

## New *Ceratocystis* species from *Eucalyptus* and *Cunninghamia* in South China

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**Abstract** During routine surveys for possible fungal pathogens in the rapidly expanding plantations of *Eucalyptus* and *Cunninghamia lanceolata* in China, numerous isolates of unknown species in the genus *Ceratocystis* (Microascales) were obtained from tree wounds. In this study we identified the *Ceratocystis* isolates from *Eucalyptus* and *Cunninghamia* in the GuangDong, GuangXi, FuJian and HaiNan Provinces of South China based on morphology and through comparisons of DNA sequence data for the ITS, partial  $\beta$ -tubulin and TEF-1 $\alpha$  gene regions. Morphological and DNA sequence comparisons revealed two previously unknown species residing in the Indo-Pacific Clade. These are described here as *C. cercfabiensis* sp. nov. and *C. collisensis* sp. nov. Isolates of *C. cercfabiensis* showed intragenomic variation in their ITS sequences and four strains were selected for cloning of the ITS gene region. Twelve ITS haplotypes were obtained from 17 clones selected for sequencing,

differing in up to seven base positions and representing two separate phylogenetic groups. This is the first evidence of multiple ITS types in isolates of *Ceratocystis* residing in the Indo-Pacific Clade. Caution should thus be exercised when using the ITS gene region as a barcoding marker for *Ceratocystis* species in this clade. This study also represents the first record of a species of *Ceratocystis* from *Cunninghamia*.

**Keywords** Ceratocystidaceae · Fungal barcoding genes · Multiple ITS types · Plantation forestry

## Introduction

The Ascomycete genus *Ceratocystis* (Microascales, Ceratocystidaceae), occurs on a wide range of hosts and has a wide global distribution (Harrington 2004; Roux and Wingfield 2009; De Beer et al. 2014). It was first characterized by Halsted (1890) to accommodate the causal agent of black rot on sweet potato and the type of the genus, *Ceratocystis fimbriata*. Species of *Ceratocystis* are characterised by dark, globose ascomata with elongated necks, from which sticky ascospore masses exude at their apices (Upadhyay 1981). Most of the species in the genus, as defined recently by De Beer et al. (2014), are important pathogens of woody plants, including many agricultural, fruit and forest tree crops (Kile 1993; Roux and Wingfield 2009). These pathogens result in a multiplicity of symptoms such as branch and stem cankers, vascular staining, wilt, root rot, die-back, fruit rot and mortality (Kile 1993; Harrington 2004; Roux and Wingfield 2009).

The taxonomy of the genus *Ceratocystis* has been a source of confusion since the description of *C. fimbriata* by Halstead (1890). For many years *Ceratocystis* species and other genera in the Ceratocystidaceae were confused with fungi in the Ophiostomatales (*Ophiostoma*, *Grosmannia*,

*Ceratocystiopsis*) due to similarities in their morphology (Wingfield et al. 1993, 2012). It was only with the advent of DNA sequence data that it was conclusively shown that these two groups represent multiple and distinct genera, and reside in separate families (Hausner et al. 1993; Zipfel et al. 2006). Most recently, species treated in the single genus *Ceratocystis*, but differing based on morphology, ecology and phylogenetic inference were re-classified in discrete genera (Wingfield et al. 2012; De Beer et al. 2014). *Ceratocystis* as it now stands represents a genus of mostly plant pathogens previously treated in the *Ceratocystis fimbriata* complex where species can be defined in the South American, African, Indo-Pacific and North American Clades (Johnson et al. 2005; Mbenoun et al. 2014). Others have been re-classified as species in the genera *Ambrosiella*, *Chalaropsis*, *Davidsoniella*, *Endoconidiophora*, *Huntella* and *Thielaviopsis* (De Beer et al. 2014).

Despite recent advances in developing generic concepts for *Ceratocystis*, the taxonomy of some species in the genus remains unresolved. For example, the delimitation of species in the South American Clade lack sufficient markers for their clear delimitation (Al Adawi et al. 2013; Harrington et al. 2014; Fourie et al. 2015). The ITS region, which has been selected as the universal barcoding region for fungal species (Schoch et al. 2012), has been shown to be present as multiple ITS types in single isolates of some *Ceratocystis* species (Al Adawi et al. 2013; Naidoo et al. 2013; Harrington et al. 2014). It is hoped that with full genome sequences of representatives of each of these species (Wilken et al. 2013), and in some cases multiple species, robust and reliable taxonomic markers will be found to define species in the genus.

Several *Ceratocystis* species have been associated with serious diseases of trees in forests or grown in plantations or orchards. These have been treated in various previous studies (Roux et al. 2000, 2001; Harrington 2004; Tsopeles and Angelopoulos 2004; van Wyk et al. 2007a; Engelbrecht et al. 2007; Roux and Wingfield 2009; Li et al. 2014a, 2014b). Some examples include *C. albifundus*

that causes a canker and wilt disease of Australian *Acacia mearnsii* trees in Africa (Morris et al. 1993; Roux et al. 2005), *C. manginecans* that causes a serious wilt of mango in Oman and Pakistan (van Wyk et al. 2007a), *C. cacaofunesta* that causes a lethal wilt disease of cacao (*Theobroma cacao*) in the Caribbean and Central and South America (Engelbrecht et al. 2007) and *C. platani*, that causes a canker stain and die-back of *Platanus* spp. in the USA and Europe (Tsopelas and Angelopoulos 2004). New *Ceratocystis* species, often associated with serious disease problems, are regularly being discovered and it can be argued that this group of fungi represent an assemblage of pathogens that are rapidly rising in importance globally.

During the past twenty years, the establishment of commercial forestry plantations has increased in China to meet the needs of a rapidly growing national economy. Plantations of both native and introduced tree species have been established across the country (Xie 2011). China is, for example, currently the third largest producer of *Eucalyptus* tree-products globally, with more than three and a half million hectares planted with this non-native tree species (Xie 2011). A similar situation is true for *Cunninghamia lanceolata* (Lamb.) Hook, which is native in China and is being used in large afforestation projects, particularly in southern China (Camille and Morrell 2006) and due to its rapid growth and naturally durable heartwood (Liu et al. 2010). Despite the growing importance of *Eucalyptus* and other plantation species in China, information regarding fungal diseases affecting these plantations is limited (Zhou and Wingfield 2011). This is also true regarding the possible occurrence and impact of *Ceratocystis* species associated with trees in these plantations, where there have been only two previous studies treating this topic (Chen et al. 2013; Li et al. 2014b).

In order to develop disease management strategies to ensure sustainable plantation forestry in China, an inventory of pathogenic and potentially pathogenic fungi on trees is being assembled. As part of this effort, the aim of this study was to identify *Ceratocystis* isolates obtained from freshly cut

stumps or wounds in plantations of *Eucalyptus* species and *Cunninghamia lanceolata* growing in the GuangDong, GuangXi, FuJian and HaiNan Provinces of South China.

## Materials and methods

### Isolates

*Ceratocystis* species were isolated from wounds on *Eucalyptus* and *Cunninghamia lanceolata* in plantations in the GuangDong, GuangXi, FuJian and HaiNan Provinces of South China, between September 2013 and April 2014. Sampling was conducted at different sites from the stumps of recently (less than 1 month) harvested trees and fresh wounds on the branches and stems of trees. Ascomata of the fungi were identified by 10 × magnification hand lens and samples of wood or bark bearing fresh fruiting bodies resembling those of *Ceratocystis* species were placed into individual paper bags and transported to the laboratory for isolation.

Samples were incubated in humid chambers at 25°C to induce sporulation. Single ascospore masses exuding from the tips of ascomata were transferred to 2 % malt extract agar plates (MEA: 20 g/l malt extract, 20 g/l agar, Biolab, Midrand, South Africa), containing 100 mg streptomycin sulphate (Sigma, Steinheim, Germany) and incubated at 25°C for five to 10 days.

Representative isolates were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1), and in the collection of the China Eucalypt Research Centre (CERC), Chinese Academy of Forestry (CAF), ZhanJiang, GuangDong Province, China. Representative isolates of all novel species were deposited

with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. Dried specimens of sporulating cultures were deposited with the National Collection of Fungi (PREM), Pretoria, South Africa.

### Culture characteristics and morphology

All the fungal isolates collected in this study were grouped into morphotypes based on their characteristics in culture. Cultures were incubated on 2 % MEA at 25°C until sporulation and then grouped based on colour (Rayner 1970) and macro-morphology. To study the morphology of isolates, 3-week-old cultures representing each morphotype maintained at optimum growth temperature were used. Fruiting structures, including ascomata, ascospores, conidia and phialides from selected isolates were mounted in 80 % lactic acid on microscope slides and examined under a Zeiss Axioskop microscope (Carl Zeiss, Germany). Fifty measurements of each morphological structure were made for the isolates chosen to represent the holotypes, and 10 measurements for each of the two additional isolates were selected to represent paratypes of the new species. Average (mean), standard deviation (std. dev.), minimum (min), and maximum (max) measurements were made and are presented as [(min-) (mean – std. dev.) – (mean + std. dev.) (-max)] in the descriptions of the species.

### Growth in culture

Three isolates (one holotype and two paratypes) of each of the new species found in this study were used for growth studies after 10 to 14 days of growth on 2 % MEA. A 5 mm plug was removed from these cultures and transferred to the centres of 90 mm Petri dishes containing 2 % MEA. These cultures were grown in the dark for 14 days at temperatures ranging from 10 to 35°C at five degree

intervals. For each isolate and at each temperature, five replicate plates were prepared. Two diameter measurements, perpendicular to each other, were taken daily for each colony and the averages of diameter measurements for each temperature were computed. The entire experiment was repeated once.

#### DNA extraction, PCR and sequencing

For DNA sequencing, cultures of each isolate were grown on 2 % MEA at 25°C for two weeks prior to DNA extraction. Mycelium was collected from the surface of cultures grown on MEA and transferred to Eppendorf tubes using a sterile scalpel. DNA extractions were made using the CTAB (cetyl trimethyl ammonium bromide) protocol (Möller et al. 1992). DNA working concentrations were adjusted to ~100 ng/μL, using a Thermo Scientific NanoDrop® ND-1000 Spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA).

