CHAPTER 3

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Gryzenhout M, Rodas CA, Portales JM, Wingfield BD, Wingfield MJ (2006). Novel hosts of the *Eucalyptus* canker pathogen *Chrysoporthe cubensis* and a new *Chrysoporthe* species from Colombia. *Mycological Research* (in press).

Novel hosts of the *Eucalyptus* canker pathogen *Chrysoporthe cubensis* and a new *Chrysoporthe* species from Colombia

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Abstract. The pathogen *Chrysoporthe cubensis* (formerly *Cryphonectria cubensis*) is best known for the important canker disease that it causes on *Eucalyptus* species. This fungus is also a pathogen of *Syzygium aromaticum* (clove), which is native to Indonesia and, like *Eucalyptus*, in the *Myrtaceae*. Furthermore, *C. cubensis* has been found on *Miconia* spp. native to South America and residing in the *Melastomataceae*. Recent surveys have yielded *C. cubensis* isolates from new hosts, characterized in this study based on DNA sequences for the ITS and β-tubulin gene regions. These hosts include native *Clidemia sericea* and *Rhynchanthera mexicana* (*Melastomataceae*) in Mexico, and non-native *Lagerstroemia indica* (Pride of India, *Lythraceae*) in Cuba. Isolates from these hosts and areas group in the sub-clade of *C. cubensis* accommodating the South American collections of the fungus. This sub-clade also includes isolates recently collected from *Eucalyptus* in Cuba, which are used to

epitypify *C. cubensis*. New host records from South East Asia include exotic *Tibouchina urvilleana* from Singapore and Thailand and native *Melastoma malabathricum* (*Melastomataceae*) in Sumatra, Indonesia. Consistent with their areas of occurrence, isolates from the latter collections group in the Asian sub-clade of *C. cubensis*. DNA sequence comparisons of isolates from *Tibouchina lepidota* in Colombia revealed that they represent a new sub-clade within the greater *Chrysoporthe* clade. Isolates in this clade are described as *Chrysoporthe inopina* sp. nov. nom. prov., based on distinctive morphological differences.

Taxonomic novelty: *Chrysoporthe inopina* Gryzenh. & M. J. Wingf. sp. nov. nom. prov.

Key words: *Chrysoporthe cubensis, Chrysoporthe inopina, Clidemia sericea, Lagerstroemia indica, Melastoma malabathricum, Miconia, Rhynchanthera mexicana, Tibouchina urvilleana*

INTRODUCTION

Chrysoporthe cubensis (Bruner) Gryzenh. & M. J. Wingf., previously known as *Cryphonectria cubensis* (Bruner) Hodges, is a well documented pathogen of various tree species in tropical and sub-tropical areas of the world (Wingfield 2003). On *Eucalyptus* (*Myrtales*, *Myrtaceae*), the fungus causes serious damage, especially in commercial plantations of susceptible species or clones (Hodges *et al.* 1979, Van Heerden *et al.* 2005). The fungus also causes a serious canker disease on *Syzygium aromaticum* (clove, *Myrtaceae*) in Malaysia, Indonesia, Zanzibar and Brazil (Nutman

& Roberts 1952, Reid & Booth 1969, Hodges *et al.* 1986, Gryzenhout *et al.* 2004/Chapter 1 in this thesis). *Chrysoporthe cubensis* has recently been found to cause die-back and cankers on native *Miconia rubiginosa* and *Miconia theaezans* trees (*Myrtales*, *Melastomataceae*), where these trees occur naturally in Colombia (Rodas *et al.* 2005).

In recent years, numerous isolates of *C. cubensis* have been collected from different hosts and parts of the world. Large numbers of these isolates have been characterised based on DNA sequence data for the ITS region of the ribosomal DNA operon, β-tubulin genes and Histone *H3* genes (Myburg *et al.* 1999, Myburg *et al.* 2002a, 2003, Gryzenhout *et al.* 2004). These comparisons have thus shown that isolates of *C. cubensis* group in two well resolved sub-clades, roughly related to geographic distribution, within the greater *Chrysoporthe* clade (Gryzenhout *et al.* 2004). The one sub-clade encompasses isolates mainly from South America, but isolates from the Congo, Republic of the Congo and Cameroon (Africa) also group in this clade (Myburg *et al.* 2003, Roux *et al.* 2003, Nakabonge *et al.* 2006). The second clade contains isolates from many South East Asian countries and Australia (Myburg *et al.* 1999, 2002a), as well as isolates from Zanzibar in Tanzania, Kenya, Mozambique, Malawi (Africa) and Hawaii (U.S.A.) (Myburg *et al.* 2003, Nakabonge *et al.* 2006). Isolates and specimens linked to these two sub-clades are morphologically and phenotypically indistinguishable (Gryzenhout *et al.* 2004).

In addition to the two sub-clades identified for *C. cubensis*, three other phylogenetic sub-clades have also been recognized within *Chrysoporthe,* for isolates previously assigned to *Cry. cubensis* (Myburg *et al.* 1999, 2002a, Wingfield *et al.* 2001, Gryzenhout *et al.* 2004, 2005/Chapter 2 of this thesis). The fungi defining these sub-clades are morphologically distinct from each other and from *C. cubensis* and

have thus been described as novel taxa (Gryzenhout *et al.* 2004, 2005). Collections making up the South African sub-clade have been described as *Chrysoporthe austroafricana* Gryzenh. & M. J. Wingf., recognizable by the rounded apices of the ascospores (Gryzenhout *et al.* 2004). The isolates in the sub-clade from *Tibouchina* trees in Colombia, which do not have sexual structures, have been described in a new anamorph genus for *Chrysoporthe* as *Chrysoporthella hodgesiana* Gryzenh. & M. J. Wingf. (Gryzenhout *et al.* 2004). This fungus can be recognized by its optimal temperature for growth in culture, which is lower than that of other species of *Chrysoporthe*. Isolates from *Eucalyptus* spp. in Ecuador represent the third sub-clade, and this fungus has been described as *Chrysoporthe doradensis* Gryzenh. & M. J. Wingf. based on its variably shaped conidia and pale lutous spore droplets (Gryzenhout *et al.* 2005).

Several hypotheses have been formulated regarding the origin of *C. cubensis*. One of these is that the fungus originated on clove where these trees are native on the Molucca islands of Indonesia (Hodges *et al.* 1986). The fungus could thus have been moved around the world with these trees, when they were planted for the spice trade, and later adapted to infect *Eucalyptus* (Hodges *et al.* 1986). Another view is that *C. cubensis* originated in South and Central America (Wingfield *et al.* 2001). The latter hypothesis is strongly influenced by the wide-spread occurrence of the fungus in various pan-tropical countries and islands of South and Central America and the Caribbean (Gryzenhout *et al.* 2004), its high phenotypic diversity in various South America countries (Van Heerden *et al.* 1997, Van Zyl *et al.* 1998) as well as its discovery on native *Miconia* species in Colombia (Rodas *et al.* 2005). This would be consistent with observations that *C. cubensis* rapidly infected *Eucalyptus* spp. when plantations of these trees were established in South America (Hodges *et al.* 1986,

Seixas *et al*. 2004), or on islands such as Hawaii (Hodges *et al*. 1986). Whether isolates defining the Asian and South American sub-clades of *C. cubensis* represent discrete and cryptic taxa or groups of isolates in the process of speciation is not clear. Population genetic studies are required to resolve this question and thus to more fully understand the probable origin of the fungus.

