CHAPTER 5

Phylogenetic relationships of *Cryphonectria* and *Endothia* species, based on DNA sequence data and morphology.

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PHYLOGENETIC RELATIONSHIPS OF *CRYPHONECTRIA* AND *ENDOTHIA* SPECIES, BASED ON DNA SEQUENCE DATA AND MORPHOLOGY.

ABSTRACT

The fungal genera *Endothia* and *Cryphonectria* include some of the most important pathogens of forest trees. Despite available new technology, no comprehensive comparative study based on DNA sequence data and morphology has been done on all available isolates representing *Cryphonectria* and *Endothia* species. The main objectives of this study were to assess the phylogenetic relationships among species of *Cryphonectria* and *Endothia* for which cultures are available and to establish a taxonomic framework based on DNA sequence and morphological data that will aid future studies and identification of species residing in these and related genera. Comparisons were based on sequence variation found in the ITS region of the ribosomal RNA operon and two regions of the β-tubulin gene. Besides comparing sequence data, the morphology of these species was also examined. The phylogenetic data indicated that *Endothia* and *Cryphonectria* reside in two distinct phylogenetic clades. *Cryphonectria parasitica*, *C. macrospora*, *C. nitschkei*, *C. eucalypti* and *C. radicalis* represented the *Cryphonectria* clade. *Endothia* was characterised by *E. gyrosa* and *E. singul*aris isolates. An isolate representing *E. viridistroma* grouped outside the *Endothia* clade and separately from other groups. Other clades outside that encompassing

Cryphonectria, were those represented by the *C. cubensis* isolates and fungi isolated from *Elaeocarpus dentatus* originating from New Zealand. These clades could be distinguished from *Endothia* and *Cryphonectria*, based on anamorph morphology, stromatal structure and ascospore septation. *Cryphonectria* and *Endothia*, therefore, appear to be paraphyletic and taxonomic relationships for these fungi need to be revised.

INTRODUCTION

The fungal genera *Cryphonectria* and *Endothia* (order Diaporthales) have relatively few members, but include some of the most serious pathogens of forest trees in the world. These genera have been subjected to several classical taxonomic treatments (Barr 1978, Hodges 1980, Kobayashi 1970, Roane et al. 1986a, Shear et al. 1917), most of which were based on morphological comparisons. Most important of these, is Barr's (1978) monograph on the Diaporthales, which had a fundamental and important impact on the taxonomy of *Endothia*. Prior to the work of Barr, *Cryphonectria* was synonymous with the older *Endothia* (Kobayashi 1970, Shear et al. 1917, Von Höhnel 1909). Barr (1978), however, segregated *Cryphonectria* from *Endothia* based on the differences in ascospore septation and stromatal morphology. Of the thirteen species originally treated in *Endothia*, only three were retained, i.e. *E. gyrosa* (Schw.: Fr.) Fr. (type species), *E. viridistroma* Wehm. and *E. singularis* (H. & B. Syd.) Shear & Stevens. The remaining species were transferred to *Cryphonectria* and these included the type species *C. gyrosa* (Berk. & Br.) Sacc. (= *E. tropicalis* Shear & Stevens), *C. cubensis* (Bruner) Hodges, *C. havanensis* (Bruner) Barr, *C. macrospora* (Kobayashi & Ito) Barr, *C. nitschkei* (Otth.) Barr, *C. parasitica* (Murr.) Barr and *C. radicalis* (Schw.: Fr.) Barr. Other already described species, *C. longirostris* (Earle) Micales & Stipes and *C. coccolobii* (Vizioli)

Micales & Stipes, were not mentioned by Barr (1978), but were placed in *Cryphonectria* by Micales and Stipes (1987) based on similarities in morphology with other species in *Cryphonectria*.

Of the species in *Endothia* and *Cryphonectria* only *E. gyrosa*, *C. parasitica* and *C. cubensis* are known to be serious pathogens. The remaining members of these genera are considered saprophytic (Roane et al. 1986b). *Endothia gyrosa* causes cankers on various hardwood species in the USA and is known as the causal agent of pin oak (*Quercus palustris* Muench.) blight (Appel and Stipes 1986, Roane et al. 1974, Snow et al. 1974, Stipes and Phipps 1971). *Cryphonectria parasitica* is well-known for the devastation that it has caused to the American chestnut, *Castanea dentata* Borkh. (Anagnostakis 1987, Heiniger and Rigling 1994). This fungal pathogen has destroyed the American chestnut as a major forest tree and has resulted in significant change to the ecology of the eastern hardwood forests (Anagnostakis 1987). *Cryphonectria cubensis* is another important pathogen that causes a serious canker disease of plantation *Eucalyptus* species in tropical and subtropical areas of the world (Hodges et al. 1976, Hodges et al. 1979, Sharma et al. 1985a, b, Wingfield et al. 1989). This fungus is recognised as the causal agent of die-back on clove (*Syzygium aromaticum* (L.) Murr. & Perry) (Myrtaceae) (Hodges et al. 1986) and a serious canker disease of *Tibouchina* species (Melastomataceae) (Myburg et al. 2002b, Wingfield et al. 2001). *Cryphonectria eucalypti* is a canker pathogen of *Eucalyptus* trees and occurs in South Africa (Gryzenhout et al. 2003, Van der Westhuizen et al. 1993) and Australia (Old et al. 1986, Walker et al. 1985, Yuan and Mohammed 1997). This pathogen was previously known as *E. gyrosa*, but was found to represent a distinct and new species (Venter et al. 2001, 2002).

Morphologically, *Endothia* is characterised by strongly developed, widely erumpent stromata with predominantly pseudoparenchymatous tissue (Barr 1978, Micales and Stipes 1987). Perithecia are usually born in an upright, diatrypoid configuration (Barr 1978, Micales and Stipes 1987). In contrast, the stromata of *Cryphonectria* are semi-immersed in the bark and not as strongly developed as those of *Endothia* (Barr 1978, Micales and Stipes 1987). Stromatic tissue is predominantly prosenchymatous and the perithecia are often forced into a valsoid configuration by surrounding bark tissue (Barr 1978, Micales and Stipes 1987). Furthermore, *Cryphonectria* is distinguished by fusoid to ellipsoid, one-septate ascospores, while *Endothia* has cylindrical to allantoid, aseptate ascospores (Barr 1978, Micales and Stipes 1987). The phylogenetic studies of Venter et al. (2002), however, showed that ascospore septation is not a valid character at generic identification, and that stromatal morphology is more useful.

It is difficult to distinguish between species of *Endothia* and *Cryphonectria* based on morphology. Distinction is mainly restricted to size differences in fruiting structures (Roane 1986a, Kobayashi 1970). No method has been developed to distinguish unequivocally between all species of the two genera. Pigment production (Roane and Stipes 1978), disc electrophoresis of intramycelial enzymes (Stipes et al. 1982), tolerance to antibiotics (Micales and Stipes 1986) and optimal temperatures for growth (Stipes and Ratliff 1973) could only be used to distinguish between some species, especially *C. parasitica* and *E. gyrosa*.

Myburg et al. (1999) provided the first phylogenetic data on representatives of *Endothia* and *Cryphonectria*. The aim was to resolve taxonomic questions pertaining to *C. cubensis*. This study supported the conspecificity of *E. eugeniae* with *C. cubensis* and

showed clearly that *C. parasitica* is different from *E. gyrosa*. *Cryphonectria cubensis* isolates were also found to reside in two well-resolved sub-clades, reflecting a South American and a Southeast Asian group. The study of Myburg et al. (1999) was based on sequence variation within the ITS1 and ITS2 regions of the ribosomal RNA operon. A third sub-clade including isolates from South Africa, was recognised when β-tubulin and histone *H3* gene sequences were used in phylogenetic analyses (Myburg et al. 2002a).

In a phylogenetic study conducted by Venter et al. (2002), additional species of *Cryphonectria* and *Endothia* were included to examine the generic placement of the new species, *C. eucalypti*. In this study, isolates representing *C. parasitica*, *C. radicalis*, *C. macrospora*, *E. gyrosa* and *E. singularis* were included. *Endothia* and *Cryphonectria* grouped as two distinct phylogenetic clades. Only a subset of isolates currently available to us was used in the study of Venter et al. (2002). No comprehensive study including morphology and phylogenetic data has thus been undertaken on all available isolates representing the different species of these two genera. The objective of the present study was, therefore, to compare all available species of *Endothia* and *Cryphonectria* for which cultures and vouchered specimens exist. More specifically, our aim was to re-evaluate the generic distinctions between *Cryphonectria* and *Endothia* and thus to provide a taxonomic basis, based on DNA sequence data and morphological characteristics, for future studies of species assemblages residing in them.

MATERIALS AND METHODS

Isolates studied

Isolates included in this study (Table 1) represent most of the species retained in the genera *Cryphonectria* and *Endothia* (Barr 1978, Micales and Stipes 1987). A number of these isolates were obtained from the culture collection of R.J. Stipes. Authentic cultures are not available for *C. coccolobii*, *C. longirostris* or *C. havanensis* and these could not be included in this study. The isolate labelled as E40 (CMW 10453) in the collection of R.J. Stipes was previously found to be *C. cubensis* (Micales et al. 1987) and not representative of *C. havanensis* from Cuba. Isolates of *C. eucalypti*, the most recent addition to *Cryphonectria* (Venter et al. 2002), were also included in this study. All isolates (Table 1) are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and a duplicate set of sub-cultures have been deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

DNA extractions, ribosomal RNA (ITS1, 5.8S, ITS2) and β-tubulin gene amplification

DNA was extracted as described by Myburg et al. (1999). Amplification of the ITS 1, 5.8S and ITS 2 regions of the ribosomal RNA operon as well as two regions within the β-tubulin gene were as described by Myburg et al. (1999) and Myburg et al. (2002a) respectively. The primer pairs that were used to amplify the respective regions were the following: ITS1 and ITS 4 (White et al. 1990), Bt1a and Bt1b (Glass and Donaldson

1995) and Bt2a and Bt2b (Glass and Donaldson 1995). PCR products were purified using a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany).

Ribosomal RNA (ITS1, 5.8S, ITS2) and β-tubulin gene sequencing

PCR products were sequenced in both directions using the same primer pairs that were used in the amplification reactions. Sequencing reactions were achieved using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin-Elmer, Warrington, United Kingdom). The nucleotide sequences were determined with an ABI PRISM 3100™ automated DNA sequencer.

Sequence alignment and analyses

Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems, Inc., Foster City, California) software was used to analyse the DNA sequences. All sequences generated were aligned in a data matrix using CLUSTAL X (Thompson et al. 1997) and the alignment was checked manually. Sections of the ITS and the β-tubulin introns were highly variable. This resulted in difficulty when aligning sequence data. An analysis (data not shown) of only the exon regions of the β-tubulin gene produced a phylogenetic tree with a similar topology to that obtained when full data set was considered collectively. However, there was no resolution at the intraspecific level using this more conservative data set and all the sequence data were thus retained in the analyses, where gaps were treated as missing data.

Subsequent phylogenetic analyses were performed using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b (Swofford 1998). A 500 replicate partition-homogeneity test (PHT) was executed to determine if the ribosomal (ITS1, 5.8S, ITS2) and β-tubulin gene sequence data sets (1a/1b, 2a/2b) could be combined as one data set prior to phylogenetic analyses. Gene sequences were analysed using heuristic searches with tree-bisection-reconnection (TBR) and MULTREES options (saving all optimal trees) effective. The confidence levels of the tree branch nodes generated in the phylogenetic analysis were determined by a 1000 replicate bootstrap analysis. *Diaporthe ambigua* Nitschke, a known canker pathogen of stone and pome fruit trees (Smit et al. 1996, 1997) was included as outgroup taxon to root the phylogenetic tree. Sequences were deposited in Genbank and the accession numbers are listed in TABLE I. The sequence alignments and phylogenetic tree (FIG. 1) were deposited in TreeBase (submission ID number = SN 1205).

Morphological studies

General morphological features such as stromatal and spore morphology, were examined microscopically for relevant herbarium specimens of *Endothia* and *Cryphonectria* (Table 2). As far as possible, the type specimens of the different species in the phylogenetic tree were studied. Specimens from New Zealand, linked to some of the isolates used in the phylogenetic study (Table 2), were also included.

Fruiting structures were embedded in Leica mountant (Setpoint Premier, Johannesburg, South Africa) after rehydration in boiling water for 1 min. Sections were made with a Leica CM1100 cryostat (Setpoint Premier) at –20 C and were 12-16 μm thick. Sections

were dropped in water, transferred to a microscope slide, mounted in lacto-phenol and examined using phase contrast and differential interference contrast light microscopy.

RESULTS

Ribosomal RNA (ITS1, 5.8S, ITS2) and β-tubulin gene amplification and sequencing

Amplification products for the respective gene regions were between 550bp and 600bp in size (data not shown). Sequences for isolates generated in this study were aligned with sequence data from previous studies (Table 1). The PHT performed between the ribosomal and β-tubulin gene sequence data sets generated a P-value of 0.01. This indicated that there was no significant conflict between the data sets and that they could be combined in subsequent phylogenetic analyses. The ribosomal (ITS1, 5.8S, ITS2) and β-tubulin (1a/1b and 2a/2b) sequence data sets were thus analysed together in the parsimony analyses.

The resulting combined data set comprised of 28 sequences of which one, *D. ambigua*, was used as the outgroup taxon. Manual alignment of the combined β-tubulin gene and ribosomal DNA sequence data resulted in a total of 1510 characters (Appendix 4). Of these 932 characters were constant, 132 variable characters were parsimony uninformative and 446 variable characters were parsimony informative. No sequence characters were excluded. The heuristic search produced twenty-three trees, which were converted to a strict consensus tree (tree length = 1154 steps, consistency index/CI = 0.6888, retention index/RI = 0.8376).

The consensus tree (Fig. 1) showed a well-resolved clade labelled as "*Cryphonectria* spp." and representing the taxa *C. parasitica, C. nitschkei, C. macrospora, C. eucalypti* and *C. radicalis* from Europe. Isolates of *C. radicalis* formed two distinct groups. One is represented by isolates CMW 10477 and CMW 10455 while the other is represented by isolate CMW 10484 and an isolate from *Quercus* identified as *Endothiella gyrosa* Sacc. (CMW 10436) (bootstrap value = 100%).

Two groups of isolates originally identified as species of *Cryphonectria* did not group within the main *Cryphonectria* clade. The first of these included unidentified isolates from New Zealand, which were originally labelled *C. radicalis* (CMW 10469, CMW 10470) and *C. gyrosa* (CMW 10471) (bootstrap support = 100%). These isolates originated from *Elaeocarpus dentatus* Vahl.

The second group of isolates that clustered outside the *Cryphonectria* clade were those representing *C. cubensis* (bootstrap = 100%). Within this *C. cubensis sensu lato* clade, the three sub-clades as previously defined by Myburg et al. (2002a), were evident and represented *C. cubensis* originating from South America/Congo, Southeast Asia and South Africa, respectively.

Endothia was represented by *E. gyrosa* and *E. singularis* isolates from the USA. The remaining *Endothia* species, *E. viridistroma*, grouped separately from the *Endothia* and *Cryphonectria* clades. A BLAST search on the ITS sequence data generated for the *E. viridistroma* isolate revealed that it has a 97% similarity to *Cytospora eucalypticola* Van der Westh. (Genbank Accession number = AF192321, BLAST result = 835 bits), a weak

pathogen of *Eucalyptus* in Australia and South Africa (Old et al. 1986, Van der Westhuizen 1965).

Morphological studies

The different groups found in the phylogenetic tree could be distinguished based on morphology (Fig. 2). The most important distinguishing character was anamorph morphology. The conidiomata of *E. gyrosa* (Fig. 2a) and *E. singularis* (Fig. 2b) were tuberculate and locules were numerous and minute, while those of the *Cryphonectria* spp. (i.e. *C. parasitica*, *C. radicalis* from Europe, *C. nitschkei*, *C. macrospora*) (Fig. 2c) were pulvinate and locules were few in number and large. Those of *C. eucalypti* (Fig. 2d) were similar to those of the other *Cryphonectria* spp. (Fig. 2c). The conidiomata on the specimens of *C. gyrosa* and *C. radicalis* from New Zealand (Fig. 2e) were unique, since single conidiomata were ovoid, superficial and unilocular. More complex conidiomatal structures on the New Zealand specimens (containing more than one ovoid structure) were multilocular with irregular conidial locules (Fig. 2e). Conidiomata of *C. cubensis* (Fig. 2f) were also different and were convoluted, generally unilocular, superficial and pycnidia-like. The conidiomata of *C. cubensis* were blackened, unlike the orange conidiomata of the other species of *Cryphonectria* and *Endothia*.

The ascomata of *E. gyrosa* (Fig. 2a), *E. singularis* (Fig. 2b) and the New Zealand specimens (Fig. 2e) were erumpent, strongly developed and superficial. Perithecia were diatrypoid and the bases situated in fungal tissue above the level of the bark. The ascomata of the *Cryphonectria* spp. (Fig. 2c) and *C. eucalypti* (Fig. 2d) were also erumpent, but were semi-immersed and perithecia were valsoid, with the bases

surrounded by bark tissue beneath the level of the bark. Ascomata of *C. cubensis* specimens (Fig. 2f) had weak to no stromatal tissue development, and the protruding necks of the perithecia were covered with brown tissue, which was different to the orange necks of the other specimens of *Cryphonectria*.

Ascospores of *E. gyrosa* (Fig. 2a) and *E. singularis* (Fig. 2b) were aseptate, cylindrical to allantoid. Those of the *Cryphonectria* spp. (Fig. 2c) and *C. cubensis* (Fig. 2f) were one-septate, ellipsoid to fusoid. The specimens (K 109807, K 109809, BPI 614797, BPI 614526, BPI 797701) connected to the type species of *Cryphonectria*, *C. gyrosa* from Sri Lanka, also had one septate ascospores. *Cryphonectria eucalypti* (FIG. 2d) had aseptate, cylindrical to allantoid ascospores that were different to those of other *Cryphonectria* species, and more similar to those of *Endothia* species. For specimens representing the New Zealand clade (Fig. 2e), ascospores were different to those of *Cryphonectria* species and *C. cubensis* in having 1-3 septa. Conidia of *E. gyrosa* (Fig. 2a), *E. singularis* (Fig. 2b), the *Cryphonectria* species (Fig. 2c), *C. eucalypti* (Fig. 2d) and the specimens from New Zealand (Fig. 2e) were aseptate, minute and cylindrical. The conidia of *C. cubensis* (Fig. 2f) differed from those of the others in being more oval than cylindrical.

Features of *E. viridistroma* that led to its placement in *Endothia* are the large, erumpent, tuberculate, superficial stromata (Fig. 2g). Perithecia have a similar orientation to those of *E. gyrosa* (Fig. 2g) and conidial locules are numerous and irregular to ellipsoid (Fig. 2g) (Wehmeyer 1936). Ascospores are aseptate and allantoid to slightly ellipsoid, and conidia are aseptate, cylindrical to allantoid (Fig. 2g) (Wehmeyer 1936). *Endothia viridistroma* is, however, atypical of all the other species of *Endothia* and *Cryphonectria* since the stromata of this species has a dark green exterior and green interior.

DISCUSSION

This study presents a phylogenetic analysis of a large group of isolates that were identified in *Cryphonectria* and the closely related genus *Endothia*. The majority of these are linked to vouchered specimens representing different species of *Cryphonectria* and *Endothia*, e.g. *C. parasitica*, *C. radicalis* (Europe), *C. nitschkei*, *C. macrospora*, *C. eucalypti*, *E. gyrosa* and *E. singularis*. Sequence and morphological data provide evidence that *Endothia* and *Cryphonectria* represent separate genera. These data also show that *C. cubensis* should be excluded from *Cryphonectria*. Similarly, taxa labelled as *Cryphonectria* spp., occurring on *Elaeocarpus dentatus* from New Zealand, do not reside in *Cryphonectria* and the taxon representing *E. viridistroma* does not belong in *Endothia*. The sequence and morphological data that are now available should facilitate future segregation among species in these genera. Studies including more specimens and isolates for each phylogenetic group should now be undertaken to formally describe the new generic groups proposed in this study.

To the best of our knowledge, all species for which cultures are currently available were included in this study. It is unfortunate that isolates of *C. longirostris*, *C. coccolobii* and an authentic isolate of *C. havanensis* from Cuba, the described origin of *C. havanensis* (Bruner 1916), are unavailable. An isolate (CMW 10471) that was originally identified as *C. gyrosa*, isolated from specimen PDD 32619, was phylogenetically and morphologically linked to the New Zealand group studied. Specimens in this group had morphological characteristics atypical of the type species of *Cryphonectria* and other *Cryphonectria* spp. The latter two groups have one-septate ascospores (with the exception of *C. eucalypti*), while the specimens linked to the New Zealand clade are

characterised by one to three septate ascospores. We were, therefore, not able to use isolate CMW 10471 as a representative of *C. gyrosa*, which is the type species of *Cryphonectria*.

Numerous challenges exist regarding the type species of *Endothia* and *Cryphonectria*. *Endothia gyrosa* (Fries 1849) and *C. gyrosa* (Berkeley and Broome 1875) were described in the 1800's and no cultures exist that can be linked to these names. The type specimen of *E. gyrosa* has been separated and moved among a number of herbarium collections in the past (Shear et al. 1917). The only remaining fragment of the original type material of *E. gyrosa*, which is designated as a co-type, contains only conidiomata (Shear et al. 1917). Comparisons of teleomorph morphology using the current collections are thus impossible.

In the case of *C. gyrosa*, ambiguities include the existence of two sets of herbarium specimens that are linked to this fungus. The first (K 109807, K 109809) is connected to the original description of *C. gyrosa* (basionym *Diatrype gyrosa* Berk & Br.) from Sri Lanka and the host was specified only as "sticks". Shear et al. (1917) obtained an alternative set of specimens, presumably of the same fungus, from Sri Lanka (BPI 614526, BPI 614797) on *Elaeocarpus glandulifer* Mast., when they transferred *C. gyrosa* to *E. tropicalis*. This was after *Cryphonectria* had been reduced to synonymy with *Endothia* (Von Höhnel 1909). These BPI specimens (BPI 614526, BPI 614797) were thus designated as the type specimens of *E. tropicalis* (Shear et al. 1917), while one of the original specimens connected to the 1875 description of *C. gyrosa* (K 109809, designated originally as number 290) was mentioned only as an additional collection examined (Shear et al. 1917).

In subsequent reviews on the taxonomy of *C. gyrosa* hosts other than *Elaeocarpus glandulifer* have been mentioned. These include an *Elaeagnus* sp. (Barr 1978), as well as *Elaeocarpus dentatus*, *Myrsine salicina* Heward, several *Quercus* spp., *Quintinia serrata* A. Cunn. and *Shiia sieboldii* Makino (Roane 1986a). The review of *C. gyrosa* by Barr (1978) was possibly based on specimen BPI 797701 that is stated to have originated from the original host *Elaeagnus glandulifer*, and collected from the same locality (i.e. Hakgala, Sri Lanka) as BPI 614526 and BPI 614797. There has, however, never been a plant species with the name *Elaeagnus glandulifer* (International Plant Name Index Query, http://www.ipni.org/ipni/query_ipni.html), and the host for the abovementioned material given on the herbarium packet, should probably have been *Elaeocarpus glandulifer* Mast. Mention of *C. gyrosa* on *Quercus* spp. and *S. sieboldii* (Roane 1986a), possibly originates in reports of this fungus from Japan (Kobayashi and Ito 1956, Kobayashi 1970). *Quintinia serrata* and *M. salicina* are, however, not mentioned as hosts of *C. gyrosa* in Japan (Kobayashi and Ito 1956, Kobayashi 1970), and the source of these reports is unclear. These contradictions regarding the appropriate type specimen for *C. gyrosa* need to be addressed and will probably rely upon new collections from the original collection sites.

Isolates from New Zealand considered in this study and labelled as *C. radicalis* and *C. gyrosa*, were not related to other *C. radicalis* isolates within the *Cryphonectria* clade. Nor did they resemble *C. gyrosa* specimens from Sri Lanka. These isolates from New Zealand were also not similar to the group accommodating *C. cubensis* or the phyloclade representing *Endothia*. The New Zealand isolates originated from *Elaeocarpus dentatus* and it is probable that the isolate labelled *C. gyrosa* (CMW 10471) was misidentified as

this species, because it also has large, erumpent, pulvinate stromata and originated from the *Eleaocarpus* (Roane 1986a, Shear et al. 1917). Ascospores of the New Zealand specimens labelled as *C. gyrosa* and *C. radicalis* specimens were, however, unusual in being two or three septate (Fig. 2e). They are, therefore, different from the type specimens connected to *C. gyrosa* from Sri Lanka and other specimens of *C. radicalis* that have two-celled ascospores. This feature, the ovoid anamorph structures and the grouping of these isolates separately from other *Cryphonectria* and *Endothia* isolates in the phylogenetic analysis, suggests that they most likely represent a discrete genus.

Our phylogenetic and morphological results provide added evidence that *C. cubensis* represents a distinct genus closely related to *Cryphonectria* and *Endothia*. Isolates of *C. cubensis* formed a distinct group separate from other *Cryphonectria* spp. The blackened, superficial to slightly immersed, pyriform bases with attenuated neck of the anamorph of *C. cubensis* (Bruner 1917, Hodges 1980, Myburg et al. 2002a) and reduced stromatic development and extending perithecial necks with dark brown tissue (Hodges 1980, Myburg et al. 2003), furthermore distinguishes *C. cubensis* from the *Cryphonectria* spp., the *Endothia* spp., the unidentified species occurring on *Elaeocarpus dentatus* from New Zealand and the type specimens of *C. gyrosa* from Sri Lanka.

The distinct morphology of *C. cubensis*, when compared with that of other *Cryphonectria* species, has led to uncertainty as to where *C. cubensis*, previously known as *Diaporthe cubensis* Bruner, should be placed (Bruner 1917, Hodges 1980). It has been suggested that *C. cubensis* could belong in the genus *Cryptodiaporthe*, with a *Cystosporella* anamorph (Roane 1986a). Recent phylogenetic studies based on LSU rDNA, however, showed that *C. cubensis* does not group with other *Cryptodiaporthe*

species (Castlebury et al. 2002, Zhang and Blackwell 2001). One species of *Cryptodiaporthe*, *C. corni* (Wehm.) Petr., however, did group close to *C. cubensis*, but was not representative of the genus *Cryptodiaporthe* (Castlebury et al. 2002, Zhang and Blackwell 2001).

