β-Tubulin and histone *H3* gene sequences distinguish *Cryphonectria cubensis* from South Africa, Asia, and South America

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Abstract: *Cryphonectria cubensis* (Bruner) Hodges is the causal agent of an important stem canker disease of *Eucalyptus*. Previous phylogenetic studies based on sequence data have shown that *C. cubensis* is distinct from other species of *Cryphonectria* but that *C. cubensis* isolates reside in two distinct groups, consistent with geographical origin. Thus, isolates of *C. cubensis* from South America and South Africa grouped together but apart from those originating from Southeast Asia and Australia. These results were in contrast with the symptoms of Cryphonectria canker in South Africa, which are different from those observed elsewhere in the world. The aim of this study was to use more variable regions of the fungal genome to test whether South African isolates of *C. cubensis* are genetically distinct from those from other parts of the world. For this comparison, β-tubulin and histone *H3* gene sequences were used. Specimens from South America, Southeast Asia, Australia, and South Africa were also compared morphologically. The phylogram emerging from the analysis indicated that South American and Southeast Asian – Australian isolates resided in two well-resolved but closely related clades. However, isolates from South Africa were distinct from other groups. This is consistent with ecological aspects of the South African fungus, although no obvious morphological differences between the fungi from the various regions could be found. Our results suggest that the South African fungus represents a species distinct from *C. cubensis* occurring elsewhere in the world.

Key words: β-tubulin, histone *H3*, molecular phylogeny, *Cryphonectria cubensis*.

Résumé : Le *Cryphonectria cubensis* (Bruner) Hodges est l'agent causal d'un important chancre de la tige chez les *Eucalyptus*. Des études phylogénétiques précédentes, basées sur les données de séquences, montrent que le *C. cubensis* se distingue de d'autres espèces de *Cryphonectria* mais que les isolats du *C. cubensis* forment deux groupes congrus avec les origines géographiques. Ainsi, les isolats du *C. cubensis* de l'Amérique du sud et de l'Afrique du sud se regroupent ensemble, mais distinctement des isolats provenant du sud-est asiatique et de l'Australie. Ces résultats contrastent avec les symptômes des chancres cryphonectriens de l'Afrique du sud, qui sont différents de ceux observés ailleurs dans le monde. Le but de cette étude était d'utiliser d'autres régions variables du génome fongique pour vérifier si les isolats de l'Afrique du sud du *C. cubensis* sont génétiquement distincts de ceux de d'autres parties du monde. Pour cette comparaison, les auteurs ont utilisé les séquences des gènes de la β-tubuline et de l'histone *H3*. Ils ont également comparé la morphologie de spécimens provenant de l'Amérique du sud, du sud-est asiatique, de l'Australie et de l'Afrique du sud. Le phylogramme résultant de ces analyses indique que les isolats provenant de l'Amérique du sud, du sud-est asiatique et de l'Australie se retrouvent dans deux clades distincts bien qu'étroitement reliés. Cependant, les isolats de l'Afrique du sud se distinguent des autres groupes. Ceci est congruent avec les aspects écologiques du champignon sud africain, bien qu'il n'y ait pas de différences morphologiques évidentes entre les champignons des diverses régions. Les résultats suggèrent que le champignon sud-africain constitue une espèce distincte du *C. cubensis* qu'on retrouve ailleurs dans le monde.

Mots clés : β-tubuline, histone *H3*, phylogénie moléculaire, *Cryphonectria cubensis*.

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Introduction

Cryphonectria cubensis (Bruner) Hodges is an important stem canker pathogen of *Eucalyptus* trees in plantations. It causes a disease known as Cryphonectria canker that is common in many tropical and subtropical parts of the world, where *Eucalyptus* spp. are propagated commercially (Boerboom and Maas 1970; Davison and Coates 1991;

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Florence et al. 1986; Gibson 1981; Hodges and Reis 1974; Hodges et al. 1979; Sharma et al. 1985*a*, 1985*b*; Wingfield et al. 1989). *Eucalyptus* spp. are primarily cultivated as a source of fibre to produce pulp and to a lesser extent solid-wood products. In South Africa, *Eucalyptus* spp. are widely grown in plantations and Cryphonectria canker is of major concern to local forestry companies (Wingfield et al. 1989).