The hypotheses regarding the origin of *C. cubensis* are based largely on the occurrence of this fungus on hosts other than *Eucalyptus* spp. Although the fungus has been found in Australia where *Eucalyptus* spp. are native, it is not common and it occurs in a mediterranean environment quite atypical for it (Hodges *et al.* 1986, Davison & Coates 1991). Views concerning the origin of *C. cubensis* also rest firmly on our knowledge of the geographical distribution of the pathogen. An expanded view of the hosts and distribution of *C. cubensis* must contribute significantly to a better understanding of the origin of this fungus. We have, therefore, actively collected isolates of *C. cubensis* from trees other than *Eucalyptus* and also from areas where this fungus has not previously been found. In addition, collections have been made in Cuba, the locality of the type specimen, in order to obtain isolates firmly defining this species. The aim of this study was to characterise these isolates, apparently representing new collections of *C. cubensis* from previously unreported hosts and areas, using DNA sequence data and morphological characteristics. An additional aim was to define an epitype for *C. cubensis* and thus to secure the name using DNA sequence data.

MATERIALS & METHODS

Symptoms and collection of samples

Collections used in this study were made in many parts of the world (Fig. 1). In Mexico, fruiting structures of fungi resembling *C. cubensis* were found on *Clidemia sericea* (*Melastomataceae*). These plants are common as weeds and occurred at road sides and in proximity to *Eucalyptus* plantations. Structures of a *Chrysoporthe* sp. were associated with cankers on the stems or at the bases of the stems. Subsequent collections from the same area by Mr. F. Ferreira yielded fruiting structures reminiscent of *C. cubensis* on *Rhynchanthera mexicana* (*Melastomataceae*).

In Cuba, fruiting structures believed to represent *C. cubensis* were common on cankers (Fig. 2A) on *E. grandis*, *E. saligna* and *E. urophylla* trees in Parque Metropolitano (Cerro Municipality. Havana city), Santiago de las Vegas (Boyeros Municipality, Havana city) and La Habano (or Havana city). *Chrysoporthe cubensis* was originally described from this area but there is only one specimen representing the holotype and there are no isolates linked to this important specimen (Gryzenhout *et al.* 2004). Therefore, specimens and isolates were collected from Cuba in order to fortify collections of the fungus and to define an epitype for it. In Cuba, fruiting structures resembling those of *C. cubensis* were also found on the tree *Lagerstroemia indica* (*Myrtales*, *Lythraceae*), commonly known as Pride of India, or crepe myrtle, growing in the gardens of the Institute of Ecology and Systematics, Boyeros Municipality, Havana city (Figs 2E-F).

In North Sumatra, structures resembling a species of *Chrysoporthe* were found on a native *Melastoma* sp. (*Melastoma malabathricum*) also known as the Straits Rhododendron. These trees were part of the natural vegetation around Lake Toba and

in an area where both clove and *Eucalyptus* trees are planted (Figs 2B-C). *Tibouchina urvilleana* plants (Fig. 2D) in Singapore and Thailand were also found bearing fruiting structures similar to those of *Chrysoporthe* spp. This is not a certain host of *C. cubensis* since previous reports of *C. cubensis* on *Tibouchina* spp. (Wingfield *et al.* 2001, Seixas *et al.* 2004) represent *Chrysop. hodgesiana* or are of unconfirmed identity (Gryzenhout *et al.* 2004).

Additional plant material bearing structures resembling those of *Chrysoporthe* spp. and isolates derived from these specimens, were obtained from Colombia. Specimens bearing fruiting structures taken from *T. lepidota* trees were collected at the Libano farm of Smurfit Carton de Colombia, near Pereira, Colombia. This material included teleomorph structures of a fungus resembling a *Chrysoporthe* sp.

Isolations from the fruiting structures on the bark surface of field-collected specimens, were made on malt extract agar MEA [20 g/L malt extract agar (Biolab, Merck, Midrand, South Africa)]. This was done by taking conidial or ascospore drops from the necks of fruiting structures or by exposing the spore mass inside the ascomata and then removing spores from it. The resultant cultures are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, and representative isolates have been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands (Table 1). The original bark specimens connected to the isolations have been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM).

DNA sequence comparisons

Isolates for DNA sequence comparisons were grown in Malt Extract Broth [20 g/L malt extract, Biolab, Midrand, South Africa]. DNA was extracted from mycelium as

described in Myburg *et al.* (1999). The internal transcribed spacer (ITS) regions ITS1 and ITS2, as well as the conserved 5.8S gene of the ribosomal RNA (rRNA) operon, were amplified as described by Myburg *et al.* (1999). Two regions within the β-tubulin genes were also amplified following the methods of Myburg *et al.* (2002a). Purification of PCR products was done using a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany).

The purified PCR products were sequenced with the same primers that were used to amplify the respective DNA regions. An ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Perkin-Elmer, Warrington, UK) was used to sequence the amplification products on an ABI PRISM 3100™ automated DNA sequencer. The resulting raw nucleotide sequences were edited using Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems, Inc., Foster City, California, U.S.A.) software.

Sequences were added to the existing dataset (S 1211, M 2095) of Gryzenhout *et al.* (2004) and manually aligned. This dataset (Table 1) thus included isolates of *C. cubensis* from *Eucalyptus* spp. (Myburg *et al.* 2002a, Gryzenhout *et al.* 2004), *S. aromaticum* (Myburg *et al.* 1999, 2003) and *Miconia* spp. (Rodas *et al.* 2005) from different parts of the world; isolates of *C. hodgesiana* from *T. urvilleana* (Wingfield *et al.* 2001, Gryzenhout *et al.* 2004) and *M. theaezans* (Rodas *et al.* 2005); *C. austroafricana* isolates from *Eucalyptus* spp., *T. granulosa* (Myburg *et al.* 2002b, 2002b) and *S. cordatum* (Heath *et al.* 2006); and isolates representing *C. doradensis* (Gryzenhout *et al.* 2005). The outgroup consisted of species of the closely related *Cryphonectria*, namely *Cryphonectria parasitica* (Murrill) M. E. Barr, *Cryphonectria nitschkei* (G. H. Otth) M. E. Barr and *Cryphonectria macrospore* (Tak. Kobay. & Kaz. Itô) M. E. Barr.

Phylogenetic trees were inferred using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2002). A 500 replicate partition homogeneity test (PHT) (Farris *et al.* 1994) was applied to the rRNA and β-tubulin gene sequence data sets (after the exclusion of uninformative sites, heuristic search with 100 random sequence additions and tree-bisection-reconnection branch swapping, MULTREES off) to determine whether they could be analysed collectively. A phylogenetic tree was inferred from distance analyses. The correct model for the data sets was found with MODELTEST version 3.5 (Posada & Crandall 1998). The HKY85 model (Hasegawa *et al.* 1985) with the Gamma distribution shape parameter set to 0.1798 (freqA 0.1893, freqC 0.3273, freqG 0.2368, freqT 0.2465, Ti/Tv 2.0064), was shown to be the appropriate model. A 1000 replicate bootstrap analysis was executed to assess the confidence levels of the branch nodes of the phylogenetic tree. The sequence data generated in this study have been deposited in GenBank (Table 1).

Morphology

Fruiting structures were cut from the bark specimens and rehydrated in boiling water for 1 min. The structures, embedded in Leica mountant (Setpoint Premier, Johannesburg, South Africa), were sectioned approximately 12 um thick. The sections were dropped in water, transferred to microscope slides and mounted in 85% lactic acid. Fresh slides from structures were also made with lactic acid or 3% KOH. Twenty measurements of ascospores, asci, conidia and conidiophores were taken from the fresh slides for all specimens, but fifty measurements were taken for the holotype specimen. These are presented as (min–)(average - std. dev.) – (average + std. dev.)(–max) µm. A range of measurements (minimum and maximum) was obtained from at least two structures (representing the smallest and largest) for the anamorph and

teleomorph stromata on the bark, and perithecia representing the midpoint from sections, respectively. Standard colour notations provided by Rayner (1970) were applied.

Growth of isolates CMW 12727 and CMW 12729, which represent the new species from *T. lepidota* in Colombia (Table 1), was compared in culture. Cultures were grown on MEA in 90 mm diam Petri dishes in the dark at temperatures from 15 to 35 ºC at 5 º intervals. Assessment of growth was made as is described in Gryzenhout *et al.* (2004).

