MOLECULAR TAXONOMY AND MATING TYPE GENES IN *Ceratocystis sensu stricto*

by

Regina Cornelia Witthuhn (née Strydom).

In fulfillment of the requirements of the degree

Philosophiae Doctor

in the Faculty of Natural Sciences,

Department of Microbiology and Biochemistry,

University of the Orange Free State.

January 1999

Promoter: B.D. Wingfield

Co-promoters: M.J. Wingfield

T.C. Harrington

This thesis is dedicated to my father, Steve Strydom,

who once thought he would not live to see its completion

and to my mother, Ema, for her constant support.

ACKNOWLEDGEMENTS

I thank the following people and institutions for their contributions enabling the completion of this thesis.

My advisors, Brenda Wingfield, Mike Wingfield and Tom Harrington.

The members of the following departments:

Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein.

Department of Plant Pathology, Iowa State University, Ames, Iowa, USA.

Department of Genetics, University of Pretoria, Pretoria.

Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria.

The following groups or institutions for their financial support:

Members of the Tree Pathology Co-operative Programme (TPCP).

Foundation for Research Development (FRD).

United Nations Education, Science and Cultural Organization (UNESCO).

The United States Department of Agriculture (USDA).

My husband, Leon Witthuhn.

TABLE OF CONTENTS

PREFACE

The genus *Ceratocystis sensu stricto* includes numerous species that are plant pathogens. The most virulent, primary pathogens include the causal agents of oak wilt (*C. fagacearum*) and black rot of sweet potatoes (*C. fimbriata*). Most of the weaker, secondary pathogens cause blue stain in timber (Kile *et al.*, 1993). Species of *Ceratocystis sensu stricto* are elegantly adapted for the dispersal by insects (Wingfield *et al.*, 1993). These species attract bark beetles, as well as fungus- and sap-feeding insects, through the production of volatile, fruit-like odours (Hanssen, 1993). Even though the biology and morphology of this group of fungi has been studied extensively, the phylogenetic relationships between the species in the genus are poorly defined.

Species of *Ceratocystis sensu stricto* which were once considered to be single entities, have recently been shown to represent a complex of species. Based on isozyme data, Harrington *et al.* (1996) showed that *Ceratocystis coerulescens* is comprised of five morphologically similar species. Harrington *et al.* (1996) also showed that the *Ceratocystis* spp. from conifers are similar to those occurring on hardwoods. However, the phylogenetic relationships between the species in the *C. coerulescens* complex has not been defined.

A recent study of the mating behavior in strains of *C. coerulescens*, showed that a selfing event gives rise to progeny that are self-fertile (MAT-2) or self-sterile (MAT-1). This mating type switching in one direction is referred to as uni-directional mating type switching (Harrington & McNew, 1997). Furthermore, it was shown that self-sterile (MAT-1) isolates have a slower growth rate than the self-fertile (MAT-2) isolates. The current hypothesis is that the *MAT*-2 mating type idiomorph is deleted during uni-directional mating type switching (Harrington & McNew, 1997).

The aims of the studies presented in this thesis were:

- To develop a reliable and quick identification method for all the species of the genus *Ceratocystis sensu stricto* based on molecular techniques.
- To determine the phylogenetic relationships between the better known species of the genus *Ceratocystis sensu stricto* based on the ribosomal RNA gene DNA sequences.
- To determine the phylogenetic relationships between the morphologically similar species in the *Ceratocystis coerulescens* species complex.
- To provide molecular evidence for the deletion of the *MAT*-2 mating type gene during uni-directional mating type switching in species of *Ceratocystis sensu stricto*.
- To compare the phylogenetic analyses based on ITS and *MAT* HMG box DNA sequences of species in the *C. coerulescens* complex.

This thesis represents manuscripts prepared for publication in various scientific journals. Each manuscript is an independent entity, therefore, some redundancy between the chapters has been unavoidable.

REFERENCES

Hanssen, H.-P. (1993). Volatile metabolites produced by species of *Ophiostoma* and *Ceratocystis*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology, and Pathogenicity* (ed. M.J. Wingfield, K.A. Seifert & J.F. Webber), pp. 117-125. APS Press: St Paul, Minnesota.

Harrington, T.C. & McNew, D.L. (1997). Self-fertility and uni-directional mating type switching in *Ceratocystis coerulescens*, a filamentous ascomycete. *Current Genetics* **32**, 52-59.

Harrington, T.C., Steimel, J.P., Wingfield, M.J. & Kile, G. (1996). Isozyme variation in the *Ceratocystis coerulescens* complex. *Mycologia* **88**, 104-113.

Kile, G.A. (1993). Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology, and Pathogenicity* (ed. M.J. Wingfield, K.A. Seifert & J.F. Webber), pp. 173-183. APS Press: St Paul, Minnesota.

Wingfield, M.J., Seifert, K.A. & Webber, J.F. (1993). *Ceratocystis* and *Ophiostoma*. Taxonomy, Ecology, and Pathogenicity. 293 pp. APS Press: St. Paul, Minnesota.

CHAPTER 1: LITERATURE REVIEW.

THE GENUS *CERATOCYSTIS SENSU STRICTO*,

WITH REFERENCE TO MATING TYPE GENES IN ASCOMYCETES.

1. INTRODUCTION

Controversy exists concerning the complex taxonomic relationships within the ophiostomatoid fungi (Wingfield *et al.*, 1993), which includes the genera *Ceratocystis sensu stricto* Ellis & Halsted, *Ophiostoma* H. & P. Sydow, *Ceratocystiopsis* Upadhyay & Kendrick and *Gondwanamyces* Marais & Wingfield (Marais *et al.*, 1998). The genus *Ceratocystis* was first described by Halsted in 1890 based on *C. fimbriata* Ellis & Halsted, the cause of black rot of sweet potatoes. The genus was defined as having perithecia with elongated necks. A similar morphology was observed in species of *Ophiostoma*, first described in 1919. This has led to a century of confusion, where species of *Ceratocystis* and *Ophiostoma* were treated as distinct genera and at times as one genus. As recently as 1981, Upadhyay published a monograph in which all species of *Ceratocystis* and *Ophiostoma* were treated as a single genus.

Various early researchers recognized that *Ceratocystis* and *Ophiostoma* were distinct. Morphologically the genus *Ceratocystis sensu stricto* is characterised by *Chalara* anamorphs with endogenous, phialidic conidium formation, whereas *Ophiostoma* is characterized by *Sporothrix*, *Graphium*, or similar anamorph states with exogenous, blastic conidium formation (Von Arx, 1974). Weijman & De Hoog (1975) also noted that the cell walls of these two genera are distinct. *Ceratocystis* species, in contrast to *Ophiostoma*, are also intolerant to low concentrations of cycloheximide (Harrington, 1981). In recent years it has been shown that *Ceratocystis* and *Ophiostoma* represent distinct phylogenetic lineages (Hausner *et al.*, 1993a,b; Spatafora &

Blackwell, 1994), suggesting their convergent evolution (Harrington, 1987; Wingfield *et al.*, 1993).

A great body of literature exists for *Ceratocystis*. In this review I will deal exclusively with *Ceratocystis* in the strict sense. My intention is to consider all aspects of these fungi including their taxonomy, ecology and pathogenicity. Particular attention is also given to mating type genes in Ascomycetes and the early studies of these genes in *Ceratocystis*.

2. PATHOGENICITY

Ceratocystis includes necrotrophic plant pathogens of variable pathogenicity, occurring primarily on angiosperm hosts (Kile, 1993). This group of fungi primarily includes wound-infecting opportunistic pathogens. *Ceratocystis* spp. are the causal agents of diseases on economically important agricultural crops (Halsted, 1890; Wismer, 1961), as well as forest (Gibbs & French, 1980; Gremmen & De Kam, 1977; Kile *et al.* 1996; Morris *et al.*, 1993; Wood & French, 1963), and fruit trees (Ribeiro *et al.*, 1986; DeVay *et al.*, 1968).

Ceratocystis fagacearum (Bretz) Hunt and *C. fimbriata* are two well-studied species and are aggressive primary pathogens. *C. fagacearum*, first described by Henry *et al.* (1944), causes a severe vascular wilt of oak trees, and is known only in the United States. *C. fagacearum* causes disease in Chinese chestnut and 20 of the species of oak, with species in the red oak group being the most susceptible (Kile, 1993; Sinclair *et al.*, 1987). *Ceratocystis fimbriata*, first described by Halsted in 1890, causes a variety of diseases on a wide range of hosts world-wide. The fungus is the cause of vascular stain in plane trees (Grosclaude & Olivier, 1988) and mango trees (Ribeiro *et al.*, 1986), resulting in severe discoloration of the sapwood. *C. fimbriata* also causes vascular

staining and cankers on poplars (Gremmen & De Kam, 1977; Wood & French, 1963), *Prunus* species (e.g. prunes, almonds, apricot, peach), as well as walnuts (DeVay *et al.*, 1968; Teviotdale & Harper, 1991), basal rot in *Syngonium* (Vogelzang & Scott, 1990), and root rot in sweet potatoes (*Ipomoea batatas*) (Halsted, 1890; Kile, 1993). Based on the wide host range, it was hypothesized that *C. fimbriata* might include an aggregate of closely related species (Webster & Butler, 1967). Thus for example, *C. albofundus*, first thought to represent *C. fimbriata*, causes gummosis and die-back of black wattle trees (*Acacia mearnsii*) in South Africa (Morris *et al.*, 1993). *C. albofundus* is now known to be distinct from *C. fimbriata*, and they are phylogenetically closely related (Wingfield *et al.*, 1996; Witthuhn *et al.*, 1998b).

Most other species of *Ceratocystis* are known as weak, secondary pathogens. *C. coerulescens* (Münch) Bakshi (Münch, 1907), *C. pinicola* Harrington & Wingfield, *C. resinifera* Harrington & Wingfield and *C. douglasii* Wingfield, Harrington & Solheim (Wingfield *et al.*, 1997) cause blue-stain in conifers (Harrington & Wingfield, 1998). *C. laricicola* Redfern & Minter, *C. polonica* (Siemaszko) Moreau and *C. rufipenni* Wingfield, Harrington & Solheim are associated with bark-beetles and also cause blue-stain in larch and spruce (Harrington *et al.*, 1996; Harrington & Wingfield, 1998). *C. virescens* (Davidson) Moreau (Davidson, 1944) and *C. eucalypti* Yuan & Kile (Kile *et al.*, 1996) are the causal agents of vascular stain of hardwoods and *C. virescens* causes sapstreak in maples and in tulip poplars (Harrington *et al.*, 1998; Kile, 1993). *C. paradoxa* (Dade) Moreau, causes root, stem and fruit rot on monocotyledonous plants, including pineapple disease of sugar cane (*Saccharum officinarum*) (Wismer, 1961), and root rot of palms (Kile *et al.*, 1993). *C. adiposa* (Butler) Moreau causes disease in sugar cane, but also saprophytically colonizes wood, and *C. moniliformis* (Hedgcock) Moreau has been associated with diseases of dicotyledonous plants (Kile, 1993).

3. MECHANISMS FOR SPORE DISPERSAL

Various spore dispersal mechanisms exist for the species of *Ceratocystis*. They are associated with insects, such as tree wounding bark beetles (Christiansen & Solheim, 1990; Wingfield *et al.*, 1997), as well as fungal- and sap-feeding insects, including nitidulids and drosopholid flies (Chang & Jensen, 1974; Hinds, 1972; Hinds & Davidson, 1972; Jewell, 1956; Juzwik & French, 1983). The perithecial ascomata of these species are elongated and the ascospores are exuded in slimy droplets at the apices. The wet spore masses are carried above the host tissue, making these fungi morphologically well adapted for the dispersal by insects (Upadhyay, 1981). Some of the species produce fruity or sweet odours (Hanssen, 1993) that attract insects to the infected tissue, and this facilitates the dispersal of the spores of *C. fagacearum*, *C. fimbriata* and *C. paradoxa* (Kile, 1993).

Ceratocystis spp. are not only spread through their insect association. Spore dispersal may also occur by means of wind, pruning tools (Kile, 1993), and water (Grosclaude & Oliver, 1988; Vigouroux & Stojadinovic, 1990). Underground, spread of *C. fagacearum* and *C. fimbriata* occurs mainly through root grafts (Epstein, 1978; Gibbs & French, 1980; Kile, 1993).

3.1 Dispersal by Bark Beetles

Ceratocystis polonica, *C. laricicola* and *C. rufipenni* are in association with tree wounding, bark beetles. *Ceratocystis polonica* is associated with the spruce bark beetle, *Ips typographus* L. (Christiansen & Solheim, 1990; Siemaszko, 1938; Yamaoka *et al.*, 1997), and *C. laricicola* is associated with *Ips cembrae* Heer (Redfern *et al.*, 1987; Visser *et al.*, 1995). *C. rufipenni* is

closely associated with the bark beetle species, *Dendroctonus rufipennis* L. (Wingfield *et al.*, 1997).

3.2 Dispersal by Fungal- and Sap-Feeding Insects

Those *Ceratocystis* spp. that sporulate on the aerial surfaces of infected tissue, and which are spread overland by fungal- and sap-feeding insects, include *C. fagacearum* (Appel *et al.*, 1990; Himelick & Curl, 1958; Jewell, 1956; Juzwik & French, 1983), *C. paradoxa* (Chang & Jensen, 1974), *C. fimbriata* (Hinds, 1972; Hinds & Davidson, 1972; Moller & DeVay, 1968), and *C. moniliformis* (Hinds, 1972; Hinds & Davidson, 1972). The dispersal biology of the oak wilt fungus, *C. fagacearum* has been extensively studied. In the second season after the infection of a tree, *C. fagacearum* forms thick fungal mats below the bark. Through the formation of specialized pressure pads between the wood and the bark, these fungal mats exert sufficient pressure against the bark in order to crack it open, thus exposing the conidiophores and conidia. (Leach *et al.*, 1952; Staley & True, 1952). As soon as the bark is cracked open, a fruity odour is produced by the fungus in order to attract insects (Hanssen, 1993; True *et al.*, 1952). The fungus sporulating on the mats has been found to be one of two opposite mating types (Hepting *et al.*, 1952). The visiting nitidulids and bark beetles carry conidia of opposite mating type between the mats. This facilitates fertilization, followed by the formation of perithecia, and an abundance of slimy ascospores (Leach *et al.*, 1952). Perithecia can be formed before the bark cracks open, but this happens only when both mating types are present in the host (Hepting *et al.*, 1952).

The slow spread of oak wilt through North America is mainly due to the erratic and slow spread of the causal agent, *C. fagacearum* (Appel *et al.*, 1990; Gibbs & French, 1980). Much of the

spread of the oak wilt fungus is mainly dependent on root grafts. Spores are spread from diseased trees to healthy trees via the continuous xylem system present in the root grafts (Epstein, 1978; Gibbs & French, 1980). The fungus may also be spread through infrequent visits of fungal- and sap-feeding insects to the odour producing fungal mats, from which spores are transferred to the bodies of insects, and in turn to fresh wounds on healthy trees (Gibbs & French, 1980). It has been suggested that *Ophiostoma piceae* (Münch) H. & P. Sydow may serve as a biological control agent for oak wilt (Gibbs, 1980; Juzwik & French, 1983; Ruetze & Parameswaren, 1984).

4. *Ceratocystis coerulescens* COMPLEX

Ceratocystis coerulescens, the cause of blue-stain in spruce and pine, was first described by Münch (1907). Harrington *et al.* (1996), using isozyme data, identified five morphologically similar species on conifers, all previously known as *C. coerulescens*. These five species have been described as *C. coerulescens*, *C. douglasii* which causes stain in Douglas fir (Davidson, 1953; Wingfield *et al.*, 1997), *C. rufipenni* isolated from spruce attacked by the bark beetle, *Dendroctonus rufipennis* (Davidson, 1955; Wingfield *et al.*, 1997), *C. resinifera* isolated from spruce (Harrington & Wingfield, 1998), and *C. pinicola*, the cause of blue-stain in pine (Harrington & Wingfield, 1998). On the basis of phylogenetic studies and their ecology, it seems that the *Ceratocystis* spp. isolated from conifers are clearly distinct. They are however difficult to differentiate based on morphological characteristics.

Ceratocystis polonica and *C. laricicola* also form part of the *C. coerulescens* complex and differ only from *C. coerulescens* in the shape of their ascospores. *Ceratocystis polonica* was previously known as *Ophiostoma polonicum*, but has subsequently been recognized as a distinct species of *Ceratocystis* by Visser *et al.* (1995). These two species have virtually identical morphological

characteristics, and occur on conifers (Visser *et al.*, 1995; Harrington & Wingfield, 1988). *C. polonica* however occurs on spruce, and is associated with the bark beetle, *Ips typographus* L. (Christiansen & Solheim, 1990; Siemaszko, 1938; Yamaoka *et al.*, 1997). This is in contrast to *C. laricicola* that occurs on larch, and is associated with *Ips cembrae* Heer (Redfern *et al.*, 1987; Visser *et al.*, 1995). The similarity between *C. polonica* and *C. laricicola* is reflected in their identical ITS rDNA sequences (Witthuhn *et al.*, 1998a). . The differences between the two species are evident at a physiological level in that they differ at one enzyme locus (Harrington *et al.*, 1996). Crosses between *C. polonica* and *C. laricicola* result in no viable ascospores (Harrington & McNew, 1998) suggesting that these species may be intersterile.

Based on ITS DNA sequences (Witthuhn *et al.*, 1998a), all the species in the *C. coerulescens* complex isolated from conifers, including the five morphological variants of *C. coerulescens*, as well as, *C. polonica* and *C. laricicola*, appear to be closely related. In fact the species in the complex isolated from conifers are monophyletic. It would, therefore, appear that the change to conifer species as hosts evolved only once in *Ceratocystis* (Witthuhn *et al.*, 1998a).

The *C. coerulescens* complex also includes four species from hardwoods, which are phylogenetically closely related to the *Ceratocystis* spp. isolated from conifers (Harrington *et al.*, 1996; Witthuhn *et al.*, 1998a). *C. virescens*, previously considered to be a synonym for *C. coerulescens* (Hunt, 1956; Upadhyay, 1981), was first described from hardwood lumber in eastern North America, and causes sapstreak in maple (*Acer* spp.) (Davidson, 1944). The other three species from hardwoods were isolated and described from Australasia. *Ceratocystis eucalypti* occurs on *Eucalyptus* (Kile *et al.*, 1996). *Chalara australis* is a pathogen on *Nothofagus cunninghamii* (Kile *et al.*, 1996), and *Ch. neocaledoniae* was described from coffee (*Coffea robusta*) and guava (*Psidium guajava*) (Kile & Walker, 1987).

5. MOLECULAR TAXONOMY

Fungal taxonomy has been dramatically affected during the past two decades by studies at the molecular level. Molecular taxonomy has also been applied to the genus *Ceratocystis*. The genera forming part of the ophiostomatoid group of fungi are known to have very similar morphological characteristics (Wingfield *et al.*, 1993), suggesting a close phylogenetic relationship. Based on the phylogenetic analyses of the rRNA gene sequences (Hausner *et al.*, 1992; Jones & Blackwell, 1998; Spatafora & Blackwell, 1994), it has however been determined that *Ceratocystis* and *Ophiostoma* are phylogentically distantly related, suggesting that their morphological characteristics may have converged.

The phylogenetic relationships between the species of *Ceratocystis* have also been investigated with the aid of molecular techniques (Hausner, 1993a; Visser *et al.*, 1995; Wingfield *et al.*, 1994; Wingfield *et al.*, 1996; Witthuhn *et al.*, 1998a,b). Based on these phylogenetic studies it would appear that *C. fimbriata* and *C. albofundus* (Wingfield *et al.*, 1994), as well as *C. laricicola* and *C. polonica* (Visser *et al.*, 1995; Witthuhn *et al.*, 1998a) are phylogenetically closely related. The phylogenetic relationships between the species in the *C. coerulescens* complex have also been determined (Witthuhn *et al.*, 1998a), but the phylogenetic relationships between all the *Ceratocystis* species are still unclear (Wingfield *et al.*, 1994; Witthuhn *et al.*, 1998b).

Most molecular studies performed on *Ceratocystis* spp. have been restricted to ribosomal RNA (rRNA) gene sequences. However, the complex relationships within the *C. coerulescens* complex were investigated using isozyme data (Harrington *et al.*, 1996). Restriction fragment length polymorphisms (RFLPs) (Hausner *et al.*, 1993; Witthuhn *et al.*, 1998b), have also successfully been used in the study of the relationships within the genus, *Ceratocystis*.

6. MATING SYSTEMS

Three different mating systems exist amongst the Ascomycetes. Most are heterothallic, in which two strains of opposite mating type must interact for sexual reproduction. Ascomycete species can also be self-fertile or homothallic, which means that a single strain is able to reproduce sexually. Some ascomycete species appear homothallic by compartmentalising two nuclei of opposite mating type in a single spore, which is referred to as pseudohomothallism (Nelson, 1996). Homothallic and heterothallic species can be found in many related Ascomycete genera and the mating systems must have evolved independently and frequently. It has been suggested that the constraint on switching from homothallism to heterothallism is not high (Nauta & Hoekstra, 1992), thus enabeling phylogenetically closely related species to have different mating systems.

Ceratocystis ssp. have been observed to represent of one of two mating type systems. *Ceratocystis fagacearum* (Hepting *et al.*, 1952) and *C. eucalypti* (Kile *et al.*, 1996) are strictly heterothallic. Strains of these species are one of two opposite mating types, referred to as MAT-1 or MAT-2. Strains of *C. fimbriata* (Olson, 1949; Webster, 1967; Webster & Butler, 1967), *C. coerulescens* (Bakshi, 1951; Harrington & McNew, 1997), *C. paradoxa* (Harrington & McNew, 1997) and *C. douglasii* (Davidson, 1953) are also heterothallic. Thus MAT-2 strains able to self, while the MAT-1 strains cannot undergo selfing and are self-sterile.

The progeny of a selfing event in *C. coerulescens* (= *C. pinicola*) show a 1:1 segregation. Half of the progeny are self-fertile (MAT-2) and half are self-sterile (MAT-1) (Harrington & McNew, 1997). Self-sterile isolates of the members of the *C. coerulescens* complex (Harrington & McNew, 1998) and *C. fimbriata* (Webster, 1967) do not form perithecia, unless they are crossed with self-fertile strains. The expression of the *MAT*-1 mating type, entailing self-sterility, also

entails a slower growth rate of the self-sterile, MAT-1 strains when they are compared to the self-fertile, MAT-2 strains. Harrington & McNew (1997) hypothesized that the *MAT*-2 idiomorph, together with a part of the chromosome, is deleted during the switch from a self-fertile to a self-sterile strain, resulting in the slower growth of the self-sterile, MAT-1 strains. This one directional mating type switching in species of *Ceratocystis*, whereby self-fertile (MAT-2) strains can switch to self-sterile (MAT-1) strains, but self-sterile strains (MAT-1) cannot switch to self-fertile strains (MAT-2), is referred to as "uni-directional mating type switching" (Perkins, 1987). Similar mating behavior, with ascospores from the same perithecia giving rise to strains that are self-fertile or self-sterile, has also been observed in other filamentous ascomycetes (Perkins, 1987), but is still poorly understood. Uni-directional mating type switching occurs only when the self-fertile strains undergo switching in one direction, and only if the switch is irreversible (Perkins, 1987).

7. MATING TYPE GENES IN ASCOMYCETES

Although some knowledge exists regarding mating systems in *Ceratocystis*, virtually nothing is known of the mating type genes in this group of fungi. Mating type genes have, however, been intensively studied in a small number of Ascomycetes. These studies pave the way for thorough investigations of the subject in *Ceratocystis*. Some of the first studies of mating type genes in this group of fungi are included in this thesis and the following review provides an introduction to these investigations.

Most Ascomycetes have a bipolar mating type system, with two alternative mating type alleles occupying a single mating type locus (Nelson, 1996). These mating type alleles differ extensively in their DNA and amino acid sequences, and the encoded gene products may not have the same evolutionary origin (Coppin *et al.*, 1997; Nelson, 1996). The different alleles occupy the same

chromosomal position, and are referred to as "idiomorphs" (Metzenberg, 1990), thus distinguishing them from classic alleles.

The idiomorphs at the locus referred to as the mating type, determine the ability of strains to cross. In Ascomycetes a detailed understanding of the genes occupying this locus, namely the mating type genes, are restricted to five intensely studied species (Coppin *et al.*, 1997; Nelson, 1996; Turgeon *et al.*, 1993b). In question here are two yeast species, *Saccharomyces cerevisiae* Meyer ex Hausen (Herskowitz, 1988 & 1989; Hicks, 1979) and *Schizosaccharomyces pombe* Lindner (Kelly *et al.*, 1988), and three filamentous ascomycetes, i.e. *Neurospora crassa* Shear & Dodge (Glass *et al.*, 1988, 1990; Philley & Staben, 1994; Staben & Yanofsky, 1990), *Podospora anserina* (Cesati) Niessl (Debuchy & Coppin, 1992; Debuchy *et al.*, 1993) and *Cochliobolus heterostrophus* (Drechsler) Drechsler (Turgeon *et al.*, 1993a).

The mating type idiomorphs in filamentous ascomycetes have been isolated using various methods. The *N. crassa mt A* mating type gene was isolated by chromosome walking from a linked gene (Glass *et al.*, 1988). The *mat*$^{-}$ idiomorph of *P. anserina* was isolated by using the *Neurospora mt A* mating type gene in hybridization experiments (Picard *et al.*, 1991). *C. heterostrophus* mating type genes were cloned by screening for a homothallic transformant after MAT-2 strains were transformed with cosmid clones from MAT-1 strains (Turgeon *et al.*, 1993a). In *Magnaporthe grisea* (Hebert) Barr the mating type genes were isolated by genomic subtraction (Kang *et al.*, 1994).

Mating type genes all have a similar structure. Each of the two different idiomorphs consists of a unique DNA sequence, and this is flanked by identical sequences between the two different idiomorphs (Griffin, 1993). The unique DNA sequence in each idiomorph encodes master

regulatory proteins which control sexual development (Coppin *et al.*, 1997; Nelson, 1996; Turgeon *et al.*, 1993b). These regulatory proteins encoded for by *MAT*, are structurally divided into high mobility group (HMG) DNA-binding proteins, α1 domain, homeodomain and amphipathic α-helical proteins (Nelson, 1996).

Saccharomyces cerevisiae has two haploid cell types, namely **a** and α, only differing in the *MAT* locus (Herskowitz, 1988). The two mating type genes, *MAT*α (642 bp (base pair)) and *MAT***a** (737 bp), occupy the same chromosomal position which controls the mating behavior of the budding yeast. A silent copy of the mating type not found in the expressed locus is common in many isolates, which allows for bi-directional mating type switching to occur. The *MAT*α idiomorph encodes two regulatory proteins, α1 and α2 and *MAT***a** encodes for a single regulatory protein, **a**1 (Herskowitz, 1989).

In the case of *Schizosaccharomyces pombe*, the single expression site is referred to as *mat1*, with two possible idiomorphs, termed *M* (1128 bp) and *P* (1104 bp). The two silent loci are designated *mat2-P* and *mat3-M* (Kelly *et al.*, 1988). Each idiomorph encodes for two regulatory proteins. The *P* idiomorph encodes for the proteins *Pc* and *Pi*, while the *M* idiomorph encodes *Mc* and *Mi* (Kelly *et al.*, 1988). The proteins *Mc* and *Pc* are necessary for mating and all four peptides are required for meiosis in diploid cells (Kelly *et al.*, 1988).

Amongst the filamentous ascomycetes, *N. crassa* is strictly heterothallic, and the two opposite mating types are designated *A* and *a* (Perkins & Turner, 1988). The two idiomorphs, *mt A* and *mt a*, are flanked by highly similar sequences, and are 5.3 kb (Glass *et al.*, 1990) and 3.2 kb (Staben & Yanofsky, 1990) in size, respectively. Strains of *N. crassa* have only one copy of either

of the two mating type genes. No silent copies of the other mating type gene is present, as in the case of *S. cerevisiae* (Herskowitz, 1989). The fungus produces mating specific pheromones (Bistis, 1981, 1983), which are dependant on the particular mating type idiomorph present and expressed, and results in the fusion of cells of opposite mating type and in the initiation of the sexual cycle (Bistis, 1981).

In *N. crassa* the mating type locus not only controls sexual development, but is also a vegetative incompatibility locus (Staben & Yanofsky, 1990; Glass *et al.*, 1990). The *mt a* idiomorph contains the gene *mt a-1*, which encodes for the polypeptide MT a-1 (Staben & Yanofsky, 1990). This regulatory protein is responsible for both vegetative incompatibility as well as mating (Philley & Staben, 1994). The *mt A* idiomorph encodes for three regulatory proteins, namely the *mtA-1* (Glass *et al.*, 1990), *mtA-2* and *mtA-3* (Ferreira *et al.*, 1996) transcripts.

Podospora anserina is phylogenetically closely related to *N. crassa*. The fungus is pseudohomothallic, with the two opposite mating types contained in separate nuclei within the same spore. The two mating type idiomorphs in *P. anserina* are referred to as *mat-* (4.7 kb in size) and *mat+* (3.7 kb in size) (Picard *et al.*, 1990). The *mat-* idiomorph contains three regulatory genes, *FMR1*, *SMR1* and *SMR2*, essential for fertilisation and sporulation (Debuchy *et al.*, 1993). The transcript of the *mat+* gene, called *FPR1*, is an important transcriptional factor (Debuchy & Coppin, 1992).

Cochliobolus heterostrophus is heterothallic and the mating type idiomorphs are referred to as *MAT-1* (1.3 kb) and *MAT-2* (1.2 kb). The *MAT-1* idiomorph has a 52 bp intron, while the *MAT-2* idiomorph has a 55 bp intron. The mating type exons in *C. heterostrophus* are designated MAT-1 and MAT-2, respectively (Turgeon *et al.*, 1993a).

The translation of the open reading frame (ORF) of all known *MAT*-1 idiomorphs reveal an α box, with sequence similarities between the *S. cerevisiae* MATα1, *N. crassa* MT A-1, *P. anserina* FMR1 and the *C. heterostrophus* MAT-1 *MAT* proteins (Turgeon *et al.*, 1995). Translations of the *MAT*-2 ORF of *C. heterostrophus* revealed a HMG DNA binding motif, with similarity to the *N. crassa* MT a-1 and MT A-3, *P. anserina* FPR1 and SMR2, and *S. pombe* mat-Mc *MAT* proteins (Turgeon *et al.*, 1995). Arie *et al.* (1997) used these conserved DNA binding motifs in the design of degenerate primers for the PCR amplification of the *MAT*-2 idiomorph from a variety of filamentous ascomycetes.

The study of the mating type genes in fungi may prove important in future, since many fungi of economical importance have no known teleomorph state, making accurate identification difficult. These asexual fungi may be encouraged to reproduce sexually after the transformation of the opposite mating type (Turgeon *et al.*, 1993b). However, Sharon *et al.* (1996) have shown that a homolog of the *MAT*-2 gene of *C. heterotrophus* exists in the asexual fungus *Bipolaris sacchari*. The *Bipolaris MAT*-2 gene is functional in *C. heterostrophus*, suggesting that genes, other than *MAT*, may play an important role in sexual reproduction. A further application of the mating type genes is in the phylogenetic studies of fungi. Mating type genes may prove to be useful in studying closely related species, since these genes appear to evolve at a faster rate than other sequences in the genome (Turgeon, 1998). In this regard, there is much to be learned through the study of mating type genes in *Ceratocystis,* which appears to include species with a range of mating strategies.

8. CONCLUSIONS

- *Ceratocystis sensu stricto* has been well-studied, regarding morphology, ecology and molecular characteristics. The species in the genus are associated with insect-vectors, including bark-beetles and sap- and fungal-feeding insects. Some species in the genus are aggressive plant pathogens, causing devastating diseases such as oak wilt. They are thus worthy of ongoing and more intensive research.

- The phylogenetic placement of *Ceratocystis* amongst the Ascomycetes has been relatively well-studied. Amongst the species of *Ceratocystis*, only the phylogeny of species in the *C. coerulescens* complex has been defined. The phylogenetic relationships between all the species in the genus are not clearly understood and more research is required to resolve the many intriguing questions.

- Recent mating studies in *Ceratocystis* and the observed uni-directional mating type switching in this genus, have prompted studies on the mating type genes. These mating type genes have thus far been intensely studied in only three species of Ascomycetes and work on these genes in *Ceratocystis* is in its early stages. Studies of the mating type genes in *Ceratocystis* is sure to play an important role in our understanding of anamorph-teleomorph connections and in our knowledge of the taxonomy of this fascinating group of fungi.

9. REFERENCES

Appel, D.N., Kurdyla, T. & Lewis, R., Jr. (1990). Nitidulids as vectors of the oak wilt fungus and other *Ceratocystis* spp. in Texas. *European Journal of Forest Pathology* **20**, 412-417.

Arie, T., Christiansen, S.K., Yoder, O.C. & Turgeon, B.G. (1997). Efficient cloning of ascomycete mating type genes by PCR amplification of the conserved *MAT* HMG Box. *Fungal Genetics and Biology* **21**, 118-130.

Bakshi, B.K. (1951). Studies on four species of *Ceratocystis*, with a discussion on fungi causing sap-stain in Britain. *Commonwealth Mycological Institute, Mycological Paper* **35**, 1-16.

