With compliments

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Phylogeny of *Cryphonectria cubensis* and allied species inferred from DNA analysis

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Abstract: *Cryphonectria cubensis* causes a serious canker disease of *Eucalyptus* in many tropical areas of the world. The aim of this study was to determine the phylogenetic relationships among strains of *C. cubensis* and the related species *C. parasitica, Endothia eugeniae* and *E. gyrosa* using DNA sequence data. A second objective was to develop a rapid diagnostic method to distinguish among these taxa. The variable ITS1 and ITS2 regions, as well as the conserved 5.8S gene of the rRNA operon, were amplified and sequenced. The amplified PCR products were also used in *Alu* I and *Cfo* I restriction digests. Analysis of the restriction profiles indicate that it is possible to distinguish between *C. cubensis, C. parasitica, E. eugeniae* and *E. gyrosa* using RFLPs (restriction fragment length polymorphisms). This will facilitate the identification of isolates thought to represent *C. cubensis* that sporulate poorly in culture. Analysis of the sequence data showed that *C. cubensis* isolates form a distinct clade that includes a strain from clove that might otherwise have been assigned the name *E. eugeniae*. Isolates of *C. parasitica* formed a separate clade. *Endothia gyrosa* grouped separately from both *C. cubensis* and *C. parasitica*.

Key Words: *Cryphonectria parasitica, Endothia eugeniae, Endothia gyrosa, Eucalyptus,* RFLPs, ribosomal RNA

INTRODUCTION

Cryphonectria cubensis (Bruner) Hodges is an important canker pathogen of *Eucalyptus* trees (Boerboom and Maas 1970, Davison and Tay 1983, Florence et al 1986, Gibson 1981, Hodges et al 1979, Old et al 1986, Wingfield et al 1989). This pathogen is found predominantly in tropical and sub-tropical areas where infection is favoured by high temperatures and rainfall (Florence et al 1986, Sharma et al 1985b). The importance of *C. cubensis* has been intensified by the recent and wide scale planting of *Eucalyptus* species in many countries, mainly for the production of paper and pulp.

Cryphonectria cubensis was originally described in the genus *Endothia* Fries as *Endothia havanensis* Bruner, where it was reported to cause a serious canker disease of species of *Eucalyptus* in Cuba (Bruner 1916). In the following year another fungus, *Diaporthe cubensis* Bruner, was described by Bruner as the causal agent of a canker disease on several *Eucalyptus* in Cuba (Hodges 1980). About 50 years later, Boerboom and Maas (1970) reported that a canker disease on *Eucalyptus saligna* Sm. and *Eucalyptus grandis* (Hill) Maiden in Surinam was caused by *Endothia havanensis*. Based on this report, Hodges et al (1973) recognised that the same fungus caused a canker disease in Brazil. The causal agent of a canker disease in Brazil and Surinam was reported as *D. cubensis* and not *E. havanensis* (Hodges and Reis 1974).

Hodges (1980) compared specimens of *D. cubensis* and *E. havanensis* and showed that significant morphological differences exist between the two fungi. He also discussed Bruner's (1916) hesitation in placing *D. cubensis* in the genus *Diaporthe* because this species produce pseudostromata surrounded by black zone lines, a character lacking in the *Eucalyptus* canker fungus. Hodges (1980) found that *D. cubensis*, based on morphological, cultural and pathological characteristics, was more similar to species of *Cryphonectria* and transferred *D. cubensis* to the latter genus, as *C. cubensis*. This fungus is the pathogen of primary interest in the present study. *Endothia eugeniae* (Nutman and Roberts) Reid and Booth is the causal agent of die-back on clove (*Syzygium aromaticum* (L.) Merr and Perry) (Hodges et al 1986). Various studies of this fungus have shown that it is the same as *C. cubensis* (Alfenas et al 1984a, Hodges et al 1986, Micales and Stipes 1984, 1987, Micales et al 1987). The size and shape of asci, ascospores and conidia of *E. eugeniae* are similar irrespective of whether the strains

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were isolated from *Eucalyptus* or clove (Micales and Stipes 1984). The conspecificity of the two taxa has also been confirmed on the basis of their similar appearance in culture (Hodges et al 1986), equivalent pathogenicity to *Eu. grandis* (Hodges et al 1986) and near identical isozyme patterns (Alfenas et al 1984b).

