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Taxonomy and pathogenicity of *Ceratocystis* species on *Eucalyptus* trees in South China, including *C. chinaeucensis* sp. nov.

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Abstract Commercial plantations of *Eucalyptus* species have been established in South China, especially during the past 20 years, to meet the needs of a rapidly growing national economy. As part of a survey of fungal diseases affecting *Eucalyptus* species in South China, *Ceratocystis* species were collected from *Eucalyptus* plantations in the GuangDong Province. The aims of this study were to identify these *Ceratocystis* isolates and to test their pathogenicity to *Eucalyptus*. The most aggressive isolates were also used to screen different species and clones of *Eucalyptus* for susceptibility to infection under field conditions. The fungi were identified based on morphology and through comparisons of DNA sequence data of the ITS, partial β-tubulin and TEF-1α gene regions. Morphological and DNA sequence comparisons showed that isolates collected from Chinese *Eucalyptus* plantations represent two species, *C. acaciivora* in the *C. fimbriata s.l.* species complex and a previously undescribed species belonging to the *C. moniliformis s.l.* species complex, for which the name *C. chinaeucensis* sp. nov. is provided. In pathogenicity trials, both *C. acaciivora* and *C. chinaeucensis* gave rise to lesions on wounded *Eucalyptus* trees, and the former fungus was most pathogenic. Differences were also observed in the responses of different *Eucalyptus* clones to inoculation and this could be useful in reducing disease, if *C. acaciivora* should emerge as a pathogen in the future.

Keywords Ceratocystidaceae · Fungal pathogens · Microascales · Myrtales · Plantation forestry · Wounds

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Introduction

Plantation forestry using species of *Eucalyptus* is of growing importance to China with more than three and a half million hectares of plantations already established, especially in the provinces of FuJian, GuangDong, GuangXi, HaiNan and YunNan in South China (Xie 2011). *Eucalyptus* plantations have been established for various purposes in China, but especially to produce pulp to meet the needs of the rapidly growing national economy (Xie 2011). *Eucalyptus* trees are distributed in more than 600 counties in 19 Provinces/Regions throughout China, especially in the southern regions of the country (Qi 2002; Chen 2011). Around 300 *Eucalyptus* species were introduced and planted in China, of which more than ten economically important species are widely planted (Qi 2002, 2006; Qi et al. 2006). Among these species, *E. camaldulensis*, *E. dunnii*, *E. globulus*, *E. grandis*, *E. maidenii*,

E. saligna, *E. tereticornis*, *E. urophylla* and their hybrids/clones are most popular (Qi 2002, 2006; Qi et al. 2006; Xiang et al. 2006; Xie 2011). Similar to the situation in other countries, these plantations are threatened by diseases and pests (Dell et al. 2008; Wingfield et al. 2008; Zhou et al. 2008; Zhou and Wingfield 2011).

Despite the growing importance of *Eucalyptus* plantations in China during the course of the past two decades, information regarding pests and diseases affecting these trees is limited, with most previous research focused on bacterial wilt (Ran et al. 2005). Several surveys during the last five years have, however, identified a number of fungal pathogens on *Eucalyptus* trees in South China (Zhou et al. 2007; Chen et al. 2010, 2011a, b, c). Efforts to address the lack of detailed information on diseases affecting these trees in China are also increasing.

Diseases of plantation-grown hardwood tree species caused by *Ceratocystis* species have increased in number in the last two decades (Roux and Wingfield 2009). These fungi can result in stem cankers, rapid wilt and death of affected *Eucalyptus* trees, or in wood stain (Kile et al. 1996; Roux et al. 1999; Barnes et al. 2003b; Roux and Wingfield 2009). On *Eucalyptus* trees, species of *Ceratocystis* have been reported from Africa (Roux et al. 1999, 2001, 2004, 2005; Heath et al. 2009; Van Wyk et al. 2012), Asia (Van Wyk et al. 2012), Australia (Kile et al. 1996; Yuan and Mohammed 2002; Barnes et al. 2003b; Van Wyk et al. 2007; Nkuekam et al. 2012) and South America (Barnes et al. 2003a; Rodas et al. 2008; Van Wyk et al. 2009, 2011, 2012). In Asia, the only report of *Ceratocystis* species from *Eucalyptus* is of isolates collected from tree wounds in Indonesia and Thailand (Van Wyk et al. 2012). These isolates reside in the *C. fimbriata* Ellis & Halst. *sensu. lato* (*s.l.*) species complex and are most similar to a recently described species, *C. eucalypticola* M. van Wyk & M.J. Wingf. from *Eucalyptus* in South Africa (Van Wyk et al. 2012).

Ceratocystis spp. are known to require wounds for infection (Kile 1993). Inspection of stumps in recently harvested plantations as well as artificially made wounds on tree stems have thus been used to survey plantation areas for the presence of these fungi (Barnes et al. 2003b; Roux et al. 2004; Heath et al. 2009; Van Wyk et al. 2012). The aim of this study was to identify *Ceratocystis* isolates from fresh stumps in an *Eucalyptus* plantation in the GuangDong Province of China and to establish their pathogenicity by inoculation tests both in the glasshouse and field. Material was collected from both wounded trees and the surfaces of stumps of recently harvested trees, and fungi were identified using comparisons of DNA sequence data combined with morphological characteristics. The pathogenicity of the collected isolates was also tested on different species and clones of *Eucalyptus* planted in south China.

Materials and methods

Fungal isolates

Sampling was conducted in an *Eucalyptus* plantation on the QianJin Forestry Farm, ZhanJiang, GuangDong Province, in December 2006. The plantation consisted of recently harvested *Eucalyptus urophylla* × *E. grandis* trees and samples were collected from five two-week-old stumps after examining the bark and wood for the presence of *Ceratocystis* ascocarps. Where ascocarps were present, sections of bark and wood were collected, placed in individual paper bags and transported to the laboratory for further study.

Samples were incubated in Petri dishes containing moist paper at 25 °C and inspected regularly for the presence of *Ceratocystis* ascocarps. Ascospore masses from the apices of ascomata on the incubated material were transferred to 2 % malt extract agar plates (MEA: 20 g malt extract, 20 g agar; Biolab Diagnostic Ltd., Midrand, South Africa; 1 L water), containing 100 mg Streptomycin sulfate (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) and incubated at 25 °C. All the resultant cultures were purified using single hyphal tip isolations. Representative cultures were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1), and a complete set of duplicates at the China Eucalypt Research Centre (CERC), Chinese Academy of Forestry (CAF), China. Representative isolates were also deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands and dried specimens with the National Collection of Fungi (PREM), Pretoria, South Africa.