Three gene regions, namely the Internal Transcribed Spacer (ITS) regions (ITS1, ITS2) including the 5.8S rRNA gene, part of the Beta-tubulin 1 (BT1) and part of the Translation Elongation Factor-1 alpha (TEF-1α) regions were amplified using the Polymerase Chain Reaction (PCR). The ITS regions were amplified with primers ITS1 and ITS4 (White et al. 1990), the BT1 gene region using primers Bt1a and Bt1b (Glass and Donaldson 1995), and the TEF-1α gene region with primers TEF1F and TEF2R (Jacobs et al. 2004).

For all gene regions, PCR reactions were conducted in a 25 μL final volume. Each reaction comprised 2.5 μL of 10 × PCR buffer with MgCl<sub>2</sub> (25 mM), 0.2 μL of Taq polymerase (1 U/μL) (Roche Diagnostic), 0.5 μL of deoxynucleotide triphosphate (dNTPs) mix (10 mM), 1 μL of each primer (10 mM) and 1 μL of DNA template. Reactions were run on a Bio-Rad iCycler thermocycler

(BIO-RAD, Hercules, CA, USA). For the ITS and BT1 gene regions, the thermal cycling conditions were the same and consisted of an initial denaturation step at 95°C for 5 min followed by 35 cycles of 30 s at 95°C, 45 s at 56°C and 60 s at 72°C, with a final extension at 72°C for 10 min. For the TEF-1 $\alpha$ , the thermal cycling comprised an initial denaturation at 95°C for 5 min followed by 10 primary amplification cycles of 30 s at 95°C, 30 s at 56°C, and 60 s at 72°C, 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 at 72°C for 10 min. Amplification was confirmed by staining PCR products (3  $\mu$ L aliquots) with 1.5  $\mu$ L of GelRed<sup>TM</sup> Nucleic Acid Gel stain (Biotium, Hayward, CA, USA), and separating them on a 2 % agarose gel, followed by visualization under UV light. PCR products were purified by filtration using 6 % Sephadex G-50 (Sigma).

Forward and reverse sequencing reactions were performed in 12  $\mu$ L final volumes with the same primers as used for the PCR reactions. The mixtures contained 1  $\mu$ L BigDye<sup>®</sup> Terminator v. 3.1 ready reaction mixture (Perkin-Elmer, Warrington, UK), 2  $\mu$ L sequencing buffer, 1  $\mu$ L of either the forward or reverse primer (10 mM) for each gene region or 2  $\mu$ L cleaned PCR product. The thermal cycling conditions comprised 25 cycles of 10 s at 96°C, 5 s at 54°C and 4 min at 60°C. Sequencing products were cleaned using Sephadex G-50 columns and dried in an Eppendorf 5301 vacuum concentrator, at 60°C for 5 min. They were thereafter run on an ABI PRISM<sup>TM</sup> 3100 DNA Analyzer (Applied BioSystems, Foster City, CA, USA).

## Cloning

Some *Ceratocystis* strains that showed ambiguity in the sequences of the ITS region were cloned using the pGEM<sup>®</sup>-T and pGEM<sup>®</sup>-T Easy Vector System (Promega, Madison, USA) cloning kit, following the manufacturer's instructions. The primers T7 and SP6 were used for amplification and



sequencing (Invitrogen, Life technologies, Johannesburg, SA). For each ambiguous PCR product, up to five clones were sequenced. The amplification reaction mixture had a total volume of 25  $\mu$ L, consisting of 5  $\mu$ L 5  $\times$  MyTaq™ Buffer (comprise 5 mM dNTPs, 15 mM MgCl<sub>2</sub>), 0.5  $\mu$ L MyTaq™ DNA Polymerase (Bioline Ltd.UK), 1  $\mu$ L DNA, 1  $\mu$ L of each primer (10 mM) and distilled H<sub>2</sub>O. The PCR cyclers program consisted of 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 1 min and a final extension of 72°C for 10 min.

### Multi-gene phylogenetic analyses

A preliminary identity for the *Ceratocystis* isolates was obtained by performing a similarity search (standard nucleotide BLAST) of the ITS, BT1 and TEF-1 $\alpha$  sequences against the GenBank nucleotide database (<http://www.ncbi.nlm.nih.gov>). Sequences for closely related type cultures of *Ceratocystis* species were downloaded from GenBank to compile datasets for the phylogenetic analyses (Table 1). Individual data sets and a combined data set of the BT1 and TEF-1a gene regions were used for phylogenetic analyses. Since multiple ITS copies occurred in some isolates, this dataset was not combined with the other gene regions in the analyses.

Sequences for each of the three gene regions were aligned using the online interface of MAFFT v. 7 (<http://mafft.cbrc.jp/alignment/server>) (Katoh et al. 2002), with the iterative refinement method (FFT-NS-i settings) selected. Sequence alignments were edited manually in MEGA v. 6 (Tamura et al. 2007). Sequence alignments for all the datasets were deposited in TreeBASE (<http://treebase.org/treebase-web>) and sequences for the novel taxa deposited in GenBank (Table 1).

Two different phylogenetic analyses methods were used for each of the datasets and for the combined BT1 and TEF-1a dataset. Maximum parsimony (MP) analyses were performed using

**Table 1** List of *Ceratocystis* isolates used in this study

Species	CMW No. <sup>1</sup>	CERC No. <sup>1,2</sup>	Other no. <sup>1</sup>	GenBank accession no.			Hosts (or substrate)	Collectors	Geographic Origin
				ITS <sup>3</sup>	$\beta$ -tubulin	TEF-1a			
<i>C. albifundus</i>	CMW4068			DQ520638	EF070429	EF070400	<i>Acacia mearnsii</i>	J. Roux	South Africa
<i>C. albifundus</i>	CMW5329			AF388947	DQ371649	EF070401	<i>A. mearnsii</i>	J. Roux	Uganda
<i>C. atrox</i>	CMW19383		CBS 120517	EF070414	EF070430	EF070402	<i>Eucalyptus grandis</i>	M.J. Wingfield	Australia
<i>C. atrox</i>	CMW19385		CBS 120518	EF070415	EF070431	EF070403	<i>E. grandis</i>	M.J. Wingfield	Australia
<i>C. cercfabiensis</i>	<b>CMW42489</b>	<b>CERC2168</b>		N/A	KP727605	KP727630	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	HaiNan, China
<i>C. cercfabiensis</i>	<b>CMW43029</b> <sup>4,5,6</sup>	<b>CERC2170</b>	<b>CBS 139654</b>	See Table 3	KP727618	KP727643	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	HaiNan, China
<i>C. cercfabiensis</i>	<b>CMW42504</b>	<b>CERC2323</b>		N/A	KP727595	KP727620	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	GuangXi, China
<i>C. cercfabiensis</i>	<b>CMW43030</b>	<b>CERC2325</b>		N/A	KP727606	KP727631	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	GuangXi, China
<i>C. cercfabiensis</i>	<b>CMW42512</b> <sup>5,6</sup>	<b>CERC2335</b>		See Table 3	KP727607	KP727632	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	GuangXi, China
<i>C. cercfabiensis</i>	<b>CMW42515</b> <sup>4,5</sup>	<b>CERC2345</b>	<b>CBS 139655</b>	N/A	KP727596	KP727621	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	GuangXi, China
<i>C. cercfabiensis</i>	<b>CMW43033</b>	<b>CERC2471</b>		N/A	KP727597	KP727622	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	FuJian, China
<i>C. cercfabiensis</i>	<b>CMW42574</b>	<b>CERC2549</b>		N/A	KP727598	KP727623	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	GuangDong, China
<i>C. cercfabiensis</i>	<b>CMW42577</b>	<b>CERC2552</b>		N/A	KP727599	KP727624	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	GuangDong, China
<i>C. cercfabiensis</i>	<b>CMW42736</b> <sup>5,6</sup>	<b>CERC2576</b>		See Table 3	KP727600	KP727625	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	GuangDong, China
<i>C. cercfabiensis</i>	<b>CMW42741</b> <sup>5,6</sup>	<b>CERC2581</b>		See Table 3	KP727601	KP727626	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	GuangDong, China
<i>C. cercfabiensis</i>	<b>CMW42745</b>	<b>CERC2586</b>		N/A	KP727608	KP727633	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	GuangDong, China
<i>C. cercfabiensis</i>	<b>CMW42790</b>	<b>CERC2646</b>		N/A	KP727602	KP727627	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	GuangXi, China
<i>C. cercfabiensis</i>	<b>CMW42794</b>	<b>CERC2686</b>		N/A	KP727609	KP727634	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	GuangDong, China
<i>C. cercfabiensis</i>	<b>CMW42795</b> <sup>4,5</sup>	<b>CERC2687</b>	<b>CBS 139656</b>	N/A	KP727619	KP727644	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	GuangDong, China
<i>C. cercfabiensis</i>	<b>CMW42803</b>	<b>CERC2800</b>		N/A	KP727603	KP727628	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	GuangXi, China
<i>C. cercfabiensis</i>	<b>CMW42812</b>	<b>CERC2817</b>		N/A	KP727604	KP727629	<i>Eucalyptus</i> sp.	S.F. Chen & F.F. Liu	GuangXi, China
<i>C. collisensis</i>	<b>CMW43031</b>	<b>CERC2456</b>		KP727575	KP727612	KP727637	<i>Cunninghamia lanceolata</i>	S.F. Chen & F.F. Liu	FuJian, China
<i>C. collisensis</i>	<b>CMW42550</b>	<b>CERC2457</b>		KP727576	KP727613	KP727638	<i>C. lanceolata</i>	S.F. Chen & F.F. Liu	FuJian, China
<i>C. collisensis</i>	<b>CMW42551</b> <sup>5</sup>	<b>CERC2458</b>		KP727577	KP727610	KP727635	<i>C. lanceolata</i>	S.F. Chen & F.F. Liu	FuJian, China
<i>C. collisensis</i>	<b>CMW42552</b> <sup>4,5</sup>	<b>CERC2459</b>	<b>CBS 139679</b>	KP727578	KP727614	KP727639	<i>C. lanceolata</i>	S.F. Chen & F.F. Liu	FuJian, China
<i>C. collisensis</i>	<b>CMW42553</b> <sup>4,5</sup>	<b>CERC2465</b>	<b>CBS 139646</b>	KP727579	KP727611	KP727636	<i>C. lanceolata</i>	S.F. Chen & F.F. Liu	FuJian, China
<i>C. collisensis</i>	<b>CMW42554</b> <sup>4,5</sup>	<b>CERC2466</b>	<b>CBS 139647</b>	KP727580	KP727615	KP727640	<i>C. lanceolata</i>	S.F. Chen & F.F. Liu	FuJian, China
<i>C. collisensis</i>	<b>CMW43032</b>	<b>CERC2467</b>		KP727581	KP727616	KP727641	<i>C. lanceolata</i>	S.F. Chen & F.F. Liu	FuJian, China
<i>C. collisensis</i>	<b>CMW42555</b>	<b>CERC2468</b>		KP727582	KP727617	KP727642	<i>C. lanceolata</i>	S.F. Chen & F.F. Liu	FuJian, China
<i>C. corymbiicola</i>	CMW29120		CBS 127215	HM071902	HM071914	HQ236453	<i>Corymbia variegata</i>	G.K. Nkuekam	Australia
<i>C. corymbiicola</i>	CMW29349		CBS 127216	HM071919	HQ236455	HM071905	<i>Eucalyptus pilularis</i>	G.K. Nkuekam	Australia
<i>C. larium</i>	CMW25434		CBS 122512	EU881906	EU881894	EU881900	<i>Styrax benzoin</i>	M.J. Wingfield	Indonesia
<i>C. larium</i>	CMW25435		CBS 122606	EU881907	EU881895	EU881901	<i>S. benzoin</i>	M.J. Wingfield	Indonesia
<i>C. obpyriformis</i>	CMW23807		CBS 122608	EU245004	EU244976	EU244936	<i>Acacia mearnsii</i>	R.N. Heath	South Africa

