Taxonomy and pathology of a new species of *Cryphonectria* from *Eucalyptus* in South Africa

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A thesis submitted in partial fulfilment of the requirements for the degree of

MAGISTER SCIENTIAE

In the Faculty of Natural, Agricultural and Information Sciences, Department of Plant Pathology and Microbiology, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria

March 2000

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DECLARATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree Magister Scientiae to the University of Pretoria, contain my own independent work and has hitherto not been submitted for any degree at any other University.

M Venter

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March 2000

When wisdom enters your heart,

And knowledge is pleasant to your soul,

Discretion will preserve you,

Understanding will keep you.

PROVERBS 2:10-11

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

This M.Sc. thesis could not have been completed without the help of several people. These people supported me morally, aided me with their expertise, and helped me with technical aspects. I would like to mention a few people, and thank them for what they meant to me.

The Lord and my Saviour, the real first author of this thesis, guided and supported me continuously. My parents were always in the background and prayed for me. Jean, my true friend in life, supported me and helped me many times. Dr. Hanlie Meyer shared her wisdom with me to enable me to better myself during the course of this thesis.

I would like to spend some time in thanking Teresa, Mike and Brenda for the excellent job they did in leading my study. I am grateful to Teresa for always being there, for being able to run into her office for small things, and all of the advice. Mike always manages to drag out the best from you, knows so much and always makes time for you. Brenda is also always willing, and gives such good advice!

I would especially like to thank Dr. Charles S. Hodges from North Carolina State University for sharing his knowledge and time so willingly, and taking the trouble of sending us specimens and papers. Professor Gerard Adams from Michigan State University unknowingly influenced my train of thought (and my work) and also answered many questions. We are grateful to Prof. R. Jay Stipes from the Virginia Polytechnic Institute for his advise and donating his precious culture collection containing different *Endothia* and *Cryphonectria* species, slides and most of his papers on *Endothia* and *Cryphonectria*. Dr. Paul Cannon from the Kew Botanical Institute also

counselled me on some taxonomical aspects. I am also grateful for the light micrographs (Fig. 3*a*, 3*b*) provided by Prof. Pedro W. Crous from the University of Stellenbosch.

Some people were actively involved in my project. Without the unselfish help of Dr. Ben Eisenberg with my statistical analyses, I would have been lost. Dr. Hugh Glen of the National Botanical Institute of Pretoria, provided me with the Latin descriptions in Chapter Three. I would also like to thank Anita Slabbert of the Academic Information Service for obtaining the most difficult and most needed references.

Members of the FABI team were also a rich source of help. Martin Coetzee helped me with the finer details of sequence analysis, and reviewed Chapter Two. Bernard Slippers and Dr. Jolanda Roux reviewed Chapter One and Four, respectively. Riana Jacobs and Dr. Karin Jacobs advised me on the various aspects of describing a new species. Various people also assisted me with my field trials and sampling.

Most projects would not have been possible without financial support. I am, therefore, thankful to the members of the Tree Pathology Co-operative Programme (TPCP) for their financial input. The National Research Foundation (NRF) and University of Pretoria also aided in the financing of this project. I also acknowledge the staff of the Mondi Nursery at KwaMbonambi for providing us with susceptible clones for field inoculations.

PREFACE

Endothia gyrosa is a canker pathogen of several tree genera and is best known for its association with pin oak blight in the USA. In the 1980s, investigations into the canker pathogens associated with *Eucalyptus* spp. in Australia led to the discovery of a similar fungus that was at the time also identified as *E. gyrosa*. This fungus was associated with cankers, die-back and in extreme cases, death of trees. Recently, this pathogen was also found on *Eucalyptus* spp. in South Africa. Slight morphological differences existed between the fungus called *E. gyrosa* on *Eucalyptus* in the Southern Hemisphere and the fungus from North America. It was, therefore, necessary to investigate the taxonomic relationships between *E. gyrosa* from North America, Australia and South Africa. Investigations into the pathogenicity of this fungus in South Africa has been considered important, since *Eucalyptus* forms an important part of the forestry industry in South Africa.

The literature study presented in Chapter One of this thesis, aims to provide an understanding of the taxonomy of the closely related genera *Endothia* and *Cryphonectria*. This overview summarises the taxonomic history of *Endothia* and *Cryphonectria*, and compares key morphological features used in the distinction between members of these genera. The physiology, pathology, host range and geographical distribution of *E. gyrosa* are also discussed.

Phylogenetic comparisons, by means of molecular techniques, provide a reasonably unbiased method of determining the relationships between fungi. In Chapter Two, *E. gyrosa* isolates from North America are compared with isolates from Australia and South Africa at the molecular level. The comparison was carried out using Restriction Fragment Length Polymorphisms (RFLP) and DNA sequencing of Polymerase Chain Reaction (PCR) amplicons from the ITS1 (Internal Transcribed Spacer), ITS2 and 5.8S rRNA gene of the ribosomal DNA operon.

Chapter Three represents the outcome of a morphological study on *E. gyrosa* isolates and stromatal specimens from North America, Australia and South Africa. Bark specimens containing stromata of *E. gyrosa* from the various geographical areas were compared microscopically. Isolates of *E. gyrosa* from North America and South Africa were also compared in culture.

Assessing the pathogenicity of *E. gyrosa* on *Eucalyptus* in South Africa was the aim of studies presented in Chapter Four of this thesis. Fifteen isolates of the South African fungus were screened for their virulence on a clone of *E. grandis* (ZG14). A highly virulent isolate was subsequently chosen to screen different clones of *Eucalyptus* for levels of tolerance to this pathogen. It was believed that information from this trial would be useful in establishing a breeding programme against *E. gyrosa*.

The forestry industry in South Africa generates significant revenue and provides many employment opportunities. This thesis has been produced over a period of two years, and

was aimed at investigating the pathogenicity and correct identity of the canker pathogen known to us as *E. gyrosa*. Results of this thesis will hopefully also be of value in establishing potential control strategies against this pathogen in order to minimise losses. Some repetition between chapters has been unavoidable, because they are presented as independent entities suitable for publication. Chapters have been written according to the instructions of Mycological Research.

SUMMARY

The canker pathogen, *Endothia gyrosa*, was described at the beginning of the nineteenth century in North America. More recently, a similar fungus, also identified as *E. gyrosa*, was discovered in Australia and South Africa on *Eucalyptus* spp. that was able to cause tree death in severe cases. Slight morphological differences existed between the fungus from *Eucalyptus* and *E. gyrosa* from North America, raising questions as to the taxonomic position of *E. gyrosa* from *Eucalyptus*.

Eucalyptus makes up a substantial part of the forestry industry world-wide, as well as in South Africa. The pathogenicity of *E. gyrosa* on *Eucalyptus* in South Africa was investigated in this study to determine whether this pathogen poses a threat to *Eucalyptus* plantations. The correct identity of the South African and Australian fungus was also determined through molecular and morphological comparisons.

In Chapter One of this thesis, the history and morphology of *Endothia*, the closely related genus *Cryphonectria* and the Diaporthales, to which these genera belong, are considered in detail. This information aimed to provide background for the study on phylogenetic relationships between *E. gyrosa* from Northern America, and the similar fungus from Australia and South Africa. The pathology and physiology of *E. gyrosa* on all of its hosts were also considered.

Endothia gyrosa isolates from North America, Australia and South Africa were compared with each other at the molecular level in Chapter Two. This was achieved by means of

RFLPs and DNA sequencing of PCR amplicons of the ITS1, ITS2 and 5.8S rRNA gene. RFLP profiles of South African and Australian isolates were identical but different to those of the North American isolates. The Australian and South African isolates also grouped together in the phylogenetic tree based on the DNA sequences, but separately from the North American isolates. Moreover, the South African and Australian isolates grouped together with *C. parasitica*, and not in the same clade as *E. gyrosa* from North America. *Cryphonectria cubensis* grouped in a clade of its own and not together with *C. parasitica*, as expected.

The fungus from *Eucalyptus* in the Southern Hemisphere is distinct from *E. gyrosa* in Northern America. Furthermore, it appears to belong to the genus *Cryphonectria*. This hypothesis was supported by morphological comparisons and cultural characteristics presented in Chapter Three. Stromatal specimens on bark from South Africa and Australia had a similar morphology to that of *Cryphonectria*, in that the stromata were semi-immersed and consisted of erumpent ectostromatal discs and immersed entostromatal discs, containing the perithecia. In contrast, *Endothia* had widely erumpent, predominantly entostromatic stromata with perithecia borne in the fungal tissue above the bark surface. Different cultural characteristics and different growth characteristics at 10 °C and 15 °C, provided further evidence that the fungus from *Eucalyptus* is distinct from *E. gyrosa* from North America. The fungus from *Eucalyptus* was, therefore, described as a new species belonging in *Cryphonectria*, and the name *C. eucalypti* was proposed for it.

The pathogenicity of the new species, *C. eucalypti*, was assessed through a series of field inoculations presented in Chapter Four. Fifteen isolates were inoculated into *E. grandis* clone

ZG14 and lesions of varying sizes were produced after seven weeks. Statistical analyses of the data showed that isolates of *C. eucalypti* exhibit significant isolate-environment interaction. Differing degrees of pathogenicity will, therefore, be exhibited under different conditions by a particular isolate. This indicates that *C. eucalypti* could play a role in causing disease when host trees are stressed. Levels of tolerance to *C. eucalypti* were also detected in the 42 clones tested, indicating that a breeding programme for resistance to *C. eucalypti* would be possible.

The outcome of this thesis is twofold. The true identity of the fungus on *Eucalyptus* in South Africa and Australia has been established, and its pathology on *Eucalyptus* clones in South Africa has been determined. Based on these results, we now know that *C. eucalypti* has the potential to cause serious disease under conditions favourable for disease development. Canker caused by *C. eucalypti*, however, does not pose a serious enough threat to warrant the establishment of a large scale breeding programme. The pathogen should, however, be monitored closely in the field.

OPSOMMING

Die kanker patogeen, *Endothia gyrosa*, is beskryf aan die begin van die negentiende eeu in Noord-Amerika. 'n Kanker patogeen wat boomsterfte kan veroorsaak in ernstige gevalle, en soortgelyk is aan *E. gyrosa*, is onlangs ontdek in Australië en Suid-Afrika op *Eucalyptus* spesies. Klein morfologiese verskille is waargeneem tussen die fungus van *Eucalyptus* en *E. gyrosa* van Noord-Amerika. Dit het vrae laat ontstaan rakende die taksonomiese posisie van *E. gyrosa* op *Eucalyptus*.

Eucalyptus vorm 'n aansienlike deel van die bosbou industrie wêreldwyd asook in Suid-Afrika. Die patogenisiteit van *E. gyrosa* op *Eucalyptus* in Suid-Afrika is ondersoek in hierdie studie om vas te stel of hierdie patogeen 'n bedreiging inhou vir *Eucalyptus* plantasies. Die regte identiteit van die Suid-Afrikaanse en Australiese fungus is ook ondersoek deur middel van molekulêre and morfologiese vergelykings.

In Hoofstuk Een van hierdie tesis, word die geskiedenis en morfologie van *Endothia*, die naby-verwante genus *Cryphonectria* en die Diaportales in detail behandel. Die doel met hierdie inligting is om agtergrond te verskaf vir hierdie studie wat handel oor die filogenetiese verhoudings tussen *E. gyrosa* van Noord-Amerika, Australië en Suid-Afrika. Die patologie en fisiologie van *E. gyrosa* op al sy gashere is ook beskryf.

Endothia gyrosa isolate van Noord-Amerika, Australië en Suid-Afrika is vergelyk met mekaar op die molekulêre vlak in Hoofstuk Twee. Dit is gedoen deur middel van 'n RFLP analise en DNA basispaar-opeenvolgingsbepaling van PKR fragmente van die ITS1, ITS2 en

5.8S rRNA geen. RFLP profiele van die Suid-Afrikaanse en Australiese isolate was identies, maar verskillend van die profiele van die Noord-Amerikaanse isolate. Die Australiese en Suid-Afrikaanse isolate het ook bymekaar gegroepeer in die filogenetiese boom gebaseer op die DNA volgordes, maar apart van die Noord-Amerikaanse isolate. Meer nog, die Suid-Afrikaanse en Australiese isolate het saammet *C. parasitica* gegroepeer, en nie in dieselfde groep as *E. gyrosa* van Noord-Amerika nie. *Cryphonectria cubensis* het in 'n aparte groep gegroepeer en nie, soos verwag, saammet *C. parasitica* nie.

Die fungus van *Eucalyptus* in die Suidelike Halfrond verskil van *E. gyrosa* van Noord-Amerika. Dit blyk ook om tot die genus *Cryphonectria* te behoort. Hierdie hipotese was ondersteun deur die morfologiese vergelykings en kulturele eienskappe bespreek in Hoofstuk Drie. Die morfologie van stromata op bas van Suid Afrika en Australië was soortgelyk aan die morfologie van *Cryphonectria*, naamlik dat die stromata semi-ingesonke was en bestaan het uit 'n uitstaande ektostromatale skyf en ingesonke entostromatale skyf wat die peritesia bevat. In kontras, het *Endothia* wyd uitstaande, grotendeels entostromatiese stromata gehad, met peritesia wat voorgekom het in die fungale weefsel bo die bas oppervlak. Verskillende kulturele en groei eienskappe by 10 °C en 15 °C, het verder bewys dat die fungus van *Eucalyptus* verskillend is van *E. gyrosa* van Noord Amerika. Die fungus van *Eucalyptus* is dus beskryf as 'n nuwe spesie wat behoort in *Cryphonectria*, en die naam *C. eucalypti* is voorgestel.

Die patogenisiteit van die nuwe spesie, *C. eucalypti*, is ondersoek deur middel van 'n reeks veld inokulasies voorgelê in Hoofstuk Vier. Vyftien isolate is geïnokuleer in 'n *E. grandis* kloon (ZG14) en letsels van varieerbare groottes is na sewe weke gevorm. Statistiese

ontleding van die data het gewys dat isolate van *C. eucalypti* aansienlike isolaat-omgewing interaksie getoon het. Wisselende vlakke van patogenisiteit sal dus onder verskillende toestande deur 'n betrokke isolaat getoon word. Dit impliseer dat *C. eucalypti* moontlik 'n rol kan speel in die veroorsaking van siekte wanneer die gasheer onder stres is. Vlakke van toleransie tot *C. eucalypti* is waargeneem in die 42 klone wat getoets is. Dit is 'n aanduiding dat 'n telingsprogram vir weerstand teen *C. eucalypti* moontlik is.

Die uitslag van hierdie tesis is tweevoudig. Die ware identiteit van die fungus op *Eucalyptus* in Suid-Afrika en Australië is vasgestel, en die patologie daarvan op *Eucalyptus* klone in Suid-Afrika is bepaal. Ons weet dus nou dat *C. eucalypti* die potensiaal besit om ernstige siekte te veroorsaak in toestande gunstig daartoe. Kanker veroorsaak deur *C. eucalypti* hou egter nie so 'n ernstige gevaar in vir die bosbou bedryf in Suid-Afrika, om die daarstelling van 'n grootskaalse telingsprogram te regverdig nie. Die patogeen moet egter noukeurig in veld toestande gemonitor word.

CHAPTER 1

Endothia gyrosa

A review

INTRODUCTION

Endothia gyrosa (Schw.:Fr.) Fr. is a fungus that has been known in North America since the beginning of the nineteenth century (Stevens, 1917). It is closely related to the genus *Cryphonectria* that includes two important pathogens, namely *C. parasitica* (Murr.) Barr and *C. cubensis* (Bruner) Hodges. The taxonomy of *Endothia* and *Cryphonectria* has been changed often in the past and many questions have not been decisively answered.

The discovery of a fungus identified as *E. gyrosa* in South Africa (Van der Westhuizen *et al.*, 1993) and Australia on a totally new host, namely *Eucalyptus* L'Hérit, raised questions pertaining to the phylogenetic relationship between the North American and Australian fungus (Walker *et al.*, 1985). This is because some differences between the Australian fungus and the one from North America were observed (Walker *et al.*, 1985). The stromata of the Australian specimens were less developed and more immersed in the bark. Furthermore, the lower parts of the perithecial bodies were seated in the bark and not in fungal tissue as in the case of the American specimens. These differences were attributed to the different hosts and environmental conditions existing between Australia and North America (Walker *et al.*, 1985).

The pathology and ecology of *E. gyrosa* are treated briefly in this review. The taxonomy and morphology of the genera *Cryphonectria* and *Endothia* are also discussed with specific emphasis on *E. gyrosa*, the type species of *Endothia*. This is

done to acquire an understanding of the history, close relationship, similarities and differences between *Endothia* and *Cryphonectria* for the process of considering the identity of the Australian and South African fungus.

PATHOLOGY OF *E. GYROSA*

Host range and geographical distribution

Endothia gyrosa has a very diverse host range (Table) covering nine tree families: *Castanea*, *Fagus* and *Quercus* all belong to the Fagaceae, *Acer* to the Aceraceae, *Ilex* L to the Aquifoliaceae, *Liquidambar* to the Hamamelidaceae, *Prunus* to the Rosaceae, *Vitis* to the Vitaceae, *Corylus* to the Betulaceae, *Ulmus* to the Ulmaceae and *Eucalyptus* to the Myrtaceae (Farr *et al.*, 1989). *Endothia gyrosa* has also been reported on a vast array of *Quercus* spp. in North America, as well as many *Eucalyptus* spp. in Australia, South Africa and Portugal (Table 4). Previous reports of *C. havanensis* (Bruner) Barr on *Eucalyptus* in Australia (Davison, 1982; Davison & Tay, 1983), were later found to represent the anamorph of *E. gyrosa* (Davison & Coates, 1991). The report fo *E. gyrosa* from India (Ciesla, Diekman & Putter, 1996), is erroneous, since the source on which the report was based, mentioned *C. gyrosa* (Berk. & Br.) Sacc. on *Eucalyptus* (Sharma, Mohanan & Maria Florence, 1985), and not *E. gyrosa*.

Endothia gyrosa is indigenous to the United States and occurs over a wide area of southeastern USA (Appel & Stipes, 1986; Hunter & Stipes, 1978; Roane *et al.*, 1974;

Shear, Stevens & Tiller, 1917; Snow, Beland & Czabator, 1974; Stevens, 1917). There are also isolated reports from other areas, for instance Kansas, Ohio, Michigan, Maryland, New Jersey, Connecticut, New York and California (Appel & Stipes, 1986; Hunter & Stipes, 1978; Shear *et al.*, 1917; Stevens, 1917). Outside the USA, *E. gyrosa* has been reported from China (Teng, 1934), Portugal, Spain and Italy (Spaulding, 1961) on oak and beech (Table 1). Spaulding (1961) further claimed that *E. gyrosa* was also found in Germany, western Europe, Ceylon, New Zealand and the Phillipines, although he did not mention specific hosts. In mainland Australia (Davison & Coates, 1991; Walker *et al.*, 1985; White & Kile, 1993), Tasmania (Old *et al.*, 1986, Yuan & Mohammed, 1997) and South Africa (Van der Westhuizen *et al.*, 1993), *E. gyrosa* has been found only on *Eucalyptus* spp. (Table 1).

Pathology

In North America, *E. gyrosa* was initially thought of as a weak pathogen (Shear *et al.*, 1917). More recently, *E. gyrosa* was found to cause a serious canker disease of *Quercus palustris* Muenchh. (Stipes & Phipps, 1971; Stipes, Phipps & Miller, 1971). Lesions on pin oak resulted in die-back, premature defoliation, death of large and small branches, and decline of trees over a few years (Stipes & Phipps, 1971). The disease was named pin oak blight due to its similarity to chestnut blight (Roane *et al.*, 1974). Serious cankers caused by *E. gyrosa* were also reported on the exotic Formosan sweet gum (*Liquidambar formosana* Hance.) (Snow *et al.*, 1974).

In North America, *E. gyrosa* is well known to be an opportunist and stress-related pathogen. Its occurrence appears to center on opportunities to infect the host, which is influenced by the presence of a suitable host, the condition of the host, water stress and an infection court which is usually provided through wounds (Stevens, 1917). The American fungus infects through wounds such as those provided by pruning (Appel & Stipes, 1986; Hunter, Griffin & Stipes, 1976; Hunter & Stipes, 1978; Snow *et al.*, 1974) and injured, exposed roots (Stevens, 1917; Weir, 1925). *Endothia gyrosa* appears to be well-adapted to grow through pruned stubs, since it can survive at the low water potential that exists at a pruning site (Hunter *et al.*, 1976). Water stress influences susceptibility of the host to disease development (Appel & Stipes, 1984; Hunter & Stipes, 1978). Colonization by *E. gyrosa* was also found to be most active during the growing season, specifically in July and August (Hunter & Stipes, 1978). These months were also the hottest and driest (Appel & Stipes, 1984, 1986).

In North America, *E. gyrosa* is not an aggressive pathogen that results in serious disease. Although *E. gyrosa* was reported to result in a more rapid canker expansion rate under conditions of water stress than other facultative parasitic fungi (Appel & Stipes, 1984), it has not been found to be harmful to healthy trees (Appel & Stipes, 1986). Contrary to earlier hypotheses, *E. gyrosa* was not the primary or single causal agent of pin oak decline, but rather contributed to decline along with various environmental stresses. The most important of these conditions was water stress (Appel & Stipes, 1986).

In Australia, *E. gyrosa* is associated with cankers and kino exudation from cracks and wounds on trunks; unhealed branch stubs; die-back of coppice shoots, branches and stems of *Eucalyptus*; and some tree deaths (Old *et al.*, 1986; Walker *et al.*, 1985). Pathogenicity tests in Australia confirmed that *E. gyrosa* was able to cause significant lesions, kino veins and girdling of *Eucalyptus* seedlings, but the fungus caused no permanent cankers on established trees (Old *et al.*, 1986, 1990). *Endothia gyrosa* was thus considered a mild pathogen of non-stressed trees (Old *et al.*, 1990). In a subsequent study, *E. gyrosa* was found to be one of the most pathogenic species amongst the pathogenic, weakly pathogenic and non-pathogenic species tested (Yuan & Mohammed, 1999). This, together with a report of severe cankers and tree death associated with *E. gyrosa* in a stand of *E. nitens* (Deane & Maid.) Maid. in Tasmania (Wardlaw, 1999), contradicts the view that *E. gyrosa* is not a virulent pathogen.

Endothia gyrosa has a wide distribution in South Africa, and is very common in *Eucalyptus* plantations. In the field, *E. gyrosa* is associated with superficial, slightly swollen cankers causing the bark to crack (Van der Westhuizen *et al.*, 1993). Inoculations of *E. grandis* trees resulted in significant lesions. From these studies, it was, however, evident that the cankers and disease symptoms associated with *E. gyrosa* are not as severe as those caused by *C. cubensis*.

The fungus known as *E. gyrosa* on *Eucalyptus* is a facultative parasite. It is readily isolated from wounds indicating an opportunistic habit (Old *et al.*, 1986; White & Kile, 1993). No correlation was found between canker size and applied water stress on eucalypts, but this could be due to the fact that eucalypts are more drought tolerant

than the North American trees used in previous studies (Appel & Stipes, 1984; Old *et al.*, 1990). Defoliation was an important predisposing factor since cankers resulting from inoculation with *E. gyrosa*, were significantly larger on defoliated than on non-defoliated trees (Old *et al.*, 1990). *Endothia gyrosa* may thus play an important ecological role in eucalypt forests, causing die-back in trees where defoliation has occurred (Old *et al.*, 1990).

Economic implications

Pin oak blight in North America does not have the same impact as chestnut blight, which was responsible for the virtual demise of the American chestnut (Elliston, 1981; Griffin & Elkins, 1986). Declining shade trees in urban areas are most commonly affected (Appel & Stipes, 1986). Control measures include pruning of diseased branches and the removal of heavily infected trees (Appel & Stipes, 1986). Watering could also contribute to tree health, but water is not always available (Appel & Stipes, 1984, 1986). Disease management, however, results in labour and expenses and can even contribute to the further decline of trees (Appel & Stipes, 1986).

Unlike the hosts on which the North American fungus occurs, *Eucalyptus* is planted commercially on a large scale in many countries for the production of paper and pulp (Turnbull, 1991). The emergence of clonal propagation has also increased the risk of large scale disease when clones are particularly susceptible to a pathogen (Chou, 1981; Wingfield *et al.*, 1991). Although *E. gyrosa* on *Eucalyptus* is considered a mild pathogen (Old *et al.*, 1990), *E. gyrosa* could cause serious damage on trees planted in

marginal areas, or during severe stress conditions and defoliation. There are also some indications that *E. gyrosa* could be more important than previously believed (Wardlaw, 1999; Yuan & Mohammed, 1999).

PHYSIOLOGY OF *E. GYROSA*

Endothia gyrosa is able to grow at a wide range of temperatures. The minimum temperature for growth is 9 °C while the maximum temperature is 35 °C. The optimum temperature of growth is between 23-28 °C (Roane, 1986a; Stipes & Ratliff, 1973). Furthermore, *E. gyrosa* is known to utilize lactose, galactose (Roane *et al.*, 1974; Roane, 1986a) and glucose in culture (Roane *et al.*, 1974).

A unique characteristic of *E. gyrosa* is a strong guaiacol-like odour emitted in culture (Roane *et al.*, 1974; Roane, 1986a). Guaiacol, with empirical formula $CH_3OC_6H_4OH$, is a monomethyl ether of catechol and constituent of beechwood-tar (Anonymous, 1966). It has an aromatic odour and is used as an expectorant and local anaesthetic (Gove *et al.*, 1961). This odour produced by *E. gyrosa* has not been studied in any detail.

The characteristic orange colour of *Endothia* and *Cryphonectria* is based on four pigments (skyrin, skyrinol, oxyskyrin and regulosin) that occur in different combinations in the different species (Roane & Stipes, 1978). These pigments are bisanthraquinones (Roane & Stipes, 1978) and are also responsible for the discolouration of lactophenol during slide preparation (Roane, 1986a). Skyrin is a

yellow pigment while regulosin is an orange pigment (Roane & Stipes, 1978). *Endothia gyrosa* contains only three of these pigments, namely skyrin, oxyskyrin and regulosin (Roane & Stipes, 1978). *Endothia gyrosa* can also colour growth media purple and produce red crystals in the mycelium (Roane & Stipes, 1978; Shear *et al.*, 1917). This is caused by endothine red, previously known as pigment B (Micales & Stipes, 1986; Roane, 1986a), a phenolic compound also produced by *C. radicalis* Schw.:Fr.) Barr, *E. singularis* (H. & P. Syd.) Shear & Stevens and *C. nitsckei* (Otth.) Barr (Roane, 1986a).

Endothia gyrosa from *Eucalyptus* produces small amounts of ethylene (Wilkes, Dale & Old, 1989). Ethylene is a growth regulator produced by plants, and it also plays a possible role in plant resistance to disease (Agrios, 1997). Ethylene is also produced by several plant pathogenic fungi and bacteria (Agrios, 1997). It was unclear at that time whether the ethylene is produced by invading microorganisms, by surrounding host tissue or by a possible interaction between host and invaders. Whether *E. gyrosa* contributed to ethylene production during invasion was thus uncertain (Wilkes *et al.*, 1989).