Another fungus of interest in this study is the pin-oak pathogen, *Endothia gyrosa* (Schw.: Fr.) Fr. In her monograph on the Diaporthales, Barr (1978) maintained *Endothia* and *Cryphonectria* as separate genera and retained *E. gyrosa* in the genus *Endothia.* Roane et al (1974) reported that *E. gyrosa* is very similar to *C. parasitica,* which was previously treated in *Endothia.* The genus *Cryphonectria* accommodates *Cryphonectria parasitica* (Murr.) M. E. Barr the causal agent of chestnut blight (Griffin and Elkins 1986). After the first report of chestnut blight, the causal agent was described as *Diaporthe parasitica* Murrill (Murrill 1906a, b), but *D. parasitica* was later transferred to *Endothia* as *Endothia parasitica* (Murr.) P. J. and H. W. And. (Anderson and Anderson 1912). Later, a monograph of the Diaporthales by Barr (1978) included a transfer of *E. parasitica* to *Cryphonectria* as *C. parasitica* (Murr.) M. E. Barr.

The geographic origin of *C. cubensis* is enigmatic and it is hypothesised that *C. cubensis* and *E. eugeniae* are conspecific (Alfenas et al 1984a, Hodges et al 1986, Micales and Stipes 1984, 1987, Micales et al 1987). It is also speculated that this fungus originated in Indonesia, where clove is native.

The report of *C. cubensis* on roots of *Eucalyptus marginata* Donn.: Smith growing in native forest of Western Australia (Davison and Coates 1991) is difficult to explain. This occurrence of the fungus is unusual because *C. cubensis* cankers usually occur on the stems of trees (Boerboom and Maas 1970, Florence et al 1986) or as basal cankers (Wingfield et al 1989), and in tropical and subtropical areas of the world (Boerboom and Maas 1970, Bruner 1916, Conradie et al 1990, Gibson 1981, Hodges 1980, Old et al 1986). Despite this fact, the Australian fungus has been shown to be the same as *C. cubensis,* based on a comparison of isozymes (Davison and Coates 1991).

Cryphonectria cubensis is similar to *C. parasitica* in ascospore and conidial morphology (Hodges et al 1986). It has been hypothesised (M. J. Wingfield unpubl) that the two fungi might have a common ancestor. Pathogenicity tests have shown that *C. parasitica* will cause disease on *Eucalyptus* spp. (Old and Kobayashi 1988) and *C. cubensis* on chestnuts (I. P. van der Westhuizen unpubl). These two fungi differ, however, in causing serious diseases of different hosts in nature. There is a clear need for the relatedness of the two fungi to be considered further.

The aim of this study was to compare a large number of isolates of *C. cubensis* from various parts of the world and also to compare these with the related fungi, *C. parasitica, E. eugeniae* and *E. gyrosa.* Another more distantly related fungus, *Diaporthe ambigua* Nits., that causes stem cankers of fruit trees such as *Malus* Borkh. (apple), *Pyrus* L. (pear) and *Prunus* L. (plum) (Smit et al 1994, 1995a, b) was used as an outgroup. These comparisons were based on sequences from the internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S gene of the nuclear rRNA repeat. This region was chosen for study because of the high degree of sequence variation known to exist between closely related species (Mitchell et al 1995, Viljoen et al 1993, White et al 1990).

The use of PCR-RFLP analysis has proven a useful and rapid tool to differentiate between species (Borgo et al 1996, Harrington and Wingfield 1995, Steane et al 1991) and cultivars (Scallan and Harmey 1996). An additional aim, therefore, was to develop PCR-RFLP as a diagnostic tool to differentiate between the species considered in this study.