Bistis, G.N. (1981). Chemotropic interactions between trichogynes and conidia of opposite mating-type in *Neurospora crassa*. *Mycologia* **73**, 959-975.

Bistis, G.N. (1983). Evidence for diffusible, mating-type-specific trichogyne attractants in *Neurospora crassa*. *Experimental Mycology* **7**, 292-295.

Chang, V.C.S. & Jensen, L. (1974). Transmission of the pineapple disease organism of sugar cane by nitidulid beetles in Hawaii. *Journal of Economic Entomology* **67**, 190-192.

Christiansen, E. & Solheim, H. (1990). The bark beetle-associated blue-stain fungus *Ophiostoma polonicum* can kill various spruces and Douglas fir. *European Journal of Forest Pathology* **20**, 436-446.

Coppin, E., Debuchy R., Arnaise, S. & Picard, M. (1997). Mating types and sexual development in filamentous ascomycetes. *Microbiology and Molecular Biology Reviews* **61**, 411-428.

Davidson, R.W. (1944). Two American hardwood species of *Endoconidiophora* described as new. *Mycologia* **34**, 650-662.

Davidson, R.W. (1953). Two common lumber staining fungi in the western United States. *Mycologia* **45**, 579-586.

Davidson, R.W. (1955). Wood-staining fungi associated with bark beetles in Engelmann spruce in Colorado. *Mycologia* **47**, 58-67.

Debuchy, R., Arnaise, S. & Lecellier, G. (1993). The *mat-* allele of *Podospora anserina* contains three regulatory genes required for the development of fertilized female organs. *Molecular and General Genetics* **241**, 667-673.

Debuchy, R. & Coppin, E. (1992). The mating types of *Podospora anserina*: functional analysis and sequence of the fertilization domains. *Molecular and General Genetics* **233**, 113-121.

DeVay, J.E., Lukezic, F.L., English, H., Trujillo, E.E. & Moller, W.J. (1968). *Ceratocystis* canker of deciduous fruit trees. *Phytopathology* **58**, 949-954.

Epstein, A.H. (1978). Root graft transmission of tree pathogens. *Annual Reviews of Phytopathology* **16**, 181-192.

Ferreira, A.V.B., Saupe, S. & Glass, N.L. (1996). Transcriptional analysis of the *mt A* idiomorph of *Neurospora crassa* identifies two genes in addition to *mt A-1*. *Molecular and General Genetics* **250**, 767-774.

Gibbs, J.N. (1980). Role of *Ceratocystis piceae* in preventing infection by *Ceratocystis fagacearum* in Minnesota. *Transactions of the British Mycological Society* **74**, 171-174.

Gibbs, J.N. & French, D.W. (1980). Transmission of oak wilt. *USDA Forestry Service Research Paper NC-185*, 17 pp.

Glass, N.L., Grotelueschen, J. & Metzenberg, R.L. (1990). *Neurospora crassa* A mating-type region. *Proceedings of the National Academy of Science, USA* **87**, 4912-4916.

Glass, N.L., Vollmer, S.J., Staben, C., Grotelueschen, J., Metzenburg, R.L. & Yanofsky, C. (1988). DNAs of the two mating-type alleles of *Neurospora crassa* are highly dissimilar. *Science* **241**, 570-573.

Gremmen, J. & De Kam, M. (1977). *Ceratocystis fimbriata*, a fungus associated with poplar canker in Poland. *European Journal of Forest Pathology* **7**, 11-47.

Griffin, D.H. (1993). *Fungal Physiology*. Wiley-Liss, Inc.: New York, USA. 458 pp.

Grosclaude, C. & Oliver, R. (1988). Detection par piegeage du *Ceratocystis fimbriata f. platani*. *European Journal of Forest Pathology* **18**, 385-390.

Halsted, B.D. (1890). Some fungous diseases of the sweet potato. *New Jersey Agricultural Experimental Station* **76**, 1-32.

Hanssen, H.-P. (1993). Volatile metabolites produced by species of *Ophiostoma* and *Ceratocystis*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology, and Pathogenicity* (ed. M.J. Wingfield, K.A. Seifert & J.F. Webber), pp. 117-125. APS Press: St Paul, Minnesota.

Harrington, T.C. (1981). Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. *Mycologia* **73**, 1123-1129.

Harrington, T.C. (1987). New combinations in *Ophiostoma* of *Ceratocystis* species with *Leptographium* anamorphs. *Mycotaxon* **28**, 39-43.

Harrington, T.C. & McNew, D.L. (1997). Self-fertility and uni-directional mating type switching in *Ceratocystis coerulescens*, a filamentous ascomycete. *Current Genetics* **32**, 52-59.

Harrington, T.C. & McNew, D.L. (1998). Partial interfertility among the *Ceratocystis* species on conifers. *Fungal and General Biology* **25**, 44-53.

Harrington, T.C., Steimel, J. & Kile, G.A. (1998). Genetic variation in three *Ceratocystis* species with outcrossing, selfing and asexual reproductive strategies. *European Journal of Forest Pathology* **28**, 217-226.

Harrington, T.C., Steimel, J.P., Wingfield, M.J. & Kile, G. (1996). Isozyme variation and species delimitation in the *Ceratocystis coerulescens* complex. *Mycologia* **88**, 104-113.

Harrington, T.C. & Wingfield, M.J. (1998). The *Ceratocystis* species on conifers. *Canadian Journal of Botany* **In press**.

Hausner, G., Reid, J. & Klassen, G.R. (1992). Do galeate-ascospore members of the Cephaloascaceae, Endomycetaceae and Ophiostomataceae share a common phylogeny? *Mycologia* **84**, 870-881.

Hausner, G., Reid, J. & Klassen, G.R. (1993a). On the subdivision of *Ceratocystis s.l.*, based on partial ribosomal DNA sequences. *Canadian Journal of Botany* **71**, 52-63.

Hausner, G., Reid, J. & Klassen, G.R. (1993b). On the phylogeny of *Ophiostoma*, *Ceratocystis s.s.*, and *Microascus*, and relationships within *Ophiostoma* based on partial ribosomal DNA sequences. *Canadian Journal of Botany* **71**, 1249-1265.

Hausner, G., Reid, J. & Klassen, G.R. (1993c). Grouping of isolates and species of *Ceratocystis sensu lato* on the basis of molecular and morphological characters. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology, and Pathogenicity* (ed. M.J. Wingfield, K.A. Seifert & J.F. Webber), pp. 93-104. APS Press: St Paul, Minnesota.

Henry, B.W., Moses, C.S., Richards, C.A. & Riker, A.J. (1944). Oak wilt, its significance, symptoms and cause. *Phytopathology* **34**, 636-647.

Hepting, G.H., Toole, E.R. & Boyce, J.S., Jr. (1952). Sexuality in the oak wilt fungus. *Phytopathology* **42**, 438-442.

Herskowitz, I. (1988). Life cycle of the budding yeast *Saccharomyces cerevisiae*. *Microbiological Reviews* **52**, 536-553.

Herskowitz, I. (1989). A regulatory hierarchy for cell specialization in yeast. *Nature* **342**, 749-757.

Hicks, J., Strathern, J.N. & Klar, A.J.S. (1979). Transposable mating type genes in *Saccharomyces cerevisiae*. *Nature* **282**, 478-483.

Himelick, E.B. & Curl, E.A. (1958). Transmission of *Ceratocystis fagacearum* by insects and mites. *Plant Disease Reporter* **42**, 538-545.

Hinds, T.E. (1972). Insect transmission of *Ceratocystis* species associated with aspen cankers. *Phytopathology* **62**, 221-225.

Hinds, T.E. & Davidson, R.W. (1972). *Ceratocystis* species associated with the aspen ambrosia beetle. *Mycologia* **64**, 405-409.

Hunt, J. (1956). Taxonomy of the genus *Ceratocystis*. *Lloydia* **19**, 1-58.

Jewell, F.F. (1956). Insect transmission of oak wilt. *Phytopathology* **46**, 244-257.

Jones, K.G. & Blackwell, M. (1998). Phylogenetic analysis of ambrosial species in the genus *Rassaelea* based on 18S rDNA sequences. *Mycological Research* **102**, 661-665

Juzwik, J. & French, D.W. (1983). *Ceratocystis fagacearum* and *C. piceae* on the surfaces of free-flying and fungus-mat-inhabiting nitidulids. *Phytopathology* **73**, 1164-1168.

Kang, S., Chumley, F.G. & Valent, B. (1994). Isolation of the mating-type genes of the phytopathogenic fungus *Magnaporthe grisea* using genomic subtraction. *Genetics* **138**, 289-296.

Kelly, M., Burke, J., Smith, M., Klar, A. & Beach, D. (1988). Four mating-type genes control sexual differentiation in the fission yeast. *EMBO Journal* **7**, 1537-1547.

Kile, G.A. (1993). Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology, and Pathogenicity* (ed. M.J. Wingfield, K.A. Seifert & J.F. Webber), pp. 173-183. APS Press: St Paul, Minnesota.

Kile, G.A., Harrington, T.C., Yuan, Z.Q., Dudzinski, M.J. & Old, K.M. (1996). *Ceratocystis eucalypti* sp. nov., a vascular stain fungus from eucalypts in Australia. *Mycological Research* **100**, 571-579.

Kile, G.A. & Walker, J. (1987). *Chalara australis* sp. nov. (Hyphomycetes), a vascular pathogen of *Nothofagus cunninghamii* (Fagaceae) in Australia and its relationship to other *Chalara* species. *Australian Journal of Botany* **35**, 1-32.

Leach, J.G., True, R.P. & Dorsey, C.K. (1952). A mechanism for liberation of spores from beneath the bark and for diploidization in *Chalara quercina*. *Phytopathology* **42**, 537-539.

Marais, G.J., Wingfield, M.J., Viljoen, C.D. & Wingfield, B.D. (1998). A new ophiostomatoid genus from *Protea* infructescences. *Mycologia* **90**, 136-141.

Metzenberg, R.L. (1990). The role of similarity and difference in fungal mating. *Genetics* **125**, 457-462.

Moller, W.J. & DeVay, J.E. (1968). Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* **58**, 1499-1509.

Morris, M., Wingfield, M.J. & De Beer, C. (1993). Gummosis and wilt of *Acacia mearnsii* in South Africa caused by *Ceratocystis fimbriata*. *Plant Pathology* **42**, 814-817.

Münch, E. (1907). Die Blaufäule des Nadelholzes. I-II. *Naturwissenschaftliche Zeitschrift für Forst- und Landwirtschaft* **5**, 531-573.

Nauta, M.J. & Hoekstra, R.F. (1992). Evolution of reproductive systems in filamentous ascomycetes. I. Evolution of mating types. *Heredity* **68**, 405-410.

Nelson, M.A. (1996) Mating systems in ascomycetes: a romp in the sac. *TIG* **12**, 69-74.

Olson, E.O. (1949). Genetics of *Ceratostomella*. I. Strains in *Ceratostomella fimbriata* (Ell. & Halst.) Elliott from sweet potatoes. *Phytopathology* **39**, 548-561.

Perkins, D.D. (1987). Mating-type switching in filamentous ascomycetes. *Genetics* **115**, 215-216.

Perkins, D.D. & Turner, B.C. (1988). *Neurospora* from natural populations: toward the population biology of a haploid eukaryote. *Experimental Mycology* 12, 91-131.

Philley M.L. & Staben, C. (1994). Functional analyses of the *Neurospora crassa* MT a-1 mating type polypeptide. *Genetics* **137**, 715-722.

Picard, M., Debuchy, R. & Coppin, E. (1991). Cloning the mating types of the heterothallic fungus *Podospora anserina*: developmental features of haploid transformants carrying both mating types. *Genetics* **128**, 539-547.

Redfern, D.B., Stoakley, J.T., Steele, H. & Minter, M. (1987). Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae*. *Plant Pathology* **36**, 467-480.

Ribeiro, I.J.A., Rossetto, C.J., Sabino, J.C. & Gallo, P.B. (1986). Seca da mangueira: VIII. Resistência de prota-enxertos de mangueira ao fungo *Ceratocystis fimbriata* Ell. & Halst. *Bragantia* **45**, 317-322.

Ruetze, M. & Parameswaran, N. (1984). Observations on the colonization of oak wilt mats (*Ceratocystis fagacearum*) by *Pesotum piceae*. *European Journal of Forest Pathology* **14**, 326-333.

Sharon, A., Yamaguchi, K., Christiansen, S., Horwitz, B.A., Yoder, O.C. & Turgeon, B.G. (1996). An asexual fungus has the potential for sexual development. *Molecular and General Genetics* **251**, 60-68.

Siemaszko, W. (1938). Zespoly Grybow Towarzyszacych Kornikom Polskim. *Planta Polonica* 7, 1-52.

Sinclair, W.A., Lyon, H. & Johnson, W.T. (1987). Diseases of trees and shrubs. 574 pp. Cornell University Press, Ithaca and London.

Spatafora, J.W. & Blackwell, M. (1994). The polyphyletic origins of *Ophiostomatoid* fungi. *Mycological Research* **98**, 1-9.

Staben, C. & Yanofsky, C. (1990). *Neurospora crassa* a mating-type region. *Proceedings of the National Academy of Science, USA* **87**, 4917-4921.

Staley, J.M. & True, R.P. (1952). The formation of perithecia of *Chalara quercina* in nature in West Virginia. *Phytopathology* **42**, 691-693.

Sydow, H. & Sydow, P. (1919). Mykologische Mitteilungen. *Sydowia* **1**, 33-47.

Teviotdale, B. & Harper, D. (1991). Infection of pruning and small bark wounds in almond by *Ceratocystis fimbriata*. *Plant Disease* **75**, 1026-1030.

True, R.P., Staley, J.M., Leach, G.J., Barnett H.L. & Dorsey, C.K. (1952). Liberation of spores from natural reservoirs facilitates overland spread of oak wilt. *Phytopathology* **42**, 476.

Turgeon, B.D. (1998). Application of mating type gene technology to problems in fungal biology. *Annual Review of Phytopathology* **36**, 115-137.

Turgeon, B.G., Bohlmann, H., Ciuffetti, L.M., Christiansen, S.K., Yang, G., Schäfer, W. & Yoder, O.C. (1993a). Cloning and analysis of the mating type genes from *Cochliobolus heterostrophus*. *Molecular and General Genetics* **238**, 270-284.

Turgeon, B.G., Christiansen, S.K., Yoder, O.C. (1993b). Mating type genes in ascomycetes and their imperfect relatives. In *The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics* (ed. D.R. Reynolds & J.W. Taylor), pp. 199-215. Wallingford: CAB International.

Turgeon, B.G., Sharon, A., Wirsel, S., Christiansen, S.K., Yamaguchi, K. & Yoder, O.C. (1995). Structure and function of mating type genes in *Cochliobolus* spp. and asexual fungi. *Canadian Journal of Botany* **73**, S778-S783.

Upadhyay, H.P. (1981). A monograph of *Ceratocystis* and *Ceratocystiopsis*. 176 pp. The University of Georgia Press: Athens, Georgia.

Vigouroux, A. & Stojadinovic, B. (1990). Possibilities d'infection du platane pour *Ceratocystis fimbriata f. platani* aprés contamination de l'eau ou se developpent des racines blessées. *European Journal of Forest Pathology* **20**, 118-121.

Visser, C., Wingfield, M.J., Wingfield, B.D. & Yamaoka, Y. (1995). *Ophiostoma polonicum* is a species of *Ceratocystis sensu stricto*. *Systematic and Applied Microbiology* **18**, 403-409.

Vogelzang, B.K. & Scott, E.S. (1990). *Ceratocystis fimbriata*, causal agent of basal rot of *Syngonium* cultivars, and host range studies of isolates of *C. fimbriata* in Australia. *Australian Plant Pathology* **19**, 82-89.

Von Arx, J.A. (1974). Centraalbureau voor Schimmelcultures, progress report 1973. Verh. Kon. Ned. Akad. Wet. Afd. Natuurkd., Reeks 2, **63**, 1-20.

Webster, R.K. (1967). Inheritance of sexuality, color and colony type in *Ceratocystis fimbriata*. *Mycologia* **59**, 222-234.

Webster, R.K. & Butler, E.E. (1967). A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45**, 1457-1468.

Weijman, A.C.M. & De Hoog, G.S. (1975). On the sub-division of the genus *Ceratocystis*. *Antonie van Leeuwenhoek* **41**, 353-360.

Wingfield, B.D., Grant, W.S., Wolfaardt, J.F. & Wingfield, M.J. (1994). Ribosomal RNA sequence phylogeny is not congruent with ascospore morphology among species in *Ceratocystis sensu stricto*. *Molecular Biology and Evolution* **11**, 376-383.

Wingfield, M.J., De Beer, C., Visser C. & Wingfield, B.D. (1996). A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematic and Applied Microbiology* **19**, 191-202.

Wingfield, M.J., Harrington, T.C. & Solheim, H. (1997). Two new species in the *Ceratocystis coerulescens* complex from conifers in western North America. *Canadian Journal of Botany* **75**, 827-834.

Wingfield, M.J., Seifert, K.A. & Webber, J.F. (1993). *Ceratocystis* and *Ophiostoma*. Taxonomy, Ecology, and Pathogenicity. 293 pp. APS Press: St. Paul, Minnesota.

Wismer, C.A. (1961). Pineapple disease. In *Sugar cane diseases of the world* (ed. J.P. Martin, E.V. Abbott & C.G. Hughes), pp. 224-245. Elsevier, New York, USA.

Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J., Wolfaardt, M. & Harrington, T.C. (1998a). Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. *Mycologia* **90**, 96-101.

Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J. & Harrington, T.C. (1998b). PCR based identification and phylogeny of species of *Ceratocystis sensu stricto*. *Mycological Research* **In press**.

Wood, F.A. & French, D.W. (1963). *Ceratocystis fimbriata*, the cause of a stem canker of quaking aspen. *Forest Science* **9**, 232-235.

Yamaoka, Y., Wingfield, M.J., Takahashi, I. & Solheim, H. (1997). Ophiostomatoid fungi associated with the spruce bark beetle *Ips typographus japonicus* in Japan. *Mycological Research* **101**, 1215-1227.

CHAPTER 2*

PCR-BASED IDENTIFICATION AND PHYLOGENY OF SPECIES OF *Ceratocystis sensu stricto*

ABSTRACT

Most species of *Ceratocystis sensu stricto* are virulent pathogens of a wide variety of plants including forest and fruit trees, sweet potato, pineapple and sugar cane. Confusion exists regarding the taxonomy of the species in this genus. The aim of this study was to develop a rapid and reliable PCR-based RFLP identification method and to consider phylogenetic relationships among the better-known species of *Ceratocystis*. A 1.6 kb fragment within the ribosomal DNA operon was directly amplified from living fungal tissue, without extracting DNA. The amplified fragment included part of the small (SSU) and large (LSU) sub-unit rRNA genes, the 5.8S rRNA gene and the internal transcribed spacers (ITS) 1 and 2. The PCR fragments were digested with eighteen restriction enzymes. Four of these (*Alu*I, *Dra*I, *Hae*III and *Rsa*I) produced RFLPs that separated the species of *Ceratocystis*. The amplification products from the best-known species were sequenced, and the delimitation of taxa based on this phylogenetic analysis generally agreed with results of previous studies using isozymes and rDNA sequence analysis. This study provides an extended understanding of the relationships among species of *Ceratocystis* and will form a sound foundation for further taxonomic studies of the group.

*Published as R.C. Witthuhn, B.D. Wingfield, M.J. Wingfield & T.C. Harrington. (1998). PCR-based identification and phylogeny of species of *Ceratocystis sensu stricto*. *Mycological Research*: In press.

INTRODUCTION

Species of the so-called ophiostomatoid fungi are found in four genera, *Ceratocystis sensu stricto* Ellis & Halsted, *Ophiostoma* H. & P. Sydow, *Ceratocystiopsis* Upadhyay & Kendrick and *Gondwanamyces* Marais & Wingfield. These fungi are adapted to dispersal by insects, and *Ceratocystis* includes many economically important plant pathogens (Christiansen & Solheim, 1990; Teviotdale & Harper, 1991; Kile, 1993; Morris, Wingfield & de Beer, 1993). *Ceratocystis coerulescens*, the cause of sapstain on spruce and pine, is considered to be a weak pathogen. In contrast *C. fagacearum* and *C. fimbriata* are aggressive primary pathogens. *C. fagacearum* causes oak wilt disease (Bretz, 1952; Sinclair, Lyon & Johnson, 1987), while *C. fimbriata* causes vascular stain and cankers on various hosts, including plane (Grosclaude & Oliver, 1988), mango (Ribeiro *et al.*, 1986), and rubber (Olson & Martin, 1949).

Ceratocystis species have more than one means of dispersal (Kile, 1993). Some are closely associated with bark beetles (Coleoptera: Scolytidae), such as *C. polonica* (Siemaszko, 1938; Christiansen & Solheim, 1990), *C. laricicola* (Redfern *et al.*, 1987) and *C. rufipenni* (Wingfield, Harrington & Solheim, 1997). Fungal- and sap-feeding insects are also recognized as vectors of *Ceratocystis* species; for example, picnic beetles (Coleoptera: Nitidulidae) are recognized as direct vectors of *C. paradoxa* (Chang & Jensen, 1974) and *C. fagacearum* (Himelick & Curl, 1958; Juzwik & French, 1983). *Ceratocystis* species may also be dispersed in soil or in frass of ambrosia beetles, or the spores may be splashed by water (Grosclaude & Oliver, 1988; Vigouroux & Stojadinovic, 1990; Kile, 1993).

DNA sequence data from the ribosomal RNA genes have been used effectively to determine the phylogenetic relationships among ophiostomatoid fungi (Hausner, Reid & Klassen, 1992,

1993*a-c*; Spatafora & Blackwell, 1994; Wingfield *et al.*, 1994). These phylogenetic analyses suggest that ascospore morphology is an unreliable taxonomic character (at the genus level) for this group (Hausner *et al.*, 1993b; Spatafora & Blackwell, 1994; Wingfield *et al.*, 1994). Wingfield *et al.* (1994) used large sub-unit ribosomal RNA sequences to determine the phylogenetic relationships among eight species of *Ceratocystis* and found this region to be conserved for this genus.

The more variable ITS regions were used to determine the relationships between *C. fimbriata* and *C. albofundus* (Wingfield *et al.*, 1996), between *C. polonica* and *C. laricicola* (Visser *et al.*, 1995) and among species within the *C. coerulescens* complex (Witthuhn *et al.*, 1998), but the phylogenetic relationships among these groups of species, as well as their relationship to other *Ceratocystis* species, remain poorly defined. Furthermore, insufficient attention has been given to the taxonomy of *Ceratocystis sensu stricto*. This has become especially evident in recent studies (Visser *et al.*, 1995; Harrington *et al.*, 1996; Wingfield *et al.*, 1996; Witthuhn *et al.*, 1998) that have shown that species regarded as single entities in fact represent species complexes.

The aim of this study was to develop a rapid and reliable method for the identification of *Ceratocystis* species. Furthermore, DNA sequence data of the ribosomal RNA genes of the best-known species were compared in order to resolve phylogenetic relationships.

MATERIALS AND METHODS

Isolates: Isolates of *Ceratocystis* spp. used in this study were obtained from a wide range of geographical areas and diverse sources (Table 1). These were grown on malt extract agar (20 g/l malt extract and 20 g/l agar) in Petri-dishes at room temperature for 10 d.

Polymerase chain reaction: PCR reactions were performed directly from the mycelium of the isolates without extracting DNA (Harrington & Wingfield, 1995). A part of the ribosomal DNA operon was amplified using the primers ITS1 and LR6 (Table 2). The amplified fragment included the 3' end of the small sub-unit (SSU) rRNA gene, the 5.8S rRNA gene, part of the large sub-unit (LSU) rRNA gene and the internal transcribed spacer (ITS) regions 1 and 2. The PCR reactions were performed as described by Witthuhn *et al.* (1998). The PCR products were electrophoresed in 15 g/l agarose gels, using 0.5 x TBE electrophoresis buffer, stained with ethidium bromide, and visualised using UV light. Amplification reactions were repeated at least twice for each isolate.

Restriction fragment length polymorphisms (RFLPs): Eighteen restriction enzymes (*Alu*I, *Cfo*I, *Dde*I, *Dra*I, *EcoR*I, *Hae*III, *Hind*II, *Hind*III, *Hinf*I, *Hpa*II, *Pst*I, *Pvu*II, *Rsa*I, *Sau*3A, *Sau*96I, *Scr*FI, *Taq*I, *Xba*I) were tested for RFLPs of the PCR products. The digested PCR products were separated on 20 g/l agarose gels, using 0.5 x TBE electrophoresis buffer, stained with ethidium bromide, and visualised using UV light. The sizes of the restriction products were determined against a 100 bp ladder. No fragments smaller than 150 bp were scored.

DNA sequencing: One isolate of each of eleven species of *Ceratocystis* was selected for sequencing based on the results of the RFLP study. These isolates, with their GenBank Accession numbers are: *C. fimbriata* (CMW2219, AFO43604), *C. albofundus* (CMW2475, AFO43605), *C. fagacearum* (CMW2651, AFO43598), *C. moniliformis* (CMW3782, AFO43597), *C. adiposa* (CMW1622, AFO43606), *C. paradoxa* (CMW1546, AFO43607), *C. laricicola* (CMW1016, AFO43600), *C. polonica* (CMW0672, AFO43601), *C. virescens* (CMW0460, AFO43603), *C. pinicola* (CMW1323, AFO43602) and *C. radicicola* (CMW3191, AFO43599). *Petriella setifera* (J.C. Schmidt) Curzi (ATCC26490, AFO43596) was used as the outgroup taxon (Spatafora & Blackwell, 1994). In the case of *C. coerulescens*, it is recognized that this represents a complex of at least five species (Harrington *et al.*, 1996; Wingfield *et al.*, 1997; Witthuhn *et al.*, 1998; Harrington & Wingfield., 1998), but only one, *C. pinicola*, was selected to represent the complex.

The PCR fragments were purified using Wizard PCR Preps (Promega Corporation, U.S.A) or Microcon Microconcentrators (Amicon, Inc., U.S.A). Both strands of the PCR products of nine of the 12 isolates were sequenced with 13 primers (Table 2, Fig. 1) using the *f*mol DNA sequencing kit (Promega Corporation, U.S.A). Three of the isolates were sequenced using an ABI PRISM 377 DNA sequencer (Perkin-Elmer, U.S.A) at the DNA sequencing facility at Iowa State University. The DNA sequence data were submitted to GenBank. The nucleotide sequences were manually aligned. Phylogenetic relationships among species were determined using the heuristic search option in PAUP, with gaps treated as missing data (Swofford, 1993). Bootstrap values (Felsenstein, 1985) were determined from 100 replicates.

RESULTS

RFLPs: The PCR amplifications of the species of *Ceratocystis* under consideration produced PCR products that were 1.6 kb in size. Of the 18 restriction enzymes tested *Alu*I, *Dra*I, *Hae*III and *Rsa*I produced RFLP patterns that were used to distinguish the species. The restriction enzyme maps in Figures 2 and 3 are based on the actual DNA sequences.

Restriction digests using *Alu*I (Table 1, Figure 2) produced unique restriction patterns for *C. moniliformis*,*C. fagacearum* and *C. fimbriata* isolates from *Populus* and *Prunus* (Table 1). The groups of species that had the same RFLP patterns after *Alu*I digests are: *C. fimbriata* isolates from *Platanus* spp. and *C. albofundus*, *C. adiposa* and *C. paradoxa*, and species in the *C. coerulescens* complex and *C. radicicola.*

Ceratocystis adiposa and *C. paradoxa* could not be separated based on the RFLPs produced by any of the restriction enzymes tested. Many of the other closely related species that had the same RFLP patterns using *Alu*I were, however, separated from each other based on the RFLPs produced by *Dra*I, *Hae*III and *Rsa*I (Table 1, Figure 3). The restriction patterns produced after a digestion with *Dra*I enabled distinction between *Platanus* isolates of *C. fimbriata* and *C. albofundus*. Double digestions using the enzymes *Dra*I and *Hae*III were used to separate *C. coerulescens* and *C. virescens* from *C. laricicola*, *C. polonica* and *C. radicicola*. *Ceratocystis coerulescens* isolates were distinguishable from *C. virescens* isolates based on *Rsa*I digests. *Rsa*I was also used to distinguish *C. radicicola* from *C. laricicola* and *C. polonica*. The recently described *C. pinicola*, *C. resinifera* and *C. rufipenni* could not be distinguished from *C. coerulescens* based on the RFLP analyses.

DNA sequencing: The aligned DNA sequences of the representative species of *Ceratocystis* were 1731 bp in size after gaps were inserted to achieve the alignment. Within the ITS region, high variability was observed between the DNA sequence of the various species, with numerous insertions-deletions, which made the alignment of the sequences in this region very difficult. In contrast, the large sub-unit rRNA gene (1087 bases in total) was found to be relatively conserved.

A heuristic search from the aligned DNA sequence data (1081 characters) of the large sub-unit rRNA gene produced one most parsimonious tree (Figure 4) of 288 steps (CI = 0.788, HI = 0.212, RI = 0.667). The tree was rooted to *Petriella setifera*, the outgroup species. Two major clades were found within *Ceratocystis*: *C. fimbriata* and *C. albofundus* grouped together (100 % bootstrap value), sister to the clade (95 % bootstrap value) formed by the other nine *Ceratocystis* species under consideration. Relationships among the nine other species were not clear, but *C. moniliformis*, *C. fagacearum* and *C. adiposa* formed a single, weakly supported clade (75 % bootstrap value). The *C. coerulescens* complex (*C. laricicola*, *C. polonica, C. pinicola* and *C. virescens*) formed another weakly supported (83 % bootstrap value) clade.

Much of the alignment of the DNA sequence data within the ITS1 and ITS2 regions proved to be ambiguous for all the species studied. A second analysis was performed on the DNA sequence data of the ITS and LSU regions after all characters of ambiguous alignment were removed (378 of the 1731 characters removed), with most of the removed characters in the ITS1 and ITS2 regions. A single most parsimonious tree of 420 steps (CI = 0.800, HI = 0.200, RI =0.648) was produced (data not shown), and the topology was found to be similar to the tree produced when only the LSU sequence data was analysed (Figure 4). *Petriella*

setifera was again defined as the outgroup. *Ceratocystis fimbriata* and *C. albofundus* grouped together (100 % bootstrap value) and formed a clade sister to the clade formed by all the other *Ceratocystis* species studied (96 % bootstrap value). *C. moniliformis*, *C. fagacearum* and *C. adiposa* formed a single clade (60 % bootstrap value). The members of the *C. coerulescens* complex formed a clade (89 % bootstrap value), and there was support (92 % bootstrap value) for the clade of species that occur on conifers (*C. pinicola*, *C. polonica* and *C. laricicola*).

DISCUSSION

In this study, the best known species of *Ceratocystis* have been characterized based on sequence data and RFLP analyses. The results of the sequence analyses generally support those of earlier studies (Visser *et al.* 1995; Hausner, Reid & Klassen, 1993*a*,c; Harrington *et al.*, 1996; Wingfield *et al.*, 1996). The RFLP comparisons of a large number of isolates has shown that it is possible to distinguish most of the species using this reliable and quick technique.

Ceratocystis fimbriata is a well known pathogen on a wide variety of hosts, including sweet potatoes, from which it was first described (Halsted, 1890). *Ceratocystis albofundus* is a pathogen of *Acacia mearnsii* in South Africa (Morris, Wingfield & de Beer, 1993) and was recently shown by ITS sequence analysis and morphology to represent a distinct taxon similar to, but quite distinct from, *C. fimbriata* (Wingfield *et al.*, 1996). The RFLP and LSU analyses provide additional support for this distinction. Based on the RFLP analyses, isolates of *C. fimbriata* from *Platanus* could be separated from isolates from *Populus* and *Prunus*, suggesting that *C. fimbriata* represents a species aggregate, such as was previously proposed

by Webster & Butler (1967). The RFLPs of *C. albofundus* are closer to the *Platanus* isolates than to the *Prunus* isolates of *C. fimbriata.*

Ceratocystis coerulescens, *C. laricicola*, *C. polonica* and *C. virescens* are known to be very similar and related fungi, in the *C. coerulescens* complex (Harrington *et al.*, 1996). The seven species in the complex that occur on conifers appear to be monophyletic and form a strongly supported clade based on ITS sequence analysis (Witthuhn *et al.*, 1998). Three of these conifer species (*C. pinicola*, *C. laricicola* and *C. polonica*) also grouped together based on LSU data, further suggesting that this clade arose through an adaptation to conifers. These species and *C. virescens* are not easily distinguished based on RFLP data presented here. Although *C. polonica* and *C. laricicola* can be distinguished from the other species based on *Dra*I/*Hae*III digestions, these two species cannot be separated from each other. Evidence from sequence analyses (Visser *et al.*, 1995; Witthuhn *et al.*, 1998) and isozyme analysis (Harrington *et al.*, 1996) has led us to believe that *C. polonica* and *C. laricicola* are very similar, and LSU sequence analysis further shows the simlarity between these two species.

