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The *Eucalyptus* canker pathogen *Chrysoporthe cubensis* discovered in eastern Australia

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Abstract. *Chrysoporthe cubensis* is an important pathogen of commercially planted *Eucalyptus* species (Myrtaceae) in tropical and subtropical parts of the world where these trees are planted as non-natives. Although the majority of *Eucalyptus* spp. are native to Australia, *Chr. cubensis* is not common there and has been reported only once from naturally growing *Eucalyptus marginata* in Western Australia. *Chr. cubensis* is able to infect hosts in the Myrtaceae and Melastomataceae other than *Eucalyptus*, but has not been found on hosts other than *Eucalyptus* in Australia. Recently, fruiting structures resembling those of *Chr. cubensis* were discovered on *Tibouchina heteromalla*, planted as a non-native in the Botanical Gardens in Cairns, northern Queensland. These fruiting structures and resulting isolates were characterised as *Chr. cubensis* in this study. Pathogenicity studies found that *Corymbia* spp., including commercially important spotted gum, are susceptible along with *E. pilularis* and *E. dunnii*. The discovery of *Chr. cubensis* on a non-native plant in the northern part of Australia is important as it might imply that the pathogen has been introduced into the country and pose a significant threat to native *Eucalyptus* forests. Alternatively, this fungus might occur naturally in the area on hosts other than *Tibouchina*, possibly *Eucalyptus* or related species, and that Australia forms part of its native range.

Introduction

The hardwood plantation area in Australia has expanded rapidly in recent years with over 100 000 ha planted in subtropical and tropical regions of eastern Australia (Gavran and Parsons 2008). Spotted gum *Corymbia citriodora* subsp. *variegata* (hereafter referred to as *C. variegata*), *C. citriodora* subsp. *citriodora* (hereafter referred to as *C. citriodora*) and *C. henryi* are some of the priority species used in the subtropics, along with *Eucalyptus dunnii* and *E. pilularis*, for solid wood production. The expansion of eucalypt plantations has resulted in the emergence of new pathogens, such as *Kirramyces viscidus* (Andjic *et al*. 2007), and the rapid increase in incidence of diseases previously seen as insignificant, such as *Quambalaria pitereka* (Pegg *et al*. 2008). The identification and management of pathogens that threaten these plantations is paramount to the success of the forestry industry in Australia. This is especially so because current and long-term climate change may predispose Australian *Eucalyptus* forests and plantations to these pathogens, especially those that are favoured by stress (Desprez-Loustau *et al*. 2006).

Although several stem canker pathogens have been identified from plantations in subtropical and tropical regions of Australia, they have rarely been associated with severe damage (Carnegie 2007; Van Wyk *et al*. 2007). Stem pathogens known in the area include species of *Ceratocystis*, most recently *Ceratocystis atrox* associated with *Phoracantha acanthocera* on *Eucalyptus grandis* (Van Wyk *et al*. 2007) in north Queensland, *Cytospora eucalypticola* on *E. nitens*, *Botryosphaeria eucalyptorum* on *E. dunnii* and *Holocryphia eucalypti* (=*Endothia gyrosa*) (Carnegie 2007). At present these stem pathogens impart low levels of impact, which may be due to the fact that most plantations are under 10 years of age and stem diseases often manifest themselves on older trees.

Chr. cubensis was previously known as *Cryphonectria cubensis*, the causal organism of the serious disease Cryphonectria canker on *Eucalyptus* spp. (Gryzenhout *et al*. 2004). *Cryphonectria cubensis* was shown to represent a species complex in the new genus *Chrysoporthe* and currently five species have been described in the genus (Gryzenhout *et al*. 2004, 2009). These include *Chr. cubensis*, *Chr. austroafricana* (Gryzenhout *et al*. 2004), *Chr. doradensis* (Gryzenhout *et al*. 2005), *Chr. inopina* (Gryzenhout *et al*. 2006) and the anamorphic species *Chrysoporthella hodgesiana* (Gryzenhout *et al*. 2004). All of these species are pathogens occurring in tropical and subtropical areas, but only *Chr. cubensis*, *Chr. austroafricana* and *Chr. doradensis* are known as important pathogens of *Eucalyptus*.