TAXONOMY OF THE CLOSELY RELATED GENERA, *ENDOTHIA* AND *CRYPHONECTRIA*

The taxonomic status of *Endothia* and *Cryphonectria* has often been confused. *Endothia* was separated from *Sphaeria* by Fries in 1849 (Barr, 1978). This new genus, based on *S. gyrosa* Schw., had tubular, red to tawny stromata, deformed, pale

perithecia and evanescent asci (Fries, 1849). *Cryphonectria* was first described as a sub-genus of *Nectria* in 1883, but was afforded generic status by Saccardo (1905) based on *Nectria gyrosa* Berk. & Br. (Barr, 1978; Kobayashi, 1970; Roane, 1986b). Van Höhnel transferred some species of *Cryphonectria* to other genera in the Hypocreaceae in 1909 (Kobayashi, 1970; Roane, 1986b). *Cryphonectria gyrosa*, regarded as the type species by Van Höhnel, was synonymized with *Endothia*, and thus *Cryphonectria* was also reduced to synonymy with *Endothia* (Kobayashi, 1970; Roane, 1986b). *Cryphonectria gyrosa* was, however, provided with the new name *E. tropicalis* Shear & Stevens, because the name *E. gyrosa* had already been used (Barr, 1978; Shear *et al.*, 1917). The genus *Cryphonectria* was resurrected by Barr (1978) to accommodate species in this group with one-septate, ellipsoid to fusoid ascospores while those with non-septate ascospores were retained in *Endothia*. *Cryphonectria gyrosa* was again designated as the type species.

The anamorph of *E. gyrosa*, namely *Endothiella gyrosa* Sacc., was first described in 1906 (Roane, 1986b; Shear *et al.*, 1917). The only other species of *Endothia* with an anamorph connection at that time was *E. singularis* with *Calopactis singularis* Syd. as its anamorph (Roane, 1986b; Shear *et al.*, 1917). This anamorph was reduced to symonymy with *Endothiella* Sacc. (Barr, 1978; Roane, 1986b). *Endothiella* is also used as the anamorph of species of *Cryphonectria* (Barr, 1978; Walker *et al.*, 1985). Anamorph connections for the remaining species of *Endothia* and *Cryphonectria* were established by Roane (1986b) and Kobayashi (1970). This was, however, done at the time when *Cryphonectria* species were treated in *Endothia*.

The orders and families of the group of fungi in which *Endothia* and *Cryphonectria* reside, have been changed regularly. *Endothia* was first classified within the Melogrammataceae due to large ascospores and compact stromata comprising only of fungal tissue. This family was later discarded (Kobayashi, 1970). Subsequently, *Endothia* was classified in the "Eu-Diaportheen" (Van Höhnel, 1917) based on the structure of the perithecial centrum (Barr, 1978; Kobayashi, 1970). An irregularity regarding this classification was that *Endothia*, which has cylindrical to allantoid ascospores (Barr, 1978; Shear *et al.*, 1917), was classified in the "Eu-Diaportheen", which was characterized as having non-allantoid ascospores (Van Höhnel, 1917).

Nannfeldt changed Von Höhnel's classification, basing the order Diaporthales on the sub-family "Eu-Diaportheen", and the Valsales on the "Valseen" (Barr, 1990). The new order Diaporthales included fungi with ellipsoid, hyaline and variously septate ascospores, while similar taxa with allantoid ascospores were accommodated in the Valsales (Cannon, 1988). In Nannfeldt's classification (1932), *Endothia* was broadly classified in the Sphaeriales. *Endothia* was moved to the Diaporthales when Lutrell redefined the Diaporthales basing it on a *Diaporthe*-type centrum and an *Endothia*-type ascus (Barr, 1978). This centrum type distinguished the Diaporthales from other orders (Alexopoulos & Mims, 1979; Cannon, 1988).

Many changes to the composition of the Diaporthales, based on stromatal morphology, arrangement of ascomata in stroma or substrate, and ascospore characteristics, were made subsequent to Lutrell's classification (Barr, 1990). The most important of these pertaining to *Endothia*, was when Barr (1978) divided the

Diaporthales into two sub-orders and four families based on ascomatal and beak position within the stromata, and thin- or firm-walled ascospores. Moreover, the genus *Cryphonectria* was segregated from *Endothia* to accommodate species with one-septate, fusoid to ellipsoid ascospores, while species with non-septate, allantoid to cylindrical ascospores were retained in *Endothia* (Barr, 1978). Prior to Barr (1978), the only other attempt to segregate the species of *Endothia* with one-septate ascospores into a different genus, was by Orsenigo (Roane, 1986b). This was, unfortunately, not done using accepted nomenclatural rules (Roane, 1986b).

Within the Diaporthales, Barr (1978) also moved *Endothia* from the Diaporthaceae to the Gnomoniaceae, while the "new" genus *Cryphonectria* was accommodated in the Valsaceae (Barr, 1978; Micales & Stipes, 1987). Her distinction was based on arrangement of perithecia (valsoid or diatrypoid), type of stromatic tissue (pseudoparenchymatous or prosenchymatous) and ascospore shape and septation, which placed each genus in a separate family (Barr, 1978). In a subsequent classification, she moved *Endothia* to the Valsaceae and *Cryphonectria* to the Gnomoniaceae, and placed greater emphasis on ascospore morphology (Barr, 1990).

At the time when Barr (1978) segregated *Cryphonectria* from *Endothia*, *Endothia* included two sections that had different ascospore morphologies (Kobayashi, 1970; Roane, 1986b; Shear *et al.*, 1917). While *Endothia* was still classified within the Diaporthaceae, the different ascospore morphologies resulted in the different sections of *Endothia* residing in different sub-families. Species with allantoid, one-celled ascospores resided in the Valseae, while species with didymospores were

accommodated in the Diaportheae (Kobayashi, 1970). If the two sections still existed at the time of Barr's 1978 classification, they would also have resided in two families. The reason why this classification of *Endothia* was retained until 1978, despite this contradiction, was that the stromata and cultural characteristics of *Endothia* and *Cryphonectria* were so similar that these two genera appeared to represent a single genus (Kobayashi, 1970). With the new distinction between *Endothia* and *Cryphonectria* (Barr, 1978), the incongruity of the different ascospore morphologies within the genus *Endothia* was resolved.

Barr's (1978) classification of the Diaporthales, and specifically her separation of *Endothia* and *Cryphonectria*, was supported by Micales & Stipes (1987). They also transferred species not mentioned by Barr (*C. longirostris* (Earle) Micales & Stipes and *C. coccolobii* (Vizioli) Micales & Stipes), to *Cryphonectria*. Some researchers, however, ignored the separation of *Cryphonectria* and *Endothia*, and retained *Cryphonectria* as a section within *Endothia* (Roane, 1986b). Other authors regarded *Endothia* and *Cryphonectria* as so closely related that they should not be classified in two families (Chen *et al.*, 1996; Walker *et al.*, 1985).

Currently, the Diaporthales includes two families: the Valsaceae and the Melanconidaceae. The Gnomoniaceae was provided *nomen conservandum* status to the Valsaceae (Hawksworth *et al.*, 1996) based on an article by Cannon (1988), where the families Gnomoniaceae and Valsaceae were merged under the Valsaceae. This was supported primarily by the morphological similarity between *Endothia* (Gnomoniaceae) and *Cryphonectria* (Valsaceae), and *Diaporthella* Petr.

(Gnomoniaceae) and *Diaporthe* Nitschke (Valsaceae). With this new classification, *Endothia* and *Cryphonectria* are thus accommodated in a single family, namely the Valsaceae in the Diaporthales.

MORPHOLOGY

Morphology of the Diaporthales

The primary characteristic of the Diaporthales (Tables 1, 2, 3) is the *Diaporthe*-type centrum as described by Lutrell (Alexopoulos & Mims, 1979; Barr, 1978; Cannon, 1988). The *Diaporthe*-type centrum is filled with thin-walled pseudoparenchymatous tissue in the early stages of development, no paraphyses occur, and asci develop between degenerating nutritive pseudoparenchymatous tissue (Cannon, 1988). Other characters associated with the *Diaporthe*-type centrum are perithecia usually with long necks located in a pseudostroma, and short-stalked, thick-walled asci that may be evanescent or remain attached (Alexopoulos & Mims, 1978; Hawksworth *et al*, 1996).

Consistent characters within the Diaporthales, other than characteristics of the *Diaporthe*-type centrum, are a perithecium wall with *textura epidermoidea* type tissue, a periphysate ostiolar canal and beak surface with *textura porrecta* type tissue (Barr, 1978, 1990). Variable features within the order are used to delimit the families and genera (Barr, 1978, 1990, 1991). Such features include the presence or absence and type of stromatic tissue, the position of perithecia and perithecial beaks in relation to the stroma or the substrate, and ascospore shape and septation (Barr, 1978).

***Morphology of the genera* Endothia *and* Cryphonectria**

Certain distinctive characteristics exist between species of *Cryphonectria* and *Endothia* (Tables 1, 2, 3). *Cryphonectria* was assigned to the Gnomoniaceae based on well-developed entostromatic and ectostromatic tissue, valsoid ascomata and non-apiosporous, ellipsoid or fusoid, one-septate ascospores (Table 1) (Barr, 1990). In contrast, *Endothia* has non-septate, allantoid ascospores, consistent with the Valsaceae (Table 1) (Barr, 1990). According to Barr (1978), the stromata of *Cryphonectria* is made up of predominantly prosenchymatous tissue, while those of *Endothia* are predominantly pseudoparenchymatous stromatic tissue. Other authors (Micales & Stipes, 1987; Walker *et al.*, 1985) found that pseudoparenchymatous and prosenchymatous tissue is present in both *Endothia* and *Cryphonectria*. The pseudoparenchyma occurred more commonly on the surface and upper edges of the stromata, while the prosenchyma occurred in the centre.

The morphology of stromata in fungi is thought to vary greatly depending on certain external factors. For instance, some aspects of the morphology of the *Endothia-Cryphonectria* complex, can be influenced by different hosts, bark types and environmental factors, e.g. moisture (Hodges, Alfenas & Ferreira, 1986; Micales & Stipes, 1987; Roane, 1986b; Shear *et al.*, 1917; Walker *et al.*, 1985). For this reason, greater emphasis was placed on differences in ascospore morphology, which are generally not influenced by external factors (Barr, 1990, 1991).

One morphological feature of stromata influenced by host and environment, is the number of layers in which pycnidia and perithecia are arranged. This apparently depends on the nature and thickness of the bark (Shear *et al.*, 1917). The degree to which the perithecial necks are tilted depends on the degree of development of the stroma. This is indirectly influenced by the substrate and size of ascomata (Cannon, 1988). For instance, *Cryphonectria*, which has a valsoid configuration of perithecia, may have a more diatrypoid configuration (Table 1) if the stromatic disc is wide (Micales & Stipes, 1987). The slightly tilted perithecia on the edges of the stroma probably gave the impression of a valsoid configuration (Micales & Stipes, 1987; Walker *et al.*, 1985). The sequence of formation of the perithecia and pycnidia (concurrently or separately, perithecia or pycnidial locules first) appears to be influenced by climatic factors (Shear *et al.*, 1917). The size, structure and degree to which the stroma is embedded within the substrate also depends on the nature of the bark, host and possibly moisture factors (Cannon, 1988; Hodges *et al*,. 1986; Kobayashi, 1970; Micales & Stipes, 1987; Shear *et al*,. 1917; Roane, 1986b).

Despite the variable nature of the stromata, other subtle differences exist between *Endothia* and *Cryphonectria*. The stromata of *E. gyrosa* in North America, and *E. singularis* are erumpent and subglobose, while those of *C. radicalis* and *C. parasitica* are partially embedded, confluent, but also variable (Shear *et al*,. 1917). It has also been noted that the anamorphs of the species of *Endothia* do not produce tendrils of conidia, while species of *Cryphonectria* do (Shear *et al.*, 1917). Roane (1986b), however, observed tendrils for *E. viridistroma* Wehmeyer, and the presence or absence of spore tendrils may thus not be a useful generic character.

The stromatal structure also differs between *Endothia* and *Cryphonectria*. The stroma of *Cryphonectria* has a defined and erumpent ectostromatal disc, while the entostroma is immersed and extends beyond the ectostroma. Perithecial bases in the entostroma are frequently below the level of the bark surface, and perithecial necks at the edge of the entostroma are oblique, giving them a valsoid appearance (Micales & Stipes, 1987). In contrast, the entostroma and ectostroma in *Endothia* are confluent, with host cells distributed throughout the erumpent area. Thus, the stromata are primarily entostromatic. Perithecial bases are generally in the largely erumpent area, and perithecial necks are upright, hence their diatrypoid appearance (Micales & Stipes, 1987).

Some differences in texture have also been found in *Cryphonectria* and *Endothia*. *Endothia* appears to have a predominant pseudostromatic structure while that of *Cryphonectria* tends to be prosenchymatous (Micales & Stipes, 1987). This assumption was based on the fact that pseudoparenchyma stains more darkly in a safranin:fast green series than prosenchyma, because the isodiametric cells make the pseudoparenchymatic tissue more compact, while prosenchyma consists of long filaments giving it an open structure (Table 3) (Micales & Stipes, 1987).

DIFFERENTIATION BETWEEN SPECIES OF *ENDOTHIA* AND *CRYPHONECTRIA*

Morphological differentiation

Superficially, species of *Cryphonectria* and *Endothia* look very similar. Reasons for this are the similar *Endothiella* anamorphs (Davison & Coates, 1991; Micales & Stipes, 1986; Walker *et al.*, 1985), oblong elliptical to cylindrical conidia (Shear *et al.*, 1917), similar erumpent and brightly pigmented stromata (Barr, 1978, 1990), uniform globose to pyriform perithecia (Shear *et al.*, 1917) and similar cultural characteristics (Kobayashi, 1970).

Teleomorph morphology is essential to distinguish unequivocably between species of *Endothia* and *Cryphonectria* (Micales & Stipes, 1986). In a key compiled by Roane (1986b), criteria used to differentiate between species of *Endothia* are mainly stromatal colour and size, pycnidial appearance and size, and ascospore size. Ascus length divides species of *Cryphonectria* into two groups. Below this level, stromatal, conidial and ascospore size delimit the species.

Chemotaxonomic and molecular differentiation

The difficulty in distinguishing between species of *Endothia* and *Cryphonectria* has necessitated the use of a wide range of identification techniques. One such technique is to evaluate the pigment content of these species (Roane & Stipes, 1978). It has thus

been shown that every *Endothia* species has a distinct combination of pigments, while five groups can be distinguished for the *Cryphonectria* species. Quantitative differences within groups have not been determined and the different species within a group must still be distinguished morphologically.

Morphological distinction between *E. gyrosa* and the devastating chestnut blight pathogen, *C. parasitica,* is difficult in the absence of a teleomorph (Micales & Stipes, 1986). Disc electrophoresis of intramycelial enzymes, however, successfully distinguished *C. parasitica* from *E. gyrosa* (Stipes, Emert & Brown, 1982). The use of tolerance towards antibiotics, specifically cycloheximide, has also been used to differentiate between these fungi (Micales & Stipes, 1986). *Endothia gyrosa* is more sensitive to cycloheximide. This antibiotic also influences pigment production in *E. gyrosa*, but not that of *C. parasitica*. Temperature requirements for growth has also been used for differentiation since the optimum growth temperature for *E. gyrosa* in culture was 20-28 °C, while *C. parasitica* grew best at 20 °C (Stipes & Ratliff, 1973). Furthermore, *E. gyrosa* grew more rapidly and at temperatures up to 35 °C, while *C. parasitica* failed to grow at 35 °C (Stipes & Ratliff, 1973).

Isozyme analyses (Hodges *et al.*, 1986; Micales, Stipes & Bonde, 1987), protein analyses (Hodges *et al.*, 1986) and thin layer chromatography of pigments (Micales *et al.*, 1987) showed that *C. cubensis* (a serious canker pathogen of *Eucalyptus*) and *E. eugeniae* (Nutman & Roberts) Reid & Booth (a canker pathogen of clove) are conspecific. This conspecificity was also shown with PCR (Polymerase Chain Reaction) based RFLPs (Restriction Fragment Length Polymorphisms) and DNA

sequencing data (Myburg, Wingfield & Wingfield, 1999). Isozyme analyses were also used to show that *C. cubensis* was present in Australia. In addition, it was shown that certain *Endothiella* isolates represented *E. gyrosa*, and not the anamorph of *C. havanensis*, as stated in previous reports (Davison & Coates, 1991).

Molecular studies done with *Endothia* and *Cryphonectria* involved only five species, namely *E. gyrosa*, *C. parasitica*, *C. radicalis*, *C. havanensis* and *C. cubensis* (= *E. eugeniae*). PCR-based RFLPs and sequencing of the ITS (internal transcribed spacer) 1 and 2 regions, and 5.8S rRNA gene of the rRNA operon successfully differentiated between *E. gyrosa*, *C. parasitica* (Chen *et al.*, 1995) and *C. cubensis* (Myburg *et al.*, 1999). Two sub-clades were also detected within the *C. cubensis* clade that separated South American and South African isolates, and Asian isolates (Myburg *et al.*, 1999). DNA sequences of 350 bp of the 5`-end of the large rRNA subunit (Chen *et al.*, 1995) distinguished between *E. gyrosa* and *C. parasitica*. Sequencing of the 18S rRNA and ITS1 region (Chen *et al.*, 1996), showed that *C. parasitica*, *C. radicalis*, *C. cubensis*, *C. havanensis* and *E. gyrosa* are distinct species. Moreover, *Cryphonectria* and *Endothia* grouped strongly together in the resulting phylogram and it was hyphothesized that these genera diverged late in evolutionary history. Classification of *Endothia* and *Cryphonectria* into two families is thus not supported by the sequence data (Chen *et al.*, 1996).

CONCLUSIONS

- *Endothia gyrosa* is associated with canker diseases on a wide range of hardwood hosts in North Amercia, and on *Eucalyptus* spp. in Australia and South Africa. It appears to be most damaging to trees that are under stress of have been wounded, but there have been reports of severe damage on vigorous trees.
- There is some evidence that the fungus identified as *E. gyrosa* on *Eucalyptus* spp. in Australia and South Africa might be taxonomically different from the North American fungus. Further study is needed to confirm this.
- The growing importance of *Eucalyptus* plantation forestry in South Africa suggests the need to further study the susceptibility of various species and clones of *Eucalyptus* used in forest plantations in South Africa, to *E. gyrosa* isolated from this host.

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Table 1. Geographical distribution and host range of *Endothia gyrosa*.

Country	Host[a)]
USA	*Acer saccharinum* L., *Fagus grandifolia* Ehrh. (8, 5), *Castanea dentata* Borkh., *Castanea* spp. (Cultivars), *F. sylvatica* L., *Quercus coccinea* Muenchh., *Q. imbricaria* Michx., *Q. marylandica* Muenchh., *Q. velutina* Lam., *Q. alba* L., *Q. nigra* L., *Q. ilicifolia* Wangenh., *Q. georgiana* Curtis, *Q. falcata* Michx. (8, 9), *Q. prinus* L., *Q. rubra* L., *Q. virginiana* Mill. (9), *Ilex opaca* Ait. (8), *Liquidambar styraciflua* L. (8, 9, 10), *L. formosana* Hance., (8, 10), *Prunus laurocerasus* L., *Q. agrifolia* Née, *Q. bicolor* Willd., *Q. lyrata* Walt., *Q. macrocarpa* Michx., *Q. montana* Willd. (8), *Vitis* (3, 9), *Corylus*, *Ulmus* (3), *Q. suber* L. (5, 12), *Q. phellos* L. (9, 12), *Q. palustris* Muenchh. (6, 7), *Q. borealis* Michx. f. (7)
China	*Quercus* sp. (13)
Portugal	*Q. suber*, *Q. pyrenaica* Willd., *Q. lusitanica*, *Q. fruticosa*, *C. dentata*, *Eucalyptus diversicolor* F. Muell. (11)
Spain	*C. crenata* Siebald & Zucc. (11)
Italy	*C. sativa* Mill. (11)
South Africa	*E. grandis* W. Hill ex Maid., *E.* urophylla Benth. ex Lindley, E. *nitens*, *E. grandis* X *camaldulensis* Dehnh., *E. grandis* X *urophylla* (14)
Australia, Tasmania	*E. saligna* Smith (2, 4, 16), *E. delegatensis* R. T. Baker, *E. obliqua* L'Herit (2, 4), *E. calophylla* Lindley[b)], *E. marginata* Donn. ex Smith[b)], *E. blakelyi* Maid. (2), *E. maculata* Hook., *E. grandis* [d)], *E. pilularis* Sm., *E. dalrympleana* Maid., *E. dives* Schau., *E. pauciflora* Sieb. ex Spreng., *E. rossii* R. T. Baker et H. G. Sm., *E. rubida* Deane et Maid., *E. stellulata* Sieb. ex DC., *E. viminalis* Labill., *E. globulus* Labill. (4), *E. regnans* F. Muell. (4, 17), *E. wandoo* Blakely[c)], *E. camaldulensis* [c)] (1), *E. nitens* (15)

[a)] References used are those cited in the literature and numbers are as follows:

1 Davison (1982), 2 Davison & Coates (1991), 3 Farr *et al.* (1989), 4 Old *et al.* (1986), 5 Micales & Stipes (1986), 6 Phipps *et al.* (1972), 7 Roane *et al.* (1974), 8 Roane (1986b), 9 Shear *et al.* (1917), 10 Snow *et al.* (1974), 11 Spaulding (1961), 12 Stipes *et al.* (1982), 13 Teng (1934), 14 Van der Westhuizen *et al.* (1993), 15 Wardlaw (1999), 16 Walker *et al.* (1985), 17 White & Kile (1993).

[b)] Anamorph of *C. havanensis* confirmed later to be *E. gyrosa* .

[c)] Reported as anamorph of *C. havanensis* on host, but probably *E. gyrosa*.

[d)] Inoculations produced lesions on this species, but not reported before on this host.

Table 2. Morphological features of the teleomorphic members of the Diaporthales including *Endothia gyrosa* (from North America) and *Cryphonectria gyrosa*.

	Diaporthales[1]	***E. gyrosa***[2]	***C. gyrosa***[2]
Stroma	lacking/present, prosenchymatous with pseudostroma sometimes, or pseudoparenchymatous	erumpent through bark, corticular or subcorticular, regulose, scattered or gregarious, occasionally confluent	erumpent through bark, corticular, usually scattered or gregarious, rarely confluent
Shape		pulvinate to tubercular	pustulate to pulvinate
Colour		orange-chrome when young, almost black when old	orange chrome when fresh to brown when old and weathered
Size		1·5-3 mm diam., 1·5-2 mm high	1-5 mm diam., 1-2 mm high
Perithecia	immersed/erumpent, two-layered peridium, often *textura epidermoidea* surface view, no paraphyses	mostly 25-50, usually arising in lower portion of stroma, very irregularly arranged in one to several layers	collapsed when dry, 5-50 or more in stroma, irregularly arranged in one to three layers
Shape	globose or sphaeroid		
Size	small to large	150-300 µm diam.	250-500 µm diam.
Colour		dark brown to black	black
Necks	apex central or lateral to eccentric, beaked or papillate, ostiole periphysate	slender, penetrating stroma, protruding sometimes, terminate in short conical ostiole	slender, penetrate stroma and terminate in acute ostioles
Size		up to 1100 µm long	up to 1410 µm long, projecting 0·25-1 mm above surface
Asci	unitunicate, rarely remaining attached, octo-/polysporous/ fewer than 8, refractive apical ring, chitinoid, non-amyloid	very short stipitate	
Shape	ellipsoid, oblong, inflated, clavate/cylindrical	oblong fusoid or subclavate	oblong or subclavate, nearly sessile
Size		25-30 X 6-7 µm	40-50 X 7 µm
Ascospores	hyaline, yellowish or brown	one-celled, irregularly biseriate	two-celled, not constricted at septum, irregularly biseriate,
Shape	variable in shape and septation, a-/symmetric	cylindric to allantoid	subelliptical, obtuse
Size		7-11 X 2-3 µm	7·5-12·5 X 3·5-5 µm

[1] Barr (1990)

[2] Taken from Roane (1986b) and Shear *et al.* (1917).

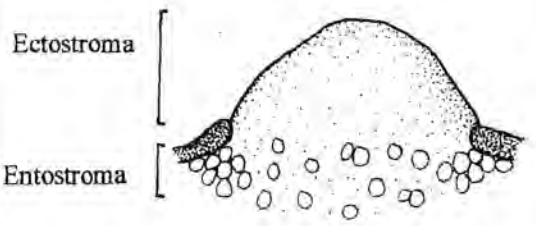
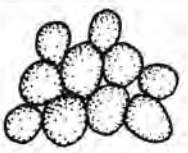
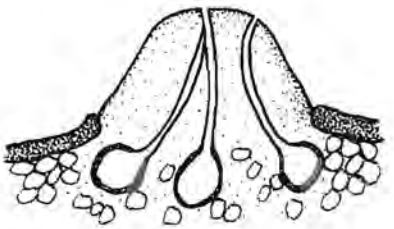
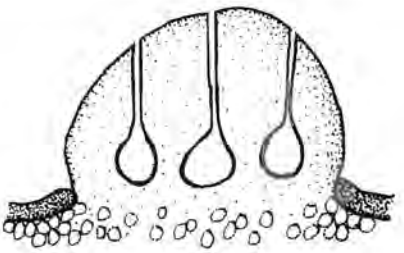
Table 3. Morphological features of the anamorphic members of the Diaporthales including *Endothia gyrosa* and *Cryphonectria gyrosa*.

	Diaporthales[1]	***E. gyrosa***[2]	***C. gyrosa***[2]
Stroma	mostly acervular, pycnidial or stromatic coelomycetes	similar to teleomorph	similar to teleomorph
Shape		similar to teleomorph	similar to teleomorph
Colour		similar to teleomorph	similar to teleomorph
Size		similar to teleomorph	similar to teleomorph
Conidia	variable in pigmentation, shape and septation	exuded in droplets	variable in size and shape
Shape		one-celled, cylindric to allantoid, oblong	one-celled, oblong to cylindric
Colour		hyaline	pale yellowish in mass
Size		3-4 X 1·5-2 µm	3·5-7 X 1·5-2·5 µm
Locules		numerous, irregular labyrinthiform chambers in stroma, opening by irregular pores in surface of stroma	numerous, irregular cavities in stroma
Conidio-genous cells	enteroblastic phialidic/holoblastic determinate/proliferating percurrently	cylindric/slightly tapering towards apex	simple, clavate, tapering above
Size		6-9 µm long	6-10 µm long

[1] Barr (1990)

[2] Taken from Roane (1986b) and Shear *et al.* (1917).

Table 4. Terminology used to describe the morphology of members of the Diaporthales.

Terminology	**Definition**[a)]	**Illustration**
Ectostroma	uppermost portion that breaks through bark, composed primarily of fungal tissue[2,3]	
Entostroma	lower portion of stroma formed under ectostroma, composed of fungal and host tissue[2,3]	
Prosenchyma	tissue composed of interwoven, parallel, elongated hyphal cells[1]	
Pseudoparenchyma	tissue composed of closely packed, isodiametric hyphal cells[2]	
Valsoid	perithecia clumped together with necks convergent, in primarily ectostromatic stroma with disc not as strongly developed and widely erumpent as for a diatrypoid configuration[3]	
Diatrypoid	perithecia grouped more effusely with necks separate, in primarily entostromatic stroma, but stroma more strongly developed and widely erumpent[3]	

a) References used are as follows: 1 Alexopoulos & Mims (1979); 2 Hawksworth *et al.* (1996) and 3 Micales & Stipes (1987).