The analyses of the LSU DNA sequence data loosely grouped *C. fagacearum*, *C. adiposa* and *C. moniliformis*. SSU analysis (Hausner, Reid & Klassen, 1993a,c) showed similarity between *C. fagacearum* and *C. adiposa*. *C. moniliformis* is, however, more similar to *C. fimbriata* than to *C. adiposa* and *C. fagacerum* based on the SSU data. *C. fimbriata*, *C. albofundus* and *C. moniliformis* are the only *Ceratocystis* species with hat shaped ascospores, and a closer phylogenetic relationship between *C. moniliformis* and *C. fimbriata*, as shown by the analyses of the SSU data (Hausner *et al.*, 1993a,c), seems more probable than the close relationship between *C. moniliformis*, *C. adiposa* and *C. fagacearum* based on LSU data.

Although the statistical support was not strong, *C. radicicola* and *C. paradoxa* appeared to be phylogenetically related. These two species are morphologically similar and their separation is based on their asexual states. *C. radicicola* was isolated from date palms in the U.S.A. (Bliss, 1941), while *C. paradoxa* has been isolated from a wide host range of monocotyledonous hosts, including palms (Kile, 1993). The similarity of the hosts, morphology and DNA sequence data supports the contention that they are phylogenetically closely related.

Ceratocystis species included in this study are those that are best known and for which cultures are readily available. The isolates used were chosen based on careful morphological comparisons and we believe that the results contribute to our further understanding of this important group of fungi. Results of this study will lay a firm foundation for the description and characterisation of new species in the future. The RFLP results will also provide a rapid means to accurately identify most of these species.

ACKNOWLEDGMENTS

We thank the Foundation for Research Development (FRD), South Africa, members of the Tree Pathology Co-operative Programme (TPCP), South Africa, the United States Department of Agriculture (USDA/FAS/ICD/Research & Scientific Exchanges, Agreement No. 58-3148-6-019) and UNESCO for financial support. We further thank Dr Meredith Blackwell for supplying DNA from *P. setifera*. Marianne Wolfaardt and Cassi Myburg are thanked for their assistance in dealing with the large collection of DNA sequence data.

REFERENCES

Bliss, D. E. (1941). A new species of *Ceratostomella* on the date palm. *Mycologia* **33**, 468-482.

Bretz, T.W. (1952). The ascigerious stage of the oak wilt fungus. *Phytopathology* **42**, 435-437.

Chang, V.C.S. & Jensen, L. (1974). Transmission of the pineapple disease organism of sugarcane by nitidulid beetles in Hawaii. *Journal of Economical Entomology* **67**, 190-192.

Christiansen, E. & Solheim, H. (1990). The bark beetle-associated blue-stain fungus *Opiostoma polonicum* can kill various spruces and Douglas fir. *European Journal of Forest Pathology* **20**, 436-446.

Felsenstein, J. (1985). Confidence intervals on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783-791.

Grosclaude, C. & Oliver, R. (1988). Detection par piegeage du *Ceratocystis fimbriata* f. *platani*. *European Journal of Forest Pathology* **18**, 385-390.

Halsted, B.D. (1890). Some fungus diseases of the sweet potato. *New Jersey Agricultural College Experiment Station Bulletin* **76**, 7-14.

Harrington, T.C. & Wingfield, B.D. (1995). A PCR based identification method for species of *Armillaria*. *Mycologia* **87**, 280-288.

Harrington, T.C., Steimel, J.P. Wingfield, M.J. & Kile, G. (1996). Isozyme variation in the *Ceratocystis coerulescens* complex. *Mycologia* **88**, 104-113.

Harrington, T.C. & Wingfield, M.J. (1998). The *Ceratocystis* species on conifers. *Canadian Journal of Botany* In press.

Hausner, G., Reid, J. & Klassen, G.R. (1992). Do gleate-ascospore members of the *Cephaloascaceae*, *Eendomycetaceae* and *Ophiostomataceae* share a common phylogeny? *Mycologia* **84**, 870-881.

Hausner, G., Reid, J. & Klassen, G.R. (1993a). On the subdivision of *Ceratocystis s.l.* based on partial ribosomal DNA sequence. *Canadian Journal of Botany* **71**, 52-63.

Hausner, G., Reid, J. & Klassen, G.R. (1993b). *Ceratocystiopsis*: a reappraisal based on molecular criteria. *Mycological Research* **97**, 625-633.

Hausner, G., Reid, J & Klassen, G.R. (1993c). On the phylogeny of *Ophiostoma*, *Ceratocystis s.s.* and *Microascus* and relationships within *Ophiostoma* based on partial ribosomal DNA sequences. *Canadian Journal of Botany* **71**, 1249-1265.

Himelick, E.B. & Curl, E.A. (1958). Transmission of *Ceratocystis fagacearum* by insects and mites. *Plant disease reporter* **42**, 538-545.

Juzwik, J. & French, D.W. (1983). *Ceratocystis fagacearum* and *C. piceae* on the surfaces of free-flying and fungus-mat-inhabiting nitidulids. *Phytopathology* **73**, 1164-1168.

Kile, G.A. (1993). Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (ed. M.J. Wingfield, K.A. Seifert & J.F. Webber), pp. 173-183. APS Press: St Paul, Minnesota, U.S.A..

Morris, M., Wingfield, M.J. & de Beer, C. (1993). Gummosis and wilt of *Acacia mearnsii* in South Africa caused by *Ceratocystis fimbriata*. *Plant Pathology* **42**, 814-817.

Olson, E.O. & Martin, W.J. (1949). Relationship of *Ceratostomella fimbriata* from Hevea rubber tree and sweet potato. *Phytopathology* **39**, 17 Abstract.

Redfern, D.B., Stoakley, J.T., Steele, H. & Minter, M. (1987). Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae*. *Plant Pathology* **36**, 467-480.

Ribeiro, I.J.A., Rossetto, C.J., Sabino, J.C., Gallo, P.B. (1986). Seca da mangueira: VIII. Resistincia de porta-enxertos de mangueira ao fungo *Ceratocystis fimbriata* Ell. & Halst. *Bragantia* **45**, 317-322.

Siemaszko, W. (1938). Zespoly Grybow Towarzyszacych Kornikom Polskim. *Planta Polonica* **7**, 1-52.

Sinclair, W.A., Lyon, H. & Johnson, W.T. (1987). Diseases of Trees and Shrubs. Cornell University Press: Ithaca and London.

Spatafora, J.W. & Blackwell, M. (1994). The polyphyletic origins of the ophiostomatoid fungi. *Mycological Research* **98**, 1-9.

Swofford, D.L. (1993). PAUP Phylogenetic Analysis using Parsimony Version 3.1.1. Champaign, IL 61820, U.S.A..

Teviotdale, B. & Harper, D. (1991). Infection of pruning and small bark wounds in almond by *Ceratocystis fimbriata*. *Plant Disease* **75**, 1026-1030.

Vigouroux, A. & Stojadinovic, B. (1990). Possibilities d'infection du platane pour *Ceratocystis fimbriata* f. *platani* aprés contamination de l'eau ou se developpment des racines blessées. *European Journal of Forest Pathology* **20**, 118-121.

Vilgalys, R. & Hester, M. (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA form several *Cryptococcus* species. *Journal of Bacteriology* **172**, 4238-4246.

Visser, C., Wingfield, M.J., Wingfield, B.D. & Yamaoka, Y. (1995). *Ophiostoma polonicum* is a species of *Ceratocystis sensu stricto*. *Systematic and Applied Microbiology* **18**, 403-409.

Webster, R.K. & Butler, E.E. (1967). A morphology and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45**, 1457-1468.

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A guide to methods and applications* (ed. M.A. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White), pp. 315-322. Academic Press: San Diego, U.S.A..

Wingfield, B.D., Grant, W.S., Wolfaardt, J.F. & Wingfield, M.J. (1994). Ribosomal RNA sequence phylogeny is not congruent with ascospore morphology among species in *Ceratocystis sensu stricto*. *Molecular Biology and Evolution* **11**, 376-383.

Wingfield, M.J., de Beer, C., Visser, C. & Wingfield, B.D. (1996). A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematic and Applied Microbiology* **19**, 191-202.

Wingfield, M.J., Harrington, T.C. & Solheim, H. (1997). Two new species in the *Ceratocystis coerulescens* complex from conifers in western North America. *Canadian Journal of Botany* **75**, 827-834.

Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J., Wolfaardt, M. & Harrington, T.C. (1998). Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. *Mycologia* **90**, 96-101.

Table 1: Isolates and origins of species of *Ceratocystis* studied and the RFLP fragment sizes using the restriction enzymes *Alu*I, *Dra*I, *Hae*III and *Rsa*I. The RFLP patterns for the four restriction enzymes were uniform within species.

Isolate numbers	Origin	Host	*Alu*I	*Dra*I	*Dra*I/*Hae*III double digests	*Rsa*I
C. fagacearum (Bretz) Hunt			440,400,200,180,150			
CMW2037	MN, U.S.A.	*Quercus* sp.				
CMW2038	MN, U.S.A.	*Quercus* sp.				
CMW2039	MN, U.S.A.	*Quercus* sp.				
CMW2651	U.S.A.	*Quercus* sp.				
CMW2658	U.S.A.	*Quercus* sp.				
C. moniliformis (Hedgcock) Moreau			400,350,260,220,150			
CMW1626,IOF8667	Japan	Unknown				
CMW4458	China	*Hevea* sp.				
CMW3782	South Africa	*Erythrina* sp.				
C. fimbriata Ellis & Halsted from *Platanus*			720,220,180,150	1600		
CMW1894	Switzerland	*Platanus* sp.				
CMW1895	Switzerland	*Platanus* sp.				
CMW1896	Switzerland	*Platanus* sp.				
CMW2218	France	*Platanus* sp.				
CMW2219,PREM51642	France	*Platanus* sp.				
CMW2220,PREM51644	France	*Platanus* sp.				
CMW2228,PREM51830	Sicily	*Platanus* sp.				
CMW2242,PREM51831	Italy	*Platanus* sp.				
CMW2324	Switzerland	*Platanus* sp.				

Isolate numbers	Origin	Host	*Alu*I	*Dra*I	*Dra*I/*Hae*III *Rsa*I double digests
C. fimbriata from *Populus* and *Prunus*			560,220,180,150		
CMW1270,C89	SD, U.S.A.	*Populus* sp.			
CMW2901,C685	Quebec, Canada	*Populus* sp.			
CMW0078	CO, U.S.A.	*Populus* sp.			
CMW2911,C578	CA, U.S.A.	*Prunus* sp.			
CMW2902,C686	CA, U.S.A.	*Prunus* sp.			
C. albofundus Wingfield, de Beer & Morris			720,220,180,150	1200,320,150	
CMW2473, PREM51639	South Africa	*Acacia* sp.			
CMW2475, PREM51641	South Africa	*Acacia* sp.			
C. adiposa (Butler) Moreau			400,340,200,180,150		
CMW0066	Unknown	Unknown			
CMW0071	Unknown	Unknown			
CMW0121	Unknown	Unknown			
CMW1622,IOF9546	JapanUnknown				
CMW2573,CBS136.34	Japan*Saccharum* sp.				
CMW2575,CBS600.74	Japan*Pinus* sp.				
CMW3307,C299	U.S.A.	Wood chips			
C. paradoxa (Dade) Moreau			400,340,200,180,150		
CMW1546	Unknown	*Musa* sp.			

Isolate numbers	Origin	Host	*Alu*I	*Dra*I	*Dra*I/*Hae*III double digests	*Rsa*I
C. radicicola (Bliss) Moreau			400,280,200,180,150			
CMW3186,CBS114.47	CA, U.S.A.	*Phoenix* sp.				
CMW3191,CBS146.59	CA, U.S.A.	Unknown				
C. coerulescens (Münch) Bakshi complex:						
C.s pinicola Harrington and Wingfield			400,280,200,180,150		620,550,280	700,600,250
C488	England	*Pinus* sp.				
C489	England	*Pinus* sp.				
CMW1323,C490	England	*Pinus* sp.				
CMW3759,C798	England	*Pinus* sp.				
C. coerulescens			400,280,200,180,150		620,550,280	700,600,250
CMW3231,C313	Germany	*Picea* sp.				
CMW3235,C321	Netherlands	Unknown				
C. resinifera Harrington and Wingfield			400,280,200,180,150		620,550,280	700,600,250
CMW3227, C276	Norway	*Picea* sp.				
CMW3229, C278	Norway	*Picea* sp.				
C. rufipenni Wingfield, Harrington and Solheim			400,280,200,180,150		620,550,280	700,600,250
CMW3247, C609	Canada	*Picea* sp.				
C. virescens (Davidson) Moreau			400,280,200,180,150		620,550,280	600,480,250
C70	Unknown	Unknown				
CMW0460,C74	NY, U.S.A.	*Quercus* sp.				
C252	NY, U.S.A.	*Acer* sp.				
C253	NY, U.S.A.	*Acer* sp.				
CMW3225,C254	NY, U.S.A.	*Acer* sp.				
C256	WI, U.S.A.	*Acer* sp.				

Isolate numbers	Origin	Host	*Alu*I	*Dra*I	*Dra*I/*Hae*III double digests	*Rsa*I
C. virescens (Davidson) Moreau			400,280,200,180,150		620,550,280	600,480,250
C262	NH,U.S.A.	*Acer* sp.				
C. laricicola Redfern & Minter			400,280,200,180,150		550,510,360	700,600,250
CMW1016	Scotland	*Larix* sp.				
CMW3212,C177	Scotland	*Larix* sp.				
CMW3214,C178	Scotland	*Larix* sp.				
CMW3217,C179	Scotland	*Larix* sp.				
CMW3219,C180	Scotland	*Larix* sp.				
CMW3221,C181	Scotland	*Larix* sp.				
C. polonica Siemaszko			400,280,200,180,150		550,510,360	700,600,250
CMW3208,C123	Norway	*Picea* sp.				
CMW3235,C321	Norway	*Picea* sp.				
CMW0672,C322, CBS133.38	Poland	*Picea* sp.				

CMW - Culture collection of M. J. Wingfield; C - Culture collection of T.C. Harrington; PREM - National Collection of Fungi, South Africa; CBS - Centraal Bureau voor Schimmelcultures, Netherlands; ATCC- American Type Culture Collection, U.S.A.; IOF - Institute for Fermentation, Japan.

Table 2: Primers used for the generation and sequencing of the PCR products.

	Sequence (5'-3')	Source
ITS1	TCCGTAGGTGAACCTGCGG	White *et al.*, 1990
ITS2	GCTGCGTTCTTCATCGATGC	White *et al.*, 1990
ITS3	GCATCGATGAAGAACGCAGC	White *et al.*, 1990
ITS4	TCCTCCGCTTATTGATATGC	White *et al.*, 1990
LR1	GGTTGGTTTCTTTTCCT	Vilgalys & Hester, 1990
LR3	GGTCCGTGTTTCAAGAC	Vilgalys & Hester, 1990
LR5	ATCCTGAGGGAAACTTC	Vilgalys & Hester, 1990
LR6	CGCCAGTTCTGCTTACC	Vilgalys & Hester, 1990
LR1R	AGGAAAAGAAACCAACC	Complement of LR1
LR3R	GTCTTGAAACACGGACC	Complement of LR3
LR5R	GAAGTTTCCCTCAGGAT	Complement of LR5
404x*	CCCTTTCAACAATTTCAC	Authors, unpublished
L1	GGTCCGTGTTTCAAG	Wingfield *et al.*, 1994

*Binds to position 404 (5'-3') of the large sub-unit ribosomal RNA gene of *Saccharomyces cerevisiae*.

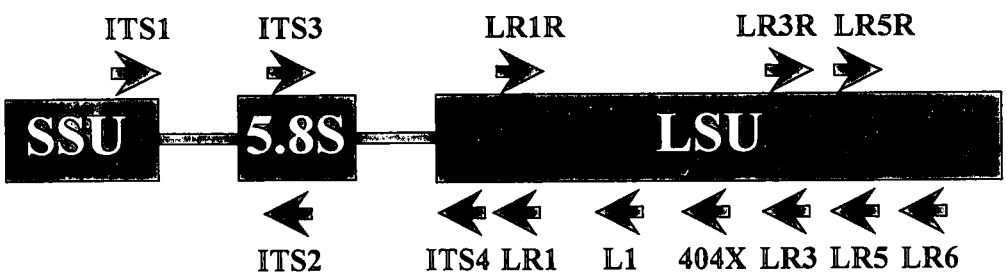


Figure 1: Diagrammatic representation of the 1600 bp PCR fragment of the species of *Ceratocystis* used in this study. The positions of the 13 sequencing primers are indicated with arrows.

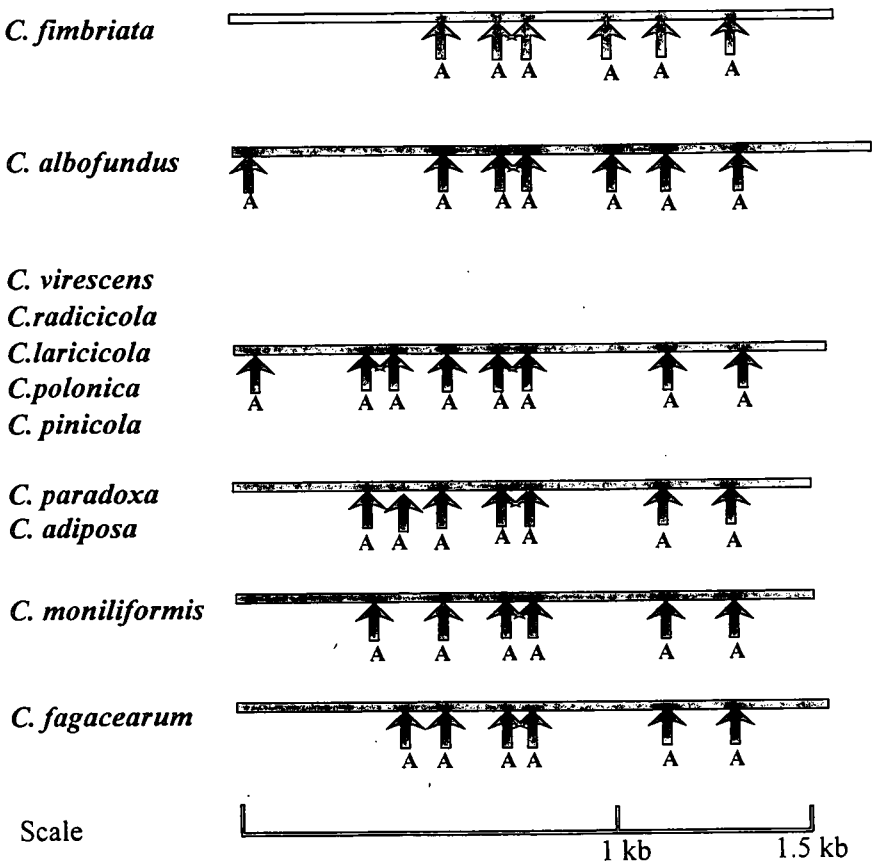


Figure 2: *Alu*I (A) restriction maps, inferred from sequence analysis, of the PCR fragments amplified from all the *Ceratocystis* species studied.

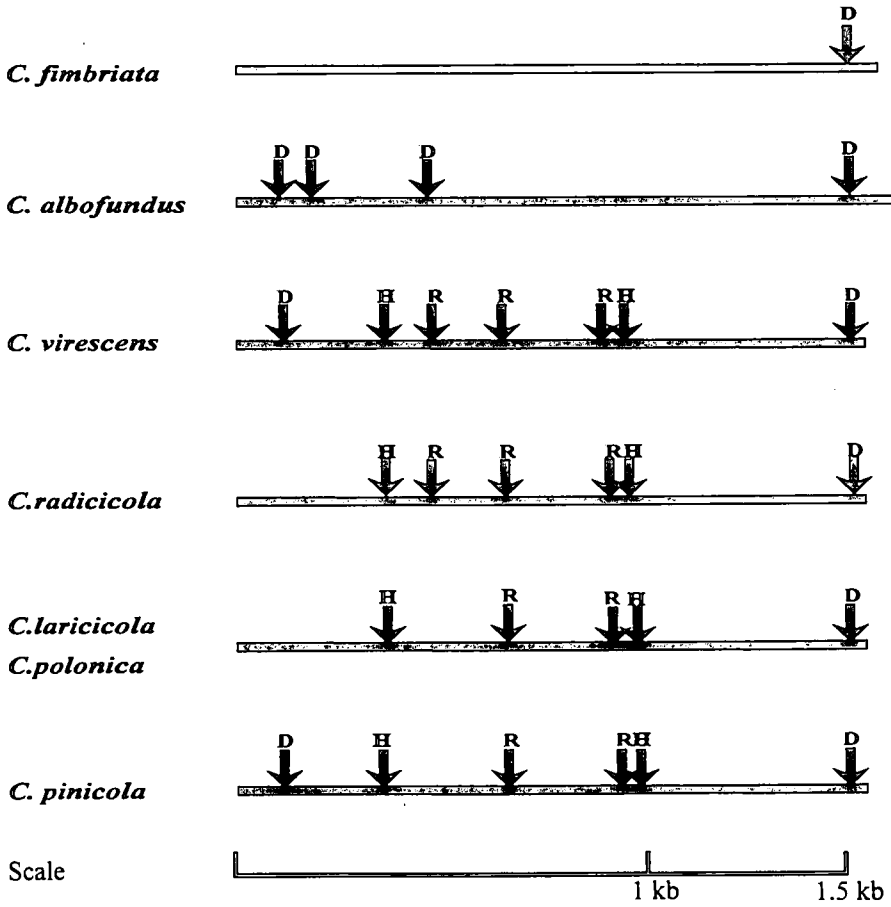


Figure 3: Restriction maps of the restriction enzymes (*Dra*I, *Hae*III and *Rsa*I), inferred from seqeunce analysis, used to distinguish closely related *Ceratocystis* species. D - *Dra*I, H - *Hae*III, R - *Rsa*I.

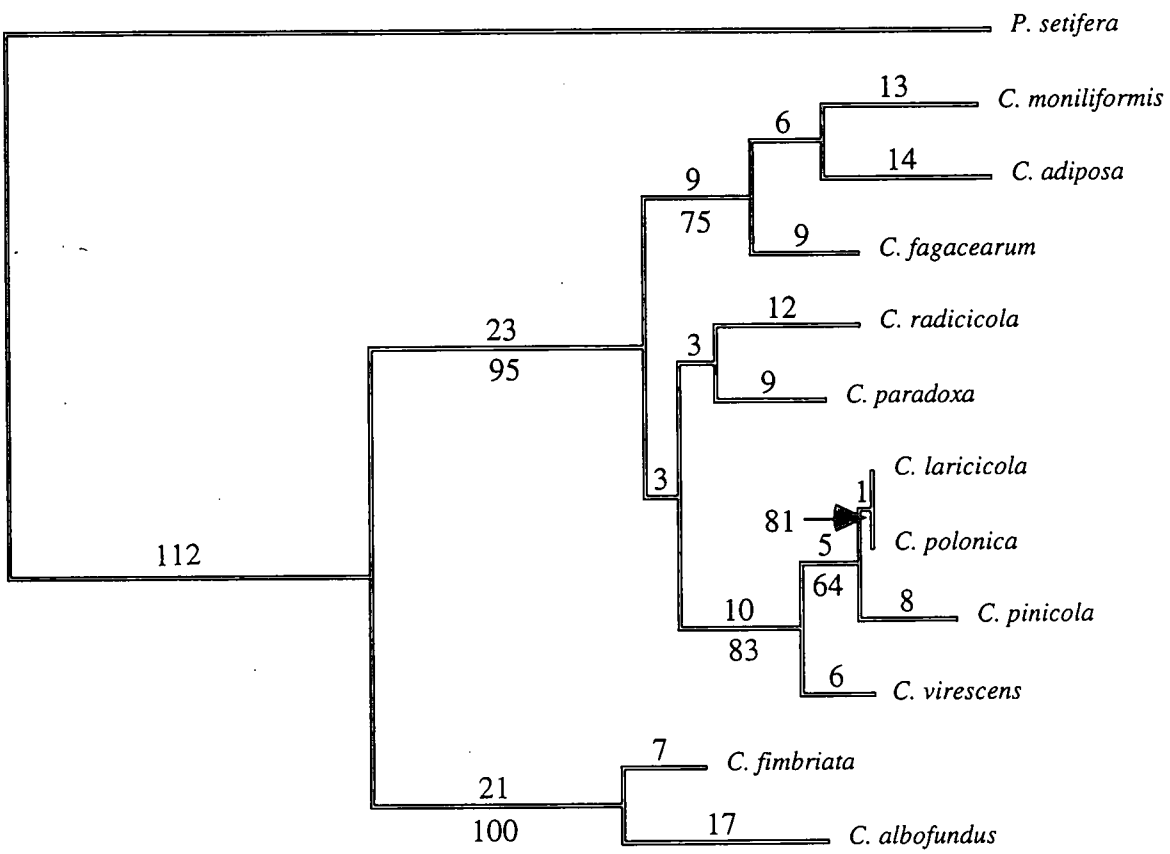
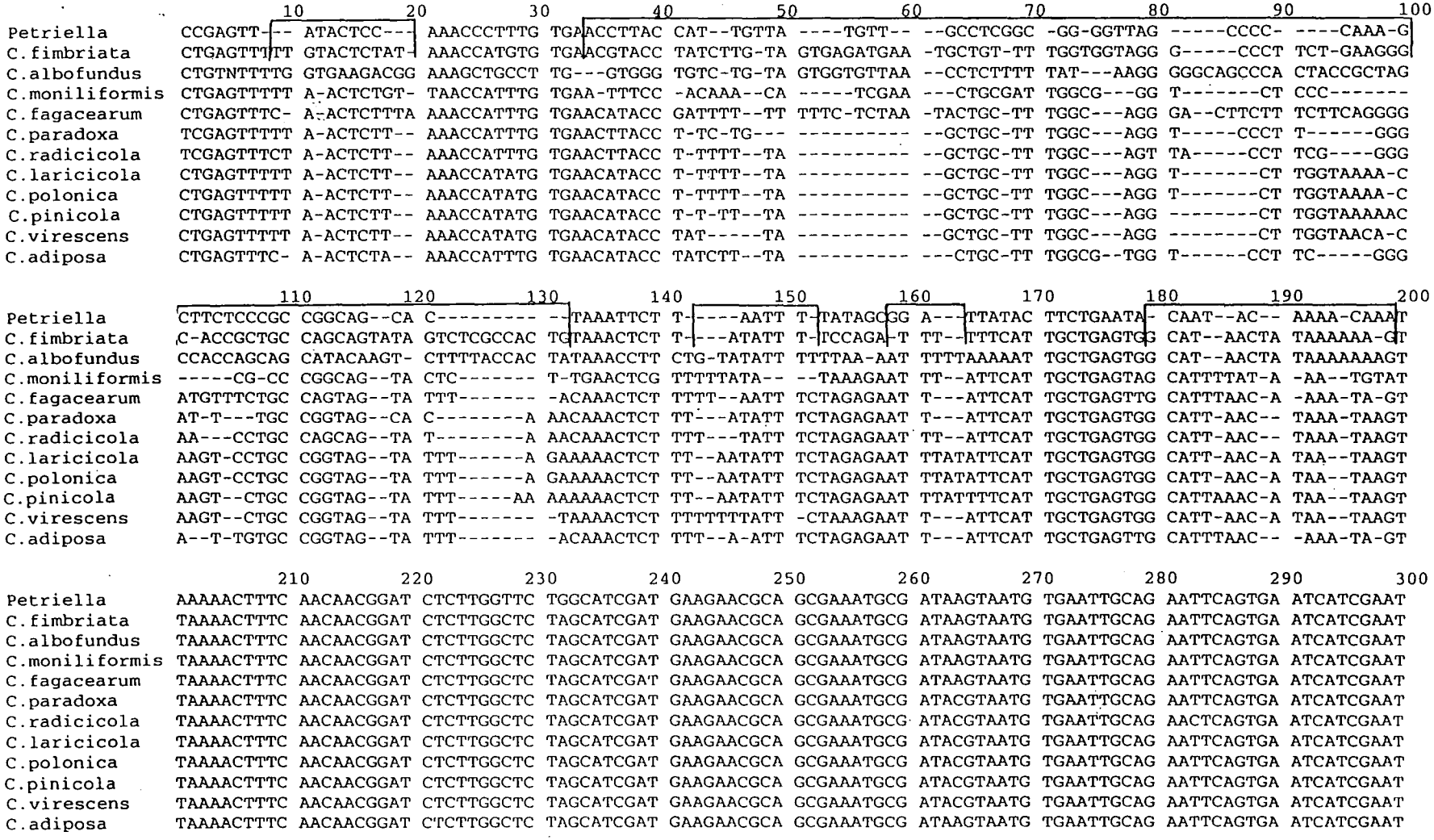
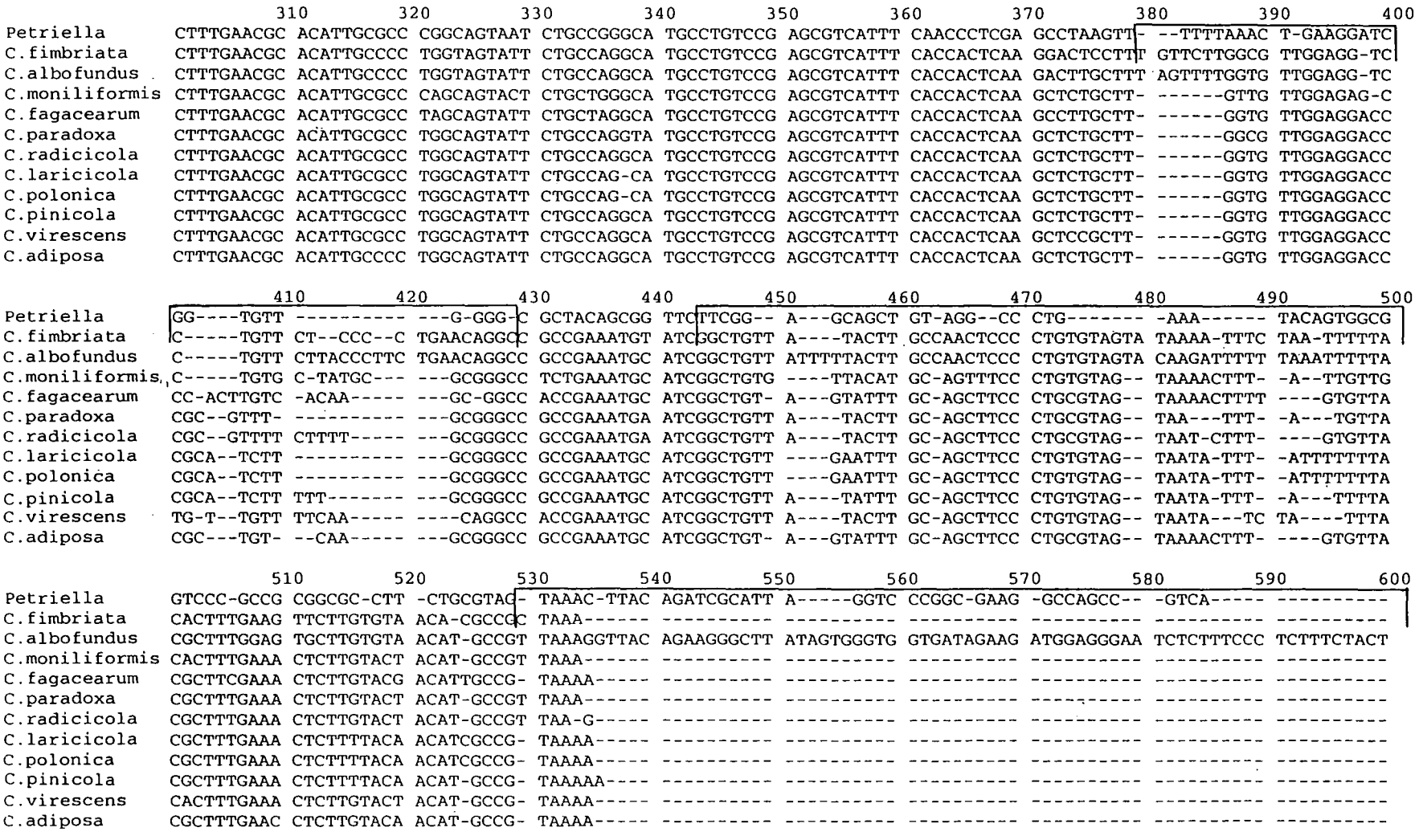


Figure 4: The most parsimonious tree (tree length = 288) produced from the DNA sequence of part of the large sub-unit ribosomal RNA gene. Bootstrap values (100 replicates) greater than 50 % are indicated at the bottom of the branches and the number of base substitutions are indicated at the top of the branches.

Figure 5: Aligned DNA sequence data (1731 bp in total), including the ITS and LSU regions of the rRNA genes, of the 12 species studied. The blocks indicate the ambiguously aligned DNA sequences that were excluded in a second phylogenetic analysis.

