CONTENTS

POPULATION BIOLOGY

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PREFACE

Bark beetles (Coleoptera: Scolytidae) are generally regarded as forest pests and act as vectors of ophiostomatoid fungi. Many *Ophiostoma* spp. can cause sapstain in lumber and logs, reducing the value of the wood. Several species are pathogenic to trees and other crops. In South Africa, sapstain fungi degrade high quality pine logs and lead to significant financial losses to the forestry industry. Three exotic pine bark beetle species have been reported from South Africa, and considerable research has been done on these insects. Little is known, however, regarding the fungi associated with them, or the interactions between fungus, bark beetle and host tree. The primary aim of the study was, therefore, to identify *Ophiostoma* spp. associated with pine bark beetles in South Africa, evaluate the pathogenicity of the most common fungal associates to pines, and gain an understanding of the population biology of the most pathogenic fungus. The study also provided the opportunity to consider the taxonomy of selected fungal isolates from pine bark beetles from other countries, obtained during the course of the study. In order to assist the reader, the thesis is divided into four sections: literature review, taxonomy, pathology, and population biology.

LITERATURE REVIEW

In Chapter 1, current knowledge regarding ophiostomatoid fungi and pine bark beetles in South Africa, as well as the association between fungi and bark beetles, is reviewed. The review also includes a brief summary of the ophiostomatoid fungi associated with pine bark beetles in other Southern Hemisphere countries such as Australia, Chile, and New Zealand.

TAXONOMY

Three species of exotic pine bark beetles, *Hylastes angustatus*, *Hylurgus ligniperda* and *Orthotomicus erosus*, have been reported to occur on *Pinus* spp. in South Africa. All these bark beetles were introduced into South Africa from Europe. In Chapter 2, I present the results of a survey of ophiostomatoid fungi associated with these three bark beetle species. During the course of the two-year survey, 1558 samples (beetles and galleries) were collected from *P. patula* and *P. elliottii* plantations in Mpumalanga and KwaZulu-Natal provinces. In total, 1254 fungal isolates were recoved and 500 of these are identified in the Chapter. Three species are recorded for the first time from South Africa.

Similar to the situation in South Africa, *Hylurgus ligniperda* and *Hylastes ater*, which are native

to Europe, commonly occur on exotic *Pinus radiata* in Chile. Specimens of these bark beetles and their galleries were obtained and the five ophiostomatoid species isolated from these specimens are illustrated and described in Chapter 3. All of these species are recorded from Chile for the first time.

In contrast to the situation in South Africa and Chile, *Ips calligraphus* and *Dendroctonus mexicanus* are native to Mexico and occur on many species of pine in that country. Chapter 4 presents the results of isolations from these two bark beetle species obtained from their natural environment. The results of this study showed that six *Ophiostoma* spp. were associated with the two beetle species. One of these is recorded for the first time from Mexico. The collection also included a previously undescribed species isolated from both beetle species, which is described in the Chapter.

In South-western China, a native bark beetle, *Tomicus piniperda*, has destroyed more than 0.5 million ha of *Pinus yunnanensis* in the past 15 years. Isolates of a blue stain fungus were obtained, but not identified, in a previous study. The fungus is morphologically similar to the anamorph of *Ophiostoma crassivaginatum* and to *Leptographium pyrinum*. Based on comparisons using light and scanning electron microscopy, this fungus is described as a new species in Chapter 5.

Ophiostoma galeiformis was isolated from the three studies on ophiostomatoid fungi associated with bark beetles from South Africa, Chile, and Mexico, respectively. The species concept of this fungus, however, has been confused over the years due to a lack of sexual structures on the type specimen and contamination of the ex-type culture. The availability of fresh *O. galeiformis* cultures provided the opportunity to study the fungus further and results are presented in Chapter 6.

PATHOLOGY

In Chapter 1 it was shown that *Ophiostoma ips*, *Leptographium serpens*, and *L. lundbergii*, occur most frequently with the three exotic pine bark beetle species in South Africa. In Chapter 7, the pathogenicity of these three fungi was tested on *Pinus elliottii* and *P. radiata* in South African plantations. Results indicated that the inoculated fungi caused resin exudation and sapwood discoloration around inoculation points. Of the three species, *O. ips* was the most pathogenic.

POPULATION BIOLOGY

A single ascospore isolate of *O. ips* was selected for the development of twelve pairs of simple sequence repeat markers (SSR). *Ophiostoma ips* was selected based on its prevalence on all three bark beetle species occurring in South Africa, and its economic importance as a sapstain agent and possible pathogen. All markers were found to be polymorphic when tested on 7 isolates of *O. ips* collected from different parts of the world. Results of this study are reported in Chapter 8.

The twelve pairs of polymorphic markers developed in the study presented in Chapter 8 were applied to examine the population structure of five populations of *O. ips*, one each from Chile, Europe, and the USA, and two from South Africa. The genetic variation, genetic distance, and mode of reproduction within and between the different populations were considered.

Summary

Opsomming

SUMMARY

The ophiostomatoid fungi include genera such as *Ophiostoma*, *Ceratocystis*, *Ceratocystiopsis*, and *Leptographium*. Several species in these genera are severe pathogens of trees and other crops. The majority of these species, however, are the causal agents of sapstain in lumber and logs, reducing the value of the wood. In South Africa, sapstain fungi degrade high quality pine logs causing significant financial losses to the forestry industry. Sapstain fungi, especially *Ophiostoma* spp., are often associated with bark beetles (Coleoptera: Scolytidae), many of which are regarded as forest pests. Three species of exotic pine bark beetles, *Hylastes angustatus*, *Hylurgus ligniperda*, and *Orthotomicus erosus*, occur on mature *Pinus* spp. in South Africa. The primary aim of this study was to identify ophiostomatoid fungi associated with pine bark beetles in South Africa, evaluate the pathogenicity of the most common of these fungal species, and gain an understanding of the population biology of the most pathogenic fungus.

During the course of the study, the opportunity arose to include introduced ophiostomatoid fungi associated with exotic pine bark beetles from Chile, and native species from Mexico and China, where little research in this field has been conducted. From Chile, five ophiostomatoid species were reported for the first time. From Mexico, six species were reported, including a new species, *Ophiostoma pulvinisporum* nom. prov. From China, another new species, *Leptographium yunnanense* sp. nov., was described.

The availability of fresh *O. galeiformis* cultures from the studies from South Africa, Chile and Mexico, as well as isolates obtained from Scotland, provided the opportunity to address the confusion that has existed in the taxonomy of this species. An epitype based on a collection from the area where *O. galeiformis* was first collected in Scotland, was designated. This study will provide a foundation for further work on the phylogeny of this species complex.

The survey on ophiostomatoid fungi associated with pine bark beetles in South Africa showed that *Ophiostoma ips*, *Leptographium serpens*, and *L. lundbergii* occur most frequently. In pathogenicity tests of these three fungi to pines, *O. ips* gave the longest lesions, and was the most pathogenic. *Ophiostoma ips* was, for this reason, selected for population studies. Twelve pairs of polymorphic simple sequence repeat markers (SSR) were then developed from *O. ips*. These markers were designed and used to examine the population structure of five populations of *O. ips* from Chile, Europe, South Africa, and the USA. Results support the hypothesis that *O. ips* was introduced into exotic pine-growing countries such as Chile and South Africa, together with the bark beetles native to Europe.

This study treats a number of questions pertaining to the ophiostomatoid fungi associated with pine-infesting beetles. It also represents the first comprehensive study of the topic in South Africa. Thus, new species have been discovered and some insight is presented into the population biology of one species of *Ophiostoma*. To the best of our knowledge this is the first population biology study of a sapstain fungus on conifers. While some important questions have been answered relating to the ophiostomatoid fungi, many remain. It is my hope that this study will provide a firm foundation for further work on the biology and taxonomy of *Ophiostoma* spp. associated with conifers-infesting bark beetles.

OPSOMMING

Die ophiostomatoïede swamme sluit genera in soos *Ophiostoma*, *Ceratocystis*, *Ceratocystiopsis*, en *Leptographium*. Verskeie van hierdie spesies is ernstige patogene van bome en ander gewasse. Die meerderheid van die spesies verkleur egter die saphout van planke en stompe, wat lei tot 'n daling in die waarde van hout. In Suid-Afrika val sapverkleuringswamme hoë kwaliteit dennestompe aan, wat beduidende finansiële verliese vir die bosbou-industrie tot gevolg het. Sapverkleuringswamme, veral *Ophiostoma* spesies, word dikwels met baskewers (Coleoptera:Scolytidae) geassosieer, waarvan baie beskou word as peste in woude en plantasies. Drie spesies van uitheemse denne-baskewers, *Hylastes angustatus*, *Hylurgus ligniperda*, en *Orthotomicus erosus*, kom voor op volwasse *Pinus* spesies in Suid-Afrika. Die primêre doel van hierdie studie was om ophiostomatoïede swamme wat met denne-baskewers in Suid-Afrika geassosieerd is, te identifiseer, die patogenisiteit van die algemeenste spesies te evalueer, en om die populasiebiologie van die mees patogeniese een te bestudeer.

Tydens die verloop van die studie het die geleentheid ontstaan om ophiostomatoïede swamme wat met uitheemse denne-baskewers in Chile geassosieerd is, asook inheemses van Mexiko en China, waar weinig navorsing in hierdie veld gedoen is, in die studie in te sluit. Van Chile is vyf ophiostomatoïede spesies vir die eerste keer gerapporteer. Van Mexiko is ses spesies gerapporteer, waaronder 'n nuwe spesie, *Ophiostoma pulvinisporum* nom. prov. Van China, is nog 'n nuwe spesie, *Leptographium yunnanense* sp. nov., beskryf.

Die beskikbaarheid van vars *O. galeiformis* kulture van die studies in Suid-Afrika, Chile en Mexiko, asook isolate wat onlangs in Skotland versamel is, het die geleentheid gebied om die verwarring wat in die taksonomie van hierdie spesie bestaan, aan te spreek. 'n Epitipe, gebaseer op 'n versameling isolate van die area waar *O. galeiformis* die eerste keer in Skotland gevind is, is aangewys. Hierdie studie sal voorts dien as 'n fondasie vir verdere werk op die filogenie van die spesiekompleks.

Die opname van ophiostomatoïede swamme wat met baskewers in Suid-Afrika geassosieer word, het gewys dat *Ophiostoma ips*, *Leptographium serpens*, en *L. lundbergii*, die meeste voorkom. In patogenisiteitstoetse van hierdie drie fungi op denne, het *O. ips* die langste letsels veroorsaak, en was dus die mees patogeniese. *Ophiostoma ips* is vir hierdie rede gekies vir populasiestudies. Twaalf pare van polimorfiese eenvoudige basispaaropeenvolgingsherhalingsmerkers (SSR) is toe

vanaf *O. ips* ontwikkel. Hierdie merkers is ontwerp en gebruik om die populasiestruktuur van vyf populasies van *O. ips* van onderskeidelik Chile, Europa, Suid-Afrika, en die VSA, te bestudeer. Die resultate ondersteun die hipotese dat *O. ips* ingevoer is saam met Europese baskewers na lande soos Chile en Suid-Afrika waar uitheemse dennebome op groot skaal aangeplant word.

Hierdie studie behandel verskeie vraagstukke met betrekking tot die ophiostomatoïede swamme wat met baskewers geassosieer word. Dit verteenwoordig ook die eerste omvattende studie van die onderwerp in Suid-Afrika. Nuwe spesies is ontdek en beskryf en insig in die populasiestruktuur van een van die *Ophiostoma* spesies is verky. Volgens ons kennis, is dit die eerste populasiestudie van 'n sapverkleuringswam op naaldhoutsoorte. Terwyl verskeie belangrike vraagstukke in verband met die ophiostomatoïede swamme beantwoord is, is daar ander vrae wat onbeantwoord bly. Ek glo dat hierdie studie 'n sterk fondasie sal vorm vir verdere werk op die biologie en taksonomie van *Ophiostoma* spesies geassosieerd met naaldhout-infesterende baskewers.

Chapter 1

The occurrence of ophiostomatoid fungi in the Southern Hemisphere, with special reference to species associated with pine bark beetles

Ophiostomatoid fungi, including species of *Ophiostoma*, *Ceratocystis*, and *Ceratocystiopsis*, can cause sapstain in lumber and logs, reducing the value of the wood. Several species are also pathogenic to trees and other crops. Ophiostomatoid fungi are often associated with bark beetles (Coleoptera: Scolytidae), many of which are regarded as forest pests. Three species of exotic bark beetles, *Hylastes angustatus*, *Hylurgus ligniperda*, and *Orthotomicus erosus*, occur on mature *Pinus* spp. in South Africa. *Hylastes angustatus* is the most aggressive of these insects, and also damages pine seedlings during its maturation feeding stage. Considerable research has been done on these three bark beetle species in South Africa. Little is known, however, regarding the fungi associated with them, or the interactions between fungus, bark beetle and host tree. Similarly, the role of bark beetles and their associated fungi in causing sapstain on pine logs in South Africa is poorly understood. This review focuses on ophiostomatoid fungi, pine bark beetles, and the association of fungi and bark beetles, with special reference to the situation in Southern Hemisphere countries such as South Africa, New Zealand, Chile, and Australia.

OPHIOSTOMATOID FUNGI

Taxonomy

Ophiostomatoid fungi represent an artificial grouping of morphologically similar genera, including *Ophiostoma* H. & P. Sydow, *Ceratocystis* Ellis & Halstead, *Ceratocystiopsis* Upadhyay & Kendrick, *Gondwanamyces* Marais & Wingfield, and *Cornuvesica* Viljoen & Wingfield (Upadhyay, 1981; Wingfield, Seifert & Webber, 1993; Marais *et al.*, 1998; Viljoen *et al.*, 2000). Although morphologically similar, these genera are phylogenetically distantly related (Spatafora & Blackwell, 1994; Viljoen, Wingfield & Wingfield, 1999). Anamorph genera associated with these teleomorph genera are: *Pesotum* Crane & Schocknecht *sensu* Okada & Seifert, *Leptographium* Lagerberg & Melin, *Sporothrix* Hektoen & Perkins ex Nicot & Mariat, *Thielaviopsis* Went, *Hyalorhinocladiella* Upadhay & Kendrick, *Knoxdaviesia* Wingfield, Van Wyk & Marasas, and *Xenochalara* Coetzee & Wingfield (Wingfield, Van Wyk & Marasas, 1988; Wingfield, 1993b; Okada *et al.*, 1998; Coetzee *et al.*, 2000; Paulin & Harrington, 2000; Paulin, Harrington & McNew, 2002).

Ophiostomatoid fungi are collectively grouped as ascomycetes with long-necked perithecia, evanescent asci and hyaline ascospores lacking pores or slits, and all of the species appear to be dispersed by arthropods (Malloch & Blackwell, 1993). This fungal group comprises more than 100 species and is distributed world-wide on a large variety of substrates (Upadhyay, 1993).

The taxonomy of ophiostomatoid fungi, especially within *Ceratocystis sensu lato*, has been confused for more than a century. The most controversial issue has been the relationship between *Ceratocystis* and *Ophiostoma*, as well as their relatedness to *Ceratocystiopsis*. For many years, *Ophiostoma* was treated as a synonym of *Ceratocystis*, based on morphological characters. However, during the 1960's, biochemical characters were found to distinguish between these taxa (Bartnicki-Garcia, 1968). The species with *Chalara* (now *Thielaviopsis*) anamorphs and without cellulose and rhamnose in their cell walls, were treated as *Ceratocystis*

sensu stricto, while those with *Leptographium*, *Sporothrix* and/or *Graphium* (now *Pesotum*) anamorph(s), with cellulose and rhamnose, were placed in *Ophiostoma* (Weijman & De Hoog, 1975). Cycloheximide tolerance was also introduced to distinguish between *Ophiostoma* and *Ceratocystis*. *Ceratocystis sensu stricto* spp. are inhibited by cycloheximide in growth media, while growth of *Ophiostoma* spp. is not affected (Harrington, 1981; Marais, 1996). In 1984, *Ceratocystis sensu lato* was officially separated into *Ophiostoma* and *Ceratocystis sensu stricto* (De Hoog & Scheffer, 1984). During the symposium on taxonomy and biology of the Ophiostomatales in Germany in August 1990, almost all contributors regarded *Ceratocystis* and *Ophiostoma* as two discrete genera and a list of known *Ophiostoma* spp. was compiled in the book that resulted from this meeting (Wingfield, Seifert & Webber, 1993).

During the past decade, advances in molecular phylogenetics have considerably improved the systematics of the higher fungi, including the ascomycetes (Reynolds & Taylor, 1993). In the ophiostomatoid fungi, DNA sequence data from the ribosomal RNA genes have been effectively used to determine their phylogenetic relationships, and have shown that *Ceratocystis* and *Ophiostoma* spp. are not closely related (Hausner, Reid & Klassen, 1993a, b, c; Spatafora & Blackwell, 1993, 1994; Wingfield, B. D. *et al.*, 1994, 1999). Based on ascospore and anamorph morphology, Wingfield (1993a) thus suggested *Ceratocystiopsis* might be a synonym of *Ophiostoma*. This was supported by partial rDNA sequences (Hausner, Reid & Klassen, 1993c). The exact taxonomic position of *Ceratocystiopsis*, however, remains uncertain (Marais *et al.*, 1998; Viljoen *et al.*, 2000).

Two new ophiostomatoid genera, *Gondwanamyces* Marais & Wingfield, and *Cornuvesica* Viljoen, Wingfield & Jacobs, were established recently. *Ceratocystiopsis proteae* Wingfield, Van Wyk & Marasas and *Ophiostoma capense* Wingfield & Van Wyk, which are both characterised by *Knoxdaviesa* anamorphs and are phylogenetically distinct from *Ophiostoma* and *Ceratocystis*, were transferred to *Gondwanamyces* (Marais *et al.*, 1998). *Cornuvesica* was erected to

accommodate *Ceratocystiopsis falcata* (Wright & Cain) Upadhyay, which has a *Chalara* anamorph but is phylogenetically distantly related to both *Ophiostoma* and *Ceratocystis* (Viljoen, Wingfield & Wingfield, 1999; Viljoen *et al.*, 2000).

Apart from the teleomorph genera, the taxonomic position of ophiostomatoid anamorphs has also been the subject of revision in recent years. Based on nuclear encoded small subunit (18S) rDNA sequences, the genus *Pesotum*, rather than *Graphium*, was emended to accommodate all *Graphium*-like anamorphs of *Ophiostoma* spp. (Okada *et al.*, 1998). The genus *Xenochalara* was established to accommodate *Chalara*-like spp. producing conidia by apical wall building (Coetzee *et al.*, 2000). In the same year, Paulin and Harrington (2000) showed that the genus *Thielaviopsis* may represent an asexual lineage within *Ceratocystis* based on sequenced portions of the 18S and 28S rDNA domain. Anamorphs of all described *Ceratocystis* spp. were thus transferred to *Thielaviopsis* (Paulin, Harrington & McNew, 2002).

Ophiostomatoid species as pathogens

Some *Ophiostoma* spp. are important tree pathogens. *Ophiostoma ulmi* (Buisman) Nannfeldt, together with its recently described sibling species, *O. novo-ulmi* Brasier, causes Dutch Elm disease, which has killed millions of elm trees during the past century in both North America and Europe (Brasier, 1979, 1990, 1991, 1996; Houston, 1991; Mitchell & Brasier, 1994; Brasier & Mehrotra, 1995). Three host-specific varieties of *Leptographium wageneri* (Kendrick) Wingfield, which cause black stain root disease of conifers, have led to severe losses in the United States and Canada (Wagener & Mielke, 1961; Cobb, 1988; Harrington, 1993a).

Some *Ceratocystis* spp. can also cause serious tree diseases. *Ceratocystis fimbriata* Ellis & Halstead causes canker on *Populus*, *Acacia* and *Prunus* (Kile, 1993), and a wilt disease of *Eucalyptus* in Central Africa (Roux *et al.*, 2000). *Ceratocystis fagacearum* (Bretz) Hunt, the cause of oak wilt in the United States, has led to significant losses of trees in forests and

parklands (Hepting, 1955; Kile, 1993). In South Africa, *Ceratocystis albofundus* Wingfield, De Beer & Morris causes a wilt disease on *Acacia mearnsii* (Roux & Wingfield, 1997). *Chalara australis* Walker & Kile, a vascular pathogen, was found to kill *Nothofagus cunninghamii* in Tasmania (Kile & Walker, 1987; Kile & Hall, 1988).

Some ophiostomatoid fungi, especially *Ceratocystis* spp., are important pathogens to food and crop plants, such as pineapple, banana, sweet potato, sugar, nut, stone fruits, beans, mangoes, dates, cotton, tobacco, rubber, coffee and cacao. *Ceratocystis fimbriata*, which causes black rot of sweet potato and canker on coffee, is an economically important pathogen (Halsted & Fairchild, 1891; Taubenhaus, 1913; Webster & Butler, 1967; Kile, 1993). *Ceratocystis paradoxa* (Dade) Moreau, which is distributed globally, can cause rot of pineapple leaves and fruit, the main and finger stalk, as well as papaw leaves (Kile, 1993).

Some *Ophiostoma*-related species can be human pathogens. *Sporothrix schenckii* Hektoen & Perkins can cause various infections in humans, such as lymphocutaneous sporotrichosis (Summerbell *et al.*, 1993). The fungus was suggested to be the anamorph of *O. stenoceras* (Robak) Nannfeldt (Mariat, Escudié & Gaxotte, 1968; Mariat, 1971; De Hoog, 1974). Berbee and Taylor (1992) showed that *S. schenckii* is phylogenetically related to the genus of *Ophiostoma*. A recent study by De Beer *et al.* (2003) confirmed, however, that *S. schenckii* and *O. stenoceras* are distinct species based on ITS rDNA sequence data.

Ophiostomatoid species as sapstain agents

Sapstain is a grey, black or bluish discoloration of sapwood caused by the presence of pigmented fungal hyphae (Seifert, 1993). The explanation by Münch (1907) that sapstain is mostly a refractive effect of hyphae in the ray parenchyma cells and resin ducts of infected wood, is still widely accepted (Seifert, 1993). The pigment reponsible for the dark colour of the hyphae is considered to be fungal melanin, 1, 8-dihydroxynaphthalene (Wheeler, 1983). Wood cell walls

are not stained and wood strength not affected (Blanchette *et al.*, 1992; Zabel & Morrell, 1992; Seifert, 1993).

Ophiostomatoid species are the most important group of sapstain fungi on many types of wood, especially in the Northern Hemisphere, together with black yeasts and dark molds (Seifert, 1993). They can greatly degrade the quality of the timber and cause significant losses to forestry world-wide (Münch, 1907; Lagerberg, Lundberg & Melin, 1927; Seifert, 1993).

Control of ophiostomatoid fungi

For many years, sapstain caused by ophiostomatoid fungi was successfully controlled by chemicals such as sodium pentachlorophenate (Croan & Highley, 1991; Byrne, 1997; Hedley, 1997). The use of such chemicals has, however, been discontinued due to environmental concerns (Croan & Highley, 1991). At present, alternative control measures are being developed. One such alternative to chemical control, is the biological control agent, Cartapip™.

Cartapip™, formulated as a dry, wettable powder, consists of a white mutant of *O. piliferum* (Fries) H. & Sydow, a well-known sapstain fungus on various softwoods throughout the world (Blanchette *et al.*, 1992; Farrell *et al.*, 1993; Behrendt *et al.*, 1995; Kay, 1997; White-McDougall, Blanchette & Farrell, 1998), and inert ingredients. When a suspension of the powder is sprayed onto freshly cut wood chips or logs, the spores germinate and the fungus penetrates the wood (Farrell *et al.*, 1989; Blanchette, 1991; Blanchette *et al.*, 1994; Grönberg, 1996). This fungal mutant can remove significant amounts of pitch/resin from the wood, which improves paper quality (Blanchette, 1991; Farrell *et al.*, 1993), and also reduces the growth of other microorganisms including staining and rotting fungi (Blanchette, 1991; Blanchette *et al.*, 1992; Farrell *et al.*, 1993; White-McDougall, Blanchette & Farrell, 1998). It is regarded as the first successful commercial biopulping process (Farrell *et al.*, 1992, 1993).

Ophiostoma spp. other than *O. piliferum*, are currently being considered in attempts to improve the efficacy of Cartapip™. Selected isolates of *O. piceae* (Münch) H. & P. Sydow and *O. pluriannulatum* (Hedgcock) H. & P. Sydow, which lack hyphal pigmentation, successfully prevented staining of sterile wood in the laboratory when challenged with a wild, staining isolate of *O. pluriannulatum* (White-McDougall, Blanchette & Farrell, 1998). Field trials also showed that selected fungal isolates significantly reduced sapstain on pulpwood and logs (White-McDougall, Blanchette & Farrell, 1998).

In contrast to ophiostomatoid fungi causing sapstain, the control of phytopathogenic ophiostomatoid fungi is more complex. Apart from standard control measures such as quarantine and eradication of infected plants (Agrios, 1997), the exploitation of fungal viruses as a possible control measure is increasingly gaining research interest (Nuss & Koltin, 1990; Smart & Fulbright, 1996). *Ophiostoma novo-ulmi*, the Dutch elm disease fungus, was, for example, reported to contain 12 unencapsilated mitochondrical virus-like double-stranded (ds) RNAs (Hong *et al.*, 1999). These dsRNAs, and virus-like dsRNAs associated with other diseased phenotypes of *O. novo-ulmi*, have potential for the development of biological control agents against Dutch elm disease (Nuss & Koltin, 1990; Hong *et al.*, 1998, 1999).

Ophiostomatoid fungi reported from Southern Hemisphere countries

Since the beginning of the last century, at least 23 ophiostomatoid species have been reported from South Africa (Table 1) (De Beer, Wingfield & Wingfield, 2003). In other Southern Hemisphere countries such as Australia, New Zealand and Chile, limited research on ophiostomatoid fungi has been carried out, and to the best of our knowledge, only 29 species have been recorded from these countries (Table 2).

BARK BEETLES

Introduction

The bark beetles (Coleoptera: Scolytidae) reside in two subfamilies, 25 tribes, and 225 genera containing more than 5800 species (Wood & Bright, 1992). Some species are among the most damaging of forest insects. Bark beetles often act as vectors of pathogenic fungi, particularly *Ophiostoma* spp. (Harrington, 1988). Most bark beetles infesting conifers carry various species of ophiostomatoid fungi (Perry, 1991; Harrington, 1993b).

