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## Novel hosts of the *Eucalyptus* canker pathogen *Chrysosporthe cubensis* and a new *Chrysosporthe* species from Colombia

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### ABSTRACT

The pathogen *Chrysosporthe cubensis* (formerly *Cryphonectria cubensis*) is best known for the important canker disease that it causes on *Eucalyptus* species. This fungus is also a pathogen of *Syzygium aromaticum* (clove), which is native to Indonesia, and like *Eucalyptus*, is a member of *Myrtaceae*. Furthermore, *C. cubensis* has been found on *Miconia* spp. native to South America and residing in *Melastomataceae*. Recent surveys have yielded *C. cubensis* isolates from new hosts, characterized in this study based on DNA sequences for the ITS and  $\beta$ -tubulin gene regions. These hosts include native *Clidemia sericea* and *Rhynchanthera mexicana* (*Melastomataceae*) in Mexico, and non-native *Lagerstroemia indica* (Pride of India, *Lythraceae*) in Cuba. Isolates from these hosts and areas group in the sub-clade of *C. cubensis* accommodating the South American collections of the fungus. This sub-clade also includes isolates recently collected from *Eucalyptus* in Cuba, which are used to epitypify *C. cubensis*. New host records from Southeast Asia include exotic *Tibouchina urvilleana* from Singapore and Thailand and native *Melastoma malabathricum* (*Melastomataceae*) in Sumatra, Indonesia. Consistent with their areas of occurrence isolates from the latter collections group in the Asian sub-clade of *C. cubensis*. DNA sequence comparisons of isolates from *Tibouchina lepidota* in Colombia revealed that they represent a new sub-clade within the greater *Chrysosporthe* clade. Isolates in this clade are described as *Chrysosporthe inopina* sp. nov., based on distinctive morphological differences.

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### Introduction

*Chrysosporthe cubensis*, previously known as *Cryphonectria cubensis*, is a well-documented pathogen of various tree species in tropical and sub-tropical areas of the world (Wingfield 2003). On *Eucalyptus* (*Myrtales*, *Myrtaceae*), the fungus causes

serious damage, especially in commercial plantations of susceptible species or clones (Hodges *et al.* 1979; Van Heerden *et al.* 2005). The fungus also causes a serious canker disease on *Syzygium aromaticum* (clove, *Myrtaceae*) in Malaysia, Indonesia, Zanzibar and Brazil (Nutman & Roberts 1952; Reid & Booth 1969; Hodges *et al.* 1986; Gryzenhout *et al.* 2004). *C. cubensis*

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has recently been found to cause die-back and cankers on native *Miconia rubiginosa* and *M. theaezans* trees (Myrtales, Melastomataceae), where these trees occur naturally in Colombia (Rodas et al. 2005).

In recent years, numerous isolates of *C. cubensis* have been collected from different hosts and parts of the world. Large numbers of these isolates have been characterised based on DNA sequence data for the ITS region of the ribosomal DNA operon,  $\beta$ -tubulin genes and Histone H3 genes (Myburg et al. 1999; Myburg et al. 2002a, 2003; Gryzenhout et al. 2004). These comparisons have thus shown that isolates of *C. cubensis* group in two well-resolved sub-clades, roughly related to geographic distribution, within the greater *Chrysosporthe* clade (Gryzenhout et al. 2004). The one sub-clade encompasses isolates mainly from South America, but isolates from the Congo, Republic of the Congo and Cameroon (Africa) also group in this clade (Myburg et al. 2003; Roux et al. 2003; Nakabonge et al. 2006). The second clade contains isolates from many South-east Asian countries and Australia (Myburg et al. 1999; 2002a), as well as isolates from Zanzibar in Tanzania, Kenya, Mozambique, Malawi (Africa) and Hawaii (USA) (Myburg et al. 2003; Nakabonge et al. 2006). Isolates and specimens linked to these two sub-clades are morphologically and phenotypically indistinguishable (Gryzenhout et al. 2004).

In addition to the two sub-clades identified for *C. cubensis*, three other phylogenetic sub-clades have also been recognized within *Chrysosporthe*, for isolates previously assigned to *Cryphonectria cubensis* (Myburg et al. 1999, 2002a; Wingfield et al. 2001; Gryzenhout et al. 2004, 2005). The fungi defining these sub-clades are morphologically distinct from each other and from *C. cubensis* and have thus been described as novel taxa (Gryzenhout et al. 2004, 2005). Collections making up the South African sub-clade have been described as *C. austro-africana*, recognizable by the rounded apices of the ascospores (Gryzenhout et al. 2004). The isolates in the sub-clade from *Tibouchina* trees in Colombia, which do not have sexual structures, have been described in a new anamorph genus for *Chrysosporthe* as *Chrysosporthella hodgesiana* (Gryzenhout et al. 2004). This fungus can be recognized by its optimal temperature for growth in culture, a temperature which is lower than that of other species of *Chrysosporthe*. Isolates from *Eucalyptus* spp. in Ecuador represent the third sub-clade, and this fungus has been described as *C. doradensis* based on its variably shaped conidia and pale luteous spore droplets (Gryzenhout et al. 2005).

Several hypotheses have been formulated regarding the origin of *C. cubensis*. One is that the fungus originated on clove on the Molucca islands of Indonesia where these trees are native (Hodges et al. 1986). The fungus could thus have been moved around the world with these trees, when they were planted for the spice trade, and later adapted to infect *Eucalyptus* (Hodges et al. 1986). Another view is that *C. cubensis* originated in South and Central America (Wingfield et al. 2001). The latter hypothesis is strongly influenced by the widespread occurrence of the fungus in various pan-tropical countries and islands of South and Central America and the Caribbean (Gryzenhout et al. 2004), its high phenotypic diversity in various South America countries (Van Heerden et al. 1997; Van Zyl et al. 1998), as well as its discovery on native *Miconia* species in Colombia (Rodas et al. 2005). This would be consistent

with observations that *C. cubensis* rapidly infected *Eucalyptus* spp. when plantations of these trees were established in South America (Hodges et al. 1986; Seixas et al. 2004), or on islands such as Hawaii (Hodges et al. 1986). Whether isolates defining the Asian and South American sub-clades of *C. cubensis* represent discrete and cryptic taxa or groups of isolates in the process of speciation is not clear. Population genetic studies are required to resolve this question and thus to more fully understand the probable origin of the fungus.

The hypotheses regarding the origin of *C. cubensis* are based largely on the occurrence of this fungus on hosts other than *Eucalyptus* spp. Although the fungus has been found in Australia where *Eucalyptus* spp. are native, it is not common but does occur in a quite atypical Mediterranean environment (Hodges et al. 1986; Davison & Coates 1991). Views concerning the origin of *C. cubensis* also rest firmly on our knowledge of the geographical distribution of the pathogen. An expanded view of the hosts and distribution of *C. cubensis* must contribute significantly to a better understanding of the origin of this fungus. Therefore we actively collected isolates of *C. cubensis* from trees other than *Eucalyptus* and also from areas where this fungus has not previously been found. In addition, specimens were collected in Cuba, the locality of the type specimen, in order to obtain isolates firmly defining this species. The aim of this study was to characterise these isolates, apparently representing new collections of *C. cubensis* from previously unreported hosts and areas, using DNA sequence data and morphological characteristics. An additional aim was to define an epitype for *C. cubensis* and thus to secure the name using DNA sequence data.