MATERIALS AND METHODS

Fungal isolates.—Isolates used in this study originated from a variety of sources and culture collections (TABLE I). They are maintained in and available from the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0001, South Africa. The isolates include those of *C. cubensis, C. parasitica, E. eugeniae, E. gyrosa.* In our study we have chosen *D. ambigua* as an outgroup for a number of reasons. This fungus belongs to a genus known to be phylogenetically related to *Cryphonectria* (Barr 1978) and it also causes a serious canker disease of apples and pears (Smit et al 1994, 1995a, b).

DNA isolations.—Isolates were grown on malt extract agar (2% wt/vol, Biolab), incubated at 28 C for 2–5 d and transferred to 1 L Erlenmeyer flasks containing 500 mL malt extract broth (2% wt/vol, Biolab) augmented with yeast extract (0.1% wt/vol, Biolab) and glucose (0.1% wt/vol, Biolab). Cultures were incubated at 30 C on rotary shakers for 7 d. The mycelium was harvested by filtration through No. 1 Whatman filter paper, lyophilized and stored at −70 C.

DNA was isolated using a modified version for DNA extraction as described by Raeder and Broda (1985). Lyophilized mycelium was ground to a fine powder in liquid nitrogen and incubated in 1000 μL extraction buffer (200 mM Tris-HCl, pH 8; 25 mM EDTA; 250 mM NaCl; 0.5% SDS) at 65 C for ten min. The suspension was then centrifuged at 13 000 rpm for 15 min and the liquid phase extracted twice with equal volumes of phenol and chloroform (1:1). Excess phenol was removed by extracting once with 500 μL chloroform and the aqueous phase transferred to new Eppendorf tubes. DNA was precipitated by adding 0.1 vol of 3 M sodium acetate and 0.6 vol of isopropanol and the mixture was stored at −20 C for 16 h. The precipitate

TABLE 1. Isolates used in this study

I.D.	Culture no.[a]	Host	Origin	Collected by:	GenBank accession number
Cryphonectria cubensis	*CRY 0127*	*Eucalyptus camaldulensis*	China	unknown	AF 046890
Cryphonectria cubensis	*CRY 0140*	*Eu. grandis*	South Africa	M. J. Wingfield	AF 046892
Cryphonectria cubensis	*CRY 46*	*Eu. marginata*	Australia	E. Davison	AF 046893
Cryphonectria cubensis	*CRY 33*	*Eu. grandis*	Australia	E. Davison	AF 046894
Cryphonectria cubensis	*CRY 126*	*Eu. marginata*	Australia	E. Davison	AF 046895
Cryphonectria cubensis	*CRY 289*	*E. grandis*	Indonesia	M. J. Wingfield	AF 046896
Cryphonectria cubensis	*CRY 268*	*E. grandis*	Venezuela	M. J. Wingfield	AF 046897
Cryphonectria cubensis	*CRY 243*	*E. grandis*	Venezuela	M. J. Wingfield	AF 046898
Cryphonectria cubensis	*CRY 82*	*Eu. deglupta*	Thailand	M. J. Wingfield	AF 046899
Cryphonectria cubensis	*CRY 138*	*Eugenia carophyllus*	Brazil	unknown	AF 046891
Cryphonectria cubensis	*CRY 0129*	*Psidium spp.*	Brazil	F. A. Ferreira	AF 046900
C. parasitica	CRY 66	*Castanea dentata*	USA	P. J. Bedker	AF 046901
C. parasitica	CRY 44	*Castanea dentata*	USA	P. J. Bedker	AF 046902
C. parasitica	CRY 67	*Castanea dentata*	USA	P. J. Bedker	AF 046903
Endothia eugeniae	CMW 3839	*Syzigium aromaticum*	Indonesia	M. J. Wingfield	AF 046904
E. gyrosa	CMW 2091	*Quercus palustris*	USA	R. J. Stipes	AF 046905
Diaporthe ambigua	CMW 2498	*Malus sylvestris*	Netherlands	S. Truter	AF 046906

[a] Culture collections of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0001, South Africa. CMW and CRY refer to the general and *Cryphonectria* culture collections.

was collected by centrifugation for 20 min at 13 000 rpm. The DNA pellets were washed with 70% ethanol, centrifuged again for 10 min at 13 000 rpm and dried under vacuum. The DNA was resuspended in 200 µL sterile SABAX water and the DNA concentration was estimated spectrophotometrically (Maniatis et al 1982).