There are generally three life history strategies for bark beetles: primary, secondary and saprophytic (Paine, Raffa & Harrington, 1997). Primary bark beetles such as *Dendroctonus frontalis* (Zimmermann), *D. ponderosae* (Hopkins) and *Ips typographus* (Linnaeus), can attack healthy living trees and eventually kill the trees as a result of mass colonization. Secondary bark beetles are those that are only capable of colonizing weakened, stressed and recently killed trees, or freshly harvested logs, such as *I. pini* (Say) and *D. rufipennis* (Kirby). The greatest number of the bark beetle species, however, are saprophytes that can only colonize dead host trees, and these include genera such as *Hylurgops* (LeConte), *Orthotomicus* (Ferrari), *Hylurgus* (Latreille), and *Xyloterus* (Erichson) (Raffa, Phillips & Salom, 1993; Paine, Raffa & Harrington, 1997). Freshly harvested logs can be infested by both secondary and saprophytic bark beetles.

Bark beetles in South Africa

Three exotic pine bark beetle species, *Hylastes angustatus* (Herbst), *Hylurgus ligniperda* (Fabricius), and *Orthotomicus erosus* (Wollaston), native to Europe and the Mediterranean Basin, infest *Pinus* spp. in South Africa (Tribe, 1990a, b, 1991, 1992). *Hylurgus ligniperda* and *O. erosus* are generally considered as secondary pests. *Hylastes angustatus*, however, is more aggressive than the other two species, and is considered as a primary pest. This insect damages pine seedlings during maturation feeding and causes significant losses in newly established pine

plantations (Anonymous, 1946; Tribe, 1992). In South Africa, considerable research has been done on the phenology, biology, control, natural enemies, phylogeny, management, and parasites of bark beetles (Anonymous, 1946; Kfir, 1986; Tribe, 1990a, b, 1991, 1992; Erasmus & Chown, 1994; Zwolinski, Swart & Wingfield, 1995). Key elements of this knowledge are summarised in the following sections:

***Hylastes angustatus* (Fig. 1)**

This species was first recorded in South Africa in 1930 from *Pinus radiata* in the southern Cape Province (Tribe, 1990a). It usually feeds on the cambium and inner bark of conifers, mainly on *Pinus* spp. such as *P. patula*, *P. taeda*, *P. montezumae*, *P. pseudostrobus*, *P. radiata* and *P. pinaster* (Bevan & Jones, 1971). The beetle can commonly be found in the roots and stumps of dead or dying conifers, and above ground level in buried trapping logs (Tribe, 1990a).

Adults of *H. angustatus* are dark brown and about 4 mm long. Their life cycle can be divided into two phases: the breeding and feeding phases, and the whole process lasts an average of 38 days. During the breeding period, beetles, from sexually mature adults, through egg and larva, to young virgin imago, can be found on damaged, dying or dead material like logs and stumps. In the feeding phase, the virgin adults seek out young healthy plants and feed on them in the root-collar region, which can cause serious losses to pine seedlings at establishment. The beetles are active for approximately 277 days, which allows for up to five generations a year (Bevan & Jones, 1971).

***Hylurgus ligniperda* (Fig. 2)**

This species was first recorded in South Africa from *Pinus* spp. in 1885. It was restricted mainly to *Pinus* spp. in the western and southern Cape Province, but has more recently been recorded from KwaZulu-Natal (Tribe, 1991). It is a secondary pest colonizing stressed and dying pine

trees, and can usually be found beneath thick bark near the bases of stems or in large roots (Tribe, 1991).

Adults of *H. ligniperda* are dark brown and about 6 mm long. This species is the largest of the three bark beetle species occurring in South Africa, but carries out very little maturation feeding. Its life history is about 45 days and there are probably four to five generations per year in South Africa (Tribe, 1991).

***Orthotomicus erosus* (Fig. 3)**

This species was first recorded in South Africa from *Pinus radiata* in 1968 at Stellenbosch (Tribe, 1990b). It is usually considered a secondary pest colonizing only stressed pine trees such as *P. patula*, *P. taeda*, *P. canariensis*, *P. radiata*, *P. elliottii*, *P. halepensis* and *P. pinaster* (Bevan, 1984). Significant losses have been reported where there has been an interaction between fire damage, fungal diseases and beetle infestation (Tribe, 1990b). The beetle can commonly be found beneath the bark of logs lying on the ground in plantations and in the above ground portions of buried trapping logs (Tribe, 1990b).

Adults of *O. erosus* are dark brown and about 3 mm long. The rear ends of the beetles are hairy and excavated into a so-called elytral declivity, which is characteristic of the species. During its life history, which is about 35 days long, the beetle normally does very little maturation feeding and spends most of its time beneath the bark of dying trees. *Orthotomicus erosus* can have up to four generations per year (Bevan, 1984; Tribe, 1990b).

Bark beetles reported from other Southern Hemisphere countries

Four pine bark beetle species, *Hylurgus ligniperda*, *Hylastes ater* (Payk.), *Ips grandicollis* (Eichoff), and *O. erosus*, have been reported from other Southern Hemisphere countries such as Australia, Chile, and New Zealand (Swan, 1942; Serez, 1987; Neumann, 1987). The first reports

from the Southern Hemisphere were of: *H. ater* from New Zealand in 1929 (Swan, 1942) and *I. grandicollis* from Australia in 1943 (Neumann, 1987). All the pine beetle species present in the Southern Hemisphere were introduced from Europe, except for *I. grandicollis*, which originated in North America (Swan, 1942; Neumann 1987). Records of these four bark beetle species in the Southern Hemisphere, together with *H. angustatus* from South Africa, are listed in Table 3.

Spread of bark beetles in the Southern Hemisphere

In Southern Hemisphere countries such as Australia, Chile, New Zealand, and South Africa, at least five pine bark beetle species, *Hylurgus ligniperda*, *Hylastes ater*, *I. grandicollis*, *O. erosus*, and *Hylastes angustatus*, have been recorded. Apart from spreading within the various countries after the initial reports, introduced pine bark beetles have subsequently also been introduced to the other pine-growing countries of the Southern Hemisphere. *Hylurgus ligniperda* was reported from Australia in the early 1940's (Swan, 1942), from New Zealand in 1974 (Anonymous, 1974), and from Chile in 1985 (Ciesla, 1988). *Hylastes ater* was reported from New Zealand in 1929 (Swan, 1942), from Australia in 1937 (Swan, 1942), and Chile in 1983 (Ciesla, 1988). *Orthotomicus erosus*, initially known only from South Africa, has since been reported from Chile (Ciesla, 1988). Only *H. angustatus* (South Africa) and *I. grandicollis* (Australia) have not been reported from other countries since their initial introduction in the Southern Hemisphere. Thus, there is some evidence to suggest lateral transfer of these insects, once they have become established in an exotic situation.

Other than *I. grandicollis*, the bark beetles introduced into the Southern Hemisphere are not considered as major forest pests in their native countries (Ciesla, 1988; Tribe, 1992). In the Southern Hemisphere, however, *H. angustatus* can girdle and cause death of pine seedlings (Tribe, 1992), *H. ater* can attack *Pinus radiata* (Swan, 1942), and *I. grandicollis* can feed and

breed on living trees (Morgan, 1967). These examples indicate that there is continuous movement of pine bark beetles and adaptations to their life strategies in the Southern Hemisphere countries.

OPHIOSTOMATOID FUNGI AND BARK BEETLES

The association between fungi and bark beetles

Bark beetles are well-known vectors of fungi, particularly *Ophiostoma* and *Ceratocystis* spp. (Münch, 1907; Whitney, 1982; Harrington & Cobb, 1988; Beaver, 1989; Wingfield, Seifert & Webber, 1993; Paine, Raffa & Harrington, 1997; Jacobs & Wingfield, 2001). These fungi generally sporulate in the galleries of the bark beetle vectors and they are either carried in mycangia, on the exoskeletons, or in the guts of the beetles (Francke-Grosmann, 1967; Whitney, 1982; Beaver, 1989; Paine, Raffa & Harrington, 1997).

The relationship between ophiostomatoid fungi and their bark beetle vectors varies among different hosts, fungal species and bark beetle species (Harrington, 1993b; Wingfield, Harrington & Solheim, 1995; Paine, Raffa & Harrington, 1997). Although the relationship between bark beetles and fungi has been the topic of much debate and reviewed several times (Francke-Grosmann, 1967; Graham, 1967; Whitney, 1971, 1982; Dowding, 1984; Harrington, 1988; Beaver, 1989; Lieutier *et al.*, 1989; Paine, Raffa & Harrington, 1997), the benefits of fungal associates of bark beetles are still not completely understood (Solheim, 1994; Wingfield, Harrington & Solheim, 1995; Krokene & Solheim, 1998; Solheim, Krokene & Långström, 2001). Generally, it has been hypothesised that some of the bark beetles depend on fungi as a source of food, or for killing trees through mycelial penetration and toxin release, thus making the habitat more favourable for insect development, while the fungi rely on the beetle for dispersal to new host trees (Paine, Raffa & Harrington, 1997). The contrary argument is that fungi are casual

contaminants of bark beetles and that they do not afford any advantage to the insects (Hobson, Parmeter & Wood, 1994; Wingfield, Harrington & Solheim, 1995).

Bark beetles belonging to the genera *Hylurgops*, *Hylastes*, *Ips* and *Scolytus* have developed fungus-carrying structures called mycangia (Francke-Grosmann, 1967; Whitney & Farris, 1970; Paine, Raffa & Harrington, 1997). Usually only one or a very few specific fungal species are carried in the mycangia of a particular beetle species and many other fungal species present in the bark beetle habitat are excluded (Francke-Grosmann, 1967; Whitney & Farris, 1970; Paine & Birch, 1983; Six & Paine, 1998, 1999a).

Several possibilities have been suggested regarding the beetles' association with mycangial fungi. Mycangial fungi may provide protection to the beetle brood (Barras, 1970; Franklin, 1970; Whitney, 1971; Ross, Fenn & Stephen, 1992; Klepzig & Wilkens, 1997), and aid the beetles in overcoming the defenses of living host trees (Berryman, 1972). The fungi may provide nutrients required for reproduction and/or development (Barras, 1973; Bridges, 1983; Goldhammer, Stephen & Paine, 1990), or alter the chemical or moisture composition of the phloem (Nelson, 1934). The fungi presumably benefit by being consistently disseminated by the beetle to suitable host trees (Six & Paine, 1999b).

The association between fungi and bark beetles could be the result of their co-evolution, and it would be an advantage for both partners (Whitney, 1982; Dowding, 1984). Primitive bark beetles probably arose in or near the Cretaceous era from arthribid-like ancestors living close to various fungi in decaying or dead plant tissue. Due to the inter-species competition and environmental stress, ethanol produced in stressed trees and derived from microbial fermentation most likely became a primary attractant for some bark beetles (Berryman, 1989; Bright, 1993). This would have facilitated both the beetle's ability to utilize decomposing host resources, as well as interaction between beetles and various microorganisms, including phytopathogenic fungi. Ophiostomatoid fungi, which often have relatively long anamorph structures, melanized

ascomata of variable sizes, and adhesive spores with concave surfaces, adapted for insect dispersal (Malloch & Blackwell, 1993).

Bark beetle - host tree - fungus interaction

The process of tree colonization by bark beetles can be divided into four phases: dispersal, host selection, concentration, and establishment (Wood, 1972). The production of aggregation pheromones during the concentration phase continues as long as a tree resists beetle colonization (Raffa & Berryman, 1983; Berryman *et al.*, 1989; Lorio, Stephen & Paine, 1995). The establishment phase begins when the host resistance stops (Berryman, 1972) and the tree starts dying (Berryman, 1972). During this stage, beetles construct galleries and initiate oviposition (Berryman, 1972). The mechanism by which the host attempts to resist the infection of bark beetles and their associated fungi has two recognized components: the constitutive resin system and the induced hypersensitive response (Reid, Whitney & Watson, 1967; Berryman, 1969; Russell & Berryman, 1976; Christiansen & Horntvedt, 1983; Cook & Hain, 1986). The constitutive resin system is highly developed in conifers which are capable of producing large quantities of resin to pitch out beetles (Christiansen, Waring & Berryman, 1987). The induced defense system has, however, been referred to as secondary resinosis (Reid, Whitney & Watson, 1967), wound response (Shrimpton, 1978), dynamic reaction zone (Shain, 1967), and hypersensitive response (Berryman, 1972). According to Nebeker, Hodges and Blanche (1993), secondary resinosis consists of: "(a) localized autolysis of parenchyma cells accompanied by rapid cellular dessication, (b) tissue necrosis, (c) secondary resinosis by adjacent secretory and parenchyma cells, and (d) formation of wood periderm to physically isolate the lesion from the mainstream of metabolism and transport".

Successful colonization of living trees by bark beetles usually results in the death of trees. Bark beetle associated fungi could facilitate this process by toxin production, mycelial plugging

of the tracheids, release of gas bubbles into the tracheids, and production of particles that block the pit openings by causing torus aspiration (Paine, Raffa & Harrington, 1997). *Ophiostoma* spp. can reduce stored food in the parenchyma cells and restrict water conduction by destroying the ray parenchyma cells that partially control water movement (Nebeker, Hodges & Blanche, 1993).

Scolytid - conifer - microbial interactions show a high level of complexity at multiple levels of biological organization. A proper understanding of the natural interactions among the beetles, pathogens and conifer hosts will contribute to better management of conifer ecosystems.

Bark beetles and sapstain

In the Northern Hemisphere, ophiostomatoid species, most of which are associated with bark beetles, are the most important sapstain agents. Indications are that these insect-associated fungi also play a significant role in sapstain problems associated with plantation forestry in the Southern Hemisphere. For effective control of sapstain, it is necessary to understand the biology of the fungal species involved, as well as the role of possible bark beetle vectors during the infection process. All these should be considered in the development of new approaches to sapstain control.

Ophiostomatoid fungi associated with bark beetles in the Southern Hemisphere

All five pine bark beetle species, *Hylurgus ligniperda*, *Hylastes ater*, *I. grandicollis*, *O. erosus*, and *Hylastes angustatus*, reported in the Southern Hemisphere countries, can vector pathogenic fungi. Some of these vectored fungal species are important sapstain agents. *Ophiostoma ips* (Rumbold) Nannfeldt and *Cop. minuta* (Siemaszko) Upadhyay & Kendrick have been isolated from *I. grandicollis* in Australia (Stone & Simpson, 1990), and *O. huntii* (Robinson-Jeffrey) de Hoog & R. J. Scheffer from *H. ater* both in Australia and New Zealand (Jacobs *et al.*, 1998).

Ophiostoma galeiformis (Bakshi) Mathiesen-Käärik has been found in New Zealand (Harrington, personal communication).

Of the bark beetles introduced into the Southern Hemisphere, only the fungal associates of *H. ater* and *I. grandicollis* have been studied in the Northern Hemisphere. In Sweden, the following fungal species have been isolated from *H. ater*: *O. ips*, *O. penicillatum* Grossman, *O. piceae* , *L. lundbergii* Lagerb. & Melin, *O. piliferum* [= *O. coeruleum*], and *Graphium areum* Hedgc. (Mathiesen, 1950; Mathiesen-Käärik, 1953). To our knowledge, only *O. ips* has been reported from *I. grandicollis* in the USA (Rumbold, 1931).

These examples show that species from this group of fungi have already been introduced into new environments. Studies on bark beetles and their associated fungi are, therefore, essential in order to understand risks linked to new introductions and to develop meaningful quarantine procedures.

Pine bark beetles and their associated fungi in South Africa

Considerable research has been done on the three exotic bark beetle species in South Africa, *Hylastes angustatus*, *O. erosus*, and *Hylurgus ligniperda*. However, the fungal associates of these beetles have been the subject of limited study in the Western Cape province (Wingfield & Knox-Davies, 1980; Wingfield & Marasas, 1980a, b; Wingfield & Marasas, 1983; Wingfield, Strauss & Tribe, 1985; Wingfield & Swart, 1989; Wingfield, Van Wyk & Marasas, 1988). Preliminary pathogenicity studies with *O. ips*, *L. serpens* (Goid.) Wingf., *L. lundbergii*, were carried out by inoculating healthy branches or freshly cut bolts of *P. radiata*. Results showed that the fungi caused lesions and could be considered as pathogens (Wingfield & Knox-Davies, 1980; Wingfield & Marasas, 1980a; Wingfield & Marasas, 1983; Wingfield & Swart, 1989).

OBJECTIVES OF THIS STUDY

In South Africa, sapstain fungi degrade high quality pine logs exported to South East Asian countries, which leads to the loss of millions of Rands every year (De Beer *et al.*, 2001). For effective management and control of sapstain in the South African context, it is necessary (1) to investigate the role of bark beetles in fungal infection, and (2) to understand the biology of the ophiostomatoid fungi which contribute to the sapstain problem. Fungal population diversity studies will also be necessary before possible biological control measures can be considered. This study, therefore, focuses on ophiostomatoid fungi associated with bark beetles in South Africa and attempts to meet the following goals:

1) To isolate and identify ophiostomatoid species associated with the three exotic pine bark beetle species in South Africa.
2) To assess the pathogenicity of these fungi and to consider their roles in sapstain.
3) To develop microsatellite markers for *Ophiostoma ips* and to use these to consider questions relating to the population genetics and origin of the fungus.
4) To consider the taxonomy and phylogeny of selected *Ophiostoma* spp., particularly within the context of those occurring in South Africa.

REFERENCES

Agrios, G. N. (1997) *Plant Pathology*. 4th edn. Academic Press, Florida, USA.

Anonymous. (1946) The black pine beetle (*Hylastes ater*) and other closely allied beetles. *Forestry Commission Leaflet No. 4*. His Majesty's Stationery Office, London: 1-10.

Anonymous. (1974) New bark beetle *Hylurgus ligniperda* (Scolytidae) discovered in New Zealand. *New Zealand Forestry Service*. 4 pp.

Barras, S. J. (1970) Antagonism between *Dendroctonus frontalis* and the fungus *Ceratocystis minor*. *Annals of the Entomological Society of America* **63**: 1187-1190.

Barras, S. J. (1973) Reduction in progeny and development in the southern pine beetle following removal of symbiotic fungi. *The Canadian Entomologist* **105**: 1295-1299.

Bartnicki-Garcia, S. (1968) Cell wall chemistry, morphogenesis and taxonomy of fungi. *Annual review of Microbiology* **22**: 87-108.

Beaver, R. A. (1989) Insect-fungus relationships in the bark and ambrosia beetles. In *Insect-Fungus Interactions* (N. Wilding, N. M. Collins, P. M. Hammond & J. F. Webber, eds): 121-143. Academic Press, London.

Behrendt, C. J., Blanchette, R. A. & Farrell, R. A. (1995) An integrated approach, using biological and chemical control, to prevent blue stain in pine logs. *Canadian Journal of Botany* **73**: 613-619.

Berbee, M. L. & Taylor, J. W. (1992) 18S ribosomal RNA gene sequence characters place the human pathogen *Sporothrix schenckii* in the genus *Ophiostoma*. *Experimental Mycology* **16**: 87-91.

Berryman, A. A. (1969) Responses of *Abies grandis* to attack by *Scolytus ventralis* (Coleoptera: Scolytidae). *The Canadian Entomologist* **101**: 1033-1041.

Berryman, A. A. (1972) Resistance of conifers to invasion by bark beetle-fungus associations. *Bioscience* **22**: 598-602.

Berryman, A. A. (1989) Adaptive pathways I Scolytid - Fungus associations. In *Insect-Fungus Interactions* (N. Wilding, N. M. Collins, P. M. Hammond & J. F. Webber, eds): 145-159. Academic Press, London.

Berryman, A. A., Raffa, K. F., Millstein, J. A. & Stenseth, N. C. (1989) Interaction dynamics of bark beetle aggregation and conifer defense rates. *Oikos* **56**: 256-263.

Bevan, D. (1984) *Orthotomicus erosus* (Wollaston) in Usutu pine plantations, Swaziland. *Forest Research Report* **64**: 1-34.

Bevan, D. & Jones, T. (1971) Report on the beetle *Hylastes angustatus* in pine plantations of Swaziland and adjacent areas of the Republic of South Africa. *Research and Development Paper* **84**: 1-12.

Blanchette, R. A. (1991) Biotechnological uses of forest fungi in the pulp and paper industry. In *Hans Merensky Fellowship Lectures*, pp. 1-20. University of Stellenbosch, South Africa.

Blanchette, R. A., Behrendt, C. J., Farrell, R. L., Iverson, S. & Brush, T. S. (1994) Industrial uses for colorless strains of *Ophiostoma*: bioprocessing and biocontrol. In *Abstracts from the*

Fifth International Mycological Congress, 14-21 August 1994, Vancouver, British Columbia, Canadia. p. 17.

Blanchette, R. A., Farrell, R. L., Burnes, T. A., Wendler, P. A., Zimmerman, W., Brush, T. S. & Snyder, R. A. (1992) Biological control of pitch in pulp and paper production by *Ophiostoma piliferum*. *Tappi Journal* **75**: 102-106.

Brasier, C. M. (1979) Dual origin of recent Dutch elm disease outbreaks in Europe. *Nature* **281**: 78-79.

Brasier, C. M. (1990) China and the origins of Dutch elm disease: an appraisal. *Plant Pathology* **39**: 5-16.

Brasier, C. M. (1991) *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. *Mycopathologia* **115**: 151-161.

Brasier, C. M. (1996) Low genetic diversity of the *Ophiostoma novo-ulmi* population in North America. *Mycologia* **88**: 951-964.

Brasier, C. M. & Mehrotra, M. D. (1995) *Ophiostoma himal-ulmi* sp. nov., a new species of Dutch elm disease fungus endemic to the Himalayas. *Mycological Research* **99**: 205-215.

Bridges, J. R. (1983) Mycangial fungi of *Dendroctonus frontalis* (Coleoptera: Scolytidae) and their relationship to beetle population trends. *Environmental Entomology* **12**: 858-861.

Bright, D. E. (1993) Systematics of bark beetles. In *Beetle-Pathogen Interactions in Conifers Forests* (T. D. Schowalter & G. M. Filip, eds): 23-36. Academic Press, New York.

Brown, R., Weintroub, D. & Simpson, M. W. (1947) Timber as a source of sporotrichosis infection. In *Sporotrichosis infection on Mines of the Witwatersrand.* A symposium. *Proceedings of the Transvaal Mine Medical Officers' Association*, Transvaal Chamber of Mines, Johannesburg, South Africa, 5-33.

Butin, H. & Aquilar, A. M. (1984) Blue-stain fungi on *Nothofagus* from Chile - including two new species of *Ceratocystis* Ellis & Halst. *Phytopathologische Zeitschrift* **109**: 80-89.

Byrne, T. (1997) Sapstain control in Canada – Can New Zealand learn anything from the frozen north? In *Strategies for Improving Protection of Logs and Lumber* (B. Kreber, ed): 7-15. *Proceedings of Symposium at the Forest Research Institute, Rotorua, New Zealand, 21-22 November. FRI Bulletin No. 204.*

Christiansen, E. & Horntvedt, R. (1983) Combined *Ips / Ceratocystis* attack on Norway spruce, and defensive mechanisms of the trees. *Zeitschrift für Angewandte Entomologie* **96**: 110-118.

Christiansen, E., Waring, R. H. & Berryman, A. A. (1987) Resistance of conifers to bark beetle attack: searching for general relationships. *Forest Ecology and Management* **22**: 89-106.

Ciesla, W. M. (1988) Pine bark beetles: a new pest management challenge for Chilean foresters. *Journal of Forestry* **86**: 27-31.

Coetzee, C., Wingfield, M. J., Crous, P. W. & Wingfield, B. D. (2000) *Xenochalara*, a new genus of dematiaceous hyphomycetes for *Chalara*-like fungi with apical wall building conidial development. *South African Journal of Botany* **66**: 99-103.

Cobb, F. W. (jr). (1988) *Leptographium wageneri*, cause of black-stain root disease: a review of its discovery, occurrence and biology with emphasis on pinyon and ponderosa pine. In *Leptographium root diseases on conifers* (T. C. Harrington & Jr. F. W. Cobbs., eds): 41-62. American Phytopathological Society Press, St. Paul, Minnesota.

Cook, S. P. & Hain, F. P. (1986) Defensive mechanisms of loblolly and shortleaf pine against attack by southern pine beetle, *Dendroctonus frontalis* Zimmermann, and its fungal associate, *Ceratocystis minor* (Hedgecock) Hunt. *Journal of Chemical Ecology* **12**: 1397-1406.

Croan, S. C. & Highley, T. L. (1991) Biological control of the blue stain fungus *Ceratocystis coerulescens* with fungal antagonists. *Material und Organismen* **25**: 255-266.

De Beer, Z. W., Wingfield, B. D. & Wingfield, M.J. (2003) The occurrence of *Ophiostoma piliferum*-like fungi on pulpwood chips and other wood sources in South Africa. *South African Journal of Science* (In press).

De Beer, Z. W., Harrington, T. C., Vismer, H. F., Wingfield, B. D. & Wingfield, M. J. (2003) Phylogeny of the *Ophiostoma stenoceras – Sporothrix schenckii* complex. *Mycologia* (In press).

De Beer, Z. W., Zhou, X. D., Wingfield, M. J. & Wingfield B. D. (2001) Assessment of sapstain on pine logs in South Africa. Proceedings of BIOY2K Combined Millennium Meeting of Southern Africa, Grahamstown, South Africa. *South African Journal of Science* **97**: xi.

De Hoog, G. S. (1974) The genera *Blastobotrys*, *Sporothrix*, *Calcarisporium* and *Calcarisporiella* gen. nov. *Studies in Mycology* **7**: 1-84.

De Hoog, G. S. & Scheffer, R. J. (1984) *Ceratocystis* versus *Ophiostoma*: a reappraisal. *Mycologia* **76**: 292-299.

Doidge, E. M. (1950) The South African fungi and lichens to the end of 1945. *Bothalia* **5**: 1-1094

Dowding, P. (1984) The evolution of insect - fungus relationships in the primary invasion of forest timber. In *Invertebrate - Microbial Interactions* (J. M. Anderson, A. D. M. Raynor & D. W. H. Walton, eds): 133-153. Cambridge University Press, New York.

Eicker, A. (1974) The mycoflora of an alkaline soil of the open-savannah of the Transvaal. *Transactions of the British Mycological Society* **63**: 281-288.

Erasmus, M. J. & Chown, S. L. (1994) Host location and aggregation behaviour in *Hylastes angustatus* (Herbst) (Coleoptera: Scolytidae). *African Entomology* **2**: 7-11.

