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Discovery of the *Eucalyptus* canker pathogen *Chrysoporthe cubensis* on native *Miconia* (Melastomataceae) in Colombia

C. A. Rodas[a]‡, M. Gryzenhout[a]*†, H. Myburg[b], B. D. Wingfield[b] and M. J. Wingfield[a]

[a]*Department of Microbiology and Plant Pathology, and* [b]*Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa*

Chrysoporthe cubensis is one of the most serious canker pathogens on commercially grown *Eucalyptus* species in the tropics and subtropics. During recent surveys for native hosts of *C. cubensis* in Colombia, fungi with fruiting structures similar to those of *C. cubensis* were found on native *Miconia theaezans* and *Miconia rubiginosa*, both members of the Melastomataceae. These fungi were identified based on morphology and DNA sequences of the ITS1/ITS2 region of the rDNA operon and the β-tubulin genes. The majority of isolates from *M. theaezans* and *M. rubiginosa* grouped together with South American *C. cubensis* isolates from *Eucalyptus* species and *Syzygium aromaticum* (clove). However, some of the isolates from *M. theaezans* grouped with isolates of *Chrysoporthella hodgesiana*, another anamorph species linked to *Chrysoporthe*, from *Tibouchina* spp. in Colombia. Pathogenicity of these fungi was assessed on various Melastomataceae. *Miconia rubiginosa* was more susceptible to infection by *C. cubensis* than two *Eucalyptus* clones. Isolates of *C. cubensis* and *Chrysop. hodgesiana* were mildly pathogenic on the various hosts included in the pathogenicity trials, and most pathogenic on *Tibouchina urvilleana* and *Tibouchina lepidota*.

Keywords: *Chrysoporthe cubensis*, *Chrysoporthella hodgesiana*, Colombia, *Diaporthales*, *Miconia rubiginosa*, *Miconia theaezans*

Introduction

Chrysoporthe cubensis, previously known as *Cryphonectria cubensis* (Gryzenhout *et al*., 2004), is one of the most serious pathogens of *Eucalyptus* spp. (Myrtaceae) in various South American countries (Boerboom & Maas, 1970; Hodges *et al*., 1976, 1979; Hodges, 1980), including Colombia (Van der Merwe *et al*., 2001). The associated canker disease has also been reported from other parts of the world with tropical or subtropical climates, mostly Africa (Gibson, 1981; Myburg *et al*., 2003; Roux *et al*., 2003), Southeast Asia (Sharma *et al*., 1985; Florence *et al*., 1986; Hodges *et al*., 1986; Myburg *et al*., 2003), Hawaii (Hodges *et al*., 1979; Myburg *et al*., 2003) and Australia (Davison & Coates, 1991; Myburg *et al*., 1999, 2003). In these regions, canker caused by *C. cubensis* is most severe in areas with high rainfall and temperature (Boerboom & Maas, 1970; Hodges *et al*., 1976, 1979; Sharma *et al*., 1985).

Cankers caused by *C. cubensis* are usually found at the base or on the lower stems of trees, but they may also occur higher up on the trunks (Hodges *et al*., 1976, 1979; Sharma *et al*., 1985). The pathogen kills the cambium, and in severe cases can result in tree death (Hodges *et al*., 1976, 1979; Sharma *et al*., 1985). The only practical management option for the disease is to plant resistant *Eucalyptus* species and clones (Hodges *et al*., 1976; Alfenas *et al*., 1983; Sharma *et al*., 1985).

Until recently, *C. cubensis* was known as *Cryphonectria cubensis*, and the disease that it causes was commonly referred to as cryphonectria canker of *Eucalyptus* (Wingfield, 2003; Gryzenhout *et al*., 2004). The fungus was transferred to the new genus *Chrysoporthe*, distinct from *Cryphonectria*, based on phylogenetic groupings arising from comparisons of ribosomal operon and β-tubulin gene sequences (Gryzenhout *et al*., 2004; Myburg *et al*., 2004). Species of *Chrysoporthe* are also morphologically distinct from *Cryphonectria* and are characterized by their dark-coloured, pyriform conidiomata and extending perithecial necks, covered in dark tissue (Gryzenhout *et al*., 2004; Myburg *et al*., 2004). *Cryphonectria* species have orange, pulvinate conidiomata, and perithecial necks extending

*To whom correspondence should be addressed.

†E-mail: Marieka.Gryzenhout@fabi.up.ac.za

‡Current address: Smurfit Cartón de Colombia, Investigación Forestal, Carrera 3 no. 10–36, Cali, Valle, Colombia.

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from the stromatal surface are covered in orange tissue (Myburg *et al*., 2004).

In addition to *C. cubensis*, two other species have been described in *Chrysoporthe* (Gryzenhout *et al*., 2004). Isolates of the fungus previously known as *Cry. cubensis* from South Africa, have been named *Chrysoporthe austroafricana*, and isolates from *Tibouchina* spp. in Colombia are known as *Chrysoporthella hodgesiana*. The latter species could not be described in *Chrysoporthe* because no teleomorph is known for it. However, DNA sequence data clearly show that *Chrysop. hodgesiana* resides in *Chrysoporthe*, and it was thus necessary to describe the new anamorph genus, *Chrysoporthella*, to accommodate this species (Gryzenhout *et al*., 2004).

Until recently, *C. cubensis* has been known to occur only on trees belonging to the Myrtaceae. These hosts are predominantly species of *Eucalyptus* and also include clove (*Syzygium aromaticum*) (Hodges *et al*., 1986). *C. cubensis* also occurs naturally on strawberry guava (*Psidium cattleianum*) (Hodges, 1988), but it is not known whether isolates from this host reside in the same phylogenetic group as other isolates of *C. cubensis* from South America.

Previously, *C. cubensis* was believed to be present on *Tibouchina urvilleana* and *Tibouchina lepidota* in Colombia, which are members of the Melastomataceae native to South America (Wingfield *et al*., 2001). More recent studies have shown that the fungus considered by Wingfield *et al*. (2001) represents *Chrysop. hodgesiana* and not *C. cubensis* (Gryzenhout *et al*., 2004). There has been a subsequent report of a fungus resembling *C. cubensis* on *Tibouchina granulosa* in Brazil (Seixas *et al*., 2004). However, it is not yet known whether this fungus from Brazil represents *C. cubensis* or *Chrysop. hodgesiana*. Furthermore, *C. austroafricana* is also known to occur on ornamental *T. granulosa* in South Africa (Myburg *et al*., 2002a).

Recent surveys of cankers on native Melastomataceae in Colombia have led to the discovery of fungi resembling *Chrysoporthe* spp. on a number of tree species that have not previously been implicated as natural hosts. The aim of this study was to identify these fungi based on morphology and DNA sequences. Pathogenicity of representative isolates was also tested on the hosts of origin and on *Eucalyptus grandis*.