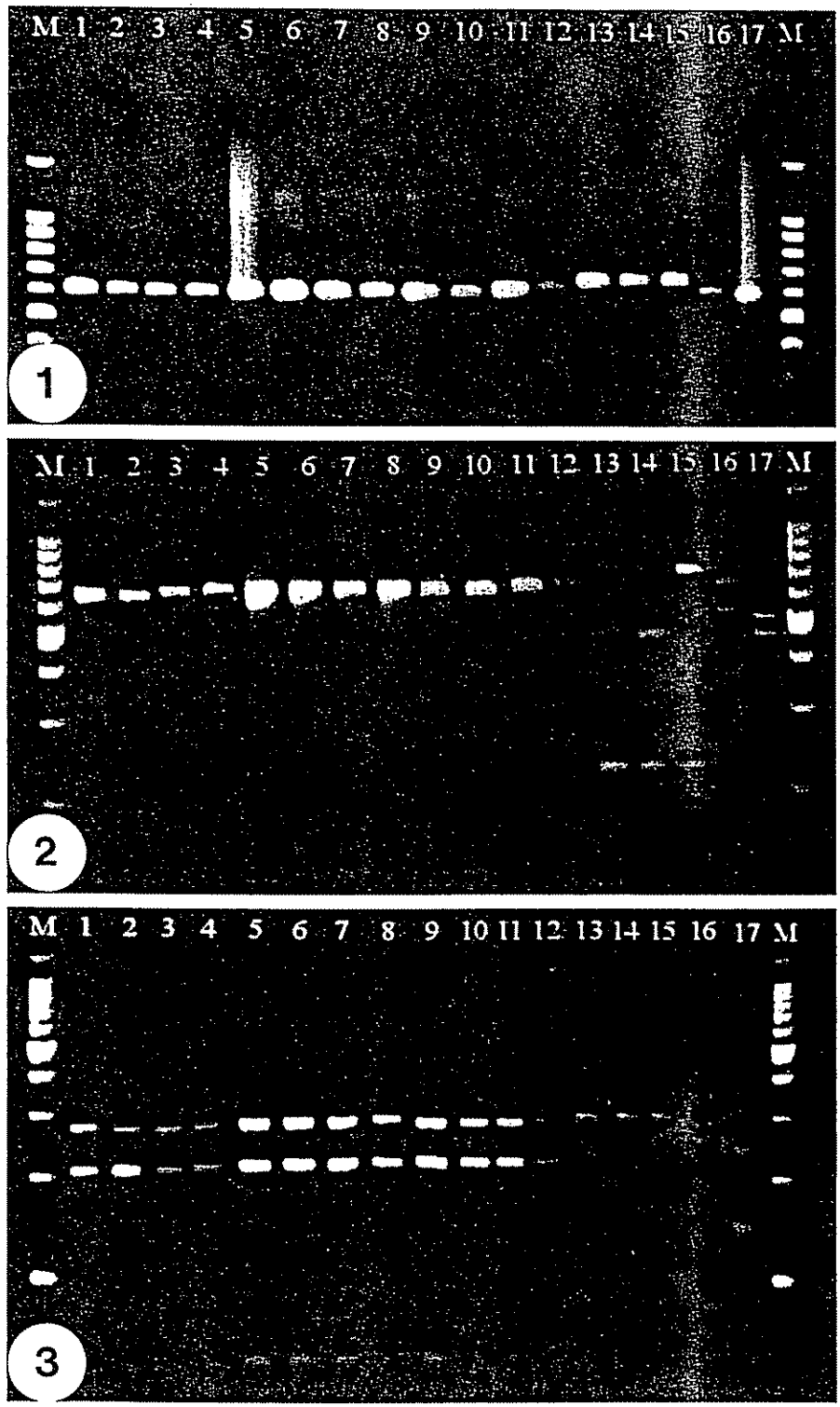
DNA amplification.—The internal transcribed spacer region of the ribosomal RNA operon was amplified using the polymerase chain reaction (PCR) (Saiki et al 1988) employing primers ITS1 and ITS4 (White et al 1990). The PCR reaction mix included five units of *Taq* polymerase (Boehringer Mannheim, Mannheim, Germany), reaction buffer, 4.5 mM $MgCl_2$, 250 µM dNTPs and 0.25 µL of each primer (0.5 µM). The PCR reaction mixtures (100 µL) were overlaid with mineral oil to prevent evaporation. Amplification reactions were done in a Hybaid Omnigene Temperature Cycler (Hybaid, Middlesex, UK). The thermal cycler was programmed for one denaturing cycle at 95 C for 10 min, followed by 35 cycles of denaturation at 95 C for 30 s, followed by an annealing step for 45 s at 55 C and then primer extension at 72 C for 2 min. A final step at 72 C for 7 min was included to ensure complete elongation of the fragment. The PCR products were electrophoresed in 1.4% agarose (Promega, Madison, Wisconsin) gels containing ethidium bromide and visualised using a UV lightsource. PCR products were purified using the Wizard PCR Preps DNA Purification System (Promega) to remove excess primers. A rapid method of obtaining DNA for amplification (Harrington and Wingfield 1995) was also used in this study.