Farrell, R. L., Blanchette, R. A., Brush, T. S., Gysin, B., Hadar, Y., Perollaz, J. J., Wendler, P. A. & Zimmerman, W. (1992) Cartapip™: a biopulping product for control of pitch and resin acid problems in pulp mills. In *Biotechnology in the Pulp and Paper Industry, Proceedings of the Fifth International Conference in Biotechnology on Pulp and Paper Industry* (M. Kuwahara & M. Shimada, eds): 27-32. Uni Publishers: Tokyo, Japan.

Farrell, R. L., Blanchette, R. A., Brush, T. S., Hadar, Y., Iverson, S., Krisa, K., Wendler, P. A. & Zimmerman, W. (1993) Cartapip™: a biopulping product for control of pitch and resin acid problems in pulp mills. *Journal of Biotechnology* **30**: 115-122.

Farrell, R. L., Hadar, E., Kay, S. J., Blanchette, R. A. & Harrington, T. C. (1997) Survey of sapstain organisms in New Zealand and albino anti-sapstain fungi. Biology and prevention of sapstain. A conference sponsored by the Department of Forest Products, Oregon State University, 25 May, 1997, Delta Whistler Resort, Whistler, British Columbia, Canada: 57-62.

Farrell, R. L., Hadar, Y., Brush, T. S., Ho, C., Blanchette, R. A., Snyder, R., Merritt, J. & Wendler, P. A. (1989) Cartapip™: A product for the biological control of pitch problems. *Unpublished*, 4 pp.

Francke-Grosmann, H. (1967) Ectosymbiosis in wood-inhabiting insects. In *Symbiosis. Vol II* (S. M. Henry, ed): 141-205. Academic Press, New York.

Franklin, R. T. (1970) Observations on the blue stain - southern pine beetle relationship. *Journal of the Georgia Entomological Society* **5**: 53-57.

Geertsema, H. (1979) Insect problems in South African forest plantations. *Wood South Africa (August)*: 33-36.

Goldhammer, D. S., Stephen, F. M. & Paine, T. D. (1990) The effect of the fungi *Ceratocystis minor* (Hedgcock) Hunt, *Ceratocystis minor* (Hedgcock) Hunt var. *barrasii* Taylor, and SJB on reproduction of the southern pine beetle, *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytidae). *The Canadian Entomologist* **122**: 407-418.

Gorter, G. J. M. A. (1977) Index of plant pathogens and the diseases they cause in cultivated plants in South Africa. *Science Bulletin No. 392.* Plant Protection Research Institute, Department of Agricultural Technical Services, Pretoria, South Africa, 177 pp.

Gorter, G. J. M. A. (1979) An annotated check list and selected bibliography of South African fungi for the period 1946 - 1977. *Technical Communication No. 163.* Department of Agricultural Technical Services, Pretoria, South Africa, 34 pp.

Graham, K. (1967) Fungal - insect mutualism. *Annual Review of Entomology* **12**: 105-126.

Grönberg, V. (1996) The use of a fungus as a pulping aid for improving chip quality and reducing pitch problems. *Unpublished*, 9 pp.

Halsted, B. D. & Fairchild, D. G. (1891) Sweet potato black rot (*Ceratocystis fimbriata* Ellis & Halstead). *Journal of Mycology* **7**: 1-11.

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

Harrington, T. C. (1988) *Leptographium* species, their distributions, hosts and insect vectors. In *Leptographium root diseases on conifers* (T. C. Harrington & Jr. F. W. Cobbs., eds): 1-39. American Phytopathological Society Press, St. Paul, Minnesota.

Harrington, T. C. (1993a) Diseases of conifers caused by species of *Ophiostoma* and *Leptographium*. In *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 161-172. American Phytopathological Society Press, St. Paul, Minnesota.

Harrington, T. C. (1993b) Biology and taxonomy of fungi associated with bark beetles. In *Beetle-Pathogen Interactions in Conifers Forests* (T. D. Schowalter & G. M. Filip, eds): 37-58. Academic Press, New York.

Harrington, T. C. & Cobb Jr., F. W. (1988) *Leptographium* root diseases on conifers. American Phytopathological Society Press, St. Paul, Minnesota.

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

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

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

Hedley, M. (1997) Development of antisapstain technologies in New Zealand – an historical perspective. In *Strategies for Improving Protection of Logs and Lumber* (B. Kreber, ed): 1-5. *Proceedings of Symposium at the Forest Research Institute, Rotorua, New Zealand, 21-22 November. FRI Bulletin No. 204.*

Hepting, G. H. (1955) The current status of oak wilt in the United States. *Forest Science* **1**: 95-103.

Hobson, K. R., Parmeter, J. R. Jr & Wood, D. L. (1994) The role of fungi vectored by *Dendroctonus brevicomis* LecConte (Coleoptera: Scolytidae) in occlusion of ponderosa pine xylem. *Canadian Entomologist* **126**: 277-282.

Hong, Y., Cole, T. E., Brasier, C. M. & Buck, K. W. (1998) Novel structures of two virus-like RNA elements from a diseased isolate of the Dutch Elm disease fungus, *Ophiostoma novo-ulmi*. *Virology* **242**: 80-89.

Hong, Y., Dover, S. L., Cole, T. E., Brasier, C. M. & Buck, K.W. (1999) Multiple mitochondrial viruses in an isolate of the Dutch Elm disease fungus *Ophiostoma novo-ulmi*. *Virology* **258**: 118-127.

Houston, D. R. (1991) Changes in non-aggressive and aggressive subgroups of *Ophiostoma ulmi* within two populations of American elm in New England. *Plant Disease* **75**: 720-722.

Hutchison, L. J. & Reid, J. (1988) Taxonomy of some potential wood-staining fungi from New Zealand: 1. Ophiostomataceae. *New Zealand Journal of Botany* **26**: 63-81.

Jacobs, K. & Wingfield, M. J. (2001) *Leptographium* species - tree pathogens, insect associates and agents of blue-stain. American Phytopathological Society Press, St. Paul, Minnesota.

Jacobs, K., Wingfield, M. J, Wingfield, B. D. & Yamaoka, Y. (1998) Comparison of *Ophiostoma huntii* and *O. europhioides* and description of *Ophiostoma aenigmaticum* sp. nov. *Mycological Research* **102**: 289-294.

Jooste, W. J. (1978) *Leptographium reconditum* sp. nov. and observations on conidiogenesis in *Verticicladiella*. *Transactions of the British Mycological Society* **70**: 152-155.

Kay, S. (1997) Biological control of sapstain in New Zealand. In *Strategies for Improving Protection of Logs and Lumber* (B. Kreber, ed): 75-79. *Proceedings of Symposium at the Forest Research Institute, Rotorua, New Zealand, 21-22 November. FRI Bulletin No. 204.*

Kfir, R. (1986) Releases of natural enemies against the pine bark beetle *Orthotomicus erosus* (Wollaston) in South Africa. *Journal of the Entomological Society of Southern Africa* **2**: 391-392.

Kile, G. A. (1993) Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K.

A. Seifert & J. F. Webber, eds): 173-183. American Phytopathological Society Press, St. Paul, Minnesota.

Kile, G. A. & Hall, M. F. (1988) Assessment of *Platypus subgranosus* as a vector of *Chalara australis*, causal agent of a vascular disease of *Nothofagus cunninghamii*. *New Zealand Journal of Forestry Science* **18**: 166-186.

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

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

Klepzig, K. D. & Wilkens, R. T. (1997) Competitive interactions among symbiotic fungi of the southern pine beetle. *Applied and Environmental Microbiology* **63**: 621-627.

Krokene, P. & Solheim, H. (1998) Pathogenicity of four blue-stain fungi associated with aggressive and non aggressive bark beetles. *Phytopathology* **88**: 39-44.

Lagerberg, T., Lundberg, G. & Melin, E. (1927) Biological and practical researches into blueing in pine and spruce. *Svenska Skogsvardsföreningens Tidskrift* **25**: 145-272.

Lanfranco, D., Sandra, I., Peredo, H., Ruiz, C., Muñoz, X. & Vives, I. (1999) *Hylurgus ligniperda* e *Hylastes ater*: Asociación especifica? *Detección identificación de agentes entomopatógenos* **12**: 16-17.

Laughton, E. M. (1937) The incidence of fungal disease on timber trees in South Africa. *South African Journal of Science* **33**: 377-382.

Lieutier, F., Yart, A., Garcia, J., Ham, M. C., Morelet, M. & Levieux, J. (1989) Champignons phytopathogènes associés à deux coléoptères Scolytidae du Pin sylvestre (*Pinus sylvestris* L.) et étude préliminaire de leur agressivité envers l'hôte. *Annales des Sciences Forestieres* **46**: 201-216.

Lorio, P. L. Jr., Stephen, F. M. & Paine, T. D. (1995) Environment and ontogeny modify loblolly pine response to induced acute water deficits and bark beetle attack. *Forest Ecology and Management* **73**: 97-110.

Malloch, D. & Blackwell, M. (1993) Dispersal biology of the ophiostomatoid fungi. In *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 195-206. American Phytopathological Society Press, St. Paul, Minnesota.

Marais, G. J. (1996) Fungi associated with infructescences of *Protea* species with special reference to the Ophiostomatales. Ph.D. Thesis, University of Orange Free State, Bloemfontein, South Africa.

Marais, G. J. & Wingfield, M. J. (1994) Fungi associated with infructescences of *Protea* species in South Africa, including a new species of *Ophiostoma*. *Mycological Research* **98**: 369-374.

Marais, G. J. & Wingfield, M. J. (1997) *Ophiostoma protearum* sp. nov. associated with *Protea caffra* infructescences. *Canadian Journal of Botany* **75**: 362-367.

Marais, G. J. & Wingfield, M. J. (2001) *Ophiostoma africanum* sp. nov., and a key to ophiostomatoid species from *Protea* infructescences. *Mycological Research* **105**: 240-246.

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

Marasas, W. F. O., Van der Westhuizen, G. C. A., Van Warmelo, K. T. & Papendorf, M. C. (1966) New and interesting records of South African fungi, Part V. *Bothalia* **9**: 229-243.

Mariat, F. (1971) Adaptation de *Ceratocystis* a la vie parasitaire chez l'animal – Etude de l'acquisition d'un pouvoir pathogene comparable a celui de *Sporothrix schenckii*. *Sabouraudia* **9**: 191-205.

Mariat, F., Escudié, A. & Gaxotte, P. (1968) Isolement de souches de *Ceratocystis* sp. à forme conidienne *Sporotrichum*, se cuis chevelus humains et de poils de rats. Comparaison avec l'espèce pathogène *Sporotrichum schenckii*. *Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences* **267**: 974-976.

Mathiesen, A. (1950) Über einige mit Borkenkäfern assoziierte Bläuepilze in Schweden. *Oikos* **2**: 275-308.

Mathiesen-Käärik, A. (1953) Eine Übersicht über die gewöhnlichsten mit Borkenkäfern assoziierten Bläuepilze in Schweden und einige für Schweden neue Bläuepilze. *Meddelanden från Statens Skogsforskningsinstitut* **43**: 1-74.

Mitchell, A. G. & Brasier, C. M. (1994) Contrasting structure of European and North American populations of *Ophiostoma ulmi*. *Mycological Research* **98**: 576-582.

Morgan, F. D. (1967) *Ips grandicollis* in South Australia. *Australian Forestry* **31**: 137-155.

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

Mouton, M., Wingfield, M. J., Van Wyk, P. S. & Van Wyk, P. W. (1994) *Graphium pseudormiticum* sp. nov.: a new hyphomycete with unusual conidiogenesis. *Mycological Research* **98**: 1272-1276.

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

Nebeker, T. E., Hodges, J. D. & Blanche, C. A. (1993) Host response to bark beetle and pathogen colonization. In *Beetle-pathogen interactions in conifer forests* (T. D. Schowalter & G. M. Filip, eds): 157-173. Academic Press, Oregon State University, Corvallis, USA. .

Nelson, R. M. (1934) Effect of bluestain fungi on southern pines attacked by bark beetles. *Phytopathologische Zeitschrift* **7**: 327-353

Neumann, F. G. (1987) Introduced bark beetles on exotic trees in Australia with special reference to infestations of *Ips grandicollis* in pine plantations. *Australian Forestry* **50**:166-178.

Nuss, D. L. & Koltin, Y. (1990) Significance of dsRNA genetic elements in plant pathogenic fungi. *Annual Review of Phytopathology* **28**: 37-58.

Okada, G., Seifeit, K. A., Takematsu, A., Yamaoka, Y., Miyazaki, S. & Tubaki, K. (1998) A molecular phylogenetic reappraisal of the *Graphium* complex based on 18S rDNA sequences. *Canadian Journal of Botany* **76**: 1495-1506.

Paine, T. D. & Birch, M. C. (1983) Acquistion and maintenance of mycangial fungi by *Dendroctonus brevicomis* LeConte (Coleoptera: Scolytidae). *Enviromental Entomology* **12**: 1384-1386.

Paine, T. D., Raffa, K. F. & Harrington, T. C. (1997) Interactions among Scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* **42**: 179-206.

Paulin, A. E. & Harrington, T. C. (2000) Phylogenetic placement of anamorphic species of *Chalara* among *Ceratocystis* species and other ascomycetes. *Studies in Mycology* **45**: 209-222.

Paulin, A. E., Harrington, T. C. & McNew, D. (2002) Phylogenetic and taxonomic evaluation of *Chalaropsis* and *Thielaviopsis* anamorphs associated with *Ceratocystis*. *Mycologia* **94**: 62-72.

Peredo, H. L. & Alonso, O. (1988) Mycoflora of *Pinus radiata* pulpwood and outdoor storing in central Chile. *Material und Organismen* **23**: 11-19

Perry, T. J. (1991) A synopsis of the taxonomic revisions in the genus *Ceratocystis* including a review of blue-staining species associated with *Dendroctonus* bark beetles. *General Technical Report*, SO-86. New Orleans, LA: U. S. Department of Agriculture, Forest Service, Southern Forest Experiment Station, pp. 1-16.

Raffa, K. F. & Berryman, A. A. (1983) Physiological aspects of lodgepole pine wound responses to a fungal symbiont of the mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *The Canadian Entomologist* **115**: 723-734.

Raffa, K. F., Phillips, T. W. & Salom, S. M. (1993) Strategies and mechanisms of host colonization by bark beetles. In *Beetle-Pathogen Interactions in Conifers Forests* (T. D. Schowalter & G. M. Filip, eds): 103-128. Academic Press, New York.

Reid, R. W., Whitney, H. S. & Watson, J. A. (1967) Reactions of lodgepole pine to attack by *Dendroctonus ponderosae* Hopkins and blue stain fungi. *Canadian Journal of Botany* **45**: 1115-1126.

Reynolds, D. R. & Taylor, J. W. (1993) The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics. CAB International, Wallingford, UK.

Ross, D. W., Fenn, P. & Stephen, F. M. (1992) Growth of southern pine beetle associated fungi in relation to the induced wound response in loblolly pine. *Canadian Journal of Forest Research* **22**: 1151-1159.

Roux, J. & Wingfield, M. J. (1997) Survey and virulence of fungi occurring on diseased *Acacia mearnsii* in South Africa. *Forest Ecology and Management* **99**: 327-336.

Roux, J., Wingfield, M. J., Bouillette, J. P., Wingfield, B. D. & Alfenas, A. C. (2000) A serious new wilt disease of *Eucalyptus* caused by *Ceratocystis fimbriata* in Central Africa. *Forest Pathology* **30**: 175-184.

Rumbold, C. T. (1931) Two blue-staining fungi associated with bark-beetle infestation of pines. *Journal of Agricultural Research* **43**: 847-873.

Russell, C. E. & Berryman, A. A. (1976) Host resistance to the fir engraver beetle. I. Monoterpene composition of *Abies grandis* pitch blisters and fungus-infected wounds. *Canadian Journal of Botany* **54**: 14-18.

Seifert, K. A. (1993) Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 141-151. American Phytopathological Society Press, St. Paul, Minnesota.

Serez, M. (1987) Use of the aggregation pheromone preparation 'Ipslure' against the Mediterranean pine bark beetle *Ips* (*Orthotomicus*) *erosus* (Woll.) (Col. Scol.). *Anzeiger-fur-Schadlingkunde, Pflanzenschutz, -Umweltschutz* **60**: 94-95.

Shain, L. (1967) Resistance of sapwood in stems of loblolly pine to infection by *Fomes annosus*. *Phytopathology* **57**: 1034-1045.

Shrimpton, D. M. (1978) Resistance of lodgepole pine to mountain pine beetle infestation. In *Theory and practice of mountain pine beetle management in lodgepole pine forests – a symposium* (A. A. Berryman, G. D. Amman, R. W. Stark & D. L. Kibbee, eds): 64-76. College of Forest Resources, University of Idaho, Moscow.

Six, D. L. & Paine, T. D. (1998) Effects of mycangial fungi and host tree species on progeny survival and emergence of *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *Environmental Entomology* **27**: 1393-1401.

Six, D. L. & Paine, T. D. (1999a) Phylogenetic comparison of Ascomycete mycangial fungi and *Dendroctonus* bark beetles (Coleoptera: Scolytidae). *Annals of the Entomological Society of America* **92**: 159-166.

Six, D. L. & Paine, T. D. (1999b) Allozyme diversity and gene flow in *Ophiostoma clavigerum* (Ophiostomatales: Ophiostomataceae), the mycangial fungus of the Jeffrey pine beetle, *Dendroctonus jeffreyi* (Coleoptera: Scolytidae). *Canadian Journal of Forest Research* **29**: 324-331.

Smart, C. D. & Fulbright, D. W. (1996) Molecular biology of fungal diseases. In *Molecular biology of the biological control of pests and diseases of plants*. (M. Gunasekaran & Weber, D. J., eds): 57-70. CRC Press, Florida.

Solheim, H. (1994) Early stages of blue-stain fungus invasion of lodgepole pine sapwood following mountain pine beetle attack. *Canadian Journal of Botany* **73**: 70-74.

Solheim, H., Krokene, P. & Långström, B. (2001) Effect of growth and virulence of associated blue-stain fungi on host colonization behaviour of the pine shoot beetles *Tomicus minor* and *T. piniperda*. *Plant Pathology* **50**: 111-116.

Spatafora, J. W. & Blackwell, M. (1993) Molecular systematics of unitunicate perithecial Ascomycetes: The Clavicipitales - Hypocreales connection. *Mycologia* **85**: 912-922.

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

Stone, C. & Simpson, J. A. (1990) Species associations in *Ips grandicollis* galleries in *Pinus taeda*. *New Zealand Journal of Forestry Science* **20**: 75-96.

Summerbell, R. C., Kane, J., Krajden, S. & Duke, E. E. (1993) Medically important *Sporothrix* species and related ophiostomatoid fungi. In *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 185-192. American Phytopathological Society Press, St. Paul, Minnesota.

Swan, D. C. (1942) The bark beetle - *Hylastes ater* Payk. (Coleoptera: Scolytidae) attacking pines in South Australia. *Journal of Agriculture* **12**: 86-90.

Talbot, P. H. B. (1956) New and interesting records of South African fungi. Part II. *Bothalia* **6**: 489-500.

Taubenhaus, J. (1913) The black rots of sweet potato. *Phytopathology* **3**: 159-166.

Taylor, J. J. (1970) A comparison of some *Ceratocystis* species with *Sporothrix schenckii*. *Mycopathologia et Mycologia Applicata* **42**: 233-240.

Tribe, G. D. (1990a) Phenology of *Pinus radiata* log colonization and reproduction by the European bark beetle *Orthotomicus erosus* (Wollaston) (Coleoptera: Scolytidae) in the south-western Cape Province. *Journal of the Entomological Society of Southern Africa* **2**: 117-126

Tribe, G. D. (1990b) Phenology of *Pinus radiata* log colonization by the pine bark beetle *Hylastes angustatus* (Herbst) (Coleoptera: Scolytidae) in the south-western Cape Province. *Journal of the Entomological Society of Southern Africa* **1**: 93-100

Tribe, G. D. (1991) Phenology of *Pinus radiata* log colonization by the red-haired pine bark beetle *Hylurgus ligniperda* (Fabricius) (Coleoptera: Scolytidae) in the south-western Cape Province. *Journal of the Entomological Society of Southern Africa* **1**: 1-7

Tribe, G. D. (1992) Colonization sites on *Pinus radiata* logs of the bark beetles, *Orthotomicus erosus*, *Hylastes angustatus* and *Hylurgus ligniperda* (Coleoptera: Scolytidae). *Journal of the Entomological Society of Southern Africa* **1**: 77-84.

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

Upadhyay, H. P. (1993) Classification of the ophiostomatoid fungi. In *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 7-13. American Phytopathological Society Press, St. Paul, Minnesota.

Vaartaja, O. (1963) Fungi associated with insects in pine trees. In *Woods and Forests Department Annual Report*, 1961-1962 (B. H. Bednall, ed): 11. Adelaide.

Vaartaja, O. (1966) Wood inhabiting fungi in a pine plantation in Australia. (Not seen.)

Vaartaja, O. (1967) The common fungal associates of the bark beetle, *Ips grandicollis*, in *Pinus radiata* in South Australia. *Australian Forest Research* **2**: 40-43.

Viljoen, C. D., Wingfield, B. D. & Wingfield, M. J. (1999) Relatedness of *Custingophora olivaceae* to *Gondwanamyces* spp. from *Protea* spp. *Mycological Research* **103**: 497-500.

Viljoen, C. D., Wingfield, M. J., Jacobs, K. & Wingfield. B. D. (2000) *Cornuvesica*, a new genus to accommodate *Ceratocystiopsis falcata*. *Mycological Research* **104**: 365-367.

Wagener, W. W. & Mielke, J. L. (1961) A staining fungus root disease of ponderosa, jeffrey and pinyon pines. *Plant Disease Reporter* **45**: 831-835.

Webster, R. K. & Butler, E. E. (1967) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45**: 1457-1467.

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

Wheeler, M. H. (1983) Comparisons of fungal melanin synthesis in ascomycetous, imperfect and basidiomycetous fungi. *Transactions of the British Mycological Society* **81**: 29-36.

White-McDougall, W. J., Blanchette, R. A. & Farrell, R. L. (1998) Biological control of blue stain fungi on *Populus tremuloides* using selected *Ophiostoma* isolates. *Holzforschung* **52**: 234-240.

Whitney, H. S. (1971) Association of *Dendroctonus ponderosae* (Coleoptera: Scolytidae) with blue stain fungi and yeasts during brood development in lodgepole pine. *The Canadian Entomologist* **103**: 1495-1503.

Whitney, H. S. (1982) Relationships between bark beetles and symbiotic organisms. In *Bark beetles in North American conifers* (J. B. Mitton & K. B. Sturgeon, eds): 183-211. University of Texas, Austin.

Whitney, H. S. & Farris, S. H. (1970) Maxillary mycangium in the mountain pine beetle. *Science* **167**: 54-55.

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

Wingfield, B. D., Viljoen, C. D. & Wingfield, M. J. (1999) Phylogenetic relationships of ophiostomatoid fungi associated with *Protea* infructescences in South Africa. *Mycological Research* **103**: 1616-1620.

Wingfield, M. J. (1993a) Problems in delineating the genus *Ceratocystiopsis*. In *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 21-26. American Phytopathological Society Press, St. Paul, Minnesota.

Wingfield, M. J. (1993b) *Leptographium* species as anamorphs of *Ophiostoma*: progress in establishing acceptable generic and species concepts. In *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 43-51. American Phytopathological Society Press, St. Paul, Minnesota.

Wingfield, M. J., Crous, P. W. & Swart, W. J. (1993) *Sporothrix eucalypti* (sp. nov.), a shoot and leaf pathogen of *Eucalyptus* in South Africa. *Mycopathologia* **123**: 159-164.

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

Wingfield, M. J., Harrington, T. C. & Solheim, H. (1995). Do conifer bark beetles require fungi to kill trees? In *Proceedings from a symposium held at the Norwegian Forest Research Institute (NISK) in Ås, Norway, 31 July – 2 August, 1995* (E. Christiansen, ed). *Aktuelt fra Skogforsk* 6/95: 6.

Wingfield, M. J. & Knox-Davies, P. S. (1980) Root-disease, associated with *Verticicladiella alacris*, of pines in South Africa. *Plant Disease* **64**: 569-571.

Wingfield, M. J. &. Marasas, W. F. O. (1980a) *Ceratocystis ips* associated with *Orthotomicus erosus* (Coleoptera: Scolytidae) on *Pinus* spp. in the Cape Province of South Africa. *Phytophylactica* **12**: 65-69.

Wingfield, M. J. &. Marasas, W. F. O. (1980b) *Verticicladiella alacris* sp. nov., associated with root disease of pines in South Africa. *Transactions of the British Mycological Society* **75**: 21-28.

Wingfield, M. J. & Marasas, W. F. O. (1983) Some *Verticicladiella* species, including *V. truncata* sp. nov., associated with root diseases of pine in New Zealand and South Africa. *Transactions of the British Mycological Society* **80**: 231-236.

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

Wingfield, M. J., Strauss, L. A. & Tribe, G. D. (1985) Fungi associated with three bark beetles in South Africa (Abstract). *Phytopathology* **75**: 1338.

Wingfield, M. J. & Swart, W. J. (1989) Relative pathogenicity of fungi associated with pine root-infesting insects in South Africa. In *Proceedings of the Seventh International Conference on Root and Butt Rots* (D. J. Morrison, ed): 381-391. British Columbia, Canada.

Wingfield, M. J. & Van Wyk, P. S. (1993) A new species of *Ophiostoma* from *Protea* infructescences in South Africa. *Mycological Research* **97**: 709-716.

Wingfield, M. J., Van Wyk, P. S. & Marasas, W. F. O. (1988) *Ceratocystiopsis proteae* sp. nov., with a new anamorph genus. *Mycologia* **80**: 23-30.

Wood, D. L. (1972). Selection and colonization of ponderosa pine by bark beetles. *Symposia of the Royal Entomological Society of London* **6**: 110-117.

Wood, S. L., & Bright, D. E. (1992) A catalog of Scolytidae and Platypodidae (Coleoptera), part 2: Taxonomic index. *Great Basin Naturalist Memoirs* **A** (13): 1-6.

Zabel, R. A. & Morrell, J. J. (1992) Wood microbiology: Decay and its prevention. Academic Press, San Diego.