CHAPTER 2

Molecular characterization of *Endothia gyrosa* isolates from *Eucalyptus* in South Africa and Australia

ABSTRACT

Endothia gyrosa is a canker pathogen best known as the causal agent of pin oak blight in North America. It also causes cankers on other woody hosts, such as *Castanea* spp. and *Liquidambar* spp. In South Africa, Australia and Tasmania, a fungus identified as *E. gyrosa* has been recorded on *Eucalyptus* spp. Some differences in morphology between the North American and the *Eucalyptus* fungus have, however, been noted. The aim of this study was to consider the phylogenetic relationship between *E. gyrosa* from North America and *E. gyrosa* from South Africa and Australia, as well as that of related fungi, namely *Cryphonectria parasitica* and *C. cubensis*. Isolates were compared using PCR-based Restriction Fragment Length Polymorphisms (RFLP) and sequences of the ITS region of the rRNA operon. *E. gyrosa* isolates from South Africa produced the same RFLP banding patterns as those from Australia, but these differed markedly from those for the American *E. gyrosa* isolates. In a phylogram based on the DNA sequences, the Australian and South African isolates of *E. gyrosa* resided in a single, well-resolved clade, while the American isolates were distinctly different. Isolates of *C. parasitica* grouped in the same clade as the South African and Australian isolates of *E. gyrosa*, but *C. cubensis* was distantly related to them. The molecular data suggest that the *Endothia* isolates from South Africa and Australia represent a distinct taxon, and probably belong to *Cryphonectria*. Of particular interest is the fact that *C. parasitica* is more closely related to the *Endothia* isolates than to *C. cubensis*.

INTRODUCTION

Endothia gyrosa (Schw.:Fr.) Fr. is a fungal pathogen best known for its association with pin oak (*Quercus palustris* Muenchh.) blight in North America (Appel & Stipes, 1986; Roane *et al.*, 1974; Stipes & Phipps, 1971). This fungus, native to North America, also causes serious cankers on exotic Formosan sweetgum (*Liquidambar formosana* Hance.) (Snow, Beland & Czabator, 1974), as well as on other *Quercus* spp., *Acer saccharinum* L. (Roane *et al.*, 1974), *Liquidambar styraciflua* L. (Snow *et al.*, 1974), *Castanea* spp., *Ilex opaca* Aiton (Appel & Stipes, 1986), *Fagus grandiflora* Ehrh., *Fagus sylvatica* L., *Prunus laurocerasus* L. (Roane, 1986), *Corylus* and *Vitis* (Farr *et al.*, 1989). In North America, *E. gyrosa* is reported to occur widely, but is particularly well-known in the southeastern parts of the USA (Appel & Stipes, 1986; Hunter & Stipes, 1978; Roane *et al.*, 1974; Shear, Stevens & Tiller, 1917; Snow *et al.*, 1974; Stevens, 1917). *Endothia gyrosa* has also been reported from China (Teng, 1934) and Europe (Spaulding, 1961).

A fungus also identified as *E. gyrosa* has been reported in Australia and Tasmania on various species of *Eucalyptus* L'Hérit, including *E. saligna* Smith, *E. maculata* Hook, *E. delegatensis* R. T. Baker, *E. regnans* F. Muell. and *E. grandis* W. Hill ex Maid. (Old *et al.*, 1986; Walker *et al.*, 1985; White & Kile, 1993). A similar fungus was recently reported from South Africa where it was associated with cankers on several species of *Eucalyptus*, such as *E. grandis*, *E. nitens* (Deane & Maid.) Maid., *E. urophylla* Benth. Ex Lindley, and hybrids of *E. grandis* with *E. camaldulensis* Dehnh. and *E. urophylla* (Van der Westhuizen *et al.*, 1993).

Endothia gyrosa has been known to occur in the USA for a considerable period of time (Barr, 1978; Shear *et al.*, 1917; Stevens, 1917). Its recent discovery in Australia and South Africa on a very different host to those known in North America, was enigmatic. The identity of the North American fungus and the one from the Southern Hemisphere was discussed by Walker *et al.* (1985), who noted morphological differences between them. These were mainly that the stromata in the Australian specimens were less developed, and that the perithecial bases were seated in the bark and not in the fungal tissue, such as occurred in specimens from North America. However, the size and shape of the perithecia, asci and ascospores of the two groups were indistinguishable and this led to the conclusion that the Australian and South African fungus represents *E. gyrosa* (Van der Westhuizen *et al.*, 1993; Walker *et al.*, 1985).

Members of the genera *Endothia* and *Cryphonectria* have long been regarded as being very closely related (Barr, 1990; Roane, 1986; Shear *et al.*, 1917). They also share a common *Endothiella* anamorph (Barr, 1978; Davison & Coates, 1991). *Cryphonectria* was separated from *Endothia* by Barr (1978) based on differences in ascospore and stromatal morphology. *Cryphonectria* has one-septate ascospores and valsoid stromata with ectostromatic and entostromatic areas in predominantly prosenchymatous tissue. This is in contrast to the ascospores of *Endothia* that are non-septate, and the stromata diatrypoid with predominantly pseudoparenchymatous, entostromatic tissue (Barr, 1978, 1990; Micales & Stipes, 1987). This distinction was maintained in later studies (Barr, 1990; Micales & Stipes, 1987).

Cryphonectria parasitica (Murr.) Barr, which causes chestnut blight, is one of the best known and important pathogens of forest trees (Elliston, 1981; Griffin & Elkins, 1986). *Cryphonectria parasitica* and *E. gyrosa* are difficult to distinguish in the absence of a teleomorph, since both produce red to orange stromata (Stipes, Emert & Brown, 1982). The fact that *C. parasitica* originally resided in *Endothia* as *E. parasitica* (Roane *et al.*, 1974; Shear *et al.*, 1917), probably also led to further confusion. *Endothia gyrosa* and *C. parasitica* have, however, been differentiated by many researchers using molecular and chemotaxonomic techniques (Micales & Stipes, 1986; Myburg, Wingfield & Wingfield, 1999; Stipes *et al.*, 1982), and the fact that they reside in distinct genera is unequivocal.

Cryphonectria cubensis (Bruner) Hodges is a serious canker pathogen of plantation grown *Eucalyptus* spp. that occur in most tropical and sub-tropical areas of the world (Davison & Coates, 1991; Florence, Sharma & Mohanan, 1986; Sharma, Mohanan & Florence, 1985; Hodges, Geary & Cordell, 1979). The fungus also causes canker of clove [*Syzygium aromaticum* (L.) Merr. & Perry] in Africa, Brazil and Indonesia, but on this host does not cause serious damage (Hodges, Alfenas & Ferreira, 1986). In South Africa, it shares the same host and approximately the same geographical distribution as *E. gyrosa* on *Eucalyptus* (Wingfield, Swart & Abear, 1989). The morphology of *C. cubensis* is quite different than that of the other *Cryphonectria* species. On *Eucalyptus* spp., *C. cubensis* lacks the prominant orange stromata typical of other *Cryphonectria* and *Endothia* spp., and forms distinct pycnidia as opposed to pycnidial locules within a stroma, as is the case for *Cryphonectria* and *Endothia*

(Hodges, 1980). On clove, *C. cubensis*, sometimes form orange stromata containing both pycnidial locules and perithecia, but the stromata are mostly embedded in the bark and are not as readly visible as those of other species of *Cryphonectria* (Hodges *et al.*, 1986).

In this study, we have used RFLP analysis based on a technique developed by Myburg *et al.* (1999), as well as partial sequence of the rRNA operon for some key isolates, to determine whether *E. gyrosa* isolates from North America, South Africa and Australia are the same or different. Furthermore, we have been intrigued by the fact that the morphology of *C. cubensis* is quite different to species of *Cryphonectria*, and that *E. gyrosa* from South Africa and Australia on *Eucalyptus* share morphological features with *C. parasitica*. Some of these shared characters include a stromatal structure more like that of *C. parasitica* than of *E. gyrosa*, and the presence of long hyphal elements between the conidiogenous cells (M. Venter, unpublished data). In this study we have thus included isolates of *C. parasitica* and *C. cubensis* to investigate the relationship of *E. gyrosa* with *C. parasitica* and *C. cubensis*.

MATERIALS AND METHODS

Source of isolates

Isolates of *E. gyrosa* from North America, South Africa and Australia were used in this study (Tables 1, 2). These isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria,

Pretoria, South Africa. Isolates were maintained on 2% malt extract agar (MEA, Biolab, Merck, Midrand, South Africa) at 4 °C.

DNA isolation

Mycelia from isolates (Table 1) were grown in 250 ml malt extract broth (2% w/v malt extract, Biolab) in the light at 25 °C. After two weeks, the mycelia were harvested by means of filtration (Whatman No. 1 filter paper) and dried between sterilized paper towels. DNA was extracted from the dried mycelium with a modified version of the DNA extraction method as developed by Raeder & Broda (1985). Dried mycelium was transferred to sterile Eppendorf tubes with 100 µl of extraction buffer [200 mM Tris-HCl (pH 8·5), 250 mM NaCl, 25 mM EDTA and 0·5% SDS]. The mixture was frozen in liquid nitrogen, ground to a fine powder using a mortar and pestle and incubated at 65 °C for five min. The freezing, grinding and incubation steps were repeated with an added 400 µl of extraction buffer until a homogenous mixture was obtained. The suspension was mixed twice with phenol and chloroform (3:1) and centrifuged at 13 000 rpm (18 000 g). All centrifugations were conducted at 4 °C. One volume of chloroform was then added to the aqueous phase, followed by centrifugation at 13 000 rpm for 10 minutes. This step was repeated until the interphase was clean. The DNA in the aqueous phase was precipitated overnight at -20 °C with 0·54 volumes of isopropanol and 0·1 volumes of 3 M sodium acetate (pH 8). This mixture was subsequently centrifuged for 30 min at 10 000 rpm. The resulting pellet was rinsed with 100 µl ice-cold 70% ethanol, centrifuged for 10 min at 13 000

rpm, and dried in a SpeedVac SC100 (Savant Instruments Inc., Farmingdale, N.Y., USA). The dried pellet was resuspended in 100 µl ddH_2O and stored at -20 °C.

DNA amplification

The variable ITS1 (internal transcribed spacer) and ITS2 regions, and conserved 5.8S rRNA gene of the ribosomal RNA operon were amplified with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990) using the polymerase chain reaction (PCR). The PCR reaction consisted of 1·25 µl (2·5 units/µl) of Boehringer Mannheim (Indianapolis, USA) *Taq* polymerase, 10 X PCR buffer, 0·3 mM dNTP, 0·5 µl of each primer (500 µg/ml) and 20-120 ng/µl template DNA. The reaction mix was made up to 100 µl and overlaid with mineral oil. The PCR was done on a HYBAID Touch Down thermal cycler (HYBAID, Middlesex, UK). The PCR conditions were as follows: 95 °C for 5 min (denaturation), followed by 30 cycles of 45 s each (annealing), 72 °C for 2 min (polymerization) and 45 s at 95 °C (denaturation). An annealing temperature of 59 °C was used for the American and Australian isolates, and 56 °C was used for the South African isolates. (The reason for the different annealing temperatures was a two bp deletion for the South Africa isolates in the area where the ITS1 primer bound to the template DNA.) A final elongation step was conducted at 72 °C for 7 min. The PCR products were separated on a 1·4% agarose (Promega, Madison, USA) gel stained with ethidium bromide (10 mg/ml), and visualized under UV illumination. PCR products were purified using the High Pure™ PCR Product Purification Kit (Boehringer Mannheim) to remove excess primers and dNTPs.

Restriction Fragment Length Polymorphisms (RFLP)

Restriction enzymes *Cfo*I and *Eco*RI were used to cut the amplified PCR products. The digested DNA fragments were separated on a 3 % agarose gel containing ethidium bromide (10 mg/ml), and visualized under UV light.

DNA sequencing

DNA sequences of the amplified PCR products were determined using an automated sequencer (ABI Prism, model 377, Perkin Elmer). The sequences of the *C. parasitica* (CRY66, CRY67), *C. cubensis* (CRY 289, CRY140) and *Diaporthe ambigua* Nits. (CMW2498) isolates were obtained from Genbank based on Myburg *et al.* (1999) (Table 2). Primers ITS1 and ITS4 (White *et al.*, 1990), and internal primers CS2 (5'-CAATGTGCGTTCAAAGATTCG-3') and CS3 (5'-CGAATCTTTGAACGCACATT G-3') (Wingfield *et al.*, 1996), which binds within the 5.8S rRNA gene, were used to sequence both strands of the amplified DNA. The sequencing reactions were done with the Big Dye sequencing system (ABI Advanced Biotechnological Institute, Perkin-Elmer Corporation, Foster City, USA) according to the manufacturer's instructions.

Phylogenetic analysis

The sequences obtained were manually aligned with Sequence Navigator version 1.0.1 (ABI Prism, Perkin Elmer, 1986) by inserting gaps. Aligned sequences were analyzed with PAUP* version 4.0b2 (Phylogenetic Analysis Using Parsimony) (Swofford, 1998). All characters were treated as unordered and were equally weighted. Gaps were treated as missing data. *Diaporthe ambigua* was defined as a monophyletic outgroup with respect to the other isolates, since it belongs to the same family as *Cryphonectria* and *Endothia* (Barr, 1990; Hawksworth *et al.*, 1996).

Both the branch and bound algorithm ('as is' addition sequence, MAXTREES set to prompt for new value), and the tree bisection-reconnection (TBR) swapping option of the heuristic search algorithm, were used to search for the most parsimonious tree. The confidence intervals for each of the branches were estimated by bootstrap analyses (1000 replications). A total of 69 ambiguous characters (bases 1-22, 31-40, 63-76, 111-118, 231-236 and 506-514) were also excluded in order to determine whether these ambiguities would have an influence on the topology of the tree. The consistency index (CI) and retention index (RI) were also calculated using PAUP* to establish the phylogram that best reflected the true phylogeny of this group.

RESULTS

DNA amplification

Differences in size could be observed for the amplification products obtained for the North American (607 bp) and South African isolates (640 bp), while the PCR product of the Australian isolate (644 bp) differed by only 4 bp from the South African isolates (Fig. 1). The fragment size of the PCR product of the *D. ambigua* isolate was estimated to be approximately 600 bp, and is, therefore, different in size to the *E. gyrosa* isolates.

Restriction Fragment Length Polymorphisms (RFLP)

The Australian and South African isolates had the same RFLP banding patterns when either *Cfo*I (Fig. 2) or *Eco*RI (Fig. 3) were used to digest the PCR product. These patterns differed from those of *E. gyrosa* isolates from North America and from *D. ambigua* for both enzymes (Figs 2, 3). The restriction maps (Fig. 4) generated from the DNA sequence, reflect these differences.

DNA sequencing and analysis

The length of the sequences aligned to those of *C. parasitica*, *C. cubensis* and *D. ambigua* obtained from Genbank, was 474 bp for the North American isolates, 506 bp for the South African isolates and 509 bp for the Australian isolates (Fig. 5). A total of

563 characters for each isolate were aligned after the inclusion of gaps. Trees identical regarding CI and RI values (0·9495 and 0·9367 respectively), number of constant and parsimonious informative characters (476 and 38 respectively), g1 values (0·860094), number of base changes per branch and tree length (99 steps), were obtained with both the TBR swapping option and the branch and bound option of PAUP. Only the number of trees (2 for branch and bound option, 3 for TBR option), bootstrap values and branch lengths differed between the trees obtained with the different options. These differences were due to a few single base differences that existed between isolates of the same species.

Exclusion of ambiguous regions did not have any influence on the phylogenetic groupings of the isolates. The tree obtained with the TBR swapping option when data were excluded, was identical to the tree obtained with the branch and bound option. The CI and RI values (0·9438 and 0·9324) and g1 value (0·853969) were slightly lower when ambiguous regions were excluded, than when such regions were included. Fewer trees (1) with a lower number of steps (89) were also obtained.

Gaps in the sequence were also treated as newstate to determine whether it would have any effect. One difference in the grouping of the isolates was observed from trees generated with gaps treated as 'missing data'. Here, the *C. cubensis* isolates did not group separately, but formed a sub-clade in the greater *C. parasitica*, Australian and South African clade. Trees were much longer (301 steps and 240 steps when bases were excluded), and CI and RI values were lower than when gaps were treated as missing data (0·8272 and 0·8729 respectively, and 0·825 and 0·8743 when bases

were excluded). Treating gaps in the sequence as missing, and not as newstate, was, therefore, preferred as the resulting trees had higher CI and RI values, and fewer steps were needed to obtain the trees.

The phylogram obtained using the branch and bound option of PAUP without the exclusion of ambigious regions was chosen to illustrate the relationships between the taxa (Fig. 6). The topology of the tree reflected the same similarities and differences seen in the restriction digests. The Australian and South African isolates of *E. gyrosa* resided in a single, well resolved clade (bootstrap support 94%). In contrast, North American isolates of *E. gyrosa* resided in a different and reasonably distinct clade (bootstrap support 99%). The *C. parasitica* isolates grouped in the same clade as the *E. gyrosa* isolates from South Africa and Australia (bootstrap support 73%), while *C. cubensis* did not group in this particular clade. The *C. cubensis* isolates had a separate, basal grouping with respect to all the *C. parasitica* and different *E. gyrosa* isolates (bootstrap support 62%).

DISCUSSION

Results of this study have shown that the South African and Australian isolates identified as *E. gyrosa* are different from those from North America. This suggests that the morphological differences observed by Walker *et al.* (1985), are taxonomically relevant. Different hosts sometimes influence the variability of stromatal morphology (Fernández & Hanlin, 1996; Micales & Stipes 1987; Micales, Stipes & Bonde, 1987). The differences observed between the Australian and North

American specimens could, therefore, be due to different hosts. An example of the influence of host on morphology is the case where *C. cubensis* from clove, and *C. cubensis* from eucalypts, were classified as two species based on their different morphology. Later, they were shown to be conspecific by means of cross-inoculations, cultural studies and electrophoretic studies on proteins (Hodges *et al.*, 1986). In our case, however, molecular data showed clearly that the South African and Australian fungus represents a taxon distinct from the North American fungus. Thus, the morphological differences are not only due to the different hosts on which the fungus occurs.

This distinction between the North American isolates of *E. gyrosa*, and those from Australia and South Africa, should be investigated further. If additional morphological evidence can be found to support the molecular evidence, the South African and Australian species of *Endothia* should be described as new. If this is necessary, choosing the correct genus in which to place the new species, poses an interesting dilemma. DNA evidence suggests that the *Eucalyptus* fungus, which has non-septate, allantoid ascospores (Van der Westhuizen *et al.*, 1993; Walker *et al.*, 1985), is more closely related to *C. parasitica*, which has one-septate, elliptical ascospores than to *E. gyrosa*, which has non-sepatate, elliptical ascospores (Barr, 1978). It is possible that the South African and Australian fungus represent a species of *Cryphonectria* rather than *Endothia*. This would suggest that ascospore septation and shape are not valid morphological characters on which to base these genera. More detailed morphological studies paired with further molecular comparisons are required to resolve this question.

Most of the species now included in *Cryphonectria* was once classified as *Endothia*, but Barr (1978) separated these two genera. She also moved them into two different families of the Diaporthales (Barr, 1978), namely *Endothia* in the Valsaceae, and *Cryphonectria* in the Gnomoniaceae (Barr, 1990). Separation into these families was based mainly on ascospore and stromatal morphology (Barr, 1990, 1991). The separation of *Cryphonectria* from *Endothia* was not used by Roane (1986), who retained *Cryphonectria* species in *Endothia*. Furthermore, it was noted by Chen *et al.* (1996), that the two genera appeared insufficiently different to be separated into two families based on sequences of the 18S rRNA and ITS1 region. Our results support this view, as *E. gyrosa* and *C. parasitica* isolates, excluding *C. cubensis* and *D. ambigua*, formed a single clade. Therefore, the genera *Cryphonectria* and *Endothia* appear to belong in a single family, and not two as suggested by Barr (1978; 1990). This would also support the views of Cannon (1988), and Hawksworth *et al.* (1989), where the Gnomoniaceae was given *nomen conservandum* status to the Valsaceae.

The phylogenetic relationships between members of the genera *Cryphonectria* and *Endothia* require additional study. For instance, the correct taxonomic placement of *C. havanensis* (Bruner) Barr is unclear. *Cryphonectria havanensis* and *C. cubensis* have been repeatedly confused with each other in the past (Hodges, 1980), and *C. havanensis* and *C. gyrosa* (Berk. & Br.) Sacc. are also thought to be synonymous (Hodges, 1980; Kobayashi, 1970). Furthermore, phylogenetic relationships of *C. cubensis* with other species of *Cryphonectria* are unclear. The basal grouping of *C.*

cubensis to the other *Endothia* and *Cryphonectria* isolates suggests that *C. cubensis* may reside in a genus other than *Cryphonectria*.

Morphological studies, done in close association with molecular comparisons, will be necessary to re-define the criteria for the differentiation of *Endothia* and *Cryphonectria*. Such studies will also lead to a better understanding of the taxonomy of species of *Endothia* and *Cryphonectria*. One of the major impediments to progress with this group of fungi is the lack of cultures linked to field collections bearing morphological structures of the fungi. As these collections become available, it should be possible to resolve remaining taxonomic questions pertaining to *Cryphonectria* and *Endothia*.

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Table 1. Isolates used in the PCR-RFLPs and DNA sequencing. DNA fragments of isolates in bold were sequenced.

Culture number[1]	Alternative designation[2]	Identification	Host	Origin	Collector
CRY1	ATCC48192	*Endothia gyrosa*	*Quercus palustris*	USA	R. J. Stipes
CRY2	ATCC48192	*E. gyrosa*	*Q. palustris*	USA	R. J. Stipes
CRY39	CBS 510.76	*E. gyrosa*	*Q. suber* L.	USA	M. K. Roane
CRY70	CBS 510.76	*E. gyrosa*	*Q. suber*	USA	M. K. Roane
CRY37	CBS 510.76	*E. gyrosa*	*Q. suber*	USA	M. K. Roane
CRY9		*E. gyrosa*	*Q. palustris*	USA	S. Anagnostakis
CRY38		*E. gyrosa*	*Q. palustris*	USA	S. Anagnostakis
CRY12		*E. gyrosa*	*Q. borealis* Michx. f.	USA	S. Anagnostakis
CRY21		*E. gyrosa*	*Q. borealis*	USA	S. Anagnostakis
CRY518		*E. gyrosa*	*Fagus* sp.	USA	C. S. Hodges
CRY103		*E. gyrosa*	*Eucalyptus*	South Africa	I. van der Westhuizen
CRY62		*E. gyrosa*	*Eucalyptus*	South Africa	I. van der Westhuizen
CRY287		*E. gyrosa*	*Eucalyptus*	South Africa	H. Smith
CRY286		*E. gyrosa*	*Eucalyptus*	South Africa	I. van der Westhuizen
CRY232		*E. gyrosa*	*Eucalyptus*	South Africa	I. van der Westhuizen
CRY45		*E. gyrosa*	*E. delegatensis*	Australia	K. Old
CRY909		*E. gyrosa*	*E. globulus* Labill.	Australia	M. J. Wingfield
CMW2498	CBS134.42	*Diaporthe ambigua*	*Malus sylvestris* Mill.	Netherlands	S. Truter

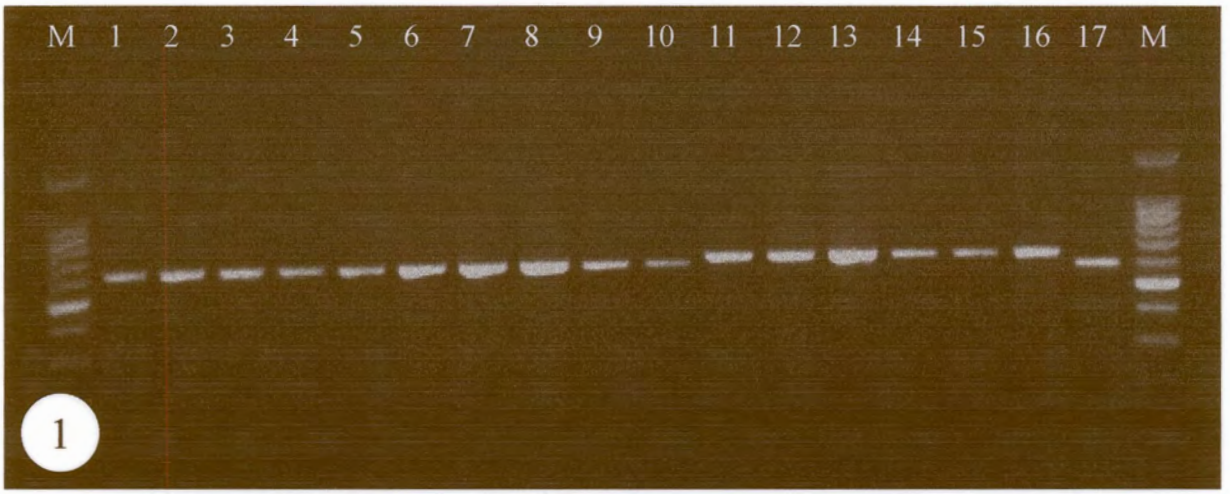
[1] Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa. CRY refers to a collection specifically of *Endothia* and *Cryphonectria* spp., whereas CMW refers to a more general collection of the Institute.

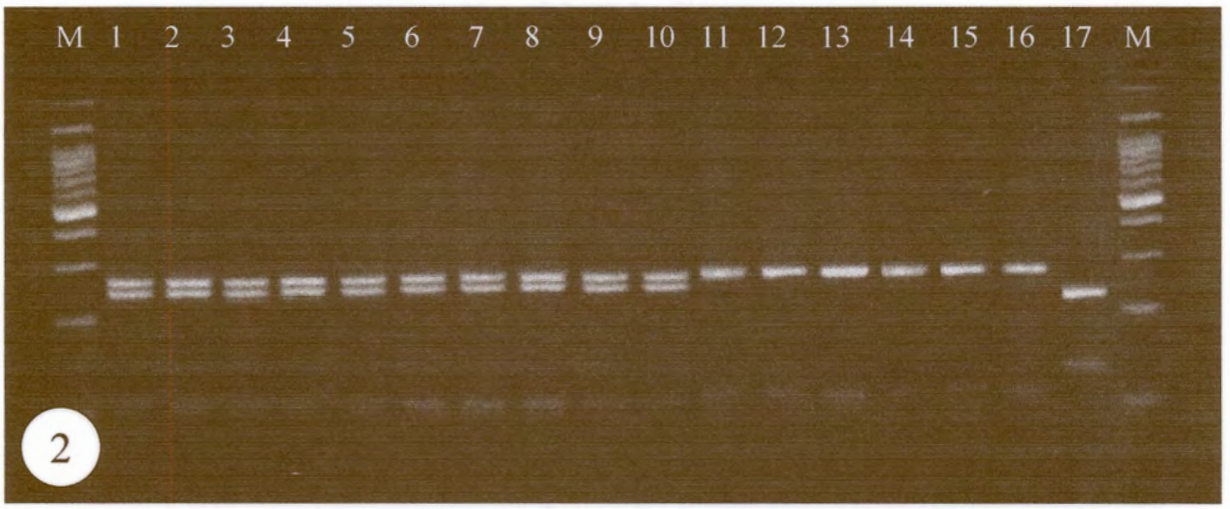
[2] American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209; Centraalbureau voor Schimmelcultures, Fungal and Yeast Collection, P. O. Box 273, 3240 AG, Baarn, Netherlands.

