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Holocryphia eucalypti on *Tibouchina urvilleana* in Australia

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Abstract. *Tibouchina* spp. (Melastomataceae) are native to South America, but have been planted as ornamentals in many southern hemisphere countries. The Melastomataceae are members of the Myrtales and are close relatives of the Myrtaceae, to which the genus *Eucalyptus* belongs. The recent discovery of several *Chrysoporthe* spp. on *Tibouchina* spp. has prompted a more detailed survey for other *Eucalyptus* pathogens that might occur on this tree. In this study, the discovery in Australia of *Holocryphia eucalypti*, a *Eucalyptus* pathogen, on diseased stems of *Tibouchina urvilleana* is reported. Characterisation of this fungus was based on morphology and comparisons of β-tubulin gene sequences. Greenhouse pathogenicity tests, including isolates of *H. eucalypti* from *Eucalyptus* spp. in Australia and South Africa, showed that the isolates of *H. eucalypti* from *T. urvilleana* were significantly more pathogenic on *T. urvilleana* than isolates from *Eucalyptus*.

Additional keywords: canker disease, *Chrysoporthe*, *Cryphonectria*, *Endothia*, forestry.

Introduction

Holocryphia eucalypti (M. Venter & M.J. Wingf.) Gryzenh. & M.J. Wingf. is generally a mild canker pathogen of *Eucalyptus* spp. in Australia (Walker *et al*. 1985; Old *et al*. 1986; Yuan and Mohammed 1997, 1999, 2000; Wardlaw 1999; Carnegie 2007*a*, 2007*b*) and South Africa (Van der Westhuizen *et al*. 1993; Gryzenhout *et al*. 2003). Typical symptoms of *H. eucalypti* infection are basal stem cankers, kino exudation from depressed cankers and in severe cases, branch and shoot dieback (Walker *et al*. 1985; Old *et al*. 1986; Van der Westhuizen *et al*. 1993; Carnegie 2007*a*). Cankers caused by *H. eucalypti* are often covered in orange fruiting structures, which make them very conspicuous on the stems of trees (Van der Westhuizen *et al*. 1993). In Australia, the fungus has been reported to kill trees under stressful conditions (Walker *et al*. 1985; Old *et al*. 1986; Wardlaw 1999; Carnegie 2007*a*, 2007*b*).

H. eucalypti was previously known as *Endothia gyrosa* (Schwein.: Fr.) Fr., a canker pathogen of many hardwood species in the United States (Shear *et al*. 1917; Stipes and Phipps 1971; Roane *et al*. 1974; Appel and Stipes 1986). It was later described as the new species *Cryphonectria eucalypti* M. Venter & M.J. Wingf. based on morphological characteristics and DNA sequence data that distinguished it from *E. gyrosa* (Venter *et al*. 2001, 2002). Recently, more extensive DNA sequence comparisons led to the description of a new genus for *C. eucalypti*, namely *Holocryphia*. The *Eucalyptus* canker pathogen in South Africa and Australia is thus known as *H. eucalypti* (Gryzenhout *et al*. 2006*a*).

A recent and potentially important discovery has been that the serious *Eucalyptus* canker pathogens, *Chrysoporthe austroafricana* Gryzenh. & M.J. Wingf. and *Chr. cubensis*, occur not only on members of the Myrtaceae but also on *Tibouchina urvilleana* (DC.) Cogn., which resides in the Melastomataceae (Myburg *et al*. 2002*a*; Gryzenhout *et al*. 2006*b*). The presence of *C. austroafricana* and *C. cubensis* on both *Eucalyptus* spp. and *Tibouchina* spp. has raised the question whether these plants might not share other *Eucalyptus* pathogens.

Although the origin of *H. eucalypti* is not known for certain, its common occurrence in *Eucalyptus* forests in Australia (Walker *et al*. 1985; Old *et al*. 1986) suggests that it is native in that area. *Tibouchina* spp. are native to South America but are relatively widely planted as ornamentals in cities in the eastern parts of Australia. The aim of the present study was to consider whether these ornamental plants have become infected with the commonly occurring *H. eucalypti*.

Methods

Fungal isolates

T. urvilleana trees growing as ornamentals in Melbourne, Victoria, and Coffs Harbour, New South Wales, were examined during November 2000 for stem canker symptoms. Cankers covered in structures resembling a *Holocryphia* sp. were found on *T. urvilleana* trees in both areas. Isolations were made directly

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from fungal structures by transferring spore masses to 2% malt extract agar (MEA) (20 g malt extract, Biolab, Merck, Midrand, South Africa and 15 g agar). Isolates were maintained at 25°C.