Zwolinski, J. B, Swart, W. J. & Wingfield, M. J. (1995) Association of *Sphaeropsis sapinea* with insect infestation following hail damage of *Pinus radiata*. *Forest Ecology and Management* **72**: 293-298.

Table 1. Ophiostomatoid fungi reported from South Africa (* doubtful identification).

Year	Species	Host	Reference
1927	*Sporothrix schenckii* Hektoen & Perkins	*Homo sapiens*	Doidge, 1950
	=*Sporotrichum beurmanii* Matr. & Ramond		
1931	*Thielaviopsis basicola* (Berk. & Br.) Ferraris	*Nicotiana tabacum*	Gorter, 1977
1937	*Ophiostoma piliferum* (Fr.) H. & P. Sydow	Logs of *Pinus radiata*	Laughton, 1937
	=*Ceratostomella pilifera* (Fr.) Winter		
1937	*Ceratocystis paradoxa* (Dade) Moreau	*Saccharum officinarum*	Doidge, 1950
	=*Thielaviopsis paradoxa* (de Seyn) Hohne		
1947	*Graphium* sp. associated with *Sporotrichum* sp.	Timber and air	Brown, Weintroub & Simpson, 1947
1956	*Ceratocystis adiposa* (Butl.) Moreau	Shoots of *Pinus* sp.	Talbot, 1956
1965	*Chalara terrestris* Agnih. & Bama	*Eucalyptus saligna*	Marasas *et al.*, 1966
1974	*Graphium putredinis* (Corda) Hughes	Soil	Eicker, 1974
1974	*Ceratocystis fimbriata* Ell. & Halst.	*Protea gigantea*	Gorter, 1979
1978	*Leptographium reconditum* Jooste	*Triticum* rhizosphere	Jooste, 1978
1980	*Ophiostoma ips* (Rumb.) Nannf.	*Orthotomicus erosus*	Wingfield & Marasas, 1980a
	=*Ceratocystis ips* (Rumb.) Moreau		
1980	*Ophiostoma serpens* (Goid.) Siem.	Roots of *Pinus pinaster*	Wingfield & Marasas, 1980b
	=*Verticicladiella alacris* Wingfield & Marasas	Roots of *Pinus radiata*	
1983	*Leptographium lundbergii* Lag. & Melin	Roots of *Pinus taeda*	Wingfield & Marasas, 1983
	=*Verticicladiella truncata* Wingfield &Marasas		
1985	*Ophiostoma* sp.	*Orthotomicus erosus*	Wingfield, Strauss & Tribe, 1985
	Leptographium sp.	*Hylurgus ligniperda*	Wingfield & Swart, 1989
	Graphium sp.	*Hylastes angustatus*	
1988	*Gondwanamyces proteae* (Wingfield & Marasas) Marais & Wingfield	*Protea repens*	Wingfield, Van Wyk & Marasas, 1988
	=*Ceratocystiopsis proteae* Wingfield, V. Wyk & Marasas		
1993	*Sporothrix eucalypti* Wingfield, Crous & Swart	*Eucalyptus grandis*	Wingfield, Crous & Swart, 1993
1993	*Gondwanamyces capensis* (Wingfield & V. Wyk) Marais & Wingfield	*Protea* spp.	Wingfield & Van Wyk, 1993
	=*Ophiostoma capensis* Wingfield & V. Wyk		
1993	*Ceratocystis fimbriata* Ellis & Halstead	*Acacia mearnsii*	Morris, Wingfield & De Beer, 1993
1994	*Ophiostoma splendens* Marais & Wingfield	*Protea* spp.	Marais & Wingfield, 1994
1994	*Graphium pseudormiticum* Mouton & Wingfield	*Orthotomicus erosus*	Mouton *et al.*, 1994
1996	*Ceratocystis albofundus* Wingfield, de Beer & Morris	*Protea* sp.	Wingfield *et al.*, 1996
1997	*Ophiostoma protearum* Marais & Wingfield	*Protea caffra*	Marais & Wingfield, 1997
2001	*Ophiostoma africanum* Marais & Wingfield	*Protea gaguedi*	Marais & Wingfield, 2001

Table 2. Ophiostomatoid fungi reported from Australia, New Zealand and Chile.

Country	Species	Host or Vector	Reference
Australia	*Ophiostoma ips* (Rumb.) Nannf.	*P. radiata*, *Ips grandicollis*	Vaartaja, 1963, 1967
	O. piliferum (Fr.) H. & P. Sydow	unknown	
	Ceratocystiopsis minuta (Siem.) Upadhyay & Kendr.	Galleries of *P. taeda* infested by *I. grandicollis*	Stone & Simpson, 1990
	Cop. sp.		
	Graphium sp.		Stone & Simpson, 1990; Vaartaja, 1967
	O. huntii (Rob.-Jeffr.) de Hoog & R. J. Scheff.	*Hylastes ater*	Jacobs *et al.*, 1998
	Ceratocystis eucalypti Z. Q. Yuan & Kile	*Eucalyptus* spp.	Kile *et al.*, 1996
	Chalara eucalypti Z. Q. Yuan & Kile	*Eucalyptus* spp.	
	Ch. australis J. Walker & Kile	*Nothofagus cunninghamii*	
	Leptographium sp.	*P. radiata*	Vaartaja, 1966
New Zealand	*O. huntii*	*H. ater*	Jacobs *et al.*, 1998
	O. piliferum (Fr.) H. & P. Sydow	Galleries of an unknown bark beetle	Hutchison & Reid, 1988
	O. coronata Olchow. & Reid	Galleries of an unknown bark beetle infesting *P. radiata*, *P. nigra* and *Eucalyptus* sp.	
	O. novae-zelandiae (Hutchison & Reid) Rulamort	Galleries of an unknown bark beetle infesting *Podocarpus spicatus*, *Podocarpus* sp., *P. radiata*, and *Pseudotsuga menziessi*	
	O. piceae (Münch) H. & P. Syd.	Galleries of an unknown bark beetle infesting *Eucalyptus* sp., *Dacrydium cupressinum*, *Larix* sp., *Podocarpus spicatus*, *Podocarpus* sp., *Pseudotsuga menziessi*.	
	O. ips (Rumb.) Nannf.	*P. elliotii*, and *P. radiata*	
	O. piceaperda (Rumb.) von Arx	Galleries of an unknown bark beetle infesting *P. radiata*, *P. nigra*, and *P. taeda*.	
	Cop. falcata (Wright & Cain) Upadhyay	Galleries of an unknown bark beetle infesting *P. radiata*, and *Larix* sp.	
	O. rostrocoronata (Davids. & Eslyn) de Hoog & Scheffer	Galleries of an unknown bark beetle infesting *Eucalyptus* sp.	
	O. pluriannulatum (Hedgcock) H. & P. Sydow	*P. radiata*	Farrell *et al.*, 1997
	O. stenoceras (Robak) Melin & Nannf.		
	O. querci (Georgew.) Moreau		
	Leptographium procerum (Kendr.) Wingfield		
	L. lundbergii Lagerb. & Melin		
	Graphium sp. A		
	Graphium sp. B		
Chile	*Sporothrix* sp.	Pulpwood of *P. radiata*	Peredo & Alonso, 1988
	S. curviconia de Hoog		
	Pesotum sp.		
	O. piliferum	Sawn timber of *Nothofagus pumilio*	Butin & Aquilar, 1984
	O. valdiviana (Butin) Rulamort	Bark and wood of *N. alpina*	
	O. piceae	Sawn timber of *N. pumilio*	
	O. nothofagi (Butin) Rulamort	Bark and wood of *N. dombeyi*	
	Graphium sp.	*H. ligniperda*	Lanfranco *et al.*, 1999

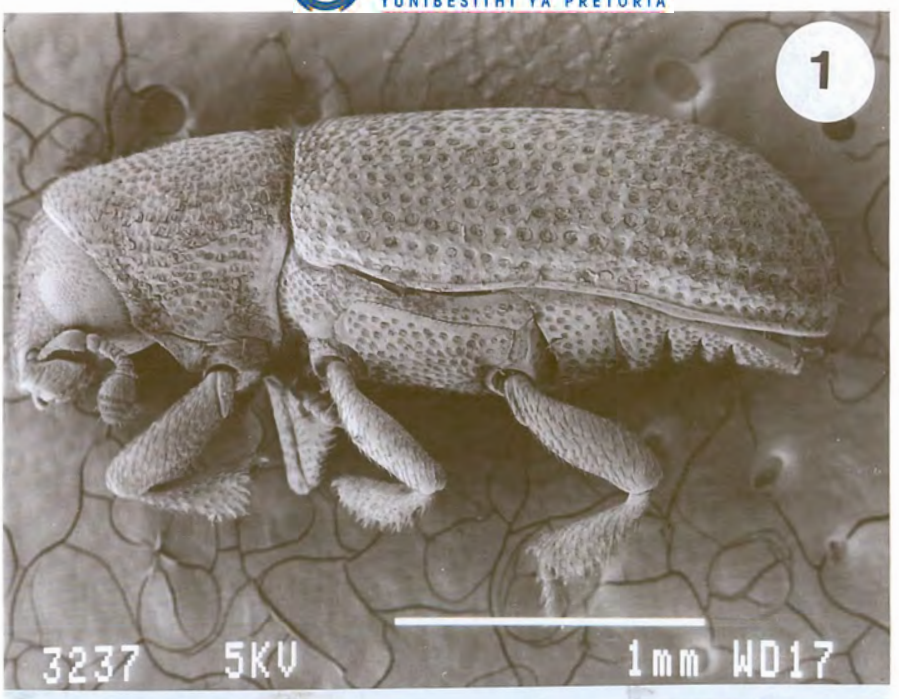
Table 3. Records of five pine bark beetle species in the Southern Hemisphere.

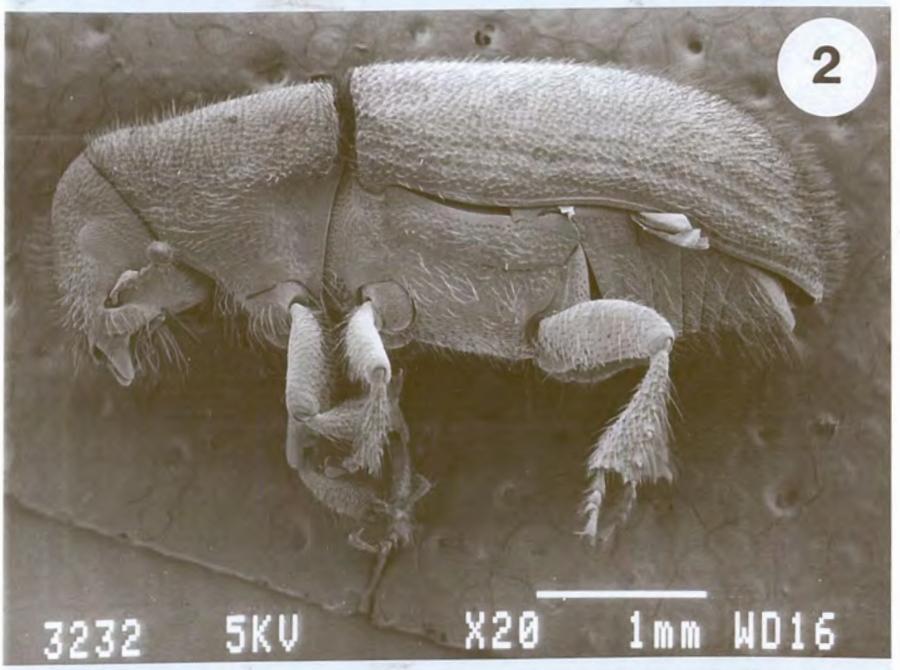
Country	Beetle Species	Host	First Record	Reference
South Africa	*Orthotomicus erosus*	*Pinus radiata*	1968	Geertsema, 1979
	Hylurgus ligniperda	*Pinus* spp.	1885	Tribe, 1991
	Hylastes angustatus	*P. radiata*	1930	Tribe, 1990b
Australia	*H. ligniperda*	*P. radiata*	1940's	Swan, 1942
	Hylastes ater	*P. radiata*	1937	Swan, 1942
	Ips grandicollis	*P. nigra* var. *cabrica*	In 1943	Neumann, 1987
Chile	*H. ligniperda*	*P. radiata*	In 1983	Ciesla, 1988
	H. ater	*P. radiata*	In 1983	Ciesla, 1988
	O. erosus	*P. radiata*	In 1983	Ciesla, 1988
New Zealand	*H. ater*	*P. radiata*	In 1929	Swan, 1942
	H. ligniperda	*Pinus* spp.	In 1974	Anonymous, 1974

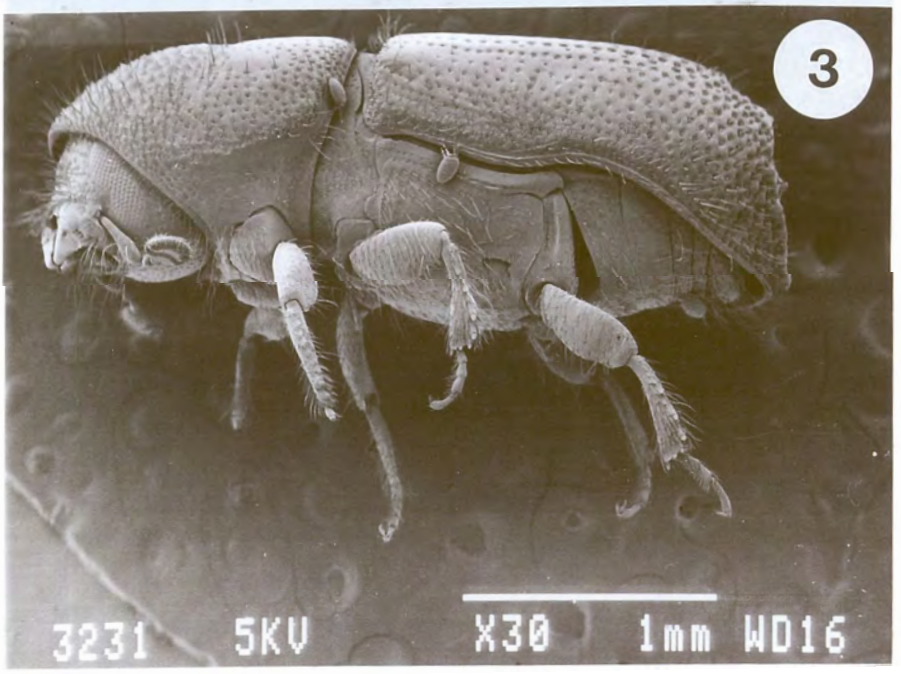
Fig. 1. *Hylastes angustatus* (Herbst).

Fig. 2. *Hylurgus ligniperda* (Fabricius).

Fig. 3. *Orthotomicus erosus* (Wollaston).







Chapter 2

Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa*

Three species of exotic bark beetles, *Hylastes angustatus*, *Hylurgus ligniperda* and *Orthotomicus erosus*, occur on *Pinus* spp. in South Africa. Although these bark beetles have been reasonably intensively studied in South Africa, little is known regarding their associated fungi. In this study, 1558 samples (beetles and galleries) were collected from *P. patula* and *P. elliottii* plantations. In total, 1254 fungal isolates were encountered and 500 of them are maintained. Forty additional isolates previously collected and stored in a culture collection were also included. Nine different ophiostomatoid species were identified. Among these, *Leptographium serpens*, *L. lundbergii*, and *Ophiostoma ips*, were most frequently encountered. *Ophiostoma galeiformis*, *O. piceae* and *L. procerum* are newly recorded from South Africa.

Keywords: sapstain, *Ophiostoma*, *Leptographium*.

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INTRODUCTION

The ophiostomatoid fungi represent an artificial grouping of morphologically similar genera, including *Ophiostoma* H. & P. Sydow, *Ceratocystis* Ell. & Halst., *Sphaeronaemella* Karsten, *Ceratocystiopsis* Upadhyay & Kendrick, *Gondwanamyces* Marais & Wingfield, and *Cornuvesica* Viljoen & Wingfield (Upadhyay, 1981; Wingfield, Seifert & Webber, 1993; Marais & al., 1998; Viljoen & al., 2000). Although morphologically similar, these genera are phylogenetically distantly related (Spatafora & Blackwell, 1994; Viljoen, Wingfield & Wingfield, 1999). Anamorph genera associated with these teleomorph genera are: *Pesotum* Crane & Schocknecht *sensu* Okada & Seifert, *Leptographium* Lagerb. & Melin, *Sporothrix* Hektoen & Perkins ex Nicot & Mariat, *Thielaviopsis* Went, *Hyalorhinocladiella* Upadhay & Kendrick, *Knoxdaviesia* Wingfield, Van Wyk & Marasas, and *Xenochalara* Coetzee & Wingfield (Wingfield, Van Wyk & Marasas, 1988; Okada & al:, 1998; Coetzee & al., 2000; Paulin & Harrington, 2000).

Many ophiostomatoid fungi are economically important because they can cause plant diseases and sapstain on logs, lumber and pulpwood. Sapstain is a grey, black or bluish discoloration of sapwood caused by the presence of pigmented fungal hyphae in the tracheids (Seifert, 1993). In South Africa, sapstain fungi degrade high quality pine logs exported to South East Asian countries, which leads to significant financial loss to the local forestry industry each year.

Many sapstain fungi, especially ophiostomatoid species, are associated with bark beetles (Coleoptera: Scolytidae). Most bark beetles are secondary pests that invade stressed trees, but some are primary forest pests (Wood & Bright, 1992) that can kill healthy living trees (Paine, Raffa & Harrington, 1997). The association between bark beetles and fungi suggests that there is mutual benefit to both partners (Whitney, 1982), although this matter is the subject of considerable debate (Wingfield, Harrington & Solheim, 1995).

Three species of exotic bark beetles, *Hylastes angustatus* (Herbst), *Hylurgus ligniperda* (Fabricius) and *Orthotomicus erosus* (Wollaston) native to Europe and the Mediterranean Basin, occur on mature *Pinus* spp. in South Africa (Tribe, 1992). Although they are generally considered secondary pests, *H. angustatus* undergoes maturation feeding on healthy pine seedlings and thus causes serious damage (Tribe, 1992).

Considerable research has been done on the three exotic bark beetles in South Africa (Kfir, 1986; Tribe, 1992; Erasmus & Chown, 1994). However, the fungal associates of these beetles have been the subjects of limited study (Wingfield & Knox-Davies, 1980; Wingfield & Marasas, 1980; Wingfield & Swart, 1989). Therefore, the aim of this investigation was to do a more detailed study and identify the fungi associated with these three bark beetle species.

MATERIALS AND METHODS

Collection of bark beetles and galleries

During the course of 1998 and 1999, beetles and galleries representing *H. angustatus*, *H. ligniperda* and *O. erosus*, were obtained from infested stumps, root collars, and trap logs of *P. patula* and *P. elliottii* in Mpumalanga and Kwazulu-Natal provinces. Trap logs, 1.5 m long and 0.2 m in diameter, were set out using the technique described by Tribe (1992). Twenty *P. patula* logs in Mpumalanga and 20 *P. elliottii* logs in Kwazulu-Natal were placed in plantations every two months from Oct. 1998 to Oct. 1999. Ten of the logs from each locality were buried at an angle of 45° and the other ten were placed on the ground surface to trap different beetle species, according to different niches they occupy (Tribe, 1992). Logs were inspected for the presence of entrance holes of beetles about six weeks after being placed in plantations. Bark surrounding the entrance holes was cut and peeled from the logs. All beetles from a single gallery were removed using a sterilized tweezer and placed in an autoclaved McCartny bottle. The complete gallery (around 1

cm away from the tunnel) was removed and placed in a separate, clean paper bag. The gallery, together with the beetles present in it, was treated as a single sample.

Isolation and identification of fungi from bark beetles and galleries

In the laboratory, each beetle was taken out of the bottle using sterilized tweezers and squashed onto the surface of selective medium for *Ophiostoma* spp. (20 g Biolab malt extract, 20 g Biolab agar and 1000 ml distilled water, amended with 0.05% cycloheximide and 0.04% streptomycin) (Harrington, 1981). Crushed beetles were left on the surface of the medium. Beetles from different galleries were incubated on separate Petri dishes at 25°C in the dark for two weeks, during which they were regularly examined for fungal growth and sporulation. Cultures were purified by transferring hyphal tips from the edges of individual colonies, or spore masses from emerging perithecia or conidiophores to fresh 2% MEA (20 g Biolab malt extract, 20 g Biolab agar and 1000 ml distilled water). Pure, sporulating cultures were examined and identified using a light microscope.

Galleries were maintained in humid chambers at 25 °C in the dark for three to four weeks. During this period, galleries were carefully examined using a dissection microscope. Spore masses accumulating at the tips of perithecia or conidiophores produced in the galleries, were carefully lifted using a fine sterile needle and transferred to 2 % MEA. These cultures were incubated at 25 °C in the dark for two weeks, and purified when necessary by transferring hyphal tips from the edges of individual colonies to fresh 2 % MEA. Perithecia and conidiophores, were mounted in lactophenol on glass slides. Fruiting structures were examined and described using light microscopy.

Frequency of occurrence

From each sample, only one isolate per fungal species was included in calculation. Frequencies of occurrence of fungi collected from bark beetles were computed using the following formula (Yamaoka & al., 1997):

$$F = (NF / NT) \times 100\ \%,$$

where F represents the frequency of occurrence (%) of the fungus from each niche; NT represents the total number of samples from which isolations were made, and NF represents the number of samples from which fungi were isolated. E. g. 312 *L. serpens* isolates (NF) were obtained from 694 samples (NT) of *H. angustatus*. The frequency of occurrence of *L. serpens* on *H. angustatus* was, therefore, F = (312 / 694) X 100 % = 45.0 %.

Maintenance of cultures

All cultures used in this study have been stored in the Culture Collection (CMW) of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, Republic of South Africa. Representative material of each species has been deposited with the National Collection of Fungi, Pretoria, South Africa (PREM).

Other fungal isolates

In 1984, a limited study was conducted on the three bark beetle species on *P. radiata* and *P. pinaster* in the Western Cape province of South Africa. Isolations were conducted in the same way as for the current study, and isolates are maintained in the CMW culture collection. Since records of these fungi have not been published elsewhere, and given the fact that they are directly related to the current study, they are included here.

RESULTS

Collection of bark beetles and galleries

A total of 1558 samples, representing the three bark beetle species in South Africa, were collected. Of these, 665 were *O. erosus*, 694 *H. angustatus*, and 199 *H. ligniperda*.

Isolation and identification of fungi from bark beetles and galleries

At least eight species of ophiostomatoid fungi were identified as associates of the three beetle species. They are: *Ophiostoma ips* (Rumb.) Nannf., *O. stenoceras* (Robak) Nannf., *O. piceae* (Münch) H. & P. Sydow, *O. galeiformis* (Bakshi) Mathiesen-Käärik, *O. pluriannulatum* (Hedgc.) H. & P. Sydow, *L. lundbergii* Lagerb. & Melin, *L. serpens* (Goid.) M. J. Wingfield, *Ceratocystiopsis minuta* (Siem.) Upadh. & Kendr., and some, as yet unidentified, *Pesotum* spp., a *Sporothrix* sp. and a *Hyalorhinocladiella* sp.. The fungal associates of the respective beetle species are listed in Tab. 1.

Among the eight identified fungal species, *O. ips* (Figs. 1-3) was the most frequently encountered on *O. erosus*, while *L. serpens* (Figs. 4-5), together with *L. lundbergii* (Figs. 6-8), were commonly found on both *H. angustatus* and *H. ligniperda*. Frequency of occurrence of *O. ips* from *O. erosus* was 60.0 %. For *L. lundbergii* and *L. serpens*, it was 44.8 % and 45.0 % for each species respectively from *H. angustatus*, and 21.6 % and 21.1 % for each species respectively from *H. ligniperda*. Frequencies of occurrence of each fungal species are also included in Tab. 1. Among the 436 isolates of *O. ips* collected, 267 were from *P. patula* and 169 from *P. elliottii*. Likewise, 345 isolates of *L. serpens* were from *P. patula* and 12 were from *P. elliottii*. In the case of *L. lundbergii*, 358 isolates originated from *P. patula* and this species was not found on *P. elliottii*.

Besides the eight identified species, a number of isolates resembling *Pesotum*, *Sporothrix* and *Hyalorhinocladiella* were isolated from the three bark beetles. Two non-ophiostomatoid sapstain

fungi, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. and *Sphaeropsis sapinea* (Fr.) Dyko & Sutton, were occasionally isolated from beetle galleries during this survey. Both these species are well-known causes of sapstain on pines in South Africa.

Other fungal isolates

In the Western Cape, five ophiostomatoid species (*O. stenoceras*, *O. pluriannulatum*, *L. serpens*, *L. lundbergii*, and *L. procerum* (Kendr.) M. J. Wingfield) were identified from *H. angustatus*, four (*O. ips*, *O. pluriannulatum*, *L. serpens*, and *Cop. minuta*) from *O. erosus*, and two (*L. lundbergii* and *Cop. minuta*) from *H. ligniperda*. In addition, a small number of *Pesotum* , *Sporothrix* and *Hyalorhinocladiella* spp., which could not be identified to species level, were included in this group. *Leptographium procerum* was the only species from this region that was not isolated from Mpumalanga and Kwazulu-Natal as the main part of this study.

DISCUSSION

At least 12 species of ophiostomatoid fungi, including unidentified *Pesotum*, *Sporothrix* and *Hyalorhinocladiella* spp., were isolated as associates of the three exotic beetles, *H. angustatus*, *H. ligniperda* and *O. erosus* in South Africa. This is the first comprehensive survey of the fungi associated with these insects in South Africa. *Ophiostoma galeiformis*, *O. piceae*, and *L. procerum* are recorded for the first time from South Africa.

Results of this study indicate that the most commonly encountered fungal associates of the bark beetles are *O. ips*, *L. serpens* and *L. lundbergii*. The difference in common associates between the three bark beetle species could be, to an extent, linked to the different niches that these beetles occupy. *Ophiostoma ips* is the species most frequently isolated from *O. erosus*,

which preferentially occupies above ground parts of stems. *Leptographium lundbergii,* together with *L. serpens*, are commonly found on both *H. angustatus* and *H. ligniperda*, which occur in the bark just above or below the ground. A number of other species, including *O. ips*, *O. pluriannulatum*, *O. stenoceras*, *Cop. minuta*, some *Pesotum* , *Sporothrix* and *Hyalorhinocladiella* spp., were also isolated from both *H. angustatus* and *H. ligniperda.* These two beetle species often share the same niche, which would explain the overlap in their fungal associates. In the field, we observed that these two beetle species constructed galleries in close proximity to each other, which might result in fungal co-infection of galleries.