Culture morphology and characteristics

Culture morphology was assessed after three to 9 days of growth on 2 % MEA amended with Streptomycin sulfate (100 mg/L). All the fungal isolates collected in this study were grouped according to their culture morphology and representatives from each group were selected for DNA sequence analyses.

To study the morphology of isolates, perithecia were induced to form by growing isolates on 2 % MEA mixed with autoclaved pine branch chips at 25 °C for some days. Fruiting structures were mounted in lactic acid on glass slides and 50 measurements of each morphological structure were made for the isolate chosen to represent the type of the unknown species. Twenty more measurements of related structures were made from two additional isolates. Average (mean), standard deviation (std.), minimum (min), and maximum (max) measurements were made and are presented as (min−) (average − std. dev.) − (average + std. dev.) (−max) in the descriptions of species.

Table 1 *Ceratocystis* spp. for which isolates or sequences were used in this study

Species	CMW No.	Other no.	GenBank[d]			Hosts	Collectors	Origin
			ITS	β-tubulin	TEF-1a			
Ceratocystis moniliformis s.l. species complex								
C. bhutanensis	CMW8242	CBS112907	AY528951	AY528956	AY528961	*Picea spinulosa*	T. Kirisits & D.B. Chhetri	Jelekha, Bhutan
"	CMW8217	CBS114289	AY528952	AY528957	AY528962	"	"	"
C. chinaeucensis	[a]CMW24653		**JQ862725**	**JQ862713**	**JQ862737**	*E. urophylla*×*E. grandis* clone	M.J. Wingfield, X.D. Zhou & S.F. Chen	GuangDong, China
"	[ab]CMW24655		**JQ862727**	**JQ862715**	**JQ862739**	"	"	"
"	[abc]CMW24658	CBS127185	**JQ862729**	**JQ862717**	**JQ862741**	"	"	"
"	[a]CMW24659		**JQ862730**	**JQ862718**	**JQ862742**	"	"	"
"	[abc]CMW24661	CBS127186	**JQ862731**	**JQ862719**	**JQ862743**	"	"	"
C. inquinana	CMW21106		EU588587	EU588666	EU588674	*Acacia mangium*	M. Tarigan	Indonesia
"	CMW21107	CBS124009	EU588588	EU588667	EU588675	"	"	"
C. microbasis	CMW21115	CBS124015	EU588592	EU588671	EU588679	"	"	"
"	CMW21117	CBS124013	EU588593	EU588672	EU588680	"	"	"
C. moniliformis	CMW4114		AY528986	AY528997	AY529007	*Schizolobium parahybum*	M.J. Wingfield	Ecuador
"	CMW8240		AY528989	AY529000	AY529010	*Cassia fistula*	M.J. Wingfield & D.B. Chhetri	Punakha, Bhutan
"	CMW8379		AY528995	AY529005	AY529016	"	M.J. Wingfield, T. Kirisits & D.B. Chhetri	"
"	CMW9590	CBS116452	AY528985	AY528996	AY529006	*Eucalyptus grandis*	J. Roux	Mpumalanga, South Africa
"	CMW9990	CBS155.62	FJ151423	FJ151457	FJ151479	*Theobroma cacao*	A.J. Hansen	Costa Rica
"	CMW10134	CBS118127	FJ151424	FJ151456	FJ151478	*E. grandis*	M. Van Wyk,	South Africa
C. moniliformopsis	CMW9986	CBS109441	AY528998	AY528987	AY529008	*Eucalyptus obliqua*	Z.Q. Yuan	Australia
"	CMW10214	CBS115792	AY528999	AY528988	AY529009	*Eucalyptus sieberi*	M.J. Dudzinski	"
C. oblonga	CMW23802		EU245020	EU244992	EU244952	*Acacia mearnsii*	R.N. Heath	South Africa
"	CMW23803		EU245019	EU244991	EU244951	"	"	"
"	CMW23804		EU245021	EU244993	EU244953	"	"	"
C. omanensis	CMW11048	CBS115780	DQ074742	DQ074732	DQ074737	*Mangifera indica*	A.O. Al-Adawi	Oman
"	CMW3800		DQ074743	DQ074733	DQ074738	"	"	"
C. savannae	CMW17300		EF408551	EF408565	EF408572	*Acacia nigrescens*	G. Kamgan & J. Roux	South Africa
"	CMW17298		EF408553	EF408567	EF408574	*Terminalia sericea*	"	"
"	CMW17297		EF408552	EF408566	EF408573	*Combretum zeyheri*	"	"
C. sumatrana	CMW21109	CBS124011	EU588589	EU588668	EU588676	*A. mangium*	M. Tarigan	Indonesia
"	CMW21111	CBS124012	EU588590	EU588669	EU588677	"	"	"
C. tribiliformis	CMW13011		AY528991	AY529001	AY529012	*Pinus merkusii*	M.J. Wingfield	Sumatra, Indonesia
"	CMW13012		AY528992	AY529002	AY529013	"	"	"
C. virescens	CMW 11164		DQ520639	EF070441	EF070413	*Fagus americanum*	D. Houston	[e]N/A

Table 1 (continued)