Isolates labelled as *C. radicalis* from Europe formed two sub-groups within the greater *Cryphonectria* clade. The one sub-clade (CMW 10477, CMW 10455) presumably represents *C. radicalis*, but the identity of isolates in the other sub-clade (CMW 10436, CMW 10484) is unknown. *Cryphonectria radicalis* has been reported to occur widely in Europe (Anagnostakis 1983, Hoegger et al. 2002, Shear et al. 1917), and also in the USA (Shear et al. 1917) and Japan (Kobayashi 1970). Despite this fact, few isolates exist and it is reportedly difficult to find the fungus in the United States (M. Milgroom, personal communication). This might be due to its displacement by the virulent *C. parasitica* that was previously not present in its natural habitat (Anagnostakis 1983, Hoegger et al. 2002). An alternative hypothesis is that *C. radicalis* is not easily noticed due to the presence of the more commonly found and pathogenic *C. parasitica* (Hoegger et al. 2002). The correct taxonomic placement of isolates in the two sub-clades representing *C. radicalis* from Europe is currently impossible, since there are no herbarium specimens linked to European isolates of *C. radicalis*.

An isolate labelled as *Endothiella gyrosa* from Portugal (CMW 10436), grouped within the European *C. radicalis* clade. *Endothiella* is currently the recognized anamorph genus for both *Endothia* and *Cryphonectria* (Hawksworth et al. 1996). This specimen was isolated from *Quercus suber* L., the same host as that of the Italian *C. radicalis* isolates. We believe that this isolate was misidentified and should have been designated as *C.*

radicalis. This, however, illustrates the difficulty of identifying species of *Endothia* and *Cryphonectria,* in the absence of teleomorph specimens. Conidia of these two genera are similar and stromatal morphology of *Cryphonectria* species can sometimes be superficial and strongly developed, similar to those of *E. gyrosa*. This could be due to host tissue characteristics and environmental conditions (Cannon 1988, Hodges et al. 1986, Shear et al. 1917).

The ascospores of *C. eucalypti* differ from those of other *Cryphonectria* spp. in being aseptate. In this study, isolates of this fungus grouped together with *Cryphonectria* species and not close to those in the *Endothia* clade. This finding supports a previous report (Venter et al. 2002) that stromatal structure is an important taxonomic feature for this group of fungi. The aseptate ascospores of *C. eucalypti,* in contrast to septate ascospores of other *Cryphonectria* species, however, raise the question as to whether this fungus represents a distinct genus. At present, phylogenetic data are insufficient to support transferring *C. eucalypti* to a discrete genus.

The *E. viridistroma* specimens included in this study have green stromata (Roane 1986a, Wehmeyer 1936), which is unlike other species of *Endothia,* which have orange stromata (Barr 1978, Shear et al. 1917, Roane 1986a). Results of a BLAST search on the ITS ribosomal sequence data generated for this *E. viridistroma* isolate, showed sequence similarities with *Cytospora eucalypticola*. *Endothia viridistroma*, however, has large, widely erumpent, pulvinate stromata with diatrypoid perithecia (Roane 1986a, Wehmeyer 1936). This is in contrast to the immersed, typically valsoid, blackened stromata of *Valsa* species and their multilocular *Cytospora* anamorphs (Spielman 1984). It is, therefore, unlikely that *E. viridistroma* can be accommodated in *Valsa*. We believe

that the *E. viridistroma* isolate in our collection was misidentified and does not represent the fungus originally described as *E. viridistroma*. The taxonomic relationships of *E. viridistroma* will be difficult to resolve, since no other isolates of this species exist and herbarium specimens contain insufficient stromata for meaningful taxonomic study.

The results of this study reflect the importance of linking isolates to vouchered herbarium material in order to identify the defined taxa. The genera *Cryphonectria* and *Endothia* include very important fungal pathogens and it is essential that these species are correctly identified. One such example relates to *C. cubensis sensu lato*. Results of the present study and that of Myburg et al. (2002a) indicate that *C. cubensis sensu lato* includes isolates reflecting three geographically distinctive groups. Of these, the South African *C. cubensis* isolates are different from *C. cubensis* in other parts of the world and they are also more pathogenic (Myburg et al. 2002a, Roux et al. 2003). This discovery has important implications for the global security of *Eucalyptus* species, both in their native range and in countries where these trees are propagated t commercially. Further studies and possibly the development of rapid techniques to identify these fungi should thus be undertaken.

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Table 1. Isolates used in this study.

Isolate no.[a]	Additional numbers[b]	Original label name of taxon[c]	Host	Origin	Collector	Genbank Accession no.
CMW 2113	-	*Cryphonectria cubensis*	*Eucalyptus grandis*	South Africa	M.J. Wingfield	AF 046892, AF 273067, AF 273462
CMW 8755	-	*C. cubensis*	*Eucalyptus grandis*	South Africa	M.J. Wingfield	AF 292040, AF 273064, AF 273459
CMW 62	-	*C. cubensis*	*Eucalyptus grandis*	South Africa	M.J. Wingfield	AF 292041, AF 273063, AF 273458
CMW 1840	-	*C. cubensis*	*Eucalyptus camaldulensis*	China	unknown	AF 046890, AF 273071, AF 273466
CMW 1853	-	*C. cubensis*	*Syzygium aromaticum*	Brazil	unknown	AF 036891, AF 273070, AF 273465
CMW 8757	-	*C. cubensis*	*Eucalyptus*	Venezuela	M.J. Wingfield	AF 046897 AF 273069 AF 273464
CMW 8758	-	*C. cubensis*	*Eucalyptus*	Venezuela	M.J. Wingfield	AF 046898 AF 273068 AF 273463
CMW 8756	-	*C. cubensis*	*Eucalyptus*	Indonesia	M.J. Wingfield	AF 046896, AF 273077, AF 375606
CMW 2632	-	*C. cubensis*	*Eucalyptus marginata*	Australia	E. Davison	AF 046893, AF 273078, AF 375607
CMW 10453	E40, CBS 505.63	*C. havanensis*	*Eucalyptus saligna*	Congo	unknown	AY 063476, AY 063478, AY 063480
CMW 10463	E54	*C. macrospora*	*Castanopsis cuspidata*	Japan	T. Kobayashi	AF 368331, AF 368351, AF 368350

Table 1. (continued)

Isolate no.[a]	Additional numbers[b]	Original label name of taxon[c]	Host	Origin	Collector	Genbank Accession no.
CMW 10518	E53	*C. nitschkei*	*Quercus*	Japan	T. Kobayashi	AF 452118 AF 525706, AF 525713
CMW 1651	-	*C. parasitica*	*Castanea dentata*	USA	unknown	AF 046901, AF 273074, AF 273467
CMW 1652	-	*C. parasitica*	*Castanea dentata*	USA	unknown	AF 046902, AF 273075, AF 273468
CMW 10455	E42, CBS 238.54	*C. radicalis*	*Quercus suber*	Italy	A. Biraghi	AF 452113, AF 525705, AF 525712
CMW 10477	E76, CBS 240.54	*C. radicalis*	*Castanea sativa*	Italy	A. Biraghi	AF 368328, AF 368347, AF 368346
CMW 10484	E83, CBS 240.54	*C. radicalis*	*Castanea sativa*	Italy	A. Biraghi	AF 368327, AF 368349, AF 368349
CMW 10469	E67	*C. radicalis*	*Elaeocarpus dentatus*	New Zealand	G. Samuels	AF 452111, AF 525707, AF 525714
CMW 10470	E68	*C. radicalis*	*Elaeocarpus dentatus*	New Zealand	G. Samuels	AF 452112, AF 525708, AF 525715
CMW 10471	E70	*C. gyrosa*	*Elaeocarpus dentatus*	New Zealand	G. Samuels	AF 452116, AF 525709, AF 525716
CMW 7037	-	*C. eucalypti*	*Eucalyptus*	Australia	M.J. Wingfield	AF 232880, AF 368343, AF 368342
CMW 7036	-	*C. eucalypti*	*Eucalyptus*	South Africa	M.J. Wingfield	AF 232878, AF 368341, AF 368340

Table 1. (continued)

Isolate no.[a]	Additional numbers[b]	Original label name of taxon[c]	Host	Origin	Collector	Genbank Accession no.
CMW 10436	E14, CBS 165.30	*Endothiella gyrosa*	*Quercus suber*	Portugal	B. d'Oliveira	AF 452117, AF 525703, AF 525710
CMW 2091	E13	*Endothia gyrosa*	*Quercus palustris*	USA	R.J. Stipes	AF 046905, AF 368337, AF 368336
CMW 10442	-	*E. gyrosa*	*Quercus palustris*	USA	R.J. Stipes	AF 368326, AF 368339, AF 368338
CMW 10465	E58	*E. singularis*	unknown	USA	unknown	AF 368323, AF 368333, AF 368332
CMW 10454	E41, CBS 202.36	*E. viridistroma*	*Cercis canadensis* Castigl.	USA	J.H. Miller	AF 452120, AF 525704, AF 525711
CMW 2498	-	*Diaporthe ambigua*	*Malus sylvestris*	Netherlands	S. Truter	AF 046906, AF 273072, AF 273471

[a] Taxa presented in bold represent those for which sequences were generated in this study. Sequences for the other taxa were obtained from the previous studies of (Myburg et al. 1999, Myburg et al. 2002a, Roux et al. 2003, Venter et al. 2002). Isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa.

[b] Alternative numbers refer to those deposited in the Centraalbureau voor Schimmelcultures (**CBS**), P.O. Box 85167, 3508 AD Utrecht, The Netherlands and those from the culture collection (**E**) of Prof. R. J. Stipes now housed in the culture collection (CMW) of FABI (see a).

[c] Names of taxa are those on the original labels. As a result of this study "*C. havanensis*" (CMW 10453) now represents *C. cubensis* and "*C. radicalis*" (CMW 10469, CMW 10470), "*C. gyrosa*" (CMW 10471), "*Endothiella gyrosa*" (CMW 10436) and "*E. viridistroma*" (CMW 10454) unidentified taxa.

Table 2. Hebarium specimens examined in this study.

Herbarium number*	Current name of taxon	Original label name on specimen	Original host name on label	Origin	Collector	Date
BPI 631857	*Cryphonectria cubensis* (type)	*Diaporthe cubensis* Bruner	*Eucalyptus botryoides* Sm.	Cuba	S.C. Bruner	1916
PREM 57297	*C. cubensis*	*Cryphonectria cubensis*	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	2001
PREM 57294	*C. cubensis*	*C. cubensis*	*E. grandis*	Colombia	M.J. Wingfield	2000
PREM 57293	*C. cubensis*	*C. cubensis*	*E. grandis*	South Africa	M. Venter	2001
K 109807	*C. gyrosa* (type)	*Nectria gyrosa* (#638)	Bark	Sri Lanka	n.a.	1868
K 109809	*C. gyrosa*	n.a. (#290)	Bark	Mount Eliya, Sri Lanka	G.H.K. Thwaites	n.a.
BPI 614797	*C. gyrosa*	*E. tropicalis* (type)	*Elaeocarpus glandulifer* Mast.	Hakgala, Sri Lanka	T. Petch	1913
BPI 614526	*C. gyrosa*	*E. tropicalis* (type)	*Elaeocarpus glandulifer*	Hakgala, Sri Lanka	T. Petch	1913
BPI 797701	*C. gyrosa*	*E. tropicalis*	*Elaeagnus glandulifer*	Hakgala, Sri Lanka	n.a.	n.a.
PDD 32619[1]	*C. gyrosa*	*E. tropicalis*	*Elaeocarpus dentatus*	Auckland, New Zealand	G.J. Samuels	1973

Table 2. (continued)

Herbarium number*	Current name of taxon	Original label name on specimen	Original host name on label	Origin	Collector	Date
PDD 20056	*C. gyrosa*	*E. tropicalis*	*Elaeocarpus hookerianus* Raoul	Southland, New Zealand	J.M. Dingley	1948
PDD 21944	*C. gyrosa*	*E. tropicalis*	*Elaeocarpus dentatus*	Auckland, New Zealand	J.M. Dingley	1963
NYBG 31874 [2]	*C. radicalis*	*E. radicalis* (Schw.: Fr.) Ces. & de Not.	Dead tree	Auckland, New Zealand	R.E. Beever	1973
TFM 1057	*C. macrospora* (type)	*E. macrospora* Kobayashi & Ito (type)	*Shiia siebordii* Makino	Japan	T. Kobayashi	1954
TFM 1045	*C. nitschkei* (type)	*E. nitschkei* Otth	*Quercus grosseserrata* Bl.	Japan	T. Kobayashi	1954
CUP 2926	*C. parasitica*	*Diaporthe parasitica* Murrill	*Castanea dentata*	New York, USA	W.A. Murrill	1907
CUP 47983	*C. parasitica*	*E. parasitica* (Murrill) P.J. & H. W. Anderson	*Castanea dentata*	Md., USA	D.S. Welch	1938
BPI 797697	*C. radicalis*	*E. radicalis* (Schw.: Fr.) Fr.	*Castanea vesca*	Locarno, Switzerland	n.a.	1862
BPI 613739	*C. radicalis*	*E. fluens* (Sow.) Shear & Stevens	*Castanea vesca*	Stresa, Italy	C.L. Shear	1913

Table 2. (continued)

Herbarium number*	Current name of taxon	Original label name on specimen	Original host name on label	Origin	Collector	Date
PREM 56211	*C. eucalypti* (type)	*C. eucalypti* (type)	*Eucalyptus grandis* X *camaldulensis*	Nyalazi, South Africa	M. Venter	1998
PREM 56218	*E. gyrosa*	*E. gyrosa*	*Q. phellos* L.	Raleigh, USA	L. Grand	1997
BPI 614515	*E. singularis* (type)	*Calopactis singularis* Syd.	*Q. gambelli*	Colorado, USA	E. Bethel	1911
DAR 11235	*E. singularis*	*E. singularis*	*Q. gunnisonii*	Colorado, USA	G.G. Hedgcock and E. Bethel	1917
DAOM 3634	*E. viridistroma* (type)	*E. viridistroma* (type)	*Cercis canadensis*	Georgia, USA	J.H. Miller	1934
BPI 797702	*E. viridistroma*	*E. viridistroma*	*C. canadensis*	Georgia, USA	J.H. Miller	1934

***BPI**, U. S. National Fungus Collections, Systematic Botany and Mycology, Rm. 304, Bldg. 011A, 10300 Baltimore Avenue, Beltsville, MD 20705-2350, USA.

PREM, National Collection of Fungi, Pretoria, South Africa.

TFM, Forestry and Forest Products Research Institute, P. O. Box 16, Tsukuba Norin Kenkyu, Danchi-Nai, Ibaraki, 305 Japan.

CUP, Plant Pathology Herbarium, Cornell University, 334 Plant Science Building, Ithaca, New York 14853-4203 USA.

PDD, Landcare Research New Zealand Limited, Private Bag 92 170, 120 Mt. Albert Road, Mt. Albert, Auckland, New Zealand.

DAR, Plant Pathology Herbarium, Orange Agricultural Institute, Forest Road, Orange, N. S. W. 2800, Australia.

DAOM, National Mycological Herbarium, Eastern Cereal and Oilseed Center (ECORC), Agriculture and Agri-Food Canada, Edifice Wm. Saunders Building. #49, Ottawa, Ontario, Canada, KIA 0C6.

K, Herbarium, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3AE, England, U.K.

[1] Specimen linked to isolate CMW 10471 (Table 1).

[2] Specimen linked to isolates CMW 10469 and CMW 10470 (Table 1).

Fig. 1. A strict consensus tree (tree length = 1918 steps, CI = 0.7106, RI = 0.8703) generated from a combined data set comprising ribosomal and β-tubulin gene sequences. Confidence levels of the tree branch nodes (>50%) are indicated above the nodes and were determined by a 1000 replicate bootstrap analysis. Branch lengths are indicated below the nodes. *Diaporthe ambigua* was used as the outgroup taxon.

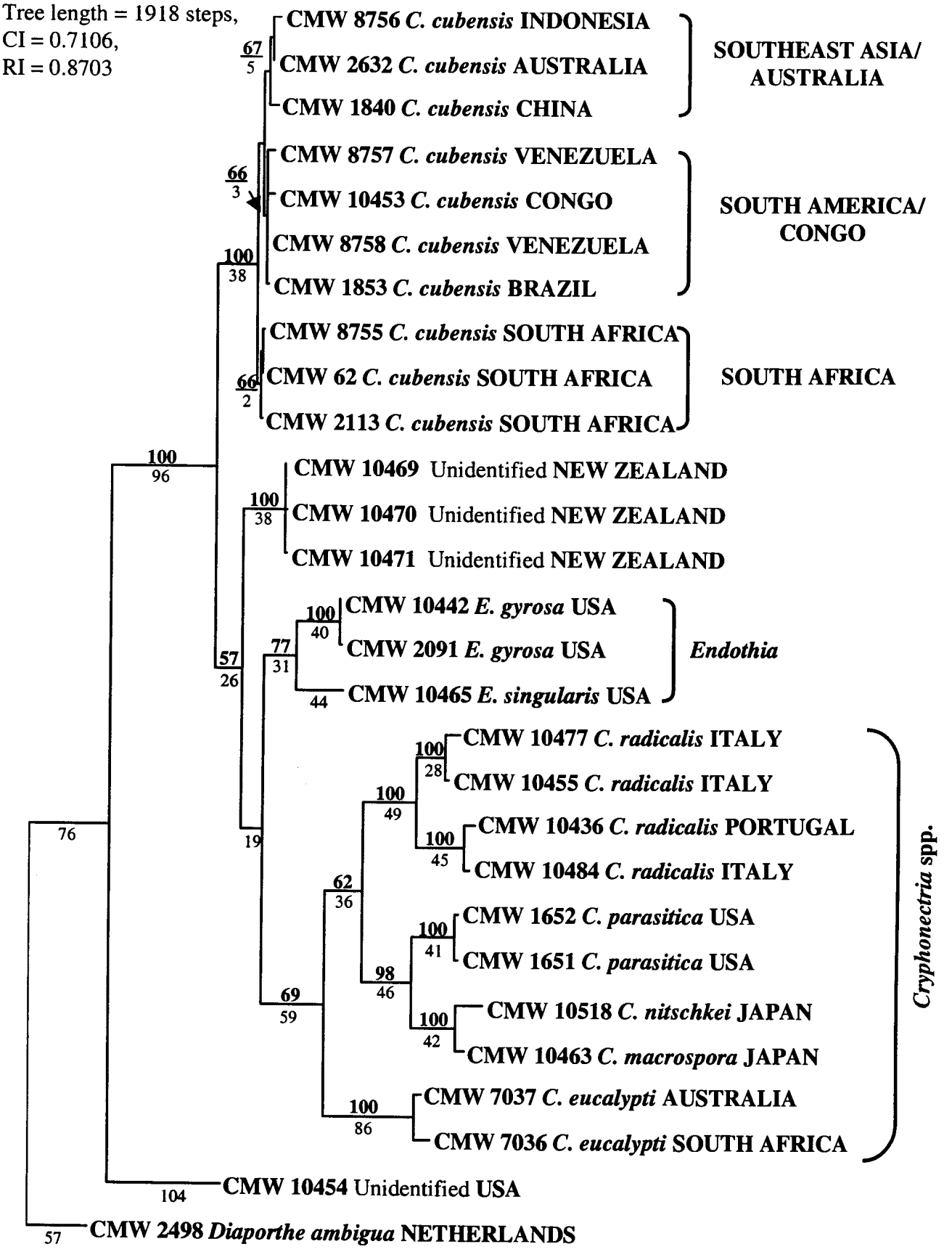
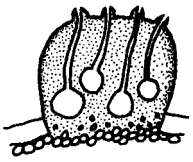
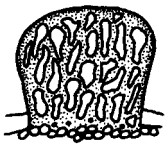
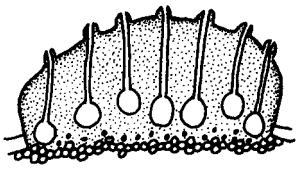
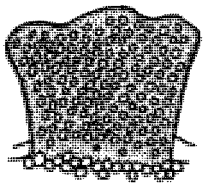
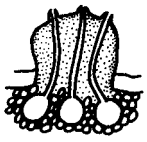
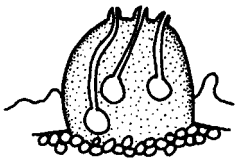
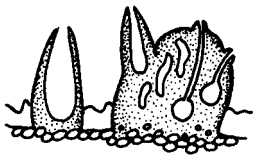
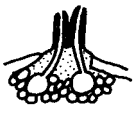
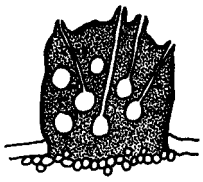


Fig. 2. Schematic drawings of the ascomata, conidiomata, ascopores and conidia of the fungi representing the different phylogenetic clades. **a.** *Endothia gyrosa*. **b.** *Endothia singularis*. **c.** *Cryphonectria* spp. representing *C. parasitica*, *C. radicalis* (Europe), *C. nitschkei*, *C. macrospora*. **d.** *Cryphonectria eucalypti*. **e.** Specimens labeled as *C. radicalis* and *C. gyrosa* from New Zealand. **f.** *Cryphonectria cubensis*. **g.** *Endothia viridistroma*.

	Ascomata	Conidiomata	Ascospores	Conidia
a) *Endothia gyrosa*				
b) *E. singularis*				
c) *Cryphonectria* spp.				
d) *C. eucalypti*				
e) **Unidentified fungus from New Zealand**				
f) *C. cubensis*				
g) *E. viridistroma*				

CHAPTER 6

DNA sequence data and morphology define *Cryphonectria* species on Fagaceae in Europe and Asia.

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DNA SEQUENCE DATA AND MORPHOLOGY DEFINE *CRYPHONECTRIA* SPECIES ON FAGACEAE IN EUROPE AND ASIA.

ABSTRACT

The genus *Cryphonectria* includes important tree pathogens as well as species believed to be saprophytes. While recent taxonomic studies have concentrated on North American and southern hemisphere *Cryphonectria* spp., little is known about Asian and European taxa found on *Castanea* and *Quercus*. A collection of isolates and specimens from woody hosts in Greece, Japan and China that could not be identified to the species level, has become available to us in recent years. In this study we identify these fungi and differentiate between the species occurring on *Quercus* and *Castanea* in Europe and Asia. Identification was achieved by morphological and phylogenetic comparisons between the unidentified fungi and established *Cryphonectria* and *Endothia* species. Phylogenetic comparisons were based on sequence data from the ribosomal ITS operon and two regions in the β-tubulin gene. Japanese and Chinese isolates showing different cultural features to those of *C. parasitica* from Japan and the USA, showed no sequence similarity with previously identified *C. parasitica* or *C. radicalis* isolates. They were related to *C. nitschkei* and *C. macrospora* from Japan, and specifically to an isolate assumed to be *C. havanensis* from

Japan. Specimens linked to these Japanese and Chinese isolates, had morphological features different from any of the Japanese herbarium specimens available to us, including specimens labelled as *C. havanensis* from Japan. A description and the name *C. clavata* are provided for this fungus. Furthermore, the fungal specimens in Japan that have been labelled as *C. havanensis* appeared to represent more than one undescribed species. Additional collections, more detailed morphological studies as well as phylogenetic analyses will be needed to fully resolve the identity of these fungi. Sequence and morphological data also suggested that there are two species currently representing *C. radicalis* in Europe. It is, however, not clear which of these represents the authentic *C. radicalis* and further investigations will be necessary to resolve this question.

INTRODUCTION

Cryphonectria and *Endothia* include fungal species that are both saprophytes and serious tree pathogens. A number of challenges exist when attempting to distinguish between these genera and species assigned to them. For example, the orange stromata of *Cryphonectria* and *Endothia* species are superficially similar. Additionally, differentiation of *Cryphonectria* and *Endothia* species is hindered by the fact that their ranges of spore size commonly overlap, and that ascospores for specimens are not always fully developed, resulting in variable measurements. Identification is further complicated when specimens or cultures do not produce spores. Taxonomic information is needed from both teleomorph and anamorph states because when only one morph is present, conclusive identification is difficult. Furthermore, fruiting structures, especially perithecia, are rarely produced in

culture, and cultural morphology is not sufficient to be used as the only means of identification.

Recent taxonomic studies, based on DNA sequence comparisons, have resolved a number of questions pertaining to the identification and differentiation of *Cryphonectria* and *Endothia* species (Myburg et al. 1999, Myburg et al. 2003, Venter et al. 2002). A comprehensive phylogenetic study on representative species of *Cryphonectria* and *Endothia*, for which cultures were available, indicated that these genera should be considered as separate taxonomic entities, even though they are closely related (Venter et al. 2002). However, studies such as those of Myburg et al. (1999), Myburg et al. (2003) and Venter et al. (2002) focussed primarily on species of *Cryphonectria* and *Endothia* that originated from the USA, Europe and countries in the Southern Hemisphere. Therefore, a similar study that focuses on the taxonomic and phylogenetic relationships of *Cryphonectria* and *Endothia* species with Asian origins is necessary.

The best-known species in *Cryphonectria* is *C. parasitica* (Murr.) Barr, the causal agent of chestnut blight, which practically eliminated the American chestnut (*Castanea dentata* Borkh.) during the last century (Anagnostakis 1987, Griffin 1986) after being introduced from eastern Asia, where it is native (Anagnostakis 1992, Milgroom et al. 1996, Shear and Stevens 1913, Shear and Stevens 1916). *Cryphonectria parasitica* also occurs on European chestnuts (*Castanea sativa* Mill.), although the disease has not been as severe as it has been in North America (Bazzigher and Miller 1991, Bissegger and Heiniger 1991, Heiniger and Rigling 1994). This is attributed to greater resistance in European chestnuts (Heiniger and

Rigling 1994, Metcalf 1908, Clapper 1952), differences in environmental conditions and the presence of naturally occurring hypovirulent *C. parasitica* strains in Europe (Grente 1965, Grente 1975, Heiniger and Rigling 1994).

