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Ceratocystis species, including two new taxa, from *Eucalyptus* trees in South Africa

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Abstract The ascomycete genus *Ceratocystis* (Microascales, Ceratocystidaceae) includes important fungal pathogens of trees, including *Eucalyptus* species. *Ceratocystis* species and their *Thielaviopsis* asexual states are typically associated with insects, such as nitidulid beetles, that spread them over long distances. *Eucalyptus* trees comprise a substantial component of the forestry industry in South Africa, however, limited information is available regarding *Ceratocystis* species that infect these trees. In this study, *Ceratocystis* species were collected from wounds on *Eucalyptus* trees in all the major plantation regions of South Africa, as well as from insects associated with these wounds. Both morphology and multigene DNA sequence analyses, using three nuclear loci, were used to identify the *Ceratocystis* species. Of the 260 isolates collected, nine *Ceratocystis* species, of which two were represented only by their *Thielaviopsis* anamorph states were identified. These species were *C. eucalypticola*, *C. pirilliformis*, *C. savannae*, *C. oblonga*, *C. moniliformis*, *T. basicola*, *T. thielavioides* and two *Ceratocystis* species that are described here as *C. salinaria* sp. nov. and *C. decipiens* sp. nov. Insects associated with these *Ceratocystis* species were *Brachypeplus depressus* (Nitidulidae), *Carpophylus bisignatus*, *C. dimidiatus* (Nitidulidae), *Xyleborus affinis* (Scolytidae), *Litargus* sp. (Mycetophagidae) and a Staphylinid (Staphylinidae) species.

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Introduction

Species in the fungal genus *Ceratocystis* Ellis & Halsted (Ascomycetes: Microascales, Ceratocystidaceae), and their *Thielaviopsis* Went anamorphs, include important pathogens of agricultural and forestry crops (Kile 1993; Roux and Wingfield 2009). These fungi can cause diseases including stem cankers, root and fruit rot, as well as vascular wilts. The type species of *Ceratocystis*, *C. fimbriata* Ellis & Halsted *sensu stricto* (*s.s.*), is the causal agent of sweet potato black rot disease (Halsted 1890; Halsted and Fairchild 1891). Other important diseases caused by *Ceratocystis* species include canker stain of plane trees caused by *C. platani* (Walter) Engelbrecht & Harrington (Walter et al. 1952; Panconesi 1999), oak wilt caused by *C. fagacearum* (Bretz) J. Hunt (Juzwik et al. 2008; Sinclair and Lyon 2005) and wilt of *Acacia mearnsii* de Wild trees caused by *C. albifundus* De Beer, Wingfield & Morris (Morris et al. 1993; Wingfield et al. 1996).

Ceratocystis species require wounds to infect their hosts (Moller and Devay 1968; Walter et al. 1952; Kile 1993) and are associated with insects that act as their vectors. Most *Ceratocystis* species are vectored by sap-feeding nitidulids and flies in what is considered a non-specific association (Cease and Juzwik 2001; Moller and Devay 1968). There is, however, growing evidence that the association between some nitidulid beetles and *Ceratocystis* species, such as *C.*

fagacearum, are not entirely casual as was previously believed (Juzwik et al. 2004; Hayslett et al. 2007). Some *Ceratocystis* species, such as *C. polonica* (Siemaszko) C. Moreau, *C. laricicola* Redfern & Minter, *C. rufipenni* Wingfield, Harrington & Solheim and *C. fujiensis* M. J. Wingf., Yamaoka & Marin, occur on conifers and are vectored by bark beetles in what is considered a specific association (Harrington and Wingfield 1998; Wingfield et al. 1997; Marin et al. 2005).

There have been increasing numbers of reports of *Ceratocystis* species infecting or causing diseases of *Eucalyptus* during the course of the last 10 years (Roux and Wingfield 2009). Thirteen *Ceratocystis* species have been reported infecting wounds on non-native *Eucalyptus* trees in plantations worldwide. *C. atrox* M. Van Wyk & M.J. Wingfield (Van Wyk et al. 2007a), *C. eucalypti* Z.Q. Yuan & Kile (Kile et al. 1996), *C. corymbiicola* Kamgan-Nkuek. & Jol. Roux and *C. tyalla* Kamgan-Nkuek. & Jol. Roux are known only from Australia (Kamgan Nkuekam et al. 2012), *C. neglecta* M. van Wyk, Jol. Roux & C. Rodas, *C. ecuadoriana* M. Van Wyk & M.J. Wingf. and *C. curvata* M. Van Wyk & M.J. Wingf. from Colombia (Rodas et al. 2008; Van Wyk et al. 2011), *C. fimbriatomima* M. van Wyk & M.J. Wingf. from Venezuela (Van Wyk et al. 2009), *C. zombamontana* R.N. Heath & Jol. Roux from Malawi (Heath et al. 2009a), *C. moniliformis* (Hedgcock) Moreau from South Africa and Tanzania (Heath et al. 2009a), *C. pirilliformis* I. Barnes & M.J. Wingf. from Australia and South Africa (Barnes et al. 2003a; Roux et al. 2004; Kamgan Nkuekam et al. 2009), *C. eucalypticola* M. van Wyk & M.J. Wingf. from South Africa (Van Wyk et al. 2012) and *C. fimbriata sensu lato* (*s.l.*) from Brazil (Ferreira et al. 1999), Uganda (Roux et al. 2001), Uruguay (Barnes et al. 2003b), Thailand and Indonesia (Van Wyk et al. 2012). Of these, *C. fimbriata s.l.* has been shown to cause wilt and death of *Eucalyptus* trees in Brazil (Ferreira et al. 1999), Uganda (Roux et al. 2001) and Uruguay (Barnes et al. 2003b), while *C. eucalypticola* appears to be the cause of disease on *Eucalyptus* trees in the Republic of Congo (Roux et al. 1999).

Three *Ceratocystis* species have been reported from wounds on *Eucalyptus* trees in South Africa. These are *C. eucalypticola*, first reported as *C. fimbriata s.l.* (Roux et al. 2004), *C. moniliformis* and *C. pirilliformis* (Roux et al. 2004; Kamgan Nkuekam et al. 2009). *C. eucalypticola* and *C. pirilliformis* have not been associated with naturally dying *Eucalyptus* trees in South Africa, but artificial inoculation with these fungi in both the field and greenhouse resulted in distinct lesions, suggesting that they have the potential to kill these trees (Roux et al. 2004). Other *Ceratocystis* species known from trees in South Africa occur on non-native *A. mearnsii* (Morris et al. 1993; Wingfield et al. 1996) or indigenous trees (Roux et al. 2007; Kamgan Nkuekam et al. 2008). In this regard, there is growing concern that *Ceratocystis* species can shift hosts, such as is the case for *C. albifundus*, first isolated from native *Protea* species in South Africa (Gorter 1977) and later found causing disease on non-native *A. mearnsii* trees in plantations (Morris et al. 1993; Wingfield et al. 1996; Roux and Wingfield 2009).

Very little is known regarding the insect associates of *Ceratocystis* species in South Africa. In a recent study considering the epidemiology of the wattle wilt pathogen, *C. albifundus*, in the country, this fungus and *Ceratocystis oblonga* R.N. Heath & Jol. Roux were isolated from three nitidulid (Coleoptera, Nitidulidae) beetle species namely, *Brachypeplus depressus* Erichson, *Carpophilus bisignatus* Boheman and *Ca. hemipterus* L. (Heath et al. 2009b). These insects were collected from both indigenous woodlands and from commercial plantations of non-native *A. mearnsii* trees, where they were either caught in insect traps or collected from beneath bark flaps on cut stumps (Heath et al. 2009b). The presence of these fungi on free-flying nitidulid beetles and on insects occurring on fungal mats growing under bark flaps suggested that nitidulid beetles are vectors of *C. albifundus* and *C. oblonga* on both native trees and *A. mearnsii* in its non-native range in South Africa (Heath et al. 2009b).

Previous studies of *Ceratocystis* species on *Eucalyptus* in South Africa have been limited to a small number of geographic and climatic areas and a limited number of *Eucalyptus* species. The recent discoveries of previously undescribed *Ceratocystis* species from native trees in South Africa, as well as from numerous *Eucalyptus* spp. in Australia and South America, suggest that additional species could occur on *Eucalyptus* trees in South Africa. This, together with the limited information regarding the biology and epidemiology of *Ceratocystis* species on *Eucalyptus* prompted this study, aimed at expanding the base of knowledge of the diversity of *Ceratocystis* species infecting these trees in the country. The nitidulid vectors of these fungi in commercial *Eucalyptus* plantations were also identified.

Materials and methods

Collection of fungal isolates

Ceratocystis species were collected from wounds on *Eucalyptus* trees in South African plantations over a 2 year period from February 2007 to December 2008. Collection sites covered the majority of the *Eucalyptus* growing areas of the country and included localities near Louis Trichardt and Tzaneen (Limpopo Province), Lothair and Sabie (Mpumalanga Province), George, Cape Town and Stellenbosch (Western Cape Province), Kumbo and Lotobeni (Eastern Cape Province) and localities near

KwaMbonambi and Pietermaritzburg (KwaZulu-Natal Province). Samples were mainly collected from the stumps of freshly harvested trees and from logs, either on the plantation floor or at the harbor.

Pieces of bark or wood were collected from cut stumps and transported to the laboratory as described by Kamgan Nkuekam et al. (2009). Isolation and purification of fungi from samples followed the protocol described by Kamgan Nkuekam et al. (2009). Isolates were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative specimens have also been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. Dried specimens of representative isolates were deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

Collection of insects

Insects were collected from beneath bark flaps on cut stumps of *Eucalyptus* trees. This was done using an aspirator (Fergusson 1982). Insects were stored in separate Eppendorf tubes and transported to the laboratory following the method described by Kamgan Nkuekam et al. (2012). The insects were grouped based on morphological characteristics and viewed using an Axiocam dissection microscope (Carl Zeiss Ltd., Germany). Representatives of each insect group were preserved in 70 % ethanol prior to identification by Dr. Andrew Cline, Senior Insect Biosystematist, Plant Pest Diagnostics Center, California Department of Food and Agriculture, United States of America.

Isolation of fungi from insects was done using carrot baiting (Moller and Devay 1968; Heath et al. 2009b). Mycelial strands, ascomata or ascospores of putative *Ceratocystis* species were then transferred from the carrot surfaces to 2 % malt extract agar (MEA: 20 gl^{-1} malt extract and 15 gl^{-1} agar, Biolab, Midrand, South Africa and 1,000 ml sterile deionised water) containing 0.05 gl^{-1} of the antibiotic streptomycin sulphate (SIGMA-ALDRICH, Steinheim, Germany).

Morphological characterization

Ceratocystis isolates were incubated at 25 °C until sporulation and then grouped based on colour (Rayner 1970) and macro-morphology on MEA. Morphological structures including ascomata and ascospores, phialides and conidia from isolates representing each morphotype were mounted in 80 % lactic acid on glass microscope slides and examined using a Zeiss Axiocam light microscope (München-Hallbergmoos, Germany). Fifty measurements of all characteristic morphological features were made for isolates chosen to represent the types of new species and ten measurements were made for additional isolates. Measurements were computed as (minimum -) mean minus st. dev. — mean plus st. dev. (- maximum).

Scanning Electron Microscopy (SEM) was used to examine spores and the asexual states of the *Ceratocystis* species. Specimens were prepared for SEM as described by Grobbelaar et al. (2009). The specimens were critical point dried (Bio-Rad E3000, Watford, England), then mounted and coated with gold in a sputter coater (Emitech K550X, Ashford, England) and examined using a JEOL JSM-840 scanning electron microscope (JEOL, Tokyo, Japan).

Growth in culture

Growth in culture was examined for two isolates of each new species identified in this study. A disk of agar (9 mm diam.) bearing mycelium of the test isolates was transferred from the actively growing margins of seven-day-old cultures and placed with the mycelial surface facing downwards, at the centres of 90 mm Petri dishes containing 2 % MEA. The plates were incubated in the dark for 10 days at temperatures ranging from 5 to 35 °C at 5° intervals. Five replicate plates were used for each isolate at each temperature considered. Two diameter measurements, perpendicular to each other, were taken daily for each colony and the averages of ten diameter measurements for each temperature were computed.

DNA sequence comparisons

Single spore drops collected from the apices of ascomata in pure cultures were transferred to 2 % MEA and allowed to grow for 7–10 days. Mycelium was scraped from the surfaces of the actively growing cultures and transferred to 1.5 ml Eppendorf tubes using a sterile hypodermic needle. DNA was extracted from all isolates using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, California, USA) following the manufacturer's instructions.

The internal transcribed spacer regions (ITS1, ITS2) and 5.8S gene of the ribosomal RNA operon were amplified with an Eppendorf Mastercycler (Merck, Germany) using primers ITS1 and ITS4 (White et al. 1990). Part of the β-tubulin gene (BT1) and the transcription elongation factor-1α gene (TEF) were also amplified using the primers βt1a and βt1b (Glass and Donaldson 1995), EF1F and EF2R (Jacobs et al. 2004) respectively.

Due to the poor resolution in species delineation recently observed for the *C. pirilliformis* clade of *Ceratocystis*, two additional gene regions were tested on species in this clade, to determine whether they could be used to better define taxa in this group. These gene regions comprised of a portion of the RNA polymerase II subunit (RPB2)

recommended by the AFTOL database, using the primers fRPB2-5F (5′-GAYGAYMGWGATCAYTTYGG-3′) and RPB2-6R (5′-GCAGGRCARACCAWMCCCCA-3′) ((www.AFTOL.org), and the BT2 region of the β-tubulin gene using the primers βt2A and βt2B (Glass and Donaldson 1995).

The PCR reaction mixtures, as well as the thermal cycling conditions, were the same as those described previously (Kamgan Nkuekam et al. 2008). A 5 μl aliquot of the PCR products was pre-stained with GelRed^{TM} Nucleic Acid Gel stain (Biotium, Hayward, USA) and separated on a 1 % agarose gel and visualized under UV light. PCR products were purified using Sephadex G-50 Gel (Sigma-Aldrich), following the manufacturer's instructions. The concentrations of the purified PCR products were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, USA). Sequencing reactions were performed using the Big Dye cycle sequencing kit with Amplitaq DNA polymerase, FS (Perkin-Elmer, Warrington, UK) following the manufacturer's protocol on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequencing PCR was prepared as described by Kamgan Nkuekam et al. (2008) and both DNA strands were sequenced.

A preliminary identity for the isolates was obtained by performing a similarity search (standard nucleotide BLAST) against the GenBank database (http://www.ncbi.nlm.nih.gov). Sequences of both DNA strands for each isolate were examined visually and combined using the programme Sequence Navigator v. 1.01 (ABI PRISM, Perkin Elmer). Additional sequences of the ex-type species of related *Ceratocystis* species and *Thielaviopsis* species were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) for comparisons. Sequences were aligned using the E-INS-i option in the online version of MAFFT 6 (Katoh and Toh 2008).

Phylogenetic analyses

Phylogenetic analyses of sequences for each group of isolates separated based on morphology were performed independently of each other. Phylogenetic analyses of data sets for each of the three nuclear loci (ITS, BT1, TEF) were performed both separately and as combined data sets. For each data set, maximum parsimony (MP), Bayesian analyses (MB), and maximum likelihood (ML) analyses were done.

MP analyses were performed in PAUP 4.0b10 (Swofford 1998), using the following settings: 100 random sequence addition replicates, tree bisection-recognition (TBR) branch swapping, and 'multrees' option in effect. Confidence levels of the MP phylogenies were estimated with the bootstrap method (1,000 replications). The same parameters were used for the RPB2 and BT2 gene regions in the analyses of the *C. pirilliformis* clade.

