

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

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**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 the available isolates representing these two genera. 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, which will aid future studies and identification of species 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  $\beta$ -tubulin gene. In addition, the morphology of these species was 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 gyrosa* and

*E. singularis* were included in the *Endothia* clade. An isolate representing *E. viridistroma* grouped outside the *Endothia* clade and separately from other groups. Other clades outside the one 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.

**Key words:**  $\beta$ -tubulin genes, *Cryphonectria*, Diaporthales, *Endothia*, phylogeny, ribosomal DNA

### INTRODUCTION

*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), all 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*. Before the work of Barr, *Cryphonectria* was synonymous with the older *Endothia* (Kobayashi 1970, Shear et al 1917, von Höhnelt 1909). Barr (1978), however, segregated *Cryphonectria* from *Endothia* based on the differences in ascospore septation and stromatal morphology. Of the 13 species originally treated in *Endothia*, only three were retained, i.e., *E. gyrosa* (Schwein. : Fr.) Fr. (type species), *E. viridistroma* L.E. Wehmeyer and *E. singularis* (H. & B. Syd.) Shear & N.E. Stevens. The remaining species were transferred to *Cryphonectria*, and these included the type species *C. gyrosa* (Berk. & Broome) Sacc. (= *E. tropicalis* Shear & N.E. Stevens), *C. cubensis* (Bruner) Hodges, *C. havanensis* (Bruner) M.E. Barr, *C. macrospora* (Tak-Kobay & Kaz-Itô) M.E. Barr, *C. nitschkei* (Oth) M.E. Barr, *C. parasitica* (Murrill) M.E. Barr and *C. radicalis* (Schwein. : Fr.) M.E. 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*.

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*tria* by Micales and Stipes (1987) based on similarities in morphology with other species in *Cryphonectria*.

Of the species in *Cryphonectria* and *Endothia*, only *E. gyrosa*, *C. parasitica*, *C. cubensis* and the recently described *C. eucalypti* M. Venter & M.J. Wingf. (Venter et al 2002) are known to be serious pathogens. The remaining members of these genera are considered saprophytic (Roane et al 1986b). *Endothia gyrosa* causes cankers on 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). *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, 1979, Sharma et al 1985a, b, Wingfield et al 1989). This fungus also causes die-back on clove (*Syzygium aromaticum* [L.] Murrill & Perry) (Myrtaceae) (Hodges et al 1986) and a serious canker disease of *Tibouchina* species (Melastomataceae) (Myburg et al 2002a, Wingfield et al 2001). *Cryphonectria eucalypti* is a canker pathogen of *Eucalyptus* spp. 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 previously was known as *E. gyrosa* but was found to represent a distinct and new species (Venter et al 2001, 2002).

*Endothia* is characterized morphologically by strongly developed, widely erumpent stromata with predominantly pseudoparenchymatous tissue (Barr 1978, Micales and Stipes 1987). Perithecia usually are borne in an upright, diatrypod 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 often are 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). Venter et al (2002), however, showed that ascospore septation is not the only important and supportive character for generic identification and that stromatal morphology is more useful.

It is difficult to distinguish among species of *Endothia* and *Cryphonectria* based on morphology. Distinction is restricted mainly to size differences in

fruiting structures, but these often overlap (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), disk 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 be used only to distinguish among some species, especially *C. parasitica* and *E. gyrosa*.

Myburg et al (1999) provided the first phylogenetic data on representatives of *Endothia* and *Cryphonectria*, while attempting to resolve taxonomic questions pertaining to *C. cubensis*. This study supported the conspecificity of *E. eugeniae* (Nutman & Roberts) Reid & Booth with *C. cubensis* and showed clearly that *C. parasitica* is different from *E. gyrosa*. *Cryphonectria cubensis* isolates also were found to reside in two well-resolved subclades, reflecting a South American and a southeastern 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 subclade including isolates from South Africa was recognized when  $\beta$ -tubulin and histone *H3* gene sequences were used in phylogenetic analyses (Myburg et al 2002b).

