Identification and Pathogenicity of *Chrysoporthe cubensis* on *Eucalyptus* and *Syzygium* spp. in South China

S. F. Chen, Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, and China Eucalypt Research Centre (CERC), Chinese Academy of Forestry (CAF), China; **M. Gryzenhout** and **J. Roux,** Department of Microbiology and Plant Pathology, FABI; **Y. J. Xie,** CERC, CAF; **M. J. Wingfield,** Department of Microbiology and Plant Pathology, FABI; and **X. D. Zhou,** FABI; CERC, CAF

ABSTRACT

Chen, S. F., Gryzenhout, M., Roux, J., Xie, Y. J., Wingfield, M. J., and Zhou, X. D. 2010. Identification and pathogenicity of *Chrysoporthe cubensis* on *Eucalyptus* and *Syzygium* spp. in South China. Plant Dis. 94:1143-1150.

The genus *Chrysoporthe* includes important pathogens of plantation-grown *Eucalyptus* spp. and has been reported from several tree genera in the order Myrtales in tropical and subtropical areas of the world. During disease surveys in South China, fruiting structures typical of *Chrysoporthe* spp. were observed on cankers on *Eucalyptus* spp. and *Syzygium cumini* trees. The aim of this study was to confirm the identity of the *Chrysoporthe cubensis* infecting the *Eucalyptus* spp. and *S. cumini* and to test the pathogenicity of the fungus. Following glasshouse trials to select virulent isolates, field inoculations were undertaken to screen different commercial *Eucalyptus* genotypes for their susceptibility to the fungus. Isolates were characterized based on their morphology and DNA sequence data for the β-tubulin and internal transcribed spacer regions of the ribosomal DNA. Results showed that the putative pathogen represented the Asian form of *C. cubensis*, which occurred on numerous different *Eucalyptus* spp. and hybrid clones as well as *S. cumini*. Field inoculations showed that all six of the *Eucalyptus* genotypes tested are susceptible to infection by *C. cubensis*. Significant differences were observed between them, providing prospects to select disease-tolerant planting stock in the future.

Stem canker diseases caused by *Chrysoporthe* spp. are considered among the most important diseases of plantation-grown *Eucalyptus* spp. in the tropics and subtropics (12,38). Infection of susceptible young trees can lead to rapid tree death while cankers weaken the stems of older trees, often resulting in stem breakage (4,24,38,40). The stem canker diseases caused by *Chrysoporthe* spp. have had a substantial impact on the development of *Eucalyptus* forestry in the tropics and Southern Hemisphere (1,2,24,35,38); vegetative propagation of these trees emerged from efforts to avoid the disease using resistant hybrid clones (38).

It is well established that Chrysoporthe canker, previously known as Cryphonectria canker, of *Eucalyptus* spp. that once was thought to be caused by a single fungus, *Cryphonectria cubensis* (Bruner) Gryzenh. & M.J. Wingf., is caused by a number of different species of *Chrysoporthe* in different parts of the world (9). These include *Chrysoporthe cubensis*, with a wide distribution and representing two distinct phylogenetic lineages, one in South and Central America and a second in Southeast Asia (12). Other species of *Chrysoporthe* on *Eucalyptus* spp. are *C. austroafricana* Gryzenh. & M.J. Wingf. in Southern Africa (9), *C. doradensis* Gryzenh. & M.J. Wingf. in Ecuador (10), and *C. zambiensis* Chungu, Gryzenh. & Jol. Roux in Zambia (3).

Apart from *Eucalyptus* spp., *Chrysoporthe* spp. have been shown to be especially prevalent on other tree species in the order Myrtales (12). These fungi have been found on *Syzygium* spp. (*Myrtaceae*) in Africa (13,22,23), Brazil (14), Indonesia (14,20), and Malaysia (25); on *Tibouchina* spp. (*Melastomataceae*) in South Africa (18), South America (11,29,39), and Southeast Asia (11); and on *Lagerstroemia indica* (Lythraceae) in Cuba (11). *C. cubensis* is also known from other trees in the family *Melastomataceae*, including *Miconia* spp., *Rhynchanthera mexicana*, and *Clidemia sericea* in Central and South America (11,26) and *Melastoma melabathricum* in Indonesia (11).

Chrysoporthe cubensis has been reported from different hosts in several regions of Southeast Asia. These reports include those from *Eucalyptus* spp. in India (30), Indonesia (24), Malaysia (24), Thailand (21), Vietnam (24), and Hong Kong, China (14,21,30). The fungus has also been reported from native *Syzygium aromaticum* in Sulawesi, Indonesia (14,20); from native *M. melabathricum* (*Melastomataceae*) in Sumatra, Indonesia (11); and on non-native *Tibouchina urvilleana* (*Melastomataceae*) in Singapore and Thailand (11).

During the course of the past two decades, China has invested significantly in the establishment of plantations of *Eucalyptus* spp. as a source of pulp for the manufacture of paper, particle board, and plywood, as well as timber for construction. Approximately two million hectares of *Eucalyptus* plantations have already been established in South China (41). Similar to the situation in other countries, these trees have rapidly been affected by diseases (42). Recent surveys of *Eucalyptus* plantations in South China identified several pathogens affecting these trees, including *C. cubensis* associated with a stem canker disease (42). Early work in the country was conducted based only on fungal morphology, and there was no extensive survey of the species diversity, host range of the fungus, or consideration of its pathogenicity. The aim of this study was to survey *Eucalyptus* and related species for the presence of *Chrysoporthe* spp. and to identify these fungi based on DNA sequence data and morphological characters. In addition, the relative susceptibility of commercially planted *Eucalyptus* genotypes to Chrysoporthe canker was evaluated in field inoculation trials.

MATERIALS AND METHODS

Sampling. *Eucalyptus* plantations in three provinces in South China (Fig. 1), where most plantations of these trees have been established, were surveyed for the presence of stem cankers in November and December 2006 and June 2007 as well as between September and November 2008. Where present in these areas, *S. cumini* trees that are related to *Eucalyptus* spp. in the family *Myrtaceae* were also examined for the presence of stem cankers. Sections of bark from the surface of stem cankers bearing fruiting structures resembling *Chrysoporthe* spp., including ascostromata and conidiomata, were collected and trans-

Corresponding author: X. Zhou
E-mail: cerc.zhou@gmail.com
or xu.zhou@fabi.up.ac.za

Accepted for publication 28 May 2010.

doi:10.1094/PDIS-94-9-1143

ported to the laboratory for isolations. Samples were incubated in moist chambers for 1 to 3 days to induce the production of spores from the fruiting bodies. Single spore tendrils were transferred to 2% malt extract agar (MEA; 20 g of Biolab malt extract, 20 g of Biolab agar, and 1 liter water; Biolab, Merck, Midrand, South Africa) and incubated at 25°C. From the resultant cultures, single hyphal tips were transferred to fresh 2% MEA to obtain pure cultures. All cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, and the China Eucalypt Research Centre (CERC), Chinese Academy of Forestry (CAF), China.

Morphology. To study the morphology of the fungi collected, fruiting structures were taken from bark specimens and sectioned using a Leica CM1100 cryostat (Setpoint Technologies) at –20°C following the method described by Gryzenhout et al. (9). Samples from China were compared with those of *Chrysoporthe* spp. (9,12). For measurements, structures from the bark on five trees of *Eucalyptus* clones W5 and U6, respectively, and 10 from *S. cumini* trees from GuangDong Province (Table 1; Fig. 1), were selected. Measurements were made from 25 conidiophores, basal cells of conidiophores, conidia, asci, and ascospores. The results are presented as minimum – (mean – standard deviation) – (mean + standard deviation) – maximum. To obtain an indication of the minimum and maximum size ranges of the stromata, measurements were obtained from structures representing the smallest and largest for the anamorph and teleomorph stromata on both *Eucalyptus* and *S. cumini* trees.