RESULTS

DNA sequence comparisons

The PHT analyses showed that the rDNA and β-tubulin sequence data sets did not have any significant conflict (P = 0. 012) and could thus be combined. The dataset consisted of 39 taxa with the aligned ribosomal DNA sequence dataset (538 characters) consisting of 473 constant characters, 20 parsimony-uninformative and 45 parsimony-informative characters (g1 = –3.671). The β-tubulin gene dataset (894 characters) consisted of 716 constant characters, 68 parsimony-uninformative and 110 parsimony-informative characters (g1 = –3.5959). After combination, the data set was comprised of 1432 characters.

The various isolates originating from Mexico, Cuba, Sumatra and Singapore grouped in the two sub-clades representing *C. cubensis*, separately from those representing *C. austroafricana*, *Chrysop. hodgesiana* and *C. doradensis* (Fig. 3). The isolates from *Cli. sericea* (CMW 12471, CMW 13046) and *R. mexicana* (CMW 12734, CMW 12736) in Mexico, grouped in the sub-clade including Mexican and

other South American isolates (bootstrap support 93%). Specifically, the isolates from *E. grandis* (CMW 14394, CMW 14404) and *L. indica* (CMW 16199, CMW 16200) in Cuba, also grouped in the South American sub-clade of *C. cubensis*. The isolates from *T. urvilleana* in Singapore (CMW 12745) and Thailand (CMW 17172, CMW 17178), and *M. malabathricum* (CMW 16192, CMW 18515) in Sumatra grouped in the South East Asian sub-clade (bootstrap support 96%).

Other than the four previously characterised sub-clades in *Chrysoporthe* (Myburg *et al.* 2002a, Gryzenhout *et al.* 2004, 2005), an unexpected sub-clade emerged in the phylogenetic tree (Fig. 3). The clade included isolates representing the new collection (CMW 12727, CMW 12729, CMW 12731) from *T. lepidota* in Colombia (bootstrap support 72%).

Morphology

Fruiting structures produced on specimens from different hosts in Mexico, Cuba, Singapore and Sumatra (Table 2) included both teleomorph and anamorph structures. These structures all resembled features previously described for *C. cubensis* (Bruner 1917, Hodges 1980, Myburg *et al.* 2003, Gryzenhout *et al.* 2004). The morphological characterisation of this material as *C. cubensis* is consistent with the results of the DNA sequence comparisons.

Fruiting structures on specimens collected from *Eucalyptus* spp. in Cuba were similar to those described for *C. cubensis* from other parts of the world. They were also indistinguishable from those of the type specimen (BPI 631857) of *C. cubensis* (Bruner 1917, Gryzenhout *et al.* 2004). The sizes of the asci on the newly collected material from Cuba differed somewhat from those reported by Bruner (1917) for Cuban specimens. They were thus up to 28 µm long, and not up to 34 µm as reported

by Bruner (1917). Gryzenhout *et al.* (2004) also reported that ascus dimensions as reported by Bruner (1917) were inconsistent with those of specimens from other countries, but they could not find asci on the type specimen to confirm the ascus dimensions. The new measurements obtained for Cuban material from *Eucalyptus* in this study are similar to ascus sizes reported by Gryzenhout *et al.* (2004) for specimens connected to *C. cubensis* from South America and South East Asia. The newly confirmed ascus size of (19–)22–26.5(–28) µm for *C. cubensis* represents an additional characteristic to distinguish *C. cubensis* from *C. austroafricana*, which has longer asci [(25–)27–32(–34) µm; Gryzenhout *et al*. 2004).

Fruiting structures on the bark material (PREM 58800) linked to the isolates from *T. lepidota* in Libano, Colombia (CMW 12727, CMW 12729, CMW 12731), which formed a separate sub-clade based on DNA data, could be distinguished from those of existing *Chrysoporthe* species. Asci (Figs 4E, 5C) were longer [(27.5–)29.5–34(–35.5) µm] than those of *C. cubensis* [(19–)22–26.5(–28) µm; Gryzenhout *et al*. (2004)] and *C. doradensis* [(19.5–)21.5–24(–25); Gryzenhout *et al*. (2005)], and corresponded with those of *C. austroafricana* [(25–)27–32(–34) µm; Gryzenhout *et al.* (2004)]. The new species could also be distinguished from *C. austroafricana*, *C. cubensis* and *C. doradensis*, because its ascospores (Figs 4F, 5C) were slightly wider (2.5–3.5 µm) than those of *C. cubensis* [(2–2.5(–3) µm; Gryzenhout *et al*. (2004)], *C. austroafricana* [(2–)2.5 µm; Gryzenhout *et al*. (2004)] and *C. doradensis* [2-2.5 µm; Gryzenhout *et al*. (2005)]. Furthermore, isolates in this group grew optimally at 25 ºC, different to the temperature for optimal growth of 30 ºC for *C. cubensis*, *C. austroafricana* (Gryzenhout *et al*. 2004) and *C. doradensis* (Gryzenhout *et al.* 2005). Conidiomata also had variable shapes, varying from subulate with no attenuated neck, to pyriform with an attenuated neck, to globose (Figs 4G, 5D). These characteristics

distinguished the new species from *Chrysop. hodgesiana*, which is the only other species growing optimally at 25 ºC (Gryzenhout *et al*. 2004). However, since shape and size of the conidiomata can be quite variable between samples (Hodges *et al.* 1986, Rodas *et al.* 2005), the shape of the conidiomata cannot always be used with confidence to distinguish *Chrysop. hodgesiana* from the new species.

Taxonomy

In this study we have characterised collections of *C. cubensis* from *Eucalyptus* spp. from Cuba (PREM 58788–PREM 58791), which is the type locality of *C. cubensis*. Based on morphology, these specimens were similar to other specimens of *C. cubensis*. Isolates connected to these specimens also grouped in the South American sub-clade of *C. cubensis* based on DNA sequence comparisons. In the absence of isolates it has been impossible to tell whether the type specimen of *C. cubensis* from Cuba would group in the South East Asian or South American sub-clade of the fungus (Gryzenhout *et al.* 2004). Certain morphological features such as ascus morphology could also not be studied, due to the poor quality of the type specimen. Epitypification of *C. cubensis* using newly collected Cuban specimens will greatly aid future taxonomic studies of *Chrysoporthe* species. We, therefore, designate specimen PREM 58788 (ex-type culture CMW 14394/CBS 118654, living culture CMW 14404/CBS 118647), which originates from the same locality as the type specimen, as epitype of *C. cubensis*. Additional specimens and linked isolates (Table 2; specimen PREM 58789 = isolate CMW 14378/CBS 118655; PREM 58790 = CMW 14362/CBS 118657; PREM 58791 = CMW 14395/CBS 118648) from Cuba have also been deposited to fortify material defining this species.

Comparisons of specimens from *T. lepidota* collected on the Libano farm in Colombia, showed that this fungus represents a previously undescribed taxon. Although the sub-clade representing this fungus is supported with a relatively low bootstrap value based on the regions of the genome sequenced, the fungus clearly does not group in the sub-clades representing other species. This new species can also be differentiated from other species of *Chrysoporthe* based on morphology. We, therefore, provide the following description for it.

Chrysoporthe inopina Gryzenh. & M. J. Wingf., **sp. nov.**, **nom. prov.** Figs 4-5.

Etymology: Latin, *inopina*, unexpected, referring to the unexpected discovery of this species.

Ascosporae fusoideae vel ovales, utrinque rotundatae, (4.5–)6–7.5(–8) × 2.5–3.5 µm. *Conidiomata* subulata vel pyriformia vel pulvinata.