<i>C. obpyriformis</i>	CMW23808	CBS 122511	EU245003	EU244975	EU244935	<i>A. mearnsii</i>	R.N. Heath	South Africa
<i>C. pirilliformis</i>	CMW6569		AF427104	DQ371652	AY528982	<i>Eucalyptus nitens</i>	M.J. Wingfield	Australia
<i>C. pirilliformis</i>	CMW6579	CBS 118128	AF427105	DQ371653	AY528983	<i>E. nitens</i>	M.J. Wingfield	Australia
<i>C. polychroma</i>	CMW11424	CBS 115778	AY528970	AY528966	AY528978	<i>Syzygium aromaticum</i>	M.J. Wingfield	Indonesia
<i>C. polychroma</i>	CMW11436	CBS 115777	AY528971	AY528967	AY528979	<i>S. aromaticum</i>	M.J. Wingfield	Indonesia
<i>C. polyconidia</i>	CMW23809	CBS 122289	EU245006	EU244978	EU244938	<i>Acacia mearnsii</i>	R.N. Heath	South Africa
<i>C. polyconidia</i>	CMW23818	CBS 122290	EU245007	EU244979	EU244939	<i>A. mearnsii</i>	R.N. Heath	South Africa
<i>Davidsoniella virescens</i>	CMW11164	CBS 123166	DQ520639	EF070441	EF070413	<i>Fagus americanum</i>	D. Houston	USA

<sup>1</sup> CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa;

CERC = Culture collection of China Eucalypt Research Centre (CERC), Chinese Academy of Forestry (CAF), ZhanJiang, GuangDong Province, China; CBS = the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

<sup>2</sup> Isolates indicated in bold are newly described in this study

<sup>3</sup> NA (not applicable) indicates isolates where multiple ITS types exist for species of *C. cercfabiensis* and where a single clean ITS sequence could not be obtained without first cloning

<sup>4</sup> Isolates used for the growth study

<sup>5</sup> Isolates obtained and used in the phylogenetic analyses

<sup>6</sup> Isolates used for the ITS cloning

PAUP v. 4.0b10 (Swofford 2002) and maximum likelihood (ML) tests were conducted with PhyML v. 3.0 (Guindon and Gascuel 2003). For MP analyses, gaps were treated as a fifth character and the characters were unordered and of equal weight with 1000 random addition replicates. A partition homogeneity test (PHT) was used to determine the congruence of the BT1 and TEF-1a datasets. For the analyses of each dataset, the most parsimonious trees were obtained with the heuristic search function and tree bisection and reconstruction (TBR) as branch swapping algorithms. MAXTREES were unlimited and branch lengths of zero were collapsed. A bootstrap analysis (50 % majority rule, 1000 replicates) was done to determine the confidence levels of the tree-branching points (Felsenstein 1985). Tree length (TL), consistency index (CI), retention index (RI) and the homoplasy index (HI) were used to assess the trees (Hillis and Huelsenbeck 1992).

For ML (maximum likelihood) analysis of each dataset, the best models of nucleotide substitution were determined with Modeltest v. 3.7 (Posada and Crandall 1998). The analyses were conducted with PhyML v. 3.0 (Guindon and Gascuel 2003). Parameters in PhyML included the retention of the maximum number of 1000 trees and the determination of nodal support by non-parametric bootstrapping with 1000 replicates. The phylogenetic trees were viewed using MEGA v.6 (Tamura et al. 2007). For both MP and ML analyses, *Davidsoniella virescens* (CMW11164) was used as the outgroup taxon (Table 2).

**Table 2** Statistics resulting from phylogenetic analyses

Dataset	No. of taxa	No. of bp <sup>1</sup>	Maximum parsimony					
			PIC <sup>2</sup>	Number trees	Tree length	CI <sup>3</sup>	RI <sup>4</sup>	HI <sup>5</sup>
ITS	35	553	228	3	499	0.858	0.945	0.142
BT1	27	534	85	7	214	0.888	0.933	0.112
TEF-1a	27	701	83	6	281	0.9	0.909	0.099
BT1/TEF-1a	27	1235	168	2	501	0.884	0.912	0.116

Dataset	Maximum likelihood							
	Subst model <sup>6</sup>	NST <sup>7</sup>	Rate matrix			Ti/tv ratio <sup>8</sup>	Rates <sup>9</sup>	
ITS	HKY+G	2				1.3092	gamma	
BT1	TrN+I	6	1.0000	2.4023	1.0000	1.0000	4.9894	equal
TEF-1a	TrNef+G	6	1.0000	1.5358	1.0000	1.0000	3.7338	gamma
BT1/TEF-1a	TrN+G	6	1.0000	1.8794	1.0000	1.0000	4.1007	gamma

<sup>1</sup> bp = base pairs. <sup>2</sup> PIC = number of parsimony informative characters. <sup>3</sup> CI = consistency index. <sup>4</sup> RI = retention index. <sup>5</sup>

HI = homoplasy index. <sup>6</sup> Subst. model = best fit substitution model. <sup>7</sup> NST = number of substitution rate categories. <sup>8</sup>

Ti/Tv ratio = transition/transversion ratio. <sup>9</sup> I = proportion of invariable sites

## Results

### Isolates

A total of 180 isolates resembling species of *Ceratocystis* were obtained from 30 different trees in 13 different forestry plantations (Fig. 1), including 11 *Eucalyptus* plantations and two *Cunninghamia lanceolata* plantations in the GuangDong, GuangXi, HaiNan and FuJian Provinces in South China. *Ceratocystis* ascocarps were commonly found on the stumps and branch/stem wounds. Slight discolouration was observed on the wood where fruiting bodies were present.

Isolates could be assigned to two broad groups based on culture morphology and the appearance of the fruiting bodies produced on MEA. One group, comprising the majority of isolates (172), had a strong fruity (banana) odour. These isolates produced sexual fruiting structures abundantly in culture. All isolates had dark, globoid ascomata with necks ~1100 µm in length and they produced

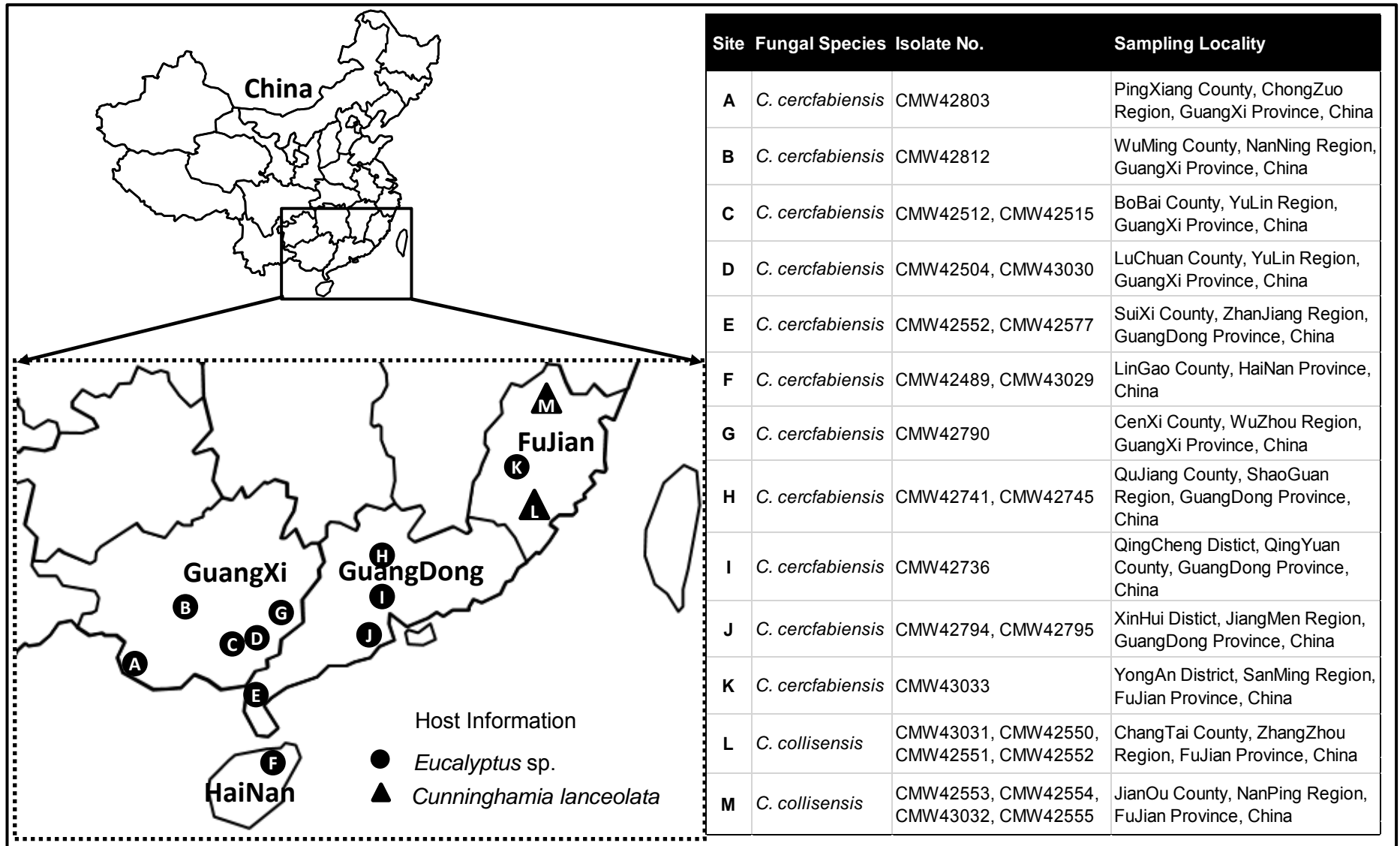


Fig. 1 Distribution of *Ceratocystis cercfabiensis* and *C. collisensis* in South China

hat-shaped, sheathed ascospores. Only flask-shaped conidiophore producing bacilliform, hyaline conidia were found and broader conidiophores producing barrel-shaped conidia appeared to be absent for isolates in this group. All isolates in this group were from *Eucalyptus* trees.