Species	CMW No.	Other no.	GenBank[d]			Hosts	Collectors	Origin
			ITS	β-tubulin	TEF-1a			
"	CMW3276		AY528984	AY528990	AY528991	*Quercus* sp.	T. Hinds	USA
Thielaviopsis ceramica	CMW15245		EU245022	EU244994	EU244926	*Eucalyptus* sp.	R.N. Heath & J. Roux	Malawi
"	CMW15246		EU245023	EU244995	EU244927	"	"	"
"	CMW15248		EU245024	EU244996	EU244928	"	"	"
C. fimbriata s.l. species complex								
C. acaciivora	CMW22562		EU588655	EU588635	EU588645	*Acacia mangium*	M. Tarigan	Indonesia
"	CMW22563		EU588656	EU588636	EU588646	"	"	"
"	CMW22564		EU588657	EU588637	EU588647	"	"	"
"	CMW22595		EU588660	EU588639	EU588649	"	"	"
"	[ab]CMW24664		**JQ862732**	**JQ862720**	**JQ862744**	*E. urophylla*×*E. grandis* clone	M.J. Wingfield, X.D. Zhou & S.F. Chen	GuangDong, China
"	[abc]CMW24667		**JQ862733**	**JQ862721**	**JQ862745**	"	"	"
"	[abc]CMW24671		**JQ862734**	**JQ862722**	**JQ862746**	"	"	"
"	[a]CMW24672		**JQ862735**	**JQ862723**	**JQ862747**	"	"	"
"	[a]CMW24673		**JQ862736**	**JQ862724**	**JQ862748**	"	"	"
C. cacaofunesta	CMW15051	CBS152.62	DQ520636	EF070427	EF070398	*Theobroma cacao*	A.J. Hansen	Costa Rica
"	CMW14809	CBS115169	DQ520637	EF070428	EF070399	"	C. Suarez	Ecuador
C. colombiana	CMW5751	CBS121792	AY177233	AY177225	EU241493	*Coffea arabica*	N/A	Colombia
"	CMW9565	CBS121790	AY233864	AY233870	EU241487	Soil	N/A	"
"	CMW9572		AY233863	AY233871	EU241488	*Mandarin*	N/A	"
C. curvata	CMW22432		FJ151439	FJ151451	FJ151473	*E. deglupta*	M.J. Wingfield	"
"	CMW22442	CBS122603	FJ151436	FJ151448	FJ151470	"	"	"
C. fimbriata s.s.	CMW15049	CBS141.37	DQ520629	EF070442	EF070394	*Ipomoea batatas*	C.F. Andrus	USA
"	CMW1547		AF264904	EF070443	EF070395	"	E.C.H. McKenzie	Papua New Guinea
C. fimbriatomima	CMW24174		EF190963	EF190951	EF190957	*Eucalyptus* sp.	M.J. Wingfield	Venezuela
"	CMW24176	CBS121787	EF190964	EF190952	EF190958	"	"	"
C. manginecans	CMW13851		AY953383	EF433308	EF433317	*Mangifera indica*	N/A	Oman
"	CMW13852		AY953384	EF433309	EF433318	*Hypocryphalus mangifera*	N/A	"
C. neglecta	CMW17808	CBS121789	EF127990	EU881898	EU881904	*Eucalyptus* sp.	C. Rodas	Colombia
"	CMW18194		EF127991	EU881899	EU881905	"	"	"
C. papillata	CMW10844		AY177238	AY177229	EU241481	*Coffea Arabica*	N/A	"
"	CMW8857		AY233868	AY233878	EU241483	*Annona muricata*	N/A	"
"	CMW8856	CBS121793	AY233867	AY233874	EU241484	*Citrus lemon*	N/A	"

Table 1 (continued)

Species	CMW No.	Other no.	GenBank[d]			Hosts	Collectors	Origin
			ITS	β-tubulin	TEF-1a			
C. platani	CMW14802	CBS115162	DQ520630	EF070425	EF070396	*Platanus occidentalis*	T.C. Harrington	USA
"	CMW23918		EU426554	EU426555	EU426556	"	M.J. Wingfield	Greece
C. virescens	CMW11164		DQ520639	EF070441	EF070413	*Fagus americanum*	D. Houston	N/A
"	CMW 3276		AY528984	AY528990	AY528991	*Quercus* sp.	T. Hinds	USA

[a] Isolates obtained and used in phylogenetic analysis in this study

[b] Isolates used for glasshouse pathogenicity tests at FABI

[c] Isolates used for field pathogenicity tests in China

[d] GenBank numbers in boldface were sequenced in this study

[e] *N/A* not available

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

CBS Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

Culture characteristics of the *Ceratocystis* isolates representing the apparently unknown species were described after isolates were grown on 2 % MEA. Representative isolates were selected from different trees and grown on 2 % MEA for 3 days. 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 at different incubation temperatures, ranging between 5 °C to 35 °C at five degree intervals. Five replicate plates of each isolate were used at each temperature. The plates were incubated in the dark, and two measurements of colony diameter, at right angles to each other, were taken daily until the fastest growing culture had covered the plate, and the averages were then calculated for each of the seven temperatures. Colony colour was determined using the colour charts of Rayner (1970).

DNA extraction, PCR and sequence reactions

Representative isolates, selected based on the morphology of their cultures, and obtained from different *Eucalyptus* trees in the GuangDong Province, were selected for DNA sequence comparisons. Cultures of each isolate were grown on 2 % MEA for 2 weeks prior to DNA extraction. Mycelium was scraped from the surfaces of cultures grown on MEA, transferred to Eppendorf tubes and freeze-dried. DNA extractions were made as described by Van Wyk et al. (2006b).

Three gene regions, namely the Internal Transcribed Spacer (ITS) regions (ITS1, ITS2) including the 5.8S rDNA operon, part of the Beta-tubulin 1 (BT1) and Transcription Elongation Factor-1 alpha (TEF-1α) regions were amplified using the Polymerase Chain Reaction. The ITS regions were amplified with primers ITS1 and ITS4 (White et al. 1990), the β-tubulin gene region using primers Bt1a and Bt1b (Glass and Donaldson 1995), and the transcription elongation factor-1α gene with primers TEF1F and TEF2R (Jacobs et al. 2004) respectively. The conditions for PCR were as described by Van Wyk et al. (2006a). The amplified products were cleaned with 6 % Sephadex G-50 columns as described by the manufacturers (Steinheim, Germany).

The same primers that were used for PCR were used for DNA sequencing with an ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied BioSystems, Foster City, California). Sequencing reactions were established and run as described by Van Wyk et al. (2006a) on an ABI PRISM™ 3100 Autosequencer (Applied BioSystems, Foster City, California, USA). The sequencing products were cleaned using 6 % Sephadex G-50 columns. Sequences were analysed using MEGA4 (Tamura et al. 2007).

Phylogenetic analyses

The ITS, β-tubulin and TEF-1α sequences of the selected isolates were subjected to BLAST searches using the NCBI

(http://www.ncbi.nlm.nih.gov) nucleotide database to obtain preliminary identifications. Sequences for closely related *Ceratocystis* species were obtained from GenBank (http://www.ncbi.nlm.nih.gov) to compile datasets for phylogenetic analyses. The sequences for the three gene regions were aligned using the online interface of MAFFT v. 5.667 (Katoh et al. 2002), with the iterative refinement method (FFT-NS-i settings). The sequence alignments were edited manually in MEGA4 (Tamura et al. 2007) and deposited in TreeBASE (http://www.treeBASE.org).

Sequence datasets were analysed with PAUP version 4.0b10 (Swofford 2002). A partition homogeneity test (PHT) in PAUP (Swofford 2003) was used to determine whether data for the three gene regions could be combined. The most parsimonious trees were obtained using the heuristic search option with stepwise addition and tree bisection and reconstruction (TBR). All equally most parsimonious trees were saved. Gaps were treated as a fifth character state. A 1,000 bootstrap replicates were calculated for the confidence intervals of the tree-branching points. *Ceratocystis virescens* (R.W. Davidson) C. Moreau was used as the out-group taxon.