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*. Isolates representing *C. parasitica*, *C. radicalis*, *C. macrospora*, *E. gyrosa* and *E. singularis* were included. *Endothia* and *Cryphonectria* grouped as two distinct phylogenetic clades (Venter et al 2002). Only a subset of isolates then available was used in the study of Venter et al (2002). No comprehensive study including morphology and phylogenetic data thus has been undertaken on available isolates representing the different species of these two genera. The objective of the present study, therefore, was to compare all available species of *Endothia* and *Cryphonectria* for which cultures and voucher 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 I) 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

TABLE I. Isolates used in this study

Isolate No. <sup>a</sup>	Alternative numbers <sup>b</sup>	Original label name of taxon <sup>c</sup>	Host	Origin	Collector	Genbank Accession No.
CMW 2113	CBS 112916	<i>Cryphonectria cubensis</i>	<i>Eucalyptus grandis</i>	South Africa	M.J. Wingfield	AF 046892, AF 273067, AF 273462
CMW 8755	—	<i>C. cubensis</i>	<i>E. grandis</i>	South Africa	M.J. Wingfield	AF 292040, AF 273064, AF 273459
CMW 62	—	<i>C. cubensis</i>	<i>E. grandis</i>	South Africa	M.J. Wingfield	AF 292041, AF 273063, AF 273458
CMW 1840	—	<i>C. cubensis</i>	<i>E. camaldulensis</i>	China	unknown	AF 046890, AF 273071, AF 273466
CMW 1853	—	<i>C. cubensis</i>	<i>Syzygium aromaticum</i>	Brazil	unknown	AF 036891, AF 273070, AF 273465
CMW 8757	—	<i>C. cubensis</i>	<i>Eucalyptus</i> sp.	Venezuela	M.J. Wingfield	AF 046897, AF 273069, AF 273464
CMW 8758	—	<i>C. cubensis</i>	<i>Eucalyptus</i> sp.	Venezuela	M.J. Wingfield	AF 046898, AF 273068, AF 273463
CMW 8756	—	<i>C. cubensis</i>	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	AF 046896, AF 273077, AF 375606
CMW 2632	—	<i>C. cubensis</i>	<i>E. marginata</i>	Australia	E. Davison	AF 046893, AF 273078, AF 375607
CMW 10453	E40, CBS 505.63	<i>C. cubensis</i>	<i>E. saligna</i>	Congo	unknown	AY 063476, AY 063478, AY 063480
CMW 10463	E54, CBS 112920	<i>C. macrospora</i>	<i>Castanopsis cuspidata</i>	Japan	T Kobayashi	AF 368331, AF 368351, AF 368350
CMW 10518	E53, CBS 112919	<i>C. nitschkei</i>	<i>Quercus</i> sp.	Japan	T. Kobayashi	AF 452118, AF 525706, AF 525713
CMW 1651	CBS 112913	<i>C. parasitica</i>	<i>Castanea dentata</i>	USA	unknown	AF 046901, AF 273074, AF 273467
CMW 1652	CBS 112914	<i>C. parasitica</i>	<i>Castanea dentata</i>	USA	unknown	AF 046902, AF 273075, AF 273468
CMW 10455	E42, CBS 238.54	<i>C. radicalis</i>	<i>Q. suber</i>	Italy	A. Biraghi	AF 452113, AF 525705, AF 525712
CMW 10477	E76, CBS 240.54	<i>C. radicalis</i>	<i>Castanea sativa</i>	Italy	A. Biraghi	AF 368328, AF 368347, AF 368346
CMW 10484	E83, CBS 112918	<i>C. radicalis</i>	<i>Castanea sativa</i>	Italy	A. Biraghi	AF 368327, AF 368349, AF 368349
CMW 10469	E67, CBS 112922	<i>C. radicalis</i>	<i>Elaeocarpus dentatus</i>	New Zealand	G. Samuels	AF 452111, AF 525707, AF 525714
CMW 10470	E68, CBS 112923	<i>C. radicalis</i>	<i>Elaeocarpus dentatus</i>	New Zealand	G. Samuels	AF 452112, AF 525708, AF 525715
CMW 10471	E70, CBS 112924	<i>C. gyrosa</i>	<i>Elaeocarpus dentatus</i>	New Zealand	G. Samuels	AF 452116, AF 525709, AF 525716
CMW 7037	—	<i>C. eucalypti</i>	<i>Eucalyptus</i> sp.	Australia	M.J. Wingfield	AF 232880, AF 368343, AF 368342
CMW 7036	—	<i>C. eucalypti</i>	<i>Eucalyptus</i> sp.	South Africa	M.J. Wingfield	AF 232878, AF 368341, AF 368340
CMW 10436	E14, CBS 165.30	<i>Endothia gyrosa</i>	<i>Q. suber</i>	Portugal	B. d'Oliviera	AF 452117, AF 525703, AF 525710
CMW 2091	E13, CBS 112915	<i>Endothia gyrosa</i>	<i>Q. palustris</i>	USA	R.J. Stipes	AF 046905, AF 368337, AF 368336
CMW 10442	—	<i>E. gyrosa</i>	<i>Q. palustris</i>	USA	R.J. Stipes	AF 368326, AF 368339, AF 368338
CMW 10465	E58, CBS 112921	<i>E. singularis</i>	unknown	USA	unknown	AF 368323, AF 368333, AF 368332
CMW 10454	E41, CBS 202.36	<i>E. viridistroma</i>	<i>Cercis canadensis</i> Castigl.	USA	J.H. Miller	AF 452120, AF 525704, AF 525711
CMW 2498	—	<i>Diaporthe ambigua</i>	<i>Malus sylvestris</i>	Netherlands	S. Truter	AF 046906, AF 273072, AF 273471

<sup>a</sup> Taxa presented in bold represent those for which sequences were generated in this study. Sequences for the other taxa were obtained from previous studies (Myburg et al 1999, Myburg et al 2002b, Roux 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.

