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FULL PAPER

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Conspecificity of *Endothia eugeniae* and *Cryphonectria cubensis*: a re-evaluation based on morphology and DNA sequence data

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Abstract *Cryphonectria cubensis* and *Endothia eugeniae* are fungal pathogens of *Eucalyptus* and clove that were reduced to synonymy on the basis of results of cross-inoculation studies, isozyme analysis, cultural studies, and morphology. A previous phylogenetic study on *Cryphonectria*, based on sequence variation in the ITS region of the ribosomal RNA operon, also supported the conspecificity of *C. cubensis* and *E. eugeniae*, but was based on only one *E. eugeniae* isolate. New collections from clove in Brazil and Indonesia have become available, providing the opportunity to reconsider the conspecificity of *C. cubensis* and *E. eugeniae*. The occurrence of *C. cubensis* on clove was confirmed based on morphological comparisons and phylogenetic analyses of ribosomal DNA and β-tubulin gene sequence data. In addition to *C. cubensis*, other fungi morphologically similar to *Cryphonectria* species on the basis of their orange stromata were present on some clove specimens, but no isolates were available for these fungi. Furthermore, some isolates, for which no herbarium material exists, grouped separately from the *C. cubensis* clade and closer to the *Cryphonectria* clade. The presence of more than one closely related fungus on clove raises questions relating to the legitimacy of the synonymy of *E. eugeniae* and *C. cubensis.* Based on the presence of *C. cubensis* on the type specimen of *E. eugeniae*, we recognize the synonymy of the two fungi but provide evidence that other fungi, more closely related to *Cryphonectria* spp. than to *C. cubensis*, are present on clove.

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Introduction

Cryphonectria cubensis (Bruner) Hodges is a well-known and important canker pathogen of *Eucalyptus* species (Boerboom and Maas 1970; Hodges 1980; Wingfield et al. 1989). The fungus is present in tropical and subtropical areas of the world where high temperatures and rainfall favor infection and disease development (Alfenas et al. 1982). Management of Cryphonectria canker is primarily achieved by the propagation of disease-tolerant *Eucalyptus* clones and *Eucalyptus* hybrids (Alfenas et al. 1983; Van Zyl and Wingfield 1999).

Endothia eugeniae (Nutman & Roberts) Reid & Booth was first reported from Zanzibar, Tanzania, as causing acute dieback of clove (*Syzygium aromaticum* (L.) Merr. & Perry) (Nutman and Roberts 1952). The pathogen infected trees through wounds and caused die-back of branches or death of whole trees by girdling of trunks. At the point of infection, the wood was stained reddish-brown (Nutman and Roberts 1952). The disease has also been reported from Malaysia (Anonymous 1954; Heath 1956; Reid and Booth 1969), which is the region in which cloves are native (Hodges et al. 1986).

The clove pathogen, now known as *C. cubensis*, was first described as *Cryptosporella eugeniae* Nutman & Roberts (1952), but was later transferred to the genus *Endothia* (Reid and Booth 1969). Hodges et al. (1986) reduced *E. eugeniae* to synonymy with *Cryphonectria cubensis*. This synonymy was based on morphological comparisons, cultural characteristics, and inoculation studies as well as analysis of isozyme banding patterns. Micales et al. (1987) confirmed this synonymy using additional isozyme analyses, general protein patterns, and identification of pigments in culture.

Previous descriptions of *E. eugeniae* describe a fungus with brown pycnidia, immersed in the bark and emerging through the periderm, to assume a flattened conical shape (Nutman and Roberts 1952). Reid and Booth (1969) and Booth and Gibson (1973) describe immersed, becoming erumpent, conical, and orange to rust-brown stromata containing more than one convoluted to irregular conidial cavity. This picture is in contrast to *C. cubensis*, which has superficial to slightly immersed, pyriform pycnidia-like eustromata with attenuated necks (Bruner 1917; Hodges 1980; Myburg et al. 2002a). These pycnidia are reddish-brown when young but turn black with age (Bruner 1917; Hodges et al. 1979; Hodges 1980). These inconsistencies in the descriptions of the two fungi continue to raise questions pertaining to the validity of their synonymy.

Descriptions suggest that a fungus morphologically similar but different to *C. cubensis* could be present on clove. The possibility thus exists that the second fungus on clove, and not *C. cubensis*, might represent the originally described *E. eugeniae*. A phylogenetic study of isolates of *C. cubensis* based on sequence variation within the internal transcribed spacer (ITS) region of the ribosomal RNA operon (Myburg et al. 1999) provided support for the synonymy of *C. cubensis* and *E. eugeniae*. These authors, however, noted that their conclusion was based on a single isolate of *E. eugeniae* and that this question should be addressed more closely using additional isolates from clove. Recently, a larger collection of isolates from clove has become available to us. The objective of the present study was, therefore, to reconsider the conspecificity of *E. eugeniae* with *C. cubensis*, based on DNA sequence data from two different gene regions. In addition, a comprehensive morphological study was undertaken on the original herbarium specimens from clove, as well as newly obtained, fresh specimens from this host.

