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).

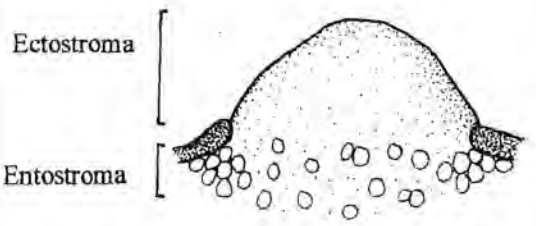
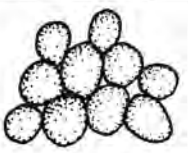
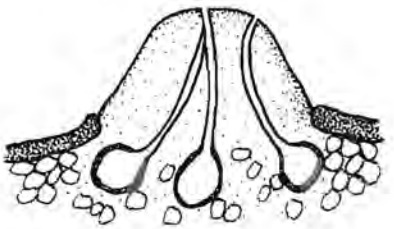
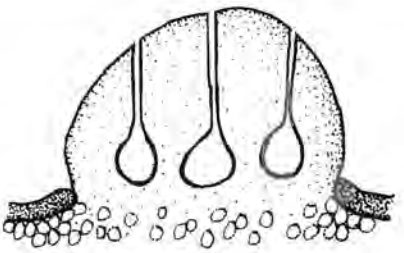
Table 3. Morphological features of the anamorphic members of the Diaporthales including *Endothia gyrosa* and *Cryphonectria gyrosa*.

	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

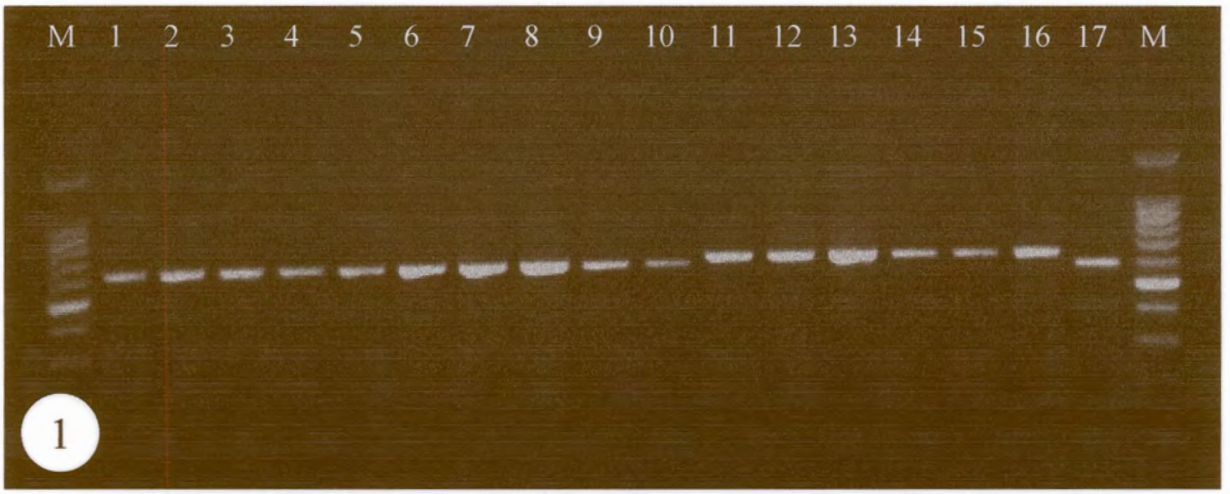
[1] Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa. CRY refers to a collection specifically of *Endothia* and *Cryphonectria* spp., whereas CMW refers to a more general collection of the Institute.