Eight isolates were collected from two *C. lanceolata* trees. These isolates were slower growing than those from *Eucalyptus* and had an irregular colony shape in culture. Limited numbers of ascomata were produced by these isolates. Ascomata were dark and globoid but with much shorter necks (~300 µm) than the *Eucalyptus* isolates and they produced hat-shaped, sheathed ascospores. Both bacilliform and barrel-shaped conidia were observed and these isolates from *C. lanceolata* also produced dark aleurioconidia in chains.

#### DNA extraction, PCR and sequencing

Twenty eight isolates were selected for DNA sequencing. These included all eight isolates from *C. lanceolata* and 20 from *Eucalyptus* species. Care was taken to select isolates representing each of the different sampling sites. PCR produced fragments of ~550 bp in length for the ITS, ~530 bp for the BT1 and ~700 bp for the TEF-1a gene regions.

The PCR amplification of the ITS gene region for some of the *Eucalyptus* isolates showed intragenomic variation in sequences. The sequence chromatograms of the ITS region for these isolates showed clear peaks up to ~140 bp and after that the base calling revealed conflicting sequence data (Fig. 2). Four strains (CMW43029, CMW42512, CMW42736 and CMW42741) that had conflicting ITS sequences were selected for cloning.





## Cloning

PCR amplification of the cloned ITS sequences produced fragment sizes of about 1000 bp and the sequence reactions for these amplicons resulted in clear, readable chromatograms. Twelve ITS haplotypes were obtained for the 17 sequenced clones derived from the four isolates studied. These haplotypes differed at up to seven base positions (Table 3, Fig. 2), and represented two separate phylogenetic groups when analysed using PAUP v. 4.0b10. This is typical of *Ceratocystis* species that have multiple ITS types (Al Adawi et al. 2013; Naidoo et al. 2013).

## Multi-gene phylogenetic analyses

All sequences obtained for the *Ceratocystis* isolates in this study were deposited in GenBank (Table 1). The BLAST search results using the NCBI nucleotide database showed that the *Ceratocystis* isolates from China resided in two distinct groups: one from *Eucalyptus*, the other from *C. lanceolata*. Isolates from *Eucalyptus* were most similar to *C. corymbiicola*, *C. polychroma* and *C. atrox*, and those from *C. lanceolata* most similar to *C. larium*, both residing in the Indo-Pacific clade defined for the genus.

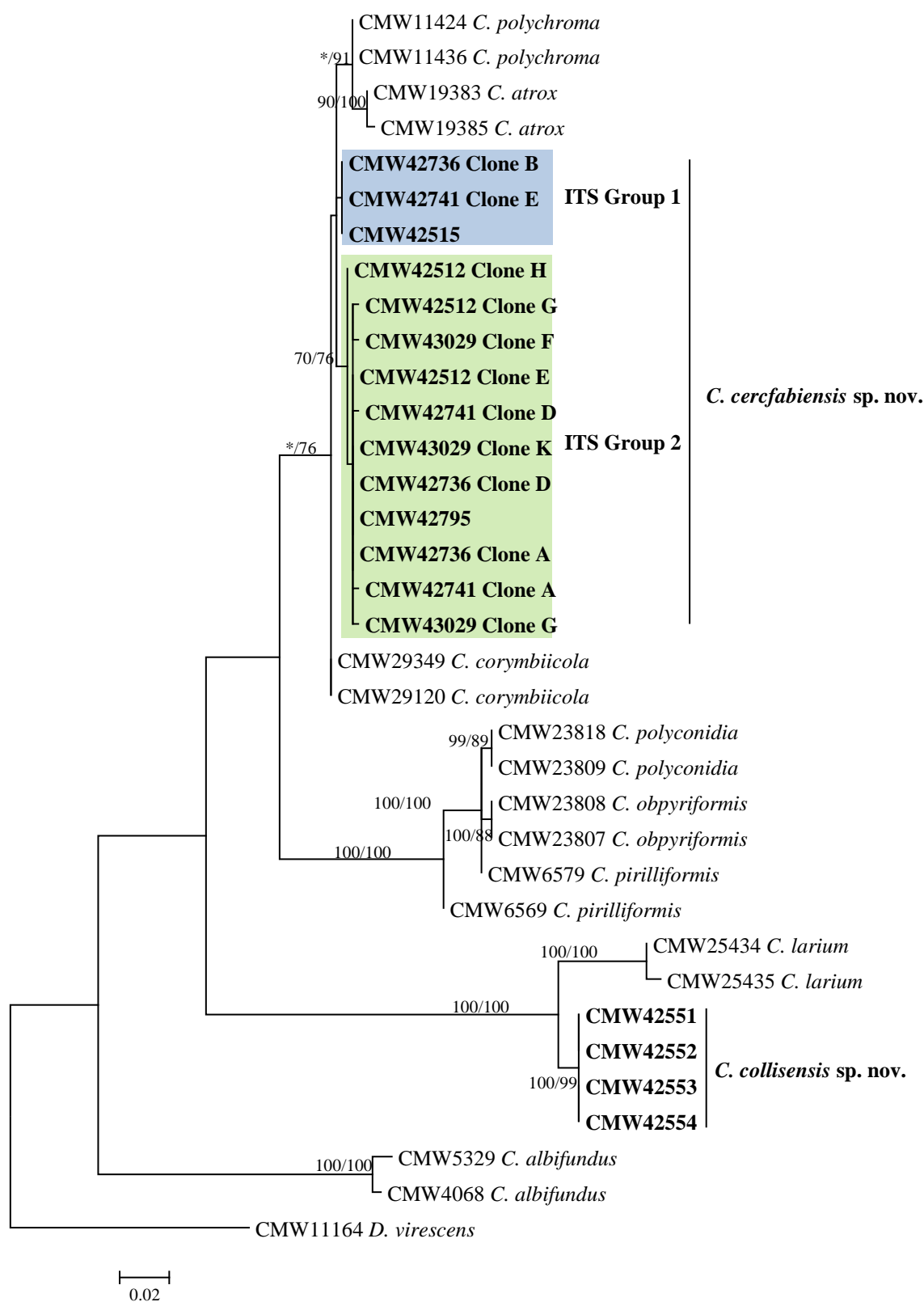
The partition homogeneity test (PHT) comparing the BT1 and TEF-1 $\alpha$  gene datasets gave a PHT value of  $P = 0.005$  showing that the data for these two gene regions could be combined for the phylogenetic analyses (Cunningham 1997). The aligned sequences for the ITS (35 taxa, 553 characters), BT1 (27 taxa, 534 characters), TEF-1 $\alpha$  (27 taxa, 701 characters), and the combined BT1 and TEF-1 $\alpha$  (27 taxa, 1235 characters) datasets were deposited in TreeBASE (No. S17259). Statistical values for the resultant phylogenetic trees for the maximum parsimony analyses and parameters for the best fit substitution models of maximum likelihood are provided in Table 2.

**Table 3** Nucleotide differences observed in the ITS region between the cloned sequences in four isolates of *Ceratocystis cercfabiensis*<sup>1</sup>

Isolate No.	Clone No.	Clone type	GenBank accession no.	ITS															
				28	41	64	86	148	155	167	226	302	310	325	396	448	491	527	
CMW42736	CMW42736_Clone A	1	KP727583	A	G	G	G	C	-	A	T	T	A	A	T	T	T	C	
	CMW42736_Clone E	1	NA	A	G	G	G	C	-	A	T	T	A	A	T	T	T	C	
	CMW42736_Clone B	2	KP727584	A	A	A	G	T	A	A	T	T	A	A	-	C	T	C	
	CMW42736_Clone D	3	KP727585	A	G	G	G	C	-	A	T	T	A	A	T	T	C	C	
CMW42741	CMW42741_Clone A	4	KP727586	A	G	G	G	C	-	G	T	T	A	A	T	T	T	C	
	CMW42741_Clone C	5	NA	A	A	A	G	T	A	A	T	T	A	A	-	C	T	-	
	CMW42741_Clone E	5	KP727588	A	A	A	G	T	A	A	T	T	A	A	-	C	T	-	
	CMW42741_Clone D	6	KP727587	A	G	G	G	C	-	A	T	T	A	G	T	T	T	C	
	CMW42741_Clone G	6	NA	A	G	G	G	C	-	A	T	T	A	G	T	T	T	C	
CMW42512	CMW42512_Clone C	1	NA	A	G	G	G	C	-	A	T	T	A	A	T	T	T	C	
	CMW42512_Clone D	1	NA	A	G	G	G	C	-	A	T	T	A	A	T	T	T	C	
	CMW42512_Clone E	7	KP727589	A	G	G	G	C	A	A	T	T	A	A	T	T	T	-	
	CMW42512_Clone G	8	KP727590	A	G	G	G	C	A	A	C	T	A	A	T	T	T	-	
	CMW42512_Clone H	9	KP727591	A	A	G	-	C	A	A	T	T	A	A	T	T	T	C	
CMW43029	CMW43029_Clone F	10	KP727592	A	G	G	G	C	A	A	T	C	A	A	T	T	T	-	
	CMW43029_Clone G	11	KP727593	A	G	G	G	C	-	A	T	T	G	A	T	T	T	C	
	CMW43029_Clone K	12	KP727594	G	G	G	G	C	-	A	T	T	A	A	T	T	T	C	
CMW42515	NA	5	NA	A	A	A	G	T	A	A	T	T	A	A	-	C	T	-	
CMW42795	NA	1	NA	A	G	G	G	C	-	A	T	T	A	A	T	T	T	C	