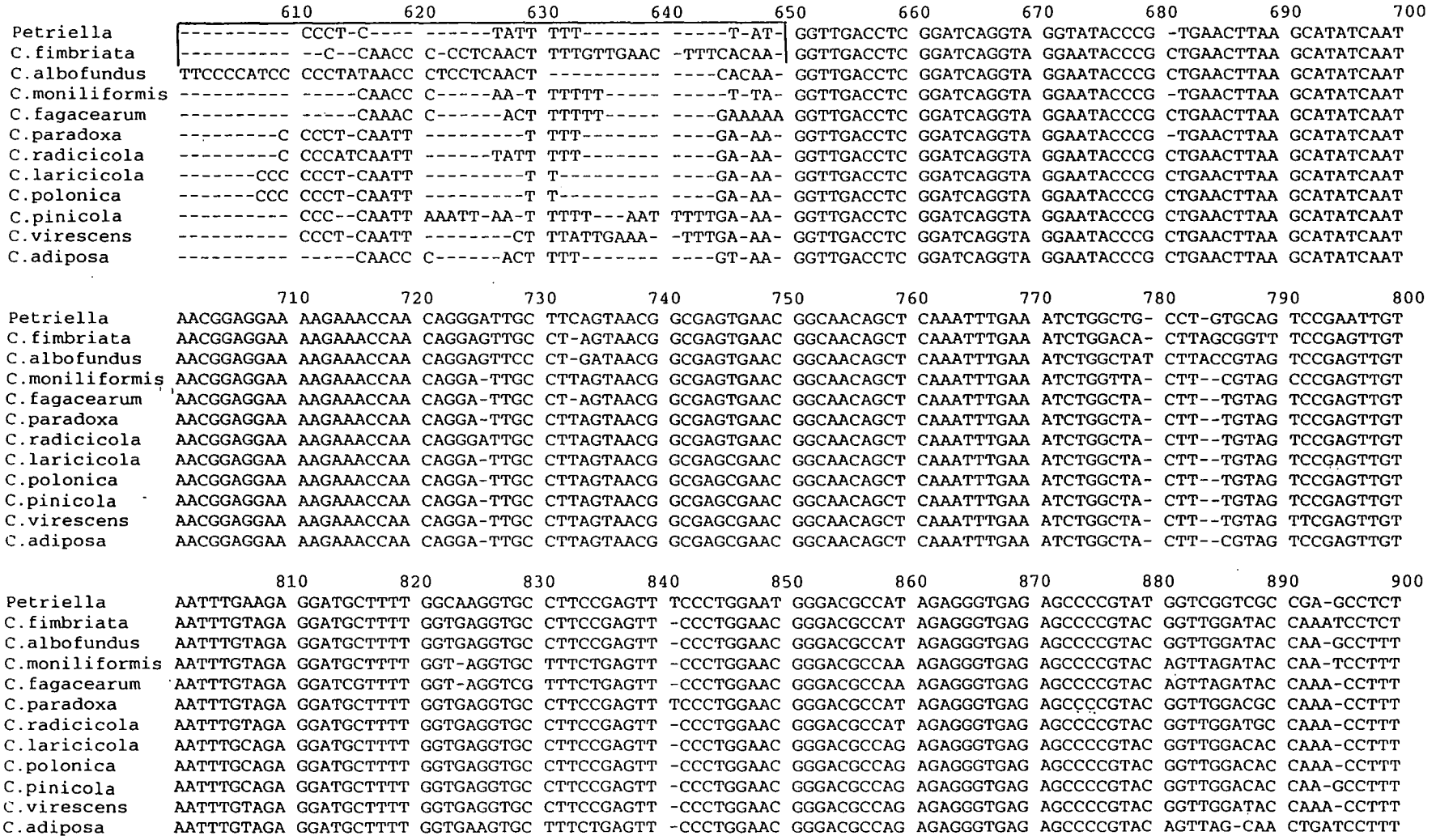

10 20 30 40 50 60 70 80 90 100
Petriella CCGAGTT-- ATACTCC--- AAACCCTTTG TGAACCTTAC CAT--TGTTA ----TGTT-- --GCCTCGGC -GG-GGTTAG -----CCCC- ----CAAA-G
C.fimbriata CTGAGTTTT GTACTCTAT- AAACCATGTG TGAACGTACC TATCTTG-TA GTGAGATGAA -TGCTGT-TT TGGTGGTAGG G-----CCCT TCT-GAAGGG
C.albofundus CTGTNTTTTG GTGAAGACGG AAAGCTGCCT TG---GTGGG TGTC-TG-TA GTGGTGTTAA --CCTCTTTT TAT---AAGG GGGCAGCCCA CTACCGCTAG
C.moniliformis CTGAGTTTTT A-ACTCTGT- TAACCATTTG TGAA-TTTCC -ACAAA--CA -----TCGAA ---CTGCGAT TGGCG---GG T-------CT CCC-------
C.fagacearum CTGAGTTTC- A-ACTCTTTA AAACCATTTG TGAACATACC GATTTT--TT TTTC-TCTAA -TACTGC-TT TGGC---AGG GA--CTTCTT TCTTCAGGGG
C.paradoxa TCGAGTTTTT A-ACTCTT-- AAACCATTTG TGAACTTACC T-TC-TG--- ---------- --GCTGC-TT TGGC---AGG T-----CCCT T------GGG
C.radicicola TCGAGTTTCT A-ACTCTT-- AAACCATTTG TGAACTTACC T-TTTT--TA ---------- --GCTGC-TT TGGC---AGT TA-----CCT TCG----GGG
C.laricicola CTGAGTTTTT A-ACTCTT-- AAACCATATG TGAACATACC T-TTTT--TA ---------- --GCTGC-TT TGGC---AGG T-------CT TGGTAAAA-C
C.polonica CTGAGTTTTT A-ACTCTT-- AAACCATATG TGAACATACC T-TTTT--TA ---------- --GCTGC-TT TGGC---AGG T-------CT TGGTAAAA-C
C.pinicola CTGAGTTTTT A-ACTCTT-- AAACCATATG TGAACATACC T-T-TT--TA ---------- --GCTGC-TT TGGC---AGG --------CT TGGTAAAAAC
C.virescens CTGAGTTTTT A-ACTCTT-- AAACCATATG TGAACATACC TAT-----TA ---------- --GCTGC-TT TGGC---AGG --------CT TGGTAACA-C
C.adiposa CTGAGTTTC- A-ACTCTA-- AAACCATTTG TGAACATACC TATCTT--TA ---------- ---CTGC-TT TGGCG--TGG T------CCT TC-----GGG
110 120 130 140 150 160 170 180 190 200
Petriella CTTCTCCCGC CGGCAG--CA C--------- --TAAATTCT T-----AATT T-TATAGCGG A---TTATAC TTCTGAATA- CAAT--AC-- AAAA-CAAAT
C.fimbriata C-ACCGCTGC CAGCAGTATA GTCTCGCCAC TGTAAACTCT T-----ATATT T-TCCAGA-T TT--TTTCAT TGCTGAGTGG CAT--AACTA TAAAAAA-GT
C.albofundus CCACCAGCAG CATACAAGT- CTTTTACCAC TATAAACCTT CTG-TATATT TTTTAA-AAT TTTTAAAAAT TGCTGAGTGG CAT--AACTA TAAAAAAAGT
C.moniliformis -----CG-CC CGGCAG--TA CTC------- T-TGAACTCG TTTTTATA-- --TAAAGAAT TT--ATTCAT TGCTGAGTAG CATTTTAT-A -AA--TGTAT
C.fagacearum ATGTTTCTGC CAGTAG--TA TTT------- -ACAAACTCT TTTT--AATT TCTAGAGAAT T---ATTCAT TGCTGAGTTG CATTTAAC-A -AAA-TA-GT
C.paradoxa AT-T---TGC CGGTAG--CA C--------A AACAAACTCT TT---ATATT TCTAGAGAAT T---ATTCAT TGCTGAGTGG CATT-AAC-- TAAA-TAAGT
C.radicicola AA---CCTGC CAGCAG--TA T--------A AACAAACTCT TTT---TATT TCTAGAGAAT TT--ATTCAT TGCTGAGTGG CATT-AAC-- TAAA-TAAGT
C.laricicola AAGT-CCTGC CGGTAG--TA TTT------A GAAAAACTCT TT--AATATT TCTAGAGAAT TTATATTCAT TGCTGAGTGG CATT-AAC-A TAA--TAAGT
C.polonica AAGT-CCTGC CGGTAG--TA TTT------A GAAAAACTCT TT--AATATT TCTAGAGAAT TTATATTCAT TGCTGAGTGG CATT-AAC-A TAA--TAAGT
C.pinicola AAGT--CTGC CGGTAG--TA TTT-----AA AAAAAACTCT TT--AATATT TCTAGAGAAT TTATTTTCAT TGCTGAGTGG CATTAAAC-A TAA--TAAGT
C.virescens AAGT--CTGC CGGTAG--TA TTT------- -TAAAACTCT TTTTTTTATT -CTAAAGAAT T---ATTCAT TGCTGAGTGG CATT-AAC-A TAA--TAAGT
C.adiposa A--T-TGTGC CGGTAG--TA TTT------- -ACAAACTCT TTT--A-ATT TCTAGAGAAT T---ATTCAT TGCTGAGTTG CATTTAAC-- -AAA-TA-GT
210 220 230 240 250 260 270 280 290 300
Petriella AAAAACTTTC AACAACGGAT CTCTTGGTTC TGGCATCGAT GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT
C.fimbriata TAAAACTTTC AACAACGGAT CTCTTGGCTC TAGCATCGAT GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT
C.albofundus TAAAACTTTC AACAACGGAT CTCTTGGCTC TAGCATCGAT GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT
C.moniliformis TAAAACTTTC AACAACGGAT CTCTTGGCTC TAGCATCGAT GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT
C.fagacearum TAAAACTTTC AACAACGGAT CTCTTGGCTC TAGCATCGAT GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT
C.paradoxa TAAAACTTTC AACAACGGAT CTCTTGGCTC TAGCATCGAT GAAGAACGCA GCGAAATGCG ATACGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT
C.radicicola TAAAACTTTC AACAACGGAT CTCTTGGCTC TAGCATCGAT GAAGAACGCA GCGAAATGCG ATACGTAATG TGAATTGCAG AACTCAGTGA ATCATCGAAT
C.laricicola TAAAACTTTC AACAACGGAT CTCTTGGCTC TAGCATCGAT GAAGAACGCA GCGAAATGCG ATACGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT
C.polonica TAAAACTTTC AACAACGGAT CTCTTGGCTC TAGCATCGAT GAAGAACGCA GCGAAATGCG ATACGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT
C.pinicola TAAAACTTTC AACAACGGAT CTCTTGGCTC TAGCATCGAT GAAGAACGCA GCGAAATGCG ATACGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT
C.virescens TAAAACTTTC AACAACGGAT CTCTTGGCTC TAGCATCGAT GAAGAACGCA GCGAAATGCG ATACGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT
C.adiposa TAAAACTTTC AACAACGGAT CTCTTGGCTC TAGCATCGAT GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT



```
                    310        320        330        340        350        360        370        380        390        400
Petriella           CTTTGAACGC ACATTGCGCC CGGCAGTAAT CTGCCGGGCA TGCCTGTCCG AGCGTCATTT CAACCCTCGA GCCTAAGTT- --TTTTAAAC T-GAAGGATC
C.fimbriata         CTTTGAACGC ACATTGCCCC TGGTAGTATT CTGCCAGGCA TGCCTGTCCG AGCGTCATTT CACCACTCAA GGACTCCTTT GTTCTTGGCG TTGGAGG-TC
C.albofundus        CTTTGAACGC ACATTGCCCC TGGTAGTATT CTGCCAGGCA TGCCTGTCCG AGCGTCATTT CACCACTCAA GACTTGCTTT AGTTTTGGTG TTGGAGG-TC
C.moniliformis      CTTTGAACGC ACATTGCGCC CAGCAGTACT CTGCTGGGCA TGCCTGTCCG AGCGTCATTT CACCACTCAA GCTCTGCTT- ------GTTG TTGGAGAG-C
C.fagacearum        CTTTGAACGC ACATTGCGCC TAGCAGTATT CTGCTAGGCA TGCCTGTCCG AGCGTCATTT CACCACTCAA GCCTTGCTT- ------GGTG TTGGAGGACC
C.paradoxa          CTTTGAACGC ACATTGCGCC TGGCAGTATT CTGCCAGGTA TGCCTGTCCG AGCGTCATTT CACCACTCAA GCTCTGCTT- ------GGCG TTGGAGGACC
C.radicicola        CTTTGAACGC ACATTGCGCC TGGCAGTATT CTGCCAGGCA TGCCTGTCCG AGCGTCATTT CACCACTCAA GCTCTGCTT- ------GGTG TTGGAGGACC
C.laricicola        CTTTGAACGC ACATTGCGCC TGGCAGTATT CTGCCAG-CA TGCCTGTCCG AGCGTCATTT CACCACTCAA GCTCTGCTT- ------GGTG TTGGAGGACC
C.polonica          CTTTGAACGC ACATTGCGCC TGGCAGTATT CTGCCAG-CA TGCCTGTCCG AGCGTCATTT CACCACTCAA GCTCTGCTT- ------GGTG TTGGAGGACC
C.pinicola          CTTTGAACGC ACATTGCGCC TGGCAGTATT CTGCCAGGCA TGCCTGTCCG AGCGTCATTT CACCACTCAA GCTCTGCTT- ------GGTG TTGGAGGACC
C.virescens         CTTTGAACGC ACATTGCGCC TGGCAGTATT CTGCCAGGCA TGCCTGTCCG AGCGTCATTT CACCACTCAA GCTCCGCTT- ------GGTG TTGGAGGACC
C.adiposa           CTTTGAACGC ACATTGCCCC TGGCAGTATT CTGCCAGGCA TGCCTGTCCG AGCGTCATTT CACCACTCAA GCTCTGCTT- ------GGTG TTGGAGGACC

                          410        420        430        440        450        460        470        480        490        500
Petriella           GG----TGTT ---------- ---G-GGG-C GCTACAGCGG TTCTTCGG-- A---GCAGCT GT-AGG--CC CTG------- -AAA------ TACAGTGGCG
C.fimbriata         C-----TGTT CT--CCC--C TGAACAGGCC GCCGAAATGT ATCGGCTGTT A----TACTT GCCAACTCCC CTGTGTAGTA TAAAA-TTTC TAA-TTTTTA
C.albofundus        C-----TGTT CTTACCCTTC TGAACAGGCC GCCGAAATGC ATCGGCTGTT ATTTTTACTT GCCAACTCCC CTGTGTAGTA CAAGATTTTT TAAATTTTTA
C.moniliformis      C-----TGTG C-TATGC--- ---GCGGGCC TCTGAAATGC ATCGGCTGTG ----TTACAT GC-AGTTTCC CTGTGTAG-- TAAAACTTT- -A--TTGTTG
C.fagacearum        CC-ACTTGTC -ACAA----- ---GC-GGCC ACCGAAATGC ATCGGCTGT- A---GTATTT GC-AGCTTCC CTGCGTAG-- TAAAACTTTT ----GTGTTA
C.paradoxa          CGC--GTTT- ---------- ---GCGGGCC GCCGAAATGA ATCGGCTGTT A----TACTT GC-AGCTTCC CTGCGTAG-- TAA---TTT- -A---TGTTA
C.radicicola        CGC--GTTTT CTTTT----- ---GCGGGCC GCCGAAATGA ATCGGCTGTT A----TACTT GC-AGCTTCC CTGCGTAG-- TAAT-CTTT- ----GTGTTA
C.laricicola        CGCA--TCTT ---------- ---GCGGGCC GCCGAAATGC ATCGGCTGTT ----GAATTT GC-AGCTTCC CTGTGTAG-- TAATA-TTT- -ATTTTTTTA
C.polonica          CGCA--TCTT ---------- ---GCGGGCC GCCGAAATGC ATCGGCTGTT ----GAATTT GC-AGCTTCC CTGTGTAG-- TAATA-TTT- -ATTTTTTTA
C.pinicola          CGCA--TCTT TTT------- ---GCGGGCC GCCGAAATGC ATCGGCTGTT A----TATTT GC-AGCTTCC CTGTGTAG-- TAATA-TTT- -A---TTTTA
C.virescens         TG-T--TGTT TTCAA----- ----CAGGCC ACCGAAATGC ATCGGCTGTT A----TACTT GC-AGCTTCC CTGTGTAG-- TAATA---TC TA----TTTA
C.adiposa           CGC---TGT- --CAA----- ---GCGGGCC GCCGAAATGC ATCGGCTGT- A---GTATTT GC-AGCTTCC CTGCGTAG-- TAAAACTTT- ----GTGTTA

                    510        520        530        540        550        560        570        580        590        600
Petriella           GTCCC-GCCG CGGCGC-CTT -CTGCGTAG- TAAAC-TTAC AGATCGCATT A-----GGTC CCGGC-GAAG -GCCAGCC-- -GTCA----- ----------
C.fimbriata         CACTTTGAAG TTCTTGTGTA ACA-CGCCGC TAAA------ ---------- ---------- ---------- ---------- ---------- ----------
C.albofundus        CGCTTTGGAG TGCTTGTGTA ACAT-GCCGT TAAAGGTTAC AGAAGGGCTT ATAGTGGGTG GTGATAGAAG ATGGAGGGAA TCTCTTTCCC TCTTTCTACT
C.moniliformis      CACTTTGAAA CTCTTGTACT ACAT-GCCGT TAAA------ ---------- ---------- ---------- ---------- ---------- ----------
C.fagacearum        CGCTTCGAAA CTCTTGTACG ACATTGCCG- TAAAA----- ---------- ---------- ---------- ---------- ---------- ----------
C.paradoxa          CGCTTTGAAA CTCTTGTACT ACAT-GCCGT TAAA------ ---------- ---------- ---------- ---------- ---------- ----------
C.radicicola        CGCTTTGAAA CTCTTGTACT ACAT-GCCGT TAA-G----- ---------- ---------- ---------- ---------- ---------- ----------
C.laricicola        CGCTTTGAAA CTCTTTTACA ACATCGCCG- TAAAA----- ---------- ---------- ---------- ---------- ---------- ----------
C.polonica          CGCTTTGAAA CTCTTTTACA ACATCGCCG- TAAAA----- ---------- ---------- ---------- ---------- ---------- ----------
C.pinicola          CGCTTTGAAA CTCTTTTACA ACAT-GCCG- TAAAAA---- ---------- ---------- ---------- ---------- ---------- ----------
C.virescens         CACTTTGAAA CTCTTGTACT ACAT-GCCG- TAAAA----- ---------- ---------- ---------- ---------- ---------- ----------
C.adiposa           CGCTTTGAAC CTCTTGTACA ACAT-GCCG- TAAAA----- ---------- ---------- ---------- ---------- ---------- ----------
```