Cryphonectria radicalis (Schw.: Fr.) Barr is a colonist of *Castanea* and *Quercus* species in the Northern Hemisphere (Anderson and Anderson 1912, Shear et al 1917). The fungus was first described in 1814 from England (Sowerby 1814) and later (1828) from the USA (Fries 1828, Shear et al. 1917). It was reported from southern Europe in 1863 (Shear et al. 1917) and from Japan in 1914 (Shear et al. 1917, Kobayashi 1970). *Cryphonectria radicalis* was, therefore, known in North America and Europe before *C. parasitica* was introduced. *Cryphonectria radicalis* has a special association with *C. parasitica* (Anagnostakis 1995, Hoegger et al. 2002) in that both species occur on the same host genera. Previous studies report species of *Castanea* and *Quercus* (Family: *Fagaceae*) as the most important hosts of *C. parasitica* and *C. radicalis* in Europe, North America (Shear et al. 1917, Roane 1986a) and eastern Asia (Kobayashi and Ito 1956, Kobayashi 1970). In a recent study aimed at isolating *C. parasitica* from dead chestnut stems in Switzerland, *C. radicalis* isolates were unintentionally collected (Hoegger et al. 2002). Identification of these isolates as *C. radicalis* was based on comparisons of morphology in culture, ascospore dimensions, mating behaviour and pathogenicity to chestnut plants.

In addition to *C. radicalis* and *C. parasitica*, *C. havanensis* (Bruner) Barr and *C. nitschkei* (Otth.) Barr have also been recorded on *Castanea* and *Quercus* spp. (Kobayashi and Ito 1956, Kobayashi 1970) making identification of the fungi on these hosts a challenge.

Furthermore, *Endothia singularis* (H. & B. Syd.) Shear and Stevens, a fungus with orange stromata typical of *Cryphonectria* species, but with aseptate ascospores, also occurs on *Castanea* and *Quercus* spp. in Japan (Kobayashi and Ito 1956, Kobayashi 1970) and a fungus reminiscent of *E. gyrosa* (Schw.: Fr.) Fr has been reported on *Quercus* from China (Teng 1934). Minor host species for this group of fungi in eastern Asia include *Castanopsis cuspidata* Schottky, a reported host for *Cryphonectria macrospora* (Kobayashi and Ito) Barr and *E. singularis* (Kobayashi 1970). Of the abovementioned species, only *E. gyrosa* is regarded as a pathogen, while *C. havanensis, C. nitschkei, C. macrospora* and *E. singularis* are considered opportunists or saprophytes (Roane et al. 1986, Kobayashi 1970 Shear et al. 1917).

A collection of isolates and specimens identified as *Cryphonectria* spp. and originating from *Quercus* and *Castanea* spp. in Greece, Japan and China form the basis of this study. Some of these isolates are those that Liu et al. (2003) treated in a recent study, which have not been described taxonomically. The objectives of this study were therefore to identify the isolates in the collection from Greece, Japan and China, to provide DNA sequence and morphological data to facilitate differentiation between the species occurring on *Quercus* and *Castanea* spp. in Europe and Asia, and to provide a full morphological description and name for the undescribed fungus mentioned in Lui et al. (2003).

MATERIALS AND METHODS

Collection of isolates and specimens

This study includes twenty-nine isolates (Table 1). Eight (CMW 10782 to CMW 10789) of these represented an unidentified fungus sampled from *Castanea* and *Quercus* spp. from Japan, China and Greece that produce less orange pigmentation than is characteristic of *C. parasitica*; three of these (CMW 10785 to CMW 10787) are from Liu et al. (2003). One Japanese isolate (CMW 10790) was morphologically similar to *C. parasitica*. Species of *Cryphonectria* and *Endothia* studied previously (Myburg et al. 2003, Venter et al. 2002) were included for comparative purposes. These are *C. parasitica* (CMW 1651, CMW 1652, CMW 10427, CMW 10431), *C. radicalis* (CMW, 10436, CMW 10455, CMW 10477), *C. macrospora* (CMW 10463), *E. gyrosa* (CMW 10442, CMW 2091) and *E. singularis* (CMW 10465). Four *C. radicalis* isolates (CMW 10791 to CMW 10794) from Europe recently studied by Hoegger et al. (2002) were incorporated as well as one isolate (CMW 11294) putatively identified as *C. havanensis* from Japan. Two *Diaporthe ambigua* Nitschke isolates (CMW 5288, CMW 5587) were included to serve as outgroup taxa in the phylogenetic analyses. Isolates of *C. radicalis* from Asia and North America are unfortunately not available and could not be included. All isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and duplicate cultures have been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

Herbarium specimens of the undescribed *Cryphonectria* species occurring in Japan, China and Europe (Table 2) were examined. Unfortunately only two of these specimens (FPH 7609, FPH 7610) are linked to isolates, i.e. CMW 10786 and CMW 10787. These specimens linked to the two isolates of unknown identity from *Castanea crenata* Sieb. & Succ. in Japan (Table 2) have been deposited in the herbarium of the Forestry and Forest Products Research Institute (FPH), P. O. Box 16, Tsukuba Norin Kenkyu, Danchi-Nai, Ibaraki, 305 Japan.

DNA isolations and amplification

DNA was isolated from cultures as described in Myburg et al. (1999). The ITS1 and ITS2 region of the ribosomal RNA operon, as well as the conserved 5.8S gene, were amplified using the primer set ITS1 and ITS4 (White et al. 1990). Two regions within the β-tubulin gene were amplified using primer pairs Bt1a with Bt1b and Bt2a with Bt2b (Glass and Donaldson 1995). The amplification reaction mixes, as well as the reaction conditions, were the same as those described in Myburg et al. (2002).

DNA sequencing and analyses

PCR products were sequenced in both directions using the same primer pairs that were used in the amplification reactions. Sequencing reactions were done 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 (Perkin Elmer, UK).

Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems, Inc., Foster City, California) software was used to edit the DNA sequences. The sequences were manually aligned with sequence data sets from previous studies (Venter et al. 2002, Myburg et al. 2002). Phylogenetic analyses were performed using PAUP (Phylogenetic Analysis Using Parsimony) software version 4.0b (Swofford 1998). The ribosomal DNA (ITS1, 5.8S, ITS2) and the β-tubulin sequence data sets were subjected to a Templeton nonparametric Wilcoxon Signed Ranked (WSR) test to assess whether the two data sets could be combined in the subsequent phylogenetic analyses (Kellogg et al. 1996). Phylogenetic analyses were done using heuristic searches with tree-bisection-reconnection (TBR) and MULTREES (saving all optimal trees) options effective. Gaps were treated as fifth characters (NEWSTATE) in the heuristic searches. The confidence levels of the branching nodes were determined by a bootstrap analysis (1000 replications). Sequence data for two *D. ambigua* isolates were used to root the phylogenetic tree. Sequences generated in this study have been deposited in GenBank and accession numbers are listed in Table 1, together with accession numbers of previously deposited sequences (Venter et al. 2002, Myburg et al. 2002, Myburg et al. 2003).

Morphological comparisons

Fruiting structures on herbarium specimens were cut from the bark and rehydrated for one min. in boiling water. The structures were sectioned at –20 °C with a Leica CM1100 cryostat after embedding in Leica mountant (Setpoint Premier, Johannesburg, South Africa). Sections, 12-16 µm thick, were mounted on microscope slides in lactophenol. Spores from the various specimens were measured in 3% KOH and lactophenol. At least twenty spores for each specimen were measured, but fifty measurements of ascospores, asci, conidia and conidiophores were made for the holotype specimen of the new species described here. The distribution of measurements (µm) for each specimen is expressed as the range and the mean (± standard deviation) using the notation: (min-)(mean-std. dev.) – (mean+std. dev.)(-max). A range of measurements was taken from two structures for the conidiomata and ascomata and used in the species description. Standard colour notations of Rayner (1970) were used.

Growth in culture of isolates CMW 10786 and CMW 10787, which are linked to specimens FPH 7609 and FPH 7610, respectively, and representing the new species described in this study (Tables 1, 2), was studied on MEA [20 g/l malt extract agar (Biolab)]. These cultures were grown in the dark at temperatures ranging from 10 °C to 30 °C at 5 °C intervals and evaluated using the technique described by Venter et al. (2002).

RESULTS

DNA sequencing and analyses

Amplification of the ITS1/ITS2 ribosomal RNA operon and β-tubulin gene regions resulted in PCR products of between 550bp and 600bp in size (data not shown). The Templeton nonparametric Wilcoxon Signed Ranked (WSR) test performed on the combined ribosomal DNA and β-tubulin sequence data sets indicated that the respective gene regions could be analysed as a combined data set in the subsequent PAUP analyses. A total of twenty-nine isolates were included in the combined data set with the two *D. ambigua* isolates serving as outgroup taxa. The combined data set consisted of a total of 1541 characters (Appendix 5), of which 929 were constant, 27 were parsimony-uninformative and 585 parsimony-informative. The heuristic search produced one most parsimonious tree (tree length= 1203 steps, consistency index = 0.77 and retention index = 0.92) (Fig. 1).

Cryphonectria and *Endothia* were resolved into distinct groups in the phylogenetic tree (Fig. 1). The *Cryphonectria* group includes three clades (Fig. 1). The first of these (**Clade 1**) includes *C. parasitica* isolates originating from the USA and Japan. **Clade 2** incorporates the unidentified fungus represented by the isolates (CMW 10782 to CMW 10787) from *Quercus* and *Castanea* spp. in China and Japan. A Japanese isolate (CMW 11294) previously assigned the name *C. havanensis*, grouped with the abovementioned isolates from *Quercus* and *Castanea* spp. This isolate and those of unknown identity are most closesly related to isolates of *C. nitschkei* and *C. macrospora* from Japan.

The third clade (**Clade 3**) in the *Cryphonectria* group included isolates that have been referred to as *C. radicalis* from Europe. The *C. radicalis* isolates from Italy (CMW 10455, CMW 10477, CMW 10791), Greece (CMW 10788, CMW 10789) and Switzerland (CMW 10792, CMW 10793, CMW 10794) showed a high degree of sequence similarity. The isolates from Switzerland are the *C. radicalis* isolates obtained from Hoegger et al. (2002). However, two isolates, CMW 10436 from Portugal and CMW 10484 from Italy, which had been identified as *C. radicalis* (Myburg et al. 2003), grouped separately from the isolates representing *C. radicalis* from Italy, Greece and Switzerland.

Morphological comparisons

Identification of the unknown *Cryphonectria* sp.

The morphology of the fungus represented by isolate CMW 10786 (annotated KB1 in Liu et al. 2003) and residing in **Clade 2** of the phylogenetic tree could be described since specimen FPH 7609 is linked to this isolate. In this fungus, ascospores were (8.5-)10-11.5-(-12.5) µm long, (3.5-)4-4.5(-5) µm wide and conidia were 4-5.5(-6) µm long, (1-)1.5(-2) µm wide (Table 3). Specimen FPH 7610, linked to isolate CMW 10787 (annotated CD28 in Liu et al. 2003), had slightly longer conidia [(4.5-)5-6.5(-7) µm long, 1.5 µm wide] (Fig. 3), but this isolate also grouped in **Clade 2** of the phylogenetic tree.

Of all of the species previously reported on woody hosts in Japan (Kobayashi and Ito 1956, Kobayashi 1970), the ascospore and conidial dimensions of the unidentified specimens

(FPH 7609, FPH 7610) most closely resembled those of *C. nitschkei* and *C. havanensis*, determined in this study (Table 3). Furthermore, both isolates CMW 10786 and CMW 10787 grouped closely with an isolate annotated as *C. havanensis* (CMW 11294) in **Clade 2** of the phylogenetic tree, but distinct from the isolate of *C. nitschkei* (Fig. 1). Unfortunately the isolate labelled as *C. havanensis* is not linked to herbarium material. Herbarium specimens, labeled *C. havanensis* in our collection, that were studied are likewise not connected to isolates currently in our collection and could thus not be included in the phylogenetic tree.

Specimens labeled as *C. havanensis* originated from fagaceous and non-fagaceous hosts. Two of these specimens from *Quercus* spp. [FPH 1203 from *Q. variabilis* Blume (Fig. 2a) and FPH 1047 from *Q. glandulifera* Blume (Fig. 2b)], specimen FPH 2300 from a *Betula* sp. (*Betulaceae*) (Fig. 2c) and specimen FPH 1270 from *Pyrus sinensis* Lindl. (*Rosaceae*) (Fig. 2d) had ascospores comparable in size (Table 3). These measurements were similar to those given by Kobayashi (1970), which was given as an average size of 8-12.5 x 3-4 µm. Specimen FPH 633 from *Eucalyptus globulus* Labill. (*Myrtaceae*) also had structures with ascospores similar to the other specimens labelled *C. havanensis* (designated as "A. *C. havanensis*" in Table 3, Fig. 2e). Smaller fruiting structures were, however, also found on specimen FPH 633 (designated as "B. *C. havanensis*" in Table 3) with ascospores (Fig. 2f) different [(6-)6.5-9(-11) µm long, 3-2.5 µm wide] from those of the other fruiting structures on the same specimen.

Specimen FPH 1047 was the only specimen labeled as *C. havanensis* that contained conidiomata. The conidia (Table 3) were comparable to measurements (3.5-4.2 µm long, 0.5-1 µm wide, 4 x 0.8 µm in average) given by Kobayashi (1970), and it was also similar to conidia of the unknown fungus. Phialide morphology, however, differentiated between the specimens of the unidentified fungus and the *C. havanensis* specimen from *Q. glandulifera* (FPH 1047). It also distinguished the unidentified fungus from *C. nitschkei* (FPH 1045). Conidiogenous cells of *C. nitschkei* in general had inflated bases with the apices of the cells strongly attenuated (Fig. 3a). Conidiogenous cells of the specimen (FPH 1047) annotated as *C. havanensis* did not have inflated bases, but were evenly attenuated (Fig. 3b). Conidiogenous cells of specimens of the unidentified fungus (FPH 7609 and FPH 7610) could be distinguished from the other two specimens by the inflated apical cells of the branched conidiophores were often inflated (Figs. 4i, 4j, 5e).

Taxonomy of the unknown *Cryphonectria* sp.

The unknown fungus found on *Quercus* spp. and *Castanea crenata* in Japan and China grouped separately from other species in the phylogenetic tree (Fig. 1). The close grouping of the isolate (CMW 11294) that has been referred to as *C. havanensis*, to isolates of the unknown fungus, is, however, not supported by morphological characteristics of the conidiogenous cells. Since the *C. havanensis* isolate (CMW 11294) is not connected to herbarium material, we propose that this isolate is not truly representative of *C. havanensis*, but is similar to the unknown fungus.

The unknown fungus could be distinguished from specimens of putative *C. havanensis* and *C. nitschkei*, the species that it resembled most closely, based on its clavate conidiogenous cells. One specimen (FPH 7610) linked to the unknown fungus also had longer conidia than any of the other Japanese species (Table 3). We, therefore, propose that the unknown fungus from *Castanea crenata* in Japan and *Quercus* spp. in China, characterised by isolates CMW 10786 and CMW 10787 and the corresponding herbarium material (FPH 7609, FPH 7610), represents a distinct and new species of *Cryphonectria*, different from the fungus in Japan identified as *C. havanensis* (Kobayashi and Ito 1956, Kobayashi 1970). The following description is provided for the fungus:

PLEASE NOTE THAT THE FOLLOWING SPECIES DESCRIPTION IS PRESENTED HERE IN PRELIMINARY FORM AND SHOULD NOT BE CITED. THE DESCRIPTION WILL BE SUBMITTED FOR PUBLICATION IN SCIENTIFIC LITERATURE.

Cryphonectria clavata M. Gryzenhout & M. J. Wingfield, prov.nom. – Figs. 4, 5.

Etym.: Latin, clavata, refers to the inflated apices of the conidiogenous cells that give the impression of a club.

Stromata semi-immersa, erumpentia, pulvinata, sphaerica vel elongata, aurantiaca. Bases peritheciorum saepe a textura corticis circumcinctae, parietibus atratis. Colla peritheciorum atrata, basi perithecii centralia, in superficie stromatis pro ostiolis nigris in papillis brevibus, textura stromatali aurantiaca tectis emergentia. Asci

fusiformes, solum immaturi stipitati, octospori. Ascosporae fusiformes vel ovatae, interdum subfalcatae, semel septatae, in septo cum aut sine strictura parva, extremis obtusis. Stromata anamorpha multilocularia convoluta, loculis saepe cum peritheciis dispositis. Conidiophora cylindrica vel basin versus bulbosa, apicibus attenuatis vel cellula apicali inflata, saepe septata, infra septo cum aut sine ramis lateralibus. Cellulae conidiogenae enteroblasticae, phialidicae, collariculo incrassatioque periclinali inconspicuo. Conidia cylindrica, interdum subfalcata, aseptata. Coloniae in MEA lanuginosae, margine integro vel crenato, albae conidiomatibus aurantiacis, catillum 90 mm diametro octo diebus tegentes, incremento optimo ad 25°C.

HOLOTYPUS: JAPONIA, in ditione "Yamanashi", loco dicto Kobuchizawa: in cortice Castaneae crenatae, Aprilio 1998, M. G. Milgroom et S. Kaneko, FPH 7609; cultura viva CMW 10786.

EPITYPUS: JAPONIA, in ditione Kyoto", loco dicto Chudai: in cortice *Castaneae crenatae*, Aprilio 1998, M. G. Milgroom et S. Kaneko, FPH 7610, cultura viva CMW 10787.

Stromata erumpent, pulvinate, spherical to elongated (Figs. 4a, 4g, 5a, 5d), 230 – 330 μm high, 250 – 1630 μm long and 210 – 1010 μm wide above the level of the bark, orange (colour 15). Ascomata stromatic, semi-immersed in bark, region above level of bark ectostromatic, lower region entostromatic containing host cells and perithecial bases (Figs. 4b, 5a), pseudoparenchymatous tissue at edge of stromata (Fig. 4c), prosenchymatous tissue in center (Fig. 4d). Perithecial bases 162 – 286 μm long, 160 – 379 μm wide, globose to sub-globose, dark-walled, up to 16 per stroma (Fig. 4b, 5a), perithecial walls 15 – 20 μm diam. Perithecial necks 55 – 72 μm wide, length depending on depth of perithecium in stroma, slender, dark, periphysate, with a central position on base of perithecium (Fig. 4b, 5a), necks emerging at the stromatal surface as black ostioles in short papillae covered with orange stromatal tissue (Fig. 4a, 5a), papillae 104 – 168 μm in diam. Asci (42-)44.5-50.5(-

56) μm long, 7-8.5(-9.5) μm wide, fusiform, numerous, floating freely in perithecial cavity, stipitate only when immature, unitunicate with non-amyloid, refractive apical rings; asci with eight ascospores (Fig. 4e, 5b). Ascospores (8.5-)9.5-11.5(-12.5) μm long, (3-)3.5-4.5(-5) μm wide, fusiform to oval, sometimes slightly curved, with or without slight constriction at septum, ends obtuse, hyaline, one septate (Figs. 4f, 5c).

Anamorphic stromata multilocular and convoluted, locules often occurring in same stroma that contains perithecia, conidia expelled through opening at stromatal surface (Figs. 4g, 4h, 5d). Conidiophores (5.5-)7.5-17(-24) μm long, (1-)1.5-2 μm wide, cylindrical or bulbous base, apices attenuated or inflated, often septated with or without lateral branches beneath septum, hyaline (Figs. 4i, 4j, 5f). Conidiogenous cells enteroblastic, phialidic, determinate, apical or lateral on branches, hyaline, collarette and periclinal thickening inconspicuous (Figs. 4i, 4j, 5f). Conidia (3.5-)4-5.5(-6.5) μm long, (1-)1.5(-2) μm wide, hyaline, cylindrical, occasionally slightly curved, hyaline, aseptate (Figs. 4k, 5g).

CULTURAL CHARACTERISTICS: Cultures on MEA fluffy with a smooth to crenate margin, white with orange (colour 15) conidiomata, covering a 90 mm plate after minimum of eight days, optimum temperature for growth 25 °C.

HOLOTYPE: JAPAN, Yamanashi Prefecture, Kobuchizawa: bark of *Castanea crenata*, April 1998, M. G. Milgroom and S. Kaneko, FPH 7609; living culture CMW 10786.

EPITYPE: JAPAN, Kyoto Prefecture, Chudai: bark of *Castanea crenata*, April 1998, M. G. Milgroom and S. Kaneko, FPH 7610, living culture CMW 10787. This herbarium

specimen was chosen as epitype because the holotype specimen contains very few anamorph structures.

SUBSTRATE: Bark of *Quercus* and *Castanea* spp.

DISTRIBUTION: China, Japan,

Distinction between *C. radicalis* groups in Europe

Based on ascospore size, two groups could be distinguished for European specimens labelled as *C. radicalis* (Table 3). This is in agreement with the phylogenetic analysis (Fig. 1) showing two distinct groups for *C. radicalis* isolates from Europe. Although no specific connections between groups defined by sequence data and spore morphology are possible because none of the specimens are linked to isolates in the phylogenetic tree, the first group of specimens (BPI 797697, BPI 613739, BPI 612672 and BPI 797693) (Table 2), originating from *Castanea sativa* in Italy and Switzerland, had ascospores (6-)7-8.5 µm long, (2-)2.5-3 µm wide (Table 3). These dimensions are similar to those given for the European *C. radicalis* isolates (CMW 10792, CMW 10793 and CMW 10794) by Hoegger et al. (2002), and to the Japanese *C. radicalis* specimens (Table 3). Conidiomata in this first group of European specimens also had pale luteous (colour 19d) cells lining the conidial locules, similar to Japanese *C. radicalis* specimens.

Specimens of *C. radicalis* from North America were similar to the group of *C. radicalis* specimens with smaller ascospores from Europe. Ascospore sizes for a specimen from a *Quercus* sp. (NYBG 1963) and another specimen (CUP 6178) fell within the size range

given for *C. radicalis* specimens from Japan and the European group with smaller ascospores (Table 3). Furthermore, cells giving rise to conidiophores were pale luteous in colour. These features were similar to those in Japanese and the European specimens with smaller ascospores. Conidial sizes for the North American *C. radicalis* specimens (CUP 6178, NYBG 2018) were comparable with those from the rest of the world (Table 3).

The second group of specimens from Europe labelled *C. radicalis* (BPI 797696, BPI 797692, BPI, 1112743, BPI 797698, BPI 612660; Table 2), originating from Italy, Abkhazia and France on *Castanea sativa*, a *Quercus* sp. and *Carpinus betulus* Linn, respectively, had longer ascospores than those of the first group, but were similar in width (Table 3). The ascospore sizes of the fungus residing in the second group did not resemble those of any other *Cryphonectria* species examined in this study (Table 3). Conidia were similar in size to the first group of *C. radicalis* from Europe with smaller ascospores (Table 3).

DISCUSSION

The present study has provided a set of DNA sequences for the ribosomal ITS region and two regions of the β-tubulin gene representing the majority of *Cryphonectria* species known to occur on woody host species in Europe and Asia. Analysis of sequence data and morphological comparisons shows that various *Cryphonectria* species occur on *Quercus* and *Castanea* species in Europe and Asia, including *C. parasitica* and *C. radicalis.* We also describe a new species of *Cryphonectria*, *C. clavata*, from Japan and China, which is phylogenetically distinct from the other *Cryphonectria* species. *Cryphonectria clavata* is

phylogenetically most closely related to *C. nitschkei* and *C. macrospora* and can be morphologically distinguished from *C. macrospora* by its smaller ascospores, and from *C. nitschkei* by its inflated apical conidiogenous cells. The combination of morphological and DNA sequence data presented in this study should aid future researchers in making correct identifications of *Cryphonectria* species found in Eurasia. This is particularly so given the difficulty of making firm identifications of these fungi based on morphology alone.

The newly described species, *C. clavata*, was mentioned in a recent study of interspecies transmission of hypoviruses (Liu et al. 2003). While sampling *C. parasitica* isolates in Japan, the authors recognised that their collections included another *Cryphonectria* species that produced less pigment in culture than *C. parasitica*, which in general were more orange. This unknown sympatric species, along with *C. parasitica*, also contained *Cryphonectria hypovirus 1* (CHV-1), which could be transmitted between the two species in culture. Liu et al. (2003) showed through DNA sequencing and RFLP data of the ribosomal ITS DNA region sequence, that CMW 10785, CMW 10786, CMW 10787 (isolates 09494, KB1 and CD28, respectively, in Lui et al. [2003]) of this unknown species grouped separately from their *C. parasitica* isolates. In our study, we have been able to show conclusively, by additional sequence data and morphological comparisons, that this unknown *Cryphonectria* species is new, and provided the name *C. clavata* for it. This confirmed the discovery by Liu et al. (2003) that the virus transmission they observed both in the laboratory and in nature was between different fungal species.

Other than *C. parasitica*, *C. radicalis*, *C. nitschkei*, *C. macrospora* and *C. clavata*, a fungus identified as *C. havanensis* was previously reported from various hosts in Japan (Kobayashi and Ito 1965, Kobayashi 1970). These hosts, including *Quercus* spp, a *Betula* sp., *P. sinensis* and *E. globulus*, are members of different plant families. Morphological comparisons of the specimens from these host species showed that spore sizes overlap with each other, although additional morphological criteria, such as conidiophore morphology, should be included to determine whether these different specimens really represent a single taxon. For example, based on ascospore size, it seems likely that two different species occur on *E. globulus* in Japan.

Apart from the fact that specimens from Japan identified as *C. havanensis* possibly represent more than one species, the name for Japanese specimens of *C. havanensis* also needs to be revised. *Cryphonectria havanensis* from Japan was at first thought to be two species (Kobayashi and Ito 1965, Kobayashi 1970). The first of these was a fungus on dead bark of *Eucalyptus globulus* annotated as *E. havanensis* Bruner (Kobayashi and Ito 1956). The second fungus occurred on fagaceous hosts in Japan and was identified as *Endothia tropicalis* Shear & Stevens (Kobayashi and Ito 1956). *Endothia tropicalis* is originally known from Sri Lanka (Berkeley and Broome, 1875, Shear et al. 1917) and is currently the type species of *Cryphonectria*, *C. gyrosa* (Barr 1978). Prior to the work of Barr (1978), Kobayashi (1970) reduced *E. tropicalis* to synonymy with *C. havanensis*, which was originally described from *Eucalyptus* spp. in Cuba (Bruner 1916). Hence Japanese specimens of *E. tropicalis* and *E. havanensis* were amalgamated under the single name *E. havanensis* (Kobayashi 1970). Although not the aim of this study, collections of these fungi

from Japan should be compared with the type specimens from Sri Lanka and Cuba, to establish the true identity of the specimens annotated as *C. havanensis* in Japan. Additional collections of *C. havanensis* from Cuba and the fungus from Japan that include sequences for cultures linked to specimens, should also be sought to fully resolve this question.