Materials and methods

Fungal isolates

Isolates used in this study, obtained from culture collections, supplied by colleagues, or collected during field studies by the last author (Table 1), include *C. cubensis* isolated from *Eucalyptus* and *S. aromaticum* from various parts of the world. Sequence data generated for other members of *Cryphonectria* (Myburg et al. 2002b; Venter et al. 2002), i.e., *C. parasitica* (Murr.) Barr, *C. macrospora* (Kobayashi & Ito) Barr, *C. nitschkei* (Otth.) Barr, and *C. radicalis*

Table 1. Isolates used in sequencing analyses

Culture no.[a]	Species	Host	Origin	Collector	Genbank accession numbers for ITS and β-tubulin sequence data
CMW 10774	*Cryphonectria cubensis*	*Syzygium aromaticum*	Tanzania, Zanzibar	C.S. Hodges	AF 492130, AF 492131, AF 492132
CMW 10775	*Cryphonectria cubensis*	*Syzygium aromaticum*	Brazil	C.S. Hodges	AY 084003, AY 084015, AY 084027
CMW 10776	*Cryphonectria cubensis*	*Syzygium aromaticum*	Brazil	C.S. Hodges	AY 084004, AY 084016, AY 084028
CMW 10777	*Cryphonectria cubensis*	*Syzygium aromaticum*	Brazil	C.S. Hodges	AY 084005, AY 084017, AY 084029
CMW 10778	*Cryphonectria cubensis*	*Syzygium aromaticum*	Brazil	C.S. Hodges	AY 084006, AY 084018, AY 084030
CMW 3839	*Cryphonectria cubensis*	*Syzygium aromaticum*	Indonesia	M.J. Wingfield	AF 046904, AY 084011, AY 084023
CMW 8649	*Cryphonectria cubensis*	*Syzygium aromaticum*	Sulawesi, Indonesia	M.J. Wingfield	AY 084000, AY 084012, AY 084025
CMW 8650	*Cryphonectria cubensis*	*Syzygium aromaticum*	Sulawesi, Indonesia	M.J. Wingfield	AY 084001, AY 084013, AY 084024
CMW 8651	*Cryphonectria cubensis*	*Syzygium aromaticum*	Sulawesi, Indonesia	M.J. Wingfield	AY 084002, AY 084014, AY 084026
CMW 8756	*Cryphonectria cubensis*	*Syzygium aromaticum*	Indonesia	M.J. Wingfield	AF 046896, AF 273077, AF 285165
CMW 9903	*Cryphonectria cubensis*	*Syzygium aromaticum*	Kalimantan	C.S. Hodges	AF 292044, AF 273066, AF 273461
CMW 9906	*Cryphonectria cubensis*	*Syzygium aromaticum*	Kalimantan	C.S. Hodges	AF 292045, AF 273065, AF 273460
CMW 1853	*Cryphonectria cubensis*	*Syzygium aromaticum*	Brazil	NA	AF 046891, AF 273070, AF 273465
CMW 8757	*Cryphonectria cubensis*	*Eucalyptus* sp.	Venezuela	M.J. Wingfield	AF 046897, AF 273069, AF 273464
CMW 10667	*Cryphonectria cubensis*	*Eucalyptus* sp.	Republic of Congo	J. Roux	AY 063477, AY 063479, AY 063481
CMW 10668	*Cryphonectria cubensis*	*Eucalyptus* sp.	Republic of Congo	J. Roux	AF 535121, AF 535123, AF 535125
CMW 1856	*Cryphonectria cubensis*	*Eucalyptus* sp.	Hawaii	NA	AY 083999, AY 084010, AY 084022
CMW 2631	*Cryphonectria cubensis*	*Eucalyptus marginata*	Australia	E. Davison	AF 543823, AF 543824, AF 523825
CMW 2632	*Cryphonectria cubensis*	*Eucalyptus marginata*	Australia	E. Davison	AF 046893, AF 273078, AF 375607
CMW 2113	*Cryphonectria cubensis*	*Eucalyptus grandis*	South Africa	M.J. Wingfield	AF 046892, AF 273067, AF 273462
CMW 62	*Cryphonectria cubensis*	*Eucalyptus grandis*	South Africa	M.J. Wingfield	AF 292041, AF 273063, AF 273458
CMW 8755	*Cryphonectria cubensis*	*Eucalyptus grandis*	South Africa	M.J. Wingfield	AF 292040, AF 273064, AF 273458
CMW 10463	*Cryphonectria macrospora*	*Castanopsis cuspidata*	Japan	T. Kobayashi	AF 368331, AF 368351, AF 368350
CMW 10518	*Cryphonectria nitschkei*	*Quercus* sp.	Japan	T. Kobayashi	AF 452118, AF 525706, AF 525713
CMW 10455	*Cryphonectria radicalis*	*Quercus suber*	Italy	A. Biraghi	AF 452113, AF 525705, AF 525712
CMW 10477	*Cryphonectria radicalis*	*Quercus suber*	Italy	A. Biraghi	AF 368328, AF 368347, AF 368346
CMW 7047	*Cryphonectria parasitica*	*Quercus virginiana*	USA	R.J. Stipes	AF 368329, AF 273073, AF 273469
CMW 7048	*Cryphonectria parasitica*	*Quercus virginiana*	USA	R.J. Stipes	AF 368330, AF 273076, AF 273470
CMW 10779	*Cryphonectria* sp.	*Syzygium aromaticum*	Somosir, Indonesia	M.J. Wingfield	AY 084007, AY 084019, AY 084031
CMW 10780	*Cryphonectria* sp.	*Syzygium aromaticum*	Somosir, Indonesia	M.J. Wingfield	AY 084008, AY 084020, AY 084032
CMW 10781	*Cryphonectria* sp.	*Syzygium aromaticum*	Kalimantan	M.J. Wingfield	AY 084009, AY 084021, AY 084033
CMW 5288	*Diaporthe ambigua*	*Malus domestica*	South Africa	W.A. Smit	AF 543817, AF 543819, AF 543821
CMW 5587	*Diaporthe ambigua*	*Malus domestica*	South Africa	W.A. Smit	AF 543818, AF 543820, AF 543822

ITS, internal transcribed spacer

[a] Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa

(Schw.: Fr.) Barr, were also included in this study. *Diaporthe ambigua* Nitschke, the causal agent of stem cankers on stone fruit trees (Smit et al. 1996, 1997), was used as the outgroup taxon to root the phylogenetic trees (Table 1). Cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Morphological comparisons

Herbarium specimens from clove listed in the original descriptions (Nutman and Roberts 1952; Reid and Booth 1969) were studied (Table 2). These specimens originated from Zanzibar and Malaysia. New clove material was also collected from Sulawesi, Indonesia (Table 2) and has been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM). Isolates CMW 8649, CMW 8650, and CMW 8651 (Table 1) originated from these specimens. Specimens of *C. cubensis* from *Eucalyptus* spp., used in the study of Myburg et al. (2002a), were also included (Table 2).