Chr. cubensis is primarily known from *Eucalyptus* (Myrtaceae, Myrtales), but several other hosts have been reported for the pathogen. In the Myrtaceae, this fungus also occurs on *Syzygium aromaticum* (clove), which is native to the Molucca islands in Indonesia (Myburg *et al*. 2003). Recently, *Chr. cubensis* has been reported from several tree species in the Melastomataceae, another family in the Myrtales. These hosts

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include *Miconia theaezans*, *M. rubiginosa* (Rodas *et al*. 2005), *Rhynchanthera mexicana*, *Clidemia sericea*, *Tibouchina urvilleana* and *Melastoma melabathricum* (Gryzenhout *et al*. 2006). Of these hosts the report on *T. urvilleana* was from a non-native tree in Thailand, but the other trees were native in the South American or Asian countries where they were found.

Several hypotheses have been proposed concerning the origin of *Chr. cubensis*. These have predominantly been based on the occurrence of the fungus on native trees related to *Eucalyptus*. This is because *Chr. cubensis* is not common in countries such as Australia, where the *Eucalyptus* spp. it infects are native (Gryzenhout *et al*. 2006, 2009). There is only a single report of *Chr. cubensis* from Australia where it was found in Western Australia on *E. marginata* roots (Davison and Coates 1991). This is not a host of the pathogen elsewhere in the world and the area of occurrence has a Mediterranean climate that is atypical for the pathogen (Gryzenhout *et al*. 2006).

The first hypothesis regarding the origin of *Chr. cubensis* was by Hodges *et al*. (1986) who suggested that the fungus might have originated in the Moluccas (Indonesia) on clove. From here, it was hypothesised to have moved to other clove-growing areas in Indonesia, and subsequently transported to other parts of the world during the slave trade. An alternative hypothesis emerged from the discovery of a fungus thought to represent *Chr. cubensis*, namely *Chr. hodgesiana*, on *T. urvilleana* in Colombia (Wingfield *et al*. 2001). *Chr. cubensis* and its closest relatives have subsequently been found on many members of the Melastomataceae growing as natives in Central and South America, and the fungus is also found on non-native *Eucalyptus* spp. and *S. cordatum* in those countries (Gryzenhout *et al*. 2006). This adds support for the view that *Chr. cubensis* is native to South and Central America where it has undergone a host shift (Slippers *et al*. 2005) to *Eucalyptus*.

Chr. cubensis resides in two very distinct phylogenetic subclades that clearly divide isolates from South America and South-East Asia (Myburg *et al*. 2002*b*; Gryzenhout *et al*. 2004, 2006). *Chr. cubensis*, therefore, either represents two cryptic species or a taxon in the process of speciation. Because the fungus has been found on native trees in both regions, a third hypothesis regarding the origin of *Chr. cubensis* suggests that isolates representing the two subclades could have separate South American and Asian origins (Gryzenhout *et al*. 2006).

During health surveys of eucalypt plantings in northern Queensland, Australia, fruiting structures from dead, woody stems resembling those of *Chr. cubensis* were observed on *Tibouchina heteromalla* in the Cairns Botanical Gardens. Because the fungus and other *Chrysoporthe* spp. are well known on *Tibouchina* spp. elsewhere in the world, and due to its importance as a *Eucalyptus* pathogen, a study was undertaken to identify the fungus conclusively. In addition, the pathogenicity of isolates was tested on *Eucalyptus* spp. important in the region, in order to assess its possible threat to forestry in Australia.

Materials and methods

Survey

In 2006, surveys aimed at identifying pathogens of significance to the timber plantation industry were conducted in northern Queensland. Surveys included trial plantings of *Eucalyptus* and *Corymbia* spp. and their hybrids as well as *Tibouchina* spp. in botanical gardens, along roads and in private gardens in areas from Cairns south to Cardwell, and west to Mareeba and the Atherton Tablelands. *Tibouchina* species were included in the surveys because they are known hosts of various *Eucalyptus* pathogens such as *Chr. cubensis* (Rodas *et al*. 2005; Gryzenhout *et al*. 2006), *H. eucalypti* (Heath *et al*. 2007) and *Botryosphaeriaceae* (Heath *et al*. 2009).