## Materials and methods

### Symptoms and collection of samples

Specimens used in this study were collected in many parts of the world (Fig 1). In Mexico, fruiting structures of fungi resembling *C. cubensis* were found on *Clidemia sericea* (Melastomataceae). These plants are common as weeds and occurred at roadsides and in proximity to *Eucalyptus* plantations. Structures of a *Chrysosporthe* sp. were associated with cankers on the stems or at the bases of the stems. Subsequent collections from the same area by Mr. F. Ferreira yielded fruiting structures reminiscent of *C. cubensis* on *Rhynchanthera mexicana* (Melastomataceae).

In Cuba, fruiting structures believed to represent *C. cubensis* were common on cankers (Fig 2A) on *E. grandis*, *E. saligna* and *E. urophylla* trees in Parque Metropolitano (Cerro Municipality, Havana city), Santiago de las Vegas (Boyeros Municipality, Havana city) and La Habano (or Havana city). *C. cubensis* was originally described from this area but there is only one specimen representing the holotype and there are no isolates linked to this important specimen (Gryzenhout et al. 2004). Therefore, specimens and isolates were collected from Cuba in order to fortify collections of the fungus and to define an epitype for it. In Cuba, fruiting structures resembling those of *C. cubensis* were also found on the tree *Lagerstroemia indica* (Myrtales, Lythraceae), commonly known as Pride of India, or crepe myrtle, growing in the gardens of the Institute of



**Fig 1 – Map of world showing the locations of different collections of *Chrysosporthe cubensis* (distinguishing between two sub-clades), *C. austroafricana*, *C. inopina*, *C. doradensis* and *Chrysosporthe hodgesiana*. Only collections verified with DNA sequence data are shown. Different hosts are also shown for collections of *C. cubensis sensu lato*.**

Ecology and Systematics, Boyeros Municipality, Havana city (Fig 2E–F).

In North Sumatra, structures resembling a species of *Chrysosporthe* were found on a native *Melastoma* sp. (*Melastoma malabathricum*) also known as the Straits rhododendron. These trees were part of the natural vegetation around Lake Toba and in an area where both clove and *Eucalyptus* trees are planted (Fig 2B–C). *Tibouchina urvilleana* plants (Fig 2D) in Singapore and Thailand were also found bearing fruiting structures similar to those of *Chrysosporthe* spp. This is not a certain host of *C. cubensis* as previous reports of *C. cubensis* on *Tibouchina* spp. (Wingfield et al. 2001, Seixas et al. 2004) represent *C. hodgesiana* or are of unconfirmed identity (Gryzenhout et al. 2004).

Additional plant material bearing structures resembling those of *Chrysosporthe* spp. and isolates derived from these specimens, were obtained from Colombia. Specimens bearing fruiting structures taken from *T. lepidota* trees were collected at the Libano farm of Smurfit Carton de Colombia, near Pereira, Colombia. This material included teleomorph structures of a fungus resembling a *Chrysosporthe* sp.

Isolations from the fruiting structures on the bark surface of field-collected specimens, were made on malt extract agar MEA [20 g l<sup>-1</sup> malt extract agar (Biolab, Merck, Midrand, South Africa)]. This was done by taking conidial or ascospore drops from the necks of fruiting structures or by exposing the spore mass inside the ascumata and then removing spores from it. The resultant cultures are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, and representative isolates have been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands (Table 1). The original bark specimens connected to the isolations have

been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM).

#### DNA sequence comparisons

Isolates for DNA sequence comparisons were grown in malt extract broth (20 g l<sup>-1</sup> malt extract, Biolab, Midrand, South Africa). DNA was extracted from mycelium as described in Myburg et al. (1999). The ITS regions ITS1 and ITS2, as well as the conserved 5.8 S gene of the ribosomal RNA (rRNA) operon, were amplified as described by Myburg et al. (1999). Two regions within the  $\beta$ -tubulin genes were also amplified following the methods of Myburg et al. (2002a). Purification of PCR products was done using a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany).

The purified PCR products were sequenced with the same primers that were used to amplify the respective DNA regions. An ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Perkin-Elmer, Warrington, UK) was used to sequence the amplification products on an ABI PRISM 3100™ automated DNA sequencer. The resulting raw nucleotide sequences were edited using Sequence Navigator version 1.0.1 (Perkin-Elmer Applied Biosystems, Inc., Foster City, California) software.

Sequences were added to the existing dataset (S 1211, M 2095) of Gryzenhout et al. (2004) and manually aligned. This dataset (Table 1) thus included isolates of *Chrysosporthe cubensis* from *Eucalyptus* spp. (Myburg et al. 2002a; Gryzenhout et al. 2004), *Syzygium aromaticum* (Myburg et al. 1999, 2003) and *Miconia* spp. (Rodas et al. 2005) from different parts of the world; isolates of *Chrysosporthe hodgesiana* from *Tibouchina urvilleana* (Wingfield et al. 2001; Gryzenhout et al. 2004) and *M. theaezans* (Rodas et al. 2005); *C. austroafricana* isolates from



**Fig 2** – Different hosts susceptible to *Chrysosporthe cubensis*. (A) Basal canker on *Eucalyptus grandis* in Cuba. (B) Native *Melastoma malabathricum* trees growing in a plantation in Sumatra. (C) Flowers of *M. malabathricum*. (D) *Tibouchina urvilleana*. (E)–(F) *Lagerstroemia indica*.