Bayesian analyses based on Markov chain Monte Carlo (MCMC) were performed with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) as outlined previously (Kamgan Nkuekam et al. 2012). Appropriate substitution models were determined using the Akaike Information Criterion (AIC) in MrModeltest 2.2 (http://www.abc.se/~nylander/). The best fit model of evolution applied to ITS, BT1 and TEF are summarized in Table 1. Burn-in values were determined using Tracer 1.4 (http://beast.bio.ed.ac.uk/Tracer) to discard trees that formed before the point of convergence, and the posterior probability in the majority rule consensus trees were calculated by MCMC sampling in MrBayes V3.1.2, using the best-fit model of evolution (Table 1).

Maximum likelihood (ML) analyses were conducted online using PhyML 3.0 (Guindon and Gascuel 2003). The AIC was used in ModelTest 3.7 (Posada and Crandall 1998) to select appropriate substitution models for the three data sets (Table 1).

The level of polymorphism in sequence variation between closest related species was analyzed with the genetic software programme MEGA V4 (Molecular Evolutionary Genetics Analysis) (Tamura et al. 2007). Sequences for each gene region considered were examined to determine the number of fixed base pair differences that separate closest related taxa. Allele networks were constructed with the programme TCS (Clement et al. 2000) to illustrate the relationship between isolates of closely related species.

Table 1 Best fit models of evolution for each gene region used in distance analyses (Bayesian and Maximum Likelihood)

	Type of Analyses	ITS	BT1	TEF	Combined tree
C. fimbriata s.l.	Bayesian	GTR + G	HKY + I	HKY	GTR + I + G
	Maximum likelihood	TVM + G (Rates = gamma, Shape=0.3029, Pinvar=0)	TrN + I (Rates = equal, Pinvar=0.5812)	TrN (Rates = equal, Pinvar=0)	TVM + I + G (Rates = gamma, Shape=0.5904, Pinvar=0.3169)
C. moniliformis s. l.	Bayesian	HKY + I	HKY + I	K80 + I	(HKY + I)
	Maximum likelihood	HKY + I (Rates = equal, Pinvar=0.9261)	TrN + I (Rates = equal, Pinvar=0.8006)	K80 + I (Rates = equal, Pinvar=0.4669)	HKY + I (Rates = equal, Pinvar=0.8017)

Pathogenicity tests

The pathogenicity of two new *Ceratocystis* species identified in this study was tested in a quarantine greenhouse. Two strains of each species were used to inoculate ten, approximately two-year-old (~1 cm diameter), *Eucalyptus grandis* (clone TAG5) trees. Two additional trees of the same age were inoculated with a sterile agar disc to serve as controls. The experimental design and conditions for inoculation were the same as those described by Kamgan Nkuekam et al. (2008). Six weeks (42 days) after inoculation, the lengths of lesions on the bark surface and in the xylem of each tree were measured. Re-isolations were made from the lesions to meet the requirements of Koch's postulates. All lesion length data were analyzed using the GLM procedure in SAS/STAT (SAS Institute Inc. 1999).

Results

Collection of fungal isolates

A total of 100 *Ceratocystis* isolates were obtained from wounds on *Eucalyptus* trees sampled. More than 300 trees were sampled in the process and isolates were obtained from all the areas sampled. These spanned six different Provinces and a wide variety of climatic conditions (Table 2). *Eucalyptus* species from which *Ceratocystis* isolates were

Table 2 Fungi isolated from *Eucalyptus* trees during surveys in South Africa

Provinces	Locations	Climatic Types	CMW	Number of Isolates	ID	Morpho-group	Hosts
Eastern Cape	Lotobeni	temperate	28204, 28205, 28206	3	*C. pirilliformis*	A2	*E. grandis*
"	Kumbo	"	27181, 27182	2	"	A2	"
KwaZulu-Natal	KwaMbonambi	subtropical	24984, 24975, 24976, 24979, 24980, 24974, 24952	7	*C. eucalypticola*	A1	*Eucalyptus* sp.
"	Pietermaritzburg	"	24955, 24957, 24958, 24960, 24961, 24962, 24963, 24965, 24967, 24969, 24970, 24972	12	"	A1	"
Limpopo	Goedehoop	"	26472, 26466	2	*C. pirilliformis*	A2	*E. cloeziana*
"	Soutpansberg	"	30888, 30860, 30889, 30861, 30890, 30891	6	*C. eucalypticola*	A1	*E. saligna*
"	"	"	30701	1	*C. decipiens*	B	"
"	"	"	25914	1	"	B	*E. maculata*
"	Goedehoop	"	25918, 25919	2	"	B	*E. cloeziana*
"	"	"	25920	1	*C. savannae*	B	"
"	Soutpansberg	"	25909, 25915, 25916	3	"	B	*E. maculata*
"	"	"	25910, 25911, 25913	3	*C. salinaria*	B	"
"	Goedehoop	"	25917	1	"	B	*E. cloeziana*
"	Soutpansberg	"	30702, 30703, 30704	3	"	B	*E. saligna*
"	"	"	25912	1	*C. moniliformis*	B	*E. maculata*
Mpumalanga	Sabie	"	30892, 30893, 30894, 30895, 30896, 30897, 30898	7	*C. eucalypticola*	A1	*E. grandis*
"	"	"	30698	1	*C. oblonga*	B	"
"	"	"	30699, 30700	2	*C. moniliformis*	B	"
Limpopo	Tzaneen	"	25001, 24991, 24989, 24998, 24994, 25012, 25008, 25025, 25021, 25019, 25017, 25015, 25023	13	*C. eucalypticola*	A1	"
Western Cape	Cape Town	mediteranean	28200, 29822	2	*C. pirilliformis*	A2	*Eucalyptus* logs
"	"	"	27162, 27163	2	"	A2	*E. diversicolor*
"	"	"	27183, 27184, 27185, 27186, 27187, 27188	6	"	A2	*E. saligna*
"	George	"	27047, 27155, 27048, 27157, 27049, 27050, 27051, 27052, 27053, 27054, 27259, 27055, 27153, 27158, 27156, 27056, 27154	17	"	A2	*Eucalyptus* sp.
"	"	"	27006, 27007	2	*C. salinaria*	B	"
Total				100			

Table 3 Fungi isolated from insects infesting *Eucalyptus* stumps during surveys in South Africa

Provinces	Locations	Climatic types	CMW nbers.	Number of Isolates	ID	Morpho-group	Insect hosts	Tree Hosts
Eastern Cape	Lotobeni	temperate	29728, 29729, 29730	3	*T. basicola*	C	*B. depressus*	*E. grandis*
"	"	"	29825	1	*C. pirilliformis*	A2	"	"
KwaZulu-Natal	KwaMbonambi	subtropical	25037, 25040, 25041, 25045, 25046	5	*C. eucalypticola*	A1	"	*Eucalyptus* sp.
"	Pietermaritzburg	"	25073	1	"	A1	"	*Eucalyptus* sp.
"	KwaMbonambi	"	25052, 25053, 25054, 25056, 25057, 25062, 25065, 25067, 25069, 25070	10	"	A1	*Carpophilus* spp.	*Eucalyptus* sp.
"	"	"	25039, 25043, 25044	3	*T. thielavioides*	C	*B. depressus*	"
"	"	"	25063, 25047, 25049	3	"	C	*Carpophilus* spp.	"
Limpopo	Soutpansberg	"	26360	1	*C. eucalypticola*	A1	*Xyleborus affinis*	*E. maculata*
"	"	"	26355, 26356, 26357, 26358, 26359	5	"	A1	*Litargus* sp.	"
"	"	"	31211, 31222, 31212, 31215, 31216, 31219, 31220, 31213, 31197, 31217, 31221, 31214, 31218	13	"	A1	*B. depressus*	*E. saligna*
"	"	"	26341, 26332, 26339, 26337, 26340, 26338, 26335, 26336, 26326, 26333, 26330, 26334, 26331, 26329	14	"	A1	"	*E. maculata*
"	"	"	31228, 31231, 31232	3	"	A1	*Ca. bisignatus*	*E. saligna*
	"	"	31230, 31234, 31227	3	"	A1	*Ca. dimidiatus*	"
	"	"	31224, 31223, 31200, 31229, 31198, 31225, 31233, 31199, 31226	9	"	A1	*Carpophilus* sp.	"
"	"	"	26235, 26236, 26237, 26238, 26351	5	"	A1	"	*E. maculata*
"	"	"	26244, 26246, 26241, 26243, 26245, 26239, 26240, 26242	8	"	A1	*Ca. bisignatus*	"
"	"	"	26352, 26353, 26354, 26247, 26248, 26249, 26250, 26251	8	"	A1	*Ca. dimidiatus*	"
"	"	"	31241, 31204, 31203, 31247, 31240, 31252, 31246, 31239, 31251, 31245, 31238, 31202, 31250, 31244, 31201, 31237, 31249, 31248, 31243, 31235, 31236, 31242, 31253	23	"	A1	Staphilinid sp.	*E. saligna*
"	"	"	30846, 30836	2	*C. savannae*	B	*Ca. dimidiatus*	"
"	"	"	30839, 30847, 30848, 30849	4	"	B	*Ca. bisignatus*	"
"	"	"	30850, 30844, 30842, 30841, 30851, 30852	6	"	B	*Carpophilus* sp.	"
"	"	"	30824, 30825, 30828, 30831, 30832, 30833	6	"	B	*B. depressus*	"
"	"	"	30857	1	"	B	Staphilinid sp.	"
"	"	"	30835, 30837	2	*C. oblonga*	B	*Ca. dimidiatus*	"
"	"	"	30838, 30840	2	"	B	*Ca. bisignatus*	"
"	"	"	30845	1	"	B	*Carpophilus* sp.	"
"	"	"	30827	1	"	B	*B. depressus*	"
"	"	"	30834, 30829, 30830	3	*C. decipiens*	B	"	"
"	"	"	30855, 30853	2	"	B	Staphilinid sp.	"
Mpumalanga	Sabie	"	31196	1	*C. eucalypticola*	A1	*B. depressus*	*E. grandis*
"	"	"	31207, 31208	2	"	A1	*Ca. bisignatus*	"
"	"	"	31259	1	"	A1	*Carpophilus* sp.	"
"	"	"	31206, 31258, 31257, 31205, 31256, 31255, 31254	7	"	A1	Staphilinid sp.	"
"	"	"	30856, 30858, 30859	3	*C. moniliformis*	B	"	"
Total				162				

Table 4 List of *Ceratocystis* isolates used in comparative morphological and phylogenetic studies

Isolate designation	Isolate number	Genbank	Gene regions	Other numbers	Hosts	Collectors	Origin
C. acaciivora	CMW22563	EU588656	ITS	NA	*Acacia mangium*	M. Tarigan	Indonesia
		EU588636	BT1	"	"	"	"
		EU588646	TEF	"	"	"	"
C. albifundus	CMW4068	DQ520638	ITS	CBS 128992	*A. mearnsii*	J. Roux	South Africa
		EF070429	BT1	"	"	"	"
		EF070400	TEF	"	"	"	"
C. aracearum	CMW14805	AY526297	ITS	CBS 115165	*Colocasia esculenta*	C.J.B. Engelbrecht	Costa Rica
		GU810160	BT1	"	"	"	"
		GU810166	TEF	"	"	"	"
C. atrox	CMW19385	EF070415	ITS	CBS 120518	*E. grandis*	M.J. Wingfield	Australia
		EF070431	BT1	"	"	"	"
		EF070403	TEF	"	"	"	"
C. belula	CMW14811	AY526288	ITS	CBS 115171	*C. esculenta*	T.C. Harrington	Brazil
		GU810162	BT1	"	"	"	"
		GU810168	TEF	"	"	"	"
C. bhutanensis	CMW8399	AY528959	ITS	CBS 115772, BH 8/8	*Picea spinulosa*	T. Kirisits & D.B. Chhetri	Bhutan
		AY528964	BT1	"	"	"	"
		AY528954	TEF	"	"	"	"
	CMW8215	AY528958	ITS	CBS114290, PREM57805	"	T. Kirisits & D.B. Chhetri	Bhutan
		AY528963	BT1	"	"	"	"
		AY528953	TEF	"	"	"	"
C. cacaofunesta	CMW15051	DQ520636	ITS	CBS 152.62	*Theobroma cacao*	A.J. Hansen	Costa Rica
		EF070427	BT1	"	"	"	"
		EF070398	TEF	"	"	"	"
C. caryae	CMW14793	EF070424	ITS	CBS 114716	*Carya cordiformis*	J. Johnson	USA
		EF070439	BT1	"	"	"	"
		EF070412	TEF	"	"	"	"
C. colocasiae	CMW14796	AY526307	ITS	CBS 114720	*Colocasia esculenta*	J. Uchida	USA
		GU810164	BT1	"	"	"	"
		GU810170	TEF	"	"	"	"
C. colombiana	CMW5751	AY177233	ITS	CBS 121792	*Coffea arabica*	M. Marin	Colombia
		AY177225	BT1	"	"	"	"
		EU241493	TEF	"	"	"	"
C. corymbiicola	CMW29120	HM071902	ITS	CBS 127215	*Corymbia variegata*	G.N. Kamgan	Australia
		HM071914	BT1	"	"	"	"
		HQ236453	TEF	"	"	"	"
C. curvata	CMW22442	FJ151436	ITS	CBS 122603	*E. deglupta*	M.J. Wingfield	Colombia
		FJ151448	BT1	"	"	"	"
		FJ151470	TEF	"	"	"	"
C. decipiens	CMW30855	HQ203216	ITS	CBS 129736	Staphilinid. sp.	G.N. Kamgan & J. Roux	South Africa
		HQ203233	BT1	"	"	"	"
		HQ236435	TEF	"	"	"	"
	CMW25918	HQ203218	ITS	CBS129735	*E. cloeziana*	"	"
		HQ203235	BT1	"	"	"	"
		HQ236437	TEF	"	"	"	"
	CMW25914	HQ203219	ITS	CBS 129737	*E. maculata*	"	"
		HQ203236	BT1	"	"	"	"

Table 4 (continued)

Isolate designation	Isolate number	Genbank	Gene regions	Other numbers	Hosts	Collectors	Origin
		HQ236438	TEF	"	"	"	"
	CMW30830	HQ203217	ITS	"	*B. depressus*	"	"
		HQ203234	BT1	"	"	"	"
		HQ236436	TEF	"	"	"	"
C. diversiconidia	CMW22445	FJ151440	ITS	CBS 123013	*Terminalia ivorensis*	M.J. Wingfield	Colombia
		FJ151452	BT1	"	"	"	"
		FJ151474	TEF	"	"	"	"
C. ecuadoriana	CMW22092	FJ151432	ITS	CBS 124020	*E. deglupta*	M.J. Wingfield	Colombia
		FJ151444	BT1	"	"	"	"
		FJ151466	TEF	"	"	"	"
C. eucalypticola	CMW11536	FJ236723	ITS	CBS 124016	*Eucalyptus* sp.	M. van Wyk & J. Roux	South Africa
		FJ236783	BT1	"	"	"	"
		FJ236753	TEF	"	"	"	"
	CMW25015	HQ203224	ITS	NA	*E. grandis*	G.N. Kamgan & J. Roux	"
		HQ203241	BT1	"	"	"	"
		HQ236443	TEF	"	"	"	"
	CMW24984	HQ203225	ITS	NA	*Eucalyptus* sp.	"	"
		HQ203242	BT1	"	"	"	"
		HQ236444	TEF	"	"	"	"
C. fimbriata	CMW15049	DQ520629	ITS	CBS 141.37	*I. batatas*	C.F. Andrus	USA
		EF070442	BT1	"	"	"	"
		EF070394	TEF	"	"	"	"
C. fimbriatomima	CMW24174	EF190963	ITS	CBS 121786	*Eucalyptus* sp.	M.J. Wingfield	Venezuela
		EF190951	BT1	"	"	"	"
		EF190957	TEF	"	"	"	"
C. inquinans	CMW21106	EU588587	ITS	CBS 124388	*A .mangium*	M. Tarigan	Indonesia
		EU588666	BT1	"	"	"	"
		EU588674	TEF	"	"	"	"
	CMW21107	EU588588	ITS	CBS 124009	"	"	"
		EU588667	BT1	"	"	"	"
		EU588675	TEF	"	"	"	"
C. larium	CMW25436	EU881908	ITS	CBS 122607	*Styrax benzoin*	M.J. Wingfield	Indonesia
		EU881896	BT1	"	"	"	"
		EU881902	TEF	"	"	"	"
C. manginecans	CMW13851	AY953383	ITS	CBS 121659	*Mangifera indica*	M. Deadman	Oman
		EF433308	BT1	"	"	"	"
		EF433317	TEF	"	"	"	"
C. microbasis	CMW21115	EU588592	ITS	CBS 124015	*A. mangium*	M. Tarigan	Indonesia
		EU588671	BT1	"	"	"	"
		EU588679	TEF	"	"	"	"
	CMW21117	EU588593	ITS	CBS 124017	"	"	"
		EU588672	BT1	"	"	"	"
		EU588680	TEF	"	"	"	"
C. moniliformis	CMW9590	AY431101	ITS	CBS 116452	*Eucalyptus grandis*	J. Roux	South Africa
		AY528985	BT1	"	"	"	"
		AY529006	TEF	"	"	"	"
	CMW8379	AY528995	ITS	NA	*Cassia fistula*	M.J. Wingfield	Bhutan
		AY529005	BT1	"	"	"	"