Sequencing and analysis of data.—A *f*mol DNA Sequencing Kit (Promega) was used to sequence the PCR products. Both DNA strands were sequenced using the primers ITS1, ITS4 (White et al 1990), CS2 and CS3 (Wingfield et al 1996). The sequence data were manually aligned by insertings gaps. A heuristic search using PAUP version 3.1.1 (Swofford 1993) was used to analyse the aligned sequences. The gaps were ignored in the PAUP analysis. One parsimonious tree was produced of 320 steps (CI = 0.788, HI = 0.212, RI = 0.667). A bootstrap analysis (1000 replications) was performed to assess the confidence intervals of the branch points (Felsenstein 1985). Sequence data from *D. ambigua* were used in outgroup analysis. Sequences are available from GenBank and accession numbers for the sequenced strains are listed in TABLE I. The sequence alignments are available from TreeBASE accession no. S306 and the matrix as accession no. M398.

Restriction fragment length polymorphisms.—PCR products generated by the amplification of the ITS1, 5.8S and ITS2 regions of the rRNA operon were digested using *Alu* I (Boehringer Mannheim) and *Cfo* I (Boehringer Mannheim) according to the manufacturer's recommendations. The digested PCR products were separated on 3% (wt/vol.) agarose (Promega) gels in 0.5× TBE (44.5 mM tris-borate, 44.5 mM boric acid, 4 mM EDTA) electrophoresis buffer. Ethidium bromide was included in the gels in order to visualise the restriction fragments under UV light.

RESULTS

DNA amplification.—A single fragment of approximately 620 base pairs (bp) in length was obtained for *C. cubensis* and the *E. eugeniae* (FIG. 1). A slightly larger fragment (approximately 640 bp in length)



FIGS. 1–3. Agarose gels. 1. PCR amplification products of the ITS1, ITS2 and the 5.8S rRNA gene. 2, 3. The internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP) profiles generated by the restriction of the ITS1 and ITS2 PCR products with restriction enzymes *Alu* I and *Cfo* I respectively. Lanes 1–11 represent the *C. cubensis* isolates (CRY 0127, CRY 0129, CRY 138, CRY 0140, CRY 46, CRY 33, CRY 2126, CRY 289, CRY 268, CRY 243, CRY 82), lane 12 represents the *E. eugeniae* isolate (CMW 3839), lanes 13–15 represent the *C. parasitica* isolates (CRY 66, CRY 44, CRY 67), lane 16 represents the *E. gyrosa* isolate (CMW 2091) and lane 17 represents the outgroup, *D. ambigua* (CMW 2498). Lane M is a 100 bp ladder (Promega) and serves as a molecular weight marker. Molecular weight marker band sizes are as follows: 100, 200, 300, 400, 500 (brightest band), 600, 700, 800, 900, 1000.