Morphological characterisation

Fruiting structures on *T. urvilleana* bark were compared with herbarium specimens of *H. eucalypti* from *Eucalyptus* spp. (Table 1; Venter *et al*. 2002). Fruiting structures were sectioned using a Leica CM1100 cryostat and Leica embedding medium (Setpoint Technologies, Johannesburg, South Africa) at −20°C to a thickness of 12 µm. Conidia and conidiophores were mounted in 3% KOH and measured. Ten measurements were taken of conidiophores and conidia for each collection and these are presented as (min–)(average – s.d.)–(average + s.d.)(–max) µm. A bark specimen bearing fruiting structures from one of the Victorian collections (Table 1) has been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM).

DNA isolation and amplification

Mycelium from actively growing cultures was inoculated into 1.5-mL microcentrifuge tubes containing 750 µL 3% (w/v) malt extract broth. DNA was isolated from 5-day-old cultures using the method described by Murray and Thompson (1980). Two β-tubulin gene regions were amplified using primer pairs Bt1a, Bt1b and Bt2a, Bt2b (Glass and Donaldson 1995).

Each polymerase chain reaction (PCR) was performed using the method described by Myburg *et al*. (2002*b*). Amplification reactions were performed on a Perkin Elmer GeneAmp PCR System 9700 thermocycler (Perkin-Elmer Applied Biosystems Inc., Foster City, CA). PCR products were visualised on a 2% agarose–ethidium bromide gel using ultraviolet light. Product sizes were estimated with a 100-bp (base pair) standard size marker (Promega, Annandale, NSW).

DNA sequencing and analyses

PCR products were purified using a High Pure PCR Product Purification Kit (Roche Diagnostic GmbH, Mannheim, Germany) and sequenced with the same primers used in the PCR amplification reactions. An ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin-Elmer, Warrington, UK) was used for sequencing with an ABI PRISM 3100 automated sequencer. Sequences were aligned using ClustalX (Thompson *et al*. 1997) and manually adjusted using Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems Inc.). All sequences obtained in this study have been deposited in GenBank.

Data analyses were performed using PAUP* (Phylogenetic Analysis Using Parsimony version 4.0b 10*, Swofford 2002). A partition-homogeneity test (PHT) was performed for the two β-tubulin gene region datasets. Analyses were done using the heuristic search option with tree-bisection-reconnection branch swapping. Gaps inserted during sequence alignment were treated as fifth base (NEWSTATE). A bootstrap analysis (50% majority rule, 1000 replications) was done to determine the confidence levels of the tree branching points (Felsenstein 1985). Previously published sequences of various closely related genera were included for comparative purposes. *Diaporthe ambigua* Nitschke, a genus known to be phylogenetically closely related to *Holocryphia* (Gryzenhout *et al*. 2006*a*, 2006*b*), was treated as a monophyletic outgroup taxon to root the phylogenetic tree.

Pathogenicity

To determine the relative pathogenicity of the *Holocryphia* sp. isolated from *T. urvilleana* in Australia, pathogenicity trials were performed under controlled greenhouse conditions. The tests were performed using a complete randomised design. All *Tibouchina* trees were maintained under greenhouse conditions for 2 weeks to acclimatise them before inoculation. The greenhouse was subjected to natural day/night conditions and a temperature setting of ∼25°C. Tree diameters varied from 20 to 30 mm. The two most rapidly growing and healthy isolates of the test fungus from *T. urvilleana* [CMW6245, CMW6246 (CMW– culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa)] were each inoculated onto 25 *T. urvilleana* trees. Twenty-five trees were also each inoculated with the isolates of *H. eucalypti* from *Eucalyptus* in South Africa (CMW7036) and Australia (CMW7038). Twenty trees were inoculated with sterile MEA plugs to serve as a control.

Wounds were made on the test trees using a 10-mm diameter cork borer. Mycelial plugs of a similar size were taken from the actively growing edges of 7-day-old cultures and placed in the wounds with the mycelium facing the cambium. Wounds were sealed with laboratory film (Parafilm 'M', American National Can Chicago, IL, USA) to protect the inoculated fungus and cambium from desiccation. After 10 weeks, lesion lengths were measured and compared. Data were subjected to ANOVA using the General Linear Model procedure of SAS (SAS Statistical

Table 1. Specimens used in the morphological comparisons

Herbarium allocation[A]	Identification	Host	Origin	Collector	Date
PREM56211	*Holocryphia eucalypti*	*Eucalyptus grandis* × *E. camaldulensis*	Nyalazi, South Australia (SA)	M. Venter	1998
PREM56212	*H. eucalypti*	*E. grandis*	Sabie, SA	J. Roux	1998
PREM56305	*H. eucalypti*	*E. saligna*	Tzaneen, SA	M. Venter	1999
PREM56214	*H. eucalypti*	*E. grandis*	Dukuduku, SA	M. Venter	1998
PREM56215	*H. eucalypti*	*E. grandis*	Amangwe, SA	M. Venter	1998
PREM56216	*H. eucalypti*	*E. grandis*	Dukuduku, SA	M. Venter	1998
PREM57595	*H. eucalypti*	*Tibouchina urvilleana*	Melbourne, Victoria	M.J. Wingfield	2001

[A]PREM, National Collection of Fungi, Pretoria, South Africa.