Table 4 (continued)

Isolate designation	Isolate number	Genbank	Gene regions	Other numbers	Hosts	Collectors	Origin
		AY529016	TEF	"	"	"	"
	CMW30856	HQ203211	ITS	"	Staphilinid sp.	G.N. Kamgan & J. Roux	South Africa
		HW203228	BT1	"	"	"	"
		HQ236430	TEF	"	"	"	"
	CMW30700	HQ203212	ITS	"	*E. grandis*	"	"
		HQ203229	BT1	"	"	"	"
		HQ236431	TEF	"	"	"	"
C. moniliformopsis	CMW10214	AY528999	ITS	CBS 115792	*E. sieberi*	M.J. Dudzinski	Australia
		AY528988	BT1	"	"	"	"
		AY529009	TEF	"	"	"	"
	CMW9986	AY528998	ITS	CBS 109441	*E. obliqua*	Z.Q. Yuan	Australia
		AY528987	BT1	"	"	"	"
		AY529008	TEF	"	"	"	"
C. neglecta	CMW17808	EF127990	ITS	CBS 121789	*Eucalyptus* sp.	M.J. Wingfield	Colombia
		EU881898	BT1	"	"	"	"
		EU881904	TEF	"	"	"	"
C. oblonga	CMW23802	EU245020	ITS	CBS 122820	*A. mearnsii*	R.N. Heath	South Africa
		EU244992	BT1	"	"	"	"
		EU244952	TEF	"	"	"	"
	CMW23803	EU245019	ITS	CBS 122291	"	"	"
		EU244991	BT1	"	"	"	"
		EU244951	TEF	"	"	"	"
	CMW30698	HQ203220	ITS	NA	*E. nitens*	G.N. Kamgan & J. Roux	South Africa
		HQ203237	BT1	"	"	"	"
		HQ236439	TEF	"	"	"	"
	CMW30835	HQ203221	ITS	"	*C. dimidiatus*	"	"
		HQ203238	BT1	"	"	"	"
		HQ236440	TEF	"	"	"	"
C. obpyriformis	CMW23807	EU245004	ITS	CBS 122608	*A. mearnsii*	R.N. Heath	South Africa
		EU244976	BT1	"	"	"	"
		EU244936	TEF	"	"	"	"
	CMW23808	EU245003	ITS	CBS 122511	"	"	"
		EU244975	BT1	"	"	"	"
		EU244935	TEF	"	"	"	"
C. omanensis	CMW11048	DQ074742	ITS	CBS 115780, PREM57815	*Mangifera indica*	A.O. Al-Adawi	Oman
		DQ074732	BT1	"	"	"	"
		DQ074737	TEF	"	"	"	"
	CMW3777	DQ074740	ITS	NA	*M. indica*	A.O. Al-Adawi	Oman
		DQ074730	BT1	"	"	"	"
		DQ074735	TEF	"	"	"	"
	CMW11046	DQ074739	ITS	CBS 118112, PREM57814	*M. indica*	A.O. Al-Adawi	Oman
		DQ074729	BT1	"	"	"	"
		DQ074734	TEF	"	"	"	"
C. papillata	CMW8856	AY233867	ITS	CBS121793	*Citrus lemon*	M.J. Wingfield	Colombia
		AY233874	BT1	"	"	"	"
		EU241484	TEF	"	"	"	"

Table 4 (continued)

Isolate designation	Isolate number	Genbank	Gene regions	Other numbers	Hosts	Collectors	Origin
C. pirilliformis	CMW6569	AF427104	ITS	PREM57322, DAR75993	*E. nitens*	M.J. Wingfield	Australia
		DQ371652	BT1	"	"	"	"
		AY528982	TEF	"	"	"	"
	CMW6579	AF427105	ITS	CBS 118128, PREM57323, DAR75996	*E. nitens*	M.J. Wingfield	Australia
		DQ371653	BT1	"	"	"	"
		AY528983	TEF	"	"	"	"
	CMW29822	HQ203227	ITS	NA	*Eucalyptus* log	G.N. Kamgan & J. Roux	South Africa
		HQ203244	BT1	"	"	"	"
		HQ236446	TEF	"	"	"	"
	CMW29825	HQ203226	ITS	"	*B. depressus*	"	"
		HQ203243	BT1	"	"	"	"
		HQ236445	TEF	"	"	"	"
C. platani	CMW14802	DQ520630	ITS	CBS 115162	*Platanus occidentalis*	T.C. Harrington	USA
		EF070425	BT1	"	"	"	"
		EF070396	TEF	"	"	"	"
C. polychroma	CMW11424	AY528983	ITS	CBS 115778, PREM57818	*Syzygium aromaticum*	E.C.Y. Liew & M.J. Wingfield	Indonesia
		AY528966	BT1	"	"	"	"
		AY528970	TEF	"	"	"	"
C. polyconidia	CMW23809	EU245006	ITS	CBS 122289	*A. mearnsii*	R.N. Heath	South Africa
		EU244978	BT1	"	"	"	"
		EU244938	TEF	"	"	"	"
	CMW23818	EU245007	ITS	CBS 122290	"	"	"
		EU244979	BT1	"	"	"	"
		EU244939	TEF	"	"	"	"
C. populicola	CMW14789	EF070418	ITS	CBS 119.78	*Populus* sp.	J. Gremmen	Poland
		EF070434	BT1	"	"	"	"
		EF070406	TEF	"	"	"	"
C. salinaria	CMW25911	HQ203213	ITS	CBS 129733	*E. maculata*	G.N. Kamgan & J. Roux	South Africa
		HQ203230	BT1	"	"	"	"
		HQ236432	TEF	"	"	"	"
	CMW30702	HQ203215	ITS	NA	*E. saligna*	"	"
		HQ203232	BT1	"	"	"	"
		HQ236434	TEF	"	"	"	"
	CMW30703	HQ203214	ITS	CBS 129734	*E. saligna*	"	"
		HQ203231	BT1	"	"	"	"
		HQ236433	TEF	"	"	"	"
C. savannae	CMW17300	EF408551	ITS	CBS 121151	*Acacia nigrescens*	G.N. Kamgan & J. Roux	South Africa
		EF408565	BT1	"	"	"	"
		EF408572	TEF	"	"	"	"
	CMW17297	EF408552	ITS	CBS 121021	*Combretum zeyheri*	G.N. Kamgan & J. Roux	South Africa
		EF408566	BT1	"	"	"	"
		EF408573	TEF	"	"	"	"
	CMW30828	HQ203223	ITS	"	*B. depressus*	G.N. Kamgan & J. Roux	South Africa

Table 4 (continued)

Isolate designation	Isolate number	Genbank	Gene regions	Other numbers	Hosts	Collectors	Origin
		HQ203240	BT1	"	"	"	"
		HQ236442	TEF	"	"	"	"
	CMW30846	HQ203222	ITS	"	*C. dimidiatus*	"	"
		HQ203239	BT1	"	"	"	"
		HQ236441	TEF	"	"	"	"
C. smalleyi	CMW14800	EF070420	ITS	CBS 114724	*Carya cordiformis*	G. Smalley	USA
		EF070436	BT1	"	"	"	"
		EF070408	TEF	"	"	"	"
C. sumatrana	CMW21109	EU588589	ITS	CBS 124011	*A.mangium*	M. Tarigan	Indonesia
		EU588668	BT1	"	"	"	"
		EU588676	TEF	"	"	"	"
	CMW21111	EU588590	ITS	CBS 124012"	"	"	"
		EU588669	BT1	"	"	"	"
		EU588677	TEF	"	"	"	"
C. tanganyicensis	CMW15992	EU244999	ITS	CBS 122293	*A. mearnsii*	R.N. Heath & J. Roux	Tanzania
		EU244971	BT1	"	"	"	"
		EU244931	TEF	"	"	"	"
C. tribiliformis	CMW13015	AY529004	ITS	CBS 115949	*Pinus mercusii*	M.J. Wingfield	Indonesia
		AY528994	BT1	"	"	"	"
		AY529015	TEF	"	"	"	"
	CMW13013	AY529003	ITS	CBS 115866	"	M.J. Wingfield	Indonesia
		AY528993	BT1	"	"	"	"
		AY529014	TEF	"	"	"	"
C. tsitsikammensis	CMW14276	EF408555	ITS	CBS 121018	*Rapanea melanophloeos*	G.N. Kamgan & J. Roux	South Africa
		EF408569	BT1	"	"	"	"
		EF408576	TEF	"	"	"	"
C. tyalla	CMW28925	HM071897	ITS	CBS 127211	*E. pilularis*	G.N. Kamgan	Australia
		HM071911	BT1	"	"	"	"
		HQ236450	TEF	"	"	"	"
	CMW28932	HM071900	ITS	CBS 128703	*E. dunnii*	"	"
		HM071913	BT1	"	"	"	"
		HQ236452	TEF	"	"	"	"
C. variospora	CMW20935	EF070421	ITS	CBS 114715	*Quercus alba*	J. Johnson	USA
		EF070437	BT1	"	"	"	"
		EF070409	TEF	"	"	"	"
C. virescens	CMW3276	DQ061281	ITS	NA	*Quercus* sp.	T. Hinds	USA
		AY528990	BT1	"	"	"	"
		AY529011	TEF	"	"	"	"
C. zombamontana	CMW15235	EU245002	ITS	CBS 122297	*Eucalyptus* sp.	R.N. Heath & J. Roux	Malawi
		EU244974	BT1	"	"	"	"
		EU244934	TEF	"	"	"	"
	CMW15236	EU245000	ITS	CBS 122296	"	"	"
		EU244972	BT1	"	"	"	"
		EU244932	TEF	"	"	"	"

obtained included six different species, namely *E. grandis* W. Hill: Maiden, *E. nitens* H.Deane & Maiden, *E. saligna* Sm., *E. maculata* Hook., *E. cloeziana* F. Muell and *E. diversicolor* F. Muell (Table 2).

Fungal isolates from insects

A wide variety of insects were found in four of the six Provinces sampled. More than 385 insects, spanning five genera in three different families were collected. Members of the Nitidulidae were the most common insects found, accounting for 255 specimens collected. These nitidulids were identified as *Brachypeplus depressus* Erichson (120 specimens), *Carpophilus bisignatus* Boheman (20 specimens) and *C. dimidiatus* Fabricius (25 specimens). Ninety other nitidulid beetles were of a *Carpophilus* sp. that could not be identified to species level. Other insects collected resided in the Staphylinidae (100 specimens), *Lithargus* sp. (Coleoptera: Mycetophagidae) (10 specimens) and *Xyleborus affinis* (Coleoptera: Scolytidae) (20 specimens) (Table 3). A total of 162 isolates of *Ceratocystis* were obtained from the insects collected in this study (Table 3).

Morphological characterization

Based on colony colour and the morphology of the ascomata, ascospores, conidiogenous cells and conidia produced on MEA, three main morphological groups in the genus *Ceratocystis* were identified. The first set of isolates produced colonies and structures typical of species in the *C. fimbriata* (*s.l.*) complex and these are referred to as the *C. fimbriata* group. These isolates could be further subdivided into two sub-groups. One of these sub-groups resembled those of *C. fimbriata s.s.*, the other was typical of those of *C. pirilliformis* as described by Barnes et al. (2003a). The second set of isolates produced colonies with morphologies typical to those in the *C. moniliformis s.l.* complex and were treated as such. The third set of isolates produced colonies having only an asexual *Thielaviopsis* state of *Ceratocystis*.

DNA sequence comparisons and phylogenetic analyses

The ITS gene region for all isolates in the *C. fimbriata s.l.* group generated contigs of ~600 bp. For the BT1 and TEF gene regions, only a few representative isolates were selected for DNA sequencing. Amplification generated contigs of ~550 and ~900 bps for the BT1 and TEF, respectively. A preliminary nucleotide Blast against the GenBank database using data sets from each of the three main gene regions revealed that some of the isolates were similar to either *C. fimbriata s.s.*, *C. manginecans* M. van Wyk, Al Adawi & M.J. Wingf. or *C. eucalypticola* and the others were closely related to *C. pirilliformis*.