The frequency of occurrence of bark beetle associated fungi could reflect the intimacy of the relationship between bark beetles and their fungal associates. Apart from *L. lundbergii* and *L. serpens* on *H. angustatus* and *H. ligniperda*, and *O. ips* on *O. erosus*, all other fungal species could be considered infrequent associates, based on their low frequencies of occurrence.

Host tree species can also be an important determinant of the relationship between beetles and their associated fungi. Some beetles are host specific and only carry specific fungi (Six & Paine, 1999). In our study, results indicate that the three most common fungi on the bark beetles were more frequently isolated from *P. patula* than *P. elliottii*. This could be due to a preference of the insects to infest the former species.

Ophiostoma ips is a fungus commonly found in association with bark beetles that infest above ground parts of trees, wherever pines are native (Raffa & Smalley, 1988; Parmeter & al., 1989). The fungus thus appears to have a very wide distribution. Other than in South Africa, it has also been introduced with bark beetles into Australia (Stone & Simpson, 1989) and Chile (Wingfield, personal communication), where pines are exotic. This fungus is also common in New Zealand (Hutchison & Reid, 1988), although no stem-infesting insects have been reported there. It is not known how this fungus was introduced into New Zealand.

Leptographium lundbergii is one of many *Leptographium* spp. that can cause sapstain (Jacobs & Wingfield, 2001), while *L. serpens* has been associated with a root disease of pines in Italy and South Africa (Lorenzini & Gambogi, 1976; Wingfield & Knox-Davies, 1980). Both these species are associated with insects and distributed throughout the world (Harrington, 1988; Jacobs & Wingfield, 2001).

Leptographium procerum, which is commonly associated with root and root collar insects, is also implicated in white pine root decline in the eastern United States, Europe and New Zealand (Kendrick, 1962; Shaw & Dick, 1980; Jacobs & Wingfield, 2001). However, the pathogenicity of the fungus, and particularly its role in root disease, has been extensively debated. Some authors suggest that it is a pathogen causing severe disease symptoms (Halambek, 1981; Lackner & Alexander, 1982), while others regard it as weakly pathogenic and relatively unimportant (Towers, 1977; Wingfield, 1986). The pathogenicity of this fungus and its role in root disease deserves to be tested in South Africa.

Of the several unidentified *Pesotum* spp. collected in this study, none resembled *Graphium pseudormiticum* Mouton & Wingfield. This fungus was isolated once from *O. erosus* in the Western Cape province, South Africa (Mouton & al., 1994), and should, therefore, be considered an occasional or infrequent associate. Further studies, which will include DNA sequencing, will be conducted to fully identify the *Pesotum* spp., as well as the unidentified *Sporothrix* and *Hyalorhinocladiella* spp. obtained.

There are only a few reports of ophiostomatoid fungi in other Southern Hemisphere countries (Butin & Aquilar, 1984; Hutchison & Reid, 1988; Stone & Simpson, 1989; Kile & al., 1996; Jacobs & al., 1998). Some of them are known to be associated with bark beetles. *Ophiostoma ips* and *Cop. minuta* have been isolated from *Ips grandicollis* (Eichhoff) in Australia (Stone & Simpson, 1989), and *O. huntii* (Rob.-Jeffr.) de Hoog & R. J. Scheff. from *H. ater* (Payk.) in both Australia and New Zealand (Jacobs & al., 1998). It is interesting to note that *O. galeiformis*

occurs both in New Zealand (Harrington, personal communication) and South Africa. The fungus was probably introduced with *H. ligniperda*, since it occurs in both countries and *H. ater* has not been reported in South Africa. These examples indicate that species from this group of fungi have already been introduced into new environments. Studies on bark beetles and their associated fungi are, therefore, essential for quarantine purposes.

The association of *Lasiodiplodia theobromae* and *S. sapinea* with beetles in this study could be considered incidental, since the biology and ecology of these fungi are somewhat different from the ophiostomatoid fungi. Both species are disseminated primarily by wind and rain (Swart & Wingfield, 1991; Cilliers, Swart & Wingfield, 1995). *L. theobromae* was, however previously identified as the main cause of sapstain on pine logs exported from South Africa (De Beer, Zhou & Wingfield, 2000). Apart from these two species, species like *L. lundbergii*, *O. ips*, *O. pluriannulatum*, *O. piceae* and *Cop. minuta*, could also be considered as potentially serious sapstain agents. These species should, therefore, together with the associated bark beetles, be taken into consideration when control measures for sapstain are developed for the South African forestry industry.

REFERENCES

Butin, H. & Aquilar, A. M. (1984) Blue-stain fungi on *Nothofagus* from Chile - including two new species of *Ceratocystis* Ellis & Halst. *Phytopathologische Zeitschrift* **109**: 80-89.

Cilliers, A. J., Swart, W. J. & Wingfield, M. J. (1995) The occurrence of *Lasiodiplodia theobromae* on *Pinus elliottii* seeds in South Africa. *Seed Science and Technology* **23**: 851-860

Coetzee, C., Wingfield, M. J., Crous, P. W. & Wingfield, B. D. (2000) *Xenochalara*, a new genus of dematiaceous hyphomycetes for *Chalara*-like fungi with apical wall building conidial development. *South African Journal of Botany* **66**: 99-103.

De Beer, Z. W., Zhou, X. D., Wingfield, M. J. & Wingfield B.D. (2001) Assesment of sapstain on pine logs in South Africa. Proceedings of BIOY2K Combined Millennium Meeting of Southern Africa, Grahamstown, South Africa. *South African Journal of Science* **97**: xi.

Erasmus, M. J. & Chown, S. L. (1994) Host location and aggregation behaviour in *Hylastes angustatus* (Herbst) (Coleoptera: Scolytidae). *African Entomology* **2**: 7-11.

Halambek, M. (1981) *Verticicladiella procera* Kendrick causal organism of eastern white pine wilting in conifers cultures . *Plant Protection* **32**: 313-323. [English summary]

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

Harrington, T. C. (1988) *Leptographium* species, their distributions, hosts and insect vectors. In *Leptographium root diseases on conifers* (T. C. Harrington & Jr. F. W. Cobbs., eds): 1-39. American Phytopathological Society Press, St. Paul, Minnesota.

Hutchison, L. J. & Reid, J. (1988) Taxonomy of some potential wood-staining fungi from New Zealand: 1. Ophiostomataceae. *New Zealand Journal of Botany* **26**: 63-81.

Jacobs, K., Wingfield, M. J, Wingfield, B. D. & Yamaoka, Y. (1998) Comparison of *Ophiostoma huntii* and *O. europhioides* and description of *Ophiostoma aenigmaticum* sp. nov. *Mycological Research* **102**: 289-294.

Jacobs, K. & Wingfield, M. J. (2001) *Leptographium* species - tree pathogens, insect associates and agents of blue-stain. American Phytopathological Society Press, St. Paul, Minnesota.

Kendrick, W. B. (1962) The *Leptographum* complex. *Verticicladiella* S. Hughes. *Canadian Journal of Botany* **40**: 771-797.

Kfir, R. (1986) Releases of natural enemies against the pine bark beetle *Orthotomicus erosus* (Wollaston) in South Africa. *Journal of the Entomological Society of Southern Africa* **2**: 391-392.

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

Lackner, A. L. & Alexander, S. A. (1982) Occurrence and pathogenicity of *Verticicladiella procera* in Christmas tree plantations in Virginia. *Plant Disease* **66**: 211-212.

Lorenzini, G. & Gambogi, P. (1976) Decline of *Pinus pinea* associated with the presence of *Verticicladiella* sp. (preliminary note) . *Informatore Fitopatologico* **5**: 5-8. [In Italian].

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

Mouton, M., Wingfield, M. J., Van Wyk, P. S. & Van Wyk, P. W. (1994) *Graphium pseudormiticum* sp. nov.: a new hyphomycete with unusual conidiogenesis. *Mycological Research* **98**: 1272-1276.

Okada, G., Seifeit, K. A., Takematsu, A., Yamaoka, Y., Miyazaki, S. & Tubaki, K. (1998) A molecular phylogenetic reappraisal of the *Graphium* complex based on 18S rDNA sequences. *Canadian Journal of Botany* **76**: 1495-1506.

Paine, T. D., Raffa, K. F. & Harrington, T. C. (1997) Interactions among Scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* **42**: 179-206.

Parmeter, J. R., Slaughter, G. W., Chen, M-M., Wood, D. L. & Stubbs, H. A. (1989) Single and mixed inoculations of Ponderosa pine with fungal associates of *Dendroctonus* spp. *Phytopathology* **79**: 768-772.

Paulin, A. E. & Harrington, T. C. (2000) Phylogenetic placement of anamorphic species of *Chalara* among *Ceratocystis* species and other ascomycetes. *Studies in Mycology* **45**: 209-222.

Raffa, K. F. & Smalley, E. B. (1988) Response of red and jack pines to inoculation with microbial associates of the pine engraver *Ips pini* (Coleoptera: Scolytidae). *Canadian Journal of Forestry Research* **18**: 581-586.

Seifert, K. A. (1993) Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 141-151. American Phytopathological Society Press, St. Paul, Minnesota.

Shaw, C. G. & Dick, M. (1980) *Verticicladiella* root disease of *Pinus strobus* in New Zealand. *Plant Disease* **64**: 96-98.

Six, D. L. & Paine, T. D. (1999) Allozyme diversity and gene flow in *Ophiostoma clavigerum* (Ophiostomatales: Ophiostomataceae), the mycangial fungus of the Jeffrey pine beetle, *Dendroctonus jeffreyi* (Coleoptera: Scolytidae). *Canadian Journal of Forest Research* **29**: 324-331.

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

Stone, C. and Simpson, J. A. (1989) Species associations in *Ips grandicollis* galleries in *Pinus taeda*. *New Zealand Journal of Forestry Science* **20**: 75-96.

Swart, W. J. & Wingfield, M. J. (1991) Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. *Plant Disease* **75**: 761-766.

Towers, B. (1977) The occurrence of *Verticicladiella procera* in Pennsylvania: 1976. *Plant Disease Reporter* **61**: 477.

Tribe, G. D. (1992) Colonization sites on *Pinus radiata* logs of the bark beetles, *Orthotomicus erosus*, *Hylastes angustatus* and *Hylurgus ligniperda* (Coleoptera: Scolytidae). *Journal of the Entomological Society of Southern Africa* **1**: 77-84.

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

Viljoen, C. D., Wingfield, B. D. & Wingfield, M. J. (1999) Relatedness of *Custingophora olivaceae* to *Gondwanamyces* spp. from *Protea* spp. *Mycological Research* **103**: 497-500.

Viljoen, C. D., Wingfield, M. J., Jacobs, K. & Wingfield. B. D. (2000) *Cornuvesica*, a new genus to accommodate *Ceratocystiopsis falcata*. *Mycological Research* **104**: 365-367.

Whitney, H. S. (1982) Relationships between bark beetles and symbiotic organisms. In *Bark beetles in North American conifers* (J. B. Mitton & K. B. Sturgeon, eds): 183-211. University of Texas, Austin.

Wingfield, M. J. (1986) Pathogenicity of *Leptographium procerum* and *L. terebrantis* on *Pinus strobus* seedlings and established trees. *European Journal of Forest Pathology* **16**: 299-308.

Wingfield, M. J., Harrington, T. C. & Solheim, H. (1995). Do conifer bark beetles require fungi to kill trees? In *Proceedings from a symposium held at the Norwegian Forest Research Institute (NISK) in Ås, Norway, 31 July – 2 August, 1995* (E. Christiansen, ed). *Aktuelt fra Skogforsk* 6/95: 6.

Wingfield, M. J. & Knox-Davies, P. S. (1980) Root-disease, associated with *Verticicladiella alacris*, of pines in South Africa. *Plant Disease* **64**: 569-571.

Wingfield, M. J. & Marasas, W. F. O. (1980) *Ceratocystis ips* associated with *Orthotomicus erosus* (Coleoptera: Scolytidae) on *Pinus* spp. in the Cape Province of South Africa. *Phytophylatica* **12**: 65-69.

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

Wingfield, M. J. & Swart, W. J. (1989) Relative pathogenicity of fungi associated with pine root-infesting insects in South Africa. In *Proceedings of the Seventh International Conference Root and Butt Rots* (D. J. Morrison, ed): 381-391. British Columbia, Canada.

Wingfield, M. J., Van Wyk, P. S. & Marasas, W. F. O. (1988) *Ceratocystiopsis proteae* sp. nov., with a new anamorph genus. *Mycologia* **80**: 23-30.

Wood, S. L., & Bright, D. E. (1992) A catalog of Scolytidae and Platypodidae (Coleoptera), part 2: Tamxonomic index. *Great Basin Naturalist Memoirs* **A** (13): 1-6.

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

Table 1. Fungal species isolated from three species of exotic bark beetles occurring in South Africa.

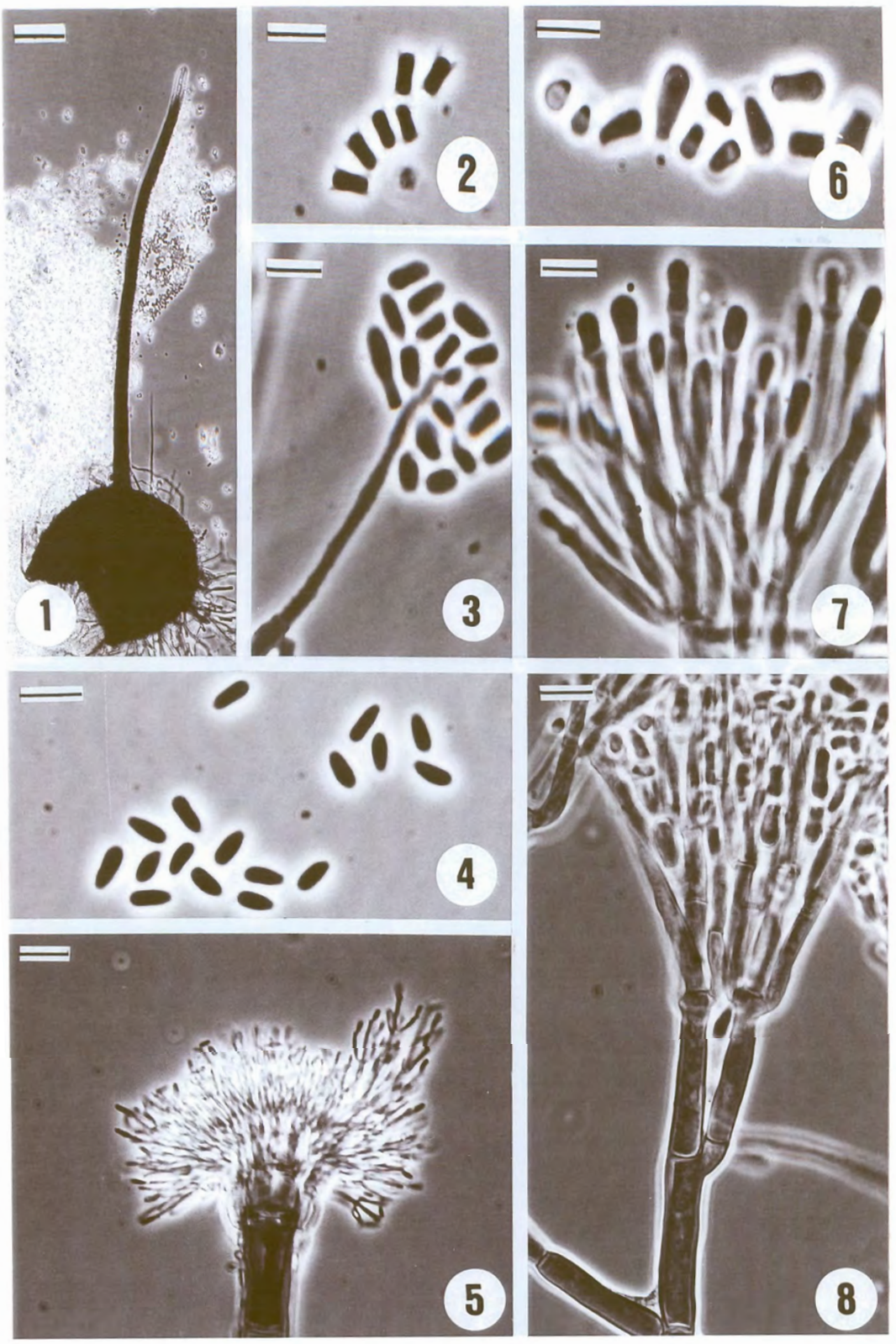
	Orthotomicus erosus	*Hylastes angustatus*	*Hylurgus ligniperda*
Ophiostoma ips	399 (60.0 %)	12 (1.7 %)	25 (12.6 %)
Leptographium lundbergii	4 (0.6 %)	311 (44.8 %)	43 (21.6 %)
L. serpens	3 (0.5 %)	312 (45.0 %)	42 (21.1 %)
O. galeiformis	-	-	5 (2.5 %)
O. pluriannulatum	2 (0.3 %)	12 (1.7 %)	2 (1.0 %)
O. stenoceras	-	5 (0.7 %)	1 (0.5 %)
O. piceae	-	-	3 (1.5 %)
Ceratocystiopsis minuta	-	6 (0.9 %)	2 (1.0 %)
Pesotum spp.	11 (1.7 %)	27 (3.9 %)	12 (6.0 %)
Sporothrix sp.	2 (0.3 %)	6 (0.9 %)	3 (1.5 %)
Hyalorhinocladiella sp.	1 (0.2 %)	2 (0.3 %)	1 (0.5 %)
Total no. of samples[1]	665	694	199
Total no. of isolates[2]	422	693	139

Notes:
[1] All beetles from a single gallery, together with the gallery, were treated as a single sample.
[2] From each sample, only one isolate per fungal species was included in the calculation.
- Not present.

Figs. 1-3. *Ophiostoma ips*. - 1. Ascocarp (Bar = 105 µm). - 2. Ascospores (Bar = 10 µm). - 3. *Hyalorhinocladiella* anamorph (Bar = 10 µm).

Figs. 4-5. *Leptographium serpens*. - 4. Conidia (Bar = 10 µm). - 5. Conidiogenous apparatus (Bar = 15 µm).

Figs. 6-8. *Leptographium lundbergii*. - 6. Conidia (Bar = 10 µm). - 7. Conidiogenous apparatus (Bar = 10 µm). - 8. Conidiophore (Bar = 10 µm).



Chapter 3

Ophiostoma spp. associated with two pine-infesting bark beetles from Chile

Bark beetles (Coleoptera: Scolytidae) are common vectors of *Ophiostoma* spp., which include primary tree pathogens as well as important sapstain agents. In Chile, *Hylurgus ligniperda* and *Hylastes ater*, which are native to Europe, commonly occur on the exotic *Pinus radiata*. Little research has been done on *Ophiostoma* spp. associated with bark beetles in Chile and especially those carried by introduced pine-infesting insects. We recently obtained specimens of these bark beetles and their galleries, and the aim of this study was to isolate and identify *Ophiostoma* spp. associated with the two beetle species. Identification was achieved using morphological characteristics and where appropriate, DNA sequencing. A total of five ophiostomatoid species, *Ceratocystiopsis minuta*, *O. galeiformis*, *O. huntii*, *O. ips*, and *O. quercus*, were found associated with the bark beetles, all of which are recorded from Chile for the first time.

Keywords: *Leptographium*, Ascomycetes, Scolytidae, *Hylurgus*, *Hylastes*.

INTRODUCTION

Pinus spp. are native to the Northern Hemisphere, where species diversity is most pronounced in Central America and Southeast Asia (Richardson, 1998). Many *Pinus* spp., however, have been introduced into Southern Hemisphere countries such as New Zealand, Australia, Chile and South Africa. In these countries, some pine species are grown in very large commercial plantations (Le Maitre, 1998; Richardson, 1998).

Many bark beetle species (Coleoptera: Scolytidae) infest *Pinus* spp. (Wood & Bright, 1992). Most of these bark beetles are not considered as pests in their native environment, but when introduced into new areas and particularly where uniform stands of *Pinus* spp. are planted, they can become problematic (Wingfield & Swart, 1994; Wingfield *et al.*, 2001). In Chile, *Hylurgus ligniperda* (Fabricius) and *Hylastes ater* (Paykull) are exotic pests of European origin which infest exotic *P. radiata* (Wood & Bright, 1992; Billings, 1993). Both of these insects can infest fresh stumps and slash shortly after trees are felled (Ciesla, 1988).

Many bark beetles are also vectors of ophiostomatoid fungi, which include a number of primary pathogens and sapstain agents (Whitney, 1982; Harrington, 1988; Seifert, 1993; Brasier & Mehrotra, 1995; Paine, Raffa & Harrington, 1997). In Chile, at least eight ophiostomatoid species have been reported from different hosts (Table 1). Little research has, however, been conducted on the fungal associates of pine-infesting bark beetles in this country.

In Chile, as is the case in South Africa, exotic pine plantations constitute a significant section of the forestry industry. Considerable research has been conducted on pine bark beetle-associated fungi in exotic pine plantations of South Africa in recent years (Wingfield & Swart, 1989; Zhou *et al.*, 2001, 2002). A comparison of the fungi associated with introduced bark beetles in Chile, with fungi from the same niche in South Africa, could provide insight into the spread of the bark beetles and their fungi south of the equator.

Recently, we have had the opportunity to examine bark beetles and their galleries from Chile, and to isolate *Ophiostoma* spp. occurring on the beetles and in their galleries. The aim of this study was to identify these fungi based on morphology and comparisons of ITS rDNA sequences.

MATERIALS AND METHODS

Isolation of fungi

In the Valdivia area of Chile, 34 specimens of *H. ater* were collected from roots of dying *P. radiata* trees, and 80 specimens of *H. ligniperda* were collected from felled trees of the same species in log stacks. Four fungal isolates were collected directly from the galleries of *H. ater*. Isolation of fungi from bark beetles and their galleries was conducted in a similar way to that described by Zhou *et al.* (2001). All cultures used in this study are maintained in the culture collection (CMW) of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Morphological studies

Both teleomorph and anamorph fruiting structures, when present, were mounted in lactophenol cotton blue on glass slides, examined microscopically, and characteristic structures measured. To induce the production of perithecia, isolates presenting only anamorphs were grown on 2 % WA (20 g Biolab agar and 1000 ml distilled water), to which sterilised pine twigs had been added.

DNA sequencing and phylogenetic analysis

Isolates used. Some isolates produced only a *Pesotum*-like anamorph in culture, resembling that of *O. piceae* (Münch) H. & P. Sydow, *O. quercus* (Georgévitch) Nannfeldt, and *O. floccosum*

Mathiesen. These isolates were difficult to identify based on morphology, and for two of them (CMW9480 and CMW9481), single hyphal tip cultures, were prepared for sequencing (Table 2).

DNA extraction. Each culture was grown in 50 ml of malt extract broth (20 g Biolab malt extract, and 1000 ml distilled water) at 25 °C in the dark for 10 days. Mycelium was then harvested by filtration (Whatman no. 1 filter paper) and freeze-dried.

DNA was extracted using a modified version of the extraction method developed by Raeder and Broda (1985). Freeze-dried mycelium was grounded to fine powder in liquid nitrogen. Approximately 0.5 ml of mycelial powder was suspended in 800 µl of extraction buffer (200 mM Tris-HCl pH 8.0, 150 mM NaCl, 25 mM EDTA pH 8.0, 0.5 % SDS). Phenol (500 µl) and 300 µl of chloroform were added to the suspension, and the mixture was votexed, then centrifuged in a Beckman JA 25.50 rotor (12,000 rpm, 60 min, 4 °C). The upper aqueous layer was transferred to sterilized Eppendorf tubes. 200 µl of phenol and an equal volume of chloroform were added, vortexed, and then centrifuged for 5 minutes. The aqueous phase was transferred again, and the chloroform extraction (400 µl) was repeated once or twice until the interface was clear. Nucleic acid was then precipitated with 0.1 vol. of 3 M NaAc (pH 5.4) and 1 vol. of isopropanol. The nucleic acid was pelleted using centrifugation (12,000 rpm, 30 min, 4 °C), and the salt removed by washing with 70 % ethanol. The vacuum-dried pellet was resuspended in 50 µl of sterile water and 2 µl of RNAase (10 mg / ml, Roche Molecular Biochemicals) was added to digest any RNA. The reaction was incubated in a water bath overnight at 37 °C. Agarose (Promega, Madison, CT, USA) gel electrophoresis (1%) was used to determine the presence of the DNA. The DNA was visualized using Ethium bromide and UV light. The concentration of the DNA was determined using UV spectroscopy (Beckman Du Series 7500 Spectrophotometer).

PCR amplification. The ITS1 and ITS2 (internal transcribed spacer) regions, including the 5.8S gene of the ribosomal RNA operon, were amplified, using primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes & Bruns, 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). The template DNA was amplified in a 50 µl PCR reaction volume, consisting of 0.5 µl of DNA solution (100-200 ng μl^{-1}), 0.5 µl of Expand High Fidelity PCR System enzyme mix (1.7 U) (Roche Molecular Biochemicals, Alameda, CA), 5 µl of Expand HF buffer (10x) without $MgCl_2$, 3 µl of $MgCl_2$ (25 mM), and 1.5 µl of each primer (10 mM). PCR reactions were performed on an Eppendorf Mastercycler® Personal (PerkinElmer, Germany). The PCR conditions were as follows: 95 °C for 2 min, followed by 40 cycles, where each cycle included 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C. A final elongation step was conducted for 8 min at 72 °C. A negative control, using water without DNA, was included with each PCR. PCR products were visualized on a 1 % agarose gel stained with ethidium bromide (10 mg ml^{-1}) under UV illumination. Amplification products were purified using the High Pure PCR Product Purification Kit (Boehringer, Mannheim, Germany).

DNA Sequencing. Sequencing reactions were carried out with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PerkinElmer Applied BioSystems) following the manufacturer's instructions. Sequencing was performed on an ABI PRISM 377 Autosequencer (PerkinElmer Applied BioSystems, Foster City, CA, USA). PCR products were sequenced with the same primers used for PCR, as well as two additional internal primers, CS2 (5'-CAATGTGCGTTCAAAGATTCG-3') (Wingfield *et al.*, 1996), and ITS3 (5'-GCATAGATGAAGAAGCAGC-3') (White *et al*, 1990).