610 620 630 640 650 660 670 680 690 700
Petriella ---------- CCCT-C---- ------TATT TTT------- -----T-AT- GGTTGACCTC GGATCAGGTA GGTATACCCG -TGAACTTAA GCATATCAAT
C.fimbriata ---------- --C--CAACC C-CCTCAACT TTTGTTGAAC -TTTCACAA- GGTTGACCTC GGATCAGGTA GGAATACCCG CTGAACTTAA GCATATCAAT
C.albofundus TTCCCCATCC CCCTATAACC CTCCTCAACT ---------- ----CACAA- GGTTGACCTC GGATCAGGTA GGAATACCCG CTGAACTTAA GCATATCAAT
C.moniliformis ---------- -----CAACC C------AA-T TTTTT----- -----T-TA- GGTTGACCTC GGATCAGGTA GGAATACCCG -TGAACTTAA GCATATCAAT
C.fagacearum ---------- -----CAAAC C------ACT TTTTT----- ----GAAAAA GGTTGACCTC GGATCAGGTA GGAATACCCG CTGAACTTAA GCATATCAAT
C.paradoxa ---------C CCCT-CAATT ---------T TTT------- ----GA-AA- GGTTGACCTC GGATCAGGTA GGAATACCCG -TGAACTTAA GCATATCAAT
C.radicicola ---------C CCCATCAATT ------TATT TTT------- ----GA-AA- GGTTGACCTC GGATCAGGTA GGAATACCCG CTGAACTTAA GCATATCAAT
C.laricicola -------CCC CCCT-CAATT ---------T T--------- ----GA-AA- GGTTGACCTC GGATCAGGTA GGAATACCCG CTGAACTTAA GCATATCAAT
C.polonica -------CCC CCCT-CAATT ---------T T--------- ----GA-AA- GGTTGACCTC GGATCAGGTA GGAATACCCG CTGAACTTAA GCATATCAAT
C.pinicola ---------- CCC--CAATT AAATT-AA-T TTTT---AAT TTTTGA-AA- GGTTGACCTC GGATCAGGTA GGAATACCCG CTGAACTTAA GCATATCAAT
C.virescens ---------- CCCT-CAATT --------CT TTATTGAAA- -TTTGA-AA- GGTTGACCTC GGATCAGGTA GGAATACCCG CTGAACTTAA GCATATCAAT
C.adiposa ---------- -----CAACC C------ACT TTT------- ----GT-AA- GGTTGACCTC GGATCAGGTA GGAATACCCG CTGAACTTAA GCATATCAAT
710 720 730 740 750 760 770 780 790 800
Petriella AACGGAGGAA AAGAAACCAA CAGGGATTGC TTCAGTAACG GCGAGTGAAC GGCAACAGCT CAAATTTGAA ATCTGGCTG- CCT-GTGCAG TCCGAATTGT
C.fimbriata AACGGAGGAA AAGAAACCAA CAGGAGTTGC CT-AGTAACG GCGAGTGAAC GGCAACAGCT CAAATTTGAA ATCTGGACA- CTTAGCGGTT TCCGAGTTGT
C.albofundus AACGGAGGAA AAGAAACCAA CAGGAGTTCC CT-GATAACG GCGAGTGAAC GGCAACAGCT CAAATTTGAA ATCTGGCTAT CTTACCGTAG TCCGAGTTGT
C.moniliformis AACGGAGGAA AAGAAACCAA CAGGA-TTGC CTTAGTAACG GCGAGTGAAC GGCAACAGCT CAAATTTGAA ATCTGGTTA- CTT--CGTAG CCCGAGTTGT
C.fagacearum AACGGAGGAA AAGAAACCAA CAGGA-TTGC CT-AGTAACG GCGAGTGAAC GGCAACAGCT CAAATTTGAA ATCTGGCTA- CTT--TGTAG TCCGAGTTGT
C.paradoxa AACGGAGGAA AAGAAACCAA CAGGA-TTGC CTTAGTAACG GCGAGTGAAC GGCAACAGCT CAAATTTGAA ATCTGGCTA- CTT--TGTAG TCCGAGTTGT
C.radicicola AACGGAGGAA AAGAAACCAA CAGGGATTGC CTTAGTAACG GCGAGTGAAC GGCAACAGCT CAAATTTGAA ATCTGGCTA- CTT--TGTAG TCCGAGTTGT
C.laricicola AACGGAGGAA AAGAAACCAA CAGGA-TTGC CTTAGTAACG GCGAGCGAAC GGCAACAGCT CAAATTTGAA ATCTGGCTA- CTT--TGTAG TCCGAGTTGT
C.polonica AACGGAGGAA AAGAAACCAA CAGGA-TTGC CTTAGTAACG GCGAGCGAAC GGCAACAGCT CAAATTTGAA ATCTGGCTA- CTT--TGTAG TCCGAGTTGT
C.pinicola AACGGAGGAA AAGAAACCAA CAGGA-TTGC CTTAGTAACG GCGAGCGAAC GGCAACAGCT CAAATTTGAA ATCTGGCTA- CTT--TGTAG TCCGAGTTGT
C.virescens AACGGAGGAA AAGAAACCAA CAGGA-TTGC CTTAGTAACG GCGAGCGAAC GGCAACAGCT CAAATTTGAA ATCTGGCTA- CTT--TGTAG TTCGAGTTGT
C.adiposa AACGGAGGAA AAGAAACCAA CAGGA-TTGC CTTAGTAACG GCGAGCGAAC GGCAACAGCT CAAATTTGAA ATCTGGCTA- CTT--CGTAG TCCGAGTTGT
810 820 830 840 850 860 870 880 890 900
Petriella AATTTGAAGA GGATGCTTTT GGCAAGGTGC CTTCCGAGTT TCCCTGGAAT GGGACGCCAT AGAGGGTGAG AGCCCCGTAT GGTCGGTCGC CGA-GCCTCT
C.fimbriata AATTTGTAGA GGATGCTTTT GGTGAGGTGC CTTCCGAGTT -CCCTGGAAC GGGACGCCAT AGAGGGTGAG AGCCCCGTAC GGTTGGATAC CAAATCCTCT
C.albofundus AATTTGTAGA GGATGCTTTT GGTGAGGTGC CTTCCGAGTT -CCCTGGAAC GGGACGCCAT AGAGGGTGAG AGCCCCGTAC GGTTGGATAC CAA-GCCTTT
C.moniliformis AATTTGTAGA GGATGCTTTT GGT-AGGTGC TTTCTGAGTT -CCCTGGAAC GGGACGCCAA AGAGGGTGAG AGCCCCGTAC AGTTAGATAC CAA-TCCTTT
C.fagacearum AATTTGTAGA GGATCGTTTT GGT-AGGTCG TTTCTGAGTT -CCCTGGAAC GGGACGCCAA AGAGGGTGAG AGCCCCGTAC AGTTAGATAC CAAA-CCTTT
C.paradoxa AATTTGTAGA GGATGCTTTT GGTGAGGTGC CTTCCGAGTT TCCCTGGAAC GGGACGCCAT AGAGGGTGAG AGCCCCGTAC GGTTGGACGC CAAA-CCTTT
C.radicicola AATTTGTAGA GGATGCTTTT GGTGAGGTGC CTTCCGAGTT -CCCTGGAAC GGGACGCCAT AGAGGGTGAG AGCCCCGTAC GGTTGGATGC CAAA-CCTTT
C.laricicola AATTTGCAGA GGATGCTTTT GGTGAGGTGC CTTCCGAGTT -CCCTGGAAC GGGACGCCAG AGAGGGTGAG AGCCCCGTAC GGTTGGACAC CAAA-CCTTT
C.polonica AATTTGCAGA GGATGCTTTT GGTGAGGTGC CTTCCGAGTT -CCCTGGAAC GGGACGCCAG AGAGGGTGAG AGCCCCGTAC GGTTGGACAC CAAA-CCTTT
C.pinicola AATTTGCAGA GGATGCTTTT GGTGAGGTGC CTTCCGAGTT -CCCTGGAAC GGGACGCCAG AGAGGGTGAG AGCCCCGTAC GGTTGGACAC CAA-GCCTTT
C.virescens AATTTGTAGA GGATGCTTTT GGTGAGGTGC CTTCCGAGTT -CCCTGGAAC GGGACGCCAG AGAGGGTGAG AGCCCCGTAC GGTTGGATAC CAAA-CCTTT
C.adiposa AATTTGTAGA GGATGCTTTT GGTGAAGTGC TTTCTGAGTT -CCCTGGAAC GGGACGCCAG AGAGGGTGAG AGCCCCGTAC AGTTAG-CAA CTGATCCTTT

```
                    910        920        930        940        950        960        970        980        990       1000
Petriella      GTAAAGCTCC TTCGACGAGT CGAGTAGTTT GGGAATGCTG CTCAAAATGG GAGGTAAACC CCTTCTAAAG CTAAATATTG GCCAGAGACC GATAGCGCAC
C.fimbriata    GTATAGCTCC TTCAACGAGT CGAGTAGTTT GGGAATGCTG CTCTAAATGG GAGGTATATC TCTTCTAAAG CTAAATATAG GCTAGAGACC GATAGCGCAC
C.albofundus   GTATAGCTCC TTCAACGAGT CGAGTAGTTT GGGAATGCTG CTCTAAATGG GAGGTATATC TCTTCTAAAG CTAAATATAG GCTAGAGACC GATAGCGCAC
C.moniliformis GTATAGCTCC TTCGACGAGT CGAGTAGTTT GGGAATGCTG CTCTAAATGG GAGGTATATC TCTTCTAAAG CTAAATATAG GCTAGAGACC GATAGCGCAC
C.fagacearum   GTATAGCTCC TTCGACGAGT CGAGTAGTTT GGGAATGCTG CTCTAAATGG GAGGTATATC TCTTCTAAAG CTAAATATAG GCTAGAGACC GATAGCGCAC
C.paradoxa     GTATAGCTCC TTCAACGAGT CGAGTAGTTT GGGAATGCTG CTCTAAATGG GAGGTATATC TCTTCTAAAG CTAAATATAG GCTAGAGACC GATAGCGCAC
C.radicicola   GTATAGCTCC TTCAACGAGT CGAGTAGTTT GGGAATGCTG CTCTAAATGG GAGGTATATC TCTTCTAAAG CTAAATACCG GCTAGAGACC GATAGCGCAC
C.laricicola   GTATAGCTCC TTCAACGAGT CGAGTAGTTT GGGAATGCTG CTCTAAATGG GAGGTATATC TCTTCTAAAG CTAAATACCG GCTAGAGACC GATAGCGCAC
C.polonica     GTATAGCTCC TTCAACGAGT CGAGTAGTTT GGGAATGCTG CTCTAAATGG GAGGTATATC TCTTCTAAAG CTAAATACCG GCTAGAGACC GATAGCGCAC
C.pinicola     GTATAGCTCC TTCAACGAGT CGAGTAGTTT GGGAATGCTG CTCTAAATGG GAGGTATATC TCTTCTAAAG CTAAATACCG GCTAGAGACC GATAGCGCAC
C.virescens    GTATAGCTCC TTCAACGAGT CGAGTAGTTT GGGAATGCTG CTCTAAATGG GAGGTATATC TCTTCTAAAG CTAAATACCG GCTAGAGACC GATAGCGCAC
C.adiposa      GTATAGCTCC TTCGACGAGT CGAGTAGTTT GGGAATGCTG CTCTAAATGG GAGGTATATC TCTTCTAAAG CTAAATATAG GCTAGAGACC GATAGCGCAC

                   1010       1020       1030       1040       1050       1060       1070       1080       1090       1100
Petriella      AAGTAGAGTG ATCGAAAGAT GAAAAGCACT TTGAAAAGAG AGTTAAACAG CACGTGAAAT TGTTGAAAGG GAAGCGCTTG CGACCAGACT TGTGCTCGTC
C.fimbriata    AAGTAGAGTG ATCGAAAGAT GAAAAGCACT TTGAAAAGAG AGTTAAACAG CACGTGAAAT TGTTGAAAGG GAAGCGCCTA TGACCAGACT TGT-TTC---
C.albofundus   AAGTAGAGTG ATCGAAAGAT GAAAAGCACT TTGAAAAGAG AGTTAAACAG CACGTGAAAT TGTTGAAAGG GAAGCGCCTA TGACCAGACT TGT-TTC---
C.moniliformis AAGTAGAGTG ATCGAAAGAT GAAAAGCACT TTGAAAAGAG AGTTAAACAG CACGTGAAAT TGTTGAAAGG GAAGCGCCTA TGACCAGACT TGT-TTC---
C.fagacearum   AAGTAGAGTG ATCGAAAGAT GAAAAGCACT TTGAAAAGAG AGTTAAACAG CACGTGAAAT TGTTGAAAGG GAAGCGCCTA TGACCAGACT TGT-CTC---
C.paradoxa     AAGTAGAGTG ATCGAAAGAT GAAAAGCACT TTGAAAAGAG AGTTAAACAG CACGTGAAAT TGTTGAAAGG GAAGCGCCTA TGACCAGACT TGT-CTC---
C.radicicola   AAGTAGAGTG ATCGAAAGAT GAAAAGCACT TTGAAAAGAG AGTTAAACAG CACGTGAAAT TGTTGAAAGG GAAGCGCCTA TGACCAGACT TGT-CTC---
C.laricicola   AAGTAGAGTG ATCGAAAGAT GAAAAGCACT TTGAAAAGAG AGTTAAACAG CACGTGAAAT TGTTGAAAGG GAAGCGCCTA TGACCAGACT TGT-TTC---
C.polonica     AAGTAGAGTG ATCGAAAGAT GAAAAGCACT TTGAAAAGAG AGTTAAACAG CACGTGAAAT TGTTGAAAGG GAAGCGCCTA TGACCAGACT TGT-TTC---
C.pinicola     AAGTAGAGTG ATCGAAAGAT TAAAAGCACT TTGAAAAGAG AGTTAAACAG CACGTGAAAT TGTTGAAAGG GAAGCGCCTA TGACCAGACT TGT-TTC---
C.virescens    AAGTAGAGTG ATCGAAAGAT GAAAAGCACT TTGAAAAGAG AGTTAAACAG CACGTGAAAT TGTTGAAAGG GAAGCGCCTA TGACCAGACT TGT-TTC---
C.adiposa      AAGTAGAGTG ATCGAAAGAT GAAAAGCACT TTGAAAAGAG AGTTAAACAG -ACGTGAAAT TGTTGAAAGG GAAGCGCCTA TGACCAGACT TGT-CTC---

                   1110       1120       1130       1140       1150       1160       1170       1180       1190       1200
Petriella      GAATCAGCCG TCGCTCGTC- -GGGGCGCAT TT-CGGCG-G G-CTTCAGGC CGGCATCAGT TCGCTGTAGG GGAGAAAGGC GGTAGG--AT GGGGTT----
C.fimbriata    -TGGCAGTTT -CGTTAGCCT TCGGGCTGAT TTACTCTGTC CAGTACAGGC CAGCATCAGT TTGCTGTCGG GGAGAAAGGC TCTGGG-AAC GTAGCTCCCC
C.albofundus   -TGGCAGTTT -CGTTAGCCC TCGGGCTGAT TTACTCTGTC CAGTACAGGC CAGCATCAGT TTGCTGTCGG GGAGAAAGGC TCTGGG-AAC GTAGCTCCCC
C.moniliformis -TATCAGTTT TGG-TAGTTT TCGGACTGCT TTACTCTGT- TAGTACAGGC CAACATCAGT TTGTTGTCGG GGAGAAAGGT T-TAGGGAAT GTGGCTCC--
C.fagacearum   -TATCAGTTT TGG-TAGTTT TCGGACTGCT TTACTCTGT- TAGTACAGGC CAACATCAGT TTGTTGTTGG GGAGAAAGGC T-TAGGGAAT GTGGCTCC--
C.paradoxa     -TATCAGTTT TGC-TAGTTT TCGGACTGGT TTACTCTGT- TAGTACAGGC CAGCATCAGT TTGTTGTCGG GGAGAAAGGC T-TAGGGAAT GTGGCTCC--
C.radicicola   -TATCAGTTT TGC-TAGTTT TCGGACTGGT TTACTCTGT- TAGTACAGGC CAGCATCAGT TTGTTGTTAG GGAGAAAGGC T-TAGGGAAT GTGGCTCCCC
C.laricicola   -TGTCAGTTT TGC-TTGTTT TCGGACTTGT TTACTCTGT- CAGTACAGGC CAACATCAGT TTGTTGTTTG GGAGAAAGGC T-TAGGGAAT GTGGCTCCC-
C.polonica     -TGTCAGTTT TGC-TTGTTT TCGGACTTGT TTACTCTGT- CAGTACAGGC CAACATCAGT TTGTTGTTTG GGAGAAAGGC T-TAGGGAAT GTGGCTCCC-
C.pinicola     -TGTCAGTTT TGC-TTGTTT TCGGACTTGT TTACTCTGT- CAGTACAGGC CAACATCAGT TTGTTGTTTG GGAGAAAGGC T-TAGGGAAT GTGGCTCCC-
C.virescens    -TGTCAGTTT TGC-TCGTTC TCGGACTTGT TTACTCTGT- CAGTACAGGC CAACATCAGT TTGTTGTTTG GGAGAAAGGC T-TAGGGAAT GTGGCTC---
C.adiposa      -TATCAGTTT TGG-TAGTTT TCGGACTGCT TTACTCTGT- TAGTACAGGC CAACATCAGT TTGTTGTCGG GGAGAAAGGC T-TAGGGAAT GTGGCTC---
```

```
                    1210        1220        1230        1240        1250        1260        1270        1280        1290        1300
Petriella       -TTTC----- -GGAGTGTTA TAGCCTACCG TATAATACCC TTCGGTGGAC TGAGGACCGC GCATCTGCAA GGATGCTGGC GTAATGGTTG TCAGGGACCC
C.fimbriata     CCTTCT-GGG GGGAGTGTTA TAGCCCTCTG CATAATACCC TTCGGCAGAC TGAGGACCGC GCTTCGGCAA GGATGCTGGC GTAATGG--C ACAGACGCCC
C.albofundus    CCTTCTGGGG GGGAGTGTTA TAGCCCTCTG CATAATACCC TTCGGCAGAC TGAGGACCGC GCTTCGGCAA GGATGCTGGC GTAATGG--C ACAGACGCCC
C.moniliformis  --TCC----- GGGAGTGTTA TAGCCCTTTA CATAATACCC TTCGGCAGAC TGAGGACCGC GCTTCGGCAA GGATGTTGGC GTAATGG--C ACACACGCCC
C.fagacearum    -TTTC---GT GGGAGTGTTA TAGCCCTTTG CATAATACC- TTCGGCAGAC TGAGGACCGC GCTTCGGCAA GGATGTTGGC GTAATGG--C ACAGACGCCC
C.paradoxa      --TTC----- GGGAGTGTTA TAGCCCTTTG CATAATACCT TTCGACAGAC TGAGGACCGC GTTTCGGCAA GGATGTTGGC GTAATGG--C ACAGACGCCC
C.radicicola    ---TC----- GGGAGTGTTA TAGCCCTTTG CATAATACCT TTCGGCAGAC TGAGGACCGC GCTTCGGCAA GGATGCTGGC GTAATGTC-C AC-GACGCCC
C.laricicola    ---TC----- GGGAGTGTTA TAGCCCTTTG CATAATACCA TTCGGCAGAC TGAGGACCGC GCTTCGGCAA GGATGTTGGC GTAATGG--C ACAGACGCCC
C.polonica      ---TC----- GGGAGTGTTA TAGCCCTTTG CATAATACCA TTCGGCAGAC TGAGGACCGC GCTTCGGCAA GGATGTTGGC GTAATGG--C ACAGACGCCC
C.pinicola      ---TC----- GGGAGTGTTA TAGCCCTTTG CATAATACCA TTCGGCAGAC TGAGGACCGC GCTTCGGCAA GGATGTTGGC GTAATGG--C ACAGACGCCC
C.virescens     -TTTC----- GGGAGTGTTA TAGCCCTTTG CATAATACCA TTCGGCAGAC TGAGGACCGC GCTTCGGCAA GGATGTTGGC GTAATGG--C ACACACGCCC
C.adiposa       --TTC----- GGGAGTGTTA TAGCCCTTTG CATAATACC- TTCGGCAGAC TGAGGACCGC GCTTCGGCAA GGATGTTGGC GTAATGG--C ACAACGACCC

                    1310        1320        1330        1340        1350        1360        1370        1380        1390        1400
Petriella       GTCTTGAAAC ACGGACCAAG GAGTCGTCCT AAATATGCGA GTGTTCGGGT GTAAAACCCC TACGCGTAAT GAAA-GTGAA CGGAGGTGAG AGCTTCGGCG
C.fimbriata     GTCTTGAAAC ACGGACCAAG GAGTCAACCT TA-TGTGCGA GTGTTTGGGT GTAAAACCCC AGCGCGTAAT GAAA-GTGAA CGTAGGTGAG AGCTTCGGCG
C.albofundus    GTCTTGAAAC ACGGACCAAG GAGTCAACCT TA-TGTGCGA GTGTTTGGGT GTAAAACCCC AGCGCGTAAT GAAA-GTGAA CGTAGGTGAG AGCTTCGGCC
C.moniliformis  GTCTTGAAAC ACGGACCAAG GAGTCAACCT TA-TATGCGA GTGTTTGGGT GTAAAACCCC AGCGCGTAAT GAAA-GTGAA CGTAGGTGAG AGCTTCGGCG
C.fagacearum    GTCTTGAAAC ACGGACCAAG GAGTCAACCT TA-TGTGCAA GTGTTTGGGT GTAAAACCCC AGCGCGTAAT GAA-CGTGAA CGTAGGTGAG AGCTTCGGCG
C.paradoxa      GTCTTGAAAC ACGGACCAAG GAGTCAACCT TAATGTGCGA ATGTTTGGGT GTAAAACCCC AGCGCGTAAT GAA-CGTGAA CGTAGGTGAG AGCTTCGGCG
C.radicicola    GTCTTGAAAC ACGGACCAAG GAATCAACCT TA-TGTGCGA GTGTTTGGGT GTAAAACCCC AGCGCGTAAT GAAACGTGAA CGTAGGTGAG AGCTTCGGCG
C.laricicola    GTCTTGAAAC ACGGACCAAG GAGTCAACCT TA-TGTGCGA GTGTTTGGCT GTAAAACCCC AGCGCGTAAT GAA-CGTGAA CGTAGGTGAG AGCTTCGGCG
C.polonica      GTCTTGAAAC ACGGACCAAG GAGTCAACCT TA-TGTGCGA GTGTTTGGGT GTAAAACCCC AGCGCGTAAT GAA-CGTGAA CGTAGGTGAG AGCTTCGGCG
C.pinicola      GTCTTGAAAC ACGGACCAAG GAGTCAACCT TA-TGTGCGA GTGTTTGGGT GTAAAACCCC AGCGCGTAAT GAAA-GTGAA CGTAGGTGAG AGCTTCGGCG
C.virescens     GTCTTGAAAC ACGGACCAAG GAGTCAACCT TA-TATGCGA GTGTTTGGGT GTAAAACCCC AGCGCGTAAT GAA-CGTGAA CGTAGGTGAG AGCTTCGGCG
C.adiposa       GTCTTGAAAC ACGGACCAAG GAGTCAACCT TA-TGTGCGA GTGTTTGGGT GTAAAACCCC AGCGCGTAAT GAA-CGTGAA CGTAGGTGAG AGCTTCGGCG

                    1410        1420        1430        1440        1450        1460        1470        1480        1490        1500
Petriella       CATCATCGAC CGATCCTGAT GTTCTCGGAT GGATTTGAGT ATGAGCATAT TGGGCCGGAC CCGAAAGAAG GTGAACTATG CCTGTATAGG GTAAAGCCAG
C.fimbriata     CATCATCGAC CGATTCTGAT GTTTTCGGAT GGATTTGAGT AAGAGCACAC AGGGTTGGAC CCGAAAGAAG GTGAACTATG CTTGTATAGG GTGAAGCCAG
C.albofundus    GATCATCGAC CGATTCTGAT GTTTTCGGAT GGATTTGAGT AAGAGCACAC AGGGTTGGAC CCGAAAGAAG GTGAACTATG CTTGTATAGG GTGAAGCCAG
C.moniliformis  CATCATCGAC CGATTCTGAT GTTCTCGGAT GGATTTGAGT AAGAGCATAT AGGGTTGGAC CCGAAAGAAG GTGAACTATG CTTGTATAGG GTGAAGCCAG
C.fagacearum    CATCATCGAC CGATTCTGAT GTTCTCGGAT GGATTTGAGT AAGAGCACAC AGGGTTGGAC CCGAAAGAAG GTGAACTATG CTTGTATAGG GTGAAGCCAG
C.paradoxa      CATCATCGAC CGATTCTGAT GTTCTCGGAT GGATTTGAGT AAGAGCACAC AGGGTTGGAC CCGAAAGAAG GTGAACTATG CTTGTATAGG GTGAAGCCAG
C.radicicola    CATCATCGAC CGATTCTGAT GTTCTCGGAT GGATTTGAGT AAGAGCACAC AGGGTTGGAC CCGAAAGAAG GTGAACTATG CTTGTATAGG GTGAAGCCAG
C.laricicola    CATCATCGAC CGATTCTGAT GTTCTCAAGT GGATTTGAGT AAGAGCATAT AGGGTTGGAC CCGAAAGAAG GTGAACTATG CTTGTATAGG GTGAAGCCAG
C.polonica      CATCATCGAC CGATTCTGAT GTTCTCGGAT GGATTTGAGT AAGAGCATAT AGGGTTGGAC CCGAAAGAAG GTGAACTATG CTTGTATAGG GTGAAGCCAG
C.pinicola      CATCATCGAC CGATTCTGAT GTTTTCGGAT GGATTTGAGT AAGAGCACAC AGGGTTGGAC CCGAAAGAAG GTGAACTATG CTTGTATAGG GTGAAGCCAG
C.virescens     CATCATCGAC CGATTCTGAT GTTCTCGGAT GGATTTGAGT AAGAGCATAT AGGGTTGGAC CCGAAAGAAG GTGAACTATG CTTGTATAGG GTGAAGCCAG
C.adiposa       CATCATCGAC CGATTCTGAT GTTCTCGGAT GGATTTGAGT AAGAGCATAT AGGGTTGGAC CCGAAAGAAG GTGAACTATG CTTGTATAGG GTGAAGCCAG
```

```
                1510        1520        1530        1540        1550        1560        1570        1580        1590        1600
Petriella       AGGAAACTCT GGTGGAGGCT CGCAGCGGTT CTGACGTGCA AATCGATCGT CAAATATGGG CATGGGGGCG AAAGACTAAT CGAACCTTCT AGTAGCTGGT
C.fimbriata     AGGAAACTCT GGTGGAGGCT CGCAGCGGTT CTGACGTGCA AATCGATCGT CAAATATGAG CATGGGGGCG AAAGACTAAT CGAACCTTCT AGTAGCTGGT
C.albofundus    AGGAAACTCT GGTGGAGGCT CGCAGCGGTT CTAGCGTGCA AATCGATGCT CAAATATGAG CATGGGGGCG AAAGACTAAT CGAACCTTCT AGTAGCTGGT
C.moniliformis  AGGAAACTCT GGTGGAGGCT CGCAGCGGTT CTGACGTGCA AATCGATCGT CAAATATGAG CATGGGGGCG AAAGACTAAT CGAACCTTCT AGTAGCTGGT
C.fagacearum    AGGAAACTCT GGTGGAGGCT CGCAGCGGTT CTGACGTGCA AATCGATCGT CAAATATGAG CATGGGGGCG AAAGACTAAT CGAACCTTCT AGTAGCTGGT
C.paradoxa      AGGAAACTCT GGTGGAGGCT CGCAGCGGTT CTGACGTGCA AATCGATCGT CAAATATGAG CATGGGGGCG AAAGACTAAT CGAACCTTCT AGTAGCTGGT
C.radicicola    AGGAAACTCT GGTGGAGGCT CGCAGCGGTT CTGACGTGCA AATCGATCGT CAAATATGAG CATGGGGGCG AAAGACTAAT CGAACCTTCT AGTAGCTGGT
C.laricicola    AGGAAACTCT GGTGGAGGCT CGCAGCGGTT CTGACGTGCA AATCGATCGT CAAATATGAG CATGGGGGCG AAAGACTAAT CGAACCTTCT AGTAGCTGGT
C.polonica      AGGAAACTCT GGTGGAGGCT CGCAGCGGTT CTGACGTGCA AATCGATCGT CAAATATGAG CATGGGGGCG AAAGACTAAT CGAACCTTCT AGTAGCTGGT
C.pinicola      AGGAAACTCT GGTGGAGGCT CGCAGCGGTT CTGACGTGCA AATCGATCGT CAAATATGAG CATGGGGGCG AAAGACTAAT CGAACCTTCT AGTAGCTGGT
C.virescens     AGGAAACTCT GGTGGAGGCT CGCAGCGGTT CTGACGTGCA AATCGATCGT CAAATATGAG CATGGGGGCG AAAGACTAAT CGAACCTTCT AGTAGCTGGT
C.adiposa       AGGAAACTCT GGTGGAGGCT CGCAGCGGTT CTGACGTGCA AATCGATCGT CAAATATGAG CATGGGGGCG AAAGACTAAT CGAACCTTCT AGTAGCTGGT

                1610        1620        1630        1640        1650        1660        1670        1680        1690        1700
Petriella       TTCCGCCGAA GTTTCCCTCA GGATAGCAGT GTTGAA---T TCT-CAGTTT TATGAGGTAA AGCGAATGAT TAGGGACTCG GGGGCGCTAT TAAGCCTTCA
C.fimbriata     TCCAGCCGAA GTTTCCCTCA GGATAGCAGT GTTGAA-TTT TCTTCAGTTT TATGAGGTAA AGCGAATGAT TAGGGACTCG GGGGCGCTAT ATTGCCTTCA
C.albofundus    TCCAGTTGAA GTTTCCCTCA GGATAGCAGT GTTGAATTTT TCTTCAGTTT TATGAGGTAA AGCGAATGAT TAGGGACTCG GGGGCGCTAT ATTGCCTTCA
C.moniliformis  TCCAGCCGAA GTTTCCCTCA GGATAGCAGT GTTGAA---- -CTTCAGTTT TATGAGGTAA AGCGAATGAT TAGGGACTCG GGGGCGCTAT ATTGCCTTCA
C.fagacearum    TCCAGCCGAA GTTTCCCTCA GGATAGCAGT GTTGAA---- -CTTCAGTTT TATGAGGTAA AGCGAATGAT TAGGGACTCG GGGGCGCTAT ATTGCCTTCA
C.paradoxa      TCCAGCCGAA GTTTCCCTCA GGATAGCAGT GTTGAA---- -CTTCAGTTT TATGAGGTAA AGCGAATGAT TAGGGACTCG GGGGCGCTAT ATTGCCTTCA
C.radicicola    TCCAGCCGAA GTTTCCCTCA GGATAGCAGT GTTGAA---- -CTTCAGTTT TATGAGGTAA AGCGAATGAT TAGGGACTCG GGGGCGCTAT ATTGCCTTCA
C.laricicola    TCCAGCCGAA GTTTCCCTCA GGATAGCAGT GTTGAA---- -CTTCAGTTT TATGAGGTAA AGCGAATGAT TAGGGACTCG GGGGCGCTAT ATTGCCTTCA
C.polonica      TCCAGCCGAA GTTTCCCTCA GGATAGCAGT GTTGAA---- -CTTCAGTTT TATGAGGTAA AGCGAATGAT TAGGGACTCG GGGGCGCTAT ATTGCCTTCA
C.pinicola      TCCAGCCGAA GTTTCCCTCA GGATAGCAGT GTTGAA---- -CTTCAGTTT TATGAGGTAA AGCGAATGAT TAGGGACTCG GGGGCGCTAT ATTGCCTTCA
C.virescens     TCCAGCCGAA GTTTCCCTCA GGATAGCAGT GTTGAA---- -CTTCAGTTT TATGAGGTAA AGCGAATGAT TAGGGACTCG GGGGCGCTAT ATTGCCTTCA
C.adiposa       TCCAGCCGAA GTTTCCCTCA GGATAGCAGT GTTGAA---- -CTTCAGTTT TATGAGGTAA AGCGAATGAT TAGGGACTCG GGGGCGCTAT ATTGCCTTCA

                1710        1720        1730
Petriella       TCCATTCTCA AACTTTAAAT ATGTAAGAAG C
C.fimbriata     TCCATTCTCA AACTTTAAAT ATGTAAGAAG C
C.albofundus    TCCATTCTCA AACTTTAAAT ATGTAAGAAG C
C.moniliformis  TCCATTCTCA AACTTTAAAT ATGTAAGAAG C
C.fagacearum    TCCATTCTCA AACTTTAAAT ATGTAAGAAG C
C.paradoxa      TCCATTCTCA AACTTTAAAT ATGTAAGAAG C
C.radicicola    TCCATTCTCA AACTTTAAAT ATGTAAGAAG C
C.laricicola    TCCATTCTCA AACTTTAAAT ATGTAAGAAG C
C.polonica      TCCATTCTCA AACTTTAAAT ATGTAAGAAG C
C.pinicola      TCCATTCTCA AACTTTAAAT ATGTAAGAAG C
C.virescens     TCCATTCTCA AACTTTAAAT ATGTAAGAAG C
C.adiposa       TCCATTCTCA AACTTTAAAT ATGTAAGAAG C
```

CHAPTER 3*

MONOPHYLY OF THE CONIFER SPECIES IN THE *Ceratocystis coerulescens* COMPLEX BASED ON DNA SEQUENCE DATA

ABSTRACT

Ceratocystis sensu stricto includes numerous species of insect-vectored, wood-staining and plant pathogenic fungi. Among these, *Ceratocystis coerulescens* is a well-known cause of blue-stain in spruce and pine. Previous investigations, using morphological characteristics and isozyme comparisons, have shown that *C. coerulescens* encompasses at least five morphological types. The aim of this study was thus to compare isolates of *C. coerulescens sensu lato* and morphologically similar species, including *C. laricicola*, *C. polonica*, *C. virescens*, *C. eucalypti*, *Chalara australis* and *Ch. neocaledoniae*, on the basis of DNA sequence data. Using the polymerase chain reaction (PCR), a 600 base pair fragment within the ribosomal DNA operon was amplified, and the PCR products were sequenced. The analyzed sequence included the 5.8S rRNA gene and the internal transcribed spacers (ITS) 1 and 2. Relationships were determined by parsimony analysis. Using *C. fimbriata* as the outgroup taxon, the five morphological types previously known as *C. coerulescens* and the two other taxa from conifers formed a strongly-supported monophyletic group that includes all the *Ceratocystis* species occurring primarily on conifers. The species from hardwood trees, *C. eucalypti*, *Ch. australis* and *Ch. neocaledoniae*, also formed a monophyletic group, sister to the conifer group. The fourth species from hardwoods, *C. virescens,* formed a group basal to the two sister groups.

*Published as R.C. Witthuhn, B.D. Wingfield, M.J. Wingfield, M. Wolfaardt & T.C.

Harrington. (1998a). Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. *Mycologia* **90**, 96-101.

INTRODUCTION

Ceratocystis sensu stricto Ellis & Halsted includes many economically important species, which are pathogens on a wide variety of plants, including forest and fruit trees, sweet potatoes, rubber and sugar cane (Kile, 1993). Most *Ceratocystis* species occur on angiosperms, but *Ceratocystis coerulescens* (Münch) Bakshi, first described from Germany (Münch, 1907), is the cause of sapstain on spruce and pine. Based on isozyme analysis Harrington *et al.* (1996) demonstrated five morphologically distinct species previously known as *C. coerulescens*. The *C. coerulescens* complex thus includes species A, B and C from logs or wounds on *Picea* or *Pinus* (Harrington *et al.*, 1996) and two newly recognized species, *C. rufipenni* Wingfield, Harrington and Solheim and *C. douglasii* (Davidson) Wingfield and Harrington (Wingfield, Harrington & Solheim, 1997). *Ceratocystis douglasii* causes a stain in wood of Douglas-fir (*Pseudotsugae menziesii*) (Davidson, 1953; Wingfield, Harrington & Solheim, 1997), and *C. rufipenni* occurs on spruce attacked by the bark beetle *Dendroctonus rufipennis* (Davidson, 1955; Wingfield, Harrington & Solheim, 1997).

Ceratocystis laricicola Redfern and Minter and *C. polonica* (Siemaszko) Moreau also occur on conifers and are morphologically similar to *C. coerulescens* but have smaller and more ellipsoid ascospores (Harrington *et al.*, 1996). *Ceratocystis polonica* is associated with the bark beetle *Ips typographus* on spruce in Europe (Siemaszko, 1938; Christiansen & Solheim, 1990) and Japan (Yamaoka *et al.*, 1997). Harrington *et al.* (1996) found *C. laricicola*, which occurs on larch infested with the bark beetle *Ips cembrae* in Europe (Redfern *et al.*, 1987) and

Japan (Visser *et al.*, 1995), to be morphologically indistinguishable from *C. polonica.* Comparisons of these species using DNA sequence data also suggest that they may be synonyms (Visser *et al.*, 1995), although they differ at a single isozyme locus (Harrington *et al.*, 1996) and are not sexually compatible (Harrington and McNew, unpublished).

Ceratocystis virescens (Davidson) Moreau, described by Davidson (1944) from hardwood lumber, has been considered a synonym of *C. coerulescens* (Hunt, 1956; Upadhyay, 1981). More recent studies have shown that it is restricted to hardwood hosts, as a pathogen on maple or as a saprophyte, and is morphologically distinct from *C. coerulescens* (Harrington *et al.*, 1996; Kile & Walker, 1987). *Ceratocystis eucalypti* Yuan and Kile is morphologically similar to *C. virescens* and colonizes wounds in *Eucalyptus* in Australia (Kile *et al.*, 1996). Two *Chalara* species, namely *Ch. neocaledoniae* Kiffer and Delon and *Ch. australis* Walker and Kile, have no known teleomorphs, but their anamorphs are similar in morphology to the anamorphs of *C. virescens* and *C. eucalypti* (Harrington *et al.*, 1996) and are thus included in the present study of the *C. coerulescens* complex. Both these *Chalara* species cause diseases in hardwoods that are similar to sap streak of maple caused by *C. virescens* (Kile, 1993). *Chalara australis* occurs in Australia on *Nothofagus cunninghamii* and *Ch. neocaledoniae* was described from coffee and guava in New Caledonia (Kile *et al.*, 1996).

Sequence data from the ribosomal RNA genes has successfully been used in determining the phylogenetic relationships among species of *Ceratocystis* (Hausner, Reid & Klassen, 1993; Wingfield *et al.*, 1994; Visser *et al.*, 1995; Wingfield *et al.*, 1996). High variability in DNA sequence exists among these species in the internal transcribed spacer (ITS) regions of the ribosomal RNA genes (Visser *et al.*, 1995; Wingfield *et al.*, 1996). The aim of this study was, therefore, to compare species of the *Ceratocystis coerulescens* complex using the DNA

sequence from the variable ITS regions in the ribosomal RNA operon.

MATERIALS AND METHODS

All isolates (Table I) were grown on malt extract agar (20 g/L ME and 20 g/L agar) in Petri dishes at room temperature for 10 d. Template DNA for amplification was obtained by scraping the mycelium with a pipette tip (Harrington & Wingfield, 1995). Amplifications were performed using the primers ITS1 and ITS4 (White *et al.*, 1990) or ITS1F (Gardes & Bruns, 1993) and ITS4. The amplified fragments include the 3' end of the small sub-unit (SSU) rRNA gene, the 5.8S rRNA gene, part of the large sub-unit (LSU) rRNA gene and the internal transcribed spacer (ITS) regions 1 and 2. The PCR reaction mixture included 2.5 units of *Taq* polymerase (Boehringer Mannheim, Mannheim, Germany), the buffer supplied with the enzyme, 250 μM dNTPs, 6.25 mM $MgCl_2$ and 0.5 μM of each primer. Initial denaturation was performed at 96 C for 60 s, followed by 35 cycles of primer annealing at 55 C for 30 s, chain elongation at 72 C for 60 s and denaturation at 92 C for 60 s. Final chain elongation took place at 72 C for 5 min. The PCR products were separated on 1.5 % agarose gels, stained with ethidium bromide and visualized using UV light.

The 600 base pair PCR fragments were purified using Wizard PCR Mini-Preps (Promega Corporation, USA) or Microcon Microconcentrators (Amicon, Inc., USA). Both strands of the PCR products were sequenced using the *f*mol DNA Sequencing Kit (Promega Corporation., USA). Seven of the isolates were sequenced using the ABI PRISM 377 DNA sequencer and ABI PRISM 310 Genetic Analyzer (Perkin Elmer, USA) at the DNA Sequencing Facility at Iowa State University. The primers ITS1, ITS1F, ITS2, ITS3 and ITS4 were used in the sequencing reactions. The DNA sequence data were deposited in GenBank.

The nucleotide sequences were manually aligned, then analyzed using PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1993). The ITS sequence of *Ceratocystis fimbriata* Ellis & Halsted, isolated from sweet potatoes, was used as an outgroup. Confidence intervals of the branch points were determined using the bootstrap technique (Felsenstein, 1985) and decay indexes (AUTODECAY 2.9.4., a computer program developed by Torsten and Eriksson in 1996).

RESULTS

The PCR amplifications from living fungal mycelium of isolates studied consistently produced amplification products of approximately 600 bp, and the DNA sequences of the species in the *C. coerulescens* complex were found to be similar. It was possible to align the data manually by inserting gaps in the sequence data. With *C. fimbriata* as the outgroup taxon, two most parsimonious trees of 124 steps were produced from the aligned 565 bp sequence data, including ITS 1 and 2 and the 5.8S gene, using the Heuristic search option of PAUP (Fig. 1) (CI=0.887, HI=0.113, RI=0.899, RC=0.797). Gaps were treated as missing data. The two trees differed only in the relationship between *Ceratocystis coerulescens* species A and B and *C. douglasii*. One tree (Fig. 1) grouped *C. coerulescens* species A and B, while the second tree grouped *C. coerulescens* species B and *C. douglasii*. The same topology was obtained when midpoint rooting was used.

All the species associated with conifers, that is, *Ceratocystis coerulescens* spp. A, B and C, *C. douglasii*, *C. laricicola*, *C. polonica* and *C. rufipenni*, formed a single clade, supported by a bootstrap value of 98 % and a decay index of d3. Among the conifer species, three isolates of *C. rufipenni* grouped closely together into one well supported sub clade (91.%, d3). A second

distinctive sub clade was formed by the three isolates of *C. coerulescens* species C (71 %, d1). *C. laricicola* and *C. polonica* had identical sequences and grouped together (85 %, d1), forming a sub clade sister to the clade formed by *C. coerulescens* sp. A and sp. B and *C. douglasii* (62 %, d1). The three Australasian species from hardwoods, *C. eucalypti*, *Ch. neocaledoniae* and *Ch. australis*, grouped in a clade sister to the conifer clade (59 %, d1). The fourth species from hardwoods, namely *C. virescens*, represented by four isolates with identical sequence, was basal to the conifer and Australasian sub groups (100 %, d6). Numerous gaps were created to achieve the alignment and there were regions of ambiguity, particularly in aligning the outgroup with the ingroup. The same tree topology was observed when ten ambiguously aligned regions (157 bases in total) were removed from the data set.

Since most of the alignment ambiguity was between the ingroup and the outgroup, *C. fimbriata*, the data set was re-aligned and re-analyzed after the removal of the *C. fimbriata* sequence. *C. virescens* was defined as the outgroup and two ambiguously aligned regions (52 bases in total) near the large sub-unit gene were removed, with the gaps treated as a fifth character (gapmode=newstate). Two parsimonious trees of 90 steps were produced from the aligned 477 bp sequence data (CI=0.811, HI=0.189, RI=0.919, RC=0.776). Both these trees reflected the same topology as found when *C. fimbriata* was used as an outgroup, with *C. coerulescens* species A and B grouping together (69 %, d3). In the one tree *C. coerulescens* species C was basal to the rest of the conifer species (95 %, d3). The conifer sub clade (98 %, d4), the Australasian sub clade (92 %, d2), as well as the sub clade including *C. coerulescens* species A and B, *C. douglasii*, *C. laricicola* and *C. polonica* (99 %, d5) were better supported after the removal of *C. fimbriata* than in the analysis with *C. fimbriata* as the outgroup taxon.

DISCUSSION

The phylogenetic analyses show that species of *Ceratocystis* from conifers form a distinct clade, suggesting that these species are monophyletic. This is despite the fact that they differ somewhat in their morphology and more noticeably in their ecology, except that they all occur on conifers in the family *Pinaceae*. In fact these are the only *Ceratocystis* species that commonly occur on conifers. It appears that the adaptation to conifers is a derived character in *Ceratocystis*, which otherwise occur on dicots and monocots. This change in host range may have evolved only once in the genus.

In this study it was possible to determine a phylogenetic relatedness of species in the *C. coerulescens* complex based on DNA sequence data from the variable region in the ribosomal RNA operon. The results confirmed the recent delineation of species in this complex based on morphology and isozyme analyses (Harrington *et al.*, 1996). There was less variation than expected in the DNA sequence data of the ITS regions among species in the *C. coerulescens* complex. Little or no variation exists between these species in the highly conserved large sub-unit rRNA gene (authors, unpublished), which indicates close relationships in the complex as a whole.

Ceratocystis coerulescens has been recognized as a causal agent of blue-staining of conifers in Europe and in North America (Kile, 1993). In the years subsequent to its description, this species had become a repository for many wound and lumber colonizing fungi that generally resemble the fungus originally described by Münch (1907). Species such as *C. virescens* were reduced to synonymy with *C. coerulescens* (Hunt, 1956; Upadhyay, 1981), and the taxa recently described as *C. douglasii* and *C. rufipenni* (Wingfield, Harrington & Solheim, 1997)

were treated under the name *C. coerulescens*. In a recent study based on isozyme analyses, Harrington *et al.* (1996) showed that these species and three additional species, designated *C. coerulescens* species A, B and C, represent distinct taxa. The sequence data support these delimitations.

Our results indicate that *C. coerulescens* sp. A and *C. coerulescens* sp. B are closely related but distinct, and these species form a clade with *C. douglasii*. *C. douglasii* was originally described as *C. coerulescens* f. *douglasii* by Davidson (1953), who noted differences in the anamorphs of *C. douglasii* and *C. coerulescens*. He also noted that the former causes stain in Douglas-fir rather than spruce and pine. The isolates of *C. laricicola* and *C. polonica* had identical ITS sequences. Harrington *et al.* (1996) and Visser *et al.* (1995) showed that these fungi are very similar and may be conspecific. These bark beetle associates differ from the other conifer species in the *C. coerulescens* complex in ascospore morphology (Harrington *et al.*, 1996) but form a weakly-supported clade with *C. coerulescens* sp. A, sp. B and *C. douglasii*. Isozyme analyses also showed similarity between *C. laricicola* and *C. polonica* and *C. coerulescens* species A and B (Harrington *et al.*, 1996).

Included in the conifer clade were *C. coerulescens* sp. C and *C. rufipenni*, which is associated with the bark beetle *Dendroctonus rufipennis*, which attacks spruce in North America. The three isolates of *C. coerulescens* species C, which have perithecial necks shorter than those of *C. coerulescens* sp. A but otherwise are morphologically similar (Harrington *et al.*, 1996), form a distinct clade separate from the other conifer species. Relationships among *C. rufipenni*, *C. coerulescens* sp. C and the other conifer species are not well resolved.

Ceratocystis eucalypti appears to be very closely related to *Ch. australis* and *Ch. neocaledoniae*. These fungi are common to Australasia, and their apparent relatedness is not surprising. It has also been shown that *Ch. australis* is partially interfertile with *MAT*-1 strains of *C. eucalypti* (Kile *et al.*, 1996). *C. virescens*, also on hardwoods, has a morphology similar to that of *C. eucalypti* (except the latter has much larger ascospores) and was found to be basal to the Australasian hardwood species and to the conifer species.