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

<sup>c</sup> 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), “*Endothia gyrosa*” (CMW 10436) and “*E. viridistroma*” (CMW 10454) unidentified taxa.



available for *C. coccolobii*, *C. longirostris* or *C. havanensis*, and these could not be included in this study. The *C. havanensis* isolate labeled as E40 (CMW 10453) in the collection of R.J. Stipes previously was found to be *C. cubensis* (Micales et al 1987) and not representative of *C. havanensis*. Isolates of *C. eucalypti*, the most recent addition to *Cryphonectria* (Venter et al 2002), also were included in this study. All isolates (TABLE I) 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 subcultures have been deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

**DNA extractions, ribosomal RNA (ITS1, 5.8S, ITS2) and  $\beta$ -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  $\beta$ -tubulin gene were as described by Myburg et al (1999) and Myburg et al (2002b) respectively. The primer pairs that were used to amplify the respective regions were 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  $\beta$ -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<sup>®</sup> Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq<sup>®</sup> DNA Polymerase, FS (Perkin-Elmer, Warrington, United Kingdom). The nucleotide sequences were determined with an ABI PRISM 3100<sup>®</sup> 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 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  $\beta$ -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  $\beta$ -tubulin gene produced a phylogenetic tree with a similar topology to that obtained when the full dataset was considered collectively. However, there was no resolution at the intraspecific level using this more conservative dataset and all the sequence data thus were 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 whether the ribosomal (ITS1, 5.8S, ITS2) and  $\beta$ -tubulin gene sequence datasets (1a/1b, 2a/2b) could be combined as one dataset before phylogenetic analyses. Gene sequences were analyzed using heuristic searches with tree-bisection-reconnection (TBR) and MulTrees option (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 stomatal and spore morphology, were examined microscopically for relevant herbarium specimens of *Endothia* and *Cryphonectria* (TABLE II). 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 II), also were 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  $\mu$ m thick. Sections were dropped in water, transferred to a microscope slide, mounted in lactophenol and examined with phase contrast and differential interference contrast light microscopy.

## RESULTS

**Ribosomal RNA (ITS1, 5.8S, ITS2) and  $\beta$ -tubulin gene amplification and sequencing.**—Amplification products for the respective gene regions were 550–600 bp in size (data not shown). Sequences for isolates generated in this study were aligned with sequence data from previous studies (TABLE I). The PHT performed between the ribosomal and  $\beta$ -tubulin gene sequence datasets generated a *P*-value of 0.01. This indicated that there was no significant conflict between the datasets and that they could be combined in subsequent phylogenetic analyses. The ribosomal (ITS1, 5.8S, ITS2) and  $\beta$ -tubulin (1a/1b and 2a/2b) sequence datasets thus were analyzed together in the parsimony analyses.

The resulting combined dataset comprised 28 sequences of which one, *D. ambigua*, was used as outgroup taxon. A total of 1520 characters were included in the phylogenetic analyses. Of these, 942 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 23 trees, which were converted to a strict consensus tree (tree length = 1154 steps, consistency index = 0.6888, retention index = 0.8376).

The consensus tree (FIG. 1) showed a well-resolved clade labeled as “*Cryphonectria* spp.” and representing the taxa *C. parasitica*, *C. nitschkei*, *C. macrospora*,