Materials and methods

Symptoms and collection of samples

Disease surveys were conducted in two areas of Colombia having a wide range of different altitudes and precipitation (Fig. 1). Specimens were collected from *Miconia theaezans* (niguito) in a natural forest, with no *Eucalyptus* plantations nearby, alongside the farm La Selva [5°35′34″W and 4°47′26″N, 3143 mm year^{-1}, 2048 masl (m above sea level)]. This farm, belonging to Smurfit Carton de Colombia, is situated near the city of Pereira (Risaralda province). Cankers covered in conidiomata and ascomata were also found on *M. rubiginosa* trees (mortiño) of different ages on the farm Vanessa (76°35′15″W and 3°5′42″N, 2365 mm year^{-1}, 1000 masl), near the city of Timba (Cauca province). These trees were coppiced and occurred within a *Eucalyptus* plantation where *C. cubensis* has previously been collected (Van der Merwe *et al*., 2001).

Figure 1 Map of Colombia showing the farms where *Chrysoporthe cubensis* was discovered on various Melastomataceae, and where field trials were conducted.

Disease symptoms on the *Miconia* spp. included branch die-back, and cankers on branches, trunks or the tree bases that often resulted in the death of trees or tree parts. In the case of *M. rubiginosa*, trees were not killed by the disease but many *E. grandis* trees in plantations adjacent to naturally occurring *M. rubiginosa* were seriously affected by cankers. Cankers on the *Miconia* spp. were generally associated with parts of plants where branches and stems were physically wounded. Fruiting structures were abundant around the edges of the actively growing canker margins.

Specimens collected from cankers were transported to the laboratory for further analysis. Isolations from single conidia were made from the fruiting structures using malt extract agar MEA (20 g L^{-1} malt extract agar, Biolab). Isolates used in this study have been preserved at 5°C in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, and representative isolates have also been deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands (Table 1). The original bark specimens from which isolations were made have been deposited (Table 2) in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM).

Table 1 Isolates of *Chrysoporthe*, *Chrysoporthella* and *Cryphonectria* spp. included in this study

Isolate number[a]	Alternative isolate number[a]	Species identity	Host	Origin	Collector	GenBank accession numbers[b]
CMW 1856	–	*Chrysoporthe cubensis*	*Eucalyptus* sp.	Kauai, Hawaii	–	AY 083999, AY 084010, AY 084022
CMW 9903	–	*C. cubensis*	*Syzygium aromaticum*	Kalimantan, Indonesia	C. S. Hodges	AF 292044, AF 273066, AF 273461
CMW 11288	CBS 115736	*C. cubensis*	*Eucalyptus* sp.	Indonesia	M. J. Wingfield	AY 214302, AY 214230, AY 214266
CMW 11289	CBS 115737	*C. cubensis*	*Eucalyptus* sp.	Indonesia	M. J. Wingfield	AY 214303, AY 214231, AY 214267
CMW 11290	CBS 115738	*C. cubensis*	*Eucalyptus* sp.	Indonesia	M. J. Wingfield	AY 214304, AY 214232, AY 214268
CMW 8650	CBS 115719	*C. cubensis*	*S. aromaticum*	Sulawesi, Indonesia	M. J. Wingfield	AY 084001, AY 084013, AY 084024
CMW 8651	CBS 115718	*C. cubensis*	*S. aromaticum*	Sulawesi, Indonesia	M. J. Wingfield	AY 084002, AY 084014, AY 084026
CMW 10774	–	*C. cubensis*	*S. aromaticum*	Zanzibar, Tanzania	–	AF 492130, AF 492131, AF 492132
CMW 2631	–	*C. cubensis*	*Eucalyptus marginata*	Australia	E. Davison	AF 543823, AF 543824, AF523825
CMW 2632	–	*C. cubensis*	*E. marginata*	Australia	E. Davison	AF 046893, AF 273078, AF 375607
CMW 10453	CBS 505.63	*C. cubensis*	*Eucalyptus saligna*	Democratic Republic of Congo	–	AY 063476, AY 063478, AY 063480
CMW 10669	CBS 115751	*C. cubensis*	*Eucalyptus* sp.	Republic of Congo	J. Roux	AF 535122, AF 535124, AF 535126
CMW 10671	CBS 115752	*C. cubensis*	*Eucalyptus* sp.	Republic of Congo	J. Roux	AF 254219, AF 254221, AF 254223
CMW 8757	–	*C. cubensis*	*Eucalyptus* sp.	Venezuela	M. J. Wingfield	AF 046897, AF 273069, AF 273464
CMW 1853	–	*C. cubensis*	*S. aromaticum*	Brazil	–	AF 046891, AF 273070, AF 273465
CMW 10778	CBS 115755	*C. cubensis*	*S. aromaticum*	Brazil	C. S. Hodges	AY 084006, AY 084018, AY 084030
CMW 9432	CBS 115724	*C. cubensis*	*Eucalyptus grandis*	Mexico	M. J. Wingfield	AY 692321, AY 692324, AY 692323
CMW 10638[c]	CBS 115746	*C. cubensis*	*E. grandis*	Colombia	C. A. Rodas	AY 956966, AY 956971, AY 956972
CMW 10639	CBS 115747	*C. cubensis*	*E. grandis*	Colombia	C. A. Rodas	AY 263419, AY 263420, AY 263421
CMW 10640[c]	CBS 115748	*C. cubensis*	*E. grandis*	Colombia	C. A. Rodas	AY 956967, AY 956973, AY 956974
CMW 9996[c]	CBS 115731	*C. cubensis*	*M. rubiginosa*	Colombia	C. A. Rodas	AY 214292, AY 214220, AY 214256
CMW 10022[c]	–	*C. cubensis*	*M. rubiginosa*	Colombia	C. A. Rodas	AY 262389, AY 262393, AY 262397
CMW 10024[c]	CBS 115739	*C. cubensis*	*M. rubiginosa*	Colombia	C. A. Rodas	AY 262390, AY 262394, AY 262398
CMW 10025[c]	–	*C. cubensis*	*M. rubiginosa*	Colombia	C. A. Rodas	AY 214293, AY 214221, AY 214257
CMW 10026[c]	–	*C. cubensis*	*M. rubiginosa*	Colombia	C. A. Rodas	AY 214294, AY 214222, AY 214258
CMW 10028[c]	–	*C. cubensis*	*M. rubiginosa*	Colombia	C. A. Rodas	AY 214295, AY 214223, AY 214259
CMW 9980[c]	–	*C. cubensis*	*Miconia theaezans*	Colombia	C. A. Rodas	AY 214297, AY 214225, AY 214261
CMW 9993[c]	CBS 115728	*C. cubensis*	*M. theaezans*	Colombia	C. A. Rodas	AY 214298, AY 214226, AY 214262
CMW 62	–	*Chrysoporthe austroafricana*	*E. grandis*	South Africa	M. J. Wingfield	AF 292041, AF 273063, AF 273458
CMW 2113	CBS 112916	*C. austroafricana*	*E. grandis*	South Africa	M. J. Wingfield	AF 046892, AF 273067, AF 273462
CMW 8755	–	*C. austroafricana*	*E. grandis*	South Africa	M. J. Wingfield	AF 292040, AF 273064, AF 273458
CMW 9327	CBS 115843	*C. austroafricana*	*Tibouchina granulosa*	South Africa	M. J. Wingfield	AF 273473, AF 273060, AF 273455
CMW 9328	–	*C. austroafricana*	*T. granulosa*	South Africa	M. J. Wingfield	AF 273474, AF 273061, AF 273456
CMW 9932	–	*C. austroafricana*	*T. granulosa*	South Africa	M. J. Wingfield	AF 273472, AF 273062, AF 273457
CMW 9927	–	*Chrysoporthella hodgesiana*	*Tibouchina urvilleana*	Colombia	C. A. Rodas & M. J. Wingfield	AF 265653, AF 292034, AF 292037
CMW 9928	–	*Chrysop. hodgesiana*	*T. urvilleana*	Colombia	C. A. Rodas & M. J. Wingfield	AF 265654, AF 292035, AF 292038
CMW 9929	–	*Chrysop. hodgesiana*	*T. urvilleana*	Colombia	C. A. Rodas & M. J. Wingfield	AF 265656, AF 292036, AF 292039
CMW 10641	CBS 115854	*Chrysop. hodgesiana*	*Tibouchina semidecandra*	Colombia	R. Arbaleaz	AY 692322, AY 692326, AY 692325
CMW 9994[c]	CBS 115729	*Chrysop. hodgesiana*	*T. semidecandra*	Colombia	R. Arbelaez	AY 956968, AY 956975, AY 956976

