees in natural ecosystems, in the absence of disease (Roux et al. 2007; Kamgan Nkuekam et al. 2008). This, in addition to supporting evidence from population genetic studies (Roux et al. 2001a; Barnes et al. 2005), has led to the view that the wattle wilt pathogen is native to Africa. The WWD is the most serious *Ceratocystis* disease affecting plantation forestry using *A. mearnsii* in Africa (Roux and Wingfield 2009), and, as an emerging "new encounter disease" (Parker and Gilbert 2004), could result in terrible ecological consequences if it were to be introduced into the natural range of wattle trees in Australia (Roux and Wingfield 2013).

Ceratocystis species are ascomycete fungi residing in the order Microascales (Schoch et al. 2009; Réblová et al. 2011). Their morphological characteristics typically combine bulbous ascomatal bases with extended necks in their sexual states and deep-seated, tubular phialides in their asexual states (Nag Raj and Kendrick 1975; Upadhyay 1981). DNA sequence data and molecular phylogenetics have profoundly impacted on the taxonomy of this group of fungi, starting with the recognition of *C. albifundus* as a novel species, distinct from *C. fimbriata* Ellis & Halst. (Wingfield et al. 1996). This group is now recognized, based on DNA sequence comparison, morphology and ecology, to include very distinct evolutionary lineages and species complexes for which discrete genera will be established (Wingfield et al. 2013). One of these will accommodate species in the *C. fimbriata* complex that includes *C. albifundus* and many other, mainly pathogenic species (Baker et al. 2003; Johnson et al. 2005; Van Wyk et al. 2013). However, taxonomic studies of *Ceratocystis* spp. are still compounded by the lack of distinctive morphological characters between closely related species and the limited resolution of molecular markers available (Van Wyk et al. 2010, 2011a, b, 2012; Kamgan Nkuekam et al. 2012a, b), limiting quick and accurate identification of these pathogens.

One of the first studies to consider the identity of *Ceratocystis* spp. in natural woody ecosystems in South Africa resulted in the discovery of *C. savannae* Kamgan & Jol. Roux in the savanna dominated Kruger National Park (KNP) and *C. tsitsikammensis* Kamgan & Jol. Roux in the Garden Route National Park (GRNP) of South Africa (Kamgan Nkuekam et al. 2008). The latter fungus showed considerable virulence when inoculated onto its native host, *Rapanea melanophloeos* (L.) Mez (Kamgan Nkuekam et al. 2008). In an attempt to explore the extent and determinants of the diversity of *Ceratocystis* spp. in the savanna ecosystem in South Africa, an extensive survey of animal-induced tree wounds was conducted throughout the KNP over 2009 and 2010. The aim of the present study was to ascertain the taxonomic and phylogenetic status of the fungi collected during this survey. This was achieved by comparing our isolates with well-known species of *Ceratocystis* using multi-gene DNA phylogenies, together with morphological characterization of representative isolates for novel taxa.

Materials and methods

Collection of isolates

Plant material for the isolation of *Ceratoystis* species was obtained from various native savanna trees, damaged by animals, especially elephants, in the KNP during 2009 and 2010. Collections were made in four areas inside KNP (including Letaba, Punda Maria, Satara and Skukuza), from fresh (less than 1-month-old) wounds on branches and stems of all trees showing damage. Wounds were inspected for the presence of fruiting bodies resembling species of *Ceratocystis* using a 10× magnifying hand lens to determine the suitability of material for collection. Samples were placed into brown paper bags, one bag for each tree sampled, and transported to the laboratory for isolation. When present, nitidulid beetle associates of *Ceratocystis* species were collected using an aspirator and they were transported to the laboratory in glass vials.

Isolation from plant material was done by placing infected wood and bark in humid chambers to encourage the sporulation of fungal fruiting structures. Small sections (~1–2 cm^2) of plant material were also wrapped between carrot discs to bait for *Ceratocystis* spp. (Moller and De Vay 1968). The same method was used to isolate *Ceratocystis* spp. from nitidulid beetles, by crushing the insects onto carrot discs.

All isolations were incubated at 25 °C for 5–10 days. They were regularly inspected under a dissecting microscope, and, where *Ceratocystis* structures had developed, purification was done by lifting a few mycelial strands or single ascospore droplets using a sterile needle and transferring these to sterile 2 % malt extract agar (MEA) (Biolab, Midrand, South Africa) supplemented with ~0.01 g/L streptomycin sulphate (Sigma, Steinheim, Germany).

Purified fungal strains were obtained by sub-culturing from single hyphal tips or spore droplets and these were maintained on MEA. Fungal strains obtained from single sampled trees were sorted into morphotypes based on cultural characteristics and when possible two representatives of each morphotype were selected for molecular typing. All selected strains were eventually broadly divided into three morphogroups representing the *C. fimbriata* (including *C.*

albifundus) complex (*C. fimbriata* s.l. group), the *C. moniliformis* (Hedgc.) C. Moreau complex (*C. moniliformis* s.l. group) and the *Thielaviopsis thielavioides* (Peyr.) A.E. Paulin, T.C. Harr. & McNew complex (*T. thielavioides* s.l. group).

DNA extraction, PCR and sequencing

DNA extraction was based on the CTAB (cetyl trimethyl ammonium bromide) protocol developed by Möller et al. (1992). All the selected fungal strains were maintained on MEA at 25 ° C for 7–14 days, whereafter mycelium was scraped from the surfaces of cultures, freeze-dried and ground, using a Retsch cell disrupter (Retsch, Germany), to a fine powder that was used as starting material for total genomic DNA isolation. Final DNA working concentrations were adjusted to ~75 ηg μL^{-1}, using a Thermo Scientific NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

For first level species delineation and identification, two nuclear gene regions were selectively amplified with polymerase chain reactions (PCR) and sequenced for all selected fungal strains. These were the internally transcribed spacer (ITS) region including the 5.8S rDNA of the ribosomal RNA gene cluster for isolates representing the *C. fimbriata* s.l. and *T. thielavioides* s.l. groups, and a portion of the beta-tubulin (β-tubulin) gene for isolates representing the *C. moniliformis* s.l. group. Additional sequences were generated for the translation elongation factor 1-alpha (TEF-1α) gene and, where applicable, the ITS and β-tubulin and used for in-depth multigene phylogenetic analyses. These involved only a few representatives of each of the putatively distinct taxa. The oligonucleotide primer combinations utilized were, respectively, the ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) for the ITS (White et al. 1990), βt1a (5′-TTCCCCCGTCTCCAC TTCTTCATG-3′) and βt1b (5′-GACGAGATCGTTCA TGTTGAACTC-3′) for the β-tubulin (Glass and Donaldson 1995) and EF1F (5′-TGCGGTGGTATCGACAAGCGT-3′) and EF2R (5′-AGCATGTTGTCGCCGTTGAAG-3′) for the (Jacobs et al. 2004) gene regions.

For all gene regions, PCR reactions were performed in a 25 μL final volume. Each reaction contained 2.5 μL of 10× Expand HF buffer with $MgCl_2$ (25 mM) (Roche Diagnostic, Mannheim, Germany), 2.5 μL of deoxynucleotide triphosphate (dNTP) mix (10 mM), 0.5 μL of each primer (10 mM), 1 μL of *Taq polymerase* (1 U/μL) (Roche Diagnostic) and 1 μL of DNA template. Reactions were run on a Bio-Rad iCycler thermocycler (BIO-RAD, Hercules, CA, USA). The same thermal cycling conditions were applied for the ITS and β-tubulin regions, which included an initial denaturation step at 96 °C for 2 min followed by 35 cycles of 30 s at 94 °C, 60 s at 54 °C and 90 s at 72 °C and a final extension step at 72 °C for 10 min. For the TEF-1α, the thermal cycle comprised an initial denaturation at 96 °C for 4 min followed by 10 primary amplification cycles of 40 s at 94 °C (denaturation), 40 s at 55 °C (annealing), and 45 s at 72 °C (extension), then 30 additional cycles of the same reaction sequence, with a 5-s increase in the annealing step per cycle. Reactions were completed with a final extension step at 72 °C for 10 min. Amplification was confirmed by staining PCR products (4 μL aliquots) with 1.5 μL of GelRed™ (Biotium, USA) nucleic acid dye and performing electrophoresis along with a DNA molecular weight marker (100 bp ladder) (Fermentas O' Gene Ruler™) on 2 % agarose gels, followed by visualization under UV light. PCR products were purified by gel filtration using 6 % Sephadex G-50 (50–150 μm bead size) (Sigma).

Forward and reverse sequencing reactions were performed in 12 μL final volumes with the same primers as used for amplification reactions. The mixtures contained 2.5 μL sequencing buffer, 0.5 μL Big Dye ready reaction mixture with Amplitaq DNA polymeraze (Perkin-Elmer, Warrington, UK), 1 μL of the selected primer (10 mM) and 4 μL purified PCR product. The thermal cycling conditions comprised 25 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C. Sequencing products were purified through Sephadex G-50 gel columns and concentrated in an Eppendorf 5301 vacuum concentrator, at 45 °C. They were thereafter run on an ABI PRISM™ 3100 DNA Analyzer (Applied BioSystems, Foster City, CA, USA). Some fungal strains in the *C. fimbriata* group, including those representing *C. albifundus,* necessitated the cloning of amplified PCR products for the ITS prior to sequencing. This was done using the pGM®-T Easy Vector System (Promega, Madison, WI, USA) following the manufacturer's instructions.

Species delineation and primary identification

Consensus sequences were assembled from forward and reverse sequencing reads using MEGA v.5 (Tamura et al. 2011). Multiple sequence alignments were constructed using MAFFT (http://www.align.bmr.kyushu-u.ac.jp/mafft/online/server/) v.6 (Katoh et al. 2005) and edited manually in MEGA. The ITS sequence dataset was used for species delineation in the *C. fimbriata* s.l. and *T. thielavioides* s.l. groups, while the β-tubulin dataset was used for the *C. moniliformis* s.l. group. For this purpose, we applied the General Mixed Yule Coalescent (GMYC) model statistical approach developed by Pons et al. (2006). The GMYC model uses branching patterns in an ultrametric phylogenetic tree to delineate species, by identifying the point of transition between micro- and macro-evolutionary processes when diversification rates are plotted against evolutionary times. This is followed by a log likelihood ratio (LR) test to assess the goodness-of-fit of the GMYC model as compared

to a null model that assumes a single population under neutral coalescence.

We implemented the GMYC model using the SPLITS (http://r-forge.r-project.org/projects/splits/) package (Ezard et al. 2009) of the statistical software R (R Development Core Team 2011). Ultrametric trees were constructed through Bayesian Markov Chain Monte Carlo (MCMC) algorithms as implemented in BEAST v.1.5.4. (Drummond and Rambaut 2007), under a strict molecular clock, constant population size and coalescent prior settings. Prior to this, duplicate sequences were excluded from the alignments for the *C. moniliformis* group, using the 'unique.seqs' command of MOTHUR v.1.21.1 (Schloss et al. 2009) and best-fit models of nucleotide substitution were estimated using JModel Test v.2.2 (Posada 2008). For each sequence alignment, two parallel MCMC runs were set for 10^7 generations, starting from a UPGM tree. Trees were selected every 1,000 generations. Convergence of the two chains was checked using Tracer v.1.5 (Rambaut and Drummond 2009). The resulting tree files were combined using Logcombiner (included in the Beast package), discarding the first 10 % of the generations from each run as burn-in. Maximum credibility trees were generated in TreeAnnotator (also included in the Beast package) enforcing the 5 % posterior probability limit.

Representative sequences of each GMYC independent entity were evaluated against published authenticated sequences from GenBank, using NCBI-Blast (http://www.ncbi.nlm.nih.gov/Blast.cgi), for possible matching with sequences of known species. From this primary identification process, 24 isolates form KNP (Table 1), including two or three representative isolates for each known species and four for putative knew species, were selected for in depth, multigene phylogenetic analyses.

Multi-gene phylogenetic analyses

Parallel analyses were conducted for the three *Ceratocystis* species complexes represented in our collection, namely *C. fimbiata* s.l., *C. moniliformis* s.l. and *T. thielavioides* s.l. For each of these groups, three sequence datasets representing each of the ITS, β-tubulin and TEF-1α gene regions were constructed. These datasets included sequences generated in this study as well as reference sequences sourced from GenBank for two representative strains of all known species in the respective complexes (Table 1). The *C. fimbriata* s.l. and *T. thielavioides* s.l. group datasets were supplemented with reference sequences for *C. virescens* (R.W. Davidson) C. Moreau, which was chosen as the outgroup taxon in phylogenetic reconstructions. *C. moniliformopsis* Yuan & Mohammed was used as the outgroup taxon for the *C. moniliformis* s.l. group.