DNA sequence comparisons. Representative isolates collected from different *Eucalyptus* species or clones and *S. cumini* in different geographic regions of South China (Table 1; Fig. 1) were selected and used for DNA sequence comparisons. Prior to DNA extraction, isolates were grown in 2% MEA at 25°C for 5 to 7 days. For each isolate, actively growing mycelium from one MEA plate per isolate was scraped from the surface of the media using a sterile scalpel and transferred to 1.5-µl Eppendorf tubes. DNA was extracted from the mycelium following the method used by Myburg et al. (21). DNA was separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. Samples were treated with 3 µl of RNase (1 mg/ml) and left overnight at 37°C to degrade RNA.

The internal transcribed spacer (ITS) regions, including the 5.8S ribosomal DNA operon and two regions within the β-tubulin gene region, were amplified using the primer pairs ITS1 and ITS4 (37) and βt1a/βt1b and βt2a/βt2b, respectively (8). Polymerase chain reaction (PCR) conditions were as outlined by Myburg et al. (19). PCR products were visualized with UV light on 1% agarose (ethidium bromide-stained) gels. The amplified products were purified using 6% Sephadex G-50 columns (Steinheim, Germany) as described by the manufacturers.

Each PCR product was sequenced in both directions with the same primers that were used for PCR reactions. The ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Foster City, CA) was used to perform the sequencing reactions. The sequence products were purified using 6% Sephadex G-50 columns, whereafter electropherograms were generated on an ABI PRISM 3100 autosequencer (Perkin-Elmer Applied Biosystems). Nucleotide sequences were analyzed using MEGA4 (33). All sequences obtained in this study have been deposited in GenBank (Table 1).

Sequences were aligned with the online version of MAFFT v. 5.667 (16), using the iterative refinement method (FFT-NS-i settings), and adjusted manually. The sequence data were analyzed using phylogenetic analyses using phylogeny (PAUP; version 4.0b10) (32). The combinability of the ITS and β-tubulin 1 and 2 gene sequence data sets was determined with a partition homogeneity test (PHT) in PAUP (6,32). Most parsimonious trees were obtained with heuristic searches using stepwise addition and tree bisection and reconstruction (TBR) as the branch-swapping algorithms. All equally parsimonious trees were saved and all branches equal to zero were collapsed. Gaps were treated as the fifth character. A bootstrap analysis (50% majority rule, 1,000 replications) was done to determine the confidence levels of the tree-branching points (7). Previously published sequences for *C. austroafricana, C. cubensis*, *C. doradensis*, *C. hodgesiana* (Gryzenh. & M.J. Wingf.) Gryzenh. & M.J. Wingf., and *C. inopina* Gryzenh. & M.J. Wingf. (3,9–11) were used for comparative purposes. *Cryphonectria parasitica* (Murrill) M.E. Barr (*Cryphonectriaceae*) was used as the outgroup taxon to root the phylogenetic trees (12).

Pathogenicity tests. *Glasshouse trials.* Six isolates (CMW26888, CMW26891, CMW26892, CMW26895, CMW26929, and CMW26932) from different locations and *Eucalyptus* genotypes in China (Table 1) were selected for inoculations. These isolates were inoculated into trees of a susceptible *Eucalyptus grandis* clone under glasshouse conditions. This was done to select the most virulent isolates for field inoculations. The trees were approximately 2 m tall and had diameters of approximately 10 mm. Before inoculation, the trees were allowed to acclimatize to the glasshouse conditions of 25°C and 14 h of daylight with 10 h of darkness. Fungal isolates were grown at 25°C under con-

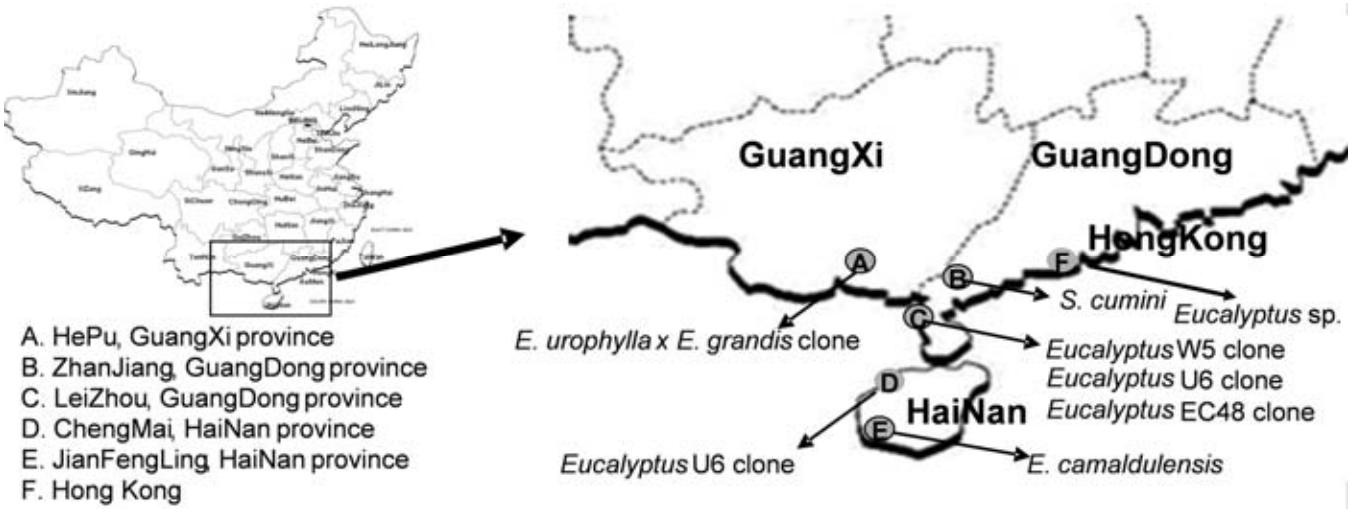

Fig. 1. Map of China indicating areas and species of *Eucalyptus* and *Syzygium* from which *Chrysoporthe cubensis* isolates were collected and identified. Records of *C. cubensis* on *Eucalyptus* spp. in Hong Kong are from previous studies (15,21,30).

tinuous fluorescent light for 6 days prior to inoculation.

In order to expose the cambium, wounds were made in the bark at a constant height (about 300 mm above the seedling medium) using a cork borer (7 mm in diameter). Discs of the same size from the actively growing colonies were inserted into the wounds with the mycelium facing the xylem. To prevent desiccation and contamination, wounds were covered with Parafilm (Pechiney Plastic Packing, Chicago). The six isolates were inoculated into the stems of 10 trees each. Ten trees were also inoculated with sterile MEA plugs to serve as controls. The 70 inoculated trees were arranged randomly in the glasshouse.

Trees were inoculated in April 2008 and results evaluated after 6 weeks by measuring the lengths of lesions in the cambium. Reisolations were made from the resultant lesions by plating small pieces of discolored xylem onto 2% MEA at 25°C. Reisolations were made from four randomly selected trees per isolate and from all trees inoculated as controls. Results were ana-

Table 1. Isolates used for phylogenetic analysis and pathogenicity trials in this study