Table 2. Genbank accession numbers of isolates sequenced in this study and those obtained from Myburg *et al.* (1999).

Culture number	GenBank accession number	Identification	Host	Geographic Origin	Collector
CRY1	AF232874	*Endothia gyrosa*	*Quercus palustris*	USA	R. J. Stipes
CRY9	AF232875	*E. gyrosa*	*Q. palustris*	USA	S. Anagnostakis
CRY39	AF232876	*E. gyrosa*	*Q. suber*	USA	M. K. Roane
CRY103	AF232877	*E. gyrosa*	*Eucalyptus*	South Africa	I. van der Westhuizen
CRY62	AF232878	*E. gyrosa*	*Eucalyptus*	South Africa	I. van der Westhuizen
CRY287	AF232879	*E. gyrosa*	*Eucalyptus*	South Africa	H. Smith
CRY45	AF232880	*E. gyrosa*	*E. delegatensis*	Australia	K. Old
CRY909	AF232881	*E. gyrosa*	*E. globulus*.	Australia	M. J. Wingfield
CRY66	AF 046901	*Cryphonectria parasitica*	*Castanea dentata* Borkh.	USA	P. J. Bedker
CRY67	AF 046903	*C. parasitica*	*C. dentata*	USA	P. J. Bedker
CRY289	AF 046896	*C. cubensis*	*E. grandis*	Indonesia	M. J. Wingfield
CRY140	AF 046892	*C. cubensis*	*E. grandis*	South Africa	M. J. Wingfield
CMW2498	AF 046909	*Diaporthe ambigua*	*Malus sylvestris*	Netherlands	S. Truter

Fig. 1. Agarose gel (1·4%) containing PCR amplification products of the ITS1, ITS2 and 5.8S rRNA gene of the rRNA operon. **Figs 2, 3.** Restriction profiles containing restriction digests of the ITS1, ITS2 and 5.8S rRNA gene (ITS-RFLP) generated by restriction enzymes *Cfo*I and *Eco*RI respectively. Digests were separated on a 3% agarose gel stained with ethidium bromide. Lanes 1 to 10 represent the RFLP digests of the North American isolates of *Endothia gyrosa* (CRY1, CRY2, CRY70, CRY37, CRY39, CRY12, CRY21, CRY38, CRY9, CRY518), lane 11 represents an Australian *E. gyrosa* isolate (CRY45) and lanes 12 to 16 are South African isolates of *E. gyrosa* (CRY286, CRY232, CRY103, CRY62, CRY287). Lane 17 represents *Diaporthe ambigua* (CMW2498), which was used as outgroup. Lanes M are a 100 base pair molecular weight marker (Promega, Madison, USA) with the following band sizes: 100, 200, 300, 400, 500 (brightest band), 600, 700, 800, 900, 1000 bp.





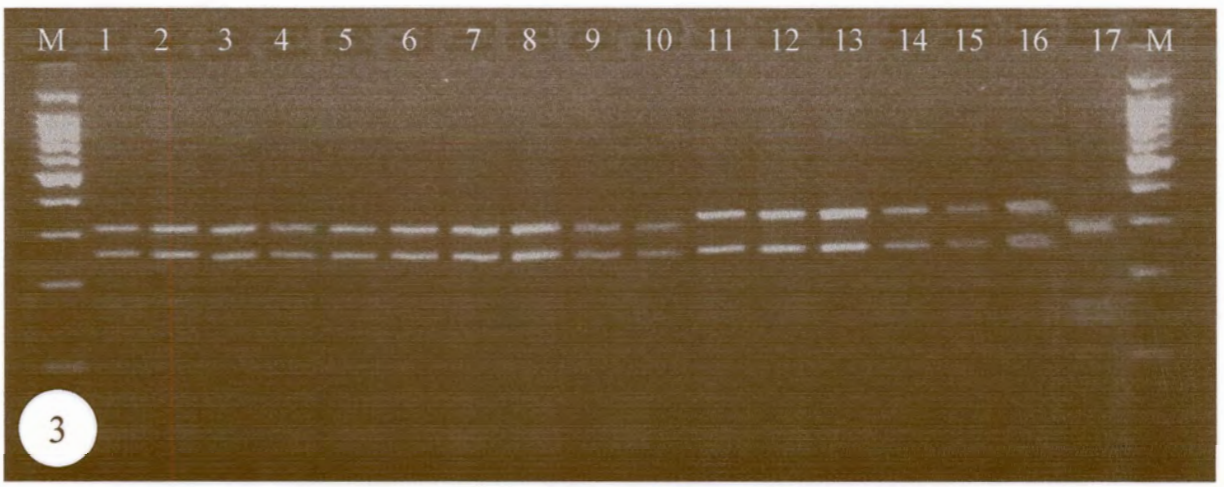


Fig. 4. Restriction map based on RFLP profiles and complete DNA sequences of PCR amplification products of the ITS1, ITS2 and 5.8S rRNA gene. PCR products were cut with restriction enzymes *Cfo*I and *Eco*RI. North American, Australian and South African isolates of *Endothia gyrosa* were used and a *Diaporthe ambigua* isolate were chosen as outgroup. The *Cfo*I restriction sites are indicated with a grey arrow pointing downward, and the *Eco*RI restriction sites are indicated with a black arrow pointing upward.

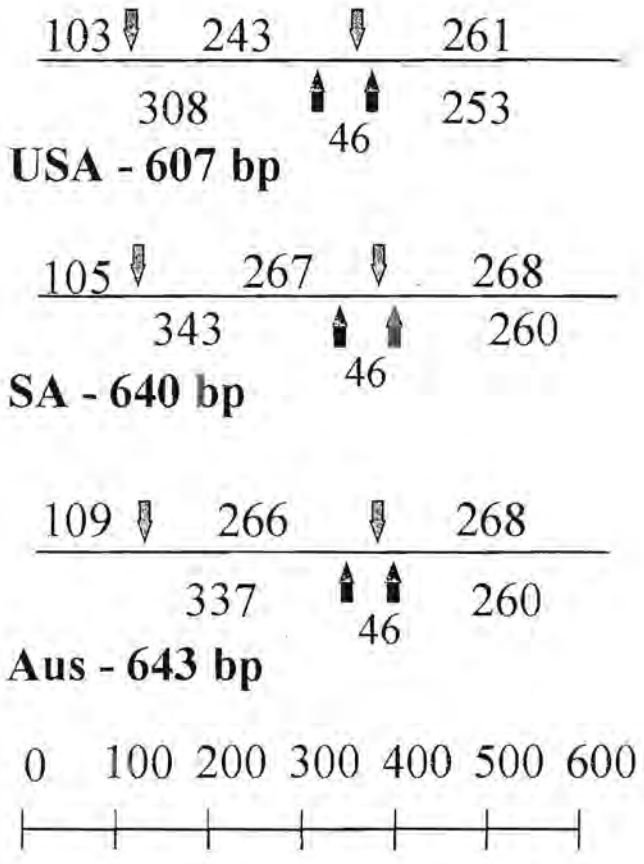

103
243
261
308
253
46
USA - 607 bp
105
267
268
343
260
46
SA - 640 bp
109
266
268
337
260
46
Aus - 643 bp
0
100
200
300
400
500
600

Fig. 5. Aligned DNA sequences of the ITS (Internal Transcribed Spacer) 1 and 2 regions, and 5.8S rRNA gene of the ribosomal operon obtained with PAUP* version 4.0b2. Isolates CRY1, CRY9 and CRY39 are *Endothia gyrosa* isolates from North America, isolates CRY62, CRY103 and CRY287 are from South Africa and isolates CRY45 and CRY909 are from Australia. Sequences of isolates CRY66 and CRY67 (*Cryphonectria parasitica*), CRY289 and CRY140 (*C. cubensis*) and CMW2498 (*Diaporthe ambigua*) were obtained from Genbank. An unknown base is indicated by "N", gaps inserted to achieve alignment by "-", and a base identical to the corresponding base of the sequence of CRY1, by "·".

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CRY1     E. gyrosa (USA)   CCAGATACCC -TTTGTGAAC TTATA-CCAT TTT---A-TC GTTGCCTCGG
CRY9     E. gyrosa (USA)   .......... -......... .....-.... ...---.-.. ..........
CRY39    E. gyrosa (USA)   .......... -......... .....-.T.. ...---.-.. ..........
CRY103   E. gyrosa (SA)    .......... -.A....... .....-..-. ...TTT.-.. ..........
CRY62    E. gyrosa (SA)    .......... -.A....... .....-..-. ...TTT.-.. ..........
CRY287   E. gyrosa (SA     .......... -.A....... .....-..-. ...TTT.-.. ..........
CRY45    E. gyrosa (Aus.)  .......... C.A....... .....-..-. ...TTT.A.. ..........
CRY909   E. gyrosa (Aus.)  .......... C.A....... .....-..-. ...TTT.-.. ..........
CRY67    C. parasitica     NNNNNNNNN. C.NN...... .....A.... ...---.-.. ..........
CRY66    C. parasitica     NNNNNNNNNN NNNNNNNNNN N....A.... ...---.-.. ..........
CRY140   C. cubensis       .......... -......... .....-..-. ...T--.-.. ..........
CRY289   C. cubensis       .......... -......... .....-..-. ...T--.-.. ..........
CMW2498  D. ambigua        .....A.... -......... .....-..-. .-----.TCT ..........

CRY1     E. gyrosa (USA)   CGCTGAGC-- -TGGGGG--- ---------- -CA------- -------CTC
CRY9     E. gyrosa (USA)   ........-- -......--- ---------- -..------- -------...
CRY39    E. gyrosa (USA)   ........-- -......--- ---------- -..------- -------...
CRY103   E. gyrosa (SA)    ........CC --.....G-- ------GAAG --.GAAAG-C TT----G..T
CRY62    E. gyrosa (SA)    ........CC --.....G-- ------GAAG --.GAAAG-C TT----G..T
CRY287   E. gyrosa (SA)    ........CC --.....G-- ------GAAG --.GAAAG-C TT----G..T
CRY45    E. gyrosa (Aus.)  ........CC --.....G-- ------GAAG --.GAAAG-C TT----G..T
CRY909   E. gyrosa (Aus.)  ........CC --.....G-- ------GAAG --.GAAAG-C TT----G..T
CRY67    C. parasitica     ........CT C......GGG GTTGGCGAAG G..GATTTTC TTCCTT-..-
CRY66    C. parasitica     ........CT C......GGG GTTGGCGAAG G..GATTTTC TTCCTT-..-
CRY140   C. cubensis       ...C....C- --..------ ------GA-G -----TGCTC TTCTGTG..-
CRY289   C. cubensis       ...C....C- --..------ ------GA-G -----TGCTC TTCTGTG..-
CMW2498  D. ambigua        ....-...-- -...------ ---------- ---------- ----------

CRY1     E. gyrosa (USA)   TCC-TG--TG CCCCCC---- ---CACC--G TG-------C AAGCGGTGG-
CRY9     E. gyrosa (USA)   ...-..--.. ......---- ---....--. ..-------. .........-
CRY39    E. gyrosa (USA)   ...-..--.. ......---- ---....--. ..-------. .........-
CRY103   E. gyrosa (SA)    ...C.CCC.- ......---- TT-..-.GG. ..TAAAAA-. C..T.T...-
CRY62    E. gyrosa (SA)    ...C.CCC.- ......---- TT-..-.GG. ..TAAAAA-. C..T.T...-
CRY287   E. gyrosa (SA)    ...C.CCC.- ......---- TT-..-.GG. ..TAAAAA-. C..T.T...-
CRY45    E. gyrosa (Aus.)  ...C.CCC.- .....----- TT-..-.GG. ..TAAAAAC. C..T.T...-
CRY909   E. gyrosa (Aus.)  ...C.CCC.- .....----- TT-..-.GG. ..TAAAAAC. C..T.T...-
CRY67    C. parasitica     C..C.CCC.- ......CCTC TTC....G-- ..CAAA---. GGTT.T...G
CRY66    C. parasitica     C..C.CCC.- ......CCTC TTC....G-- ..CAAA---. GGTT.T...G
CRY140   C. cubensis       C..C------ ---------- ---....G-- C.CAA----G C..T.-----
CRY289   C. cubensis       C..C------ ---------- ---....G-- C.CAA----G C..T.-----
CMW2498  D. ambigua        ----.CCC.- .GGGG.CCT- ---....C-- .--------. GG.T.T.---

CRY1     E. gyrosa (USA)   -AG-CAGGCC CGCCGGCGGC CCACCAAACT CTTTGTTTTT -AGACCGT-A
CRY9     E. gyrosa (USA)   -..-...... .......... .......... .......... -.......-.
CRY39    E. gyrosa (USA)   -..-...... .......... .......... .......... -.......-.
CRY103   E. gyrosa (SA)    -..--..... .......... ...TT..... ...-...... T.T.A.C.-.
CRY62    E. gyrosa (SA)    -..-...... .......... ...TT..... ...-...... T.T.A.C.-.
CRY287   E. gyrosa (SA)    -..-...... .......... ...TT..... ...-...... T.T.A.C.-.
CRY45    E. gyrosa (Aus.)  -..-...... .......... ...TT..... ...-...... T.T.A.C.-.
CRY909   E. gyrosa (Aus.)  -..-...... .......... ...TT..... ...-...... T.T.A.C.-.
CRY67    C. parasitica     G..-...... .......... ....T..... .......... -.T.A.C.-.
CRY66    C. parasitica     G..-...... .......... ....T..... .......... -.T.A.C.-.
CRY140   C. cubensis       G..-...... .......... .......... .......... -...A...-.
CRY289   C. cubensis       G..-...... .......... .......... .......... -...A...-.
CMW2498  D. ambigua        G..A...-.. ..T....... .A...T..A. ...-...... -.C.-.-.G.
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CRY1     E. gyrosa (USA)   --TCTCCTCT GAGTGTTTAC AAAAA-CAAA ------TGAA TCAAAACTTT
CRY9     E. gyrosa (USA)   --........ .......... .....-.... ------.... ..........
CRY39    E. gyrosa (USA)   --........ .......... .....-.... ------.... ..........
CRY103   E. gyrosa (SA)    --....T... ...--...-. .T...-.... AA----.... ..........
CRY62    E. gyrosa (SA)    --....T... ...--...-. .T...-.... AA----.... ..........
CRY287   E. gyrosa (SA)    --....T... ...--...-. .T...-.... AA----.... ..........
CRY45    E. gyrosa (Aus.)  --....T... ...--...-. .T...-.... AA----.... ..........
CRY909   E. gyrosa (Aus.)  --....T... ...--...-. .T...-.... AA----.... ..........
CRY67    C. parasitica     --....T... ....A----. .T...-.... AAAAAA.... ..........
CRY66    C. parasitica     --....T... ....A----. .T...-.... AAAAAA.... ..........
CRY140   C. cubensis       --....T... .........-- .T..--.... CAAA--.... ..........
CRY289   C. cubensis       --....T... .........-- .T..--.... CAAA--.... ..........
CMW2498  D. ambigua        AA-...T--- ...------. .C...A..T. A-----.... ..........

CRY1     E. gyrosa (USA)   CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC AGCGAAATGC
CRY9     E. gyrosa (USA)   .......... .......... .......... .......... ..........
CRY39    E. gyrosa (USA)   .......... .......... .......... .......... ..........
CRY103   E. gyrosa (SA)    .......... .......... .......... .......... ..........
CRY62    E. gyrosa (SA)    .......... .......... .......... .......... ..........
CRY287   E. gyrosa (SA)    .......... .......... .......... .......... ..........
CRY45    E. gyrosa (Aus.)  .......... .......... .......... .......... ..........
CRY909   E. gyrosa (Aus.)  .......... .......... .......... .......... ..........
CRY67    C. parasitica     .......... .......... .......... .......... ..........
CRY66    C. parasitica     .......... .......... .......... .......... ..........
CRY140   C. cubensis       .......... .......... .......... .......... ..........
CRY289   C. cubensis       .......... .......... .......... .......... ..........
CMW2498  D. ambigua        .......... .......... .......... .......... ..........

CRY1     E. gyrosa (USA)   GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG
CRY9     E. gyrosa (USA)   .......... .......... .......... .......... ..........
CRY39    E. gyrosa (USA)   .......... .......... .......... .......... ..........
CRY103   E. gyrosa (SA)    .......... .......... .......... .......... ..........
CRY62    E. gyrosa (SA)    .......... .......... .......... .......... ..........
CRY287   E. gyrosa (SA)    .......... .......... .......... .......... ..........
CRY45    E. gyrosa (Aus.)  .......... .......... .......... .......... ..........
CRY909   E. gyrosa (Aus.)  .......... .......... .......... .......... ..........
CRY67    C. parasitica     .......... .......... .......... .....G.... ..........
CRY66    C. parasitica     .......... .......... .......... .....G.... ..........
CRY140   C. cubensis       .......... .......... .......... .......... ..........
CRY289   C. cubensis       .......... .......... .......... .......... ..........
CMW2498  D. ambigua        .......... .......... .......... .......... ..........

CRY1     E. gyrosa (USA)   CACATTGCGC CCGCTGGAAT TCCAGCGGGC AT-GCCTGTT CGAGCGTCAT
CRY9     E. gyrosa (USA)   .......... .......... .......... ..-....... ..........
CRY39    E. gyrosa (USA)   .......... .......... .......... ..-....... ..........
CRY103   E. gyrosa (SA)    .......... .......... .......... ..-....... ..........
CRY62    E. gyrosa (SA)    .......... .......... .......... ..-....... ..........
CRY287   E. gyrosa (SA)    .......... .......... .......... ..-....... ..........
CRY45    E. gyrosa (Aus.)  .......... .......... .......... ..-....... ..........
CRY909   E. gyrosa (Aus.)  .......... .......... .......... ..-....... ..........
CRY67    C. parasitica     .........-- -..-..-... ....-...-. ..C..N.... ..........
CRY66    C. parasitica     .........-- -..-..-... ....-...-. ..C....... ..........
CRY140   C. cubensis       .........-- -..-..-... ....-...-. ..C....... ..........
CRY289   C. cubensis       .........-- -..-..-... ....-...-. ..G....... ..........
CMW2498  D. ambigua        .......... ..T....T.. ...G.A.... ..-....... ..........
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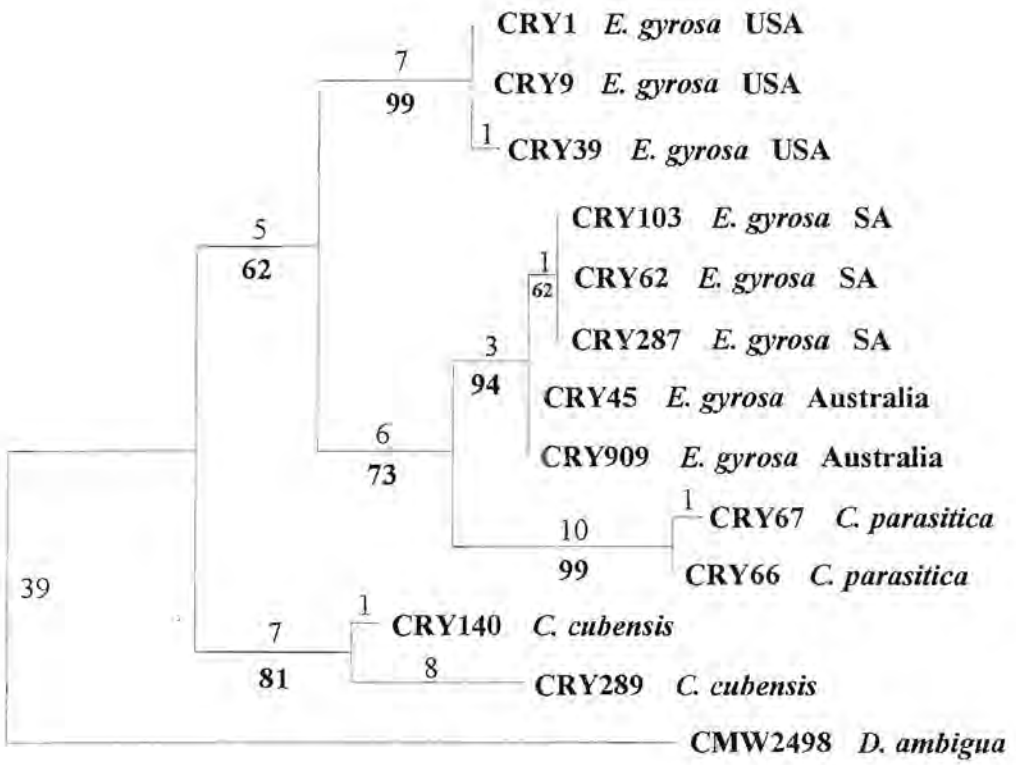
```
CRY1     E. gyrosa (USA)   TTCAACCCTC AAGCC---TG GCTTGGTGTT GGGGCACTAC CTGTA---CA
CRY9     E. gyrosa (USA)   .......... .....---.. .......... .......... .....---..
CRY39    E. gyrosa (USA)   .......... .....---.. .......... .......... .....---..
CRY103   E. gyrosa (SA)    .......... .....CCT.. .......... .......... .C...A--..
CRY62    E. gyrosa (SA)    .......... .....CCT.. .......... .......... .C...A--..
CRY287   E. gyrosa (SA)    .......... .....CCT.. .......... .......... .C...A--..
CRY45    E. gyrosa (Aus.)  .......... .....CCT.. .......... .......... .C...AAA.-
CRY909   E. gyrosa (Aus.)  .......... .....C-T.. .......... .......... .C...AAA.-
CRY67    C. parasitica     .......... ....---T.. .......... ....T..... .C...AAA--
CRY66    C. parasitica     .......... ....---T.. .......... ....T..... .C...AAA--
CRY140   C. cubensis       .......... .....---.. .......... .......... ....T-CA..
CRY289   C. cubensis       .......... .....---.. .......... ........G. ....TTTA..
CMW2498  D. ambigua        .......... .....---.. ........A. ........G. T.C.TACC..

CRY1     E. gyrosa (USA)   ACGG-TAGGC CCTGAAATTT AGTGGCGGGC TCGCTAAGAC TCTGAGCGTA
CRY9     E. gyrosa (USA)   ....-..... .......... .......... .......... ..........
CRY39    E. gyrosa (USA)   ....-..... .......... .......... .......... ..........
CRY103   E. gyrosa (SA)    -...-..... .........G .......... .......... ..........
CRY62    E. gyrosa (SA)    -...-..... .........G .......... .......... ..........
CRY287   E. gyrosa (SA)    -...-..... .........G .......... .......... ..........
CRY45    E. gyrosa (Aus.)  --..-..... .......... .......... .......... ..........
CRY909   E. gyrosa (Aus.)  --..-..... .......... .......... .......... ..........
CRY67    C. parasitica     -...-..... .......... .......... .......... .......C..
CRY66    C. parasitica     -...-..... .......... .......... .......... ..........
CRY140   C. cubensis       G...G..... .......... .A........ .......... ..........
CRY289   C. cubensis       G...G..... .......... .......... .......... .....A....
CMW2498  D. ambigua        .GAAGC.... .........C .......A.. ....C.G... C.C.....C.

CRY1     E. gyrosa (USA)   GTAGTTT--- ----ATCA-C CTCGCTTTGG AAGGATTA-G CGGT-GCTCT
CRY9     E. gyrosa (USA)   .......--- ----....-. .......... .........-. ....-.....
CRY39    E. gyrosa (USA)   .......--- ----....-. .......... .........-. ....-.....
CRY103   E. gyrosa (SA)    .......TT- ----....A. .......... .........-. ....T.....
CRY62    E. gyrosa (SA)    .......TT- ----....A. .......... .........-. ....T.....
CRY287   E. gyrosa (SA)    .......TT- ----....A. .......... .........-. ....T.....
CRY45    E. gyrosa (Aus.)  .......TT- ----....A. .......... .........-. ....T.....
CRY909   E. gyrosa (Aus.)  .......TT- -----...A. .......... .........-. ....T.....
CRY67    C. parasitica     .......TTT TTTCT...A. .......... .........-. ....T.....
CRY66    C. parasitica     .......TTT TTTCT...A. .......... .........-. ....T.....
CRY140   C. cubensis       .......TT- ----....-. .......... .........-. ....-.....
CRY289   C. cubensis       .......TT- ----....-. .......... .........-C GA..-..C..
CMW2498  D. ambigua        ......---- -----AA.C. ......C... ....CCCTG. ....-..C..

CRY1     E. gyrosa (USA)   TGCCGT-AAA ACC
CRY9     E. gyrosa (USA)   ......-... ...
CRY39    E. gyrosa (USA)   ......-... ...
CRY103   E. gyrosa (SA)    ......-... ...
CRY62    E. gyrosa (SA)    ......-... ...
CRY287   E. gyrosa (SA)    ......-... ...
CRY45    E. gyrosa (Aus.)  ......-... ...
CRY909   E. gyrosa (Aus.)  ......-... ...
CRY67    C. parasitica     ......-... ...
CRY66    C. parasitica     ......-... ...
CRY140   C. cubensis       ......-... ...
CRY289   C. cubensis       ......-... ...
CMW2498  D. ambigua        -.....T... -..
```

Fig. 6. The most parsimonious tree obtained from sequences of the ITS1, ITS2 and 5.8S rRNA gene of the ribosomal operon for isolates of *Endothia gyrosa* (USA, Australia and South Africa), *Cryphonectria parasitica, C. cubensis* and the outgroup *Diaporthe ambigua*. The tree was obtained using the branch and bound algorithm of PAUP* 4.0b2 without the exclusion of ambiguous regions (tree length = 99, CI = 0·9495, RI = 0·9367, g1 = 0·809149). Percentage confidence levels (1000 bootstrap replications) are indicated in bold below the branches, and the amount of steps are indicated above the branches.


CRY1 E. gyrosa USA
CRY9 E. gyrosa USA
CRY39 E. gyrosa USA
CRY103 E. gyrosa SA
CRY62 E. gyrosa SA
CRY287 E. gyrosa SA
CRY45 E. gyrosa Australia
CRY909 E. gyrosa Australia
CRY67 C. parasitica
CRY66 C. parasitica
CRY140 C. cubensis
CRY289 C. cubensis
CMW2498 D. ambigua
7
99
1
5
62
1
62
3
94
6
73
1
10
99
39
1
7
81
8

CHAPTER 3

A new species of *Cryphonectria* from South Africa and Australia pathogenic to *Eucalyptus*

ABSTRACT

Endothia gyrosa is known as the pin oak blight pathogen in North America, and also causes cankers on other trees. In South Africa and Australia the fungus has been recorded only on *Eucalyptus* spp. In a previous study, based on molecular data, Australian and South African isolates of *E. gyrosa* grouped with *C. parasitica* rather than with North American isolates of *E. gyrosa*. The aim of this study was, therefore, to compare *E. gyrosa* isolates from North America with those from South Africa and Australia and to consider whether differences observed at the molecular level can be substantiated based on morphology. Morphological features were compared from a large collection of specimens. Distinct morphological differences were observed between North American specimens and those from South Africa and Australia. In addition, growth in culture at 10 °C and 15 °C was different for those sets of isolates. Moreover, stromatal morphology in the South African and Australian specimens was more similar to that of *Cryphonectria gyrosa* than to *Endothia*. Morphological data thus confirm the differences observed at the molecular level. The fungus known as *Endothia gyrosa* from *Eucalyptus* in South Africa and Australia is consequently described as a new taxon in *Cryphonectria*.