Samples with fruiting bodies resembling those of *Chr. cubensis* were collected from dead and dying stem material and transported back to the laboratory in Brisbane for isolation. Isolations from the bark material were made on 20 g/L malt extract agar (MEA; Bacto Laboratories Pty Ltd, Liverpool, NSW, Australia) by taking spore drops from the apices of fruiting structures. The identity of the fruiting structures was verified microscopically to represent *Chr. cubensis* using standard techniques. Isolates from infected *T. heteromalla* from northern Queensland are preserved at 5°C in the culture collections of Brisbane Plant Pathology, DPI&F (BRIP 52368A–C) and that of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa (CMW 18855–18860). The original bark material has been deposited with the National Collection of Fungi, Pretoria, South Africa (PREM 60250 = CMW 18855–18858; PREM 60251 = CMW 18859–18860).

DNA sequence characterisation

DNA was extracted from three representative isolates (BRIP 52368A–C) of the fungus representing a *Chrysoporthe* sp. collected form *T. heteromalla* in the Cairns Botanical Garden. PCR products were obtained for the part of the β-tubulin gene region using primer pairs BT1a/1b and BT2a/2b (Glass and Donaldson 1995) following the protocols described by Gryzenhout *et al*. (2004). The PCR products were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase (Perkin-Elmer, Warrington, UK) on an ABI PRISM 3100 automated DNA sequencer. The resultant sequences (GenBank accession numbers BRIP 52368A = GQ204311; BRIP 52368B = GQ204312; BRIP 52368C = GQ204313) were compared with representative sequences for the five described species of *Chrysoporthe* (Gryzenhout *et al*. 2004, 2005, 2006). Sequences of *Cryphonectria parasitica*, *Cry. japonica* and *Cry. macrospora*, three closely related species, were used as outgroup taxa (Gryzenhout *et al*. 2009).

Using the PAUP program version 4.0b10, a parsimony analysis was done on the dataset (uninformative sites excluded, heuristic search with 100 random sequence additions and tree-bisection-reconnection branch swapping, MULTREES off, base pairs reweighted according to the consistency index). A 70% bootstrap analysis (1000 replicates) was also done on the dataset to determine the confidence levels of the branches.

Host range and pathogenicity

To determine the host range and potential importance of the isolated fungus, a selection of *Eucalyptus* spp. and *Corymbia* spp. grown in subtropical and tropical regions of Australia, were tested for their susceptibility to the fungus isolated from

T. heteromalla in northern Queensland. These trees are used in plantation development and occur naturally in the subtropical and tropical regions of eastern Australia. *Eucalyptus* spp. tested included *E. tereticornis*, *E. cloeziana*, *E. argophloia*, *E. longirostrata*, *E. dunnii*, *E. grandis*, *E. pilularis* and an *E. grandis* × *E. camaldulensis* hybrid. *Corymbia* spp. included *C. variegata*, *C. torelliana* and a *C. torelliana* × *C. variegata* hybrid. Cuttings of *T. heteromalla* were also established, and were included in the study along with a hybrid *Tibouchina* var. Carol Lyn and *Tibouchina* var. Alstonville, which are sold commercially in Queensland.

Seedlings and cuttings were grown in steam-sterilised soil mix, fertilised with slow release Scott's Osmocote Plus Native Trees (Baulkham Hills, NSW, Australia) as required and irrigated twice a day for 10 min each day using overhead sprinklers. Glasshouse temperatures were maintained at 25–28°C during the day and 20−22°C overnight. Trees were grown for 6 months, to allow the development of a woody stem, before inoculation.

A culture of the fungus from *T. heteromalla* in Cairns (BRIP 52368A) was established on MEA and grown in the dark at 25°C for 4 weeks. A sterilised cork borer, 10 mm in diameter, was used to wound the stems of trees, exposing the cambium. A 10-mm-diameter piece of malt agar with *Chr. cubensis* was then placed on the wound site and covered with parafilm. As a control, sterile MEA was placed in the wound sites and covered with parafilm. Each treatment was replicated six times.

To measure the lesion length, the outer layer of bark was scraped away above and below the inoculation point to expose the cambium. Lesion lengths were measured 6 weeks after inoculation. An occlusion rating was also given to each treatment based on a 1–4 rating where 1 = totally occluded; 2 = partial occlusion; 3 = no occlusion evident; and 4 = cankering evident.

