MOLECULAR CHARACTERISATION OF SELECTED OPHIOSTOMATOID FUNGI

A thesis submitted in fulfilment of the requirements for the degree of Magister Scientiae in the Faculty of Natural Sciences, Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein

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Declaration

I, the undersigned, hereby declare that the dissertation herewith submitted for the degree of Magister Scientiae to the University of the Orange Free State, contains my own independent work and has hitherto not been submitted for any degree at any other University.

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TABLE OF CONTENTS

Chapter 1

Literature Review

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PREFACE

Ceratocystis sensu lato includes over a hundred different species that are accommodated in four genera, namely *Ophiostoma, Ceratocystis sensu stricto, Ceratocystiopsis* and *Gondwanomyces*. These fungi are widely distributed throughout the world. The ophiostomatoid fungi include insect-associated plant pathogens, human pathogens, wood staining fungi as well as fungi of biotechnological significance.

The teleomorphs produced by the ophiostomatoid fungi are morphologically similar, but a remarkably diversity is observed in the morphology of anamorphs produced by these fungi. The diversity of anamorphs, their adaptation to insect dispersal and different habitats occupied by these fungi has resulted in a number of taxonomic controversies, many of which persist today.

In 1981, Upadhyay published a well-documented monograph of *Ceratocystis sensu lato*. However, even today, the gathering of new information on these fungi, demands a re-evaluation of the classification and placement of many species, new species are being discovered and others are being increasingly better understood. The use of molecular characters to clarify the phylogenetic relationships between fungi has proven successful and is gaining substantial support. Through these approaches, a deeper understanding of the taxonomy and phylogeny of the ophiostomatoid fungi is emerging.

This thesis includes four chapters in which the taxonomy of selected species of ophiostomatoid fungi are reconsidered using partial DNA sequence data from the ribosomal DNA (rDNA) genes. The aim has been to resolve various controversies often based on morphological classifications of these species.

The first chapter presents an overview of the literature pertaining to the taxonomy of the ophiostomatoid fungi, the use of the ribosomal RNA operon in taxonomy and the value of molecular techniques in the classification of fungi. Due to the diversity of all the available morphological characters for fungal classification, it is often very difficult to correctly place a species or genus. Molecular techniques have enabled researchers to reconsider earlier problematic or difficult taxonomic placements. However, it is important to recognise that neither molecular nor morphological data alone will resolve problems and combined data sets are most desireable.

The second chapter of this thesis deals with the re-classification of *Ophiostoma polonicum*. *Ophiostoma polonicum* was first isolated in 1939 in Poland and described by Siemaszko. The fungus was later described as producing a *Leptographium* anamorph similar to that of *Ophiostoma penicillatum*. The presence of a *Chalara* anamorph was, however, recently observed in collections of the fungus from Europe and Japan. This led to a need, to reconsider the taxonomic placement of *O. polonicum*.

Ceratocystis autographa is unusual amongst the ophiostomatoid fungi in that it is reported to produce two anamorphs with distinct modes of conidial development. The anamorphs described for this fungus were a *Chalara*-like state, characteristic of the genus *Ceratocystis s.str.* and a *Sporothrix* anamorph typical of *Ophiostoma*. The aim of chapter three was to restudy the taxonomic placement of *C. autographa* and cultures from *Juniperus*, thought to represent this fungus.

In chapter four a species of *Leptographium* that has been thought to be related to *Leptographium penicillatum*, is reconsidered. Although it is morphologically quite distinct from that species. Comparisons were thus made between this fungus from the insect *Hylurgops palliatus* and other well-defined *Leptographium* species.

The studies included in this thesis form part of an ongoing process to correctly classify fungi in *Ceratocystis s.l.* The valuable incorporation of molecular tehniques when morphological characters alone are not sufficient for classification purposes, is demonstrated. Using partial DNA sequence data obtained from the rDNA operon, we were able to clarify some controversial issues pertaining to the ophiostomatoid fungi.

CHAPTER 1

LITERATURE REVIEW

"Among the fungi, identification and classification have always presented some special challenges. Molecular biological approaches can do much to answer some perennial questions about how fungi are organised."

Metzenberg, 1991

1. INTRODUCTION

The ophiostomatoid fungi are comprised of four genera, *Ceratocystis sensu stricto* Ellis & Halst., *Ophiostoma* Syd. & P. Syd., *Gondwanomyces* Marais & Wingfield and *Ceratocystiopsis* Upadh. & Kend. These fungi have a cosmopolitan distribution on a wide variety of substrates (Upadhyay, 1993). The group is economically important due to the pathogenicity of some species on important crops and trees (Upadhyay, 1993). The ophiostomatoid fungi are adapted to insect dispersal of which the most common insect vectors are bark beetles (Coleoptera: Scolytidae) (Malloch & Blackwell, 1993). These fungi have adapted conidia, which are produced in slimy masses, enabling them to be carried by insects (Upadhyay & Kendrick, 1974).

Researchers have found that morphological characteristics alone are insufficient to reveal the phylogenetic relationships between most of the ophiostomatoid fungi. They have concluded that the incorporation of additional characters into taxonomic issues would be valuable for comparative purposes. The utilisation of biochemical and molecular techniques into the classification of the ophiostomatoid fungi has been a major area of activity in recent years (Hausner, Reid & Klassen, 1992; Seifert, Wingfield & Wingfield, 1995). Taxonomists have found that not all areas of the genome are equally suitable for the use of molecular techniques to deduce the taxonomy and phylogeny of particular groups of organisms (Bruns, White & Taylor, 1991). Kendrick (1993) stated that sequence data could provide decisive evidence on taxonomic placings, especially in the ophiostomatoid fungi. Morphological studies would, however, always be of importance and should be used concurrently with molecular studies. It is thus important for taxonomists to recognise that a good classification must utilise every known aspect of an organism (Kendrick, 1993).

2. THE GENUS *CERATOCYSTIS SENSU LATO*

The genus *Ceratocystis sensu stricto* Ellis & Halst. was first established in 1890, for species producing *Chalara* anamorphs through ring wall building (Halsted, 1890; Samuels, 1993). *Ceratocystis fimbriata* Ellis & Halst. was designated as the type species for this genus (Halsted, 1890). In 1919, another genus, *Ophiostoma* Syd. & P. Syd., was established for species with holoblastic sympodial conidium development. The designated type for this genus was *Ophiostoma piliferum* (Fr.) C. Moreau (Sydow & Sydow, 1919). A third genus, *Ceratocystiopsis* Upadh. & Kend., was later added to accommodate taxa with sheathed, elongate to falcate ascospores (Upadhyay & Kendrick, 1975).

Species of *Ceratocystis sensu lato* mostly occur in the Northern Hemisphere (Griffin, 1966; Lagerberg, Lundberg & Melin, 1927; Mathiesen-Kaärik, 1960; Solheim, 1986; Upadhyay, 1981). Some species have, however, also been discovered in various places in the Southern Hemisphere such as Brazil, Mexico, New Zealand, South Africa and Chile (Butin & Aquilar, 1984; Gorter, 1977; Hutchinson & Reid, 1988; Marais & Wingfield, 1994; Samuels & Müller, 1978). Species have also been recorded from various parts in Asia (Yamaoka, Wingfield &Takahashi, 1994).

2.1. Morphological characters

The shape and size of ascocarps, asci and ascospores was first used as a primary characteristic for identification of the ophiostomatoid fungi (De Hoog & Scheffer, 1984; Hunt, 1956; Upadhyay, 1993). The availability of teleomorph states was, therefore, very important for classification purposes. Although the teleomorphs produced by the ophiostomatoid fungi are morphologically similar, a diversity of anamorphs are produced (Mouton, Wingfield & Van Wyk, 1994). Characters associated with the production of anamorphs are also considered important i.e. type of conidiogenesis and the shape and size of the conidia produced (Hunt, 1956; Nag Raj & Kendrick, 1975; Wingfield, Kendrick & Van Wyk, 1991).

There are two distinct anamorph types occurring in the ophiostomatoid fungi namely the *Chalara* type with enteroblastic conidiogenesis and the *Graphium, Sporothrix* type with holoblastic conidiogenesis (De Hoog & Scheffer, 1984; Hunt, 1956; Mouton *et al.*, 1994; Samuels, 1993). Most mycologists accept the concepts of Von Arx (1974), restricting species with phialidic anamorphs (*Chalara*) to *Ceratocystis s.str.* In contrast species with *Sporothrix, Graphium, Leptographium* or similar states forming conidia through blastic sympodial development, are included in the genus *Ophiostoma* (Upadhyay, 1993).

2.2. Cell Wall and Physiological characters

Rosinski & Campana (1964), reported the occurrence of cellulose in the hyphal cell walls of fungi. Cellulose was found to be present in the cell walls of *Ophiostoma ulmi* (Buismann) Nannf., but absent in the cell walls of *Ceratocystis fagacearum* (Bretz) Hunt (Rosinski & Campana, 1964). Smith, Patik & Rosinski (1967), further found cellulose to be absent in the cell walls of *C. fimbriata* and suggested that the absence or presence of cellulose could be correlated to the type of conidiogenesis of each of these fungi. These authors further concluded that cellulose is present only in species with exoconidial development (ring wall building described by Minter, Kirk & Sutton, 1982). After investigation of several *Ophiostoma* and *Ceratocystis* species, Spencer & Gorin (1971), observed a relationship between the polysaccharides present in the cell walls and the type of anamorph. Species producing *Graphium* or *Sporothrix* anamorphs were found to contain rhamnose in their cell walls, but this compound was absent in species with *Chalara* anamorphs (Spencer & Gorin, 1971). Jewell (1974), also observed the distribution of cellulose in the cell walls of certain ophiostomatoid fungi and confirmed the findings of Smith *et al.* (1967), that a correlation exists between the type of conidium development (exoconidial or endoconidial) and the presence or absence of cellulose in the cell walls. Likewise, Weijman & De Hoog (1975) found a correlation between the contents of the cell walls and the type of anamorph produced for species of the ophiostomatoid fungi. These findings supported the work of De Hoog (1974) and Von Arx (1974) who separated *Ophiostoma* and *Ceratocystis* based on their anamorphs. Therefore, presence or absence of certain polysaccharides is one of the most important characters, after conidium development, to distinguish between species of *Ophiostoma* and *Ceratocystis* (De Hoog & Scheffer, 1984).

The discovery of the effect of the antibiotic, cycloheximide, by Fergus (1956), on species of the ophiostomatoid fungi, has also had a profound effect on the classification of these fungi. Fergus (1956) noted that cycloheximide had a very low degree of inhibition on species of *Ophiostoma*. Species with *Chalara* anamorphs are sensitive to cycloheximide, while species with anamorphs other than *Chalara* are tolerant to this antibiotic (Harrington, 1981). Harrington (1981) thus used cycloheximide tolerance to support the division of the genera *Ceratocystis* and *Ophiostoma* by correlating this character with cell wall composition and anamorph produced.

2.3. Pathogenicity

The ophiostomatoid fungi not only have a wide distribution, but also occur on a variety of substrates (Upadhyay, 1993). These substrates include trees (Griffin, 1966; Lagerberg *et al.*, 1927; Solheim, 1986), *Protea* spp. (Marais & Wingfield, 1994; Wingfield, Van Wyk, & Marasas, 1988), dung (Upadhyay, 1981) and agricultural crops such as sweet potato (*Ipomoea batatas* [L.] Lam.) (Halsted, 1890; Olsen & Martin, 1949). Some ophiostomatoid fungi are known as primary pathogens causing death and destruction of their hosts, while most species in this group are opportunistic pathogens and mainly saprophytes (Kile, 1993).

Ophiostoma ulmi Buism. and *O. novo-ulmi* Brasier are the best known and also most destructive pathogens amongst the *Ophiostoma* species. These fungi have been responsible for the destruction of the elm trees in Europe as well as Great Britain and parts of North America (Brasier, 1991; Gibbs, 1974; Gibbs, Burdekin & Brasier, 1977). The destruction of elm trees was significant because of their important uses for people in Britain. Outbreaks spread through trade with infected wood (Brasier, 1991; Gibbs, 1978).

Three different varieties of *Leptographium wageneri* (Kendrick) Wingfield [the anamorph of *Ophiostoma wageneri* (Goheen & Cobb) Harrington] are responsible for considerable losses of conifers in North America and are found on pine as well as fir trees (Harrington & Cobb, 1987). Introduction of these fungi into the host occurs by root feeding bark beetles. The developing symptoms are similar to wilt diseases of deciduous trees with radial black streaks being observed in the wood (Harrington & Cobb, 1983; Smith & Graham, 1975).

Ceratocystis fimbriata is an important pathogen on a variety of hosts, including sweet potatoes and rubber trees (Halsted, 1890; Halsted & Fairchild, 1891; Olsen & Martin, 1949; Wood & French, 1963). *Ceratocystis fagacearum*, also known as the oak wilt fungus, is another pathogen causing major damage to oak trees in the United States (Appel, Drees & Johnson, 1985). This fungus has also been considered as a biological control agent to kill oak trees competing with more economically important pine trees (French & Schroeder, 1969). The diseases caused by species of *Ceratocystis* are varied and listed fully in other publications (Bramble & Holst, 1940; Davidson, 1979; Davidson, Francke-Grosmann & Kaärik, 1967; Gibbs, 1974; Kile, 1993).

Sapstain or blue stain is caused by pigmented ophiostomatoid fungi in the sapwood of trees resulting in the discoloration of the wood (Gibbs, 1993; Lagerberg *et al.*, 1927; Seifert, 1993). The damages caused is, however, only visual and not structural as in the case of soft rot or decay fungi and the colonising fungi have no effect on the strength of the wood (Gibbs, 1993; Seifert, 1993). The seriousness of blue stain is of great concern in timber industry (Seifert, 1993). Ironically, stained wood products are sometimes in demand due to the pigmented patterns caused by the invading fungi (Gibbs, 1993).

2.4. Insect Associations

Ophiostomatoid fungi are closely associated with and frequently carried by insects of which the bark beetles (Coleoptera: Scolytidae) are the most common vectors (Lagerberg *et al.*, 1927; Malloch & Blackwell, 1993; Upadhyay & Kendrick, 1974; Wingfield, 1993). Many of the bark beetles have adapted specialised structures (mycangia) to hold and culture these fungi (Paine, Stephen & Cates, 1993; Solheim, 1993). The elongated ascocarp necks, bearing masses of slimy ascospores at their apices, also provide an important adaptation for distribution by insects (Wingfield, 1993; Wingfield, Wingfield & Kendrick, 1994). The fruiting structures of these fungi are often found in bark beetle galleries under the bark of the host trees. The emerging beetles may then serve as the spore dispersal agents. Although neck length is thought to be an aid for insect dispersal, even species without necks or with very short necks are associated with insects (Wingfield, 1993).

Upadhyay and Kendrick (1974) observed that specific insect vectors carry the conidia of species of *Ceratocystis s.str.* Species of *Ceratocystis s.str.*, are also associated with non-specific insect vectors such as flies and nitidulid beetles (Dowding, 1973; Juzwik & French, 1983). Species of *Ophiostoma*, in contrast, have very specific associations with insect vectors, especially bark beetles (Upadhyay, 1981). *Ophiostoma penicillatum* (Grosmann) Siemaszko is an example of a very specific relationship, as this fungus is always found associated with *Ips typographus* L. on spruce (Solheim, 1986). The fungus-vector relationships are sometimes more casual, as in the case of *O. piceae* (Münch) Syd. & P. Syd., which has been observed in association with many different bark beetle-fungus complexes (Mathiesen, 1950; Mathiesen-Kaärik, 1953). Many of the sapstaining ophiostomatoid fungi are also closely linked with bark beetles acting as vectors (Gibbs, 1993; Harrington, 1993).

3. PROBLEMS IN CLASSIFICATION

Sneath (1989) stated that the ultimate aims of a taxonomist are to classify an organism for further use by scientists, to identify an organism and to produce data-bases that summarise as much relevant information about the organisms as possible.

Morphological characteristics have always been used for the classification of organisms (Lane *et al.*, 1985). Until recently, the ophiostomatoid fungi were identified by the shape and size of their ascocarps, asci and ascospores (Upadhyay, 1993). Anamorphs have also been used in the taxonomy of the ophiostomatoid fungi due to their diversity (De Hoog & Scheffer, 1984). Anamorphs were, however, found to be highly sensitive to the age of the organism or the accumulation of staling products in the growth medium or substrate on which they had been incubated (De Hoog & Scheffer. 1984; Nag Raj & Kendrick, 1975).

The use of morphological characters for the classification of microorganisms does not always correlate well with natural relationships (Lane *et al.*, 1985). Phenotypic characters (physiological reactions and morphology) have an unknown genetic basis and intuition must sometimes be relied on by mycologists for interpretation in classifications (Kurtzman, 1989). Also, a single anamorph species could be connected to more than one teleomorph and it is clear that the grouping of anamorphs by morphological similarity alone, is often misleading (Kendrick, 1981). The need has, therefore, arisen for the incorporation of molecular characters in the classification of eukaryotic and prokaryotic organisms (Bruns, White & Taylor, 1991; Kendrick, 1993; Lane *et al.*, 1985). DNA sequence data can be a very useful and valuable tool for the determination of the phylogenetic placement of a group of organisms in the presence of doubtful morphological information (Brasier, 1993; Halanych, 1996; Hillis, 1987; Kohn, 1992).

4. MOLECULAR SYSTEMATICS

Molecular techniques have provided us with reliable tools for the identification of individuals within a species, varieties of the same fungal species or different species of the same genus (Brasier, 1993; Kohn, 1992; Taylor, 1986; Trigiana, Caetanoanollea, Bassam & Windham, 1995). Berbee and Taylor (1992a) found that phylogenetic trees obtained using data from the ribosomal DNA (rDNA) genes, could clarify relationships between fungi, even though no sexual structures were produced by the organisms in question.

The discovery of the powerful Polymerase Chain Reaction (PCR) technique (Saiki *et al.*, 1988), has facilitated the application of molecular technology in sytematic studies. Molecular techniques are now used extensively to clarify phylogenetic relationships in the fungi (Berbee & Taylor, 1992a; b; Bruns, White & Taylor, 1991; Kohn, 1992). Researchers have found that the use of morphological characters are not sufficient for phylogenetic studies and that molecular characters are very helpful in resolving phylogenetic relationships in the fungi (Berbee & Taylor, 1992a; b; Bruns *et al.*, 1991; Halanych, 1996; Kohn, 1992; Patterson, Williams & Humphries, 1993; Visser *et al.*, 1995; Wingfield, De Beer, Visser & Wingfield, 1996; Wingfield, Grant, Wolfaardt & Wingfield, 1994). Hausner *et al.* (1993) confirmed the separation of *Ceratocystis* and *Ophiostoma* based on their Restriction Fragment Length Polymorphism (RFLP) and sequence studies. Sibling species are also more readily identified using molecular than morphological methods (Brasier, 1993; Peterson & Kurtzman, 1991).

When using molecular techniques, it is first necessary to determine the level at which a character varies and then to decide which technique is applicable (Kohn, 1992). Careful consideration is thus of utmost importance when choosing a specific molecular technique. The costs of a specific technique must also be considered, as sequencing is for example a lot more expensive than RFLP studies. It is sometimes, wise to first use a "cheaper" technique and then proceed with more expensive and extensive techniques depending on the level of clarification desired. Partial sequencing of the rDNA genes has proven to be very successful for the identification of individual ophiostomatoid fungi (Berbee & Taylor, 1992a; Hausner *et al.* 1993; Visser *et al.*, 1995; Witthuhn *et al.*, 1998).

4.1. The ribosomal DNA (rDNA) genes

Ribosomal DNA genes are present in every living cell and play an important role in protein synthesis. These genes are thus relatively stable (Blanz & Unseld, 1988). Although conserved, these genes contain highly variable regions, even amongst related taxa (McCarroll *et al.*, 1983; Woese, 1987) (Fig. 1). These genes are found in a high copy number and are located in tandem repeats on a single chromosome

(Kohn, 1992; Lane *et al.*, 1985; Peterson & Kurtzman, 1991; Watson *et al.*. 1987; Woese, 1987). The rDNA gene operon is thus ideal for studying the relationship between distant as well as closely related species, and have been extensively and effectively used in phylogenetic studies, especially in fungi (Berbee & Taylor, 1992a:b; Bowman *et al.*, 1992; Bruns *et al.*, 1991; McCarroll, *et al.*, 1983; Metzenberg, 1991; Olsen *et al.*, 1986; White, Bruns & Taylor, 1990; Woese, 1987). It would appear that using partial sequence data from a gene results in the same inferred phylogeny compared to the sequence of the entire gene (Peterson & Kurtzman, 1991).

4.1.1. The LSU and SSU rDNA genes

Although the large subunit (LSU) and the small subunit (SSU) rDNA genes are highly conserved regions, nucleotide substitutions can take place at a very low rate (Blanz & Unseld, 1988). These regions are, therefore, very suitable for determining phylogenetic relationships amongst distantly related species (McCarroll *et al.*, 1983; Schegel, 1991). The larger the genetic distance to be calculated, however, the less accurate are the phylogenies determined for these regions (Guého, Kurtzman & Peterson, 1990). The rate of nucleotide substitution in the small and large subunits is relatively constant, even among closely related species. (Guého *et al.*, 1990; Ritland, Ritland & Straus, 1993). Studies by Blanz & Unseld (1988) have, however, shown that the_SSU is more conserved than the LSU. DNA sequence from a 200 base pair (bp) region of the LSU rRNA, enabled Peterson & Kurtzman (1991) to separate pairs of sibling species from each other. The SSU has been used for comparisons between major classes of fungi, namely Basidiomycetes, Ascomycetes and Chytridiomycetes (Bowman *et al.*, 1992), Chytridiomycetes and Oömycetes (Forster, Coffey, Elwood & Sogin, 1990) and within classes of Ascomycetes and Pyrenomycetes (Berbee & Taylor, 1992 a; b). The LSU gene has not been sequenced extensively for fungal phylogenetic studies and studies have shown that this gene is more suitable for distinguishing between related species and genera than the SSU gene (Guèho *et al.*, 1990; Yamada *et al.*, 1989; 1990).