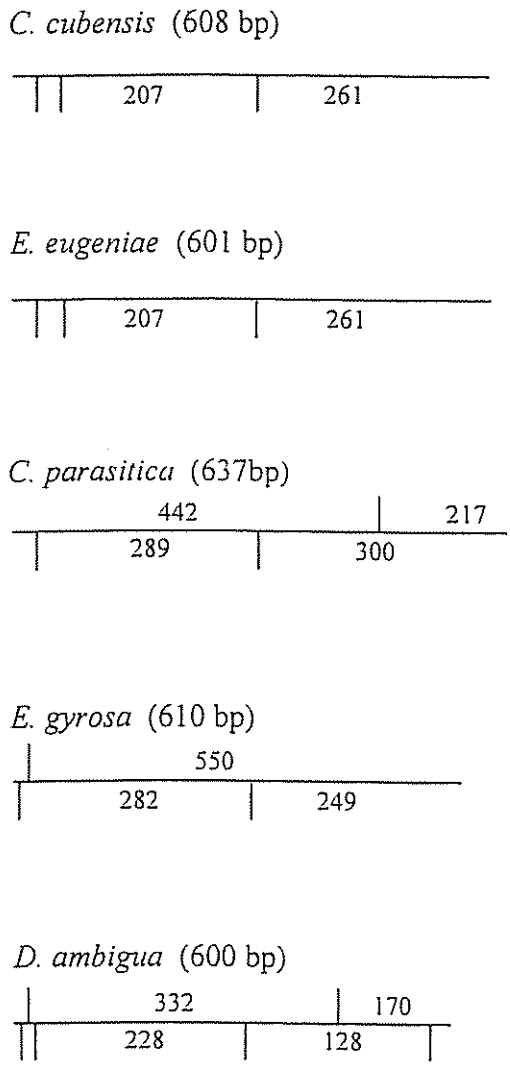


FIG. 4. Restriction map of PCR products containing ITS regions from *C. cubensis, C. parasitica, E. eugeniae, E. gyrosa* and *D. ambigua*. These maps were drawn from the DNA sequence data as well as the observed restriction profiles. *Alu* I restriction sites are indicated above the line and *Cfo* I restriction sites below the line.

was generated for the *C. parasitica* isolates. PCR products of *E. gyrosa* and *D. ambigua* were smaller than those of *C. cubensis* (approximately 610 bp and 600 bp, respectively). It was, therefore, possible to distinguish between species based on the size differences of specific amplified regions.

Restriction fragment length polymorphisms.—The restriction enzymes *Alu* I and *Cfo* I cut at different sites (FIG. 4) and produced RFLP profiles that revealed differences between *C. cubensis* and the other species used in this study (FIGS. 2, 3). The sequence data given is not entirely complete and therefore certain fragment sizes indicated on the restriction map (FIG. 4) are an estimate based on the restriction profiles (FIGS. 2, 3) and sequence data.

Phylogenetic analysis of sequence data.—It was possible to align all sequence data manually by inserting gaps. Sequence alignments are available on request from the corresponding author. From the aligned DNA sequences, it was clear that all *C. cubensis* isolates fall within a defined group. This included an isolate from clove canker in Indonesia that had been assigned the name *E. eugeniae*. The DNA sequence from the outgroup, *D. ambigua,* was considerably different from that of all the other isolates included in this study.

A phylogram was generated from the heuristic search done on the aligned DNA sequence data (FIG. 5). Isolates of *C. cubensis* and *C. parasitica* form two well-supported clades (bootstrap support 100%) to the exclusion of *E. gyrosa* and *D. ambigua* (bootstrap support 74%). Within the *C. cubensis* clade was a subclade, containing isolates from China, Australia, Indonesia and Thailand (bootstrap support 71%), and excluding isolates from Venezuela, South Africa and Brazil.