**Table 1 – Isolates included in this study**

Species identity	Isolate number <sup>a</sup>	Alternative isolate number <sup>a</sup>	Host	Origin	Collector	GenBank accession numbers <sup>b</sup>
<i>Chrysoporthe cubensis</i>	CMW 1856	–	<i>Eucalyptus</i> sp.	Kauai, Hawaii	–	AY 083999, AY 084010, AY 084022
	CMW 9903	–	<i>Syzygium aromaticum</i>	Kalimantan, Indonesia	C.S. Hodges	AF 292044, AF 273066, AF 273461
	CMW 11289	CBS 115737	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	AY 214303, AY 214231, AY 214267
	CMW 8650	CBS 115719	<i>S. aromaticum</i>	Sulawesi, Indonesia	M.J. Wingfield	AY 084001, AY 084013, AY 084024
	CMW 10774	–	<i>S. aromaticum</i>	Zanzibar, Tanzania	–	AF 492130, AF 492131, AF 492132
	CMW 2632	–	<i>E. marginata</i>	Australia	E. Davison	AF 046893, AF 273078, AF 375607
	CMW 12745	CBS 117837	<i>Tibouchina urvilleana</i>	Singapore	M.J. Wingfield	DQ368764, DQ368780, DQ368781
	CMW 17172	CBS 118664	<i>T. urvilleana</i>	Thailand	M.J. Wingfield	DQ368765, DQ368782, DQ368783
	CMW 17178	CBS 118665	<i>T. urvilleana</i>	Thailand	M.J. Wingfield	DQ368766, DQ368784, DQ368785
	CMW 16192	CBS 119474	<i>Melastoma malabathricum</i>	Sumatra	M.J. Wingfield	DQ368767, DQ368786, DQ368787
	CMW 186515	CBS 118651	<i>M. malabathricum</i>	Sumatra	M.J. Wingfield	DQ368768, DQ368788, DQ368789
	CMW 10669	CBS 115751	<i>Eucalyptus</i> sp.	Republic of Congo	J. Roux	AF 535122, AF 535124, AF 535126
	CMW 1853	–	<i>S. aromaticum</i>	Brazil	–	AF 046891, AF 273070, AF 273465
	CMW 10778	CBS 115755	<i>S. aromaticum</i>	Brazil	C.S. Hodges	AY 084006, AY 084018, AY 084030
	CMW 9432	CBS 115724	<i>E. grandis</i>	Mexico	M.J. Wingfield	AY 692321, AY 692324, AY 692323
	CMW 12734	CBS 115853	<i>Rhynchanthera mexicana</i>	Mexico	F. Ferreira	DQ368769, DQ368790, DQ368791
	CMW 12736	CBS 115847	<i>R. mexicana</i>	Mexico	F. Ferreira	DQ368770, DQ368792, DQ368793
	CMW 13046	CBS 115762	<i>Clidemia sericeae</i>	Mexico	F. Ferreira	DQ368772, DQ368796, DQ368797
	CMW 12471	CBS 115849	<i>C. sericeae</i>	Mexico	F. Ferreira	DQ368771, DQ368794, DQ368795
	CMW 14394 (ex-epitype culture designated here)	CBS 118654	<i>E. grandis</i>	Cuba	M.J. Wingfield	DQ368773, DQ368798, DQ368799
	CMW 14404	CBS 118647	<i>E. grandis</i>	Cuba	M.J. Wingfield	DQ368774, DQ368800, DQ368801
	CMW 16199	CBS 118652	<i>Lagerstroemia indica</i>	Cuba	M.J. Wingfield	DQ368775, DQ368802, DQ368803
	CMW 16200	CBS 118650	<i>L. indica</i>	Cuba	M.J. Wingfield	DQ368776, DQ368804, DQ368805
	CMW 10639	CBS 115747	<i>E. grandis</i>	Colombia	C.A. Rodas	AY 263419, AY 263420, AY 263421
	CMW 10026	–	<i>Miconia rubiginosa</i>	Colombia	C.A. Rodas	AY 214294, AY 214222, AY 214258
	CMW 10028	–	<i>M. rubiginosa</i>	Colombia	C.A. Rodas	AY 214295, AY 214223, AY 214259
	CMW 9980	–	<i>M. theaezans</i>	Colombia	C.A. Rodas	AY 214297, AY 214225, AY 214261
CMW 9993	CBS 115728	<i>M. theaezans</i>	Colombia	C.A. Rodas	AY 214298, AY 214226, AY 214262	
<i>C. austroafricana</i>	CMW 2113	CBS 112916	<i>E. grandis</i>	South Africa	M.J. Wingfield	AF 046892, AF 273067, AF 273462
	CMW 9327	CBS 115843	<i>T. granulosa</i>	South Africa	M.J. Wingfield	AF 273473, AF 273060, AF 273455
	CMW 10192	CBS 118649	<i>S. cordatum</i>	South Africa	M. Gryzenhout	AY 214299, AY 214227, AY 214263
<i>Chrysoporthella hodgesiana</i>	CMW 10641	CBS 115854	<i>T. semidecandra</i>	Colombia	R. Arbelaez	AY 692322, AY 692326, AY 692325
	CMW 9995	CBS 115730	<i>T. semidecandra</i>	Colombia	R. Arbelaez	AY956969, AY956977, AY956978
	CMW 10625	CBS 115744	<i>M. theaezans</i>	Colombia	C.A. Rodas	AY956970, AY956979, AY956980
<i>C. inopina</i>	CMW 12727 (ex-type culture designated here)	CBS 118659	<i>T. lepidota</i>	Colombia	R. Arbelaez	DQ368777, DQ368806, DQ368807
	CMW 12729	CBS 118658	<i>T. lepidota</i>	Colombia	R. Arbelaez	DQ368778, DQ368808, DQ368809
	CMW 12731	CBS 118656	<i>T. lepidota</i>	Colombia	R. Arbelaez	DQ368779, DQ368810, DQ368811
<i>C. doradensis</i>	CMW 11286	CBS 115734	<i>E. grandis</i>	Ecuador	M.J. Wingfield	AY 214289, AY 214217, AY 214253
	CMW 11287	CBS 115735	<i>E. grandis</i>	Ecuador	M.J. Wingfield	AY 214290, AY 214218, AY 214254
	CMW 9123	CBS 115717	<i>E. deglupta</i>	Ecuador	M.J. Wingfield	DQ 224034, DQ 224038, DQ 224039

(continued on next page)

**Table 1 – (continued)**

Species identity	Isolate number <sup>a</sup>	Alternative isolate number <sup>a</sup>	Host	Origin	Collector	GenBank accession numbers <sup>b</sup>
<i>Cryphonectria parasitica</i>	CMW 1652	CBS 112914	<i>Castanea dentata</i>	U.S.A.	–	AF 046902, AF 273075, AF 273468
<i>C. nitschkei</i>	CMW 10518	CBS 112919	<i>Quercus sp.</i>	Japan	T. Kobayashi	AF 452118, AF 525706, AF 525713
<i>C. macrospora</i>	CMW 10463	CBS 112920	<i>Castanopsis cuspidata</i>	Japan	T. Kobayashi	AF 368331, AF 368351, AF 368350

a CMW = Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria; CBS = Centraalbureau voor Schimmelcultures (Utrecht). Isolates in bold were sequenced in this study.

b Accession numbers refer to sequence data of the ITS,  $\beta$ -tubulin 1 (primers Bt1a/1b) and  $\beta$ -tubulin 2 (primers Bt2a/2b) regions respectively.

*Eucalyptus* spp., *T. granulosa* (Myburg et al. 2002a,b) and *S. cordatum* (Heath et al. 2006); and isolates representing *C. doradensis* (Gryzenhout et al. 2005). The outgroup consisted of species of the closely related *Cryphonectria*, namely *C. parasitica*, *C. nitschkei* and *C. macrospora*.

Phylogenetic trees were inferred using PAUP version 4.0b10 (Swofford 2002). A 500 replicate partition homogeneity test (PHT) (Farris et al. 1994) was applied to the rRNA and  $\beta$ -tubulin gene sequence data sets (after the exclusion of uninformative sites, heuristic search with 100 random sequence additions and tree bisection-reconnection branch swapping, MULTREES off) to determine whether they could be analysed collectively. A phylogenetic tree was inferred from distance analyses. The correct model for the data sets was found with MODELTEST version 3.5 (Posada & Crandall 1998). The HKY85 model (Hasegawa et al. 1985) with the gamma distribution shape parameter set to 0.1798 (freqA 0.1893, freqC 0.3273, freqG 0.2368, freqT 0.2465, Ti/Tv 2.0064), was shown to be the appropriate model. A 1000 replicate BS analysis was executed to assess the confidence levels of the branch nodes of the phylogenetic tree. The sequence data generated in this study have been deposited in GenBank (Table 1).