Sequences were aligned as previously described using MAFFT. A first round of analyses involving whole datasets included maximum parsimony (MP) and Bayesian inferences of phylogeny applied to concatenated, multi-locus sequence data of the three gene regions. In a second tier of analyses, only the apparently new species in the *C. fimbriata* s.l. and *C. moniliformis* s.l. groups and their closest relatives were considered. The phylogenetic relationship of these taxa based on single locus data of the ITS, β-tubulin and TEF-1α genes was investigated using MP analyses.

Maximum Parsimony analyses were performed using PAUP v.4.0b10* (Swofford 2002). Uninformative characters were excluded and all informative characters were unordered and of equal weight. For the *C. fimbriata* s.l. group, two gap treatments were considered, first as "new character" state and then as "missing data", while for the *C. moniliformis* s.l. and *T. thielavioides* s.l. groups only the "new character state" was applied. MP trees were generated via a heuristic tree search involving 100 random stepwise addition replicates and tree-bisection-reconstruction (TBR) branch-swapping. Statistical support for branch nodes of most parsimonious trees (MPTs) was assessed using 1,000 bootstrap replicates. Other parameters estimated for MPTs included the tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency indexes (RC). The PAUP settings, as well as tree parameters estimated for single locus analyses, were the same as those implemented in combined multi-gene analyses. Furthermore, the genealogical concordance of the three genes was tested using partition homogeneity tests (PHT) with 1,000 heuristic search replicates in PAUP (Swofford 2002).

Bayesian phylogenies were inferred based on Markov Chain Monte Carlo (MCMC) analyses with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). Best-fit models of nucleotide substitutions were selected using JModeltest v,2.2 (Posada 2008) under the Akaike information criterion (AIC). All the models selected were of the standard General-Time-Reversible (GTR) type with gamma-distribution of substitution rates. The MCMC procedure involved four chains and started from a random tree topology. Sampling every 100th generation, 1 million random tree generations were performed for each of the *C. moniliformis* s. l. and *T. thielavioides* s.l. datasets, whereas for the *C. fimbriata* s.l. dataset, 5 million generations were necessary to reach a stationary phase in the distribution of sample likelihoods. Posterior probability distributions were assessed with Tracer v.1.5 (Rambaut and Drummond 2009), and the default burn-in setting of the first 10 % of the generations was enforced in the construction of maximum credibility consensus trees. Final consensus trees were visualized using FigTree (Morariu et al. 2008).

Culture characteristics and morphology

Two isolates representing each of the purported new *Ceratocystis* species emerging from the phylogenetic analyses

Table 1 List of *Ceratocystis* species included in this study

Species	Isolate no		Gene region/GeneBank accession no			Host (or substrate)	Geographic origin	Collectors	Relevant references
			ITS	BT	TFF				
Ceratocystis fimbriata complex									
C. acaciivora	CMW22562		EU588655	EU588635	EU588645	*Acacia mangium*	Indonesia	M. Tarigan	Tarigan et al. (2010a)
C. acaciivora	CMW22563		EU588656	EU588636	EU588646	*Acacia mangium*	Indonesia	M. Tarigan	Tarigan et al. (2010a)
C. albifundus	CMW5329		AF388947	DQ371649	EF070401	*Acacia mearnsii*	Uganda	J. Roux	Roux et al.(2001b)
C. albifundus	CMW23825	CBS119681	EU245010	EU244982	EU244942	*Acacia mearnsii*	South Africa	R.N. Heath	Heath et al. (2009)
C. albifundus	**CMW37312**		**KC691452**	**KC691476**	**KC691500**	***Terminalia sericea***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. albifundus	**CMW37313**		**KC691453**	**KC691477**	**KC691501**	***Combretum zeyheri***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. atrox	CMW19383	CBS 120517	EF070414	EF070430	EF070402	*Eucalyptus grandis*	Australia	M.J. Wingfield	Van Wyk et al. (2007b)
C. atrox	CMW19385	CBS 120518	EF070415	EF070431	EF070403	*Eucalyptus grandis*	Australia	M.J. Wingfield	Van Wyk et al. (2007b)
C. cacaofunesta	CMW15051	CBS 152.62	DQ520636	DQ520636	EF070398	*Theobroma cacao*	Costa Rica	A.J. Hansen	Baker Engelbrecht and Harrington (2005)
C. cacaofunesta	CMW14809	CBS 115169	DQ520637	EF070428	EF070399	*Theobroma cacao*	Ecuador	C. Suarez	Baker Engelbrecht and Harrington (2005)
C. caryae	CMW14793	CBS 114716	EF070424	EF070439	EF070412	*Carya cordiformis*	USA	J. Johnson	Johnson et al. (2005)
C. caryae	CMW14808	CBS 115168	EF070423	EF070440	EF070411	*Carya ovata*	USA	J. Johnson	Johnson et al. (2005)
C. colombiana	CMW5751	CBS 121792	AY177233	AY177225	EU241493	*Coffea arabica*	Colombia	M. Marin	Van Wyk et al. (2010)
C. colombiana	CMW9572		AY233863	AY233871	EU241488	*Citrus reticulata* (Mandarin)	Colombia	M. Marin	Van Wyk et al. (2010)
C. corymbiicola	CMW29120	CBS 127215	HM071902	HM071914	HQ236453	*Corymbia variegata*	Australia	G.K. Kamgan	Kamgan Nkuekam et al. (2012a)
C. corymbiicola	CMW29349	CBS 127216	HM071919	HQ236455	HM071905	*Eucalyptus pilularis*	Australia	G.K. Kamgan	Kamgan Nkuekam et al. (2012a)
C. curvata	CMW22433	CBS 122513	FJ151438	FJ151450	FJ151472	*Eucalyptus deglupta*	Ecuador	M.J. Wingfield	Van Wyk et al. (2011b)
C. curvata	CMW22435	CBS 122604	FJ151437	FJ151449	FJ151471	*Eucalyptus deglupta*	Ecuador	M.J. Wingfield	Van Wyk et al. (2011b)
C. diversiconidia	CMW22445	CBS 123013	FJ151440	FJ151452	FJ151474	*Terminalia ivorensis*	Ecuador	M.J. Wingfield	Van Wyk et al. (2011b)
C. diversiconidia	CMW22447	CBS 122818	FJ151442	FJ151454	FJ151476	*Terminalia ivorensis*	Ecuador	M.J. Wingfield	Van Wyk et al. (2011b)
C. eucalypticola	CMW10000	CBS 124019	FJ236722	FJ236782	FJ236752	*Eucalyptus* sp.	South Africa	M. Van Wyk & J. Roux	Van Wyk et al. (2012)
C. eucalypticola	CMW11536	CBS 124016	FJ236723	FJ236783	FJ236753	*Eucalyptus* sp.	South Africa	M. Van Wyk & J. Roux	Van Wyk et al. (2012)
C. ecuadoriana	CMW22092	CBS 124020	FJ151432	FJ151444	FJ151466	*Eucalyptus deglupta*	Ecuador	M. Van Wyk & J. Roux	Van Wyk et al. (2011b)
C. ecuadoriana	CMW22093	CBS 124021	FJ151433	FJ151445	FJ151467	*Eucalyptus deglupta*	Ecuador	M. Van Wyk & J. Roux	Van Wyk et al. (2011b)
C. fimbriata s.s	CMW1547	CBS 123010	AF264904	EF070443	EF070395	*Ipomoea batatas*	Papua N. G.	E.C.H. McKenzie	Roux et al. (2000); Van Wyk et al. (2007a)
C. fimbriata s.s	CMW15049	CBS 141.37	DQ520629	EF070442	EF070394	*Ipomoea batatas*	USA	C.F. Andrus	Van Wyk et al. (2006b; 2007a)
C. fimbriatomima	CMW24174	CBS 121786	EF190963	EF190951	EF190957	*Eucalyptus* sp.	Venezuela	M.J. Wingfield	Van Wyk et al. (2009b)
C. fimbriatomima	CMW24176	CBS 121787	EF190964	EF190952	EF190958	*Eucalyptus* sp.	Venezuela	M.J. Wingfield	Van Wyk et al. (2009b)
C. larium	CMW25434	CBS 122512	EU881906	EU881894	EU881900	*Styrax benzoin*	Indonesia	M.J. Wingfield	Van Wyk et al. (2009a)

Table 1 (continued)

Species	Isolate no		Gene region/GeneBank accession no			Host (or substrate)	Geographic origin	Collectors	Relevant references
			ITS	BT	TFF				
C. larium	CMW25435	CBS 122606	EU881907	EU881895	EU881901	*Styrax benzoin*	Indonesia	M.J. Wingfield	Van Wyk et al. (2009a)
C. mangicola	CMW14797	CBS 114721	AY953382	EF433307	EF433316	*Mangifera indica*	Brazil	C.J. Baker	Van Wyk et al. (2011a)
C. mangicola	CMW27306		FJ200256	FJ200269	FJ200282	*Mangifera indica*	Brazil	C.J. Rosetto	Van Wyk et al. (2011a)
C. manginecans	CMW13851	CBS 121659	AY953383	EF433308	EF433317	*Mangifera indica*	Oman	A.O. Al Adawi	Van Wyk et al. (2007a)
C. manginecans	CMW13852	CBS 121660	AY953384	EF433309	EF433318	*Mangifera indica*	Oman	A.O. Al Adawi	Van Wyk et al. (2007a)
C. mangivora	CMW27305	CBS 128340	FJ200262	FJ200275	FJ200288	*Mangifera indica*	Brazil	C.J. Rosetto	Van Wyk et al. (2011a)
C. mangivora	CMW27304	CBS 127204	FJ200261	FJ200274	FJ200287	*Mangifera indica*	Brazil	M. Barreto Figueiredo	Van Wyk et al. (2011a)
C. neglecta	CMW17808	CBS 121789	EF127990	EU881898	EU881904	*Eucalyptus* sp.	Colombia	C. Rodas & J. Roux	Rodas et al. (2008)
C. neglecta	CMW18194	CBS 121017	EF127991	EU881899	EU881905	*Eucalyptus* sp.	Colombia	C. Rodas & J. Roux	Rodas et al. (2008)
C. obpyriformis	CMW23807	CBS 122608	EU245004	EU244976	EU244936	*Acacia mearnsii*	South Africa	R.N. Heath	Heath et al. (2009)
C. obpyriformis	CMW23808	CBS 122511	EU245003	EU244975	EU244935	*Acacia mearnsii*	South Africa	R.N. Heath	Heath et al. (2009)
C. papillata	CMW8856	CBS 121793	AY233867	AY233874	EU241484	*Citrus limon*	Colombia	M.J. Wingfield	Van Wyk et al. (2010)
C. papillata	CMW10844		AY177238	AY177229	EU241481	*Coffea arabica*	Colombia	M.J. Wingfield	Van Wyk et al. (2010)
C. pirilliformis	CMW6569		AF427104	DQ371652	AY528982	*Eucalyptus nitens*	Australia	M.J. Wingfield	Barnes et al. (2003)
C. pirilliformis	CMW6579	CBS 118128	AF427105	DQ371653	AY528983	*Eucalyptus nitens*	Australia	M.J. Wingfield	Barnes et al. (2003)
C. platani	CMW14802	CBS 115162	DQ520630	EF070425	EF070396	*Platanus occidentalis*	USA	T.C. Harrington	Baker Engelbrecht and Harrington (2005)
C. platani	CMW23918		EF070426	EF070397	EU426554	*Platanus* sp.	Greece	M.J. Wingfield	Van Wyk et al. (2006b, 2007a)
C. polychroma	CMW11424	CBS 115778	AY528970	AY528966	AY528978	*Syzygium aromaticum*	Indonesia	M.J. Wingfield	Van Wyk et al. (2004b)
C. polychroma	CMW11436	CBS 115777	AY528971	AY528967	AY528979	*Syzygium aromaticum*	Indonesia	M.J. Wingfield	Van Wyk et al. (2004b)
C. polyconidia	CMW23809	CBS 122289	EU245006	EU244978	EU244938	*Acacia mearnsii*	South Africa	R.N. Heath	Heath et al. (2009)
C. polyconidia	CMW23818	CBS 122290	EU245007	EU244979	EU244939	*Acacia mearnsii*	South Africa	R.N. Heath	Heath et al. (2009)
C. populicola	CMW14789	CBS 119.78	EF070418	EF070434	EF070406	*Populus* sp.	Poland	J. Gremmen	Johnson et al. (2005)
C. populicola	CMW14819	CBS 114725	EF070419	EF070435	EF070407	*Populus* sp.	USA	T. Hints	Johnson et al. (2005)
C. smalleyi	CMW14800	CBS 114724	EF070420	EF070436	EF070408	*Carya cordiformis*	USA	G. Smalley	Johnson et al. (2005)
C. smalleyi	CMW26383	CBS 114724	EU426553	EU426555	EU426556	*Carya cordiformis*	USA	Unknown	Johnson et al. (2005)
C. tanganyicensis	CMW15991	CBS 122295	EU244997	EU244969	EU244929	*Acacia mearnsii*	Tanzania	R.N. Heath & J. Roux	Heath et al. (2009)
C. tanganyicensis	CMW15999	CBS 122294	EU244998	EU244970	EU244939	*Acacia mearnsii*	Tanzania	R.N. Heath & J. Roux	Heath et al. (2009)
C. thulamelensis	**CMW35970**		**KC691454**	**KC691478**	**KC691502**	***Combretum zeyheri***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. thulamelensis	**CMW35971**	**CBS 131283**	**KC691455**	**KC691479**	**KC691503**	***Combretum zeyheri***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. thulamelensis	**CMW35972**	**CBS 131284**	**KC691456**	**KC691480**	**KC691504**	***Colophospermum mopane***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. thulamelensis	**CMW35973**		**KC691457**	**KC691481**	**KC691505**	***Colophospermum mopane***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. tsitsikammensis	CMW14276	CBS 121018	EF408555	EF408569	EF408576	*Rapanea melanophloeos*	South Africa	G.N. Kamgan & J. Roux	Kamgan Nkuekam et al. (2008)