4.1.2. The 5S rDNA gene

The 5S rDNA gene was the first gene to be studied extensively, its short length (120 nucleotides), making it easy to sequence the whole gene (Blanz & Unseld, 1988). The small number of nucleotides has, however, limited the use of this gene for phylogenetic studies (Guého *et al.*, 1990; Kurtzman, 1989; Olsen *et al.*, 1986). The secondary structure of the 5S rRNA gene is highly conserved, although, different types of the 5S rRNA gene can occur within a single strain of some fungi, thus making phylogenies based on this gene uncertain (Blanz & Unseld, 1988; Kurtzman, 1989).

4.1.3. The Intergenic Spacer region (IGS)

The IGS region is a large and highly variable region, sometimes up to 2000 bp in size (Chambers, Dutta & Crouch, 1986; Kohn, 1992; Martin, 1990). Harrington & Wingfield (1995) used this region to study 11 *Armillaria* (Fr.:Fr) Staude species from Europe and North America. The IGS region has also been used as a species-specific probe (Hendriques, Sá-Nogueira, Giménez-Jurando & Van Uden, 1991). Use of this region has also made it possible to identify polymorphic restriction sites within and between species (Adachi *et al.*, 1993).

4.1.4. The Internal Transcribed Spacers regions (ITS1 & ITS2)

The ITS regions have variable sequences due to their noncoding facility, but are shorter than the IGS region (Kohn, 1992; Ritland *et al.*, 1993). The ITS regions separates the more conserved LSU, SSU and 5.8S rRNA genes (Fig. 1) (Ritland *et al.*, 1993). The ITS1 spacer shows a significant base length variation and an A + T rich region could be found from bases 135 – 168 (Ritland *et al.*, 1993).

The ITS spacer regions are used for studying more recent divergence between organisms, as they are subject to more rapid evolutionary change (Chambers *et al.*, 1986; Hillis & Dixon, 1991; Metzenberg, 1991). RFLP studies of these regions are useful for the determination of phylogenies of closely related species and even for the determination of phylogenies between closely related individuals and populations (Ritland *et al.*, 1993). The ITS spacer regions together with the 5.8S rRNA gene have been very successfully sequenced and applied in phylogenetic studies on ophiostomatoid fungi (Visser *et al.*, 1995; Wingfield *et al.*, 1996; Witthuhn *et al.*, 1998).

4.1.5. The 5.8S rDNA gene

The 5.8S gene, despite being very small, only about 160 nucleotides, shows very little sequence divergence between closely related species (Blanz & Unseld, 1988; Olsen *et al.*, 1986; Ritland *et al.*, 1993). The use of this region in phylogenetic studies is, therefore, very limited.

5. ANALYSIS OF MOLECULAR DATA

Even though there have been major breakthroughs in the taxonomy of the ophiostomatoid fungi using molecular techniques in the last few years, few guidelines are available as to which phylogenetic data are the best to use. Little research has been done on the problem of distinguishing data containing relevant phylogenetic information from data containing so much "noise" that the real data remain hidden (Huelsenbeck, 1991). Woese (1987) stated that not all sequence data are of equal value for the determination of phylogenetic relationships. McCarroll *et al.* (1983) further found that the incorporation of conserved sequences is essential for correct and meaningful alignments in the rDNA operon. However, these conserved sequences do not contribute to the inferred phylogenies. It is not necessary to sequence the whole gene, but it is important to sequence a relevant fragment. In other words, the fragment sequenced needs to show enough variation at the appropriate level for a meaningful phylogeny (Kohn, 1992; Peterson & Kurtzman, 1991).

Sequence data must be analysed using the method most suited for the testing of the hypothesis (Kohn, 1992). There are two types of statistical analysis for inferring phylogenetic trees. Firstly, distance analysis or cladistics, for example, UPGMA (unweighted group-pair method with arithmetic mean) (Sneath & Sokal, 1973). Distance methods involve fitting of a tree to a matrix of pairwise distances (Felsenstein, 1988). Distance analysis is more reliable when large sequence divergences are observed (Olsen *et al.*, 1986).

Parsimony analysis is based on the analysis of characters using programs such as PAUP (Phylogenetic analysis Using Parsimony, Swofford, 1985) to construct phylogenetic trees of minimum length. Thus the tree that contains the smallest number of nucleotide changes (Felsenstein, 1988; Lake, 1987; Olsen *et al.*, 1986; Wheeler & Honeycutt, 1988). Parsimony analysis is one of the most widely used and generally accepted methods of phylogenetic inference (Lake, 1987).

The analysis of sequence data requires the alignment of the nucleotides. It is important that the number of nucleotides compared is statistically meaningful (Lane *et al.*, 1985). The compared sequences should be appropriately aligned and only truly homologous nucleotides should be considered (Lane *et al.*, 1985). To obtain sequence alignment, gaps corresponding to insertions or deletions, are inserted (Kohn, 1992; Swofford & Olsen, 1990). Sequences can be visually aligned, or by using computerised programmes to achieve alignments (Needleman & Wunsch, 1970). Computerised alignments should, however, not always be assumed to be correct and should always be visually checked (Kohn, 1992). It is important to note that sequence alignment can affect resulting tree topologies (Lake, 1987). Regions of sequences showing excessive divergence or which are too conserved to make meaningful alignments, should be excluded from analyses (Swofford & Olsen, 1990).

An "outgroup" is brought into analyses to provide a broader phylogenetic context to aid in the determination of the root of the "ingroup" (Maddison & Maddison, 1993). The outgroup should be as closely related to the ingroups as possible. This is, however, not easy with the fungi, as close relationships may not continue outside a group of taxa and the selected outgroup may, therefore, be too distantly related (Kohn, 1992). Ideally, more than one outgroup should be used to test a hypothesis.

Once a phylogeny has been obtained using a data set, it is then important to determine the validity of this phylogeny in terms of the data itself. There are currently two highly successful techniques available, namely Bootstrap and g_1 statistics (Felsenstein, 1985; Huelsenbeck, 1991). DNA Bootstrapping is used to assess the reliability of the sequence data and to determine the strength of the branch points in the tree (Bruns, White & Taylor, 1991; Felsenstein, 1988; Sanderson, 1995). Confidence in a clustering of the tree could be determined by the percentage of times it is supported by the Bootstrap replicates (Maddison & Maddison, 1993; Sanderson, 1995). For example, Berbee & Taylor (1992a), used Bootstrap to determine the strength with which the data support the branches in the most parsimonious tree or trees they obtained in their studies, using partial sequence data from the 18S rDNA.

The g_1 statistic is an indicator of the tree-length distribution skewness of a phylogenetic hypothesis (Huelsenbeck, 1991). The length of a tree is determined by the number of nucleotide changes required to fit the actual data onto the best possible tree topology (Berbee & Taylor, 1992b). Huelsenbeck (1991) found that tree-length distribution skewness provides a useful criterion for the judging of the phylogenetic information of the character data. Character data that are found to be consistent with one phylogenetic hypothesis will result in tree length distributions that are skewed to the left, or in other words they are highly negative (Huelsenbeck, 1991). The g_1 statistic is, therefore, a good indicator of the quality of the sequence data used to draw a phylogeny. Parsimony can be trusted in cases where the sequence data produce significantly left-skewed tree-length distributions.

To provide more definitive relationships between fungal species, many researchers are using both molecular and morphological characters in phylogenetic studies (Lutzoni & Vilgalys, 1995). Congruence between morphological and molecular datasets, however, could present problems in analyses and care needs to be taken when combining these data sets (Huelsenbeck, 1991; Lutzoni & Vilgalys, 1995; Maddison & Maddison, 1993; Patterson, Williams & Humphries, 1993). Phylogenetic trees obtained using different data sets are seldom identical (Lutzoni & Vilgalys, 1995). The type of analysis used, different sampling techniques, alignment and human error are all factors affecting congruence (Lutzoni & Vilgalys, 1995; Patterson, Williams & Humphries, 1993).

6. CONCLUSIONS

Although many breakthroughs have been made in the taxonomy of the ophiostomatoid fungi, there are many problems that remain to be resolved. New techniques are being developed readily to aid in the classification of these fungi and these will enable taxonomists to test and resolve old and new hypotheses concerning the ophiostomatoid fungi.

The use of morphological characters alone in classifications is often not sufficiently reliable due to the diversity observed in the ophiostomatoid fungi. Teleomorphs are not always available and classifications based on only anamorph characters do not always reflect the genetic relatedness of species. It is, therefore, necessary to re-evaluate the morphological characters used in the taxonomy of the ophiostomatoid fungi, using molecular techniques.

The rDNA operon has proved to be successful when used to resolve fungal phylogenies. Different regions of the rDNA operon are more suitable for use in classifications at certain taxonomic levels. Many different techniques are available to study the rDNA operon; the choice of technique depends on the question that needs to be answered.

Molecular techniques have proven to be indispensable in resolving taxonomic and phylogenetic relationships between fungi. The combination of molecular and morphological data sets, although not free of problems, are leading to the development of an effective taxonomic and phylogenetic system for all fungi. In this dissertation, it is clear that the combination of molecular and morphological data have done much to correct troublesome classifications in the ophiostomatoid fungi. It is, however, always important for the taxonomist to incorporate as many characters as possible to ensure a reliable and trustworthy classification.

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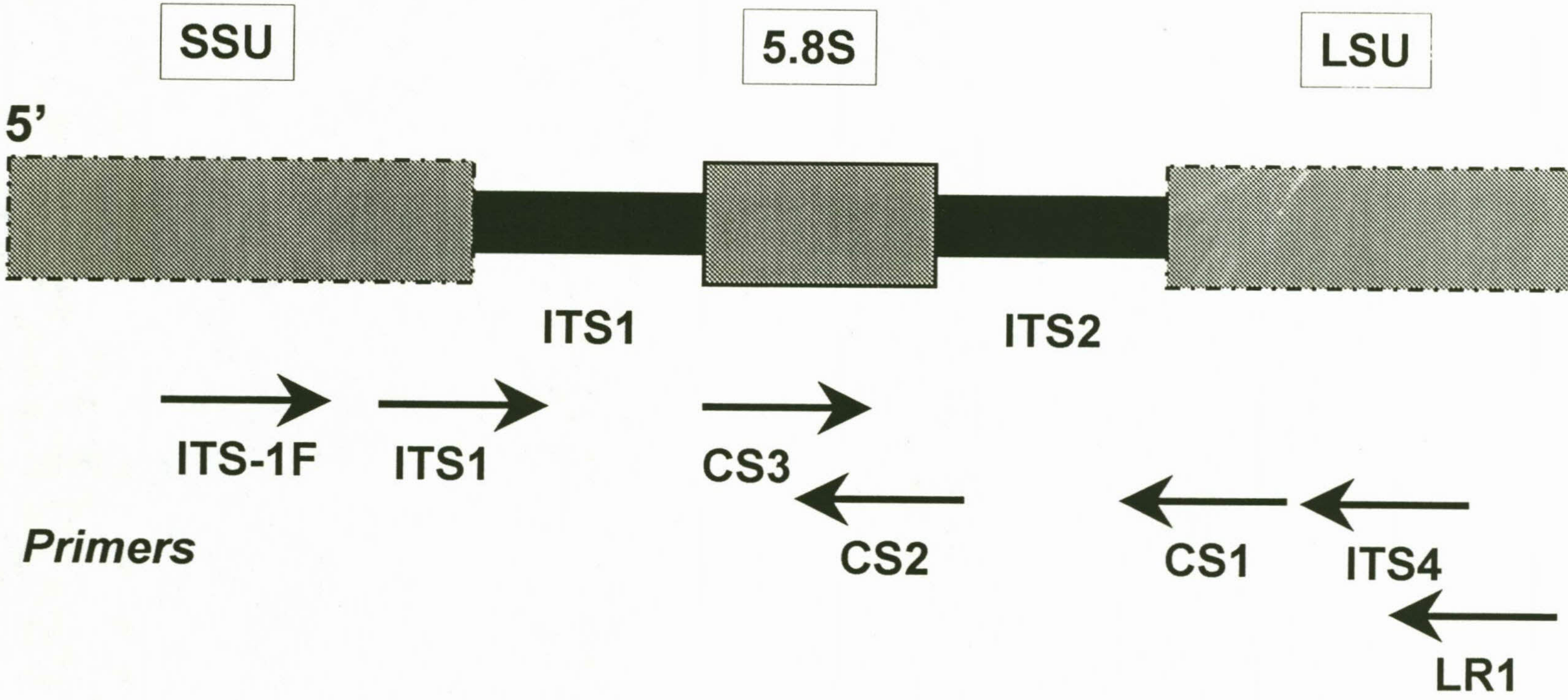

Fig. 1. Ribosomal DNA operon, with primers used in molecular studies (White et al., 1990)

CHAPTER 2

Ophiostoma polonicum is a species of *Ceratocystis sensu stricto*

Ophiostoma polonicum was first isolated in Poland and described in 1939 by Siemaszko. The fungus is a serious pathogen of spruce and is vectored by the bark beetle *Ips typographus*. *Ophiostoma polonicum* was described as producing a *Leptographium* anamorph similar to that of *Ophiostoma penicillatum*. The fungus was later transferred to *Ceratocystis penicillata* but is currently treated as a species of *Ophiostoma*. Examination of the culture collected by Siemaszko and also from recent collections from spruce in Europe and Japan have shown the presence of a *Chalara* state. Cell polysaccharide analysis revealed the absence of rhamnose in the cells of *O. polonicum* which is more typical of *Ceratocystis* species. The fungus was also found to be sensitive to cycloheximide, which is characteristic of species of *Ceratocystis sensu stricto*. Comparisons of partial sequence data of the ribosomal DNA operon have also revealed that isolates of *O. polonicum* group together with *Ceratocystis laricicola*, which is a well defined species of *Ceratocystis sensu stricto*. We, therefore, conclude from this study that *O. polonicum* is a typical species of *Ceratocystis s.str.* and also that the species is very likely conspecific with *C. laricicola*.

Keywords: rRNA, *Ophiostoma polonicum*, phylogeny, *Ceratocystis*, *Ips typographus*

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The ophiostomatoid fungi include the genera *Ceratocystis* Ell. & Halst. and *Ophiostoma* Sydow and have been known since the early part of this Century. Most species in these genera have ascomata with elongate necks and they are generally considered to be vectored by insects (Wingfield *et al.* 1993). Numerous species of these fungi also include serious pathogens of trees of which *Ophiostoma ulmi* Buismann and *Ophiostoma novo-ulmi* Brasier (Brasier, 1993) and *Ceratocystis fagacearum* Bretz (Kile, 1993) are perhaps best known.

The taxonomy of *Ophiostoma* and *Ceratocystis* has had a confused history. These genera have been regarded as synonyms or as separate entities by various authors in the past. In the most recent and comprehensive treatment of the group, Upadhyay (1981) treated the genera as synonyms but included the new genus *Ceratocystiopsis* Upadhyay and Kendrick to accommodate species with falcate ascospores. Convincing evidence based on cell wall composition (Jewell, 1974; Rosinski & Campana, 1964; Spencer & Gorin, 1971; Weijman & De Hoog, 1975) has, however, been presented to support the notion that *Ceratocystis* and *Ophiostoma* are distinct. Species of *Ceratocystis* also produce conidia by ring wall building and have *Chalara* anamorphs (Minter, Kirk & Sutton, 1982) as opposed to apical wall building (*Graphium* Corda, *Leptographium* Lagerberg & Melin, *Sporothrix* Hektoen & Perkins, *Hyalorhinocladiella* Upadhyay & Kendrick) in *Ophiostoma* anamorphs (Mouton *et al.*, 1994). Unlike *Ceratocystis*, species of *Ophiostoma* are also resistant to high concentrations of the antibiotic cycloheximide (Harrington, 1981).

Recent studies at the molecular level have provided additional evidence for the fact that *Ceratocystis* and *Ophiostoma* are distinct. These studies have been based on analysis of the 18S ribosomal RNA gene and have shown that the genera are distantly related (Hausner, Reid & Klassen, 1992; 1993a; 1993b; 1993c; Spatafora & Blackwell, 1994). Morphological similarities in teleomorph structures in these phylogenetically distinct groups have apparently developed as an adaptation to an insect-associated habitat.

Ophiostoma polonicum Siemaszko was first described in 1938 associated with the bark beetle (Coleoptera: Scolytidae) *Ips typographus* L. that infests spruce, *Picea abies* (L.) Karst. in Europe (Siemaszko, 1938). The fungus was described as having a

Leptographium anamorph. Moreau (1952) later treated this fungus in the genus *Ceratocystis* as *Ceratocystis polonica*. No type material exists for *O. polonicum* but, based on the *Leptographium* state described by Siemaszko (1938), Upadhyay (1981) reduced this species to synonymy with *Ceratocystis penicillata* (Grosm.) Moreau (=*Ophiostoma penicillatum* (Grosm.) Siemaszko). Later, Solheim (1986) considered *O. penicillatum* and *O. polonicum* to be distinct species (Solheim, 1991; 1993; Christiansen, 1985). Solheim (1986) was, however, unable to detect the *Leptographium* state or any other anamorph in *O. polonicum*.

Considerable confusion surrounds the generic placement of the fungus that Siemaszko (1938) named *O. polonicum*. During a recent survey of fungi associated with *Ips typographus japonicus* infesting *Picea jezoensis* (Sieb. *et.* Zucc.) Carr. in Hokaido, Japan, we noted an apparent *Chalara* state in some isolates of a fungus otherwise resembling *O. polonicum*. This would indicate that the fungus is a *Ceratocystis* species as opposed to a species of *Ophiostoma*. The aim of this study was, therefore, to examine isolates of *O. polonicum* from Japan and Europe using a variety of techniques and, thus, to establish an appropriate generic placement for this fungus.

MATERIAL AND METHODS

Isolates examined

For the purposes of this study, we made morphological examinations of four isolates of a fungus matching the teleomorph characteristics of *O. polonicum* from Japan and Europe including the isolate collected by Siemaszko in Poland obtained from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands (CBS). Isolates from Europe were from *I. typographus*-infested *P. abies* and supplied by Dr. Solheim (Norwegian Forest Research Institute, Section of Forest Ecology, Division of Forest Pathology, N-1423 Ås-NLH, Norway). Those from Japan were collected by Dr. Yamaoka from *I. typographus japonicus*-infested *P. jezoensis* in Hokkaido, Japan. All isolates included in this study are maintained in the culture collection of Prof. M.J. Wingfield, with representative isolates also available in other collections.

For the experimental part of this study, four isolates of *O. polonicum* were utilised. These include one from Japan (YCC118 [CMW 2274]) and three from Europe (ATCC 62335 [CMW 1164], CBS 133.38 [CMW 672], CMW 2443). The European isolates included two isolates supplied by H. Solheim as well as an isolate (CMW 672) collected by Siemaszko who originally described this fungus. Two isolates of *Ceratocystis laricicola* Redfern & Minter (CMW 1016 and CMW 1017) supplied by Dr. R.B. Redfern (Forestry Commission, Northern Research Station, Roslin, Midlothian EH25 9SY, UK) were also included given the fact that this fungus is very similar to *O. polonicum*, with the exception of the purported *Leptographium* state of the latter species.

Morphological examination of *O. polonicum* was done using light microscopy. Perithecia were abundant in numerous isolates including CMW 2274 from Japan and CMW 1164 from Europe and teleomorph characteristics could be compared with those described for this fungus by Siemaszko (1938). Cultures were also examined thoroughly for the presence of anamorph characters.

Cycloheximide tolerance and Cell polysaccharide analysis

All isolates of *O. polonicum* used in the experimental part of this study were tested for their ability to tolerate Cycloheximide in culture. In addition, an isolate of *Ceratocystis fimbriata* Ell. & Halst. and *Ophiostoma piceae* Münch were included for comparative purposes. Five different concentrations (0.00, 0.05, 0.10, 0.50, 1.00 and 2.50 %) of cycloheximide in 2 % Malt extract agar (MEA; 20 g malt extract and 20 g agar/l H_2O) were tested. Agar discs, 4.2 mm in diameter were cut from the actively growing margin of cultures and placed at the centres of three Petri dishes containing the different concentrations of cycloheximide for each isolate. Two diameter measurements were taken for each colony after incubation in the dark for six days. Averages of these measurements were calculated and this experiment was repeated once.

Isolates of *O. polonicum*, for the cell polysaccharide analysis, were grown in 15 g ME (malt extract) and 5 g Glucose/1l dH_2O at 20 °C for two weeks. Cell material was collected by centrifugation at 10 000 rpm for 10 min, washed twice with dd H_2O and lyophilised. The freeze-dried cell material was hydrolised with 72 % H_2SO_4 for 12 h at 4 °C , followed by boiling for 2.5 h in 2 N H_2SO_4 and then neutralised with NaOH. Presence of cell saccharides was determined by column chromatography using a Technicon Auto Analyser System (Alsa Tech, Isando, Johannesburg, South Africa) as previously described by Van Biljon & Olivier (1989).