Software 1989). To determine whether the inoculated fungi were responsible for the lesion development, Koch's Postulates were applied by reisolating from the lesions and confirming the identity of the isolated fungi based on morphology.

Results

Fungal isolates

A *Holocryphia* sp. was found on various *T. urvilleana* trees. Four isolates (CMW6244, CMW6245, CMW6246, CMW6249) were obtained from trees in Melbourne, Victoria, and one isolate (CMW10729) was isolated from a tree at Coffs Harbour, New South Wales. All isolates are maintained in the CMW.

Morphological characterisation

Only asexual fruiting bodies were found on *T. urvilleana* trees (PREM57595). Stromata were 180–330 µm wide, orange, pulvinate, semi-immersed in the bark, uni to multilocular with strongly convoluted locules. Long paraphyses [(18.5–) 31.5–60.5 × 1–1.5 (–2) µm] similar to the paraphyses found between conidiophores of *H. eucalypti* (Walker *et al*. 1985; Venter *et al*. 2002) were observed. Conidia were cylindrical

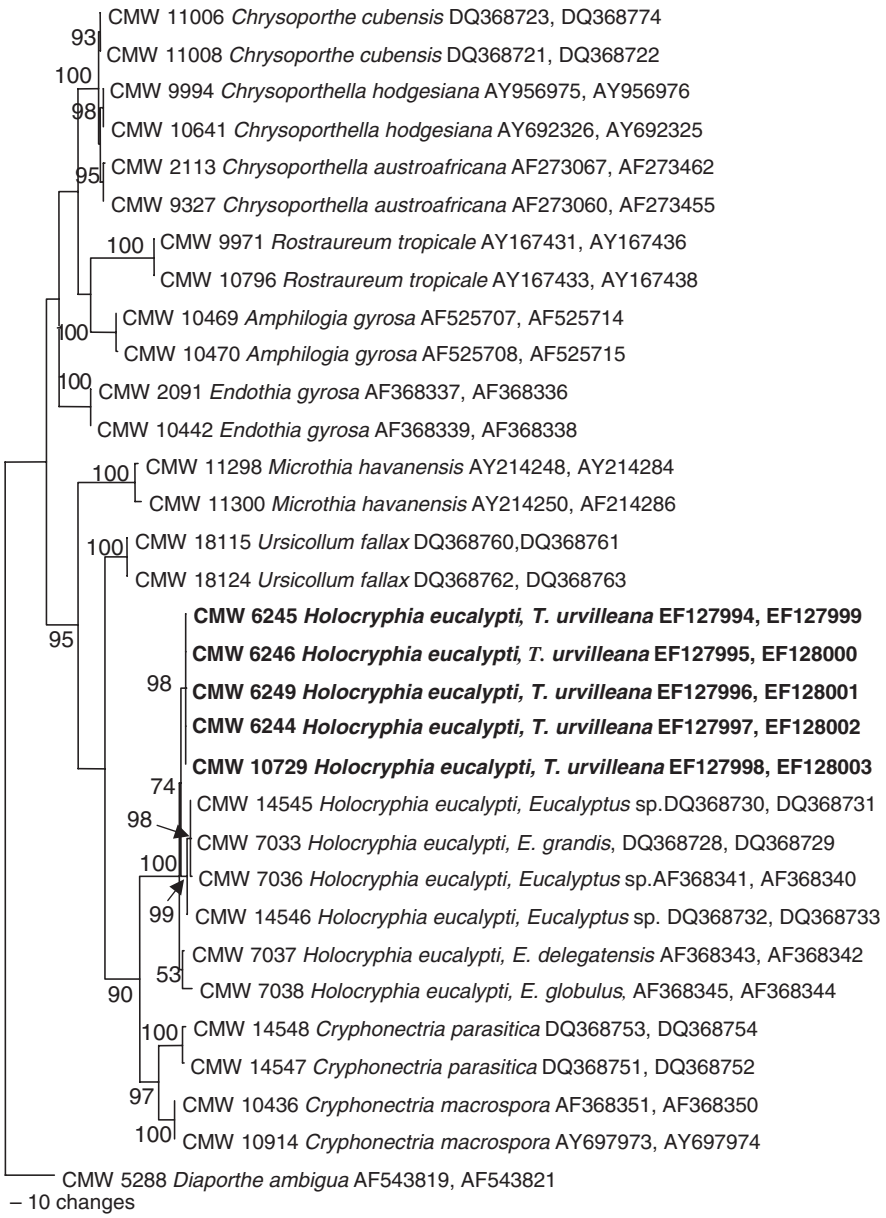

Fig. 1. Phylogenetic tree generated from the β-tubulin gene sequence dataset [tree length = 1045 steps, consistency index (CI) = 0.820, retention index (RI) = 0.0.88664, rescaled consistency index (RC) = 0.727 and homoplasy index (HI) = 0.180] generated from heuristic searches. Bootstrap values (1000 replicates) are indicated above the branches with those lower than 50% not shown. *Diaporthe ambigua* was used to root the tree.

and similar in size [(3.0–) 3.5–4.5 (–5.0) × 1.0–1.5 µm] to those of *H. eucalypti* (Walker *et al*. 1985; Venter *et al*. 2002). Conidiophores were (6.0–)8.5–18.0(–25) × 1.0–1.5 (–2.0) µm and corresponded with those of *H. eucalypti* (Venter *et al*. 2002).

DNA sequencing and analyses

PCR amplification of the five isolates from *Tibouchina* in Australia with the two primer pairs resulted in fragments of ~500 bp (Bt1a/Bt1b) and ~440 bp (Bt2a/Bt2b), respectively. Aligned sequences of the combined data resulted in a dataset of 944 characters, consisting of 538 constant characters, 370 parsimony informative characters and 36 variable characters that were parsimony uninformative. After alignment, a partition homogeneity test on the two separate datasets gave a PHT value of $P = 0.1$ showing that the two datasets are congruent and could be combined in the phylogenetic analysis. The heuristic search produced one most parsimonious tree (tree length = 1077 steps, consistency index = 0.6509, retention index = 0.8754, rescaled consistency index = 0.5698 and homoplasy index = 0.3491) (Fig. 1). A strict bootstrap consensus tree showed that most branches were well supported with high bootstrap values (Fig. 1). All the isolates from *T. urvilleana* from Australia grouped in the same clade as other *H. eucalypti* isolates from South Africa and Australia isolated from *Eucalyptus* spp.