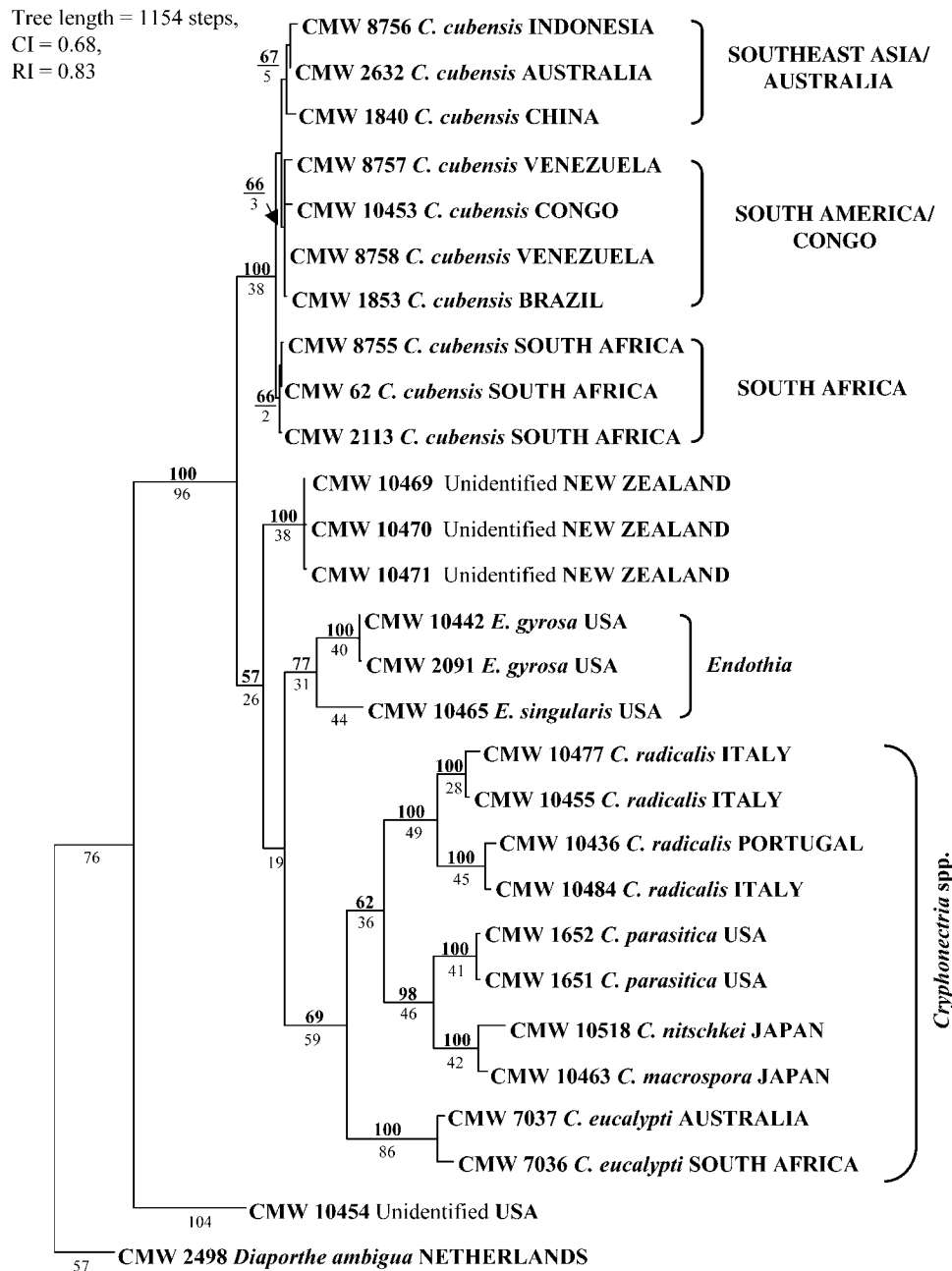


FIG. 1. A strict consensus tree (tree length = 1154 steps, consistency index = 0.6888, retention index = 0.8376) generated from a combined dataset comprising ribosomal and  $\beta$ -tubulin gene sequences. Confidence levels of the tree branch nodes (>50%) are indicated above the nodes and were determined by a 1000 replicate bootstrap analysis. Branch lengths are indicated below the nodes. *Diaporthe ambigua* was used as outgroup taxon.

*C. eucalypti* and European *C. radicalis*. 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 = 100%).

Two groups of isolates identified originally as species of *Cryphonectria* did not group within the main *Cryphonectria* clade. The first of these included un-

identified isolates from New Zealand, which originally were labeled *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 subclades as previously de-

TABLE II. Herbarium specimens examined in this study.