DISCUSSION

The aims of this study were to resolve the taxonomic confusion surrounding the identity of *C. cubensis* and fungi considered to be related to it. The spacer regions in the rRNA operon have been shown previously to differ between different species of fungi (Mitchell et al 1995, Viljoen et al 1993, White et al 1990) but within a single species are often constant, as we have found in this study (Mitchell et al 1995). It is important, however, to also note that instances have been found where ITS sequence data within a single species are highly variable (Seifert et al 1995).

The differences in size of the amplified DNA fragments for the isolates used in this study gave us an initial indication that differences in RFLPs and DNA sequence could be expected. The amplified products from all the *C. cubensis* isolates were thus the same size. In contrast, amplification products from *C. parasitica* DNA were larger in size than those generated for *C. cubensis*. It was also of interest that the amplification fragment for the *E. eugeniae* isolate was similar in size to those of the *C. cubensis* isolates.

Whether *C. cubensis* occurs in Australia is an intriguing question which has already raised some interest (Davison and Coates 1991, Walker et al 1985). The isolates of *C. cubensis* from Australia originate from the roots of Jarrah (*E. marginata*) in Western Australia (Davison and Coates 1991), an unusual host for this fungus. This occurrence is also unusual in the sense that Western Australia has a mediterranean

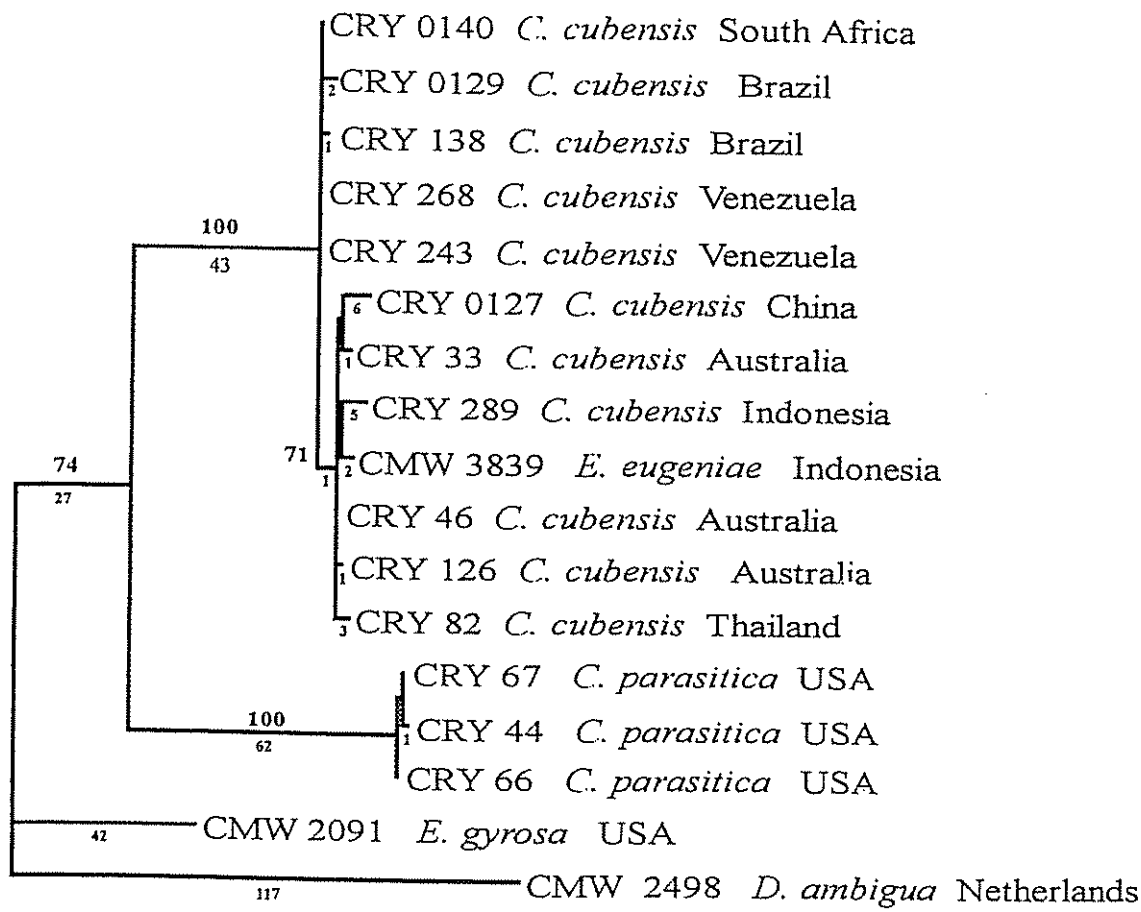


FIG. 5. One parsimonious tree (tree length = 288), based on the sequence data from the ITS1, ITS2 and the 5.8S RNA gene, that was generated using the Heuristic search option within PAUP 3.1.1. *Diaporthe ambigua* was used as the outgroup in this analysis. Percentage confidence intervals (1000 bootstrap replications) are indicated above the branches and the number of steps (substitutions) along the branches below the branches.

climate that is very different to the hot and humid areas where *C. cubensis* is best known (Florence et al 1986, Sharma et al 1985a, b). Using isozyme comparisons, Davison and Coates (1991) showed that isolates from Western Australia grouped with typical isolates of *C. cubensis* and were distinct from the related fungus, *Endothia gyrosa*. Our sequence data confirm the latter. *Cryphonectria cubensis* is one of the most damaging pathogens known to occur on *Eucalyptus*. In this sense it is considered to be of quarantine importance and it could, in theory, threaten native vegetation in Australia. However, if the fungus already occurs throughout Australia, it might be of less concern as a potentially damaging introduced pathogen. Alternatively, it is possible that the fungus is restricted to Western Australia where it might be geographically isolated from susceptible *Eucalyptus* spp. in the eastern part of the country. This matter deserves further and more intensive study.