Phylogenetic analysis. The obtained sequences were aligned using Sequence Navigator version 1.01 (ABI PRISM, PerkinElmer). This alignment was checked manually, and compared with data

of related isolates from other studies, obtained from GenBank (Table 2). Aligned data were analysed using PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1998). Uninformative characters were excluded from the analyses. The most parsimonious trees were produced using a heuristic search with TBR (Tree Bisection and Reconstruction) branch swapping. Bootstrap analysis (1000 replicates) was run to determine confidence intervals of the branching points.

RESULTS

Isolation of fungi

In total, 45 fungal isolates were obtained from the specimens collected. Of these, 30 were isolated from *H. ligniperda*, and 15 from *H. ater*. Eleven isolates, representing all the morphological groups present, were selected for further investigation (Table 2).

Morphological studies

Morphological study showed that two ophiostomatoid fungi, *Ceratocystiopsis minuta* (Siem.) Upadhyay & Kendrick and *Ophiostoma ips* (Rumbold) Nannfeldt, were commonly associated with *H. ligniperda*. Three ophiostomatoid fungi, *O. galeiformis* (Bakshi) Mathiesen-Käärik, *O. huntii* (Rob.-Jeffr.) de Hoog & R. J. Scheff., and a *Pesotum* sp. resembling the anamorphs of *O. piceae*, *O. quercus*, and *O. floccosum*, were found associated with *H. ater*.

Sequence analysis

DNA fragments (574 bp) were amplified for the isolates with *Pesotum* anamorphs. Manual alignment of these sequences resulted in a total of 579 characters. Of these, 45 were parsimony informative, 6 parsimony uninformative, and 528 were constant. Heuristic searches without using the outgroup taxon, resulted in one most parsimonious tree (CI = 1.000, RI = 1.000, HI = 0.000),

in which three main clades were well resolved (Fig. 1). Two isolates obtained in this study (CMW 9480 and CMW 9481) resided in the first clade representing *O. quercus* group. The second and third clade represented *O. piceae* and *O. floccosum* groups, respectively, with bootstrap supports of 90 % and 100%.

DISCUSSION

In this study, three ophiostomatoid species were found associated with each of the two bark beetle species from Chile. From *Hylurgus ligniperda*, *Ceratocystiopsis minuta*, *Ophiostoma galeiformis* and *O. ips* were isolated, while *O. galeiformis, O. huntii*, and *O. quercus* were found with *Hylastes ater*. *Ophiostoma galeiformis* was the only fungal species present on both bark beetle species. This study represents the first report of these five fungal species from Chile, and this list considerably increases the number of ophiostomatoid fungi known from the country.

Ceratocystiopsis minuta was first described by Siemaszko (1939) from *Picea abies* infested by *Ips typographus* L. in Poland. The fungus is associated with different conifer-infesting bark beetles from many parts of the world (Davidson, 1942; Mathiesen-Käärik, 1953; Upadhyay, 1981; Solheim, 1986; Stone & Simpson, 1990; Yamaoka *et al.*, 1998). In South Africa, *Cop. minuta* has been found on the exotic *Hylastes angustatus* (Herbst) and *Hylurgus ligniperda* (Zhou *et al.*, 2001). The presence of *Cop. minuta* on *H. ligniperda* infesting *P. radiata* in Chile is not surprising, given its association with the European bark beetles in South Africa.

Ophiostoma galeiformis is associated with many different bark beetle species. The fungus was first described by Bakshi (1951) in Scotland, where it was isolated from *Larix kaempferi* infested by *Hylurgops palliatus* (Gyll.), *Dryocoetes autographus* (Ratzeburg), and *Trypodendron lineatum* (Olivier) (Bakshi, 1951). In Sweden, *O. galeiformis* has been isolated from *Picea*

infested with *Hylastes cunicularius* (Errichson) (Mathiesen-Käärik, 1953), as well as from pine-infesting bark beetles (Hunt, 1956). The discovery of the fungus from Chile in the present study is not unusual as is has also been found associated with the exotic pine-infesting *H. ligniperda* in South Africa (Zhou *et al.*, 2001). The *Hylastes* vectors of these fungi, however, differ in the two areas, although both originate in Europe.

Ophiostoma huntii has been associated with several different bark beetle species on *Pinus* and *Picea* spp. (Jacobs & Wingfield, 2001). This species was originally isolated from pine infested with a *Dendroctonus* sp. in Canada (Robinson-Jeffrey & Grinchenko, 1964), and has been reported to be associated with *D. ponderosae* (Hopk.), *Ips pini* (Say), *Hylastes macer* (LeConte), and *Tomicus piniperda* (Linnaeus) in Europe (Jacobs *et al.*, 1998) and the USA (Davidson & Robinson-Jeffrey, 1965; Harrington, 1988; Gibbs & Inman, 1991; Wingfield & Gibbs, 1991). *Ophiostoma huntii* occurs also in Australia and New Zealand (Jacobs *et al.*, 1998), where it is associated with the European root-infesting bark beetle, *H. ater*. Its presence on *H. ater* in Chile suggests that this insect was introduced into Chile from New Zealand, Australia, or at least from a common source. Studies on populations of fungi such as *O. huntii* in Chile, Australia and New Zealand, might provide useful information on how the bark beetles and fungi have been distributed throughout the Southern Hemisphere.

Ophiostoma ips was first described from *Ips calligraphus* on *P. echinata*, *P. sylvestris*, and *P. rigida* in the USA (Rumbold, 1931), and has since been widely reported to be the associate of many conifer-infesting bark beetles in the Northern Hemisphere (Rumbold, 1931; Nisikado & Yamauti, 1933; Mathiesen-Käärik, 1953; Hunt, 1956; Mathre, 1964; Upadhyay, 1981; Rane & Tattar, 1987; Lieutier *et al.*, 1991; Perry, 1991; Masuya *et al.*, 1999). In the Southern Hemisphere, it has been reported in Australia from galleries of *Ips grandicollis* (Eichhoff) on *P. taeda* (Vaartaja, 1966; Stone & Simpson, 1990), and from New Zealand on *P. elliottii* and *P. radiata* (Hutchison & Reid, 1988; Farrell *et al.*, 1997). The fungus has also been reported from

South Africa associated with *O. erosus*, *H. angustatus* and *H. ligniperda* occurring on *P. radiata*, *P. patula* and *P. elliottii* (Wingfield & Marasas, 1980; Zhou *et al.*, 2001). In this study, *O. ips* was isolated from *H. ligniperda* on *P. radiata* in Chile, which is similar to the situation in South Africa.

Ophiostoma quercus (De Beer *et al.*, 2003) occurs primarily on hardwoods, but occasionally also on conifers, while *O. piceae* occurs almost exclusively on conifers (Harrington *et al.*, 2001; De Beer, Wingfield & Wingfield, 2003). These two species are morphologically almost indistinguishable, but can be separated based on ITS rDNA sequence data (Harrington *et al.*, 2001; De Beer, Wingfield & Wingfield, 2003). DNA sequence comparisons in the present study have confirmed the presence of *O. quercus* in association with *H. ater* on *P. radiata* in Chile. In South Africa, *O. quercus*, but not *O. piceae*, has, however, been reported from various hardwoods, and those identifications were confirmed with ITS rDNA sequencing (De Beer, Wingfield & Wingfield, 2003). The results of the present study thus suggest that previous reports of *O. piceae* from *Nothofagus*, *Laurelia*, and *Pinus* spp. in Chile (Butin & Aquilar, 1984; Butin & Peredo, 1986; Billings, 1993), might have represented *O. quercus*, and not *O. piceae*.

As in Chile, *H. ligniperda* and *H. ater* occur in Australia and New Zealand (Swan, 1942; Anonymous, 1974), whereas only *H. ligniperda* has been reported from South Africa (Tribe, 1991). While both these bark beetle species were introduced from Europe to these Southern Hemisphere countries, the pine species planted there originate from North America. Unfortunately very little is known regarding the fungal associates of these bark beetle species in their native environments in the Northern Hemisphere. Only from Sweden five ophiostomatoid species have been recorded from *H. ater*: *O. ips*, *O. penicillatum* Grossman, *O. piceae* , *L. lundbergii* Lagerb. & Melin, *O. piliferum* [= *O. coeruleum*], and *Graphium areum* Hedgc. (Mathiesen, 1950; Mathiesen-Käärik, 1953). None of these species have, however, been found in association with *H. ater* in the Southern Hemisphere to date. It appears that *H. ater* forms associations with different fungal

species in different environments. This might indicate that the relationship between bark beetle vector and fungus in this case is coincidental, rather than specific.

REFERENCES

Anonymous. (1974) New bark beetle *Hylurgus ligniperda* (Scolytidae) discovered in New Zealand. *New Zealand Forestry Service*. 4 pp.

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

Billings, R. F. (1993) Pest risk assessment of the importation of *Pinus radiata*, *Nothogagus domebeyi*, and *Laurelia philippina* logs from Chile. USDA, Forest Service, Miscellaneous Publication No. 1517.

Brasier, C. M. & Mehrotra, M. D. (1995) *Ophiostoma himal-ulmi* sp. nov., a new species of Dutch elm disease fungus endemic to the Himalayas. *Mycological Research* **99**: 205-215.

Butin, H. & Aquilar, A. M. (1984) Blue-stain fungi on *Nothofagus* from Chile - including two new species of *Ceratocystis* Ellis & Halst. *Phytopathologische Zeitschrift* **109**: 80-89.

Butin, von H. & Peredo, H. L. (1986) Hongos parasitos en coniferas de America del Sur. *Bibliotheca Mycol.* **101**: 1-100. (Not seen)

Ciesla, W. M. (1988) Pine bark beetles: a new pest management challenge for Chilean foresters. *Journal of Forestry* **86:** 27-31.

Davidson, R. W. (1942) Some additional species of *Ceratostomella* in the United States. *Mycologia* **34**: 650-662.

Davidson, R. W. & Robinson-Jeffrey, A. H. H. (1965) New records of *Ceratocystis europhioides* and *C. huntii* with *Verticicladiella* imperfect stages from conifers. *Mycologia* **57**: 488-490.

De Beer, Z. W., Glen, H. F., Wingfield, B. D. & Wingfield, M. J. (2003) *Ophiostoma quercus* or *Ophiostoma querci? Mycotaxon* (In press).

De Beer, Z. W., Wingfield, B. D. & Wingfield, M. J. (2003) The *Ophiostoma piceae* complex in the Southern Hemisphere: a phylogenetic study. *Mycological Research* (In press).

Farrell, R. L., Hadar, E., Kay, S. J., Blanchette, R. A. & Harrington, T. C. (1997) Survey of sapstain organisms in New Zealand and albino anti-sapstain fungi. Biology and prevention of

sapstain. A conference sponsored by the Department of Forest Products, Oregon State University, 25 May, 1997, Delta Whistler Resort, Whistler, British Columbia, Canada: 57-62.

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

Gibbs, J. N. & Inman, A. (1991) The pine shoot beetle *Tomicus piniperda* as a vector of blue-stain fungi to windblown pine. *Forestry* **64**: 239-249.

Harrington, T. C. (1988) *Leptographium* species, their distributions, hosts and insect vectors. In *Leptographium root diseases on conifers* (T. C. Harrington & Jr. F. W. Cobbs., eds): 1-39. American Phytopathological Society Press, St. Paul, Minnesota.

Harrington, T. C., McNew, D., Steimel, J., Hofstra, D. & Farrell, R. (2001) Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia* **93**: 111-136.

Hunt, J. (1956) Taxonomy of the genus *Ceratocystis*. *Lloydia* **19**: 1-59.

Hutchison, L. J. & Reid, J. (1988) Taxonomy of some potential wood-staining fungi from New Zealand: 1. Ophiostomataceae. *New Zealand Journal of Botany* **26**: 63-81.

Jacobs, K. & Wingfield, M. J. (2001) *Leptographium* species - tree pathogens, insect associates and agents of blue-stain. American Phytopathological Society Press, St. Paul, Minnesota.

Jacobs, K., Wingfield, M. J., Wingfield, B. D. & Yamaoka, Y. (1998) Comparison of *Ophiostoma huntii* and *O. europhioides* and description of *O. aenigmaticum* sp. nov. *Mycological Research* **102**: 289-294.

Le Maitre, D. C. (1998) Pines in cultivation: a global view. In *Ecology and biogeography of Pinus* (D. M. Richardson, ed): 407-431. Cambridge University Press.

Lieutier, F., Yart, A., Garcia, J., Ham, M. C., Morelet, M. & Levieux, J. (1989) Champignons phytopathogènes associés à deux coléoptères Scolytidae du Pin sylvestre (*Pinus sylvestris* L.) et étude préliminaire de leur agressivité envers l'hôte. *Annales des Sciences Forestieres* **46**: 201-216.

Masuya, H., Kaneko, S., Yamaoka, Y. & Ohsawa, M. (1999) Comparisons of ophiostomatoid fungi associated with *Tomicus piniperda* and *T. minor* in Japan red pine. *Journal of Forestry Research* **4**: 131-135.

Mathiesen-Käärik, A. (1953) Eine Übersicht über die gewöhnlichsten mit Borkenkäfern assoziierten Bläuepilze in Schweden und einige für Schweden neue Bläuepilze. *Meddelanden från Statens Skogsforskningsinstitut* **43:** 1-74.

Mathre, D. E. (1964) Survey of *Ceratocystis* spp. associated with bark beetles in California. *Contributions of the Boyce Thompson Institute* **22**: 353-362.

Nisikado, Y. & Yamauti, K. (1933). Contributions to the knowledge of the sap stains of wood in Japan. I. Studies on *Ceratostomella ips* Rumbold, the cause of blue stain of pine trees in western Japan. *Berichte des Ohara Instituts für Landwirtschaftliche Forschungen* **5**: 501-538.

Paine, T. D., Raffa, K. F. & Harrington, T. C. (1997) Interactions among Scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* **42**: 179-206.

Peredo, H. L. & Alonso, O. (1988) Mycoflora of *Pinus radiata* pulpwood and outdoor storing in central Chile. *Material und Organismen* **23**: 11-19.

Perry, T. J. (1991) A synopsis of the taxonomic revisions in the genus *Ceratocystis* including a review of blue-staining species associated with *Dendroctonus* bark beetles. *General Technical Report*, SO-86. New Orleans, LA: U. S. Department of Agriculture, Forest Service, Southern Forest Experiment Station, pp. 1-16.

Raeder, U. & Broda, P. (1985) Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**: 17-20.

Rane, K. K. & Tattar, T. A. (1987) Pathogenicity of blue stain fungi associated with *Dendroctonus terebrans*. *Plant Disease* **71**: 879-883.

Richardson, D. M. (1998) Ecology and biogeography of *Pinus*. Cambridge University Press.

Robinson-Jeffrey,. R. C. & Grinchenko, A. H. H. (1964) A new fungus in the genus *Ceratocystis* occurring on blue-stained lodgepole pine attacked by bark beetles. *Canadian Journal of Botany* **46**: 1523-1527.

Rumbold, C. T. (1931) Two blue-staining fungi associated with bark-beetle infestation of pines. *Journal of Agricultural Research* **43**: 847-873.

Seifert, K. A. (1993) Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In *Ceratocystis* and *Ophiostoma* Taxonomy, Ecology and Pathogenicity (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 141-151. American Phytopathological Press, St. Paul, Minnesota.

Siemaszko, W. (1939) Fungi associated with bark beetles in Poland. *Planta Polonica* **7**:1-52.

Solheim, H. (1986) Species of Ophiostomataceae isolated from *Picea abies* infested by the bark beetle *Ips typographus* . *Nordic Journal of Botany* **6**: 199-207.

Stone, C. & Simpson, J. A. (1990) Species associations in *Ips grandicollis* galleries in *Pinus taeda*. *New Zealand Journal of Forestry Science* **20**: 75-96.

Swan, D. C. (1942) The bark beetle - *Hylastes ater* Payk. (Coleoptera: Scolytidae) attacking pines in South Australia. *Journal of Agriculture* **12**: 86-90.

Swofford, D. L. (1998) PAUP*: Phylogenetic Analysis Using Parsimony (* and other methods). Version 4.0. Sinaur Associates: Sunderland, Massachusetts.

Travassos, L.R. & Lloyd, K.O. (1980) *Sporothrix schenckii* and related species of *Ceratocystis*. *Microbiological Reviews* **44**: 683-721.

Tribe, G. D. (1991) Phenology of *Pinus radiata* log colonization by the red-haired pine bark beetle *Hylurgus ligniperda* (Fabricius) (Coleoptera: Scolytidae) in the south-western Cape Province. *Journal of the Entomological Society of Southern Africa* **1**: 1-7.

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

Vaartaja, O. (1966) The common fungal associates of the bark beetle, *Ips grandicollis* in *Pinus radiata* in South Australia. *Australian Forestry Research* **2**: 40-43.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols: genes for phylogenetics* (M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 315-322. Academic Press, San Diego, California.

Whitney, H. S. (1982) Relationships between bark beetles and symbiotic organisms. In *Bark beetles in North American conifers* (J. B., Mitton & K. B., Sturgeon, eds): 183-211. Austin: University of Texas.

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

Wingfield, M. J. & Gibbs, J. N. (1991) *Leptographium* and *Graphium* species associated with pine-infesting bark beetles in England. *Mycological Research* **95**: 1257-1260.

Wingfield, M. J. &. Marasas, W. F. O. (1980) *Ceratocystis ips* associated with *Orthotomicus erosus* (Coleoptera: Scolytidae) on *Pinus* spp. in the Cape Province of South Africa. *Phytophylactica* **12**: 65-69.

Wingfield, M. J., Slippers, B., Zhou, X. D., De Beer, W., Govender, P. & Wingfield, B. D. (2001) Global spread of insect-associated fungi on exotic plantation pines. In *Protection on world forests from insect pests: advances in research* (R. Alfaro, K. Day, S. Salom, K. S. S. Nair, H. Evans, A. Liebhold, F. Lieutier, M. Wagner, K. Futai & K. Suzuki, eds): 107-114 (IUFRO World Series Vol. 11). Papers presented at the XXI IUFRO World Congress 7-12 August 2000, Malaysia.

Wingfield, M. J. & Swart, W. J. (1989) Relative pathogenicity of fungi associated with pine root-infesting insects in South Africa. In *Proceedings of the Seventh International Conference on Root and Butt Rots* (D. J. Morrison, ed): 381-391. British Columbia, Canada.

Wingfield, M. J. & Swart, W. J. (1994) Integrated management of forest tree diseases in South Africa. *Forest Ecology and Management* **65**: 11-16.

Wood, S. L., & Bright, D. E. (1992) A catalog of Scolytidae and Platypodidae (Coleoptera), Part 2: Taxonomic Index. *Great Basin Naturalist Memoirs* **A(13)**: 1-6.

Yamaoka, Y., Wingfield, M. J., Ohsawa, M. & Kuroda, Y. (1998) Ophiostomatoid fungi associated with *Ips cembrae* in Japan and pathogenicity to Japanese larch. *Mycoscience* **39**: 367-378.

Zhou X. D., De Beer Z. W., Wingfield B. D. & Wingfield M. J. (2001) Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. *Sydowia* **53**: 290-300.

Zhou, X. D., De Beer, Z. W., Wingfield, B. D & Wingfield, M. J. (2002) Infection sequence and pathogenicitiy of *Ophiostoma ips*, *Leptographium serpens* and *L. lundbergii* to pines in South Africa. In *Fungal Succession* (K. D. Hyde & E. B. G. Jones, eds). *Fungal Diversity* **10**: 229-240.

Table 1. Ophiostomatoid fungi previously reported from Chile.

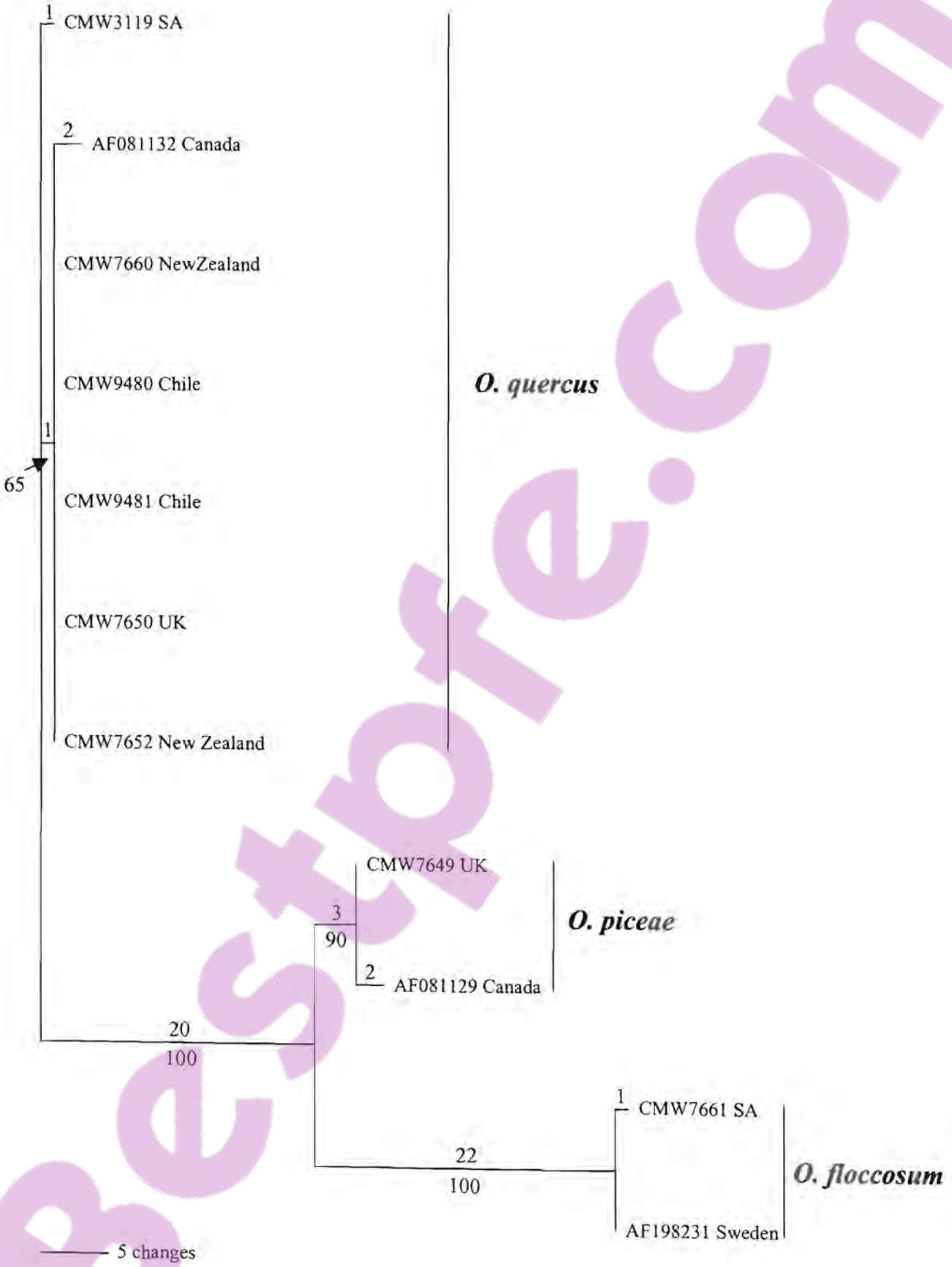
Fungal species	Host	Reference(s)
Ophiostoma nothofagi (Butin) Rulamort	*Nothofagus dombeyi*	Butin & Aguilar, 1984.
O. piceae (Münch) H. & P. Sydow	*Nothofagus* spp.; *Pinus* spp.; *Laurelia* spp.	Butin & Aguilar, 1984; Butin & Peredo, 1986; Billings, 1993; Harrington *et al.*, 2001.
O. piliferum (Fries) H. & P. Sydow	*N. pumilio*	Butin & Aguilar, 1984.
O. valdivianum (Butin) Rulamort	*N. alpina*; *N. dombeyi*	Butin & Aguilar, 1984.
Pesotum sp.	*P. radiata*	Peredo & Alonso, 1988.
Sporothrix curviconia de Hoog	*P. radiata*	Peredo & Alonso, 1988.
Sporothrix schenckii Hekt. & Perkins	Human	Travassos & Lloyd, 1980.
Sporothrix sp.	*P. radiata*	Peredo & Alonso, 1988.

Table 2. Fungi isolated from bark beetles and their galleries in Chile and isolates of selected species used as reference material in this study.

Species	Isolate No.	GenBank No.	Collector/supplier	Origin	Host	Insect
Ceratocystiopsis minuta	[a]CMW10770			Chile	*Pinus radiata*	*Hylurgus ligniperda*
Ophiostoma galeiformis	CMW9478, CMW9479			Chile	*P. radiata*	*Hylastes ater*
	CMW9482, CMW9483			Chile	*P. radiata*	*H. ligniperda*
O. huntii	CMW10768 CMW10769			Chile	*P. radiata*	*H. ater*
O. ips	CMW5089 CMW6402			Chile	*P. radiata*	*H. ligniperda*
O. quercus	[b]CMW9480 [b]CMW9481			Chile	*P. radiata*	*H. ater*
O. floccosum	CMW7661	AF493253	ZW de Beer	South Africa	*P. elliottii*	
		AF198231	A Kåårik	Sweden	*Picea* or *Pinus*	
O. piceae	CMW7649	AF081130	JN Gibbs	UK	*P. sylvestris*	
		AF081129	SH Kim *et al.*	Canada	*Picea mariana*	
O. quercus	CMW7650	AF198238	PT Scard, JF Webber	UK	*Quercus* sp.	
		AF081132	SH Kim *et al.*	Canada	*Tsuga*	
	CMW3119	AF493244	ZW de Beer	South Africa	*Pinus* chips	
	CMW7660	AF493252	ZW de Beer	New Zealand	*Pinus* chips	
	CMW7652		RA Blanchette	New Zealand	*P. radiata*	

[a] CMW = Culture Collection Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
[b] Isolates used in rDNA sequence analyses.

Fig. 1. Phylogram of *Pesotum* group based on analyses of ITS sequences (ITS1 and ITS2 regions, as well as 5.8S rRNA gene). *Ophiostoma ips* was used as an outgroup. Base substitution numbers are indicated above the branches and the bootstrap values (1000 bootstrap repeats) below the branches.



Chapter 4

Characterisation of *Ophiostoma* spp. associated with pine bark beetles from Mexico, including *O. pulvinisporum* sp. nov.