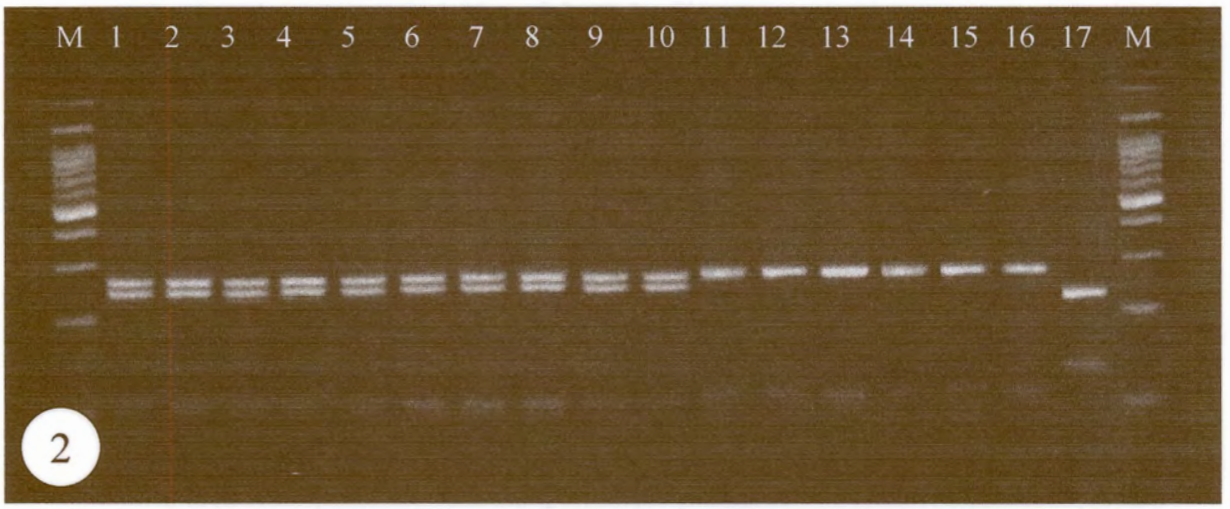
[2] American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209; Centraalbureau voor Schimmelcultures, Fungal and Yeast Collection, P. O. Box 273, 3240 AG, Baarn, Netherlands.

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.





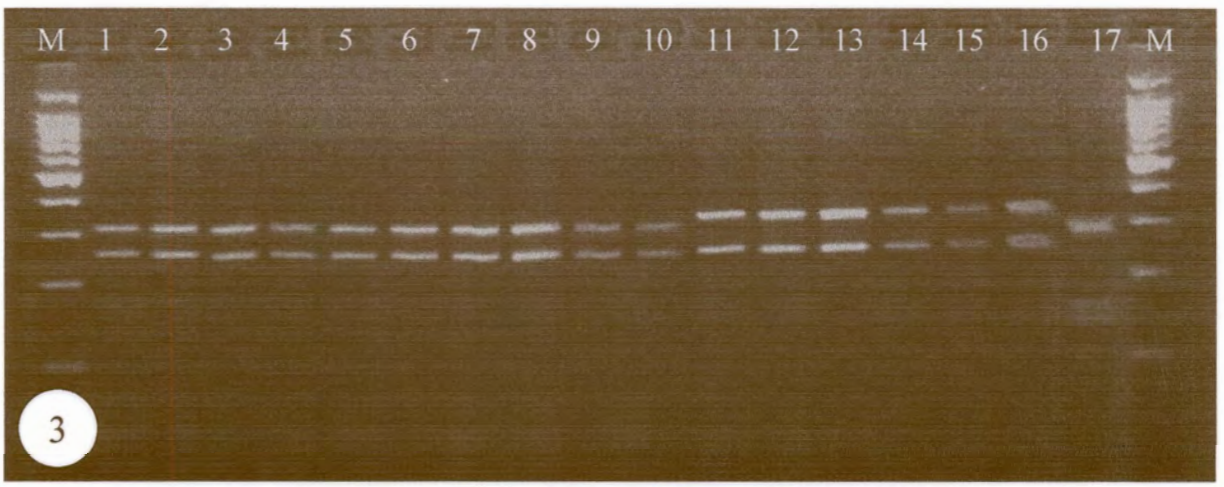


Fig. 4. Restriction map based on RFLP profiles and complete DNA sequences of PCR amplification products of the ITS1, ITS2 and 5.8S rRNA gene. PCR products were cut with restriction enzymes *Cfo*I and *Eco*RI. North American, Australian and South African isolates of *Endothia gyrosa* were used and a *Diaporthe 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.