Sequence comparisons

Cultures were grown on cellophane discs placed on 2 % MEA and incubated at 20 °C until the mycelium covered the disc. These cellophane discs were then transferred to sterile Petri dishes, lyophilised and stored at -20 °C. Nucleic acid was extracted from the freeze-dried material using a modified Guanidinium Thiocyanate procedure of Chirgwin *et al.* (1979). A region within the ribosomal DNA operon, including the variable Internal Transcribed Spacer regions (ITS1 and ITS2) as well as the conserved 5.8S gene, were amplified using the Polymerase Chain Reaction (PCR), (Saiki *et al.*, 1988). Primers ITS1 (5'TCCgTAggTgAACCTgCgg3') and ITS4 (5'TCCTCCgCTTATTgATATgC3') (10 OD/ml) were used for amplification (White *et al.*, 1990). Reactions were performed in a Hybaid Omnigene Temperature Cycler (Hybaid, Middlesex, UK) for 35 cycles using *Taq* DNA polymerase (Promega Corporation, Madison, U.S.A.) with the Magnesium free 10x Buffer supplied by the manufacturer. The final buffer composition was 6.25 mM $MgCl_2$, 1.6 mM of each dNTP and 360 pmol of each primer. An initial 5 min denaturation step at 96 °C was performed, followed by 35 cycles of 92 °C for 60 seconds (denaturation), 55 °C for 30 seconds (annealing) and 72 °C for 60 seconds (extension) with a final elongation step of 5 min at 72 °C. The PCR products were visualised on a 1 % (w/v) agarose gel to assess the amplification. The amplified DNA products were purified using the Magic PCR Preps (Promega Corporation, Madison, U.S.A.) and sequenced with the *f*mol Sequencing System (Promega Corporation, Madison, U.S.A.). Primers ITS4, ITS1, CS2 (5'CAATgTgCgTTCAAAgATTCg3') and CS3 (5'CgAATCTTTgAACgCACATTg3') [constructed by B.D. Wingfield, University of the Orange Free State] were used to determine the DNA sequence in both directions. The sequence data were visually aligned and the phylogenetic relationships the Orange Free State] were used to determine the DNA sequence in both directions. The sequence data were visually aligned and the phylogenetic relationships determined using PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1993). Both the branch and bound and the heuristic options were used in the PAUP analysis. DNA sequence of *C. fimbriata*, the type species of *Ceratocystis s.str.* was included in this study for comparative purposes. *Neurospora crassa* sequence (Chambers, Dutta & Crouch, 1986) was used as an outgroup.

RESULTS

Morphological comparisons

Cultural characteristics of the four *O. polonicum* isolates were similar and differed markedly from those previously described for *O. penicillatum*. The former fungus has a light grey colour with profuse aerial mycelium as opposed to the dark green-grey colonies of *O. penicillatum*. Isolate CMW 672 collected by Siemaszko (1938) produced no fruiting structures. Other isolates of the fungus produced distinct ophiostomatoid ascomata with single-celled, reniform, sheathed, two gutulate ascospores identical to those described by Siemaszko (1938). Although these structures were extremely rare, conidiophores (Fig. 1) and conidia (Fig. 2) typical of *Chalara* species were occasionally found associated with the bases of the ascomata in many isolates.

Cycloheximide tolerance and cell wall polysaccharides

All four isolates of *O. polonicum* tested for tolerance to cycloheximide, proved to be sensitive to all concentrations of this antibiotic, even to as low as 0.05 g/l of cycloheximide (Table 1). In this characteristic they were similar to *C. fimbriata* (type species of *Ceratocystis s.str.*), but differed markedly from *O. piliferum* (typical species of *Ophiostoma*). Rhamnose was found to be absent in all the isolates of *O. polonicum* tested for cell polysaccharides.

Sequence comparisons

A single DNA fragment of about 550 bp was observed using gel electrophoresis for all isolates amplified. For each isolate amplified, a total of about 500 bp were sequenced and read (Fig. 3). The DNA sequences obtained were visually aligned and edited using MacClade 3.0 (Fig 3) (Maddison & Maddison, 1993). The sequence of *Neurospora crassa* which was included in this study for comparative purposes was obtained from Chambers *et al.* (1986). The bootstrap analysis for each option resulted in the same tree configuration and a strict consensus tree was constructed (Fig 4). The g1 statistics determined from the bootstrap trees obtained for the branch and bound and the heuristic searches were -1.89 and -1.74, respectively. The *O. polonicum* isolates formed a cluster with *C. fimbriata* with a confidence interval of a 100 %, confirming other evidence that these isolates are most likely species of *Ceratocystis*. The isolate from Japan grouped together with Siemaszko's isolate with a confidence interval of 100 % and these two clustered with the two *C. laricicola* isolates with 74 % confidence (Fig 4). The Norwegian isolate of *O. polonicum* was less closely related to the above-mentioned isolates, although more closely related to these than to *C. fimbriata.*

DISCUSSION

Results of this study show that the fungus that was described as *O. polonicum* is a typical species of *Ceratocystis s.str.* The most definitive characteristics that lead us to this conclusion are the presence of a *Chalara* anamorph (Minter *et al.*, 1982; De Hoog & Scheffer, 1984), sensitivity to cycloheximide (Harrington, 1981) and the absence of rhamnose in the cell walls (Rosinski & Campana, 1964; Weijman & De Hoog, 1975; Spencer & Gorin, 1971). This fungus can easily be separated from *O. penicillatum*, which has a *Leptographium* anamorph and is tolerant to cycloheximide. We suggest, therefore, that the name *Ceratocystis polonica* should be used for this fungus.

In addition to the above characteristics, based on rDNA sequence analyses, it was possible to confirm that isolates of *C. polonica* form a distinct group of fungi, closely related to *C. fimbriata* and distinct from *N. crassa*. The fact that two isolates of *C. laricicola*, a very well defined species of *Ceratocystis*, grouped together with *C. polonica* also supports the new generic placement of *O. polonicum* in *Ceratocystis s.str.* These data also strongly suggest that *C. laricicola* and *C. polonica* are the same species although detailed morphological comparisons are required before a synonymy can be presented.

Past confusion pertaining to the generic placement of *C. polonica* is relatively easy to explain. This fungus is one of numerous ophiostomatoid fungi including *O. penicillatum*, that is carried by *Ips typographus* infesting spruce in Europe (Solheim, 1986). It, thus, occurs in close association with the latter fungus and the two are often isolated as mixed cultures (Wingfield, unpublished). It is our view that when this fungus was first collected, Siemaszko was dealing with a mixed culture of the two fungi and that he incorrectly identified the *Leptographium* state as being connected with ascomata of *C. polonica*. Upadhyay (1981) did not have access to type material of this fungus and evidently chose to synonymise it with *O. penicillatum* based on reported presence of a *Leptographium* state by Siemaszko (1938). Solheim (1986) later recognised that *C. polonica* was distinct from *O. penicillatum* but did not recognise that it might belong in *Ceratocystis* rather than in *Ophiostoma*.

Species of *Ophiostoma* and *Ceratocystis* represent excellent examples of distinct taxa that have evolved convergently in adaption to insect dispersal (Hausner *et al.*, 1993c; Spatafora & Blackwell, 1994; Wingfield *et al.*, 1994; Wingfield, Wingfield & Kendrick, 1994). The discovery that the fungus previously known as *O. polonicum*, is in fact a species of *Ceratocystis s.str.*, makes it the first example of these two phylogenetically distinct groups occurring associated with the same insect in a specialised niche.

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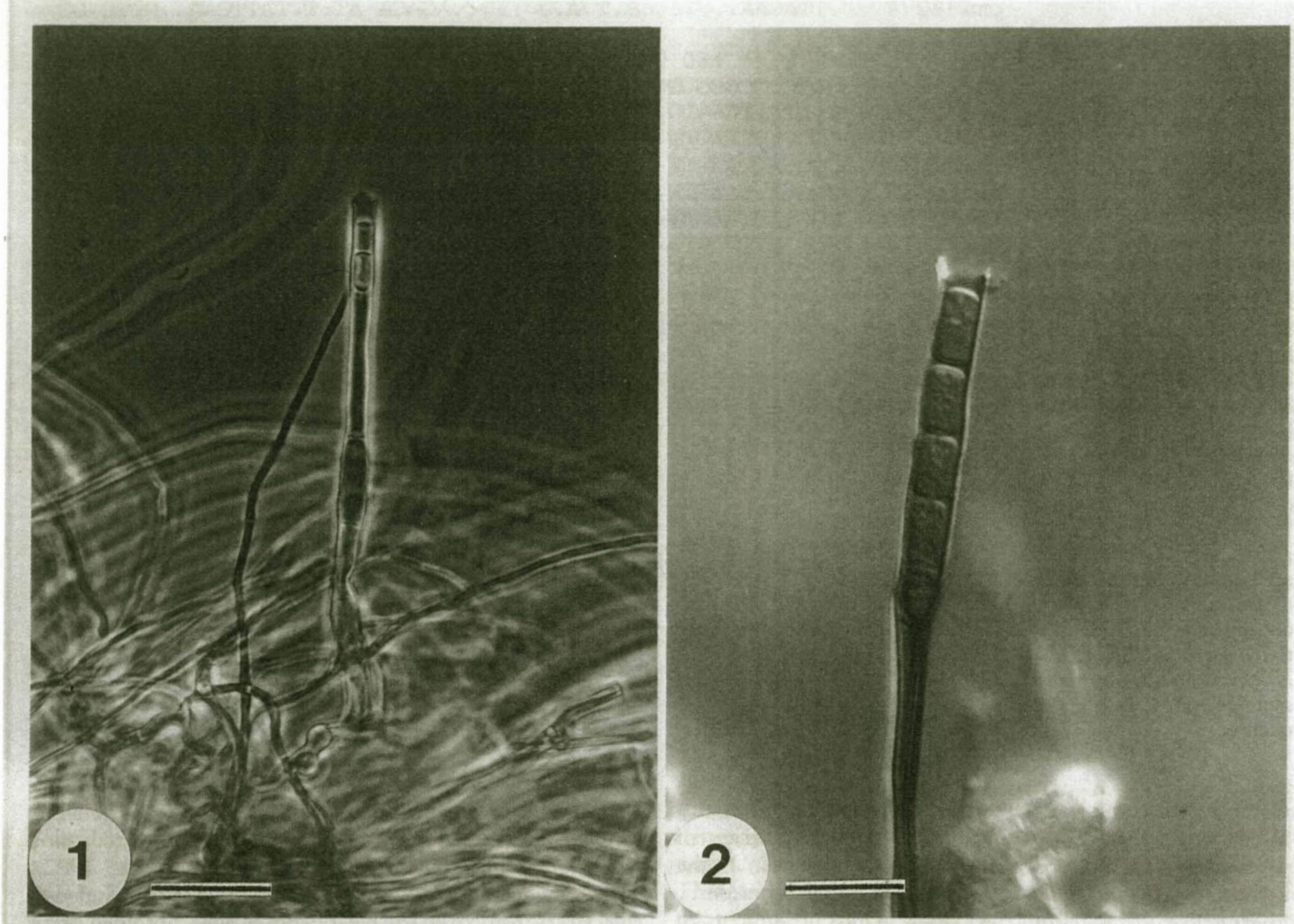
Table 1. Average growth in mm of *Ophiostoma polonicum* isolates on various concentrations of cycloheximide. *Ophiostoma piceae* and *Ceratocystis fimbriata* were included for comparative purposes

	% Cycloheximide (g/l)					
Fungus	0.00	0.05	0.10	0.50	1.00	2.50
Ophiostoma polonicum						
CMW 1164 (Sweden)	44.8	-	-	-	-	-
CMW 2443 (Sweden)	21.2	-	-	-	-	-
CMW 2274 (Japan)	19.5	-	-	-	-	-
CMW 672 (Poland)	34.1	-	-	-	-	-
Ceratocystis fimbriata						
CMW 2220	33.0	-	-	-	-	-
Ophiostoma piceae						
CMW 153	48.2	40.9	41.0	38.3	35.0	37.1

Fig. 1-2. Conidiophore and conidia of *O. polonicum*. **Fig. 1**. Light micrograph showing the typical *Chalara* state observed for *O. polonicum* (Bar: 15mm = 20μm) and **Fig. 2.** showing the conidia produced by the conidiophore of *O. polonicum* (Bar: 15mm = 10μm).



Figs. 1–2. Conidiophore and conidia of *O. polonicum*. Fig 1. Light micrograph showing the typical *Chalara* state observed for *O. polonicum* (Bar: 15 mm = 20µ) and Fig 2. showing the conidia produced by the conidiophore of *O. polonicum* (Bar: 15 mm = 10µ).

Fig 3. Alignment of the DNA sequences obtained for the ITS1, ITS2 regions and the 5.8S rRNA gene for three isolates of *O. polonicum* (CMW 672, CMW 2274 & CMW 2443), two isolates of *C. laricicola* (CMW 1016 & CMW 1017), one isolate of *C. fimbriata* (CMW 2220) and *N. crassa* which was used as an outgroup. N indicates unknown bases; a dot indicates bases identical to the corresponding base in *N. crassa* and dashes represent deletions in the sequence.

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                   10         20         30         40         50
N. crassa  TCATTACAGA GTTGCAAAAC TCCCACAAAC CATCGCGAAT CTTACCCGTA
CMW 2220   ---------- C.GATTCTTG .AGTGAG.TG A..G.T.TT. TGGTGGTAGG
CMW 672    .......T.- AG.NNNNGGA -.T.TT.... ...AT-.TGA ACATA..TNN
CMW 1017   .......T.G AG.TTTT--A A.T.TT.... ...AT-.TGA ACATA..T.T
CMW 2443   .......T.- AG.TTTT--A A.T.TT.... ...AT-..GA ACATA..T.T
CMW 1016   .......T.- AG.TTTT--A A.T.TT.... ...AT-.TGA ACATA..T.T
CMW 2274   .......T.- AG.NNNNGGA -.T.TT.... ...AT-.TGA ACATA..TNN

                   60         70         80         90        100
N. crassa  CGGTTGCCTC GGCGCTGGCG GTCC-GGA-- -AAGG-CCTT CGGGCCTCCC
CMW 2220   GCCC.T.TGA A.G.-CAC.. C.G.CA.CAG T.TA.T.--. ..CCA..GT-
CMW 672    .CCA-..TG. TTT.GCA.GT C.TGG-T.AA AC.A.T-.C. GCC.GTAGTA
CMW 1017   TTTA-..TG. TTT.GCA.GT C.TGG-T.AA AC.A.T-.C. GCC.GTAGTA
CMW 2443   NTTA-..TG. TTT.GCA.GT C.TGG-T.AA AC.A.T-.C. GCC.GTAGTA
CMW 1016   TTTA-..TG. TTT.GCA.GT C.TGG-T.AA AC.A.T-.C. GTC.GTAGTA
CMW 2274   .CCA-..TG. TTT.GCA.GT C.TGG-T.AA AC.A.T-.C. GCC.GTAGTA

                  110        120        130        140        150
N. crassa  T------AAA CTCTTGA-TA TTTATGTCTC TCTGAGTAAA CTTTTAAATG
CMW 2220   -......... .....-.... ...TCCAGAT .T.TTCATTG ..GAGTGGCA
CMW 672    .TTAGAA... .....T.A.. ...C.AGAGA AT.T.TATTC A..GCTG.GC
CMW 1017   .TTAGAA... .....T.A.. ...C.AGAGA AT.T.TATTC A..GCTG.G-
CMW 2443   .TTACAA... .....T.A.. ...C.AGAGA AT.T.T.TTC A..GCTG.G-
CMW 1016   .TTAGAA... .....T.A.. ...C.AGAGA AT.T.TATTC A..GCTG.G-
CMW 2274   .TTAGAA... .....T.A.. ...C.AGAGA AT.T.TATTC A..GCTG.GC

                  160        170        180        190        200
N. crassa  GATCCTCGGG TCTCCGCTCG CGGCTGCCCG CCGGAGTGCC GAAACAAGTC
CMW 2220   T.A.--TA-- ---------- ---------- ---------- T...A....T
CMW 672    TGG.A.TAA- ---------- ---------- ---------.A T..TA.GC.T
CMW 1017   TGG.A.TAA- ---------- ---------- ---------.A T..TA.G-.T
CMW 2443   TGG.A.TAA- ---------- ---------- ---------.A T..TA.G-.T
CMW 1016   TGG.A.TAA- ---------- ---------- ---------.A T..TA.G-.T
CMW 2274   TGG.A.TAA- ---------- ---------- ---------.A T..TA.GC.T

                  210        220        230        240        250
N. crassa  AAAACTTTCA ACAACGGATC TCTTGGTTCT GGCATCGATG AAGAACGCAG
CMW 2220   .......... .......... ......C... A......... ..........
CMW 672    .......... .......... ......C... A......... .....-....
CMW 1017   .......... .......... ......C... A......... ..........
CMW 2443   .......... .......... ......C... A......... ..........
CMW 1016   .......... .......... .T....C... A......... ..........
CMW 2274   .......... .......... ......C... A......... ..........
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                     260        270        280        290        300
N. crassa   CGAAATGCGA TA-GGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT
CMW 2220    .......... ..A.-..... .......... .......... ..........
CMW 672     .......... ..C..C.... .......... .-.-...... -.......-.
CMW 1017    .......... ..C-...... .-........ .......... .......NN.
CMW 2443    .......... ..C-...... .......... .......... ..........
CMW 1016    .......... ..C-...... .......... .......... ..........
CMW 2274    .......... ..C..C.... .......... .--....... -.......-.


                     310        320        330        340        350
N. crassa   CTTTGAACGC ACATTGCGC- TCGCCAGTAT TCTGGCGAGC ATGCCTGTTC
CMW 2220    ..N....... .......C.C .G.N-..... ....C.AG.. ........-.
CMW 672     ..C...--.. ....-.GC.C .G-.-..... ...-C.AGCA -.........
CMW 1017    ..N...N... .......N.C .G..-..... ....C.AGCA C.......C.
CMW 2443    .......... .-....GC.. .G..-..... ....C-AG-A N.......C.
CMW 1016    .......... .........C .G..-..... ....C.AGCA -.......C.
CMW 2274    ..C...--.. ....-.GC.C .G-.-..... ...-C.AGCA ..........


                     360        370        380        390        400
N. crassa   --GAGCGTCA TTTCAACCAT CAAGCTCTGC TT--GCG-TT GGGGATCCGC
CMW 2220    C......... .....C.AC. ....GA..C. ..TGNNC... ..C.T.GGAG
CMW 672     C.-.-..... .....C.AC. .......... ...G.T.... ..A.GA..CG
CMW 1017    G.-...N... .....C.AC. .......... ...GN-.... ..A.GA..CG
CMW 2443    G.-....... .....C.AC. .........- ...G.T.... ..A.GN..CG
CMW 1016    G.-....... .....C.AC. .......... ...G-T.... ..A.GA..AG
CMW 2274    C.-.-..... .....C.AC. .......... ...G.T.... ..A.GA..CG


                     410        420        430        440        450
N. crassa   G-GCTGTCCG CTCAAAATCA GTGGCGGGCT CGTCAGTCAC ACCGAGCGTG
CMW 2220    .TC....T.T .C.CTG.A.. .GCCGCC.AA ATGT.TC-GG CTGTTATACT
CMW 672     CA--.C.T-- -----G--.G .GCCGCC.AA ATG..TC-GG CTGTT-GAAT
CMW 1017    CA--.C.T-- -----G--.G .-CCGCC.AA ATG..TC-GG CTGTTCGAAT
CMW 2443    CA--.C.T-- -----G--.G .-CCGCC.AA ATG..TC-GG CTGTT-GAAT
CMW 1016    -.-------- ---------- ---------- ---------- ----------
CMW 2274    CA--.C.T-- -----G--.G .GCCGCC.AA ATG..TC-GG CTGTT-GAAT