INTRODUCTION

Endothia gyrosa (Schw.:Fr.) Fr. is a fungal pathogen best known for its association with the disease known as pin oak (*Quercus palustris* Muenchh.) blight in North America (Appel & Stipes, 1986; Roane *et al.*, 1974; Stipes & Phipps, 1971). This fungus, apparently native to North America, also causes serious cankers on exotic Formosan sweetgum (*Liquidambar formosana* Hance.) (Snow, Beland & Czabator, 1974) and other trees, including several *Quercus* spp., *Acer saccharinum* L. (Roane *et al.*, 1974), *Liquidambar styraciflua* L. (Snow *et al.*, 1974), *Castanea* spp., *Ilex opaca* Aiton (Appel & Stipes, 1986), *Fagus sylvatica* L. (Shear, Stevens & Tiller, 1917), *F. grandiflora* Ehrh., *Prunus laurocerasus* L. (Roane, 1986), *Ulmus*, *Corylus* and *Vitis* (Farr *et al.*, 1989).

In the USA, *E. gyrosa* has been reported to occur in various southeastern states (Stevens, 1917), as well as isolated areas of Kansas (Shear *et al.*, 1917; Stevens, 1917), Ohio (Appel & Stipes, 1986; Hunter & Stipes, 1978; Stevens, 1917), Michigan, Maryland, New Jersey, Connecticut, New York, California (Shear *et al.*, 1917, Stevens, 1917) and on the Pacific coast (Stevens, 1917). *Endothia gyrosa* has also been reported in China on *Quercus* (Teng, 1934) and Spaulding (1961) reported the fungus in Portugal, Spain and Italy on *Quercus* spp. and *Fagus* L. spp. Spaulding (1961) also noted that *E. gyrosa* occurred in Germany, western Europe, Ceylon, New Zealand and the Phillipines, but no hosts were recorded.

Endothia gyrosa has been reported as a pathogen of *Eucalyptus* L'Hérit from Australia (Davison & Coates, 1991; Walker *et al.*, 1985), Tasmania (Old *et al.*, 1986; Yuan & Mohammed, 1997) and South Africa (Van der Westhuizen *et al.*, 1993). There is also a single report of the fungus on *E. diversicolor* F. Muell. in Portugal (Spaulding, 1961). In southeastern and western Australia, *E. gyrosa* is found on numerous species of *Eucalyptus* (Davison & Coates, 1991; Old *et al.*, 1986; Walker *et al.*, 1985; White and Kile, 1993). The fungus was recently reported from South Africa associated with cankers on several species of *Eucalyptus*, including *E. grandis* W. Hill ex Maid., *E. nitens* (Deane & Maid.) Maid., *E. urophylla* R. T. Blake and hybrids of *E. grandis* with *E. camaldulensis* Dehnh. and *E. urophylla* (Van der Westhuizen *et al.*, 1993).

Endothia gyrosa was not originally regarded as a serious pathogen in North America (Shear *et al.*, 1917), but more recent reports describe it as relatively important (Roane *et al.*, 1974; Snow *et al.*, 1974; Stipes & Phipps, 1971). Cankers are discreet, sunken and elongated (Stipes & Phipps, 1971) with the characteristic orange stromata formed on the surface of the bark (Snow *et al.*, 1974; Stipes & Phipps, 1971). Common symptoms associated with the fungus, and more particularly with pin oak blight, are die-back, defoliation (Roane *et al.*, 1974; Stipes & Phipps, 1971), death of branches and overall tree decline (Stipes & Phipps, 1971). *Endothia gyrosa* is an opportunistic pathogen, and pruning or other wounds appear to provide infection courts (Roane *et al.*, 1974; Snow *et al.*, 1974; Stevens, 1917; Stipes & Phipps, 1971; Weir, 1925). Disease appears to be stress-related and becomes more serious when trees are

predisposed by environmental factors, such as water stress (Appel & Stipes, 1984 1986; Hunter & Stipes, 1978; Stevens, 1917).

In Australia and Tasmania, *E. gyrosa* has been found associated with elongated vertical cankers on *Eucalyptus* spp. (Walker *et al.*, 1985), cracking of the bark, kino veins (Old *et al.*, 1986), die-back of branches (Old *et al.*, 1990; Walker *et al.*, 1985), severe cankers and death of trees (Wardlaw, 1999). In artificial inoculations, the fungus was found to be pathogenic (Old *et al.*, 1986, 1990; Walker *et al.*, 1985; Yuan & Mohammed, 1999), and was especially serious in seedling inoculations (Old *et al.*, 1990). In South Africa, cankers tend to be superficial and characterized by bark cracking and slightly swollen areas on the stems (Van der Westhuizen *et al.*, 1993). The fungus on eucalypts is also an opportunist, readily infecting through wounds (Old *et al.*, 1986; White & Kile, 1993). Although water stress has not been found to predispose eucalypts to infection by *E. gyrosa*, defoliation had an aggravating effect on lesion length (Old *et al.*, 1990). Based on those results, *E. gyrosa* in Australia has been considered a mild pathogen, having no serious effect on healthy trees (Old *et al.*, 1990).

Endothia gyrosa has been known to occur in the USA from the first half of the nineteenth century (Barr, 1978; Shear *et al.*, 1917; Stevens, 1917). Its recent discovery in Australia and South Africa on a completely different host to those known in North America, is unusual. The question of the similarities and differences between the North American fungus and Southern Hemisphere *Eucalyptus* fungus was raised by Walker *et al.* (1985), who noted morphological differences between those two groups

of fungi. Those differences were mainly that the stromata of the Australian specimens were less well developed, and that the lower part or the entire perithecial body is seated in the bark and not in fungal tissue, as is observed in specimens from North America. Nonetheless, the North American and Australian specimens had similar perithecia, asci and ascospores, leading to the identification of the Australian fungus as *E. gyrosa*.

In a previous study (Chapter 2), the South African and Australian fungus was found to be different to *E. gyrosa* from North America based on PCR-based RFLPs and DNA sequence comparisons. Furthermore, the South African and Australian isolates grouped more closely with *Cryphonectria parasitica* (Murr.) Barr than with *E. gyrosa* from North America. The aim of this study was, therefore, to determine, based on morphology, whether collections of *E. gyrosa* from Australia and South Africa represent the same taxon as those from North America, and to consider their respective generic positions.

MATERIALS AND METHODS

Source of isolates

Bark samples from South Africa were collected from different localities in commercial plantations of *Eucalyptus* exhibiting typical canker symptoms (Table 1). Samples from trees in North America, used for comparative purposes, were sent by Dr. C. S. Hodges, Dept. Plant Pathology, NC State University, Raleigh, NC, USA.

Specimens of the Australian fungus, deposited by Walker *et al.* (1985) in the Plant Pathology Herbarium, Orange Agricultural Institute, were also examined (Table 1). All samples considered have been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM) (Table 1). The type specimen of *E. gyrosa*, which had a North American origin, was found not to be useful for study (Shear *et al.*, 1917) and the co-type as listed by Shear *et al.* (1917), could not be located despite attempts to do so. Specimens of *C. gyrosa* (Berk. & Br.) Sacc., the type of *Cryphonectria*, were also examined (Table 1).

Light microscopy

Stromata together with surrounding bark tissue were removed from all bark samples using a scalpel, sectioned vertically with a razor blade, and mounted on microscope slides in lactophenol. Fifty measurements were made of all structures believed to be of taxonomic significance, including perithecia, asci, ascospores, pycnidial locules, conidiogenous cells and conidia. Due to the limited occurrence of stromata, only 30 stromata were measured for the holotype. The means and ranges of all measurements were computed. The colour designations of Rayner (1970) were assigned to standardize colours to structures.

Growth study

The growth in culture of isolates CRY 910, CRY 911, CRY 900 (South Africa) and CRY 21, CRY 906, CRY 907 (North America) were compared (Table 2). Two types

of media were used, namely 20 g/l malt extract agar (Biolab, Merck, Midrand, South Africa) and 50 g/l potato dextrose agar (Difco Laboratories, Detroit, USA). A disc 6 mm in diameter, taken from the edge of actively growing colonies, was placed in the centre of each of four 90 mm Petri dishes per isolate. Growth studies were conducted at 10 to 30 °C, with 5 °C intervals, in the dark. The colony diameters (mm) were measured daily from the third day of the experiment until the first isolates completely covered the plate on day nine. Colony diameter of each isolate was compared as an average of the four readings per isolate. Isolates are preserved in the culture collection of the Forestry & Agricultural Biotechnology Institute (FABI), University of Pretoria, Hillcrest, Pretoria, 0002 South Africa.

RESULTS

Morphological comparisons

The South African and North American fungi were found to be distinctly different from each other. The South African and Australian specimens from *Eucalyptus*, however, were morphologically indistinguishable from each other. The stromata of the North American specimens were much larger [(911-1562(-1800) μm high, (1250-)1296-1881(-2050) μm in diameter] than those of the South African specimens [(202-)389-741(-940) μm high, (186-)343-704(-868) μm in diameter]. The stromata from the North American specimens were also superficial and subclavate (Fig. 1) compared with the South African specimens where stromata were usually semi-immersed (although fully immersed and superficial stromata also occurred occasionally) (Fig.

2*a*, *d*). The ratio of the part of the stromata that are erumpent to the entire length of the stroma, is 1:2 for the South African and Australian fungus (sometimes a much as 1:4), while the ratio for the North American fungus is 1:1, since the stromata of the North American fungus are almost completely erumpent. Stromata from *Eucalyptus* were more variable in shape than those from trees in North America. Furthermore, the lower half of the stromata of the North American specimens had a typical pseudostromatic appearance with host cells distributed thoughout the stromatal tissue (Fig. 1*a*, *d*). The ectostroma and entostroma were also continuous with each other, but predominantly entostromatic. The lower parts of the stromata of the South African and Australian specimens consisted predominantly of host tissue, while the upper parts of the stromata in these specimens consisted primarily of fungal tissue (Fig. 2*a*, *d*). The South African and Australian stromata thus had typical ectostromatic discs forming the erumpent parts, and entostromatic discs containing the perithecia.

The stromatal structure of the North American *E. gyrosa* specimens were consistent with those described for *Endothia* by Micales & Stipes (1987). In contrast, the stromatal structure of the South African and Australian specimens resembled that for species of *Cryphonectria* (Micales & Stipes, 1987) rather than *Endothia*. These specimens also had stromata that were similar to the specimens of *C. gyrosa*, which is the type of *Cryphonectria*.

The pycnidial locules in the South African and Australian specimens were usually less than 10 in number, irregular and convoluted in shape (Fig. 2*a*, 3*a*). In contrast, the pycnidial stromata of the North American specimens had more than 30 locules, and

the locules were not convoluted (Fig. 1a). In most cases, the pycnidial locules in the South African specimens were large [(56-)104-368(-558) μm long, (50-)82-230(-350) μm wide] relative to the stromata, with some smaller locules also being present. The locules in the North American specimens [(47-)67-163(-279) μm long, (35-)51-107(-175) μm wide] were small relative to the stromata and were dispersed thoughout the stromata, giving them a labyrinthiform appearance (Fig. 1*a*). Conidia in the specimens from North America were exuded from pycnidia in large orange (15) droplets becoming sienna (15i) in colour when older. Conidia of the South African and Australian specimens were exuded either as brilliantly orange (15) or brilliantly luteous (19) spore tendrils or in orange droplets.

The conidiogenous cells in the South African specimens were longer [(4-)8-17·5(-27) μm long, 0·5-1·5(-2) μm wide; mean length/width ratio 13:1] (Fig. 2*b*, 3*b*) than those of the North American specimens [(4-)5·5-9·5(-12) μm long, 1-1·5(-2) μm wide; mean length/width ratio 7·5:1]. Furthermore, hyphae up to 71·5 μm long occurred between the conidiogenous cells in the South African specimens (Fig. 2*b*, 3*c*), but not in the North American specimens. These long hyphae often terminated in phialides, but they were never seen to produce conidia and were probably sterile. No conidiogenous cells could be seen in the Australian specimens due to the age of the specimens. The long sterile hyphae were, however, noted by Walker *et al.* (1985). Similar long hyphae have also been reported in *C. parasitica* (Shear *et al.*, 1917), although not in any other species of this group (Kobayashi, 1970; Roane, 1986; Shear *et al.*, 1917).

The perithecial bases in the South African and Australian specimens were generally dark with dark necks (Fig. 2*d*, 3*d*), while the bases of the young perithecia were usually light coloured. The perithecial bases in the North American specimens were not always as dark, or only had darkened areas around the bases of the necks (Fig. 1*b*), similar to young perithecia in the South African and Australian samples. The perithecial bases of the North American specimens were also situated in the stromatal tissue above the level of the bark surface, while those of the South African and Australian perithecia generally occurred below the level of the bark surface, frequently surrounded with bark tissue, and not exclusively surrounded with fungal tissue. Furthermore, the North American specimens had perithecia that often occurred together with active pycnidial locules. Perithecia were seldom found together with pycnidial locules in the South African specimens. When they did occur together, the pycnidial locules were barren.

Ascus and ascospore morphology are similar for the South African and North American fungus (Fig. 1*f*, 2*f*). Asci of the South African specimens were (12-)17·5-26·5(-34) µm long and 4·5-7(-9) µm wide. Asci of the North American specimens were shorter [(9·5-)11·5-18(-20) µm long, (2·5-)3·5-6 µm wide] than the South African asci, but longer measurements up to 30 µm has been reported for asci of the North American fungus (Barr, 1978; Shear *et al.*, 1917). Ascospore size for the South African specimens were similar to those of the North American specimens [(4·5-)6-9(-12·5) µm long, (0·5-)1-1·5(-2) µm wide for the South African specimens; (3·5-)7·5-11(-13) µm long, 0·5-1·5(-2) µm wide for the North American specimens]. The South

African specimens also had the allantoid to cyllindrical shape of the North American specimens.

When mounting *E. gyrosa* specimens from North America in lactophenol, this mounting medium always became orange-coloured. In contrast, South African and Australian specimens did not discolour lactophenol. This discolouration of lactophenol was previously noted by Roane (1986) and is due to the pigments found in *Endothia* and *Cryphonectria*. Pigments of *E. gyrosa* from North America and other species of *Endothia* and *Cryphonectria* have previously been studied (Roane & Stipes, 1978) and differences have been observed in pigment composition for different species of *Endothia* and *Cryphonectria*. Differences in discolouration of lactophenol indicates that there are probably distinct differences in pigment contents in the North American isolates and the South African and Australian fungus from *Eucalyptus* known as *E. gyrosa*.

Growth in culture

Clear differences in colony morphology were observed between the South African and North American isolates of *E. gyrosa*. Colonies of isolates from North America were fuscous black (13''''m) to buff (19''f) to cinnamon (15'') (Fig. 4*a*, *b*) and had either a uniformly fluffy or sectored appearance (Fig. 4*b*). Colonies of the South African isolates were white and fluffy (Fig. 4*a*). Similar differences were observed on both MEA and PDA. Although not produced during the growth study, South African isolates usually produce orange pycnidia on MEA and PDA, while North American

isolates seldomly produce pycnidia. The South African isolates also grew faster at 10 °C and 15 °C than the North American isolates (Fig. 5) on both MEA and PDA.

DISCUSSION

In this study we have shown conclusively that the fungus known as *E. gyrosa* from *Eucalyptus* in South Africa and Australia is morphologically different to *E. gyrosa* from North America. This is both in terms of a wide range of structures on host tissue as well as characteristics in culture. The South African and Australian fungus is also different from other species of *Endothia* in that *E. singularis* (H. & P. Syd.) Shear & Stevens has large, red stromata [2-4 mm high, 3-5 mm in diameter (Shear *et al.*, 1917)], and *E. viridistroma* Wehm. has green stromata (Barr, 1978; Roane, 1986). These morphological observations support previously presented DNA sequence and RFLP data showing that South African and Australian isolates are distinct from North American isolates (Chapter 2).

In Chapter Two of this thesis, DNA sequnces of the ITS region of the ribosomal operon, suggested that the South African and Australian fungus is more closely related to *Cryphonectria* than to *Endothia*. Our morphological observations support this view. *Endothia* species have strongly developed, widely erumpent, sub-globose, primarily entostromatic stromata consisting primarily of pseudoparenchyma, and with perithecia occurring generally above the bark surface in a diatrypoid configuration. In contrast, *Cryphonectria* species, as well as the South African and Australian *Eucalyptus* fungus, have semi-immersed stromata consisting primarily of prosenchyma and with

distinct ectostromatal and entostromatal discs. The perithecia occur underneath the bark surface and have a valsoid appearance near the periphery of the stroma when the stroma is weakly developed. This is in accordance with previous observations by Micales & Stipes (1987).

Different hosts, bark types and environments may influence the stromatal morphology of *Endothia* and *Cryphonectria*. More specifically, the number of layers of pycnidia and perithecia can be influenced by the bark of the host, while the sequence of formation of perithecia and pycnidia appears to be influenced by climatic factors (Shear *et al.*, 1917). Moreover, the degree to which perithecia are positioned to assume a diatrypoid or valsoid configuration, appears to depend on the degree of stromatal development (Cannon, 1988). The size, structure and degree to which stromata are embedded, depends on the bark, host and external factors, such as moisture (Cannon, 1988; Hodges, Alfenas & Ferreira, 1986; Kobayashi, 1970; Micales & Stipes, 1987; Roane, 1986; Shear *et al*,. 1985). This variability due to external factors and host, was the primary argument provided by Walker *et al.* (1985) to explain the differences between Australian and North American specimens. The magnitude of differences between these fungi, is, however, great and we are convinced that these are not due to host or environmental factors. Furthermore, the fact that molecular data support our morphological observations leaves us with little doubt that these fungi represent distinct taxa.

Ascospores of the South African and Australian specimens are clearly non-septate and cylindrical to allantoid (Fig. 2*f*). This is typical of the genus *Endothia* (Roane, 1986;

Shear *et al.*, 1917). However, stromatal morphology in this fungus closely resembles that of *Cryphonectria* as described by Micales & Stipes (1987). They also resemble the structure of those in *C. gyrosa*, which is the type species of *Cryphonectria*. This is consistent with the grouping of the South African and Australian isolates with *C. parasitica* and not with *E. gyrosa* in a phylogram based on DNA sequence data (Chapter 2). We, therefore, propose that the fungus from *Eucalyptus* in South Africa and Australia should reside in *Cryphonectria* and not in *Endothia* as initially suggested. The following description is thus provided for the new taxon.

PLEASE NOTE THAT THE FOLLOWING SPECIES DESCRIPTION IS PRESENTED HERE IN PRELIMINARY FORM AND SHOULD NOT BE CITED. THE DESCRIPTION WIL BE PUBLISHED FORMALLY IN SCIENTIFIC LITERATURE AT A LATER DATE.

Cryphonectria eucalypti Venter et M. J. Wingfield, sp. nov.

Etym.: refers to the occurrence of this fungus exclusively on *Eucalyptus* spp.

Coloniae albae, plumeae, margine laevi, interdum maculis stramineis (21'd), celeriter crescentes, 90 mm diametro in minime novem diebus attingentes, temperatura faustissima 25–30 °C. Stromata in areis papulosis nunc turmis gregariis nunc individuis, interdum confluentes, in cortice subimmersa, aliquando erumpentia vel omnino immersa, multilocularia, (202-)389-741(-940) μm alta, (186-)343-704(-868) μm lata; aurantiaca (15); parte superiori eustromatica, pseudoparenchymatica, parte inferiori pseudostromatica, in centro cum prosenchymate e basibus peritheciorum vel

loculis pycnidiorum in textura hospitis formato (Fig. 2*a*, 2*d*, 3*a*, 3*d*). Infra 10 loculi pycnidiorum in quoque stromate, statura variabile (56-)104-368(-558) μm longi, (50-)82-230(-350) μm lati, forma et directione irregulari, saepe convoluti, non-ostiolati. Cellulae in parietatibus loculorum cellulas conidiogenas efficientes, hyalinae cum textura globosa, tristromaticae, (5-)6-14(-20) μm crassae. Cellulae conidiogenae phialidosae, septatae, ramosae, hyalinae, cylindricae, apicem versus plerumque subcontractae, nonnullae basibus inflatis, (4-)8-17·5(-27) μm longae, 0·5-1·5(-2) μm latae, longitudo latitudoque in ratione fere 13:1 (Fig. 2*b*, 3*b*). Hyphae steriles inter cellulis conidiogenis, interdum apicibis phialidosis, longae, septatae, cylindricae, apicem versus subcontractae, (22-)28·5-50(-71·5) μm longae, 1-1·5 μm latae (Fig. 2*b*, 3*c*). Conidia amerosporae, oblongo-cylindraceae, allantoideae, oblongae vel ovoideae, (2·5-)3-4(-5) longa, 0·5-1 μm lata, hyalinae, longitudo latitudoque in ratione fere 3·5:1 (Fig. 2*c*, 3*b*). Perithecia sub superficie corticis ad basin stromatum immersa, 1–12 in quoque stromate, globosa, subglobosa vel triangularia, parietibus fuscis, (105-)161-245(-288) μm longa, (82-)157-291(-257) μm latae (Fig. 2*d*, 3*d*), cellulis basalibus textura epidermoidea cum a superficie videntur; parietatibus (8-)13-22(-31) μm diametro. Colla fusca, gracilia, periphysata, in stromate centralia, (65-)118-385(-630) μm longa, (31-)33-53(-65) μm lata, longitudine secundum in stromate perithecii profunditatem; canale ostiolari 20-26(-28) μm lato; pariete colli (10-)11-14(-15) μm diametro, cellulis textura intricata vel porrecta cum a superficie videntur, apicem colli versus textura porrecta; colla per texturam stromatis tortuosa, ad superficiem stromatis ut ostiola, papillae vel rostra longa cum textura stromatis aurantiaca tecta erumpentia, usque ad 170 μm supra superficie stromatis extensa. Asci multi, diffluentes, in cavitate perithecii libere natantes, cylindrici vel fusiformes, (12-)17·5-26·5(-34) μm

longi, 4·5-7(-9) μm lati, longitudo latitudoque in ratione fere 4:1, dum immaturi stipitates, unitunicati, annulis apicalibus non-amyloideis refractivis, in quoque asco 8 ascosporis (Fig. 2*e*, 3*e*). Ascosporae non-septatae, hyalinae virido-tinctae, cylindricae vel fusiformes, allantoideae vel in uno extremitate sublaxatae, extremitatibus rotundatis vel subcontractis, (4·5-)6-9(-12·5) μm longae, (0·5-)1-1·5(-2) μm latae, longitudo latitudoque in ratione fere 7·5:1 (Fig. 2*f*, 3*e*).

Colonies white, fluffy with a smooth margin, sometimes with straw yellow (21'd) patches, fast growing, covering a 90 mm plate after minimum of nine days, optimum temperature 25 – 30 °C. Stromata in papulose areas either as gregarious groups or individually, sometimes confluent; semi-immersed in bark, occasionally erumpent or totally emmersed, multilocular, (202-)389-741(-940) μm high, (186-)343-704(-868) μm wide; orange (15); upper region eustromatic, pseudoparenchymatous; lower region pseudostromatic with prosenchyma in centre, comprised mainly of perithecial bases or pycnidial locules within host tissue (Fig. 2*a*, 2*d*, 3*a*, 3*d*). Less than 10 pycnidial locules per stroma, variable in size, (56-)104-368(-558) μm long, (50-)82-230(-350) μm wide, shape and orientation irregular, often convoluted, non-ostiolate. Cells in locule walls hyaline with textura globulosa, 1-3 layers thick, (5-)6-14(-20) μm. Conidiogenous cells phialidic, septate, branched, hyaline, cylindrical, usually slightly tapered towards apex, some with inflated bases, (4-)8-17·5(-27) long, 0·5-1·5(-2) μm wide, mean conidiogenous cell length/width ratio 13:1 (Fig. 2*b*, 3*b*). Sterile hyphae, occasionally with phialidic apices present amongst conidiogenous cells, long, septate, cylindrical, slightly tapered towards apex, (22-)28·5-50(-71·5) long, 1-1·5 μm wide (Fig. 2*b*, 3*c*). Conidia amerospores, oblong-cylindrical, allantoid,

or oblong to obovoid, (2·5-)3-4(-5) long, 0·5-1 μm wide, hyaline, mean length/width ratio 3·5:1 (Fig. 3*b*, 2*c*). Perithecia embedded beneath surface of bark at base of stromata, 1-12 per stroma, globose to sub-globose to triangular, dark-walled, (105-) 161-245(-288) long, (82-)157-291(-257) μm wide (Fig. 2*d*, 3*d*), basal cells with *textura epidermoidea* in surface view; perithecial walls (8-)13-22(-31) μm in diameter. Necks dark, slender, periphysate, with a central position in the stroma, (65-) 118-385(-630) μm long, (31-)33-53(-65) μm wide, length depending on depth of perithecium in stroma; ostiolar canal 20-26(-28) μm wide, neck wall (10-)11-14(-15) μm in diameter, neck cells with *textura intricata* to *textura porrecta* in surface view, with textura porrecta at apex of neck, necks wind through stromatic tissue and emerge at stromatal surface as ostioles, papillae or long beaks covered with orange stromatal tissue, extending up to 170 μm above stromatal surface. Asci numerous, evanescent, floating freely in perithecial cavity, cylindrical to fusiform, (12-)17·5-26·5(-34) μm long, 4·5-7(-9) μm wide, mean length/width ration 4:1, stipitate when immature, unitunicate with non-amyloid, refractive apical rings; asci with 8 ascospores (Fig. 2*e*, 3*e*). Ascospores aseptate, hyaline with greenish tint, cylindrical to fusiform, allantoid or slightly wider at one end, ends rounded or slightly tapered, (4·5-)6-9(-12·5) μm long, (0·5-)1-1·5(-2) μm mean ascopore length/width ratio 7·5:1 (Fig. 2*f*, 3*e*).

Host. *Eucalyptus* species

Distribution. South Africa, Tasmania and mainland Australia.

Specimens examined

Herbarium types: Cryphonectria eucalypti: South Africa, Nyalazi, Northern Kwazulu/Natal, bark of GC747 clone of *Eucalyptus*, M. Venter, 25 February 1998, PREM 56211 (holotype); South Africa, Sabie, Mpumalanga, bark of *E. grandis*, J. Roux, August 1998, PREM 56212 (paratype); South Africa, Tzaneen, Mpumalanga, bark of *E. saligna* Smith, M. Venter, 6 February 1999, PREM 56305 (paratype); South Africa, Dukuduku, Northern Kwazulu/Natal, bark of *E. grandis*, M. Venter, October 1998, PREM 56214; South Africa, Amangwe, Northern Kwazulu/Natal, bark of *E. grandis*, M. Venter, October 1998, PREM56215; South Africa, Dukuduku, Northern Kwazulu/Natal, bark of *E. grandis*, M. Venter, October 1998, PREM 56216.