Results

Survey

The only trees of the *Eucalyptus* and *Tibouchina* spp. included in the surveys that had structures similar to those of *Chrysoporthe* spp., were those of *T. heteromalla* growing in the Cairns Botanical Gardens (Fig. 1*a*). These plants displayed severe dieback and fruiting structures were abundant on the dead and dying stems. Morphological characteristics of the fungus including conidiomata, conidiophores and conidia, were typical of those of *Chr. cubensis* (Gryzenhout *et al*. 2004). While not included in the original surveys, *T. heteromalla* trees growing in the Brisbane Botanical Gardens (Mt Cootha) were also assessed for symptoms associated with species of *Chrysoporthe* but none were identified at the time of inspection.

Fig. 1. (*a*) Branch dieback and fruiting bodies of *Chrysoporthe cubensis* was detected on *Tibouchina heteromalla* in Cairns Botanical Gardens, northern Queensland, Australia. (*b*) Lesions caused by *Chr. cubensis* isolates in glasshouse trials on *Corymbia torelliana* × *C. variegata* (*c*) *Tibouchina* var. Alstonville (*d*) *Eucalyptus dunnii* (*e*) and *C. torelliana*. Arrow heads indicate inoculation points and arrows indicate lesion limits. (*f*) Mature (white arrows) and immature (black arrows) indicates fruiting bodies of *Chr. cubensis* above the inoculation point on *C. variegata*.

DNA sequence comparisons

The DNA sequence dataset of the β-tubulin gene region consisted of 1007 characters (757 constant, 47 uninformative, 203 informative) and 24 taxa. Phylogenetic analyses resulted in two trees that were identical except for lengths of individual branches (tree length = 251.5, consistency index = 0.911, retention index = 0.940). In the phylogenetic trees, the isolates from *T. heteromalla* in Cairns grouped in the South-East Asian subclade (Fig. 2) of *Chr. cubensis* (bootstrap 76%). The different isolates also had the same previously defined signature base pairs as other isolates from South-East Asia (Gryzenhout *et al*. 2004), including the Australian isolate from *E. marginata*, CMW 2632 from Western Australia (data not shown).

Pathogenicity tests

Inoculations with *Chr. cubensis* prevented wound occlusion on all *Eucalyptus* and *Corymbia* spp. and their hybrids, and the lesions were significantly ($P < 0.0001$) different from the control treatments, where wounds occluded either partially or fully (Fig. 3). *E. tereticornis* was significantly less affected by the inoculated fungus in comparison to other *Eucalyptus* and *Corymbia* spp. and hybrids. Wounds on treated and untreated *T. heteromalla* and *Tibouchina* hybrids either partially or totally occluded within 5 weeks of inoculation but failed to occlude on *Tibouchina* var. Alstonville.

Lesion lengths varied on the *Corymbia* spp. with the *Corymbia* hybrid *C. torelliana* × *C. variegata* being most susceptible (Figs 2*b*, 4) and significantly more susceptible to inoculation with *Chr. cubensis* than *C. variegata* ($P < 0.0001$) and *C. torelliana* ($P = 0.0041$) (Figs 2*e*, 4). Three of six *Corymbia* hybrid trees were dead within 3 weeks of inoculation. Tree deaths were also recorded for *C. variegata*. Although no deaths were recorded for *C. torelliana*, lesion lengths were significantly greater ($P = 0.0093$) than those on *C. variegata*. Ascomata of *Chr. cubensis* were present above wound sites on *Corymbia* species 5 weeks after inoculation.

Lesion development was recorded on all species of *Eucalyptus* except *E. tereticornis*. Of the species of *Eucalyptus* tested, *E. pilularis* was significantly more susceptible ($P > 0.0001$) to inoculation with *Chr. cubensis* than other species. Lesion lengths for inoculated *E. dunnii* (Figs 2*d*, 4) and *E. argophloia* were significantly ($P = 0.0147$, $P = 0.0318$) different from the uninoculated controls. Lesion lengths on *E. grandis*, *E. grandis* × *E. camaldulensis*, *E. cloeziana* and *E. longirostrata* were not significantly different from those on the untreated controls.