Isolate[a]						GenBank no.		
CMW no.	Alternative	Species identity	Host	Origin	Collector[b]	ITS[c]	β-tubulin 1	β-tubulin 2
CMW12746[d]	…	*Chrysoporthe cubensis*	*Eucalyptus* sp.	GuangDong, China	TIB	HM142105	HM142121	HM142137
CMW12748[d]	…	*C. cubensis*	*Eucalyptus* sp.	GuangDong, China	TIB	HM142106	HM142122	HM142138
CMW12749[d]	…	*C. cubensis*	*Eucalyptus* sp.	GuangDong, China	TIB	HM142100	HM142116	HM142132
CMW24693[d]	…	*C. cubensis*	*Eucalyptus* sp.	GuangDong, China	MJW	HM142101	HM142117	HM142133
CMW24695[d]	…	*C. cubensis*	*Eucalyptus grandis*	GuangDong, China	MJW	HM142093	HM142109	HM142125
CMW24697[d]	…	*C. cubensis*	*Eucalyptus* sp.	GuangDong, China	MJW	HM142102	HM142118	HM142134
CMW24909[d,f,g]	…	*C. cubensis*	*Syzygium cumini*	GuangDong, China	MJW & XDZ	HM142094	HM142110	HM142126
CMW24919[d,f,g]	…	*C. cubensis*	*S. cumini*	GuangDong, China	MJW & XDZ	HM142104	HM142120	HM142136
CMW24921[d,f,g]	…	*C. cubensis*	*S. cumini*	GuangDong, China	MJW & XDZ	HM142103	HM142119	HM142135
CMW26888[d,e]	…	*C. cubensis*	*E. urophylla* × *E. grandis*	GuangXi, China	MJW & XDZ	HM142095	HM142111	HM142127
CMW26890[d]	…	*C. cubensis*	*E. urophylla* × *E. grandis*	GuangXi, China	MJW & XDZ	HM142096	HM142112	HM142128
CMW26891[d,e]	…	*C. cubensis*	*Eucalyptus* U6 clone	GuangDong, China	MJW & XDZ	HM142107	HM142123	HM142139
CMW26892[d,e,f,g]	…	*C. cubensis*	*Eucalyptus* U6 clone	GuangDong, China	MJW & XDZ	HM142108	HM142124	HM142140
CMW26895[d,e]	…	*C. cubensis*	*Eucalyptus* W5 clone	GuangDong, China	MJW & XDZ	HM142097	HM142113	HM142129
CMW26929[d,e,f,g]	…	*C. cubensis*	*E. camaldulensis*	HaiNan, China	MJW & XDZ	HM142098	HM142114	HM142130
CMW26932[d,e]	…	*C. cubensis*	*Eucalyptus* U6 clone	HaiNan, China	MJW & XDZ	HM142099	HM142115	HM142131
CMW1856	…	*C. cubensis*	*Eucalyptus* sp.	Kauai, Hawaii	NA	AY083999	AY084022	AY084010
CMW9903	…	*C. cubensis*	*S. aromaticum*	Kalimantan, Indonesia	CSH	AF292044	AF273066	AF273461
CMW11288	CBS115736	*C. cubensis*	*Eucalyptus* sp.	Indonesia	MJW	AY214302	AY214230	AY214266
CWM11290	CBS115738	*C. cubensis*	*Eucalyptus* sp.	Indonesia	MJW	AY214304	AY214232	AY214268
CMW8650	CBS115719	*C. cubensis*	*S. aromaticum*	Sulawesi, Indonesia	MJW	AY084001	AY084024	AY084013
CMW8651	CBS115718	*C. cubensis*	*S. aromaticum*	Sulawesi, Indonesia	MJW	AY084002	AY084014	AY084026
CMW10774	…	*C. cubensis*	*S. aromaticum*	Zanzibar, Tanzania	NA	AF492130	AF492131	AF492132
CMW2631	…	*C. cubensis*	*E. marginata*	Australia	ED	AF543823	AF543824	AF543825
CMW2632	…	*C. cubensis*	*E. marginata*	Australia	ED	AF046893	AF273078	AF375607
CMW10453	CBS505.63	*C. cubensis*	*E. saligna*	Republic of Congo	Unknown	AY063476	AY063478	AY063480
CMW10669	CBS115751	*C. cubensis*	*Eucalyptus* sp.	Republic of Congo	JR	AF535122	AF535124	AF535126
CMW10671	CBS115752	*C. cubensis*	*Eucalyptus* sp.	Republic of Congo	JR	AF254219	AF254221	AF254223
CMW10639	CBS115747	*C. cubensis*	*E. grandis*	Colombia	CAR	AY263419	AY263420	AY263421
CMW14394	CBS118654	*C. cubensis*	*E. grandis*	Cuba	MJW	DQ368773	DQ368798	DQ368799
CMW1853	…	*C. cubensis*	*S. aromaticum*	Brazil	NA	AF046891	AF273070	AF273465
CMW10777	…	*C. cubensis*	*S. aromaticum*	Brazil	CSH	AY084005	AY084029	AY084017
CMW10778	CBS115755	*C. cubensis*	*S. aromaticum*	Brazil	CSH	AY084006	AY084030	AY084018
CMW2113	CBS112916	*C. austroafricana*	*E. grandis*	South Africa	MJW	AF046892	AF273067	AF273462
CMW9327	CBS115843	*C. austroafricana*	*Tibouchina granulosa*	South Africa	MJW	AF273473	AF273060	AF273455
CMW14561	…	*C. austroafricana*	*S. cordatum*	South Africa	GN	DQ246605	DQ246559	DQ246582
CMW13976	…	*C. austroafricana*	*S. cordatum*	Zambia	JR	DQ246614	DQ246568	DQ246591
CMW11286	CBS115734	*C. doradensis*	*E. grandis*	Ecuador	MJW	AY214289	AY214217	AY214253
CMW11287	CBS115735	*C. doradensis*	*E. grandis*	Ecuador	MJW	AY214290	AY214218	AY214254
CMW10625	CBS115744	*C. hodgesiana*	*Miconia theaezans*	Colombia	CAR	AY956970	AY956979	AY956980
CMW10641	CBS115854	*C. hodgesiana*	*T. semidecandra*	Colombia	RA	AY692322	AY692326	AY692325
CMW12727	CBS118659	*C. inopina*	*T. lepidota*	Colombia	RA	DQ368777	DQ368806	DQ368807
CMW12729	CBS118658	*C. inopina*	*T. lepidota*	Colombia	RA	DQ368778	DQ368808	DQ368809
CMW7048	…	*Cryphonectria parasitica*	*Quercus virginiana*	United States	FFL	AF368330	AF273076	AF273470
CMW13749	…	*C. parasitica*	*Castanea mollisima*	Japan	NA	AY697927	AY697943	AY697944

[a] CMW = culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; CBS = the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

[b] TIB = T. I. Burgess, MJW = M. J. Wingfield, XDZ = X. D. Zhou, NA = not applicable, CSH = C. S. Hodges, ED = E. Davison, JR = J. Roux, CAR = C. A. Rodas, GN = G. Nakabonge, RA = R. Arbaleaz, and FFL = F. F. Lombard.

[c] Internal transcribed spacer.

[d] Isolates obtained and used in phylogenetic analysis in this study.

[e] Isolates used for pathogenicity tests on *E. grandis* clone seedlings in glasshouse.

[f] Isolates used for pathogenicity tests on *Eucalyptus* plantations in field in China.

[g] Isolates used for pathogenicity tests on *S. cumini* branches in field in China.

lyzed in SAS (version 8) using the PROC general linear model (GLM) (28). Analysis of variance (ANOVA) was used to determine the effects of fungal strain on lesion length. Prior to ANOVA, homogeneity of variance across treatments was verified. For significance tests among means, Fisher's protected test was used. F values with $P < 0.05$ were considered significant.

Field trials. To evaluate the relative susceptibility of commercially planted *Eucalyptus* spp. and clones grown in South China, field inoculations were done using isolates (CMW26892 and CMW26929) identified in the glasshouse inoculations as most virulent. Three isolates (CMW24909, CMW24919, and CMW24921) originating from *S. cumini* (Table 1) were also used in the study. The field trial was situated in the ZhanJiang area of GuangDong Province and consisted of seven *Eucalyptus* genotypes, including pure species and hybrids. These included an *E. grandis* clone (CEPT-1), an *E. grandis* × *E. tereticornis* clone (CEPT-2), an *E. pellita* genotype (CEPT-3), two *E. urophylla* × *E. grandis* clones (CEPT-4 and CEPT-7), an *E. urophylla* clone (CEPT-5), and an *E. wetarensis* clone (CEPT-6). The isolates originating from *S. cumini* were inoculated onto two of the *Eucalyptus* genotypes (CEPT-6 and CEPT-7) due to limited availability of trees for inoculation. At the time of inoculation, trees were 1 year old and 6 to 10 trees of each genotype were inoculated per isolate. An equal number of trees were inoculated with sterile MEA discs to serve as negative controls. Wounds were made in the bark at a constant height (about 400 to 800 mm above the ground) using a cork borer (9 mm in diameter). The inoculation trail was conducted in October 2008.