Some isolates originating from clove (CMW 10779, CMW 10780, CMW 10781) had culture morphology different from clove isolates that were thought to represent *C. cubensis*. The cultures were buff (19″f) to hazel (11′k) in contrast to those of *C. cubensis* that were creamy white with cinnamon (15″) patches. Unfortunately, no vouchered specimens exist for these isolates.

Fruiting structures of *Cryphonectria* spp. are infrequently produced in culture and are not representative of fruiting structures occurring naturally on bark. Isolate CMW 10781 from clove was, therefore, inoculated into wax-sealed sticks of another member of the Myrtaceae, a *Eucalyptus grandis* W. Hill: Maiden clone (ZG 14), to gain additional information on its morphology. Isolates CMW 8649 and CMW 8650 from clove, known to be *C. cubensis*, were also inoculated into *E. grandis* sticks for comparative purposes. These inoculations were done using the technique described by Van Heerden and Wingfield (2001). Specimens resulting from these inoculations (Table 2) have been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM).

Hodges et al. (1986) performed inoculations on *E. saligna* and clove sticks using *C. cubensis* isolates from

Table 2. Specimens used in morphological comparisons

Herbarium no.[a]	Identity	Host	Origin	Date	Collector
IMI 44954 (holotype)	*Endothia eugeniae*	*Syzygium aromaticum*	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 44945	*Endothia eugeniae*	*Syzygium aromaticum*	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 45440	*Endothia eugeniae*	*Syzygium aromaticum*	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 45445	*Endothia eugeniae*	*Syzygium aromaticum*	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 45443	*Endothia eugeniae*	*Syzygium aromaticum*	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 45448a	*Endothia eugeniae*	*Syzygium aromaticum*	Zanzibar	1951	NA
IMI 45446	*Endothia eugeniae*	*Syzygium aromaticum*	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 44953	*Endothia eugeniae*	*Syzygium aromaticum*	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 44951	*Endothia eugeniae*	*Syzygium aromaticum*	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 45452	*Endothia eugeniae*	*Syzygium aromaticum*	Zanzibar	1951	J. Nutman & F.M. Roberts
IMI 49266	*Endothia eugeniae*	*Syzygium aromaticum*	Zanzibar	NA	J. Nutman & F.M. Roberts
IMI 45449	*Endothia eugeniae*	*Syzygium aromaticum*	Zanzibar	1951	NA
IMI 45450	*Endothia eugeniae*	*Syzygium aromaticum*	Zanzibar	1951	NA
IMI 279614	*Cryphonectria cubensis*	*Eucalyptus urophylla*	Cameroon	1983	I.A.S. Gibson
IMI 56425a	*Endothia eugeniae*	Isolate ex *Eugenia* sp. on elm twigs	Malaysia	1954	W.J. Cherewick
IMI 58569	*Endothia eugeniae*	*Syzygium aromaticum*	Malaysia	1954	A. Johnston
IMI 58388	*Endothia eugeniae*	*Syzygium aromaticum*	Malaysia	1954	A. Johnston
IMI 58567	*Endothia eugeniae*	*Eugenia* sp.	Malaysia	NA	A. Johnston
IMI 58568	*Endothia eugeniae*	*Eugenia* sp.	Malaysia	1954	A. Johnston
IMI 350626	*Cryphonectria cubensis*	*Syzygium aromaticum*	Singapore	1991	C.P. Yik
PREM 57469	*Cryphonectria cubensis*	Inoculations into *E. saligna* and *S. aromaticum*	NA	1986	C.S. Hodges
PREM 57470[b]	*Cryphonectria cubensis* and unknown fungus	*Syzygium aromaticum*	Sulawesi, Indonesia	2001	M.J. Wingfield
PREM 57471	*Cryphonectria cubensis*	Inoculation of CMW 8649 into *E. grandis*	NA	2002	M. Gryzenhout
PREM 57472	*Cryphonectria cubensis*	Inoculation of CMW 8650 into *E. grandis*	NA	2001	M. Gryzenhout
PREM 57473	Unknown	Inoculation of isolate CMW 10781 into *E. grandis*	NA	2001	M. Gryzenhout
IMI 304273	*Cryphonectria cubensis*	*Eucalyptus aromatica*	Malaysia	1986	Low Chow Fong
PREM 57297	*Cryphonectria cubensis*	*Eucalyptus* sp.	Indonesia	2001	M.J. Wingfield
IMI 284438	*Cryphonectria cubensis*	*Eucalyptus grandis*/*Eugenia* sp.	Venezuela	1983	C.S. Hodges
PREM 57294	*Cryphonectria cubensis*	*Eucalyptus grandis*	Colombia	2000	M.J. Wingfield

[a] PREM, National Collection of Fungi, Pretoria, South Africa; IMI, Herbarium, CABI Bioscience, Bakeham Lane, Egham, Surrey TW20 9TY, UK

[b] Vouchered specimens linked to isolates CMW 8649 (PREM 57471), CMW 8650 (PREM 57472), and CMW 8651

Eucalyptus and clove. The aim of that study was to consider the effect of clove and *Eucalyptus* bark on the morphology of the infecting fungus. The specimens from these inoculations were made available to us by Dr. C.S. Hodges, Department of Plant Pathology, North Carolina State University, Raleigh, NC, USA. These specimens (Table 2) have also been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM).

Structures for morphological study were mounted in Leica mountant (Setpoint Premier, Johannesburg, South Africa) after boiling in water for 1 min. Specimens were sectioned at 12–16 μm using a Leica CM1100 cryostat (Setpoint Premier) at −20°C. Sections were mounted in lacto-phenol and examined microscopically. Ten measurements were taken for conidia and ascospores, presented as (min−) (mean − SD) − (mean + SD) (−max). The color notations of Rayner (1970) were used throughout this study.