Herbarium No. <sup>a</sup>	Current name of taxon	Original label name on specimen	Original host name on label	Origin	Collector	Date
BPI 631857	<i>Cryphonectria cubensis</i> (type)	<i>Diaporthe cubensis</i>	<i>Eucalyptus botryoides</i> Sm.	Cuba	S.C. Bruner	1916
PREM 57297	<i>C. cubensis</i>	<i>Cryphonectria cubensis</i>	<i>Eucalyptus</i> sp.	Indonesia	M.J. Wingfield	2001
PREM 57294	<i>C. cubensis</i>	<i>C. cubensis</i>	<i>E. grandis</i>	Colombia	M.J. Wingfield	2000
MASS	<i>C. cubensis</i>	<i>C. gyrosa</i>	<i>E. grandis</i>	Minas Gerais, Brazil	C.S. Hodges	1973
PREM 57293	<i>C. cubensis</i>	<i>C. cubensis</i>	<i>E. grandis</i>	South Africa	M. Venter	2001
IMI 45450	<i>C. cubensis</i>	<i>Endothia eugeniae</i>	<i>Syzygium aromaticum</i>	Zanzibar	unknown	1951
K 109807	<i>C. gyrosa</i> (type)	<i>Nectria gyrosa</i> Berk. & Broome (#638)	Bark	Sri Lanka	unknown	1868
K 109809	<i>C. gyrosa</i>	unknown (#290)	Bark	Mount Eliya, Sri Lanka	G.H.K. Thwaites	n.a.
BPI 614797	<i>C. gyrosa</i>	<i>E. tropicalis</i> (type)	<i>Elaeocarpus glandulifer</i>	Hakgala, Sri Lanka	T. Petch	1913
BPI 614526	<i>C. gyrosa</i>	<i>E. tropicalis</i> (type)	<i>Elaeocarpus glandulifer</i>	Hakgala, Sri Lanka	T. Petch	1913
BPI 797701	<i>C. gyrosa</i>	<i>E. tropicalis</i>	<i>Elaeagnus glandulifer</i>	Hakgala, Sri Lanka	unknown	n.a.
PDD 32619 <sup>b</sup>	<i>C. gyrosa</i>	<i>E. tropicalis</i>	<i>Elaeocarpus dentatus</i>	Auckland, New Zealand	G.J. Samuels	1973
PDD 20056	<i>C. gyrosa</i>	<i>E. tropicalis</i>	<i>Elaeocarpus hookerianus</i>	Southland, New Zealand	J.M. Dingley	1948
PDD 21944	<i>C. gyrosa</i>	<i>E. tropicalis</i>	Raoul	Auckland, New Zealand	J.M. Dingley	1963
NY 31874 <sup>c</sup>	<i>C. radicalis</i>	<i>E. radicalis</i> (Schwein.: Fr.) Ces. & de Not.	Dead tree	Auckland, New Zealand	R.E. Beaver	1973
TFM 1057	<i>C. macrospora</i> (type)	<i>E. macrospora</i> Kobayashi & Ito (type)	<i>Shiia sieboldii</i>	Japan	T. Kobayashi	1954
TFM 1045	<i>C. nilschkei</i> (type)	<i>E. nilschkei</i> Oth.	<i>Quercus grosseserrata</i> Bl.	Japan	T. Kobayashi	1954
CUP 2926	<i>C. parasitica</i>	<i>Diaporthe parasitica</i> Murrill	<i>Castanea dentata</i>	New York, USA	W.A. Murrill	1907
CUP 47983	<i>C. parasitica</i>	<i>E. parasitica</i> (Murrill) P.J. & H.W. Anderson	<i>Castanea dentata</i>	Md., USA	D.S. Welch	1938
BPI 797697	<i>C. radicalis</i>	<i>E. radicalis</i> (Schwein.: Fr.)	<i>Castanea vesca</i>	Locarno, Switzerland	unknown	1862
BPI 613739	<i>C. radicalis</i>	<i>E. fluens</i> (Sow.) Shear & Stevens	<i>Castanea vesca</i>	Stresa, Italy	C.L. Shear	1913
PREM 56211	<i>C. eucalypti</i> (type)	<i>C. eucalypti</i> (type)	<i>Eucalyptus grandis</i> × <i>ca-maldulensis</i>	Nyalazi, South Africa	M. Venter	1998
PREM 56218	<i>E. gyrosa</i>	<i>E. gyrosa</i>	<i>Q. phellos</i> L.	Raleigh, USA	L. Grand	1997
BPI 614515	<i>E. singularis</i> (type)	<i>Calopactis singularis</i> Syd.	<i>Q. gambelli</i>	Colorado, USA	E. Bethel	1911
DAR 11235	<i>E. singularis</i>	<i>E. singularis</i>	<i>Q. gunnisonii</i>	Colorado, USA	G.G. Hedgcock & E. Bethel	1917
DAOM 3634	<i>E. viridistroma</i> (type)	<i>E. viridistroma</i> (type)	<i>Cercis canadensis</i>	Georgia, USA	J.H. Miller	1934
BPI 797702	<i>E. viridistroma</i>	<i>E. viridistroma</i>	<i>Cercis canadensis</i>	Georgia, USA	J.H. Miller	1934

<sup>a</sup>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; MASS, Herbarium, Biology Department, University of Massachusetts, Amherst, Massachusetts 01003-5810, USA; IMI, Herbarium, CABI Bioscience, Bakeham Lane, Egham, Surrey TW20 9TY, UK; NY, William and Lynda Steere Herbarium, New York Botanical Garden, Bronx, New York 10458-5126, USA; 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.