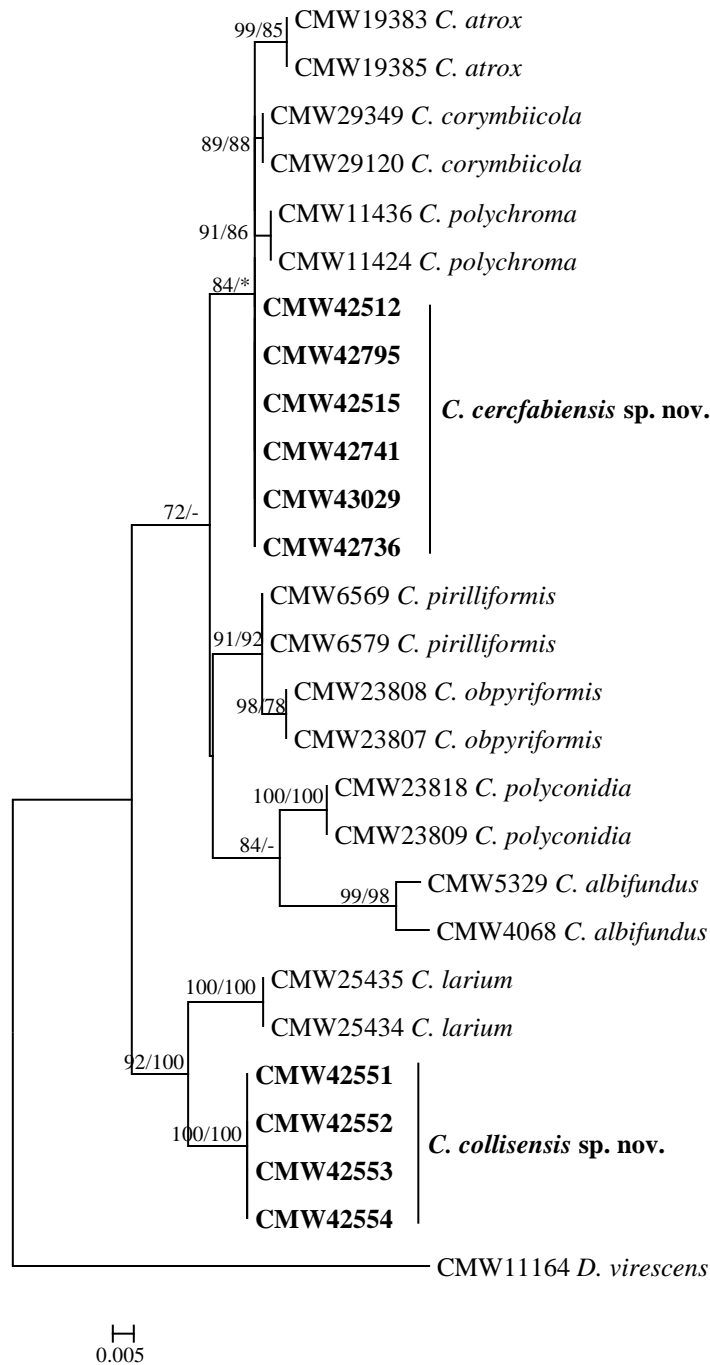
<sup>1</sup> Nucleotides that are different from the majority consensus sequence are highlighted in grey blocks

## ITS

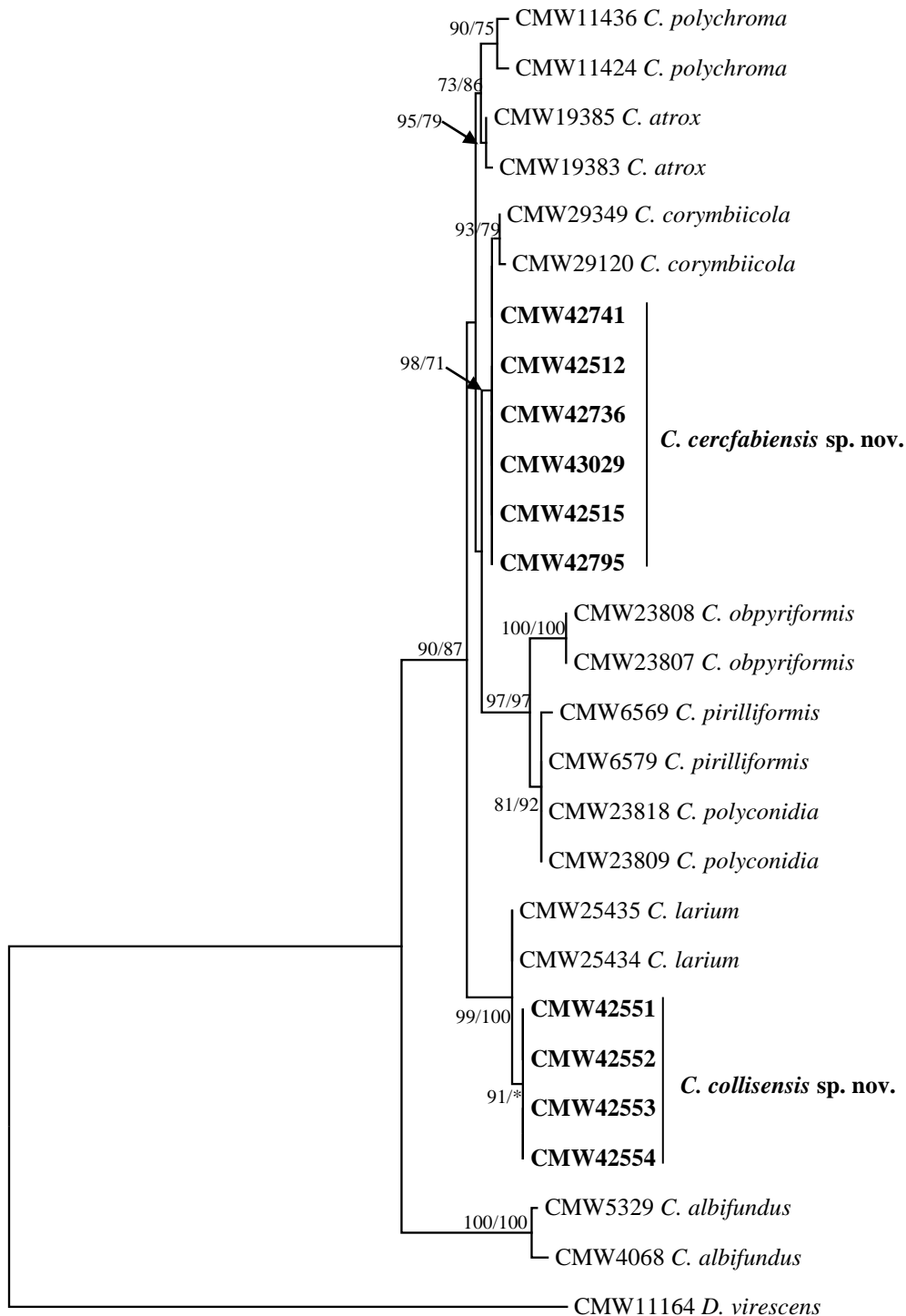


**Fig. 3** Phylogenetic tree of the ITS nuclear ribosomal DNA for *Ceratocystis* species in the Indo-Pacific clade including the new species *C. cercfabiensis* and *C. collisensis*. All cloned sequences obtained for *C. cercfabiensis* representing multiple ITS types (twelve in total) are also included and fall into two groups. Tree based on maximum likelihood (ML) analysis. Isolates in bold were sequenced in this study. Bootstrap values > 70 % for ML and maximum parsimony (MP) are presented above branches as follows: ML/MP, bootstrap values lower than 70 % are marked with \*. *Davidsoniella virescens* (CMW11164) represents the outgroup

## BT1



**Fig. 4** Phylogenetic tree based on maximum likelihood (ML) analysis of BT1 gene sequences for various *Ceratocystis* species in the Indo-Pacific clade. Isolates in bold were sequenced in this study. Bootstrap values > 70% for ML and maximum parsimony (MP) are presented above branches as follows: ML/MP, bootstrap values lower than 70% are marked with \*. *Davidsoniella virescens* (CMW11164) represents the outgroup

TEF-1 $\alpha$ 

**Fig. 5** Phylogenetic tree based on maximum likelihood (ML) analysis of TEF-1 $\alpha$  gene sequences for various *Ceratocystis* species in the Indo-Pacific clade. Isolates in bold were sequenced in this study. Bootstrap values > 70 % for ML and maximum parsimony (MP) are presented above branches as follows: ML/MP, bootstrap values lower than 70 % are marked with \*. *Davidsonsia virescens* (CMW11164) represents the outgroup



**Fig. 6** Phylogenetic tree based on maximum likelihood (ML) analysis of a combined dataset of BT1 and TEF-1 $\alpha$  gene sequences for various *Ceratocystis* species in the Indo-Pacific clade. Isolates in bold were sequenced in this study. Bootstrap values > 70% for ML and maximum parsimony (MP) are presented above branches as follows: ML/MP, bootstrap values lower than 70% are marked with \*. *Davidsoniella virescens* (CMW11164) represents the outgroup

Phylogenetic analyses of the ITS (Fig. 3), BT1 (Fig. 4), TEF-1 $\alpha$  (Fig. 5) and combined BT1 and TEF-1 $\alpha$  (Fig. 6) gene sequences for both the maximum likelihood (ML) and maximum parsimony (MP) analyses consistently showed that the isolates from China represent two previously unknown species. The position of the fungal species in each phylogenetic clade (species), in relation to each other, differed slightly, but the overall topologies were similar. Isolates obtained from *Eucalyptus* species in South China were phylogenetically closest to *C. corymbiicola*. It clearly showed that there were two sub-clades of the ITS type for the isolates in this clade (Fig. 3), but both were distinct from previously described *Ceratocystis* species and more similar to each other than to other species in the genus. Isolates collected from *C. lanceolata* were most closely related to *C. larium*. Only a single ITS type was found for the eight isolates of this species.