The phylogenetic relationships as determined here differ somewhat from the distance analysis derived from isozyme data (Harrington *et al.*, 1996). Our phylogenetic approach shows taxa on conifers to be monophyletic, possibly evolving from a hardwood ancestor. The hardwood species, *Ch. neocaledoniae*, *Ch. australis* and *C. eucalypti*, grouped more closely with each other based on the DNA sequence data than on the isozyme markers. Also, *C. douglasii* differed substantially from all other species in the complex based on isozymes but was near *C. coerulescens* sp. A and sp. B based on the ITS sequence data. In both analyses, *C. laricicola* and *C. polonica* were closer to *C. coerulescens* sp. A and sp. B than to *C. coerulescens* sp. C and *C. rufipenni.*

In this study we have shown that it is possible to distinguish taxa in *Ceratocystis* based on DNA sequence data from the ribosomal RNA operon. The fact that various species that we have considered have been compared using other techniques, with very similar results, gives us substantial confidence in this approach. The data pointed to the importance of host range in the evolution of *Ceratocystis* species. We believe that there is considerable opportunity to compare other species of *Ceratocystis* and related fungi based on sequence data of the ribosomal RNA operon and expect that this will result in a considerable improvement in our understanding of the relationships of the group.

ACKNOWLEDGMENTS

We thank the Foundation for Research Development (FRD) and the Members of the Tree Pathology Co-operative Program (TPCP), South Africa for financial support.

REFERENCES

Christiansen, E. & Solheim, H. (1990). The bark beetle-associated blue-stain fungus *Opiostoma polonicum* can kill various spruces and Douglas fir. *European Journal of Forest Pathology* **20**, 436-446.

Davidson, R.W. (1944). Two American hardwood species of *Endoconidiophora* described as new. *Mycologia* **34**, 650-662.

Davidson, R.W. (1953). Two common lumber staining fungi in the western United States. *Mycologia* **45**, 579-586.

Davidson, R.W. (1955). Wood-staining fungi associated with bark beetles in Engelmann spruce in Colorado. *Mycologia* **47**, 58-67.

Felsenstein, J. (1985). Confidence intervals on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783-791.

Gardes, M. & Bruns, T.D. (1993). ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**, 113-118.

Harrington, T.C., Steimel, J.P., Wingfield, M.J. & Kile, G. (1996). Isozyme variation in the *Ceratocystis coerulescens* complex. *Mycologia* **88**, 104-113.

Harrington, T.C. & Wingfield, B.D. (1995). A PCR based identification method for species

of *Armillaria*. *Mycologia* **87**, 280-288.

Hausner, G., Reid, J. & Klassen, G.R. (1993). On the subdivision of *Ceratocystis* s.l., based on partial ribosomal DNA sequences. *Canadian Journal of Botany* **71**, 52-63.

Hunt, J. (1956). Taxonomy of the genus *Ceratocystis*. *Lloydia* **19**, 1-58.

Kile, G.A. (1993). Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (ed. M.J. Wingfield, K.A. Seifert & J.F. Webber), pp. 173-183. APS Press: St Paul, Minnesota, USA.

Kile, G.A., Harrington, T.C., Yuan, Z.Q, Dudzinski, M.J. & Old, K.M. (1996). *Ceratocystis eucalypti* sp. nov., a vascular stain fungus from eucalypts in Australia. *Mycological Research* **100**, 571-579.

Kile, G.A. & Walker, J. (1987). *Chalara australis* sp. nov. (Hyphomycetes), a vascular pathogen of *Nothofagus cunninghamii* (Fagaceae) in Australia and its relationship to other *Chalara* species. *Australian Journal of Botanay* **35**, 1-32.

Münch, E. (1907). Die blaufaule des nadelhoises. *Naturwissenschaftliche Zeitschrift fur Forst- und Landwirtschaft* **5**, 531-573.

Redfern, D.B., Stoakley, J.T., Steele, H. & Minter, M. (1987). Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae*. *Plant Pathology* **36**, 467-480.

Siemaszko, W. (1938). Zespoly Grybow Towarzyszacych Kornikom Polskim. *Planta Polonica* 7, 1-52.

Swofford, D.L. (1993). *PAUP Phylogenetic Analysis using Parsimony Version 3.1.1.* Champaign, IL 61820, USA.

Upadhyay, H.P. (1981). *A monograph of Ceratocystis and Ceratocystiopsis*, 176 pp. The University of Georgia Press: Athens, Georgia, USA.

Visser, C., Wingfield, M.J., Wingfield, B.D. & Yamaoka, Y. (1995). *Ophiostoma polonicum* is a species of *Ceratocystis sensu stricto*. *Systematic and Applied Microbiology* **18**, 403-409.

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A guide to methods and applications* (ed. M.A. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White), pp. 315-322. Academic Press: San Diego, USA.

Wingfield, M.J., de Beer, C., Visser, C. & Wingfield, B.D. (1996). A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematic and Applied Microbiology* **19**, 191-202.

Wingfield, M.J., Harrington, T.C. & Solheim, H. (1997). Two new species in the *Ceratocystis coerulescens* complex from conifers in western North America. *Canadian Journal of Botany* **75**, 827-834.

Yamaoka, Y., Wingfield, M.J., Takahashi, I. & Solheim, H. (1997). Ophiostomatoid fungi associated with the spruce bark beetle *Ips typographus* in Japan. *Mycological Research* **101**, 1215-1227.

Table I: Isolates of *Ceratocystis* used for DNA sequencing, their origins and GenBank Accession Numbers of ITS sequence of DNA.

Isolate number[a]	Species	Host	Origin	Genbank Accession Number
C490, CMW1323	*C. coerulescens* sp. A	*Pinus* sp.	England	U75614
C313, CBS140.37	*C. coerulescens* sp. B	*Picea abies*	Germany	U75615
C50, CMW0451	*C. coerulescens* sp. C	*Picea engelmannii*	USA	U75616
C662	*C. coerulescens* sp. C	*Picea abies*	Norway	U75617
C666	*C. coerulescens* sp. C	*Picea abies*	Norway	U75618
C609	*C. rufipenni*	*Picea engelmannii/D. rufipennis*[b]	Canada	U75619
C610	*C. rufipenni*	*Picea engelmannii/D. rufipennis*[b]	Canada	U75620
C612	*C. rufipenni*	*Picea engelmannii/D. rufipennis*[b]	Canada	U75621
CMW1016	*C. laricicola*	*Larix decidua/I. cembrae*[b]	Scotland	U75622
C708, CMW0672, CBS228.83	*C. polonica*	*Picea abies/I. typographus*[b]	Norway	U75623
C74, CMW0460	*C. virescens*	*Quercus* sp.	USA	U75624
C251	*C. virescens*	*Acer* sp.	USA	U75625
C69	*C. virescens*	*Fagus americanum*	USA	Sequence = U75625
C203, ATCC11066	*C. virescens*	*Liriodendron tulipifera*	USA	Sequence = U75625
C324, CBS142.53	*C. douglasii*	*Pseudotsuga menziesii*	USA	U75626
C639	*C. eucalypti*	*Eucalyptus sieberi*	Australia	U75627
C694, CBS149.83	*Chalara neocaledoniae*	*Coffea robusta*	New Caledonia	U75628
C619	*Chalara australis*	*Nothofagus cunninghamii*	Australia	U75629
C854	*C. fimbriata*	*Ipomoea batatas*	USA	AF007749

[a]CMW - Culture collection of M. J. Wingfield. C - Culture collection of T.C. Harrington. CBS - Centraal Bureau voor Schimmelcultures, Baarn, Netherlands. ATCC - American Type Culture Collection.

[b]Bark beetle associates, where known, are in the genera *Ips* and *Dendroctonus*.

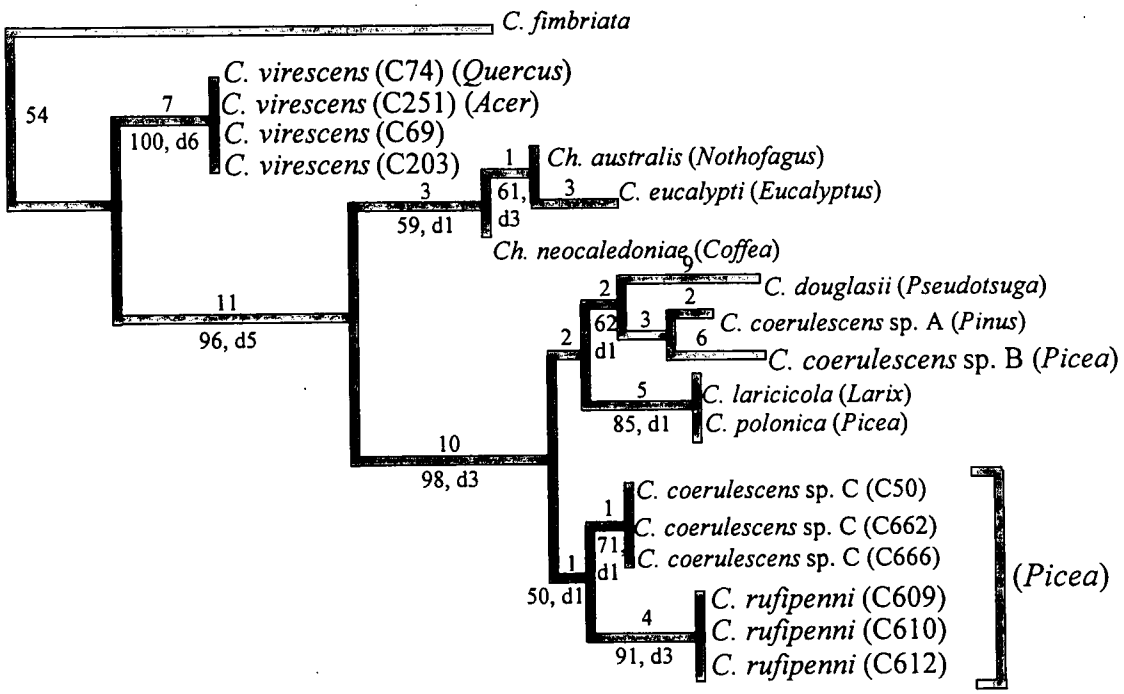


Fig. 1. One of the two most parsimonious trees produced from the DNA sequence of part of the ribosomal RNA operon (565 bp in size) using PAUP. Tree length = 124. The number of base substitutions are indicated above the branches and the bootstrap percentages (100 bootstrap replicates) and decay index (100 replicates) are indicated below the branches. Host species are indicated in brackets.

Figure 2: Aligned DNA sequence data (565 bp in total), including ITS1, ITS2 and the 5.8S rRNA gene, of the species studied.

```
Cfimria    CTGAGTTTTT GTACTCTATA AACCATGTGT GAACGTACCT ATCTTGTAGT GAGATGAATG CTGTTTTGGT -GGTAGGGCC CTTCTGAAGG GAGGGCACCG
c74vire    CTGAGTTTTT A-ACTCT-TA AACCATATGT GAACATACCT ATT------- -------A-G CTGCTTTGGC AGG-CTTGGT ---------- AACACAAGTC
C251vir    CTGAGTTTTT A-ACTCT-TA AACCATATGT GAACATACCT ATT------- -------A-G CTGCTTTGGC AGG-CTTGGT ---------- AACACAAGTC
C69vire    CTGAGTTTTT A-ACTCT-TA AACCATATGT GAACATACCT ATT------- -------A-G CTGCTTTGGC AGG-CTTGGT ---------- AACACAAGTC
C203vir    CTGAGTTTTT A-ACTCT-TA AACCATATGT GAACATACCT ATT------- -------A-G CTGCTTTGGC AGG-CTTGGT ---------- AACACAAGTC
Chaustr    CCGAGTTTTT A-ACTCTTTA AACCATATGT GAACATACCT -TCT------ -------A-G CTGCTTTGGC AGG-CTTGGT ---------- AACACAAGTC
Ceucaly    CCGAGTTTTT A-ACTCTTTA AACCATATGT GAACATACCT -TCT------ -------A-G CTGCTTTGGC AGG-CTTGGT ---------- AACACAAGTC
Chneoca    CTGAGTTTTT A-ACTCT-TA AACCATATGT GAACATACCT -TCT------ -------A-G CTGCTTTGGC AGG-CTTGGT ---------- AACACAAGTC
Cdougla    CCGAGTTTTT A-ACTCT-TA AACCATATGT GAACATACCC -TTTT----- -------A-G CTGCTTTGGC AGG-CTTGGT ---------A AAAACAAGTC
CcoeruA    CTGAGTTTTT A-ACTCT-TA AACCATATGT GAACATACCT -TTTT----- -------A-G CTGCTTTGGC AGG-CTTGGT ---------A AAAACAAGTC
CcoeruB    CTGAGTTTTT A-ACTCT-TA AACCATATGT GAACAT-CCT -TTTT----- -------A-G CTGCTTTGGC AGG-CTTGGT TT---GAA-A AAAACAAGTC
CcoCC50    CTGAGTTTTT A-ACTCT-TA AACCATATGT GAACATACCT -TCT------ -------A-G CTGCTTTGGC AGG-CTTGGT ---------- AAAACAAGTC
CcCC662    CTGAGTTTTT A-ACTCT-TA AACCATATGT GAACATACCT -TCT------ -------A-G CTGCTTTGGC AGG-CTTGGT ---------- AAAACAAGTC
CcCC666    CTGAGTTTTT A-ACTCT-TA AACCATATGT GAACATACCT -TCT------ -------A-G CTGCTTTGGC AGG-CTTGGT ---------- AAAACAAGTC
CruC609    CTGAGTTTTT A-ACTCT-TA AACCATATGT GAACATACCT -TCT------ -------A-G CTGCTTTGGC AGG-CTTGGT ---------- AAAACAAGTC
CruC610    CTGAGTTTTT A-ACTCT-TA AACCATATGT GAACATACCT -TCT------ -------A-G CTGCTTTGGC AGG-CTTGGT ---------- AAAACAAGTC
CruC612    CTGAGTTTTT A-ACTCT-TA AACCATATGT GAACATACCT -TCT------ -------A-G CTGCTTTGGC AGG-CTTGGT ---------- AAAACAAGTC
Clarici    CTGAGTTTTT A-ACTCT-TA AACCATATGT GAACATACCT -TTTTT---- -------A-G CTGCTTTGGC AGGTCTTGGT ---------- AAAACAAGTC
Cpoloni    CTGAGTTTTT A-ACTCT-TA AACCATATGT GAACATACCT -TTTTT---- -------A-G CTGCTTTGGC AGGTCTTGGT ---------- AAAACAAGTC

Cfimria    CTGCCAGCAG TATTAGTCTC ACCACTATAA A-CTCTTTT- ATTATTTTCT AGA---TT-- -TTTCATTGC TGAGTGGCAT --AACTATAA AAAAAGTTAA
c74vire    -TGCCGGTAG TATTT----- -------TAA AACTCTTTTT TTTATT--CT AAAGAATT-A --TTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
C251vir    -TGCCGGTAG TATTT----- -------TAA AACTCTTTTT TTTATT--CT AAAGAATT-A --TTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
C69vire    -TGCCGGTAG TATTT----- -------TAA AACTCTTTTT TTTATT--CT AAAGAATT-A --TTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
C203vir    -TGCCGGTAG TATTT----- -------TAA AACTCTTTTT TTTATT--CT AAAGAATT-A --TTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
Chaustr    TTGCCGGTAG TATTT----- ------ACAA A-CTCTTTT- --TATTT-CT AGAGAATT-A -TTTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
Ceucaly    TTGCCGGTAG TATTT----- ------ACAA A-CTCTTTT- --TATTT-CT AGAGAATT-A --TTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
Chneoca    TTGCCGGTAG TATTT----- ------ACAA A-CTCTTTT- --TATTT-CT AGAGAATT-A --TTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
Cdougla    -TGCCGGTAG TATAT----- -----AAAAA AACTCTTT-- AATATTT-CT ACAGAATTTA -TTTCATTGC TGAGTGGCAT AAAAC-ATAA --TAAGTTAA
CcoeruA    -TGCCGGTAG TATTT----- ----AAAAAA AACTCTTT-- AATATTT-CT AGAGAATTTA TTTTCATTGC TGAGTGGCAT TAAAC-ATAA --TAAGTTAA
CcoeruB    -TGCCGGCAG TATAT----- ----AAAAAA CACTCTTT-- AATATTT-CT AGAGAATTTA TTTTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
CcoCC50    -TGCCGGTAG CATTT----- -------AAA AACTCTTT-- -ATATTT-CT AGAGAATT-A --TTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
CcCC662    -TGCCGGTAG CATTT----- -------AAA AACTCTTT-- -ATATTT-CT AGAGAATT-A --TTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
CcCC666    -TGCCGGTAG CATTT----- -------AAA AACTCTTT-- -ATATTT-CT AGAGAATT-A --TTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
CruC609    -TGCCGGTAG CATTT----- -------AAA AACTCTT--- AATATTT-CT AGAGAATT-A --TTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
CruC610    -TGCCGGTAG CATTT----- -------AAA AACTCTT--- AATATTT-CT AGAGAATT-A --TTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
CruC612    -TGCCGGTAG CATTT----- -------AAA AACTCTT--- AATATTT-CT AGAGAATT-A --TTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
Clarici    CTGCCGGTAG TATTT----- -----AGAAA AACTCTTT-- AATATTT-CT AGAGAATTTA TATTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
Cpoloni    CTGCCGGTAG TATTT----- -----AGAAA AACTCTTT-- AATATTT-CT AGAGAATTTA TATTCATTGC TGAGTGGCAT T-AAC-ATAA --TAAGTTAA
```

```
Cfimria    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA AGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
c74vire    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
C251vir    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
C69vire    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
C203vir    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
Chaustr    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
Ceucaly    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
Chneoca    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
Cdougla    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
CcoeruA    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
CcoeruB    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
CcoCC50    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
CcCC662    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
CcCC666    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
CruC609    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
CruC610    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
CruC612    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
Clarici    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT
Cpoloni    AACTTTCAAC AACGGATCTC TTGGCTCTAG CATCGATGAA GAACGCAGCG AAATGCGATA CGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT

Cfimria    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAGAT TATTCTTT-C TTGGCGTTGG AGGTCC---T
c74vire    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCCGC TTGGTGTTGG AGGACCTGTT
C251vir    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCCGC TTGGTGTTGG AGGACCTGTT
C69vire    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCCGC TTGGTGTTGG AGGACCTGTT
C203vir    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCCGC TTGGTGTTGG AGGACCTGTT
Chaustr    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCCGC TTGGTGTTGG AGGACCCG-C
Ceucaly    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCCGC TTGGTGTTGG AGGACTCG-C
Chneoca    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCCGC TTGGTGTTGG AGGACCCG-C
Cdougla    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCTGC TTGGTGTTGG AGGACCCG-C
CcoeruA    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCTGC TTGGTGTTGG AGGACCCG-C
CcoeruB    TGAACGCACA TTGGCCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCTGC TTGGTGTTGG AGGACCCG-C
CcoCC50    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCTGC TTGGTGTTGG AGGACCCG-C
CcCC662    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCTGC TTGGTGTTGG AGGACCCG-C
CcCC666    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCTGC TTGGTGTTGG AGGACCCG-C
CruC609    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCTGC TTGGTGTTGG AGGACCCG-C
CruC610    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCTGC TTGGTGTTGG AGGACCCG-C
CruC612    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAGGCATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCTGC TTGGTGTTGG AGGACCCG-C
Clarici    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAG-CATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCTGC TTGGTGTTGG AGGACCCG-C
Cpoloni    TGAACGCACA TTGCGCCTGG CAGTATTCTG CCAG-CATGC CTGTCCGAGC GTCATTTCAC CACTCAAG-- ----CTCTGC TTGGTGTTGG AGGACCCG-C
```

```
Cfimria    GTTCTCCCCT G-AACAGGCC GCCGAAATGC ATCGGCTGTT ATACTTGCCA ACTCCCCTGT GTAGTATAAA ATTTCTAATT TTTACACTTT GAAGTTCTTG
c74vire    GTTTTC---- --AACAGGCC ACCGAAATGC ATCGGCTGTT ATACTTGC-A GCTTCCCTGT GTAGT--AAT ATCTAT---- -TTACACTTT GAAACTCTTG
C251vir    GTTTTC---- --AACAGGCC ACCGAAATGC ATCGGCTGTT ATACTTGC-A GCTTCCCTGT GTAGT--AAT ATCTAT---- -TTACACTTT GAAACTCTTG
C69vire    GTTTTC---- --AACAGGCC ACCGAAATGC ATCGGCTGTT ATACTTGC-A GCTTCCCTGT GTAGT--AAT ATCTAT---- -TTACACTTT GAAACTCTTG
C203vir    GTTTTC---- --AACAGGCC ACCGAAATGC ATCGGCTGTT ATACTTGC-A GCTTCCCTGT GTAGT--AAT ATCTAT---- -TTACACTTT GAAACTCTTG
Chaustr    GTCTTT---- -ATGCGGGCC GCCGAAATGC ATCGGCTGTT ATACTTGC-A GCTTCCCTGT GTAGT--AAT ATTTAT---- TTTACGCTTT GAAACTCTTG
Ceucaly    ATCTT---AT GATGCGGGCC GCCGAAATGC ATCGGCTGTT ATACTTGC-A GCTTCCCTGT GTAGT--AAT ATTTAT---- -TTACGCTTT GAAACTCTTG
Chneoca    GTCTT----- -ATGCGGGCC GCCGAAATGC ATCGGCTGTT ATACTTGC-A GCTTCCCTGT GTAGT--AAT ATTTAT---- -TTACGCTTT GAAACTCTTG
Cdougla    ATCT------ --TGCGGGCC GCCGAAATGC ATCGGCTGTT ATATTTGC-A GCTTCCCTGT GTAGT--AAT ATTTAT---- TTTACGCTTT GAAACTCTTT
CcoeruA    ATCTTTT--- --TGCGGGCC GCCGAAATGC ATCGGCTGTT ATATTTGC-A GCTTCCCTGT GTAGT--AAT ATTTAT---- TTTACGCTTT GAAACTCTTT
CcoeruB    GTCTTTTTTG TTTGCGGGCC GCCGAAATGC ATCGGCTGTT ATATTTGC-A GCTTCCCTGT GTAGT--AAT ATTTAT---- TTTACGCTTT GAAACTCTTT
CcoCC50    ATCT------ --TGCGGGCC ACCGAAATGC ATCGGCTGTT ATATTTGC-A GCTTCCCTGT GTAGT--AAT ATTTAT---- -TTACGCTTT GAAACTCTTA
CcCC662    ATCT------ --TGCGGGCC ACCGAAATGC ATCGGCTGTT ATATTTGC-A GCTTCCCTGT GTAGT--AAT ATTTAT---- -TTACGCTTT GAAACTCTTA
CcCC666    ATCT------ --TGCGGGCC ACCGAAATGC ATCGGCTGTT ATATTTGC-A GCTTCCCTGT GTAGT--AAT ATTTAT---- -TTACGCTTT GAAACTCTTA
CruC609    ATCT------ --TGCGGGCC GCCGAAATGC ATCGGCTGTT GAATTTGC-A GCTTCCCTGT GTAGT--AAT ATTTAT---- -TTACGCTTT GAAACTCTTA
CruC610    ATCT------ --TGCGGGCC GCCGAAATGC ATCGGCTGTT GAATTTGC-A GCTTCCCTGT GTAGT--AAT ATTTAT---- -TTACGCTTT GAAACTCTTA
CruC612    ATCT------ --TGCGGGCC GCCGAAATGC ATCGGCTGTT GAATTTGC-A GCTTCCCTGT GTAGT--AAT ATTTAT---- -TTACGCTTT GAAACTCTTA
Clarici    ATCT------ --TGCGGGCC GCCGAAATGC ATCGGCTGTT GAATTTGC-A GCTTCCCTGT GTAGT--AAT ATTTAT-TTT TTTACGCTTT GAAACTCTTT
Cpoloni    ATCT------ --TGCGGGCC GCCGAAATGC ATCGGCTGTT GAATTTGC-A GCTTCCCTGT GTAGT--AAT ATTTAT-TTT TTTACGCTTT GAAACTCTTT

Cfimria    TGTAACAC-G CCGCTAAA-- -CCC-GCTCA A--------- CTTTTGTTGA A---CTTTTC -ACAA
c74vire    TACTACAT-G CCG-TAAAA- -CCC---TCA A--------T TCTTTATTGA A--ATTT--- GA-AA
C251vir    TACTACAT-G CCG-TAAAA- -CCC---TCA A--------T TCTTTATTGA A--ATTT--- GA-AA
C69vire    TACTACAT-G CCG-TAAAA- -CCC---TCA A--------T TCTTTATTGA A--ATTT--- GA-AA
C203vir    TACTACAT-G CCG-TAAAA- -CCC---TCA A--------T TCTTTATTGA A--ATTT--- GA-AA
Chaustr    TACTACAT-G CCG-TAAAA- CCCC---TCA A--------- ---------- ----TTTTTT GA-AA
Ceucaly    TACTACAT-G CCG-TAAAAA -CCC---TCT A--------- ---------- ----TTTTTT GA-AA
Chneoca    TACTACAT-G CCG-TAAAA- -CCC---TCA A--------- ---------- ---TTTTTTT GA-AA
Cdougla    TATAACAT-G CCG-TAAAA- -CCC--ATCA A----TAT-A TATATA-TGT ATTATTTTT- GAAAA
CcoeruA    TACAACAT-G CCG-TAAAAA CCCC-AATTA AA--TTAA-- TTTTTA---- ---ATTTTT- GA-AA
CcoeruB    TACAACAT-G CCG-TAAAA- CCCTAGATTA AATTTTAATT TTTTTAAAGT TTTATTTTTT GA-AA
CcoCC50    TACAACAT-G CCGTTAAA-- -CCC---TCA A--------- ---------- ---ATTTTT- GA-AA
CcCC662    TACAACAT-G CCGTTAAA-- -CCC---TCA A--------- ---------- ---ATTTTT- GA-AA
CcCC666    TACAACAT-G CCGTTAAA-- -CCC---TCA A--------- ---------- ---ATTTTT- GA-AA
CruC609    TATAACAT-G CCG-TAAAA- -CCC---TCA A--------- ---------- ---ACTTTT- GA-AA
CruC610    TATAACAT-G CCG-TAAAA- -CCC---TCA A--------- ---------- ---ACTTTT- GA-AA
CruC612    TATAACAT-G CCG-TAAAA- -CCC---TCA A--------- ---------- ---ACTTTT- GA-AA
Clarici    TACAACATCG CCG-TAAAAC CCCC--CTCA A--------- ---------- ----TTTT-- GA-AA
Cpoloni    TACAACATCG CCG-TAAAAC CCCC--CTCA A--------- ---------- ----TTTT-- GA-AA
```

CHAPTER 4

DELETION OF THE *MAT*-2 GENE DURING UNI-DIRECTIONAL MATING TYPE SWITCHING IN *Ceratocystis sensu stricto.*

ABSTRACT

Filamentous ascomycetes are generally heterothallic, with single ascospore isolates representing one of two opposite mating types. This mating system is observed in the strictly heterothallic *Ceratocystis eucalypti*. Most other *Ceratocystis* species including *C. virescens*, *C. coerulescens* and *C. pinicola* are homothallic. The MAT-2 strains are self-fertile, while MAT-1 strains are self-sterile and grow more slowly than MAT-2 strains. The current hypothesis is that self-sterility is due to the deletion of the *MAT*-2 mating type gene and the resulting expression of the *MAT*-1 mating type. Part of the *MAT*-2 idiomorph in *C. eucalypti*, *C. virescens* and *C. pinicola* was amplified using degenerate primers designed from the conserved *MAT*-2 HMG DNA binding motif. The expected ~300 base pair (bp) PCR products were cloned and sequenced. Specific primers were designed that amplified a 210 bp fragment only in MAT-2 isolates of *C. eucalypti*, *C. virescens* and *C. pinicola*. This fragment was absent from the self-sterile (MAT-1) progeny of *C. virescens* and *C. pinicola*, confirming the deletion of *MAT*-2 during uni-directional mating type switching. The known DNA sequence data for the *C. eucalypti MAT*-2 mating type idiomorph was also increased to 1 371 bp, using TAIL-PCR and uneven PCR.

INTRODUCTION

A detailed understanding of mating type genes in ascomycetes is mainly restricted to two yeast species, namely *Saccharomyces cerevisiae* (Herskowitz, 1988 & 1989) and *Schizosaccharomyces pombe* (Kelly *et al.*, 1988), and three genera of filamentous ascomycetes, i.e. *Neurospora* (Glass *et al.*, 1990; Staben & Yanofsky, 1990), *Podospora* (Debuchy & Choppin, 1992; Debuchy *et al.*, 1993) and *Cochliobolus* (Turgeon *et al.*, 1993). Research in this field has been restricted by the fact that mating type genes differ extensively between genera and even amongst closely related fungal species.

Turgeon *et al.* (1995) reported that the *Cochliobolus heterostrophus MAT*-2 idiomorph encodes for a mating specific DNA-binding protein, which includes a high mobility group (HMG) DNA binding site, similar to HMG regions found in *Neurospora crassa mt a*-1 (Staben & Yanofsky, 1990) and *Podospora anserina* FPR1 (Debuchy & Choppin, 1992). These conserved amino acid sequences have been used in the design of degenerate primers for the amplification of the *MAT*-2 HMG box in the pyrenomycetes and in the loculoascomycetes (Arie *et al.*, 1997).

Two different mating type systems exist amongst the species of *Ceratocystis sensu stricto* Ellis & Halsted. A number of species in this genus are strictly heterothallic, such as *C. eucalypti* Yuan and Kile (Kile *et al.*, 1996) and *C. fagacearum* (Bretz) Hunt (Hepting *et al.*, 1952), with single ascospore isolates being one of two opposite mating types. Most species of *Ceratocystis*, including members of the *C. coerulescens* (Münch) Bakshi complex (Bakshi, 1951; Harrington & McNew, 1997; 1988) and *C. fimbriata* Ellis & Halsted (Andrus & Harter, 1933; Olson, 1949) are homothallic, with selfings giving rise to self-fertile and self-sterile ascospores. The self-fertile isolates are MAT-2 and the self-sterile isolates are MAT-1. Progeny of a selfing event in these

species generally segregate in a 1:1 ratio, half of the progeny are self-fertile (MAT-2) and the other half are the slower growing self-sterile (MAT-1) strains (Harrington & McNew, 1997). This apparent switching of the expression of mating type in *Ceratocystis sensu stricto* is referred to as uni-directional mating type switching (Perkins, 1987; Harrington & McNew, 1997). Crosses involving the self-sterile isolates cannot revert to the self-fertile phenotypes (Harrington & McNew, 1997). The switching event is not reversible (Harrington & McNew, 1997), as is the case in *S. cerevisiae* (Hicks *et al.*, 1979).

Self-sterile (MAT-1) isolates of *C. coerulescens*, which is phylogenetically closely related to *C. pinicola* Harrington and Wingfield (Witthuhn *et al.*, 1998), have a slower growth rate than the self-fertile (MAT-2) isolates (Harrington & McNew, 1997). Harrington & McNew (1997) suggested that the *MAT-2* idiomorph is deleted during mating type switching. We further hypothesize that mating type switching results in the deletion or loss of expression of more than a single gene (the MAT-2 idiomorph). This might explain the differences in the growth rate of the self-sterile (MAT-1) isolates. The aim of this study was to amplify part of the *MAT*-2 idiomorph from selected species of *Ceratocystis sensu stricto* and to test whether the *MAT*-2 mating type gene is deleted during uni-directional mating type switching.

MATERIALS AND METHODS

Ceratocystis eucalypti strains used in this study included 50 single ascospore progeny resulting from a cross between C639 (MAT-1) and C642 (MAT-2). The *C. virescens* strains consisted of 20 single-ascospore progeny from a selfing event of the strain C74, and the *C. pinicola* strains included 20 single-ascospore progeny from a selfing event of the strain C795. All fungal isolates are maintained in the culture collection of T.C. Harrington.

DNA was extracted according to the method described by DeScenzo & Harrington (1994). Degenerate primers, NcHMG1 (5'-CCYCGYCCYCCYAAYGCNTAYAT-3') and NcHMG2 (5'-CGNGGRTTRTARCGRTARTNRGG-3') (Figure 1), designed for the amplification of the conserved *MAT*-2 HMG box in *Neurospora crassa* and *Podospora anserina* (Arie *et al.*, 1997) were used in PCR amplification reactions. The PCR reactions were performed as described by Arie *et al.* (1997) using *Taq* DNA Polymerase (Promega Corporation, USA) or Expand High-Fidelity *Taq* DNA Polymerase (Boehringer Mannheim, Germany), with primer annealing at 55 °C. The PCR products were separated on 2 % agarose gels, stained using ethidium bromide and visualised under UV light.

The expected ~300 bp PCR products were extracted from gel slices using the QIAquick Gel Extraction Kit (Qiagen Inc., USA). These products were cloned using the pGEM-T Easy Vector System (Promega Corporation, USA) or the pCR-Script Amp SK(+) Cloning Kit (Stratagene, USA.). Both stands of the cloned fragments were sequenced with the M13 Reverse and M13 Universal primers, using the ABI PRISM 377 DNA sequencer and the ABI PRISM 377 Genetic Analyzer (Perkin-Elmer, USA) at Iowa State University, USA and at the University of the Orange Free State, South Africa.