Bark beetles (Coleoptera: Scolytidae) are common vectors of *Ophiostoma* spp. These fungi include primary tree pathogens and important sapstain agents. In Mexico, *Ips calligraphus* and *Dendroctonus mexicanus* are found on many species of pine. *Pinus maximinoi* and *P. pseudostrobus* are the hosts of both species of insects. Little research has, however, been done on ophiostomatoid fungi associated with pine bark beetles in Mexico. We recently obtained specimens of these bark beetles and their galleries from Mexico. The aim of the study was to isolate and identify *Ophiostoma* spp. associated with the two beetle species. In total, six *Ophiostoma* spp. were found to be associated with them and of which *O. nigrocarpum* is recorded from Mexico for the first time. The collection also included a previously undescribed species isolated from both beetles, and for which we provide the name *O. pulvinisporum*.

Keywords: *Ips*, *Dendroctonus*, rRNA.

INTRODUCTION

Pinus spp. are native to the Northern Hemisphere, and the genus is one of the largest groups of conifers (Richardson, 1998). Pine trees usually comprise a significant component of the ecosystems where they grow. The greatest number of *Pinus* spp. occurs in Central and North American countries such as Mexico (Price, Liston & Struss, 1998). Many bark beetle species (Coleoptera: Scolytidae) infest pines. In Mexico, two of the bark beetle species, *Ips calligraphus* (Germar) and *Dendroctonus mexicanus* (Hopkins), occur on indigenous *Pinus* spp. (Wood & Bright, 1992). *Dendroctonus mexicanus* is known to infest and kill 21 species of pines, of which *Pinus pseudostrobus* is one of the most important (Marmolejo-Moncivais, 1989; Marmolejo & García-Ocañas, 1993), while *I. calligraphus* is known to infest six species of pine, in some cases as a primary insect but in others as secondary agent, with *P. maximinoi* as one of its most common hosts in tropical environments.

A large number of bark beetle species are considered as pests, also because they act as vectors of fungi, particularly ophiostomatoid fungi (Münch, 1907; Whitney, 1982; Beaver, 1989; Paine, Raffa & Harrington, 1997). Some ophiostomatoid species are important plant pathogens (Harrington, 1988; Brasier & Mehrotra, 1995), and many are the causal agents of sapstain (Lagerberg, Lundberg, & Melin, 1927; Seifert, 1993). To the best of our knowledge, at least 14 ophiostomatoid species have been reported from Mexico in five research papers (Table 1). Recently, we had the opportunity to examine bark beetles and their galleries from Mexico, and to isolate ophiostomatoid fungi occurring on the beetles and in the galleries. The aim of this study was to identify the *Ophiostoma* spp. associated with two beetle species. Light microscopy and sequence of the ITS region of the rRNA operon were employed to identify the isolates.

MATERIALS AND METHODS

Isolation of fungi

Fungi were isolated from bark beetles as well as from their galleries. In Chiapas, Mexico, 35 galleries of *D. mexicanus* infesting dying *P. pseudostrobus*, and 20 of *I. calligraphus* infesting dying *P. maximinoi*, were collected.

Galleries were carefully examined using a dissection microscope. Spore masses accumulating at the tips of perithecia or conidiophores were carefully lifted using a fine sterile needle and transferred to a medium selective for *Ophiostoma* spp. (20 g Biolab malt extract, 20 g Biolab agar and 1000 ml distilled water, amended with 0.05 % cycloheximide and 0.04 % streptomycin).

Beetles from the same gallery were squashed directly onto the selective medium in Petri dishes. Cultures were incubated at 25 °C in the dark, and purified by transferring mycelium from the edges of single colonies to fresh 2 % MEA (20 g Biolab malt extract, 20 g Biolab agar and 1000 ml distilled water). All cultures used in this study are maintained in the Culture Collection (CMW) of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Morphological studies

Both teleomorph and anamorph fruiting structures, when present, were mounted in lactophenol cotton blue on glass slides, examined microscopically, and characteristic structures were measured. Isolates with *Hyalorhinocladiella* anamorphs resembling the anamorph of *O. ips* (Rumbold) Nannfeldt, were also grown on 2 % WA (20 g Biolab agar and 1000 ml distilled water), with sterilised pine twigs, to induce production of perithecia. For *O. ips*–like cultures, 50 measurements were made for each structure, and the ranges and averages were computed.

Growth studies

The optimal growth temperature for selected isolates resembling the anamorph of *O. ips* (CMW 9023, CMW 9024, and CMW 9028) was determined by growing the isolates at temperatures ranging from 5 to 35 °C, at 5 °C intervals. Each isolate was inoculated onto the agar surface in six 2 % MEA plates for each temperature, with a 6.0 mm diameter agar disk taken from the actively growing margin of a fresh isolate. Colony diameters were measured after four and eight days, and an average was calculated from six random measurements. Growth rates of three authenticated *O. ips* isolates (CMW 6445, CMW 9013, and CMW 9319) were determined in a similar way.

Mating experiments

Mating experiments were conducted on isolates from Mexico resembling *O. ips* (CMW 9024 and CMW 9028) to determine thallism and to obtain perithecia. Ten single ascospore cultures were prepared from perithecia obtained in a cross between the two isolates. The single ascospore cultures were crossed in every possible combination. To induce production of perithecia, these cultures were incubated at 25 °C in the dark on 2 % WA with sterilised pine twigs for three weeks. Some crosses gave rise to sexual structures and it was thus possible to select tester strains of opposite mating type. Ten single conidium cultures were also prepared from each of the two isolates, and the tester strains were then crossed with single conidium cultures to confirm the authenticity of the tester strains. The thallism of authentic *O. ips* isolates (CMW 6418 and CMW 6463) were determined in a similar way, except that 30 single ascospore and 30 single conidium cultures were made of each isolate.

DNA sequencing and phylogenetic analysis

Some isolates only reproduced asexually in culture and were difficult to identify based on morphology. Of these, 12 single hyphal tip isolates resembling *Sporothrix* or *Hyalorhinocladiella*, were selected for sequencing (Table 2). Since some isolates resembled the anamorph of *O. ips*, and only one reference sequence for this species was available from GenBank, ten authentic isolates of *O. ips* originating from other parts of the world were also included in the study (Table 3). DNA was extracted using a modified version of the extraction method developed by Raeder and Broda (1985). PCR amplification, DNA Sequencing, and phylogenetic analysis were conducted in a similar way as described in Chapter 3. The trees were rooted using the GenBank sequence of *O. ulmi* (Buisman) Nannf. (AF198232) for the *Hyalorhinocladiella* group, and sequence of *O. stenoceras* (Robak) Nannf. (AF484462) for the *Sporothrix* group. Bootstrap analysis (1000 replicates) was run to determine confidence intervals of the branching points.

RESULTS

Isolation of fungi

In total, 25 fungal isolates were obtained from the specimens collected. Of these, 16 isolates were from *D. mexicanus*, and nine from *I. calligraphus*. Eighteen isolates, representing all the morphological groups present, were selected for further investigation (Table 2).

Morphological studies

Morphological study of isolates showed that five ophiostomatoid species, *Ceratocystiopsis minuta* Upadhyay & Kendrick, an *Ophiostoma galeiformis*-like sp., *O. pluriannulatum* (Hedgc.) H. & P. Sydow, a *Hyalorhinocladiella* sp., and *Sporothrix* spp., were associated with *D. mexicanus*. Two

species, *O. pluriannulatum* and a *Hyalorhinocladiella* sp., were collected from the galleries of *I. calligraphus*.

Growth studies

The *O. ips*–like isolates from Mexico grew optimally at 30 °C, while authentic strains of *O. ips* grew best at 25 °C, reaching 58 mm and 50 mm in diameter in four days, respectively. No growth was obtained for any of the isolates at 5 °C, and minimal growth occurred at 10 and 35 °C.

Mating experiments

None of the 10 single ascospore or 20 single conidium cultures of the *O. ips*-like isolates produced perithecia. However, when crossed with each other, 30 of the 45 crosses produced perithecia. Two most vigorously growing strains were selected for mating between isolates. Twenty of 30 single ascospore cultures (67 %), and 24 of 30 single conidium cultures (80 %) of *O. ips* produced perithecia.

Sequence analysis

The *Sporothrix* group. DNA fragments approximately 540 bp in size were amplified for the isolates with *Sporothrix* anamorphs (Table 2). Manual alignment of these sequences resulted in a total of 545 characters. Of these, 8 were parsimony-informative, 25 parsimony-uninformative, and 512 constant. Heuristic searches using *O. stenoceras* as the outgroup taxon, resulted in one most parsimonious tree (Fig. 1) with a length of 37 (CI = 0.946, RI = 0.895, HI = 0.054), in which two main clades were evident. The first clade, which had a bootstrap support of 93 %, included sequences of two isolates (AF484452 and AF484474) of authenticated *O. nigrocarpum* (Davidson) de Hoog, and four Mexican isolates (CMW9485, CMW9486, CMW9488, and CMW9491). The second clade, which had the bootstrap support of 83 %, included two Mexican isolates

(CMW9487 and CMW9489), which are distinct from *O. abietinum* Marmolejo & Butin, *O. stenoceras*, and the other Mexican isolate (CMW9492).

The *Hyalorhinocladiella* group. DNA fragments of approximately 590 bp in size were amplified for the isolates with *Hyalorhinocladiella*-like anamorphs. Manual alignment of these sequences resulted in a total of 624 characters. Of these, 24 were parsimony informative, 115 parsimony uninformative, and 485 constant. Heuristic searches using *O. ulmi* as the outgroup taxon resulted in four most parsimonious trees (CI = 0.968, RI = 0.936, HI = 0.032). Two main clades (Fig. 2) were evident in all phylogenetic trees. The first clade represented the *O. ips* group, with a bootstrap support of 99 %. The second clade, with a bootstrap support of 100 %, represented an undescribed taxon.

TAXONOMY

Based on ITS sequence comparisons, mating reactions, growth studies, and morphology, we conclude that the fungus that superficially resembled *O. ips* represents a distinct taxon. This is described as follows:

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.

Ophiostoma pulvinisporum X. D. Zhou & M. J. Wingfield sp. nov. Figs. 3A - 3G.

Etym.: derived from the Latin *pulvinus* (cushion) and *sporus* (spore); the epithet refers to the pulvinate ascospores of this species.

Perithecia in su perficie 2 % WA efficientur ubi proles aliae aluntur. Bases peritheciorum globosae, atratae, (150-) 276 (-400) µm diametro, hyphis aseptatis laete griseis ornatae; colla

atrobrunnea vel nigra, laevia, (400-) 1520 (-3520) µm longa, basin versus (28-) 50 (-80) µm, apicem versus (10-) 17 (-48) µm lata. Hyphae ostiolares desunt; asci non visi. Ascosporae hyalinae, aseptatae, vaginatae, a latere fronteque visae pulviniformes, (3-) 4 (-6) x (1-) 2 (-3) µm, ab extremo visae quadrangulares.

Anamorpha *Hyalorhinocladiella* dominans, conidiophoris 70 – 160 (- 230) µm longis, conidiis hyalinis, ellipsoideis vel ovoideis, (3-) 6 (-21) x (1-) 2 (-4) µm. Anamorpha *Leptographium* conidiophoris 60 – 120 (- 170) µm longis, conidiis hyalinis, oblongis vel ellipsoideis, basibus truncatis, (2-) 4 (-6) x (1-) 1.5 (-2.5) µm. Anamorpha *Pesotum* conidiophoris 240 – 260 (- 280) µm longis, conidiis hyalinis, bacillaribus, (3-) 4 (-7) x (1-) 1.3 (-2.5) µm.

Coloniae crescunt optime ad 30 °C in 2 % MEA, 58 mm diametro quattuor diebus attingentes; laete griseae vel aetate atrobrunneae. Ad 5 °C non crescunt, et ad 10 et 35 °C minime crescunt.

Anamorphs: *Pesotum* (Fig. 3D), *Leptographium* (Fig. 3E), and *Hyalorhinocladiella* (Fig. 3F, 3G). Perithecia produced superficially on 2 % WA when strains of opposite mating type are crossed. Perithecial bases globose, dark, (150-) 276 (-400) µm in diameter (Fig. 3A), ornamented with aseptate light grey hyphae. Perithecial necks dark brown to black, smooth, (400-) 1520 (-3520) µm long, (28-) 50 (-80) µm wide at base, (10-) 17 (-48) µm wide at the apex (Figs.3A, 3B). Ostiolar hyphae absent. Asci not observed. Ascospores hyaline, aseptate, with sheaths, pillow shaped in side and face view, (3-) 4 (-6) x (1-) 2 (-3) µm (Fig. 3C), quadrangular in end view.

Hyalorhinocladiella anamorph predominant, conidiophores: 70 – 160 (- 230) µm long; conidia hyaline, ellipsoid to ovoid, (3-) 6 (-21) x (1-) 2 (-4) µm. *Leptographium* anamorph: conidiophores 60 – 120 (- 170) µm long, conidia hyaline, oblong to ellipsoid with truncate bases,

(2-) 4 (-6) x (1-) 1.5 (-2.5) µm. *Pesotum* anamorph: conidiophores 240 – 260 (- 280) µm long, conidia hyaline, rod-shaped, (3-) 4 (-7) x (1-) 1.3 (-2.5) µm.

Colonies with optimal growth at 30 °C on 2 % MEA, reaching 58 mm in diameter in four days. Colonies light grey (19''d) to dark mouse grey (13'''''k) with age (Rayner, 1970). No growth at 5 °C, and minimal growth at 10 and 35 °C.

Specimens examined: Cultures on 2 % MEA, isolated from *D. mexicanus* infesting *P. pseudostrobus*, Chiapas, Mexico, February, 2001, collected by M. J. Wingfield, CMW 9022 (Holotype: PREM***, dried culture). Paratypes: isolated from *D. mexicanus* infesting *P. pseudostrobus*, Chiapas, Mexico, February, 2001, collected by M. J. Wingfield, CMW 9020 (Paratype: PREM***, dried culture) ; isolated from *I. calligraphus* infesting *P. maximinoi*, Chiapas, Mexico, February, 2001, collected by M. J. Wingfield, CMW 9026 (Paratype: PREM***, dried culture) and CMW 9493 (Paratype: PREM***, dried culture).

DISCUSSION

In this study, six ophiostomatoid species were found associated with *D. mexicanus* and *I. calligraphus* from Mexico. These included *Cop. minuta*, *O. pluriannulatum*, an *O. galeiformis*-like species, *O. nigrocarpum* and two species closely related to, but distinct from *O. nigrocarpum*, as well as the new species similar to *O. ips* that we have named *O. pulvinisporum*. Apart from *O. pulvinisporum*, *O. nigrocarpum* is also recorded for the first time from Mexico.

Ceratocystiopsis minuta was first described by Siemaszko from *Picea abies* infested by *Ips typographus* (Linnaeus) in Poland (Siemaszko, 1939). The fungus is commonly associated with *I. typographus* infesting Norway spruce, *Tomicus* spp. infesting *Pinus* spp. in Europe (Mathiesen-

Käärik, 1953; Solheim, 1986), as well as with various conifer-infesting *Dendroctonus* and *Ips* spp. in North America, Australia, and Japan (Davidson, 1942; Upadhyay, 1981; Stone & Simpson, 1990; Yamaoka *et al.*, 1998). In South Africa, the fungus has been found on the exotic *Hylastes angustatus* (Herbst) and *Hylurgus ligniperda*, and it was evidently introduced into the country from Europe (Zhou *et al.*, 2001). The presence of the fungus in Mexico is not surprising given its wide distribution in the Northern Hemisphere.

Ophiostoma pluriannulatum was first described by Hedgcock (1906) from *Quercus borealis* in the USA. The fungus is known as a sapstain agent, especially of hardwoods, and as a fungal associate of many insects in the Northern Hemisphere (Lagerberg, Lundberg & Melin, 1927; Hedgcock, 1933; Hunt, 1956), including Mexico, where it has been found on both *Quercus* and *P. pseudostrobus* (Marmolejo & García-Ocañas, 1993). In the Southern Hemisphere, the fungus occurs in New Zealand on *P. radiata* (Farrell *et al.*, 1997), and in South Africa it is associated with three pine-infesting bark beetle species (Zhou *et al.*, 2001). This study represents the first report of the fungus from *P. maximinoi*. It is also the first time that *O. pluriannulatum* has been associated with a *Dendroctonus* species, and although it has been found on *Ips typographus* in Sweden (Mathiesen-Käärik, 1953), it has not previously been reported from *Ips calligraphus*.

Ophiostoma galeiformis (Bakshi) Mathiesen-Käärik was first described by Bakshi (1951) in Scotland, and is associated with a wide variety of bark beetle species (Bakshi, 1951; Mathiesen-Käärik, 1953; Hunt, 1956; Zhou *et al.*, 2001). The isolate from Mexico (CMW9490) that we have tentatively assigned to this species, closely resembles *O. galeiformis*, but the culture differs slightly from published descriptions. This fungus has not been reported from North America before and it is possible that the isolate represents a distinct taxon. The taxonomy of this species is confused since the type material has apparently been lost. The identity of the isolate from Mexico will thus be considered in a future study focussing on isolates of resembling this species from different parts of the world.

Ophiostoma nigrocarpum was first described by Davidson (1966) from *P. ponderosa* infested with *D. brevicomis* (LeConte) in the USA. The fungus has been reported on both conifers and hardwoods and has been collected in North America, New Zealand and Australia (Schirp *et al.*, 1999). Ribosomal DNA sequence data have, however, shown that what is presently regarded as the *O. nigrocarpum*-complex consists of a number of distinct taxa (De Beer *et al.*, 2003). A fungus that has been referred to as *O. abietinum* Marmolejo & Butin, originally described from *Abies vejari* in Mexico (Marmolejo & Butin, 1990), is also included in this complex (De Beer *et al.*, 2003). Comparison of DNA sequence data in the present study showed that four Mexican isolates (CMW9491, CMW9486, CMW9488, CMW9485) from *D. mexicanus* infesting *P. pseudostrobus*, grouped together with two *O. nigrocarpum* isolates (CBS408.77 and ATCC22391), isolated from *P. ponderosa* and a *Dendroctonus* sp. respectively in the USA. This group had strong bootstrap support of 93 % and we believe it represents *O. nigrocarpum*. Two other Mexican isolates from *P. pseudostrobus* (CMW9489 and CMW9787) formed a clade distinct from the remaining isolates, with bootstrap support of 83 %. The single isolate (CMW9492) from *P. maximinoi*, also grouped separately from *O. nigrocarpum*, as did *O. abietinum*. Our results, therefore, suggest that *O. abietinum*, the isolate from *P. maximinoi*, and the two *P. pseudostrobus* isolates grouping separately, represent taxa distinct from *O. nigrocarpum*. Further studies, including additional isolates, will be needed to clarify the identity of these fungi.

Ophiostoma pulvinisporum and *O. ips* are morphologically similar. Both *O. pulvinisporum* and *O. ips* are characterised by pillow-shaped ascospores with distinct sheaths. The anamorphs of both species form a continuum of conidiophore structures varying from single mononematous structures terminating in penicillately branched apices similar to *Leptographium*, to synnematous structures reminiscent of *Pesotum*. Anamorphs of *O. ips* have in the past been referred to the genera *Hyalorhinocladiella*, *Graphium* (now *Pesotum*), and *Leptographium*, since no single genus can accommodate the variety of structures produced by this species (Rumbold, 1931, 1941; Hunt,

1956; Davidson, 1978; Upadyhay, 1981; Wingfield, Seifert & Webber, 1993). In our opinion, the *Hyalorhinocladiella* form of *O. pulvinisporum* is predominant and if an anamorph genus is required, we would preferentially refer to it as *Hyalorhinocladiella*.

Ophiostoma pulvinisporum and *O. ips* can be distinguished from each other based on their different growth rates, mating systems, and ITS rDNA sequences. *Ophiostoma pulvinisporum* grows optimally at 30 °C and *O. ips* at 25 °C. Our results also showed that *O. pulvinisporum* is clearly heterothallic. In contrast, *O. ips* is considered homothallic. ITS rDNA sequence data comparisons in this study, furthermore, supported the separation of *O. pulvinisporum* and *O. ips*.

Ophiostoma pulvinisporum was isolated from *D. mexicanus* and *I. calligraphus,* occurring on *P. pseudostrobus* and *P. maximinoi* respectively in Mexico. Previously, *O. ips* has been reported from *D. mexicanus* and an *Ips* sp. in Mexico, infesting *P. pseudostrobus* and *P. teocote* (Marmolejo-Moncivais, 1989; Marmolejo & García-Ocañas, 1993). These reports of *O. ips* from Mexico might, have represented *O. pulvinisporum*, but this can only be confirmed if cultures from the original studies are obtained.

The presence of a large number of species of fungi on the limited collection of material obtained for this study, indicates that a large diversity of ophiostomatoid species are probably associated with pine bark beetles in Mexico. This is also to be expected since Mexico is rich in native pine species (Richardson, 1998). Further studies with additional bark beetles and pine species from this country will most likely reveal many more undescribed ophiostomatoid species.

REFERENCES

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

Beaver, R. A. (1989) Insect-fungus relationships in the bark and ambrosia beetles. In *Insect-Fungus Interactions* (N. Wilding, N. M. Collins, P. M. Hammond & J. F. Webber, eds): 121-143. Academic Press, London.

Brasier, C. M. & Mehrotra, M. D. (1995) *Ophiostoma himal-ulmi* sp. nov., a new species of Dutch elm disease fungus endemic to the Himalayas. *Mycological Research* **99**: 205-215.

Davidson, R. W. (1942) Some additional species of *Ceratostomella* in the United States. *Mycologia* **34**: 650-662.

Davidson, R. W. (1966) New species of *Ceratocystis* from conifers. *Mycopathologia et Mycologia applicata* **28**: *273-286.*

Davidson, R. W. (1978) Staining fungi associated with *Dendroctonus adjunctus* in pines. *Mycologia* **70**:35-40.

De Beer, Z. W., Harrington, T. C., Vismer, H. F., Wingfield, B. D. & Wingfield, M. J. (2003) Phylogeny of the *Ophiostoma stenoceras – Sporothrix schenckii* complex. *Mycologia* (In press).

Farrell, R. L., Hadar, E., Kay, S. J., Blanchette, R. A. & Harrington, T. C. (1997) Survey of sapstain organisms in New Zealand and albino anti-sapstain fungi. Biology and prevention of sapstain. A conference sponsored by the Department of Forest Products, Oregon State University, 25 May, 1997, Delta Whistler Resort, Whistler, British Columbia, Canada: 57-62.

Harrington, T. C. (1988) *Leptographium* species, their distributions, hosts and insect vectors. In *Leptographium root diseases on conifers* (T. C. Harrington & Jr. F. W. Cobbs., eds): 1-39. American Phytopathological Society Press, St. Paul, Minnesota.

Hedgcock, G. G. (1906) Studies upon some chromogenic fungi which discolor wood. *Missouri Botanical Gardens Annual Report* **17**: 59-114.

Hedgcock, G. G. (1933) The prevention of wood-staining in basket veneers. *Journal of Forestry* **31**:416-420.

Hunt, J. (1956) Taxonomy of the genus *Ceratocystis*. *Lloydia* **19**: 1-59.

Lagerberg, T., Lundberg, G. & Melin, E. (1927) Biological and practical researches into blueing in pine and spruce. *Svenska Skogsvardsföreningens Tidskrift* **25**: 145-272.

Marmolejo-Moncivais, J. G. (1989) Forstpathologische untersuchungen zur pilzflora Nordost-Mexikos. Ph. D. Dissertation, Universität Göttingen. pp. 37-55

Marmolejo, J. G. & Butin, H. (1990) New conifer-inhabiting species of *Ophiostoma* and *Ceratocystiopsis* (Ascomycetes, Microascales) from Mexico. *Sydowia* **42**: 193-199.

Marmolejo, J. G. & García-Ocañas, F. (1993) Contribuciones micologicas en homenaje al biologo Jose Castillo Tovar por su labor en pro de la micologia mexicana. *Reporte Cientifico, No. Especial* **13**: 155-170.

Mathiesen-Käärik, A. (1953) Eine Übersicht über die gewöhnlichsten mit Borkenkäfern assoziierten Bläuepilze in Schweden und einige für Schweden neue Bläuepilze. *Meddelanden från Statens Skogsforskningsinstitut* **43:** 1-74.

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

Paine, T. D., Raffa, K. F. & Harrington, T. C. (1997) Interactions among Scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* **42**: 179-206.

Price, R. A., Liston, A. & Struss, S. H. (1998) Phylogeny and systematics of *Pinus*. In *Ecology and biogeography of Pinus* (D. M. Richardson, ed): 49-65. Cambridge University Press.

Raeder, U. & Broda, P. (1985) Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**: 17-20.

Rayner, R. W. (1970) A mycological colour chart. Commonwealth Mycological Institute and British Mycological Society, Kew.

Reyes, J. & Castillo, J. (1981) Micromicetos de la rizosfera del sorgo. *Bol. Soc. Mex. Mic.* **15**: 5-8.

Richardson, D. M. (1998) Ecology and biogeography of *Pinus*. Cambridge University Press.

Rumbold, C. T. (1931) Two blue-staining fungi associated with bark-beetle infestation of pines. *Journal of Agricultural Research* **43**: 847-873.

Rumbold, C. T. (1941) A blue stain fungus, *Ceratostomella montium* n. sp. And some yeasts associated with two species of *Dendroctonus*. *Journal of Agricultural Research* **62**: 589-601.

Schirp, A., Farrell, R. L. & Kreber, B. (1999) Effect of New Zealand staining fungi on structural wood integrity of radiata pine. In *The 2nd New Zealand Sapstain Symposium, Proceedings of Symposium, Rotorua, New Zealand, 18-19 November,* (B. Kreber, ed): 99-104. Forest Research Bulletin No. 215.

Seifert, K. A. (1993) Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In *Ceratocystis* and *Ophiostoma* Taxonomy, Ecology and Pathogenicity (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 141-151. American Phytopathological Press, St. Paul, Minnesota.

Siemaszko, W. (1939) Fungi associated with bark beetles in Poland. *Planta polonica* **7**:1-52.

Solheim, H. (1986) Species of Ophiostomataceae isolated from *Picea abies* infested by the bark beetle *Ips typographus*. *Nordic Journal of Botany* **6**: 199-207.

Stone, C. & Simpson, J. A. (1990) Species associations in *Ips grandicollis* galleries in *Pinus taeda*. *New Zealand Journal of Forestry Science* **20**: 75-96.