<sup>b</sup>Specimen linked to isolate CMW 10471 (TABLE I).

<sup>c</sup>Specimen linked to isolates CMW 10469 and CMW 10470 (TABLE I).

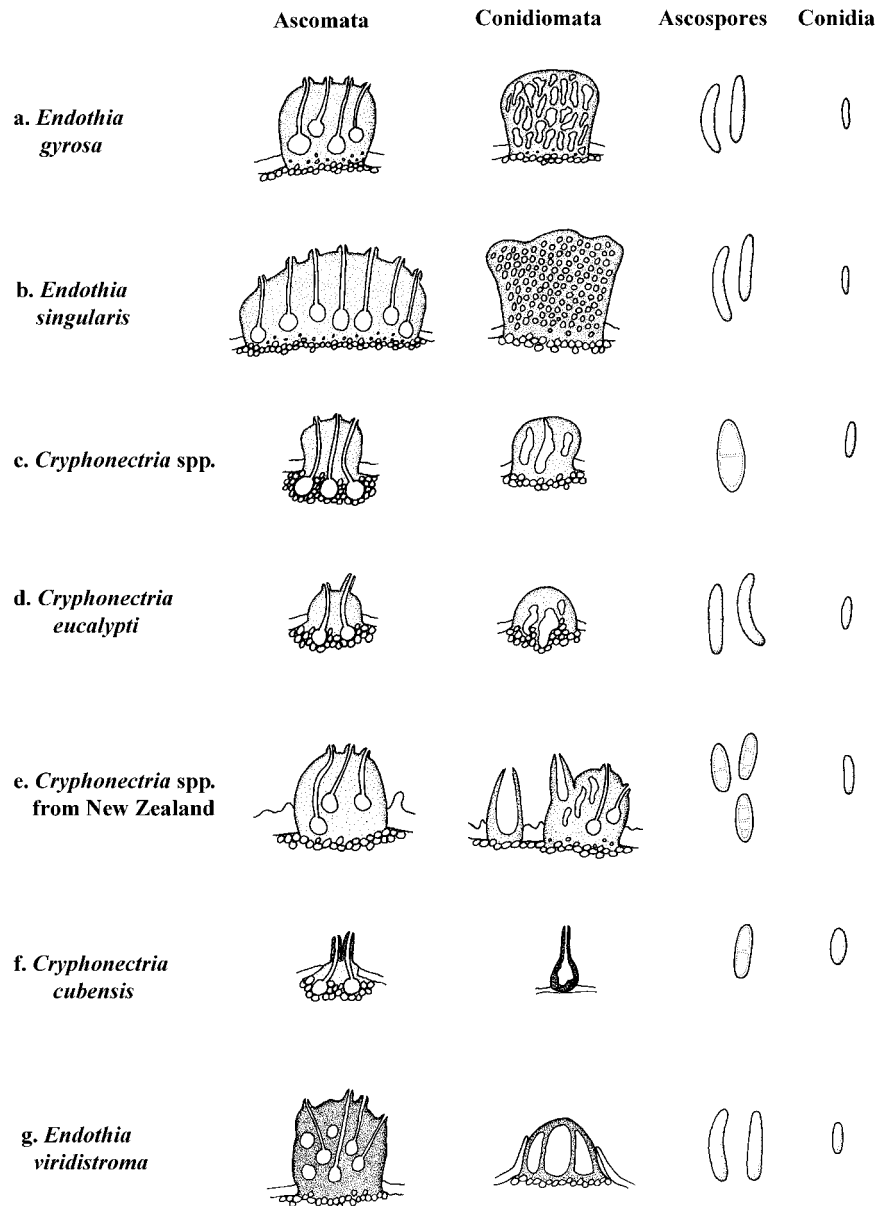


FIG. 2. Schematic drawings of the ascomata, conidiomata, ascospores and conidia of the fungi representing the different phylogenetic clades. a) *Endothia gyrosa*. b) *Endothia singularis*. c) *Cryphonectria* spp. representing *C. parasitica*, *C. radicalis* (Europe), *C. nitschkei*, *C. macrospora*.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

fined by Myburg et al (2002b) were evident and represented *C. cubensis* originating from South America/Congo, southeastern 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 re-

sult = 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. nit-*



*schkei*, *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 because 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) also were different and generally were unilocular, superficial and pyriform with attenuated necks. The conidiomata of *C. cubensis* were blackened, unlike the orange conidiomata of 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 to superficial and strongly developed. 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) also were erumpent but were semi-immersed and perithecia were valsoid, with the bases surrounded by bark tissue beneath the level of the bark. Ascumata of *C. cubensis* specimens (FIG. 2f) had weak to no stromatal tissue development, and the black perithecial necks, as they protruded through the stromatal surface, were covered with brown tissue, which was different from the black necks of the other specimens of *Cryphonectria* that were covered with orange tissue.