Pathogenicity

Greenhouse inoculations on *T. urvilleana* trees resulted in distinct lesions within 10 weeks. The control inoculations produced no lesions. All isolates tested produced lesions statistically significant from the control inoculations ($P < 0.001$). There were no significant differences in pathogenicity between the Australian (CMW7038) and South African (CMW7036) *H. eucalypti* isolates from *Eucalyptus* spp. ($P = 0.1699$). Isolates from *T. urvilleana* were, however, significantly more pathogenic than the *H. eucalypti* isolates from *Eucalyptus* spp. in Australia and South Africa ($P < 0.001$). The test organism was consistently isolated from the lesions.

Discussion

This study presents the first report of *H. eucalypti* from a host plant other than *Eucalyptus*. Identification of isolates from *T. urvilleana* was achieved using morphological and phylogenetic comparisons. β-tubulin sequences were chosen for comparison of isolates in the present study as previous studies have shown that this region accurately reflects phylogenetic relationships in *Holocryphia* and allied genera (Venter *et al*. 2002; Myburg *et al*. 2004; Gryzenhout *et al*. 2006*a*, 2006*b*).

In the present study it has been shown that the *H. eucalypti* isolates from *T. urvilleana* were pathogenic on this species. This strongly suggests that the fungus was responsible for the die-back observed on the ornamental trees sampled. The results also show that the *H. eucalypti* isolates from *T. urvilleana* were more pathogenic on *T. urvilleana*, than isolates from South Africa and Australia, isolated from *Eucalyptus* spp. The pathogenicity of the *Tibouchina* isolates on *Eucalyptus* and other related Myrtales remains to be tested.

If *H. eucalypti* originated in Australia with *Eucalyptus* as its native host, it is possible that the fungus may have made a host jump and cross-infected the non-native *T. urvilleana*. Such a host jump seems plausible because *Chr. austroafricana* and *Chr. cubensis* are also able to cross-infect these two hosts (Myburg *et al*. 2002*a*; Gryzenhout *et al*. 2006*b*). Results from the present study support the fact that *Tibouchina* spp. could harbour *Eucalyptus* pathogens. This could have major implications for quarantine, as a pathway for the spread of the pathogen could be masked as it is moved on an inconspicuous ornamental tree plant between countries. It also raises the concern that if *H. eucalypti* is introduced into South America it poses a major threat to the native *Tibouchina* population and possibly other native Melastomataceae. Further studies on other members of the Melastomataceae both in Australia and other parts of the world are needed to confirm this hypothesis.

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