Ascostromata semi-immersed in bark, recognizable by extending, fuscous-black, cylindrical perithecial necks and in some cases, erumpent, limited ascostromatic tissue appearing orange, 90–540 µm high above the bark, 200–770 µm diam (Figs 4A–B, 5A–B). *Perithecia* valsoid, 1–6 per stroma, bases immersed in the bark, black, globose, 306–390 µm diam, perithecial wall 21–25 µm thick (Figs 4B, 5B). Top of perithecial bases covered with cinnamon to orange, predominantly prosenchymatous, stromatic tissue, which is occasionally visible above the bark surface (Figs 4B–C, 5B). *Perithecial necks* black, periphysate, 77–131 µm wide (Figs 4B, 4D, 5B). Necks emerging through the bark covered in umber, stromatic tissue of *textura porrecta*, thus appearing fuscous-black (Figs 4B, 5A–B), extending necks up to 2330

µm long, 90–180 µm wide. *Asci* 8-spored, biseriate, unitunicate, free when mature, non-stipitate with a non-amyloid refractive ring, fusoid to ellipsoid, (27.5–)29.5–34(–35.5) × (4.5–)5.5–6.5(–7) µm (Figs 4F, 5C). *Ascospores* hyaline, one-septate, fusoid to oval, with rounded apices, (4.5–)6–7.5(–8) × 2.5–3.5 µm (Figs 4E, 5C).

Conidiomata eustromatic, superficial to slightly immersed, subulate to pyriform to pulvinate, with neck attenuated or not, usually with one neck per structure (Figs 4G–H, 5D–E), fuscous-black, inside umber when young, conidiomatal base above the bark surface 100–650 µm high above level of bark, 70–710 µm diam, necks up to 780 µm long, 50–190 µm wide. *Conidiomatal locules* with even to convoluted inner surface, occasionally multilocular, single locule connected to 1 or several necks (Figs 4H, 5E). *Stromatic tissue* of base of *textura globulosa*, walls of outer cells thickened (Fig. 4I), neck tissue of *textura porrecta* (Fig. 4J). *Conidiophores* hyaline, with basal cells of irregular shape and (2.5–)3.5–6(–7) × 2–3.5(–4) µm, branched irregularly at the base or above into cylindrical cells, with or without separating septa, total length of conidiophore (11–)12.5–22.5(–29.5) µm (Figs 4K, 5F). *Conidiogenous cells* phialidic, determinate, apical or lateral on branches beneath a septum, cylindrical to flask-shaped with attenuated apices, (1.5–)2–2.5(–3) µm wide, collarette and periclinal thickening inconspicuous (Figs 4K, 4L, 5F). *Conidia* hyaline, non-septate, oblong, (3–)3.5–4 × (1.5–)2–2.5 µm (Figs 4L, 5F), masses exuded as orange to luteous droplets.

Cultural characteristics: on MEA (CMW 12727, CMW 12729) white with cinnamon to hazel patches, fluffy with a smooth margin, fast-growing, covering a 90 mm diam plate after a minimum of five days at the optimum temperature of 25 ºC. Cultures rarely sporulating after sub-culturing, teleomorph not produced in culture.

Substrate: Bark of *Tibouchina lepidota.*

Distribution: Colombia

Specimens examined: **Colombia,** Risaralda, Libano farm near Pereira (75° 35′ 49″ W and 40° 43′ 13″ N, 2102 meters above sea level, 3143 mm/y), *Tibouchina lepidota*, Jan. 2003, R. Arbelaez, **holotype** PREM 58800, ex-type culture CMW 12727 = CBS 118659, living cultures CMW 12729 = CBS 118658, CMW 12731 = CBS 118656.

DISCUSSION

Results of this study encompass reports of several new host species for *C. cubensis* from various parts of the world (Fig. 1). Many of these hosts are native to the countries in which they have been found. Thus, isolates of *C. cubensis* residing in the South American sub-clade, originated on native *Cli. sericea* and *R. mexicana* in Mexico. These collections add to those on *Miconia* spp. that have been reported before as native hosts of *C. cubensis* in Colombia (Rodas *et al.* 2005). Isolates from the native melastome, *M. malabathricum*, were shown to group in the South East Asian clade of *C. cubensis*. This represents the first ever collection of *C. cubensis* from a native plant growing in a natural situation in South East Asia. The only other equivalent collection is that of *C. cubensis* from clove collected in Malaysia (Reid & Booth 1969), Sulawesi (Myburg *et al.* 2003) and elsewhere in Indonesia (Hodges *et al*. 1986). Although clove occurs naturally in the Molucca islands, which is relatively close to areas in Indonesia and Malaysia where *C. cubensis* has been found to occur, the trees in the latter areas are planted and not native.

Other hosts of *C. cubensis* reported in this study are exotics in the countries where the fungus was collected. *Lagerstroemia indica* is native to China, but is

planted world-wide as a garden ornamental. In the case of this study, *C. cubensis* was found on this tree in Cuba. Isolates from this collection in Cuba group in the South American sub-clade of *C. cubensis*, which is the same as collections of the fungus now available from *Eucalyptus* in Cuba. The origin of the fungus on *Eucalyptus* and *L. indica* is not known but it probably originated on a native plant in the area. It is, however, also possible that *L. indica* represents a native host of *C. cubensis* in China where the tree is native. If that were the case, isolates of the fungus from China would most probably group in the South East Asian sub-clade. That was also true in previous studies (Myburg *et al.* 1999, Myburg *et al.* 2002a), where an isolate from *E. camaldulensis* in China grouped in the South East Asian clade.

Tibouchina urvilleana is a native of South America, but it is also popular as an ornamental in various countries, because of its conspicuous and attractive flowers. In this study, *C. cubensis* was found on *T. urvilleana* planted as a non-native ornamental in Singapore and Thailand. Isolates from these collections group in the South East Asian clade of *C. cubensis*. The fungus is also known to occur on *Eucalyptus* in Thailand (Old *et al.* 2004). Presumably, the fungus in the area originally came from a native South East Asian plant, possibly a species of *Melastoma* of which there are numerous native species in the area. *Tibouchina* spp. are known to be highly susceptible to infection by *Chrysoporthe* spp. (Wingfield *et al.* 2001, Myburg *et al.* 2002b, Seixas *et al.* 2004) and trees planted as exotics could easily become infected, as appears to have occurred in this situation.

Prior to this study, *Tibouchina* spp. had not been confirmed as hosts of *C. cubensis*. Previous collections from *Tibouchina*, thought to represent *C. cubensis* (Wingfield *et al.* 2001), have recently been shown to represent *Chrysop. hodgesiana* (Gryzenhout *et al.* 2004). The collections of Seixas *et al.* (2004) from *Tibouchina*

were identified based only on morphological characters and before *Chrysoporthe* was described (Gryzenhout *et al.* 2004) It is thus unclear whether these represent *C. cubensis* or one of the related fungi such as *Chrysop. hodgesiana* or *C. inopina*, described in this study. It is, however, likely that *C. cubensis* occurs on native *Tibouchina* spp. in South America due to the susceptibility of these trees to *Chrysoporthe* spp.

The collection of *C. cubensis* from native *M. malabathricum* supports the view that South East Asia could be an area of origin for *C. cubensis.* The fact that the isolates group in the discrete South East Asian clade of the fungus, however, supports the DNA based view that there are two discrete populations of the fungus. Isolates representing both clades have now been found on native plants (species of *Melastoma*, *Miconia*, *Clidemia* and *Rhynchanthera*) and non native plants (*S. aromaticum*, *Eucalyptus*, *Lagerstroemia*, *Tibouchina*) in two geographically distant parts of the world. Either of these areas could represent the area of origin of *C. cubensis*, or the isolates might represent two discrete taxa that have speciated recently.

Ideally, isolates from various hosts should be included in studies at the population level aimed at determining the world-wide population structure and origin of *C. cubensis sensu lato*. Such studies are, however, frustrated by the difficulty of obtaining sufficient numbers of isolates from native hosts in the various countries where they are known to occur. Although there is substantial phylogenetic support for separating the isolates of the two sub-clades of *C. cubensis* into two taxa, these fungi appear to be morphologically identical. They also share hosts in the two regions in which they occur. We thus believe that a population-based species concept is required if these isolates from the two regions are to be treated as discrete taxa. Such studies will be greatly aided by evidence presented in this study that the type

specimen of *C. cubensis* phylogenetically represents the South American form of the fungus. This was previously unknown and the question could only be resolved with collections of *C. cubensis* from Cuba. These specimens and cultures, including an epitype linked to them, should provide a robust basis for future studies.