Isolates from *Eucalyptus* were phylogenetically most similar to *C. corymbiicola*, *C. polychroma* and *C. atrox* in the Indo-Pacific clade (Fig. 3, 4, 5, 6), however, they could be distinguished from these three groups using single nucleotide polymorphism (SNP) analyses for each of the three gene regions sequenced (Table 4, 5). Comparisons of these four groups showed that each group could be separated from the other three by 10–28 unique SNPs for all three gene regions (Table 5). Isolates from *Eucalyptus* differed from other three species (*C. corymbiicola*, *C. polychroma* and *C. atrox*) by 10, 16, 22 unique SNPs, respectively (Table 4, 5).

## Taxonomy

Based on morphological comparisons and multigene sequence phylogenies, the *Ceratocystis* isolates from *Eucalyptus* and *C. lanceolata* trees in South China represent two undescribed species residing





Species	Isolate number	<i>BTI</i>							
		39	163	191	246	251	254	261	485
<i>C. cercfabiensis</i>	CMW42736	T	A	C	C	T	C	T	C
	CMW42741	T	A	C	C	T	C	T	C
	CMW42512	T	A	C	C	T	C	T	C
	CMW43029	T	A	C	C	T	C	T	C
	CMW42515	T	A	C	C	T	C	T	C
	CMW42795	T	A	C	C	T	C	T	C
<i>C. corymbiicola</i>	CMW29120	T	A	C	C	G	-	T	C
	CMW29349	T	A	C	C	G	-	T	C
<i>C. atrox</i>	CMW19383	C	G	T	C	T	C	T	T
	CMW19385	C	G	T	C	T	C	T	T
<i>C. polychroma</i>	CMW11424	T	A	C	T	T	C	C	C
	CMW11436	T	A	C	T	T	C	C	C

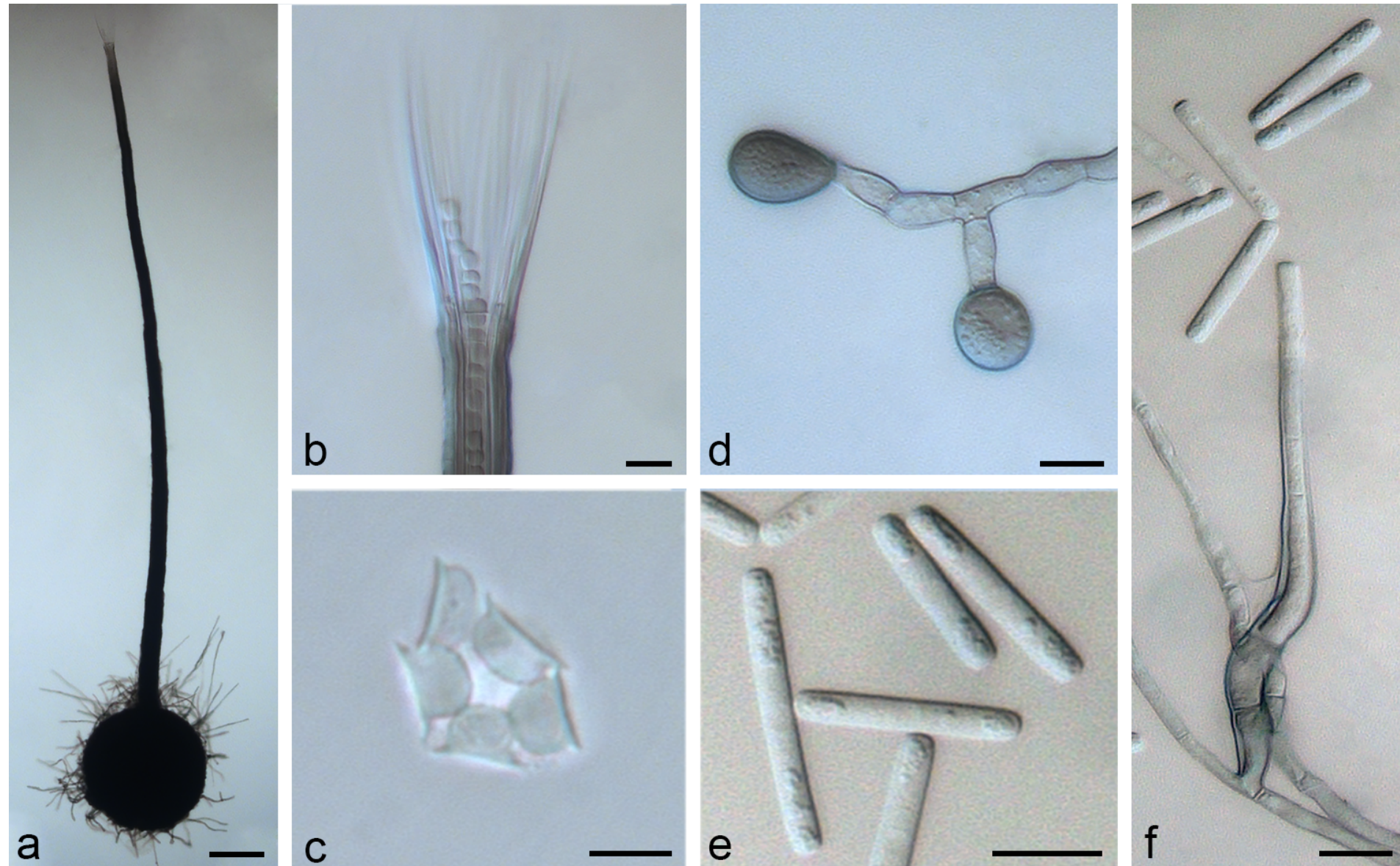
Species	Isolate number	<i>TEF</i>										
		14	135	136	137	325	361	362	508	636	646	670
<i>C. cercfabiensis</i>	CMW42736	T	-	-	T	G	C	C	G	A	T	C
	CMW42741	T	-	-	T	G	C	C	G	A	T	C
	CMW42512	T	-	-	T	G	C	C	G	A	T	C
	CMW43029	T	-	-	T	G	C	C	G	A	T	C
	CMW42515	T	-	-	T	G	C	C	G	A	T	C
	CMW42795	T	-	-	T	G	C	C	G	A	T	C
<i>C. corymbiicola</i>	CMW29120	T	-	-	-	G	C	A	G	A	T	C
	CMW29349	T	-	-	-	G	C	A	G	A	T	C
<i>C. atrox</i>	CMW19383	T	-	T	T	G	C	C	A	T	C	T
	CMW19385	T	-	T	T	G	C	C	A	T	C	T
<i>C. polychroma</i>	CMW11424	C	T	T	T	A	T	C	G	T	C	T
	CMW11436	C	T	T	T	A	T	C	G	T	C	T

<sup>1</sup> Only polymorphic nucleotides occurring in all of the isolates are shown. Fixed polymorphisms for each group (or fixed but shared between two groups) are highlighted

**Table 5** Number of unique alleles found in *Ceratocystis cercfabiensis*, *C. corymbiicola*, *C. atrox* and *C. polychroma*<sup>1</sup>

<i>ITS/BT1/TEF-1<math>\alpha</math></i>	<i>C. corymbiicola</i>	<i>C. atrox</i>	<i>C. polychroma</i>
<i>C. cercfabiensis</i>	10 (6/2/2)	22(13/4/5)	16 (6/2/8)
<i>C. corymbiicola</i>		28 (15/6/7)	22 (8/4/10)
<i>C. atrox</i>			25 (14/6/5)

<sup>1</sup> The order of the three genes: Total numbers (ITS/BT1/TEF-1 $\alpha$ )



**Fig. 7** Morphological characteristics of *Ceratocystis cercfabiensis*. a. Globose ascomata. b. Divergent ostiolar hyphae. c. Hat-shaped ascospores. d. Thick-walled chlamydospores. e. Bacilliform conidia. f. Flask-shaped conidiophores. Scale Bars: a = 100 μm, c = 5 μm, b, d, e, f = 10 μm

in the Indo-Pacific Clade, clearly separated from other *Ceratocystis* species. These novel species are described as follows:

*Ceratocystis cercfabiensis* F.F. Liu, Jol. Roux & S.F. Chen sp. nov. (Fig. 7)

MycoBank No. MB811888

*Etymology* the name “*cercfabiensis*” refers to the CERC-FABI Tree Protection Programme (CFTPP) that represents a co-operative research venture established between the China Eucalypt Research Centre (CERC), an Institute of the Chinese Academy of Forestry and the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa (<http://www.fabinet.up.ac.za>).

*Culture characteristics* Colonies on MEA greenish olivaceous (23’’’), reverse greenish olivaceous (23’’’). Mycelium immersed and superficial. *Hyphae* smooth, septate, without constriction at septa. Colony surfaces scattered with black ascomata. Optimal temperature for growth 25°C, covering the 90 mm plates after 14 days, no growth at 5°C or 35°C. After 14 days, colonies at 10°C, 15°C, 20°C, 25°C and 30°C reached 7 mm, 29 mm, 50 mm, 73 mm and 57 mm, respectively.

*Sexual state* *Ascomata* scattered, with bulbous bases and long necks formed superficially or partially submerged in the substrate. *Ascomatal bases* dark brown to black, globose, (100-) 137 – 218.8 (-302) µm long and (79-) 138 – 231 (-286) µm wide in diameter. Spines or ornamentations absent. *Ascomatal necks* brown to black, erect, slender, (473-) 829 – 1400 (-1756) µm long, (14-) 17 – 26 (-33) µm wide at apices, (22-) 30 – 43 (-55) µm wide at bases. *Ostiolar hyphae* present, hyaline, divergent, (32-) 48 – 70 (-82) µm long. *Asci* not observed. *Ascospores* hat-shaped, invested in sheaths, aseptate, (4.1-) 5.7 – 6.8 (-7.5) µm long and (2.5-) 3.1 – 3.9 (-4.6) µm wide with sheaths in

side view. Ascospores accumulating in buff yellow (19 d) mucilaginous masses at the apices of ascomatal necks.