Previously, *Endothia* and *Cryphonectria* resided in the Valsaceae and the Gnomoniaceae, respectively, based on arrangement of perithecia (valsoid or diatrypoid), stromatic tissue type and ascospore shape and septation (Barr, 1990). Nonetheless, the stromatal morphology of these two genera is very similar (Cannon, 1988). Others were also of the opinion that these two genera are too close to reside in different families (Chen *et al.*, 1996; Walker *et al*,. 1985). The *nom. cons*. status of the Gnomoniaceae to the Valsaceae (Hawksworth *et al.*, 1996), however, placed *Endothia* and *Cryphonectria* in the same family, and supports the view that these genera are closely related. The fact that the new species, *C. eucalypti* described in this study, shares *Endothia*-like and *Cryphonectria*-like characteristics, confirms that *Endothia* and *Cryphonectria* are closely related and probably diverged recently.

In the past, emphasis has been placed on ascospore septation to differentiate *Endothia* and *Cryphonectria*. In this study, we have shown that stromatal structure is the more useful characteristic to separate the genera, and is more conserved than ascospore morphology. This is also consistent with results of molecular comparisons. Molecular studies done parallel with morphogical comparisons of available stromatal collections and herbarium specimens, should be carried out to redefine phylogenetic relationships between these two genera.

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Table 1. Specimens of *Endothia gyrosa*, *Cryphonectria eucalypti* and *C. gyrosa* used in morphological comparisons.

Identification	State	Host	Origin	Collector	Date	Herbarium allocation[1)]
E. gyrosa	Anamorph, teleomorph	*Quercus phellos* L.	Raleigh, USA	L. Grand	April 1997	PREM 56218
C. eucalypti (holotype)	Teleomorph	*Eucalyptus grandis* X *camaldulensis*	Nyalazi, SA	M. Venter	February 1998	PREM 56211
C. eucalypti (paratype)	Teleomorph	*Eucalyptus grandis* W. Hill ex Maid.	Sabie, SA	J. Roux	August 1998	PREM 56212
C. eucalypti (paratype)	Teleomorph	*E. saligna* Smith	Tzaneen, SA	M. Venter	February 1999	PREM 56305
C. eucalypti	Anamorph	*E. grandis*	Dukuduku, SA	M. Venter	October 1998	PREM 56214
C. eucalypti	Anamorph	*E. grandis*	Amangwe, SA	M. Venter	October 1998	PREM 56215
C. eucalypti	Anamorph	*E. grandis*	Dukuduku, SA	M. Venter	October 1998	PREM 56216
C. eucalypti	Anamorph	*E. globulus* Labill.	Perth, Australia	M. Wingfield	August 1997	PREM 56217
C. gyrosa	Teleomorph	*Elaeocarpus dentatus* Vahl.	Omahuta Forest, New Zealand	S. J. Hughes	June 1964	DAR 14534 DAOM 93506A
C. gyrosa	Teleomorph	Dead twigs	Olinda pipe line, Maui, USA	C. L. Shear	December 1927	DAR 49895
E. gyrosa	Teleomorph	*E. saligna*	Termeil, Australia	K. Old	October 1983	DAR 49904
E. gyrosa	Teleomorph	*E. saligna*	Currowan, Clyde Mountain, Australia	K. Old	June 1984	DAR 49905
E. gyrosa	Teleomorph	*E. saligna*	Currowan, Clyde Mountain, Australia	K. Old	September 1983	DAR 49906
E. gyrosa	Teleomorph	*E. saligna*	Currowan, Clyde Mountain, Australia	K. Old	October 1983	DAR 49907
E. gyrosa	Teleomorph	*E. saligna*	Termeil, Australia	K. Old	October 1983	DAR 49909
E. gyrosa	Teleomorph	*Liquidambar* sp.	Grenada, USA	C. L. Shear & N. E. Stevens	February 1914	DAR 49897

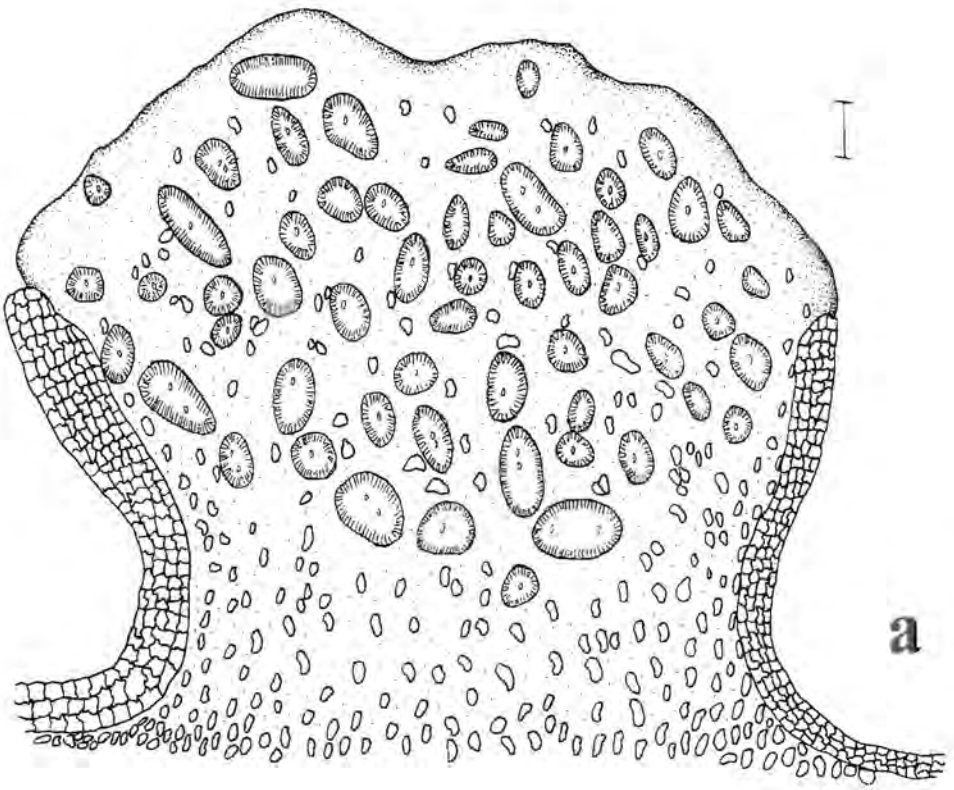
1) PREM, National Collection of Fungi, Pretoria, South Africa; DAR, Plant Pathology Herbarium, Orange Agricultural Institute, Forest Road, Orange, N. S. W. 2800, Australia; DAOM, National Mycological Herbarium, Eastern Cereal and Oilseed Center (ECORC), Agriculture and Agri-Food Canada, Edifice Wm. Saunders Bldg. #49, Ottawa, Ontario, Canada, K1A 0C6.

Table 2. Isolates of *Endothia gyrosa* and *Cryphonectria eucalypti* used in culture comparisons.

Culture number[1)]	Identification	Host	Origin	Collector	Date
CRY910	*C. eucalypti*	*Eucalyptus grandis*	Amangwe, SA	M. Venter	October 1998
CRY911	*C. eucalypti*	*E. grandis* X *camaldulensis*	Nyalazi, SA	M. Venter	February 1998
CRY900	*C. eucalypti*	*E. saligna*	Tzaneen, SA	M. Venter	February 1999
CRY906	*E. gyrosa*	*Quercus falcata.* Michx.	Raleigh, USA	C. Vernia	September 1998
CRY907	*E. gyrosa*	*Q. falcata*	Raleigh, USA	C. Vernia	September 1998
CRY21	*E. gyrosa*	*Q. borealis* Michx.	Lipman Drive, Raleigh, USA	S. Anagnostakis	-

1) Culture collection of the Forestry & Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, 0002.

Fig. 1. Anamorph and teleomorph states of *Endothia gyrosa*. (*a*) Anamorphic stroma (Bar = 100 μm). (*b*) Conidiogenous cells (Bar = 10 μm). (c) Conidia (Bar = 10 μm). (d) Teleomorphic stroma (Bar = 100 μm). (e) Asci (Bar = 10 μm). (f) Ascospores (Bar = 10 μm).



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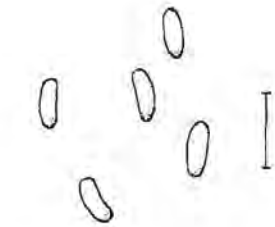

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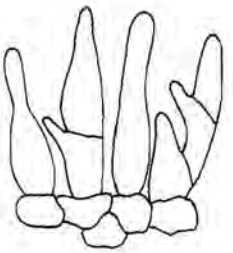

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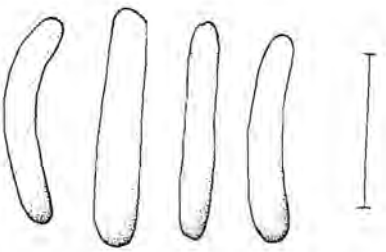

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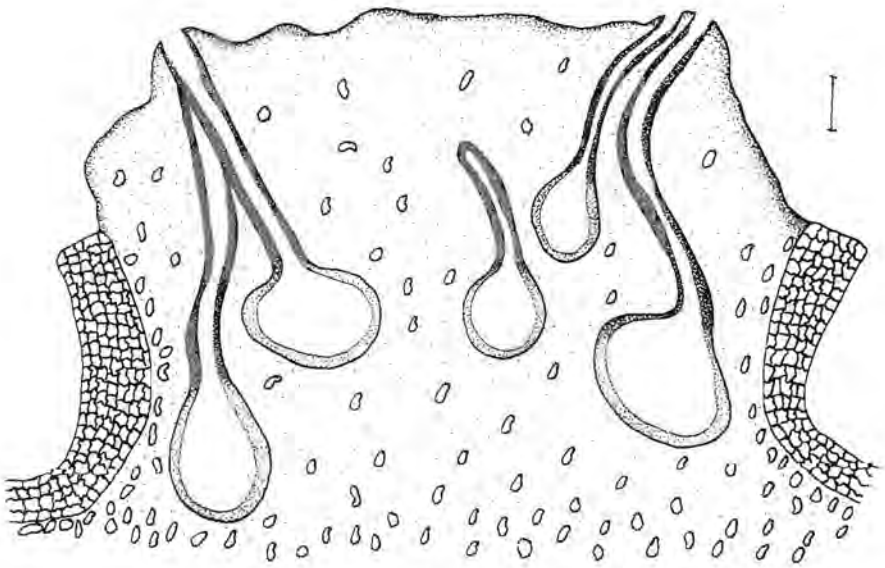

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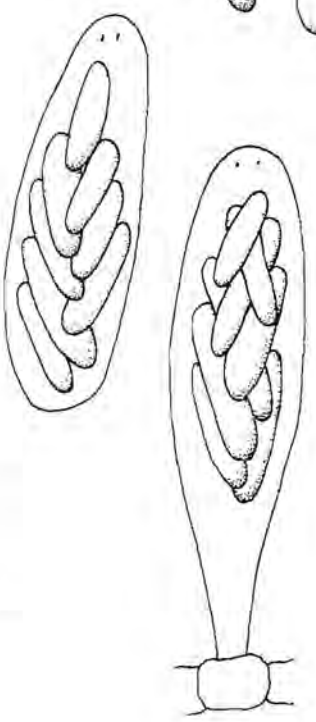
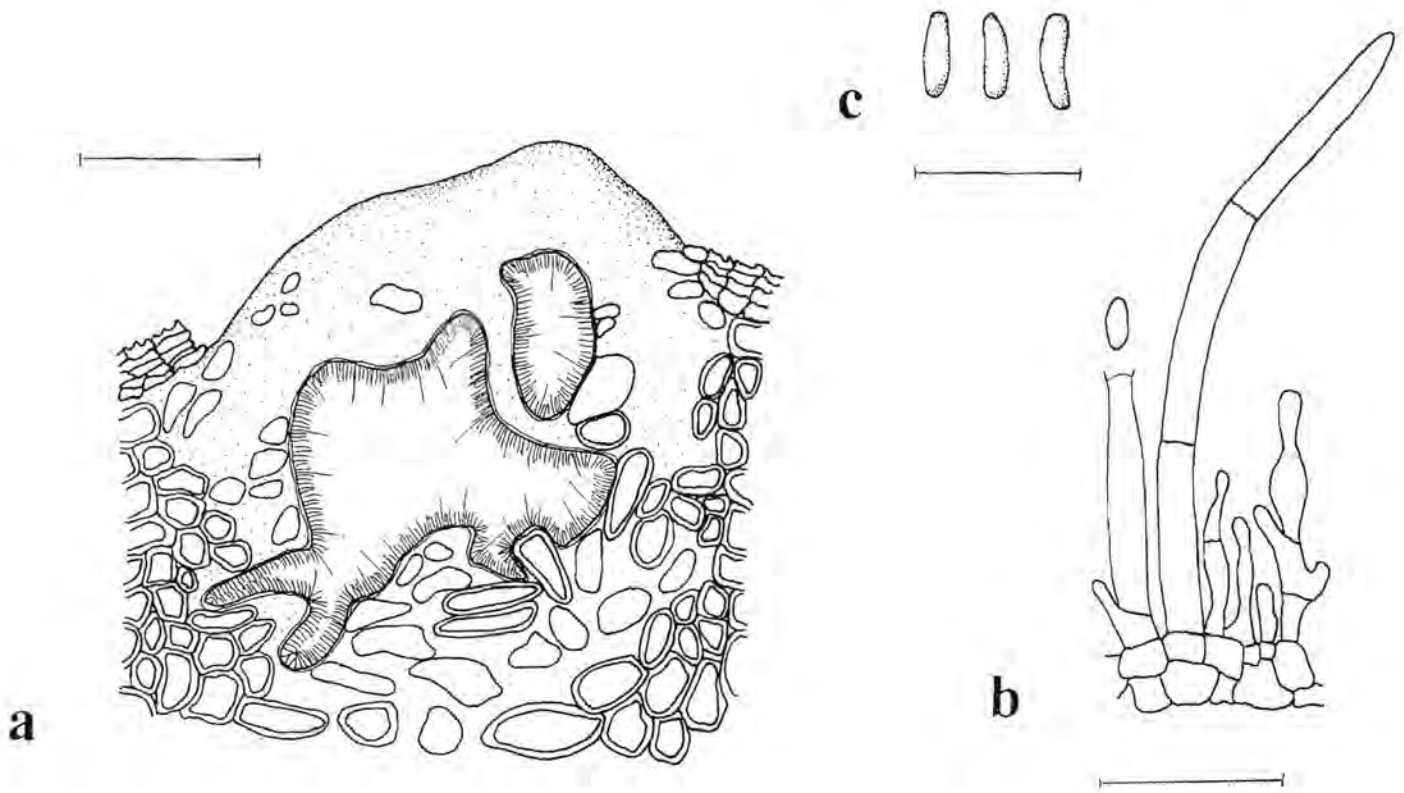


e

Fig. 2. Anamorph and teleomorph states of *Cryphonectria eucalypti*. (*a*) Anamorphic stroma (Bar = 100 μm). (*b*) Conidiogenous cells and sterile hyphal cell (Bar = 10 μm). (*c*) Conidia (Bar = 10 μm). (*d*) Teleomorphic stroma (Bar = 100 μm). (*e*) Asci (Bar = 10 μm). (*f*) Ascospores (Bar = 10 μm).



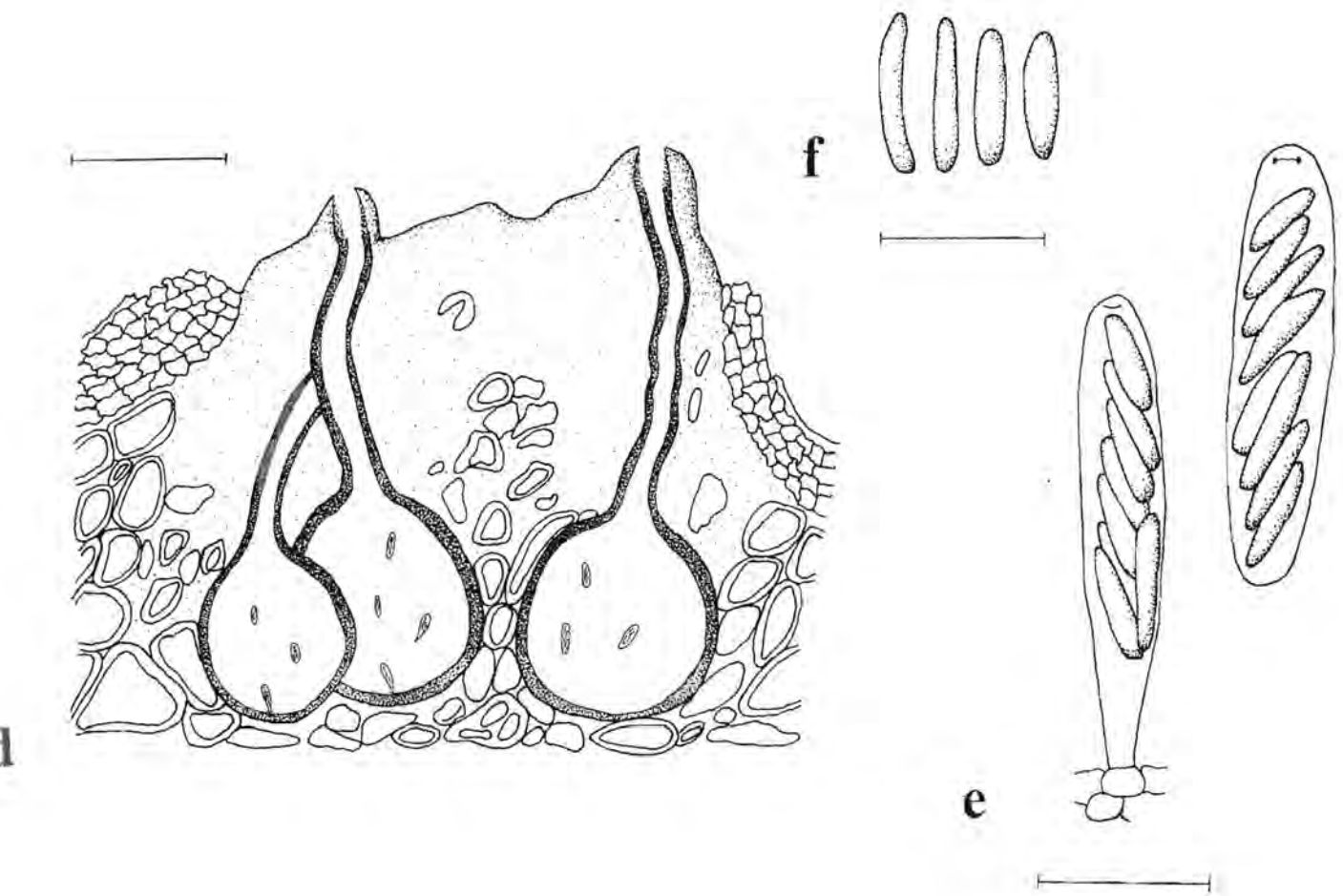


Fig. 3. Light micrographs of the anamorph and teleomorph states of *Cryphonectria eucalypti*. (*a*) Anamorph stroma containing pycnidial locules (indicated with arrow) lined with conidiogenous cells (Bar = 100 μm). (*b*) Conidiogenous cells producing conidia (Bar = 10 μm). (*c*) Sterile hyphal cell indicated with arrow (Bar = 10 μm). (*d*) Teleomorphic stroma containing perithecium (Bar = 100 μm). (*e*) Ascus containing ascospores (Bar = 10 μm).

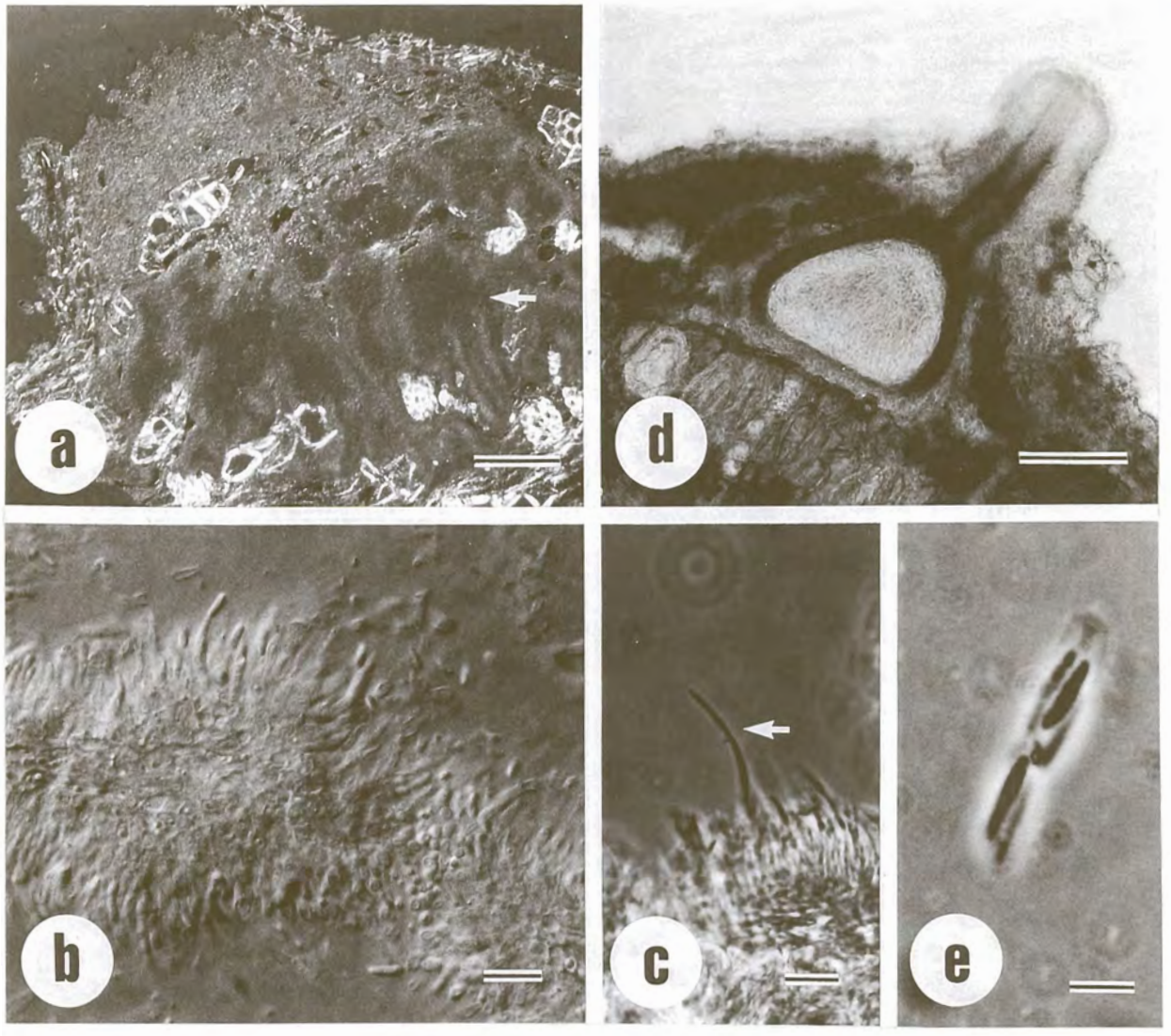


Fig. 4. (*a*) Cultures of *Endothia gyrosa* from North America and *Cryphonectria eucalypti* from South Africa on MEA and PDA. (*b*) Different growth patterns of *E. gyrosa* isolates.