The *MAT*-2 HMG box sequence data of *C. eucalypti* and *C. pinicola* were used to design specific, non-degenerate primers. PCR reactions were performed as described for the degenerate primers (Arie *et al.*, 1997), using Expand High-Fidelity *Taq* DNA Polymerase (Boehringer Mannheim, Germany), with primer annealing performed at 58 °C. The *C. eucalypti MAT*-2 specific primers, EUM2-1 (5'-GACATCAAGCCGTCAAGACCG-3') and EUM2-2 (5'-GTCTTTTTGTATGCTTCGGCC-3') (Figure 1) were tested against MAT-1 and MAT-2 single-ascospore progeny of *C. eucalypti* and *C. virescens*. The *C. pinicola MAT*-2 specific primers,

COER2-1 (5'-GACACCAAGACGTCAAAGCC-3') and COER2-2 (5'-GCTTTTCTTGTAAGTTTCAGC-3') were tested against MAT-1 and MAT-2 single-ascospore progeny of *C. pinicola.*

Thermal Asymmetric Interlaced (TAIL)-PCR (Liu & Whittier, 1995) was used for the amplification of the flanking regions of the *MAT*-2 HMG box in *C. eucalypti.* The PCR reaction mixtures and primers for TAIL-PCR were compiled as described by Arie *et al.* (1996) and the PCR cycling conditions were done as described by Liu & Whittier (1995), using the primers EUM2-1 and EUM2-2 (Figure 1) in the primary TAIL-PCR reaction. This PCR reaction was followed by secondary TAIL-PCR reaction using the primers ETP1 (5'-AATCAGCTAGCATCCGTG-3') and ETP2 (5'-GAATCTGAAGAGCTATGG-3') (Figure 1). Single PCR products produced by the secondary TAIL-PCR reactions were cloned and sequenced.

Further flanking regions of the known DNA sequence of the *MAT*-2 idiomorph of *C. eucalypti* were obtained using the uneven PCR technique (Chen & Wu, 1997). The primary reactions were performed with the primer ETP1, followed by a secondary reaction with the primer ETP3 (5'-CGATACGACTATCTGGTTGC-3') (Figure 1), using 0.5 µl of the 25 µl ETP1 primary PCR reaction mixture as template DNA. The primary PCR reaction mixture contained 50 ng of template DNA, 2 units *Taq* DNA Polymerase (Advanced Biotechnologies Ltd., UK), the buffer supplied with the enzyme, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM ETP1 and 2 pg of the OPB12 primer (5'-CCTTGACGCA-3') (Operon Technologies, Inc., USA). Thermal cycling conditions for the primary and secondary PCR reactions are as described by Chen & Wu (1997). Single bands produced by the secondary uneven-PCR reactions were cloned and sequenced.

The DNA sequence data from the 3' conserved flanking region of the *MAT*-1 and *MAT*-2 idiomorphs of *C. eucalypti* were amplified using the primers ETP3 and EUM2-6I (5'GGAACCAACCATATGGACTG3') (Figure 1). PCR reaction mixtures were compiled as described by Arie *et al.* (1997), with the PCR primer annealing performed at 55 °C. The expected 130 bp single PCR fragments were cleaned and sequenced.

RESULTS

Degenerate primers designed for the amplification of the *MAT*-2 HMG box (Arie *et al.*, 1995) were successfully used in the amplification of the expected ~300 bp PCR fragment in *Ceratocystis eucalypti*, *C. pinicola* and *C. virescens*. The *C. eucalypti* and *C. pinicola MAT*-2 HMG box DNA sequence data were used for the design of specific *MAT*-2 HMG box primers. The *C. eucalypti* primers only produced amplification products in *MAT*-2 strains of *C. eucalypti*, *C. virescens*, *Chalara neocaledoniae* and *Ch. australis*, while the *C. pinicola* specific primers amplified the *MAT*-2 region in only *C. pinicola* MAT-2 strains. No *MAT*-2 HMG box amplification products were observed by amplification with *MAT*-2 primers in *MAT*-1 strains of species of *Ceratocystis sensu stricto*.

The specific primers (EUM2-1 and EUM2-2) (Figure 1) designed for the amplification of the *MAT*-2 HMG box in the strictly heterothallic *C. eucalypti*, amplified the expected 210 bp PCR fragment only in the 25 MAT-2 progeny and not in the 25 MAT-1 progeny. The same primers were tested against progeny of a selfing event in the MAT-2 strain of *C. virescens*. The expected fragment was only amplified in the 10 self-fertile, MAT-2 strains. No amplification products were observed in the 10 self-sterile, MAT-1 strains (Figure 2). The specific primers (COER2-1 and COER2-2), designed for the amplification of the *MAT*-2 HMG box in *C. pinicola*, only amplified

the expected 210 bp products in the 10 self-fertile, MAT-2 progeny, with no amplification products observed in the 10 self-sterile, MAT-1 progeny.

The known DNA sequence data of the *MAT*-2 idiomorph of *C. eucalypti* was increased from 280 bp to 1 371 bp (Figure 3) with the use of TAIL-PCR (Liu & Whittier, 1995) and uneven-PCR (Chen & Wu, 1997) techniques. The known DNA sequence of the *MAT-2* idiomorph of *C. eucalypti* encodes for two introns (base number 134 to 448 and base 647-702). The total DNA coding region is 371 bp in size and codes for a protein which is 122 amino acids in length. An internet search in Genbank (BLAST) of homologous amino acid sequences showed homology to the *Fusarium oxysporum* MAT-2 protein, the *N. crassa* MT a-1 protein, the *P. anserina* FPR1 protein and the SMTa-1 protein from *Sordaria.*

A portion of the 3' conserved flanking region within the *MAT*-2 and *MAT*-1 idiomorphs of *C. eucalypti* was amplified using the primers ETP3 and EUM2-6I (Figure 1). The same sized fragment (130 bp in total) was amplified in the 25 *MAT*-1 and 25 *MAT*-2 progeny of *C. eucalypti*. The DNA sequence data of this 130 bp region amplified from the MAT-1 and MAT-2 strains were found to be identical.

DISCUSSION

Degenerate primers, designed for the amplification of the *MAT*-2 HMG box in all pyrenomycetes (Arie *et al.*, 1995), were successfully used in the amplification of the *MAT*-2 HMG box, a part of the *MAT*-2 idiomorph, in these species of *Ceratocystis sensu stricto*. The specific primer pairs designed for the amplification of part of the *MAT*-2 HMG box only produced amplification products in *MAT*-2 strains of species of *Ceratocystis virescens, C. eucalypti* and *C. pinicola*. No

amplification products were observed in *MAT*-1 strains. The molecular data presented here provide evidence for the deletion of the *MAT*-2 idiomorph during uni-directional mating type switching in homothallic species of *Ceratocystis sensu stricto*.

Ceratocystis eucalypti is strictly heterothallic (Kile *et al.*, 1997), with two strains of opposite mating type. The *MAT*-2 HMG box was amplified in the MAT-2 strains, but no amplification products were observed in the MAT-1 strains. Therefore, no silent copies of the *MAT*-2 idiomorph was detected in *C. eucalypti MAT*-1 strains, as is the case with *S. cerevisiae* (Herskowitz, 1988 & 1989).

Ceratocystis pinicola (Harrington & McNew, 1997) and *C. virescens* (Harrington *et al.*, 1998), unlike *C. eucalypti*, undergo uni-directional mating type switching (Perkins, 1987; Harrington & McNew, 1997). The *MAT*-2 HMG box was amplified from self-fertile (MAT-2) isolates of *C. virescens* and *C. pinicola*, which is phylogenetically closely related to *C. coerulescens* (Witthuhn *et al.*, 1998). However, no amplification products were observed in the self-sterile (MAT-1) isolates. This suggests that the *MAT*-2 idiomorph is deleted during the switching event from a self-fertile to a self-sterile strain during uni-directional mating type switching, and further explains why this switching event is uni-directional.

The *MAT*-2 HMG box was amplified from half of the progeny of a selfing event in *C. virescens* and *C. pinicola*, suggesting a Mendelian segregation of the *MAT*-2 HMG box during uni-directional mating type switching. Furthermore, the *MAT*-2 mating type gene was only amplified in the self-fertile progeny and not in the self-sterile progeny, suggesting that *MAT*-2 co-segregates with the self-fertile mating type phenotype.

The mating type idiomorphs in all the studied ascomycetes consist of a variable region, flanked by two conserved sequences (Nelson, 1996; Coppin *et al.*, 1997). The 3' conserved flanking regions of the *MAT*-1 and *MAT*-2 idiomorphs in *C. eucalypti* were amplified and the flanking DNA sequences of the two idiomorphs were found to be identical. Further evidence for the fact that the 3' end of the known *C. eucalypti MAT*-2 DNA sequence includes the flanking regions of the gene is based on Southern hybridization experiments (data not shown). DNA hybridizations were done using a 1 kb PCR fragment of the *C. eucalypti MAT*-2 idiomorph with a portion of the flanking region as a probe against MAT-1 and MAT-2 strains of *C. eucalypti*. It was possible to detect hybridization in both MAT-1 and MAT-2 strains. The genomic DNA was digested with a combination of the restriction enzymes, *Eco*RV and *Bam*HI. DNA fragments that hybridize to the probe were detected in both mating types, the fragment was 1.9 kb in the case of the *MAT*-2 strain and 2.1 kb for the MAT-1 strain. When genomic DNA was digested with a combination of *Hind*III and *Xba*I, hybridization products of 2.7 kb and 5.3 kb were detected for the MAT-2 and MAT-1 strains, respectively.

Results from this study provide molecular evidence for the deletion of the *MAT*-2 mating type idiomorph during uni-directional mating type switching in species of *Ceratocystis sensu stricto*. Furthermore, the known DNA sequence data of the *MAT*-2 mating type idiomorph of *C. eucalypti* was increased to 1.3 kb (Figure 3), including a part of the conserved flanking regions of the mating type idiomorphs, forming a firm foundation for further studies of the mating type genes in *Ceratocystis sensu stricto*.

ACKNOWLEDGMENTS

Financial support for this study was provided by the Foundation for Research Development (FRD), South Africa, the United Nations Education, Science and Cultural Organization (UNESCO), the United States Department of Agriculture (USDA) and the members of the Tree Pathology Co-operative Programme (TPCP), South Africa. The technical assistance of Joe Steimel and Doug McNew is greatly appreciated.

REFERENCES

Andrus, C.F. & Harter, L.I. (1933). Morphology of reproduction in *Ceratostomella fimbriata*. *Journal of Agricultural Research* **46**, 1059-1078.

Arie, T., Christiansen, S.K., Yoder, O.C. & Turgeon, B.G. (1997). Efficient cloning of Ascomycete mating type genes by PCR amplification of the conserved *MAT* HMG box. *Fungal Genetics and Biology* **21**, 118-130.

Bakshi, B.K. (1951). Studies on four species of *Ceratocystis*, with a discussion on fungi causing sap-stain in Britain. *Commonwealth Mycological Institute, Mycological Paper* **35**, 1-16.

Chen, X. & Wu, R. (1997). Direct amplification of unknown genes and fragments by uneven Polymerase Chain Reaction. *Gene* **185**, 195-197.

Coppin, E., Debuchy R., Arnaise, S. & Picard, M. (1997). Mating types and sexual development in filamentous ascomycetes. *Microbiology and Molecular Biology Reviews* **61**, 411-428.

Debuchy, R. & Coppin, E. (1992). The mating types of *Podospora anserina*: functional analysis and sequence of the fertilization domains. *Molecular and General Genetics* **233**, 113-121.

Debuchy, R., Arnaise, S. & Lecellier, G. (1993). The *mat*- allele of *Podospora anserina* contains three regulatory genes required for the development of fertilized female organs. *Molecular and General Genetics* **241**, 667-673.

DeScenzo, R.A. & Harrington, T.C. (1994). Use of $(CAT)^5$ as a DNA fingerprinting probe for fungi. *Phytopathology* **84**, 534-540.

Glass, N.L., Grotelueschen, J. & Metzenberg, R.L. (1990). *Neurospora crassa* A mating-type region. *Proceedings of the National Academy of Science, USA* **87**, 4912-4916.

Harrington, T.C. & McNew, D.L. (1997). Self-fertility and unidirectional mating type switching in *Ceratocystis coerulescens*, a filamentous ascomycete. *Current Genetics* **32**, 52-59.

Harrington, T.C. & McNew, D.L. (1998). Partial interfertility among the *Ceratocystis* species on conifers. *Fungal and General Biology* **25**, 44-53.

Harrington, T.C., Steimel, J. & Kile, G.A. (1998). Genetic variation in three *Ceratocystis* species with outcrossing, selfing and asexual reproductive strategies. *European Journal of Forest Pathology* **28**, 217-226.

Hepting, G.H., Toole, E.R. & Boyce, J.S., Jr. (1952). Sexuality in the oak wilt fungus. *Phytopathology* **42**, 438-442.

Herskowitz, I. (1988). Life cycle of the budding yeast *Saccharomyces cerevisiae*. *Microbiological Reviews* **52**, 536-553.

Herskowitz, I. (1989). A regulatory hierarchy for cell specialization in yeast. *Nature* **342**, 749-757.

Hicks, J., Strathern, J.N. & Klar, A.J.S. (1979). Transposable mating type genes in *Saccharomyces cerevisiae*. *Nature* **282**, 478-483.

Kelly, M., Burke, J., Smith, M., Klar, A. & Beach, D. (1988). Four mating-type genes control sexual differentiation in the fission yeast. *EMBO Journal* **7**, 1537-1547.

Kile, G.A., Harrington, T.C., Yuan, Z.Q., Dudzinski, M.J. & Old, K.M. (1996). *Ceratocystis eucalypti* sp. nov., a vascular stain fungus from eucalypts in Australia. *Mycological Research* **100**, 571-579.

Liu, Y.-G. & Whittier, R.F. (1995). Thermal Asymmetric Interlaced PCR: Automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. *Genomics* **25**, 674-681.

Nelson, M.A. (1996) Mating systems in ascomycetes: a romp in the sac. *TIG* **12**, 69-74.

Olson, E.O. (1949). Genetics of *Ceratostomella*. I. Strains in *Ceratostomella fimbriata* (Ell. & Halst.) Elliot from sweet potatoes. *Phytopathology* **39**, 548-561.

Perkins, D.D. (1987). Mating Type Switching in Filamentous Ascomycetes. *Genetics* **115**, 215-216.

Staben, C. & Yanofsky, C. (1990). *Neurospora crassa* a mating-type region. *Proceedings of the National Academy of Science, USA* **87**, 4917-4921.

Turgeon, B.G., Bohlmann, H., Ciuffetti, L.M., Christiansen, S.K., Yang, G., Schäfer, W. & Yoder, O.C. (1993). Cloning and analysis of the mating type genes from *Cochliobolus heterostrophus*. *Molecular and General Genetics* **238**, 270-284.

Turgeon, B.G., Sharon, A., Wirsel, S., Christiansen, S.K., Yamaguchi, K. & Yoder, O.C. (1995). Structure and function of mating type genes in *Cochliobolus* spp. and asexual fungi. *Canadian Journal of Botany* **73**, S778-S783.

Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J., Wolfaardt, M. & Harrington, T.C. (1998a). Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. *Mycologia* **90**, 96-101.

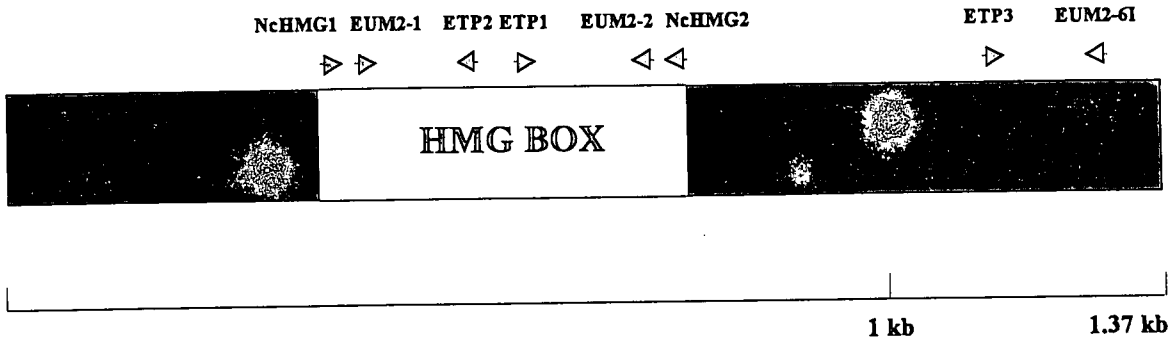


Figure 1: A diagramatic representation of the primers used in the amplification of part of the *MAT*-2 mating type idiomorph in *Ceratocystis eucalypti*.

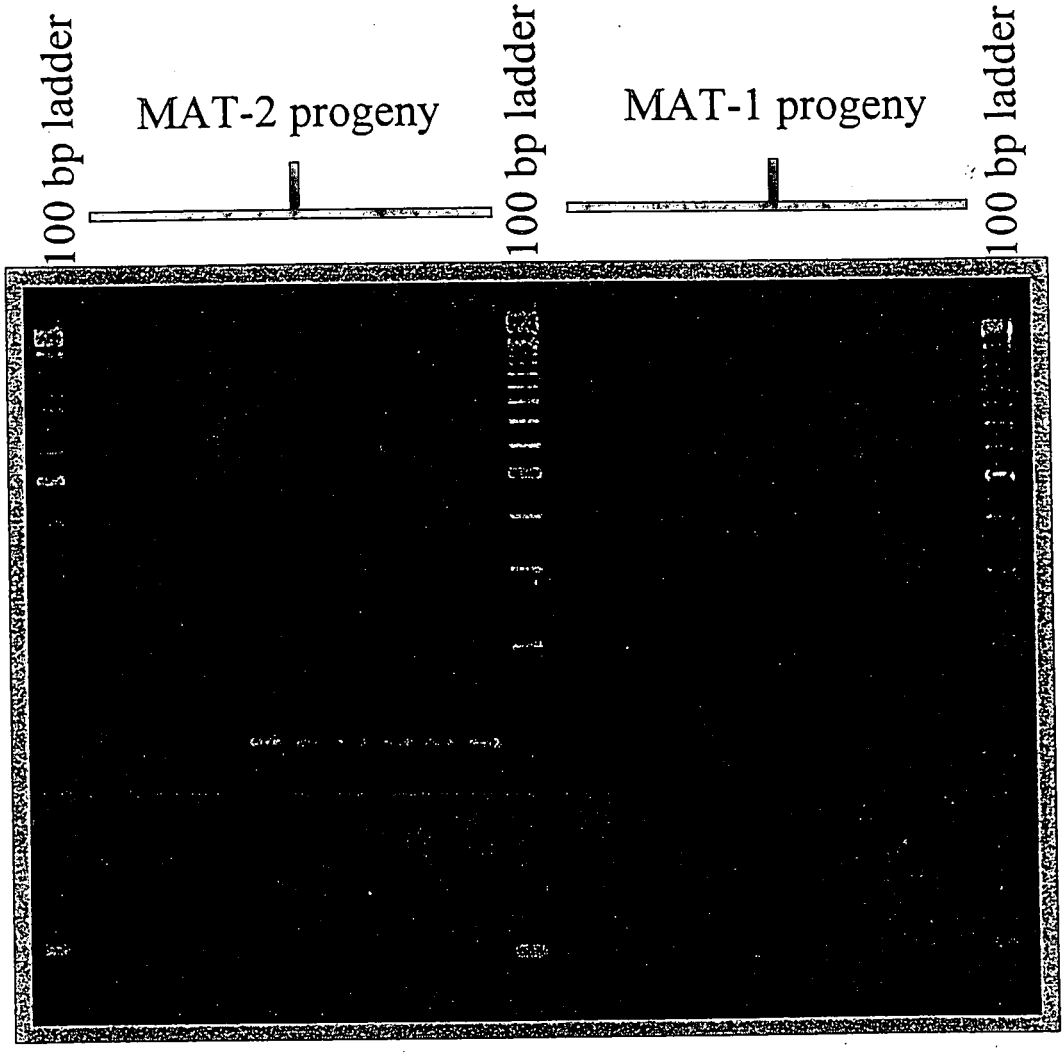


Figure 2: The PCR amplification products, using the primers EUM2-1 and EUM2-2 (Figure 1), of the *MAT*-2 HMG box of progeny of a selfing in *Ceratocystis virescens*, separated on a 2 % agarose gel. Lanes 1, 12 and 23: 100 bp DNA ladder (Promega Corporation, USA). Lanes 2-11: *C. virescens* MAT-2 progeny. Lanes 13-22: *C. virescens* MAT-1 progeny.

```
      10        20        30        40        50
12345678901234567890123456789012345678901234567890
TGTCGAGTGAGATGAATCAATAAAGAAGCTACTACATGACTTCGGGTAAG
TTTAACAATCAGATAGATAGTGGGGGCCACGAAAAGTAATTAAGGTCCTA
TCAGTGGTATAATCGGGGCGTCTGCTTTATTAGTACGAGATGCTGTCGAT
ACTGATCGCTTTTTCATCGGTTCTATTCAAGATTTTAACACCGAAGATAA
ATCCTTGATATCAGTTCCTGGTTGCGCATATTTTGTCCTCGTAACATCAA
ACCCTATCACAGGTTCAGGCTGCGTCATCGGTCCTGATATGATGGTACTA
GGCGATGATATGTTTGATACCTTACAAAGCAAAGAAGATGAAATTCAGCC
AAAGATTCCGCGCCCACCGAATGCATATATATTGTATAGAATAGACAGAC
ATCAAGCCGTCAAGACCGACTTTCCAAACATATCGAATAATGAAATTTGT
AAGTATCCATAGCTCTTCAGATTCTCAACATACGGAAGAAACATGTTCGC
TAATCTATATTCATTCAGCAAAAATTCTCGGCAAACGTTGGAGAGAAGAA
TCAGCTAGCATCCGTGAATTTTACAGAGAGCAGGCCGAAGCATACAAAAA
GACATTTATGGAAATGTATCCTGATTATCGGTACAAACCTCGAAAGGCGA
GCGAGAAGAAGCGCCGTCGGCGAAATATTACATCTATCGCACTCGACAAT
GAAGGCCGTTCCCCATCTACCGTAACCGCTAGTTCTCCAGAAACCATGTT
TCTAGAGGAGTTTACCCAATCACCAATCTAACTCACTTCCTTTCTTTTTA
TTTAACATCATCTCCCGGCGACTCGTTACATTATTTATTCATCATAGCTA
GACTATATATCCTTTACAGTCATGACATATATAACTATATTTTTACGCAT
ACTACTATTCAAGTACCAACAATATAATACCCTCACCTTTCTACAATTAA
CTGAAAATGGTATAAAATAAGTCCTCGTATCTATAAAAATTACACGTAGC
GTGTTGAACAACTGTTTGCAATATTCTTTTCAAAATGATGTGTTGTCATG
GCTCTATAAGACATCGTTCAAGTAATCAGTCAAGCCAAAAAACCGGTGAT
GAAACAAGGGTATGAATTGAAAATGGCAGATACTTGTATTCATCGATACA
ACTATCTGGTTGCTACGTTAAATCTCAAACTTTAACCAAGATCCTTCTCA
CCGCTTGGTAGGGTTACTATACACGATGGATATGATGTTCATCTCATCTA
CAAACAGTCCATATGGTTGGTTCCAACAAAGTCGTAATAATTGATATATA
TGAGACCTTAACCCTTACATGTCCGTGAATTCCGTTTTCGTTGACTTAGA
AAGTGGGGCCGTCAACCATTGTATTATGGCTTTCTCTACTTTCTTCATTG
GCGTCACAATATGGCAATTATATTCGGTGGCCTAAATGCTGGTTGCTAAC
TATAGAAACTTCTAACTATAGCTATCTCGACAATATTCAAAACATGGTAC
TTGCGGTAAGTTTGGGCTTATAGAGCGTCATGTTGATGTTTGAAATAACG
CTGACCCGTGCAAAAATAGCAGTTGGGACAAATGACATCAACGCTATTGC
TTTATTGCCAAACTGTTATCAGCAGGCAGTCTAGCTGGGTGCAAAGCAAT
TACAAACCTTGAAACTTGTTGTCTGTTCGCCACAG
```

Figure 3: Partial DNA sequence data (1 371 bp) of the *MAT*-2 mating type idiomorph in *Ceratocystis eucalypti*.

CHAPTER 5

A PHYLOGENETIC COMPARISON OF THE RIBOSOMAL DNA AND *MAT*-2 HMG BOX DNA SEQUENCES OF SPECIES IN THE *Ceratocystis coerulescens* COMPLEX.

ABSTRACT

The genus *Ceratocystis sensu stricto* includes species that are insect-vectored, plant pathogens, occurring mainly on angiosperm hosts world-wide. Species in the *C. coerulescens* complex form a morphologically distinct group that are pathogens on conifers and hardwoods. In recently years, species in the *C. coerulescens* complex have been intensively studied based on their morphology, as well as on molecular characteristics. The aim of this study was to compare the phylogeny of these species based on partial rDNA sequences with a phylogenetic analysis of the *MAT*-2 HMG box DNA and translated amino acid sequence data. A 210 bp PCR fragment of part of the *MAT*-2 HMG box of species in the *C. coerulescens* complex was amplified, sequenced and phylogenetically analysed using PAUP. *C. fimbriata* was used as the outgroup taxon and was found to be distinct from the species in the *C. coerulescens* complex. Species in the *C. coerulescens* complex from conifers formed a single clade, sister to a clade that included the species from hardwoods. The phylogeny based on the *MAT*-2 HMG box sequences differed from that based on ITS rDNA sequences only in that *C. virescens* was basal to the other species in the *C. coerulescens* complex. Furthermore, *C. laricicola* and *C. polonica* could not be separated from each other based on ITS rDNA sequences, but were distinguished based on the *MAT*-2 HMG box sequences, which clearly separated *C. polonica* and *C. laricicola* as distinct.

INTRODUCTION

Ceratocystis coerulescens (Münch) Bakshi, first described by Münch (1907), cause blue stain in spruce (*Picea*) and pine (*Pinus*) wood. Harrington *et al.* (1996) identified five morphological variants of *C. coerulescens* on conifers. These five morphologically similar taxa have been described as *C. coerulescens* (Harrington & Wingfield, 1998), *C. pinicola* Harrington & Wingfield (Harrington & Wingfield, 1998) *C. douglasii* (Davidson) Wingfield & Harrington (Wingfield *et al.*, 1997), *C. rufipenni* Wingfield, Harrington & Solheim (Wingfield *et al.*, 1997) and *C. resinifera* Harrington & Wingfield (Harrington & Wingfield, 1998).

Ceratocystis laricicola Redfern & Minter and *C. polonica* Siemaszko are morphologically similar to *C. coerulescens* and form part of the *C. coerulescens* complex (Harrington *et al.*, 1996). *C. laricicola* occurs on larch (*Larix*) and is associated with the bark beetle, *Ips cembrae* (Redfern *et al.*, 1987; Visser *et al.*, 1995). *C. polonica* is an important pathogen associated with spruce (*Picea*) infested with the bark beetle, *Ips typographus* (Siemazko, 1938; Christian & Solheim, 1990; Yamaoka *et al.*, 1997)

Ophiostoma polonicum has recently been recognised as a typical species of *Ceratocystis sensu stricto*, based on the presence of a *Chalara* anamorph and on ribosomal DNA (rDNA) sequences (Visser *et al.*, 1995). *C. polonica* and *C. laricicola* cannot be separated based on morphology (Harrington *et al.*, 1996; Harrington & Wingfield, 1998) or ITS rDNA sequences (Witthuhn *et al.*, 1998b). However, Harrington & Wingfield (1998) separated *C. laricicola* and *C. polonica* as two distinct species based on the fact that they differ in a single isozyme locus (Harrington *et al.*, 1996) and occupy two different ecological niches. Mating studies (Harrington & McNew, 1998) further suggested that these species are intersterile.

Species in the *C. coerulescens* complex primarily occur on conifers, but various species are also known from hardwoods. *C. virescens* (Davidson) Moreau has been considered a synonym of *C. coerulescens* (Hunt, 1956; Upadhyay, 1981), but has in the past been shown to be distinct (Harrington *et al.*, 1996; Witthuhn *et al.*, 1998a,b). *C. eucalypti* Yuan & Kile, isolated from *Eucalyptus* in Australia (Kile & Walker, 1987), together with two *Chalara* species from hardwoods, *Ch. neocaledoniae* Kiffer & Delon and *Ch. australis* Walker & Kile, also form part of the *C. coerulescens* complex (Harrington *et al.*, 1996; Witthuhn *et al.*, 1998b).

The aim of this study was to determine the phylogenetic relationships between the species in the *Ceratocystis coerulescens* complex based on the *MAT*-2 HMG box DNA (Turgeon *et al.*, 1998) and translated amino acid sequences. The phylogeny based on the *MAT*-2 HMG box sequences was compared to that based on the ITS rDNA sequence data.

MATERIALS AND METHODS

Isolates used in this study are from the culture collections of the Forestry and Agricultural Biotechnology Institute (FABI) and T.C. Harrington (Table 1). The isolates were grown on 20 g/L malt extract for 10 days. Mycelium was harvested and the DNA was extracted using the method by DeScenzo & Harrington (1994).

The PCR reactions for the amplification of the *MAT*-2 HMG box were performed as described by Arie *et al.* (1997), using Expand High-Fidelity *Taq* DNA Polymerase (Boehringer Mannheim, Germany), with primer annealing performed at 58 °C. The *C. eucalypti MAT*-2 specific primers, EUM2-1 (5'-GACATCAAGCCGTCAAGACCG-3') and EUM2-2 (5'-GTCTTTTTGTATGCTTCGGCC-3') (authors, publication in preparation) were used for *C.*

eucalypti, *C. virescens*, *Ch. neocaledoniae* and *Ch. australis*. The *C. pinicola MAT*-2 specific primers, COER2-1 (5'-GACACCAAGACGTCAAAGCC-3') and COER2-2 (5'-GCTTTTCTTGTAAGTTTCAGC-3') (authors, publication in preparation) were used for *C. pinicola*, *C. douglasii*, *C. laricicola* and *C. polonica*. The expected 210 bp (base pair) *MAT*-2 HMG box PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., USA). Both strands of the single PCR products were sequenced with both PCR primers, using an ABI PRISM 377 DNA sequencer and the ABI PRISM 377 Genetic Analyser (Perkin-Elmer, USA) at Iowa State University, USA and at the University of the Orange Free State, South Africa. The *MAT*-2 HMG box DNA sequence data for the outgroup taxon, *C. fimbriata*, were obtained from Harrington & Steimel (unpublished data).

The DNA sequence data and the translated amino acid sequences were compared to all known *MAT*-2 HMG box sequences. The DNA and amino acid sequence data were manually aligned and phylogenetic analyses were performed using PAUP (Swofford, 1993). The ITS rDNA sequence data of the species studied (Witthuhn *et al.*, 1998b) were manually aligned and phylogenetically analyzed. Confidence intervals were determined using 100 bootstrap replicates (Felsenstein, 1985).

RESULTS

The primers, EUM2-1 and EUM2-2, were used to amplify the *MAT*-2 HMG box in *C. eucalypti* and *C. virescens*. These primers also amplified a 120 bp fragment from the phylogenetically closely related anamorphic species, *Chalara australis* and *Ch. neocaledoniae* (Harrington *et al.*, 1996; Witthuhn *et al.*, 1998b). The use of primers, COER2-1 and COER2-2, resulted in the amplification of a 210 bp PCR product in *C. pinicola*, *C. douglasii*, *C. laricicola* and *C. polonica*.

The *MAT*-2 HMG box DNA sequences were translated into amino acid sequence and compared to the *MAT*-2 HMG box amino acid sequence of other pyrenomycetes (Arie *et al.*, 1997) (Figure 1). Twenty three conserved amino acids were identified throughout the 71 total amino acids of the pyrenomycetes (Figure 1). The *MAT*-2 HMG box of the species of *Ceratocystis* contained an intron (72 bp in size) at the conserved position in the serine (S) codon (Figure 1), similar to the introns present in most other pyrenomycetes (Arie *et al.*, 1997).

The translated amino acid sequence homology among the species of *Ceratocystis* was ~80 %, and the homology between the *Ceratocystis* species and other known pyrenomycetes *MAT*-2 HMG box amino acid sequences was ~55 %. The translated amino acid sequences of the *MAT*-2 HMG box of *C. eucalypti*, *C. virescens* and the two *Chalara* species, all from hardwoods, were found to consist only of single amino acid differences. The amino acid sequences of *Ch. neocaledoniae* and *Ch. australis* were identical. As with the hardwood species, the amino acid sequences of the conifer species (*C. pinicola*, *C. laricicola*, *C. polonica* and *C. douglasii*) were found to be very similar.

A phylogenetic analysis of the manually aligned *MAT*-2 DNA sequence data (208 bp in total) of nine species of *Ceratocystis*, including *C. fimbriata* as the outgroup taxon, produced a single most parsimonious tree (tree length = 247, CI = 0.899, HI = 0.101 and RI = 0.887) (Figure 3). *C. eucalypti*, *Ch. neocaledoniae*, *Ch. australis* and *C. virescens*, all from hardwoods, formed a distinct clade (100 % bootstrap value), with *C. virescens* basal to the other three species in the clade (78 % bootstrap value) and *Ch. neocaledoniae* and *Ch. australis* grouped closely together (100 % bootstrap value). *C. pinicola*, *C. douglasii*, *C. laricicola* and *C. polonica*, all from conifers, formed a clade (100 % bootstrap) sister to the clade formed by the hardwood species.

The *C. pinicola* and *C. douglasii* grouped together (72 % bootstrap), while *C. laricicola* and *C. polonica* grouped together (100 % bootstrap value).