*Asexual state* producing phialides, typical of *Thielaviopsis* with enteroblastic conidium ontogeny. *Conidiophores* of only one type, flask-shaped, hyaline at apices, becoming brown towards bases, multi-septate, phialidic, tubular, tapering at apices (42-) 60 – 145 (-291)  $\mu\text{m}$  long, (2.7-) 3.9 – 5.5 (-7.4)  $\mu\text{m}$  wide at apices and (3.2-) 4 – 6 (-7.7)  $\mu\text{m}$  wide at bases. *Conidia* hyaline, aseptate, bacilliform to dumbbell shaped, (8.8-) 16.2 – 25.6 (-49.9)  $\mu\text{m}$  long and (2.7-) 3.4 – 4.6 (-2.7)  $\mu\text{m}$  wide. *Chlamydospores* ovoid, smooth, formed singly, terminal, hyaline when young, becoming dark brown when mature, (9.9-) 12.1 – 15.0 (-16.7)  $\times$  (7.0-) 9.2 – 11.5 (-13.0)  $\mu\text{m}$  in size.

*Habitat* stumps of recently felled (less than one month) *Eucalyptus* trees in China.

*Known distribution* GuangDong, GuangXi, FuJian and HaiNan Provinces, China.

*Specimens examined* China, HaiNan Province, LinGao County, *Eucalyptus* plantation. Isolated from recently harvested tree stumps, September 2013, S.F. Chen, F.F. Liu & T. Huang, HOLOTYPE PREM 61229, culture ex-type CMW43029 = CERC2170 = CBS 139654.

*Additional specimens* China, GuangXi Province, YuLin Region, BoBai County, *Eucalyptus* plantation. Isolated from recently harvested tree stumps, October 2013, S.F. Chen, F.F. Liu & G.Q. Li, PARATYPE PREM 61230, culture ex-type CMW42515 = CERC2345 = CBS 139655; China, GuangDong Province, JiangMen Region, XinHui District, *Eucalyptus* plantation. Isolated from recently harvested tree stumps, January 2014, S.F. Chen, F.F. Liu & G.Q. Li, PARATYPE PREM 61231, culture ex-type CMW42795 = CERC2687 = CBS 139656.

**Table 6** Morphological comparisons of *C. cercfabiensis* and other phylogenetically closely related species<sup>1</sup>

	<i>C. cercfabiensis</i>	<i>C. corymbicola</i>	<i>C. polychroma</i>	<i>C. atrox</i>
Ascomata base	(100-) 137 – 218.8 (-302) × (79-) 138 – 231 (-286) <sup>2</sup>	(159-) 189 – 241 (-290) × (160.5-) 185.0 – 237.5 (-272.5)	(208-) 217 – 261 (-269) diam	(120-) 140 – 180 (-222) diam
Ascomata base Average	177.9 × 184.5 <sup>3</sup>	215.0 × 211.0	239.0 × 239.0	160.0 × 160.0
Ascomata neck	(473-) 829 – 1400 (-1756)	(603.0-) 755.0 – 1009.0 (-1097.5)	(837-) 849 – 1071 (-1187)	(277-) 313 – 401 (-451)
Ascomata neck Average	1114.5	882.0	960.0	357.0
Ascospores	(4.1-) 5.7 – 6.8 (-7.5) × (2.5-) 3.1 – 3.9 (-4.6)	(4.5-) 5.0 – 5.5 (-6.0) × (2.5-) 3.0 – 3.5 (-4.0)	5–7 × 3–4	4–6 × 3–4
Ascospores Average	6.3 × 3.5	5.3 × 3.3	6.0 × 3.5	5.0 × 3.5
Bacilliform conidia	(8.8-) 16.2 – 25.6 (-49.9) × (2.7-) 3.4 – 4.6 (-2.7)	(11.0-) 15.0 – 21.5 (-27.5) × (3.0-) 3.5 – 4.5 (-5.5)	(13-) 16 – 24 (-26) × 3–5	(9-) 11 – 15 (-17)×3–5
Bacilliform conidia Average	20.9 × 4.0	18.3 × 4.0	20.0 × 4.0	13.0 × 4.0
Barrel-shaped conidia	not present	(7.5-) 8.5 – 12.0 (-14.5) × (3.5-) 4.0 – 5.5 (-6.5)	9–11 × 6–8	(7-) 8 – 12 (-14) × (5-) 6 – 8 (-9)
Barrel-shaped conidia Average		10.3 × 4.8	10.0 × 7.0	10.0 × 7.0
Chlamydospore	(9.9-) 12.1 – 15.0 (-16.7) × (7.0-) 9.2 – 11.5 (-13.0)	(8.5-) 11.0 – 12.0 (-16.5) × (6.5-) 8.0 – 11.0 (-16.5)	11–14 × 8–14	not present
Chlamydospore Average	13.6 × 10.4	11.5 × 9.5	12.5 × 11.0	

<sup>1</sup> All measurements are in  $\mu\text{m}$

<sup>2</sup> Measurements are presented in the format [(minimum-) (average – standard deviation) – (average + standard deviation) (-maximum)]

<sup>3</sup> Measurements are presented in the format minimum × maximum

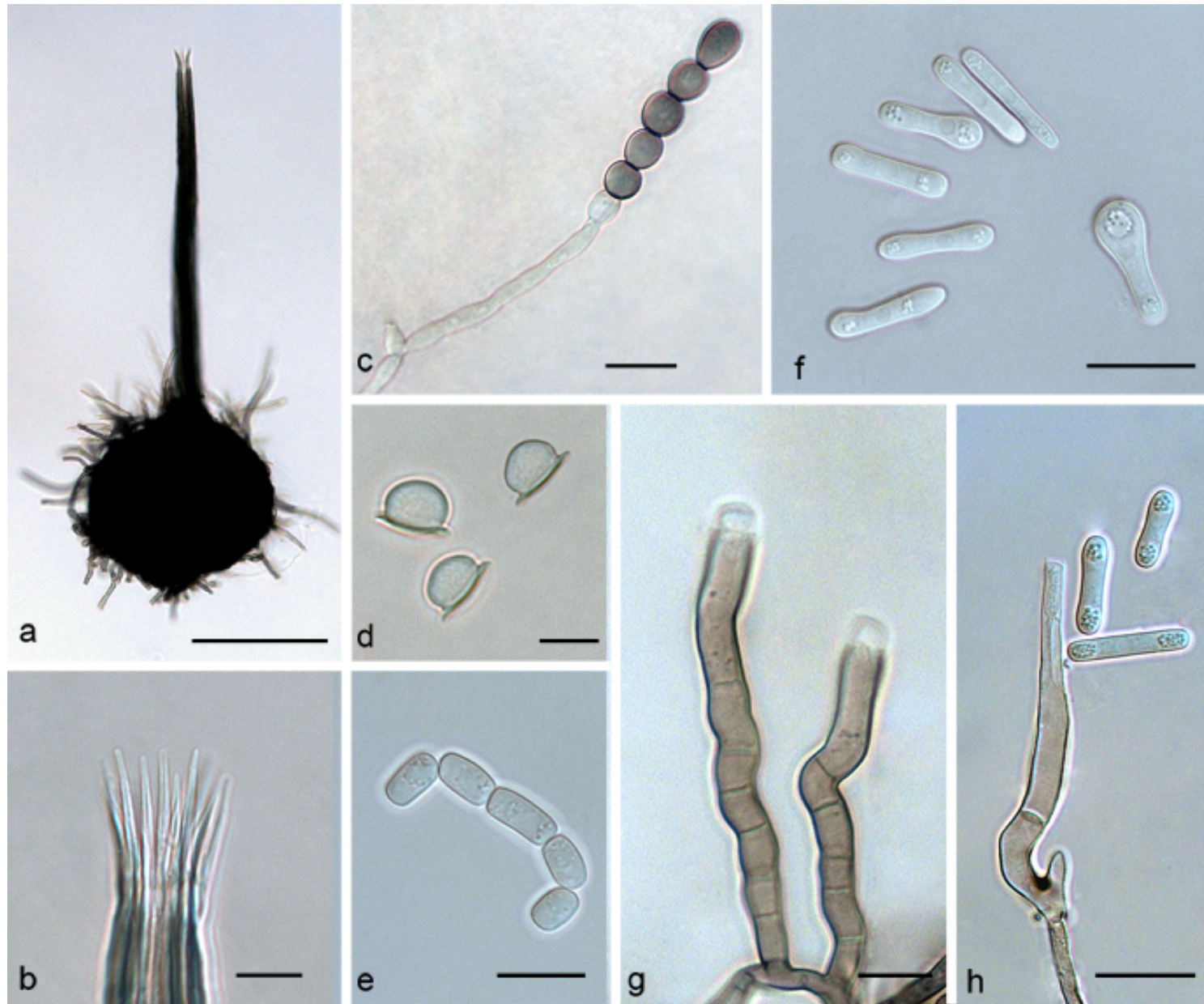
*Notes* *Ceratocystis cercfabiensis* is phylogenetically most closely related to *C. corymbiicola* (Nkuekam et al. 2012), *C. polychroma* (Van Wyk et al. 2004) and *C. atrox* (Van Wyk et al. 2007b). It can be distinguished from these species by the size of their ascomatal bases, necks and ascospores (Table 6). When grown on 2 % MEA, the ascomatal bases of *C. cercfabiensis* (average 178×184 µm) are smaller than those of *C. corymbiicola* (average 215×211 µm) and *C. polychroma* (average 239×239 µm), but larger than those of *C. atrox* (average 160×160 µm). Ascomatal necks of *C. cercfabiensis* (average 1115 µm) are much longer than those of *C. corymbiicola* (average 882 µm), *C. polychroma* (average 960 µm) and *C. atrox* (average 357 µm). Ascospores of *C. cercfabiensis* (average 6.3×3.5 µm) are also larger than those of *C. corymbiicola* (average 5.3×3.3 µm), *C. polychroma* (average 6.0×3.5 µm) and *C. atrox* (average 5×3.5 µm).

*Ceratocystis collisensis* F.F. Liu, M.J. Wingf. & S.F. Chen sp. nov. (Fig. 8)

MycoBank No. MB811889

*Etymology* the name “collis” is derived from that Latin word “mountain”, reflecting the fact that the samples were collected from the WuYi Mountains in China.