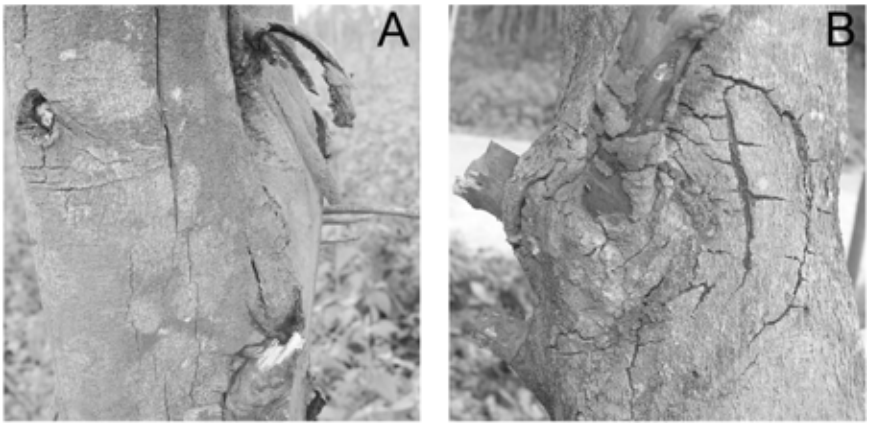

Fig. 2. Symptoms of infection by *Chrysoporthe cubensis* on **A,** *Eucalyptus* spp. and **B,** *Syzygium cumini* in China.

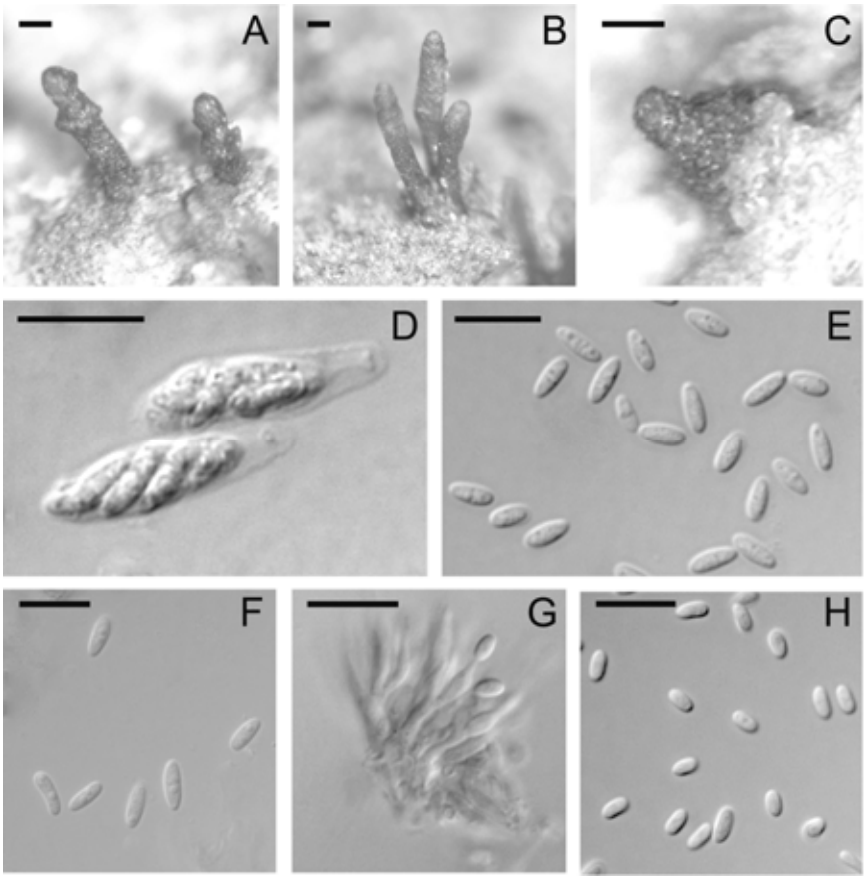

Fig. 3. Fruiting structures of *Chrysoporthe cubensis* from a *Eucalyptus* sp. and *Syzygium cumini* in China. **A,** Ascostroma on bark of *Eucalyptus* sp. **B,** Ascostroma on bark of *S. cumini*. **C,** Conidioma on bark of *Eucalyptus* sp. **D,** Asci from *S. cumini* tree. **E,** Ascospores from *S. cumini* bark. **F,** Ascospores from *Eucalyptus* sp. **G,** Conidiophores from *Eucalyptus* sp. **H,** Conidia from *Eucalyptus* sp. Bars: A, B, and C = 100 µm; D, E, F, G, and H = 10 µm.

To evaluate the susceptibility of *S. cumini* to the *Chrysoporthe cubensis* isolates collected from South China, the same isolates as those used in field inoculations of *Eucalyptus* genotypes were inoculated onto the branches of *S. cumini* trees in ZhanJiang. For the five selected isolates, 10 branches from each of five *S. cumini* trees were inoculated per isolate. An equal number of branches were inoculated with sterile MEA discs to serve as negative controls. Inoculations were conducted in a similar way to those for the field and glasshouse inoculations, using a 9-mm-diameter cork borer. Branches were inoculated in September 2008, and the inoculation trial was repeated once, 7 days after the first inoculations on five different *S. cumini* trees.

Lesion lengths were recorded after 5 weeks for the *Eucalyptus* trees and 6 weeks for the *S. cumini* trees. To evaluate the pathogenicity of the isolates of *C. cubensis* to *Eucalyptus* genotypes and *S. cumini*, the lengths of the lesions in the cambium below the bark were recorded. Results from the experiments were analyzed separately in SAS (version 8) using the PROC GLM (28) and in a similar manner as for the greenhouse inoculation tests.

RESULTS

Sampling. Symptoms typical of infection by *C. cubensis*. were commonly observed on *Eucalyptus* and *S. cumini* trees in South China. These included dying branches, cracked bark, and cankers girdling the stems (Fig. 2A and B). Fruiting structures typical of *C. cubensis*, including ascostromata and conidiomata, were found on the surface of the cankers on *Eucalyptus* trees, whereas only ascostromata were observed on *S. cumini*. Isolates were obtained from a total of 25 *Eucalyptus* trees and 19 *S. cumini* trees. Of these, isolates were obtained from three *E. camaldulensis* and three *E. urophylla* × *E. grandis* trees in HaiNan Province, four *E. urophylla* × *E. grandis* trees in GuangXi Province, one each of three trees representing *Eucalyptus* clones W5, U6, and EC48, and one tree of an unknown *Eucalyptus* sp. in GuangDong

Province (Table 1). Isolates were also collected from cankers on five *Eucalyptus* trees, including *E. grandis*, in GuangDong Province. All isolates of *C. cubensis* from *S. cumini* were collected from GuangDong Province (Fig. 1; Table 1).

Morphology. The ascostromata on the bark taken from cankers on *Eucalyptus* trees were semi-immersed, 120 to 200 µm (average: 150 µm) high above the level of the bark, and 250 to 410 µm (average: 350 µm) wide above the surface of the bark (Fig. 3A). The perithecial stromata were valsoid with perithecia partially embedded in the bark tissue. Perithecia were fuscous black in color and the tops of the perithecial bases were covered with cinnamon to orange fungal tissue, which was occasionally visible above the bark surface. The necks of the perithecia emerging from the stromata were covered with fuscous black tissue and were cylindrical, up to 520 µm (average: 410 µm) in length and 100 to 150 µm (average: 120 µm) wide (Fig. 3A). Asci were 19.5–22 to 26–27 × 4.5–5 to 6–6.5 µm, fusoid to ellipsoidal, containing eight ascospores. Ascospores were 5–5.5 to 7–8 × 2–3 µm, hyaline, 1-septate, fusoid to oval, with the ends tapering (Fig. 3F).