Table 1 (continued)

Species	Isolate no		Gene region/GeneBank accession no			Host (or substrate)	Geographic origin	Collectors	Relevant references
			ITS	BT	TFF				
C. tsitsikammensis	CMW14278	CBS 121019	EF408556	EF408570	EF408577	*Rapanea melanophloeos*	South Africa	G.N. Kamgan & J. Roux	Kamgan Nkuekam et al. (2008)
C. variospora	CMW20935	CBS 114715	EF070421	EF070437	EF070409	*Quercus alba*	USA	J. Johnson	Johnson et al. (2005)
C. variospora	CMW20936	CBS 114714	EF070422	EF070438	EF070410	*Quercus robur*	USA	J. Johnson	Johnson et al. (2005)
C. zambeziensis	**CMW35958**	**CBS 131280**	**KC691458**	**KC691482**	**KC691506**	***Combretum imberbe***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. zambeziensis	**CMW35959**		**KC691459**	**KC691483**	**KC691507**	***Combretum imberbe***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. zambeziensis	**CMW35962**		**KC691460**	**KC691484**	**KC691508**	***Schotia brachypetala***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. zambeziensis	**CMW35963**	**CBS 131282**	**KC691461**	**KC691485**	**KC691509**	***Acacia nigrescens***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
Ceratocystis moniliformis complex									
C. bhutanensis	CMW8217	CBS 114289	AY528957	AY528962	AY528952	*Picea spinulosa*	Bhutan	T. Kirisits & D.B.Chhetri	Van Wyk et al. (2004a)
C. bhutanensis	CMW8242	CBS 112907	AY528956	AY528961	AY528951	*Picea spinulosa*	Bhutan	T. Kirisits & D.B.Chhetri	Van Wyk et al. (2004b)
C. cryptoformis	**CMW36826**	**CBS 131277**	**KC691462**	**KC691486**	**KC691510**	***Terminalia sericea***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. cryptoformis	**CMW36827**		**KC691463**	**KC691487**	**KC691511**	***Combretum zeyheri***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. cryptoformis	**CMW36828**	**CBS 131279**	**KC691464**	**KC691488**	**KC691512**	***Ziziphus mucronata***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. cryptoformis	**CMW36870**		**KC691465**	**KC691489**	**KC691513**	***Combretum hereroense***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. decipiens	CMW25914	CBS 129737	HQ203219	HQ203236	HQ236438	*Eucalyptus maculata*	South Africa	G.N. Kamgan & J. Roux	Kamgan Nkuekam et al. (2012b)
C. decipiens	CMW25918	CBS 129735	HQ203218	HQ203235	HQ236437	*Eucalyptus cloeziana*	South Africa	G.N. Kamgan & J. Roux	Kamgan Nkuekam et al. (2012b)
C. inquinans	CMW21106		EU588587	EU588666	EU588674	*Acacia mangium*	Indonesia	M. Tarigan	Tarigan et al. (2010b)
C. inquinans	CMW21107	CBS 124009	EU588588	EU588667	EU588675	*Acacia mangium*	Indonesia	M. Tarigan	Tarigan et al. (2010b)
C. microbasis	CMW21115	CBS 124015	EU588592	EU588671	EU588679	*Acacia mangium*	Indonesia	M. Tarigan	Tarigan et al. (2010b)
C. microbasis	CMW21117	CBS 124013	EU588593	EU588672	EU588680	*Acacia mangium*	Indonesia	M. Tarigan	Tarigan et al. (2010b)
C. moniliformis	CMW4114	CBS118151	AY528997	AY528986	AY529007	*Shizolobium parahyba*	Ecuador	M.J. Wingfield	Van Wyk et al. (2006a)
C. moniliformis	CMW9590	CBS116452	AY431101	AY528985	AY529006	*Eucalyptus grandis*	South Africa	J. Roux	Van Wyk et al. (2006a)
C. moniliformopsis	CMW9986	CBS109441	AY528998	AY528987	AY529008	*Eucalyptus obliqua*	Australia	Z.Q. Yuan	Yuan and Mohammed (2002])
C. moniliformopsis	CMW10214	CBS115792	AY528999	AY528988	AY529009	*Eucalyptus sieberi*	Australia	M.J. Dudzinski	Yuan and Mohammed (2002)
C. oblonga	CMW23803	CBS122291	EU245019	EU244991	EU244951	*Acacia mearnsii*	South Africa	R.N. Heath	Heath et al. (2009)
C. oblonga	CMW30835		HQ203221	HQ203238	HQ236440	*Carpophilus dimidiatus*	South Africa	G.N. Kamgan & J. Roux	Kamgan Nkuekam et al. (2012b)
C. oblonga	**CMW36836**		**KC691466**	**KC691490**	**KC691514**	***Ziziphus mucronata***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. oblonga	**CMW36853**		**KC691467**	**KC691491**	**KC691515**	***Colophospermum mopane***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. omanensis	CMW3800	CBS117839	DQ074743	DQ074733	DQ074738	*Mangifera indica*	Oman	A.O. Al Adawi	Al-subhi et al. (2006)

Table 1 (continued)

Species	Isolate no		Gene region/GeneBank accession no			Host (or substrate)	Geographic origin	Collectors	Relevant references
			ITS	BT	TFF				
C. omanensis	CMW11048	CBS115787	DQ074742	DQ074732	DQ074737	*Mangifera indica*	Oman	A.O. Al Adawi	Al-Subhi et al. (2006)
C. salinaria	CMW25911	CBS 129733	HQ203213	HQ203230	HQ236432	*Eucalyptus maculata*	South Africa	G.N. Kamgan & J. Roux	Kamgan Nkuekam et al. (2012b)
C. salinaria	CMW30703	CBS 129734	HQ203214	HQ203231	HQ236433	*Eucalyptus saligna*	South Africa	G.N. Kamgan & J. Roux	Kamgan Nkuekam et al. (2012b)
C. savannae	CMW17300	CBS121151	EF408551	EF408565	EF408572	*Acacia nigrescens*	South Africa	G.N. Kamgan & J. Roux	Kamgam Nkuekam et al. (2008)
C. savannae	CMW30828		HQ203223	HQ203240	HQ236442	*Brachypeplus depressus*	South Africa	G.N. Kamgan & J. Roux	Kamgan Nkuekam et al. (2012b)
C. savannae	**CMW36829**		**KC691468**	**KC691492**	**KC691516**	***Combretum imberbe***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. savannae	**CMW36858**		**KC691469**	**KC691493**	**KC691517**	***Colophospermum mopane***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. sublaevis	CMW22444	CBS122518	FJ151430	FJ151464	FJ151486	*Terminalia ivorensis*	Ecuador	M.J. Wingfield	Van Wyk et al. (2011b)
C. sublaevis	CMW22449	CBS122517	FJ151431	FJ151465	FJ151487	*Terminalia ivorensis*	Ecuador	M.J. Wingfield	Van Wyk et al. (2011b)
C. sumatrana	CMW21109	CBS 124011	EU588589	EU588668	EU588676	*Acacia mangium*	Indonesia	M. Tarigan	Tarigan et al. (2010b)
C. sumatrana	CMW21111	CBS 124012	EU588590	EU588669	EU588677	*Acacia mangium*	Indonesia	M. Tarigan	Tarigan et al. (2010b)
C. tribiliformis	CMW13011	CBS115867	AY528991	AY529001	AY529012	*Pinus merkusii*	Indonesia	M.J. Wingfield	Van Wyk et al. (2006a)
C. tribiliformis	CMW13012	CBS118242	AY528992	AY529002	AY529013	*Pinus merkusii*	Indonesia	M.J. Wingfield	Van Wyk et al. (2006a)
C. tyalla	CMW28917		HM071899	HM071909	HQ236448	*Eucalyptus grandis*	Australia	G.K. Kamgan	Kamgan Nkuekam et al. (2012a)
C. tyalla	CMW28920		HM071896	HM071910	HQ236449	*Eucalyptus grandis*	Australia	G.K. Kamgan	Kamgan Nkuekam et al. (2012a)
T. ceramica	CMW15245	CBS122299	EU245022	EU244994	EU244926	*Eucalyptus grandis*	Malawi	R.N. Heath & J. Roux	Heath et al. (2009)
T. ceramica	CMW15248	CBS122300	EU245024	EU244996	EU244928	*Eucalyptus grandis*	Malawi	R.N. Heath & J. Roux	Heath et al. (2009)
Thielaviopsis thielavioides complex									
T. basicola	CMW6714		FJ411331	FJ411357	FJ411305	Daucus carota (carrot)	Australia		Van Wyk et al. (2009a)
T. basicola	CMW25439		FJ411334	FJ411360	FJ411308	*Styrax benzoin*	Indonesia	M.J. Wingfield	Van Wyk et al. (2009a)
T. basicola	**CMW35968**		**KC691470**	**KC691494**	**KC691518**	***Acacia grandicornuta***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
T. basicola	**CMW35969**		**KC691471**	**KC691495**	**KC691519**	***Acacia grandicornuta***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
T. basicola	**CMW35974**		**KC691472**	**KC691496**	**KC691520**	***Colophospermum mopane***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
T. ovoidea	CMW22733	CBS 354.76	FJ411343	FJ411369	FJ411317	Fire wood	Netherlands	Unknown	Van Wyk et al. (2009a)
T. populi	CMW26387	CBS 484.71	FJ411336	FJ411362	FJ411310	*Populus robusta*	Belgium	Unknown	Van Wyk et al. (2009a)
T. populi	CMW26388	CBS 486.71	FJ411337	FJ411363	FJ411311	*Populus gelrica*	Belgium	Unknown	Van Wyk et al. (2009a)
T. thielavioides	CMW22736	CBS 148.37	FJ411342	FJ411367	FJ411315	*Lupinus albus*	Italy	Unknown	Van Wyk et al. (2009a)
T. thielavioides	CMW22737	CBS 180.75	FJ411341	FJ411366	FJ411314	*Populus* sp.	Belgium	Unknown	Van Wyk et al. (2009a)
T. thielavioides	**CMW37309**		**KC691473**	**KC691497**	**KC691521**	***Pseudolachnostylis* sp.**	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**

Table 1 (continued)

Species	Isolate no		Gene region/GeneBank accession no			Host (or substrate)	Geographic origin	Collectors	Relevant references
			ITS	BT	TFF				
T. thielavioides	**CMW37310**		**KC691474**	**KC691498**	**KC691522**	***Philenoptera violacea***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
T. thielavioides	**CMW37311**		**KC691475**	**KC691499**	**KC691523**	***Carpophilus hemipterus***	**South Africa**	**M. Mbenoun & J. Roux**	**Present study**
C. virescens	CMW11164	CBS123166	DQ520639	EF070441	EF070413	*Fagus americana*	USA	D. Houston	Van Wyk et al. (2007b)

Material highlighted in bold was used to generate new sequence data

were selected for culture and morphological studies. These isolates were maintained on 2 % MEA at room temperature. Optimum growth temperatures were determined by comparing colony diameters at six different temperatures, ranging from 10 to 35 °C at 5 °C intervals. For each isolate and at each temperature five replicate plates were prepared by transferring 8-mm-diameter agar plugs from the margins of actively growing cultures to the centers of Petri dishes (90 mm) containing fresh, sterile 2 % MEA. Plates were incubated in the dark for 3 or 14 days depending on whether they were related to *C. moniliformis* s.l. or *C. fimbriata* s.l.. Colony diameters were measured along two perpendicular axes centred on the plugs, and averages and standard deviations were computed.