DNA isolations and PCR

DNA was isolated as previously described by Myburg et al. (1999). Amplification of the ITS region of the ribosomal RNA operon, as well as the two regions within the β-tubulin gene, was carried out as described in Myburg et al. (1999) and Myburg et al. (2002a), respectively. The primer pairs used to amplify the two β-tubulin regions were Bt1a with Bt1b and Bt2a with Bt2b (Glass and Donaldson 1995); ITS 1 and ITS 4 (White et al. 1990) were used to amplify the ITS 1 and ITS 2 regions of the ribosomal RNA operon. PCR products were separated on 1% agarose (Promega, Madison, WI, USA) gels containing ethidium bromide and visualized using an UV light.

Sequencing

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The PCR products were sequenced in both directions using the primers already mentioned. Sequencing reactions were carried out using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin-Elmer, Warrington, United Kingdom). DNA sequences were determined using an ABI PRISM 3100 automated DNA sequencer. DNA sequences were verified with Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems, Foster City, CA, USA) and aligned using CLUSTAL X (Thompson et al. 1997). The resulting alignment was checked manually.

A Templeton nonparametric Wilcoxon signed ranked (WSR) test (Kellogg et al. 1996) was done on a combined sequence data set including aligned β-tubulin and ITS sequences. Results from this test indicated that the data sets could be combined and considered as one in subsequent phylogenetic analyses.

A heuristic search was executed on the aligned data set using PAUP* version 4.0b (Swofford 1998). The TBR (tree-bisection-reconnection) algorithm of the heuristic search (MulTrees option effective, saving all optimal trees) was chosen. Seventeen trees were generated and a strict consensus tree was computed. Gaps were treated as fifth characters (Newstate), and characters were unordered and equally weighted. A bootstrap analysis of 1000 replicates was done to assess the confidence levels of the internodes. The consensus tree was rooted with the two *D. ambigua* isolates. Sequences generated in this study were deposited in GenBank, and accession numbers are listed in Table 1. Sequence alignments are available from TreeBase (matrix SN 1251-353). Accession numbers of sequence data obtained from previous studies (Myburg et al. 1999, 2002a,b; Roux et al. 2003) are also listed in Table 1.

Results

Morphological comparisons

More than one fungus residing in *Cryphonectria* was observed on the various clove specimens included in this study. These fungi had conidia and ascospores similar in size and shape and were difficult to distinguish on the bark, but differed based on position relative to the bark, stromatic tissue types, and internal morphology of the stromata. *Cryphonectria cubensis* occurred on bark specimens from Zanzibar, Malaysia, and Indonesia. In addition, a fungus with orange (15) to sienna (15i) stromata was found on the material from Zanzibar. Furthermore, herbarium materials originating from inoculation with isolate CMW 10781 from Indonesia contained fruiting structures with different characteristics to *C. cubensis* or the other fungus with the orange to sienna-colored stromata. These different fungi are discussed in greater detail under the following sections, and morphological features are summarized in Table 3.

Cryphonectria cubensis on clove

Structures typical of *C. cubensis* (Table 3) were found on the clove specimens from Zanzibar (IMI 45449, IMI 45450, IMI 45440), Malaysia (IMI 56425a, IMI 58569, IMI 58388, IMI 58567, IMI 58568), and Indonesia (PREM 57470). Conidiomata were either pyriform with attenuated necks (Fig. 1a,c), or pulvinate because necks were broken or the structures were not fully developed (Fig. 1b,d). The tissue type in these stromata was characteristic of *C. cubensis* (Table 3) with base tissue *textura globulosa* (Fig. 1e) and neck tissue *textura porrecta* (Fig. 1f). Structures with a tissue type resembling that of *C. cubensis* were found on the type specimen of *E. eugeniae* (IMI 44954), but these were too brittle for thorough examination. Conidia (Fig. 1g, Table 3) were similar to those on specimens of *C. cubensis* on *Eucalyptus* (IMI 279614, IMI 304273, PREM 57297, IMI 284438, PREM 57294) and those previously described for *C. cubensis* (Bruner 1917; Hodges 1980; Myburg et al. 2002a,b).

The internal structure of the conidiomata of *C. cubensis* was variable on clove. Pulvinate, blackened, multilocular

Table 3. Key morphological characteristics of the different fungi found on herbarium material of clove

Fungus	Origin	Conidioma				Ascoma			
		External color	Structure	Stromatic tissue	Conidia	Stroma color	Structure	Stromatic tissue	Ascopores
Cryphonectria cubensis	Zanzibar, Malaysia, Indonesia	Dark mouse gray (15k)	Pyriform with attenuated neck, or pulvinate, unilocular occasionally multilocular, convoluted	Umber (15 m), base *textura globulosa*, neck *textura porrecta*	Oval to ovoid, aseptate, 3–4 (–4.5) × 1–1.5 (–2) µm	Orange (15) to pale luteous (19d) stroma with blackened perithecial necks	Semi-immersed, slightly erumpent, frequently formed underneath conidioma	Limited, diffuse, prosenchymatous	Fusoid, one-septate, (5–) 6–7.5 (–8) × 1.5–2.5 µm
Orange to sienna fungus	Zanzibar	Orange (15) to sienna (15i)	Erumpent, elongated pulvinate, convoluted multilocular chambers underneath bark surface	Orange (15) to sienna (15i), lower part often lighter, dense, prosenchymatous	Oval to ovoid, aseptate, (3–) 3.5–4 (–4.5) × 1–1.5 (–2) µm	NA	NA	NA	NA
CMW 10781 artificial inoculation	Indonesia	Blackened	Generally ovoid	Umber (15 m), pseudoparenchymatous	Cylindrical, aseptate, (2.5–) 3–3.5 (–4) × 1 µm	NA	NA	NA	NA

structures with convoluted and multilocular conidial chambers below the bark (Fig. 1d) were observed on clove tissue from Zanzibar (IMI 45440). The tissue type of the erumpent parts, as well as the spore shape and size [3.5–4(–4.5) × 1–1.5 µm], were similar to those of *C. cubensis*. The same extent of differences was observed for the clove and *Eucalyptus* material inoculated with *C. cubensis* (PREM 57469) and studied by Hodges et al. (1986). A small number of conidiomata were observed on this material, with structures on clove semi-immersed and conidial locules strongly convoluted and occurring underneath the bark.