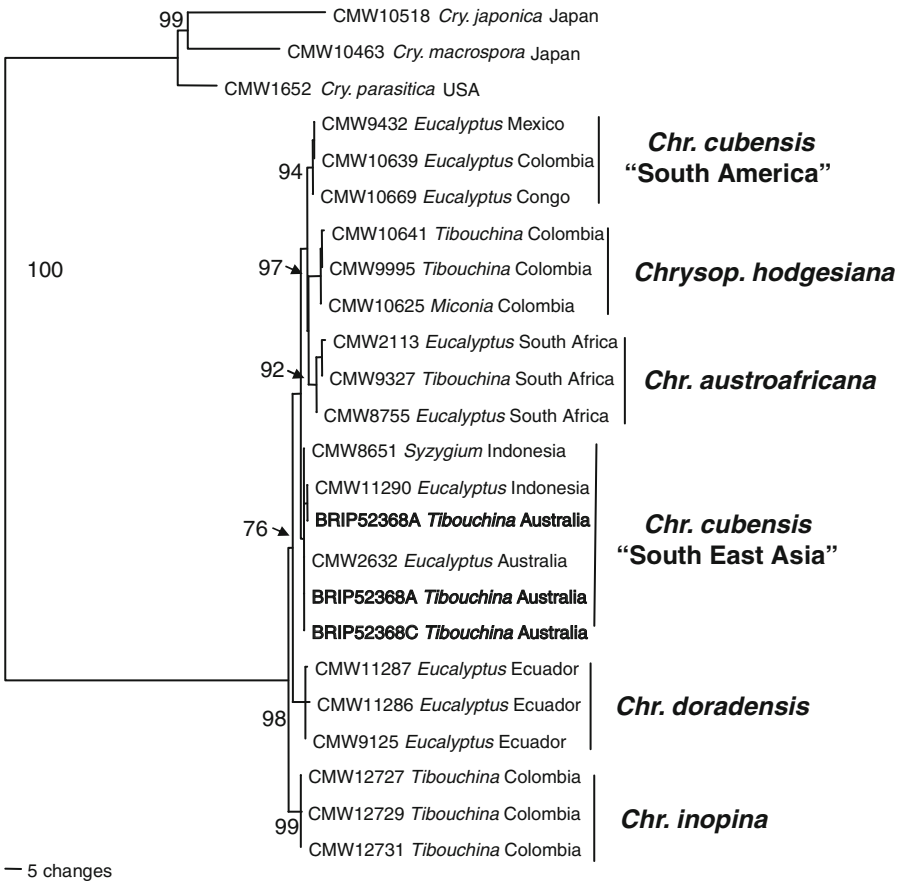

Fig. 2. Parsimony-based phylogram based on DNA sequences of the β-tubulin gene. Confidence levels are based on a 70% bootstrap analysis. Isolates sequenced in this study are shown in bold. The three *Cryphonectria* species were defined as outgroup.

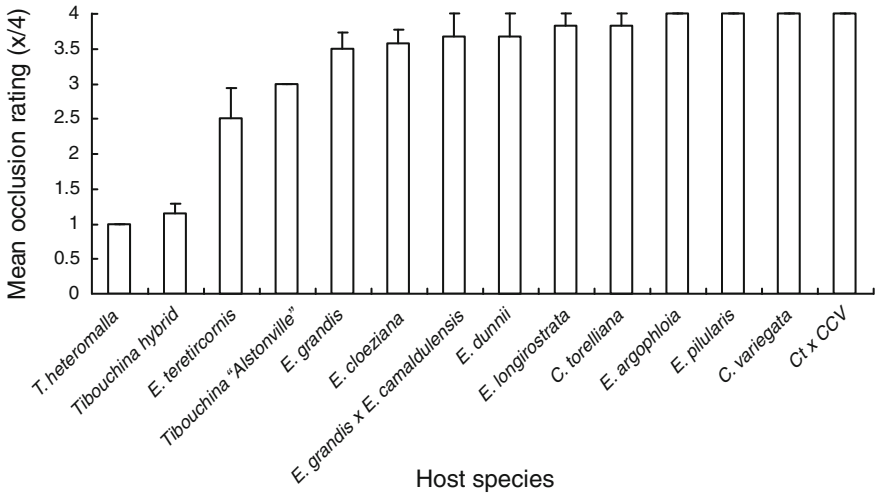

Fig. 3. Comparison of wound occlusion ratings on *Eucalyptus*, *Corymbia* and *Tibouchina* spp. inoculated with *Chrysoporthe cubensis* in comparison to uninoculated controls under glasshouse conditions. Mean wound occlusion rating is shown with standard errors.