Table 1 *Continued*

Isolate number[a]	Alternative isolate number[a]	Species identity	Host	Origin	Collector	GenBank accession numbers[b]
CMW 9995[c]	CBS 115730	*Chrysop. hodgesiana*	*T. semidecandra*	Colombia	R. Arbelaez	AY 956969, AY 956977, AY 956978
CMW 10625[c]	CBS 115744	*Chrysop. hodgesiana*	*M. theaezans*	Colombia	C. A. Rodas	AY 956970, AY 956979, AY 956980
CMW 10626[c]	CBS 115745	*Chrysop. hodgesiana*	*M. theaezans*	Colombia	C. A. Rodas	AY 262392, AY 262396, AY 262400
CMW 1652	CBS 112914	*Cryphonectria parasitica*	*Castanea dentata*	U.S.A.	–	AF 046902, AF 273075, AF 273468
CMW 10518	CBS 112919	*Cryphonectria nitschkei*	*Quercus* sp.	Japan	T. Kobayashi	AF 452118, AF 525706, AF 525713
CMW 10463	CBS 112920	*Cryphonectria macrospora*	*Castanopsis cuspidata*	Japan	T. Kobayashi	AF 368331, AF 368351, AF 368350

[a]CMW, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
[b]Accession numbers refer to sequence data of the ITS, β-tubulin 1 (primers Bt1a/1b) and β-tubulin 2 (primers Bt2a/2b) regions, respectively.
[c]Isolates sequenced in this study.

Table 2 Specimens of *Chrysoporthe cubensis* used in morphological comparisons

Identity	Herbarium no.[a]	Linked culture[b,c]	Host	Origin	Date	Collector
Chrysoporthe cubensis	PREM 57294	CMW 10639	*Eucalyptus grandis*	Vanessa, Colombia	2000	M. J. Wingfield
C. cubensis	PREM 57517	CMW 2357	*Miconia rubiginosa*	Vanessa, Colombia	2001	C. A. Rodas
	PREM 58307	CMW 9996				
	PREM 58308	CMW 10025				
	PREM 58309	CMW 10026				
	PREM 58311	CMW 10028				
	PREM 58312	CMW 10022				
	PREM 58313	CMW 10024				
	PREM 58314	–				

[a]PREM, National Collection of Fungi, Pretoria, South Africa.
[b]CMW refers to the culture collections of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa.
[c]Isolates did not originate from the PREM specimens, but both specimens and isolates were collected from the same location and host.

DNA sequence comparisons

Isolates from *Miconia* spp. and *E. grandis* in Colombia were included in the DNA sequence comparisons (Table 1). Previously characterized *C. cubensis* isolates from *Eucalyptus* spp. (Myburg *et al*., 2002b; Gryzenhout *et al*., 2004) and *S. aromaticum* (Myburg *et al*., 1999, 2003) from different parts of the world, were included for comparative purposes. Isolates of *Chrysop. hodgesiana* from *T. urvilleana* (Wingfield *et al*., 2001; Gryzenhout *et al*., 2004) and *C. austroafricana* from *Eucalyptus* spp. and *T. granulosa* (Myburg *et al*., 2002a, 2002b) were also included. Species of the closely related *Cryphonectria*, namely *Cry. parasitica*, *Cry. nitschkei* and *Cry. macrospora*, were included as outgroup taxa to root the phylogenetic trees.

Isolates for DNA sequence comparisons were grown in malt extract broth (20 g L^{-1} malt extract). DNA was extracted from mycelium as described in Myburg *et al*. (1999). The internal transcribed spacer (ITS) regions ITS1 and ITS2 as well as the conserved 5·8S gene of the ribosomal RNA (rRNA) operon, and two regions within the β-tubulin genes were amplified using the primer pairs and reaction conditions as given by Myburg *et al*. (1999) and Myburg *et al*. (2002b), respectively. PCR products were visualized on ethidium bromide-stained 1% agarose gels, using a UV light. Purification of PCR products was done using a QIAquick PCR Purification Kit (Qiagen GmbH, 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, 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., California, USA) software. Sequences were added to the existing dataset (S 1211, M 2095) of Gryzenhout *et al*. (2004) and manually aligned. Phylogenetic trees were inferred using PAUP (Phylogenetic Analysis Using Parsimony) version 4·0b (Swofford, 1998). A 500 replicate partition homogeneity test (PHT) was done on the rRNA and β-tubulin gene sequence data sets (after the exclusion of uninformative sites) to determine whether they could be analysed collectively (Farris *et al*., 1994).

A phylogenetic tree was inferred from distance analyses. The correct model for the datasets was found with MODELTEST version 3·5 (Posada & Crandall, 1998), namely the HKY85 model (Hasegawa *et al*., 1985) with the gamma distribution shape parameter set to 0·2202 (HKY + G). A 1000 replicate bootstrap 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 and accession numbers are listed in Table 1.

Morphology

For morphological identifications, fruiting structures from bark specimens were sectioned and studied under the microscope according to the method of Gryzenhout *et al*. (2004). Colour notations of Rayner (1970) were used. Growth studies were also conducted because *Chrysop. hodgesiana* can only be distinguished from *C. cubensis* based on its optimal growth temperature (25°C): *C. cubensis* and *C. austroafricana* grow optimally at 30°C (Gryzenhout *et al*., 2004). Growth rate of representative isolates (CMW 10625, CMW 10626, CMW 10022) from the *Miconia* spp. was therefore compared in culture to isolates from *E. grandis* (CMW 10638, CMW 10640) and *T. semidecandra* (CMW 9994, CMW 9995) to confirm the identifications based on DNA sequence comparisons. For the comparisons of growth in culture, the procedure described by Gryzenhout *et al*. (2004) was used, except that the temperature range tested in the current study was from 20°C to 35°C.