A PAUP analysis of the translated *MAT*-2 HMG box amino acid sequences (45 characters in total) of the *Ceratocystis* spp. studied produced a single most parsimonious tree (tree length = 44, CI = 0.977, HI = 0.023, RI = 0.963) (data not shown). The phylogram based on the translated *MAT*-2 HMG box amino acid sequences had a similar topology to the phylogram based on the *MAT*-2 HMG box DNA sequences. The species from hardwoods formed a single clade (70 % bootstrap value), and *Ch. neocaledoniae* and *Ch. australis* had identical amino acid sequences. The species from conifers formed a single clade (99 % bootstrap value) sister to the clade formed by the hardwood species. *C. laricicola* and *C. polonica* grouped together (74 % bootstrap value) and were distinct from each other.

A phylogenetic analysis of the DNA sequence data of the ITS rDNA region (560 bp in total) of the studied *Ceratocystis* species produced a single most parsimonious tree (length of the shortest tree = 104, CI=0.942, HI=0.058, RI=0.864) (Figure 3). *C. virescens*, isolated from hardwoods, was basal to the other species in the *C. coerulescens* complex (91 % bootstrap value). *C. eucalypti*, *Ch. neocaledoniae* and *Ch. australis*, all isolated from hardwoods, formed a single clade (73 % bootstrap value), with *Ch. australis* and *C. eucalypti* grouping closer together (60 % bootstrap value). The clade formed by the species from conifers, including *C. pinicola*, *C. laricicola* and *C. polonica,* formed a sister clade (100 % bootstrap value) to the clade formed by the hardwood species. *C. douglasii* and *C. pinicola* grouped together (60 % bootstrap value) *and* *C. laricicola* and *C. polonica* grouped together (100 % bootstrap value). The ITS DNA sequence data of *C. laricicola* and *C. polonica* are identical.

DISCUSSION

Phylogenetic analysis of the *MAT*-2 HMG box DNA sequences from species in the *C. coerulescens* complex resulted in a phylogram with a topology similar to the phylogram based on the ITS rDNA sequences previously published by Witthuhn *et al.* (1998b). The phylogram based on *MAT*-2 HMG box DNA sequences further confirmed the monophyletic origin of the *Ceratocystis* species from conifers. The hardwood species formed a monophyletic group only based on the *MAT*-2 HMG box sequences.

The specific primers, EUM2-1 and EUM2-2, designed from the DNA sequence of the *MAT*-2 HMG box in *C. eucalypti*, amplified the *MAT*-2 HMG box from the phylogenetically closely related species, *C virescens*, *Ch. neocaledoniae* and *Ch. australis*, all from hardwoods. *Chalara australis and Ch. neocaledoniae* have no known teleomorphs (Kile & Walker, 1987), but behave as MAT-2 strains in crosses (Kile *et al.*, 1996). It was, therefore, not surprising that the *MAT*-2 HMG box was amplified from these two *Chalara* species. The *C. pinicola MAT*-2 HMG box specific primers, COER2-1 and COER2-2, produced PCR products in *C. douglasii*, *C. laricicola* and *C. polonica*.

A PAUP analysis of the *MAT*-2 mating type gene DNA and amino acid sequences separates the species from hardwoods into a single clade. Based on the ITS rDNA sequences *Ch. australis*, *Ch. neocaledoniae* and *C. eucalypti* formed a single clade, with *C. virescens* basal to all the other species in the *C. coerulescens* complex (Witthuhn *et al.*, 1998b). The phylogram produced from the *MAT*-2 HMG box DNA sequences and translated amino acid sequences suggests the monophyletic origin of the four species from hardwoods. All the species from hardwoods have similar morphological characteristics and it would appear that the monophyletic origin of the

species from hardwoods, including *C. virescens*, may reflect the true phylogenetic relationships within the group.

The phylogenetic analysis of the *MAT*-2 HMG box DNA and translated amino acid sequences of the species from conifers, namely *C. pinicola*, *C. douglasii*, *C. laricicola* and *C. polonica*, formed a clade sister to the clade formed by the hardwood species. The same clustering was observed when a phylogenetic analysis was performed using ITS rDNA sequences. The topology based on the translated amino acid sequences of the *MAT*-2 HMG box was similar to the topology based on the *MAT*-2 DNA sequences. The similar nature of the two phylograms reflects the fact that mutations in the 3rd base position of the translated amino acids are not saturated and thus suitable for phylogenetic analysis.

Harrington & Wingfield (1998) were unable to separate *C. laricicola* from *C. polonica* based on morphological differences. Despite the absence of distinguishing morphological characters the authors considered these fungi to represent distinct taxa, based on their different host species and insect vectors. Crosses between the two species give rise to no perithecia or perithecia with inviable ascospores (Harrington & McNew, 1998). The *MAT*-2 HMG box DNA and translated amino acid sequence differences enables the separation of *C. laricicola* and *C. polonica.*

The *MAT*-2 HMG box DNA sequences of species of *Ceratocystis* are considerably more variable than the ITS rDNA sequence data. The *MAT*-2 HMG box sequences are, therefore, more useful in comparing closely related species, such as *C. laricicola and C. polonica*, than the ITS rDNA sequences. The *MAT*-2 HMG box sequences might be useful in future phylogenetic studies of the species of *Ceratocystis sensu stricto*.

ACKNOWLEDGMENTS

Financial support for this study was provided by the Foundation for Research Development (FRD), South Africa, the United Nations Education, Science and Cultural Organization (UNESCO), the United States Department of Agriculture (USDA) and the members of the Tree Pathology Co-operative Programme (TPCP). The technical assistance of Joe Steimel is greatly appreciated.

REFERENCES

Arie, T., Christiansen, S.K., Yoder, O.C. & Turgeon, B.G. (1997). Efficient cloning of ascomycete mating type genes by PCR amplification of the conserved *MAT* HMG Box. *Fungal Genetics and Biology* **21**, 118-130.

Christiansen, E. & Solheim, H. (1990). The bark beetle-associated blue-stain fungus *Ophiostoma polonicum* can kill various spruces and Douglas fir. *European Journal of Forest Pathology* **20**, 436-446.

DeScenzo, R.A. & Harrington, T.C. (1994). Use of $(CAT)^5$ as a DNA fingerprinting probe for fungi. *Phytopathology* **84**, 534-540.

Harrington, T.C. & McNew, D.L. (1998). Partial interfertility among the *Ceratocystis* species on conifers. *Fungal and General Biology* **25**, 44-53.

Harrington, T.C., Steimel, J.P., Wingfield, M.J. & Kile, G. (1996). Isozyme variation and species delimitation in the *Ceratocystis coerulescens* complex. *Mycologia* **88**, 104-113.

Harrington, T.C. & Wingfield, M.J. (1998). The *Ceratocystis* species on conifers. *Canadian Journal of Botany* **In press**.

Hunt, J. (1956). Taxonomy of the genus *Ceratocystis*. *Lloydia* **19**, 1-58.

Kile, G.A., Harrington, T.C., Yuan, Z.Q., Dudzinski, M.J. & Old, K.M. (1996). *Ceratocystis eucalypti* sp. nov., a vascular stain fungus from eucalypts in Australia. *Mycological Research* **100**, 571-579.

Kile, G.A. & Walker, J. (1987). *Chalara australis* sp. nov. (Hyphomycetes), a vascular pathogen of *Nothofagus cunninghamii* (Fagaceae) in Australia and its relationship to other *Chalara* species. *Australian Journal of Botany* **35**, 1-32.

Münch, E. (1907). Die Blaufäule des Nadelholzes. I-II. *Naturwissenschaftliche Zeitschrift für Forst- und Landwirtschaft* **5**, 531-573.

Felsenstein, J. (1985). Confidence intervals on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783-791.

Redfern, D.B., Stoakley, J.T., Steele, H. & Minter, M. (1987). Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae*. *Plant Pathology* **36**, 467-480.

Siemaszko, W. (1938). Zespoly Grybow Towarzyszacych Kornikom Polskim. *Planta Polonica* **7**, 1-52.

Swofford, D.L. (1993). *PAUP Phylogenetic Analysis using Parsimony Version 3.1.1.* Champaign, IL 61820, USA.

Turgeon, B.D. (1998). Application of mating type gene technology to problems in fungal

biology. *Annual Review of Phytopathology* **36**, 115-137.

Visser, C., Wingfield, M.J., Wingfield, B.D. & Yamaoka, Y. (1995). *Ophiostoma polonicum* is a species of *Ceratocystis sensu stricto*. *Systematic and Applied Microbiology* **18**, 403-409.

Upadhyay, H.P. (1981). A monograph of *Ceratocystis* and *Ceratocystiopsis*. 176 pp. The University of Georgia Press: Athens, Georgia.

Wingfield, M.J., Harrington, T.C. & Solheim, H. (1997). Two new species in the *Ceratocystis coerulescens* complex from conifers in western North America. *Canadian Journal of Botany* **75**, 827-834.

Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J. & Harrington, T.C. (1998a). PCR based identification and phylogeny of species of *Ceratocystis sensu stricto*. *Mycological Research* **In press**.

Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J., Wolfaardt, M. & Harrington, T.C. (1998b). Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. *Mycologia* **90**, 96-101.

Yamaoka, Y., Wingfield, M.J., Takahashi, I. & Solheim, H. (1997). Ophiostomatoid fungi associated with the spruce bark beetle *Ips typographus* in Japan. *Mycological Research* **101**, 1215-1227.

Table 1: Isolates of *Ceratocystis* used in this study, their hosts, and the ITS DNA sequence Genbank accession numbers.

Isolate number[a]	Species	Host	ITS Genbank Accession Number[b]
C490, CMW1323	*C. pinicola*	*Pinus* sp.	U75614
C795	*C. pinicola*	*Pinus* sp.	Not submitted
CMW1016	*C. laricicola*	*Larix decidua*	U75622
C708, CMW0672, CBS228.83	*C. polonica*	*Picea abies*	U75623
C74, CMW0460	*C. virescens*	*Quercus* sp.	U75624
C324, CBS142.53	*C. douglasii*	*Pseudotsuga menziesii*	U75626
C639	*C. eucalypti*	*Eucalyptus sieberi*	U75627
C694, CBS149.83	*Chalara neocaledoniae*	*Coffea robusta*	U75628
C619	*Chalara australis*	*Nothofagus cunninghamii*	U75629
C854	*C. fimbriata*	*Ipomoea batatas*	AF007749

[a]CMW - Culture collection of M. J. Wingfield, Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa. C - Culture collection of T.C. Harrington, Department of Plant Pathology, 351 Bessey Hall, Iowa State University, Ames, Iowa, 50011, USA.. CBS - Centraal Bureau voor Schimmelcultures, Baarn, Netherlands.
[b]Witthuhn *et al.* (1998b).

Figure 1: The *MAT-2* HMG box amino acid sequence data for species of *Ceratocystis sensu stricto* aligned with the *MAT-2* HMG box amino acid sequences for other known pyrenomycetes (Arie *et al.*, 1997). The Genbank Accession numbers of the two *Fusarium* species are indicated in brackets. Amino acids that are homologous between the species are indicated in bold and the position of the intron is indicated by an arrow.

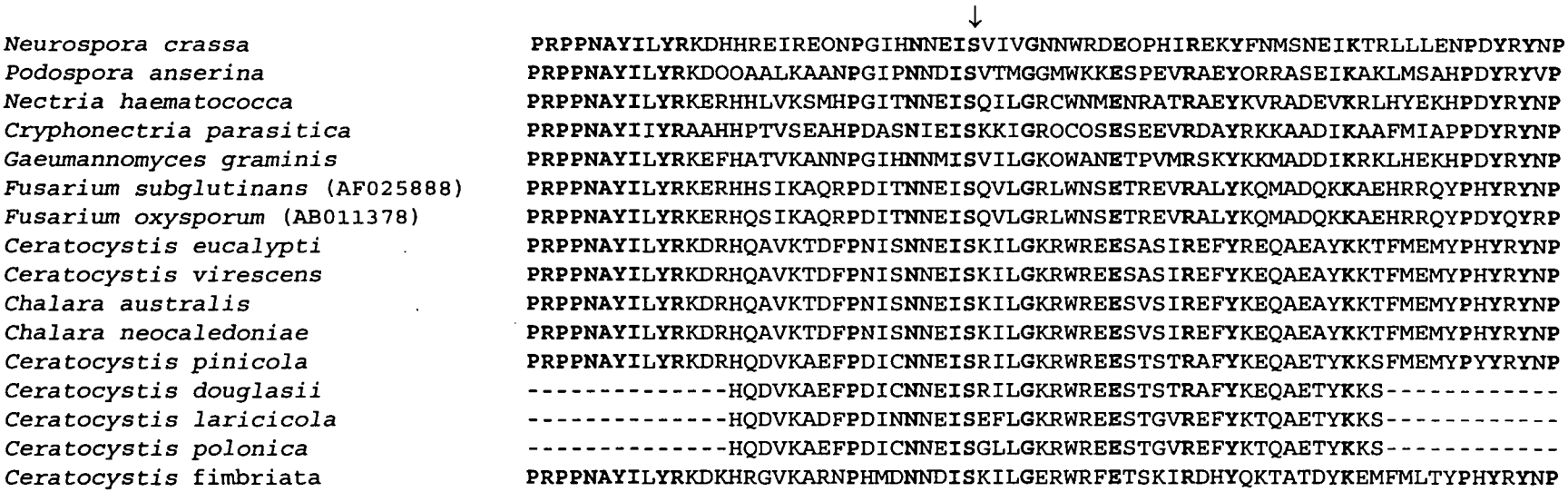

Neurospora crassa PRPPNAYILYRKDHHREIREONPGIHNNEISVIVGNNWRDEOPHIREKYFNMSNEIKTRLLLENPDYRYNP
Podospora anserina PRPPNAYILYRKDOOAALKAANPGIPNNDISVTMGGMWKKESPEVRAEYORRASEIKAKLMSAHPDYRYVP
Nectria haematococca PRPPNAYILYRKERHHLVKSMHPGITNNEISQILGRCWNMENRATRAEYKVRADEVKRLHYEKHPDYRYNP
Cryphonectria parasitica PRPPNAYIIYRAAHHPTVSEAHPDASNIEISKKIGROCOSESEEVRDAYRKKAADIKAAFMIAPPDYRYNP
Gaeumannomyces graminis PRPPNAYILYRKEFHATVKANNPGIHNNMISVILGKOWANETPVMRSKYKKMADDIKRKLHEKHPDYRYNP
Fusarium subglutinans (AF025888) PRPPNAYILYRKERHHSIKAQRPDITNNEISQVLGRLWNSETREVRALYKQMADQKKAEHRRQYPHYRYNP
Fusarium oxysporum (AB011378) PRPPNAYILYRKERHQSIKAQRPDITNNEISQVLGRLWNSETREVRALYKQMADQKKAEHRRQYPDYQYRP
Ceratocystis eucalypti PRPPNAYILYRKDRHQAVKTDFPNISNNEISKILGKRWREESASIREFYREQAEAYKKTFMEMYPHYRYNP
Ceratocystis virescens PRPPNAYILYRKDRHQAVKTDFPNISNNEISKILGKRWREESASIREFYKEQAEAYKKTFMEMYPHYRYNP
Chalara australis PRPPNAYILYRKDRHQAVKTDFPNISNNEISKILGKRWREESVSIREFYKEQAEAYKKTFMEMYPHYRYNP
Chalara neocaledoniae PRPPNAYILYRKDRHQAVKTDFPNISNNEISKILGKRWREESVSIREFYKEQAEAYKKTFMEMYPHYRYNP
Ceratocystis pinicola PRPPNAYILYRKDRHQDVKAEFPDICNNEISRILGKRWREESTSTRAFYKEQAETYKKSFMEMYPYYRYNP
Ceratocystis douglasii ----------HQDVKAEFPDICNNEISRILGKRWREESTSTRAFYKEQAETYKKS----------
Ceratocystis laricicola ----------HQDVKADFPDINNNEISEFLGKRWREESTGVREFYKTQAETYKKS----------
Ceratocystis polonica ----------HQDVKAEFPDICNNEISGLLGKRWREESTGVREFYKTQAETYKKS----------
Ceratocystis fimbriata PRPPNAYILYRKDKHRGVKARNPHMDNNDISKILGERWRFETSKIRDHYQKTATDYKEMFMLTYPHYRYNP

Figure 2: The aligned *MAT-2* HMG box DNA sequences (208 bp total) of the species in the *Ceratocystis coerulescens* complex.

```
                          10        20        30        40        50
                 12345678901234567890123456789012345678901234567890
C.eucalypti      GACATCAAGCCGTCAAGACCGACTTTCCAAACATATCGAATAATGAAATT
C.virescens      GACATCAAGCAGTCAAGACTGACTTTCCAAACATATCGAATAATGAAATC
Ch.neocaledoniae GACATCAAGCCGTCAAGACCGACTTTCCGAACATATCGAATAATGAAATT
Ch.australis     GACATCAAGCCGTCAAGACCGACTTTCCGAACATATCGAATAATGAAATT
C.pinicola       GACACCAAGACGTCAAAGCCGAATTTCCAGACATTTGCAACAATGAAATT
C.douglasii      GACACCAAGACGTCAAAGCCGAATTTCCAGACATTTGCAACAATGAAATT
C.laricicola     GACACCAAGACGTCAAAGCCGATTTTCCAAATATCAACAACAATGAAATC
C.polonica       GACACCAAGACGTCAAAGCCGAATTTCCAAATATCAACAACAATGAAATT
C.fimbriata      AACATCGTGGCGTTAAGGCTAGGAATCCTCATATGGACAATAATGATATT

                          60        70        80        90       100
                 12345678901234567890123456789012345678901234567890
C.eucalypti      TGTAAGTATCCATAGCTCTTCAGATTCTCAACATAC-GGAAGAAACATGT
C.virescens      TGTAAGTATCCATGGCTTTTCAGATTTTCTACACAT-GGAGGTGATTGGT
Ch.neocaledoniae TGTAAGTATCTATAGCTCTTCAAATTTTCAACATGTGGGAAGAAATCCAT
Ch.australis     TGTAAGTATCTATAGCTCTTCAAATTTTCAACATGC-GGAAGAAATCCAT
C.pinicola       TGTAAGCGCTCACAGCTCATTAA--------TCTATATAGGCCTATAATT
C.douglasii      TGTAAGCACCCACAGCTCATTAA--------TCTTTATAGGCCTATAATC
C.laricicola     TGTAAGCGCCCACAGCTCACTAA--------TCTATATAAGCCTTTTATT
C.polonica       TGTAAGCGCCCACAGCTCACTAA--------TCTATATAGGTCTCTATTT
C.fimbriata      TGTAAG----CTTTCCCCAGCCTGCCACGACCGAGTAAATGCTGACAACC

                         110       120       130       140       150
                 12345678901234567890123456789012345678901234567890
C.eucalypti      TCGCTAATCTATATTCATTCAGCAAAAATTCTCGGCAAACGTTGGAGAGA
C.virescens      TCGCTAATCTATGTCCAATCAGCCAAAATTCTAGGTAAACGTTGGAGAGA
Ch.neocaledoniae TCGCGAATCTATATTCATTCAGCAAAAATTCTCGGCAAACGTTGGAGAGA
Ch.australis     TCGCTAATCTATATTCATTCAGCAAAAATTCTCGGCAAACGTTGGAGAGA
C.pinicola       CTCTGACACGTCTTTGTTTTAGCAAGAATTCTCGGTAAACGCTGGAGAGA
C.douglasii      TTCTGACACGTCTTTGATCCAGCAA-AATTCTCGGTAAACGCTGGAGAGA
C.laricicola     CTCTGACACGTTTGATTT-AGCGAGAGTTTCTCGGTAAACGCTGGAGAGA
C.polonica       CTCTGACACGTTTGATTT-AGCAAGGGCTTCTCGGTAAACGCTGGAGAGA
C.fimbriata      TTCTAGCAATATGGCTAGGCGAGCGGTGGAGATTTGAAAC-CTC----GA

                         160       170       180       190       200
                 123456789012345678901234567890123456789012345678901234567890
C.eucalypti      AGAATCAGCTAGCATCCGTGAATTTTACAGAGAGCAGGCCGAAGCATACAAAAAGACA
C.virescens      AGAATCAGCCAGCATTCGTGAATTTTACAAAGAGCAGGCGGAAGCATACAAAAAGACA
Ch.neocaledoniae AGAATCAGTCAGCATCCGTGAATTTTACAGAGAGCAGGCCGAAGCATACAAAAAGACA
Ch.australis     AGAATCAGTCAGCATCCGTGAATTTTACAGAGAGCAGGCCGAAGCATACAAAAAGACA
C.pinicola       AGAATCTACTAGCACTCGAGCATTCTATAAAGAACAAGCTGAAACTTACAAGAAAAGC
C.douglasii      AGAGTCTACTAGTATTCGAGATTTCTATAAAGAACAAGCTGAAACTTACAAGAAAAGC
C.laricicola     AGAGTCTACTGGCGTTCGAGAATTCTATAAAACGCAAGCTGAAACTTACAAGAAAAGC
C.polonica       AGAGTCTACTGGCGTTCGAGAATTCTATAAAACGCAAGCTGAAACTTACAAGAAAAGC
C.fimbriata      AGA-----------TTCGGGACCATTACCAAAAGACGGCCACAGATTACAAGGAAATG
```

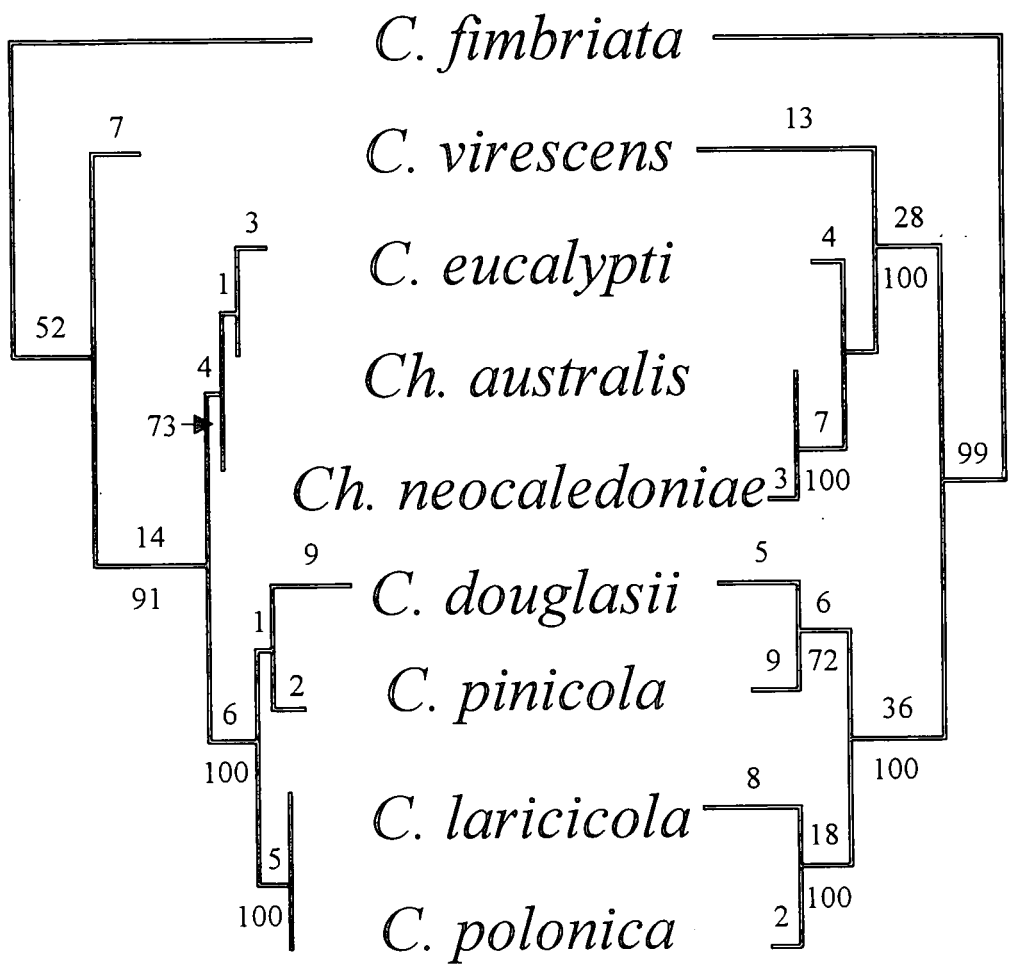


Figure 3: The single most parsimonious tree produced from the aligned 208 bp of the *MAT*-2 HMG box DNA sequences (right) shows a similar topology to the single most parsimonious tree produced from the aligned 560 bp ITS rDNA sequences (left). The number of base substitutions are indicated above the branch points. Bootstrap values (100 replicates) are indicated below the branches.

CHAPTER 6: SUMMARY

Most species of *Ceratocystis sensu stricto* are virulent pathogens of a wide variety of plants. In the research presented in this thesis, I have developed a rapid and reliable PCR-based RFLP identification method for species of *Ceratocystis*. A 1.6 kb fragment within the ribosomal DNA operon was directly amplified from living fungal tissue, without extracting DNA. The amplified fragment included part of the small and large sub-unit rRNA genes, the 5.8S rRNA gene and the internal transcribed spacers 1 and 2. The PCR fragments were digested with eighteen restriction enzymes. Four of these (*Alu*I, *Dra*I, *Hae*III and *Rsa*I) produced RFLPs that separated the species of *Ceratocystis*. The 1.6 kb PCR products, from the better known species of *Ceratocystis*, were sequenced and phylogenetically analyzed. The delimitation of taxa was consistent with results of previous studies using isozymes and rDNA sequence analysis.

Ceratocystis coerulescens is a well-known cause of blue stain in spruce and pine. It was shown that *C. coerulescens* encompasses at least five morphological types. I compared isolates of *C. coerulescens sensu lato* and morphologically similar species on the basis of ITS DNA sequences. A 600 bp fragment within the ribosomal DNA operon, including the 5.8S rRNA gene and ITS 1 and 2, was amplified, sequenced and analyzed using PAUP. The five morphological types previously known as *C. coerulescens*, and the two other taxa from conifers, formed a strongly-supported monophyletic group that includes all the *Ceratocystis* species occurring primarily on conifers. The species from hardwood trees, *C. eucalypti*, *Ch. australis* and *Ch. neocaledoniae*, also formed a monophyletic group, sister to the conifer group. The fourth species from hardwoods, *C. virescens,* formed a group basal to the two sister groups.

The phylogeny of species in the *C. coerulescens* complex based on ITS DNA sequences were compared to the phylogeny based on the *MAT*-2 HMG box DNA sequences. A 210 bp PCR fragment of part of the *MAT*-2 HMG box of species in the *C. coerulescens* complex was amplified. *C. fimbriata* was used as the outgroup taxon and was distinct from the other *Ceratocystis* species studied. The species from conifers formed a single clade, sister to the clade formed by the species from hardwoods. Phylogenetic analysis of the *MAT*-2 HMG box DNA sequences differed slightly from the phylogenetic analysis based on ITS DNA sequences. Based on ITS DNA sequences *C. virescens* was basal to the other species in the *C. coerulescens* complex, while *C. laricicola* and *C. polonica* could not be separated from each other. Differences in the *MAT*-2 HMG box DNA sequences for the latter two species clearly showed them to be as distinct from each other.

Recent mating studies on *C. coerulescens* have prompted a study of the expression of mating type genes in species of *Ceratocystis sensu stricto*. *C. eucalypti* is strictly heterothallic. Most other *Ceratocystis* species, including *C. virescens*, *C. coerulescens* and *C. pinicola* are homothallic. The MAT-2 strains are self-fertile, while MAT-1 strains are self-sterile and grow more slowly than MAT-2 strains. Part of the *MAT*-2 idiomorph in *C. eucalypti*, *C. virescens* and *C. pinicola* was amplified using degenerate primers designed from the conserved *MAT*-2 HMG DNA binding motif. The expected ~300 bp PCR products were cloned and sequenced. Specific primers were designed that amplified a 210 bp fragment only in MAT-2 isolates of *C. eucalypti*, *C. virescens* and *C. pinicola*. This fragment was absent from the self-sterile (MAT-1) progeny of *C. virescens* and *C. pinicola*, confirming the deletion of *MAT*-2 during uni-directional mating type switching. The known DNA sequence data for the *C. eucalypti MAT*-2 mating type idiomorph was increased from 280 bp to 1 371 bp, using TAIL-PCR and uneven PCR.

CHAPTER 7: OPSOMMING

Die meeste spesies in *Ceratocystis sensu stricto* is virulente patogene van 'n wye verskeidenheid plante. Die navorsing saamgevat in hierdie tesis sluit die ontwikkeling van 'n vinnige en betroubare PKR-gebaseerde, RFLP-identifikasie van die *Ceratocystis* spesies in. 'n 1.6 kb fragment binne die ribosomale DNA operon was direk vanaf lewendige fungus materiaal geamplifiseer, sonder dat die ekstraksie van DNA plaasgevind het. Die geamplifiseerde fragment het die klein en groot sub-eenhede van die rRNA gene, asook die ITS 1 en 2 gebiede ingesluit. Die PKR fragmente was met agtien beperkingsensieme gesny. Vier van hierdie ensieme (*Alu*I, *Dra*I, *Hae*III en *Rsa*I) het RFLPs geproduseer wat die species in *Ceratocystis* van mekaar onderskei het. Die basispaaropeenvolging van die 1.6 kb PKR produkte van die bekendste *Ceratocystis* spesies was bepaal en filogeneties ontleed. Die filogenie van die taksa was ooreenstemmend met resultate van vorige studies gebaseer op iso-ensieme en die analise van rDNA basispaaropeenvolgings.

Ceratocystis coerulescens is 'n bekende oorsaak van blou vlek in sparden en dennebome. Daar is reeds uitgewys dat *C. coerulescens* uit ten minste vyf morfologies soortegelyke tipes bestaan. Die isolate van *C. coerulescens sensu lato* en morfologies soortgelyke spesies was op die basis van ITS DNA basispaaropeenvolgings met mekaar vergelyk. Die basispaaropeenvolging van 'n 600 bp fragment binne die ribosomale DNA operon, insluitende die ITS 1 en 2 gebiede, sowel as die 5.8 S rRNA gene, was bepaal en filogeneties ontleed. Die vyf morfologiese tipes, voorheen bekend as *C. coerulescens*, asook die ander twee taksa vanaf konifers, het 'n sterk ondersteunde monofiletiese groep gevorm, wat al die spesies van konifers ingesluit het. Die spesies van hardehoutbome, *C. eucalypti*, *Ch. australis* en *Ch. neocaledoniae*, het ook 'n monofiletiese groep

gevorm, en was 'n sustersgroep van die spesies van konifers. Die vierde spesie van hardehout, *C. virescens*, was basaal tot die twee sustersgroepe.

Die filogenie van spesies in die *C. coerulescens* kompleks, gebaseer op ITS DNA basispaaropeenvolging, is vergelyk met die filogenie gebaseer op die *MAT*-2 HMG boks DNA basispaaropeenvolging. 'n 210 bp PKR fragment van 'n gedeelte van die *MAT*-2 HMG boks van spesies in die *C. coerulescens* komplek was geamplifiseer. *C. fimbriata* was gebruik as die buite groep en het afsonderlik van die ander bestudeerde *Ceratocystis* spesies gegroepeer. Die spesies vanaf konifers het 'n enkele groep gevorm en was 'n sustersgroep van die hardehout spesiegroep. Filogenetiese analise van die *MAT*-2 HMG boks DNA basispaaropeenvolging het effens verskil van die filogenetiese studie wat op die ITS DNA basispaaropeenvolging gebaseer was. Volgens die ITS DNA basispaaropeenvolging was *C. virescens* basaal tot die ander spesies in die *C. coerulescens* kompleks, terwyl *C. laricicola* en *C. polonica* nie van mekaar onderskei kon word nie. Verskille in die *MAT*-2 HMG boks DNA basispaaropeenvolging van die twee laasgenoemde spesies onderskei diè as twee afsonderlike spesies.

Onlangse parings van *C. coerulescens* het 'n studie aangaande die uitdrukking van die paringstipe gene van spesies in *Ceratocystis sensu stricto* geïnisieer. *C. eucalypti* is streng heterotallies. Die meeste ander *Ceratocystis* spesies, insluitende *C. virescens, C. coerulescens* en *C. pinicola*, is homotallies. Die MAT-2 stamme is self-fertiel, terwyl die MAT-1 stamme self-steriel is en stadiger groei as die MAT-2 stamme. 'n Gedeelte van die *MAT*-2 idiomorf in *C. eucalypti*, *C. virescens* en *C. pinicola* was geamplifiseer deur gebruik te maak van gedegenereerde peilers. Die peilers was ontwerp vanaf die gekonserveerde *MAT*-2 HMG DNA bindingsmotief. Die verwagte ~300 bp PKR fragmente was gekloneer en die DNA basispaaropeenvolging was daarna bepaal. Spesifieke peilers was ontwerp vir die amplifikasie van 'n 210 bp fragment in slegs MAT-2 isolate

van *C. eucalypti*, *C. virescens* en *C. pinicola*. Hierdie fragment was nie teenwoordig in die self-steriele (MAT-1) nageslag van *C. virescens* en *C. pinicola* nie. Hierdie bevinding bevestig die verwydering van *MAT*-2 gedurende een-rigting paringstipe omskakeling. Die bekende DNA basispaaropeenvolging van die *C. eucalypti MAT*-2 paringstipe idiomorf was vanaf 280 bp tot 1 371 bp uitgebrei, deur van TAIL-PKR en ongelyke PKR gebruik te maak.