Phylogenetic trees were constructed based on Bayesian probabilities in MrBayes version 3.1.1 using a Markov Chain Monte Carlo (MCMC) algorithm (Ronquist and Huelsenbeck 2003). For each of the datasets (ITS, BT1 and TEF-1α), MrModeltest v.2.3 (Nylander 2004) was used to determine a suitable model of nucleotide substitution. The MCMC procedure with four chains were run for 1,000,000 generations and trees were sampled every 100th generation. To confirm the number of trees that were created before stabilization, tree likelihood scores were evaluated to avoid including trees that were sampled before convergence.

Pathogenicity tests

Glasshouse trials

Isolates from the two distinct *Ceratocystis* groups identified based on morphology and phylogenetic inference were selected for pathogenicity trials in the glasshouse. Three isolates of each of the species representing the *C. moniliformis s.l.* complex (CMW24664, CMW24667, CMW24671) and *C. fimbriata s.l.* complex (CMW24665, CMW24658, CMW24661) were selected. Isolates were grown on 2 % MEA at 25 °C for six days before inoculation.

Eucalyptus grandis clone TAG-5, which is moderately sensitive to many *Eucalyptus* pathogens, was selected for inoculations. Ten trees (~10 mm diameter) of clone TAG-5 were inoculated with the six test isolates after acclimatization in a glasshouse for four weeks. The glasshouse was subjected to a constant temperature of approximately 25 °C and natural day/night conditions. Ten *E. grandis* trees were inoculated with sterile MEA plugs as controls. Inoculations were conducted on the tree stems 300 mm above soil level using a 6 mm diameter sterile metal cork borer following the method described by Chen et al. (2010). The 70 inoculated trees, 10 for each of the *Ceratocystis* isolates and 10 as controls, were randomly arranged in the glasshouse.

Results were obtained after six weeks by measuring the lengths of the lesions in the cambium for each tree. Re-isolations were made from resultant lesions to show that the inoculated fungi had resulted in the lesions that developed. This was done by cutting small pieces of wood from the lesion edges and placing them on 2 % MEA at 25 °C. Re-isolations were conducted from four randomly selected trees for each isolate and all control trees. Results were analysed in SAS/STAT using the general linear model (GLM) procedure (SAS Institute 1999).

Field trials

Based on the results of pathogenicity tests in the glasshouse trials, the most aggressive isolates which produce relatively longer lesions of each species were selected for use in field inoculation trials, two isolates (CMW24658, CMW24661) representing the most virulent *C. moniliformis s.l.* species complex and two isolates (CMW24667, CMW24671) representing the *C. fimbriata s.l.* species complex were selected for pathogenicity tests under field conditions in China. Field inoculations were carried out in ZhanJiang, GuangDong Province, on six *Eucalyptus* genotypes. These included an *E. grandis* clone (CEPT-1), an *E. grandis*×*E. tereticornis* Sm. clone (CEPT-2), an *E. pellita* F. Muell. genotype (CEPT-3), an *E. urophylla* S. T. Blake×*E. grandis* clone (CEPT-4), an *E. urophylla* clone (CEPT-5) and an *E. wetarensis* L.D. Pryor clone (CEPT-6). For every clone/species, 50 one-year-old trees were selected for inoculation, with 10 trees of each *Eucalyptus* genotype used for each of the four *Ceratocystis* isolates. Ten trees of each *Eucalyptus* genotype were inoculated with sterile MEA plugs to serve as controls.

Inoculations were conducted on the tree stems 50–100 cm above the ground using a 9 mm diameter sterile metal cork borer similar to the technique described in Chen et al. (2010). The inoculation wounds were covered with masking tape to protect them from desiccation. After five weeks, lesions in the cambium were measured and the results were analysed in SAS/STAT with the general linear model (GLM) procedure (SAS Institute 1999).

Results

Fungal isolates

A total of 54 isolates resembling species of *Ceratocystis* were obtained from stumps of recently cut *Eucalyptus* trees

in GuangDong Province. All the isolates were collected from the same *Eucalyptus urophylla*×*E. grandis* clone in the same plantation. *Ceratocystis* ascocarps were commonly found on the tissue collected from the stumps. Slight discolouration was observed on the wood of stumps where fruiting bodies were present.

Culture morphology and characteristics

Isolates could be grouped into two broad groups based on culture morphology. One group including 32 isolates grew fast, with white mycelium. These isolates, with hat-shaped, sheathed ascospores, disc-shaped plates at the bases of the ascomatal necks where they connect to the ascomatal bases and short conical spines on the ascomatal bases, resembled species in the *C. moniliformis s.l.* complex. These isolates could be induced to produce sexual fruiting structures only by incubating them on a mixture of 2 % MEA with pine branch chips, and by alternating incubation temperatures between 25 °C and 5 °C every two days. Only primary phialides producing cylindrical, hyaline conidia were observed for isolates in this group, and no secondary phialides were observed.

The remaining 22 isolates grew slowly and had dark green colonies. These isolates had hat-shaped, sheathed ascospores and the ascomata were without the disc shaped attachments or spines on the ascomatal bases and were typical of fungi in the *C. fimbriata s.l.* complex. They produced chlamydospores for both the anamorphic and teleomorphic structures.

Four isolates (CMW24655, CMW24658, CMW24659, CMW24661) later identified as undescribed species residing in the *C. moniliformis s.l.* species complex were selected for growth studies. The four isolates showed very similar growth patterns in culture and grew optimally at 30 °C, with no growth at 5 °C, and only slow growth at 10 °C. They reached an average of 79 mm after 3 days at 25 °C and 89 mm at 30 °C. At 35 °C, colonies reached a diameter of 47 mm after 3 days.

Phylogenetic analyses

PCR of *Ceratocystis* isolates from China resulted in amplicons of approximately 500 bps for the ITS and partial β-tubulin gene regions, and 800 bps for the TEF-1α gene region. Blast searches for sequences of the ITS, β-tubulin and TEF-1α gene regions all showed that the isolates belonged to two different clades within the genus *Ceratocystis*. One group of isolates was most similar to those in the *C. moniliformis s.l.* species complex, while the second group was most similar to isolates in the *C. fimbriata s.l.* species complex. These isolates were subsequently treated in two separate datasets in the phylogenetic analyses.