The teleomorph of *C. cubensis* (Table 3, Fig. 1h,i) on specimens IMI 45450 and IMI 45440 was frequently observed developing underneath anamorph structures (Fig. 1i). Stromatal development (Table 3) was prosenchymatous, orange (15) to luteous (17), and restricted to the area around the base of the perithecial necks (Fig. 1j). Ascospores (Fig. 1k) were similar to those of *C. cubensis* (Table 3) as previously observed (Bruner 1917; Hodges 1980).

Other fungi on clove specimens

A fungus with stromatal structure, color, and stromatal tissue different to that of *C. cubensis* (Table 3) was found on some specimens from Zanzibar (IMI 45452, IMI 44951), studied in the original descriptions of *E. eugeniae* (Nutman and Roberts 1952; Reid and Booth 1969). These structures were only conidiomatal and occurred between structures of *C. cubensis.* They were erumpent, pulvinate (Fig. 2a), with several convoluted locules underneath the bark (Fig. 2b). Stromatic tissue (Table 3) was densely prosenchymatous (Fig. 2c), different from that of *C. cubensis*, which is *textura globulosa* (Fig. 1e). Conidia from the orange structures (Fig. 2d) were similar in size and shape to those of *C. cubensis* (Table 3). No isolates exist for these specimens, and it is impossible to study them further.

Specimens from Indonesia (PREM 57470) that gave rise to isolates of *C. cubensis* (CMW 8649, CMW 8650, CMW 8651) also contained ascomata different from those of *C. cubensis*. These ascomata superficially resembled the teleomorph of *C. cubensis* and also had one-septate, fusoid ascospores. They differed from *C. cubensis* because the stromatic tissue was densely prosenchymatous and orange to sienna. The latter characteristics were similar to those of the orange to sienna fungus on specimens from Zanzibar, but thorough comparisons between the fungus from Zanzibar and the Indonesian structures were hindered by the fact that stromata for the Indonesian specimens were inordinately few in number. Furthermore, no isolates exist for these structures. For the present, we are unable to draw a definitive conclusion regarding the identity of the fungus associated with these structures on the Indonesian material.

Eucalyptus sticks (PREM 57473) that had been inoculated with isolate CMW 10781 from Indonesia showed structures different from those (PREM 57471, PREM 57472) arising from the *C. cubensis* isolates (CMW 8649 CMW 8650). Conidiomata (Table 3) were blackened