Cryphonectria cubensis was first observed in South Africa in 1988 (Wingfield et al. 1989). Surveys for this fungus in the late 1970s did not detect the pathogen, and it thus appeared that the pathogen had been recently introduced into the country. Consistent with this view, Van Heerden and Wingfield (2001) showed that *C. cubensis* in South Africa is represented by a relatively low number of genets and thus a narrow genetic base. This is in contrast with the high level of genetic variation that is found in *C. cubensis* populations elsewhere (Van Heerden et al. 1997).

Cryphonectria canker in South Africa is different from the disease occurring elsewhere in the world. While cankers in South America and Southeast Asia are generally at various heights on stems, those in South Africa form exclusively at the bases of trees (Conradie et al. 1990). Another unusual aspect of the disease in South Africa is that only asexual reproductive structures are found on cankers. This is in contrast with those in Southeast Asia and South America where sexual reproductive structures are predominant on cankers (Van Heerden and Wingfield 2001).

In a previous phylogenetic study, *C. cubensis* isolates from various parts of the world were compared using ribosomal RNA gene sequence data (Myburg et al. 1999). Results showed that isolates from South Africa and South America group together and separately from those originating in Southeast Asia and Australia. This result was inconsistent with morphological and pathological data, which suggested that *C. cubensis* from South Africa might be different from the fungus occurring elsewhere in the world.

Previous studies have shown that β-tubulin and histone *H3* gene regions are polymorphic (Donaldson et al. 1995; Glass and Donaldson 1995; Steenkamp et al. 1999, 2000) and useful in phylogenetic studies on fungi. The objective of this study was therefore to compare isolates of *C. cubensis* from South Africa with those from other parts of the world using DNA sequences likely to have higher resolution than the ITS region of the ribosomal RNA operon. Morphological comparisons of asexual fruiting structures were also made to determine whether specimens from South Africa could be distinguished from those originating in Southeast Asia and South America.

Materials and methods

Fungal isolates

Isolates used in this study included *Cryphonectria parasitica* (Murr.) Barr, the causal agent of chestnut blight (Elliston 1981), and *C. cubensis* isolates from Southeast Asia, Australia, South America, and South Africa (Table 1). *Diaporthe ambigua* Nits., a canker pathogen of stone and pome fruit trees (Smit et al. 1996, 1997), was included as the outgroup. All isolates used in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa.

DNA isolation and amplification

DNA isolations were performed as described by Myburg et al. (1999). Polymerase chain reaction (PCR) amplifications were performed as described by Glass and Donaldson (1995). Amplification reactions of the β-tubulin gene and the histone *H3* gene were done using primer pairs Bt1a/1b and Bt2a/2b and H3 1a/1b, respectively. Each 50-μL amplification reaction consisted of the following: 1 mM dNTPs (0.25 mM of each), 1× reaction buffer (supplied with the enzyme), 2.5 mM $MgCl_2$, 0.1 μM of each primer, 5 units of Expand *Taq* polymerase (Roche Biochemicals, Mannheim, Germany), and DNA template. Amplifications were done on a Perkin-Elmer GeneAmp PCR System 9700 thermocycler (Perkin-Elmer Applied BioSystems, Inc., Foster City, Calif.). Amplification of the histone *H3* gene was done using the following reaction conditions: an initial denaturing step at 94°C (1 min) followed by 30 cycles of denaturing at 94°C (1 min), annealing at 68°C (1 min), and elongation at 72°C (1 min). Amplification of the Bt1a/1b region of the β-tubulin gene was done using the same reaction conditions except that the annealing temperature was adjusted to 60°C. Amplification of the Bt2a/2b region was done over a range of annealing temperatures (55–68°C) because the Bt2a/2b primers annealed at different temperatures for the respective isolates used in this study. PCR products were visualised on 1% agarose (Promega, Madison, Wis.) gels containing ethidium bromide.