Stromatic conidiomata formed on the surfaces of the ascostromata or as separate structures, and were superficial to slightly immersed, pyriform to clavate (Fig. 3C). Stromatic conidiomatal bases were 130 to 430 µm (average: 170 µm) high above the level of the bark and 190 to 750 µm (aver-

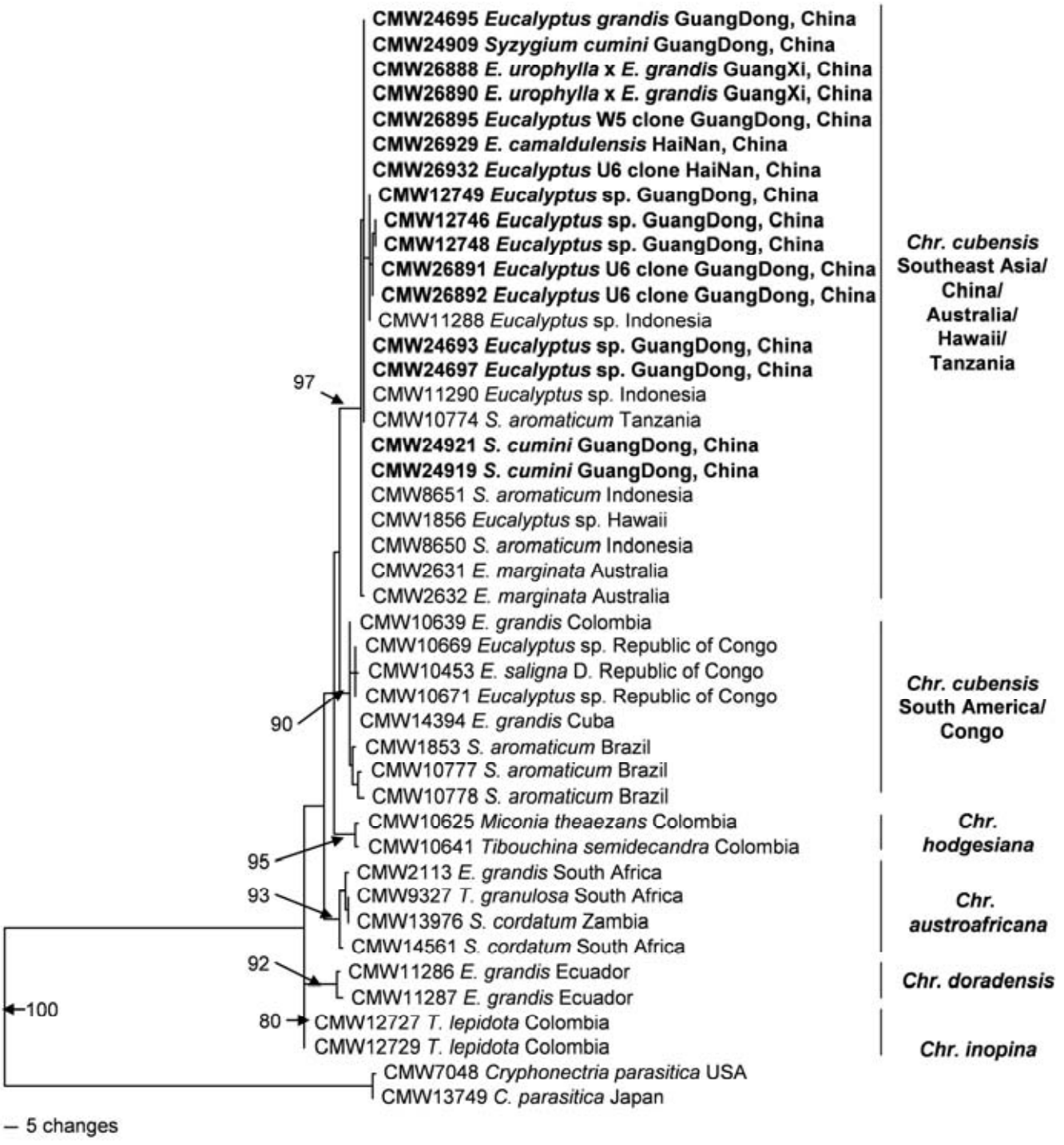

Fig. 4. One of 36 most parsimonious trees generated from combined sequence data of the internal transcribed spacer ribosomal DNA and β-tubulin gene sequence data and generated from heuristic searches performed on the combined data set (tree length of 348, CI of 0.937, and RI of 0.954). Bootstrap values (1,000 replicates) above 75% are indicated on the branches. Isolates sequenced in this study are in bold. *Cryphonectria parasitica* was used to root the tree.

age: 320 µm) wide. Stromatic conidiomatal necks were up to 320 µm long and 80 to 160 µm wide (Fig. 3C). Stromatic conidiomatal locules with even to convoluted inner surface were occasionally multilocular, with single locules connected to one or several necks. Conidiophores were hyaline with globose to rectangular basal cells that were rounded off, 2.5–3 to 6.5–9 × 2–2.5 to 4.5–6 µm, and branched irregularly at the base or above into cylindrical cells that were delimited by septa or not; conidiophores were 13–14.5 to 21.5–26.5 µm in length and conidiogenous cells were cylindrical to flask-shaped with attenuated apices, 1.5–2 to 3–3.5 µm wide (Fig. 3G). Conidia were 3–3.5 to 4.5–5 × 1.5–2 to 2.5–3 µm, hyaline, oblong, aseptate, and exuded as bright luteous tendrils or droplets (Fig. 3H).

The morphology of ascostromata on *S. cumini* trees was similar to that on the *Eucalyptus* trees (Fig. 3B), and conidiomata were not observed. The emerging necks of the perithecia from stromata were up to 900 µm (average: 610 µm) long, 80 to 140 µm (average: 110 µm) wide, and covered with fuscous black tissue (Fig. 3B). Asci were 19.5–21.5 to 25.5–27.5 × 4.5–5 to 6–6.5 µm, fusoid to ellipsoidal in shape, and eight spored (Fig. 3D). Ascospores were 5–5.5 to 6.5–7 × 2 to 3 µm, hyaline, 1-septate, fusoid to oval, with tapered ends and septa that could be in various positions in the spore but usually central (Fig. 3E).

Specimens examined were China, GuangDong Province, bark of *Eucalyptus* clone, December 2006, M. J. Wingfield and X. D. Zhou, PREM60451, living culture CMW26891; and GuangDong Province, bark of *S. cumini*, December 2006, M. J. Wingfield and X. D. Zhou, PREM60452, living culture CMW24921.

DNA sequence comparisons. After alignment of sequence data, a partition homogeneity test on the three separate data sets gave a PHT value of $P = 0.014$, showing that the data sets (ITS and βt) were significantly incongruent. However, based on the results of previous studies (9,11), they were combined in the phylogenetic analysis. The combined ITS and β-tubulin data set had 1,327 characters of equal weight, with 1,019 constant characters, of which 31 were parsimony uninformative and 277 were parsimony informative. In all, 36 most parsimonious trees were retained, and one of them (50% majority rule), with tree length = 312 steps, CI = 0.939, RI = 0.956, RC = 0.897, and HI = 0.061, was chosen to obtain a representative tree of the data (Fig. 4).

Results of the DNA sequence analyses showed that the isolates collected from *Eucalyptus* spp. in South China represented *C. cubensis* (Fig. 4). Isolates from the *S. cumini* trees were similar to those from *Eucalyptus* spp. Isolates from China grouped within the Asian clade of *C. cubensis* (9,11,19) together with isolates originating from Australia, Hawaii, Indonesia, and Tanzania, with 97% bootstrap support, and separate from the South American clade of *C. cubensis*, with a 90% bootstrap (Fig. 4).