Comparison of the ITS, BT1 and TEF sequence data of selected isolates in the *C. fimbriata s.l.* group with those of related *Ceratocystis* species from GenBank (Table 4) showed that one group of isolates represented *C. eucalypticola* and the other *C. pirilliformis* (Fig. 1). These results

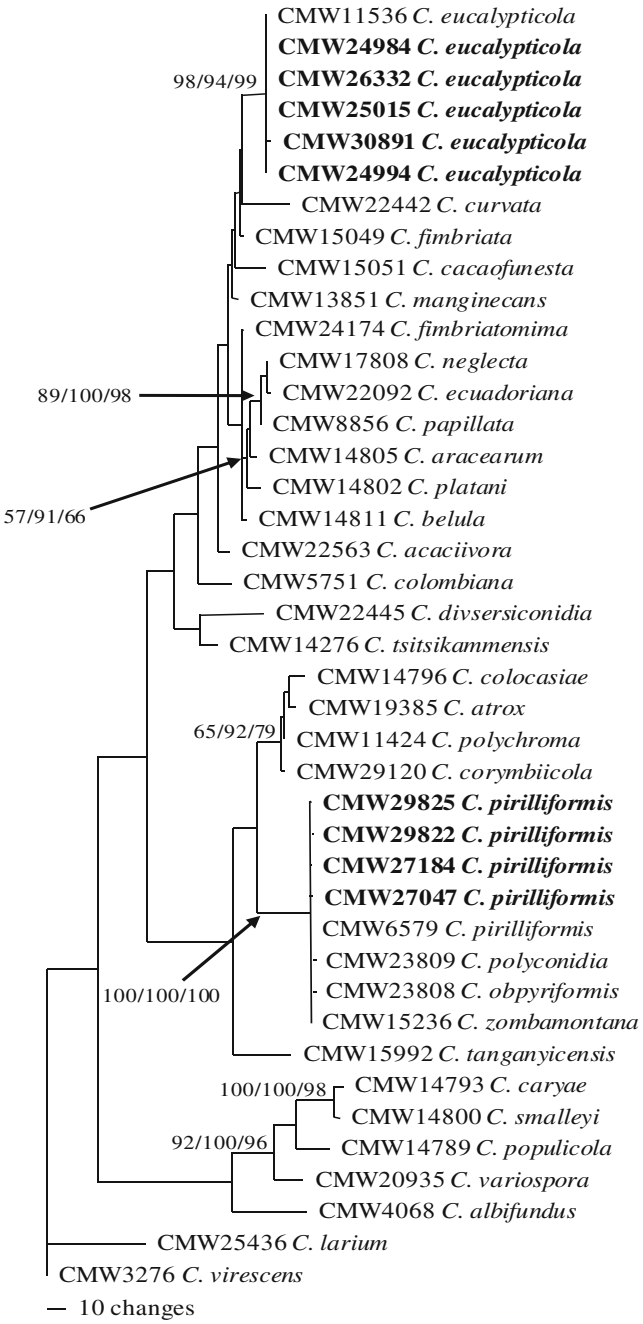

Fig. 1 Phylogenetic tree produced from a heuristic search of the ITS sequence data, showing the relationship between members of *C. fimbriata s.l.* Isolates sequenced in this study are in bold font type. *C. virescens* was used as out-group taxon. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node

were confirmed using maximum parsimony, Bayesian and maximum likelihood analyses (ML). Tree statistics for maximum parsimony analysis are summarized in Table 5. Comparison of sequence data for all three gene regions confirmed the identities of the isolates as either *C. pirilliformis* or *C. eucalypticola* (Fig. 2). These isolates had

Table 5 Tree statistics for Maximum Parsimony analyses

		ITS	BT	TEF	Concatenated tree
C. fimbriata s.l.	Total Characters	533	541	197	1251
	Parsimony informative characters	189	98	50	337
	Parsimony uninformative characters	28	35	27	90
	Constant characters	316	388	120	824
	Tree length	449	192	99	766
	CI	0.686	0.812	0.899	0.722
	RI	0.895	0.938	0.960	0.902
C. moniliformis s.l.	Total Characters	448	442	296	1186
	Parsimony informative characters	16	40	97	153
	Parsimony uninformative characters	0	4	6	10
	Constant characters	432	398	193	1023
	Tree length	20	62	150	245
	CI	0.900	0.887	0.807	0.792
	RI	0.985	0.980	0.956	0.955

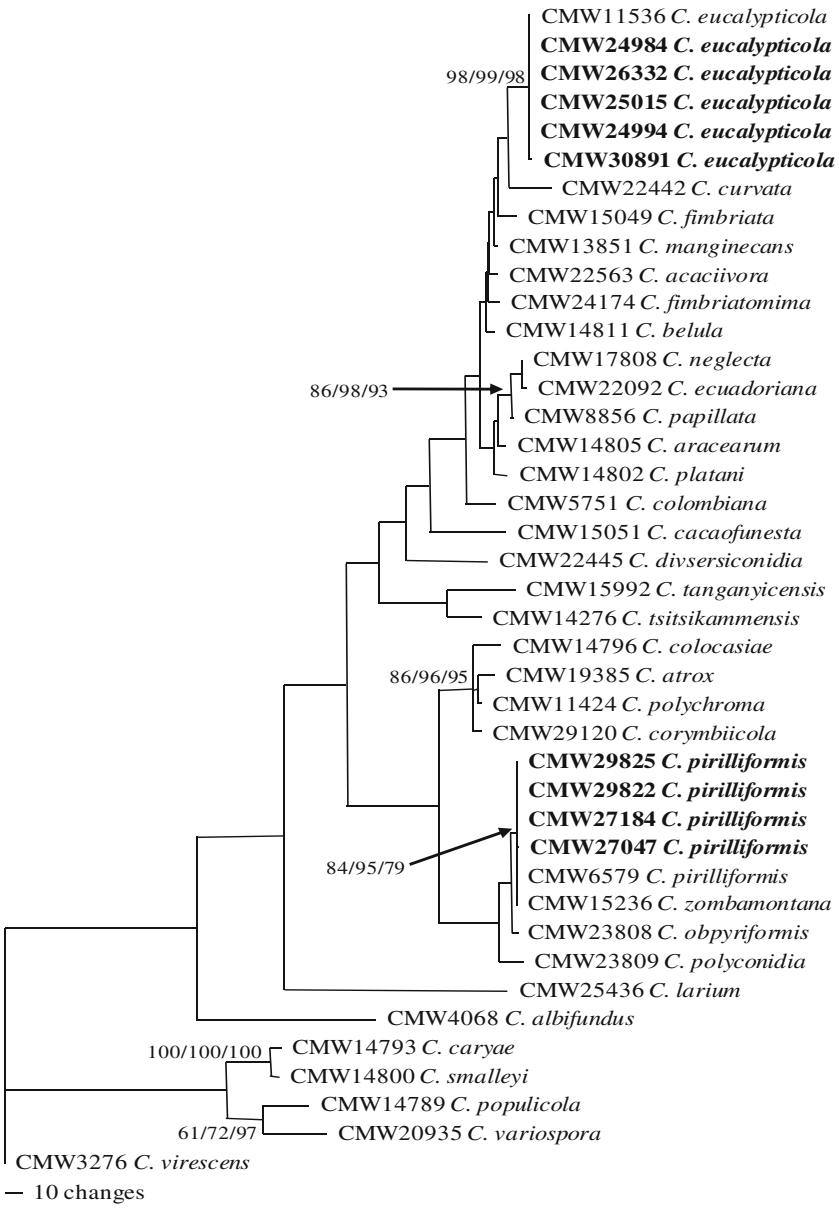

Fig. 2 Phylogenetic tree produced from a heuristic search of the combined ITS, BT1 and TEF sequence data, showing the relationship between members of *C. fimbriata s.l.* Isolates sequenced in this study are in bold font type. *C. virescens* was used as out-group taxon. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node

identical BT1 sequences and the TEF sequences differed only in a small number of bases in multiple base repeat regions (data not shown).

Isolates identified as *C. pirilliformis* fell within a clade comprising *C. zombamontana*, *C. obpyriformis* and *C. polyconidia*. The resolution between the four species was poor, especially between *C. pirilliformis* and *C. zombamontana*. For example, there were seven base pair differences between the ex-type strain of *C. pirilliformis* and the ex-type strain of *C. zombamontana* (Tables 6 and 7). These differences resided mainly in the T-rich multiple repeat regions of the ITS gene and were not informative in the analyses (Fig. 1). Some isolates identified as *C. pirilliformis* had ITS sequences the same as those for the ex-type strain of *C. zombamontana* (Tables 6 and 7). An unrooted tree showing relationships between the ex-type strain of *C. pirilliformis* and its closest phylogenetic neighbors was also constructed, based on three gene regions (ITS, BT1, TEF) (Fig. 3) and this showed that strains of *C. zombamontana* were identical to those of *C. pirilliformis*.

Polymerase chain reactions using RPB2 primers were unspecific resulting in multiple DNA fragments when resolved on agarose gels. DNA fragments of expected size were extracted from the gel and sequenced directly using the same primers used for PCR amplification. Sequence data obtained were used for phylogenetic analyses using maximum parsimony. Based on this analysis, *C. pirilliformis*, *C. zombamontana* and *C. obpyriformis* formed a single monophyletic clade with 100 % homology supported by a bootstrap of 100 % (data not shown). However, *C. polyconidia* fell within a single well resolved and highly supported clade and was the only species that could be separated from other taxa using this marker.

BT2 primers used in polymerase chain reactions were specific resulting in single DNA fragments when resolved on agarose gels. However, in phylogenetic analyses using maximum parsimony in PAUP, all known species in *C. pirilliformis s.l.* resided in a single and highly supported clade with 100 % homology (data not shown).

Seven haplotypes were identified within the *C. pirilliformis s.l.* clade in multilocus analysis using TCS (Table 8, Fig. 4). These comprised *C. obpyriformis* (CMW23807, CMW23808) and *C. polyconidia* (CMW23809, CMW23818), which each formed a single haplotype. The ex-type strain of *C. pirilliformis* (CMW6569) and its paratype strain (CMW6579) fell within two different haplotypes (Fig. 4). *C. zombamontana* strains were intermingled with isolates sequenced in this study and these were closely related to *C. pirilliformis*. The seven haplotypes resulting from the analysis were interconnected and formed a single allelic network.

Isolates resembling those in the *C. moniliformis* complex could be distinguished only based on sequence data for the BT1 (Fig. 5) and TEF (Fig. 6) gene regions, with no resolution obtained for the ITS gene region (data not shown). This is similar to previous reports for the *C. moniliformis s.l.* complex (Van Wyk et al. 2006a; Kamgan Nkuekam et al. 2008). Sequence data for the BT1 gene region grouped isolates into five clades (Fig. 5, Table 4) that represented *C. savannae*, *C. moniliformis*, *C. oblonga* and two unidentified species. The identities were supported by Bayesian, MP and ML analyses. Isolates representing *C. moniliformis* were 100 % identical to those of the type species. Minor differences of up to two base pairs were found between some isolates identified as *C. savannae* and the ex-type isolate of the fungus (Tables 9 and 10). Some isolates in the larger *C. oblonga* clade differed from the ex-type isolate in up to three base pairs (Tables 9 and 10). Unrooted trees illustrating relationships between the two unidentified

Table 6 Number of fixed base pairs across the ITS gene region showing differences between species in the *C. pirilliformis s.l.* clade of *Ceratocystis*

Isolates	ITS														
	19	23	95	110	122	123	157	176	192	193	280	283	350	352	421
CMW6569-*C. pirilliformis*	C	–	G	T	T	T	–	–	–	–	G	G	A	A	T
CMW6579-*C. pirilliformis*	.	.	–	.	.	.	.	A	–	–	.	.	.	.	–
CMW29825-*C. pirilliformis*	.	.	–	.	–	–	T	.	T	T	.	.	.	.	–
CMW29822-*C. pirilliformis*	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
CMW27184-*C. pirilliformis*	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
CMW27047-*C. pirilliformis*	.	.	–	.	.	.	.	.	.	.	A	.	.	.	.
CMW15235-*C. zombamontana*	T	.	–	.	–	–	T	.	T	T	.	.	.	.	–
CMW15236-*C. zombamontana*	T	.	–	.	–	–	T	.	T	T	.	.	.	.	–
CMW23807-*C. obpyriformis*	.	A	.	G	.	.	.	.	.	.	.	A	.	.	–
CMW23808-*C. obpyriformis*	.	A	.	G	.	.	.	.	.	.	.	A	.	.	–
CMW23809-*C. polyconidia*	.	.	–	.	.	.	.	.	.	.	.	.	C	C	.
CMW23818-*C. .polyconidia*	.	.	–	.	.	.	.	.	.	.	.	.	C	C	.

Table 7 Number of fixed base pairs across the ITS gene region showing differences between species in the *C. pirilliformis s.l.* clade of *Ceratocystis*

Isolates	CMW6569	CMW6579	CMW29825	CMW29822	CMW27184	CMW27047	CMW15235	CMW15236	CMW23807	CMW23808	CMW23809	CMW23818
CMW6569-*C. pirilliformis*	0											
CMW6579-*C. pirilliformis*	3	0										
CMW29825-*C. pirilliformis*	7	6	0									
CMW29822-*C. pirilliformis*	1	2	6	0								
CMW27184-*C. pirilliformis*	1	2	6	0	0							
CMW27047-*C. pirilliformis*	1	3	7	1	1	0						
CMW15235-*C. zombamontana*	8	7	1	7	7	8	0					
CMW15236-*C. zombamontana*	8	7	1	7	7	8	0	0				
CMW23807-*C. obpyriformis*	4	5	9	5	5	6	10	10	0			
CMW23808-*C. obpyriformis*	4	5	9	5	5	6	10	10	0	0		
CMW23809-*C. polyconidia*	3	4	8	2	2	3	9	9	7	7	0	
CMW23818-*C.polyconidia*	3	4	8	2	2	3	9	9	7	7	0	0

species and their closest phylogenetic neighbors showed that they represented two undescribed taxa (Fig. 7).

Analyses of the TEF data set for the *C. moniliformis s.l.* isolates were not concordant with those for the BT1 data set, but also revealed five different clades (Fig. 6, Table 4). These clades represented *C. savannae* and *C. oblonga*, which could not be differentiated from each other using TEF sequence data, *C. moniliformis* and three separate clades including isolates from *Eucalyptus* and insects. All clades were supported by Bayesian, MP and ML analyses (Fig. 6, Table 4). There were three base pair differences between *Eucalyptus* isolates identified as *C. moniliformis* and the ex-type isolate of the species. Minor variations were found among isolates identified as *C. savannae* and the ex-type isolate of the species. Isolates residing in Clade 1 of the *C. moniliformis s.l.* group, identified as representing an undescribed taxon based on the BT1 gene region split into two well-supported clades in the TEF analyses, different from other *Ceratocystis* reference strains (Fig. 6). A total of 11 bp differences (10 indels and 1 fixed bp) separated the two clades (Table 10). Isolates residing in Clade 2 formed a single well resolved and strongly supported clade in the TEF tree, similar to the results for the BT1 data set (Figs. 5 and 6). Analyses of a TEF data set including only *C. savannae*, *C. oblonga* and their closest relatives and visualization of results in an unrooted tree confirmed the unique nature of Clade 2 isolates (Figs. 7 and 8). Isolates of *C. savannae* and *C. oblonga* could not be distinguished from each other based on the TEF gene region (Fig. 6). For the TEF data set, all analyses, including Bayesian, MP as well as ML were concordant.

Parsimony analysis of the combined dataset for the ITS, BT1 and TEF gene regions for isolates in the *C. moniliformis s.l.* group, and including related *Ceratocystis* species from GenBank (Table 4) resolved the isolates into five different clades (Fig. 9, Table 4). Sequence discordance found in TEF data sets remained present except that the unidentified species that split in two clades in the TEF tree, resided in a larger well-supported clade showing considerable sequence variation among isolates within the clade (Fig. 9). These data were confirmed by the 50 % majority rule tree obtained from Bayesian analyses, a bootstrap tree obtained from MP as well as from ML analyses.

Isolates residing in the group where only a *Thielaviopsis* state was present were considered only based on sequence data for the ITS gene region. This region generated a contig of ~500 bp. A preliminary nucleotide Blast against the GenBank database confirmed that isolates reside in *Thielaviopsis*. Comparison of the ITS data set for these isolates with sequences for other *Thielaviopsis* species, using parsimony analysis, resulted in a phylogenetic tree where one set of the isolates grouped with *T. basicola* (Berk. Et Br.) Ferr (AF275490, AF275494), and the second

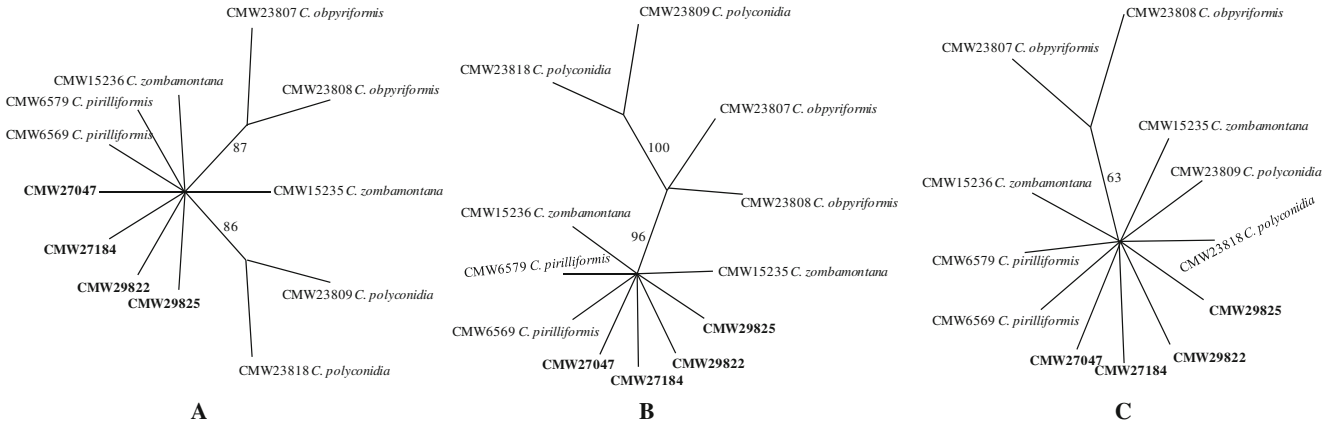

Fig. 3 Unrooted maximum parsimony trees produced from a heuristic search of the ITS (**a**) BT1 (**b**) and TEF (**c**) sequence data respectively, showing the relationship between species within the *C. pirilliformis s.l.* clade. All isolates sequenced are in *bold font type*. Bootstrap values were derived from 1000 replicates and are indicated next to each clade

set of isolates grouped with *T. thielavioides* (Peyr.) A.E. Paulin, T.C. Harr. & McNew (AF275487, AF275488) strains with 100 % bootstrap support at the nodes (Fig. 10).