DNA sequencing

PCR products were purified using a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). The PCR products were sequenced in both directions using the same primers used in the amplification reactions. Sequencing reactions were conducted using an ABI PRISM™ dye terminator cycle sequencing ready reaction kit with AmpliTaq® DNA polymerase, FS (Perkin-Elmer, Warrington, U.K.). DNA sequences were determined using an ABI PRISM 377™ automated DNA sequencer.

Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems, Inc., Foster City, Calif.) was used to translate the β-tubulin and histone *H3* DNA sequences into putative amino acid sequences. The amino acid sequence of the respective β-tubulin gene was compared with the β-tubulin amino acid sequence of *Neurospora crassa* Shear and B.O. Dodge (Genbank accession No. M13630, Orbach et al. 1986). The amino acid sequence of the histone *H3* gene was compared with that of *N. crassa* (Genbank accession No. CAA25761, Woudt et al. 1983; Glass and Donaldson 1995). Conserved exon and variable intron sites were determined for *C. cubensis*, *C. parasitica*, and *D. ambigua*. DNA sequences were aligned using CLUSTAL W (Thompson et al. 1997) and the alignment was checked manually.

Phylogenetic analyses were performed using PAUP, version 4.0b (Swofford 1998). A partition-homogeneity test was performed on a combined data set including β-tubulin and histone *H3* gene sequences. Analyses were done using heuristic searches with no branch swapping and MULTREES (saving all optimal trees) options effective. Gaps were

Table 1. Taxa used in this study.

Species	Culture No.*	Origin	Genbank accession No.
Cryphonectria cubensis	CMW 2113	South Africa	AF273067†, AF273462†, AF281805‡
Cryphonectria cubensis	CMW 62	South Africa	AF273063†, AF273458†, AF281806‡
Cryphonectria cubensis	CMW 8755	South Africa	AF273064†, AF273459†, AF281807‡
Cryphonectria cubensis	CMW 8757	Venezuela	AF273069†, AF273464†, AF281810‡
Cryphonectria cubensis	CMW 8758	Venezuela	AF273068†, AF273463†, AF281811‡
Cryphonectria cubensis	CMW 1853	Brazil	AF273070†, AF273465†, AF281808‡
Cryphonectria cubensis	CMW 8756	Indonesia	AF273077†, AF375606†, AF285165‡
Cryphonectria cubensis	CMW 1840	China	AF273071†, AF273466†, AF281814‡
Cryphonectria cubensis	CMW 2632	Australia	AF273078†, AF375607†, AF466697‡
Cryphonectria parasitica	CMW 1652	U.S.A.	AF273468§, AF273075§, AF281802‡
Cryphonectria parasitica	CMW 7047	U.S.A.	AF273469§, AF273073§, AF281803‡
Cryphonectria parasitica	CMW 7048	U.S.A.	AF273470§, AF273076§, AF281804‡
Diaporthe ambigua	CMW 2498	Netherlands	AF273471§, AF273072§, AF281815‡

*Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa.
†β-Tubulin 1a/1b and 2a/2b sequence data generated in this study.
‡Histone *H3* sequence data generated in this study.
§β-Tubulin 1a/1b and 2a/2b sequence data obtained from Venter et al. (2002).

treated as fifth characters (NEWSTATE) in the heuristic searches. The confidence levels of the branching points were determined by a bootstrap analysis (1000 replications). *Diaporthe ambigua* was used as the outgroup taxon to root trees. Sequences were deposited in Genbank and accession numbers are listed in Table 1.

Morphological comparisons

Anamorph structures of *C. cubensis* occurring on *Eucalyptus grandis* bark from South Africa were examined microscopically. These specimens, PREM49377, PREM49378, and PREM49379 (Table 2), were those deposited as part of the study by Wingfield et al. (1989). Specimens of bark with pycnidia from Colombia, Mexico, Vietnam, Indonesia, and Hong Kong were also examined (Table 2). Pycnidia were sectioned in length using a Leitz 1310K freezing microtome and KRYOMAT 1700 generator following a method described by Venter et al. (2002). Sections of pycnidia, as well as other anamorph structures, were subsequently examined using a Zeiss Axioskop compound microscope. Measurements were presented as (min–)(mean – SD) – (mean + SD)(–max).