Partition homogeneity tests with 1,000 replicates on the three gene regions for the *C. moniliformis s.l.* dataset including isolates CMW24653, CMW24655, CMW24658, CMW24659, CMW24661 showed that they could be combined (P=0.01) (Cunningham 1997). The combined dataset for the three gene regions (1,783 characters, 1,242 constant, 541 parsimony informative; TreeBASE: 12579) produced two equally most parsimonious trees, the first of which was selected for presentation (Fig. 1). The tree had a length of 774 steps, a Consistency Index=0.859, Retention Index=0.942 and Homoplasy Index=0.141. For Bayesian analyses, model test analysis recommended a HKY+G model [Prset statefreqpr=dirichlet (1,1,1,1); Lset nst=2 rates=gamma] for the ITS gene region, GTR+G model [Prset statefreqpr=dirichlet (1,1,1,1); Lset nst=6 rates=gamma] for the BT1 gene region, and model HKY+I+G [Prset statefreqpr=dirichlet (1,1,1,1); Lset nst=2 rates=invgamma] for the TEF-1α gene region. The first 1,000 trees were discarded since they were outside the convergence (burn-in) point. The Bayesian posterior probability values offered strong support to the bootstrap values obtained (Fig. 1). Based on the two phylogenetic analyses, the isolates residing in the *C. moniliformis s.l* complex formed a well-resolved and unique clade (Fig. 1), separate from all the previously described *Ceratocystis* species in the *C. moniliformis s.l.* complex, with strong phylogenetic support (bootstrap support value=96 %, Bayesian posterior probabilities=1.00). This indicated that these isolates represent a previously undescribed species. The phylogenetically most closely related species were *C. inquinans*, *C. microbasis*, C. *omanensis* and *C. sumatrana* (Fig. 1).

Partition homogeneity tests with 1,000 replicates for the *C. fimbriata s.l.* datasets based on isolates CMW24664, CMW24667, CMW24671, CMW24672, CMW24673 showed that the sequences for the three gene regions could be combined (P=0.03) (Cunningham 1997). The combined dataset for the three gene regions (1,698 characters, 1,236 constant, and 462 parsimony informative; TreeBASE: 12579) produced three equally most parsimonious trees of which the first tree was chosen for presentation (Fig. 2). The tree had a length of 594 steps, a Consistency Index=0.892, Retention Index=0.927 and Homoplasy Index=0.108. For Bayesian analyses, model test analysis recommended a HKY+G model [Prset statefreqpr=dirichlet(1,1,1,1); Lset nst=2 rates=gamma] for the ITS gene region, GTR+I model [Prset statefreqpr=dirichlet (1,1,1,1); Lset nst=6 rates=propinv] for the BT1 gene region, and model HKY+G [Prset statefreqpr=dirichlet (1,1,1,1); Lset nst=2 rates=gamma] for the TEF-1α gene region. The first 1,000 trees were discarded since they were outside the convergence (burn-in) point. The Bayesian posterior probability values offered strong support to the bootstrap values obtained (Fig. 2). Based on the two phylogenetic analyses, the *Ceratocystis* isolates in the *C. fimbriata s.l.* group were identified as *C. acaciivora* (Fig. 2).

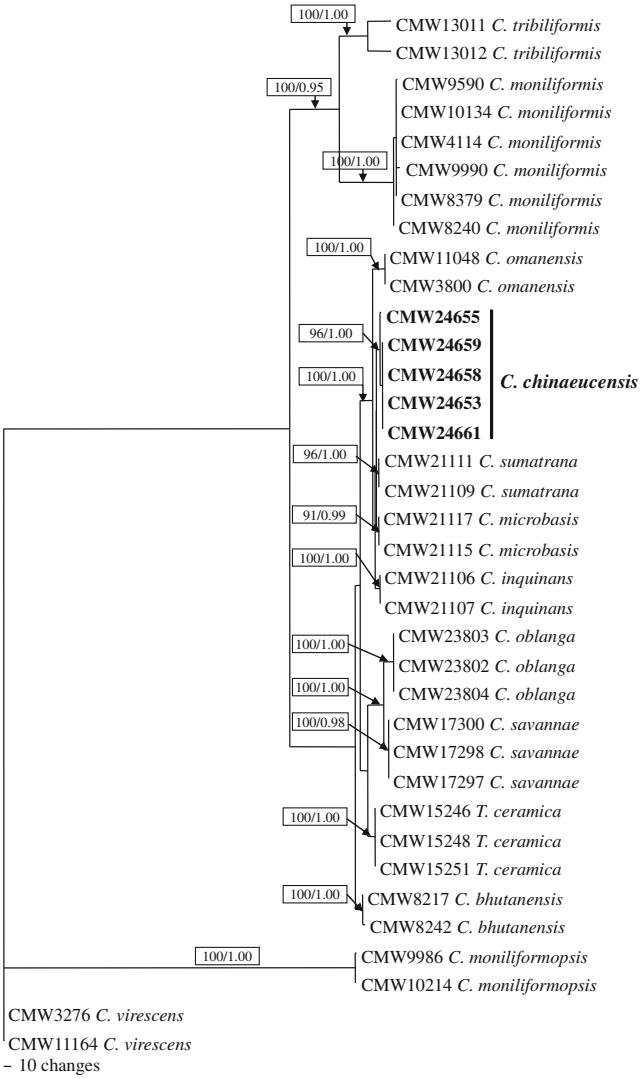

Fig. 1 Phylogenetic tree produced from a heuristic search of the combined ITS, β-tubulin and Translation Elongation factor-1α sequence data, showing the relationship between *C. chinaeucensis* sp. nov. from China and other species in the *C. moniliformis s.l.* species complex. *C. virescens* was used as out-group taxon. Bootstrap values and Bayesian values are indicated at the branch nodes