Morphological characteristics were determined using 2-week and 3-week-old cultures maintained at their optimum growth temperature, respectively for the *C. moniliformis* s.l. and *C. fimbriata* s.l. groups. The mycological colour charts of Rayner (1970) were used to record colony colours. Fungal structures were mounted on microscope slides in 85 % lactic acid and examined under a Zeiss Axioskop microscope (Carl Zeiss, Germany). Images of structures were captured with a HRc Axiocam digital camera fitted to the microscope and structure sizes were determined with the Axiovision 3.1 software also fitted to the microscope. Where possible, 50 measurements were taken for each taxonomically informative morphological character for isolates chosen to represent holotypes, and 10 measurements for isolates chosen as paratypes of new taxa. Specific means and standard deviation values were computed for each character. These measurements are presented as the extremes in parentheses and the range represented by the mean over all holotype and paratype measurements, plus or minus the standard deviations.

All isolates designated as holotypes and paratypes in morphological descriptions are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. The same isolates have also been deposited with the Centraalbureau voor Schimmelcultures (CBS), the Netherlands, and dried herbarium specimens were deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

Results

Isolates

A total of 308 fungal isolates representing three species lineages in the broadly defined *Ceratocystis* s.l. (Wingfield et al. 2013) were collected in this study. Based on morphology, these included 186 isolates resembling species in the *C.*

moniliformis complex, 114 isolates resembling those in the *C. fimbriata* complex (including 104 isolates of *C. albifundus*) and 8 isolates resembling species in the *T. thielavioides* complex. A representative selection of this collection of isolates has been preserved in the culture collection (CMW) at FABI.

Ceratocystis spp. were isolated from various native savanna trees, representing 25 species, 17 genera and 10 plant families (Table 2). They occurred in association with at least six species of nitidulid beetles that belonged to the genera *Brachypeplus* (*B. ater* Grouvelle) and *Carpophilus* (including *Ca. apicipennis* Fairmaire, *Ca. bisignatus* Boheman, *Ca. dimidiatus* (Fabricius), *Ca. hemipterus* L. and one unidentified species.

Species delineation

Ceratocystis moniliformis s.l. group

Successful amplification and sequencing of the β-tubulin gene region was achieved for all selected isolates in the *C. moniliformis* s.l. group, producing sequences of a relatively constant length of about 497 bp. The β-tubulin dataset comprised 69 unique haplotypes and the best-fit model of nucleotide substitution selected for this dataset was TIM1ef+G. The GMYC analysis applied to the β-tubulin gene produced a model with three independent groups (Fig. 1). The model was supported by a significant LR test (likelihood of null model: 589.7; maximum likelihood of GMYC model: 594.6143; LR test: 0 .02111723*). Two of these groups were respectively identified using NCBI-BLAST as *C. oblonga* R.N. Heath & Jol. Roux and *C. savannae*, showing 99–100 % homology with GenBank accessions of respective species. The third group was closely related to, but different, from both *C. oblonga* and *C. savannae.* This group was considered to represent an undescribed species.

Table 2 List of tree species from which *Ceratocystis* species were collected in Kruger National Park

Plant family	Tree species
ANACARDIACEAE	*Lannea stuhlmannii*
	Lannea sp.
	Sclerocarya birrea
CAPPARACEAE	*Boscia albitrunca*
CAESALPINIACEAE	*Cassia abbreviata*
	Colophospermum mopane
	Peltophorum africanum
	Schotia brachypetala
COMBRETACEAE	*Combretum apiculatum*
	Combretum hereroense
	Combretum imberbe
	Combretum molle
	Combretum zeyheri
	Terminalia sericea
EBENACEAE	*Euclea divinorum*
ERYTHROXYLACEAE	*Erythroxylum emarginatum*
EUPHORBIACEAE	*Croton megalobotrys*
	Spirostachys africana
FABACEAE	*Philenoptera violacea*
MIMOSACEAE	*Acacia grandicornuta*
	Acacia nigrescens
	Acacia tortilis
	Acacia xanthophloea
	Acacia sp.
	Albizia harveyi
RHAMNACEAE	*Ziziphus mucronata*

Ceratocystis fimbriata s.l. and *T. thielaviopsis s.l. groups*

ITS sequences were generated for all isolates in the *T. thielaviopsis* s.l. group. Likewise, we were successful in generating ITS sequences for all isolates in the *C. fimbriata* s.l. group, other than *C. albifundus*. The latter could be distinguished by its unique morphological characteristics, and therefore only three isolates of this species were sequenced to supplement the ITS sequence dataset. Isolates involved in vector-cloning produced multiple polymorphic ITS copies. However, differences between sister sequences were phylogenetically insignificant, and we used the 'consensus.seqs' command in MOTHUR v.1.21.1 (Schloss et al. 2009) to create a consensus ITS sequence for each of these isolates. The ITS sequence dataset comprised 31 taxa and was heterogeneous with regards to sequence length. Two groups (509 and 517 bp) associated with *T. thielavioides* s.l. and three groups (~600, ~706 and ~740 bp) associated with *C. fimbriata* s.l. group could clearly be distinguished. The TPM2uf+G was selected as the best-fit model of nucleotide substitution.

The GMYC analysis was consistent with the ITS sequence length polymorphisms, delineating five independent entities (Fig. 2). The model was supported by a highly significant LR value (likelihood of null model: 132.0526; maximum likelihood of GMYC model: 137.8351; likelihood ratio: 11.56507; LR test: 0.00903173**). The GenBank BLAST-search identified the two *Thielaviopsis*-related groups as *T. thielavioides* and *T. basicola* (Berk. & Broome) Ferraris and confirmed the identity of *C. albifundus* with maximum similarity index. The remaining two groups of isolates related to the *C. fimbriata* complex did not match any known species from the GenBank database. They were considered to represent two new species.

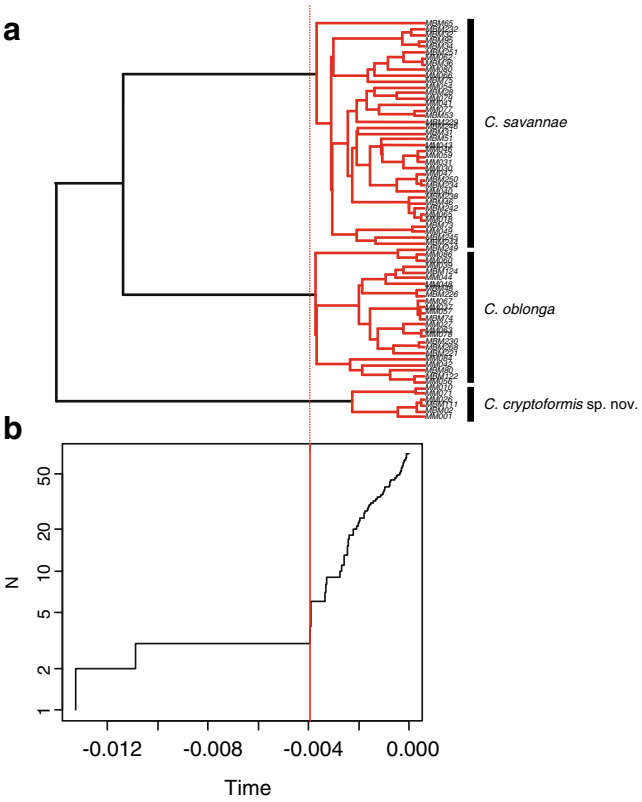

Fig. 1 Species delineation in the *Ceratocystis moniliformis* s.l. group from Kruger National Park based on General Mixed Yule coalescent model. **a** Phylogeny based on the β-tubulin gene obtained from 10^7 generations in BEAST; **b** species through time plot showing the transition between speciation and population processes

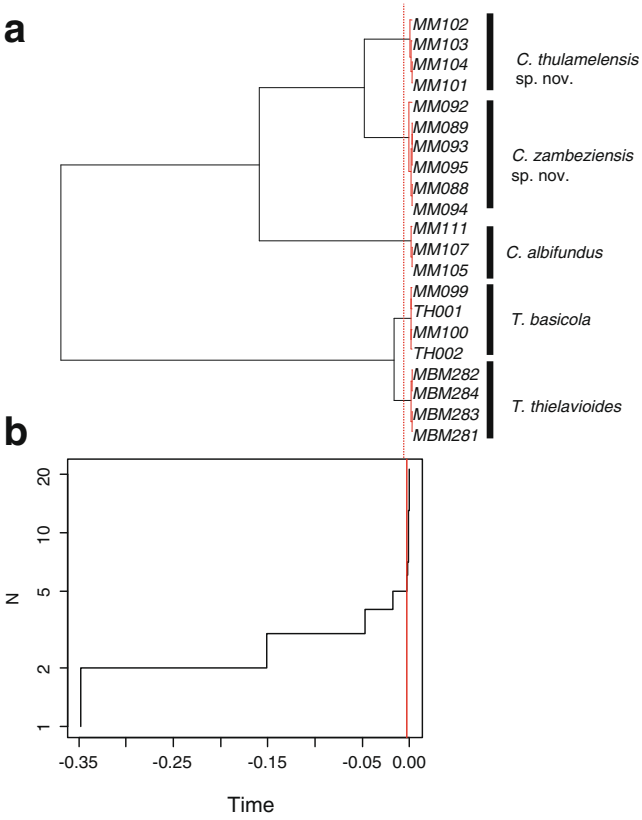

Fig. 2 Species delineation in the *Ceratocystis fimbriata* s.l. and *Thielaviopsis thielavioides* s.l. groups from Kruger National Park based on General Mixed Yule coalescent model. **a** Phylogeny based on the ITS gene obtained from 10^7 generations in BEAST; **b** species through time plot showing the transition between speciation and population processes

Multi-gene phylogenetic analyses

Ceratocystis moniliformis s.l. group

The concatenated data matrix for isolates in the *C. moniliformis* s.l. group included 36 taxa and 1,141 total characters to which the ITS, β-tubulin and TEF-1α gene partitions contributed 374, 442 and 325 characters, respectively. The total number of parsimony-informative characters was 193, whereas the number of parsimony-uninformative characters was 948, which included 14 variable parsimony-uninformative and 934 constant characters. The heuristic search resulted in nine equal MPTs of 288 steps (CI=0.83, RI=0.95, RC=0.79), (Fig. 3). The four isolates representing the putative new species from KNP formed a strongly supported (97 % bootstrap) clade, whereas the remaining KNP isolates grouped with the reference strains for *C. oblonga* and *C. savannae* in two less well-resolved sister groups. The Bayesian phylogenetic reconstruction supported the MP tree topology, producing 100 % posterior probability for the clade representing the putative new species.

In the MP analyses based on single genes, only the β-tubulin gene sequence data could resolve the isolates representing the new species with strong (99 %) bootstrap support (Fig. 4). This apparently undescribed species was monophyletic with the TEF-1α gene but showed incomplete lineage sorting with *C. oblonga*. The PHT (*p*-value= 0.001) indicated little phylogenetic congruence between the three genes. This reflected the poor resolution of the ITS and TEF-1α gene sequences, rather than any major conflict between single MP tree topologies.