Results

DNA amplification and sequencing

The β-tubulin gene fragments (1a/1b and 2a/2b) were both approximately 550 base pairs (bp) in size. Amplification of the histone *H3* gene generated a 550-bp fragment. Positions of introns and exons of the β-tubulin and histone *H3* genes amplified in this study were the same as those of *N. crassa* (Genbank accession Nos. M13630 and CAA25761, respectively). The coding regions were highly conserved with sequence variation limited to the third codon position. No insertions or deletions of coding regions were observed in the gene regions considered in this study.

The partition-homogeneity test generated a *P* value of 0.01. This indicated that the β-tubulin gene and the histone *H3* gene sequence data sets could be combined in one phylogenetic analysis. The aligned β-tubulin and histone *H3* gene sequences were therefore analysed as one data set. For each taxon, 1369 characters were included in the heuristic search. Among these, 969 characters were constant and 136 characters parsimony uninformative. One most parsimonious tree (length of tree = 499 steps, consistency index = 0.9579, and retention index = 0.9651) was produced from 263 parsimony-informative characters (Fig. 1).

The phylogenetic tree (Fig. 1) generated from the combined β-tubulin and histone *H3* gene sequence data showed that all of the *C. cubensis* isolates grouped within one clade (bootstrap = 100) separately from the *C. parasitica* isolates and the outgroup *D. ambigua*. Within the greater *C. cubensis* clade, three distinct groups were obvious. These included a South American (bootstrap = 90), a Southeast Asian – Australian (bootstrap = 80), and a South African subclade (bootstrap = 95). The South American and Southeast Asian – Australian clades were more closely related to each other (bootstrap value = 55) than they were to the South African clade (Fig. 1).

Morphological comparisons

Pycnidia from the South African specimens differed slightly in morphology from structures collected in South America and Southeast Asia. Pycnidia from South Africa had an obvious eustromatic appearance where the layer of cells giving rise to the conidiophores was convoluted (Fig. 2*a*) and tissues in the pycnidial walls were prosenchymatous. Furthermore, sections through the edge of the pycnidia often revealed more than one cavity (Fig. 2*b*), while those near the middle revealed a single cavity (Fig. 2*a*). In contrast, pycnidial cavities from South American and Southeast Asian collections (Figs. 2*c* and 2*d*) were seldom as strongly convoluted, and longitudinal sections rarely revealed more than one pycnidial cavity. Where additional conidial cavities were observed, these were usually small (Fig. 2*d*).

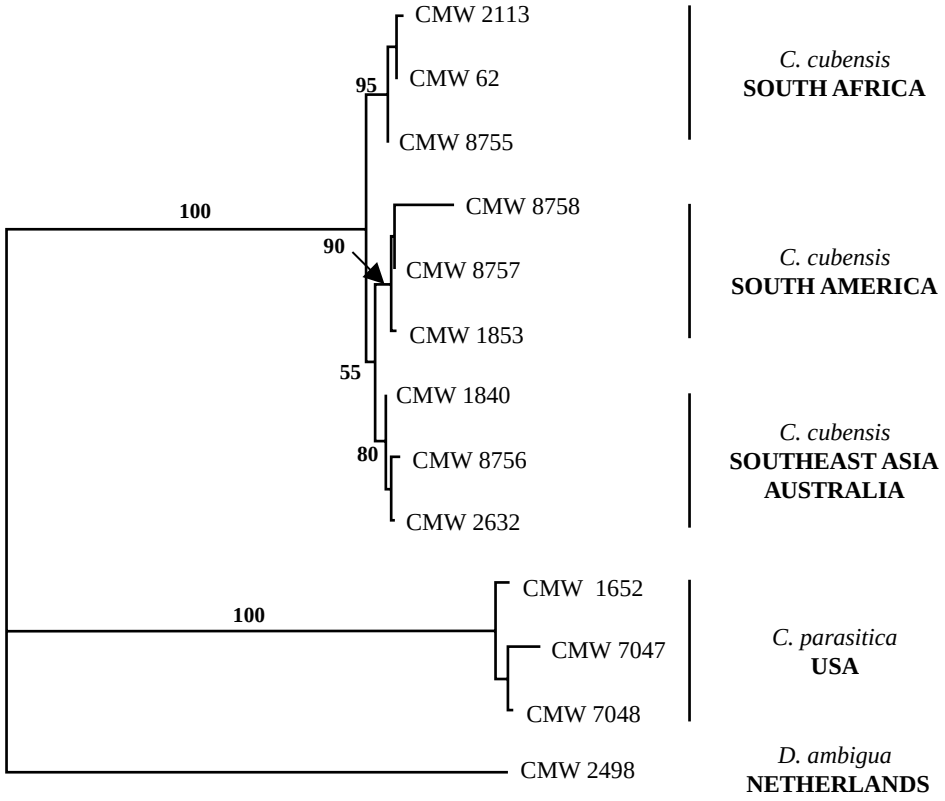
Conidia from specimens representing different geographical areas were similar in size and shape (Figs. 2*e*–2*h*).