It is evident from this study that *C. cubensis* is able to infect plants from at least three different families although all of these are in the *Myrtales*. The families include the *Myrtaceae*, *Melastomataceae* and *Lythraceae* and they represent a wide diversity of morphological and physiological characteristics (Dahlgren & Thorne 1984, Conti *et al.* 1997). Even within the families, *C. cubensis* is able to infect plants belonging to different tribes, e.g. the *Syzygieae* and the *Eucalypteae* in the *Myrtaceae* (Wilson *et al.* 2005), and the *Miconieae* and *Melastomeae* in the *Melastomataceae* (Clausing & Renner 2001). Furthermore, artificial inoculations on members of several additional families in the *Myrtales* have shown that they are also susceptible to infection by *Chrysoporthe* spp., although these have not yet been found as natural hosts (Hodges *et al.* 1986, Seixas *et al.* 2004). These hosts include members of the *Rhizophoraceae*, *Combretaceae*, *Onagraceae*, *Punicaceae*, *Sapotaceae* and *Lauraceae*, which represent distinct phylogenetic groups within the *Myrtales* (Conti *et al.* 1996, Conti *et al.* 1997). Only a limited number of genera in each of these families has been tested (Seixas *et al.* 2004), and it is likely that additional hosts of *C. cubensis* will be discovered.

Phylogenetic analyses in this study have revealed an unexpected and closely related new sub-clade in the larger *Chrysoporthe* group. This is the phylogenetic sub-clade accommodating isolates from *T. lepidota* in Colombia. The distinct grouping of the sub-clade from Colombia is clearly supported with morphological characteristics and we were able to describe the new species *C. inopina*. Fruiting structures of *C.*

inopina contained asci longer that those of *C. cubensis*, and ascospores wider than those for all other species of *Chrysoporthe*.

When *C. inopina* was first discovered, our view was that it might represent the teleomorph of *Chrysop. hodgesiana*, or a first report of *C. cubensis* on *Tibouchina* spp. in South America. This is because no teleomorph is known for *Chrysop. hodgesiana*, nor is *Tibouchina* a confirmed host for *C. cubensis* (Gryzenhout *et al.* 2004). DNA sequences of these isolates and morphological comparisons, however, showed clearly that the fungus represents an undescribed taxon. *C. inopina* thus represents a third species of *Chrysoporthe* on native *Melastomataceae* in Colombia, with the other two species *C. cubensis* (Rodas *et al.* 2005) and *Chrysop. hodgesiana* (Gryzenhout *et al.* 2004, Rodas *et al.* 2005). Of these, both *C. inopina* and *Chrysop. hodgesiana* occur on *Tibouchina* spp. and in the same area. It is thus evident that a complex of species, closely related and morphologically similar, occurs on these native trees in South America. Extreme care should thus be taken when collecting and identifying these fungi, especially where work is related to quarantine and disease management, which is clearly important for fungi such as *C. cubensis*.

Chrysoporthe cubensis was previously known almost exclusively as a *Eucalyptus* canker pathogen, but this and other recent studies (Wingfield *et al.* 2001, Myburg *et al.* 2002b, Gryzenhout *et al.* 2004, Rodas *et al.* 2005) have reported on new and diverse hosts for the fungus. These studies have radically changed our perception of the ecology of *C. cubensis*. Furthermore, besides *C. cubensis*, there are many closely related and morphologically similar species that have previously been treated under the older name as representing *Cry. cubensis*. Some of these species in *Chrysoporthe*, such as *C. cubensis*, *C. austroafricana* and *C. doradensis*, are important pathogens of *Eucalyptus*, but all species appear to be pathogens of members

of the *Myrtales*. They should thus be considered as serious potential pathogens that could cause devastating diseases if they were to be accidentally introduced into new areas with large populations of native, susceptible plants. For example, *C. austroafricana*, apparently native to South Africa (Heath *et al.* 2006), causes a serious disease of *Eucalyptus* in that country and represents a significant threat to native forests of *Eucalyptus* in Australia (Roux *et al.* 2003, Wingfield 2003). These relatively unknown and newly discovered fungi deserve more attention from pathologists and quarantine authorities.

ACKNOWLEDGEMENTS

We are grateful to Mr. F. Ferreira (Tabasco, Mexico) for supplying specimens of *C. cubensis* from *R. mexicana* and *Cli. sericea* in Mexico. We thank Me. R. Bhoora and J. Jakavula for assistance with DNA sequencing of some isolates, and Dr. Henrietta Myburg for providing the sequence of isolate CMW 10192. Dr. H.F. Glen (Natal Herbarium, Durban, South Africa) assisted us in selecting a name for *C. inopina*. This study was made possible by funding from the National Research Foundation (NRF), members of the Tree Pathology Co-operative Programme (TPCP), and the THRIP support programme of the Department of Trade and Industry, South Africa.

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Table 1. Isolates included in this study.

Species identity	Isolate number[a]	Alternative isolate number[a]	Host	Origin	Collector	GenBank accession numbers[b]
Chrysoporthe cubensis	CMW 1856	–	*Eucalyptus* sp.	Kauai, Hawaii	–	AY 083999, AY 084010, AY 084022
	CMW 9903	–	*Syzygium aromaticum*	Kalimantan, Indonesia	C.S. Hodges	AF 292044, AF 273066, AF 273461
	CMW 11289	CBS 115737	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	AY 214303**,** AY 214231, AY 214267
	CMW 8650	CBS 115719	*S. aromaticum*	Sulawesi, Indonesia	M.J. Wingfield	AY 084001, AY 084013, AY 084024
	CMW 10774	–	*S. aromaticum*	Zanzibar, Tanzania	–	AF 492130, AF 492131, AF 492132
	CMW 2632	–	*E. marginata*	Australia	E. Davison	AF 046893, AF 273078, AF 375607
	CMW 12745	CBS 117837	*Tibouchina urvilleana*	Singapore	M.J. Wingfield	DQ368764, DQ368780, DQ368781
	CMW 17172	CBS 118664	*T. urvilleana*	Thailand	M.J. Wingfield	DQ368765, DQ368782, DQ368783
	CMW 17178	CBS 118665	*T. urvilleana*	Thailand	M.J. Wingfield	DQ368766, DQ368784, DQ368785
	CMW 16192	CBS **	*Melastoma malabathricum*	Sumatra	M.J. Wingfield	DQ368767, DQ368786, DQ368787
	CMW 18515	CBS 118651	*M. malabathricum*	Sumatra	M.J. Wingfield	DQ368768, DQ368788, DQ368789
	CMW 10669	CBS 115751	*Eucalyptus* sp.	Republic of Congo	J. Roux	AF 535122, AF 535124, AF 535126
	CMW 1853	–	*S. aromaticum*	Brazil	–	AF 046891, AF 273070, AF 273465
	CMW 10778	CBS 115755	*S. aromaticum*	Brazil	C.S. Hodges	AY 084006, AY 084018, AY 084030
	CMW 9432	CBS 115724	*E. grandis*	Mexico	M.J. Wingfield	AY 692321, AY 692324, AY 692323
	CMW 12734	CBS 115853	*Rhynchanthera*	Mexico	F. Ferreira	DQ368769, DQ368790, DQ368791