                     460        470        480        490        500
N. crassa   GTGACTCTAC ATCGCTATGG TCGTGCGGCG GG-------- ----------
CMW 2220    T-.C.AACT. CC.TG.G.A. .ATAAAATTT CTAATTTTT. ACACTTTGAA
CMW 672     T..CAG..T. CC-TG.G.A. .AA.ATT-A- TTTTTTG... ACGCTTT.GA
CMW 1017    T..CAG..T. CC-TG.G.A. .AA.ATTTA- TTTTTTT... AGCGCTTTGA
CMW 2443    T..CAG..T. CC-TG.G.A. .AA.ATTTA- TTTT...... ACGCTTT.GA
CMW 1016    ------..T. CC-TG.G.A. .AA.ATTTA- TTTTTTT... ACGCTTT.GA
CMW 2274    T..CAG..T. CC-TG.G.A. .AA.ATT-A- TTTTTTG... ACGCTTT.GA
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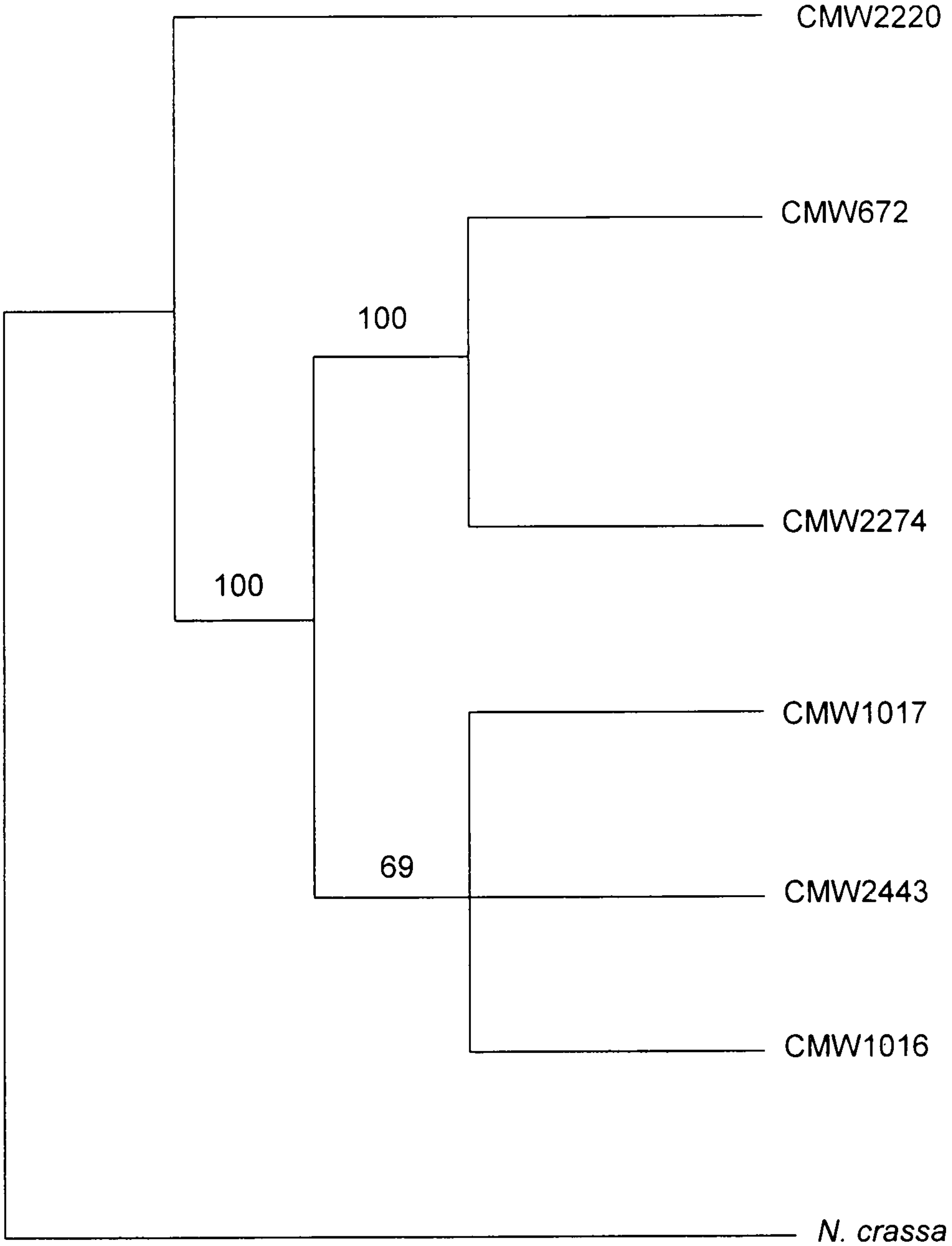
                   510        520        530        540        550
N. crassa   --TTC-TT-- --------GC CG-TAAAACC CCCC------ ----------
CMW 2220    .G......GT G.TAACAC.. ..C....-.. AA..CCCTCA ACTTTTGTTG
CMW 672     AAC.....TT A.CAACATCG .CG....... ....A..TCA ATTTT.....
CMW 1017    AAC.....TT A.CAACATCG .CG....... .......TCA ATTTT.....
CMW 2443    AAC.....T. ..CA.CATCG .CG....... .......TCA ATTTT.....
CMW 1016    AAC.....TT A.CAACATCG .CG....... .......TCA ATTTT.....
CMW 2274    AAC.....TT A.CAACATCG .CG....... ....A..TCA ATTTT.....