### Morphology

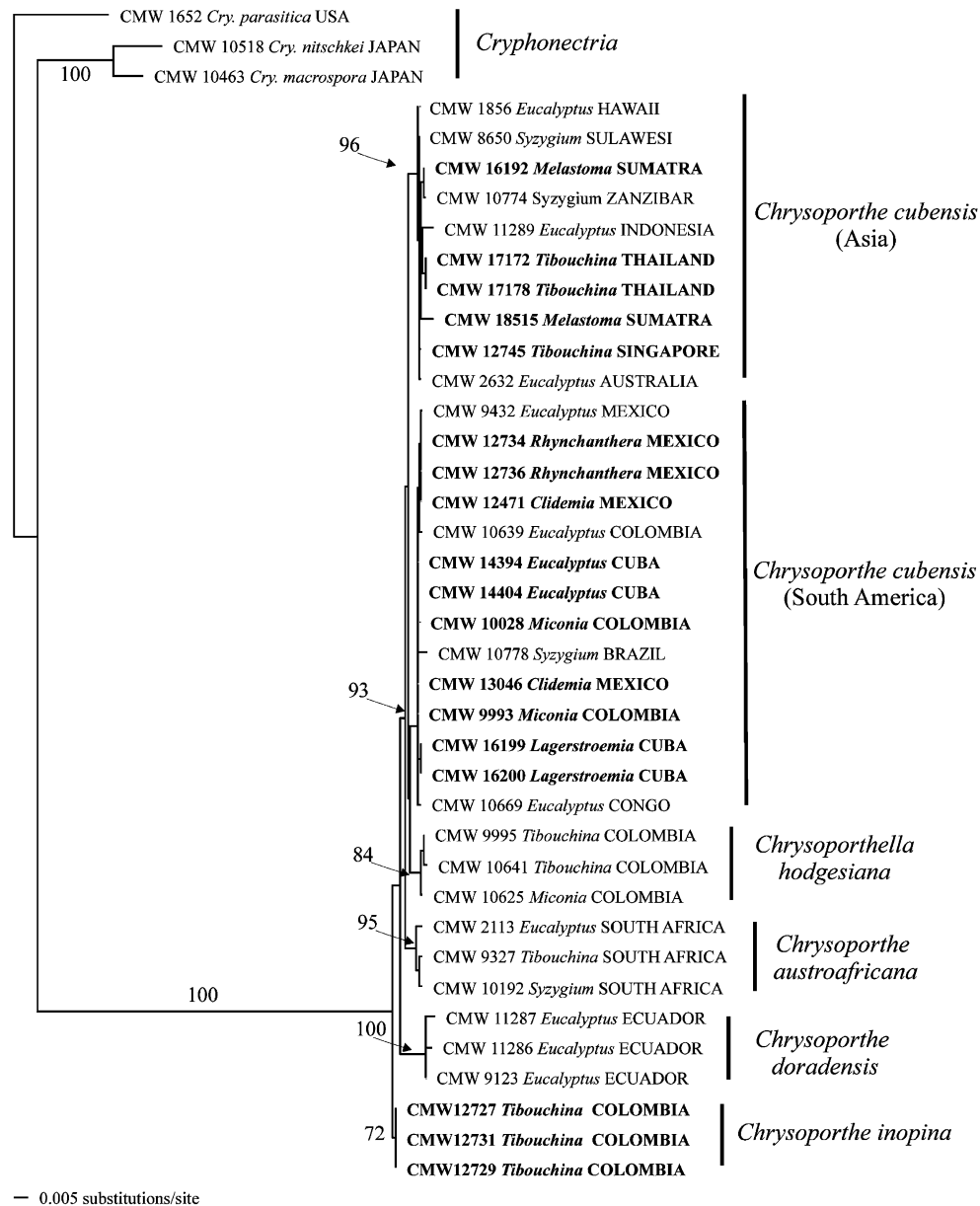
Fruiting structures were cut from the bark specimens and rehydrated in boiling water for 1 min. The structures, embedded in Leica mountant (Setpoint Premier, Johannesburg, South Africa), were sectioned approximately 12  $\mu$ m thick. The sections were dropped in water, transferred to microscope slides and mounted in 85 % lactic acid. Fresh slides from structures were also made with lactic acid or 3 % potassium hydroxide. Twenty measurements of ascospores, asci, conidia and conidiophores were taken from the fresh slides for all specimens, but 50 measurements were taken for the holotype specimen. These are presented as (min)-(average - std. dev.) - (average + std. dev.)-(max)  $\mu$ m. A range of measurements (minimum and maximum) was obtained from at least two structures (representing the smallest and largest) for the anamorph and teleomorph stomata on the bark, and perithecia representing the midpoint from sections, respectively. Standard colour notations provided by Rayner (1970) were applied.

Growth of isolates CMW 12727 and CMW 12729, which represent the new species from *T. lepidota* in Colombia (Table 1), was compared in culture. Cultures were grown on MEA in 90 mm diam Petri dishes in the dark at temperatures from 15 to 35 °C at 5 ° intervals. Assessment of growth was made as is described in Gryzenhout et al. (2004).

## Results

### DNA sequence comparisons

The PHT analyses showed that the rDNA and  $\beta$ -tubulin sequence data sets did not have any significant conflict ( $P = 0.012$ ) and could thus be combined. The dataset consisted of 39 taxa with the aligned ribosomal DNA sequence dataset (538 characters) consisting of 473 constant characters, 20 parsimony-uninformative and 45 parsimony-informative



**Fig 3 – Phylogram obtained from a combined data set comprising of ribosomal and  $\beta$ -tubulin gene sequences. The phylogram was obtained with distance analyses using the HKY85 parameter model ( $G = 0.1798$ ,  $\text{freqA } 0.1893$ ,  $\text{freqC } 0.3273$ ,  $\text{freqG } 0.2368$ ,  $\text{freqT } 0.2465$ ,  $\text{Ti/Tv } 2.0064$ ). Confidence levels, determined by a 1000 replicate BS analysis, of the tree branch nodes  $>70\%$  are indicated. Isolates sequenced in this study are bolded and host species for *Chrysosporthe cubensis* are indicated in italics. *Cryphonectria parasitica*, *C. nitschkei* and *C. macrospora* were defined as an outgroup.**

characters ( $g1 = -3.671$ ). The  $\beta$ -tubulin gene dataset (894 characters) consisted of 716 constant characters, 68 parsimony-uninformative and 110 parsimony-informative characters ( $g1 = -3.5959$ ). After combination, the data set was comprised of 1432 characters.