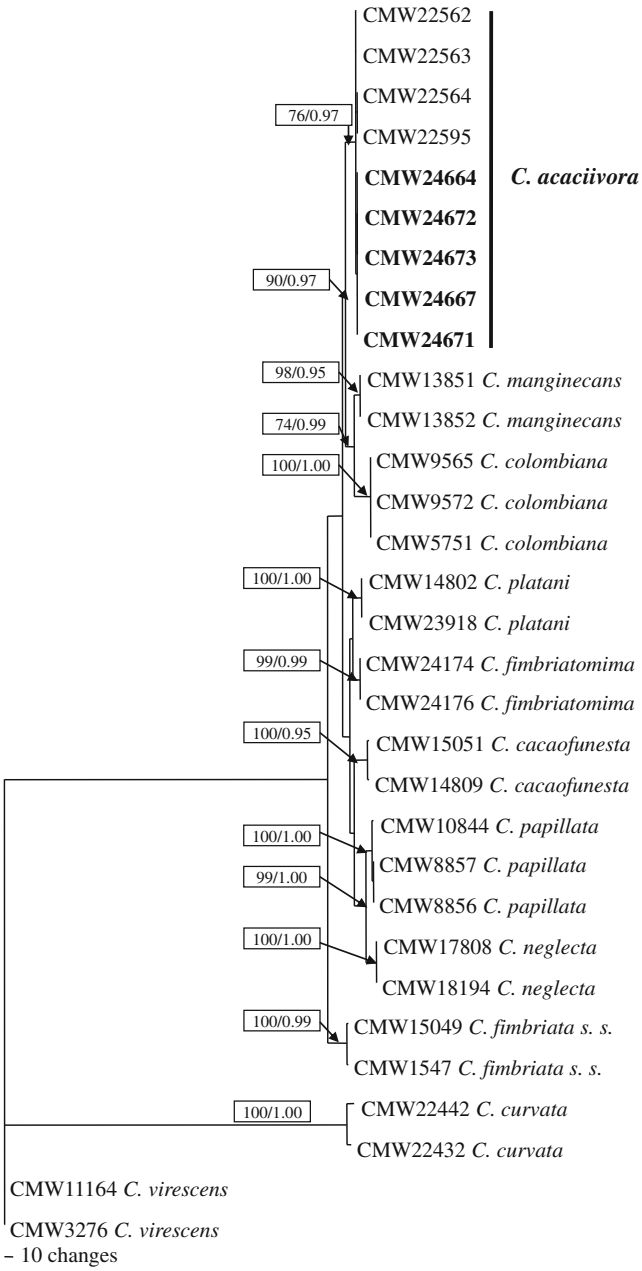

Fig. 2 Phylogenetic tree produced from a heuristic search of the combined ITS, β-tubulin and Translation Elongation factor-1α sequence data, showing the relationship between *C. acaciivora* from China and other species in the *C. fimbriata s.l.* species complex. *C. virescens* was used as out-group taxon. Bootstrap values and Bayesian values are indicated at the branch nodes

Taxonomy

Based on morphological characteristics and DNA sequence comparisons, the *Ceratocystis* isolates from *Eucalyptus* trees in China represent two species, *C. acaciivora*, and a previously undescribed species residing in the *C. moniliformis s.l.* complex, which we describe here as follows:

Ceratocystis chinaeucensis S.F. Chen, Jol. Roux, M.J. Wingf. & X.D. Zhou, sp. nov. (Fig. 3a–h)

MycoBank No. MB564849

Anamorph: *Thielaviopsis* sp.

Etymology: the name "*chinaeucensis*" refers to the country, China where this species was first found and the host genus, *Eucalyptus*, from which it was first isolated.

Colonies white on malt extract agar. *Mycelium* aerial. *Optimal growth temperature* range 25–30 °C, with no growth at 5 °C. *Hyphae* smooth or granulated, not constricted at septa. *Ascomatal bases* dark brown to black, globose to obpyriform, ornamented with spines and hyphae, spines dark brown to black, (5–) 6–13 (–17) μm long, bases (166–) 212–271 (–315) μm in width, (196–) 232–304 (–355) μm in length. *Ascomatal necks* dark brown to black,

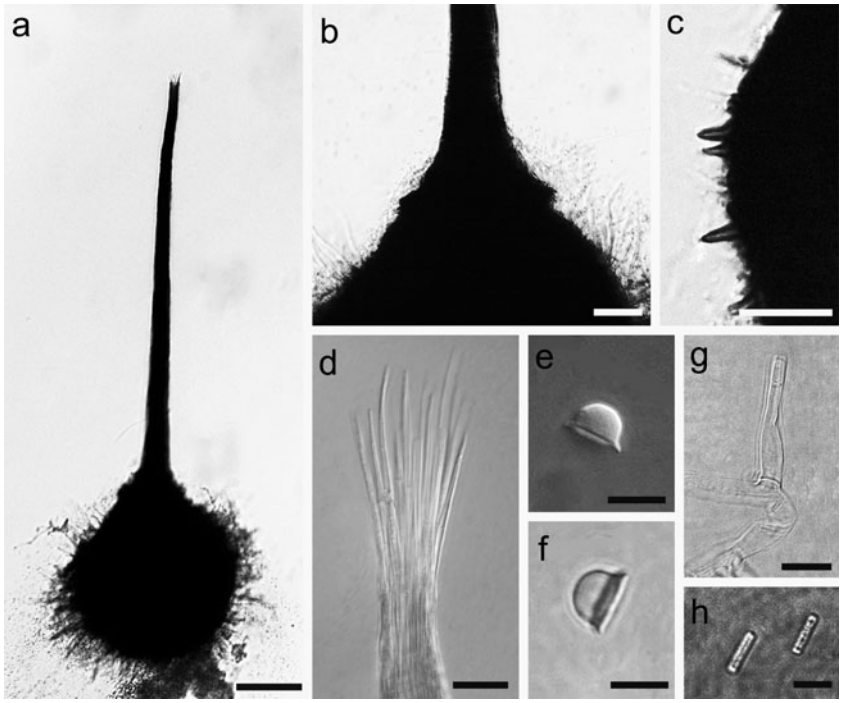

Fig. 3 Morphological features of *C. chinaeucensis* from *Eucalyptus* trees in China. **a** Ascomata with globose to obpyriform bases. **b** Ascoma neck base. **c** Ascomatal base with conical spines. **d** Divergent ostiolar hyphae. **e**, **f** Hat-shaped ascospore in side view. **g** Primary phialide. **h** Cylindrical conidia. Scale Bars: **a**=100 μm, **b**, **c**, **d**=20 μm, **e**, **f**, **h**=5 μm, **g**=10 μm

(28–) 32–51 (–72) μm wide at base, (12–) 14–22 (–28) μm wide at the apex, (333–) 410–551 (–629) μm in length, with a disc-like base. *Ostiolar hyphae* divergent, hyaline, (20–) 24–37 (–47) μm long. *Asci* not observed. *Ascospores* hat-shaped in side view, aseptate, hyaline, invested in sheath, 3–4 μm in length, 6–8 (–9) μm wide with sheath, (4–) 5–6 μm wide without sheath. Ascospores accumulating in buff-yellow (19 d) mucilaginous masses at the apices of ascomatal necks.

Thielaviopsis anamorph: *Conidiophores* of one type occurring singly on mycelium, hyaline, (16–) 21–25 (–34) μm in length, (2–) 3–4 (–5) μm wide at base, 3–5 μm wide at broadest point, 2–3 μm wide at tip. *Phialidic conidium* development through ring wall building, conidia formed singly. *Conidia* (5–) 6–8 (–9) μm in length, 1–3 μm wide. No secondary conidiophores, secondary conidia or chlamydospores observed.

Habitat: stumps of recently (two-week-old) felled *E. urophylla*×*E. grandis* hybrid.

Known distribution: GuangDong Province, China.

Material examined: CHINA, GuangDong Province, December, 2006, M.J. Wingfield, X.D. Zhou & S.F. Chen, isolated from stumps of *E. urophylla*×*E. grandis* clone. HOLOTYPE PREM60735, ex-type culture CMW24658= CBS127185; PARATYPE PREM60736, living culture CMW24661 = CBS127186; PARATYPE PREM60737, living culture CMW24659; Other living cultures, CMW24653, CMW24655.