                   560
N. crassa   -A-TTTC-TA AGGTTGACC
CMW 2220    A.C....AC. .........
CMW 672     .-.----.G. .........
CMW 1017    .-.----.A. .AC......
CMW 2443    ......-GA. .........
CMW 1016    .-.----NA. .........
CMW 2274    .-.----.G. .........
```

Fig 4. Consensus tree generated from PAUP analysis depicting the phylogenetic relationships between *C. laricicola* (CMW 1016 & CMW 1017), *O. polonicum* (CMW 672, CMW 2274 & CMW 2443) and *C. fimbriata* (CMW 2220). *Neurospora crassa* was used as an outgroup. Bootstrap values are indicated at the branch points.


CMW2220
CMW672
100
CMW2274
100
CMW1017
69
CMW2443
CMW1016
N. crassa

CHAPTER 3

Xenochalara, a new genus of dematiaceous hyphomycetes for *Chalara*-like fungi with apical wall building conidial development

Ceratocystis autographa is unusual amongst the ophiostomatoid fungi in that it produces two distinct anamorphs, typifying different teleomorph genera. A *Chalara*-like anamorph characteristic of the genus *Ceratocystis sensu stricto* and a *Sporothrix* anamorph typical of the genus *Ophiostoma*, have been described for the fungus. In a recent study, isolates resembling *C. autographa* that were collected from *Juniperus* were shown to have a different type of conidiogenesis to that of typical species of *Chalara*. Furthermore, cycloheximide tolerance and the presence of rhamnose in the cell walls of these isolates also suggested that they were more closely related to *Ophiostoma* than to *Ceratocystis s.str.* Parsimony analysis of ribosomal DNA sequence data in this study, showed that these isolates formed a monophyletic group, distinct from *Ceratocystis* isolates and from *Ophiostoma ulmi.* The *Chalara*-like anamorph associated with *C. autographa* isolates is also distinct from typical *Chalara* anamorphs of *Ceratocystis s.str.* in that it produces conidia from phialides via apical wall building, in contrast to the ring wall building typical of *Chalara* species. We, therefore, propose the establishment of a new genus, *Xenochalara* gen.nov., for these and other *Chalara*-like species producing conidia by apical wall building.

Keywords: *Ceratocystis sensu stricto, Xenochalara, Ophiostoma,* rDNA, phylogeny

The ophiostomatoid fungi include three genera namely, *Ophiostoma* H. & P. Sydow, *Ceratocystis sensu stricto* Ellis & Halst. and *Ceratocystiopsis* Upad. & Kendrick (De Hoog & Scheffer, 1984). Anamorphs of *Ceratocystis s.str.* are restricted to the genus, *Chalara* (Corda.) Rabenh., which produce conidia through ring wall-building (Minter, Kirk & Sutton, 1982), contain no cellulose (Rosinski & Campana, 1964) or rhamnose (Spencer & Gorin, 1971) in their cell walls, and are sensitive to the antibiotic, cycloheximide (Harrington, 1981). In contrast, species in *Ophiostoma* and *Ceratocystiopsis*, form conidia through apical wall building and the anamorphs are in genera other than *Chalara* (De Hoog & Scheffer, 1984; Mouton, Van Wyk & Wingfield, 1994; Upadhyay & Kendrick, 1975). These species also contain cellulose and rhamnose in their cell walls (Rosinski & Campana, 1964; Spencer & Gorin, 1971) and are tolerant to high concentrations of cycloheximide (Harrington, 1981).

Ceratocystis autographa Bakshi is unusual amongst the ophiostomatoid fungi in that it is reported to produce two anamorph forms with distinct modes of conidial development. Bakshi (1951) first isolated *C. autographa* from the galleries of the bark beetles, *Dryocoetes autographus* and *Hylurgops palliatus* infesting *Larix leptolepis* (Japanese larch) in Scotland. Bakshi described *C. autographa* as producing two distinct anamorphs (Bakshi, 1951). In the one, the hyaline, round to ovoid conidia are produced exogenously and are carried singly or in clusters. The other anamorph produces conidia endogenously in flask-shaped conidiophores. The conidia formed by the latter anamorph are hyaline, barrel-shaped, one-celled and are formed in chains (Bakshi, 1951).

Subsequent to the initial description of *C. autographa* (Bakshi, 1951), much controversy has arisen concerning the taxonomy of the *Chalara*-like anamorph. Hunt (1956) confirmed the description of Bakshi, and further observed that the conidia were cylindrical with truncate ends. Nag Raj & Kendrick (1975) and Gams & Holubová-Jechová (1976) also described the barrel-shaped endoconidia as short, clavate, rounded at the apex with truncate bases. Nag Raj & Kendrick (1975), furthermore, described the endoconidiophores as phialidic, lageniform with a distinct constriction at the base of the collarette and identified this anamorph as a species of *Chalara*. Unfortunately, no culture of the type *C. autographa* was preserved. In a subsequent study, Gams & Holubová-Jechová (1976) collected an isolate from *Juniperus* that they identified as *C. autographa* (CBS 670.75). Wingfield *et al.* (1995) re-examined the *Juniperus* isolate and observed that the *Chalara*-like anamorph was unusual. Although the conidia were formed in chains, they had single attachment points that is typical of apical wall building. This was in contrast to ring wall-building conidial development, typical of *Chalara*, where conidia that occur in chains have two attachment points (Minter *et al.*, 1982).

Wingfield *et al.*, (1995) showed that the *Juniperus* isolate had a high degree of tolerance to cycloheximide. This suggested a closer relationship to the genus *Ophiostoma* than to *Ceratocystis s.str.* Although a *Sporothrix* anamorph was commonly observed on the type (IMI 20162) of *C. autographa,* Wingfield *et al.* (1995) found that the *Juniperus* isolates very rarely formed a *Sporothrix* synanamorph, and that the latter was usually inconspicuous when present. Furthermore, in a comparison of sequence data from the SSU and LSU rDNA genes, Hausner, Reid & Klassen (1993) suggested that this isolate (CBS 670.75) could have been misidentified as it grouped separately from both *Ceratocystis s.str* and *Ophiostoma* species in their analyses. Based on its unique mode of conidiogenesis, Wingfield *et al.* (1995) suggested that this fungus belonged to a genus other than *Chalara*, and that its morphological similarity to *Chalara* is probably a result of convergent evolution.

The aim of this study was to consider the placement of isolates that have been labelled as *C. autographa,* within *Ceratocystis s.l.* We made use of the variable ITS1 and ITS2 regions, as well as the conserved 5.8S gene, of the rDNA operon in an attempt to resolve a long standing taxonomic enigma surrounding the culture labelled as *C. autographa* (CBS 670.75). Previously published sequence data of isolates known to reside in the genera *Ceratocystis s.str.* and *Ophiostoma*, were chosen for comparative purposes. *Ophiostoma ulmi* (Buisman) Nannf. was chosen as representative of the genus *Ophiostoma*. Sequence data from two isolates of *Ceratocystis fimbriata* (Witthuhn *et al.*, 1998a,b), were chosen to represent *Ceratocystis s.str.* In addition, sequence data from *Chalara australis* and *Chalara neocaledoniae* published by (Witthuhn *et al.*, 1998a), were also included in this study to provide further comparison with well-defined species of *Chalara*.

MATERIALS AND METHODS

Morphology

Isolates of the fungus from *Juniperus* in the Netherlands (CBS 670.75) and single conidial transfers of cultures initially identified as *C. autographa* (CMW 1099, CMW 1901 and CMW 2547) have been deposited in the culture collection of the Forestry and Agricultural Biotechnology Institute at the University of Pretoria, South Africa. These isolates were grown on 2 % MEA (20 g Malt Extract, 20 g Biolab Agar / 1000 ml H_2O) in Petri dishes and incubated at 25 °C under near-ultraviolet light. The holotype specimen of *C. autographa* (IMI 20162) was also examined and compared with the cultures used in this study. Light microscopy was performed on material mounted in lactophenol on glass slides. Scanning and Transmission electron micrographs from the study of Wingfield *et al*., (1995) were also used in our interpretation of the conidium development of the isolate CBS 670.75.

Molecular studies

Single conidial transfers (CMW1099, CMW 1901 and CMW2547) were grown on cellophane discs placed on 2 % MEA (20 g malt extract, 20 g Biolab Agar / 1000 ml H_2O) and incubated at 25 °C until a mycelial mat had formed. The discs were transferred to sterile Petri dishes, lyophilised and stored at -20 °C. Nucleic acid was extracted from the lyophilised material following a modification of the procedure described by Raeder & Broda (1985). The freeze-dried mycelium was transferred to sterile Eppendorf tubes and 500 µl Extraction Buffer (200 mM Tris-HCl, pH 8.5; 250 mM NaCl; 25 mM EDTA, 0.5% SDS) added. The mixture was immersed in liquid nitrogen and ground with a plastic pestle in an Eppendorf tube. Phenol (350 µl) was added after grinding and the solution homogenised. Chloroform (150 µl) was added and the suspension was rapidly mixed, after which it was centrifuged for 1 hr at 10 000 rpm and 4 °C. The aqueous phase was immediately transferred to sterile Eppendorf tubes, and 1 volume of chloroform was added. The upper phase was transferred to sterile Eppendorf tubes and the DNA precipitated by the addition of 0.54 vol. Isopropanol and 0.1 vol. 3 M NaAc. After incubation for 60 min at -20 °C, the mixture was centrifuged for 30 min at 14 000 rpm at 4 °C. The pellet was washed with 70 % Ethanol, dried and resuspended in 100 µl sterile H_2O. The DNA yield was assessed by agarose gel electrophoresis.

A region within the ribosomal DNA operon, which includes the 5.8S gene and the internal transcribed spacer regions (ITS 1 & ITS 2), was amplified using the Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1988). Primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (10 OD) were used for amplification (White *et al.*, 1990). Reactions were performed in a Hybaid Omnigene Temperature Cycler (Hybaid, Middlesex, U.K.) for 35 cycles using *Taq* DNA Polymerase (Promega Cooperation, Madison, U.S.A.) and using conditions described by Visser *et al.* (1995). The final reaction conditions were 6.25 mM $MgCl_2$, 1.6 mM dNTPs and 50 pmol of each primer in a 100 μl reaction volume.

The amplified DNA fragments were visualised on a 1% (w/v) agarose gel to assess the size and quality of amplification products. The products were purified using the Magic PCR Preps (Promega Corporation, Madison, U.S.A.) and the resulting fragments were sequenced using the Sequenase PCR Product Sequencing Kit from Amersham (USB, Cleveland, Ohio, USA). The ITS1, ITS4, CS2 (5'CAATGTGCGTTCAAAGATTCG3') and CS3 (5'CGAATCTTTGAACGCACATTG3') primers (Visser *et al.*, 1995, White *et al.*, 1990) were used to determine the DNA sequence of both strands. The sequence data were visually aligned and the phylogenetic relationships determined using PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1993). The branch and bound options, as well as the heuristic options were used in the analysis. Confidence in the clustering and tree topology was determined using Bootstrap analysis and the *g*1 statistic.

RESULTS

Morphology

Morphological characteristics observed for the *Chalara*-like state of the *Juniperus* isolate and the holotype of *C. autographa* were similar(data not shown). Single attachment points were observed on the conidia, which is consistent with the detailed observations of Wingfield *et al.* (1995). This is indicative of apical wall building conidium development

(Minter, Kirk & Sutton, 1983) and atypical of species of *Ceratocystis s.str.*, that produce conidia through ring wall building conidium development (Minter *et al.*, 1982). However, the *Juniperus* and *Larix* isolates were distinct in that the latter had a prominent and well-developed *Sporothrix* state, and formed sclerotia in culture (Bakshi, 1951). In contrast, the *Juniperus* isolate very rarely formed an inconspicuous *Sporothrix*-like synanamorph, and formed no chlamydospores or sclerotia in culture (Fig. 1).

Molecular studies

Amplification of the DNA in each of the three isolates yielded a single DNA fragment of approximately 550 base pairs (bp). Approximately 500bp were sequenced, visually aligned and edited using MacClade 3.0 (Maddison & Maddison, 1993) (Fig. 2). The exhaustive and random trees options in PAUP were used to determine the $g1$ statistic for the obtained trees (Swofford, 1993; Hillis & Huelsenbeck, 1992).

The same tree configuration was observed using the consensus tree options with the branch and bound and heuristic options (Fig. 3). Bootstrap analysis revealed high confidence intervals for each cluster branch (Fig. 3). The minimum tree length obtained was 601 steps, with a Consistency Index (CI) of 0.900, a Homoplasy Index (HI) of 0.100 and a Retention Index (RI) of 0.894. The $g1$ value obtained from the exhaustive search and the random trees option for this study is –1.00, indicating a strong support for the parsimony analysis and the resulting tree topology. The $g1$ statistics discriminate phylogenetic signals from random noise in systematic data sets (Hillis & Huelsenbeck, 1992). It is an indication of the skewness of a distribution and $g1=0$ for a perfectly symmetrical tree length distribution (Hillis & Huelsenbeck, 1992; Huelsenbeck, 1991). Character data that are consistent with one phylogenetic hypothesis produce tree-length distributions that are highly skewed to the left (Huelsenbeck, 1991). Sequence data for *C. fimbriata* and the two Chalara spp. formed a group distinct from the *Juniperus* isolates. However, the *Ceratocystis, Chalara* and *Juniperus* isolates comprised a larger group that was very distinct from the isolate of *O. ulmi*. This clustering confirmed the previous findings (Hausner *et al.*, 1993, Wingfield *et al.*, 1995) that the *Chalara*-like anamorph of the *Juniperus* isolates (incorrectly referred to as the *Chalara* anamorph of *C. autographa*) is not a typical species of *Ceratocystis s.str.*

DISCUSSION

One question that needs to be resolved is whether *C. autographa* (IMI 20162), which was collected from Larch in the U.K. is the same as the isolate (CBS 670.75) collected from *Juniperus* in the Netherlands. Although their *Chalara* states are similar, the *Sporothrix* state is much more common and prominent in the Larch specimen than in the one from *Juniperus*. Furthermore, no teleomorph was observed in the *Juniperus* isolate, nor any sclerotia which Bakshi reported as commonly occurring in *C. autographa*. Because of these uncertainties we prefer to treat the isolate from *Juniperus* as a separate taxon.

The presence of a *Chalara,* as well as a *Sporothrix* anamorph, in a species of *Ceratocystis sensu lato* is contradictory to the current accepted generic concepts applied to the group (De Hoog & Scheffer, 1984; Weijman & De Hoog, 1975). The taxonomic placement of *C. autographa* is thus clearly in dispute and is in need of further investigation.

In 1995, Wingfield and co-workers conducted a study on the culture deposited in CBS as *C. autographa* (CBS 670.75). These authors observed many unusual features for the *Chalara*-like anamorph of *C. autographa.* Species of *Chalara* are characterised by ring wall building conidiogenesis resulting in chains of conidia with two attachment points (Minter *et al.*, 1982, Nag Raj & Kendrick, 1975). However, the *Chalara*-like anamorph observed for the type specimen of *C. autographa* (IMI 20162) and also for the isolate from *Juniperus* (CBS 670.75) represent two similar species that have conidia with single attachment points, typical of apical wall building conidiogenesis (Minter *et al.*, 1982). Wingfield *et al.*, (1995), therefore, suggested that further studies were needed to determine the correct classification of the *Chalara*-like anamorph. In a comparison of sequence data, Hausner *et al.* (1993) also suggested that the isolate CBS 670.75 probably does not represent the fungus originally described from Larch by Bakshi (1951).

Comparisons of ribosomal DNA sequence data obtained in the current study, clearly showed that the *Juniperus* isolates had no affinity with either *Ceratocystis s.str*, *Ophiostoma* or typical *Chalara* species. This confirms the observations of Hausner *et al.*, (1993) and Wingfield *et al.*, (1995), although not their interpretation of the problem.

In our view, the *Chalara*-like anamorph of *C. autographa* (IMI 20162) and the *Chalara*-like species from *Juniperus* (CBS 670.75) probably do not represent the same fungus, for reasons discussed above. Furthermore, based on the molecular and morphological data, we conclude that the *Chalara*-like anamorph of the fungus isolated from *Juniperus* represents a genus other than *Chalara*. We, therefore, describe a new genus, characterised by *Chalara*-like conidiophores and conidia with single attachment points that arise through apical wall building conidium development, to represent this fungus.

THE FOLLOWING DESCRIPTION SHOULD BE SEEN AS A DRAFT FOR THE PURPOSES OF THIS THESIS AND SHOULD NOT BE CITED. THE COMPLETE DESCRIPTION WILL BE PUBLISHED ELSEWHERE.

Xenochalara M.J. Wingf. & Crous gen. nov.

Species typica ***Xenochalara juniperi*** M.J. Wingfield *et* Crous

Hyphomycetosum genus morphologia simile Chalarae, sed distinctum quia conidia apicali pariete construendo producit quodque 'cycloheximide' tolerat. Mycelium constans ex hyphis ramosis septatis; hyphae hyalinae et leves, brunnescentes asperascentesque, repertae singulatim vel in filis. Chlamydosporae absentes. Conidiophora micronematosa, exorientia ex aerio mycelio vel ex submersis hyphis, erecta, simplicia, saepe ad cellas conidiogenas reducta, vel 1-septata, subcylindrica, recta vel exigue curvata, pallide brunnea, leves. Conidiogenae cellae phialidicae, terminales, subcylindricae vel lageniformes, leves, subbrunneae vel pallide brunneae, venter conicus vel ellipsoideus, collariculum anguste obconicum vel subcylindricum. Conidia hyalina, levia, aseptata, reperta in falsis catenis usque 55; brevi-clavata, apice rotundato, basi truncata.

A hyphomycete genus morphologically similar to *Chalara*, but distinct in producing conidia through apical wall building, and being tolerant of cycloheximide. Mycelium consisting of branched, septate hyphae; hyphae hyaline and smooth, becoming brown and rough, occurring singly or in strands. Chlamydospores absent. Conidiophores micronematous, arising from aerial mycelium or submerged hyphae, erect, simple, frequently reduced to conidiogenous cells, or 1-septate, subcylindrical, straight to slightly curved, light brown, smooth. Conidiogenous cells phialidic, terminal, subcylindrical or lageniform, smooth, pale to light brown, venter conical to ellipsoid, collarette narrowly obconical to subcylindrical. Conidia hyaline, smooth, aseptate, occurring in false chains of up to 55; short clavate, apex rounded, base truncate.

THE FOLLOWING DESCRIPTION SHOULD BE SEEN AS A DRAFT FOR THE PURPOSES OF THIS THESIS AND SHOULD NOT BE CITED. THE COMPLETE DESCRIPTION WILL BE PUBLISHED ELSEWHERE.

Xenochalara juniperi M.J. Wingf. *et* Crous sp. nov.
Teleomorph: unknown.

Mycelium constans ex ramosis septatis hyphis; hyphae hyalinae et leves, brunnescentes et asperascentes, repertae singulatim vel in filis usque 10, 1.5-2.5 æm diametro. Chlamydosporae absentes. Conidiophora micronematosa, exorientia ex mycelio aerio vel hyphis submersis, erecta, simplicia, saepe redacta ad cellulas conidiogenas, vel 1-septata, subcylindrica, recta vel exigue curvata, pallide brunnea, levia, 13-35 µm longa, 2.5-4 µm lata. Conidiogenae cellulae phialidicae, terminales, subcylindricae vel lageniformes, leves, subbrunneae vel pallide brunneae, 13-20 µm longae, 2.5-3(-4) µm latae; venter conicus vel ellipsoideus, 10-15 x 2.5-3(-4) µm; collariculum anguste obconicum vel subcylindricum, paries parum obscurior et crassior quam paries ventris, 5-8 x 1.5-2 µm; transitio a ventre ad collariculum vel abrupte vel paulatim fit, saepe quoque constricta ad basilare conidiophori septum. Conidia hyalina, levia, aseptata, evenientia in falsis usque ad 55 catenis; brevi-clavata, apice rotundato, basi truncata, (3-)3.5-4(-5) x 1-1.5 µm.

Holotype: The Netherlands, Llheedorp, on decaying needles of *Juniperus communis*, Nov. 1975, W. Gams, dried specimen deposited at PREM; culture ex-type, CBS 670.75.

Mycelium consisting of branched, septate hyphae; hyphae hyaline and smooth, becoming brown and rough, occurring singly or in strands of up to 10, 1.5-2.5 μm diam. Chlamydospores absent. Conidiophores micronematous, arising from aerial mycelium or submerged hyphae, erect, simple, frequently reduced to conidiogenous cells, or 1-septate, subcylindrical, straight to slightly curved, light brown, smooth, 13-35 μm long, 2.5-4 μm wide. Conidiogenous cells phialidic, terminal, subcylindrical or lageniform, smooth, pale to light brown, 13-20 μm long, 2.5-3(-4) μm wide; venter conical to ellipsoid, 10-15 x 2.5-3(-4) μm; collarette narrowly obconical to subcylindrical, wall slightly darker and thicker than that of venter, 5-8 x 1.5-2 μm; transition from venter to collarette abrupt to gradual, also frequently constricted at basal conidiophore septum. Conidia hyaline, smooth, aseptate, occurring in false chains of up to 55; short-clavate, apex rounded, base truncate, (3-)3.5-4(-5) x 1-1.5 μm.

No other examples are known of *Ceratocystis* species having both a *Sporothrix* and a *Chalara* anamorph. It is possible, if not probable, that the *Sporothrix* sp. present on the type specimen (IMI 20162) is the anamorph linked to the teleomorph structures described as *C. autographa* by Bakshi (1951). If this is true, then the fungus would best reside in *Ophiostoma*. The *Chalara*-like state, however, would be better accommodated in *Xenochalara*. Our re-examination of the dried type studied by Bakshi showed that it had indeed been contaminated with mites, which could have contaminated the plates with different fungi, as is evident from the *Penicillium* sp. sporulating on one. However, given the absence of cultures, this matter cannot be resolved further. Fresh collections from Larch in Scotland infested with *D. autographus* and *H. palliatus* would be required to finally resolve the identity of *C. autographa*. For the present we consider it a species of dubious status.

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Fig. 1. *Xenochalara juniperi* and its *Sporothrix* synanamorph sporulating on malt extract agar. Conidiophores, lageniform conidiogenous cells and short-clavate, catenulate conidia (bar = 10 μm).

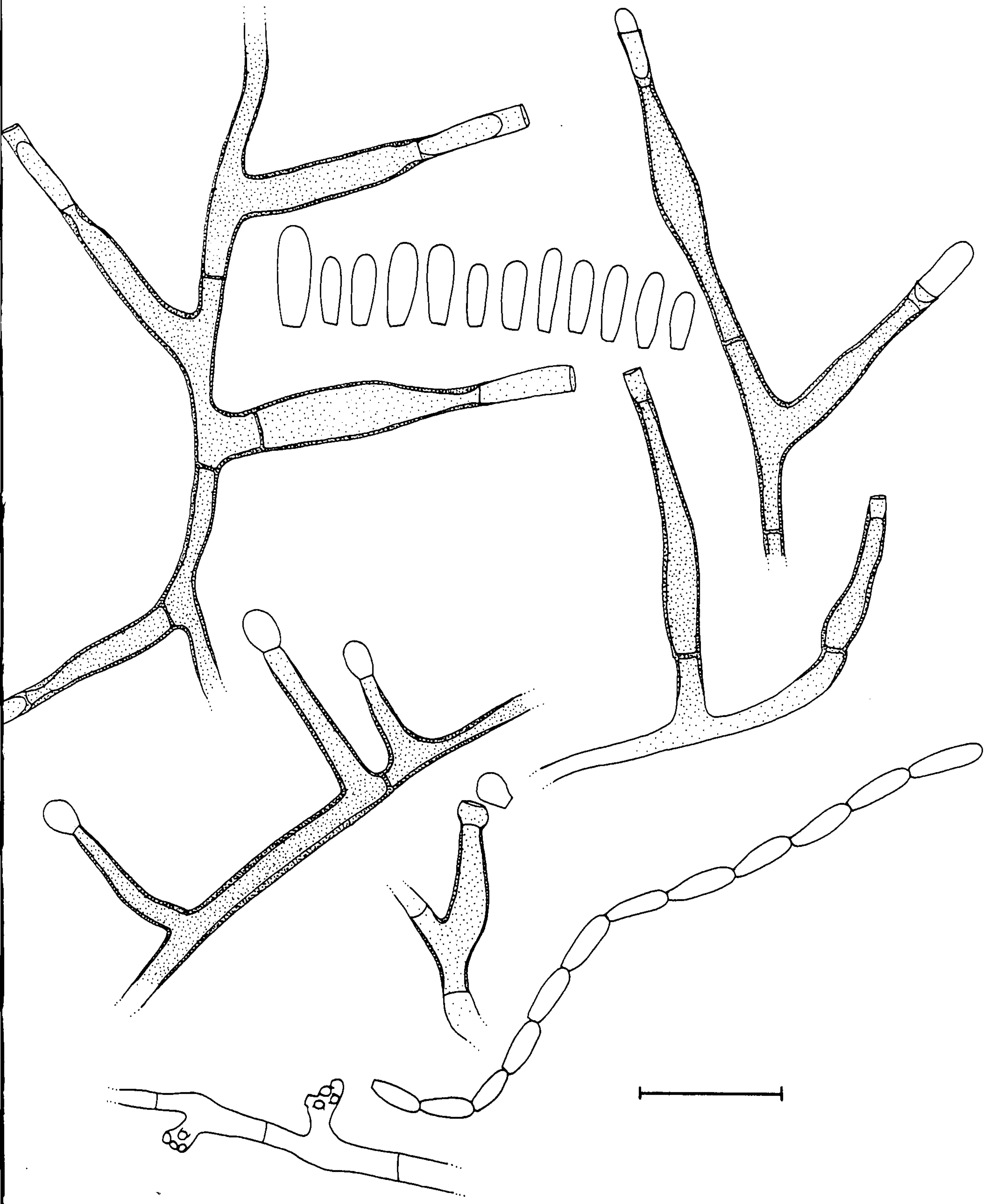

Fig. 2. DNA sequences obtained for the ITS1, ITS2 regions of the rDNA operon and the 5.8S rDNA gene from *Ceratocystis autographa* labelled isolates (CMW 1099, CMW 1901, CMW 2547) and the *Ceratocystis* and *Chalara* isolates (Witthuhn *et al.*, 1998a). Sequence data for *Ophiostoma ulmi* was obtained from the GENBANK sequence database. N indicates unknown bases; a dot indicates bases identical to the corresponding base in *Ceratocystis fimbriata* and dashes represent deletions in the sequence.

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                                       10         20         30         40         50         60         70         80
C.fimbriata                 ACTCTATAAA CCATGTGTGA ACG-TACCTA TCTTGTAGTG AGATGAATGC TGTTTTGGTG GTAGGGCCCT TCTGAAG---
C.fimbriata(C854)           .......... .......... .......... .......... .......... .......... .......... .......GGA
Ch.australis                .....T.... ....A..... ..A......- ...A.C---- -------... .---....C- --...---.. .GGT..C..A
Ch.neocaledoniae            .....-.... ....A..... ..A......- ...A.C---- -------... .---....C- --...---.. .GGT..C..A
C.douglasii                 .....-.... ....A..... ..A.....CT .T.A.C---- -------... .---....C- --...---.. .GGT..A.AA
C.autographa(cmw1099)       .....C-C.. ..C.A.--.T TAA....AAC CT....---- -------... .---....C. --..CCG... CGGCC.CC..
C.autographa(cmw2547)       .....C-C.. ..C.A.--.T TAA....AAC CT....---- -------... .---....C. --..CCG... CGGCC.CC..
C.autographa(cmw1901)       .....C-C.. ..C.A.--.T TAA....AAC CT....---- -------... .---....C. --..CCG... CGGCC.CC..
O.ulmi                      ....--CC.. ..C.-..C.. ..CG....CC GT.CTGTTCT C.T.----.. --..C...C. .G...----- -GG--..GG.

                                       90         100        110        120        130        140        150        160
C.fimbriata                 -GGCACC--G CTGCCAGCAG TAT-AGT-CT CG-CCACTGT -----AAA-C TCTT--A-TA TTTTCCA--- ------GA-T
C.fimbriata(C854)           G......... .......... ...T...... .A.....--. AT........ ....TT.T.. .....T.GA. ......--..
Ch.australis                .--..AGTC- T....G.T.. ...TT--.-- --.------- AC........ ....TT-... ...-.T.GA. ........A.
Ch.neocaledoniae            .--..AGTC- T....G.T.. ...TT--.-- --.------- AC........ ....TT-... ...-.T.GA. ........A.
C.douglasii                 .--..AGTC- -....G.T.. ...AT--.-- --.---A-A- A.......A. ....T..A.. ...-.T.CA. ........A.
C.autographa(cmw1099)       .......TC. ...GTG---- ---....G.. ......GA.G ACCCC...C. CT--..-.-- -------... ........A.
C.autographa(cmw2547)       .......TC. ...GTG---- ---....G.. ......GA.G ACCCC...C. CT--..-.-- -------... ........A.
C.autographa(cmw1901)       .......TC. ...GTG---- ---....G.. ......GA.G ACCCC...C. CT--..-.-- -------... ........A.
O.ulmi                      GC..--GTC- T.CGGG..GC .GCCTC.... .TC...G-.- ..TCCTTCGG G.GCCCGCC. CGGC.GCGAG CCGCCT..AC

                                       170        180        190        200        210        220        230        240
C.fimbriata                 T-TTTT---- -CA-T----- ----TGCTGA GTGGCATAA- -CTATAAAAA A--GTTAAAA CTTTCAACAA CGGATCTCTT
C.fimbriata(C854)           ..-....... .......... .......... .......-.A .......... .A........ .......... ..........
Ch.australis                ..A....... .......... .......... .......T.A ..-....--T .A........ .......... ..........
Ch.neocaledoniae            ..A-...... .......... .......... .......T.A ..-....--T .A........ .......... ..........
C.douglasii                 .TA....... .......... .......... .........A A.-....--T .A........ .......... ..........
C.autographa(cmw1099)       -.A-...... .-.G...... GTCG.-.... .-----..-. ......T..T .......... .......... ..........
C.autographa(cmw2547)       -.A-...... .-.G...... GTCG.-.... .-----..-. ......T..T .......... .......... ..........
C.autographa(cmw1901)       -.A-...... .-.G...... GTCG.-.... .-----..-. ......T..T .......... .......... ..........
O.ulmi                      CT...CTAAA C..G.AACGA AACG.-.... .--AA.C..A CAA.A.C.-- -...CC.... .......... ..........
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                                     250        260        270        280        290        300        310        320
C.fimbriata               GGCTCTAGCA TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT TGCAGAATTC AGTGAATCAT CGAATCTTTG
C.fimbriata(C854)         .......... .......... .......... .......... .......... .......... .......... ..........
Ch.australis              .......... .......... .......... ........C. .......... .......... .......... ..........
Ch.neocaledoniae          .......... .......... .......... ........C. .......... .......... .......... ..........
C.douglasii               .......... .......... .......... ........C. .......... .......... .......... ..........
C.autographa(cmw1099)     ..T...G... .......... .......... .......... .......... .......... .......... ..........
C.autographa(cmw2547)     ..T...G... .......... .......... .......... .......... .......... .......... ..........
C.autographa(cmw1901)     ..T...G... .......... .......... .......... .......... .......... .......... ..........
O.ulmi                    ......G... .......... .......A.. ........C. .....C.... .......... ..GAGTC... ..........

                                     330        340        350        360        370        380        390        400
C.fimbriata               AACGCACATT GC-CCC--TG GTAGTATTCT GCCAGGCATG CCTGTCCGAG CGTCATTTCA CCACTCAAGG ACTCCTTTGT
C.fimbriata(C854)         .......... ..G..-.... .C........ .......... .......... .......... .........A TTATTC..T-
Ch.australis              .......... ..G..-.... .C........ .......... .......... .......... .........C T.CG...---
Ch.neocaledoniae          .......... ..G..-.... .C........ .......... .......... .......... .........C T.CG...---
C.douglasii               .......... ..G..-.... .C........ .......... .......... .......... .........C T..G...---
C.autographa(cmw1099)     .......... ..G...CG.. .---.....C ..GG...... .....T.... .....G.A.. A........C T..G...---
C.autographa(cmw2547)     .......... ..G...CG.. .---.....C ..GG...... .....T.... .....G.A.. A........C T..G...---
C.autographa(cmw1901)     .......... ..G...CG.. .---.....C ..GG...... .....T.... .....G.A.. A........C T..G...---
O.ulmi                    .......... .GG...GCCA .---...... .G.G...... .......... .........C ..C....GAT G.C.T....G

                                     410        420        430        440        450        460        470        480
C.fimbriata               T---CTTGGC GTTGGAGGTC CTGT--TCTC CCCTGAA--C AGGCCGCCGA AATGTATCGG CTGTTATACT TGCCAACTCC
C.fimbriata(C854)         -......... .......... .......... .......... .......... ....C..... .......... ..........
Ch.australis              -...---..T ........A. .C.-CG...T TATGCG-..- -......... ....C..... .......... ...-.G..T.
Ch.neocaledoniae          -...---..T ........A. .C.-CG...T -ATGCG-..- -......... ....C..... .......... ...-.G..T.
C.douglasii               -...---..T ........A. .C.-CA...- --TGCG-..- -......... ....C..... ........T. ...-.G..T.
C.autographa(cmw1099)     -...---..T C...-..CC- .C.-..---. .GGCA.CGG. ....TTT-A. ...CAG.-.. .G.CGCCC.C ..G.T-..AA
C.autographa(cmw2547)     -...---..T C...-..CC- .C.-..---. .GGCA.CGG. ....TTT-A. ...CAG.-.. .G.CGCCC.C ..G.T-..AA
C.autographa(cmw1901)     -...---..T C...-..CC- .C.-..---. .GGCA.CGG. ....TTT-A. ...CAG.-.. .G.CGCCC.C ..G.T-..AA
O.ulmi                    GAGGGC...T .....-.C.. ...CCC...G .GTGCG-..- ...G.C.TC. ..ACC.GT.. .G.GCCCGTC ..GTTGGCT.
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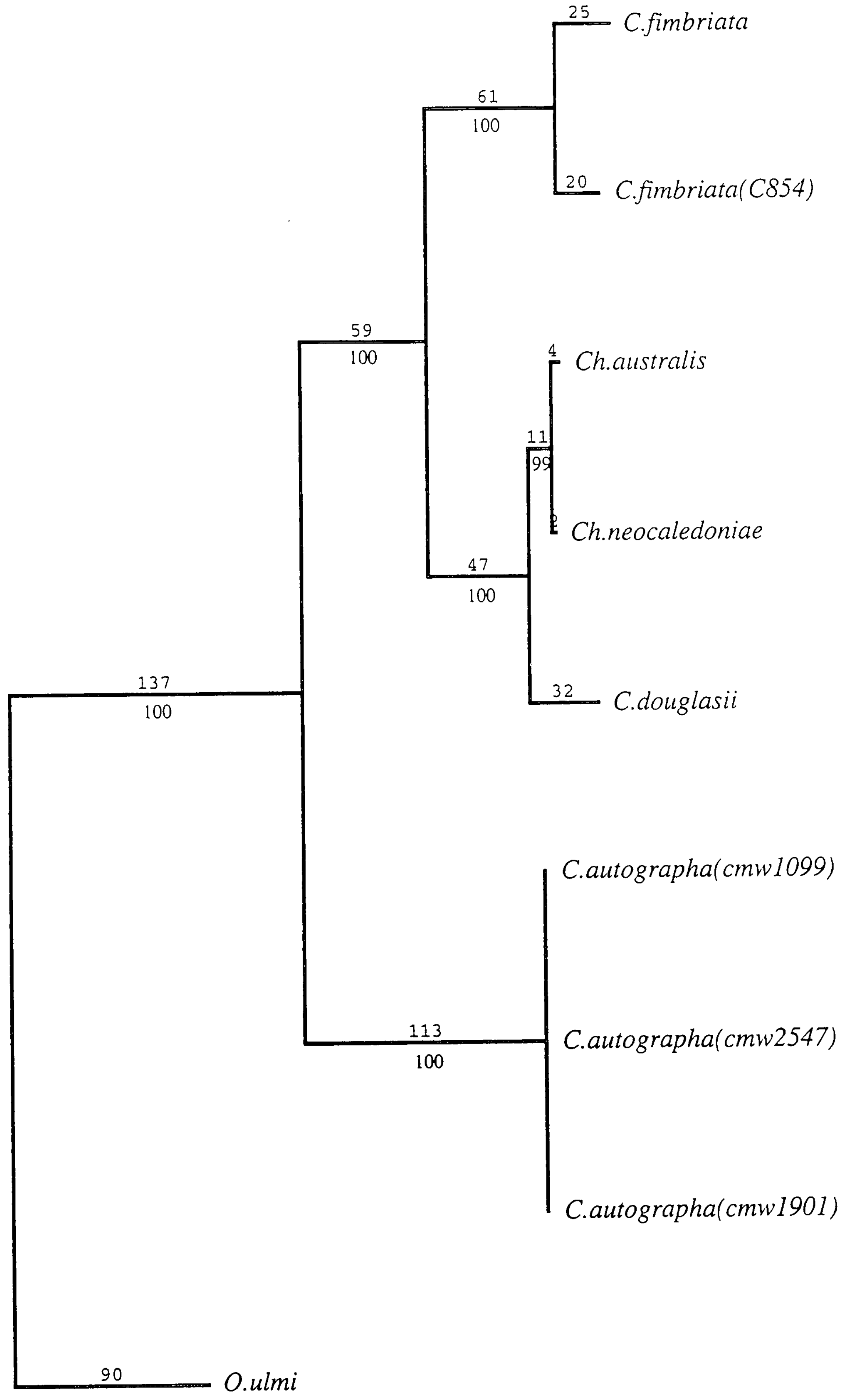
```
                                     490        500        510        520        530        540        550        560
C.fimbriata                CCTGTGTAGT A-TAAAA-TT TCTAATTTTT ACACTTTGAA GTTCTTGTGT AACA-CGCCG CTAAACCAAC CCCCTCAAC-
C.fimbriata(C854)          .......... .......T.. -......... .......... .......... ....C-.... .....----. ..G.......
Ch.australis               .......... .A..---T.. A-.----... ..G....... AC......AC T...T-.... -....A---. ...-....-.
Ch.neocaledoniae           .......... .A..---T.. A-.-----.. ..G....... AC......AC T...T-.... -....A---. ..--....-.
C.douglasii                .......... .A..---T.. A-.----... ..G....... AC....T.A. ....T-.... -....A---. ..-A....TA
C.autographa(cmw1099)      G.---..... .A..C.CCCC G...CAGAG. C.CGG.G..T .--....--- ----.----- --CC.G.... ...--.....
C.autographa(cmw2547)      G.---..... .A..C.CCCC G...CAGAG. C.CGG.G..T .--....--- ----.----- --CC.G.... ...--.....
C.autographa(cmw1901)      G.---..... .A..C.CCCC G...CAGAG. C.CGG.G..T .--....--- ----.----- --CC.G.... ...--.....
O.ulmi                     .----.-..C GC-.G--.-- ---------. ..-.---... CGCAAGT.C. CT.TCTCG.T ..GC.G.--. ..GG..GGTG