Differences were observed between isolates from China. Some of these isolates (CMW12746, CMW12748, CMW12749, CMW26891, and CMW26892) differed from the other isolates with two fixed nucleotide differences in the ITS gene region whereas, for the β-tubulin gene region, no differences were found between these isolates. The bootstrap values within the *C. cubensis* clade representing sequence differences are not significant (Fig. 4), suggesting that these reflect intraspecific sequence differences rather than interspecies variation.

Pathogenicity tests. *Glasshouse trials.* All *C. cubensis* isolates from China tested for pathogenicity on the *Eucalyptus* clone produced lesions within 6 weeks, while small lesions were produced for the control inoculations (Fig. 5). ANOVAs showed significant differences in susceptibility to the fungal isolates on the inoculated *Eucalyptus* clone ($P < 0.001$). The mean comparison tests showed that the lesion lengths produced by the *C. cubensis* isolates were significantly longer ($P < 0.0001$ to $P = 0.0036$) than those of the controls (Fig. 5). Of the isolates tested, lesions produced by isolate CMW26929 were significantly longer ($P < 0.0001$ to $P = 0.0004$) than those of the other isolates (Fig. 5). All the inoculated fungi were successfully reisolated from the lesions, whereas no *C. cubensis* was isolated from the controls. Based on the glasshouse trial, isolates CMW26929 and CMW26892 were most virulent and, therefore, used for field inoculations.

Field trials in China. The two *C. cubensis* isolates (CMW26892 and CMW26929) inoculated on seven *Eucalyptus* genotypes gave rise to distinct lesions. ANOVAs showed significant differences in susceptibility to the fungal isolates between the

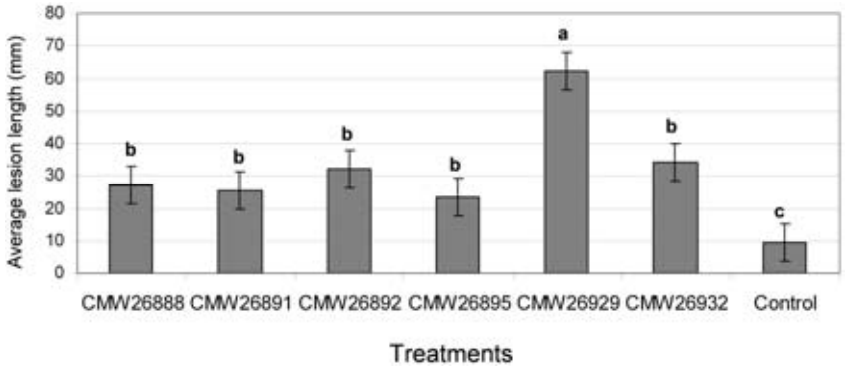

Fig. 5. Column chart indicating the average lesion length (in millimeters) resulting from inoculation trials onto an *Eucalyptus grandis* clone (TAG-5) under glasshouse conditions. Six isolates of *Chrysoporthe cubensis* were used that was identified from *Eucalyptus* trees in China. Bars represent 95% confidence limits for each treatment. Different letters above the bars indicate treatments that were statistically significantly different ($P = 0.05$).

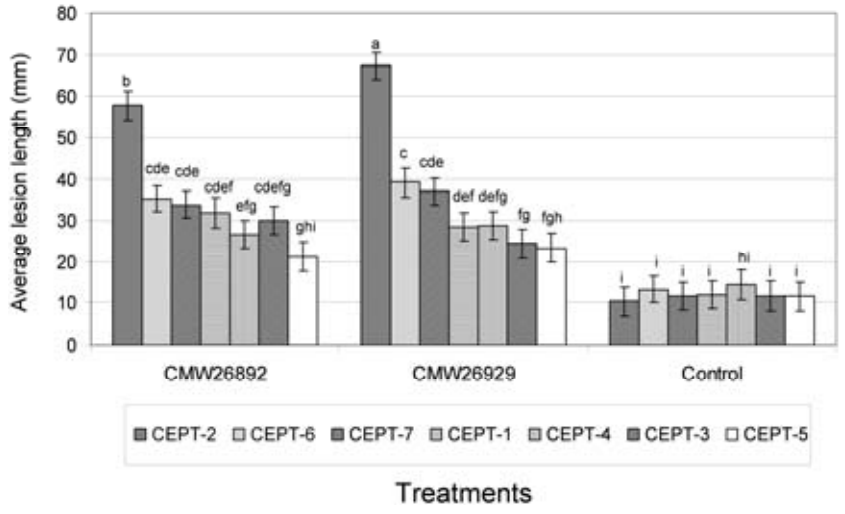

Fig. 6. Column chart indicating the average lesion lengths (in millimeters) resulting from inoculation trials on seven *Eucalyptus* genotypes. Lesion lengths were produced by two *Chrysoporthe cubensis* isolates (CMW26892 and CMW26929). Bars represent 95% confidence limits for each treatment. Different letters above the bars indicate treatments that were statistically significantly different ($P = 0.05$).

various *Eucalyptus* genotypes ($P < 0.001$). Statistical analyses of the data showed that not all the isolates of *C. cubensis* reacted in the same manner to the tested *Eucalyptus* genotypes. For example, lesions produced by CMW26929 on *Eucalyptus* genotype CEPT-2 were significantly longer ($P = 0.0437$) than those of CMW26892 (Fig. 6) whereas, for the other *Eucalyptus* genotypes, the lesions produced by the two *C. cubensis* isolates were not significantly different ($P = 0.2477$ to 0.6777) (Fig. 6). The mean comparison tests further showed that the lesions produced by the two *C. cubensis* isolates (CMW26892 and CMW26929) from *Eucalyptus* were significantly longer ($P < 0.0001$ to $P = 0.0477$) than those of the controls, except for isolate CMW26892 ($P = 0.053$) on *Eucalyptus* CEPT-5 (Fig. 6). The results further showed that *Eucalyptus* genotype CEPT-2 is the most susceptible while genotype CEPT-5 is the most tolerant to infection by *C. cubensis* (Fig. 6).

Under field conditions, five *C. cubensis* isolates (CMW26892, CMW26929, CMW24909, CMW24919, and CMW24921) inoculated on two *Eucalyptus* genotypes (CEPT-6 and CEPT-7) also resulted in lesions. ANOVAs showed significant differences in susceptibility to the fungal isolates between the two *Eucalyptus* genotypes ($P < 0.001$). The three *C. cubensis* isolates (CMW24909, CMW24919, and CMW24921) originating from *S. cumini* trees produced lesions on both *Eucalyptus* genotypes (CEPT-6 and CEPT-7). The mean comparison tests showed that lesions produced by two of the isolates (CMW24909 and CMW24919) were significantly longer ($P < 0.0001$ to $P = 0.0085$) than those produced by the controls (Fig. 7). However, lesions produced by isolates (CMW26892 and CMW26929) originating from *Eucalyptus* trees were significantly longer ($P < 0.0001$) than those produced by the two isolates (CMW24919 and CMW24921) from *S. cumini* trees (Fig. 7).

Where *S. cumini* branches were inoculated with *C. cubensis* isolates from *Eucalyptus* spp. (CMW26892 and CMW26929) and *S. cumini* (CMW24909, CMW24919, and CMW24921), distinct lesions developed after 6 weeks. For the first inoculation experiment, ANOVAs showed significant differences in susceptibility to the fungal isolates on the inoculated *S. cumini* branches ($P = 0.0516$). Statistical analyses of the data showed that not all the isolates of *C. cubensis* reacted in the same manner to all the tested *S. cumini* branches. For example, lesions produced by CMW26929 were significantly longer than those of CMW26892 ($P = 0.0269$) and CMW24909 ($P = 0.0058$) (Fig. 8). In the second test, ANOVAs showed no significant differences in susceptibility to the fungal isolates on the inoculated *S. cumini* branches ($P = 0.9730$). For each experiment, all five isolates produced significantly longer lesions ($P < 0.0001$) than those of the controls (Fig. 8).