Hoegger et al. (2002) showed that *C. radicalis* occurs sympatrically with *C. parasitica.* Our results support this finding. Both our data and those of Hoegger et al. (2002) show that *C. radicalis* continues to exist in Asia, Europe and the USA, even though it is apparently not common. This species can be distinguished from *C. parasitica* based on ascospore length and width, although in the absence of a teleomorph it will be difficult to distinguish between the two species because conidial dimensions of *C. parasitica* and *C. radicalis* overlap. *Cryphonectria parasitica* also produces mycelial fans in the wood and these are not present in the case of *C. radicalis* (Roane 1986a, Shear et al. 1917). Another important distinguishing characteristic between the two species is that *C. radicalis* colours growth medium purple due to the production of a pigment known as endothine red, while *C. parasitica* does not produce this pigment (Hoegger et al. 2002,Roane 1986b, Roane and Stipes 1978, Shear et al. 1917).

Results of the present study and those of Myburg et al. (2003) show the presence of two groups within the fungus known as *C. radicalis* in Europe. These groups were defined independently based on DNA sequence data and morphology. It is, however, difficult to resolve whether the two groups found based on DNA sequence data, corresponds with the two groups distinguished based on morphology. This is because isolates used in the

phylogenetic analyses were not linked to any specimens in the morphological comparisons. It is, furthermore, difficult to deduct from previous studies which of the morphological groups in Europe corresponded most closely with published data for *C. radicalis*. Shear et al. (1917) made comparisons of various key specimens that included European and North American material. They obtained ascospore dimensions of 6-10 µm long, 3-4.5 µm wide for *C. radicalis* that encompassed both morphological groups in Europe identified in the present study. Shear et al. (1917) also observed that *C. radicalis* had highly variable ascospores, and it is possible that they were treating the two different species that we are now able to distinguish based on DNA sequence data, as a single species. The study of Hoegger et al. (2002), however, may present a possible link because ascospore dimensions given for the isolates from Hoegger et al. (2002), correspond with measurements of the one group of *C. radicalis* specimens from Europe and Japan that had ascospores (6-)7-8.5 µm long. The isolates from Hoegger et al. (2002) were included in this study and resided in the group that includes *C. radicalis* isolates from Greece and Italy. Unfortunately, no morphological data are available for the isolates in the second phylogenetic clade, incorporating CMW 10436 from Portugal and CMW 10484 from Italy.

The presence of two groups labelled as *C. radicalis* in Europe, makes it unclear which of the groups represents the true *C. radicalis* in Europe. The type specimen of *C. radicalis* has a North American origin (Fries 1828). The specimens from the USA examined in this study, had ascospore ranges that corresponded most closely with those of the group of *C. radicalis* specimens from Europe with smaller ascospores. Furthermore, North American specimens also had pale luteous linings to the conidial locules, similar to these European specimens.

However, in order to verify the identity of the two taxa that apparently represent the fungus known as *C. radicalis* in Europe, more detailed morphological comparisons of herbarium collections from Europe with those of the type specimen from North America will be necessary. More thorough studies are also needed including collections of North American *C. radicalis* specimens since Shear et al. (1917) mentioned a second form of *C. radicalis*, named *E. fluens* var. *mississippiensis*, existing in North America. There is thus the possibility that *C. radicalis* in North America represents different fungi. Unfortunately the type specimen of *C. radicalis* and other specimens of *C. radicalis* from the USA, are not linked to living isolates that we have been able to obtain. Numerous enquiries lead us to believe that these isolates do not exist and new collections will be needed to resolve the identity of *C. radicalis* in the USA.

A number of questions relating to *C. radicalis* still remain. We have no knowledge regarding the relatedness of the fungi known as *C. radicalis* from Europe, Japan and from North America. It would be interesting to determine the relationships of the different continental groups of *C. radicalis*, in order to establish whether *C. radicalis sensu lato* has been moved around the world through human involvement, as was the case with *C. parasitica*; or whether it is the member of *Cryphonectria* with the widest geographical distribution. The data presented in this study should aid future researchers in answering these questions and making correct identifications of *Cryphonectria* species found in Eurasia. This is particularly so given the difficulty of making firm identifications of these fungi based on morphology. We recommend comparisons of DNA sequences in addition to analysis of morphological criteria in order to characterise new collections of these fungi.

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Table 1. List of isolates included in this study[a].

Isolate number[b]	Alternative isolate numbers[d]	Species	Host	Origin	Collector	GenBank Accession numbers
CMW 10782	-	*Cryphonectria clavata*	*Quercus mongolica*	Japan	M. Kusunoki	AF 140242, AF 140248, AF 140254
CMW 10783	-	*C. clavata*	*Quercus mongolica*	Japan	M. Kusunoki	AF 140244, AF 140250, AF 140256
CMW 10784	-	*C. clavata*	*Quercus mongolica*	Japan	M. Kusunoki	AF 140245, AF 140249, AF 140257
CMW 10785	09494	*C. clavata*	*Quercus* sp.	China	M. Milgroom and S. Kaneko	AF 140246, AF 140252, AF 140258
CMW 10786	KB1	*C. clavata*	*Castanea crenata*	Japan	M. Milgroom and S. Kaneko	AF 140247, AF 140251, AF 140259
CMW 10787	CD28	*C. clavata*	*Castanea crenata*	Japan	M. Milgroom and S. Kaneko	AF 214212, AF 214214, AF 214216
CMW 11294[c]	E57	*C. clavata*	*Quercus grosserata*	Japan	T. Kobayashi	AY 214211, AY 214213, AY 214215
CMW 10788	D15	*C. radicalis*	*Quercus*	Greece	P. Cortesi	AY 143075, AY 143077, AY 143079
CMW 10789	D31	*C. radicalis*	*Quercus*	Greece	P. Cortesi	AY 143076, AY 143078, AY 143080
CMW 10790	-	*C. parasitica*	*Quercus serrata*	Japan	M. Kusunoki	AF 140243, AF 140253, AF 140255
CMW 10791	M 285	*C. radicalis*	*Quercus suber*	Italy	M. Orsenigo	AF 548742, AF 548746, AF 548750
CMW 10792	M 2268	*C. radicalis*	*Castanea sativa* Mill.	Switzerland	U. Heiniger	AF 548743, AF 548747, AF 548751

Table 1. (continued)

Isolate number[b]	Alternative isolate numbers[d]	Species	Host	Origin	Collector	GenBank Accession numbers
CMW 10793	M 2269	*C. radicalis*	***Castanea sativa***	Switzerland	U. Heiniger	AF 548744, AF 548748, AF 548752
CMW 10794	M 2270	*C. radicalis*	*Castanea sativa*	Switzerland	U. Heiniger	AF 548745, AF 548749, AF 5487503
CMW 10427	ATCC 48197	*C. parasitica*	*Quercus virginiana* Mill.	USA	R.D. Wolfe	AF 368329, AF 273073, AF 273469
CMW 10431	ATCC 48198	*C. parasitica*	*Quercus virginiana*	USA	F.F. Lombard	AF 368330, AF 273076, AF273470
CMW 1651	-	*C. parasitica*	*Castanea dentata*	USA	P.J. Bedker	AF 046901, AF 273074, AF 273467
CMW 1652	-	*C. parasitica*	*Castanea dentata*	USA	P.J. Bedker	AF 046902, AF 273075, AF 273468
CMW 10436	CBS 165.30	*C. radicalis*	*Quercus suber*	Portugal	B. d'Oliviera	AF 452117, AF 525703, AF 525710
CMW 10455	CBS 238.54	*C. radicalis*	*Castanea dentata*	Italy	A. Biraghi	AF 452113, AF 525705, AF 525712
CMW 10477	CBS 240.54	*C. radicalis*	*Quercus suber*	Italy	M. Orsenigo	AF 368328, AF 368347, AF 368346
CMW 10484	-	*C. radicalis*	*Castanea sativa*	Italy	A. Biraghi	AF 368327, AF 368349, AF 368349
CMW 10518	-	*C. nitschkei*	*Quercus*	Japan	T. Kobayashi	AF 452118, AF 525706, AF 525713
CMW 10463	-	*C. macrospora*	*Castanopsis cuspidata*	Japan	T. Kobayashi	AF 368331, AF 368351, AF 368350

Table 1. (continued)

Isolate number[b]	Alternative isolate numbers[d]	Species	Host	Origin	Collector	GenBank Accession numbers
CMW 10465	-	*Endothia singularis*	unknown	USA	R.J. Stipes	AF 368323, AF 368333, AF 368332
CMW 10442	-	*E. gyrosa*	*Quercus palustris* L.	USA	R.J. Stipes	AF 368326, AF 368339, AF 368338
CMW 2091	ATCC 48192	*E. gyrosa*	*Quercus palustris*	USA	R.J. Stipes	AF 046905, AF 368337, AF 368336
CMW 5288	-	*Diaporthe ambigua*	*Malus domestica*	South Africa	W.A. Smit	AF 543817, AF 543819, AF 543821
CMW 5587	-	*D. ambigua*	*Malus domestica*	South Africa	W.A. Smit	AF 543818, AF 543820, AF 543822

[a] All isolates are maintained in the culture collection (**CMW**) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[b] Taxa presented in bold represent isolates sequenced in this study. Sequence data for the other taxa are from the studies of Venter et al. (2002), Myburg et al 2002), Myburg et al. (2003).

[c] This isolate was previously labelled as *Cryphonectria havanensis*.

[d] **ATCC** = American Type Culture Collection, P.O. Box 1549, Manassas, VA 20108, USA.

CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

FPH = Forestry and Forest Products Research Institute, P. O. Box 16, Tsukuba Norin Kenkyu, Danchi-Nai, Ibaraki, 305 Japan.

M = isolates used in Hoegger et al. (2002).

(**D15, D31**) = Isolates used in Cortesi et al. (2001).

(**09494, KB1, CD28**) = isolates used in Lui et al. (2003).

Table 2. Specimens of *Cryphonectria* species used in the morphological comparisons.

Identification	Herbarium allocation[a]	Host	Origin	Collector	Date
Cryphonectria parasitica	FPH 1326	*Castanea crenata*	Tsurukawa, Japan	T. Kobayashi	1953
C. parasitica	FPH 629	*Castanea crenata*	Koganei, Japan	T. Kobayashi	1953
C. parasitica	FPH 608	*Castanea crenata*	Matsudo, Japan	T. Kobayashi	1953
C. parasitica	FPH 600	*Castanea crenata*	Seki, Japan	T. Kobayashi	1953
C. radicalis	BPI 612660	n.a.	Como, Italy	C.L. Shear	1912
C. radicalis	BPI 612672	*Castanea sativa*	Etremblieres, Switzerland	C.L. Shear	1913
C. radicalis	BPI 613739	*Castanea sativa*	Stresa, Italy	C.L. Shear	1913
C. radicalis	BPI 1112743	*Quercus sp.*	Bois Bastard, France	F. Candoussau	1992
C. radicalis	BPI 797696	*Castanea* sp.	Rome, Italy	Prof. Liropoli	1877
C. radicalis	BPI 797697	*Castanea sativa*	Locarno, Switzerland	n.a.	1862
C. radicalis	BPI 797698	n.a.	Sciolze, Italy	n.a.	1873
C. radicalis	BPI 797692	*Carpinus betulus*	Abkehazia	Woronin	n.a.
C. radicalis	BPI 797693	*Castanea* sp.	Locarno, Switzerland	Denotaris	1862
C. radicalis	BPI 797694	*Castanea* sp.	Locarno, Switzerland	Daldini	1862
C. radicalis	BPI 797695	*Castanea* sp.	Como, Italy	n.a.	n.a.

Table 2. (continued)

Identification	Herbarium allocation[a]	Host	Origin	Collector	Date
C. radicalis	FPH 1200	*Quercus variabilis*	Meguro, Japan	T. Kobayashi	1953
C. radicalis	FPH 1072	*Quercus serrata* Thunb.	Machida, Japan	T. Kobayashi	1954
C. radicalis	FPH 2483	*Quercus salicina* Blume	Komayama, Japan	T. Kobayashi	1959
C. radicalis	FPH 601	*Alnus firma* Siebold & Zucc.	Nishina, Japan	T. Kobayashi	1955
C. radicalis	FPH 652	*Carpinus japonica* Blume	Asakawa, Japan	T. Kobayashi	1962
C. radicalis	NYBG 1963	*Quercus* sp.	Glatfelter, USA	C.L. Shear and N.E. Stevens	1913
C. radicalis	CUP 6178	Chestnut stump	Connellsville, USA	P.J. Anderson and H.W Anderson	1912
C. havanensis	FPH 633	*Eucalyptus globulus*	Meguro, Japan	T. Kobayashi	1954
C. havanensis	FPH 2300	*Betula* sp.	Yoshiwara, Japan	Zinno	1963
C. havanensis	FPH 1270	*Pyrus sinensis*	Inagi, Japan	T. Kobayashi	1960
C. havanensis	FPH 1203	*Quercus variabilis*	Seto, Japan	T. Kobayashi	1953
C. havanensis	FPH 1047	*Quercus glandulifera*	Japan	T. Kobayashi	1954

Table 2. (continued)

Identification	Herbarium allocation[a]	Host	Origin	Collector	Date
C. macrospora (type)	FPH 1057	*Castanopsis cuspidata*	Shinagawa, Japan	T. Kobayashi	1954
C. macrospora	FPH 1058	*Castanopsis cuspidata*	Shinagawa, Japan	T. Kobayashi	1954
C. nitschkei (type)	FPH 1045	*Quercus grosseserrata*	Meguro, Japan	T. Kobayashi	1954
C. clavata (holotype)	FPH 7609	*Castanea crenata*	Kobuchizawa Japan	M. Milgroom and S. Kaneko	1998
C. clavata (epitype)	FPH 7610	*Castanea crenata*	Chudai, Japan	M. Milgroom and S. Kaneko	1998

[a] **BPI** = U. S. National Fungus Collections, Systematic Botany and Mycology, Rm. 304, Bldg. 011A, 10300 Baltimore Avenue, Beltsville, MD 20705-2350, USA.

PREM = National Collection of Fungi, Pretoria, South Africa.

FPH = Forestry and Forest Products Research Institute, P. O. Box 16, Tsukuba Norin Kenkyu, Danchi-Nai, Ibaraki, 305 Japan.

CUP = Plant Pathology Herbarium, Cornell University, 334 Plant Science Building, Ithaca, New York 14853-4203 USA,

.[b] Specimen FPH 7609 is linked to isolate CMW 10786, and specimen FPH 7610 is linked to isolate CMW 10787.

Table 3. Spores sizes for the different species studied. Species, and individual specimens that was considered separately, are mentioned in the order of decreasing ascospore length.

Label name	Specimens	Ascospore length	Ascospore width	Specimens	Conidial length	Conidial width
Cryphonectria macrospora	FPH 1057	14-17(-19)	(4.5-)5.5-7(-8)	FPH 1057 FPH 1058	3.5-4.5(-5)	1-1.5
C. nitschkei	FPH 1045	(9.5-)10-11.5(-12.5)	(3-)3.5-4.5(-5)	FPH 1045	3.5-5(-6)	(1-)1.5(-2)
C. havanensis (*Quercus*)	FPH 1203 FPH 1047	(8-)9.5-11.5(-13)	(3.5-)4-5(-5.5)	FPH 1047	(3-)3.5-4.5(-5)	1.5(-2)
C. clavata	FPH 7609	(8.5-)10-11.5(-12.5)	(3.5-)4-4.5(-5)	FPH 7609	4-5.5(-6)	(1-)1.5(-2)
C. clavata	FPH 7610	n.a.	n.a.	FPH 7610	(4.5-)5-6.5(-7)	1.5
C. havanensis (*Betula* sp.)	FPH 2300	(8-)9.5-11(-12.5)	(3-)3.5-4(-4.5)	n.a.	n.a.	n.a.
C. havanensis (*Pyrus sinensis*)	FPH 1270	10-12(-13.5)	(3-)3.5-4(-4.5)	n.a.	n.a.	n.a.

Table 3. (continued)

Label name	Specimens	Ascospore length	Ascospore width	Specimens	Conidial length	Conidial width
"A. *C. havanensis*" (*Eucalyptus globulus*)	FPH 633	9.5-12(-13.5)	3-3.5(-4.5)	n.a.	n.a.	n.a.
"B. *C. havanensis*" (*Eucalyptus globulus*)	FPH 633	(6-)6.5-9(-11)	3-2.5	n.a.	n.a.	n.a.
C. parasitica	FPH 629 FPH 1326	(7.5-)8-9(-9.5)	3.5-4(-4.5)	FPH 600 FPH 608 FPH 1326	(3-)3.5-4(-4.5)	1-1.5
C. radicalis, longer ascospores (Europe)	BPI797696 BPI 797692 BPI1112743 BPI 797698 BPI 612660	(7-)8-10(-12)	(2-)2.5-3.5(-4)	BPI1112743 BPI 797698 BPI 612660	(3-)3.5-4(-4.5)	1-1.5(-2)

Table 3. (continued)

Label name	Specimens	Ascospore length	Ascospore width	Specimens	Conidial length	Conidial width
C. radicalis Japan and China	FPH 652 FPH 2483	(5.5-)6.5-8(-9.5)	(2-)2.5-3.5	FPH601 FPH 652 FPH 1072 FPH 1200	3-4(-4.5)	1-1.5
C. radicalis smaller ascospores (Europe)	BPI 797697 BPI 613739 BPI 612672 BPI 797693	(6-)7-8.5	(2-)2.5-3	BPI 613739 BPI 612672 BPI 797693	(3-)3.5-4(-4.5)	1-1.5(-2)
C. radicalis NYBG 1963 (USA)	NYBG 1963	(5.5-)6.5-8.5(-10)	(2.5-)3-4	n.a.	n.a.	
C. radicalis CUP 6178 (USA)	CUP 6178	(5-)5.5-7(7.5)	2.5-3(-3.5)	CUP 6178	(2.5-)3-3.5(-4)	1-1.5

Fig. 1. Most parsimonious phylogenetic tree (tree length= 1203 steps, CI = 0.77 and RI = 0.92) generated from sequence variation within a combined ribosomal (ITS1, 5.8S, ITS2) and β-tubulin (1a/b, 2a/b) sequence data set. Bootstrap values >50% (1000 replicates) are indicated. The *Diaporthe ambigua* isolates were used as outgroup taxa to root the phylogenetic tree.

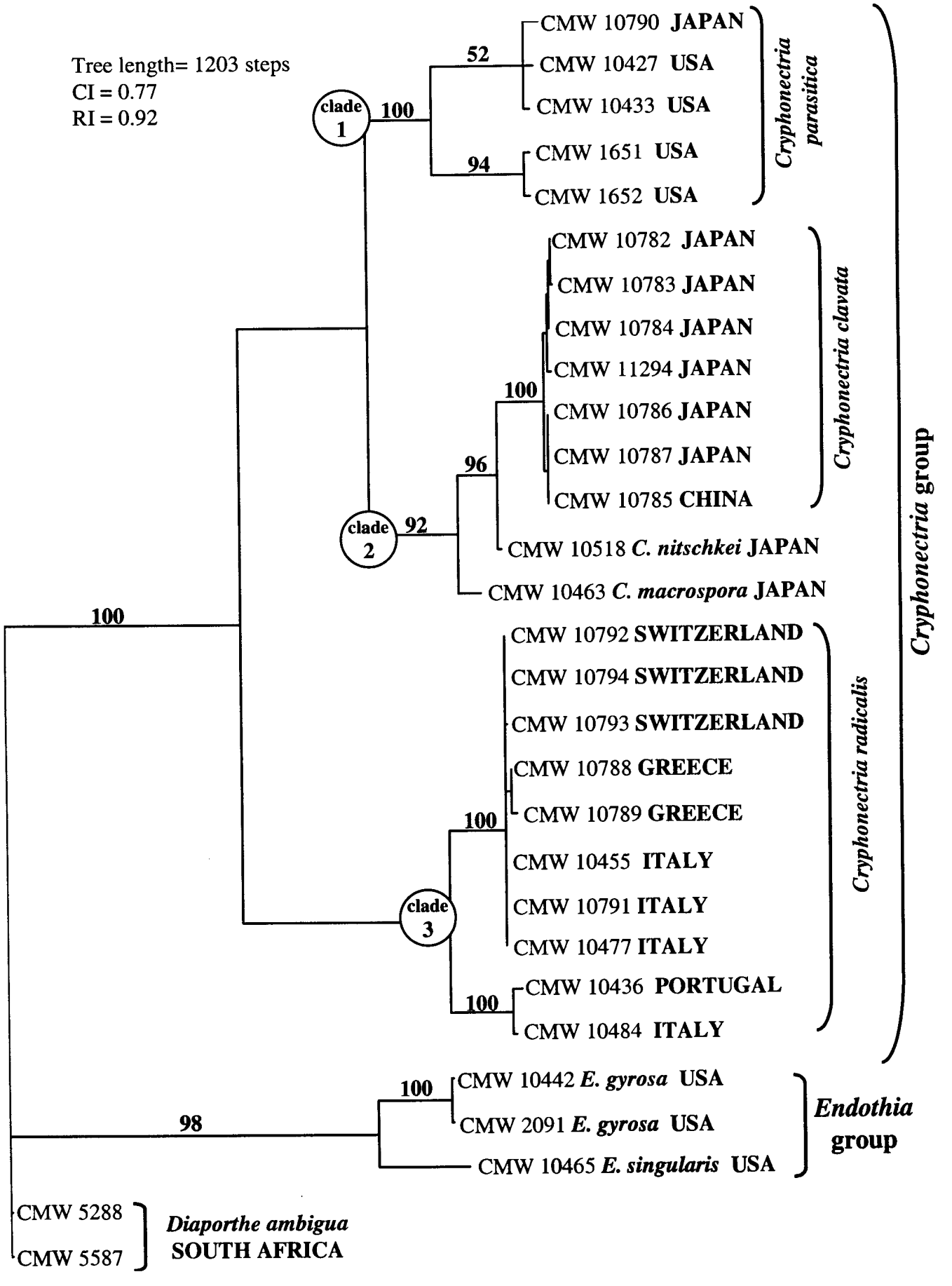


Fig. 2. Micrographs of ascospores from fruiting structures on various specimens annotated as *Cryphonectria havanensis* from different hosts in Japan. **a**. FPH 1203 from *Quercus variabilis*. **b**. FPH 2300 from a *Betula* sp. **c**. FPH 1047 from *Q. glandulifera*. **d**. FPH 1270 from *Pyrus sinensis*. **e**. Larger ascospores on specimen FPH 633 ("A. *C. havanensis*") from *Eucalyptus globulus*. **f**. Smaller ascospores on specimen from FPH 633 ("B. *C. havanensis*") from *Eucalyptus globulus*. (Scale bars for **a-f** 10 μm).

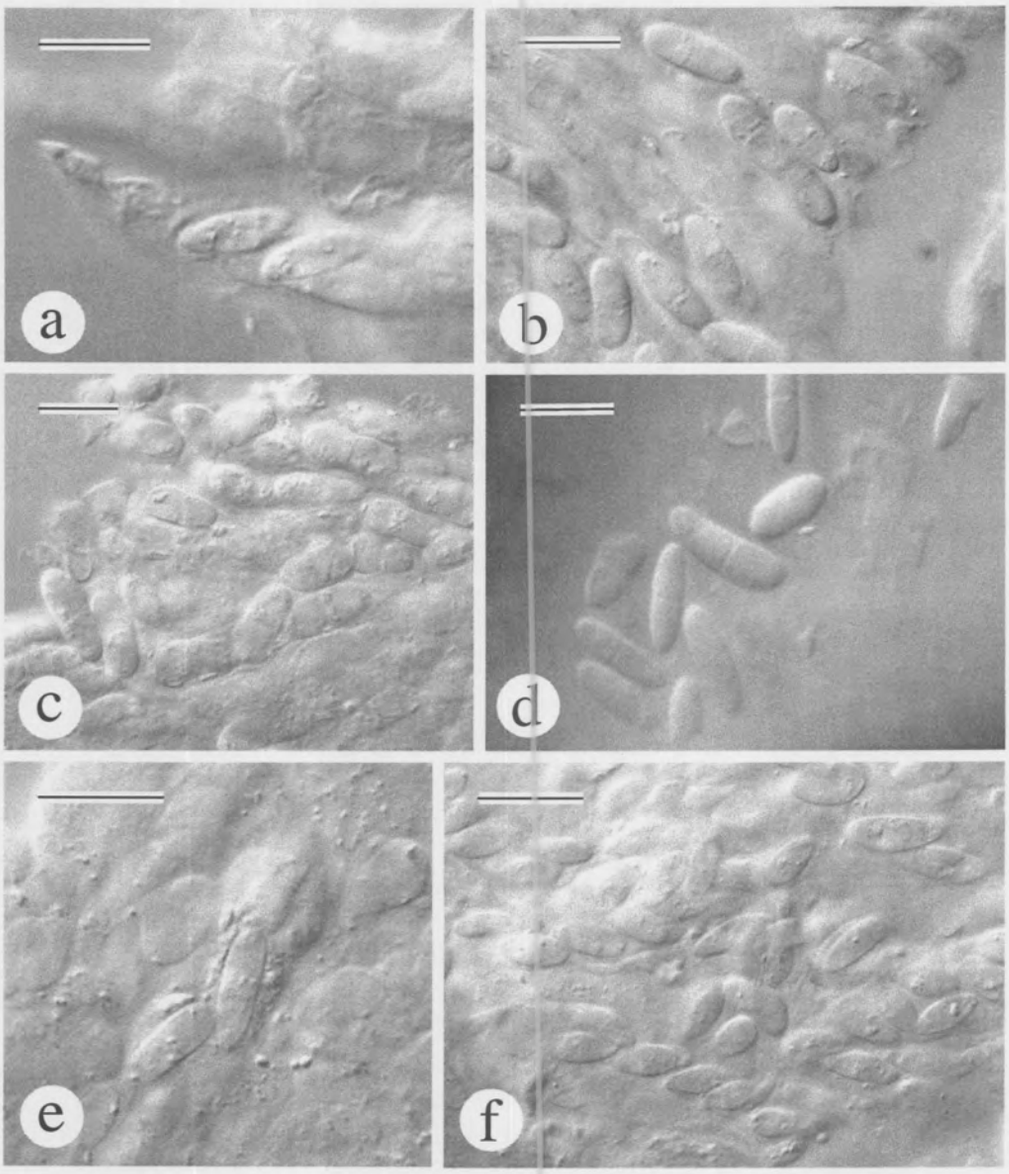


Fig. 3. Micrographs of phialides of two species of *Cryphonectria*. **a**. From type specimen (FPH 1045) of *C. nitschkei* from *Quercus grosseserrata*. **b**. From specimen (FPH 1047) of putative *C. havanensis* from *Q. glandulifera*. (Scale bars for **a**, **b** 10 μm).