Table 2. Specimens used in morphological comparisons.

Herbarium No.*	Identity	Host	Origin	Date	Collector
PREM49379	*Cryphonectria cubensis*	*Eucalyptus grandis*	South Africa	1988	M.J. Wingfield
PREM49377	*Cryphonectria cubensis*	*Eucalyptus grandis*	South Africa	1986	M.J. Wingfield
PREM49378	*Cryphonectria cubensis*	*Eucalyptus grandis*	South Africa	1987	M.J. Wingfield
PREM57293	*Cryphonectria cubensis*	*Eucalyptus grandis*	South Africa	2001	M. Venter
PREM57294	*Cryphonectria cubensis*	*Eucalyptus grandis*	Colombia	2000	M.J. Wingfield
PREM57295	*Cryphonectria cubensis*	*Eucalyptus* sp.	Mexico	2000	M.J. Wingfield
IMI263717	*Cryphonectria cubensis*	*Eucalyptus* sp.	Hong Kong	1981	C.S. Hodges
PREM57296	*Cryphonectria cubensis*	*Eucalyptus grandis* × *urophylla* clone	Vietnam	2000	M.J. Wingfield
PREM57297	*Cryphonectria cubensis*	*Eucalyptus* sp.	Indonesia	2001	M.J. Wingfield

*PREM, National Collection of Fungi, Pretoria, South Africa; IMI, Herbarium, CABI Bioscience, Bakeham Lane, Egham, Surrey TW20 9TY, U.K.

Fig. 1. One most parsimonious tree (tree length = 499 steps, consistency index = 0.9579, retention index = 0.9651) generated from sequence variation within a combined βtubulin and histone *H3* gene sequence data set. Bootstrap values (1000 replicates) are indicated.