Pathogenicity tests

Glasshouse inoculation trials

Three isolates (CMW 10638, CMW 10639, CMW 10640) of *C. cubensis* from *E. grandis* in Colombia and two isolates (CMW 10625, CMW 10626) of *Chrysop. hodgesiana* from *M. theaezans* were screened for pathogenicity on 7-month-old *T. urvilleana* plants in a contained glasshouse with natural light at ~25°C. Trees were planted in plastic containers, watered frequently and fertilized when necessary (N-P-K and Boron; 70 and 10 g, respectively, per tree). Five trees were inoculated with each of the test fungi and an equal number of trees were inoculated with sterile water agar (WA; 20 g L^{-1}) plugs. These WA inoculated trees served as negative controls. Inoculations were done by removing a plug of bark at a constant height (~30 cm above the ground) with a cork borer (9 mm diameter) to expose the cambium. Agar discs of the same size were taken from the edges of actively growing cultures and placed inside the wounds with the mycelium facing downwards. The agar discs were covered with tissue paper moistened with sterile water, and secured with masking tape, to reduce desiccation of the inoculum. The masking tape was removed after 10 days.

Trees were inoculated in October 2001 and lesion development was evaluated after 4 weeks. Lesions were exposed by scraping away the bark and the lengths of the lesions were measured. Re-isolations were done from the lesions. A pathogenic isolate of *C. cubensis* from *E. grandis* and an isolate of *Chrysop. hodgesiana* from *M. theaezans* (CMW 10639 and CMW 10625, respectively) were selected for subsequent field inoculation trials.

In a second glasshouse trial, two *C. cubensis* isolates (CMW 10022, CMW 10024) from *M. rubiginosa* were inoculated on *T. urvilleana* and *E. grandis* (clone ZG14), which were 17–24 months old and up to 1·8 m high. A highly pathogenic isolate of *C. austroafricana* from South Africa (CMW 2113), used in previous pathogenicity studies (Van Heerden & Wingfield, 2001, 2002; Myburg *et al*., 2002a), was included for comparative purposes. Inoculation procedures were the same as those in the first glasshouse trial and 10 trees were inoculated for each of the three test isolates and for the negative control using WA discs. Inoculations were done as described above, except that a cork borer with a diameter of 6 mm was used. The trees were inoculated in May 2002, and evaluated in June 2002.

Field inoculation trials (Colombia)

The first inoculation trial was conducted at Rancho Grande farm (Fig. 1), Restrepo, Valle (76°30′49″W and 3°51′43″N, 1067 mm $year^{-1}$, 1469 masl). This trial included reciprocal inoculations with an isolate of *C. cubensis* from *E. grandis* (CMW 10639) and an isolate of *Chrysop. hodgesiana* from *M. theaezans* (CMW 10625), selected in the first glasshouse trial. Five tree species were used, namely *T. semidecandra*, *T. lepidota*, *T. urvilleana*, *M. theaezans* and a clone of *E. grandis* (clone 274). Trees were 1 year old and 20 of each tree species were inoculated per isolate. An equal number of trees were inoculated with WA discs to serve as negative controls. Inoculations were conducted in a similar way to those for the glasshouse inoculations, but the diameter of the inoculation wound was 4 mm. Trees were inoculated in May 2002 and lesion development was evaluated after 12 weeks. Internal lesion length in the cambium was measured for all field trials and the test fungi were reisolated from the lesions.

The second field trial was at Vanessa farm (Fig. 1), Timba, Cauca province. The *C. cubensis* isolate CMW 10022 from *M. rubiginosa*, shown to be pathogenic in the preliminary glasshouse trial, was used. Twenty three-year-old *E. grandis* trees (clone 275), 20 trees from seeds of a cross between *E. grandis* and *E. urophylla* (*E.* '*urograndis*'

clone 212), and 20 *M. rubiginosa* trees were inoculated. The *M. rubiginosa* trees were approximately 6 years old and formed part of the native vegetation surrounding the commercial plantations. Ten trees of each host were inoculated with uninoculated MEA to serve as negative controls. The trial was initiated in June 2002 and lesion lengths were measured after 12 weeks. The same inoculation techniques used in glasshouse and other field trials were applied, except that the inoculation wounds were 6 mm in diameter.

Data for all pathogenicity trials were analysed using a one-way analysis of variance (ANOVA) with SAS (2000) and did not deviate from normality. For the analyses, trees were randomly assigned, reflecting the experimental design. Scatter plots of the lesion measurements on the 20 trees of a species–clone combination exhibited no dependence. A Levene test for homogeneity of variances was applied throughout and showed that heterogeneity was not a factor in the data.

Results

DNA sequence comparisons

Amplification of the ITS1, 5·8S and ITS2 rRNA regions as well as the two regions in the β-tubulin gene resulted in PCR products of approximately 600 and 550 bp, respectively. The aligned DNA sequence of the partial ITS1/ITS2 region (538 bp) consisted of 472 constant characters, 28 parsimony-uninformative and 38 parsimony-informative characters, while the aligned sequence of the β-tubulin gene regions (894 bp) consisted of 716 constant characters, 71 parsimony-uninformative and 107 parsimony-informative characters. The rRNA and the β-tubulin sequence data sets were not fully congruent in the phylogenetic analyses ($P = 0{\cdot}032$) because the ITS region could not differentiate *C. austroafricana*. However, the datasets were combined following Gryzenhout *et al*. (2004) to strengthen the support of the different clades. The combined data set (1432 bp) consisted of 45 taxa with the *Cry. parasitica*, *Cry. macrospora* and *Cry. nitschkei* isolates as the outgroup (Fig. 2).

The phylogenetic tree (Fig. 2) showed the same four clades as previously characterized (Myburg *et al*., 2002b, 2003; Gryzenhout *et al*., 2004). These clades represented *Chrysop. hodgesiana*, *C. austroafricana*, and the two morphologically identical clades from Southeast Asia/Zanzibar/Hawaii and South America/Congo, respectively, that define *C. cubensis* (Gryzenhout *et al*., 2004). The majority of isolates from *M. theaezans* (CMW 9980, CMW 9993) and *M. rubiginosa* (CMW 9996, CMW 10022, CMW 10024, CMW 10025, CMW 10026, CMW 10028) grouped in the South American/Congolese clade of *C. cubensis* together with isolates (CMW 10638, CMW 10639, CMW 10640) from *E. grandis* in Colombia (bootstrap support = 94%). However, two isolates from *M. theaezans* (CMW 10625, CMW 10626) grouped together with isolates of *Chrysop. hodgesiana* (bootstrap support = 81%).

Morphology

Specimens (PREM 57517, PREM 58307-58309, PREM 58311-58314) for isolates from *M. rubiginosa* (Table 2), which were identified as *C. cubensis* based on DNA sequence data (Fig. 2), were available for study. These specimens contained both anamorph and teleomorph structures similar to specimens from *Eucalyptus* spp. (PREM 57294). These fruiting structures also resembled those previously described for *C. cubensis* (Bruner, 1917; Hodges *et al*., 1979; Hodges, 1980; Myburg *et al*., 2003; Gryzenhout *et al*., 2004).