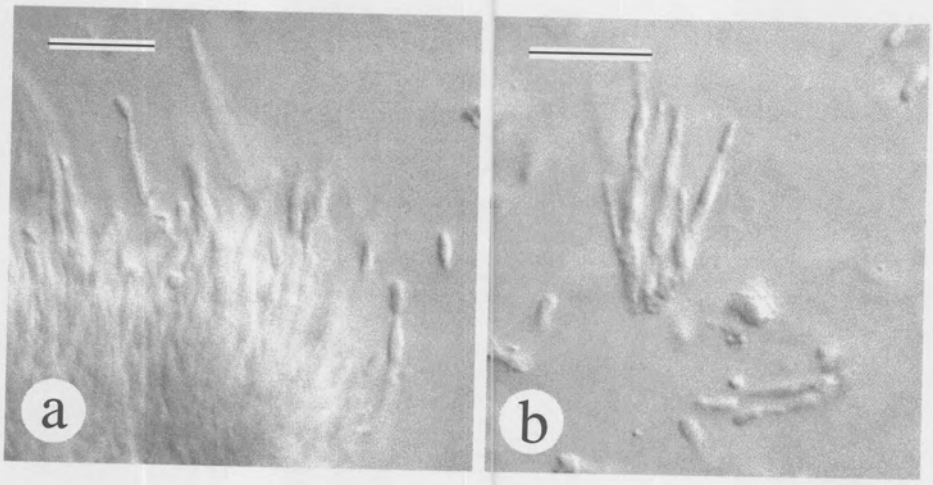


Fig. 4. Micrographs representing the fruiting structures of *Cryphonectria clavata*. **a**. Ascomata on bark. **b**. Longitudinal section through ascomata. **c**. Pseudoparenchymatous tissue. **d**. Prosenchymatous tissue. **e**. Ascus with ascospores. **f**. Ascospores. **g**. Conidioma on bark. **h**. Longitudinal section through conidioma. **i-j**. Conidiophores and conidiogenous cells. **k**. Conidia. (Scale bars for **a**, **b**, **g**, **h** 100 µm; **c**, **d** 20 µm; **e**, **f**, **i**, **j**, **k** 10 µm).

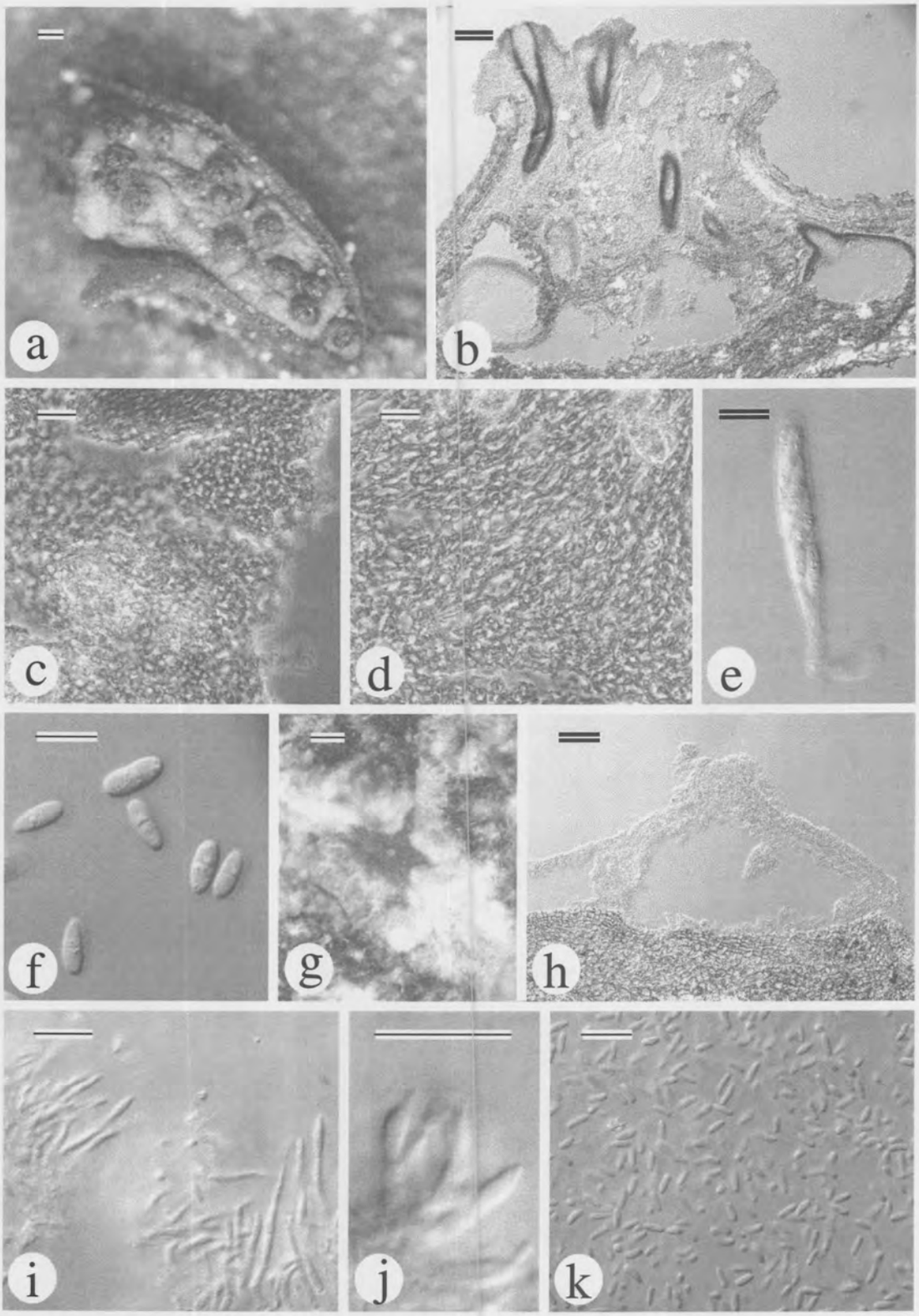
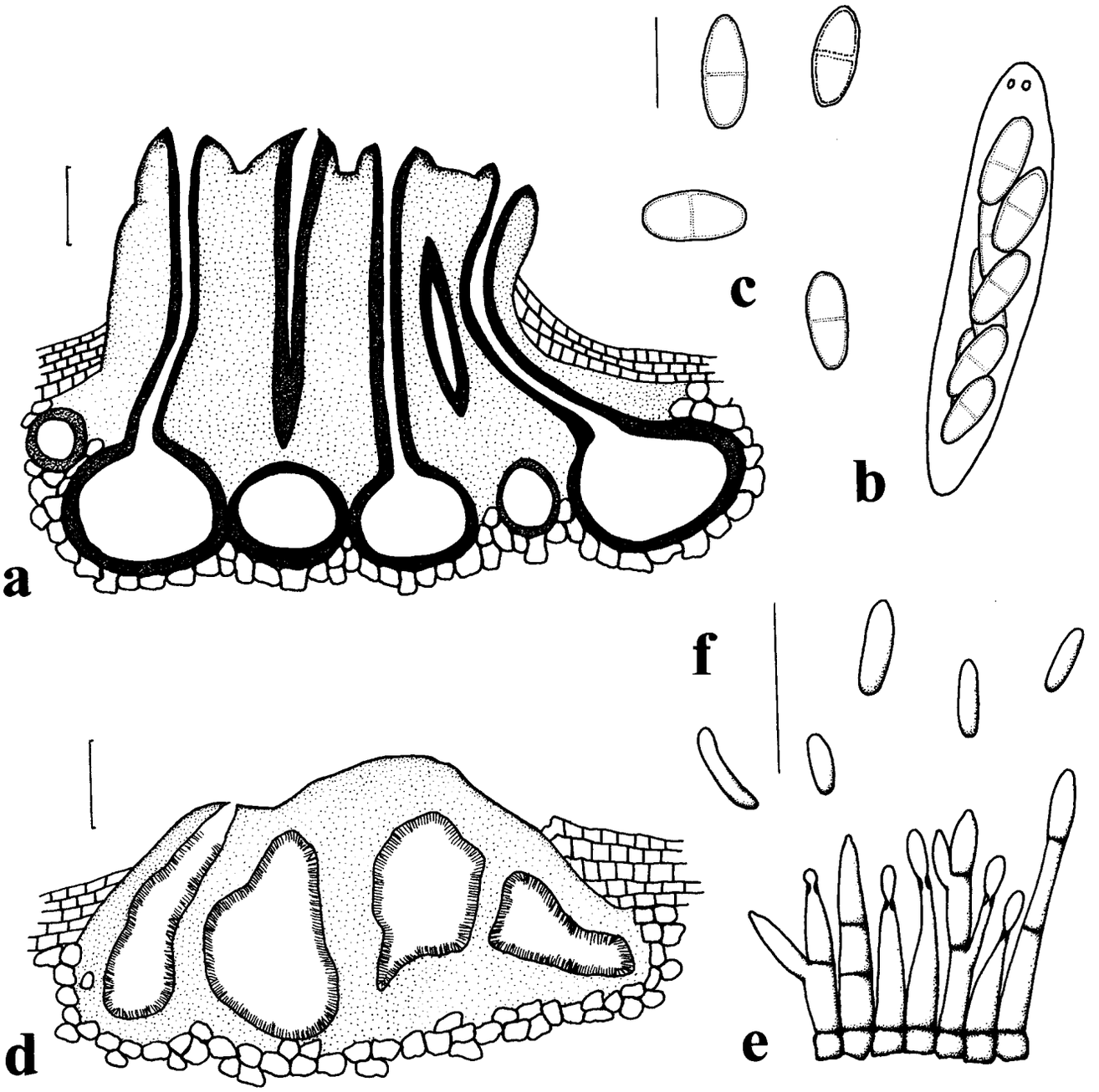


Fig. 5. Line drawings of the fruiting structures of *Cryphonectria clavata*. **a**. Ascoma. **b**. Ascus. **c**. Ascospores. **d**. Conidioma. **e**. Conidiophores and conidiogenous cells. **f**. Conidia. (Scale bars for **a**, **d** 100 μm; **b**, **c**, **e**, **f** 10 μm).



CHAPTER 7

Genera and species in the *Cryphonectria/Endothia* complex and their placement in the Diaporthales: A Molecular and Morphological synopsis.

GENERA AND SPECIES IN THE *CRYPHONECTRIA/ENDOTHIA* COMPLEX AND THEIR PLACEMENT IN THE DIAPORTHALES: A MOLECULAR AND MORPHOLOGICAL SYNOPSIS.

ABSTRACT

The order Diaporthales encompasses a number of important fungal plant pathogens, many of which reside in the genera *Cryphonectria* and *Endothia*. Collections of a number of newly described species, and as yet undescribed fungi, resembling species of *Cryphonectria* and *Endothia*, have recently been made from various hosts in different parts of the world. The aim of this study was to characterise these collections and to consider their relationships with all *Cryphonectria* and *Endothia* species available in culture. Identification and characterisation was based on morphological comparisons and sequence analyses of the ITS ribosomal DNA region and two regions in the β-tubulin gene. Sequence analyses showed that *Cryphonectria* and *Endothia* are characterised by a number of previously described as well as some presently undescribed species. The majority of the newly collected fungi resembling *Cryphonectria* spp., formed groups closely related to but distinct from *Cryphonectria*. This suggests that *Cryphonectria* is paraphyletic and includes a number of different genera. Family level relationships of *Cryphonectria* and *Endothia* were considered in terms of their relationships with recognised lineages in the Diaporthales, based on sequence data from the large subunit (LSU) nuclear rDNA. *Cryphonectria* and *Endothia*

species, including the related genera recognised in this study, formed a distinct group in the LSU rDNA phylogenetic tree. This supports the view that *Cryphonectria* and *Endothia*, as well as their closest relatives, should reside in a discrete family of the Diaporthales.

INTRODUCTION

The Diaporthales includes important plant pathogenic fungi. Members of this order are morphologically united by a *Diaporthe*-type centrum (Alexopoulos and Mims 1978, Barr 1978). Morphological criteria include perithecia with long necks, located in a pseudostroma with no paraphyses and thick-walled asci that are either evanescent with short stalks or intact (Alexopoulos and Mims 1978, Hawksworth et al. 1996). Features such as the presence or absence of stromatic tissue, stromatal tissue type, the position of the perithecia and perithecial beaks relative to the substrate, ascospore shape and ascospore septation have been used to differentiate between the families and genera in the Diaporthales (Barr 1978).

Cryphonectria and *Endothia* are amongst the better-studied genera in the Diaporthales. This is primarily because they include important tree pathogens. The best-known pathogens are *Cryphonectria parasitica* (Murr.) Barr, *C. cubensis* (Bruner) Hodges, *C. eucalypti* M. Venter and M.J. Wingf. and *Endothia gyrosa* (Schw.: Fr.) Fr. *Cryphonectria parasitica* and *E. gyrosa* are mainly found on members of the Fagaceae (Barr 1978, Kobayashi 1970, Roane 1986, Shear et al. 1917) while *C. cubensis* (Hodges 1980, Hodges et al. 1979, Hodges et al. 1986) and *C. eucalypti* mainly infect members of the Myrtaceae (Old et al. 1986, Venter et

Differentiation between species of *Cryphonectria* and *Endothia* has primarily been based on the morphological characteristics of their teleomorph states (Micales and Stipes 1987). *Cryphonectria* species have valsoid perithecia, semi-immersed stromata and one-septate ascospores, while *Endothia* species have diatrypoid perithecia, superficial stromata and aseptate ascospores (Barr 1978, Kobayashi 1970, Micales and Stipes 1987, Roane 1986, Shear et al. 1917). However, in a recent study that integrated DNA sequence data and morphology, Venter et al. (2002) showed that ascospore morphology is not a reliable characteristic to distinguish unequivocably between *Cryphonectria* and *Endothia*, but that stromatal morphology represents an important characteristic defining the two genera.

Cryphonectria was synonymised with *Endothia* by Von Höhnel (1909) and remained so until a monograph on the Diaporthales by Barr (1978). In this monograph, *Cryphonectria* was resurrected and various species were transferred from *Endothia* to *Cryphonectria.* These include *C. gyrosa* (Berk. & Br.) Sacc. (type species), *C. cubensis*, *C. havanensis* (Bruner) Barr, *C. macrospora* (Kobayashi & Ito) Barr, *C. nitschkei* (Otth.) Barr, *C. parasitica* and *C. radicalis* (Schw.: Fr.) Barr. Species currently treated in *Cryphonectria* but not included in Barr's (1978) monograph, are *C. longirostris* (Earle) Micales & Stipes and *C. coccolobii* (Vizioli) Micales & Stipes. These species were placed in *Cryphonectria* based on similar morphological characteristics (Micales and Stipes 1987). Only *Endothia gyrosa* (type species), *E. viridistroma* Wehmeyer and *E. singularis* (H. & B. Syd.) Shear and Stevens were retained in *Endothia.*

The newest member to the *Cryphonectria-Endothia* complex is *C. eucalypti* M. Venter & M. J. Wingf. (Venter et al. 2002). *Cryphonectria eucalypti*, previously considered conspecific with *E. gyrosa*, is the causal agent of a canker disease on *Eucalyptus* species in South Africa and Australia (Gryzenhout et al. 2003, Old et al. 1986, Venter et al. 2001, Venter et al. 2002, Walker et al. 1985). Venter et al. (2002) used both morphology and DNA sequence data to show that *E. gyrosa* from *Eucalyptus* in South Africa and Australia was different from *E. gyrosa* from the United States and more closely related to species of *Cryphonectria* even though the fungus had non-septate, allatoid ascospores typical of Endothia spp., and was subsequently described as *C. eucalypti*.

An additional *Cryphonectria* species will be formally described in near future. This species emerged from a study by Lui et al. (2003) and is characterised in Chapter 6 (this thesis) as *C. clavata* M. Gryzenhout & M. J. Wingf. nom. prov. This characterisation was based on ribosomal ITS and β-tubulin DNA sequence analyses as well as morphological comparisons of a large collection of *Cryphonectria* species on *Fagaceae* in Europe and Asia. Morphological and DNA sequence results indicated that *C. clavata* was different from all the *Cryphonectria* species for which cultures are currently available.

In a recent study of Myburg et al. (2003a) two new *Cryphonectria*-like fungi, other than *C. cubensis* (= *E. eugeniae* [Nutman & Roberts] Reid & Booth), were discovered on clove. This discovery was based on morphological comparisons and phylogenetic analyses. One of these newly discovered species was represented by isolates that grouped separately from the *C. cubensis* isolates in the phylogenetic tree and more closely to the clade representing

Cryphonectria. Morphological characterisation of this *Cryphonectria*-like clove fungus could not be resolved due to the lack of specimens linked to the isolates. The second species found on the clove specimens resembled the anamorph of *Cryphonectria* based on similar orange to sienna stroma. No isolates were available that could be linked to the specimens of this species. Description of these two fungi must await acquisition of isolates linked to specimens that can be connected to these fungi. The synonymy of *C. cubensis* and *E. eugeniae* (Hodges et al. 1986, Micales et al. 1987) was also confirmed in the study of Myburg et al. (2003a).

Two new genera, closely related to *Cryphonectria*, will be described in near future. The first will accommodate a fungus that is associated with a canker disease on *Terminalia ivorensis* A. Cheval. in Ecuador. Phylogenetic analyses showed that this fungus resides in a group separate from but closely related to *C. cubensis* and is distinguished by its orange, superficial, rostrate conidiomata (M. Gryzenhout, personal communication). Furthermore, morphological comparisons show that *C. longirostris* is similar to the fungus on *T. ivorensis* (M. Gryzenhout, personal communication). Nevertheless, *C. longirostris* could be differentiated from this fungus based on conidial size. *Cryphonectria longirostris* will, therefore, also be accommodated in this new genus (M. Gryzenhout, personal communication).

The second new genus related to *Cryphonectria* will include an undescribed fungal species occurring on *Miconia theaezans* Cogn. and *T. urvilleana*, trees native to Colombia, as well as on *Eucalyptus grandis* W.Hill ex Maiden in Colombia. This fungus is anamorphic, but

morphological and DNA sequence comparisons showed that it resides in the Diaporthales, close to *Cryphonectria*. The fungus is morphologically similar to *C. cubensis* based on its blackened, pyriform conidiomata, but can be distinguished from the latter species based on the distinct orange apices of the necks (M. Gryzenhout, personal communication).

Phylogenetic studies on *Cryphonectria* and *Endothia* (Myburg et al. 1999, Myburg et al. 2002a, Myburg et al. 2003b, Roux et al. 2003, Venter et al. 2001) have shown that taxonomic changes are needed for *C. cubensis*. Ribosomal ITS (Venter et al. 2001) and β-tubulin (Myburg et al. 2003b) sequence data have shown that *C. cubensis* should be considered in a genus separate but related to *Cryphonectria*. The sequence data were strongly supported by morphological characteristics such as dark brown tissue that covers the extending perithecial necks in the ascomata of *C. cubensis* as opposed to the orange tissue that covers the extending perithecial necks of typical *Cryphonectria* spp. In addition, *C. cubensis* has superficial, pyriform and blackened conidiomata different to the typical orange, pulvinate and semi-immersed conidiomata of *Cryphonectria* spp. (Gryzenhout et al. 2002, Myburg et al. 2003b). Furthermore, three phylogenetic sub-clades were observed within *C. cubensis* and these accommodated *C. cubensis* from South America/Congo, Southeast Asia/Australia and South Africa (Myburg et al. 2002a, Myburg et al. 2003b, Roux et al. 2003). Fungi residing in these sub-clades apparently represent distinct species (Myburg et al. 2002a).

Myburg et al. (2003b) studied isolates originating from *Elaeocarpus dentatus* Vahl. in New Zealand and previously thought to represent *C. gyrosa* and *C. radicalis*. DNA sequence data

comparisons revealed that this fungus should be described as a new species within a new genus, closely related to *Cryphonectria*, in the Diaporthales. Morphological features defining this group are ovoid, superficial conidiomata, and one to three septated ascospores (Myburg et al. 2003b).

In recent years, we have acquired a large number of new collections of *Cryphonectria* spp. as well as closely related species. In addition, new species or incorrectly identified species have been recognised that require further study. These fungi have been isolated from a variety of host species originating from different geographical areas of the world. The objective of this study was to provide a comprehensive synopsis for all previously described and new fungal collections awaiting description. These comparisons are based on ribosomal ITS and β-tubulin gene sequence data and morphological characteristics. Data have been extracted from a large number of previous studies (Heath et al. 2003, Myburg et al. 1999, Myburg et al. 2002a, Myburg et al. 2002b, Myburg et al. 2003a, Myburg et al. 2003b, Chapter 6 [this thesis], Roux et al. 2003, Venter et al. 2002, Wingfield et al. 2001). This study also includes fungi not previously studied as well as molecular and morphological data not published before. In order to understand the relationships among all these fungi it was necessary to determine their taxonomic position in relation to other members of the Diaporthales. This was achieved by determining LSU rDNA sequence data for these fungi and comparing it with the LSU rDNA sequence data used in publications treating the species in the Diaporthales (Castlebury et al. 2002, Zhang and Blackwell 2001).

MATERIALS AND METHODS

Isolates studied

Isolates included in this study (Table 1) represent all the species residing in *Cryphonectria* and *Endothia* for which isolates are currently available. These isolates also represent a broad range of geographical origins and hosts. As far as we are aware, no isolates exist for species such as *C. coccolobii*, authentic *C. havanensis* from Cuba, *C. gyrosa* and authentic *E. viridistroma*. These species could, therefore, not be included in the present study. The *E. viridistroma* isolate included in Myburg et al. (2003b) most likely represents a *Cytospora* sp. and has been excluded from this study. An isolate labelled as *C. gyrosa* (CMW 10471) and included in Myburg et al. (2003b) did not represent this fungus and could, therefore, not be included in the present study as *C. gyrosa*. Representatives of the newly described genera that will accommodate the fungi on *T. ivorensis* and *M. theaezans*, *T. urvilleana* and *Eucalyptus grandis* in Colombia were also included, as well as the unknown fungus found on clove in Indonesia (Myburg et al. 2003a) and waterberry in South Africa (M. Gryzenhout, personal communication) occurring together with *C. cubensis*. Furthermore, fungi thought to be species of *Cryphonectria*, but that we now believe represent new genera in the Diaporthales, have been incorporated to reflect their relationships with one another as well as with other *Cryphonectria* and *Endothia* spp. *Diaporthe ambigua* Nitschkei isolates were included as outgroup taxa to root the ITS/β-tubulin phylogenetic tree.

Representative isolates from the above mentioned groups of fungi were included in LSU sequence data analyses. These sequences were compared to the LSU sequence data sets generated in Zhang and Blackwell (2001) (Table 3) and Castlebury et al. (2002) (Table 4). The LSU sequence data for *Magnaporthe grisea* (T.T. Herbert) Yaegashi & Udugawa (AB 026819), *Pyricularia grisea* (Cooke) Sacc. (AF 362554), *Gaeumannomyces graminis* (Sacc.) Arx & D. Oliver (AF 362556) and *Gaeumannomyces graminis* (AF 362557) generated in the study of Castlebury et al. (2002), were used as outgroup taxa to root the LSU phylogenetic tree.

The isolates listed in Table 1 are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Many of the isolates are also available in other internationally recognised collections and the information is included in Tables 1 and 2.

DNA extractions

DNA was isolated from mycelium using the DNA extraction buffer of Raeder and Broda (1985). The DNA extraction buffer consisted of 200 mM Tris-HCl (pH 8.5), 250 mM NaCl, 25mM EDTA and 0.5% SDS. DNA quality was assessed by gel electrophoresis in a 1% agarose gel containing ethidium bromide. The DNA was visualised by exposing the agarose gel to a UV light source.

ITS rDNA and β-tubulin amplification

Reaction conditions to amplify the ITS1, 5.8S and ITS2 regions of the rRNA operon as well as two regions within the β-tubulin gene are described in the studies of Myburg et al (1999) and Myburg et al (2002a,b). The primer pairs used to amplify the ribosomal DNA (ITS1, 5.8S and ITS2) and β-tubulin gene regions are those designed and used by White et al. (1990) and Glass and Donaldson (1995), respectively. Amplified products were purified using a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and directly used as templates in sequencing reactions.

LSU rDNA amplification

The partial LSU rDNA gene was amplified with primers pairs ITS3 (White et al. 1990) and LR3 (Rehner and Samuels 1994, Vilgalys and Hester 1990). PCR conditions were: 95°C for 3 min (denature), 30 cycles of 95°C for 30 s (denature), 56°C for 45s (anneal), 72°C for 1 min (elongation) and a final elongation step of 72°C for 4 min. LSU amplification products were purified using a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and directly used as templates in subsequent sequencing reactions.

Sequencing

Sequencing reactions were as specified by the manufacturers of the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Warrington, United

Kingdom). Nucleotide sequence data were generated with an ABI PRISM 3100™ automated DNA sequencer (Perkin-Elmer, Warrington, United Kingdom). The primer pairs used in the respective sequencing reactions are as follows: ITS1 and ITS4 (amplifying the rDNA operon), Bt1a and Bt1b (amplifying β-tubulin region 1a/1b), Bt2a and Bt2b (amplifying β-tubulin region 2a/2b), LS1 and LR3 (amplifying LSU rDNA).

Sequence data analyses

The raw sequence data generated for the respective gene regions were edited using the Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems, Inc., Foster City, California) software package. Gaps were inserted during sequence alignment and were treated as fifth characters (NEWSTATE) in the sequence analyses. Phylogenetic analyses were executed using the software package PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1998).

Sequence data obtained from other studies

Sequence data from other studies were included in this study for comparative purposes. Ribosomal ITS and β-tubulin DNA sequence data were obtained from the studies of Heath et al. (2003), Myburg et al. (1999), Myburg et al. (2002a,b), Myburg et al. (2003a,b), Chapter 6 (this thesis), Venter et al. (2001), Venter et al. (2002), Roux et al. (2003) and Wingfield et al. (2001). Large subunit ribosomal RNA sequence data were obtained from Zhang and Blackwell (2001) and Castlebury et al. (2002). Sequence alignments used by

Zhang and Blackwell (2001) and Castlebury et al. (2002) were obtained from TreeBASE. TreeBASE study accession numbers are S 665 (Zhang and Blackwell 2001) and S 815 (Castlebury et al. 2002).