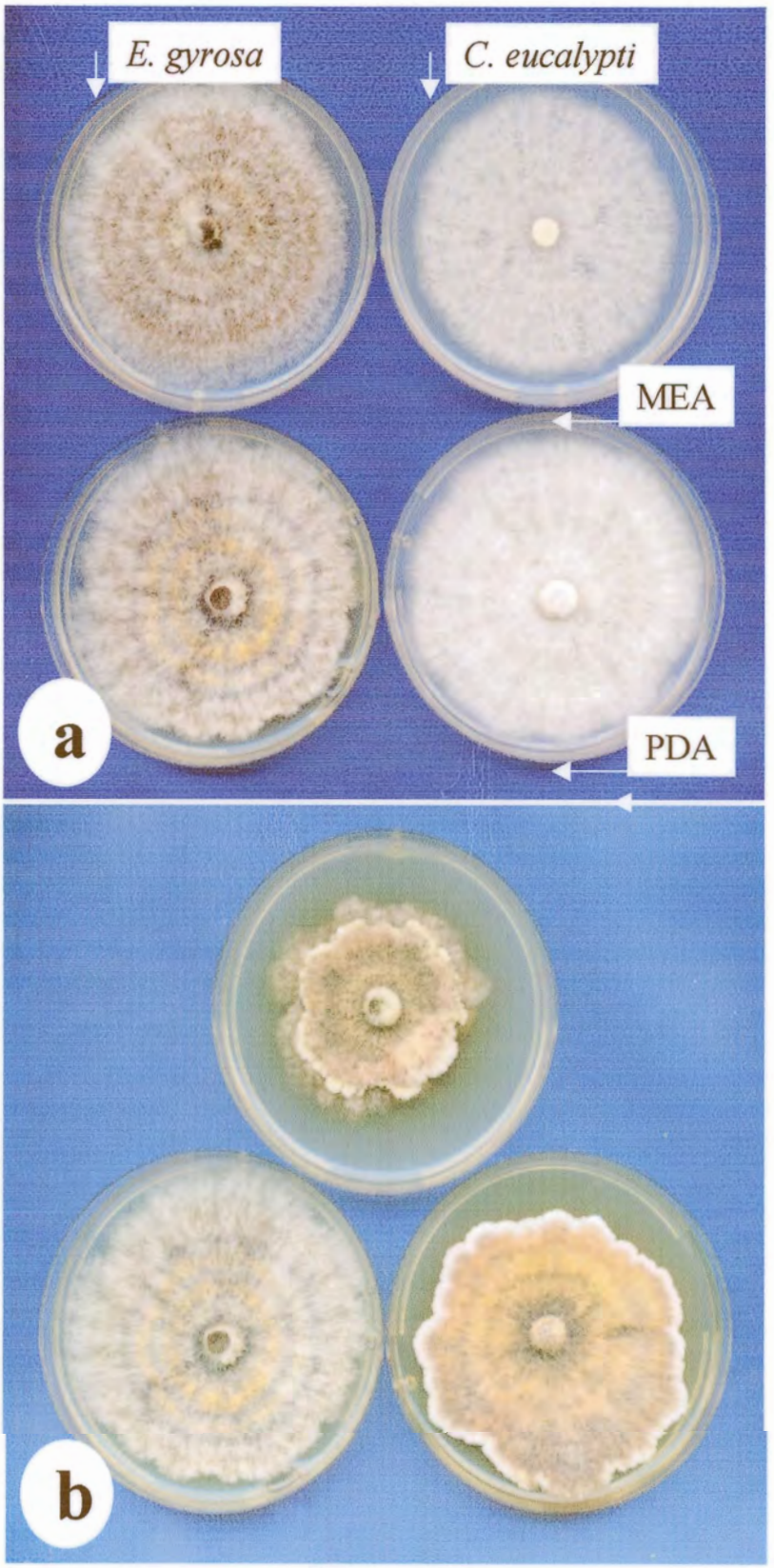
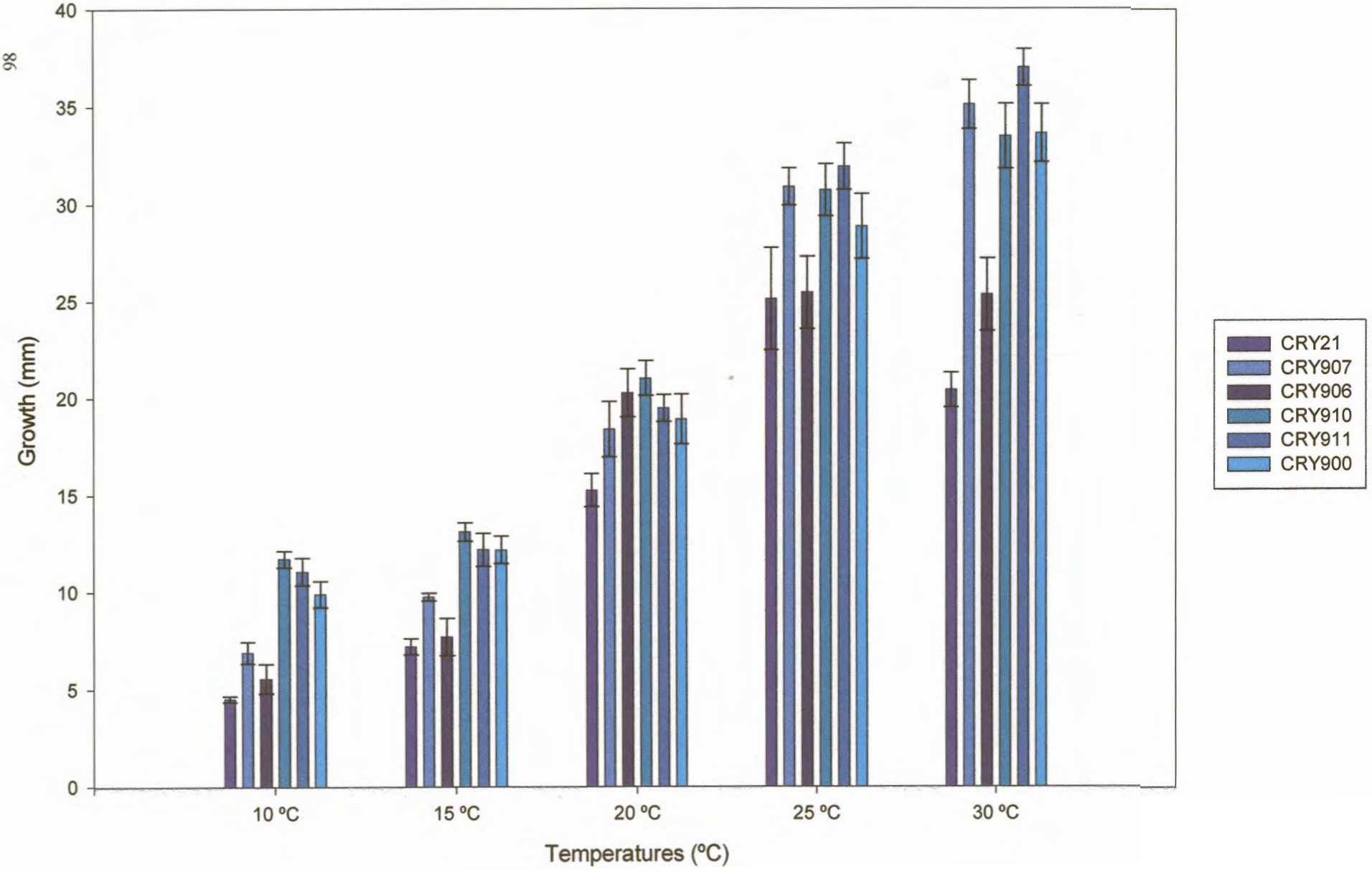


Fig. 5. Comparison of growth of isolates of *Endothia gyrosa* (CRY21, CRY 906, CRY 907) and *Cryphonectria eucalypti* (CRY 900, CRY910, CRY 911) at 10 °C, 15 °C, 20 °C, 25 °C and 30 °C on malt extract agar (MEA). Growth is reflected as a mean of four measurements in mm after 9 days. Bars represent standard error values.


Growth (mm)
0
5
10
15
20
25
30
35
40
10 °C
15 °C
20 °C
25 °C
30 °C
Temperatures (°C)
CRY21
CRY907
CRY906
CRY910
CRY911
CRY900

CHAPTER 4

Pathogenicity of *Cryphonectria eucalypti* to *Eucalyptus* clones in South Africa

ABSTRACT

Eucalyptus spp. are planted in many parts of the world for the production of timber and pulp, and in South Africa they form a major component of the forestry industry. The canker pathogen, *Cryphonectria eucalypti*, is pathogenic to *Eucalyptus* spp. in Australia and Tasmania and occurs in all of the major *Eucalyptus* growing areas of South Africa. This study was undertaken to consider the pathogenicity of *C. eucalypti* to *Eucalyptus* clones in South Africa. Fifteen isolates of *C. eucalypti* were initially screened for their virulence on a susceptible *E. grandis* clone (ZG14) in the field. A plot consisting of 42 different clones of *Eucalyptus* was subsequently challenged with a selected virulent isolate of *C. eucalypti* to determine whether clones differ in their tolerance to the pathogen. Lesions of varying size were produced by *C. eucalypti* in all trials, seven weeks after inoculation. Results showed that *C. eucalypti* is capable of causing significant lesions on *Eucalyptus* in South Africa and that disease development is strongly dependant on environmental factors. All of the clones tested were susceptible to *C. eucalypti*, but varying levels of tolerance to the pathogen existed. This should enable the establishment of a breeding programme aimed at reducing the impact of *C. eucalypti* in South Africa.

INTRODUCTION

Recent studies have shown that a fungus reported as *E. gyrosa* on *Eucalyptus* L'Hérit from Australia and South Africa, is not the same species as *E. gyrosa* from North America (Chapters 2, 3). Isolates from Australia and South Africa had identical RFLP (Restriction Fragment Length Polymorphisms) patterns for the ITS1, ITS2 and 5.8S regions of the Internal Transcribed Spacer region of the ribosomal RNA operon, but differed from those of North American isolates. In a phylogenetic analysis based on sequences of the ITS regions and 5.8S rRNA gene, Australian and South African isolates grouped separately from North American isolates. Moreover, the South African and Australian isolates grouped with *Cryphonectria parasitica* (Murr.) Barr in a different clade to *E. gyrosa* from North America. The molecular differences were substantiated when morphological and physiological characteristics of the fungi were considered. The South African and Australian fungus from *Eucalyptus* was consequently described as a new species of *Cryphonectria*, namely *C. eucalypti* Venter & M. J. Wingfield (Chapter 3). *Endothia gyrosa* from *Eucalyptus* will, therefore, be referred to as *C. eucalypti* for the remainder of this chapter.

Typical symptoms of *C. eucalypti* infection on *Eucalyptus* in Australia included bark cracks and cankers with associated exudation of kino, die-back of coppice shoots, branches and stems, and in severe cases, tree death (Old *et al.*, 1986; Walker *et al.*, 1985). Based on pathogenicity trials, *C. eucalypti* was viewed as a mild pathogen in the absence of stress, although it may cause girdling and death of seedlings (Old *et al.*, 1986, 1990). *Endothia gyrosa* has, however, been associated with severe cankers on *E. nitens* (Deane & Maid.) Maid. in Tasmania (Wardlaw, 1999). In that situation, annual cankers developed in the bark,

while more severe cankers also extended into the cambium. The annual cankers were shed from trees with the bark, thereby causing little damage. Infections reaching the cambial area, however, caused die-back and tree death. Furthermore, in pathogenicity trials in Australia, *C. eucalypti* was amongst the most pathogenic species in a test that included a *Phoma* sp. and *Seridium eucalypti* Nag Raj (Yuan & Mohammed, 1999).

In South Africa, cankers caused by *C. eucalypti* are usually superficial on the bark (Fig. 1) (Van der Westhuizen *et al.*, 1993). These cankers rarely cause kino exudation or damage to the cambium and have no apparent influence on timber quality or yield. *Cryphonectria eucalypti* has, however, recently been found associated with serious cankers extending into the cambium of *E. smithii* Denn. ex Smith in the Mooirivier area near Pietermaritzburg (Dr. J. Roux, personal communication), and it was closely associated with stunted *Eucalyptus* seedlings near Tzaneen.

Eucalyptus spp. make up approximately 40 % of forestry plantations in South Africa (Anonymous, 1998). Vegetative propagation of *Eucalyptus* clones to improve timber quality and yield, is widely practiced (Denison & Kietzka, 1993; Leakey, 1987). Clonal propagation, however, increases the threat of disease outbreaks and a clear understanding of such problems is needed in order to reduce risks (Chou, 1981; Wingfield *et al.*, 1991). The conflicting reports regarding the potential for *C. eucalypti* to cause disease and death of *Eucalyptus* spp. prompted us to investigate its pathogenicity to *Eucalyptus* clones in South Africa. The aim of this study was, therefore, to test the virulence of several isolates of *C. eucalypti* on a clone of *Eucalyptus* in field inoculations. A virulent isolate of the fungus was

then selected and used to screen a larger number of clones to determine whether they display differences in tolerance.

MATERIALS AND METHODS

Source of isolates

Stromata associated with typical cankers on *Eucalyptus* spp. from commercial plantations in various regions of South Africa, were collected. Isolations were made by placing the stromata on malt extract agar (Biolab, Merck, Midrand, South Africa). Fifteen isolates were randomly chosen for the initial inoculations (Table 1). Isolates were maintained on 2% malt extract agar at 4 °C. All isolates are preserved in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry & Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Screening of isolates

Inoculations were done on the tree trunk using the technique described by Van der Westhuizen *et al.* (1993). A negative control consisting of a sterile MEA plug was also included. Lesions were measured by removing the bark and exposing the cambial discolouration, since no external discolouration of the bark around the lesions was visible on the particular clone used. Reisolations were conducted at the time when lesion lengths were recorded.

Inoculations with fifteen isolates of *C. eucalypti* were done in a two-year-old coppice stand of a *Eucalyptus grandis* W. Hill ex Maid. clone (ZG14) at the Flatcrown farm near KwaMbonambi, northern Kwazulu/Natal. Twenty trees per isolate were inoculated in January 1998 and lesion lengths were measured seven weeks later. A second inoculation consisting of two blocks of trees of the same clone at different locations in the same compartment, was done in March 1998 with the same isolates used in the first inoculation, and results were recorded after seven weeks. Trees in two different blocks were inoculated in order to determine whether the variation in the data obtained for isolates in the first inoculation, was linked to the isolates or the environment. Combined analysis of variance were performed on all data. Combining experiments, with identical treatments, in an appropriate ANOVA, is a valid analysis (Freeman, 1973). Differences between the means of isolates were evaluated using appropriate multiple comparison methods (Tukey's Multiple Range and the t-test) (SAS, 1989).

Possible interactions between isolates and the environment were investigated by means of an AMMI analysis (Additive Main Effects and Multiplicative Interaction Model) (Gauch & Zobel, 1996). This analysis makes it possible to analyze the interaction between isolates and environment and employs a principal component analysis of the interaction sums of squares. In this way the interaction is separated into a predictable portion (usually the first principal component), the rest being relegated to unpredictable noise (Eisenberg *et al.*, 1996). The first principal component provides score values for isolates and trials and these are plotted against the overall means of the isolates and trials. The score values are represented on the Y-axis and their deviation from the zero line (positive or negative), reflects the degree of interaction which is exhibited by an isolate or a trial. All control treatments resulted in zero values for

lesion development. These were not included in the AMMI analyses because they would not contribute to the evaluation of the interaction.

Screening of Eucalyptus *clones*

One isolate with a high level of virulence (CRY920), was selected from the isolate screening trial for inoculation of 42, eighteen-month-old clones located on the Amangwe estate near KwaMbonambi, northern Kwazulu/Natal. The 42 clones were planted in a randomized block design consisting of ten blocks, in which each clone was planted once. Each block consisted of two identical rows. Four extra rows, identical to the design of the first block, were planted at the side of the trial. One of these extra rows was used to inoculate a negative control using sterile agar plugs, and all other rows (thus maximum 23 trees per clone) were inoculated with the test fungus. The trial was surrounded by two border rows of trees.

Inoculations were made in October 1998 and lesions measured in December 1998. Both external bark lesions as well as lesions at the cambium surface developed in all the inoculated trees unlike the ZG14 clones where no external bark lesions were visible. Lengths and widths of external lesions were measured for all trees, since reading of internal cambial lesions resulted in too much damage to the tree. The experiment could not be repeated due to the cost and complexity of establishing such a large clonal trial. The same trees could not be used for inoculation since *E. gyrosa* is an endophyte of *Eucalyptus* (personal communication, Dr. Dennis Wilson) and would persist in the host tissue long after inoculation. This may influence the data to be more variable.

Measurements were analyzed with one and two way analysis of variance. Differences between the clones were evaluated using multiple comparison methods (Tukey's Multiple Range and the t-test) (SAS, 1989).

RESULTS

Screening of isolates

Two weeks after inoculation, localized cracking and kino exudation were visible around the inoculation points (Fig. 2*a*). No symptoms were visible in the control trees (Fig. 2*b*). After seven weeks, *C. eucalypti* was found sporulating on the surface of most lesions that developed after inoculation. No external discolouration of the epidermis was produced and lesions consisted only of kino veins in the cambium (Fig. 2*c*) that resulted in a swelling on the bark surface. Reisolations consistently resulted in the recovery of the inoculated fungus.

The lengths of cambial lesions were measured and used in statistical analyses since the lesions remained reasonably constant in width (30 mm) throughout the trial. Significant differences ($Pr > 0{\cdot}0001$) between lesion lengths were observed for the two inoculation times and for the two separate blocks used in the second inoculation (Table 2). The first inoculation had the greatest mean lesion length for all trees (136·9 mm), while mean lesion length in the second block was lower (82·6 mm) than the first block (103·8 mm) of the second inoculation.

Significant differences existed between isolates in all inoculations ($Pr > 0{\cdot}0001$) (Table 2, Fig. 3-6). Cambial lesions varied from 15 mm (CRY924) to 285 mm (CRY920) in length for

all three inoculations. All lesions differed significantly in size from the control (Pr>0·0001), which produced no or only small lesions. Means per isolate for all inoculations combined were of the same size order and represented a continuum of values between 132 mm and 100 mm when ranked from the highest to the lowest overall mean for the three data sets combined. In this ranking, lesion lengths for isolates did not differ significantly from those directly above or below them in the ranking. Isolates that ranked low, however, did differ significantly from those with a high ranking. Due to the continuous change between values, it was not possible to establish distinct groupings based on the virulence of the isolates.

Lesion lengths were, however, grouped in arbitrary clusters based on size. These groupings were done to define the virulence of each isolate since the isolates did not form natural groupings. In these clusters, isolates fell in three relative virulence groups, namely a highly virulent group (120-132 mm), a medium virulent group (110-119 mm) and a low virulent group (100-109 mm). Based on this ranking, isolates CRY916, CRY923, CRY920 and CRY924 were the most virulent. Three groups and close size order of lesion lengths were also observed for each of the two inoculation studies separately, including the two separate blocks inoculated on the same date.

Ranking positions of isolates based on mean lesion length differed for each of the three inoculation events. For example, isolate CRY917 ranked highest in the first inoculation (Fig. 3), but was fifteenth in the first block of the second inoculation (Fig. 4), sixth in the second block of the second inoculation (Fig. 5), and eighth in the ranking based on overall mean for all three inoculations (Fig. 6). Some lesion lengths were consistently above the average lesion size for all isolates (e.g. CRY923, CRY916), while others were either below or above

average size (e.g. CRY921, CRY912, CRY902) (Fig. 3-5). Some isolates (e.g. CRY923 in the high pathogenicity group and CRY925 in the medium pathogenicity group) held constant positions in the groups, but others, such as CRY917, was different in its position in groups, for each inoculation. This shift in ranking made selection of the most pathogenic isolates difficult, and ultimately this choice needed to be relatively arbitrary.

Combined analysis of variance for the results of the three isolate inoculations showed a significant interaction of experiment with isolates (Pr>0·0001) (Table 2). This was confirmed when the different mean lesion lengths for each inoculation were plotted graphically (Fig. 7). The slopes of each graph representing an isolate, differed from those of the graphs for the other isolates because the pathogenicity of isolates did not vary uniformly between inoculations. Most graphs for isolates showed a decrease in mean, except for those representing the control, which showed an increase for block one of the second inoculation trial (Fig. 7*a*), and CRY916 (Fig. 7*b*) and CRY917, which showed a decrease for block one of the second inoculation trial. The means for each isolate in the first inoculation trial differed significantly in most cases from the means of each of the blocks of the second inoculation trial. The majority of means for the two blocks of the second inoculation trial, differed significantly from each other, even though the two blocks of the second inoculation were established at the same time. Only the control inoculation showed no significant difference between the three inoculation trials. The differences in slopes in the graphs (Fig. 7) likely indicates interaction with the environment. This finding is further supported by the difference in ranking of isolates for each inoculation.

Analysis of data using the AMMI model (Fig. 8) showed that some isolates exhibited greater interaction with external factors, while others were more stable. All isolates, having the same size order of lesions, could be divided into three groups: a group with little interaction (score values 0±1), a group with moderate interaction (±1 to ±2), and a group showing high levels of interaction (±2 to ±4). Isolates CRY916, CRY917 and CRY923 showed a high degree of interaction, while isolates CRY922, CRY913 and CRY925 showed little interaction. Each of the three inoculation trials also had high interaction with external factors. The first block of the second inoculation trial had a negative value (-5·8), while the first inoculation and the second block of the second inoculation had positive values (1·9 and 3·9 respectively) (Fig. 8).

Screening of Eucalyptus *clones*

Cambial lesions, as well as external bark discolouration (Fig. 2*d*), were produced on all of the clones. Significant differences in lesion length were observed between blocks within the trial (Pr>0·0321), as well as between the different clones (Pr>0·0001) (Table 3, Fig. 9). Based on length, width and area (length x width), means for each clone formed a continuum of values in the mean rankings. Thus, no clone differed significantly from the clone directly below or above it in ranking. Levels of tolerance to *C. eucalypti* were, however, visible because clones with a low ranking differed significantly from those with a high ranking. Four tolerance groups were identified in the rankings based on the length, width and area of lesions. For example, the four groups based on the mean lesion area were: highly susceptible (4039·5 - 4900·3 mm), moderately susceptible (3032·2 - 3776·0 mm), moderately tolerant

(2011·5 - 2951·0 mm) and highly tolerant (1216·3 - 1931·1 mm) (Fig. 9). The controls formed no lesions in any of the inoculations conducted.

Rankings of the clones based on lesion width and lesion area, were similar, especially in the highly susceptible and highly tolerant groups, but differed from the rankings of the measurements based on length. In a correlation analysis, rankings based on width and area of lesions were more similar to each other (r = 0·91) than the rankings between length and area (r = 0·71), while those between length and width were poorly correlated (r = 0·39). Lesion area appeared to be the best measure for tolerance in this case, and was thus chosen for assessing the tolerance of the clones due to the high correlation with lesion width. Based on the rankings of lesion area, clones GC 575, GU115 and GC962 were the most tolerant, while clones GC747 and GC796 were the least tolerant to infection by *E. gyrosa* (Fig. 9).

DISCUSSION

Results of this study show that *C. eucalypti* is capable of causing distinct lesions on *Eucalyptus* spp. in South Africa. High levels of interaction between isolates, host and environment also exist. In South Africa, symptoms associated with *C. eucalypti* (reported as *E. gyrosa*) in the past have been of superficial cracks on the surface of tree bark (Van der Westhuizen *et al.*, 1993), with no cambial cankers. The interaction that exists for *C. eucalypti* isolates with the environment could explain the enigma surrounding the fact that *C. eucalypti* is generally not a serious pathogen in field situations, but causes distinct disease in inoculation studies. In Australia and Tasmania, *C. eucalypti* is reported to be capable of causing tree death (Walker *et al.*, 1985; Wardlaw, 1999), although it most commonly causes

annual cankers (Yuan & Mohammed, 1997). Results of this pathogenicity study, as well as recent reports of *C. eucalypti* associated with severe stem cankers in the Tzaneen and Mooirivier areas, suggest that *C. eucalypti* is more important in South Africa than previously recognized.

In South Africa, *C. eucalypti* occurs in all the major *Eucalyptus* growing areas and has also been reported from Swaziland (Dr. J. Roux, personal communication). The fungus has also been found on numerous species of *Eucalyptus* in the country (Van der Westhuizen *et al.*, 1993). In the current inoculation trials, lesions were produced on all 42 clones tested. *Cryphonectria eucalypti* has also been found sporulating on ZG36, TAG524, GC748, GC747 and TAG555 prior to inoculation. This wide host range is consistent with the situation in Australia, where *C. eucalypti* is reported on 20 *Eucalyptus* spp. (Davison, 1982; Davison & Coates, 1991; Old *et al.*, 1986; Walker *et al.*, 1985; Wardlaw, 1999; White & Kile, 1993).

Cryphonectria eucalypti has been found to persist for one year in lesions associated with field inoculations in Australia (Old *et al.*, 1986). This is probably explained by the fact that the fungus is an endophyte in the bark and twigs of some *Eucalyptus* spp. (Dr. Dennis Wilson, personal communication). *Botryosphaeria dothidea* Ces. & De Not., which causes serious disease after the onset of stress conditions such as drought, hot winds and frost, is also an endophyte of *Eucalyptus* spp. (Smith *et al.*, 1996a; Smith, Wingfield & Wingfield, 1996b). The isolate-environment interaction exhibited by the *C. eucalypti* isolates, and its endophytic status makes this pathogen similar to *B. dothidea*.

Variation in results and low levels of reproducibility for pathogenicity tests using *E. gyrosa*, have been encountered before (Old *et al.*, 1986). The differences in lesion length for the isolate trials could be explained by interactions between isolates and external factors, including the host. This was confirmed by the AMMI analysis. Based on the production of significantly large lesions in the artificial inoculations, this fungus can thus be aggressive in conditions conducive to disease. This is typical of facultative pathogens of woody plants (Colhoun, 1973; Schoeneweiss, 1975, 1981).

The environment, for instance different temperatures and the availability of water, can interact and influence the resistance of a host to disease development by facultative pathogens (Colhoun, 1973; Schoeneweiss, 1975, 1981). *Cryphonectria eucalypti* was found on virtually every tree in a very dry compartment in the Tzaneen region of South Africa, and was also very common in one of the coldest compartments in that same area. In Australia, trees weakened by defoliation were found to be more susceptible to *C. eucalypti* (Old *et al.*, 1990). In the same study, water stress did not predispose the trees to *C. eucalypti*, although it was thought that the *Eucalyptus* trees used in that study might have had a high degree of tolerance to water stress. It is, therefore, difficult to name a single factor that may have been responsible for the interaction shown by the statistical analyses of the data in this study. It is more likely that a combination of factors, unique for each environment, may predispose a tree to disease (Colhoun, 1973).

Determining the level of interaction of an isolate with external factors is crucial when selecting an appropriate isolate for disease screening. Selection of an isolate producing large lesions may not be the best choice if that isolate shows a high level of interaction, since it

will give more variable results. Selecting an isolate with low interaction would in all likelihood yield more stable results (Eisenberg *et al.*, 1996; Gauch & Zobel, 1996). This, however, needs to be determined experimentally for each pathogen.

Isolates showing a high degree of interaction may not be pathogenic in a particular environment, but may become serious elsewhere. This is important in determining the potential damage that a pathogen might cause. Genotype x environment interaction is well recognized in forestry (Matheson & Cotterill, 1990; Basford & Cooper, 1998) and is supported by the AMMI model. The pathogenicity of an isolate with a positive score value is augmented in environments with positive score values, for instance CRY 916 in the first inoculation and second block of the second inoculation (Fig. 8). This might have been the situation in the *E. nitens* stand in Tasmania where severe cankers led to tree death (Wardlaw, 1999). Environments with negative score values will have a diminishing effect on pathogenicity for isolates with positive score values (Eisenberg *et al.*, 1996).

Different levels of susceptibility to *C. eucalypti* have previously been observed for five species of *Eucalyptus* (Old *et al.*, 1986; Yuan & Mohammed, 1999). The varying levels of tolerance observed for different clones in the present study indicates that a selection and breeding program to develop trees tolerant to *C. eucalypti* could be established. Such programmes have already been established for *Cryphonectria cubensis* (Bruner) Hodges in South Africa (Denison & Kietzka, 1993) and Brazil (Alfenas, Jeng & Hubbes, 1983) as well as for Eucalyptus rust in Brazil (Dianese, Moraes & Silva, 1984). The endophytic nature of *E. gyrosa* also requires further study.

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Table 1. Isolates of *Cryphonectria eucalypti* used in the inoculation trials.

Isolate number[a)]	Host	Origin[b)]	Collector	Date
CRY902	*Eucalyptus* sp.	Amangwe	J. Roux	July 1997
CRY912	*Eucalyptus* sp.	Amangwe	J. Roux	July 1997
CRY913	*E. grandis*	Dukuduku	J. Roux	July 1997
CRY914	*E. grandis*	Dukuduku	J. Roux	July 1997
CRY915	*E. grandis*	Sabie	M. Venter	December 1997
CRY916	*Eucalyptus* sp.	Futululu	J. Roux	July 1997
CRY917	*E. grandis*	Graskop	M. Venter	December 1997
CRY918	*Eucalyptus* sp.	KwaMbonambi area	J. Roux	July 1997
CRY919	*Eucalyptus* sp.	KwaMbonambi town	J. Roux	July 1997
CRY920	*Eucalyptus* sp.	Nseleni	J. Roux	July 1997
CRY921	*Eucalyptus* sp.	Nyalazi	J. Roux	July 1997
CRY922	*Eucalyptus* sp.	Nyalazi	J. Roux	July 1997
CRY923	*E. grandis*	Piet Retief	M. Venter	December 1997
CRY924	*E. saligna* Smith	Tzaneen	J. Roux	October 1997
CRY925	*E. saligna*	Tzaneen	J. Roux	October 1997

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

b) All areas or estates other than Tzaneen, Piet Retief, Graskop and Sabie, are in KwaZulu/Natal, while the former regions are in the Mpumalanga provence.

Table 2. Combined ANOVA for lesion length measurements of the three inoculation sets of the ZG14 trial with *Cryphonectria eucalypti*.

Source	SS	df	MS	F	p
Isolate	604184·8	15	40279·0	30·6	0·0001
Inoculation	353943·9	2	176972·0	130·4	0·0001
Isolate x inoculation	117328·6	30	3911·0	2·97	0·0001

SEM for inoculations = 9·2

SEM for isolates = 21·0

SEM for isolates x inoculation interaction = 36.3

Table 3. Two way ANOVA for lesion length, width and area (l x b) associated with *Cryphonectria eucalypti* inoculations of 42 *Eucalyptus* clones.