Ascospores of *E. gyrosa* (FIG. 2a) and *E. singularis* (FIG. 2b) were aseptate and cylindrical to allantoid. Those of the *Cryphonectria* spp. (FIG. 2c) and *C. cubensis* (FIG. 2f) were one-septate and 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 single-septate ascospores, although a few ascospores were seen with two septa. *Cryphonectria eucalypti* (FIG. 2d) had aseptate, cylindrical to allantoid ascospores that were different from 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 from 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 in the stroma 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*, however, is atypical of all the other species of *Endothia* and *Cryphonectria* because the stromata of this species are dark green to black.

#### DISCUSSION

This study presents a phylogenetic analysis of a large group of isolates that were accommodated in *Cryphonectria* and the closely related genus *Endothia*. The majority of these are linked to voucher specimens representing 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 labeled as *Cryphonectria* spp., occurring on *Eleaocarpus dentatus* from New Zealand, do not reside in *Cryphonectria* and the taxon representing *E. viridistroma* does not belong in *Endothia*. Studies including more specimens and isolates for each phylogenetic group now should be undertaken to describe formally the new generic groups proposed in this study. For instance, the relationships between the three phylogenetic subclades within *C. cubensis sensu lato* should be clarified. The sequence and morphological data that now are available also should facilitate future segregation among species in these genera.

To the best of our knowledge, all species for which cultures currently are 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 originally was identified as *C. gyrosa*, isolated from specimen PDD 32619, phylogenetically and morphologically was linked to the New Zealand group studied. This isolate most probably was identified as this species due to similarities in stromatal morphology and ascospore size and the fact that it was isolated from *Eleaocarpus* spp. (Roane 1986a, Shear et al 1917). It seems unlikely that this specimen (CMW 10471) represents the type species of *Cryphonectria*, and it will be impossible to resolve this question in the absence of isolates for *C. gyrosa* from Sri Lanka.



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 19th century, 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 was designated as a cotype by Shear et al (1917), contains only conidiomata (Shear et al 1917). Comparisons of teleomorph morphology using the current collections thus are impossible, and efforts to designate an epitype or neotype for *Endothia* will prove invaluable for future studies.

In the case of *C. gyrosa*, ambiguities include the existence of two sets of herbarium specimens that are linked to this fungus. The first (type specimen K 109807, K 109809) is connected to the original description of *C. gyrosa* (basionym *Diatrype gyrosa* Berk. & Broome) 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 Höhnelt 1909) and the new name, *E. tropicalis*, was given because the name *E. gyrosa* already existed. These BPI specimens (BPI 614526, BPI 614797) thus were 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 No. 290) was mentioned only as an additional collection examined (Shear et al 1917). The original type specimen (K 109807) of the protologue of *C. gyrosa*, however, will take priority to serve as type specimen for this species (ICBN section 7.4, Greuter et al 2000).

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). Barr (1978) also examined a specimen from *Eucalyptus grandis* Sm. in Brazil (MASS) for her treatment of *C. gyrosa*, but this specimen actually represents *C. cubensis* (TABLE II). The review of *C. gyrosa* by Barr (1978) stated that the fungus originally was described from Sri Lanka on *Elaeagnus glandulifer* and this possibly was based on specimen BPI 797701 that is stated to have occurred on the original host *Elaeagnus glandulifer* and was collected from the same locality (i.e., Hakgala, Sri Lanka) as BPI 614526

and BPI 614797. However, never has there 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 above-mentioned material given on the herbarium packet probably should have been *Elaeocarpus glandulifer*. Mention of *C. gyrosa* on *Quercus* spp. and *S. sieboldii* (Roane 1986a) possibly originated from reports of this fungus from Japan (Kobayashi and Ito 1956, Kobayashi 1970). *Quintinia serrata* and *M. salicina*, however, are 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 probably will rely on new collections from the original collection sites.