Notes: *Ceratocystis chinaeucensis* is morphologically very similar to *C. inquinans*, *C. microbasis*, *C. omanensis* and *C. sumatrana*. The fungus has white cultures, as compared to those of *C. inquinans* (brown), *C. microbasis* (cream-buff to brown), *C. omanensis* (wood brown) and *C. sumatrana* (cream-buff to brown). The ascomata of *C. chinaeucensis* [length (196–) 232–304 (–355) μm, width (166–) 212–271 (–315) μm] are larger than those of *C. inquinans* [length (116–) 149–205 (–236) μm, width (130–) 161–217 (–270) μlm], *C. microbasis* [length (65–) 82–122 (–162) μm, width (82–) 100–146 (–185) μm], *C. omanensis* [length (154–) 206–254 (–279) μm, width not available] and *C. sumatrana* [length (148–) 168–218 (–293) μm, width (158–) 187–235 (–296) μm]. The ascospores of *C. chinaeucensis* [6–8 (–9) μm] are longer than those of *C. inquinans* (5–7 μm), *C. microbasis* (5–7 μm), *C. omanensis* (5–7 μm) and *C. sumatrana* (5–7 μm). The primary conidia of *C. chinaeucensis* [length (5–) 6–8 (–9) μm, width 1–3 μm] are shorter than those of *C. inquinans* [length (5–) 6–8 (–11) μm] and *C. microbasis* [length (3–) 4–6 (–11) μm], and narrower than those of *C. inquinans* [width (2–) 3–5 (–7) μm] and *C. sumatrana* [width 2–4 (–5) μm]. Only cylindrical hyaline conidia (primary conidia) were observed for isolates of *C. chinaeucensis*, while isolates of *C. inquinans* and *C. sumatrana* always produced cylindrical hyaline conidia and barrel-shaped conidia (secondary conidia).

Pathogenicity tests

Glasshouse trials

Within 6 weeks, all *Ceratocystis* isolates tested for pathogenicity on *Eucalyptus* clone TAG-5 produced lesions, while no lesions were produced in the control inoculations (Fig. 4).

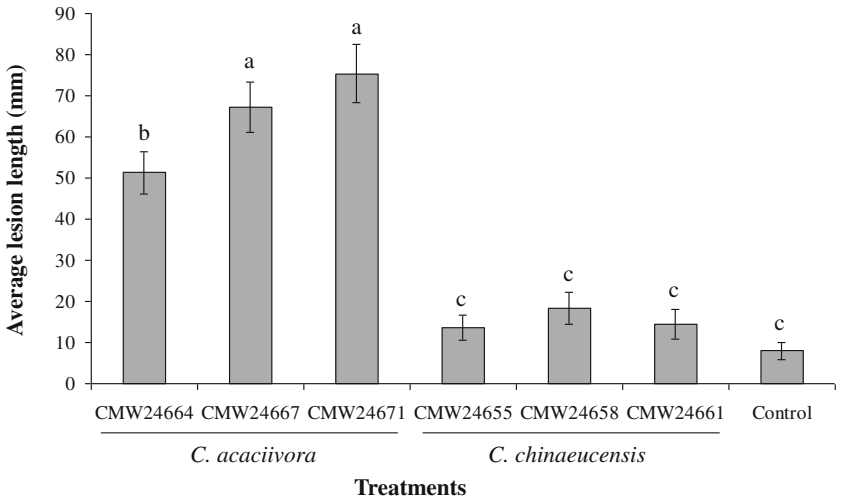

Fig. 4 Column chart indicating the average lesion length (in millimetres) resulting from inoculation trials with *C. acaciivora* (CMW24664, CMW24667 and CMW24671), *C. chinaeucensis* (CMW24655, CMW24658 and CMW24661) and the controls on an *E. grandis* clone (TAG-5) under glasshouse conditions. Vertical bars represent standard error of means. Different letters above the bars indicate treatments that were statistically different (P=0.05)

Lesions associated with inoculations using three isolates (CMW24664, CMW24667 and CMW24671) identified as *C. acaciivora* were significantly longer than those associated with *C. chinaeucensis* isolates (CMW24655, CMW24658, CMW24661) and the controls ($P<0.001$). Isolate CMW24671 (*C. acaciivora*) was the most virulent (Fig. 4). No significant differences were found between the lesion lengths caused by the three *C. chinaeucensis* isolates and the control (P=0.1553 to P=0.4291) (Fig. 4). The two most virulent isolates for each species (CMW24671 and CMW24667 for *C. acaciivora*, CMW24858 and CMW24661 for *C. chinaeucensis*) were selected for field inoculations. The inoculated *C. acaciivora* and *C. chinaeucensis* isolates were re-isolated from selected lesions, while no fungi resembling *Ceratocystis* were isolated from the controls.

Field trials

In the field inoculations, lesions produced by the *C. acaciivora* isolates (CMW24667, CMW24671) were significantly longer than those of *C. chinaeucensis* (CMW24658, CMW24661) and the controls ($P<0.0001$ to P=0.0386)