Taxonomy

Based on phylogenetic analyses of sequence data for three gene regions, two previously unknown *Ceratocystis* species are recognized from *Eucalyptus* or insects associated with these trees in South Africa. These two fungi reside in the larger *C. moniliformis s.l.* complex, and were clearly separated based on sequence data for reference strains of other species in this group. Descriptions are provided for them in the following section. Furthermore, *C. zombamontana* and *C. pirilliformis* could not be separated based on DNA sequence data or morphology. *C. zombamontana* is consequently reduced to synonymy with *C. pirilliformis*.

Table 8 Number of haplotypes and their frequencies amongst species in the *C. pirilliformis s.l.* clade of *Ceratocystis*

Haplotype numbers	Frequencies	Isolate numbers	Haplotype designation
1	3	CMW15235	*C. zombamontana*
1	3	CMW15236	″
1	3	CMW29825	*C. pirilliformis*
2	2	CMW29822	″
2	2	CMW29184	″
3	1	CMW27047	″
4	1	CMW6569	″
5	1	CMW6579	″
6	2	CMW23807	*C. obpyriformis*
6	2	CMW23808	″
7	2	CMW23809	*C. polyconidia*
7	2	CMW23818	″

Ceratocystis salinaria Kamgan-Nkuek. & Jol. Roux sp. nov. (Fig. 11) MB519695

Etymology: Salinaria “pertaining to salt-works” used by Vitruvius (1st Century AD) and reflects the fact that the fungus was found in the Soutpansberg area, South Africa, famous for its salt pans.

Colonies Buff (19”d) coloured, ascomata produced rapidly and abundantly, scattered over the colonies and embedded within aerial mycelium, giving colonies a Honey (19”b) coloured appearance. Aerial mycelium distributed evenly across the colonies giving cultures a fluffy appearance. Reverse of colonies Honey (19”b). Colony diameter reaching 45.5 mm in 3 days on MEA at 25 °C. Optimal growth at 25 °C, growth at 30 °C with colony diameter reaching 43 mm in 3 days. No growth at 5 °C or 37 °C. Mycelium forming thick mat on agar. Hyphae smooth, not constricted at septa. *Ascomata* scattered over the colonies. *Ascomatal* bases dark brown, globose to obpyriform, (138.0-) 189.0–247.5 (-272.0) μm long and (124.0-) 155.5–204.5 (-232.5) μm wide, with dark conical spines (5.0-) 6.5–9.0 (-11.0) μm and hyphal hair. *Ascomatal* necks dark brown, (297.5-) 379.5–499.5 (-592.0) μm long, middle of necks (19.0-) 23.5–28.5 (-31.5) μm wide, tips of necks (11.5-) 12.0–16.5 (-23.0) μm wide, producing sticky and hyaline spore drops at the tips of divergent *ostiolar hyphae*, (19.5-) 24.5–100.5 (-123.5) μm long and with disc-like (disciform) bases, (43.0-) 55.0–73.5 (-88.0) μm wide at bases. *Asci* not seen, evanescent, deliquescing early in the development. *Ascospores* hat-shaped, hyaline, aseptate, invested in sheaths (4.5-) 5.0–5.5 (-6.0)×(2.5-) 3.0–3.5 (-4.0) μm, accumulating in round, *straw yellow* (21’d) spore drops, becoming creamy with age.

Anamorph: *Thielaviopsis*. *Conidiophores* singly on mycelium, phialidic, hyaline, tubular (18.5-) 20.5–28.5 (-39.5)×(2.0-) 2.5–3.0 (-3.5) μm, colarettes absent. *Conidia* hyaline, aseptate, two types, oblong (5.0-) 5.5–7.5 (-9.0)× (1.5-) 2.0–3.0 (-3.5) μm and bacilliform-shaped with

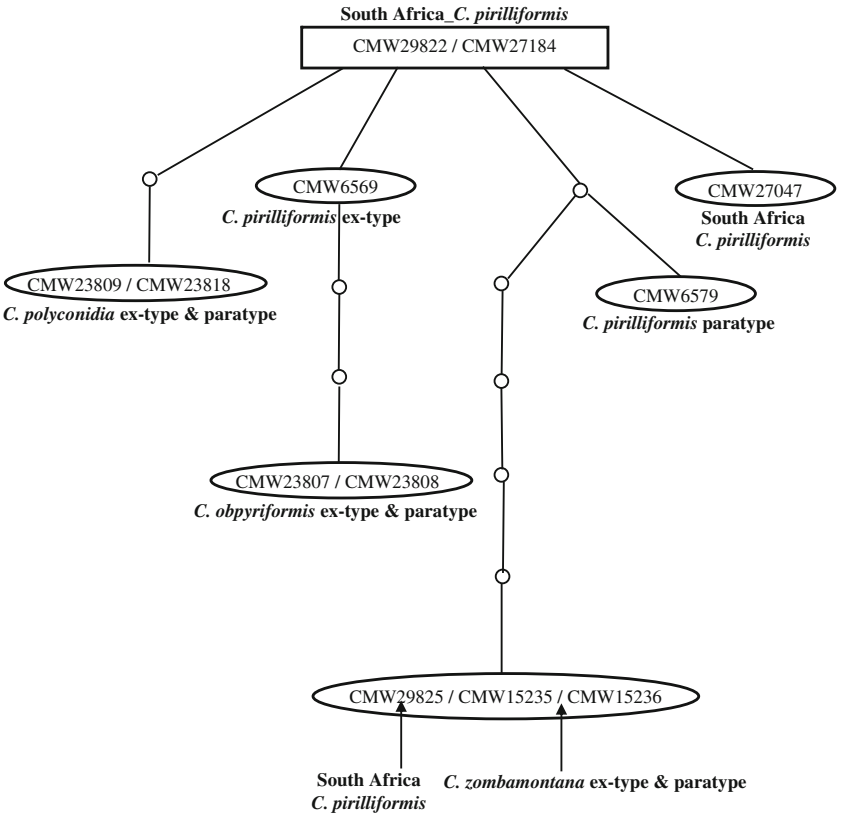

Fig. 4 Allele networks produced from ITS sequence data, showing the relationship between species in the *C. pirilliformis* s.l. clade comprising the type strains of *C. pirilliformis* (CMW6569, CMW6579), *C. zombamontana* (CMW15235, CMW15236), *C. polyconidia* (CMW23809, CMW23818), *C. obpyriformis* (CMW23807, CMW23808) and other isolates collected from South Africa (CMW29822, CMW27184, CMW27047, CMW29825) and identified as *C. pirilliformis* in this study. All four species are interconnected within the network, indicating that they originated from the same ancestral gene pool. However, *C. polyconidia* and *C. obpyriformis* each form single haplotypes distantly related from the other species

rounded bases (6.5-) 7.5–9.5 (-10.5)×(1.0-) 1.5–2.5 (-3.0) μm. Chlamydospores (aleuroconidia) not observed.

Specimen examined: South Africa, Limpopo Province, Soutpansberg area (S23° 02,350', E030° 14,209'), isolated from stumps of *Eucalyptus maculata*, 18/06/2007, G. Kamgan Nkuekam and J. Roux, holotype PREM 60557, living culture CMW25911 = CBS129733.

Additional specimens: South Africa, Limpopo Province, Soutpansberg area, from stumps of *Eucalyptus saligna*, 17/12/2008, G. Kamgan Nkuekam and J. Roux, paratype, living culture CMW30702 = PREM 60558, from stumps of *Eucalyptus saligna*, 17/12/2008, G. Kamgan Nkuekam and J. Roux, paratype, living culture CMW30703 = PREM60559 = CBS129734.

Ceratocystis decipiens Kamgan-Nkuek. & Jol. Roux sp. nov. (Fig. 12) MB519696

Etymology: Decipiens, the Latin word for "deceiving" and referring to the fact that the fungus would be seen as a single species based on BT or two species based on TEF sequence data.

Colonies Buff (19"d) coloured, ascomata often absent or produced late in small quantities, scattered over the colonies. Reverse of colonies Honey (19"b) from the edge, turning nearly Isabelline (17"i) towards the center. Colony diameters reaching 39 mm in 3 days on MEA at 30 °C. Optimal growth at 30 °C, no growth at 35 °C or at 5 °C. Mycelium forming thick mat on agar, becoming fluffy towards the center. Hyphae septate, not constricted at septa. *Ascomata* scattered over the surface of the colonies or embedded in mycelium. *Ascomatal* bases dark brown, globose to obpyriform, (132.5-) 167.5–216.5 (-258.5) μm long and (108.5-) 162.5–218.0 (-244.0) μm wide, with dark conical spines, (5.0-) 5.5–11.5 (-16.5) μm and hyphal hair ornamentations. *Ascomatal* necks dark brown, (355.0-) 401.0–500.5 (-596.5) μm long, middle of necks (17.5-) 21.0–25.5 (-27.5) μm wide, tips of necks (9.5-) 11.0–13.0 (-16.0) μm wide, producing sticky, hyaline spore drops at the tips of divergent *ostiolar hyphae*, (13.0-) 15.5–24.5 (-35.5) μm long and with disc-like (disciform) bases, (47.0-) 58.5–86.5 (-102.5) μm wide at bases. *Asci* not seen, evanescent, deliquescing early in the development. *Ascospores* hat-shaped, hyaline, aseptate, invested in sheaths (4.0-) 4.5–5.0 (-5.5)×(2.0-) 2.5–3.0 (-3.5) μm, accumulating in round, straw yellow (21'd) spore drops, becoming creamy with age.

Anamorph: *Thielaviopsis*. *Conidiophores* singly on mycelium, phialidic, hyaline, tubular (15.5-) 21.5–30.5 (-35.0)×(2.0-) 2.5–3.5 (-4.0) μm, colarettes absent. *Conidia* hyaline, aseptate, two types, oblong (4.5-) 5.5–6.5 (-7.5)×(1.5-) 2.0–2.5 (-3.5) μm and bacilliform-shaped (5.0-) 5.5–7.5 (-10.5)×(1.0-) 1.5–2.0 (-2.5) μm. Chlamydospores (aleuroconidia) not observed.

Specimen examined: South Africa, Limpopo Province, Soutpansberg area (S23° 02,350', E030° 14,209'), isolated from *Staphilinid* sp. obtained from stumps of a *Eucalyptus saligna* tree, 17/12/2008, G. Kamgan Nkuekam and J.

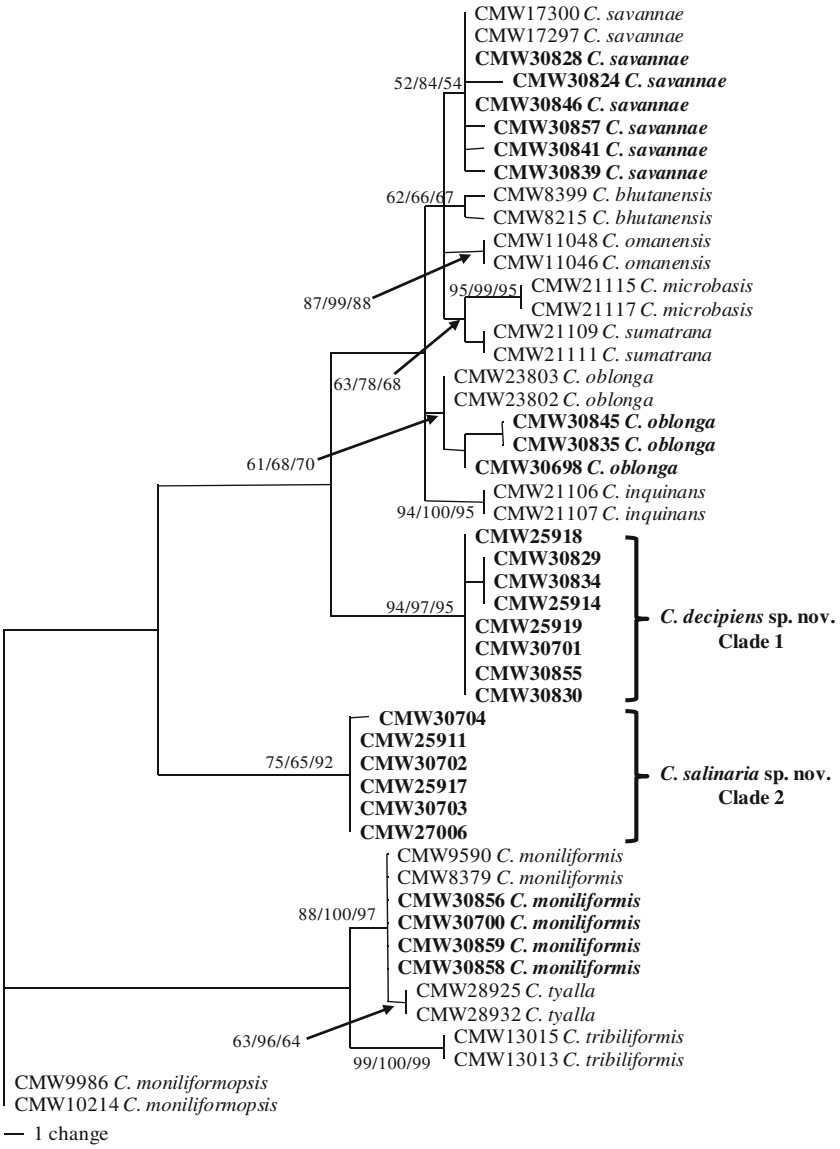

Fig. 5 Phylogenetic tree produced from a heuristic search of the BT1 sequence data, showing the relationship between members of *C. moniliformis s.l.* Isolates sequenced in this study are in bold font type. *C. moniliformopsis* was used as out-group taxon. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node

Roux, holotype PREM60560, living culture CMW30855 = CBS129736.

Additional specimens: South Africa, Limpopo Province, Soutpansberg area, from wound on *Eucalyptus cloeziana*, 21/06/2007, G. Kamgan Nkuekam and J. Roux, paratype, living culture CMW25918 = PREM60561 = CBS129735, from wound on *Eucalyptus maculata*, 21/06/2007, G. Kamgan Nkuekam and J. Roux, paratype, living culture CMW25914 = PREM60562 = CBS129737.

Ceratocystis pirilliformis I. Barnes & M.J. Wingfield, Mycologia 95:867. 2003

= *Ceratocystis zombamontana* R.N Heath & Jol. Roux, Fungal Diversity 34:53. 2009.