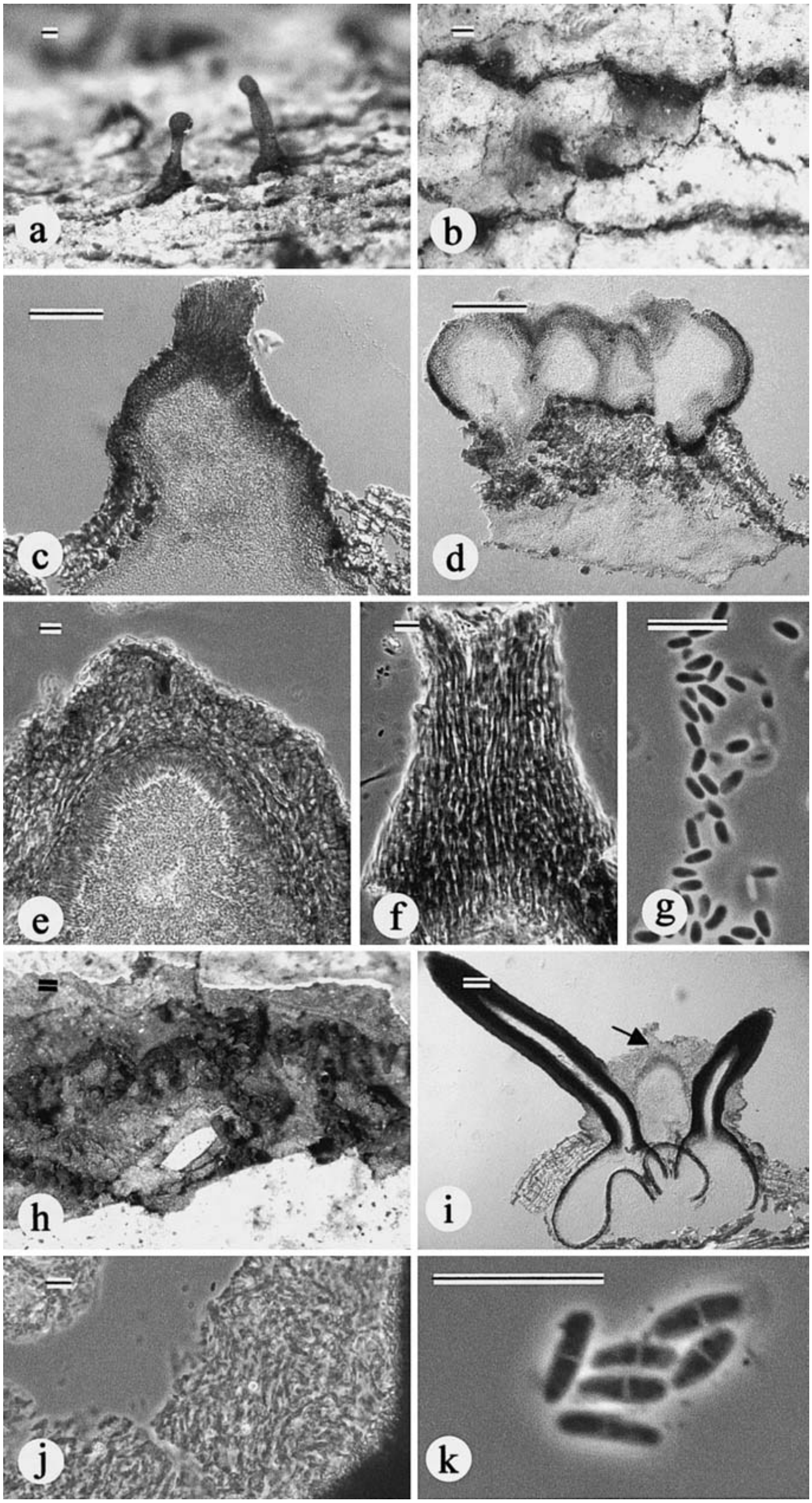

Fig. 1. Light micrographs of *Cryphonectria cubensis* occurring on clove. **a** Pyriform conidiomata. **b** Pulvinate conidiomata without necks. **c** Longitudinal section through conidioma with neck attached. **d** Longitudinal section through multilocular, pulvinate conidioma. **e** Base tissue. **f** Neck tissue. **g** Conidia. **h** Ascomata. **i** Longitudinal section through perithecia occurring underneath conidioma (*arrow*). **j** Stromatic tissue of ascoma. **k** Ascospores. *Bars* **a**, **h** 200 μm; **b–d**, **i** 100 μm; **e–g**, **j**, **k** 10 μm

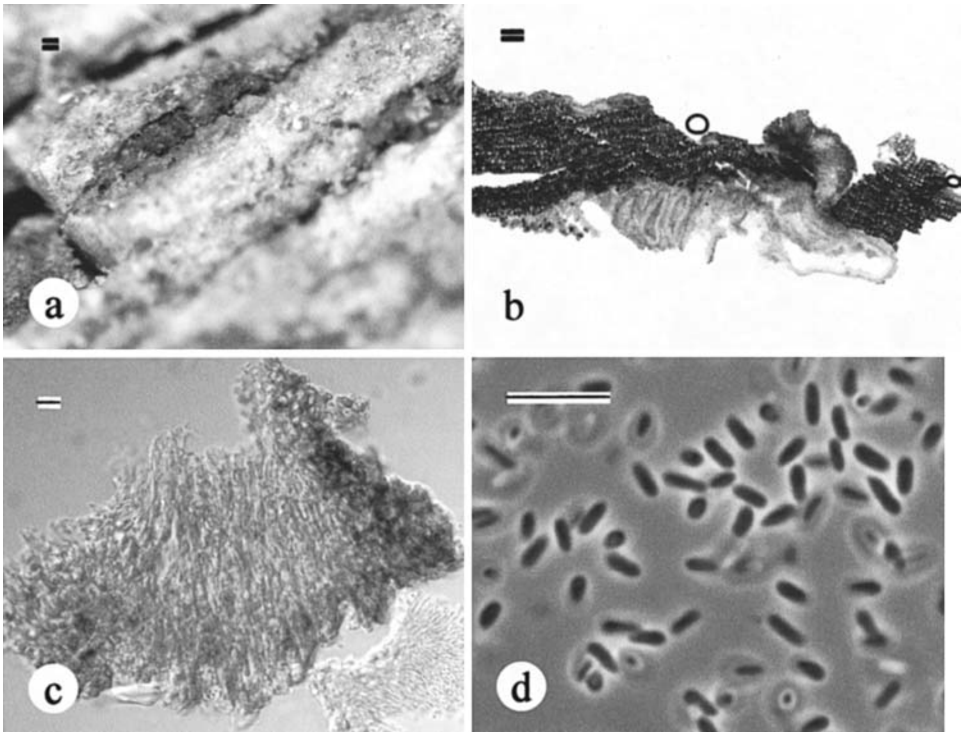

Fig. 2. Light micrographs of the unknown fungus with an orange to sienna anamorph occurring on clove in Zanzibar. **a** Conidioma. **b** Longitudinal section through conidioma. **c** Stromatic tissue. **d** Conidia. *Bars* **a**, **b** 100 μm; **c**, **d** 10 μm

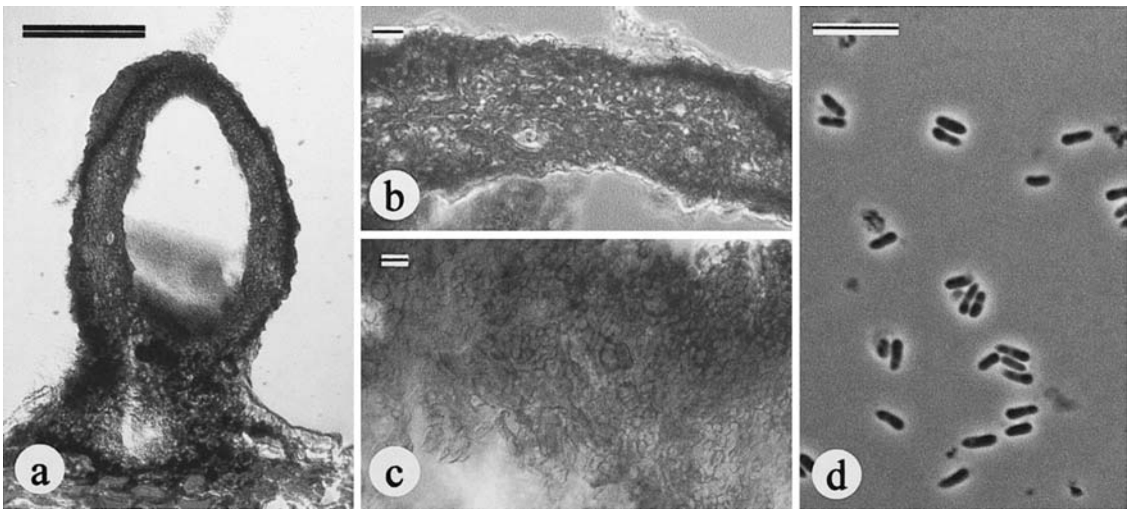

Fig. 3. Light micrographs of fruiting structures produced by isolate CMW 10781 in artificial inoculations on *Eucalyptus* sticks. **a** Longitudinal section through superficial conidioma. **b**, **c** Stromatic tissue. **d** Conidia. *Bars* **a** 100 μm; **b–d** 10 μm