A comparison of isolates based on growth in culture confirmed results of the phylogenetic analyses. Isolates CMW 10022 (*M. rubiginosa*), CMW 10638 and CMW 10640 (*E. grandis*), which had been identified as representing *C. cubensis*, grew optimally at 30°C and were able to grow at 35°C. *Chrysop. hodgesiana* isolates (CMW 9994, CMW 9995) from *T. semidecandra* displayed optimum growth at 25°C and were not able to grow at 35°C. Isolates CMW 10625 and CMW 10626 (*M. theaezans*) showed the same growth pattern as isolates CMW 9994 and CMW 9995, confirming that they represented *Chrysop. hodgesiana*. These results are consistent with those reported by Gryzenhout *et al*. (2004).

Pathogenicity tests

Glasshouse inoculations

In the first glasshouse trial, inoculation with *C. cubensis* isolates (CMW 10638, CMW 10639, CMW 10640) from *E. grandis* and *Chrysop. hodgesiana* isolates (CMW 10625, CMW 10626) from *M. theaezans* gave rise to distinct lesions (Fig. 3) from which the test isolates could be reisolated. Lesions associated with the most pathogenic of these isolates (CMW 10625, CMW 10638, CMW 10639) were not significantly different from each other, but differed significantly ($P < 0{\cdot}0014$) from the control inoculation. Isolates CMW 10639 (*C. cubensis*) from *E. grandis* and CMW 10625 (*Chrysop. hodgesiana*) from *M. theaezans* were chosen for field inoculations because they were most pathogenic for each species group.

In the second glasshouse trial, *C. cubensis* isolates (CMW 10022, CMW 10024) from *M. rubiginosa* and the South African isolate (CMW 2113) of *C. austroafricana* resulted in different size lesions (Fig. 4). The isolate of *C. austroafricana* (CMW 2113) was more pathogenic on the *E. grandis* clone than the other isolates tested (Fig. 4). This isolate was also less pathogenic on *T. urvilleana* (Fig. 4) than on the *E. grandis* clone. A *C. cubensis* isolate from *M. rubiginosa* (CMW 10024) was more pathogenic on *E. grandis* than on *T. urvilleana* (Fig. 4) and it was also more pathogenic on *E. grandis* than the other isolate from *M. rubiginosa* (CMW 10022). Isolate CMW 10022 from *M. rubiginosa* was equally pathogenic on *E. grandis* and *T. urvilleana* (Fig. 4). All isolates produced lesions significantly larger ($P = 0{\cdot}001$) than those associated with the control inoculations. Only *E. grandis* trees infected by the *C. austroafricana* isolate (CMW

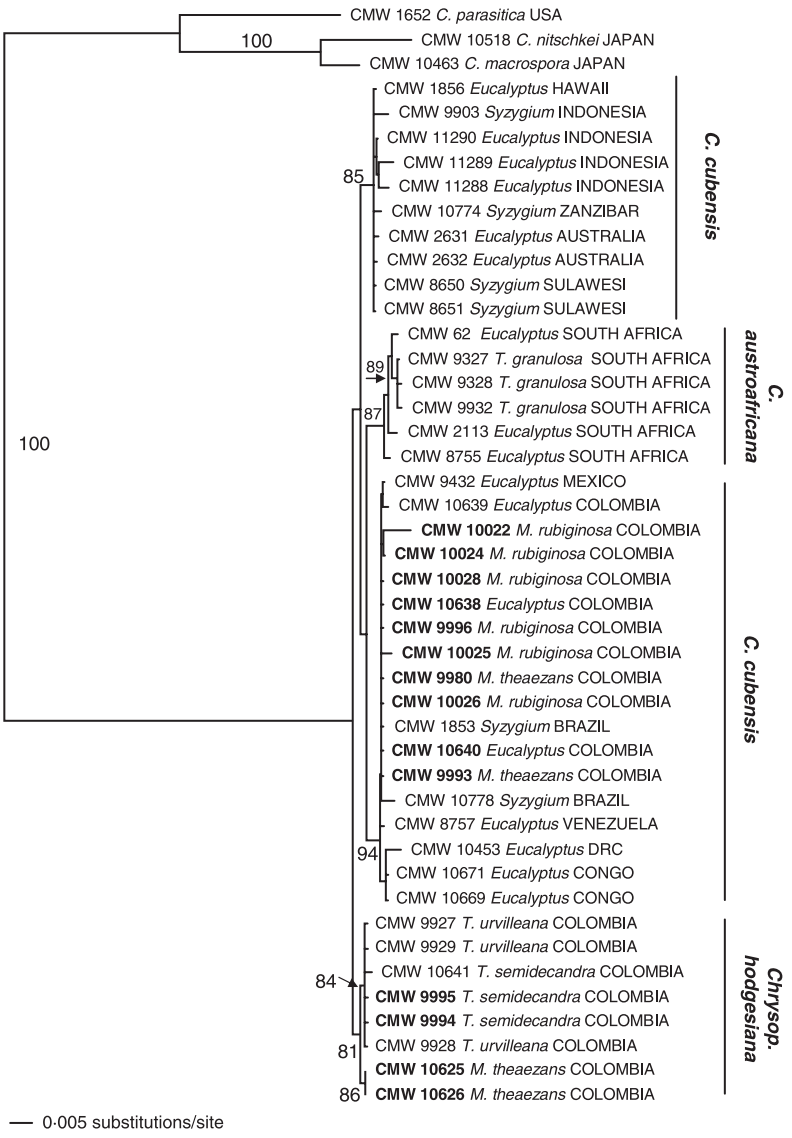

Figure 2 The phylogram generated from a combined data set comprising ribosomal and β-tubulin gene sequences of *Chrysoporthe cubensis*, *C. austroafricana* and *Chrysoporthella hodgesiana*. The phylogram was obtained with distance analyses using the HKY85 parameter model ($G = 0{\cdot}2202$). Confidence levels of the tree branch nodes > 70% are indicated and were determined by a 1000 replicate bootstrap analysis. Isolates sequenced in this study are written in bold together with source host and location. Sequences for *Cryphonectria parastica*, *Cry. nitschkei* and *Cry. macrospora* were used as outgroups.

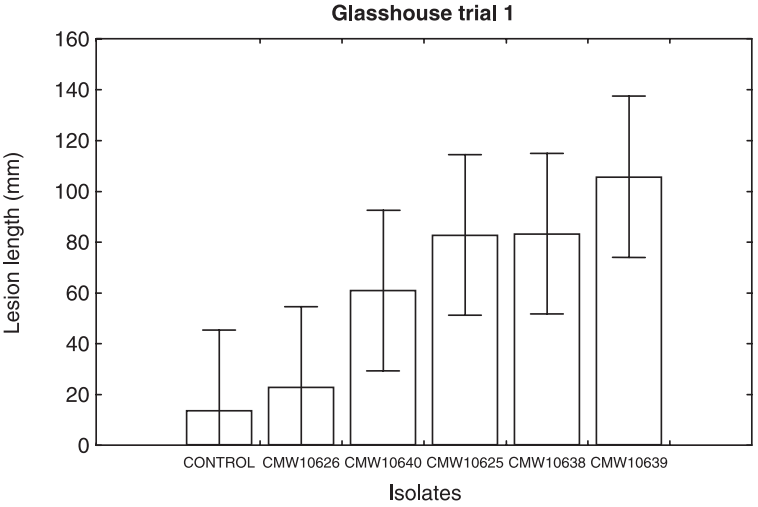

Figure 3 Comparison of lesion extension in 7-month-old *Tibouchina urvilleana* trees under glasshouse conditions. The trees were inoculated with *Chrysoporthella hodgesiana* isolates from *Miconia theaezans* (CMW 10625, CMW 10626) and *Chrysoporthe cubensis* isolates from *Eucalyptus grandis* (CMW 10640, CMW 10638, CMW 10639) in Colombia, and a negative control. Mean length of lesions is shown with 95% confidence limits.