C. zombamontana, described from *Eucalyptus* trees in Malawi (Heath et al. 2009a) was found to be phylogenetically indistinguishable from *C. pirilliformis* based on DNA sequence data for five gene regions. Strains of the two species have identical BT1, BT2, TEF and RPB2 sequences. Strains of *C. zombamontana*, including the ex-type strain, have identical ITS1, ITS2 and 5.8S sequences as strains of *C. pirilliformis* collected in the current study. A number of morphological differences were previously reported for the two species. These included the fact that chlamydospores were produced in culture by *C. pirilliformis* and not by *C. zombamontana*, and the fact that *C. zombamontana* produced flask-shaped primary phialides compared to the cylindrical to lageniform phialides of *C. pirilliformis* (Heath et al. 2009a). A re-evaluation of isolates of *C. zombamontana* collected by Heath et al. (2009a) revealed the presence of chlamydospores in culture CMW15235 (Fig. 13). The measurements of chlamydospores in culture CMW15235 and those of the ex-type species of *C. pirilliformis* described by Barnes et al. (2003a) were found to overlap. Other than

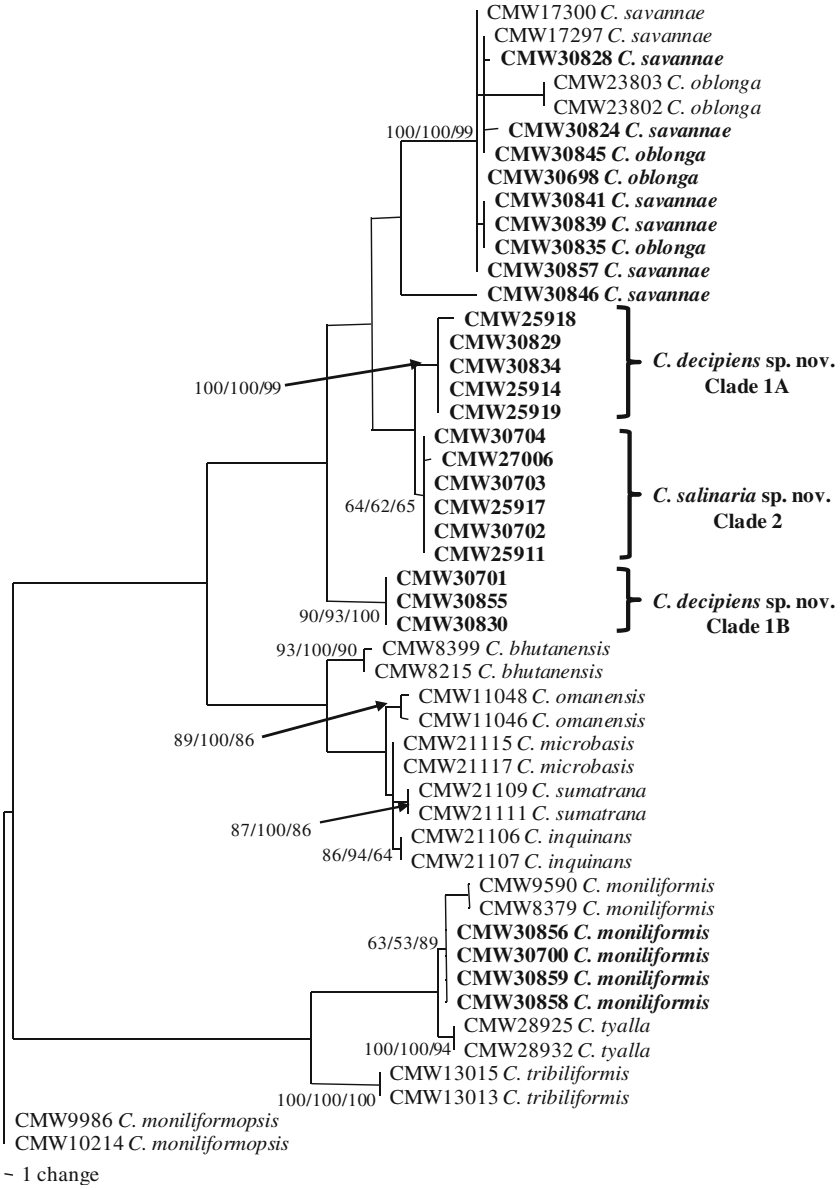

Fig. 6 Phylogenetic tree produced from a heuristic search of the TEF sequence data, showing the relationship between members of *C. moniliformis s.l.* Isolates sequenced in this study are in bold font type. *C. moniliformopsis* was used as out-group taxon. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node

reducing *C. zombamontana* to synonymy with *C. pirilliformis*, the description of *C. pirilliformis* should include the fact that some strains of the fungus might often not produce chlamydospores in culture. In addition *C. pirilliformis* produces two types of phialides, flask-shaped primary phialides and cylindrical to lageniform phialides.

This study reports nine *Ceratocystis* species, collected from six Provinces spanning various climatic conditions in South Africa. These include two species for which only the *Thielaviopsis* states were obtained. *C. eucalypticola* is the most widely spread species, occurring in four of the sampled Provinces (Fig. 14, Tables 2 and 3). It was obtained from *E. grandis* trees and from a wide variety of insects (Tables 2 and 3). This is followed by *C. pirilliformis* obtained from three Provinces on four *Eucalyptus* species (*E. cloeziana*, *E. diversicolor*, *E. grandis*, *E. saligna*) and from *B. depressus* (Tables 2 and 3). *C. oblonga* was found in two Provinces (Limpopo and Mpumalanga) on *E. grandis* trees. It was also obtained from *B. depressus*, *C. bisignatus* and *C. dimidiatus* (Table 3). *C. moniliformis* was found in both Limpopo and Mpumalanga Provinces on two *Eucalyptus* species (*E. grandis*, *E. maculata*) and from a Staphilinid species (Tables 2 and 3). *C. salinaria* was found in two Provinces (Limpopo, Western Cape) on three *Eucalyptus* species (*E. cloeziana*, *E. maculata*, *E. saligna*), while *C. decipiens* was found exclusively in the Limpopo Province on two *Eucalyptus* species (*E. cloeziana*, *E. maculata*) and on a Staphilinid species (Tables 2 and 3). *C. savannae* was found in Limpopo Province, on two *Eucalyptus* species (*E. cloeziana*, *E. maculata*) and on *B. depressus*, *C. bisignatus*, *C. dimidiatus* and a Staphilinid species (Tables 2 and 3). *T. basicola* and *T. thielavioides* were obtained from nitidulid beetles

Table 9 Summary of polymorphic nucleotides found within the BT1 gene region generated from phylogenetic analyses and showing differences between *C. salinaria*, *C. decipiens* and closest related taxa

Isolates	BT1																	
	14	116	126	127	131	134	138	139	140	141	143	150	158	167	168	254	323	335
CMW17300-*C. savannae*	C	T	C	T	T	C	A	T	C	A	A	G	C	T	C	C	T	T
CMW17297-*C. savannae*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMW30824-*C. savannae*	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	T	.	.
CMW30846-*C. savannae*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMW30839-*C. savannae*	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMW23803-*C. oblonga*	.	C	T	C	.	.	.	.	T	.	.	.	.	.	.	.	.	.
CMW23802-*C. oblonga*	.	C	T	C	.	.	.	.	T	.	.	.	.	.	.	.	.	.
CMW30845-*C. oblonga*	.	C	.	C	.	.	.	.	T	.	.	.	T	.	A	.	.	.
CMW30835-*C. oblonga*	.	C	.	C	.	.	.	.	T	.	.	.	T	.	A	.	.	.
CMW30698-*C. oblonga*	.	C	.	C	.	.	.	.	T	.	.	.	T	.	.	.	.	.
CMW25918-*C. decipiens*	.	C	.	C	.	.	.	C	.	.	.	A	.	.	.	.	C	C
CMW25914-*C. decipiens*	.	C	.	C	.	A	.	C	.	.	.	A	.	.	.	.	C	C
CMW30701-*C. decipiens*	.	C	.	C	.	.	.	C	.	.	.	A	.	.	.	.	C	C
CMW30855-*C. decipiens*	.	C	.	C	.	.	.	C	.	.	.	A	.	.	.	.	C	C
CMW30830-*C. decipiens*	.	C	.	C	.	.	.	C	.	.	.	A	.	.	.	.	C	C
CMW30704-*C. salinaria*	.	C	.	C	G	T	G	.	.	G	.	.	.	A	.	.	C	C
CMW30703-*C. salinaria*	.	C	.	C	G	T	G	.	.	G	.	.	.	A	.	.	.	C
CMW30702-*C. salinaria*	.	C	.	C	G	T	G	.	.	G	.	.	.	A	.	.	.	C
CMW25911-*C. salinaria*	.	C	.	C	G	T	G	.	.	G	.	.	.	A	.	.	.	C

collected in the Eastern Cape and KwaZulu-Natal Provinces respectively.

Pathogenicity test

Six weeks after inoculation, *Eucalyptus* trees were assessed for lesion development in the bark and cambium. *Ceratocystis salinaria* and *C. decipiens* produced very small lesions on both the bark and in the cambium of the *E. grandis* trees inoculated. On the bark, the mean lesion lengths (Lsmean) produced by *C. salinaria* and *C. decipiens* were 1.2 cm (R=0.75, CV=9.9, P<0.0001, Confidence limit =95 %) and 0.8 cm (R=0.75, CV=9.9, P<0.0001, Confidence limit= 95 %), respectively, while on the cambium, the Lsmeans were 1.5 cm (R=0.8, CV=8.9, P<0.0001, Confidence limit =95 %) and 1 cm (R=0.8, CV=8.9, P<0.0001, Confidence limit =95 %), respectively (data not shown). Lesions were present on both the bark (Lsmean =1 cm, R=0.75, CV=9.9, P<0.0001, Confidence limit =95 %) and the cambium (Lsmean =1 cm, R=0.8, CV=8.9, P<0.0001, Confidence limit =95 %) of the control trees inoculated for the *C. salinaria* treatment while there were no lesions on either the bark or the cambium of the control trees inoculated for the *C. decipiens* treatment (data not shown). At the time of assessment, trees showed no signs of disease and neither *C. salinaria* nor *C. decipiens* could be re-isolated from the small lesions associated with their inoculation.

Discussion

Nine *Ceratocystis* species were identified in this study from *Eucalyptus* species and insects associated with them, in six Provinces of South Africa. Two of these, *C. eucalypticola* and *C. pirilliformis* reside in the *C. fimbriata s.l.* complex. Five of the other *Ceratocystis* species identified are members of the *C. moniliformis s.l.* complex and the remaining two were found only in the *Thielaviopsis* anamorph state. Species in the *C. moniliformis s.l.* clade included *C. savannae*, *C. oblonga*, *C. moniliformis* and two new taxa for which the names *C. salinaria* and *C. decipiens* were provided. The asexual species included *T. basicola* and *T. thielavioides*. In pathogenicity tests, *C. salinaria* and *C. decipiens* resulted in only small lesions on both the bark and the xylem of young *Eucalyptus* trees and they are, therefore, not considered pathogens of *Eucalyptus* trees in South Africa.

Separation of *Ceratocystis* species based on morphology and phylogenetic inference from DNA sequence data is becoming increasingly difficult as new species are described. During the course of the past 10 years, sequence

Table 10 Summary of polymorphic nucleotides found within the TEF gene region generated from phylogenetic analyses and showing differences between *C. salinaria*, *C. decipiens* and closest related taxa

Isolates	TEF																															
	4	6	9	10	16	18	29	32	34	35	36	46	50	55	61	65	71	75	83	123	130	139	168	186	233	256	258	259	262	275	284
CMW17300-*C. savannae*	C	T	A	T	C	–	G	T	A	T	G	G	G	T	A	G	C	T	G	C	C	G	T	A	G	A	A	G	T	T	A
CMW17297-*C. savannae*	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMW30824-*C. savannae*	.	.	.	.	.	–	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
CMW30846-*C. savannae*	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMW30839-*C. savannae*	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
CMW23803-*C. oblonga*	T	C	G	C	T	–	.	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.	.	.	.
CMW23802-*C. oblonga*	T	C	G	C	T	–	.	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.	.	.	.
CMW30845-*C. oblonga*	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMW30835-*C. oblonga*	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
CMW30698-*C. oblonga*	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMW25918-*C. decipiens*	.	.	.	.	.	–	C	.	G	G	.	A	A	.	G	A	.	C	.	G	T	A	A	.	C	.	.	.	C	.	.
CMW25914-*C. decipiens*	.	.	.	.	.	–	C	.	G	G	.	A	A	.	G	A	.	C	.	G	T	A	A	.	C	.	.	.	C	.	.
CMW30701-*C. decipiens*	.	.	.	.	.	–	C	.	G	G	A	.	A	.	.	A	.	C	.	G	.	.	G	G	.	T	.	.	C	C	G
CMW30855-*C. decipiens*	.	.	.	.	.	–	C	.	G	G	A	.	A	.	.	A	.	C	.	G	.	.	G	G	.	T	.	.	C	C	G
CMW30830-*C. decipiens*	.	.	.	.	.	–	C	.	G	G	A	.	A	.	.	A	.	C	.	G	.	.	G	G	.	T	.	.	C	C	G
CMW30704-*C. salinaria*	.	.	.	.	.	T	C	.	G	G	.	A	A	.	.	A	.	C	.	G	T	.	A	.	.	.	G	.	C	.	.
CMW30703-*C. salinaria*	.	.	.	.	.	–	C	.	G	G	.	A	A	.	.	A	.	C	.	G	T	.	A	.	.	.	G	.	C	.	.
CMW30702-*C. salinaria*	.	.	.	.	.	–	C	.	G	G	.	A	A	.	.	A	.	C	.	G	T	.	A	.	.	.	G	.	C	.	.
CMW25911-*C. salinaria*	.	.	.	.	.	–	C	.	G	G	.	A	A	.	.	A	.	C	.	G	T	.	A	.	.	.	G	.	C	.	.

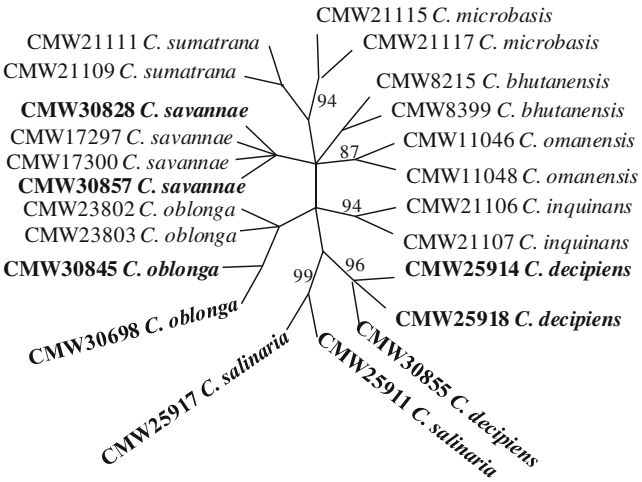

Fig. 7 Unrooted maximum parsimony tree produced from a heuristic search of the BT1 sequence data, showing the relationship between *C. salinaria* sp. nov., *C. decipiens* sp. nov. from *Eucalyptus* trees in South Africa and their most closely related neighbors in the *C. moniliformis s.l.* species complex

data for the ITS, TEF and BT1 gene regions have been used to distinguish morphologically similar species in the *C. fimbriata* and *C. moniliformis* complexes from each other (Van Wyk et al. 2006a, 2007a, b; Kamgan Nkuekam et al. 2008). In this regard, the ITS gene region has provided most information for species in the *C. fimbriata s.l.* clade and the TEF and BT1 regions are most informative for species in the *C. moniliformis s.l.* complex (Van Wyk et al. 2006a, 2012; Kamgan Nkuekam et al. 2008). In this study, we used both separate analyses for different gene regions as well as combined analyses to infer the phylogenies of members of the *C. moniliformis s.l.* and *C. fimbriata s.l.* complex. This made it possible to avoid errors in interpretation that could arise from using either separate analyses or combined analyses exclusively, as suggested by Huelsenbeck et al. (1996).