			mexicana			
	CMW 12736	CBS 115847	*R. mexicana*	Mexico	F. Ferreira	DQ368770, DQ368792, DQ368793
	CMW 13046	CBS 115762	*Clidemia sericeae*	Mexico	F. Ferreira	DQ368772, DQ368796, DQ368797
	CMW 12471	CBS 115849	*Cli. sericeae*	Mexico	F. Ferreira	DQ368771, DQ368794, DQ368795
	CMW 14394 (ex-epitype culture designated here)	CBS 118654	*E. grandis*	Cuba	M.J. Wingfield	DQ368773, DQ368798, DQ368799
	CMW 14404	CBS 118647	*E. grandis*	Cuba	M.J. Wingfield	DQ368774, DQ368800, DQ368801
	CMW 16199	CBS 118652	*Lagerstroemia indica*	Cuba	M.J. Wingfield	DQ368775, DQ368802, DQ368803
	CMW 16200	CBS 118650	*L. indica*	Cuba	M.J. Wingfield	DQ368776, DQ368804, DQ368805
	CMW 10639	CBS 115747	*E. grandis*	Colombia	C.A. Rodas	AY 263419, AY 263420, AY 263421
	CMW 10026	–	*Miconia rubiginosa*	Colombia	C.A. Rodas	AY 214294, AY 214222, AY 214258
	CMW 10028	–	*M. rubiginosa*	Colombia	C.A. Rodas	AY 214295, AY 214223, AY 214259
	CMW 9980	–	*Miconia theaezans*	Colombia	C.A. Rodas	AY 214297, AY 214225, AY 214261
	CMW 9993	CBS 115728	*M. theaezans*	Colombia	C.A. Rodas	AY 214298, AY 214226, AY 214262
Chrysoporthe austroafricana	CMW 2113	CBS 112916	*E. grandis*	South Africa	M.J. Wingfield	AF 046892, AF 273067, AF 273462
	CMW 9327	CBS 115843	*Tibouchina granulosa*	South Africa	M.J. Wingfield	AF 273473, AF 273060, AF 273455
	CMW 10192	CBS 118649	*Syzygium cordatum*	South Africa	M. Gryzenhout	AY 214299, AY 214227, AY 214263
Chrysoporthella hodgesiana	CMW 10641	CBS 115854	*Tibouchina semidecandra*	Colombia	R. Arbaleaz	AY 692322, AY 692326, AY 692325

	CMW 9995	CBS 115730	*T. semidecandra*	Colombia	R. Arbelaez	AY956969, AY956977, AY956978
	CMW 10625	CBS 115744	*M. theaezans*	Colombia	C.A. Rodas	AY956970, AY956979, AY956980
Chrysoporthe inopina	**CMW 12727** (ex-type culture designated here)	CBS 118659	*Tibouchina lepidota*	Colombia	R. Arbelaez	DQ368777, DQ368806, DQ368807
	CMW 12729	CBS 118658	*T. lepidota*	Colombia	R. Arbelaez	DQ368778, DQ368808, DQ368809
	CMW12731	CBS 118656	*T. lepidota*	Colombia	R. Arbelaez	DQ368779, DQ368810, DQ368811
Chrysoporthe doradensis	CMW 11286	CBS 115734	*E. grandis*	Ecuador	M.J. Wingfield	AY 214289, AY 214217, AY 214253
	CMW 11287	CBS 115735	*E. grandis*	Ecuador	M.J. Wingfield	AY 214290, AY 214218, AY214254
	CMW 9123	CBS 115717	*Eucalyptus deglupta*	Ecuador	M.J. Wingfield	DQ 224034, DQ 224038, DQ 224039
Cryphonectria parasitica	CMW 1652	CBS 112914	*Castanea dentata*	U.S.A.	–	AF 046902, AF 273075, AF 273468
Cryphonectria nitschkei	CMW 10518	CBS 112919	*Quercus sp.*	Japan	T. Kobayashi	AF 452118, AF 525706, AF 525713
Cryphonectria macrospore	CMW 10463	CBS 112920	*Castanopsis cuspidata*	Japan	T. Kobayashi	AF 368331, AF 368351, AF 368350

[a] CMW = Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria; CBS = Centraalbureau voor Schimmelcultures (Utrecht). Isolates in bold were sequenced in this study.

[b] Accession numbers refer to sequence data of the ITS, β-tubulin 1 (primers Bt1a/1b) and β-tubulin 2 (primers Bt2a/2b) regions respectively.

Table 2. Herbarium specimens used in this study.

Species identity	Herbarium number[a]	Linked isolate[a]	Host	Origin	Collector	Date
Chrysoporthe cubensis	PREM 58788 (epitype designated here)	CMW 14394, CMW 14404	*Eucalyptus grandis*	Santiago de las Vegas, Boyeros Municipality, Havana city Cuba	M.J. Wingfield	Jan. 2004
	PREM 58789	CMW 14378	*E. grandis*	60 km west from Havana, Cuba	M.J. Wingfield	Jan. 2004
	PREM 58790	CMW 14362	*E. saligna*	Parque Metropolitano, Cerro Municipality, Havana city.	M.J. Wingfield	Jan. 2004
	PREM 58791	CMW 14395	*Eucalyptus urophylla*	Road to Havana, Cuba	M.J. Wingfield	Jan. 2004
	PREM 58792	CMW 16199	*Lagerstroemia indica*	Havana city, Cuba	M.J. Wingfield	Jan. 2004
	PREM 58793	CMW 12734, CMW 12736	*Rhynchanthera mexicana*	Mexico	F. Ferreira	2002
	PREM 58794	CMW 12734, CMW 12736	*R. mexicana*	Mexico	F. Ferreira	2002

	PREM 58795	CMW 13046	*Clidemia sericea*	Mexico	F. Ferreira	2002
	PREM 58796	CMW 12471	*C. sericea*	Mexico	F. Ferreira	2002
	PREM 58797	CMW 12745	*Tibouchina urvilleana*	Singapore	M.J. Wingfield	Apr. 2003
	PREM 58798	CMW 16192	*Melastoma malabathricum*	Lake Toba, Aek Nauli, Sumatra	M.J. Wingfield	Feb. 2004
	PREM 58799	CMW 18515	*M. malabathricum*	Lake Toba, Sumatra	M.J. Wingfield	May 2005
Chrysoporthe inopina	PREM 58800 (holotype designated here)	CMW 12727, CMW 12729, CMW 12731	*Tibouchina lepidota*	Pereira, Colombia	R. Arbelaez	Jan. 2003

[a] PREM, National Collection of Fungi (Pretoria); CMW = Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria; CMW = Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria.

Fig. 1. Map of world showing the locations of different collections of *Chrysoporthe cubensis* (distinguishing between two sub-clades), *Chrysoporthe austroafricana*, *Chrysoporthe inopina*, *Chrysoporthe doradensis* and *Chrysoporthella hodgesiana*. Only collections verified with DNA sequence data are shown. Different hosts are also shown for collections of *C. cubensis sensu lato*.