Ceratocystis fimbriata s.l. group

The concatenated data matrix for isolates in the *C. fimbriata* s.l. group comprised 69 taxa and 1,908 total characters, including 654 characters from the ITS gene, 559 characters from the β-tubulin gene and 695 characters from the TEF-1α gene. When gaps were considered as new character states, 1001 characters were constant, 230 were variable but parsimony-uninformative and 667 were variable and parsimony-informative. Treating gaps as missing data

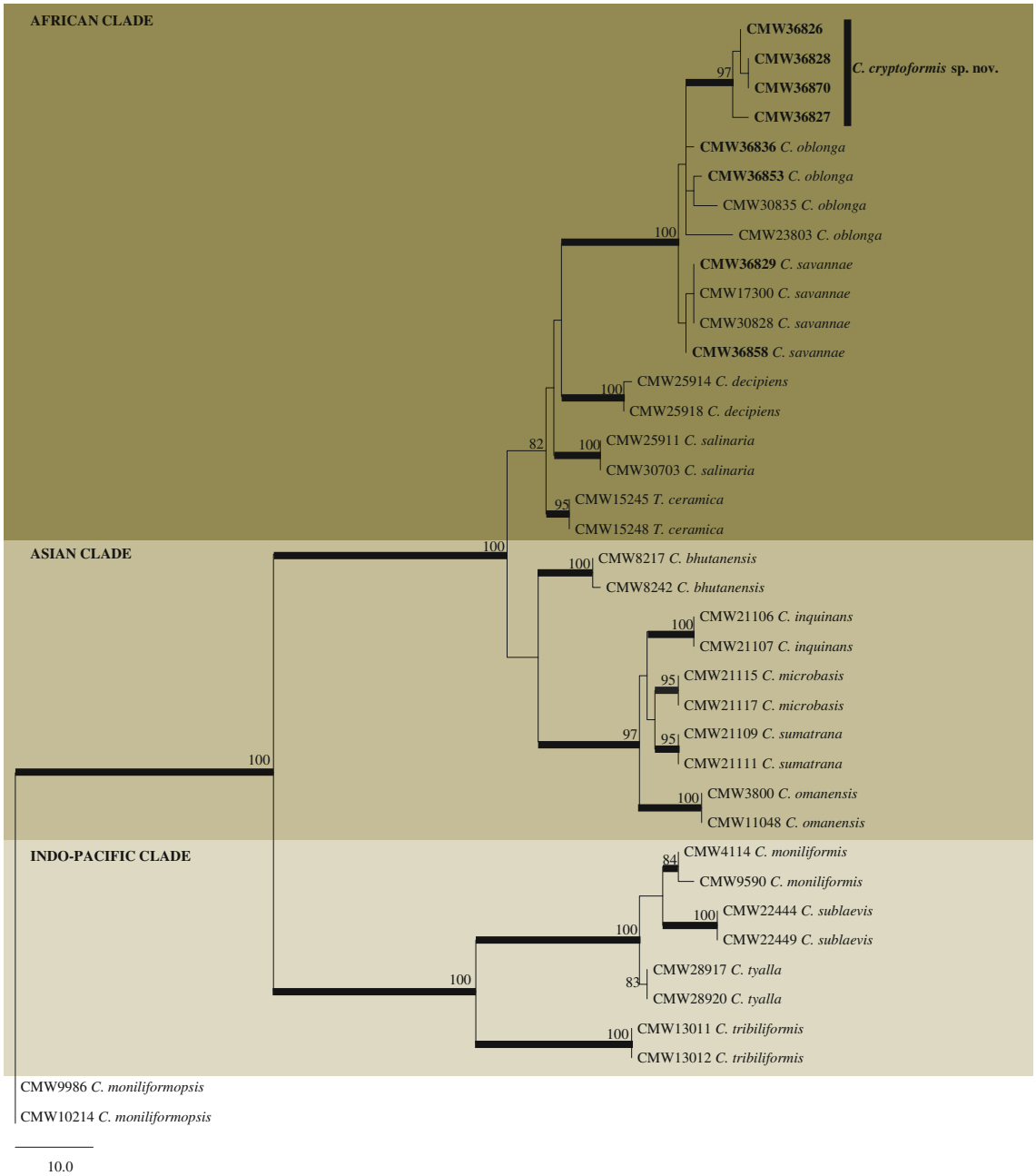

Fig. 3 Most parsimonious tree from 100 heuristic searches with combined ITS, β-tubulin and TEF-1α gene sequences showing the position of *C. cryptoformis* sp. nov. in a global phylogeographic scheme of the *C. moniliformis* species complex. Bootstrap values >70 % from 1,000 replicates are indicated above branches. Thick branches are those with >90 % posterior probability support based on Bayesian, maximum-credibility-consensus trees. Taxa from KNP are highlighted in *bold*

changed these values to 1,250, 162 and 496, respectively. Seven equally most MPTs of 1,818 steps were obtained from the heuristic search when the first gap treatment was enforced, whereas with the second gap treatment, 100 MPTs of 1,146 steps were obtained. In both cases, the same values for goodness of character fit indices (CI=0.62, RI=0.87, RC=0.54) were obtained. Isolates representing the two putative new species from KNP were consistently resolved in two distinct clusters with 100 % bootstrap support. Likewise, two isolates initially identified as *C. albifundus* clustered with reference strains of this species, also with 100 % bootstrap, but formed a distinctive sub-clade (>80 % bootstrap).

Tree topologies under the two gap treatments displayed some differences, especially regarding the placement of the KNP taxa and their relationship with *C. larium* M. Van Wyk

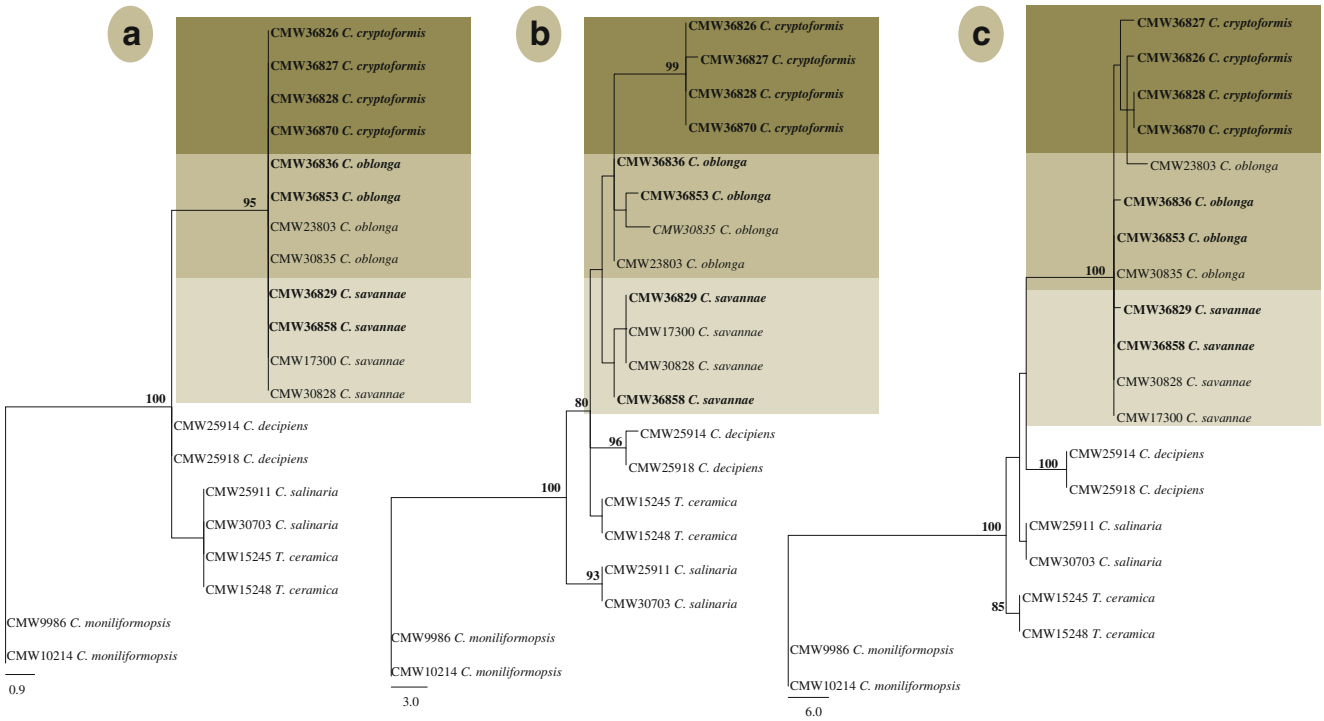

Fig. 4 Single-gene most parsimonious trees of the African clade of *C. moniliformis* s.l. based on **a** ITS (Tree no.: 1; TL: 13; CI: 1; RI: 1; RC: 1), **b** β-tubulin (Tree no.: 7; TL: 51; CI: 0.92; RI: 0.95; RC: 0.87) and **c** TEF-1α (Tree no.: 4; TL: 117; CI: 0.94; RI: 0.96; RC: 0.9) sequence data. Isolates from KNP are highlighted in *bold*. The trees were generated via 100 heuristic searches. Bootstrap values >70 % from 1,000 replicates are indicated above branches. Note: a larger sequence size (786 characters) was used for the TEF-1α gene

& M.J. Wingf. With a full consideration of gaps in the analyses, these fungi shared a relatively recent common ancestor, one of the new species forming a strong (100 % bootstrap) group with *C. tsitsikammensis,* whereas the other grouped loosely with *C. larium, C. albifundus* and *C. tangayicensis* R.N. Heath & Jol Roux. When considering gaps as missing data, a different arrangement of relatedness was derived, in which only the strong association between *C. tsitsikammensis* and one new species was retained. *C. larium* emerged as more closely related to the clade comprising *C. atrox M. van Wyk & M. J. Wingf.* and *C. pirilliformis I. Barnes & M.J. Wingf* than to *C. albifundus,* as it has previously been presented (Van Wyk et al. 2010, 2011a, b, 2012). This second scheme depicted a more biogeographically meaningful representation of the *C. fimbriata* lineage, circumscribing two biogeographic groups respectively centred in Africa and the Indo-pacific region, in addition to the well-known South and North American clades (Fig. 5). Furthermore, this representation was also supported by the results from Bayesian phylogenetic inferences (Fig. 5).

Single-gene MP trees (Fig. 6) each completely resolved the African biogeographic group into five distinct clusters representing the same clades as circumscribed in combined multilocus analyses. The clades representing the two putative new taxa from KNP were supported by bootstrap values ranging between 84 and 100 %. The β-tubulin and TEF-1α trees also separated the reference strains from KNP isolates of *C. albifundus*, with strong (88–99 %) bootstrap support. The PHT (p value = 0.5) indicated high genealogical concordance between the three genes.

Thielaviopsis thielavioides s.l. group

The concatenated data matrix for isolates in the *T. thielaviodes* s.l. group comprised 14 taxa and 1,675 characters, including 404 characters from the ITS gene, 539 characters from the β-tubulin gene and 731 characters from the TEF-1α gene. The total number of parsimony-informative characters was 343 whereas the number of parsimony-uninformative characters was 1332, of which 218 were variable-parsimony-uninformative and 1,114 were constant. The heuristic search resulted in one most parsimonious tree (Fig. 3) of 633 steps (CI=0.85, RI=0.93, RC=0.79). The six isolates from KNP were distributed between two clades, respectively related to *T. basicola* and *T. thielavioides*, with maximum bootstrap support. However, within each of these clades, the KNP isolates formed well resolved clusters. The Bayesian tree topology and posterior probabilities mirrored the MP phylogeny (Fig. 7).

All alignments and phylogenetic trees generated in this study have been uploaded to TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S14151).