(Fig. 3a), sometimes with a luteous (19) apex, with pseudoparenchymatous tissue (Fig. 3b,c). These structures and the tissues associated with them were also different from those of the other fungus (IMI 45452, IMI 44951) with orange to sienna stromata from Zanzibar (Fig. 2b,c). Conidia were also distinct in being more cylindrical (Fig. 3d), but length measurements were in the same size range as *C. cubensis* (Table 3). The shape and internal structure of the fruiting bodies were inordinately variable to draw any definite conclusions on the identity of this fungus, but were in general superficial and ovoid. The *C. cubensis* isolates, however, produced ascomata and conidiomata that were similar to those of *C. cubensis* on the clove specimens from nature and showed little variation among each other.

Sequencing

Amplification products for the DNA regions considered in this study were approximately 600 bp (ITS) and 550 bp (β-tubulin) in size. A combined sequence data set comprising ITS ribosomal and β-tubulin gene sequences included 1505

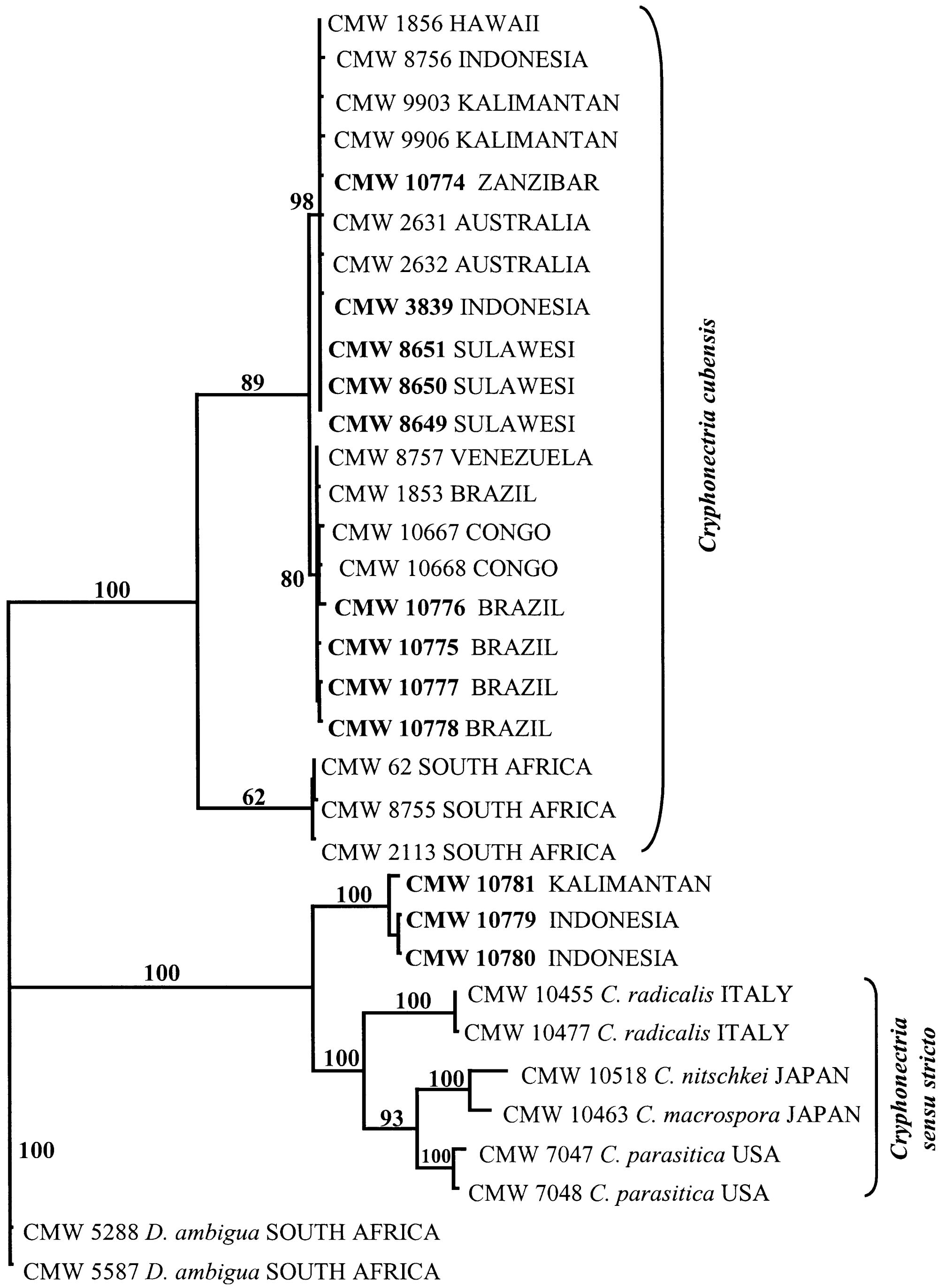

Fig. 4. Strict consensus tree [tree length = 1198 steps; consistency index (CI) = 0.8, retention index (RI) = 0.9] computed from 17 trees generated after heuristic search of a combined data set including ribosomal DNA and β-tubulin gene sequences. Bootstrap values (1000 replicates) are indicated above the internodes. Taxa in bold represent those sequenced in the present study. The *Diaporthe ambigua* isolates included in this study were used as outgroups to root the phylogenetic tree

aligned sequence characters of which 879 were constant, 40 parsimony uninformative, and 586 parsimony informative. A strict consensus tree [tree length = 1198 steps; consistency index (CI) = 0.8 and retention index (RI) = 0.9] was computed (Fig. 4) from the seventeen trees generated in the heuristic search.