The various isolates originating from Mexico, Cuba, Sumatra and Singapore grouped in the two sub-clades representing *Chrysosporthe cubensis*, separately from those representing *C. austroafricana*, *C. hodesiana* and *C. doradensis* (Fig 3). The isolates from *Clidemia sericea* (CMW 12471, CMW 13046) and *Rhynchanthera mexicana* (CMW 12734, CMW 12736) in Mexico,

grouped in the sub-clade including Mexican and other South American isolates (BS support 93%). Specifically, the isolates from *Eucalyptus grandis* (CMW 14394, CMW 14404) and *Lagerstroemia indica* (CMW 16199, CMW 16200) in Cuba, also grouped in the South American sub-clade of *C. cubensis*. The isolates from *Tibouchina urvilleana* in Singapore (CMW 12745) and Thailand (CMW 17172, CMW 17178), and *Melastoma malabathricum* (CMW 16192, CMW 18515) in Sumatra grouped in the South-east Asian sub-clade (BS support 96%).

Other than the four previously characterised sub-clades in *Chrysosporthe* (Myburg et al. 2002a; Gryzenhout et al. 2004, 2005),

an unexpected sub-clade emerged in the phylogenetic tree (Fig 3). The clade included isolates representing the new collection (CMW 12727, CMW 12729, CMW 12731) from *T. lepidota* in Colombia (BS support 72 %).

### Morphology

Fruiting structures produced on specimens from different hosts in Mexico, Cuba, Singapore and Sumatra (Table 2) included both teleomorph and anamorph structures. These structures all resembled features previously described for *Chrysosporthe cubensis* (Bruner 1917; Hodges 1980; Myburg et al. 2003; Gryzenhout et al. 2004). The morphological characterisation of this material as *C. cubensis* is consistent with the results of the DNA sequence comparisons.

Fruiting structures on specimens collected from *Eucalyptus* spp. in Cuba were similar to those described for *C. cubensis* from other parts of the world. They were also indistinguishable from those of the type specimen (BPI 631857) of *C. cubensis* (Bruner 1917; Gryzenhout et al. 2004). The sizes of the asci on the newly collected material from Cuba differed somewhat from those reported by Bruner (1917) for Cuban specimens. They were thus up to 28 µm long, and not up to 34 µm as reported by Bruner (1917). Gryzenhout et al. (2004) also reported that ascus dimensions as reported by Bruner (1917) were inconsistent with those of specimens from other countries, but they could not find asci on the type specimen to confirm the ascus dimensions. The new measurements obtained for Cuban material from *Eucalyptus* in this study are similar to ascus sizes reported by Gryzenhout et al. (2004) for specimens connected to *C. cubensis* from South America and Southeast Asia. The newly confirmed ascus size of (19–)22–26.5(–28) µm for *C. cubensis* represents an additional characteristic to distinguish *C. cubensis* from *C. austroafricana*, which has longer asci [(25–)27–32(–34) µm; Gryzenhout et al. 2004].

Fruiting structures on the bark material (PREM 58800) linked to the isolates from *Tibouchina lepidota* in Libano, Colombia (CMW 12727, CMW 12729, CMW 12731), which formed a separate sub-clade based on DNA data, could be distinguished from those of existing *Chrysosporthe* spp. Asci (Figs 4E, 5C) were longer [(27.5–)29.5–34(–35.5) µm] than those of *C. cubensis* [(19–)22–26.5(–28) µm; Gryzenhout et al. (2004)] and *C. doradensis* [(19.5–)21.5–24(–25); Gryzenhout et al. (2005)], and corresponded with those of *C. austroafricana* [(25–)27–32(–34) µm; Gryzenhout et al. (2004)]. The new species could also be distinguished from *C. austroafricana*, *C. cubensis* and *C. doradensis*, because its ascospores (Figs 4F, 5C) were slightly wider (2.5–3.5 µm) than those of *C. cubensis* [(2–)2.5(–3) µm; Gryzenhout et al. (2004)], *C. austroafricana* [(2–)2.5 µm; Gryzenhout et al. (2004)] and *C. doradensis* [2–2.5 µm; Gryzenhout et al. (2005)]. Furthermore, isolates in this group grew optimally at 25 °C, different to the temperature for optimal growth of 30 °C for *C. cubensis*, *C. austroafricana* (Gryzenhout et al. 2004) and *C. doradensis* (Gryzenhout et al. 2005). Conidiomata also had variable shapes, varying from subulate with no attenuated neck, to pyriform with an attenuated neck, to globose (Figs 4G, 5D). These characteristics distinguished the new species from *C. hodgesiana*, which is the only other species growing optimally at 25 °C (Gryzenhout et al. 2004). However, as shape and size of the conidiomata can be quite variable

between samples (Hodges et al. 1986; Rodas et al. 2005), the shape of the conidiomata cannot always be used with confidence to distinguish *C. hodgesiana* from the new species.

### Taxonomy

In this study we have characterised collections of *Chrysosporthe cubensis* from *Eucalyptus* spp. from Cuba (PREM 58788–PREM 58791), which is the type locality of *C. cubensis*. Based on morphology, these specimens were similar to other specimens of *C. cubensis*. Isolates connected to these specimens also grouped in the South American sub-clade of *C. cubensis* based on DNA sequence comparisons. In the absence of isolates it has been impossible to tell whether the type specimen of *C. cubensis* from Cuba would group in the Southeast Asian or South American sub-clade of the fungus (Gryzenhout et al. 2004). Certain morphological features such as ascus morphology could also not be studied, due to the poor quality of the type specimen. Epitypification of *C. cubensis* using newly collected Cuban specimens will greatly aid future taxonomic studies of *Chrysosporthe* species. We, therefore, designate specimen PREM 58788 (ex-type culture CMW 14394/CBS 118654, living culture CMW 14404/CBS 118647), which originates from the same locality as the type specimen, as epitype of *C. cubensis* (*lectotypus hic designatus*). Additional specimens and linked isolates (Table 2; specimen PREM 58789 syn.: isolate CMW 14378/CBS 118655; PREM 58790 syn.: CMW 14362/CBS 118657; PREM 58791 syn.: CMW 14395/CBS 118648) from Cuba have also been deposited to fortify material defining this species.

Comparisons of specimens from *Tibouchina lepidota* collected on the Libano farm in Colombia, showed that this fungus represents a previously undescribed taxon. Although the sub-clade representing this fungus is supported with a relatively low BS value based on the regions of the genome sequenced, the fungus clearly does not group in the sub-clades representing other species. This new species can also be differentiated from other species of *Chrysosporthe* based on morphology. We, therefore, provide the following description for it.

#### *Chrysosporthe inopina* Gryzenh. & M. J. Wingf., sp. nov.

(Figs 4–5)

Etym.: Latin, *inopina*, unexpected, referring to the unexpected discovery of this species.

Ascospores fusoidae vel ovaes, utrinque rotundatae, (4.5–)6–7.5(–8) × 2.5–3.5 µm. Conidiomata subulata vel pyriformia vel pulvinata.

Typus: **Colombia:** Risaralda: Libano farm near Pereira, (75° 35' 49" W and 40° 43' 13" N, 2102 m above sea level, 3143 mm/y), *Tibouchina lepidota*, Jan. 2003, R. Arbelaez (PREM 58800 – holotypus, CMW 12727/CBS 118659 – cultura ex tipo, living cultures CMW 12729/CBS 118658, CMW 12731/CBS 118656).