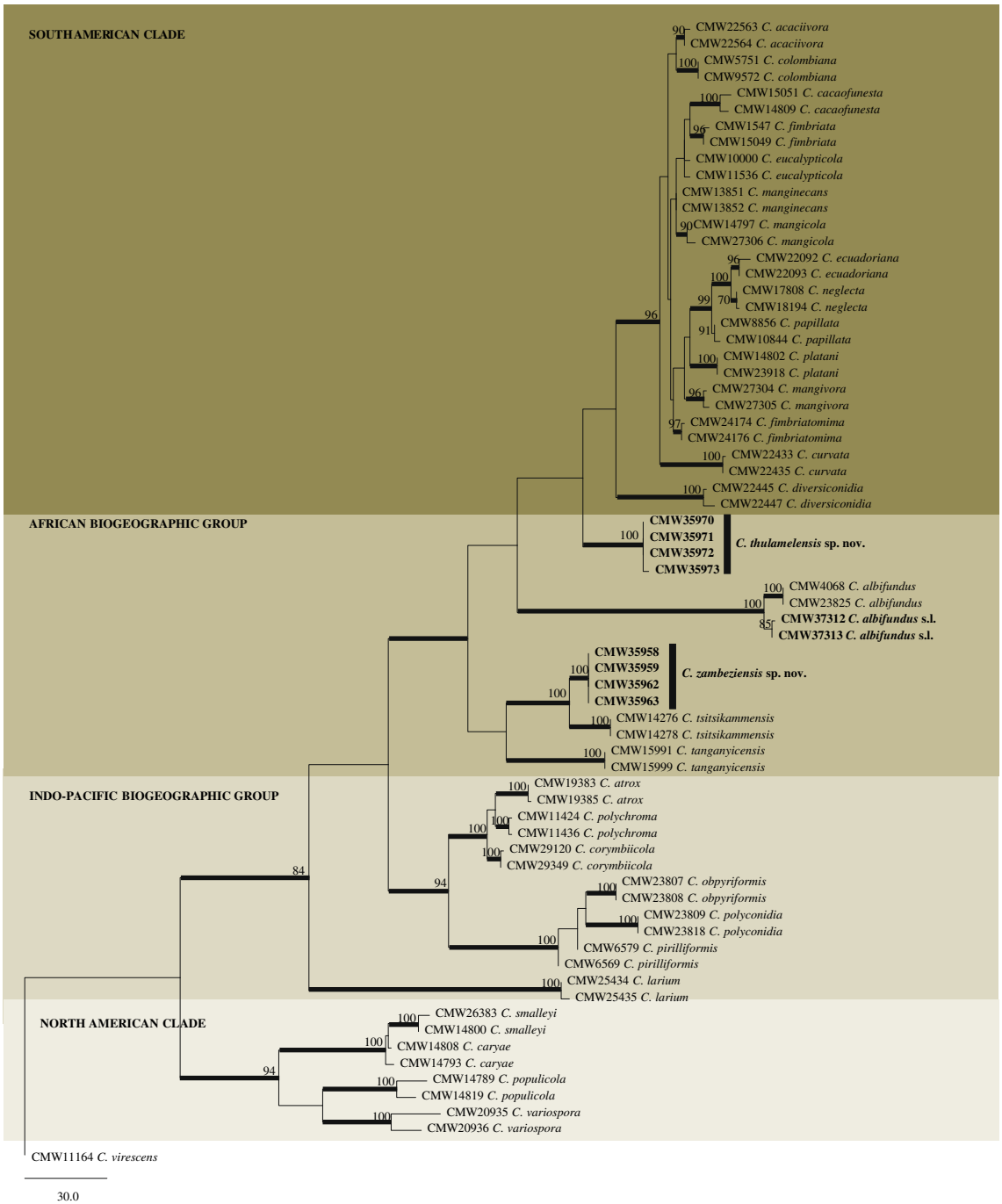

Fig. 5 Most parsimonious tree resulting from 100 heuristic searches with combined ITS, β-tubulin and TEF-1α gene sequences, showing the position of *C. thulamelensis* sp. nov. and *C. zambeziensis* sp. nov in a global phylogeographic scheme for the *C. fimbriata* species complex. Bootstrap values >70 % from 1,000 replicates are indicated above branches. *Thick branches* are those with >90 % posterior probability support based on Bayesian, maximum-credibility-consensus trees. Taxa from KNP are highlighted in *bold*

Taxonomy

Results emerging from phylogenetic analyses of the ITS, β-tubulin and TEF-1α gene sequences showed clearly that isolates of *Ceratocystis* from KNP represented several distinct taxa, including three new species for which morphological descriptions are provided as follows:

Ceratocystis cryptoformis M. Mbenoun & Jol. Roux sp. nov., Fig. 8, MB 804009

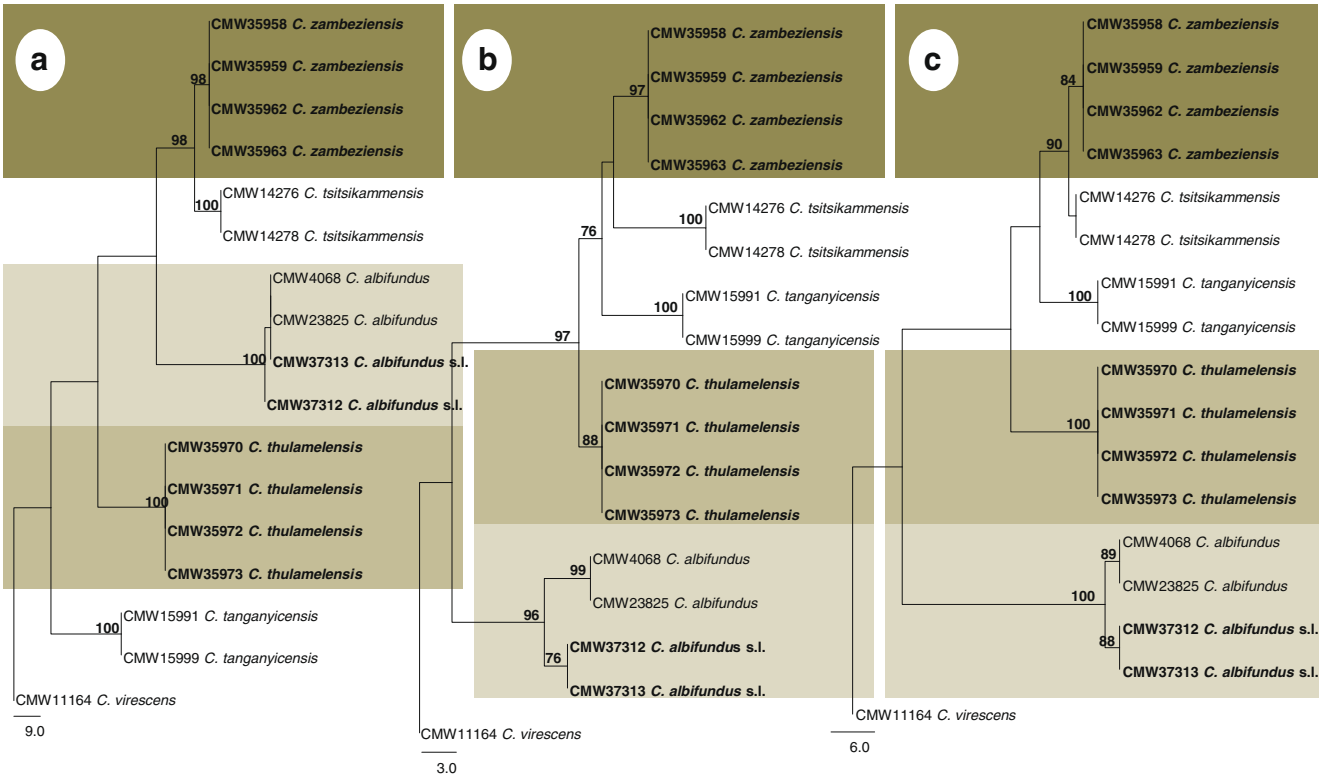

Fig. 6 Single-gene most parsimonious trees of the African biogeographic group of *C. fimbriata* s.l. based on **a** ITS (Tree no.: 4; TL: 173; CI: 0.85; RI: 0.94; RC: 0.8), **b** β-tubulin (Tree no.: 1; TL: 65; CI: 0.94; RI: 0.97; RC: 0.91) and **c**: TEF-1α (Tree no.: 1; TL: 116; CI: 0.93; RI: 0.97; RC: 0.9) sequence data. Isolates from KNP are highlighted in *bold*. The trees were generated via 100 heuristic searches. Bootstrap values >70 % from 1,000 replicates are indicated above branches

Etymology: this name reflects the lack of distinctive morphological characters for this species as compared to its closest relatives.

Culture characteristics: *Colonies* on MEA initially hyaline to white, darkening to isabella colour (19”i), reverse grayish sepia (17””i), after 10 days. *Mycelium* fluffy and superficial. *Hyphae* smooth or granular, septate, without constrictions at septa. *Optimal temperature* for growth 30 °C, growth at 35 °C but no growth at 10 °C. Fast growing, reaching 60–75 mm in diameter within 3 days.

Sexual state: *Ascomata* with bulbous bases and long necks formed superficially on substrate or suspended in aerial mycelium, with a random distribution. *Ascomatal bases* dark brown, globose to obpyriform, often ornamented with scattered conical spines, (124–)204–348(−502) μm high and (138–)185–311(−475) μm wide in diameter. *Ascomatal necks* dark brown, erect or slightly curled, forming disciform structures at the junction with basal bulbs, (322–)480–902(−1160) μm long, (12–)15–21(−25) μm wide at apices and (26–)39–63(−84) μm wide at bases. *Ostiolar hyphae* hyaline, divergent, (21–)27–43(−56) μm long. *Asci* not observed. *Ascospores* accumulating in creamy to yellow droplets at the tips of ascomatal necks, surrounded by sheaths, aseptate, cucullate (hat-shaped) in side view, (5–)6–7 μm wide and 3–4 μm long.

Asexual state: typical of *Thielaviopsis* with enteroblastic conidium ontogeny. *Conidiophores* occurring solitary or aggregated in small bundles and arising laterally from vegetative hyphae, hyaline, phialidic, lageniform, (17–)20–34(−44) μm long, 2–3 μm wide at apices and (3–)4–5 μm wide at bases. *Primary conidia* hyaline, aseptate, cylindrical, (3–)5–6(−8) μm long and 2–3 μm wide. *Secondary conidia* hyaline, aseptate, diversiform, 6–9(−12) μm long and (2–)3–4(−6) μm wide. *Aleurioconidia* not observed.

Specimen examined: South Africa, Kruger National Park, near Skukuza (25 07.283S, 31 21.296E), isolated from wound on *Ziziphus mucronata,* June 2010, M. Mbenoun & J. Roux, HOLOTYPE PREM 60824, culture ex-type CMW 36828=CBS 131279.

Additional specimens: South Africa, Kruger National Park, near Skukuza (25 03.260S, 31 34.419E), isolated from wound on *Terminalia sericea*, June 2010, M. Mbenoun & J. Roux, PREM 60822 (PARATYPE), culture ex-type CMW 36826=CBS 131277; KNP, near Skukuza (S25 06.009 S, E31 27.742), isolated from wound on *Combretum zeyheri*,

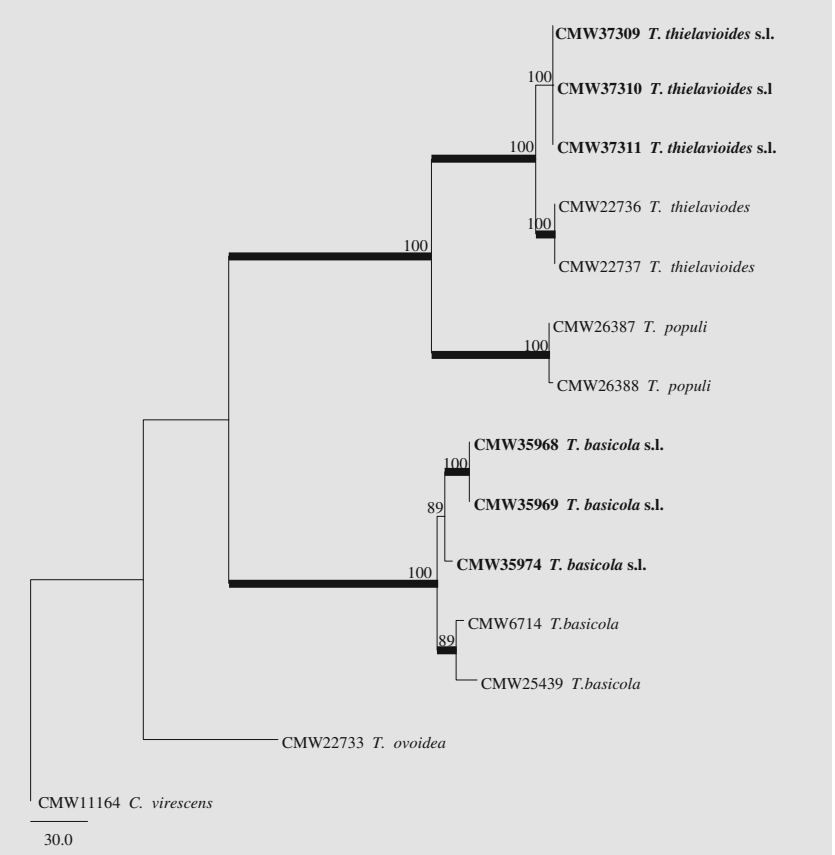

Fig. 7 Most parsimonious tree from 100 heuristic searches with combined ITS, β-tubulin and TEF-1α gene sequences depicting the relationship between KNP isolates (bold) and references of species in the *T. thielavioides* complex. Bootstrap values >70 % from 1,000 replicates are indicated above branches. *Thick branches* are those with >90 % posterior probability support based on Bayesian, maximum-credibility-consensus trees

June 2010, M. Mbenoun & J. Roux, PREM 60823, culture ex-type CMW 36827=CBS 131278.

Ceratocystis thulamelensis M. Mbenoun & Jol. Roux sp. nov., Fig. 9, MB 804010

Etymology: the name refers to the extinct civilization of THULAMELA whose vestiges are found in the same area where this fungus was first collected.

Culture characteristics: *Colonies* on MEA grayish olivaceous (21""), reverse grayish olivaceous, turning dark olive-brown with age. *Mycelium* sparse, immersed and superficial. *Hyphae* smooth, septate, without constriction at septa. *Optimal temperature* for growth 25 °C, slow-growing, 30–50 mm colony diameter in 2 weeks, no growth at 10 °C and at 35 °C.