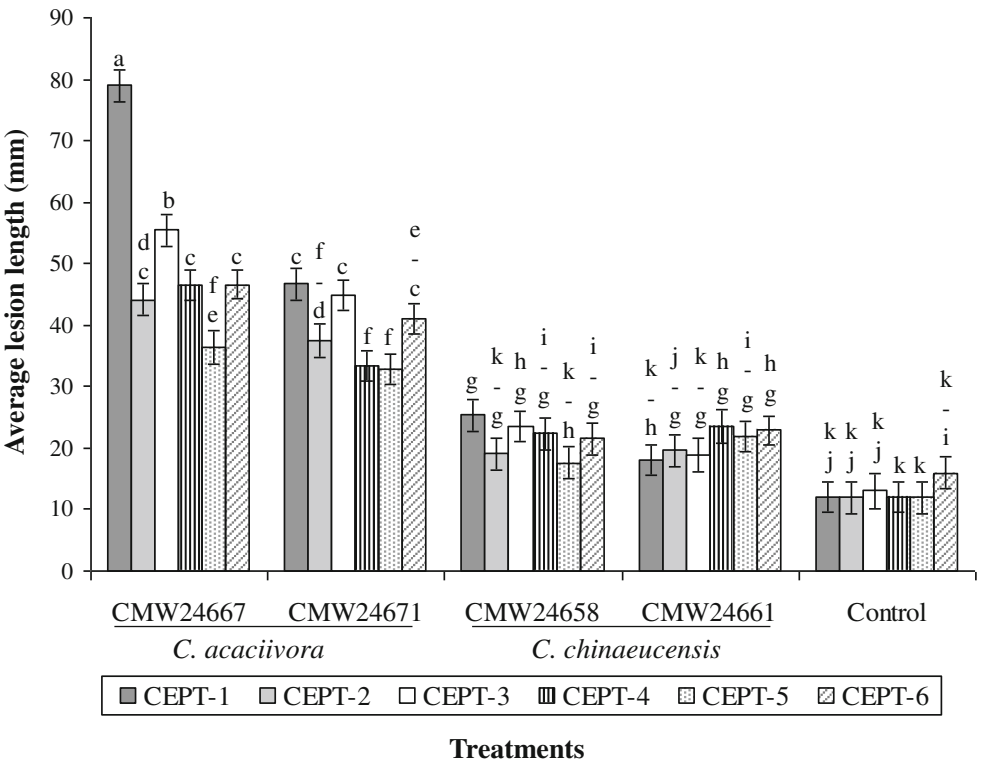

Fig. 5 Column chart indicating the average lesion length (in millimetres) resulting from inoculation trials of six *Eucalyptus* genotypes inoculated with two isolates each of *C. acaciivora* (CMW24667 and CMW24671), *C. chinaeucensis* (CMW24658 and CMW24661) and the controls. Vertical bars represent standard error of means. Different letters above the bars indicate treatments that were statistically different (P=0.05)

(Fig. 5). Lesions produced by the *C. chinaeucensis* isolates were not all significantly longer than those of the controls ($P<0.0001$ to $P=0.6531$) (Fig. 5).

There was significant isolate×genotype interaction ($P<0.0001$), suggesting that not all the *Eucalyptus* genotypes reacted similarly to all isolates. For example, in the case of *C. acaciivora*, isolate CMW24667 produced significantly longer lesions on *Eucalyptus* genotype CEPT-1, CEPT-3 and CEPT-4 than those of isolate CMW24671 ($P<0.0001$ to $P=0.0036$), but on genotype CEPT-2, CEPT-5, CEPT-6, the lesion length produced by isolates CMW24667 and CMW24671 of *C. acaciivora* were not significantly different ($P=0.0759$ to $P=0.3353$) (Fig. 5).

The six *Eucalyptus* genotypes showed significant differences of susceptibility to *C. acaciivora* (Fig. 5). For example, lesion lengths produced by isolate CMW24667 on CEPT-1 were significantly longer than those on other genotypes ($P<0.0001$ to $P=0.0148$) (Fig. 5), indicating that for isolate CMW24667, CEPT-1 is the most susceptible to infection, while genotype CEPT-5 was the most tolerant, producing the smallest lesions (Fig. 5).

Discussion

In this study, which represents the first to consider *Ceratocystis* species on *Eucalyptus* in China, two species were identified. These were *C. acaciivora* and a previously undescribed species, for which the name *C. chinaeucensis* has been provided. While both species are capable of causing lesions on *Eucalyptus* species under field conditions, only *C. acaciivora* showed clear evidence of being a pathogen. This study also provides the first data regarding the relative susceptibility of Chinese *Eucalyptus* genotypes to *Ceratocystis* species.

Ceratocystis isolates from China resided in two groups based both on morphology and DNA sequence data. *Ceratocystis chinaeucensis* was phylogenetically most closely related to *C. inquinans*, *C. microbasis*, *C. omanensis* and *C. sumatrana*. These species are all known only from Asia, with *C. omanensis* described from declining mango trees in Oman (Al-Subhi et al. 2006) and *C. inquinans*, *C. microbasis* and *C. sumatrana* from wounds on *Acacia mangium* trees in Indonesia (Tarigan et al. 2010). However, comparison of the sequence data showed that *C. chinaeucensis* isolates represent a well-resolved and distinct clade in this group. This new species could also be distinguished from other *Ceratocystis* spp. in *C. moniliformis s.l.*, based on morphology. It has especially large ascomata and ascospores and primary conidia (cylindrical hyaline conidia) that are always shorter or narrower than those of the other four species (Tarigan et al. 2010).

This study represents only the second report of the recently described *C. acaciivora* and expands its host range to include *Eucalyptus*. The origin of *C. acaciivora* is unknown and no information is available on its possible insect vectors. It was first discovered and described from young diseased *A. mangium* trees in several plantations in Indonesia where it causes a serious disease of these trees (Tarigan et al. 2011). In our study, *C. acaciivora* was isolated from fresh stumps and whether it is a pathogen of standing trees in the country is still unknown. Continued disease surveys of *Eucalyptus* in China is necessary to determine the true impact of *C. acaciivora* on these trees.

Pathogenicity tests showed that both *C. acaciivora* and *C. chinaeucensis* can produce lesions on the tested *Eucalyptus* genotypes. However, *C. acaciivora* was the only species that produced lesions significantly different to the controls, both in the glasshouse and field inoculations. This was not surprising because species in the *C. fimbriata s.l.* complex, to which *C. acaciivora* belongs, are well-known plant and tree pathogens (Kile 1993; Wingfield et al. 1996; Barnes et al. 2003a; Johnson et al. 2005; Engelbrecht et al. 2007; Roux and Wingfield 2009). *C. chinaeucensis* is best recognised as a saprophyte and this is consistent with other members of the *C. moniliformis s.l.* complex, which are not considered to be pathogens (Davidson 1935; Bakshi 1951; Hunt 1956) although some do cause sap stain (Kile 1993; Van Wyk et al. 2006a).

Field inoculation results showed that the tolerance of different *Eucalyptus* genotypes is significantly different from each. This is similar to what has been found for *Eucalyptus* clones against *C. fimbriata* s.l. in Brazil (Zauza et al. 2004). These results suggested that it may be effective to manage *Eucalyptus* diseases caused by *Ceratocystis* species through the selection of disease-resistant *Eucalyptus* planting genotypes. However, our results also show that care should be taken of isolates selected for screening trials, since an isolate by *Eucalyptus* genotype interaction exists. Results of this study provide a foundation for future work aimed at managing diseases caused by the *Ceratocystis* species in *Eucalyptus* plantations in China.

In this study, we collected *Ceratocystis* species only from fresh stumps in a single recently harvested plantation. It is clear that at least one of the species collected, *C. acaciivora*, is capable of causing lesions on *Eucalyptus* species. It will be important to expand the surveys and to continue monitoring tree deaths in China for the involvement of *Ceratocystis* species. This is especially important in light of the increasing number of reports globally of *Ceratocystis* species as pathogens of *Eucalyptus* (Roux and Wingfield 2009) and other trees.

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