The phylogram generated for the combined sequence data set (Fig. 4) showed three groups of fungi, clustering separately from the outgroup taxa represented by the *D. ambigua* isolates. The first clade (bootstrap support 100%) represents *C. cubensis* isolated from *Eucalyptus* species and clove originating from Southeast Asia (bootstrap support 98%), South America (bootstrap support 80%), and South Africa (bootstrap support 62%). The second group represents isolates that also originated on clove in Indonesia (bootstrap support 100%). A third group (bootstrap support 100%) is characterized by *C. parasitica*, *C. radicalis*, *C. macrospora*, and *C. nitschkei* and represents species that characterize the genus *Cryphonectria sensu stricto* (Venter et al. 2002).

Three subgroups of fungi make up the *C. cubensis* clade. These groups, previously identified by Myburg et al. (2002a), represent three geographic areas where *C. cubensis* is known to occur. In the present study, one group represents *C. cubensis* isolated from *Eucalyptus* and clove originating from countries in Southeast Asia and Australia (bootstrap support 98%). The clove isolates (CMW 8649, CMW 8650, CMW 8651) from Indonesia as well as the Indonesian clove isolate (CMW 3839) used in the study of Myburg et al. (1999) clustered within this Southeast Asian/Australian clade. A clove isolate from Zanzibar (CMW 10774) also grouped in the Southeast Asian clade.

The second group within the *C. cubensis* clade (bootstrap support 80%) included Brazilian isolates from clove (CMW 10775, CMW 10776, CMW 10777, CMW 10778) as well as Brazilian (CMW 1853) and Venezuelan (CMW 8757) *C. cubensis* isolates from *Eucalyptus.* This clade also contained *C. cubensis* isolates from the Congo (CMW 10667, CMW 10668) that have been reported previously to group within the South American sub-clade (Roux et al. 2003).

The third subclade in the larger *C. cubensis* group included isolates originating from South Africa (bootstrap support 62%). This clade grouped separately from the South American and Southeast Asian *C. cubensis* group (bootstrap support 100%) and appears to represent a distinct taxon, as previously shown by Myburg et al. (2002a).

A group of isolates originating on clove in Indonesia (CMW 10779, CMW 10780, CMW 10781) formed a separate and discrete clade, separately from the other clove isolates in the *C. cubensis* clade (bootstrap support 100%). This group was also separate from *C. parasitica*, *C. radicalis*, *C. macrospora*, and *C. nitschkei* (bootstrap support 100%). The isolates in this clade were those that had cultural and morphological characteristics different to those of *C. cubensis*.

Discussion

In this study, we have been able to confirm unequivocally that *C. cubensis* occurs on clove. This conclusion was based on ribosomal ITS and β-tubulin gene sequence data for fungi isolated from clove originating from South America, Indonesia, and central Africa. We have linked these results to morphological characteristics for relevant herbarium specimens collected from clove in Indonesia, Malaysia, and Zanzibar. However, morphological and phylogenetic data from this study also indicate the presence of other fungi related to *Cryphonectria*, occurring on clove.

The presence of a fungus, other than *C. cubensis*, on the clove specimens used in the original description of *E. eugeniae* raises doubt as to which fungus was referred to in the original description of *E. eugeniae. Cryphonectria cubensis* and the second fungus with orange stromata are similar and their conidia are undistinguishable. It is, therefore, likely that previous workers would have unwittingly assumed that these fungi represented a single taxon. The teleomorph description of *E. eugeniae* clearly refers to *C. cubensis*, because it describes perithecia developing below the conidiomata (Nutman and Roberts 1952; Reid and Booth 1969). The description of the anamorph of *E. eugeniae*, however, could relate to either *C. cubensis* or the fungus with the orange anamorph that we have found on the original specimens. The identity of *E. eugeniae* is connected to the type specimen of this fungus (IMI 44954), which contains structures with the same tissue type as *C. cubensis.* The synonymy of *E. eugeniae* with *C. cubensis* is, therefore, valid and the other fungi occurring on clove will require independent names.

A fungus represented by isolates CMW 10779, CMW 10780, and CMW 10781 that was different from both *C. cubensis* and the fungus with orange to sienna stromata from Zanzibar was isolated from cankers on clove in northern Sumatra and Kalimantan, Indonesia. DNA sequence data clearly showed that this fungus differs from *C. cubensis* and the other *Cryphonectria* spp., yet it is closely related. These isolates could not be connected to morphological structures on host tissue. Bark inoculations on *Eucalyptus* yielded information on conidial and tissue morphology, but structural morphology was excessively variable to be used in descriptions. Additional specimens and isolates of this third fungus on clove will be necessary before a name can be provided for it.

Results of this study have shown that *C. cubensis* occurs on clove in South America, Southeast Asia, and central Africa. Isolates from clove reside in two phylogenetic groups that were previously defined by Myburg et al. (1999, 2002a) for *C. cubensis* isolates from *Eucalyptus*. It was interesting to discover that *C. cubensis* isolates from central Africa included those from both the Southeast Asian and South American phylogenetic lineages. These phylogenetic data suggest that *C. cubensis* has been introduced into Africa on two separate occasions. South African *C. cubensis* isolates, however, clearly reside in a separate lineage with a distinct origin, as recently shown by Myburg et al. (2002a).

The presence of *Cryphonectria* spp. on clove appears to be considerably more complex than previously realized. Based on detailed comparisons of DNA sequence and morphological characteristics, we have found that at least two closely related and similar fungi can occur on a single clove specimen. The absence of either herbarium specimens or isolates has made conclusive identifications of these fungi difficult. However, there is good evidence to show that at least three different species of *Cryphonectria* occur on clove, and future collections should make it possible to provide names for the two unidentified species.

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