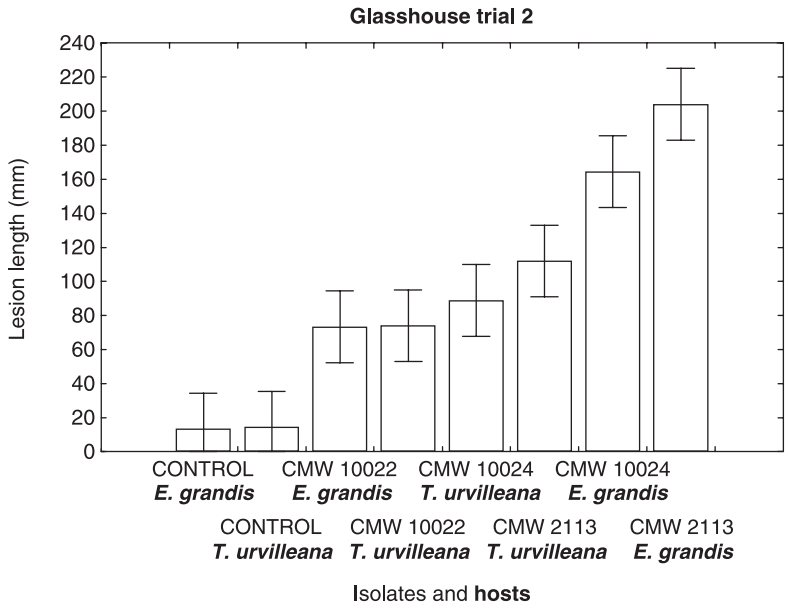

Figure 4 Comparison of lesion extension in 1-year-old trees of *Tibouchina urvilleana* and ZG14 clones of *E. grandis* under glasshouse conditions. Trees were inoculated with *Chrysoporthe cubensis* isolates from *Miconia rubiginosa* (CMW 10022, CMW 10024) and a negative control. A *Chrysoporthe austroafricana* isolate from *Eucalyptus grandis* in South Africa (CMW 2113) was also included. Mean length of lesions is shown with 95% confidence limits.

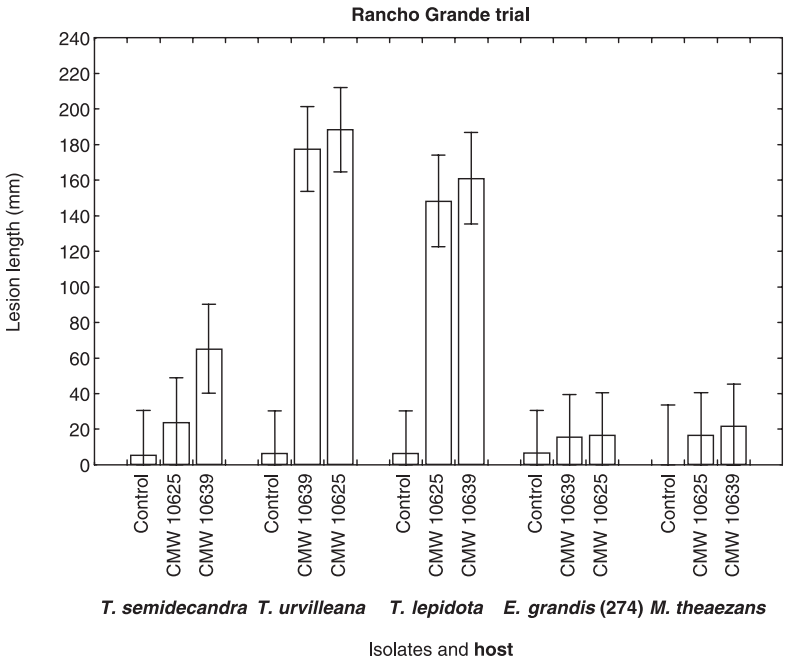

Figure 5 Comparison of lesion extension in 1-year-old trees of *Tibouchina urvilleana*, *Tibouchina lepidota*, *Tibouchina semidecandra*, *Miconia theaezans* and a *Eucalyptus grandis* clone (274) in field inoculations in Colombia. Isolates of *Chrysoporthella hodgesiana* from *M. theaezans* (CMW 10625) and *Chrysoporthe cubensis* from *E. grandis* (CMW 10639) from Colombia, and a negative control were used. Mean lesion length is shown with 95% confidence limits.

2113) produced epicormic shoots below the inoculation points, indicating that the inoculated stems had been girdled.

Field inoculation trials (Colombia)

In the first field trial, lesions were produced on all tree species (*T. urvilleana*, *T. lepidota*, *T. semidecandra*, *M. theaezans*, *E. grandis*) in response to inoculation with the *C. cubensis* isolate CMW 10693 from *E. grandis* and the *Chrysop. hodgesiana* isolate CMW 10625 from *M. theaezans*. The longest lesions were produced on *T. urvilleana* and *T. lepidota*, while lesions on *T. semidecandra*, although smaller, also differed significantly ($P = 0{\cdot}0001$) from those associated with the control inoculations (Fig. 5). Lesions on *M. theaezans* and the *E. grandis* clone were only slightly longer than the control inoculations (Fig. 5). Lesions produced by the *C. cubensis* isolate (CMW 10639) and *Chrysop. hodgesiana* isolate (CMW 10625) were similar in size on each tree species (Fig. 5).

In the second field trial, *M. rubiginosa* trees (Fig. 6) were more susceptible ($P = 0{\cdot}0001$) to the *C. cubensis*

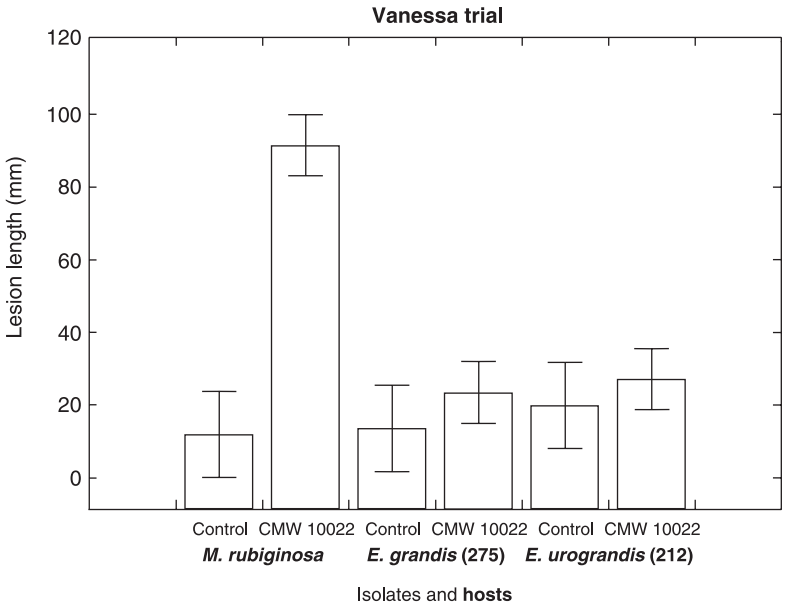

Figure 6 Comparison of lesion extension in 6-year-old *Miconia rubiginosa* trees, and trees of a 3-year-old *Eucalyptus grandis* clone (275) and a *E. urograndis* cross (clone 212), in field inoculations in Colombia. An isolate of *Chrysoporthe cubensis* from *M. rubiginosa* (CMW 10022) and a negative control were used. Mean length of lesions is shown with 95% confidence limits.