                                     570        580
C.fimbriata                ---------- ---TTTTGTT
C.fimbriata(C854)          .......... ..........
Ch.australis               .......... .......-..
Ch.neocaledoniae           .......... .......T..
C.douglasii                TATATATATG TAT.A..T..
C.autographa(cmw1099)      .......... ...-...C.-
C.autographa(cmw2547)      .......... ...-...C.-
C.autographa(cmw1901)      .......... ...-...C.-
O.ulmi                     CCC.AGCCGT CAACGGC.C.
```

Fig. 3. Phylogram generated from PAUP analysis depicting the phylogenetic relationships between the *Ceratocystis autographa* (CMW 1099, CMW 1901, CMW 2547), *Ceratocystis* isolates and *Ophiostoma ulmi*. Tree length = 601. The number of base substitutions is indicated above the branches and the bootstrap percentages (100 bootstrap replicates) are indicated below the branches.


25
C.fimbriata
61
100
20
C.fimbriata(C854)
59
100
4
Ch.australis
11
99
2
Ch.neocaledoniae
47
100
137
100
32
C.douglasii
C.autographa(cmw1099)
113
100
C.autographa(cmw2547)
C.autographa(cmw1901)
90
O.ulmi

CHAPTER 4

Leptographium guttulatus sp. nov., a new species from spruce and pine in Europe

Leptographium spp. are anamorphs of *Ophiostoma* and are characterised by conidiophores with dark mononematous stipes and complex conidiogenous apparatuses with several series of branches. These fungi are also characterised by their distinct tolerance to high concentrations of cycloheximide. Most *Leptographium* spp. cause sap stain in conifer timber and some species are root pathogens of trees. In recent years, an unknown *Leptographium* sp. has regularly been isolated from spruce and pine in Europe. This species is found in association with bark beetles that infest these conifers. These insects most probably act as vectors for this fungus and belong to the genera *Hylurgops*, *Hylastes* and *Tomicus*. Morphological comparisons, as well as partial ribosomal DNA comparisons have shown that this species represents a previously undescribed taxon. In this paper we describe this fungus as new and provide the name *Leptographium guttulatus* sp. nov. for it.

Keywords: *Leptographium*, *Ophiostoma,* ribosomal DNA, phylogeny

Species of *Leptographium* Lagerberg & Melin are characterised by dark mononematous conidiophores with complex conidiogenous apparatuses. The conidiogenous cells produce numerous conidia that accumulate in mucilaginous masses at the apex of the conidiophores (Kendrick, 1962; Wingfield, 1993). *Leptographium* spp. are morphologically adapted for dispersal by insects, especially bark beetles (Coleoptera: Scolytidae) (Lagerberg, Lundberg & Melin, 1927; Leach, Orr & Christensen, 1934; Münch, 1907; Upadhyay, 1981). Some *Leptographium* spp. are known anamorphs of *Ophiostoma* (Harrington, 1987; Wingfield, Seifert & Webber, 1993). *Leptographium* spp. are best known among plant pathologists for their ability to cause blue-stain of conifer sapwood. A few species also cause diseases of trees and the best known of these is black-stain root disease caused by three varieties of *Leptographium wageneri* (Kendrick) Wingfield (Cobb, 1988; Harrington & Cobb, 1987).

Leptographium spp. can be divided broadly into three groups based on their conidium size. These include species with conidia that are long (7 - 20 µm), medium (5 - 8 µm) and short (3 - 5 µm). Most *Leptographium* spp. can be accommodated in the group with medium sized conidia (Jacobs, unpublished). Species in this group include the anamorphs of *O. europhioides* (Wright & Cain) Solheim, *O. laricis* Van der Westhuizen *et al.* and *O. huntii* (Robinson-Jeffrey) Wingfield (Robinson-Jeffrey & Davidson, 1968; Van der Westhuizen *et al.*, 1995; Wright & Cain, 1961). This is in contrast to the anamorphs of species such as *O. penicillatum* Grosmann, *O. americanum* Jacobs, Wingfield & Bergdahl and *O. dryocoetidis* Kendrick & Molnar that have conidia that are at least twice or even three times as long as they are wide (Grosmann, 1931; 1932; Jacobs, Wingfield & Bergdahl, 1997; Kendrick & Molnar, 1965). Other characters that are useful in the identification of *Leptographium* spp. include the length of conidiophores, type of primary branches and the presence or absence of rhizoids.

Recently, an unknown *Leptographium* spp. has been isolated from spruce and pine in Europe. Wingfield & Gibbs (1991) referred to these isolates as *Leptographium* sp. "GG" and postulated that they might be the same as the fungus known as *Ophiostoma penicillatum* f. *palliati* described by Mathiesen (1950), but for which no authentic material exists. The aim of this study was to examine a collection of isolates of this fungus in detail at both molecular and morphological levels.

MATERIALS AND METHODS

Hylastes cunilaris, *Hylurgops palliatus* and *Hylurgops glabratus* adult beetles were collected from infested Norway spruce (*Picea abies*) logs. Fungal isolations were done by macerating the beetles in a few drops of sterile water and diluting the macerate on the surface of 90 mm plastic Petri dishes with Malt extract agar (MEA, 20 g Merck malt extract, 16 g Sigma agar, 100 mg Streptomycin sulphate, 1000 ml destilled water). In some cases, 10 mg/l of Cycloheximide was added. Alternatively, entire beetles or parts of the insects were placed on the plates. The dishes were incubated at room temperature in diffuse daylight. Fungi were purified by transferring mycelium of conidial masses to fresh MEA plates. Additionally, isolations were performed from stained sapwood of pine (*Pinus sylvestris*) attacked by the bark beetle *Tomicus minor*.

Isolates used for molecular comparison are listed in Table 1. Two isolates of *Leptographium* sp. "GG" were used in the molecular comparisons. These were compared with isolates of *L. penicillatum, L. americanum* and the *Leptographium* anamorph of *O. dryocoetidis.* These species were chosen to represent *Leptographium* spp. with long conidia. *Leptographium laricis* and the *Leptographium* anamorph of *O. europhioides* were chosen to represent species with short conidia and an isolate of *Leptographium procerum* was chosen as the outgroup because it is considered to be a typical *Leptographium* and is characterised by small conidia.

Molecular comparisons

Cultures used (Table 1) were grown in 2 % liquid Malt Extract (2 g/l ME per 100 ml H_2O) in 250 ml Erhlenmeyer flasks at 25 °C until the surface of the medium was covered with mycelium. A modification of the DNA extraction technique described by Raeder & Broda (1985) was used to extract total genomic DNA. Mycelium was harvested and transferred to sterile Eppendorf tubes and extraction buffer (500 µl total; 200 mM Tris-HCl, pH 8.5; 250 mM NaCl; 25 mM EDTA; 0.5 % SDS) added. The suspension was quick frozen in liquid nitrogen and ground to a fine paste with a pestle. Proteins were denatured by phenol-chloroform steps until the interphase was clear. The DNA was precipitated by the addition of 0.54 volume isopropanol and 0.1 volume 3 M NaAc, pH 5.8. After incubation for at least 60 min at -20 °C, the DNA was pelleted by centrifugation at 4 °C for 30 min at 14 000 rpm. The pellets were washed with 70 % ethanol, dried and resuspended in 100 µl sterile H_2O. The presence of DNA was determined by agarose gel electrophoresis.

The ITS1 and ITS2 internal transcribed spacer regions, as well as the 5.8S gene of the rDNA operon were amplified, using the Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1988). Primers used in the amplification reactions are CS1 (5'-TAGCTGATCCGAGGTCAA-3') (Strydom, Wingfield & Wingfield, 1997), ITS-1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes & Bruns, 1993), ITS4 (5'-TCCTCCGCTTATTGATATGC -3'), LR5 (5'-TCCTGAGGGAAACTTCG-3'), LR1 (5'-GGTTGGTTTCTTTTCCT-3') (White *et al.*, 1990). Amplifications were performed in a Hybaid Touchdown temperature cycler (Hybaid, Middlesex, UK) for 35 cycles using High Fidelity Expand *Taq* DNA polymerase (Boehringer Mannheim, Germany) with the Expand HF Buffer with $MgCl_2$, supplied by the manufacturer and 2 ng DNA for each isolate. The cycling parameters were: an initial denaturation step at 95 °C for 2 min followed by 35 cycles of 95 °C for 45 seconds, 55 °C for 45 seconds, 62 °C for 20 seconds and 72 °C for 45 seconds. The reactions were completed with a final elongation step at 72 °C for 8 min. The PCR fragments were visualised on a 1 % agarose gel containing ethidium bromide and using a UV light. A single fragment of about 600 base pairs (bp) was obtained. However, small differences in fragment size was observed for the different species.

The PCR products were purified using the Nucleon QC for PCR/oligo clean-up kit (Amersham Life Science, England). The Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham Life Science, USA) on the ABI PRISM 377 automatic sequencer (Perkin Elmer Applied Biosystems) was used for sequencing. A region of 550 bp to 590 bp was sequenced for *O. penicillatum, O. americanum*, O. europhiodes, *O. laricis* and *Leptographium* sp 'GG' using primers ITS-1F and CS1. The

ITS2 region for *O. dryocoetidis* was sequenced using primer LR1. The sequence data were edited using Sequence Navigator (PE Applied Biosystems) and manually aligned. Phylogenetic relationships were determined using PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1993). Confidence intervals for the various groups were determined using Bootstrap analysis (1000 replicates).

Morphology and growth characteristics

All measurements were done on fungal structures produced in culture on 2 % Malt extract agar, MEA, (20g Biolab malt extract, 20g Biolab agar and 1000 ml destilled water). Fungal structures for microscopic examination were mounted on slides in lactophenol. Fifty measurements of each relevant morphological structure were made and ranges and averages computed.

The optimal temperature for growth of the *Leptographium* sp. (CMW 742) was determined by inoculating eight MEA plates for each temperature with a 0.6 mm diameter agar disk taken from the actively growing margin of a fresh isolate. The plates were incubated at temperatures ranging from 5 to 35 °C at 5 °C intervals. Colony diameters were measured on the fourth and the eighth day after commencing the experiment, and the diameters of colonies were computed as an average of eight readings.

Cycloheximide tolerance of the *Leptographium* sp. was determined by growing isolate CMW 742 on 2 % MEA amended with different concentrations of cycloheximide (0, 0.05, 0.1, 0.5, 1, 2.5 and 5 g/l) in Petri dishes. Dishes were incubated in the dark at 25 °C for eight days and the colony growth was measured. Five replicates were done for each concentration and the growth rate was determined based on the average of ten readings.

RESULTS

Molecular comparisons

A single DNA fragment of approximately 600 bp was observed in the amplification reactions for all isolates. From these fragments, 550 bp were sequenced for the isolates of *O. penicillatum* (CMW 2306, CMW 2302, CMW 2642, CMW 2643), *O. americanum* (CMW 495, CMW 2963) and *O. dryocoetidis* (CMW 442) (Table 1). A total of 590 bp were sequenced for isolates of *O. laricis* (CMW 2014, CMW 2016, CMW 1980), *O. europhioides* (CMW 452, CMW 1949) and *Leptographium* spp. "GG" (CMW 1310, CMW 742) (Fig. 1). A single most parsimonious tree was generated using PAUP analysis. The phylogram obtained (Fig. 2) showed the results of a General Heuristic search, with *Leptographium procerum* as the functional outgroup. The shortest tree length was 496, with a Consistency index (CI) of 0.823, a Retention Index (RI) of 0.177 and Homoplasy Index (HI) of 0.916.

Isolates of *Ophiostoma penicillatum, O. americanum* and *O. dryocoetidis* clustered to form a single clade (Fig. 2). From the dendrogram it appeared that *O. penicillatum* and *O. americanum* are more closely related to each other than they are to *O. dryocoetidis.* The second clade was comprised of the isolates of *O. europhioides* and *O. laricis* (Fig. 2). The unknown species, *Leptographium* sp. "GG" (Wingfield & Gibbs, 1991) clustered separately to the other groups. The outgroup species, *L. procerum* appears to be more closely related to the unknown *Leptographium* sp. than to the other isolates in the study.

Morphology and growth characteristics

The *Leptographium* sp. "GG" considered here, is characterised by conidiophores typical of *Leptographium* spp. The conidiogenous apparatus superficially resembles the brush-like conidiogenous apparatus of the anamorph of *O. europhioides.* However, the *Leptographium* sp. is characterised by large guttules in its conidia, that distinguish it from other *Leptographium* spp. Wingfield & Gibbs (1991) suggested that this species could be the same as the fungus identified by Mathiesen (1950) as *Ophiostoma penicillatum* f. *palliati.* From the molecular comparison in this study, it is clear that this species has no relatedness to *O. penicillatum* and it is morphologically distinct from all other *Leptographium* spp. The fungus is, therefore, described as a new species in *Leptographium.*

THE FOLLOWING DESCRIPTION SHOULD BE SEEN AS A DRAFT FOR THE PURPOSES OF THIS THESIS AND SHOULD NOT BE CITED. THE COMPLETE DESCRIPTION WILL BE PUBLISHED ELSEWHERE.

Leptographium guttulatus **Wingfield & Jacobs sp. nov.**

Colonia atro-olivacea (21 "m). Coloniae margo levis. Hyphae submersae in medio solido paucis myceliis aeriis, olivaceae, (21 "k), leves, rectae, non constrictae ad septa, 5 - 13 (medius numerus = 9) æm diametro. Conidiophora evenientia singulatim, exorientia directe ex mycelio vel aerio mycelio, erecta, macronematosa, mononematosa, 200 - 810 (medius numerus = 415) æm longitudine, rhizoidaceae structurae absentes. Conidiogenus apparatus 60 - 200 (medius numerus = 114) æm longus, conidica massa exclusa, 2 usque 4 seriebus ramorum cylindricorum; 2 - 4 metulae primariae. Conidiogenae cellae discretae, 2 - 3 per ramum, cylindricae, exigue attenuatae ad apicem, 10 - 27 (medius numerus = 17) æm longae et 2 - 3 (medius numerus = 2)_ æm latae. Conidia hyalina, aseptata, oblonga vel exigue obovoidea, 4 - 10 (medius numerus = 7) x 2 - 3 (medius numerus = 2) æm.

Colonies with optimal growth at 25 °C on 2 % MEA, reaching 36 mm in diameter in 9 days. Little growth at 5 °C and no growth above 30 °C. Able to withstand high concentrations of cycloheximide with a 5 % increase in growth on 0.5 g/l cycloheximide after 9 days at 25 °C in the dark. Colony dark olive (21''m). Colony margin smooth. Hyphae submerged on solid medium with little aerial mycelium, olivaceous (21''k), smooth, straight, not constricted at the septa, 5 - 13 (mean = 9) µm diameter.

Conidiophores occurring singly, arising directly from the mycelium or aerial mycelium, erect, macronematous, mononematous, 200 - 810 (mean = 415) µm in length (Fig. 3), rhizoid-like structures absent (Fig. 4, 9a). Stipe olivaceous (21''k), smooth, cylindrical, simple, 2 - 7 septate, 120 - 670 (mean = 300) µm long (from first basal septum to below primary branches), 5 - 12 (mean = 8) µm wide below primary branches, apical cell not swollen; 5 - 12 (mean = 8.5) µm wide at base, basal cell not swollen. Conidiogenous apparatus 60 - 200 (mean = 114) long, excluding the conidial mass, with 2 to 4 series of cylindrical branches; 2-4 primary branches, light olivaceous (21''k) to olivaceous (21''m), smooth, cylindrical, aseptate, 18 - 40 (mean = 29) µm long and 5 - 10 (mean = 7) µm wide, secondary branches light olivaceous (21''k), aseptate, 15 - 35 (mean = 24.5) µm long, 3 - 8 (mean = 5.5) µm wide; tertiary branches hyaline, aseptate, 10 - 33 (mean = 21) µm long, 3 - 5 (mean = 4) µm wide, quaternary branches hyaline, aseptate, 9 - 25 (mean = 14.5) µm long, 2 - 4 (mean = 3) µm wide (Fig. 5, 9b). Conidiogenous cells discrete, 2-3 per branch, cylindrical, tapering slightly at the apex, 10 - 27 (mean = 17) µm long and 2 - 3 (mean = 2) µm wide. Conidium development occurring through replacement wall building with holoblastic ontogeny and percurrent proliferation and delayed secession giving a false impression of sympodial proliferation (Minter, Kirk & Sutton, 1982; 1983; Van Wyk, Wingfield & Marasas, 1987) (Fig. 6, 7). Conidia hyaline, aseptate, oblong to slightly obovoid, prominent guttulate, 4 - 10 (mean = 7) x 2 - 3 (mean = 2) µm. Conidium frill absent (Fig.8, 9c). Conidia accumulating in slimy droplets at the apices of conidiogenous apparatus, hyaline at first, becoming cream coloured (19'f) with age (Rayner, 1970). Conidial masses cream coloured when wet, remaining the same colour when dry.

SPECIMENS EXAMINED: HOLOTYPE: CMW 742, isolated from *Tomicus piniperda* from *Pinus sylvestris,* Orleans, France, 1984, M. Morelet. PARATYPES: CMW 1310, isolated from *Tomicus piniperda* from *Pinus,* Fresley Cannon Wood, Hampshire, England, 1988, J.N. Gibbs, CMW 4901, isolated from *Hylurgops palliatus* from Norway spruce, Flatz, Niederösterreich, Austria, 28 May 1997, T. Kirisits, CMW 4902, isolated from *Hylurgops glabratus* from Norway spruce, Glein, Styria, Austria, 3 August 1993, T. Kirisits, CMW 4903 isolated from *Hylurgops* *palliatus* from Norway spruce, Flatz, Niederösterreich, Austria, 28 May 1998, T. Kirisits.

Insect associates

Leptographium guttulatus was rarely isolated from adult beetles of *H. cunicularius*, *H. palliatus* and *H. glabratus* collected from Norway spruce and from the sapwood of a pine log attacked by *Tomicus minor*. Based on the limited material of this study, *L. guttulatus* appears to be a minor component of the mycobiota of these four bark beetles. *Ophiostoma europhioides* was one of the most frequent ophiostomatoid fungi isolated from *H. cunicularius*, *H. palliatus* and *H. glabratus*. *Ophiostoma penicillatum* was never isolated from the above mentioned insects.

DISCUSSION

Leptographium guttulatus can easily be distinguished from other *Leptographium* spp. based on the distinct guttules in the conidia, that are unlike those of any other *Leptographium* spp. This species is one of several *Leptographium* spp. found both on spruce and pine in Europe. Other species include the *Leptographium* anamorph of *O. europhioides* and *L. penicillatum* (Solheim, 1986). *Leptographium guttulatus* can be distinguished from *O. europhioides* primarily based on the absence of a teleomorph in the former species. The anamorph of *O. europhioides* can also readily be distinguished from *L. guttulatus* based on its considerably smaller conidiophores, although the conidiogenous apparatuses of these two species are similar in their brush-shaped appearance. The shapes and sizes of the conidia of these species are similar, but those of *L. guttulatus* can be distinguished based on the presence of guttules. The anamorph of *O. penicillatum* is characterised by large allantoid conidia, in contrast to the obovoid conidia of *L. guttulatus*. These two species can, therefore, easily be distinguished. From the molecular data it is also clear that *L.*

guttulatus is not related to *O. penicillatum* and that any reference to the latter species is misleading.

As is true with most *Leptographium* spp., *L. guttulatus, O. penicillatum and O. europhiodes* are associated with bark beetles (Coleoptera: Scolytidae). *Leptographium guttulatus* has been isolated from spruce and pine in Europe and is transmitted by a wide range of bark beetles, that belong to the genera *Hylastes*, *Hylurgops* and *Tomicus* (Tables 1 & 2) (Wingfield & Gibbs, 1991). In contrast, *O. penicillatum* is consistently associated with the spruce bark beetles *Ips typographus* (Solheim, 1986; Krokene & Solheim, 1996), *Ips duplicatus* (Krokene & Solheim, 1996) and *Ips amitinus* (Kirisits, unpublished). Mathiesen (1950) also reported *O. penicillatum* to be associated with *H. palliatus*, although this record is probably not correct. In the present study, *O. penicillatum* was neither isolated from *H. cunicularius, H. glabratus* nor *H. palliatus*, that are vectors of *Leptographium guttulatus*. Furthermore, *O. penicillatum* was not found to be associated with *H. palliatus* in Denmark (Harding, 1989) and Norway (Krokene & Solheim, 1996). The common insect associates of *L. guttulatus* thus appear to be quite different to those of *O. penicillatum*. Similar to *L. guttulatus*, *Ophiostoma europhioides* is found to be associated with various bark beetles on spruce and pine in Europe. For example, *O. europhioides* is found to be associated with *Ips typographus* (Solheim, 1986), *Ips duplicatus* (Krokene & Solheim, 1996), *Ips amitinus* (Kirisits, unpublished), *Hylurgops palliatus* (Harding, 1989; Krokene & Solheim, 1996) on Norway spruce and with *Tomicus piniperda* attacking Scots pine (Solheim & Långström, 1991). In this study *O. europhioides* was frequently isolated from *H. cunicularius, H. palliatus* and *H. glabratus*. The literature, as well as the results of the present study, thus clearly show similarities in the habitat of *L. guttulatus* and *O. europhioides*.