Source	SS	df	MS	F	P
Block (length)	4198·7	10	419·9	2·0	0·0321
Clone (length)	40121·4	41	978·6	4·7	0·0001
Block (width)	3849·1	10	384·9	2·3	0·0137
Clone (width)	40607·3	41	990·4	5·8	0·0001
Block (area)	44396136·0	10	4439613·6	2·6	0·0055
Clone (area)	319068267·8	41	7782152·9	4·5	0·0001

Length: SEM (Clones) = 4·4

Width: SEM (Clones) = 3·9

Area: SEM (Clones) = 398·2

Fig. 1. Disease symptoms associated with *Cryphonectria eucalypti* on *Eucalyptus* in South Africa. (*a*), (*b*) Longitudinal cracking of the bark associated with cankers caused by *C. eucalypti*. (*c*), (*d*) Orange stromata of *C. eucalypti* sporulating around cankers.

Fig. 2. Lesion types associated with inoculation by *Cryphonectria eucalypti* into *Eucalyptus grandis* clone ZG14. (*a*) Cracking and kino exudation around inoculation point. (*b*) Healthy control inoculation. (*c*) Cambial lesions produced in the cambium. (*d*) Discolouration of the bark.

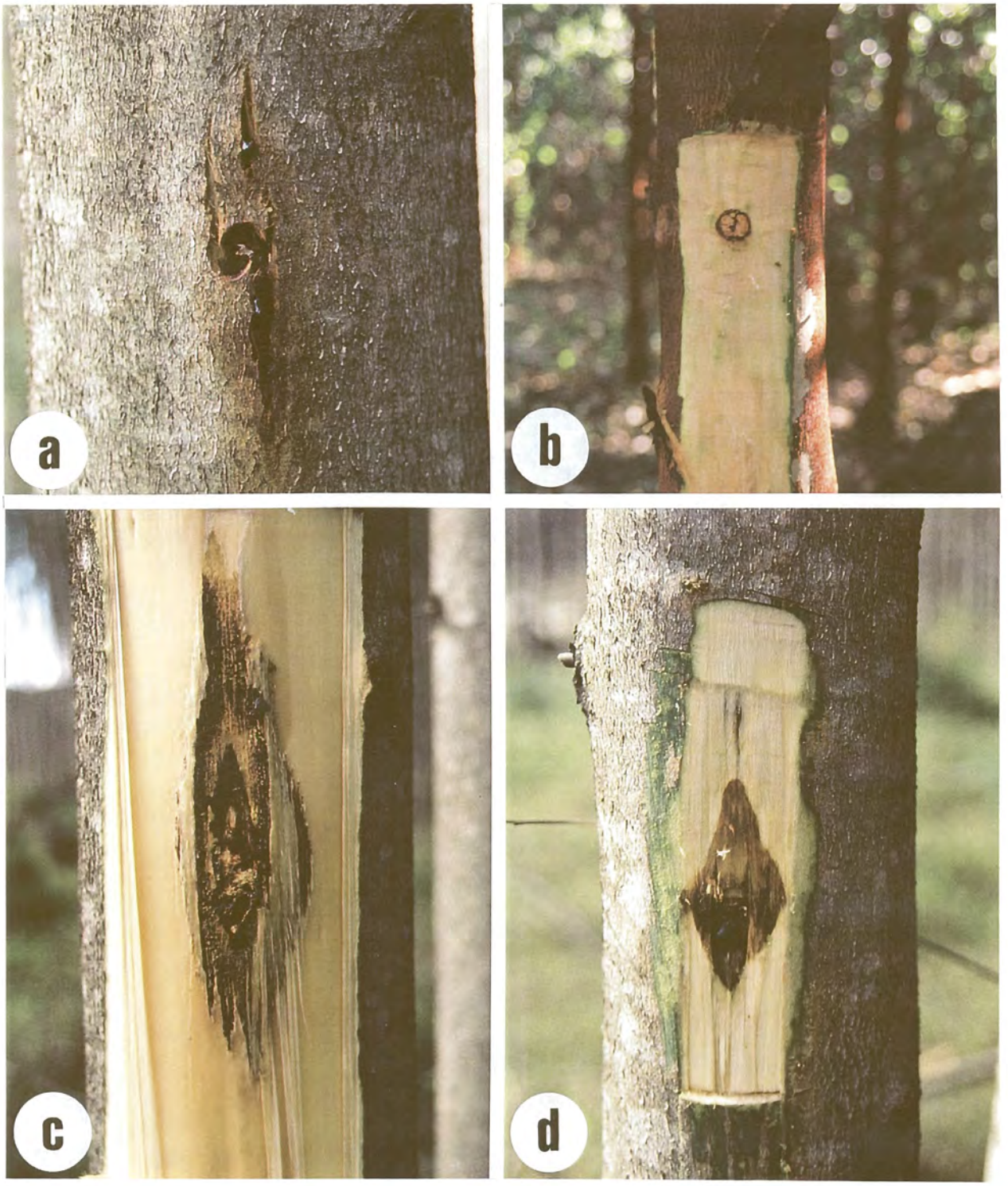


Fig. 3. Mean lesion length values, sorted from the lowest to the highest values, of the fifteen isolates of *Cryphonectria eucalypti* and a negative control for the first inoculation of the ZG14 clone of *Eucalyptus grandis* in KwaZulu/Natal. Lesions were measured seven weeks after inoculation.

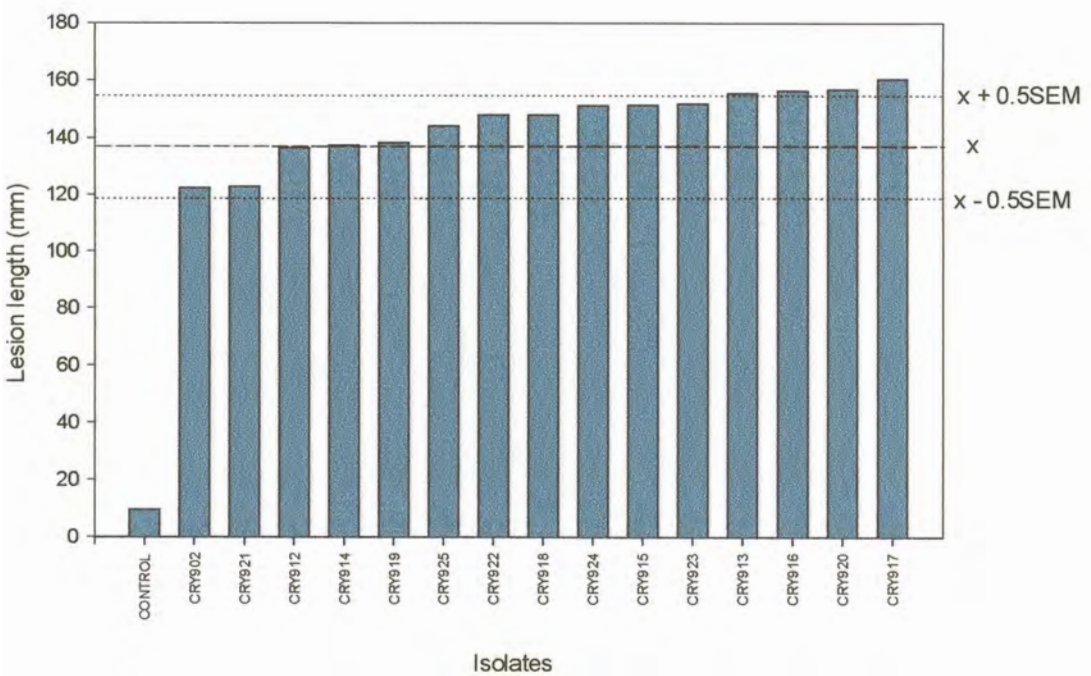


Fig. 4. Mean lesion length values, sorted from the lowest to the highest values, of the fifteen isolates of *Cryphonectria eucalypti* and a negative control for the first block of the second inoculation of the ZG14 clone of *Eucalyptus grandis* in KwaZulu/Natal. Lesions were measured seven weeks after inoculation.

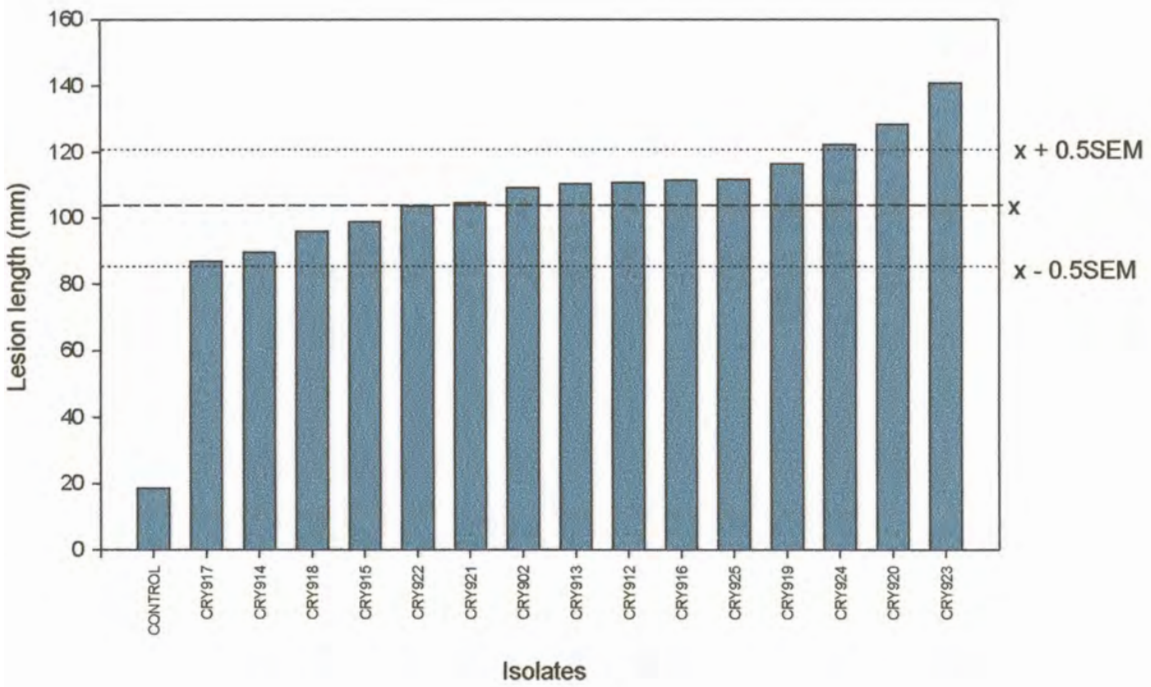


Fig. 5. Mean lesion length values, sorted from the lowest to the highest values, of the fifteen isolates of *Cryphonectria eucalypti* and a negative control for the second block of the second inoculation of the ZG14 clone of *Eucalyptus grandis* in KwaZulu/Natal. Lesions were measured seven weeks after inoculation.

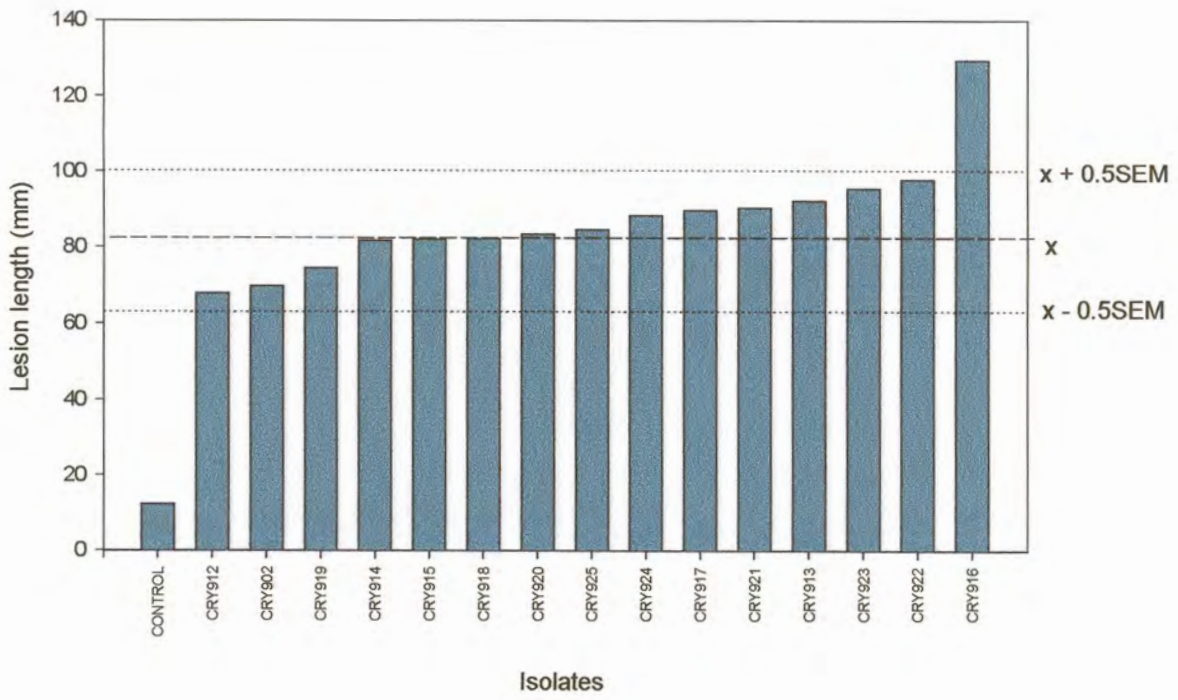


Fig. 6. Mean lesion length values, sorted from the lowest to the highest values, of the fifteen isolates of *Cryphonectria eucalypti* and a negative control over all three inoculation sets of the ZG14 clone of *Eucalyptus grandis* in KwaZulu/Natal. Lesions were measured seven weeks after inoculation.

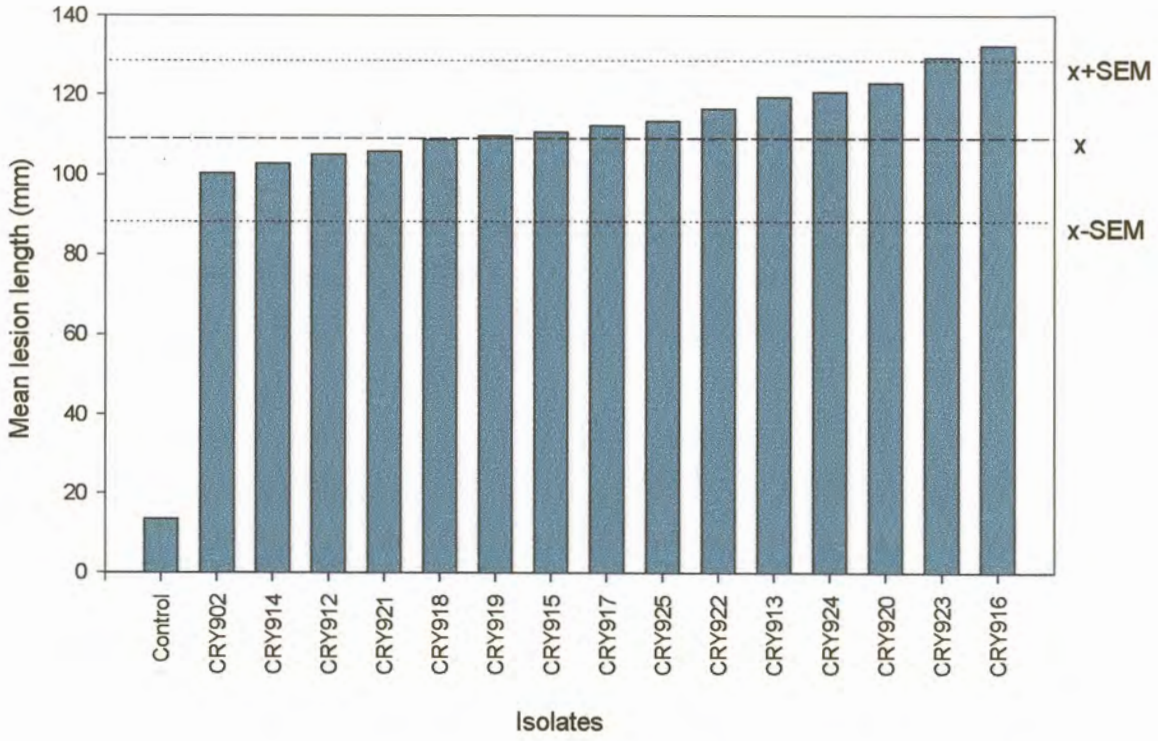
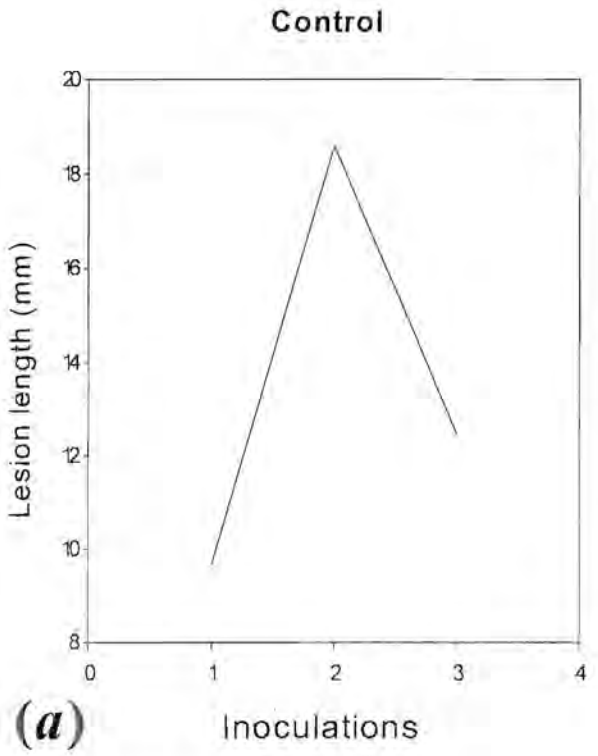
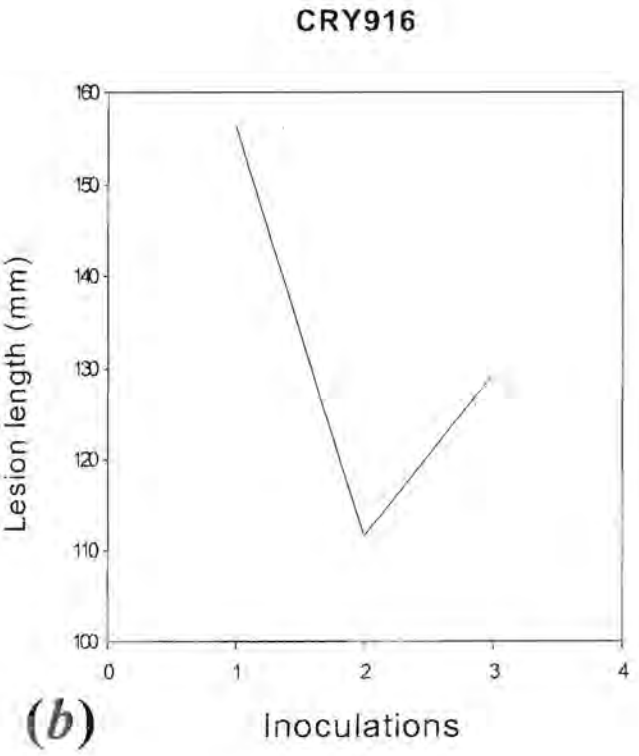


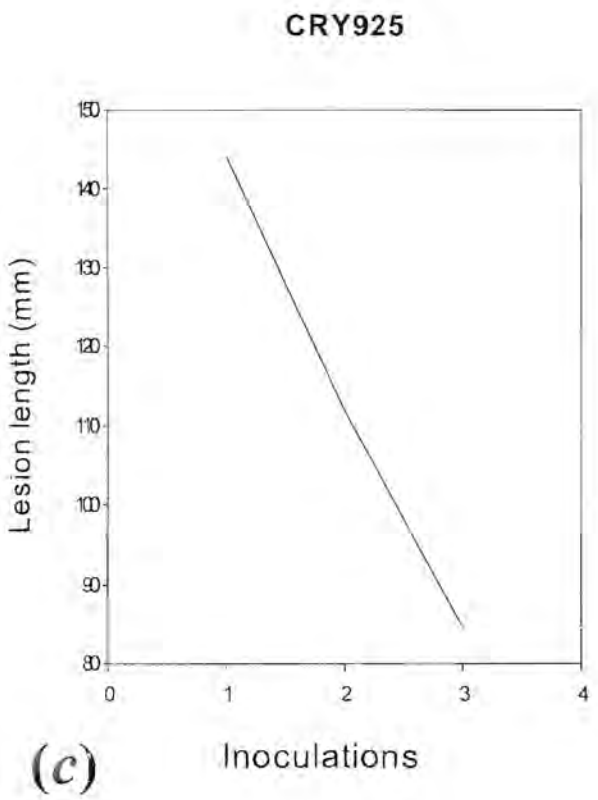
Fig. 7*a-d*. Graphs representing least square means for the three sets of inoculations of *Cryphonectria eucalypti* isolates on a clone of *Eucalyptus grandis* (ZG14). Results represent three isolates of *E. gyrosa* (CRY916, CRY925, CRY923) and a negative control inoculation. These three isolates were chosen for presentation because they represent characteristic trends for the larger group of isolates.


Control
Lesion length (mm)
20
18
16
14
12
10
8
0
1
2
3
4
Inoculations

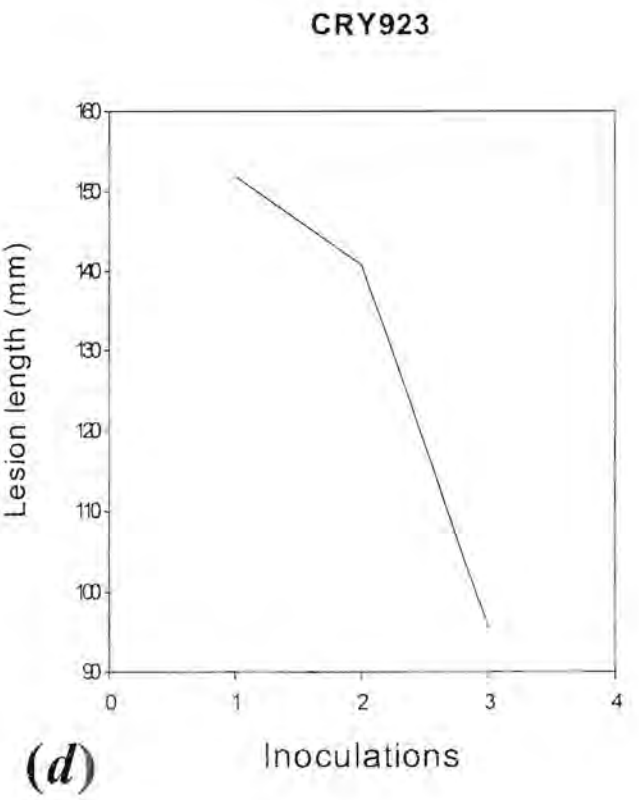
(a)


CRY916
Lesion length (mm)
160
150
140
130
120
110
100
0
1
2
3
4
Inoculations

(b)


CRY925
Lesion length (mm)
150
140
130
120
110
100
90
80
0
1
2
3
4
Inoculations

(c)


CRY923
Lesion length (mm)
160
150
140
130
120
110
100
90
0
1
2
3
4
Inoculations

(d)

Fig. 8. Score values obtained from an AMMI analysis (Additive Main Effects and Multiplicative Interaction Model) plotted against the overall lesion length for each isolate of *Cryphonectria eucalypti* in the ZG14 inoculation trial. Score values for the different inoculations of the ZG14 trial are also included: ZG1 represents the first inoculation, ZG2.1 represents the first block of the second inoculation and ZG2.2 represents the second block of the second inoculation.

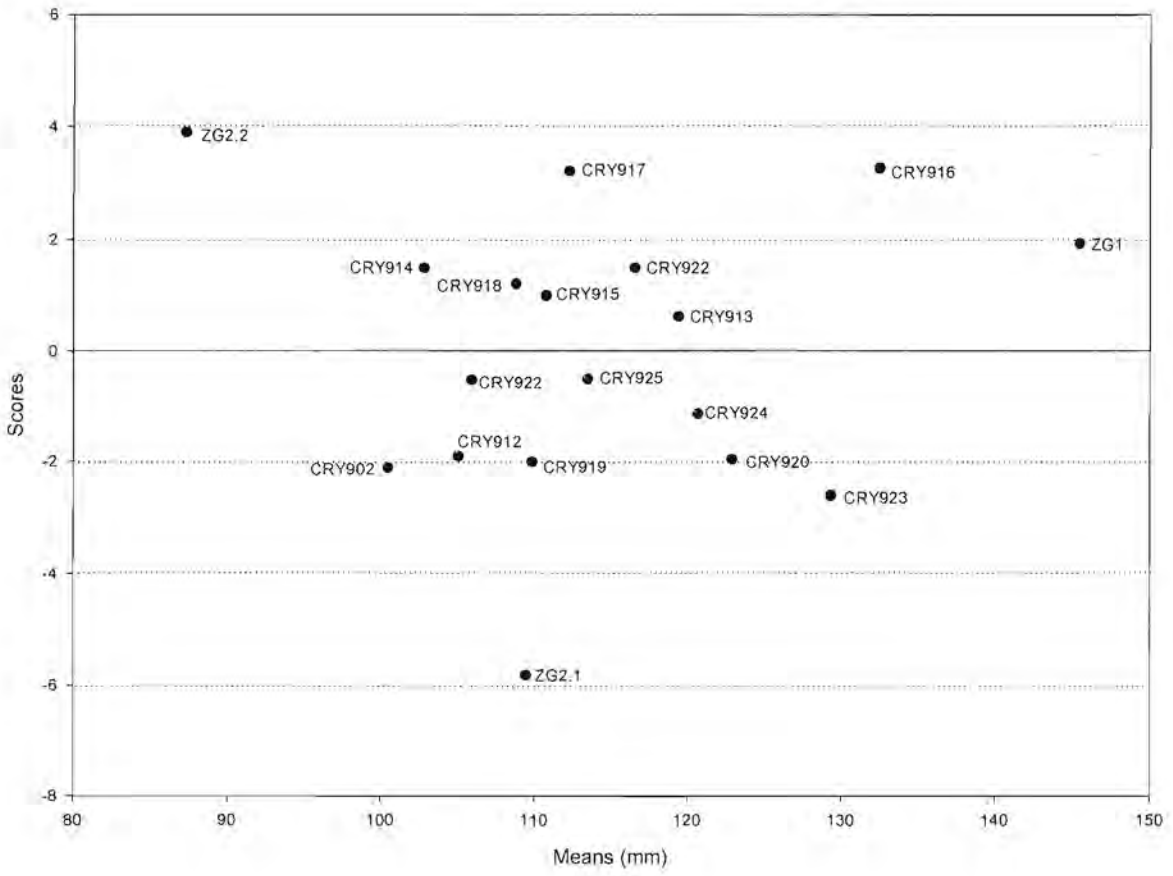

6
4
2
0
-2
-4
-6
-8
Scores
80
90
100
110
120
130
140
150
Means (mm)
ZG2.2
CRY917
CRY916
ZG1
CRY914
CRY922
CRY918
CRY915
CRY913
CRY922
CRY925
CRY924
CRY912
CRY902
CRY919
CRY920
CRY923
ZG2.1

Fig. 9. Mean lesion area (length x width) values of 42 clones of *Eucalyptus grandis* after inoculation with a highly pathogenic isolate of *Cryphonectria eucalypti*, namely CRY920. Lesions were measured six weeks after inoculation.