Delimiting species in the *C. moniliformis s.l.* complex is especially problematic. This is not only due to lack of concordance between gene regions (Van Wyk et al. 2006a; Kamgan Nkuekam et al. 2008), but could also be due to the presence of pseudogenes that are often present in more than one copy (Podlaha and Zhang 2010; Rouchka and Cha 2009). In this study, isolates of *C. decipiens* formed a single clade based on BT1 sequences, but two distinct clades based on TEF sequences and they were identical based on ITS sequences. Because of these inconsistencies we have adopted a conservative approach where these isolates have been described as representing a single species. Similarly, isolates identified as representing *C. oblonga* were identical to the ex-type and other isolates of *C. savannae* based on TEF and ITS sequences, but differed from this species based on BT1 sequences. Differences were also found amongst *C. oblonga* isolates collected in this study and those representing the ex-type isolate of the species both in the TEF and BT1 gene regions, suggesting that they represent closely related but distinct taxa. We have, however, refrained from describing them as distinct as more robust markers should be used to support this decision.

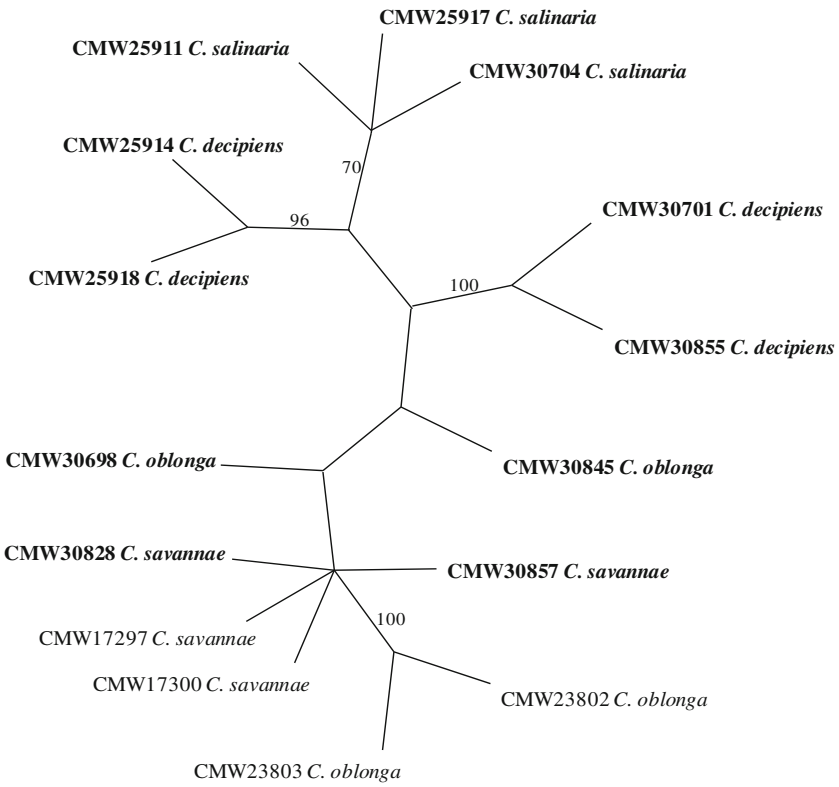

Fig. 8 Unrooted maximum parsimony tree produced from a heuristic search of the TEF sequence data, showing the relationship between *C. salinaria* sp. nov., *C. decipiens* sp. nov. from *Eucalyptus* trees in South Africa and their most closely related neighbors in the *C. moniliformis s.l.* species complex

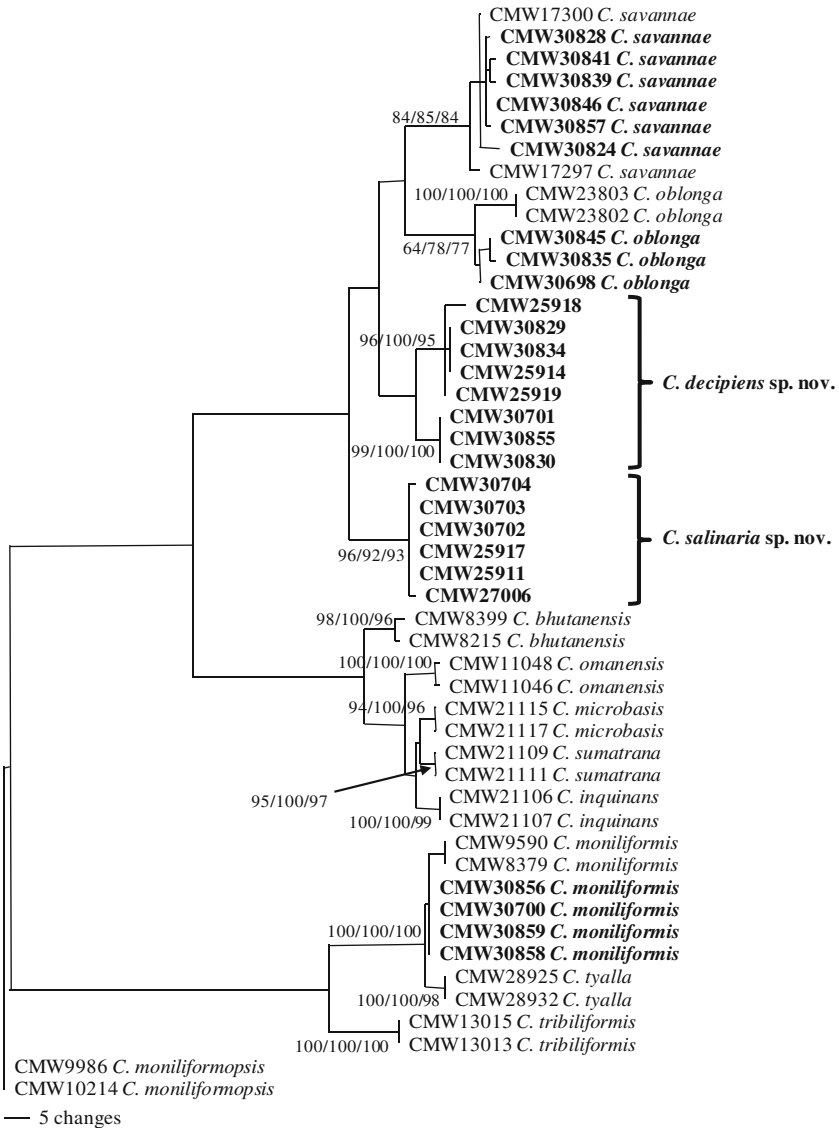

Fig. 9 Phylogenetic tree produced from a heuristic search of the combined ITS, BT1 and TEF sequence data, showing the relationship between members of *C. moniliformis s.l.* Isolates sequenced in this study are in bold font type. *C. moniliformopsis* was used as out-group taxon. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values respectively are indicated at each relevant node

The two new *Ceratocystis* species described in this study, *C. salinaria* and *C. decipiens*, are members of the *C. moniliformis s.l.* complex. Similar to other members of the *C. moniliformis s.l.* complex, *C. salinaria* and *C. decipiens* are characterized by fast growing cultures and they produce strong fruity (banana) odors on artificial media. Likewise, they produce ascomata with spiny bases and plate-like structures at the bases of the ascomatal necks as well as hat-shaped ascospores typical of species in the *C. moniliformis s.l.* complex. Most strains of *C. salinaria* sporulated readily on artificial media, producing ascomata and ascospore drops. In contrast, only one strain of *C. decipiens* sporulated on artificial media, and this strain ceased to sporulate after a single transfer to new media.

C. salinaria and *C. decipiens* differed in their growth rates, the length of their ostiolar hyphae and the widths of their necks. Minor morphological differences were also found between these newly described species and *C. oblonga*/*C. savannae*, which are their closest phylogenetic relatives. The ascomatal neck lengths of both *C. salinaria* and *C. decipiens*, as well as their bacilliform conidia were much shorter than those of *C. oblonga* and *C. savannae*. In addition, *C. decipiens* has necks with wider bases than those found in *C. oblonga* and *C. savannae*.

This study expands the host and geographic ranges of *C. savannae* to now include *Eucalyptus* trees. *C. savannae* was first described from native trees in South Africa in the absence of disease (Kamgan Nkuekam et al. 2008). In this study, *C. savannae* was isolated from wounds on two *Eucalyptus* species grown in the Soutpansberg area of South Africa, as well as from a staphylinid beetle and from two nitidulid beetles collected in this area. Previous reports of *C. savannae* were from the Kruger National Park and Leeuwfontein Collaborative Nature Reserve, both in the savanna regions in the eastern part of South Africa, similar to the Soutpansberg region. The discovery of *C. savannae*

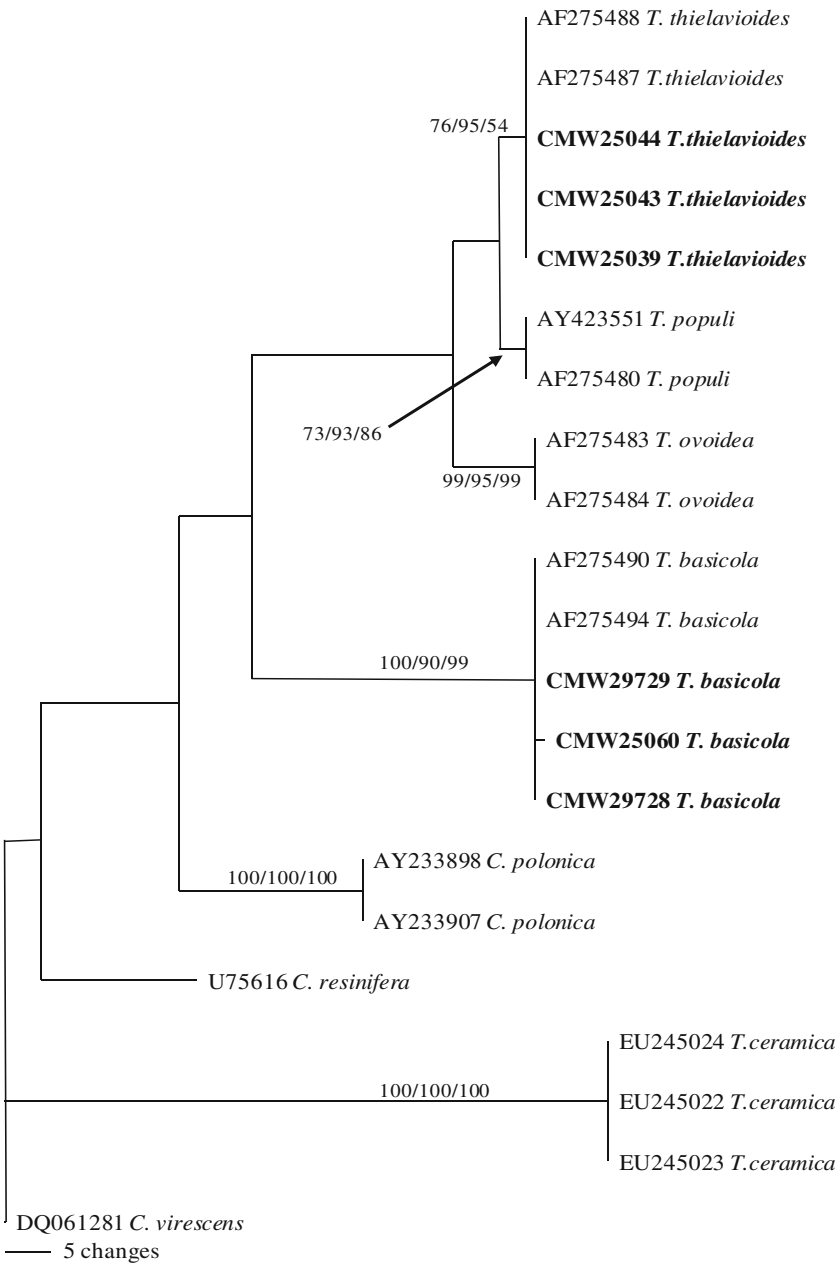

Fig. 10 Phylogenetic tree produced from a heuristic search of the ITS sequence data, showing the phylogenetic identity of *Thielaviopsis* strains collected in South Africa. Isolates sequenced in this study are in bold font type. *C. virescens* was used as out-group taxon. MP bootstrap values are indicated at each relevant node

on *Eucalyptus* brings the substrates on which the fungus occurs to seven tree species, spanning six genera and four families. The origin of *C. savannae* is unknown and it is not known whether it is a native fungus that has spread from native trees to infect non-native *Eucalyptus* trees, most probably mediated by insect dispersal, or whether it is an introduced saprophyte that has adapted to native tree species.

C. oblonga was recently described from South Africa, associated with three nitidulid species, *B. depressus*, *Ca. bisignatus* and *Ca. hemipterus* collected from both native savanna regions and from plantations of non-native *Acacia mearnsii* trees (Heath et al. 2009b). It was, therefore, not surprising to find *C. oblonga* on *Eucalyptus* associated with two nitidulid beetles, *B. depressus* and an unidentified *Carpophilus* sp. Results of this study expand the substrates on which the fungus has been found in South Africa to include *Eucalyptus* trees and confirm the findings of Heath et al. (2009b) that nitidulid beetles are vectors of this fungus in South Africa.

Isolating *C. moniliformis* from *Eucalyptus* trees in this study was not surprising. The fungus has previously been reported from wounds on *E. grandis* trees in South Africa (Roux et al. 2004; Heath et al. 2009a) and Tanzania (Heath et al. 2009a). In this study, *C. moniliformis* was isolated from *E. maculata* grown in the Soutpansberg area and from *E. grandis* grown in the Sabie area. The fungus was also isolated from a staphylinid beetle infesting *E. grandis* trees

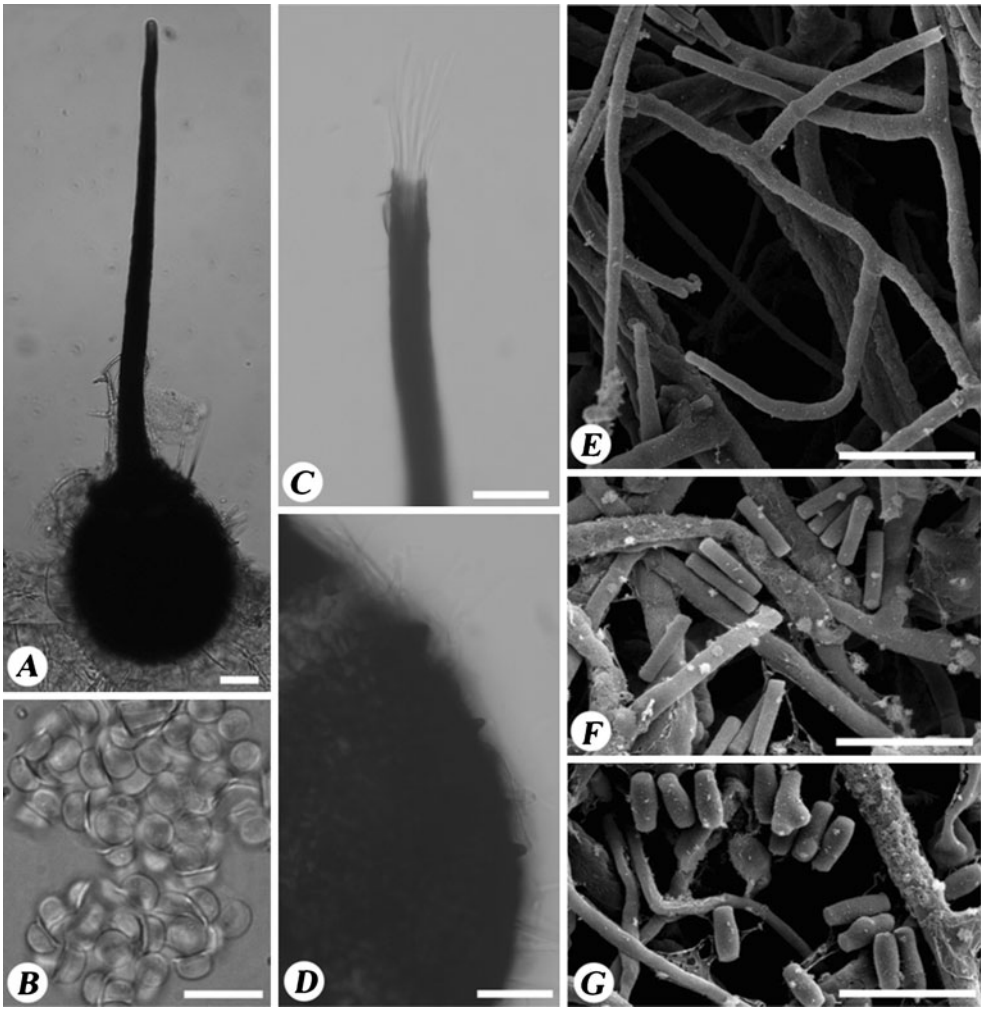

Fig. 11 Morphological characteristics of *Ceratocystis salinaria* sp. nov. **a** Globose ascomatal base (scale bar= 50 μm), **b** Hat-shaped ascospores (scale bar=10 μm), **c** Divergent ostiolar hyphae (scale bar=100 μm), **d** Ascomatal base with conical spines (scale bar=100 μm), **e** Phialidic conidiogenous cell with emerging conidia (scale bar=10 μm), **f** Bacilliform shaped conidia (scale bar=10 μm), **g** Oblong shaped conidia (scale bar= 10 μm)

in Sabie. This represents the first report of *C. moniliformis* from an insect and indicates that, like other *Ceratocystis* species, the fungus is also probably vectored by a wide variety of insects, including staphylinid beetles.