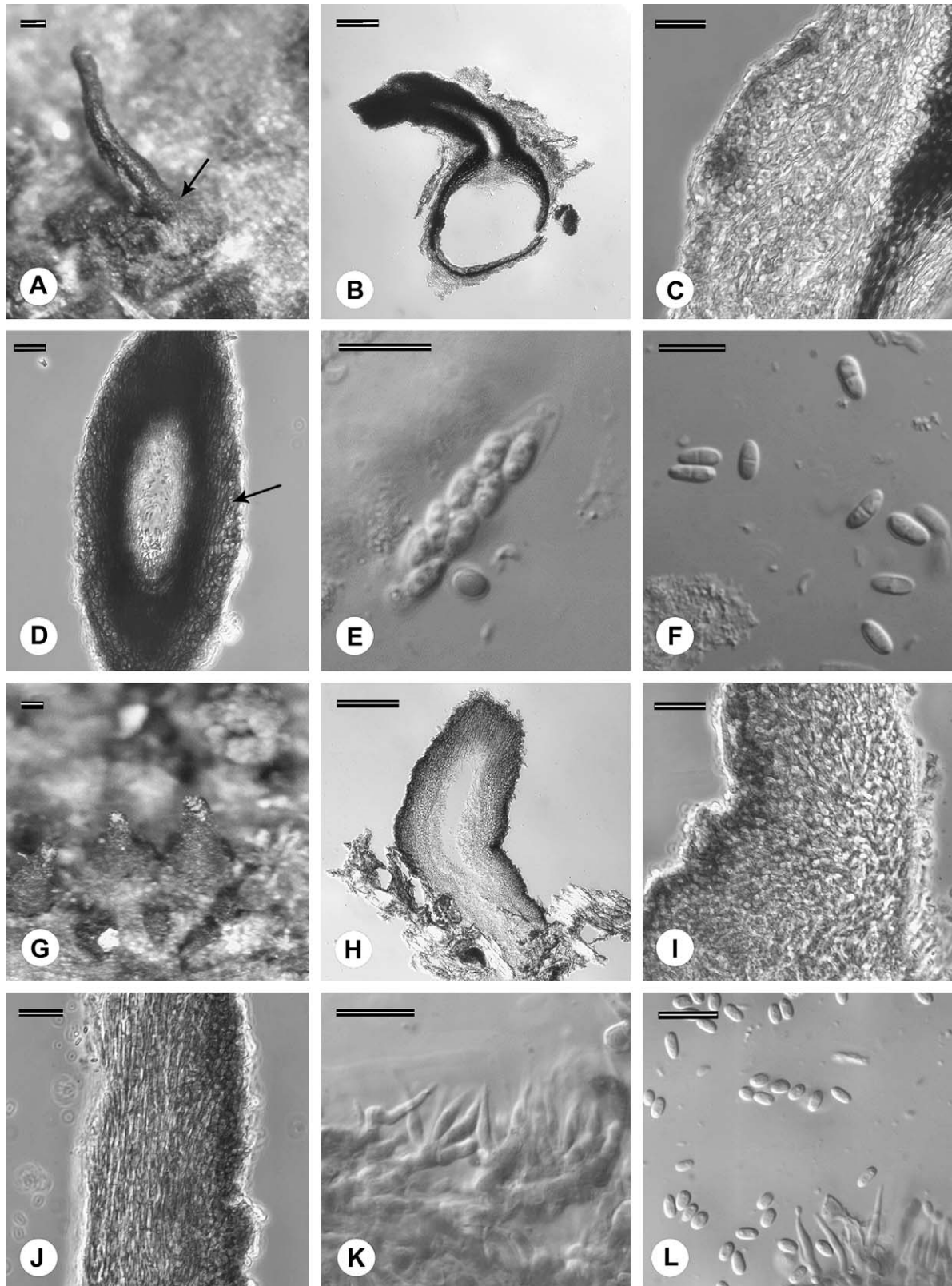
Ascstromata semi-immersed in bark, recognizable by extending, fuscous-black, cylindrical perithecial necks and in some cases, erumpent, limited ascostromatic tissue appearing orange, 90–540 µm high above the bark, 200–770 µm diam (Figs 4A–B, 5A–B). Perithecia valsoid, 1–6 per stroma, bases immersed in the bark, black, globose, 306–390 µm diam, perithecial wall



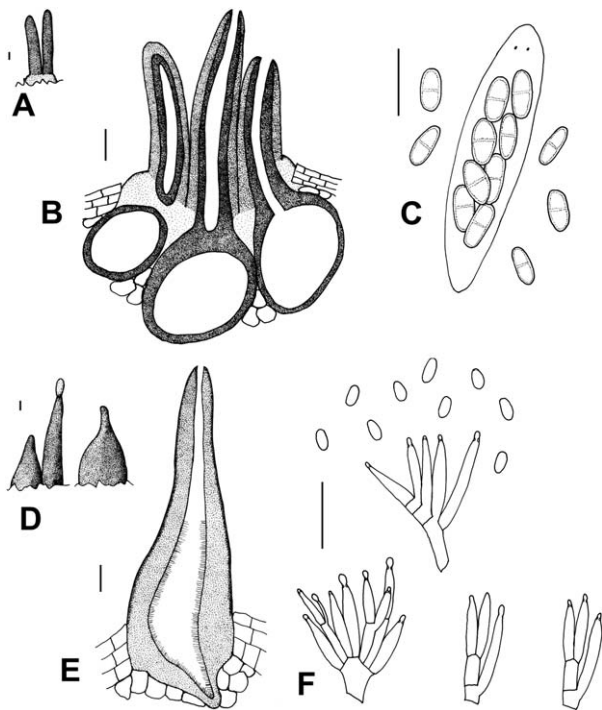
**Table 2 – Herbarium specimens used in this study**

Species identity	Herbarium number <sup>a</sup>	Linked isolate <sup>a</sup>	Host	Origin	Collector	Date
<i>Chrysosporthe cubensis</i> (epitype designated here)	PREM 58788	CMW 14394, CMW 14404	<i>Eucalyptus grandis</i>	Santiago de las Vegas, Boyeros Municipality, Havana city Cuba	M.J. Wingfield	Jan. 2004
<i>C. cubensis</i>	PREM 58789	CMW 14378	<i>E. grandis</i>	60 km west from Havana, Cuba	M.J. Wingfield	Jan. 2004
	PREM 58790	CMW 14362	<i>E. saligna</i>	Parque Metropolitano, Cerro Municipality, Havana city.	M.J. Wingfield	Jan. 2004
	PREM 58791	CMW 14395	<i>E. urophylla</i>	Road to Havana, Cuba	M.J. Wingfield	Jan. 2004
	PREM 58792	CMW 16199	<i>Lagerstroemia indica</i>	Havana city, Cuba	M.J. Wingfield	Jan. 2004
	PREM 58793	CMW 12734, CMW 12736	<i>Rhynchanthera mexicana</i>	Mexico	F. Ferreira	2002
	PREM 58794	CMW 12734, CMW 12736	<i>R. mexicana</i>	Mexico	F. Ferreira	2002
	PREM 58795	CMW 13046	<i>Clidemia sericea</i>	Mexico	F. Ferreira	2002
	PREM 58796	CMW 12471	<i>C. sericea</i>	Mexico	F. Ferreira	2002
	PREM 58797	CMW 12745	<i>Tibouchina urvilleana</i>	Singapore	M.J. Wingfield	Apr. 2003
	PREM 58798	CMW 16192	<i>Melastoma malabathricum</i>	Lake Toba, Aek Nauli, Sumatra	M.J. Wingfield	Feb. 2004
<i>C. inopina</i> (holotype designated here)	PREM 58799	CMW 18515	<i>M. malabathricum</i>	Lake Toba, Sumatra	M.J. Wingfield	May 2005
	PREM 58800	CMW 12727, CMW 12729, CMW 12731	<i>T. lepidota</i>	Pereira, Colombia	R. Arbelaez	Jan. 2003

a PREM, National Collection of Fungi (Pretoria); CMW = Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria; CMW = Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria.