Endothia eugeniae and *C. cubensis* are morphologically similar fungi (Alfenas et al 1984a, Hodges et al 1986, Micales and Stipes 1984) that also share hosts in the Myrtaceae. Thus, *C. cubensis* is a pathogen of *Eucalyptus*, while *E. eugeniae* infects clove. Isozyme studies (Alfenas et al 1984b, Hodges et al 1986, Micales and Stipes 1984) indicate that these fungi are conspecific. In the present study we included an isolate from clove and have found that it groups with the *C. cubensis* isolates. This result supports the hypothesis of conspecificity, but additional isolates from clove need to be studied.

The DNA sequence generated in this study indicates that *C. cubensis*, *C. parasitica* and *E. gyrosa* are closely related fungi. In comparative morphological studies done on teleomorphs and anamorphs (Walker et al 1985), it was concluded that *Endothia* and *Cryphonectria* are closely related genera and that they should be placed in close proximity in any classification. Species accommodated in these genera are separated based on differences in ascospore shape and septation, stromatic configuration and distribution of stromatic tissues (Micales and Stipes 1987). Our data thus support contemporary morphological opinions regarding these fungi.

In this study we have shown that it is possible to distinguish between *C. parasitica, E. gyrosa* and *C. cubensis* using RFLP and that RFLP provide a rapid method for identifying isolates thought to represent *C. cubensis*. We have also been successful in obtaining PCR fragments using mycelial scrapes in our PCR reactions. This, in association with restriction digestion, would allow rapid and accurate identification of this pathogen. *Cryphonectria cubensis* has proven to be notoriously difficult to identify with certainty due to the fact that, in many parts of the world and on various hosts, only the anamorph is found. This is typically the case in South Africa and it has also been our experience when making collections from clove (Wingfield unpubl data).

A general conclusion from this study is that our DNA-based comparisons largely support a range of previous studies, primarily based on morphology and to a lesser extent, isozyme comparisons. Numerous questions, however, remain unanswered or are partially resolved. We hope to expand our collection of *E. gyrosa* and *E. eugeniae* isolates for comparison. Additional studies and pathogenicity tests on a wide range of *Eucalyptus* spp. using Australian *C. cubensis* isolates would also be valuable.

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