Similar to previous studies involving *Eucalyptus* trees, *C. pirilliformis* was commonly encountered. The fungus was first described from wounds made on eucalypts in Australia (Barnes et al. 2003a) and was later discovered occurring in the same niche in South Africa (Roux et al. 2004; Kamgan Nkuekam et al. 2009). The current study expands the geographic and host range of *C. pirilliformis* and it is now known from four Provinces in South Africa, spanning areas more than 2,000 km distant from each other. This study represents the first report of *C. pirilliformis* from an insect. It indicates that, like other *Ceratocystis* species, the fungus is probably vectored by nitidulid insects. In recent population genetic studies of the fungus from South African collections, it was found that *C. pirilliformis* was probably introduced into South Africa, possibly from Australia, due to the low gene diversity and allelic richness of the fungus in South Africa (Kamgan Nkuekam et al. 2009).

C. zombamontana, first described from *Eucalyptus* trees in Malawi (Heath et al. 2009a) was reduced to synonymy with *C. pirilliformis* because analysis of DNA sequence data for five gene regions and morphological comparisons could not distinguish isolates from each other. The lack of resolution between isolates of *C. zombamontana* and *C. pirilliformis* in this study and not previously, most probably arose from the fact that additional strains of *C. pirilliformis*, both from Australia and South Africa have become available for comparison. This provided greater intra-species variation and thus an overlap between sequences for *C. zombamontana* and *C. pirilliformis*. Reconsideration of sequences used in previous studies to distinguish *C. zombamontana* from *C. pirilliformis* also revealed that differences in ITS for these species are found only in multiple repeat regions, thus providing little meaningful resolution. Allele networks for the ITS gene regions also supported the synonymy of *C. pirilliformis* and *C. zombamontana*.

Isolates of *C. eucalypticola* infecting eucalypts have a broad geographic distribution on these trees in South Africa. *C. eucalypticola* was first reported as *C. fimbriata* from three provinces (KwaZulu-Natal, Limpopo, Mpumalanga) spanning four different locations (KwaMbonambi,

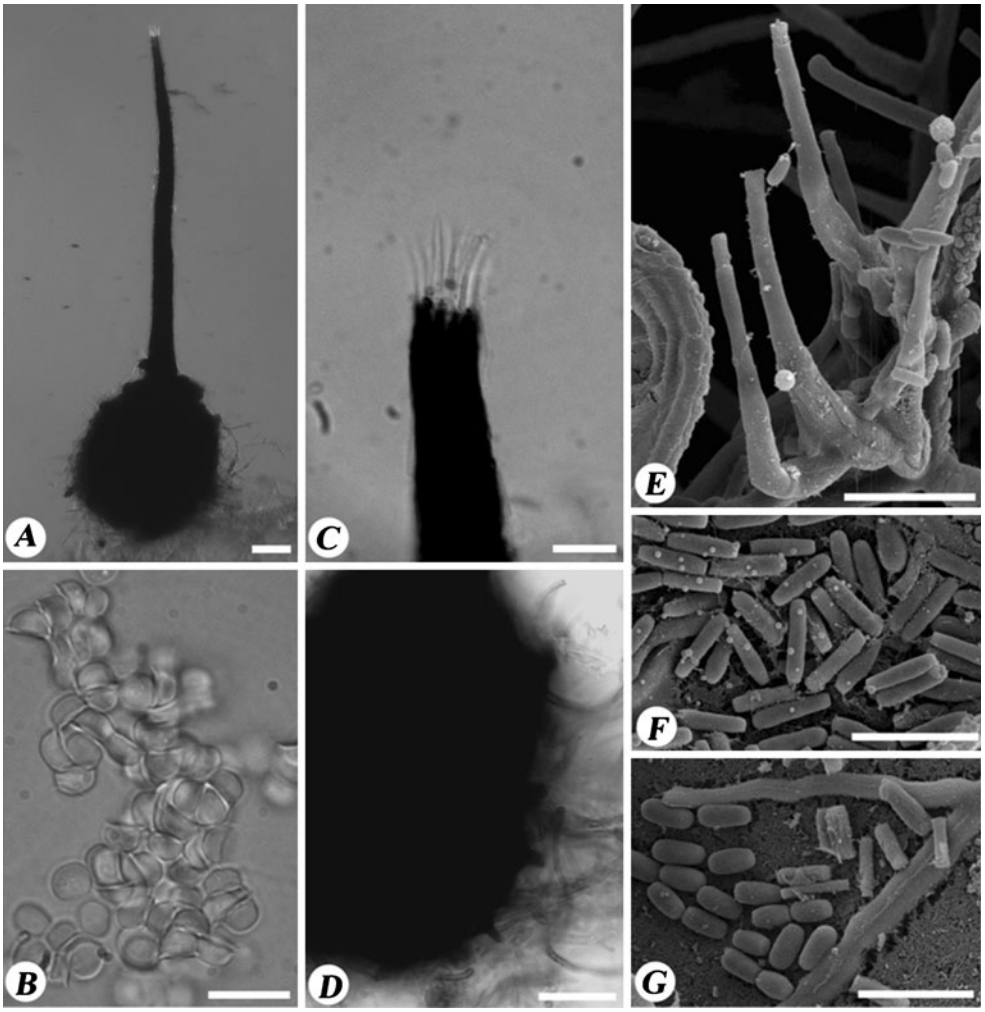

Fig. 12 Morphological characteristics of *Ceratocystis decipiens sp. nov.* **a** Globose ascomatal base (scale bar= 50 μm), **b** Hat-shaped ascospores (scale bar=10 μm), **c** Divergent ostiolar hyphae (scale bar=10 μm), **d** Ascomatal base with conical spines (scale bar=100 μm), **e** Phialidic conidiogenous cell with emerging conidia (scale bar=10 μm), **f** Bacilliform shaped conidia (scale bar=10 μm), **g** Oblong shaped conidia (scale bar= 10 μm)

Haxyview/Wilgeboom, Paulpietersburg, Tzaneen) of South Africa (Van Wyk et al. 2006b). Isolates identified in this study originated from these same provinces where they were broadly collected in five different locations (Tables 2 and 3). Collections arising from this study report the association of *C. eucalypticola* with insects for the first time. It was interesting to find this fungus associated not only with nitidulid beetles but also with *Xyleborus affinis* which is a wood boring ambrosia beetle (Wood 1982). *X. affinis* is native to tropical America, but is now widely distributed worldwide, as well as in Africa (Rabaglia et al. 2006; Wood 1982) and this is the first record of a *Ceratocystis* sp. associated with it.

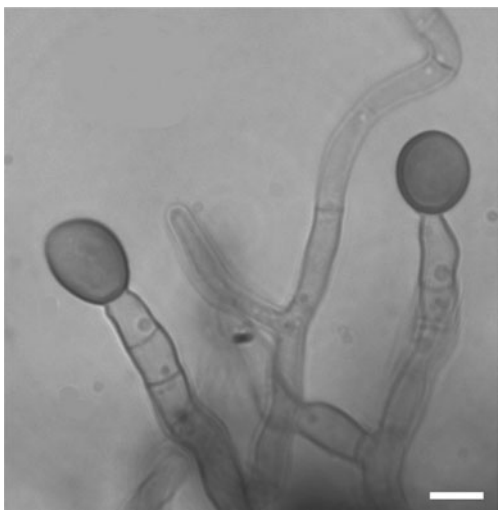

Fig. 13 Morphological characteristics of chlamydospores of *C. zombamontana* from isolate CMW15235 (scale bar=5 μm)

T. thielavioides causes root rot disease to various plant families in countries such as Brazil, Australia, Malaysia, Indonesia, North America and in Europe (Anonymous 2012). *T. thielavioides* was found for the first time in South Africa on two nitidulid species and it would be interesting to know whether it has been introduced into the country. The other *Thielaviopsis* sp. encountered, *T. basicola* was found on one nitidulid species. This is interesting because the fungus is known as a pathogen of several vegetable crops world-wide, including South Africa where it causes black pod rot of groundnuts (*Arachis hypogaea* L.) and black root rot of chicory (*Cichorium intybus* L.) (Prinsloo 1980; Prinsloo et al. 1991; Labuschagne and

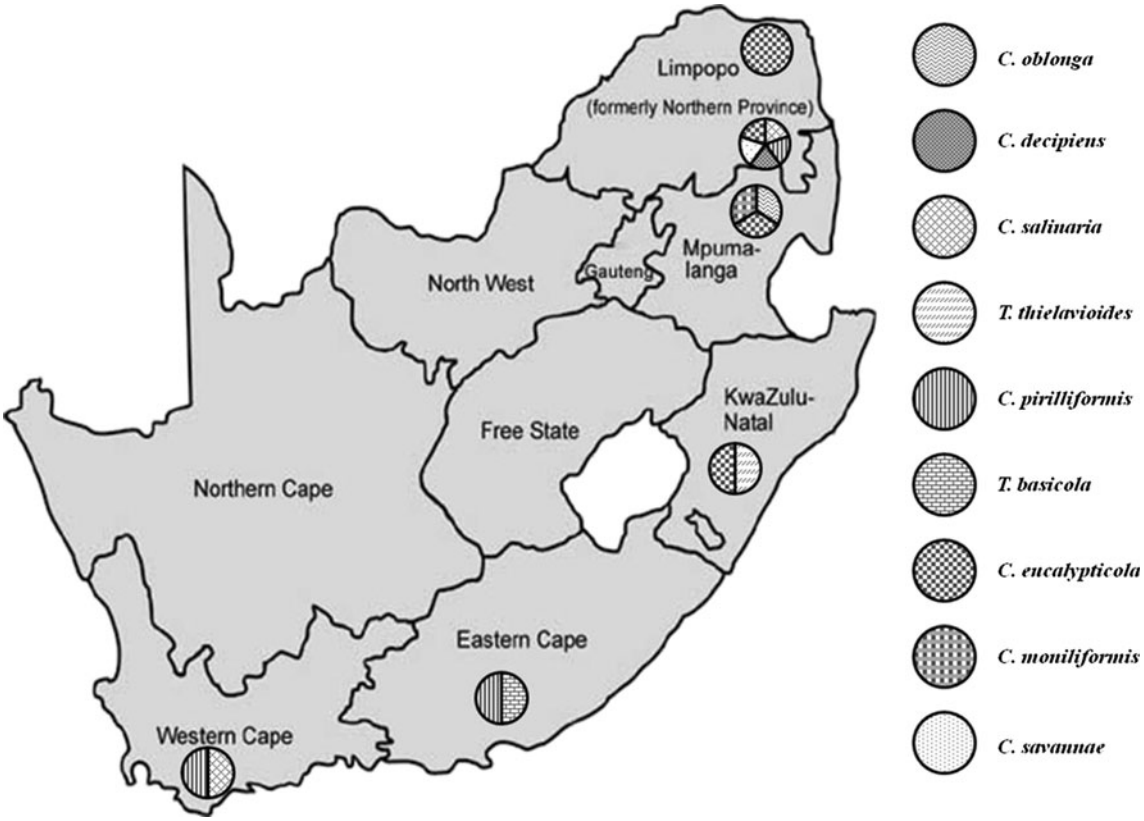

Fig. 14 Map of South Africa showing the current geographic distribution of *Ceratocystis* spp. and *Thielaviopsis* spp. identified in this study from *Eucalyptus* spp. and insects in the country

Kotze 1991; Geldenhuis et al. 2006). Population genetic studies using microsatellite markers have shown that two genotypes of *T. basicola* occur in South Africa, and that the fungus was most likely introduced into the country from Europe, probably through the distribution of root crops (Geldenhuis et al. 2006). Nitidulid beetles usually pupate in soil or in wood infested by fungi, and also feed on a wide variety of substrates such as flowers, stored crop products and fungi (Hinton 1945, Habeck 2002). These insects could have acquired the fungus from one of these substrates before flying to cut *Eucalyptus* stumps where they were collected. The results suggest that the fungus is able to leave the soil environment and infect above-ground parts of plants.

This study represents the first report of *Ceratocystis* species from insects in the Staphylinidae. Four *Ceratocystis* species, *C. eucalypticola*, *C. moniliformis*, *C. decipiens* and *C. savannae* were isolated from these insects. The Staphylinidae is one of the larger families of the Coleoptera (Lawrence and Newton 1995), which includes many predaceous and mycophagous species feeding mainly on macrofungi (Lawrence and Milner 1996). These insects are likely casual vectors of *Ceratocystis* species, with no fixed association given the number of *Ceratocystis* species that was isolated from them in a relatively limited study.

This study represents the most comprehensive consideration of *Ceratocystis* species and insects on *Eucalyptus* trees in South Africa. The number of species identified as well as insects from which they were isolated shows that the diversity of *Ceratocystis* species on *Eucalyptus* is still poorly understood in South Africa and even more so in other parts of the world. Future studies should explore the diversity of *Ceratocystis* species on native trees as well as on *Eucalyptus* trees in South Africa and in other African countries and will likely reveal numerous species of *Ceratocystis*, some of which could be important tree pathogens. Of the species collected in this study, several, including *C. pirilliformis*, *C. moniliformis*, *T. basicola* and *T. thielavioides*, have also been reported from other countries, including from *Eucalyptus* and other hosts, suggesting inter continental spread of these fungi. All the species collected in this study, as well as in a recent study of *Ceratocystis* species from *Eucalyptus* in Australia (Kamgan Nkuekam et al. 2012) produce fruity aromas, making them well-adapted for dispersal by various insects, including those living in the wood, bark and below the bark of trees. They can thus easily be spread on timber and other plant material and should all be considered important quarantine threats.

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