**Fig 4** - Light micrographs of *Chrysosporthe inopina* from Colombia (from holotype PREM 58800). (A) Black perithecial neck and orange stromatic tissue (arrow) of ascostroma on bark. (B) Vertical section through ascoma. (C) Stromatic tissue of ascostroma. (D) Perithecial neck and surrounding tissue (arrow). (E) Ascus. (F) Ascospores. (G) Conidiomata on bark. (H) Vertical section through conidioma. (I) Tissue of the conidiomal base. (J) Tissue of conidiomal neck. (K) Conidiophores. (L) Conidia. Bars of A-B and G-H = 100  $\mu\text{m}$ ; C-D and I-J = 20  $\mu\text{m}$ ; E-F and K-L = 10  $\mu\text{m}$ .



**Fig 5** – Line drawings of *Chrysosporthe inopina* (from holotype PREM 58800). (A) Shape of ascoma on bark. (B) Section through ascoma. (C) Asci and ascospores. (D) Shapes of conidiomata on bark. (E) Section through conidioma. (F) Conidiophores, conidiogenous cells and conidia. Bars of A–B and D–E = 100  $\mu$ m; C and F = 10  $\mu$ m.

21–25  $\mu$ m thick (Figs 4B, 5B). Top of perithecial bases covered with cinnamon to orange, predominantly prosenchymatous, stromatic tissue, which is occasionally visible above the bark surface (Figs 4B–C, 5B). Perithecial necks black, periphysate, 77–131  $\mu$ m wide (Figs 4B, 4D, 5B). Necks emerging through the bark covered in umber, stromatic tissue of *textura porrecta*, thus appearing fuscous-black (Figs 4B, 5A–B), extending necks up to 2330  $\mu$ m long, 90–180  $\mu$ m wide. Asci 8-spored, biseriata, unitunicate, free when mature, non-stipitate with a non-amylloid refractive ring, fusoid to ellipsoid, (27.5–)29.5–34(–35.5) fusoid to ellipsoid  $\times$  fusoid to ellipsoid (4.5–)5.5–6.5(–7)  $\mu$ m (Figs 4F, 5C). Ascospores hyaline, one-septate, fusoid to oval, with rounded apices, (4.5–)6–7.5(–8)  $\times$  2.5–3.5  $\mu$ m (Figs 4E, 5C).

Conidiomata eustromatic, superficial to slightly immersed, subulate to pyriform to pulvinate, with neck attenuated or not, usually with one neck per structure (Figs 4G–H, 5D–E), fuscous-black, inside umber when young, conidiomatal base above the bark surface 100–650  $\mu$ m high above level of bark, 70–710  $\mu$ m diam, necks up to 780  $\mu$ m long, 50–190  $\mu$ m wide. Conidiomatal locules with even to convoluted inner surface, occasionally multilocular, single locule connected to 1 or several necks (Figs 4H, 5E). Stromatic tissue of base of *textura globulosa*, walls of outer cells thickened (Fig 4I), neck tissue of *textura porrecta* (Fig 4J). Conidiophores hyaline, with basal cells of irregular shape and (2.5–)3.5–6(–7)  $\times$  2–3.5(–4)  $\mu$ m, branched irregularly at the base or above into cylindrical cells, with or without separating septa, total length of conidiophore

(11–)12.5–22.5(–29.5)  $\mu$ m (Figs 4K, 5F). Conidiogenous cells phialidic, determinate, apical or lateral on branches beneath a septum, cylindrical to flask-shaped with attenuated apices, (1.5–)2–2.5(–3)  $\mu$ m wide, collarette and periclinal thickening inconspicuous (Figs 4K, 4L, 5F). Conidia hyaline, non-septate, oblong, (3–)3.5–4  $\times$  (1.5–)2–2.5  $\mu$ m (Figs 4L, 5F), masses exuded as orange to luteous droplets.

Cultures on MEA (CMW 12727, CMW 12729) white with cinnamon to hazel patches, fluffy with a smooth margin, fast-growing, covering a 90 mm diam plate after a minimum of 5 d at the optimum temperature of 25  $^{\circ}$ C. Cultures rarely sporulating after sub-culturing, teleomorph not produced in culture.

Ecology: Bark of *Tibouchina lepidota*.

Distribution: Colombia

## Discussion

Results of this study encompass reports of several new host species for *Chrysosporthe cubensis* from various parts of the world (Fig 1). Many of these hosts are native to the countries in which they have been found. Thus, isolates of *C. cubensis* residing in the South American sub-clade, originated on native *Clidemia sericea* and *Rhynchanthera mexicana* in Mexico. These collections add to those of *Miconia* spp. that have been reported before as native hosts of *C. cubensis* in Colombia (Rodas et al. 2005). Isolates from the native melastome, *Melastoma malabathricum*, were shown to group in the Southeast Asian clade of *C. cubensis*. This represents the first ever collection of *C. cubensis* from a native plant growing in a natural situation in Southeast Asia. The only other equivalent collection is that of *C. cubensis* from clove collected in Malaysia (Reid & Booth 1969), Sulawesi (Myburg et al. 2003) and elsewhere in Indonesia (Hodges et al. 1986). Although clove occurs naturally in the Molucca islands, which is relatively close to areas in Indonesia and Malaysia where *C. cubensis* has been found to occur, the trees in the latter areas are planted and not native.

Other hosts of *C. cubensis* reported in this study are exotics in the countries where the fungus was collected. *Lagerstroemia indica* is native to China, but is planted worldwide as a garden ornamental. In the case of this study, *C. cubensis* was found on this tree in Cuba. Isolates from this collection in Cuba group in the South American sub-clade of *C. cubensis*, which is the same as collections of the fungus now available from *Eucalyptus* in Cuba. The origin of the fungus on *Eucalyptus* and *L. indica* is not known but it probably originated on a native plant in the area. However, it is also possible that *L. indica* represents a native host of *C. cubensis* in China where the tree is native. If that were the case, isolates of the fungus from China would most probably group in the Southeast Asian sub-clade, as was also found in previous studies (Myburg et al. 1999; Myburg et al. 2002a), where an isolate from *E. camaldulensis* in China grouped in the Southeast Asian clade.