Analysis of ITS and β-tubulin sequence data

The ribosomal DNA (ITS1, 5.8S, ITS2) and β-tubulin sequence data sets were subjected to the Templeton nonparametric Wilcoxon Signed Ranked (WSR) (Kellogg et al. 1996) test to assess whether they could be combined as one sequence data set in the phylogenetic analyses. Heuristic searches, with tree-bisection-reconnection (TBR) and MULTREES options (saving all optimal trees) effective, were used to analyse the DNA sequences. The confidence levels of the tree branch nodes were determined by a 1000 replicate bootstrap analysis (Felsenstein 1985). GenBank accession numbers of sequences generated in this study as well as those from previous phylogenetic studies are listed in Table 1.

Analyses of LSU sequence data

The LSU rDNA region of twenty-seven taxa was amplified (Table 1) in the present study. These taxa represent *Cryphonectria* and *Endothia*, related taxa and new species (representatives from **clades 1-10** in the ITS/β-tubulin phylogenetic tree). Phylogenetic trees were generated by maximum parsimony (MP) using the heuristic search option, with random sequence addition (1000 replications) and tree-bisection-reconnection (TBR) branch swapping options of PAUP* 4.0b8 (Swofford 1998) effective. All the sequence characters

were unordered and given equal weight. Gaps were treated as missing data in the parsimony analyses. Branch support was determined with 1000 bootstrap replications (Felsenstein 1985), MULTREES and TBR options rejected and random sequence additions for the MP bootstraps changed to 10. LSU sequence data generated in this study were deposited in GenBank and the accession numbers are listed in Table 1. Accession numbers for LSU sequences obtained from Zhang and Blackwell (2001) and Castlebury et al. (2002) are listed in Tables 3 and 4 respectively.

Morphological observations

Herbarium specimens (Table 2) linked to the different genera and ten phylogenetic groups indicated in the ITS/β-tubulin phylogenetic tree (Table 1, Fig. 1) were compared. Wherever possible, type specimens were included. Newly collected specimens linked to the undescribed taxa represented in the ITS/β-tubulin phylogenetic tree have been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM). No herbarium specimens are available for the *Endothiella* spp. on *Eucalyptus* spp. from New Zealand (isolates CMW 10010, CMW 10011 and CMW 10797) or isolate CMW 11297 annotated as *C. havanensis* from Mexico. Fruiting structures for the group of isolates from clove in Indonesia (CMW 10780, CMW 10779, CMW 10781) were obtained through artificial inoculations on stems of *E. grandis* (Myburg et al. 2003a).

Longitudinal sections of the stromata were made by rehydrating the fruiting structures in boiling water for 1 min and then embedding them in Leica mountant (Setpoint Premier,

Johannesburg, South Africa). Sections, 12-16 μm thick, were made with a Leica CM1100 cryostat (Setpoint Premier) at –20 °C. The sections were then dropped in water, transferred to microscope slides and mounted in lacto-phenol. These slides were studied using standard light microscopy. Spores were also examined in 3% KOH.

RESULTS

ITS/β-tubulin sequence analyses and morphological data

Results from the Templeton nonparametric Wilcoxon Signed Ranked test showed no significant conflict between the two data sets and the ITS ribosomal DNA and β-tubulin gene sequence data sets could be combined in the parsimony analyses. The combined ITS and β-tubulin sequence data sets included 106 taxa of which two *D. ambigua* isolates were incorporated as the outgroup taxa. A total of 1602 characters were included in the phylogenetic analyses of which 746 were constant, 96 variable but parsimony-uninformative and 760 variable and parsimony-informative. (Sequence alignments are available on request from the author as the document was too large to include in this thesis). The 'MaxTrees' limit (100) was reached in the heuristic search and a strict consensus tree (tree length = 3267 steps, consistency index/CI of = 0.51, retention index/RI of = 0.9) was computed for the 100 trees.

The phylogram derived from the combined sequence data (Fig. 1) depicts ten phylogenetically distinct clades, which have been numbered accordingly. Some of these

groups have previously been recognised while others have not yet been identified. The ten clades are each supported by unique morphological features.

Clade 1 (bootstrap support = 100%) includes *Cryphonectria cubensis* and is represented by isolates of *C. cubensis* from different parts of the world and from different hosts. Morphological features that define this clade are superficial, blackened pyriform conidiomata and ascomata with weakly developed orange to cinnamon stromatal tissue and blackened perithecial necks (Fig. 2a). Ascospores are fusoid, one-septate, and conidia are minute, aseptate, oblong to oval (Fig. 2a).

The three sub-groups of *C. cubensis* isolates from different parts of the world, previously described in Myburg et al. (2002a) were supported in the present study (Fig. 1). The South American sub-clade (bootstrap support = 77%) includes *C. cubensis* isolates from *Eucalyptus* spp. in Brazil and Venezuela. *Cryphonectria cubensis* isolates from *Eucalyptus* spp. in the Congo (Roux et al. 2003) and the *C. cubensis* isolates occurring on clove (*S. aromaticum*) in Brazil (Myburg et al. 2003a) grouped in the South American sub-clade. Recently obtained isolates from *Miconia* species (Melastomataceae) native to Colombia, i.e. *M. rubiginosa* (Bonpl.) DC. and *M. theaezans* (C. Rodas, personal communication), also resided in this sub-clade.

The Southeast Asian and Australian sub-clade (bootstrap support = 87%) included *C. cubensis* isolates from *Eucalyptus* spp. originating from Indonesia, China, Vietnam and Australia. *Cryphonectria cubensis* isolated from clove in Indonesia and Zanzibar (Myburg

et al. 2003b) grouped with those isolated from *Eucalyptus* spp. A *C. cubensis* isolate from Hawaii (CMW 1856) also grouped in this clade.

Isolates of *C. cubensis* in South Africa resided in a distinct sub-clade separately (bootstrap support = 87%) from the Southeast Asian/Australian and South American/Congo sub-clades. Isolates in this sub-clade represent those collected from *Eucalyptus* spp., *Tibouchina* spp. (Myburg et al. 2002b) and native waterberry trees (*Syzygium cordatum*) in South Africa (Heath et al. 2003).

Two additional groups, not previously recognised, are evident in the phylogram (Fig. 1). *Cryphonectria cubensis* isolates from a *Eucalyptus* sp. in Ecuador grouped separately from the other South American *C. cubensis* isolates (bootstrap support = 98%). Similarly, the *C. cubensis* isolates previously described from *T. urvilleana* in Colombia (Wingfield et al. 2001) (bootstrap support = 99%), grouped basal to the other sub-clades defined by geographical origin. A *C. cubensis* isolate from *M. theaezans* (CMW 9979) grouped together with these isolates from *T. urvilleana* in Colombia.

Clade 2 includes a group of isolates that represent the fungus occurring on *T. ivorensis* in Ecuador. This group clearly represents a distinct genus based on sequence data and morphological characteristics (bootstrap support = 100%). This fungus is more closely related to the group representing *C. cubensis sensu lato* than to any of the other groups in the phylogenetic tree. Based on morphology, this group is characterised by superficial, pyriform, orange conidiomata with long necks (Fig. 2b). Ascomata are seated in the bark,

surrounded by little stromatic tissue except for a sheath of tissue around the perithecial necks. Ascospores are fusoid to ellipsoid, one-septate, and conidia are minute, aseptate, cylindrical to oval (Fig. 2b).

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Clade 3 incorporates *C. eucalypti* isolates from South Africa and Australia (Venter et al. 2001, Venter et al. 2002) and fungi identified as *Endothiella* spp. occurring on *Eucalyptus* in New Zealand (bootstrap stupport = 100%). One of the isolates identified as an *Endothiella* sp. (CMW 10797), grouped separately (bootstrap support = 100%) from the other New Zealand *Endothiella* spp. as well as the *C. eucalypti* isolates and most probably represents a distinct species. This group of fungi have small, semi-immersed stromata containing convoluted conidial locules, and perithecia with bases surrounded by host tissue (Fig. 2c). Ascospores are cylindrical, sometimes allantoid and aseptate while conidia are minute, cylindrical and aseptate (Fig. 2c).

Isolates in **clades 4** to **6** grouped closely together in the phylogenetic tree (Fig.1). Morphology of **clades 5** and **6** is not yet fully resolved due to a lack of herbarium material or the presence of only one morph.

Clade 4 includes the isolates representing a fungus originating from *M. theaezans* in Colombia. The members of this genus have blackened, pyriform conidiomata (Fig. 2d). The apices of the conidiomatal necks are characteristically orange and conidia are minute, aseptate and oval (Fig. 2d). No teleomorph is known for this fungus.

Clade 5 represents undescribed fungi, occurring alongside *C. cubensis* on clove (*S. aromaticum*) in Indonesia (Myburg et al. 2003a) and waterberry (*S. cordatum*), a native tree species in South Africa (M. Gryzenhout, personal communication). The undescribed fungus on clove from Indonesia, represented by isolates CMW 10779, CMW 10780 and CMW 10781, is not connected to original host material. Fruiting structures have been produced on artificially inoculated *E. grandis* bark (Myburg et al. 2003a). Although these fruiting structures were too variable to draw definitive conclusions regarding the morphology of the fungus (Myburg et al. 2003a), fruiting structures were in general blackened, superficial and conical without long necks (Fig. 2e). Conidia were minute, cylindrical and aseptate (Fig. 2e). The teleomorph of this fungus is unknown. Fruiting structures of the fungus from *S. cordatum* (CMW 9978) in South Africa were only sexual and consisted of semi-immersed perithecia in well-developed, orange stromata (Fig. 2e). Ascospores are fusoid and one-septate (Fig. 2e).

Clade 6 comprises the unnamed fungus (CMW 9945, CMW 9946) isolated from *T. urvilleana* in New Zealand. Ascomatal structures representing isolates in this clade contains orange-brown stromatal tissue with semi-immersed perithecia (Fig. 2f). Ascospores are fusoid and one septate (Fig. 2f). No anamorph was found on the current herbarium specimens.

Isolates and specimens labelled as *C. havanensis* and collected on *Eucalyptus* in Mexico (CMW 11297, CMW 11298), and a fungus occurring on *Myrica faya* in Madeira (CMW 11299, CMW 11300) and the Azores (CMW 11301, CMW 11302) make up **Clade 7**. The

fungus collected from the Azores and Madeira, was associated with cankers of *M. faya* (Gardner and Hodges 1990, Hodges and Gardner 1992). Fruiting structures related to this group appear similar to those characterising *Cryphonectria sensu stricto*, but the stromata are more superficial and less developed (Fig. 2g). Ascospores are fusoid to ellipsoid, one-septate, and conidia minute, cylindrical and aseptate (Fig. 2g).

Cryphonectria sensu stricto is represented by isolates of *C. parasitica*, *C. nitschkei*, *C. radicalis* and *C. macrospora* that reside in **Clade 8**. This group was also recognised previously (Myburg et al. 2003b, Myburg et al. Chapter 6, Venter et al. 2002) and includes the new species, *C. clavata*, on *Quercus* and *Castanea* spp. from Japan (Myburg et al. Chapter 6). Two isolates, CMW 10436 and CMW 10484, identified as *C. radicalis*, grouped closely, but separately (bootstrap support = 100%) from the other *C. radicalis* isolates (CMW 10455, CMW 10477, CMW 10788-10789, CMW 10791-10794). These isolates (CMW 10436, CMW 10484) probably represent a distinct species, but due to the lack of herbarium specimens with fungal structures on host tissue, it is not possible to conclude which of the two groups represent authentic European *C. radicalis* (Myburg et al. 2003b, Myburg et al. Chapter 6). Species in **clade 8** are unified by conidiomata that are orange, multilocular and semi-immersed (Fig. 2h). Ascomata are orange, well developed, erumpent, semi-immersed, with perithecia in a valsoid orientation (Fig. 2h). Ascospores are fusoid to ellipsoid and one-septate while the conidia are minute, cylindrical and aseptate (Fig. 2h).

Endothia is represented by the two species *E. gyrosa* and *E. singularis* that reside in **Clade 9**. This group is well supported within the phylogenetic tree (bootstrap support = 91%) and

is distinct from any of the other phylogenetic groups. Species of *Endothia* are characterised by orange, superficial, strongly developed stromata with perithecia in a diatrypoid orientation, and numerous conidial locules (Fig. 2i). Ascospores are cylindrical to allantoid and aseptate, while conidia are minute, cylindrical and aseptate (Fig. 2i).

Clade 10 incorporates a group of fungi (CMW 10469, CMW 10470, CMW 10471) isolated from *Elaeocarpus dentatus* in New Zealand (Myburg et al. 2003b). This clade grouped basal to the other clades in the phylogenetic tree. The stromata of these fungi are large, superficial, orange, and perithecia are borne similar to those of *Endothia* (Fig. 2j). Conidiomata, however, have a conical shape (Fig. 2j). Ascospores are also very distinct from other species currently described in *Cryphonectria sensu lato* and have one to three septa (Fig. 2j). Conidia are cylindrical and aseptate (Fig. 2j).

Analysis of LSU rDNA sequences

The LSU sequence data set included 125 taxa, of which 20 sequences were obtained from Zhang and Blackwell (2001) (Table 3) and 76 sequences from Castlebury et al. (2002) (Table 4) of which four represented the outgroup taxa, i.e. *Magnaporthe grisea*, *Pyricularia grisea* and *Gaeumannomyces grisea* (2 representatives). Fifteen additional taxa representing the genera *Cryphonectria* and *Endothia* and fourteen isolates representing the suggested new genera and/or species were included (Table 1). The LSU sequence data set consisted of 552 total bases of which 408 were constant, 18 were parsimony-uninformative and 126 were parsimony-informative. (Sequence alignments are available on request from the author as

the document was too large to include in this thesis). The heuristic search done for the MP analyses resulted in 100 trees and a strict consensus tree (Fig. 3) were computed (tree length = 487 steps, CI = 0.37, RI = 0.87).

The LSU phylogenetic tree (Fig. 3) is similar to a portion of the tree generated by Castlebury et al. (2002), although the present study includes a substantially greater number of taxa representing the *Cryphonectria-Endothia* complex. Other lineages in the phylogram represent the families Gnomoniaceae *sensu stricto*, Melanconidaceae *sensu stricto*, a *Schizoparme* complex including the anamorph genera *Coniella* and *Pilidiella*, the Valsaceae *sensu stricto* and the Diaporthaceae *sensu stricto* (Castlebury et al. 2002, Zhang and Blackwell, 2001).

The *Cryphonectria-Endothia* complex, as it is presented in Fig. 3, includes *C. parasitica* (AF 277132), *Cryptodiaporthe corni* (AF 277133) and *Endothia eugeniae* (AF 277142) included in the study of Zhang and Blackwell (2001). *Endothiella gyrosa* (AF 362555), *Cryptodiaporthe corni* (AF 408343), *Chromendothia citrina* (AF 408335), *Cryphonectria macrospora* from Russia (AF 408340), *Cryphonectria nitschkei* from Russia (AF 408341), *Cryphonectria cubensis* (AF 408338) and *Cryphonectria havanensis* (AF 408339) were included in the study of Castlebury et al. (2002). The taxa selected as representatives of the different clades in the ITS/β-tubulin phylogram (Fig. 1) in this study, all grouped within the *Cryphonectria-Endothia* complex. The phylogenetic groupings in the LSU phylogram (Fig. 3) mirrored those of the ITS/β-tubulin phylogram (Fig. 1) and similar species grouped together.

The isolates representing the species *Cryptodiaporthe corni* (Castlebury et al. 2002, Zhang and Blackwell 2001) and *Chromendothia citrina* (Castlebury et al. 2002) grouped within the *Cryphonectria-Endothia* complex. The grouping of the *C. corni* isolate with those in the *Cryphonectria-Endothia* complex was strange as *C. corni* grouped separately from the type species of *Cryptodiaporthe*, *C. aesculi*. Castlebury et al. (2002) suggested that C. corni might reside in either *Cryphonectria* or *Endothia*. The *E. eugeniae* isolate included in the study of Zhang and Blackwell (2001) grouped with the undescribed fungi on clove (CMW 10781) (Myburg et al. 2003a) and *S. cordatum* in South Africa (CMW 9978). The isolates identified as *C. radicalis* and *C. gyrosa* (CMW 10469 and CMW 10470) from New Zealand (Myburg et al. 2003b) fall within the *Cryphonectria-Endothia* complex, even though they have three-septate ascospores, and grouped basal to those in the other clades in the ITS/β-tubulin tree (Fig.1). Isolates of *C. cubensis* grouped closely with the isolates of *C. cubensis* and the *C. havanensis* (AF 408339) included in the study of Castlebury et al. (2002). Interestingly, the *C. havanensis* (AF 408339) isolate, deposited in CBS as *C. havanensis* (CBS 505.63), is actually *C. cubensis*. Studies of Micales et al. (1987), Hodges et al. (1986) and Myburg et al. (2003b), based on cultural, morphological comparisons, protein profiles and phylogenetic analyses based on DNA sequence data have also shown that isolate CBS 503.63 was misidentified as *C. havanensis* and represents *C. cubensis*.

DISCUSSION

Ribosomal ITS and β-tubulin DNA sequence analyses show that a number of genera exist in a group of fungi that has previously been represented by *Cryphonectria* and *Endothia*. In

this study we putatively recognise eight new generic groups within the so-called *Cryphonectria-Endothia* complex. These generic groups are based on distinct morphological and/or molecular differences. These generic groups are represented by the following: *C. cubensis* (**clade 1**), the fungus occurring on *T. ivorensis* in Ecuador (**clade 2**), *C. eucalypti* and the *Endothiella* spp. from New Zealand (**clade 3**), the fungus on Colombian *M. theaezans* and *Tibouchina* spp. (**clade 4**), the fungus on Indonesian clove and South African waterberry trees occurring alongside *C. cubensis* (**clade 5**), the fungus on *T. urvilleana* from New Zealand (**clade 6**), the isolates from Mexico identified as *C. havanensis* and the fungus originating from Madeira and the Azores (**clade 7**) and the fungus on *E. dentatus* from New Zealand that is characterised by three-septate ascospores (**clade 10**). All species in these clades can be differentiated based on morphological features that are linked to stromatal shape and colour of the anamorph, position of both morphs relative to the bark and ascospore septation.

The LSU rDNA analysis of this study strongly support the view of Castlebury et al. (2002) that the *Cryphonectria-Endothia* complex based on distinct, monophyletic group within the Diaporthales and that it should be considered as a separate family in this order. The study of Castlebury et al. (2002) showed the distinct grouping of *Cryphonectria* and *Endothia* spp., although their results were based on only a small collection of isolates representing these genera. The large number of undescribed genera and species included in the present study add further justification for describing a family for these fungi as well as species of *Cryphonectria* and *Endothia*. This selection of species and genera are unified by the production of orange pigments in their stromatic tissue and in culture, as well as a purple

discolouration of the fruiting structures in 3% KOH and a yellow discolouration in lactic acid (Castlebury et al. 2002).

This study includes all the isolates currently known to us that represent species of *Cryphonectria* and *Endothia* and related genera. There are, however, some species for which no living cultures or authentic isolates, linked to previous descriptions, exist. These include *C. longirostris*, *C. coccolobii*, *C. havanensis* from Cuba or *C. gyrosa*, the type species of *Cryphonectria*. Morphological characteristics linked to clades 1-10 identified in this study, can be used to predict appropriate placements for the abovementioned taxa for which isolates are not available. These predictions would, however, be without phylogenetic support. For example, *C. longirostris* will be placed in the new genus that accommodates the fungus on *T. ivorensis* in Ecuador. This placement will be based on the fact that *C. longirostris* and the fungus on *T. ivorensis* have similar superficial rostrate conidiomata (M. Gryzenhout, personal communication).

The genera *Cryphonectria* and *Endothia* retain a significant taxonomic position in the Diaporthales, despite the fact that new generic groups have been identified. *Cryphonectria* and *Endothia* species represented in this study include some of the species that have been treated by authors such as Kobayashi (1970), Barr (1978), Roane, (1986), Micales and Stipes (1987) and Shear et al. (1917). These species are *C. parasitica*, *C. radicalis*, *C. macrospora*, *C. nitschkei*, *E. gyrosa* and *E. singularis*. It is unfortunate that no isolates linked to collections of *C. gyrosa* from Sri Lanka exist that can be used to confirm the relationship between the type species of *Cryphonectria* and the *Cryphonectria* species

included in this study. Isolates of *E. gyrosa*, the type species of *Endothia*, has been incorporated in this study although these isolates are not linked to the original type collection.

The remaining described species of *Cryphonectria*, namely *C. longirostris*, *C. cubensis*, *C. havanensis* and *C. coccolobii*, await further study. *Cryphonectria longirostris* will be transferred as a member of the new genus (**clade 2**) including the fungus on *T. ivorensis* in Ecuador (M. Gryzenhout, personal communication). *Cryphonectria cubensis sensu lato* is the only long-established species for which isolates exists, and it did not group with the other *Cryphonectria* spp. and will be described in a genus of its own. The taxonomic position of *C. havanensis* from Cuba and *C. coccolobii* still need to be considered. All of these species exhibit characteristics different from the type species *C. gyrosa* (K 109807) from Sri Lanka.

Cryphonectria cubensis sensu lato forms a well-delineated group (**clade 1**). The morphological characteristics of *Cryphonectria cubensis sensu lato*, that distinguish it from *Cryphonectria sensu stricto* and the type specimen of *C. gyrosa* (K 109807), are long, black perithecial necks and superficial, pyriform, blackened conidiomata (Myburg et al. 2002a). These morphological differences as well as the separate phylogenetic grouping support the suggestion of Myburg et al. (2002a), Myburg et al. (2003b) and Venter et al. (2001) that *C. cubensis sensu lato* should be considered in a discrete genus.

Cryphonectria cubensis sensu lato accommodates five sub-groups. These include isolates from Ecuador, South America/Congo, Colombia, South Africa and Southeast Asia/Australia. Isolates residing in these sub-groups will most likely be described as distinct species (M. Gryzenhout, personal communication). The proposed species are morphologically similar, but are well delineated based on distinct differences in the ITS and β-tubulin gene sequences.

Isolates of *C. cubensis sensu lato* have been reported to occur on a variety of host species throughout the world. These include species of *Eucalyptus* (Hodges et al. 1976), *Syzygium* (Heath et al. 2003, Hodges et al. 1986, Myburg et al. 2003a), *Miconia* (C. Rodas, personal communication) and *Tibouchina* (Myburg et al. 2002b, Wingfield et al. 2001). These tree species reside in the Myrtaceae and Melastomataceae (Myrtales). Two of these tree hosts, i.e. *Miconia* and *Tibouchina*, are native in South America, which suggests that *C. cubensis* comprising the South American/Congo phylogenetic clade (Fig. 1) might have an origin in South America. An origin in Indonesia has also been suggested by Hodges et al. (1986). This hypothesis could be valid for *C. cubensis* representing the Southeast Asian phylogenetic clade (Fig. 1) and occuring on native *Syzygium* species in Indonesia. Furthermore, it seems that *C. cubensis* isolates from South Africa have an origin on South African *Syzygium* species. Further studies at population level are needed to resolve these hypotheses regarding the origin of *C. cubensis* and its occurrence on *Eucalyptus*.

Myburg et al. (2003a) described the presence of a fungus resembling Cryphonectria occurring with *C. cubensis* on clove in Indonesia. Sequence data showed that this fungus

was closely related yet distinct from *Cryphonectria sensu stricto*. This relatedness was confirmed in the present study, but a conclusive identification of this *Cryphonectria*-like fungus could not be made due to lack of specimens on natural substrate that could be linked to these isolates. Artificial inoculations of an isolate of this fungus into *Eucalyptus* yielded conidiomata that were superficial, ovoid, black, and without necks (Myburg et al. 2003b), and which easily could be distinguished from the conidiomata of *C. cubensis* on eucalyptus which have long attenuated necks (Myburg et al. 2002a, Myburg et al. 2003a). This distinction should aid future collection and identification of this fungus on clove.

A *Cryphonectria*-like fungus was discovered occurring with *C. cubensis* on native waterberry trees (*S. cordatum*) in South Africa (M. Gryzenhout, personal communication). Phylogenetic analyses show that this fungus grouped with the *Cryphonectria*-like fungus occurring alongside *C. cubensis* on clove in Indonesia. Herbarium specimens of this fungus from waterberry contained teleomorphic stromata that were orange, semi-immersed and had short orange necks, different from the black necks of *C. cubensis*. Based on the close phylogenetic grouping of the *Cryphonectria*-like fungi from Indonesia and South Africa, we believe that this group of fungi should reside in a new genus. The teleomorph state of the fungus from Indonesia is unknown, while the anamorph state of the fungus from South Africa has not been found. Yet, the fungi represented in **clade 5** are morphologically characterised by blackened ovoid conidiomata without the long necks that is typical of *C. cubensis* on eucalyptus, and by semi-immersed ascomata with orange, well-developed stromatic tissue and orange tissue that covers the perithecial as they extend from the stromatal surface.

A canker pathogen on *Eucalyptus* in Australia and South Africa, previously known as *E. gyrosa*, recently has been described as a new species, *Cryphonectria eucalypti* (Venter et al. 2002). The placement of this fungus was justified based on stromatal similarities with *Cryphonectria* species and the close phylogenetic grouping of *C. eucalypti* isolates with species of *Cryphonectria* (Venter et al. 2002). When fungal groups that were more closely related to *Cryphonectria* were included, Myburg et al. (2003b) found that the *C. eucalypti* isolates were still more closely related to *Cryphonectria* than to species of *Endothia*, even though *C. eucalypti* has similar aseptate, allantoid ascospores usually considered characteristic of species of *Endothia*. Phylogenetic results from this study support this close relationship but also suggest that *C. eucalypti* most probably represents a distinct generic lineage (**clade 3**). The distinct grouping of *C. eucalypti* isolates results from the inclusion of isolates that possibly represents undescribed species belonging to this generic lineage, namely the isolates (CMW 10010, CMW 10011) labelled as *Endothiella* spp. from New Zealand. This generic distinction is supported by the fact that ascospores of *C. eucalypti* are unlike those of *Cryphonectria*.