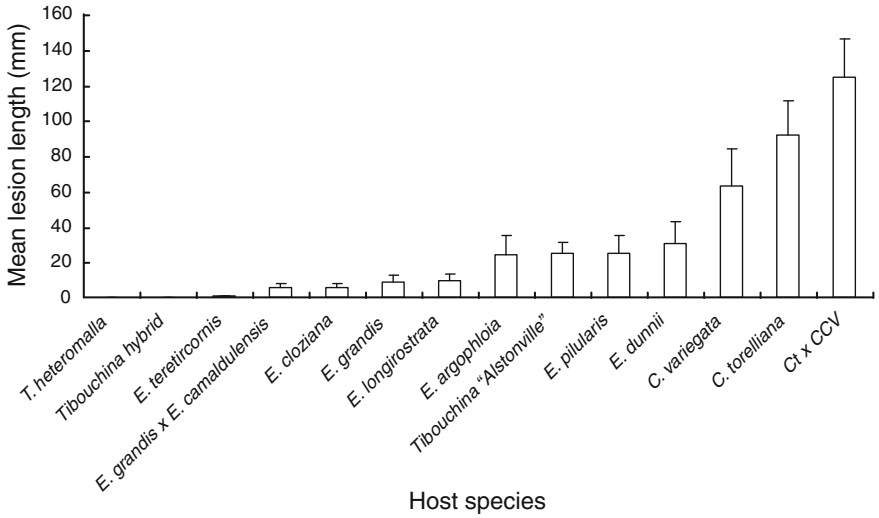

Fig. 4. Comparison of lesion length on *Eucalyptus*, *Corymbia* and *Tibouchina* species inoculated with *Chrysoporthe cubensis* and uninoculated controls under glasshouse conditions. Mean length of lesion is shown with standard errors.

External lesions did not develop on *T. heteromalla* or *Tibouchina* var. Carol Lyn. However, small lesions were observed on *Tibouchina* var. Alstonville (Figs 2*c*, 4). Although lesions were not observed on *T. heteromalla*, fruiting structures typical of *Chr. cubensis* were identified on stems of this plant 5 months after inoculation. Twelve months after inoculation, branch dieback with associated *Chr. cubensis* fruiting bodies was detected on *T. heteromalla*. Cultures were obtained from these fruiting structures and DNA sequence comparisons confirmed the presence of *Chr. cubensis*.

Discussion

This study records the first discovery of the important *Eucalyptus* canker pathogen *Chr. cubensis* in eastern Australia. This is the second record of the pathogen in Australia, but the first time that it has been found associated with disease symptoms. The discovery of *Chr. cubensis* in an area of Australia where the climate is typical of that encountered in areas of the world where the pathogen causes serious disease, is of concern and it deserves further attention.

The isolates of *Chr. cubensis* discovered on *T. heteromalla* in Cairns represent the South-East Asian subclade of the pathogen. This is particularly relevant because isolates of the fungus from around the world are phylogenetically divided into two discrete groups separating those from South-East Asia from those of South and Central America (Myburg *et al*. 2002*b*; Gryzenhout *et al*. 2004, 2006). The South-East Asian form of *Chr. cubensis* has been found on native trees in Indonesia (Hodges *et al*. 1986;

Gryzenhout *et al*. 2006), which supports the view that it is native in the region. It is, therefore, possible that the fungus found in Cairns represents a native fungus in the southern-most part of its natural range. Alternatively, it has been accidentally introduced into the region.

The fact that *Chr. cubensis* was found in a botanical garden might suggest that the fungus was introduced into Australia. Ornamental plants could represent unsuspected vessels that carry important pathogens such as *Chr. cubensis*, around the world (Gryzenhout *et al*. 2009). *Tibouchina* spp. are native to South and Central America and the fact that the South-East Asian form of the fungus was found suggests that the fungus was not introduced from the Americas directly on imported plants. In their natural range, *Tibouchina* spp. grow at relatively high altitudes and thus in cool environments, and they become seriously diseased due to infection by *Chr. cubensis* when they are planted as ornamentals in hot and humid environments. If *Chr. cubensis* was introduced into Australia, it would have come from somewhere in South-East Asia. In this case it could have been on *Tibouchina* spp. that are commonly grown in countries such as Thailand and Singapore as ornamentals and also infected by *Chr. cubensis* (Gryzenhout *et al*. 2006), or other susceptible ornamental species.