DISCUSSION

Results of the present study confirmed the presence of *C. cubensis* on *Eucalyptus* spp. in mainland China. The fungus had previously been reported only from Hong Kong, China (12,14,21,30). This is especially important because recent studies (12) have shown that there are various closely related species of *Chrysoporthe* that can be confused with each other and that multigene DNA sequence analysis is needed to obtain unequivocal identifications. As part of this study, the new host *S. cumini* is recorded for *C. cubensis*. Inoculation trials confirmed the pathogenicity of *C. cubensis* on the *Eucalyptus* genotypes used for plantation forestry in China as well as on *S. cumini*. Genotypes of *Eucalyptus* were shown to differ in their susceptibility to this pathogen.

Comparisons of sequence data for the ITS and β-tubulin gene regions provided definitive evidence that isolates of *Chrysoporthe* collected from China were those of *C. cubensis*. Previous research has shown that, based on ITS, β-tubulin, and Histone H3 gene sequence data, *C. cubensis* isolates reside in two very distinct phylogenetic clades. These clades represent isolates from Southeast Asia and South America (9,11,19). Based on sequence data for ITS and β-tubulin gene regions, isolates from South China group within the Southeast Asian clade of this species.

Field inoculation studies using different *Eucalyptus* genotypes grown in South China provided valuable data for the selection of future planting material in the country. The susceptibility of the genotypes tested differed significantly from each other. This is similar to results of screening trials in South Africa (5,34,35), which have resulted in the effective management of canker caused by *C. austroafricana* through the identification of disease-resistant planting stock (35,36,38). Thus, in South Africa, it is currently difficult to find *C. austroafricana* infections on *Eucalyptus* trees in plantations due to the success of selection and breeding programs that have been supported by disease

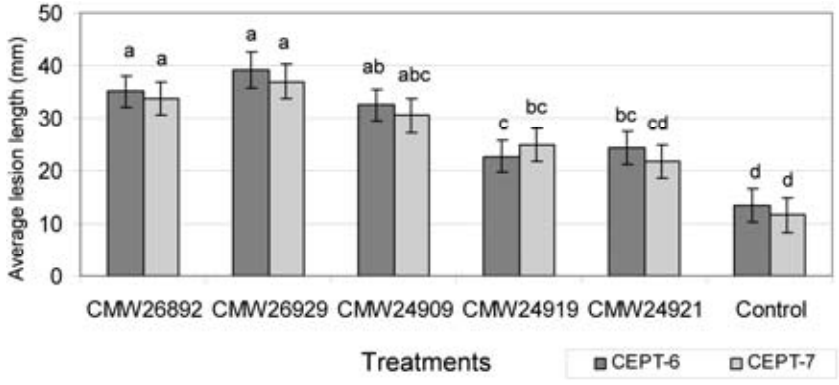

Fig. 7. Column chart indicating the average lesion lengths (in millimeters) resulting from inoculation trials on two *Eucalyptus* genotypes (CEPT-6 and CEPT-7) in the field. Five *Chrysoporthe cubensis* isolates were used, with two isolates (CMW26892 and CMW26929) identified from *Eucalyptus* trees and three isolates (CMW24909, CMW24919, and CMW24921) identified from *Syzygium cumini* trees. Bars represent 95% confidence limits for each treatment. Different letters above the bars indicate treatments that were statistically significantly different ($P = 0.05$).

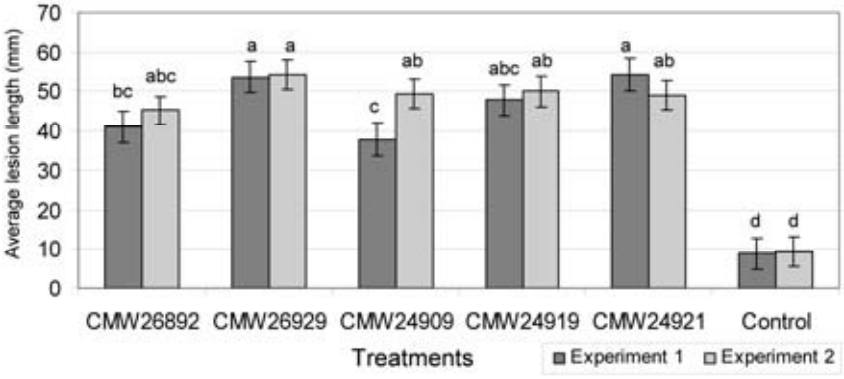

Fig. 8. Column chart indicating the average lesion lengths (in millimeters) resulting from inoculation trials on branches of *Syzygium cumini* that were inoculated with five *Chrysoporthe cubensis* isolates. Two of these isolates (CMW26892 and CMW26929) were identified from *Eucalyptus* trees and three isolates (CMW24909, CMW24919, and CMW24921) were identified from *S. cumini* trees. Bars represent 95% confidence limits for each treatment. Different letters above the bars indicate treatments that were statistically significantly different ($P = 0.05$).

screening via inoculation (J. Roux, *unpublished data*). As a further aid to the selection of disease-tolerant planting material, genetic markers have been developed in South Africa to aid in effective screening of species, hybrids, and clones (17). This is an approach that might be useful in China.

An interesting outcome of this study was the discovery of *C. cubensis* on *S. cumini*. This tree is planted as an ornamental in South China and is native to the nearby countries of Bangladesh, India, Indonesia, Nepal, and Pakistan. In this regard, it seems likely that it is also a natural host of the pathogen in Southeast Asia. This would be consistent with the fact that *C. cubensis* has been found on other native members of the order Myrtales in Southeast Asia and in South and Central America and has apparently undergone a host shift (31) to infect *Eucalyptus* spp. (11,22,27). Similarly, the closely related pathogen *C. austroafricana* is found on native *Syzygium* spp. in South Africa and it has apparently undergone a host shift to infect *Eucalyptus* spp. (13,22). Pathogenicity tests in this study also showed that *S. cumini* is susceptible to infection by *C. cubensis*. This could explain the extensive cankering and branch die-back observed regularly on *S. cumini* trees.

This study represents the first comprehensive work that investigates the pathogenicity of *C. cubensis* on *Eucalyptus* and *S. cumini* trees in China. Moreover, the identification of commercially available *Eucalyptus* genotypes tolerant to Chrysoporthe canker will promote the selection of tolerant stocks for wide-scale planting. This approach has been shown to effectively reduce the disease severity in other parts of the world (15,35,36) and it should also be useful in China.

ACKNOWLEDGMENTS

This study was initiated through the bilateral agreement between South Africa and China, and funded through projects 30771732 and 2007DFA31190. We thank the members of the Tree Protection Cooperative Programme (TPCP) for financial and logistical support; T. Burgess (Murdoch University, Australia) for providing some isolates used in this study; H. Groeneveld and M. van der Linde for assistance with the statistical analyses, and colleagues at the LeiZhou Forestry Bureau (X.-T. Mou, G.-X. Zhao, and C.-Y. Xie) for their valuable assistance in the field.