Sexual state: *Ascomata* with bulbous bases and long necks formed superficially or partially submerged in substrate, with a scattered distribution. *Ascomatal bases* dark brown to black, globose, (88–)129–219(−246) μm long and (70–)129–219(−243) μm wide in diameter. *Ascomatal necks* brown, erect, slender (203–)265–407(−487) μm long, (11–)15–25(−30) μm wide at apices and (23–)28–44(−58) μm wide at bases. *Ostiolar hyphae* hyaline, divergent, (21–)28–48(−57) μm long. *Asci* not observed. *Ascospores* accumulating in creamy to yellow droplets at the tips of ascomatal necks, embedded in sheaths, aseptate, cucullate (hat-shaped) in side view, (5–)6–7(−8) μm wide, (2–)3–4 μm high.

Asexual state: typical of *Thielaviopsis* with enteroblastic conidium ontogeny. *Conidiophores* of two types, phialidic, occurring solitary. *Primary conidiophores* hyaline at apices, turning brown towards bases with one to three basal septa when arising laterally from vegetative hyphae, lageniform, (36–)61–121(−175) μm long, (2–)3–5 μm wide at apices and (2–)5–6 wide at bases, hyaline, tubular and variable in size when terminal on hyphae. *Secondary conidiophores* borne near the bases of ascomata, light brown, flaring, size not determined (because of scarce numbers). *Primary conidia* hyaline, aseptate, cylindrical or rectangular, (9–)11–17(−22) μm long and (2–)3–4 μm wide. *Secondary conidia* hyaline, aseptate, barel-shaped, 7–10(−11) μm long and 6–8 μm wide. *Aleurioconidia* brown, thick-walled, globose to subglobose, (11–)13–15(−17) μm long and (10–)11–13(−14) μm wide.

Specimen examined: South Africa, Kruger National Park, near Punda Maria (22 40.537S, 31 06.893E), isolated from wound on *Colophospermum mopane,* June 2010, M. Mbenoun & J. Roux, HOLOTYPE PREM 60828, culture ex-type CMW 35972=CBS 131284.

Additional specimens: South Africa, Kruger National Park, near Punda Maria (22 44.022S, 31 00.956E), isolated from wound on *Combretum zeyheri*, June 2010, M.

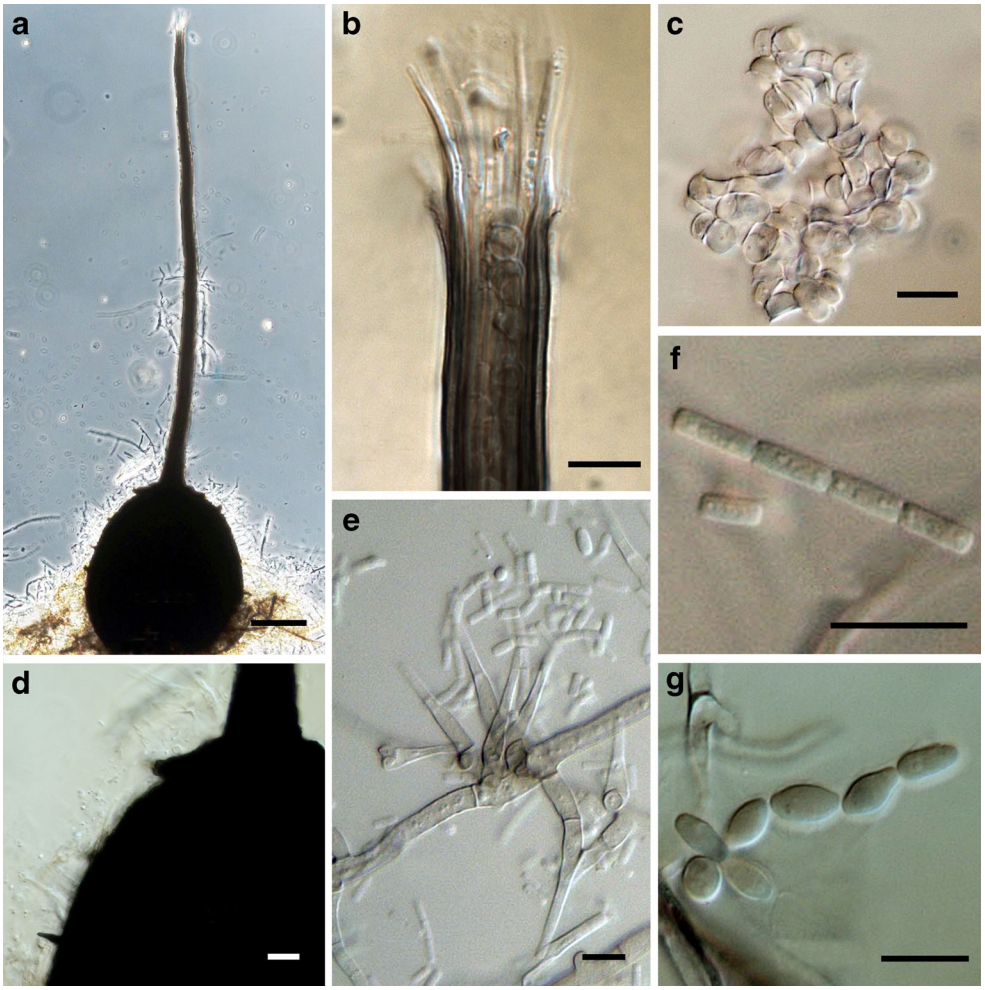

Fig. 8 Morphological characteristics of *Ceratocystis cryptoformis* sp. nov. **a** ascomata with globose base and extended neck; **b** details of neck tip showing divergent ostiolar hyphae; **c** cucullate (hat-shaped) ascospores; **d** details of ascocarp base showing disciform structure at neck base and conical ornamentations; **e** lageniform phialides; **f** cylindrical primary conidia; **g** diversiform secondary conidia

Mbenoun & J. Roux, PREM 60827 (PARATYPE), culture ex-type CMW 35971=CBS 131283; South Africa, Kruger National Park, near Punda Maria (22 40.537S, 31 06.893E), isolated from wound on *Colophospermum mopane,* June 2010, M. Mbenoun & J. Roux, CMW 35973=CBS 131285.

Ceratocystis zambeziensis M. Mbenoun & Jol. Roux sp. nov., Fig. 10, MB 804011

Etymology: the name refers to the broad Zambezian ecoregion that includes the Kruger National Park and the areas where this species was collected.

Culture characteristics: *Colonies* on MEA greenish olivaceous (23'''), reverse greenish olivaceous. *Mycelium* immersed and superficial. *Hyphae* smooth, septate, without constriction at septa. *Optimal temperature* for growth 25 °C, slow-growing, colony diameters reaching ~60 mm in diameter within 2 weeks, no growth at 10 °C and 35 °C.

Sexual state: *Ascomata* with bulbous bases and long necks formed superficially or partially submerged in substrate, with a scattered distribution. *Ascomatal bases* dark brown to black, globose, (100–)151–229(−294) μm high and (103–)147–215(−251) μm wide in diameter. *Ascomatal necks* dark brown, erect, slender, (124–)288–486(−601) μm long, (11–)16–24(−44) μm wide at apices and (20–)22–34(−50) μm wide at bases. *Ostiolar hyphae* hyaline, convergent, (35–)43–63(−77) μm long. *Asci* not observed. *Ascospores* accumulating in creamy to yellow droplets at the tips of ascomatal necks, embedded in sheaths, aseptate, cucullate (hat-shaped) in side view, (5−)6−7(−8) μm wide, 3–4 μm long.

Asexual state: typical of *Thielaviopsis* with enteroblastic conidium ontogeny. *Primary conidiophores* hyaline at apices, turning brown towards bases, multi-septate, phialidic, tubular, tapering at apices (47–)87–223(−236) μm long, (3–)4−5 μm wide at apices and (4–)5–7 μm wide at apices and (3.9–)5.4–6.6(−6.9) μm wide at bases. *Secondary conidiophores* not observed. *Primary conidia* hyaline, aseptate, bacilliform-shaped, (9–)12–18(−22) μm long and 3–4(−6) μm wide. *Secondary conidia* not observed. *Aleurioconidia* brown, thick-walled, globose to spherical, (10–)12–14(−16) μm long and (9–)12–14(−15) μm wide.

Specimen examined: South Africa, Kruger National Park, near Satara (24 21.948S, 31 45.861E), isolated from wound on *Combretum imberbe,* June 2010, M. Mbenoun & J.

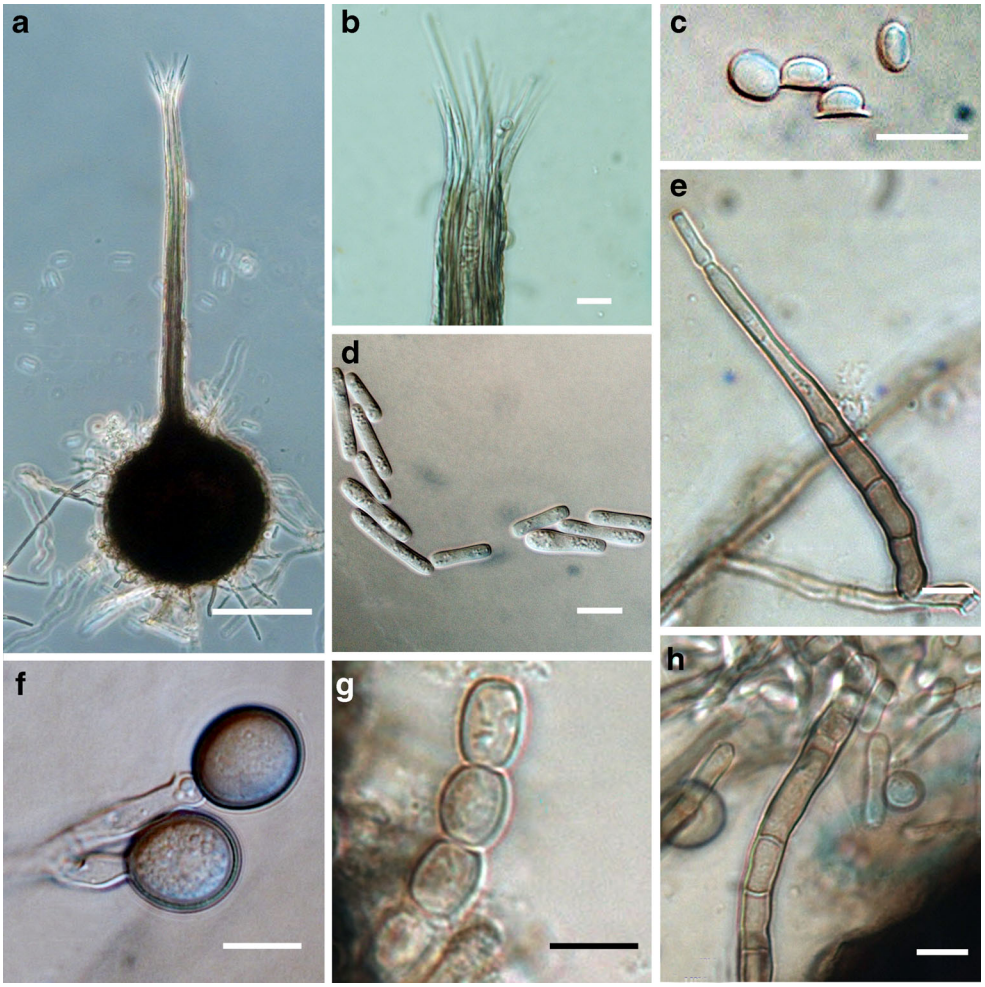

Fig. 9 Morphological characteristics of *Ceratocystis thulamelensis* sp. nov. **a** ascomata with globose base and extended neck; **b** details of neck tip showing divergent ostiolar hyphae; **c** cucullate (hat-shaped) ascospores; **d** bacilliform primary conidia; **e** lageniform phialidic conidiophores; **f** globose aleurioconidia; **g** oblong secondary conidia; **h** flaring secondary conidiophore

Roux, HOLOTYPE PREM 60825, culture ex-type CMW 35958=CBS 131280.

Additional specimens: South Africa, Kruger National Park, near Satara (24 22.026S, 31 45.897E), isolated from wound on *Acacia nigrescens*, June 2010, M. Mbenoun & J. Roux, PREM 60826 (PARATYPE), culture ex-type CMW 35963=CBS 131282; South Africa, Kruger National Park, near Satara (24 25.737S, 31 47.265E), isolated from wound on *Schotia brachypetala*, June 2010, M. Mbenoun & J. Roux, culture ex-type CMW 3596=CBS 131281.