Clade 6 accommodates the fungus on *T. urvilleana* in New Zealand. Despite the fact that this fungus is represented in this study by only two isolates, the orange-brown stromatic tissue of this fungus and sequence data justifies treating this fungus in a discrete genus. Unfortunately the anamorph of this fungus is absent on the available herbarium material. It is likely that since anamorph morphology has been one of the most important morphological characters to distinguish among the different clades in the phylogenetic tree, the anamorph

of this fungus is likely to yield better morphological criteria to separate this clade from the others.

The group of fungi residing in **Clade 7** and tentatively identified as *C. havanensis* from Mexico, and those isolated from *M. faya* in the Azores and Madeira, require further study prior to deciding on their identity. The occurrence of a fungus reminiscent of a *Cryphonectria* species but different from *C. cubensis*, has been reported before from Florida, USA on *Eucalyptus grandis* (Barnard et al. 1987, Barnard et al. 1993). It is possible that the fungus occurring on *Eucalyptus* spp. in Mexico (CMW 11297, CMW 11298) will be similar to the fungus reported from Florida. Further comparisons will also be needed to determine whether these fungi, annotated as *C. havanensis*, represents *C. havanensis* that was first described from Cuba (Bruner 1916). It is also possible that *C. coccolobii*, a fungus found on *Coccoloba uvifera* (L.) L. (seagrape) in Bermuda (Vizioli 1923, Waterston 1947) and Florida (Barnard et al. 1993) will reside in this group. These questions will, however, be difficult to pursue in the absence of additional isolates linked to specimens.

The overall results of this study reflect the importance of establishing a clear and well-defined delineation for species residing in the genera *Cryphonectria* and *Endothia*, and those identified in this study that need further characterisation. It is also clear that there are many more species and genera in this group than previously thought and we believe that there are others that have not yet been discovered. Results of this study have provided a framework for further collecting and characterising of genera and species in the family that is typified

by *Cryphonectria*. To aid future identifications the following key to the possible genera linked to the different phylogenetic clades is presented.

PLEASE NOTE THAT THE FOLLOWING KEY PRESENTED HERE IS IN PRELIMINARY FORM AND SHOULD NOT BE CITED. A DETAILED KEY WILL BE PRESENTED IN A FUTURE STUDY.

KEY:

1a. Conidiomata black.....2

1b. Conidiomata orange.....3

2a. Conidiomata pyriform, slender necks with orange apices; teleomorph unknown.....
.....Fungus on *M. theaezans* and *Tibouchina* spp., Colombia **(Clade 4)**

2b. Conidiomata pyriform with attenuated, uniformly black necks; ascomata have black necks.....*C. cubensis* **(Clade 1)**

2c. Conidiomata superficial, ovoid without a neck; ascomata with orange perithecial necks.....Fungi on *Syzygium* spp. Indonesia, South Africa **(Clade 5)**

3a. Stromata superficial.....4

3b. Stromata semi-immersed.....6

4a. Conidiomata rostrate, long slender necks; ascomata semi-immersed, no stromatic tissue except sheath around perithecial necks.....
.....Fungus on *Terminalia ivorensis*, Ecuador **(Clade 2)**

4b. Stromata rounded.....5

5a. Large, tubercular, multilocular conidiomata; aseptate ascospores.....

....*Endothia* **(Clade 9)**

5b. Large, conical conidiomata; one to three septated ascospores.....

.....Fungus on *Elaeocarpus* sp., New Zealand **(Clade 10)**

5c. Small, pulvinate stroma, one septated ascospores.....

.....*C. havanensis*-type fungi **(Clade 7)**

6a. Large, erumpent stromata, perithecial necks emerging from stromatal surface papillate, one septate ascospores..... .....*Cryphonectria* **(Clade 8)**

6b. Small stromata, aseptated ascospores.....*C. eucalypti* **(Clade 3)**

6c. Small, orange-brown stromata, perithecial necks emerging from stromatal surface long, one-septated ascospores.....

.....Fungus on *Tibouchina* spp., New Zealand **(Clade 6)**

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Table 1. Taxa included in the phylogenetic analyses. Taxa in bold represent fungal isolates sequenced in the present study. Taxa names indicated as "Undescribed" represent new fungal species awaiting description.

Isolate numbers [a]	Additional numbers [b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 11286	CRY 1471	*C. cubensis*	*Eucalyptus* sp.	Ecuador	M.J. Wingfield	AY 214289, AY 214217, AY 214253	AY 194096
CMW 11287	CRY 1473	*C. cubensis*	*Eucalyptus* sp.	Ecuador	M.J. Wingfield	AY 214290, AY 214218, AY214254	AY 194095
CMW 8757	CRY 268	*C. cubensis*	*Eucalyptus* sp.	Venezuela	M.J Wingfield	AF 046897, AF 273069, AF 273464	-
CMW 8758	CRY 243	*C. cubensis*	*Eucalyptus* sp.	Venezuela	M.J Wingfield	AF 046898, AF 273068, AF 273463	AY 194098
CMW 1853	CRY 138	*C. cubensis*	*Syzygium aromaticum*	Brazil	unknown	AF 036891, AF 273070, AF 273465	-
CMW 10667	-	*C. cubensis*	*Eucalyptus* sp.	Republic of Congo	M.J. Wingfield	AY 063477, AY 063479, AY 063481	-
CMW 10668	-	*C. cubensis*	*Eucalyptus* sp.	Republic of Congo	M.J Wingfield	AF 535121, AF 535123, AF 535125	-

Table 1. (continued)

Isolate numbers [a]	Additional numbers [b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 10669	-	*C. cubensis*	*Eucalyptus* sp.	Republic of Congo	M.J Wingfield	AF 535122, AF 535124, AF 535126	-
CMW 10453	CRY 1533, E40, CBS 505.63	*C. havanensis* [c]	*E. saligna*	Demographic Republic of Congo	R.J. Stipes	AY 063476, AY 063478, AY 063480	-
CMW 9970	CRY 2357	*C. cubensis*	*Miconia rubiginosa*	Colombia	C. Rodas M.J. Wingfield	AY 214291, AY 214219, AY 214255	-
CMW 9996	CRY 2404	*C. cubensis*	*M. rubiginosa*	Colombia	C. Rodas M.J. Wingfield	AY 214292, AY 214220, AY 214256	-
CMW 10025	-	*C. cubensis*	*M. rubiginosa*	Colombia	C. Rodas M.J. Wingfield	AY 214293, AY 214221, AY 214257	-
CMW 10026	-	*C. cubensis*	*M. rubiginosa*	Colombia	C. Rodas M.J. Wingfield	AY 214294, AY 214222, AY 214258	-
CMW 10028	-	*C. cubensis*	*M. rubiginosa*	Colombia	C. Rodas M.J. Wingfield	AY 214295, AY 214223, AY 214259	-

Table 1. (continued)

Isolate numbers [a]	Additional numbers [b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 10775	CRY 498	*Endothia eugeniae*	*Syzygium aromaticum*	Brazil	C.S. Hodges	AY 084003, AY 084015, AY 084027	-
CMW 10776	CRY 499	*E. eugeniae*	*S. aromaticum*	Brazil	C.S. Hodges	AY 084004, AY 084016, AY 084028	-
CMW 10777	CRY 500	*E. eugeniae*	*S. aromaticum*	Brazil	C.S. Hodges	AY 084005, AY 084017, AY 084029	-
CMW 10778	CRY 501	*E. eugeniae*	*S. aromaticum*	Brazil	C.S. Hodges	AY 084006, AY 084018, AY 084030	-
CMW 9979	-	*C. cubensis*	*Miconia theaezans*	Colombia	M.J. Wingfield	AY 214296, AY 214224, AY 214260	-
CMW 9980	-	*C. cubensis*	*M. theaezans*	Colombia	M.J. Wingfield	AY 214297, AY 214225, AY 214261	-
CMW 9993	-	*C. cubensis*	*M. theaezans*	Colombia	M.J. Wingfield	AY 214298, AY 214226, AY 214262	-

Table 1. (continued)

Isolate numbers [a]	Additional numbers [b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 9927	CRY 368	*C. cubensis*	*Tibouchina urvilleana*	Colombia	C. Rodas, M.J. Wingfield	AF 265653, AF 292034, AF 292037	-
CMW 9928	CRY 371	*C. cubensis*	*T. urvilleana*	Colombia	C. Rodas, M.J. Wingfield	AF 265654, AF 292035, AF 292038	-
CMW 9929	CRY 378	*C. cubensis*	*T. urvilleana*	Colombia	C. Rodas M.J. Wingfield	AF 265656, AF 292036, AF 292039	-
CMW 9932	CRY 675	*C. cubensis*	*T. granulosa*	South Africa	M.J. Wingfield	AF 273472, AF 273062, AF 273457	-
CMW 9327	CRY 782	*C. cubensis*	*T. granulosa*	South Africa	M.J. Wingfield	AF 273473, AF 273060, AF 273455	-
CMW 9328	CRY 783	*C. cubensis*	*T. granulosa*	South Africa	M.J. Wingfield	AF 273474, AF 273061, AF 273456	-
CMW 62	CRY 98	*C. cubensis*	*Eucalyptus grandis*	South Africa	M.J. Wingfield	AF 292041, AF 273063, AF 273458	AY 194097

Table 1. (continued)

Isolate numbers [a]	Additional numbers [b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 2113	CRY 0140	*C. cubensis*	*Eucalyptus grandis*	South Africa	M.J. Wingfield	AF 046892, AF 273067, AF 273462	-
CMW 8755	CRY 144	*C. cubensis*	*E. grandis*	South Africa	M.J. Wingfield	AF 292040, AF 273064, AF 273458	-
CMW 10192	-	*C. cubensis*	*Syzygium cordatum*	South Africa	M. Gryzenhout	AY 214299, AY 214227, AY 214263	-
CMW 8317	CRY 2089	*C. cubensis*	*E. camaldulensis*	Vietnam	M.J. Wingfield	AY 214300. AY 214228, AY 214264	-
CMW 8318	CRY 2090	*C. cubensis*	*E. camaldulensis*	Vietnam	M.J. Wingfield	AY 214301, AY 214229, AY 214265	-
CMW 1856	-	*C. cubensis*	*Eucalyptus* sp.	Hawaii	unknown	AY 083999, AY 084010, AY 084022	-
CMW 8756	CRY 289	*C. cubensis*	*E. grandis*	Indonesia	M.J. Wingfield	AF 046896, AF 273077, AF 375606	-

Table 1. (continued)

Isolate numbers [a]	Additional numbers [b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 9903	CRY 555	*C. cubensis*	*Eucalyptus* sp.	Kalimantan	M.J. Wingfield	AF 292044, AF 273066, AF 273461	-
CMW 2632	-	*C. cubensis*	*E. marginata*	Australia	E. Davison	AF 046893, AF 273078, AF 375607	-
CMW 3839	-	*E. eugeniae*	*Syzygium aromaticum*	Indonesia	M.J. Wingfield	AF 046904, AY 084011, AY 084023	-
CMW 8649		*E. eugeniae*	*S. aromaticum*	Sulawesi, Indonesia	M.J. Wingfield	AY 084000, AY 084012, AY 084025	-
CMW 8650	-	*E. eugeniae*	*S. aromaticum*	Sulawesi, Indonesia	M.J. Wingfield	AY 084001, AY 084013, AY 084024	-
CMW 8651	-	*E. eugeniae*	S. aromaticum	Sulawesi, Indonesia	M.J. Wingfield	AY 084002, AY 084014, AY 084026	
CMW 10774	CRY 497	*E. eugeniae*	S. aromaticum	Zanzibar, Tanzania	n.a	AF 492130, AF 492131, AF 492132	-
CMW 11288		*C. cubensis*	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	AY 214302, AY 214230, AY 214266	-

Table 1. (continued)

Isolate numbers [a]	Additional numbers [b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 11289	-	*C. cubensis*	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	AY 214303, AY 214231, AY 214267	-
CMW 11290	-	*C. cubensis*	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	AY 214304, AY 214232, AY 2143268	-
CMW 11291	-	*C. cubensis*	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	AY 214305, AY 214233, AY 2143269	-
CMW 11292	-	*C. cubensis*	*Eucalyptus* sp.	Vietnam	M.J. Wingfield	AY 214306, AY 214234, AY2143270	-
CMW 11293	-	*C. cubensis*	*Eucalyptus* sp.	Vietnam	M.J. Wingfield	AY 214307, AY 214235, AY 2143271	-
CMW 1840	CRY 0127	*C. cubensis*	*Eucalyptus camaldulensis*	China	unknown	AF 046890, AF 273071, AF 273466	-
CMW 9971	CRY 2345	Undescribed	*Terminalia ivorensis*	Ecuador	M.J. Wingfield	AY 167425, AY 167430, AY 167435	-

Table 1. (continued)

Isolate numbers [a]	Additional numbers [b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 9972	CRY 2346	Undescribed	*T. ivorensis*	Ecuador	M.J. Wingfield	AY 167426, AY 167431, AY 167436	AY 194092
CMW 9973	CRY 2348	Undescribed	*T. ivorensis*	Ecuador	M.J. Wingfield	AY 167427, AY 167432, AY 167437	-
CMW 10796	CRY 2353	Undescribed	*T. ivorensis*	Ecuador	M.J. Wingfield	AY 167428, AY 167433, AY 167438	-
CMW 9975	CRY 2355	Undescribed	*T. ivorensis*	Ecuador	M.J. Wingfield	AY 167429, AY 167434, AY 167439	-
CMW 10782	CRY 778	*Cryphonectria clavata*	*Quercus mongolica*	Japan	M. Kusunoki	AF 140242, AF 140248, AF 140254	-
CMW 10783	CRY 780	*C. clavata*	*Q. mongolica*	Japan	M. Kusunoki	AF 140244, AF 140250, AF 140256	-
CMW 10784	CRY 781	*C. clavata*	*Q. mongolica*	Japan	M. Kusunoki	AF 140245, AF 140249, AF 140257	-

Table 1. (continued)

Isolate numbers[a]	Additional numbers[b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 10785	CRY 1444	*C. clavata*	*Quercus* sp.	China	M. Milgroom and S. Kaneko	AF 140246, AF 140252, AF 140258	-
CMW 10786	CRY 1447	*C. clavata*	*Quercus* sp.	Japan	M. Milgroom and S. Kaneko	AF 140247, AF 140251, AF 140259	AY 194099
CMW 11294	E57	*C. clavata*	*Q. mongolica*	Japan	T. Kobayashi and S. Kaneko	AY 214211, AY 214213, AY 214215	-
CMW 10791	CRY 2789, E83	*C. radicalis*	*Q. suber*	Italy	M. Orsenigo	AF 548750, AF 548746, AF 548742	-
CMW 10455	CRY 1535, E42	*C. radicalis*	*Q. suber*	Italy	A. Biraghi	AF 452113, AF 525705, AF 525712	AY 194101
CMW 10477	CRY 1557, E76	*C. radicalis*	*Q. suber*	Italy	A. Biraghi	AF 368328, AF 368347, AF 368347	AY 194102
CMW 10788	CRY 809	*C. radicalis*	*Quercus* sp.	Greece	P. Cortesi	AY 143075, AY 143077, AY 143079	-

Table 1. (continued)

Isolate numbers [a]	Additional numbers [b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 10789	CRY 810	*C. radicalis*	*Quercus* sp.	Greece	P. Cortesi	AY 143076, AY 143078, AY 143080	-
CMW 10436	CRY 1516, E14	*Endothiella gyrosa* [d]	*Q. suber*	Portugal	B. d'Oliveira	AF 452117, AF 525703, AF 525710	-
CMW 10484	CRY 1564, E83	*C. radicalis*	*Q. suber*	Italy	A. Biraghi	AF 368327, AF 368349, AF 368349	-
CMW 10792	CRY 2790	*C. radicalis*	*C. sativa*	Switzerland	U. Heiniger	AF 548751, AF 548747, AF 548743	-
CMW 10793	CRY 2791	*C. radicalis*	*C. sativa*	Switzerland	U. Heiniger	AF 548752, AF 548748, AF 548744	-
CMW 10794	CRY 2792	*C. radicalis*	*C. sativa*	Switzerland	U. Heiniger	AF 548753, AF 548749, AF 548745	-
CMW 10790	CRY 779	*C. parasitica*	*Q. serrata*	Japan	M. Kusunoki	AF 140243, AF 140253, AF 140255	-

Table 1. (continued)

Isolate numbers [a]	Additional numbers [b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 7047	CRY 1507, E5	*C. parasitica*	*Q. virginiana*	USA	R.J. Stipes	AF 292042, AF 273073, AF 273469	-
CMW 7048	CRY 1511, E9	*C. parasitica*	*Q. virginiana*	USA	R.J. Stipes	AF 292043, AF 273076, AF 273470	AY 194100
CMW 1651	CRY 66	*C. parasitica*	*Q. virginiana*	USA	-	AF 046902, AF 273075, AF 273468	-
CMW 1652	CRY 44	*C. parasitica*	*Castanea dentata*	USA	-	AF 046901, AF 273074, AF 273467	-
CMW 10518	CRY 1669, E53	*C. nitschkei*	*Quercus* sp.	Japan	T. Kobayashi	AF 452118, AF 525706, AF 525713	-
CMW 10463	CRY 1543, E54	*C. macrospora*	*Castanopsis cupsidata*	Japan	T. Kobayashi	AF 368331, AF 368351, AF 368350	-
CMW 7036	CRY 62	*C. eucalypti*	*E. delegatensis*	Australia	M.J. Wingfield	AF 232878, AF 368341, AF 368340	AY 194105

Table 1. (continued)

Isolate numbers [a]	Additional numbers [b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 7037	CRY 45	*C. eucalypti*	*Eucalyptus* sp.	South Africa	M.J. Wingfield	AF 232880, AF 368343, AF 368342	AY 194106
CMW 10010	CRY 2401	*Endothiella* sp.	*E. fastigata*	New Zealand		AY 214308, AY 214236, AY 214272	AY 194112
CMW 10011	CRY 2402	*Endothiella* sp.	*Eucalyptus* sp.	New Zealand		AY 214309, AY 214237, AY 214273	AY 194113
CMW 10797	CRY 2399	*Endothiella* sp.	*E. regnans*	New Zealand		AY 214310. AY 214238. AY 214274	AY 1941011
CMW 10030	-	Undescribed	*Miconia theaezans*	Colombia	C. Rodas M.J. Wingfield	AY 214311, AY 214239, AY 214275	AY 194103
CMW 10032	-	Undescribed	*M. theaezans*	Colombia	C. Rodas M.J. Wingfield	AY 214312, AY 214240, AY 214276	AY 194104
CMW 10035	-	Undescribed	*M. theaezans*	Colombia	C. Rodas M.J. Wingfield	AY 214313, AY 214241, AY 214277	-

Table 1. (continued)

Isolate numbers [a]	Additional numbers [b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 11295	-	Undescribed	*M. theaezans*	Colombia	C. Rodas and M.J. Wingfield	AY 214314, AY 214242, AY 214278	AY 194089
CMW 11296	-	Undescribed	*M. theaezans*	Colombia	C. Rodas and M.J. Wingfield	AY 214315, AY 214243, AY 214279	AY 194090
CMW 10779	CRY 543	*Cryphonectria* sp.	*Eugenia aromatica*	Indonesia	M.J. Wingfield	AY 084007, AY 084019, AY 084031	-
CMW 10780	CRY 544	*Cryphonectria* sp.	*E. aromatica*	Indonesia	M.J. Wingfield	AY 084008, AY 084020, AY 084032	-
CMW 10781	CRY 554	*Cryphonectria* sp.	*E. aromatica*	Indonesia	M.J. Wingfield	AY 084009, AY 084021, AY 084033	AY 194093
CMW 9978	-	*Cryphonectria* sp.	*Syzygium cordatum*	South Africa	M. Gryzenhout	AY 214316, AY 214244, AY 214280	AY 194094
CMW 9945	-	Undescribed	*Tibouchina urvilleana*	New Zealand	M.J. Wingfield	AY 214317, AY 214245, AY 214281	AY 194109

Table 1. (continued)

Isolate numbers [a]	Additional numbers [b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 9946	-	Undescribed	*T. urvilleana*	New Zealand	M.J. Wingfield	AY 214318, AY 214246, AY 214282	AY 194110
CMW 11297	CRY 303	*Cryphonectria havanensis*	*Eucalyptus sp.*	Mexico	n.a	AY 214319, AY 214247, AY 214283	-
CMW 11298	CRY 514	*C. havanensis*	*Eucalyptus sp.*	Mexico	C.S. Hodges	AY 214320, AY 214248, AY 214284	AY 194091
CMW 11299	-	Undescribed	*Myrica faya*	Madeira	C.S. Hodges	AY 214321. AY 214249. AY 214285	AY 194087
CMW 11300	-	Undescribed	*M. faya*	Madeira	C.S. Hodges	AY 214322, AY 214250, AY 214286	AY 194088
CMW 11301	CRY 490	Undescribed	*M. faya*	Açores	C.S. Hodges	AY 214323, AY 214251, AY 214287	-
CMW 11302	CRY 491	Undescribed	*M. faya*	Açores	C.S. Hodges	AY 214324, AY 214252, AY 214288	-

Table 1. (continued)

Isolate numbers [a]	Additional numbers [b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 2091	CRY 1515, E13	*E. gyrosa*	*Quercus palustris*	USA	R.J. Stipes	AF 046905, AF 368337, AF 368336	AY 194114
CMW 10442	CRY 1522, E27	*E. gyrosa*	*Q. palustris*	USA	G.J. Samuels	AF 368326, AF 368339, AF 368338	AY 194115
CMW 10465	CRY 1545, E58	*E. singularis*	unknown	USA	R.J. Stipes	AF 368323, AF 368333, AF 368332	-
CMW 10469	CRY 1549, E67	*C. radicalis*	Spragg's bush	New Zealand	G.J. Samuels	AF 452111, AF 525707, AF 525714	AY 194107
CMW 10470	CRY 1550, E68	*C. radicalis*	Spragg's bush	New Zealand	G.J. Samuels	AF 452112, AF 525708, AF 525715	AY 194108
CMW 10471	CRY 1551, E70	*C. gyrosa*	*Elaeocarpus dentatus*	New Zealand	G.J. Samuels	AF 452116, AF 525709, AF 525716	-
CMW 5288	-	*Diaporthe ambigua*	*Malus domestica*	South Africa	W.A. Smit	AF 543817, AF 543819, AF 543821	-

Table 1. (continued)

Isolate numbers[a]	Additional numbers[b]	Original label name of taxon	Host	Origin	Collector	GenBank Accession numbers	
						ITS, β-tubulin	LSU
CMW 5587	-	*D. ambigua*	*M. domestica*	South Africa	W.A. Smit	AF 543818, AF 543820, AF543822	-

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

[b] Additional numbers that is linked to the CMW isolates. These isolates are also maintained in the culture collection of FABI.

CRY = *Cryphonectria* culture collection.

E = Culture numbers of isolates previously maintained in the culture collection of Prof. R.J. Stipes.

CBS = Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

[c] "*C. havanensis*" = *C. cubensis*

[d] "*Endothiella gyrosa*" = *C. radicalis*

Table 2. Herbarium specimens studied.

Linked to phylogenetic clade (Fig. 1)	Herbarium number [a]	Linked culture number	Current name of taxon	Original host name on label	Origin	Collector	Date
1	BPI 631857 (type)	n.a.	*Cryphonectria cubensis*	*Eucalyptus botryoides* Sm.	Cuba	S.C. Bruner	1916
1	PREM 57297	n.a.	*C. cubensis*	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	2001
1	PREM 57294	n.a.	*C. cubensis*	*E. grandis*	Colombia	M.J. Wingfield	2000
1	PREM 57293	n.a.	*C. cubensis*	*E. grandis*	South Africa	M. Venter	2001
1	PREM 57518	CMW 11286 CMW 11287	*C. cubensis*	*Eucalyptus* sp.	Ecuador	M.J. Wingfield	2001
1	PREM 56914	CMW 9927 CMW 9928	*C. cubensis*	*Tibouchina urvilleana*	Colombia	M.J. Wingfield	1999
2	PREM 57519	CMW 9972 CMW 10796	Undescribed	*Terminalia ivorensis*	Ecuador	M.J. Wingfield	2001
2	NYBG 4340 (type)		*C. longirostris*	Fallen tree	Puerto Rico	A. Heller	1900
3	PREM 56211	n.a.	*C. eucalypti* (type)	*Eucalyptus grandis* X *camaldulensis*	South Africa	M. Venter	1998

Table 2. (continued)

Linked to phylogenetic clade (Fig. 1)	Herbarium number [a]	Linked culture number	Current name of taxon	Original host name on label	Origin	Collector	Date
4	PREM 57520	CMW 10030 CMW 10032	Undescribed	*Miconia theaezans*	Colombia	C.A. Rodas	1998
5	PREM 57473	CMW 10781	Undescribed	Inoculation of isolate CMW 10781 into *E. grandis*	n.a.	M. Gryzenhout	2001
5	PREM 57521	CMW 9978	Undescribed	*Syzygium cordatum*	South Africa	M. Gryzenhout & R. Heath	2002
6	PREM 57522	CMW 9946	Undescribed	*T. urvilleana*	New Zealand	M.J. Wingfield	2002
7	PREM 57523	CMW 11298	*C. havanensis*	*E. saligna*	Mexico	C.S. Hodges	1998
7	PREM 57524	CMW 11299 CMW 11300	Undescribed	*Myrica faya*	Madeira	C.S. Hodges	2000
7	PREM 57525	CMW 11301 CMW 11302	Undescribed	*M. faya*	Azores	C.S. Hodges	unknown
8	K 109807	n.a.	*C. gyrosa* (type)	Bark	Sri Lanka	n.a.	1868
8	TFM 1057	n.a.	*C. macrospora* (type)	*Shiia sieboldii* Makino	Japan	T. Kobayashi	1954

Table 2. (continued)

Linked to phylogenetic clade (Fig. 1)	Herbarium number [a]	Linked culture number	Current name of taxon	Original host name on label	Origin	Collector	Date
8	TFM 1045	n.a.	*C. nitschkei* (type)	*Quercus grosseserrata* Bl.	Japan	T. Kobayashi	1954
8	CUP 2926	n.a.	*C. parasitica*	*Castanea dentata*	New York, USA	W.A. Murrill	1907
8	CUP 47983	n.a.	*C. parasitica*	*Castanea dentata*	Md., USA	D.S. Welch	1938
8	TFM 652	n.a.	*C. radicalis*	*Carpinus carpinoides*	Japan	T. Kobayashi	1962
8	BPI 797693	n.a.	*C. radicalis*	*Castanea* sp	Italy	Denotaris	1862
8	FPH 7609	n.a.	*C. clavata*	*Castanea crenata*	Japan	M. Milgroom	1998
9	PREM 56218	n.a.	*E. gyrosa*	*Q. phellos* L.	Raleigh, USA	L. Grand	1997
9	BPI 614515	n.a.	*E. singularis* (type)	*Q. gambelli*	Colorado, USA	E. Bethel	1911
10	PDD 32619	CMW 10471	Undescribed	*Elaeocarpus dentatus*	Auckland, New Zealand	G.J. Samuels	1973

Table 2. (continued)

Linked to phylogenetic clade (Fig. 1)	Herbarium number [a]	Linked culture number	Current name of taxon	Original host name on label	Origin	Collector	Date
10	PDD 20056	n.a.	Undescribed	*Elaeocarpus hookerianus* Raoul	Southland, New Zealand	J.M. Dingley	1948
10	PDD 21944	n.a.	Undescribed	*Elaeocarpus dentatus*	Auckland, New Zealand	J.M. Dingley	1963
10	NYBG 31874	CMW 10469 CMW 10470	Undescribed	Dead tree	Auckland, New Zealand	R.E. Beever	1973

[a] **BPI** = U.S. National Fungus Collections, Systematic Botany and Mycology, Rm. 304, Bldg. 011A, 10300 Baltimore Avenue, Beltsville, MD 20705-2350, USA;

PREM = National Collection of Fungi, Pretoria, South Africa;

TFM = Forestry and Forest Products Research Institute, P. O. Box 16, Tsukuba Norin Kenkyu, Danchi-Nai, Ibaraki, 305 Japan;

CUP = Plant Pathology Herbarium, Cornell University, 334 Plant Science Building, Ithaca, New York 14853-4203 USA; PDD, Landcare Research New Zealand Limited, Private Bag 92 170, 120 Mt. Albert Road, Mt. Albert, Auckland, New Zealand;

DAR = Plant Pathology Herbarium, Orange Agricultural Institute, Forest Road, Orange, N. S. W. 2800, Australia;

DAOM = National Mycological Herbarium, Eastern Cereal and Oilseed Center (ECORC), Agriculture and Agri-Food Canada, Edifice Wm. Saunders Building. #49, Ottawa, Ontario, Canada, KlA 0C6.

CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

Table 3. List of taxa included in Zhang and Blackwell (2001).

Taxon	Host	Collector	Culture no.	GenBank
Ampiporthe castanea (Tulasne) Barr	*Castanea sativa*		CBS 392.93	AF 277128
Apiognomonia supraseptata Kaneko et Kobayashi	*Quercus glauca*		ATCC 58737	AF 277127
Apioplagiostoma aceriferum (Cooke) Petr.	*Acer campestre*		CBS 781.79	AF 277129
Apiosporopsis carpinea (Fr.) Sacc.	*Carpinus hetulus*		CBS 771.79	AF 277130
Cryphonectria parasitica (Murr.) Barr	*Castanea sp.*		S. Anagnostakis 713	AF 277132
Cryptodiaporthe corni (Wehmeyer) Perr.	*Cornus alternifolia*		ATCC 66834	AF 277133
Discula campestris (Pass.) Arx	*Acer sp.*	S. Anagnostakis	S. Anagnostakis	AF 277140
Diaporthe phaseolorum	n.a.	n.a.	n.a.	U47830
Discula destructiva Redlin 254 (type)	*Cornus florida*	S. Redlin	S. Redlin	AF 277137
Discula fraxinae Peck	*Fraxinus sp.*	S. Anagnostakis	S. Anagnostakis	AF 277138
Discula quercina (Cooke) Sacc.	*Fraxinus sp.*	A. Rossman	A. Rossman	--
Discula sp.326	*Quercus sp.*	S. Anagnostakis	S. Anagnostakis	AF 277139
Endothia eugeniae (Nutman and Roberts) J. Reid and C. Booth	*Syzygium aromaticum*		CBS 534.82	AF 277142
Gnomonia padicola (Libert) Klebahn	*Prunus padus*		CBS 845.79	AF 277134
Gnomonia setaceae (Pers. Ex Fr.) Ces and de Not.	*Castanea sativa*		CBS 863.79	AF 277135
Linospora caprae (DC.) Fuckel	*Salix caprea*		CBS 372.69	AF 277143
Melanconis marginalis (Peck) Wehmeyer	*Alnus tenuifolia*		ATCC 56907	AF 277144
Plagiostoma euphorbiae Fuckel	*Euphorbia palustris*		CBS 340.78	AF 277131
Pleuroceras pleurostylum (Auerswald) Barr	*Salix Helvetica*		CBS 906.79	AF 277145
Valsa ambiens ssp. *Leucostomoides* (Peck) Spielman	*Acer rubrum*		ATCC 52280	AF 277146

ATCC = American Type Culture Collection, P.O. Box 1549, Manassas, VA 20108, USA.

CBS = Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

Table 4. List of taxa included in Castlebury et al. (2002).

Taxon [a]	Country	Host	Collector	Specimen no.[b]	Culture no. [c]	GenBank LSU
Apiognomnia errabunda (Roberge) Höhn [anamorph *Discula umbrinella* (Berk. and Broome) M. Morelet]	Switzerland	*Fagus sylvatica*	M. Monod	--	AR 2813 (= CBS 109747)	AF 408334
Chromendothia citrina Lar. N. Vassiljeva	Russia	*Quercus mongolica* Fisch. ex Ledeb.	L. Vasilyeva	BPI 747935	AR 3446 (= CBS 109758)	AF 408335
Coniella australiensis Petr.	South Africa	Leaf litter	K.T. van Warmelo	BPI 748425	IMI 261318	AF 408336
***Coniella fragariae* (Oudem.) B. Sutton**	India	Soil	V.V. Bhatt	BPI 841767	IMI 081599	AF 408391
Coniella fragariae	USA: Minnesota	*Lythrum salicaria*	n.a.	BPI 747949	AR 3382 (= ATCC PTA-275)	AF 362553
Coniella musaiensis B. Sutton var. hibisci B. Sutton	?Africa	*Hibiscus* sp.	R.R. Cervantes	BPI 748426	AR 3534 (= CBS 109757)	AF 408337
Cryphonectria cubensis (Bruner) Hodges	Cameroon	*Eucalyptus urophylla* S.T. Blake	I.A.S Gibson	BPI 841768	CBS 101281	AF 408338
Cryphonectria havanensis (Bruner) M.E. Barr	Zaire	*Eucalyptus saligna* Sm.	Unknown	BPI 748427	CBS 505.63	AF 408339
Cryphonectria macrospora (Tak. Kobay. & Kaz. Itô) M.E. Barr	Russia	*Quercus mongolica*	L. Vasilyeva	BPI 748428	AR 3444 (= CBS 109764)	AF 408340

Table 4. (continued)

Taxon [a]	Country	Host	Collector	Specimen no.[b]	Culture no. [c]	GenBank LSU
Cryphonectria nitschkei (G.H. Otto) M.E. Barr	Russia	*Quercus mongolica*	L. Vasilyeva	BPI 748429	AR 3433 (= CBS 109776)	AF 408341
Cryptodiaporthe aesculi (Fuckel) Petr.	Austria	*Aesculus hippocastanum*	W. Jaklitsch	BPI 748430	AR 3580 ex WJ 1695 (= CBS 109765)	AF 408342
Cryptodiaporthe corni (Wehm.) Petr.	USA: Maine	*Cornus alternifolia* L.f.	S. Redlin	BPI 747916	AR 2814 (= CBS 245.90)	AF 408343
Cryptodiaporthe hystrix (Tode) Petr.	Austria	*Acer pseudoplantanus*	W. Jaklitsch	BPI 748431	AR 3565 ex WJ 1491 (= CBS 109759)	AF 408344
Cryptodiaporthe salicella (Fr.) Petr.	Austria	*Salix*. sp	W. Jaklitsch	BPI 747938	AR 3455 ex WJ 1463 (= CBS 109775)	AF 408345
***Cryptosporella hypodermia* (Fr.) Sacc.**	Austria	*Ulmus minor* Mill.	W. Jaklitsch	BPI 748432	AR 3552 ex WJ 1694	AF 408346
Cryptosporella hypodermia	Austria	*Ulmus minor/laevis* Pall.	W. Jaklitsch	BPI 748433	AR 3566 ex WJ 1497 (= CBS 109753)	AF 408347
Diaporthe arctii	USA: New Jersey	*Ambrosia trifida*	n.a.	BPI 747273	AR 3450	AF 362562
Diaporthe decendens (Fr.) Fuckel	Austria	*Corylus avellana* L.	W. Jaklitsch	BPI 747942	AR 3459 ex WJ 1473 (= CBS 109772)	AF 408348
Diaporthe detrusa (Fr.) Fuckel	Austria	*Berberis vulgaris* L.	W. Jaklitsch	BPI 748434	AR 3424 ex WJ 1445 (= CBS 109770)	AF 408349

Table 4. (continued)

Taxon [a]	Country	Host	Collector	Specimen no.[b]	Culture no. [c]	GenBank LSU
***Diaporthe eres* Nitschke**	Austria	*Acer campestre* L.	W. Jaklitsch	BPI 748435	AR 3538 ex WJ 1643 (= CBS 109767)	AF 408350
Diaporthe eres	Austria	*Corylus avellana*	n.a.	BPI 747936	AR 3519	AF 362565
Diaporthe fibrosa (Pers.:Fr) Nitschke	Austria	*Rhamus catharticus* L.	W. Jaklitsch	BPI 747929	AR 3425 ex WJ 1417 (= CBS 109751)	AF 408351
Diaporthe medusae Nitschke	Austria	*Laburnum anapyroides*	W. Jaklitsch	BPI 748231	AR 3422 ex WJ 1443 (= CBS 109492)	AF 3408352
Diaporthe oncostoma (Duby) Fuckel	Russia	*Robinia pseudoacacia* L.	L. Vasilyeva	BPI 747934	AR 3445 (= CBS 109741)	AF 408353
Diaporthe padi G. H. Otto	Austria	*Prunus padus* L.	W. Jaklitsch	BPI 748436	AR 3419 ex WJ 1458 (= CBS 109784)	AF 408354
Diaporthe pardalota (Mont.) Fuckel	Canada: British Columbia	*Epilobium augustifolium* L.	M. Barr	BPI 747946	AR 3478 ex MBB 10220 (= CBS 109768)	AF 408355
Diaporthe perjuncta Niessl	Austria	*Ulmus glabra* Huds.	W. Jaklitsch	BPI 748437	AR 3461 ex WJ 1480 (= CBS 109745)	AF 408356
Diaporthe pustulata (Desm,) Sacc.	Austria	*Acer pseudoplatanus*	W. Jaklitsch	BPI 747928	AR 3430 ex WJ 1428 (= CBS 109742)	AF 408357
Diaporthe pustulata	Austria	*Acer pseudoplatanus*	W. Jaklitsch	BPI 748438	AR 3535 ex WJ 1628 (= CBS 109760)	AF 408358
Discula destructiva	USA: Maryland	*Cornus florida*	n.a.	n.a.	ATCC 76230	AF 362568

Table 4. (continued)

Taxon [a]	Country	Host	Collector	Specimen no.[b]	Culture no. [c]	GenBank LSU
Discula destructiva Redlin	USA: Washington	*Cornus nuttallii* Audubon	M. Daughtrey	BPI 1107757	AR 2596 (= CBS 109771)	AF 408359
***Ditopella ditopa* (Fr.:Fr.) J. Schröt.**	Austria	*Acer glutinosa* (L.) Gaertn.	W. Jaklitsch	BPI 748439	AR 3423 ex WJ 1443 (= CBS 109748)	AF 408360
Endothiella gyrosa	USA: Maryland	*Quercus sp.*	n.a.	n.a.	AR 3396	AF 362555
Gnomoniella fraxinae (anamorph: *Discula fraxinea*)	USA: Maryland	*Fraxinus pennsylvanica*	n.a.	n.a.	AR 2789	AF 362552
***Gnomonia gnomon* (Tode : Fr.) J. Schröt.**	Italy	*Carylus avellana*	M. Ribaldi	--	CBS 199.53	AF 408361
Gnomonia leptostyla (Fr.:Fr.) Ces. and de Not. [anamorph *Marssonina juglandis* (Lib.) Magnus)	USA: Illinois	*Juglans nigra* L.	D. Neely	BPI 747976	FAU 543	AF 408362
Gnomonia setacea	USA: New Jersey	*Quercus prinus*	n.a.	BPI 747274	AR 3451	AF 362563
Greeneria uvicola	USA: Ohio	*Vitis sp.*	n.a.	n.a.	n.a.	AF 362670
***Harkenessia eucalypti* Cooke**	Australia	*Eucalyptus regnans* F. Muell.	Z-q. Yuan	--	CBS 342.97	AF 408363
Harkenessia lythri D.F. Farr & Rossman	USA: Minnesota	*Lythrum salicaria* L.	E. Katovich	BPI 747560	AR 3383 (=ATCC PTA-2756)	AF 408363

Table 4. (continued)

Taxon [a]	Country	Host	Collector	Specimen no.[b]	Culture no. [c]	GenBank LSU
***Hercospora tiliae* (Pers.:Fr.) Fr.**	Austria	*Tilia tomentosa* Moench	W. Jaklitsch	BPI 748440	AR 3526 ex WJ 1600 (= CBS 109746)	AF 408365
Leucostoma auerswaldi Nitschke	Austria	*Frangula alnus* Mill.	W. Jaklitsch	BPI 748456	AR 3428 ex WJ 1424 (= CBS 109774	AF 408384
Leucostoma cincta (Fr.:Fr.) Höhn	Russia	*Padus maackii* Rupr.	L. Vasilyeva	BPI 748441	AR 3415 (= CBS 109766))	AF 408366
Leucostoma nivea (Hoffm.:Fr.) Höhn	Austria	*Salix pupurea* L.	W. Jaklitsch	BPI 748442	AR3512 ex WJ 1555 (= CBS 109743)	AF 408367
Leucostoma nivea	Russia:	*Populus* sp.	n.a.	BPI 748232	AR 3413	AF 362558
Mazzantia napelli (Ces.) Sacc.	Austria	*Aconitum vulparia* Rchb.	W. Jaklitsch	BPI 748443	AR 3498 ex WJ 1531 (= CBS 109769)	AF 408368
Melanconis alni Tul.	Austria	*Alnus viridis* (Vill.) Lam. & DC.	W. Jaklitsch	BPI 748444	AR 3500 ex WJ 1542 (= CBS 109773)	AF 408371
Melanconis alni	Russia: Sakhalin Island	*Duschekia maximowiczii*	n.a.	BPI 748233	AR 3529	AF 362566
Melanconis desmazierii Petr.	Austria	*Tilia* sp.	W. Jaklitsch	BPI 748445	AR 3525 ex WJ 1588 (= CBS 109780)	AF 408372
Melanconis marginalis (Peck) Wehm.	Canada: British Columbia	*Alnus rubra* Bong.	M. Barr	BPI 748446	AR 3442 ex MBB 1021A (= CBS 109744)	AF 408373

Table 4. (continued)

Taxon [a]	Country	Host	Collector	Specimen no.[b]	Culture no. [c]	GenBank LSU
***Melanconis stilbostoma* (Fr.) Tul.**	Austria	*Betula pendula* Roth	W. Jaklitsch	BPI 748447	AR 3501 ex WJ 1543 (= CBS 109778)	AF 408374
Melanconis stilbostoma	Russia: Sakhalin Island	*Betula sp.*	n.a.	BPI 748234	AR 3548	AF 362567
Ophiovalsa betulae (Tul. and C. Tul.) Petr. anamorph *Discula betulina* (Sacc.) Höhn.)	Austria	*Betula pendula*	W. Jaklitsch	BPI 748448	AR 3524 ex WJ 1610 (= CBS 109763)	AF 408375
***Ophiovalsa suffusa* (Fr.) Petr.** [anamorph Disculina vulgaris (Fr.) B. Sutton]	Austria	*Alnus incana* (L.) Moench	W. Jaklitsch	BPI 748449	AR 3496 ex WJ 1556 (= CBS 109750)	AF 408376
***Phragmaporthe conformis* (Berk. and Broome) Petr.**	Canada: British Columbia	*Alnus rubra*	M. Barr	BPI 748450	AR 3632 ex MBB 10338 (= CBS 109783)	AF 408377
***Pilidiella castaneicola* (Ellis and Everh.) Arx**	Korea	unknown	K.S. Bae	BPI 748451	CBS 143.97	AF 408378
Pilidiella granati (Sacc.) Aa	Cyprus	*Punica granatum* L.	R.M. Natrass	BPI 748452	CBS 152.33	AF 408379
Pilidiella granati	Turkey	*Punica granatum*	N. Kaskalöglu	BPI 748453	CBS 814.71	AF 408380
Plagiostoma conradii (Ellis) M.E. Barr	USA: New Jersey	*Hudsonia tomentosa* Nutt.	G. Bills	BPI 746482	AR 3488 (= CBS 109761)	AF 408381
***Plagiostoma euphorbiae* (Fuckel) Fuckel**	Netherlands	*Euphorbia palustris* L.	Unknown	--	CBS 340.78	AF 408382

Table 4. (continued)

Taxon [a]	Country	Host	Collector	Specimen no. [b]	Culture no. [c]	Genbank LSU
Shizoparme botrytidis Samuals	Puerto Rico	Dead wood	S. Huhndorf	BPI 748454	SMH 1354 (= AR 3504)	AF 408383
Shizoparme straminea	USA: Virginia	*Rosa rugosa*	n.a.	BPI 797000	CBS 149.22	AF 362569
Valsa ambiens	Austria	*Fagus sylvatica*	n.a.	BPI 748237	AR 3516	AF 362564
Valsa cenisia De Not.	Austria	*Juniperus communis* L.	W. Jaklitsch	BPI 748457	AR 3522 ex WJ 1583 (= CBS 109752)	AF 408385
Valsa ceratosperma (Tode: Fr.) Maire	Russia	*Quercus mongolica*	L. Vasilyeva	BPI 748458	AR 3416 (= CBS 109756)	AF 408386
Valsa ceratosperma	Austria	*Quercus robur* L.	W. Jaklitsch	BPI 748459	AR 3426 ex WJ 1425 (= CBS 109756)	AF 408387
Valsa germanica	Austria	*Salix alba*	n.a.	BPI 748236	AR 3427	AF 362561
Valsa mali	Russia: Primorsky Territory	*Malus sp.*	n.a.	BPI 748235	AR 3417	AF 362559
Valsella adherens Fuckel	Russia	*Betula* sp.	L. Vasilyeva	BPI 748460	AR 3549 (= CBS 109782)	AF 408388
***Valsella salicis* Fuckel**	Italy	*Salix fragilis* L.	W. Jaklitsch	BPI 748461	AR 3514 ex WJ 1580 (= CBS 109754)	AF 408389
Wuestneia molokaiensis Crous & J.D. Rogers	USA: Hawaii	*Eucalyptus robusta* Sm.	J. Rogers	BPI 748462	AR 3578 (= CBS 109779)	AF 408390

Table 4. (continued)

Taxon [a]	Country	Host	Collector	Specimen no. [b]	Culture no. [c]	Genbank LSU
[d]*Magnaporthe grisea* (T.T. Herbert) Yaegashi & Udugawa	n.a.	n.a.	n.a.	n.a.	n.a.	AB 026819
[d]*Pyricularia grisea*	USA: Pennsylvania	*Lolium perenne*	n.a.	n.a.	AR 3390	AF 362554
[d]*Gaeumannomyces graminis* (Sacc.) Arx & D. Oliver	United Kingdom	*Avena* sp.	n.a.	n.a.	AR 3400	AF 362556
[d]*Gaeumannomyces graminis*	USA: Georgia	*Glycine* sp.	n.a.	n.a.	AR 3401	AF 362557

[a] Type species of genus in bold

[b] **BPI** = U.S. National Fungus Collection.

[c] **AR** = Amy Rossman, Systematic Botany and Mycology Laboratory, USDA-ARS, 10300 Baltimore Ave., Beltsville, Maryland. USA 20705.

ATCC = American Type Culture Collection, P.O. Box 1549, Manassas, VA 20108, USA.

CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

FAU = Maintained by Amy Rossman (see address above).

IMI = International Mycological Institute, now CABI, Inc.

MBB = Margeret Barr Bigelow, Sidney, British Colombia.

SMH = Sabine M. Huhndorf, Field Museum, Chicago, IL.

WJ = Walter Jaklitsch, Van den langen Lüssen 31/2. A-1190, Vienna.

[d] Isolates included as outgroup taxa.

Fig. 1. A strict consensus tree (tree length = 3267 steps, CI = 0.51, RI = 0.9) generated from a combined data set comprising ribosomal (ITS 1, 5.8S, ITS 2) and β-tubulin gene sequences. Confidence levels of the tree branch nodes are indicated and were determined by a 1000 replicate bootstrap analysis. Taxa in bold represent the isolates that were sequenced in the present study. Species names in capital letters represent host species. *Diaporthe ambigua* isolates were used as the outgroup taxa to root the phylogenetic tree.

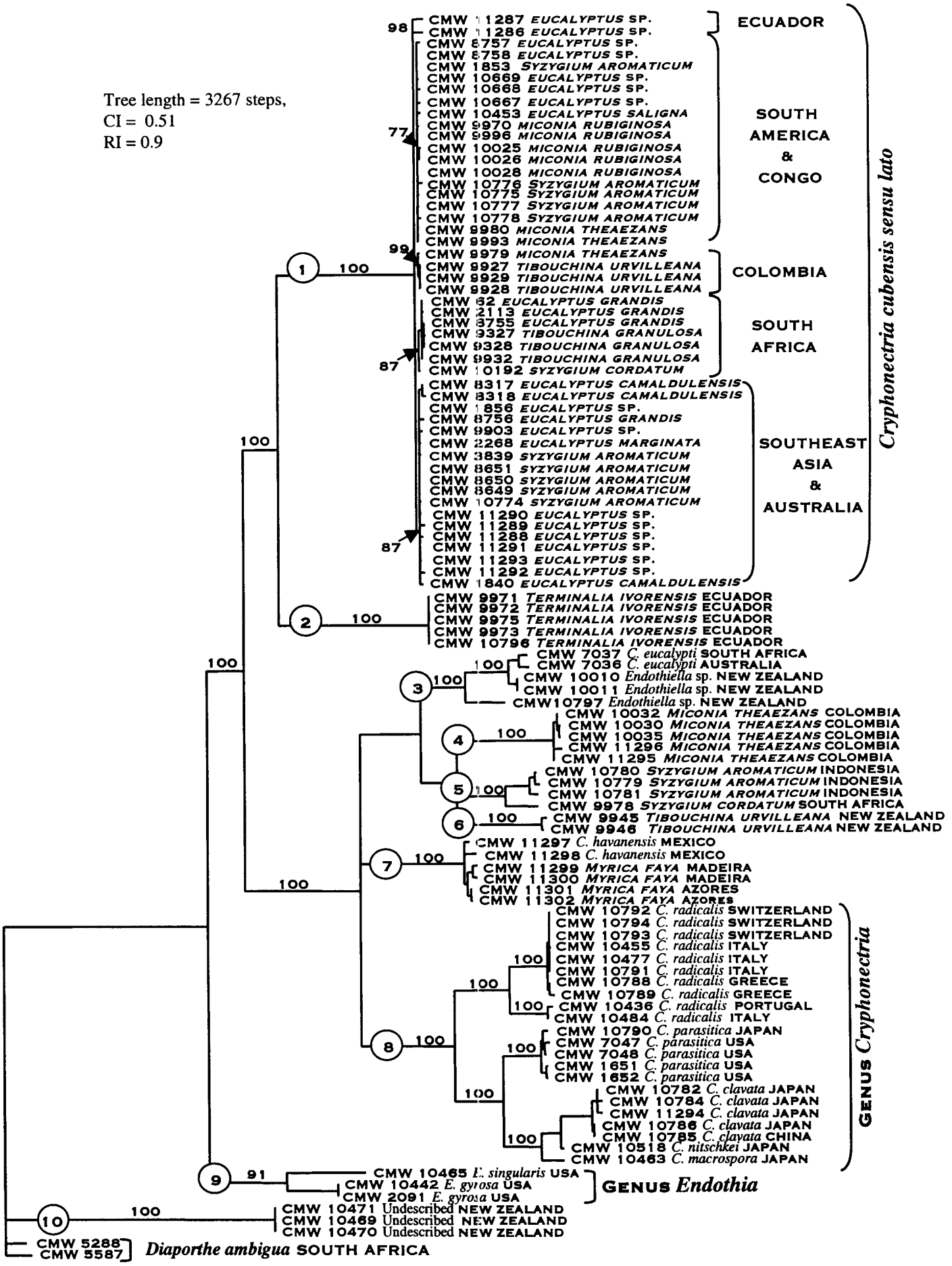
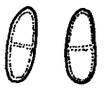
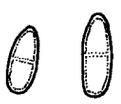


Fig. 2. Schematic drawings of the conidiomata, ascomata, ascopores and conidia for the fungal groups represented by the different phylogenetic clades. **a**. *Cryphonectria cubensis sensu lato* (**clade 1**). **b.** The fungus on *Terminalia ivorensis* from Ecuador (**clade 2**). **c.** *Cryphonectria eucalypti* and other species (**clade 3**). **d.** The fungus from Colombia (**clade 4**). **e**. Fungal species from *Syzygium* spp. in Indonesia and South Africa (**clade 5**). **f.** Fungus from *Tibouchina urvilleana* in New Zealand (**clade 6**). **g.** Fungi from Mexico, Azores and Madeira (**clade 7**). **h.** *Cryphonectria* (**clade 8,** ascospores not representing those of *C. eucalypti*). **i.** *Endothia* (**clade 9**). **j.** Fungus from *Elaeocarpus* spp. in New Zealand (**clade 10**).

Clade	Conidioma	Ascoma	Ascospores	Conidia
a) *Cryphonectria cubensis* **Clade 1**				
b) Fungus on *Terminalia ivorensis* **Clade 2**				
c) *C. eucalypti* **Clade 3**				
d) Fungus from Colombia **Clade 4**		—	—	
e) Fungus on *Syzygium* spp. **Clade 5**				
f) Fungus on *Tibouchina* from New Zealand **Clade 6**	—			—
g) Fungi from Mexico, Açores and Madeira **Clade 7**				
h) *Cryphonectria* **Clade 8**				
i) ***Endothia*** **Clade 9**				
) Fungus on *Elaeocarpus* rom New Zealand **Clade 10**