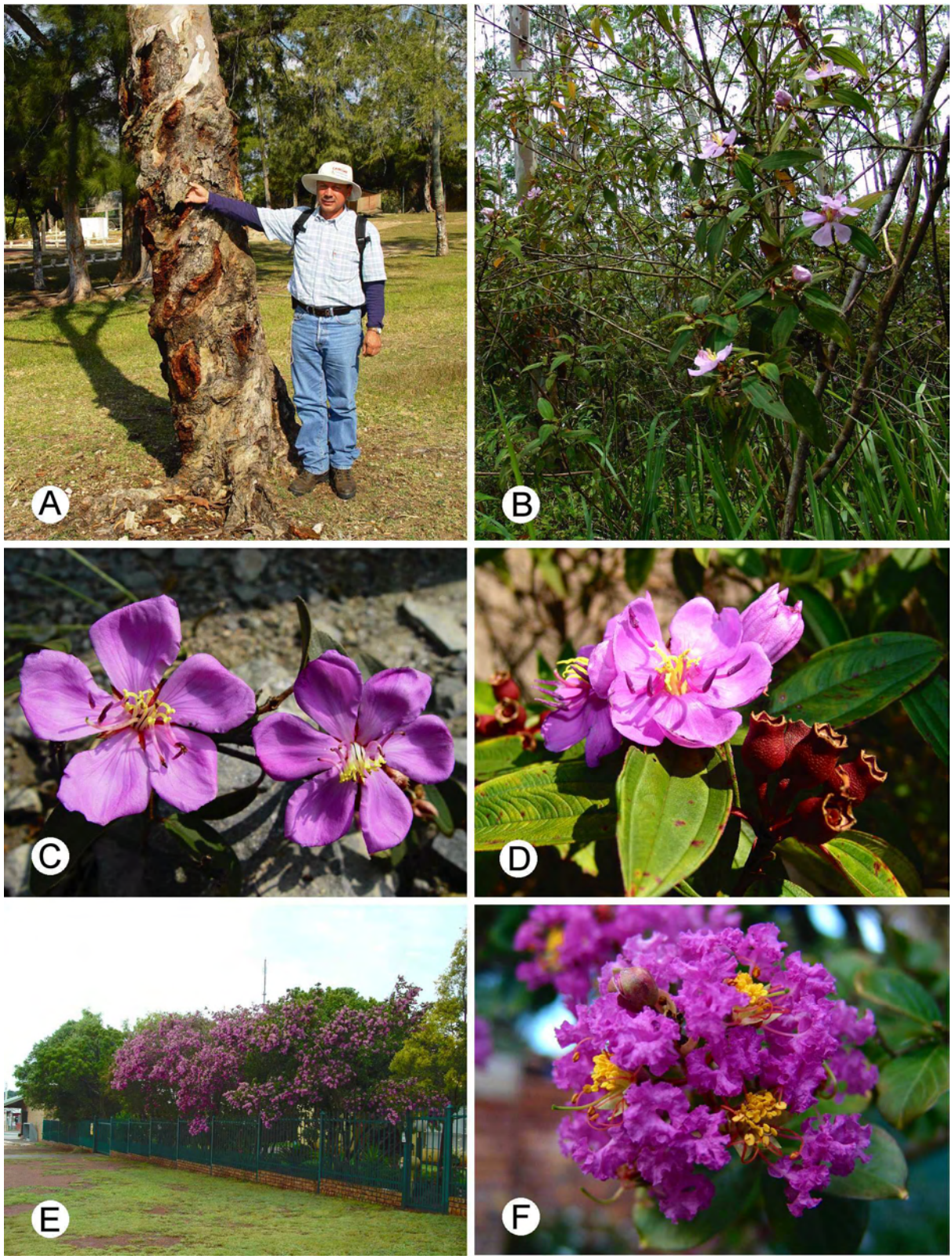

Fig. 2. Different hosts susceptible to *Chrysoporthe cubensis*. A. Basal canker on *Eucalyptus grandis* in Cuba. B. Native *Melastoma malabathricum* trees growing in a plantation in Sumatra. C. Flowers of *M. malabathricum*. D. *Tibouchina urvilleana*. E–F. *Lagerstroemia indica*.

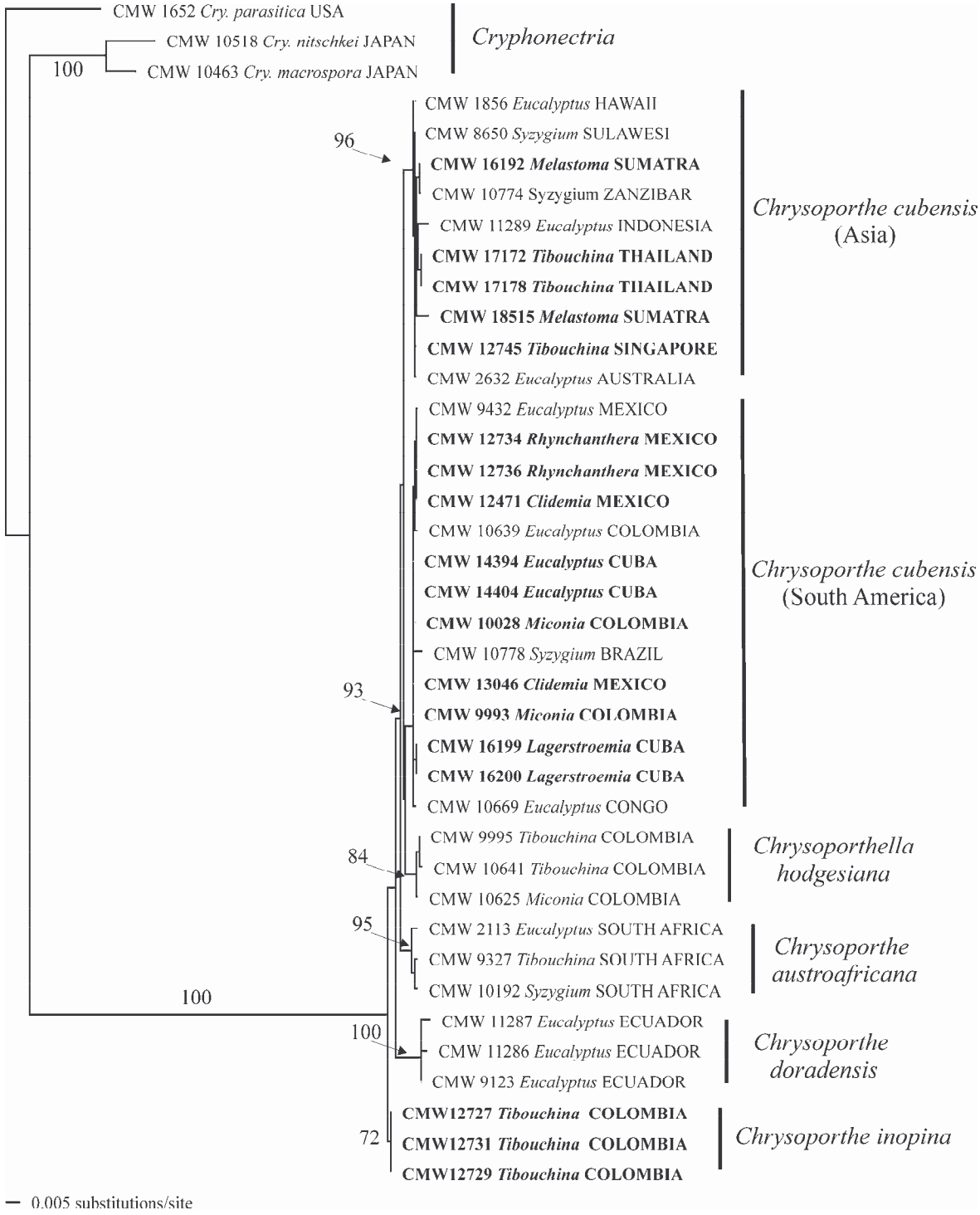

Fig. 3. Phylogram obtained from a combined data set comprising of ribosomal and β-tubulin gene sequences. The phylogram was obtained with distance analyses using the HKY85 parameter model (G = 0.1798, freqA 0.1893, freqC 0.3273, freqG 0.2368, freqT 0.2465, Ti/Tv 2.0064). Confidence levels, determined by a 1000 replicate bootstrap analysis, of the tree branch nodes >70% are indicated. Isolates sequenced in this study are bolded and host species for *Chrysoporthe cubensis* are indicated in italics. *Cryphonectria parastica*, *Cryphonectria nitschkei* and *Cryphonectria macrospora* were defined as an outgroup.