Discussion

This study encompasses the description of three previously unknown species of *Ceratocystis*, namely, *C. cryptoformis*, *C. thulamelensis* and *C. zambeziensis*. These fungi were discovered during a survey of *Ceratocystis* spp. infecting trees in a natural savanna ecosystem in South Africa. Their primary identification among other co-occurring sister species was based on single gene sequence data, applying a statistical phylogenetic approach based on the general mixed Yule coalescent (GMYC) model (Pons et al. 2006). Multi-gene phylogenetic analyses of three gene regions, and especially the genealogical concordance phylogenetic species recognition (GCPSR) concept (Taylor et al. 2000), supported the GMYC-based identification. This highlighted the reliability of the latter method for fungal species recognition. However, the GMYC model is only as good as the taxonomic resolution of the gene region used. In this study, our selection of the ITS and β-tubulin was informed by previous studies (Van Wyk et al. 2011a, b; Kamgan Nkuekam et al. 2012a, b) that have shown that these two genes are among the best available for delineating species, respectively, in the *C. fimbriata* (as well as *T. thielavioides*) and *C. moniliformis* complexes.

Ceratocystis cryptoformis resides in the *C. moniliformis* complex (Van Wyk et al. 2006a; Wingfield et al. 2013). The closest relatives of this species in the global multi-gene phylogeny of this lineage are *C. oblonga* and *C. savannae*, also occurring in KNP. The three species form a well-resolved group in what appears to represent an African clade of *C. moniliformis* s.l., in distinction to the Asian and Indo-pacific clades. This clade also includes *T. ceramica* R.N. Heath & Jol. Roux (Heath et al. 2009), *C. decipiens*

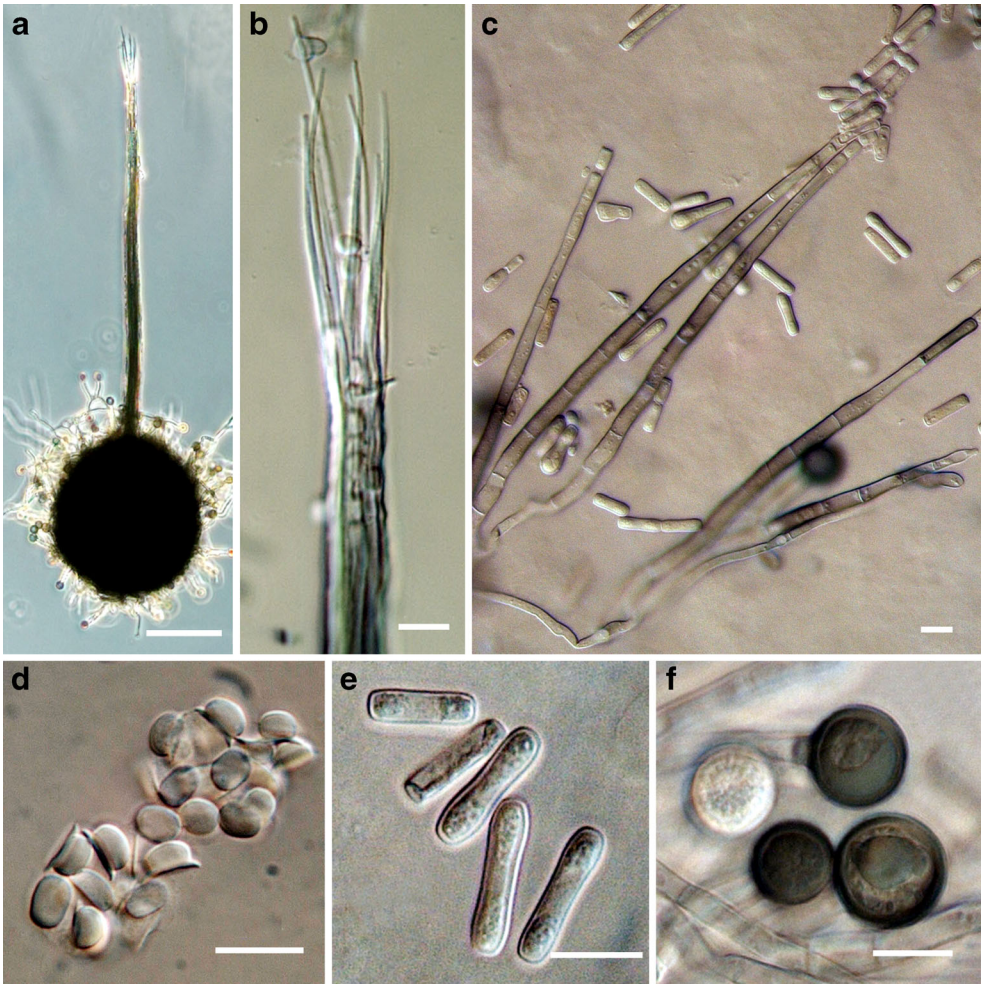

Fig. 10 Morphological characteristics of *Ceratocystis zambeziensis* sp. nov. **a** ascomata with globose base and extended neck; **b** details of neck tip showing convergent ostiolar hyphae; **c** tubular phialidic conidiophores; **d** cucullate (*hat-shaped*) ascospores; **e** bacilliform primary conidia; **f** spherical aleurioconidia

Kamgan-Nkuek. & Jol. Roux and *C. salinaria* Kamgan-Nkuek. & Jol. Roux (Kamgan Nkuekam et al. 2012b). In the description of the latter two species, it emerged that, among the three commonly used loci for inferring phylogenetic relationships in *Ceratocystis*, the β-tubulin gene performs the best for delineating cryptic species within the African clade of *C. moniliformis* s.l. The TEF-1α showed incomplete lineage sorting, whereas the ITS showed no resolution. The results obtained from our single-locus analyses are consistent with these observations. More generally, phylogenetic studies in *Ceratocystis* have been faced with the problem of limited resolution of available markers. Although in most recent cases the rule has been to follow the GCPSR, the recognition of several species has, in reality, relied on a single gene (Van Wyk et al. 2011a, b, 2012; Kamgan Nkuekam et al. 2012b). In this process, additional arguments demonstrating the robustness of such a "pseudo" GCPSR approach have been sought through haplotype networks and/or fixed nucleotide polymorphism analyses. In the present case, we adopted the view that such supplementary analyses would be superfluous, considering that the taxonomic distinctiveness of *C. cryptoformis* with respect to its two closest relatives has been demonstrated using less subjective GMYC analyses based on the β-tubulin gene sequences.

Ceratocystis cryptoformis, *C. oblonga* and *C. savannae* can be considered cryptic species. They portray the same general morphological and culture characteristics typical of *C. moniliformis* s.l., including a rapid growth on artificial media, the presence of conical ornamentations on their ascocarp bases and the seeming absence of aleurioconidia (chlamydospores). *C. cryptoformis* may be distinguished by slightly larger ascomata or smaller primary conidia, but more generally the three species have overlapping morphometric values for taxonomically informative characters. The presence of granular hyphae in its mycelium makes *C. cryptoformis* closer to *C. oblonga,* whereas its temperature optimum of 30 °C and the ability to survive at 35 °C are reminiscent of *C. savannae.* These three fungi coexist as saprophytes on tree wounds in the northern Limpopo Province of South Africa. Their growth patterns in response to various temperatures in combination with previous collection records (Kamgan Nkuekam et al. 2008, 2012b; Heath et al. 2009) suggest that the geographic distribution

of *C. cryptoformis* and *C. savannae* extends northwards while that of *C. oblonga* extends southwards.

Like its two siblings, *C. cryptoformis* is not known outside Africa. It is likely that the three fungi are native to Africa and the savanna ecosystem represents their natural habitat. However, while *C. oblonga* and *C. savannae* have invaded adjacent commercial plantations of non-native *Acacia* and/or Eucalypt tree species (Heath et al. 2009; Kamgan Nkuekam et al. 2012b), *C. cryptoformis* has not been detected beyond the natural savanna environment in KNP.

Ceratocystis thulamelensis and *C. zambeziensis* are both members of the *C. fimbriata* complex (Johnson et al. 2005; Wingfield et al. 2013). This group was the first in *Ceratocystis* in which geographical differentiation was showed in its phylogenetic structure, notably with the identification of three geographic clades, respectively centred in North America, South America and Asia (Johnson et al. 2005). As additional species are discovered and included in phylogenetic analyses, emerging evidence suggests that additional centres of diversification for *C. fimbriata* s.l. may be found elsewhere in the world. In Africa. in particular, the supplementary information emerging from the two new species described here reveals that at least three clades have radiated on the continent. *C. zambeziensis* resides in one of these clades, along with *C. tsitsikammensis* and *C. tanganyicensis,* while *C. thulamelensis* and *C. albifundus* each represent a distinct clade with no known close relatives. All these groups have a common ancestor, which is also shared by members in the South American clade, making the circumscription of a coalesced, unique African lineage for *C. fimbriata* s.l. problematic. On the other hand, genetic distances between the African clades are considerable; for instance, *C. thulamelensis* is closer to the South American clade than it is to either of the two other African clades. For all these reasons, we consider it appropriate to use the terminology "African biogeographic group" to refer to *C. fimbriata* s.l. from Africa. A similar group can be defined for the Indo-Pacific region. Our results suggest that these two biogeographic groups, as well as the South American clade, have evolved from a common ancestor.

In contrast to *C. moniliformis* s.l. species from Africa, members of the African biogeographic group of *C. fimbriata* s.l. are phylogenetically well resolved. This is distinctly reflected in each of the three genes used in this study. Moreover, the congruence of the three genes is supported by a highly significant PHT. However, with the exception of *C. albifundus*, easily distinguishable by its unique morphology, only minor morphological differences separate species of *C. fimbriata* s.l. from Africa. These fungi portray the general characteristics inherent to the *C. fimbriata* complex, including a slow growth, the absence of ornamentation on their ascocarp bases and the production of aleurioconidia. Their morphometric characteristics for taxonomically informative characters, however, generally overlap.

C. thulamelensis and *C. zambeziensis* have the same temperature optima at 25 °C, similar to *C. tsitsikammensis*, but in contrast to *C. tanganyicensis* (20 °C) and *C. albifundus* (30 °C). The only marked differences between the two new species in their colony characteristics are the colour and rate of growth. *C. thulamelensis* grows more slowly, forming darker olivaceous colonies with sparse mycelium. This fungus is also characterized by its propensity to lose the capacity to form ascomata on artificial medium, usually after the first transfer. But, colony characteristics for *C. zambeziensis* are similar to those reported for *C. tsitsikammensis*, with which it shares a lack of, or scarcity of secondary conidia.

The three new *Ceratocystis* species described in this study were collected in KNP along with five previously well-known species, including *C. oblonga* and *C. savannae* in the *C.moniliformis* s.l. lineage, *C. albifundus* in the *C. fimbriata* s.l. lineage and *T. basicola* and *T. thielalavioides* in the *T. thielavioides* s.l. lineage. While it is undoubted that KNP isolates of *C. savannae* and *C. oblonga* and their respective references from GenBank are monophyletic and represent single taxonomic entities, results of this study include some evidence suggesting that populations of *C. albifundus*, *T. basicola* and *T. thielavioides* from the KNP may represent, or include cryptic species. This is because there was substantial polymorphism within the clades representing these species in multi-gene phylogenies. Moreover, in the case of *C. albifundus*, single-locus MP analyses based on β-tubulin and TEF-1α gene sequences concordantly separated the KNP isolates from GenBank references for this species. Further investigations will be needed to clarify the taxonomic status of these isolates.

This study highlights the fact that the diversity of *Ceratocystis* species in natural ecosystems in Africa is still largely overlooked. Vast areas of natural vegetation, with similar or different ecologies, exist on the continent and have not been explored. Surveying more of these natural ecosystems will result in the discovery of more species and lineages and provide important information about the distribution and host range of these fungi, especially for the taxa with the potential to initiate disease outbreaks. These studies will also provide opportunities to investigate more evolutionary questions such as those related to ecological specialization. On the other hand, we showed that careful selection of molecular markers and phylogenetic approaches, especially the GMYC model, could efficiently assist in resolving issues regarding species boundaries in this fungal group.

Acknowledgements This study was supported financially by the Department of Science and Technology (DST)/National Research Foundation (NRF) Centre of Excellence in Tree Health Biotechnology (CTHB) of South Africa. We are grateful to the South African National Parks (SanParks) scientific services at Skukuza for technical and logistical assistance during the field survey and to Prof. Timothy Barraclough for his advice on using the GMYC model.

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