Isolates from New Zealand considered in this study and labeled as *C. radicalis* and *C. gyrosa* were not related to *C. radicalis* isolates or other *Cryphonectria* spp. within the *Cryphonectria* clade. These isolates from New Zealand also were not similar to the group accommodating *C. cubensis* or the phyloclade representing *Endothia*. Ascospores of the New Zealand specimens were one to three septate (FIG. 2e) and are different from species residing in *Cryphonectria* that have two-celled ascospores (Barr 1978). The different ascospore morphology, the ovoid anamorph structures and the phylogenetic grouping of these isolates separately from other *Cryphonectria* and *Endothia* isolates in the phylogenetic analysis suggest that they most likely represent a discrete genus.

Ascospores of specimens linked to the type species, *C. gyrosa* from Sri Lanka (K 109807, K 109809, BPI 614797, BPI 614526, BPI 797701), occasionally contained two septa and, therefore, were similar to specimens connected to the fungus in the New Zealand phyloclade. This type of septation never has been noted previously for *C. gyrosa* or for *Cryphonectria* (Barr 1978, Berkeley and Broome 1875, Saccardo 1905). In the absence of authentic isolates of *C. gyrosa* from Sri Lanka, it is impossible to tell whether this fungus will reside in the phyloclade that represents *Cryphonectria* in the current study. It equally could be possible that *C. gyrosa* will group separately from all other species of *Cryphonectria*. Ascospore septation observed for *C. gyrosa* in this study is similar to that of the New Zealand specimens, and these two fungi could represent the same taxon. This would have important consequences for the taxonomy of *Cryphonectria* species and their appropriate generic placement.

Our phylogenetic and morphological results provide added evidence that *C. cubensis* represents a distinct genus closely related to *Cryphonectria* and *En-*

*dothia*. Isolates of *C. cubensis* formed a distinct group separate from other *Cryphonectria* spp. The dark, superficial to slightly immersed, pyriform anamorphs of *C. cubensis* (Bruner 1917, Hodges 1980, Myburg et al 2002b), reduced stromatic development and blackened protruding perithecial necks (Hodges 1980, Myburg et al 2003), furthermore distinguished *C. cubensis* from the *Cryphonectria* spp., the *Endothia* spp., the unidentified species occurring on *Elaeocarpus dentatus* from New Zealand and the type specimen of *C. gyrosa* from Sri Lanka. The morphological features that distinguish *C. cubensis* from *Cryphonectria* were evident on a variety of host genera, including clove and *Eucalyptus* spp.

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 labeled as *C. radicalis* from Europe formed two subgroups within the greater *Cryphonectria* clade. The one subclade (CMW 10477, CMW 10455) presumably represents *C. radicalis*, but the identity of isolates in the other subclade (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 in the USA (Shear et al 1917) and Japan (Kobayashi 1970). Despite this fact, few isolates exist and it reportedly is difficult to find the fungus in the United States (M. Milgroom pers comm). This might be due to its displacement by the virulent *C. parasitica* that previously was 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 subclades representing *C. radicalis* from Europe currently is impossible because no herbarium specimens are linked to European isolates of *C. radicalis*.

An isolate labeled 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 *Cry-*

*phonectria* (Hawksworth et al 1995). 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 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 sometimes can 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 most closely 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. Ascospore septation, however, has been one of the criteria with which to define the phyloclade from New Zealand. This raises the question whether the aseptate ascospores of *C. eucalypti*, in contrast to septated ascospores of other *Cryphonectria* species, suggest that this fungus represents a distinct genus from *Cryphonectria*. Currently available phylogenetic data provide no evidence to support transferring *C. eucalypti* to a discrete genus. Inclusion of larger numbers of isolates of *C. eucalypti* from South Africa and Australia might help to resolve this question.

The *E. viridistroma* specimens included in this study have green stromata (Roane 1986a, Wehmeyer 1936), unlike other species of *Endothia* that have orange stromata (Barr 1978, Roane 1986a, Shear et al 1917). 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 therefore is unlikely that *E. viridistroma* could 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 because no other isolates of this species exist and herbarium specimens contain insufficient stromatal tissue for a meaningful taxonomic study.

The results of this study reflect the importance of linking isolates to voucher herbarium material to

identify the defined taxa. The genera *Cryphonectria* and *Endothia* include important fungal pathogens, and it is essential that these species be identified correctly. One such example relates to *C. cubensis sensu lato*. Results of the present study and that of Myburg et al (2002b) 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, with regard to disease symptoms (Myburg et al 2002b; Wingfield et al 1989, 2001) and the occurrence of the sexual state (Myburg et al 2002a; Wingfield et al 1989, 2001). The South African fungus is also more pathogenic than that occurring on *Eucalyptus* elsewhere in the world (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 grown commercially. Further studies and possibly the development of rapid techniques to identify these fungi thus should be undertaken.

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