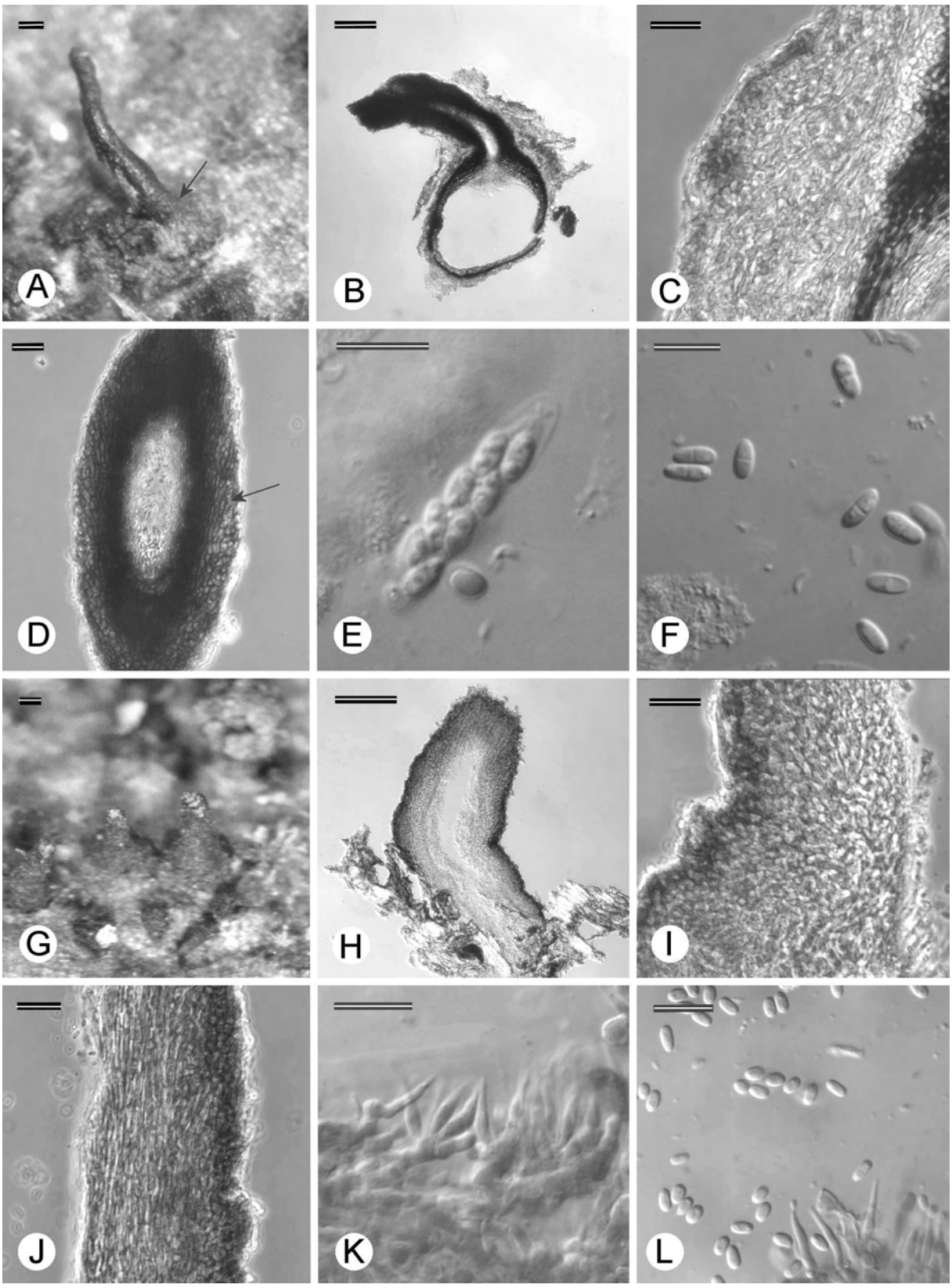

Fig. 4. Light micrographs of *Chrysoporthe inopina* from Colombia (from holotype PREM 58800). A. Black perithecial neck and orange stromatic tissue (arrow) of ascostroma on bark. B. Vertical section through ascoma. C. Stromatic tissue of ascostroma. D. Perithecial neck and surrounding tissue (arrow). E. Ascus. F. Ascospores. G. Conidiomata on bark. H. Vertical section through conidioma. I. Tissue of the conidiomal base. J. Tissue of conidiomal neck. K. Conidiophores. L. Conidia. Scale bars A–B, G–H = 100 µm; C–D, I–J = 20 µm; E–F, K–L = 10 µm.

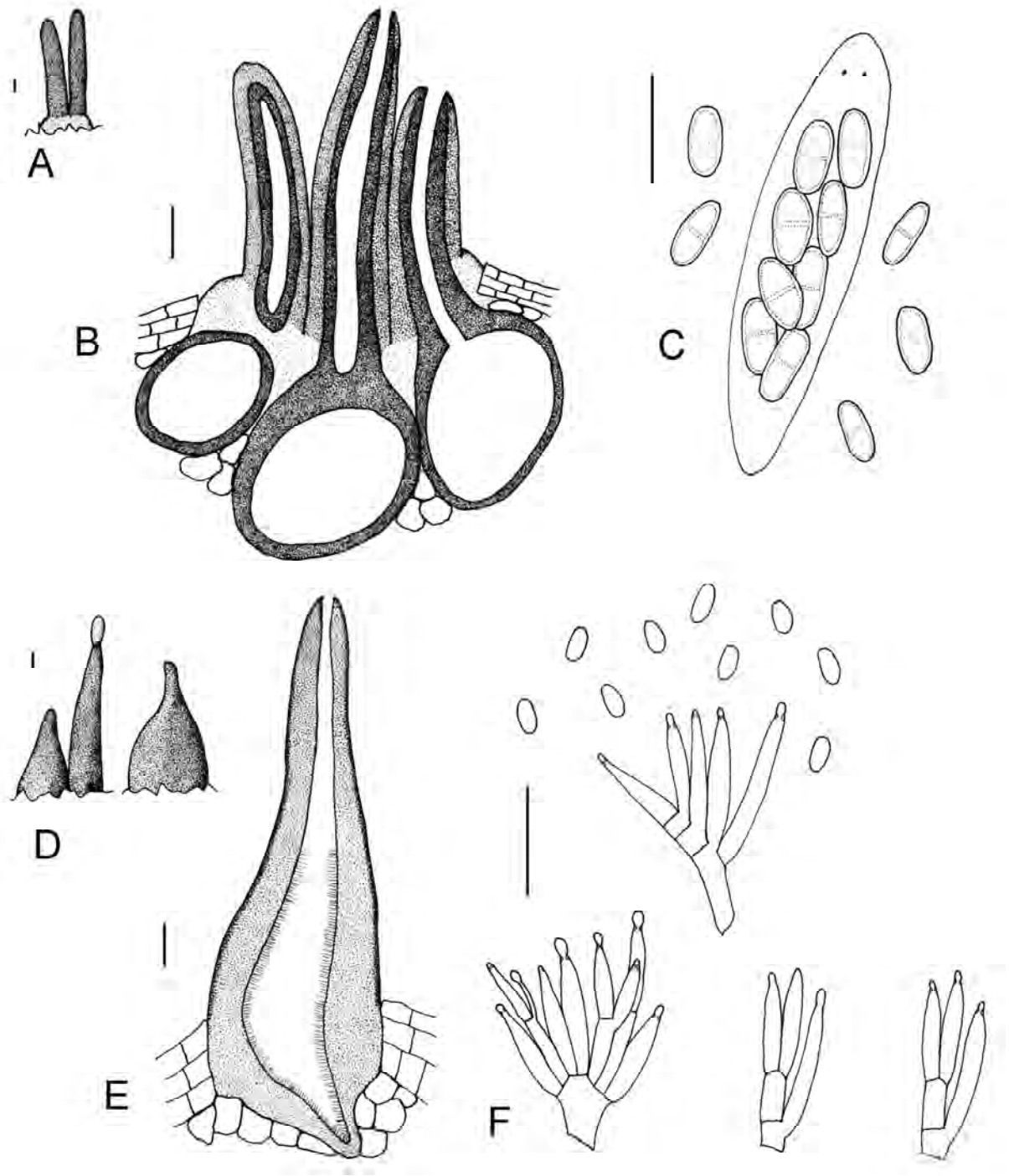

Fig. 5. Line drawings of *Chrysoporthe inopina* (from holotype PREM 58800). A. Shape of ascoma on bark. B. Section through ascoma. C. Asci and ascospores. D. Shapes of conidiomata on bark. E. Section through conidioma. F. Conidiophores, conidiogenous cells and conidia. Scale bars A–B, D–E = 100 µm; C, F = 10 µm.