*Culture characteristics* Colonies on MEA olivaceous (21’’k), reverse olivaceous (21’’k). Mycelium immersed and superficial, with white-grey mycelia at the edge. *Hyphae* smooth, septate, without constriction at septa. Colony surfaces with scattered black ascomata. Optimal temperature for growth 25°C, slow growing, no growth at 5°C or 35°C. After 14 days, colonies at 10°C, 15°C, 20°C, 25°C and 30°C reached 10 mm, 30 mm, 42 mm, 50 mm and 41 mm, respectively.



**Fig. 8** Morphological characteristics of *Ceratocystis collisensis*. a. Ascomata with globose to obpyriform bases. b. Divergent ostiolar hyphae. c. Chain of aleurioconidia. d. Hat-shaped ascospores in side view. e. Barrel-shaped conidia in a chain. f. Various shapes of bacilliform conidia. g. Broader conidiophores with emerging barrel-shaped conidia. h. Flask-shaped conidiophores. Scale Bars: a = 100  $\mu\text{m}$ , b, c, e, f, g, h = 10  $\mu\text{m}$ , d = 5  $\mu\text{m}$



*Sexual state* *Ascomatal bases* black, globose to obpyriform, (152-) 174 – 253 (-304)  $\mu\text{m}$  long and (134-) 164 – 224 (-255)  $\mu\text{m}$  wide in diameter. Spines or ornamentations absent. *Ascomatal necks* dark brown to black, erect, slender, (208-) 301 – 423 (-527)  $\mu\text{m}$  long, (12-) 15 – 20 (-25)  $\mu\text{m}$  wide at apices and (18-) 25 – 33 (-39)  $\mu\text{m}$  wide at bases. *Ostiolar hyphae* present, hyaline, divergent, (6-) 16 – 26 (-31)  $\mu\text{m}$  long. *Asci* not observed. *Ascospores* hat-shaped, invested in sheaths, aseptate, (5.6-) 6.5 – 7.8 (-8.4)  $\mu\text{m}$  long and (2.7-) 3.6 – 4.6 (-5.3)  $\mu\text{m}$  wide with sheaths in side view. Ascospores accumulating in buff yellow (19 d) mucilaginous masses at the apices of ascomatal necks.

*Asexual state* phialidic, typical of *Thielaviopsis* with enteroblastic conidium ontogeny. *Conidiophores* of two types, flask shaped producing bacilliform conidia, hyaline at apices, turning brown towards bases, multi-septate, lageniform, tubular, variable in size when terminal on hyphae, tapering at apices, (46-) 47 – 337 (-129)  $\mu\text{m}$  long, (2.3-) 3.6 – 4.9 (-6.2)  $\mu\text{m}$  wide at apices and (2.3-) 4.2 – 6.2 (-7.7)  $\mu\text{m}$  wide at bases or untapered and broader producing barrel-shaped conidia, borne near the bases of ascomata, light brown, flaring, (15-) 34 – 79 (-121)  $\mu\text{m}$  long, (3.3-) 4.5 – 7 (-9)  $\mu\text{m}$  wide at apices and (2.8-) 3.8 – 5 (-6.4)  $\mu\text{m}$  wide at bases. *Bacilliform conidia* hyaline, aseptate, cylindrical to dumbbell-shaped, (10.7-) 14.5 – 21.3 (-32.3)  $\mu\text{m}$  long and (2.9-) 3.4 – 4.4 (-5.6)  $\mu\text{m}$  wide. *Barrel-shaped conidia* hyaline, aseptate, in chains, (5.4-) 6.1 – 8.7 (-10.9)  $\mu\text{m}$  long and (3.7-) 4.4 – 6.3 (-7.9)  $\mu\text{m}$  wide. *Aleuriiconidia* ovoid, smooth, dark brown, embedded in agar, produced in chains, (9.2-) 11.3 – 14.4 (-17.6)  $\times$  (7.2-) 8.8 – 11.1 (-13.7)  $\mu\text{m}$  in size.

*Habitat* stumps of recently felled (less than one month) *Cunninghamia lanceolata* trees in China.

*Known distribution* FuJian Province, China.

*Specimen examined* China, FuJian Province, ZhangZhou Region, ChangTai County, *Cunninghamia lanceolata* plantation. Isolated from recently harvested tree stumps, October 2013, S.F. Chen, F.F. Liu & G.Q. Li, HOLOTYPE PREM 61232, culture ex-type CMW42552 = CERC2459 = CBS 139679.

*Additional specimens* China, FuJian Province, NanPing Region. JianOu County, *Cunninghamia lanceolata* plantation. Isolated from recently harvested tree stumps, November 2013, S.F. Chen, F.F. Liu & G.Q. Li, PARATYPE PREM 61233, culture ex-type CMW42553 = CERC2465 = CBS 139646; China, FuJian Province, NanPing Region. JianOu County, *Cunninghamia lanceolata* plantation. Isolated from recently harvested tree stumps, November 2013, S.F. Chen, F.F. Liu & G.Q. Li, PARATYPE PREM 61234, culture ex-type CMW42554 = CERC2466 = CBS 139647.

*Notes* *Ceratocystis collisensis* is phylogenetically most closely related to *C. larium*. It can be distinguished from this species by the size of its ascomatal bases, necks, ascospores and aleurioconidia. Ascomatal bases of *C. collisensis* (average 214×194 µm) are larger than those of *C. larium* (average 152×170 µm), but ascomatal necks (average 362 µm) are shorter than those of *C. larium* (average 460 µm). Ascospores of *C. collisensis* (average 7.2×4.1 µm) are longer and wider than those of *C. larium* (average 5.5×3.0 µm). Aleurioconidia of *C. collisensis* (average 12.9×10.0 µm) are larger than those of *C. larium* (average 11×9 µm) (Van Wyk et al. 2009).

## Discussion

This study provides descriptions for two previously unknown *Ceratocystis* species, *C. cercfabiensis* and *C. collisensis*, from China. It also represents the first record of *Ceratocystis* species from China that reside in the Indo-Pacific biogeographic group of this genus (Mbenoun et al. 2014). There are only six previous reports of *Ceratocystis* species from China and all species reside in the South American Clade. These species include *C. manginecans* from recently harvested stumps of *Eucalyptus* in GuangDong Province (Chen et al. 2013; Fourie et al. 2015), and *C. fimbriata* s.l., causing a disease on *Eucalyptus* (Li et al. 2014b), *Ipomoea batatas* (sweet potato; Sy 1956), *Punica granatum* (pomegranate; Huang et al. 2003; Xu et al. 2011), *Colocasia esculenta* (taro; Huang et al. 2008) and *Eriobotrya japonica* (loquat; Li et al. 2014a). Considering the number of *Ceratocystis* species found in other countries, and the small number of studies on species of *Ceratocystis* and other Ceratocystidaceae found in China, the discovery of *C. cercfabiensis* and *C. collisensis* suggests that many other species in the genus and family remain to be discovered in this geographic area.

*Ceratocystis cercfabiensis* was found at all *Eucalyptus* sites sampled and in four Provinces of China. This suggests that it has a wide geographic distribution in the region. In contrast, *C. collisensis* was obtained only from *C. lanceolata*, at two sites in the FuJian Province. This is the first report of a *Ceratocystis* species from a *Cunninghamia* sp. A very limited number of *C. lanceolata* trees were sampled in this study and surveys of *C. lanceolata* and related species in China should yield additional isolates of this fungus and make it possible to gain knowledge of its relative importance, especially given that this is the first *Ceratocystis* sp. to be found on a conifer subsequent to the taxonomic revision of de Beer et al. (2014). All previous reports of *Ceratocystis* species from conifers represent species of *Huntia* and *Endoconidiophora* (De Beer et al. 2014).

*Ceratocystis cercfabiensis* is phylogenetically most closely related to *C. corymbiicola* (Nkuekam et al. 2012), *C. polychroma* (Van Wyk et al. 2004) and *C. atrox* (Van Wyk et al. 2007b). All three of these species have been reported from *Syzygium* and *Eucalyptus* (Myrtaceae). *Ceratocystis corymbiicola* and *C. atrox* were reported from *Eucalyptus* trees in Australia (Nkuekam et al. 2012; Van Wyk et al. 2007b) and *C. polychroma* from dying *S. aromaticum* (clove) trees in Indonesia (Van Wyk et al. 2004). However, comparisons of sequence data showed that *C. cercfabiensis* isolates represent a distinct clade in this group. This new species could also be distinguished from these three *Ceratocystis* species based on the fact that it has distinctly longer ascomatal necks, larger ascospores and that dark barrel-shaped aleurioconidia found in some species of *Ceratocystis* appear not to be present.

*Ceratocystis collisensis* is most closely related to *C. larium*. The latter species was described from *Styrax benzoin* trees being tapped for their aromatic resin in Indonesia (Van Wyk et al. 2009). These two species form a distinct sub-clade in the Indo-Pacific Clade that has been recognised in *Ceratocystis* (De Beer et al. 2014; Mbenoun et al. 2014), but they are distinct from each other based on sequence data as well as morphology.

The delimitation of species in *Ceratocystis* has been strongly reliant on sequence data from the ITS region, which has also been identified as the universal barcoding region for fungi (Schoch et al. 2012). However, in this study, we used cloning to provide direct evidence for the occurrence of multiple ITS types in *C. cercfabiensis*. Our results revealed that more than one ITS haplotype can occur in a single isolate, which has also been reported previously for *Ceratocystis* species in the South American Clade (Naidoo et al. 2013; Harrington et al. 2014; Fourie et al. 2015). The ITS region should, thus, be used with caution for species delineation in *Ceratocystis*. Although multiple ITS types were found in *C. cercfabiensis*, both groups represented clades distinct from other

*Ceratocystis* species, thus supporting our BT1 and TEF-1 $\alpha$  data showing that *C. cercfabiensis* represents a novel species.

Neither *C. cercfabiensis* nor *C. collisensis* were associated with disease or death of trees, but were obtained from fresh harvesting wounds. The recent description of a disease of *Eucalyptus* in China, caused by *C. fimbriata* s.l. (Li et al. 2014b), and reports of disease and death of *Eucalyptus* species caused by species of *Ceratocystis* in Africa (Roux et al. 1999, 2000, 2004) and South America (Barnes et al. 2003; Rodas et al. 2008; Van Wyk et al. 2012), however, suggest that these fungi could become important to *Eucalyptus* forestry in China. They clearly deserve further study in the future.

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