isolate from *M. rubiginosa* (CMW 10022) than the *E. grandis* trees tested (Fig. 6). Inoculations with isolate CMW 10022 on the *E. grandis* clone (275) and the hybrid clone (212) gave rise to lesions that did not differ from those of the control inoculations (Fig. 6).

Discussion

This study reports on the first discovery of the *Eucalyptus* canker pathogen *C. cubensis* on native *Miconia* species (Melastomataceae) in Colombia. Isolates of the fungus from *M. theaezans* and *M. rubiginosa* grouped in the subclade that characterizes *C. cubensis* occurring in South America, as defined in previous studies (Myburg *et al*., 1999, 2002b, 2003; Roux *et al*., 2003; Gryzenhout *et al*., 2004). Fungal structures on herbarium specimens linked to these isolates had conidiomata and ascomata typical of *C. cubensis*, and spores were similar in size to those previously reported for this fungus (Hodges, 1980; Myburg *et al*., 2002b, 2003; Gryzenhout *et al*., 2004).

In a previous study, Wingfield *et al*. (2001) reported on the discovery of the *Eucalyptus* canker pathogen, *Cry. cubensis*, on native *Tibouchina* spp. in Colombia. That fungus was shown to be pathogenic to both *Eucalyptus* and *T. urvilleana* trees and it was speculated that *Tibouchina* could represent the host of origin of the fungus. A later study (Gryzenhout *et al*., 2004), including a large set of isolates collected in recent years, has shown that the fungus studied by Wingfield *et al*. (2001) represents *Chrysop. hodgesiana* and not *C. cubensis*. The present study therefore represents the first discovery in South America of *C. cubensis* on native hosts, *M. rubiginosa* and *M. theaezans*, and where the identity of the fungus has been confirmed with DNA sequence comparisons.

Not all of the isolates collected from *Miconia* spp. in this study represent *C. cubensis*. Some isolates from *M. theaezans* were identified as *Chrysop. hodgesiana* based on DNA sequences and cultural characteristics. Two species of *Chrysoporthe* thus appear on this native host in Colombia. Thus far, only one of them, *C. cubensis*, has been found causing cankers on *Eucalyptus*, although *Chrysop. hodgesiana* also appears to have the capability to do so.

Pathogenicity trials in this study included isolates representing both *C. cubensis* and *Chrysop. hodgesiana*. It was interesting that there were no significant differences in pathogenicity between isolates of *C. cubensis* (CMW 10638, CMW 10639, CMW 10640) and *Chrysop. hodgesiana* (CMW 10625, CMW 10626) in either the glasshouse or field trials. Results of this study confirm those of Wingfield *et al*. (2001) where the fungus now known as *Chrysop. hodgesiana* was shown to be able to infect *Eucalyptus* spp., although it has never been found to occur naturally on this host. Both *C. cubensis* and *Chrysop. hodgesiana* appear to represent an equal threat to commercial *Eucalyptus* plantations in Colombia.

Field inoculation trials showed that species of native Melastomataceae in Colombia differ in their susceptibility to infection by *C. cubensis* and *Chrysop. hodgesiana*. In a field trial where five different host species were tested, *T. urvilleana* and *T. lepidota* were the most susceptible to the isolates of both *C. cubensis* and *Chrysop. hodgesiana*. In contrast, *M. theaezans* trees were relatively tolerant to infection. *Tibouchina semidecandra* was less susceptible to infection by *C. cubensis* and *Chrysop. hodgesiana* than the other two species of *Tibouchina*, but it was more susceptible than *M. theaezans*. Generally, these results reflect a high level of susceptibility amongst various species of *Miconia* and *Tibouchina* to infection by *C. cubensis* and *Chrysop. hodgesiana*. *Miconia* and *Tibouchina* are native plants in Colombia and their relative susceptibility to the two pathogens might differ in different regions of the

country. Results of artificial inoculation tests give an indication of susceptibility, but they can also be misleading, and should be viewed within the context of the objectives specified for the inoculation tests.

Results of the pathogenicity trials in this study suggest that *C. cubensis* is more pathogenic on native Melastomataceae, especially *Tibouchina* spp., than on *E. grandis*. It is generally believed that pathogens are less virulent on their native hosts than susceptible exotic species (Leppik, 1970; Newhouse, 1990). Therefore, the *E. grandis* clones used in the trials were expected to be more susceptible to *C. cubensis* than the *Tibouchina* and *Miconia* spp. However, these commercially grown clones have been subjected to intensive selection for resistance to disease in recent years and it is possible that the clones or seed lots chosen for these trials have a high degree of tolerance to the pathogen.

Isolates of *C. cubensis* from native *Miconia* spp. in Colombia could have originated on these trees. However, it is possible that these fungi were introduced into the country and later adapted the capacity to infect native Melastomataceae. In this study, *C. cubensis* was found on *M. theaezans* in native vegetation that was far removed from *Eucalyptus* plantations. It therefore seems more likely that this fungus originated on *Miconia* spp. in Colombia than elsewhere. In the case of the site where *C. cubensis* was found on *M. rubiginosa*, these trees were coppiced when *Eucalyptus* stands were established and have hardly been affected by *C. cubensis*. In contrast, the *Eucalyptus* trees in the area have been seriously damaged by *C. cubensis* and it seems that the origin of *C. cubensis*, in this case, is more likely to be *M. rubiginosa* than *Eucalyptus*. However, resolving the question of original host was not an objective of this study and will need to be answered through a comprehensive genetic analysis of a population of isolates.

The occurrence of *C. cubensis* on *M. theaezans* and *M. rubiginosa*, which are native to South America, suggests that this pathogen could possibly be indigenous to that part of the world. Members of the Melastomataceae are common in South America, Central America, the Caribbean islands and Hawaii (Everett, 1981). The occurrence of *C. cubensis* on species belonging to this family could support the hypothesis (Wingfield *et al*., 2001) that the fungus occurred widely throughout South and Central America and the Caribbean, prior to the widescale planting of *Eucalyptus* species. This would explain prior observations (Hodges *et al*., 1986; Seixas *et al*., 2004) that *Eucalyptus* trees were rapidly infected by the pathogen after planting in South American countries. Besides having a possible origin in South America, several alternative hypotheses on the origin of *C. cubensis* exist (Hodges *et al*., 1986; Seixas *et al*., 2004). Although this study reports on the discovery of a potential original host for *C. cubensis* in South America, more extensive surveys in representative areas of Colombia, and including large numbers of isolates, would be necessary to determine the extent of its occurrence on these native hosts in South America. A constraint to this work is that collecting this material will be difficult due to the sociopolitical climate in this area.