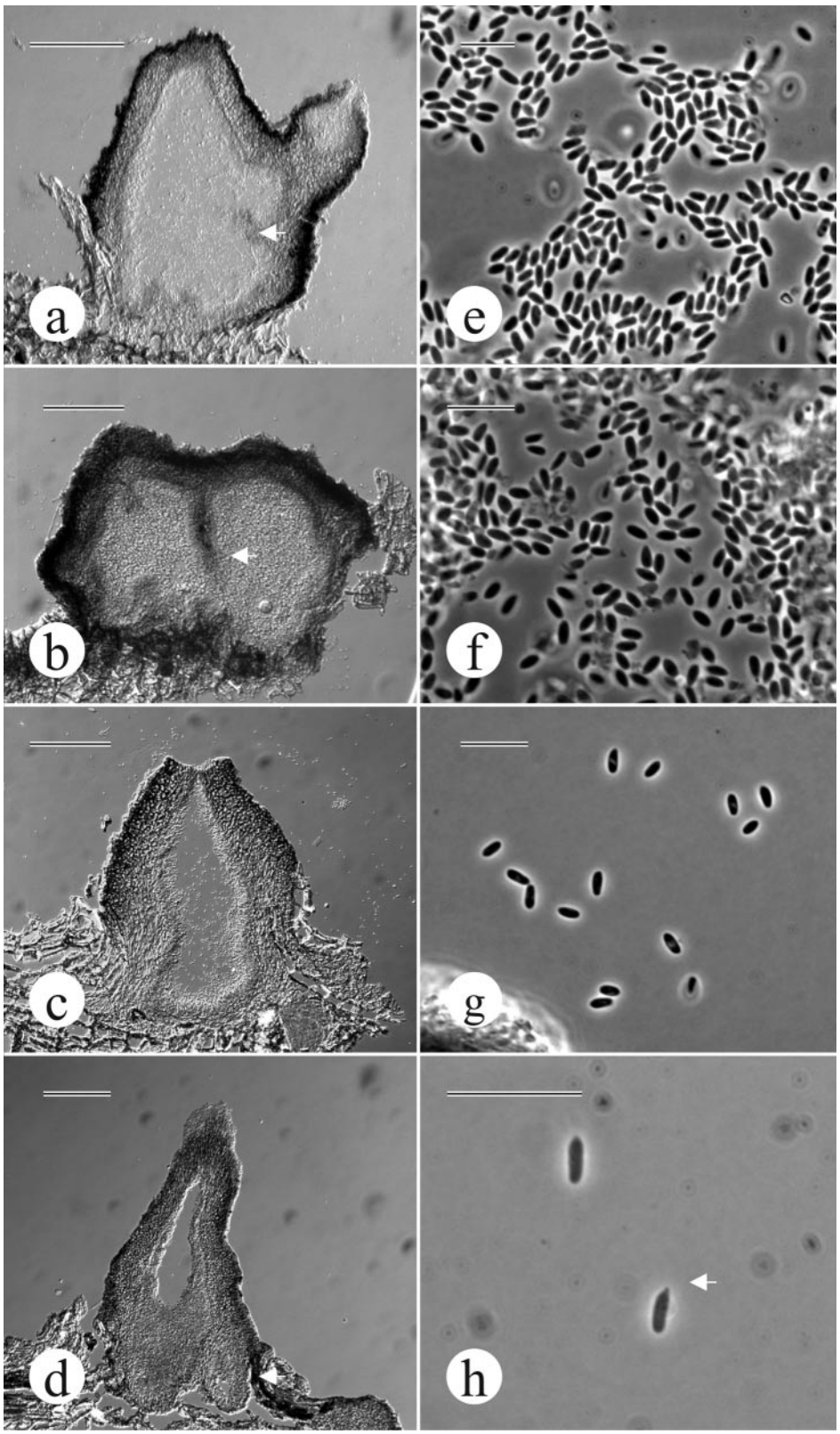
Conidia from Southeast Asia were 3–4 (–4.5) × 1–1.5 µm (Fig. 2*e*), from South American conidia were 3–4 (–4.5) × 1–1.5 µm (Fig. 2*f*), and from South Africa were (3–) 3.5–4.5 (–5) × 1–1.5 µm (Fig. 2*g*). Although rarely so, some conidia from the South African collections had papillate apices (Fig. 2*h*). These have not previously been noted in *C. cubensis* and were not found in the specimens from other parts of the world.

Discussion

Using comparisons of histone *H3* and β-tubulin gene sequences, we have been able to show conclusively that *C. cubensis* from South Africa is phylogenetically distinct from the fungus of the same name occurring in Southeast Asia – Australia and South America. This finding is consistent with the fact that the fungus in South Africa is associated with symptoms different from those found in the latter areas. Our results suggest that the South African fungus has an origin different from that of *C. cubensis* from other parts of the world.

In a previous study, Myburg et al. (1999) examined the relationships between *C. cubensis* and other *Cryphonectria* spp. as well as between *C. cubensis* from different hosts and areas. Results of the present study confirm results from Myburg et al. (1999) that the fungus from Southeast Asia –

Fig. 2. Conidiomata and conidia of *Cryphonectria cubensis* from various parts of the world. (*a* and *b*) Cross section of a conidioma from South Africa showing convoluted basal layer (arrow in Fig. 2*a*) and cross wall (arrow in Fig. 2*b*). Bars = 100 µm. (*c*) Cross section of a conidioma from Hong Kong. Bar = 100 µm. (*d*) Cross section of a conidioma from Colombia with a second, small cavity (arrow). Bar = 100 µm. (*e–g*) Conidia from Hong Kong, Mexico, and South Africa. Bars = 10 µm. (*h*) Papillate apex (arrow) on a conidium from South Africa. Bar = 10 µm.