LITERATURE CITED

1. Alfenas, A. C., Jeng, R., and Hubbes, M. 1983. Virulence of *Cryphonectria cubensis* on *Eucalyptus* species differing in resistance. Eur. J. For. Pathol. 13:197-205.
2. Campinhos, E., and Ikemori, Y. K. 1983. Mass production of *Eucalyptus* spp. by rooting cuttings. Silvicultura 8:770-775.
3. Chungu, D., Gryzenhout, M., Muimba-Kankolongo, A., Wingfield, M. J., and Roux, J. 2010. Taxonomy and pathogenicity of two novel *Chrysoporthe* species from *Eucalyptus grandis* and *Syzygium guineense* in Zambia. Mycol. Pro. DIO 10.1007/s11557-009-0646-9.
4. Conradie, E., Swart, W. J., and Wingfield, M. J. 1990. Cryphonectria canker of *Eucalyptus*, an important disease in plantation forestry in South Africa. S. Afr. For. J. 152:43-49.
5. Conradie, E., Swart, W. J., and Wingfield, M. J. 1992. Susceptibility of *Eucalyptus grandis* to *Cryphonectria cubensis*. Eur. J. For. Pathol. 22:312-315.
6. Farris, J. S., Källersjö, M., Kluge, A. G., and Bult, C. 1994. Testing significance of incongruence. Cladistics 10:315-319.
7. Felsenstein, J. 1985. Confidence intervals on phylogenetics: an approach using bootstrap. Evolution 39:783-791.
8. Glass, N. L., and Donaldson, G. C. 1995. Development of primersets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. 61:1323-1330.
9. Gryzenhout, M., Myburg, H., Van der Merwe, N. A., Wingfield, B. D., and Wingfield, M. J. 2004. *Chrysoporthe*, a new genus to accommodate *Cryphonectria cubensis*. Stud. Mycol. 50:119-142.
10. Gryzenhout, M., Myburg, H., Wingfield, B. D., Montenegro, F., and Wingfield, M. J. 2005. *Chrysoporthe doradensis* sp. nov. pathogenic to *Eucalyptus* in Ecuador. Fungal Divers. 20:39-57.
11. Gryzenhout, M., Rodas, C. A., Portales, J. M., Clegg, P., Wingfield, B. D., and Wingfield, M. J. 2006. Novel hosts of the *Eucalyptus* canker pathogen *Chrysoporthe cubensis* and a new *Chrysoporthe* species from Colombia. Mycol. Res. 110:833-845.
12. Gryzenhout, M., Wingfield, B. D., and Wingfield, M. J. 2009. Taxonomy, Phylogeny, and Ecology of Bark-Inhabiting and Tree Pathogenic Fungi in the Cryphonectriaceae. American Phytopathological Society, St. Paul, MN.
13. Heath, R., Gryzenhout, M., Roux, J., and Wingfield, M. J. 2006. Discovery of the canker pathogen *Chrysoporthe austroafricana* on native *Syzygium* spp. in South Africa. Plant Dis. 90:433-438.
14. Hodges, C. S., Alfenas, A. C., and Ferreira, F. A. 1986. The conspecificity of *Cryphonectria cubensis* and *Endothia eugeniae*. Mycologia 78:343-350.
15. Hodges, C. S., Reis, M. S., Ferreira, F. A., and Henfling, J. D. M. 1976. O cancro do eucalipto causado por *Diaporthe cubensis*. Fitopatal. Bras. 1:129-167.
16. Katoh, K., Misawa, K., Kuma, K., and Miyata, T. 2002. MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30:3059-3066.
17. Lezar, S., Myburg, A. A., Berger, D. K., Wingfield, M. J., and Wingfield, B. D. 2004. Development and assessment of microarray-based DNA fingerprinting in *Eucalyptus grandis*. Theor. Appl. Genet. 109:1329-1336.
18. Myburg, H., Gryzenhout, M., Heath, R., Roux, J., Wingfield, B. D., and Wingfield, M. J. 2002. *Cryphonectria* canker on *Tibouchina* in South Africa. Mycol. Res. 106:1299-1306.
19. Myburg, H., Gryzenhout, M., Wingfield, B. D., and Wingfield, M. J. 2002. β-Tubulin and Histone *H3* gene sequences distinguish *Cryphonectria cubensis* from South Africa, Asia, and South America. Can. J. Bot. 80:590-596.
20. Myburg, H., Gryzenhout, M., Wingfield, B. D., and Wingfield, M. J. 2003. Conspecificity of *Endothia eugeniae* and *Cryphonectria cubensis*: A re-evaluation based on morphology and DNA sequence data. Mycoscience 44:187-196.
21. Myburg, H., Wingfield, B. D., and Wingfield, M. J. 1999. Phylogeny of *Cryphonectria cubensis* and allied species inferred from DNA analysis. Mycologia 91:243-250.
22. Nakabonge, G., Roux, J., Gryzenhout, M., and Wingfield, M. J. 2006. Distribution of Chrysoporthe canker pathogens on *Eucalyptus* and *Syzygium* species in eastern and southern Africa. Plant Dis. 90:734-740.
23. Nutman, F. J., and Roberts, F. M. 1952. Acute die-back of clove trees in the Zanzibar Protectorate. Ann. Appl. Biol. 39:599-607.
24. Old, K. M., Wingfield, M. J., and Yuan, Z. Q. 2003. A Manual of Diseases of Eucalypts in South-East Asia. Centre for International Forestry Research, Jakarta, Indonesia.
25. Reid, J., and Booth, C. 1969. Some species segregated from the genera *Cryptospora*, *Cryptosporella*, and *Sillia*. Can. J. Bot. 47:1055-1060.
26. Rodas, C. A., Gryzenhout, M., Myburg, H., Wingfield, B. D., and Wingfield, M. J. 2005. Discovery of the *Eucalyptus* canker pathogen *Chrysoporthe cubensis* on native *Miconia* (Melastomataceae) in Colombia. Plant Pathol. 54:460-470.
27. Roux, J., Myburg, H., Wingfield, B. D., and Wingfield, M. J. 2003. Biological and phylogenetic analyses suggest that two *Cryphonectria* species cause cankers of *Eucalyptus* in Africa. Plant Dis. 87:1329-1332.
28. SAS Institute Inc. 1999. SAS/STAT Users Guide, Version 8. SAS Institute Inc., Cary, NC.
29. Seixas, C. D. S, Barreto, R. W., Alfenas, A. C., and Ferreira, F. A. 2004. *Cryphonectria cubensis* on an indigenous host in Brazil: a possible origin for *eucalyptus* canker disease? Mycologist 18:39-45.
30. Sharma, J. K., Mohanan, C., and Florence, E. J. M. 1985. Occurrence of *Cryphonectria* canker disease of *Eucalyptus* in Kerala, India. Ann. Appl. Biol. 106:265-276.
31. Slippers, B., Stenlid, J., and Wingfield, M. J. 2005. Emerging pathogens: fungal host jumps following anthropogenic introduction. Trends Ecol. Evol. 20:420-421.
32. Swofford, D. L. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods), Version 4. Sinauer Associates, Sunderland, MA.
33. Tamura, K., Dudley, J., Nei, M., and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596-1599.
34. Van der Westhuizen, I. P., Wingfield, M. J., Kemp, G. H. J., and Swart, W. J. 1992. Comparative susceptibility of *Eucalyptus grandis* clones and hybrids to *Cryphonectria cubensis*. (Abstr.) Phytophylactica 24:107.
35. Van Heerden, S. W., Amerson, H. V., Preisig, O., Wingfield, B. D., and Wingfield, M. J. 2005. Relative pathogenicity of *Cryphonectria cubensis* on *Eucalyptus* clones differing in the tolerance to *C. cubensis*. Plant Dis. 89:659-662.
36. Van Heerden, S. W., and Wingfield, M. J. 2002. Effect of environment on the response of *Eucalyptus* clones to inoculation with *Cryphonectria cubensis*. For. Pathol. 32:395-402.
37. White, T. J., Bruns. T., Lee. S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.
38. Wingfield, M. J. 2003. Increasing threat of diseases to exotic plantation forests in the southern hemisphere: lessons from *Cryphonectria* canker. Australas. Plant Pathol. 32:133-139.
39. Wingfield, M. J., Rodas, C., Wright, J., Myburg, H., Venter, M., and Wingfield, B. D. 2001. First report of Cryphonectria canker on *Tibouchina* in Colombia. For. Pathol. 31:297-306.
40. Wingfield, M. J., Swart, W. J., and Abear, B. 1989. First record of Cryphonectria canker of *Eucalyptus* in South Africa. Phytophylactica 21:311-313.
41. Xie, Y. J. 2006. Eucalypt research at the beginning of 21st century. China Forestry House Press, Beijing. (In Chinese)
42. Zhou, X. D., Xie, Y. J., Chen, S. F., and Wingfield, M. J. 2008. Diseases of eucalypt plantations in China: challenges and opportunities. Fungal Divers. 32:1-7.