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References

Alfenas AC, Jeng R, Hubbes M, 1983. Virulence of *Cryphonectria cubensis* on *Eucalyptus* species differing in resistance. *European Journal of Forest Pathology* **13**, 197–205.

SAS Institute, 2000. *SAS Statistical Software. SAS/STAT Users Guide*, *Version* 6, 4th edn. Cary, NC, USA: SAS Institute.

Boerboom JHA, Maas PWT, 1970. Canker of *Eucalyptus grandis* and *E. saligna* in Surinam caused by *Endothia havanensis*. *Turrialba* **20**, 94–9.

Bruner SC, 1917. Una enfermedad gangrenosa de los eucaliptos. *Estacion Experimental Agronomica Bulletin* **37**, 1–33.

Davison EM, Coates DJ, 1991. Identification of *Cryphonectria cubensis* and *Endothia gyrosa* from eucalypts in Western Australia using isozyme analysis. *Australasian Plant Pathology* **20**, 157–60.

Everett TH, 1981.*The New York Botanical Garden Illustrated Encyclopedia of Horticulture*, Vol. 7. New York, USA: Garland Publishing, Inc, 2196–7.

Farris JS, Källersjö M, Kluge AG, Bult C, 1994. Testing significance of incongruence. *Cladistics* **10**, 315–9.

Florence EJM, Sharma JK, Mohanan C, 1986. A stem canker disease of *Eucalyptus* caused by *Cryphonectria cubensis* in Kerala. *Kerala Forest Research Institute Scientific Paper* **66**, 384–7.

Gibson IAS, 1981. A canker disease of *Eucalyptus* new to Africa. *FAO, Forest Genetic Resources Information* **10**, 23–4.

Gryzenhout M, Myburg H, Van der Merwe NA, Wingfield BD, Wingfield MJ, 2004. *Chrysoporthe*, a new genus to accommodate *Cryphonectria cubensis*. In: Crous PW, Samson RA, Gams W, Summerbell RC, Boekhout T, de Hoog GS, Stalpers JA, eds. *CBS Centenary: 100 Years of Fungal Biodiversity and Ecology*. Utrecht, The Netherlands: Centraalbureau voor Schimmelcultures. *Studies in Mycology* **50**, 119–42.

Hasegawa M, Kishino H, Yano T, 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22**, 160–74.

Hodges CS, 1980. The taxonomy of *Diaporthe cubensis*. *Mycologia* **72**, 542–8.

Hodges CS, 1988. *Preliminary Exploration for Potential Biology Control Agents for* Psidium cattleianum. Honolulu, Hawaii: Cooperative National Park Resources Studies Unit, University of Hawaii at Manoa. (Technical Report 66.)

Hodges CS, Alfenas AC, Cordell CE, 1986. The conspecificity of *Cryphonectria cubensis* and *Endothia eugeniae*. *Mycologia* **78**, 334–50.

Hodges CS, Geary TF, Cordell CE, 1979. The occurrence of *Diaporthe cubensis* on *Eucalyptus*. Florida, Hawaii and Puerto Rico. *Plant Disease Reporter* **63**, 216–20.

Hodges CS, Reis MS, Ferreira FA, Henfling JDM, 1976. O cancro do eucalipto causado por *Diaporthe cubensis*. *Fitopatalogia Brasileira* **1**, 129–70.

Leppik EE, 1970. Gene centres of plants as sources of disease resistance. *Annual Review of Phytopathology* **8**, 323–40.

Myburg H, Gryzenhout M, Heath R, Roux J, Wingfield BD, Wingfield MJ, 2002a. Cryphonectria canker on *Tibouchina* in South Africa. *Mycological Research* **106**, 1299–306.

Myburg H, Gryzenhout M, Wingfield BD, Stipes RJ, Wingfield MJ, 2004. Phylogenetic relationships of *Cryphonectria* and *Endothia* species, based on DNA sequence data and morphology. *Mycologia* **96**, 990–1001.

Myburg H, Gryzenhout M, Wingfield BD, Wingfield MJ, 2002b. β-tubulin and Histone *H3* gene sequences distinguish *Cryphonectria cubensis* from South Africa, Asia and South America. *Canadian Journal of Botany* **80**, 590–6.

Myburg H, Gryzenhout M, Wingfield BD, Wingfield MJ, 2003. Conspecificity of *Endothia eugeniae* and *Cryphonectria cubensis*: a re-evaluation based on morphology and DNA sequence data. *Mycoscience* **104**, 187–96.

Myburg H, Wingfield BD, Wingfield MJ, 1999. Phylogeny of *Cryphonectria cubensis* and allied species inferred from DNA analysis. *Mycologia* **91**, 243–50.

Newhouse JR, 1990. Chestnut Blight. *Scientific American* **July**, 74–9.

Posada D, Crandall KA, 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–8.

Rayner RW, 1970. *A Mycological Colour Chart*. Kew, Surrey, UK: Commonwealth Mycological Institute and British Mycological Society.

Roux J, Myburg H, Wingfield BD, Wingfield MJ, 2003. Biological and phylogenetic analyses suggest that two *Cryphonectria* spp. cause cankers of *Eucalyptus* in Africa. *Plant Disease* **87**, 1329–32.

Seixas CDS, Barreto RW, Alfenas AC, Ferreira FA, 2004. *Cryphonectria cubensis* on an indigenous host in Brazil: a possible origin for eucalyptus canker disease? *Mycologist* **18**, 39–45.

Sharma JK, Mohanan C, Florence EJM, 1985. *Disease Survey in Nurseries and Plantations of Forest Tree Species Grown in Kerala*. Kerala, India: Kerala Forest Research Institute. (Research Report 36.)

Swofford DL, 1998. PAUP. *Phylogenetic Analysis Using Parsimony, Version 4.0b1*. Sunderland, MA, USA: Sinauer Associates.

Van der Merwe NA, Myburg H, Wingfield BD, Rodas C, Wingfield MJ, 2001. Identification of *Cryphonectria cubensis* from Colombia based on rDNA sequence data. *South African Journal of Science* **97**, 295–6.

Van Heerden SW, Wingfield MJ, 2001. Genetic diversity of *Cryphonectria cubensis* isolates in South Africa. *Mycological Research* **105**, 94–9.

Van Heerden SW, Wingfield MJ, 2002. Effect of environment on the response of *Eucalyptus* clones to inoculation with *Cryphonectria cubensis*. *Forest Pathology* **32**, 395–402.

Wingfield MJ, 2003. 2003 Daniel McAlpine Memorial Lecture. Increasing threat of diseases to exotic plantation forests in the Southern Hemisphere: lessons from Cryphonectria canker. *Australasian Plant Pathology* **23**, 133–9.

Wingfield MJ, Rodas C, Wright J, Myburg H, Venter M, Wingfield BD, 2001. First report of Cryphonectria canker on *Tibouchina* in Colombia. *Forest Pathology* **31**, 297–306.