Australia and South America forms two distinct, yet closely related groups. ITS sequences presented by Myburg et al. (1999) indicated that South African isolates were most closely related to the fungus from South America. However, the sequences from the ITS1/ITS2 regions are insufficiently variable to resolve the taxonomic questions relating to *C. cubensis* sensu lato.

Cryphonectria cubensis cankers in South America and Southeast Asia are typically covered with perithecia. Pycnidia are present but rare (M.J. Wingfield, data not shown). This is in contrast with the situation in South Africa where structures thought to be perithecia of *C. cubensis* have been observed only once (Wingfield et al. 1989) and pycnidia are the dominant structures on cankers (Van Heerden and Wingfield 2001). These differences, and the unique nature of cankers in South Africa, are consistent with the findings in this study, showing that the South African fungus is unique.

Cankers caused by *C. cubensis* in Australia have only been reported once (Davison and Coates 1991) and are different from cankers observed in Southeast Asia. These cankers on *Eucalyptus marginata* Sm. were on the roots and only pycnidia were observed (Davison and Coates 1991). This could indicate that the Australian fungus is also different. Molecular data reported in the present study, however, clearly show that the Australian fungus is part of the Southeast Asian subclade.

The overall morphology of pycnidia found on cankers in South Africa, Southeast Asia, and South America is very similar. In this study, however, slight differences between pycnidia from South Africa and those from elsewhere in the world have been detected. Small differences were also noted in conidial shape, although the papillate apices of a small number of South African conidia are an insufficiently consistent feature to note with any confidence.

Previous descriptions of *C. cubensis* have treated the anamorph fruiting structures as pycnidia (Bruner 1917; Hodges 1980; Hodges et al. 1976). This is possibly due to the fact that the anamorph has the typical shape and appearance of a pycnidium when viewed on the bark. However, when sectioned, the anamorph structures closely resemble convoluted eustromata (Hawksworth et al. 1996). We suggest that the term "eustroma" should be used in future to describe the anamorph structure of *C. cubensis*.

Although not an objective of the present study, our results confirm those of previous studies (Myburg et al. 1999; Venter et al. 2001) that *C. cubensis* is very distantly related to *C. parasitica*. This is also consistent with the fact that the two fungi can be distinguished by a number of clear morphological characteristics. The most obvious of these are that *C. cubensis* has loosely aggregated perithecia embedded in a weakly developed basal stroma. This is different from *C. parasitica* where perithecial bases are embedded in a well-developed stroma. These differences have led Venter et al. (2001) to conclude that the fungi probably reside in distinct genera. This conclusion is supported by SSU and LSU sequence data, where *C. cubensis* (= *Endothia eugeniae*) does not group in the same clade as *C. parasitica* (Zhang and Blackwell 2001). Results of the present study have also shown that β-tubulin and histone *H3* gene sequence data should be useful in future investigations aimed at providing better resolution to differentiate between various species of *Cryphonectria* and related fungi.

Substantial sequence data are now available to support the view that *C. cubensis* in South Africa is different from the fungus with the same name occurring elsewhere in the world. Available data also suggest that the South African fungus and *C. cubensis* elsewhere in the world have different origins. One commonly held hypothesis is that *C. cubensis* originated from native clove trees (*Syzigium aromaticum* (L.) Merr., and Perry (Myrtaceae)) in Indonesia (Hodges et al. 1986). There is equally strong evidence to suggest that *C. cubensis* originated on *Tibouchina* spp. trees (Melastomataceae) in South America (Wingfield et al. 2001). This raises the intriguing question of where the South African fungus might have originated. Based on the results of this study, the origin of the South African fungus is likely to be different from the origin of the fungus in South America, Southeast Asia, and Australia. Resolution of this question is likely to emerge from collections of fungi similar to *C. cubensis* on native Myrtaceae and Melastomataceae, both in Africa and elsewhere in the world.

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