Ophiostoma americanum and *O. dryocoetidis* were included in the study because they are both characterised by large conidia that are usually three times as long as they are wide and thus, similar to those of *O. penicillatum* (Grosmann, 1930; Kendrick & Molnar, 1965; Jacobs *et al.,* 1997). However, these species are not native to Europe and are known only from *Larix* spp. and *Abies lasiocarpa,* respectively (Kendrick &

Molnar, 1956; Jacobs *et al.*, 1997). Thus, they cannot easily be confused with *L. guttulatus*.

From the molecular data generated in this study, it seems that species characterised by large spores cluster together to form a well resolved clade. This phenomenon also seems true for species with smaller conidia. These include isolates of *O. laricis* and *O. europhioides*. It, therefore, appears that conidium size might be an indication of phylogenetic relationships. However, *Leptographium guttulatus* (sp. "GG") and *L. procerum*, are both characterised by similar sized conidia than those of the anamorphs of *O. laricis* and *O. europhioides* but clustered separately from the small conidia clade, thereby contradicting the hypothesis that conidium size is an indication of phylogenetic relationship. This is also apparent in the isozyme analysis of several *Leptographium* spp., done by Zambino & Harrington (1992), where there is no correlation between conidium size and phylogeny.

The taxonomy of *Leptographium* has been in disarray virtually since the establishment of the genus by Lagerberg & Melin in 1927. In recent years, efforts have been made to improve this situation. Where species have some significant ecological or political significance, they have generally been described, although many undescribed species remain. The description of *L. guttulatus* has lead to a better understanding and taxonomic placement of one species that has been isolated regularly in recent years. What must follow is a comprehensive review of the genus *Leptographium,* which is also supported by molecular comparisons. We are currently undertaking such a study and this will hopefully resolve many of the remaining taxonomic questions pertaining to species in the genus.

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Table 1. Isolates used for molecular comparisons in this study.

Isolate number[a]	Species	Origin	Host	Insect associate	Collector
CMW 2306	*O. penicillatum*	Japan	*Picea jezoensis*	*Ips typographus* f. *japonicus*	Y Yamaoka
CMW 2302	"	"	"	"	"
CMW 2642	"	Norway	*Picea abies*	*Ips typographus*	H. Solheim
CMW 2643	"	Sweden	"	*Ips typographus*	A. Käärik
CMW 495 (CBS 497.96)	*O. americanum*	North America	*Larix decidua*	*Dendroctonus simplex*	D.R. Bergdahl
CMW 2963	"	"	"	"	M.J. Wingfield
CMW 2014	*O. laricis*	Japan	*Larix kaempferi*	*Ips cembrae*	"
CMW 2016	"	"	"	"	"
CMW 1980	"	"	"	"	Y. Yamaoka
CMW 452 (CBS 275.65)	*O. europhioides*	USA			T.C. Harrington
CMW 1949	"	Japan	*Larix kaempferi*	*Ips cembrae*	Y. Yamaoka
CMW 1310	*L.* sp. "GG"	England	*Pinus sylvestris*	*Tomicus piniperda*	J.N. Gibbs
CMW 742	"	France	"	"	M. Morelet
CMW 442	*O. dryocoetidis*	British Columbia	*Abies lasiocarpa*	*Dryocoetus confusus*	A.C. Molnar
CMW 662 (CBS 929.69)	*L. procerum*	Virginia, USA	Pine forest soil		C.S. Hodges

a. CMW - Culture collection of the Tree Pathology Co-operative Programme, Forest and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, 0002, CBS -Centraalbureau voor Schimmelcultures, Baarn, Netherlands.

Table 2. Frequency of occurrence of *Leptographium guttulatus* from various bark beetle niches in Austria, Central Europe.

Bark beetle species	**Investigation year**	**Source of isolation**	**Number of samples yielding growth of *L. guttulatus***	**Total number of samples**
Hylastes cunicularius	1997[2]	Beetle	2	26
	1998[1]	"	0	9
Hylurgops palliatus	1997[1]	"	1	1
	1998[1]	"	0	6
Hylurgops glabratus	1993[1]	"	1	79
	1997[1]	"	0	28
Tomicus minor	1998[1]	Sapwood	1	150

[1] One locality, [2] Two localities

Table 3. Known insect associates of *Leptographium guttulatus*.

Bark beetle species	**Host**	**Geographic locality**	**Reference**
Hylastes ater	*Pinus sylvestris*	England	Wingfield & Gibbs (1991)
Hylastes opacus	*Pinus sylvestris*	England	Wingfield & Gibbs (1991)
Hylastes cunicularius	*Picea abies*	Austria, Central Europe	Jacobs *et al.* (this paper)
Hylurgops palliatus	*Pinus sylvestris*	England	Wingfield & Gibbs (1991)
Hylurgops palliatus	*Picea abies*	Austria, Central Europe	Jacobs *et al.* (this paper)
Hylurgops glabratus	*Picea abies*	Austria, Central Europe	Jacobs *et al.* (this paper)
Tomicus minor	*Pinus sylvestris*	Austria, Central Europe	Jacobs *et al.* (this paper)
Tomicus piniperda	*Pinus sylvestris*	France	Morelet, personal communication
Tomicus piniperda	*Pinus sylvestris*	England	Wingfield & Gibbs (1991)

Fig. 1. Aligned sequences of the ITS1, ITS2 regions and 5.8S rRNA gene of the rDNA operon for the four *O. penicillatum* (cmw2306, cmw2302, cmw2643, cmw2642), two *O. americanum* (cmw0495, cmw2963), one *O. dryocoetidis* (cmw0442), two *O. europhiodes* (cmw0452, cmw1949), three *O. laricis* (cmw2014, cmw 2016, cmw1980)and two *Leptographium* sp. 'GG' (cmw1310, CMW742). *Leptographium procerum* was used as a functional outgroup. Bases identical to the corresponding bases in the sequence of the first *O. penicillatum* isolate are indicated by dots, dashes indicates gaps in the sequence inserted in order to achieve optimum alignment.

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                                          10         20         30         40         50         60         70         80
O.penicillatum(cmw2306)           ACTCCCAACC CGTGCATACA TT-CCGCATT TCTTGCGCCC GTT----GCC --TTCCGGCG GGCGGCGCGC GCC-------
O.penicillatum(cmw2302)           .......... .......... .......... .......... .......... .......... .......... ..........
O.penicillatum(cmw2642)           .......... .......... .......... .......... .......... .......... .......... ..........
O.penicillatum(cmw2643)           .......... .......... .......... .......... .......... .......... .......... ..........
O.americanum(cmw0495)             ....A..... .......... .C........ ....------ ---..GC... CG..G.CT.C T.GC.G...G C--GCGCGCC
O.americanum(cmw2963)             .......... -........- .C....A... ....------ ---..GC... CG..G.CT.C T.GC.G...G C--GCGCGCC
L.procerum(cmw0662)               .......... ......A..C ..A......C CT.-CT.A-G AGA..CGA.. CG..G.CT.C T.AC.G...G C-GCAACCCC
L.europhioides(cmw0452)           .......... ......A..C ..A......A CT..C..A-G AGAGAGC... TG..G.CT.C T.TC.G...G C-GTG.....
O.europhioides(cmw1949)           .......... ......A..C ..A......A CT..C..A-G AGAGAGC... TG..G.CT.C T.TC.G...G A-GTG....C
O.laricis(cmw2014)                .......... ......A..C ..A......A CT..C..A-G AGAGAGC... TG..G.CT.C T.TC.G...G A-GTG....C
O.laricis(cmw2016)                .......... ......A..C ..A......A CT..C..A-G AGAGAGC... TG..G.CT.C T.TC.G...G A-GTG....C
O.laricis(cmw1980)                .......... ......A..C ..A......A CT..C..A-G AGAGAGC... TG..G.CT.C T.TC.G...G A-GTG....C
Leptographium sp. "GG" (cmw1310)  .......... ......A..C ..A......C CT..CT.A-G AGAGAGC... CG..G.CT.C T..C.G...G C-GTG..CCC
Leptographium sp. "GG" (cmw0742)  .......... ......A..C ..A......C CT..CT.A-G AGAGAGC... CG..G.TT.C T..C.G...G C-GTG..CCC
O.dryocoetidis(cmw0442)           .......... ......A..C ..A......A CT..C..AAG AGAGAGC... TG.GG.CT.C T.TCCG...G AAGTG....C

                                          90        100        110        120        130        140        150        160
O.penicillatum(cmw2306)           TCCAGC---- -TTCCGGCTG GCGGCGCCCG CCCGCCGGAA AGACCAGCGG CCGCAC---- ---------- ----------
O.penicillatum(cmw2302)           .......... .......... .......... .......... -......... .......... .......... ..........
O.penicillatum(cmw2642)           .......... .......... .......... .......... G......... .......... .......... ..........
O.penicillatum(cmw2643)           .......... .......... .......... .......... G......... .......... .......... ..........
O.americanum(cmw0495)             ......C... .-..-..... .......... ........GG GC-....... .......... .......... ..........
O.americanum(cmw2963)             ....A.C... .-..-..... ........A. ........GG GC-....... .......... .......... ..........
L.procerum(cmw0662)               C..TT.CCTG GGGG-..... -..A..G.G. G...GG..CG C.G.TC.GAC G-----.... ......CTCT CTCCCC....
L.europhioides(cmw0452)           -..TT.CCTC C.....A..A -...AAGGG. TGG.A...GC .CC.GCC..C .G.GGGCGCG GCATGGCCCG CTCCCTCTCG
O.europhioides(cmw1949)           C..TT.CCTC C........A -...A.GGG. TGG.A...GC .CC.GCC..C .G.GGGCTCC GCGTGTCCCG CTCCCTCTCG
O.laricis(cmw2014)                C..TT.CCTC C........A -...A.GGG. TGGAA...GC .CC.GCC..C .G.GGGCGCG GCGTGGCCCG CTCCCTCTCG
O.laricis(cmw2016)                C..TT.CCTC C........A -...A.GGG. TGGAA...GC .CC.GCC..C .G.GGGCGCG GCGTGGCCCG CTCCCTCTCG
O.laricis(cmw1980)                C..TT.CCTC C........A -...A.GGG. TGGAA...GC .CC.GCC..C .G.GGGCGCG GCGTGGCCCG CTCCCTCTCG
Leptographium sp. "GG" (cmw1310)  ...TC.CCTC CC..T.CGG. .G..GTTGGA .GG..GCCCG CCCG.C.G.. GT..GGCGCG G.....CCCG CTCCCT...C
Leptographium sp. "GG" (cmw0742)  ...TC.CCTC CC..T.CGG. .G..GTTGGA .GG..GCCCG CCCG.C.G.. GT..GGCGCG G.....CCCG CTCCCTCT.C
O.dryocoetidis(cmw0442)           C..TT.CCTC C........A -...AAGGG. TGGAA...GC .CC.TCC..C .G.GGGCGCG GCGTGCCCCC CTCCCTCTCG
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                                                  170        180        190        200        210        220        230        240
O.penicillatum(cmw2306)              ----TCT-GA A-CCT-CTTC ---GTATACT -GAGCAGTCT GAG-AAAAAC CA--TG--AA TCGTTAAAAC TTTCAACAAC
O.penicillatum(cmw2302)              .......... .......... .......... .......... .......... .......... .......... ..........
O.penicillatum(cmw2642)              .......... .......... .......... .......... .......... .......... .......... ..........
O.penicillatum(cmw2643)              .......... .......... .......... .......... .......... .......... .......... ..........
O.americanum(cmw0495)                .......... ........-- GC....C... .......... ...C-..... .......... .......... ..........
O.americanum(cmw2963)                .......... ........-- GC....C... .......... ...C-..... .......... .......... ..........
L.procerum(cmw0662)                  ....---... .....T...T ACA......C ATTA.C.... ...C-..... ..C....... .......... ..........
L.europhioides(cmw0452)              .......T.. .....T...T ACA......C TATAAT.... ...T-..... ..CA-..... .......... ..........
O.europhioides(cmw1949)              .......T.. .....T...T ACA......C TATAATA... ...TT..... ..CC-..... .....C.... ..........
O.laricis(cmw2014)                   .......T.. .....T...T ACA......C TATAAT.... ...T-..... ..CA-..... .......... ..........
O.laricis(cmw2016)                   .......T.. .....T...T ACA......C TATAAT.... ...T-..... ..CA-..... .......... ..........
O.laricis(cmw1980)                   .......T.. .....T...T ACA......C TATAAT.... ...T-..... ..CACC.... .......... ..........
Leptographium sp. “GG” (cmw1310)     GCCG-.-... .....T...T GCA.....A. T.TATC.... ...C-..... ..CA-..... .......... ..........
Leptographium sp. “GG” (cmw0742)     GCCG-.-... .A...T...T GCA.....A. T.TATC.... ...C-..... ..CA.-.... .......... ..........
O.dryocoetidis(cmw0442)              .......T-. .A...T...T ACAT.....C TAGAATT... ..-TTCT.G- -.CC.CAA.. ....-..C.. ..........

                                                  250        260        270        280        290        300        310        320
O.penicillatum(cmw2306)              GGATCTCTTG GTTCTGGCAT CGATGAAGAA CGCAGCGAAC TGCGATAAGT AATGTGAATT GCAGAATTCA GTGAACCATC
O.penicillatum(cmw2302)              .......... .......... .......... .......... .......... .......... .......... ..........
O.penicillatum(cmw2642)              .......... .......... .......... .......... .......... .......... .......... ..........
O.penicillatum(cmw2643)              .......... .......... .......... .......... .......... .......... .......... ..........
O.americanum(cmw0495)                .......... .......... .......... ....C..... .......... .......... ...C...... ..........
O.americanum(cmw2963)                .......... .......... .......... .......... .......... .......... .......... ..........
L.procerum(cmw0662)                  .......... .......... ....A..A.. .......... .......... ....C..... .......... .C..G.....
L.europhioides(cmw0452)              .......... .......... .......... .......... .......... ....C..... .......... .C..G.....
O.europhioides(cmw1949)              .......... .......... .......... .......... .......... ....C..... .......... .C..G.....
O.laricis(cmw2014)                   .......... .......... .......... .......... .......... ....C..... .......... .C..G.....
O.laricis(cmw2016)                   .......... .......... .......... .......... .......... ....C..... .......... .C..G.....
O.laricis(cmw1980)                   .......... .......... .......... .......... .......... ....C..... .......... .C..G.....
Leptographium sp. “GG” (cmw1310)     .......... .......... .......... .......... .......... ....C..... .......... .C..G.....
Leptographium sp. “GG” (cmw0742)     .......... .......... .......... .......... .......... ....C..... .......... .C..G.....
O.dryocoetidis(cmw0442)              .......... .......... .......... .........A .......... .........C ........T. ..........
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                                              330        340        350        360        370        380        390        400
O.penicillatum(cmw2306)               GAATCTTTGA ACGCACATTG CGCCCGCCAG CATTCTGGCG GGCATGCCTG TCCGAGCGTC ATTTCCACCC TCACGCGCCC
O.penicillatum(cmw2302)               .......... .......... .......... .......... .......... .......... .......... ..........
O.penicillatum(cmw2642)               .......... .......... .......... .......... .......... .......... .......... ..........
O.penicillatum(cmw2643)               .......... .......... .......... .......... .......... .......... .......... ..........
O.americanum(cmw0495)                 .......... .......... .......... .......... .......... .......... .....T.TTT ..........
O.americanum(cmw2963)                 .......... .......... .......... .......... .......... .......... .......... ......A...
L.procerum(cmw0662)                   .......... .......... .......... .......... .......... .......... ......T... ......AG.G
L.europhioides(cmw0452)               .......... .......... .......... .......... .......... .......... ......T... ......AG.A
O.europhioides(cmw1949)               .......... .......... .......... .......... .......... .......... ......T... ......AG.A
O.laricis(cmw2014)                    .......... .......... .......... .......... .......... .......... ......T... ......AG.A
O.laricis(cmw2016)                    .......... .......... .......... .......... .......... .......... ......T... ......AG.A
O.laricis(cmw1980)                    .......... .......... .......... .......... .......... .......... ......T... ......AG.A
Leptographium sp. "GG" (cmw1310)      .......... .......... .......... .......... .......... .......... ......T... ......AG.G
Leptographium sp. "GG" (cmw0742)      .......... .......... .......... .......... .......... .......... ......T... ......AG.G
O.dryocoetidis(cmw0442)               A......... .......... .......... .......... .......... ......A... .......... ..........

                                              410        420        430        440        450        460        470        480
O.penicillatum(cmw2306)               CTTTAGTGCG TGGTGTT-GG GGCATACTGC GG-CTGCC-G CGAGGCGCCG CAGGCCCTGA AACCCAGTGG CGGGCCGGCA
O.penicillatum(cmw2302)               .......... .......... .......... .......... .......... .......... .......... ..........
O.penicillatum(cmw2642)               .......... .......... .......... .......... ......A... .......... .......... ..........
O.penicillatum(cmw2643)               .......... .......... .......... .......... ......A... .......... .......... ..........
O.americanum(cmw0495)                 ...C...... .......... .......... .......... .A....A... ........A. .......... ..........
O.americanum(cmw2963)                 ...C...... .......... .......... .......... .A....A... ........A. .......... ..........
L.procerum(cmw0662)                   .GCC--.... .......T.. ...G.T.... ..G.A.G.CT GCGCCTAG.A -.-...GCCG C.A-----CC .CC.AAA..C
L.europhioides(cmw0452)               .GCC--.... .......T.. ...G.T.... ..C.CCG.TT GCGCCTAG.. T.T...GCCG C.A-----CC .CC.AAA..C
O.europhioides(cmw1949)               .GCC--.... .......... ...G.TT... ..C.C.G.TT GCGCCTAG.. T.T...GCCG ----...-CC .CC.AAA..C
O.laricis(cmw2014)                    .GCC--.... .......... ...G.TT... ..C.C.G.TT GCGCCTAG.. T.T...GCCG ----...-CC .CC.AAA..C
O.laricis(cmw2016)                    .GCC--.... .......... ...G.T.... ..C.C.G.TT GCGCCTAG.. T.T...GCCG ----...-CC .CC.AAA..C
O.laricis(cmw1980)                    .GCC--.... .......... ...G.T.... ..C.C.G.TT GCGCCTAG.. T.T...GCCG ----...-CC .CC.AAA..C
Leptographium sp. "GG" (cmw1310)      .GCC--.... .......... ...G.T.... ..C.A.G.CT GCGCC.AG.. ......GCCG ----...-CC .CC.AAA..C
Leptographium sp. "GG" (cmw0742)      .GCC--.... .......... ...G.T.... ..C.A.G.CT GCGCC.AG.. ......GCCG ----...-CC .CC.AAA..C
O.dryocoetidis(cmw0442)               .......... .......... ...G.T-... ....G..... ......C... .......C.. .......... ..........
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                                          490        500        510        520        530        540        550        560
O.penicillatum(cmw2306)           GAA-GGCTTC CGAGCGCAGT AAGCATCACG CCCT------ -TCGCTTCCG GACGCCCCT- GCTG-GCA-- ----------
O.penicillatum(cmw2302)           ........-. .......... .......... .......... .-........ .......... .......... ..........
O.penicillatum(cmw2642)           .......... .......... .......... .......... .-........ .......... .......... ..........
O.penicillatum(cmw2643)           ........C. .......... .......... .......... .-....C... .......... .......... ..........
O.americanum(cmw0495)             ...A-...-. ........A. ......T... .......... .-.A..-... ........-. .......... ..........
O.americanum(cmw2963)             ...A-...C. ........A. ......T... ...-...... ......-... ........-. .......... ..........
L.procerum(cmw0662)               -.GTT..-GG GCC.-...CC GG-.-..C.A GAGCATTAAG CA-CAA.--- ---.G..-.C ...CT.G.CG CCCC..GCCT
L.europhioides(cmw0452)           -.GTT..-GG GCCC-...CC GG-.-..C.A A.GCAATAAG CA-CAG.--- ---....-.C ...TT.G.CG CCCC..GCCT
O.europhioides(cmw1949)           -.GT...-GG GCC.-....C GG..T..CGA G.GCAGTAAG CAGCAG.--- ---....-.C ...TT.G.CG CCCCC.GCCT
O.laricis(cmw2014)                -.GT...-GG GCC.-....C GG..-..CGA G.GCAGTAAG CAGCAG.--- ---....-.C ...TT.G.CG CCCCC.GCCT
O.laricis(cmw2016)                -.GT...-GG GCC.-....C GG..-..CGA G.GCAGTAAG CAGCAG.--- ---....-.C ...TT.G.CG CCGCC.GCCT
O.laricis(cmw1980)                -.GT...-GG GCC.-....C GG..-..CGA G.GCAGTAAG CAGCAG.--- ---....-.C ...TT.G.CG CCCCC.GCCT
Leptographium sp. “GG” (cmw1310)  -.GT...-GG GCC.-....C GG..T..CGA G.GCAGTAAG CATCAC---- ---....-.C ...CT.G.CG CCCCC.GCCT
Leptographium sp. “GG” (cmw0742)  -.GT...-GG GCC.-....C GG.-T..CGA G.GCAGTAAG CATCAC---- ---....-.C ...CT.G.CG GTTCCCGCCT
O.dryocoetidis(cmw0442)           .......... .......... ........-. .......... ......CT-. .......... .......... ..........

                                          570
O.penicillatum(cmw2306)           GCTCGCCATG C
O.penicillatum(cmw2302)           .......... .
O.penicillatum(cmw2642)           .......... .
O.penicillatum(cmw2643)           .......... .
O.americanum(cmw0495)             .......C.. .
O.americanum(cmw2963)             .......C.. .
L.procerum(cmw0662)               ..--...C.. .
L.europhioides(cmw0452)           ..--...C.. .
O.europhioides(cmw1949)           ..--...C.. .
O.laricis(cmw2014)                ..--...T.. .
O.laricis(cmw2016)                ..--...T.. .
O.laricis(cmw1980)                ..--...T.. .
Leptographium sp. “GG” (cmw1310)  ..--...C.. .
Leptographium sp. “GG” (cmw0742)  ..--...C.. .
O.dryocoetidis(cmw0442)           ........AA G
```