It was interesting that the *T. heteromalla* plants tested for susceptibility to *Chr. cubensis* failed to develop symptoms rapidly. This is different to previous inoculations on *Tibouchina* spp. with isolates of *Chr. cubensis* (Rodas *et al*. 2005) and other *Chrysoporthe* spp. (Wingfield *et al*. 2001; Myburg *et al*. 2002*a*; Gryzenhout *et al*. 2005), where these plants typically die rapidly. However, the plants slowly became severely diseased in the inoculation trials and they are clearly susceptible to infection, which is also consistent with the plants on which they were discovered in Cairns.

Glasshouse pathogenicity tests conducted in this study showed that there is a high level of susceptibility to *Chr. cubensis*, particularly among the *Corymbia* spp. and *E. pilularis*. These are important native species in Australia and it is of concern that they are susceptible to infection by *Chr. cubensis*. Their high level of susceptibility also suggests that they are unlikely to have evolved together with the pathogen and this would suggest that it is an alien pathogen in Australia.

The fact that *Chr. cubensis* has not been detected during annual forest health surveys raises several questions as to the origin of the pathogen. This is especially so given the susceptibility of *Corymbia* species and *E. pilularis* in an area with a tropical and subtropical climate suitable for infection by *Chrysoporthe* species. While the source of the *T. heteromalla* trees planted in the Cairns Botanical Gardens is unknown, it has undoubtedly been imported at some point in time. The fact that symptom development was not apparent for some time after inoculation with *Chr. cubensis*, may have allowed for importation of infected material, which was asymptomatic at the time of importation. The other possibility is that *Chr. cubensis* is native to northern Queensland and somewhat cryptic within the native environment. Thus, plantation age and expansion of the eucalypt plantations may not yet be sufficient in this region to see the development of Cryphonectria canker. It must also be considered that *Chr. cubensis* could have an endophytic phase on certain hosts similar to species of *Botryosphaeria* on *Eucalyptus* spp. These fungi cause endophytic infections that may persist for extended periods of time and cause stem canker and dieback in trees under stress conditions (Slippers *et al*. 2009).

The natural mode of infection in native or older plantation *Corymbia* spp. may be a factor in the expression of pathogenicity and subsequent detection of *Chr. cubensis*. Both *C. torelliana* and species of spotted gum are smooth-barked and perhaps less conducive to infection. Wardlaw (1999), when investigating stem cankers associated with *H. eucalypti* (=*E. gyrosa*) in *E. nitens* plantations, found that only 11% of trees with smooth bark had cankers in comparison to 97% of rough-barked trees. Wardlaw (1999) also identified *H. eucalypti* in fine cracks in the new bark of rough-barked trees and suggested the presence of this suitable infection court differentiated rough bark from smooth bark in terms of infection. Yuan and Mohammed (2001) also found (using artificial inoculation studies) that cankers formed on smooth-barked trees and surmised that the absence of cracks in smooth-barked trees was significant at the pre-penetration stage under conditions of natural infection. *Chr. cubensis* is similar to *H. eucalypti* in that it infects trees through wounds and susceptibility might also be related to bark morphology in *Eucalyptus* spp.

In terms of quarantine and long-term forest biosecurity, it will be important to determine whether *Chr. cubensis* found in this study is a native or an introduced pathogen. The ideal route to resolving this question will be through a population genetic study on isolates of the pathogen collected in north Queensland. The fungus is clearly not common as it was only encountered once on a non-native plant. A route to collecting a population of the pathogen would be to plant a susceptible species of *Tibouchina* in north Queensland plantations and to use these as trap trees. Such a study would also provide useful information as to whether the fungus is present in the wild, but with a sufficiently low inoculum density as not to be obvious.

Clearly, further surveys of plantation and native eucalypts, and related trees, are required to elucidate the presence of *Chr. cubensis* in Australia. This study only represents the second report of *Chr. cubensis* in Australia besides that in Western Australia, and both these reports indicate a limited host and geographic range. Pathogenicity tests to identify resistance against Chrysoporthe canker within the *Corymbia* species and hybrid and *Eucalyptus* species targeted for plantation development, especially in the northern tropics, may help prevent serious losses in the near future. However, it must be taken into account that artificial wounding, particularly in very young trees, may not reflect the expression of pathogenicity in the native environment. Therefore, inoculation of older plantation trees may provide a better understanding of host susceptibility within *Corymbia* and *Eucalyptus* species in subtropical and tropical regions of Australia.

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