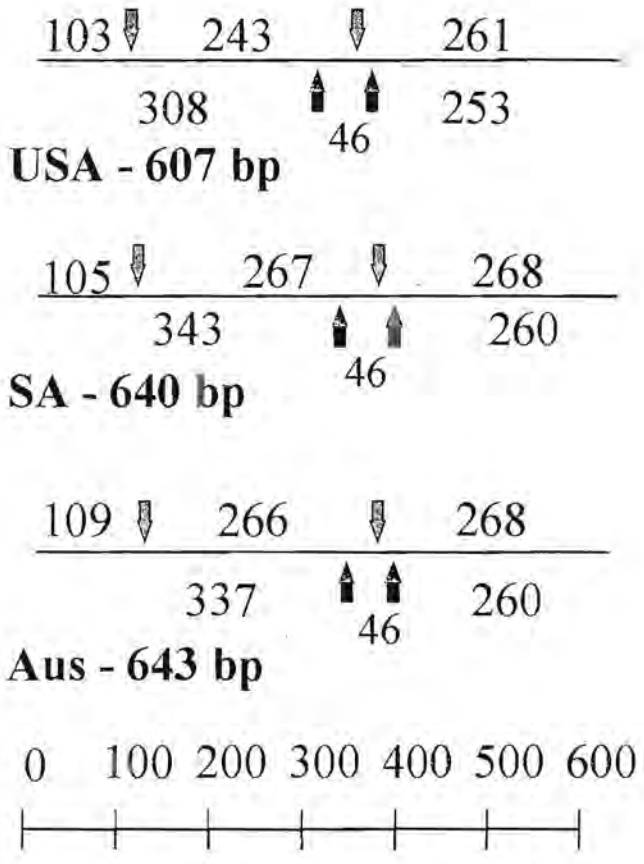

103
243
261
308
253
46
USA - 607 bp
105
267
268
343
260
46
SA - 640 bp
109
266
268
337
260
46
Aus - 643 bp
0
100
200
300
400
500
600

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 "·".

```
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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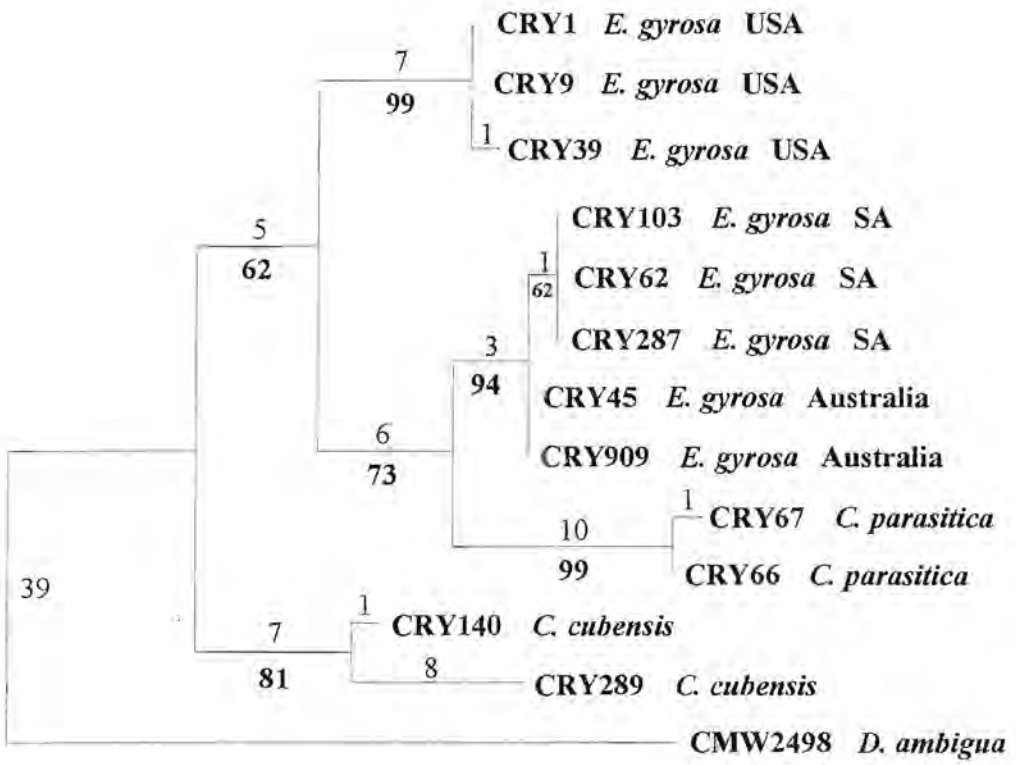
```
CRY1     E. gyrosa (USA)   TTCAACCCTC AAGCC---TG GCTTGGTGTT GGGGCACTAC CTGTA---CA
CRY9     E. gyrosa (USA)   .......... .....---.. .......... .......... .....---..
CRY39    E. gyrosa (USA)   .......... .....---.. .......... .......... .....---..
CRY103   E. gyrosa (SA)    .......... .....CCT.. .......... .......... .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