Fig. 2. Phylogram produced by PAUP analysis based on partial sequence of the ITS1 and ITS2 regions, as well as the complete sequence of the 5.8S rRNA gene. *Leptographium procerum* was used as a functional outgroup. Tree length = 496. The number of base substitutions are indicated above the branches and the bootstrap percentages (100 bootstrap repeats) are indicated below the branches.

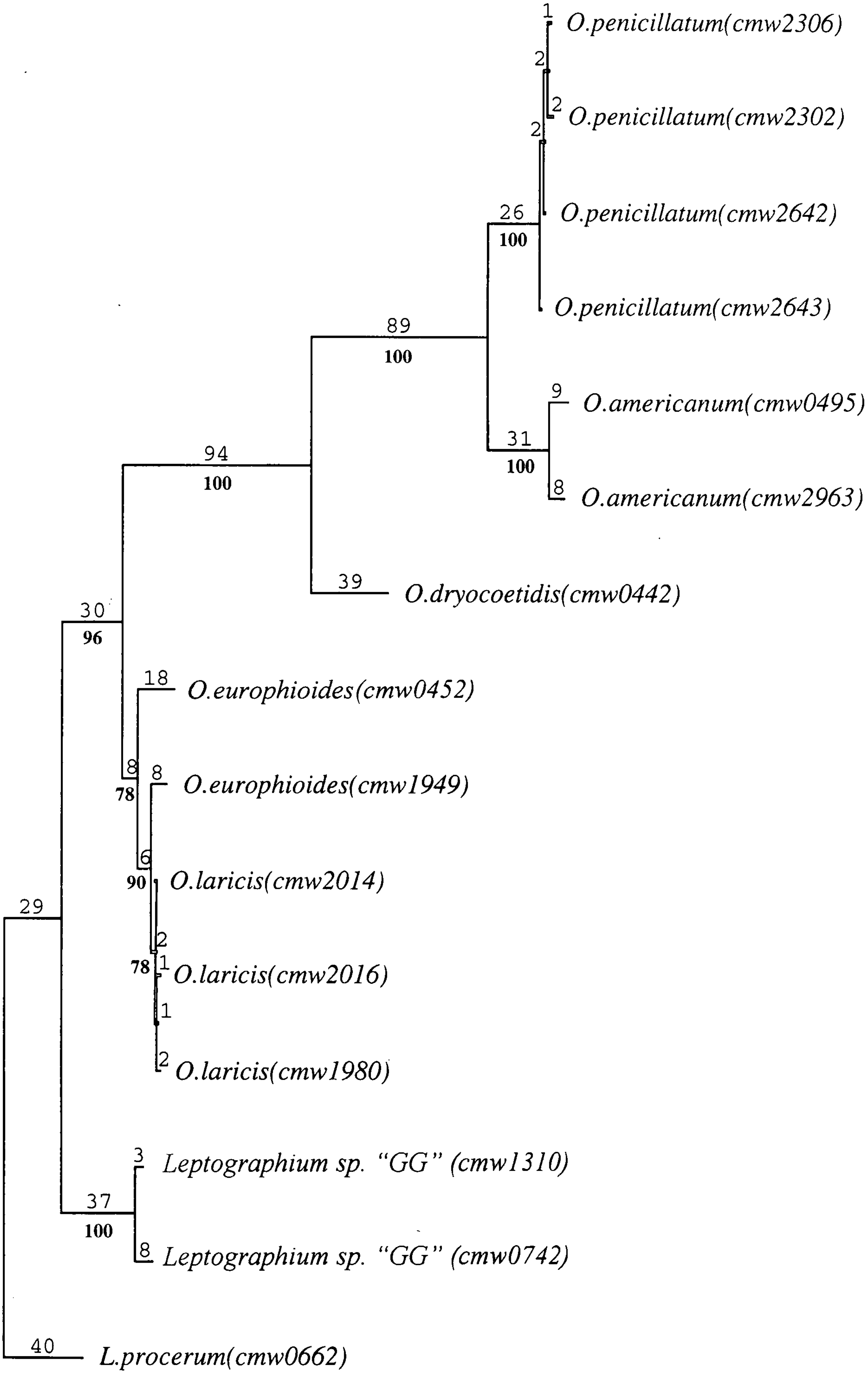
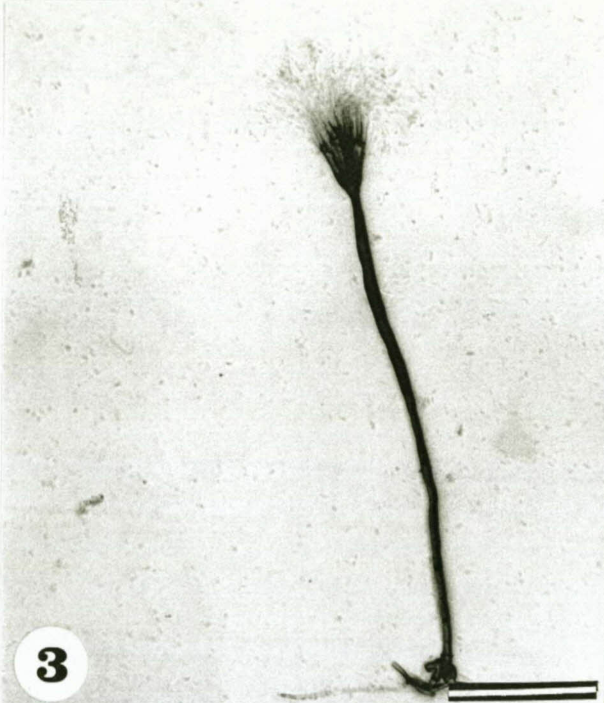

1
O.penicillatum(cmw2306)
2
2
O.penicillatum(cmw2302)
2
O.penicillatum(cmw2642)
26
100
O.penicillatum(cmw2643)
89
100
9
O.americanum(cmw0495)
31
100
8
O.americanum(cmw2963)
94
100
39
O.dryocoetidis(cmw0442)
30
96
18
O.europhioides(cmw0452)
8
78
8
O.europhioides(cmw1949)
6
90
O.laricis(cmw2014)
29
2
78
1
O.laricis(cmw2016)
1
2
O.laricis(cmw1980)
3
Leptographium sp. "GG" (cmw1310)
37
100
8
Leptographium sp. "GG" (cmw0742)
40
L.procerum(cmw0662)

Fig. 3 - 8. Light micrographs showing the morphological characters of *Leptographium guttulatus* (CMW 742). **Fig. 3.** Conidiophore with a dark olivaceous stipe and complex conidiogenous apparatus (Bar = 100 µm). **Fig. 4.** Footcell of the conidiophores without rhizoids (Bar = 10 µm). **Fig. 5.** Complex conidiogenous apparatus (Bar = 10 µm). **Fig. 6-7.** Conidiogenous cells showing false sympodial conidiogenesis (Bar = 10 µm). **Fig. 8.** Conidia with prominent vacuoles (Bar = 10 µm).



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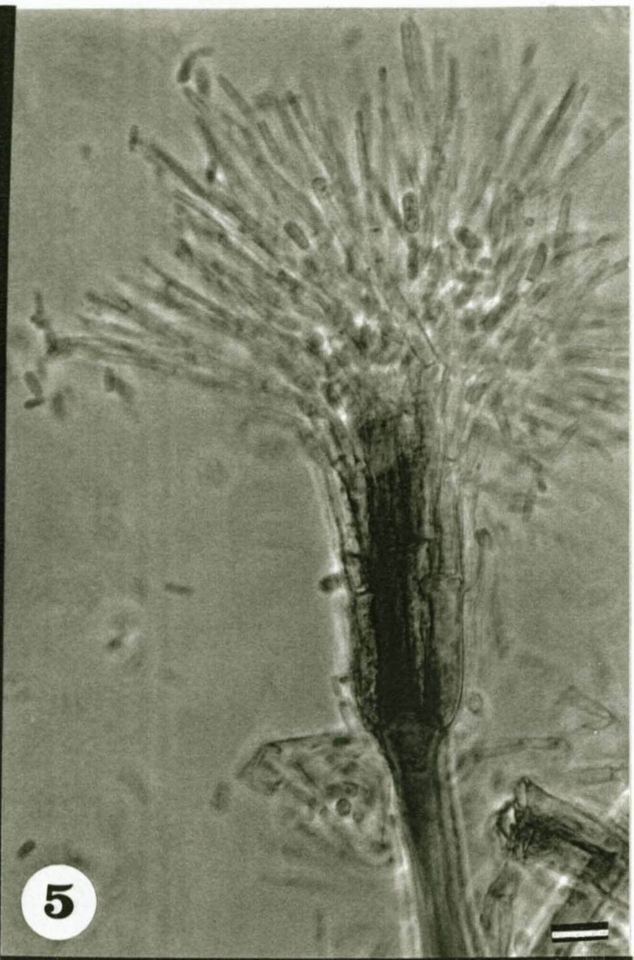

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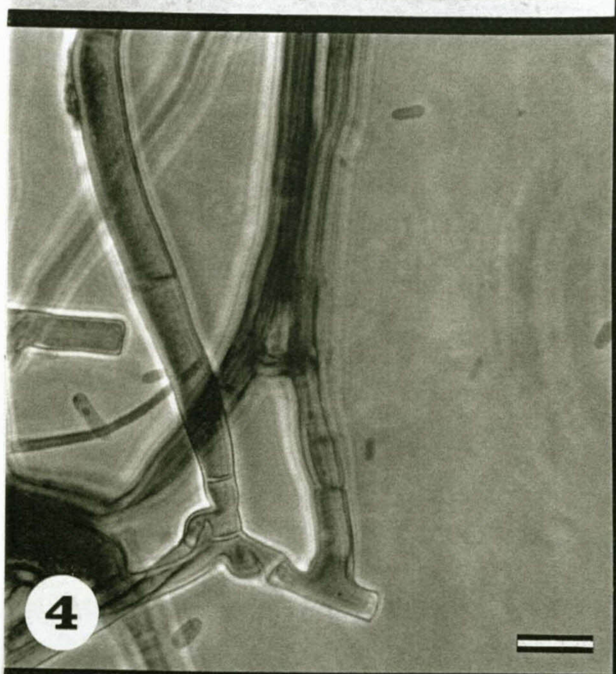

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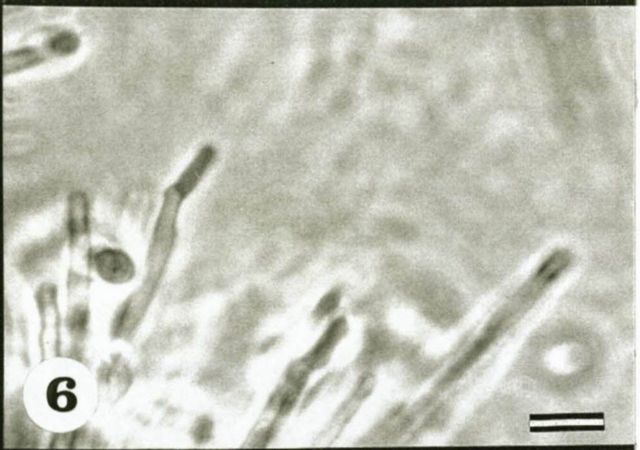

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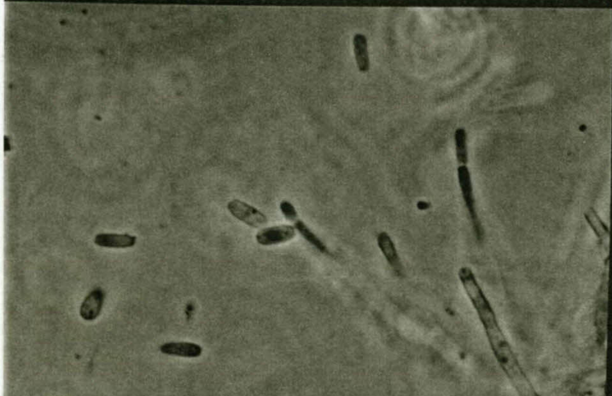

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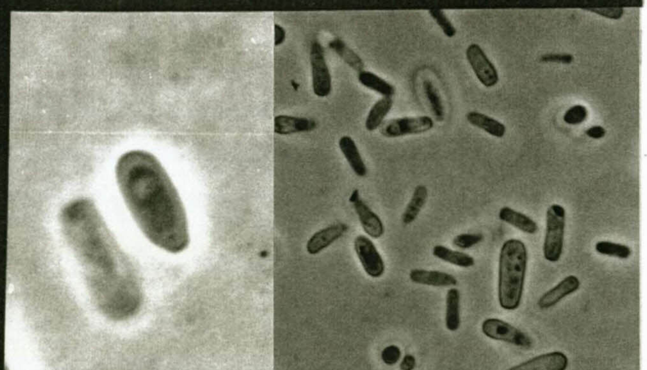
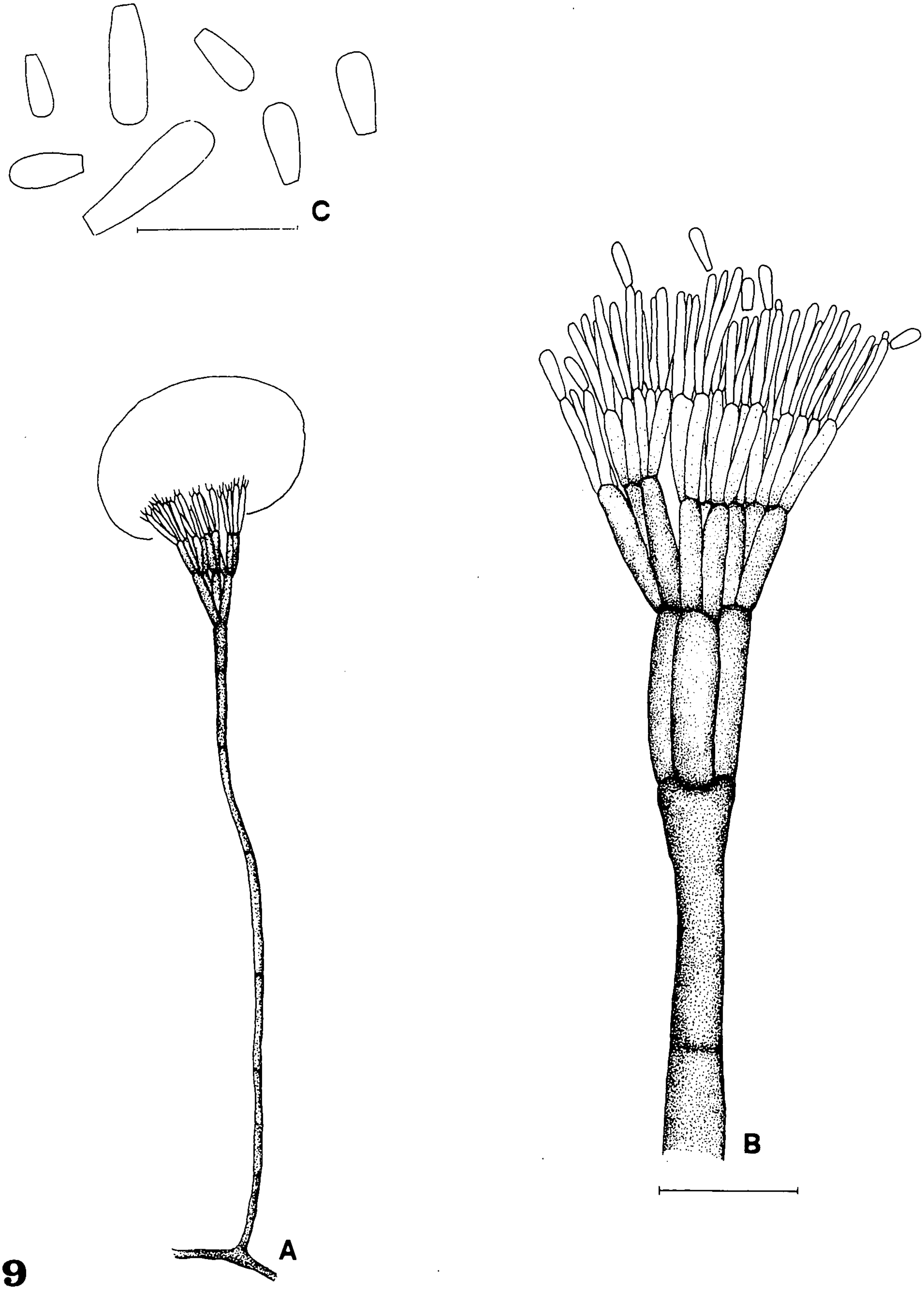


8

Fig. 9. Line drawings of *Leptographium guttulatus* (CMW 742). **A**. Habit sketch of the conidiophore. **B**. Conidiogenous apparatus (Bar = 10 μm). **C**. Conidia (Bar = 10 μm).



9

SUMMARY

The ophiostomatoid fungi are found worldwide on a wide variety of substrates. The group is economically important due to the pathogenicity of some species on important crops and trees. The success of these fungi as plant pathogens is undoubtly related, at least in part, to their association with insects, especially bark beetles.

The presence of a *Chalara* anamorph, sensitivity to cycloheximide and the absence of rhamnose in the cell wall, led us to believe that *Ophiostoma polonicum* is a typical species of *Ceratocystis s.str.* Partial sequence data from the rDNA operon presented in chapter two of this thesis, confirmed the placement of *O. polonicum* in *Ceratocystis s.str.* I have, therefore, suggested that the name *Ceratocystis polonica* should be used for this fungus in future and that the reported *Leptographium* anamorph was most probably a contaminant.

In chapter three, I have attempted to resolve the question concerning the occurrence of both *Sporothrix* and *Chalara* anamorphs in *Ceratocystis autographa.* No cultures exist for the type of *C. autographa*, although a type specimen shows the presence of two distinct and taxonomically unrelated anamorphs. Cultures identified as *C. autographa*, isolated from *Juniperus* needles are very different to the original niche of the fungus and have caused great confusion. Through sequencing the ITS1, ITS2 and 5.8S regions of the rDNA operon of these cultures and comparing them with key species, I have shown that these cultures probably have no connection to *C. autographa.* I have also concluded that they are atypical of *Chalara* and should reside in a new genus to be named *Xenochalara.*

The last chapter of this thesis relates to an unusual *Leptographium* species, isolated primarily from the bark beetle *Hylurgops palliatus.* The fungus has reasonably large conidia and has been thought to be related to *Leptographium penicillatum* as *L. penicillatum* f.sp. *palliati.* Through sequence data comparisons of this fungus with *L. penicillatum* and other key species, we have shown that it is distinct. We have, therefore, provided a full description and have also proposed the name *L. guttulatus* for it.

This thesis includes three research studies pertaining to diverse questions that have plaqued the taxonomy of *Ceratocystis sensu lato*. Problems have been resolved primarily using sequencing of the Internal Transcribed Spacer regions of the rDNA operon. Many additional problems remain in this group of fungi and I feel optomistic that approaches similar to those presented here will lead to resolving them in coming years.

OPSOMMING

Die ophiostomatoid fungi kom wêreldwyd voor en word op 'n wye verskeidenheid van substrate aangetref. Die groep het 'n groot invloed op die ekonomie weens die patogenesiteit van sekere spesies vir belangrike gewasse en bome. Die sukses van hierdie fungi as plantpatogene is gedeeltelik toe te skryf aan hul assosiasie met insekte, veral baskewers.

Die teenwoordigheid van 'n *Chalara* anamorf, sensitiwiteit vir sikloheksimied en die afwesigheid van ramnose in die selwand, het ons laat besluit dat *Ophiostoma polonicum* 'n tipiese spesie van *Ceratocystis s.str.* is. Die plasing van *O. polonicum* in die genus *Ceratocystis s.str*, word verder bevestig deur 'n basisopeenvolging studie van 'n gedeelte van die ribosomale DNA operon. Ek het dus voorgestel dat die naam *Ceratocystis polonica* vir hierdie fungus in die toekoms gebruik word. Die *Leptographium* anamorf was moontlik 'n kontaminant.

In hoofstuk drie het ek onderneem om die kwessie omtrent die voorkoms van beide 'n *Sporothrix* en 'n *Chalara* anamorf in *Ceratocystis autographa*, op te los. Geen herbarium materiaal van die tipe vir *C. autographa* is beskikbaar. Kulture wat as *C. autographa* geidentifiseer is, is vanaf die naalde van Juniperus geïsoleer en het weens die verskil in habitat groot verwarring in die taksonomie veroorsaak. Basisopeenvolging bepaling van die ITS1, ITS2 gebiede asook die 5.8S geen van die rDNA operon van hierdie kulture en die vergelyking daarvan met ander sleutel spesies het gewys dat hierdie kulture moontlik geen verwantskap met *C. autographa* het nie. Ek het ook die gevolgtrekking gemaak dat hierdie spesies nie tipies van *Chalara* is nie en dus in 'n nuwe genus, *Xenochalara*, geplaas moet word.

Die laaste hoofstuk van die tesis handel oor *Leptographium* spesies wat hoofsaaklik vanaf die baskewer, *Hylurgops palliatus* versamel is. Die fungus het redelik groot konidia en 'n moontlike verwantskap met *Leptographium penicillatum* as *L. penicillatum* f.sp. *palliati* is vermoed. Met die vergelyking van basisopeenvolgings van hierdie fungus met *L. penicillatum* en ander sleutel spesies, is hierdie fungus as afsonderlik bewys. Ons het dus 'n volledige beskrywing van hierdie fungus gedoen en ook die nuwe naam, *L. guttulatus*, voorgestel.

Hierdie tesis bevat drie navorsing studies handelend oor 'n verskeidenheid vrae met betrekking tot die taksonomie van *Ceratocystis sensu lato*. Probleme is hoofsaaklik opgelos deur basisopeenvolging bepaling van die ITS gebiede van die rDNA operon. Baie addisionele probleme wat nog in die taksonomie van *Ceratocystis s.l.* voorkom kan deur die benaderings in hierdie studie gebruik, opgelos word.