*Tibouchina urvilleana* is a native of South America, but it is also popular as an ornamental in various countries, because of its conspicuous and attractive flowers. In this study, *C. cubensis* was found on *T. urvilleana* planted as a non-native ornamental in Singapore and Thailand. Isolates from these collections group in the Southeast Asian clade of *C. cubensis*.

The fungus is also known to occur on *Eucalyptus* in Thailand (Old et al. 2004). Presumably, the fungus in the area originally came from a native Southeast Asian plant, possibly a species of *Melastoma* of which there are numerous native species in the area. *Tibouchina* spp. are known to be highly susceptible to infection by *Chrysosporthe* spp. (Wingfield et al. 2001; Myburg et al. 2002b; Seixas et al. 2004), and trees planted as exotics could easily become infected, as appears to have occurred in this situation.

Before this study, *Tibouchina* spp. had not been confirmed as hosts of *C. cubensis*. Previous collections from *Tibouchina*, thought to represent *C. cubensis* (Wingfield et al. 2001), have recently been shown to represent *C. hodgesiana* (Gryzenhout et al. 2004). The collections of Seixas et al. (2004) from *Tibouchina* were identified based only on morphological characters and before *Chrysosporthe* was described (Gryzenhout et al. 2004) It is thus unclear whether these represent *C. cubensis* or one of the related fungi such as *C. hodgesiana* or *C. inopina*, described in this study. However, it is likely that *C. cubensis* occurs on native *Tibouchina* spp. in South America due to the susceptibility of these trees to *Chrysosporthe* spp.

The collection of *C. cubensis* from native *M. malabathricum* supports the view that Southeast Asia could be an area of origin for *C. cubensis*. The fact that the isolates group in the discrete Southeast Asian clade of the fungus, however, supports the DNA-based view that there are two discrete populations of the fungus. Isolates representing both clades have now been found on native plants (species of *Melastoma*, *Miconia*, *Clidemia* and *Rhynchanthera*) and non-native plants (*S. aromaticum*, *Eucalyptus*, *Lagerstroemia*, *Tibouchina*) in two geographically distant parts of the world. Either of these areas could represent the area of origin of *C. cubensis*, or the isolates might represent two discrete taxa that have speciated recently.

Ideally, isolates from various hosts should be included in studies at the population level aimed at determining the worldwide population structure and origin of *C. cubensis sensu lato*. However, such studies are restricted by the difficulty of obtaining sufficient numbers of isolates from native hosts in the various countries where they are known to occur. Although there is substantial phylogenetic support for separating the isolates of the two sub-clades of *C. cubensis* into two taxa, these fungi appear to be morphologically identical. They also share hosts in the two regions in which they occur. We thus believe that a population-based species concept is required if these isolates from the two regions are to be treated as discrete taxa. Such studies will be greatly aided by evidence presented in this study that the type specimen of *C. cubensis* phylogenetically represents the South American form of the fungus. This was previously unknown and the question could only be resolved with collections of *C. cubensis* from Cuba. These specimens and cultures, including an epitype linked to them, should provide a robust basis for future studies.

It is evident from this study that *C. cubensis* is able to infect plants from at least three different families, although all of these are in *Myrtales*. The families include *Myrtaceae*, *Melastomataceae* and *Lythraceae* and they represent a wide diversity of morphological and physiological characteristics (Dahlgren & Thorne 1984; Conti et al. 1997). Even within the families, *C. cubensis* is able to infect plants belonging to different tribes, e.g. *Syzygiaceae* and *Eucalypteae* in *Myrtaceae* (Wilson et al. 2005),

and *Miconiaceae* and *Melastomeae* in *Melastomataceae* (Clausing & Renner 2001). Furthermore, artificial inoculations on members of several additional families in *Myrtales* have shown that they are also susceptible to infection by *Chrysosporthe* spp., although these have not yet been found as natural hosts (Hodges et al. 1986, Seixas et al. 2004). These hosts include members of *Rhizophoraceae*, *Combretaceae*, *Onagraceae*, *Punicaceae*, *Sapotaceae* and *Lauraceae*, which represent distinct phylogenetic groups within *Myrtales* (Conti et al. 1996; Conti et al. 1997). Only a limited number of genera in each of these families has been tested (Seixas et al. 2004), and it is likely that additional hosts of *C. cubensis* will be discovered.

Phylogenetic analyses in this study have revealed an unexpected and closely related new sub-clade in the larger *Chrysosporthe* group. This is the phylogenetic sub-clade accommodating isolates from *T. lepidota* in Colombia. The distinct grouping of the sub-clade from Colombia is clearly supported with morphological characteristics and we were able to describe the new species *C. inopina*. Fruiting structures of *C. inopina* contained asci longer than those of *C. cubensis*, and ascospores wider than those for all other species of *Chrysosporthe*.

When *C. inopina* was first discovered, our view was that it might represent the teleomorph of *C. hodgesiana*, or a first report of *C. cubensis* on *Tibouchina* spp. in South America. This is because no teleomorph is known for *C. hodgesiana*, and *Tibouchina* is not a confirmed host for *C. cubensis* (Gryzenhout et al. 2004). DNA sequences of these isolates and morphological comparisons, however, showed clearly that the fungus represents an undescribed taxon. *C. inopina* thus represents a third species of *Chrysosporthe* on native *Melastomataceae* in Colombia, with the other two species *C. cubensis* (Rodas et al. 2005) and *C. hodgesiana* (Gryzenhout et al. 2004; Rodas et al. 2005). Of these, both *C. inopina* and *C. hodgesiana* occur on *Tibouchina* spp. and in the same area. It is thus evident that a complex of species, closely related and morphologically similar, occurs on these native trees in South America. Extreme care should thus be taken when collecting and identifying these fungi, especially where work is related to quarantine and disease management, which is clearly important for fungi such as *C. cubensis*.

*C. cubensis* was previously known almost exclusively as a *Eucalyptus* canker pathogen, but this and other recent studies (Wingfield et al. 2001; Myburg et al. 2002b; Gryzenhout et al. 2004; Rodas et al. 2005) have reported on new and diverse hosts for the fungus. These studies have radically changed our perception of the ecology of *C. cubensis*. Furthermore, besides *C. cubensis*, there are many closely related and morphologically similar species that have previously been treated under the older name as representing *Cryphonectria cubensis*. Some of these species in *Chrysosporthe*, such as *C. cubensis*, *C. austroafricana* and *C. doradensis*, are important pathogens of *Eucalyptus*, but all species appear to be pathogens of members of *Myrtales*. They should thus be considered as serious potential pathogens that could cause devastating diseases if they were to be accidentally introduced into new areas with large populations of native, susceptible plants. For example, *C. austroafricana*, apparently native to South Africa (Heath et al. 2006), causes a serious disease of *Eucalyptus* in that country and represents a significant threat to native forests of *Eucalyptus* in

Australia (Roux *et al.* 2003; Wingfield 2003). These relatively unknown and newly discovered fungi deserve more attention from pathologists and quarantine authorities.

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