Travassos, L.R. & Lloyd, K.O. (1980) *Sporothrix schenckii* and related species of *Ceratocystis*. *Microbiological Reviews* **44**: 683-721.

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

Whitney, H. S. (1982) Relationships between bark beetles and symbiotic organisms. In *Bark beetles in North American conifers* (J. B., Mitton & K. B., Sturgeon, eds): 183-211. Austin: University of Texas.

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

Wood, S. L., & Bright, D. E. (1992) A catalog of Scolytidae and Platypodidae (Coleoptera), part 2: Taxonomic index. *Great Basin Naturalist Memoirs* **A (13)**: 1-6.

Yamaoka, Y., Wingfield, M. J., Ohsawa, M. & Kuroda, Y. (1998) Ophiostomatoid fungi associated with *Ips cembrae* in Japan and pathogenicity to Japanese larch. *Mycoscience* **39**: 367-378.

Zhou X. D., De Beer Z. W., Wingfield B. D. & Wingfield M. J. (2001) Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. *Sydowia*. **53**: 290-300.

Table 1. Ophiostomatoid fungi reported from Mexico.

Fungal species	Host	Insect	[a]Ref.
Ophiostoma abietinum Marmolejo & Butin	*Abies vejari*	*Pseudohylesinus* sp.	**1, 2, 3**
O. conicolum Marmolejo & Butin	*Pinus cembroides*	*Conophthorus cembroides*	**1, 2, 3**
O. hyalothecium Davidson	*P. pseudostrobus*		**1**
O. ips (Rumbold) Nannfeldt	*P. teocote*;	*Dendroctonus mexicanus*; *Ips*	**1**
	P. pseudostrobus	sp.	**3**
O. minus (Hedgcock) H. & P. Sydow	*P. arizonica*		**3**
O. piceae (Münch) H. & P. Sydow	*Quercus affinis*		**3**
O. piliferum (Fries) H. & P. Sydow	*P. hartwegii*		**3**
O. pluriannulatum (Hedgcock) H. & P. Sydow	*Q. affinis*; *P. pseudostrobus*		**3**
[b]*Ceratocystis tubicollis* Olchow. & Reid	*P. teocote*	*D. valens*	**1**
C. adiposa (Butler) Moreau	Soil		**4**
Cop. colliferu Marmolejo & Butin	*P. teocote*; *P. hartwegii*	*D. valens*	**1, 2, 3**
Cop. fasciata (Wright & Cain) Upadhyay	*P. pseudostrobus*	*D. mexicanus*	**1, 3**
Cop. minuta (Siem.) Upadhyay & Kendrick	*P. pseudostrobus*	*D. mexicanus*	**1, 3**
Sporothrix schenckii Hekt. & Perkins	Human		**5**

[a] References: **1.** Marmolejo-Moncivais, 1989; **2.** Marmolejo & Butin, 1990; **3.** Marmolejo & García-Ocañas, 1993; **4.** Reyes & Castillo, 1981; **5.** Travassos & Lloydd, 1980.

[b] Should be transferred to *Ophiostoma* according to Wingfield, Seifert & Webber, 1993.

Table 2. Fungi isolated in this study from bark beetles and their galleries in Mexico.

Species	Host	Insect	[a]Isolation Number
Ceratocystiopsis minuta	*Pinus pseudostrobus*	*Dendroctonus mexicanus*	CMW10771
Ophiostoma galeiformis-like	*P. pseudostrobus*	*D. mexicanus*	CMW9490
O. nigrocarpum	*P. pseudostrobus*	*D. mexicanus*	[b]**CMW9485**; **CMW9486**; **CMW9488**; **CMW9491**
O. nigrocarpum-like 1	*P. maximinoi*	*Ips calligraphus*	[b]**CMW9492**
O. nigrocarpum-like 2	*P. pseudostrobus*	*D. mexicanus*	[b]**CMW9487**; **CMW9489**
O. pluriannulatum	*P. pseudostrobus*	*D. mexicanus*	CMW10772
	P. maximinoi	*I. calligraphus*	CMW10773
O. pulvinisporum	*P. pseudostrobus*	*D. mexicanus*	[c]**CMW9020**; **CMW9022**; CMW9023
	P. maximinoi	*I. calligraphus*	CMW9024; **CMW9026**; CMW9028; **CMW9493**

[a] Isolate numbers in bold type refer to isolates used for rDNA sequence analyses.
[b] Isolate with *Sporothrix* anamorphs.
[c] Isolates with *Hyalorhinocladiella* anamorphs.

Table 3. Isolates of selected species used for comparative purpose in this study.

Species	[a]Isolate	Other no.	GenBank	Collector/supplier	Origin	Host/insect
O. abietinum		CBS125.89	AF484453	JG Marmolejo	Mexico	*Abies vejari*
O. ips		C327	AF198244	TC Harrington	USA	-
	CMW312			P Bedker	USA	*Pinus resinosa*
	CMW1173			Mendel	Israel	*Crypturgus mediteranous*
	CMW5089				Chile	*P. radiata, H. ligniperda*
	CMW6402				Chile	*P. radiata, H. ligniperda*
	CMW6418				South Africa	*P. elliottii, O. erosus*
	CMW6463				South Africa	*P. elliottii, H. ligniperda*
	CMW7075	CBS137.36		CT Rumbold	USA	*Ips integer*
	CMW7076	CBS151.54		A Käärik	Sweden	*O. proximus*
	CMW7079	CBS438.94		T Kirisits	Austria	*I. sexdentatus*
	CMW9005				Sweden	*P. sylvestris, I. acuminatus*
O. nigrocarpum		CBS408.77	AF484452	HS Whitney	USA	*P. ponderosa*
		ATCC22391	AF484474	RW Davidson	USA	*Dendroctonus* sp.
O. stenoceras	CMW3202	CBS237.32	AF484462	H Robak	Norway	Pine pulp
O. ulmi		CBS102.63	AF198232	FW Holmes, HM Heybroek	Nethelands	*Ulmus hollandica*

[a] CMW = Culture Collection Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Fig. 1. Phylogram of the *Sporothrix* group based on analyses of ITS sequences (ITS1 and ITS2 regions, as well as 5.8S rRNA gene). *Ophiostoma stenoceras* was used as outgroup. Base substitution numbers are indicated above the branches and the bootstrap values (1000 bootstrap repeats) below the branches.

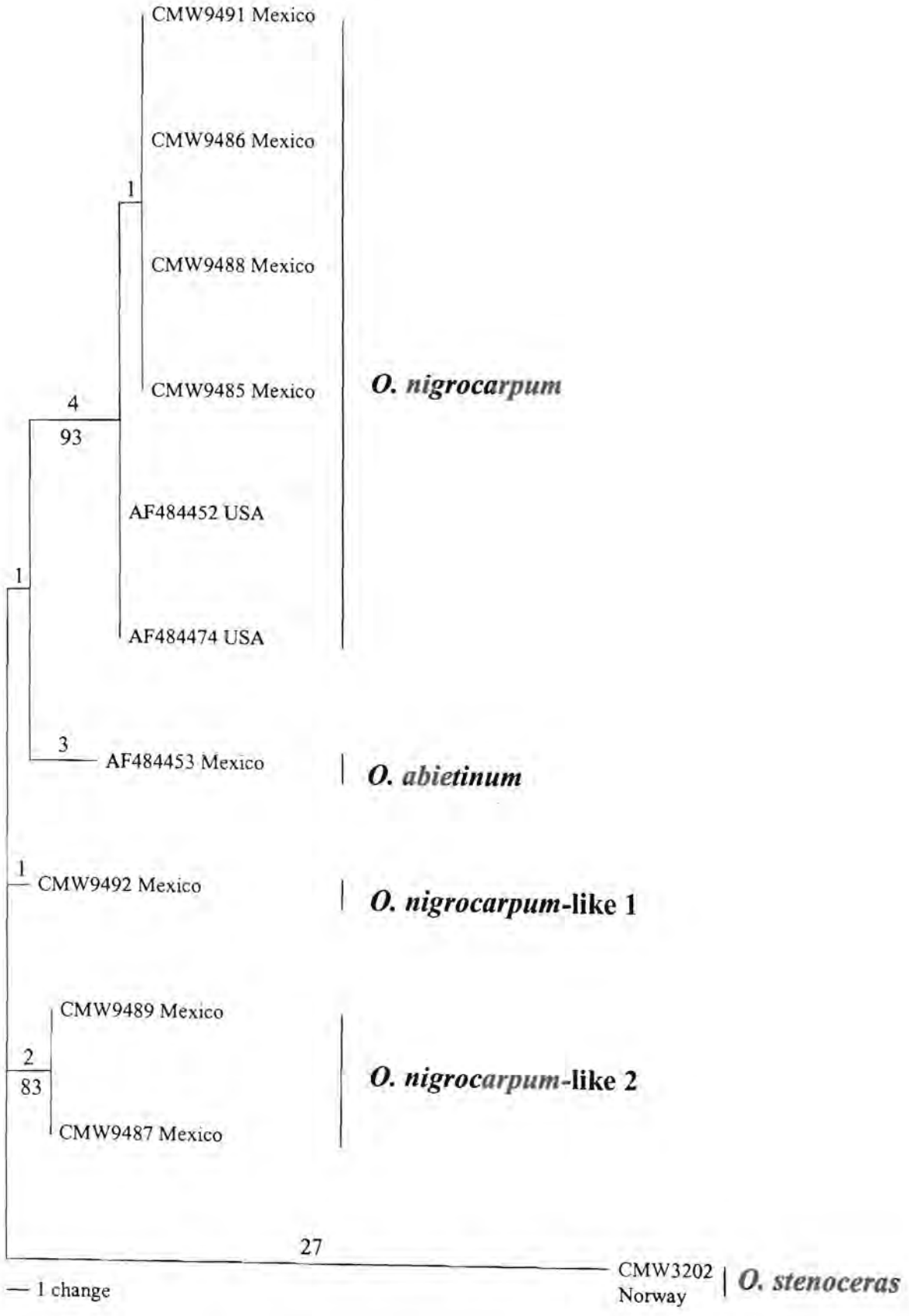
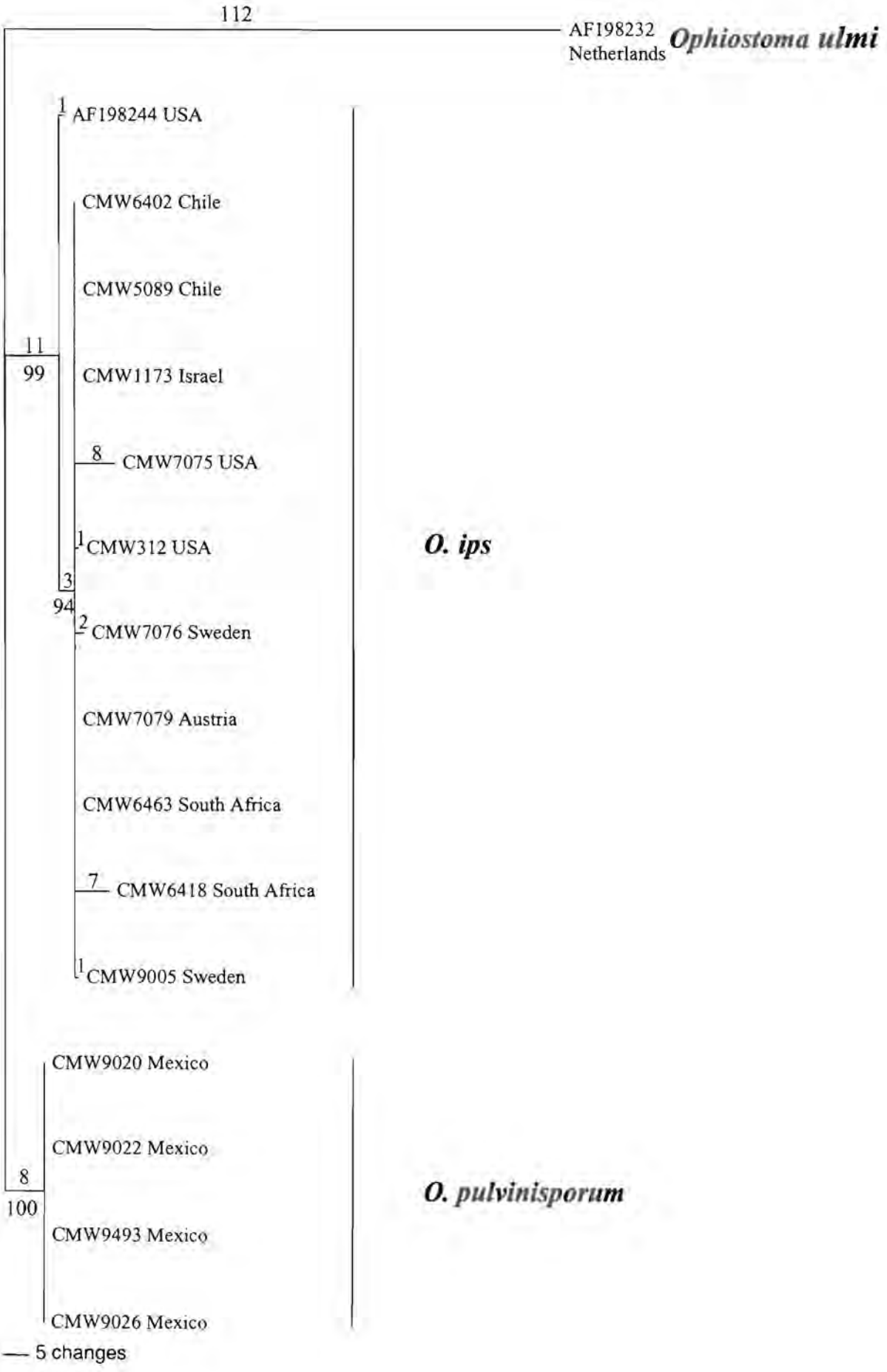
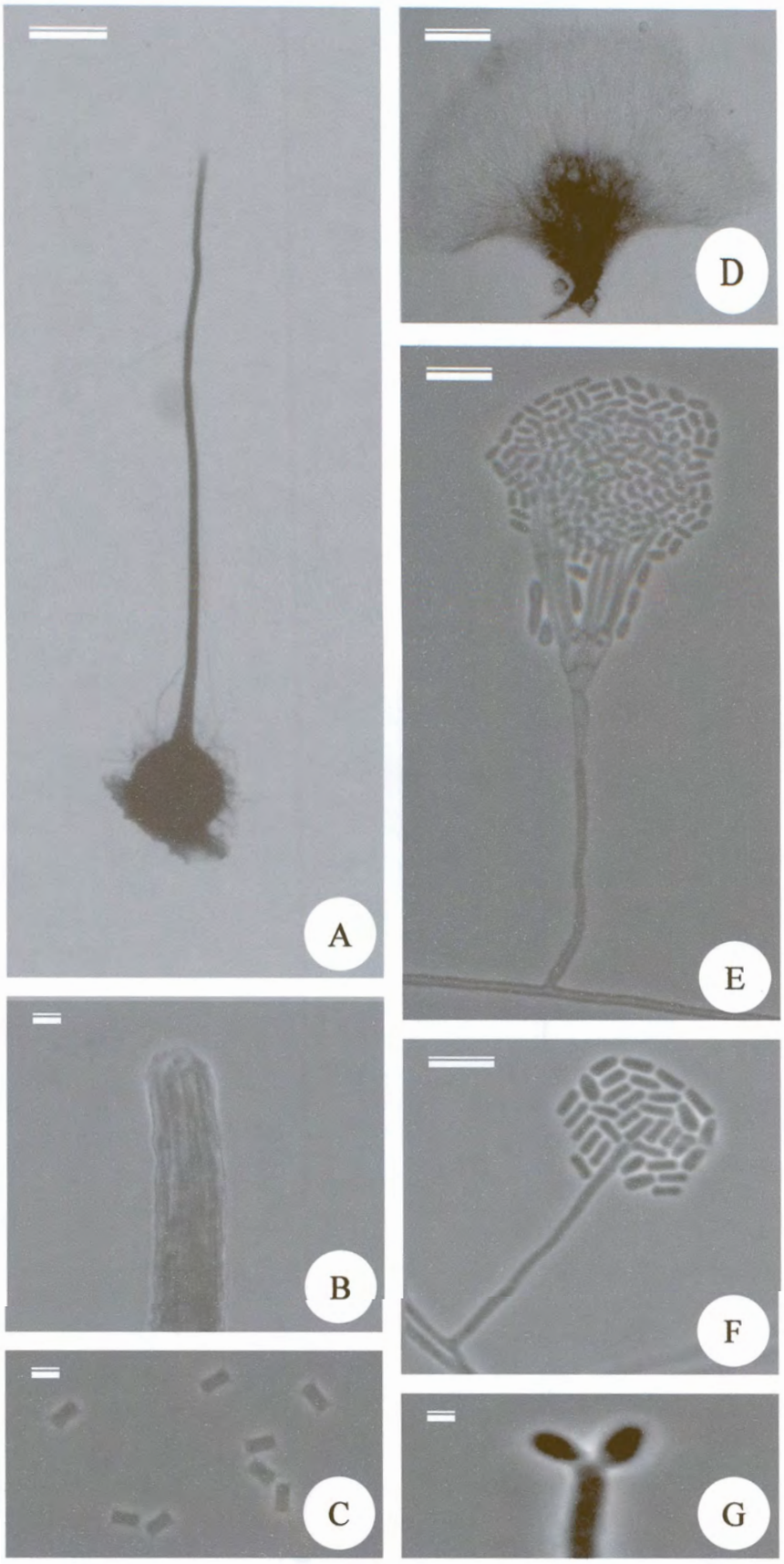


Fig. 2. Phylogram of the *Hyalorhinocladiella* group based on analyses of ITS sequences (ITS1 and ITS2 regions, as well as 5.8S rRNA gene). *Ophiostoma ulmi* was used as an outgroup. Base substitution numbers are indicated above the branches and the bootstrap values (1000 bootstrap repeats) below the branches.


112
AF198232 Netherlands
Ophiostoma ulmi
1
AF198244 USA
CMW6402 Chile
CMW5089 Chile
11
99
CMW1173 Israel
8
CMW7075 USA
1
CMW312 USA
O. ips
3
94
2
CMW7076 Sweden
CMW7079 Austria
CMW6463 South Africa
7
CMW6418 South Africa
1
CMW9005 Sweden
CMW9020 Mexico
CMW9022 Mexico
8
100
O. pulvinisporum
CMW9493 Mexico
CMW9026 Mexico
5 changes

Figs. 3A - 3G. *Ophiostoma pulvinisporum* (CMW 9022) on 2 % MEA. A. Dark perithecia with long neck (Bar = 210 µm). B. Apex of the neck without ostiolar hyphae (Bar = 8 µm). C. Pillow shaped ascospores (Bar = 4 µm). D. *Pesotum* anamorph (Bar = 11µm). E. *Leptographium* anamorph (Bar = 17 µm). F. *Hyalorhinocladiella* anamorph (Bar = 9 µm). G. Conidia of *Hyalorhinocladiella* anamorph (Bar = 2 µm).



Chapter 5

A new *Leptographium* species associated with *Tomicus piniperda* in South-western China*

Tomicus species (Coleoptera: Scolytidae) are serious pests of pines with a wide distribution in Europe, Asia and America. In Yunnan, South-western China, *T. piniperda* has destroyed more than 0.5 million ha of *Pinus yunnanensis* in the past 15 years. A blue stain fungus belonging to the genus *Leptographium* is associated with both the shoot-feeding and trunk-attacking stages of the beetle's life cycle. The fungus is morphologically similar to the anamorph of *Ophiostoma crassivaginatum* and to *L. pyrinum,* which are both characterised by short robust conidiophores and hyphae covered by a granular layer. Both these species have been isolated from conifers and are associated with insects. After comparing the fungus from *T. piniperda* with similar *Leptographium* species, using light and scanning electron microscopy, we concluded that it represents a new taxon, which is described here as *L. yunnanense* sp. nov.

Keywords: morphology, taxonomy, *Pinus yunnanensis,* Yunnan.

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INTRODUCTION

Tomicus piniperda L. (Coleoptera: Scolytidae), one of the world's major pine forest pests, is widely distributed throughout Europe, North America and Asia (Bakke, 1968; Haack & Kucera, 1993; Yin, Huang & Li, 1984). In Europe, it is the principal bark beetle that attacks Scots pine (*Pinus sylvestris* L.) and is responsible for severe growth losses (Långström & Hellqvist, 1990). Recently, the beetle has been reported in North America, where it has contributed to a decline in quality of Christmas trees in the Great Lake region (Haack & Lawrence, 1997). In China, it is regarded as one of five major pests of pines. In the past 15 years, it has destroyed more than 0.5 million ha of Yunnan pine (*P. yunnanensis* Franchet) (Ye, 1991), representing 52 % of the total 5 million ha of forests.

Bark beetles that infest conifers are commonly known to carry *Leptographium* spp. (Kendrick, 1962; Harrington, 1988; Wingfield & Gibbs, 1991; Wingfield, 1993;). In some cases this association can lead to considerable losses to forestry (Harrington, 1993). The insects appear to be vectors of the fungi, while the fungi might serve as a source of food for the insect and contribute to the death of trees through mycelial penetration and toxin release (Paine, Raffa & Harrington, 1997). The nature of this association is, however, still actively being debated (Wingfield, Harrington & Solheim, 1995).

Leptographium species can generally be recognised by their long mononematous conidiophores with dark stipes and complex conidiogenous apparatuses, consisting of a series of branches. These branches terminate in conidiogenous cells that produce numerous conidia through annellidic conidium development (Kendrick, 1962). In most cases the conidiogenous cells are characterised by delayed secession of the conidia, which gives them the appearance of sympodial conidium development (Van Wyk, Wingfield & Marasas, 1988). The hyaline and

aseptate conidia of *Leptographium* species are accumulated in slimy masses at the apices of the conidiophores, making these fungi ideal for insect dispersal.

Limited research has been done on the taxonomy of fungi associated with bark beetles in Asia (Kaneko & Harrington, 1990; Yamaoka et al., 1997, 1998; Masuya, Kaneko & Yamaoka, 1998; Masuya *et al.*, 1999) and no previous study has been conducted in China. During the course of a study to isolate and identify the blue stain fungi associated with *T. piniperda* attacking *P. yunnanensis* in South-western China, a *Leptographium* species was isolated in association with *T. piniperda*. The aim of this study was to identify and name this fungus.

MATERIALS AND METHODS

Sampling and fungal isolation

In excess of 1200 adult *T. piniperda* beetles were collected during the beetle's shoot-feeding and trunk-attacking stages in four different localities of Yunnan, South-western China, approximately 250 kilometers apart. Collections were made during one season of the beetle's life cycle. Each infested shoot, including a beetle within it, and each gallery including a pair of beetles within the gallery, were placed in a separate clean plastic bag. All beetle samples were inoculated into 1 m long (15-20 cm diam.) freshly cut and uninfected Yunnan pine logs waxed at both ends using the technique described by Furniss, Solheim and Christiansen (1990), and maintained at room temperature. After 4 weeks, fungi were isolated from the lesions that developed in the phloem and transferred to potato dextrose agar (PDA, 150 g potatoes, 15 g dextrose in 1000 ml distilled water) amended with 0.04% streptomycin. Purified colonies were transferred to 2 % malt extract agar (MEA, 20 g Biolab malt extract, 20 g Biolab agar and 1000 ml distilled water) plates and

incubated at 25 °C until the onset of sporulation. Fungal structures for microscopic examination were mounted on glass slides in lactophenol. Fifty measurements of each relevant morphological structure were made. Colours were determined with the aid of colour charts (Rayner, 1970). All cultures used in this study are maintained in the Culture Collection (CMW) of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, Republic of South Africa and representative material including dried and living cultures has been deposited in the National Collection of Fungi (PREM, PPRI), South Africa.

Scanning electron microscopy

For scanning electron microscopy, small blocks of agar cut from sporulating colonies were fixed in 3% glutaraldehyde and 0.5 % osmium tetroxide in a 0.1 M phosphate buffer, dehydrated in a graded acetone series and critical-point dried. Specimens were mounted and coated with gold palladium alloy and examined using a JEOL JSM 840 scanning electron microscope.

Optimal temperature for growth

The optimal growth temperature for the isolates (CMW 5152, CMW 5153, CMW 5304 and CMW 5305) was determined by inoculating eight MEA plates for each temperature with a 6.0 mm diam. 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 an average was calculated from eight random readings.

Cycloheximide sensitivity

Cycloheximide tolerance of isolates (CMW 5152, CMW 5153, CMW 5304 and CMW 5305) was determined by growing them on 2 % MEA amended with 0.5 g/l cycloheximide. Dishes

were incubated in the dark at 25 °C for seven days, and two colony diameters were measured at right angles. Five replicate plates were used and the growth rate (mm/day) was determined based on the average of ten diameter readings.

RESULTS

A *Leptographium* species was isolated from 12.3 % of adult *T. piniperda* that had been collected from both shoot-feeding and trunk-attacking stages. Isolates of the *Leptographium* species are characterised by small robust conidiophores and an optimal growth temperature of 25 °C. This species also proved to be tolerant to cycloheximide, with no reduction in growth when grown on 0.5 g/l of the antibiotic. Isolates of this species produced a considerable number of conidia. In older cultures, the spore masses become hardened, making the observation of the sporulating structures difficult. The hyphae of the *Leptographium* species were characterised by granular surfaces. Comparison with known species of *Leptographium* led us to conclude that this is a previously undescribed *Leptographium* species, and it is described herein.

Leptographium yunnanense X. D. Zhou, K. Jacobs, M. J. Wingfield & M. Morelet, sp. nov. Figs. 1-7.

Coloniae in 2 % MEA ad 25 °C optime crescentes et post 7 dies 13 mm diam attingentes, margine integrae. Mycelia immersa vel emersa, hyphis aeriis sparsis emittentia, atro-olivacea vel hyalina, ad exterius granulares. Conidiophora singula, erecta, macronematosa, mononematosa, 74.0 – 233.0 µm longa; structura rhizoideiformis absens. Apparatus conidiogeni praeter massam conidii (40.0 -) 83.0 - 88.0 (- 127.0) µm longi, ex ramis cylindricis 2 vel 4-seriatis compositi; rami primarii 2 vel 3, pallide olivacei vel hyalini, cylindrici, laeves, 0 - 1-septati, (9.0 -) 12.0 -

15.0 (- 20.0) x 3.0 - 7.0 µm; rami secundarii pallide olivacei vel hyalini, aseptati, (9.0 -) 13.0 x 15.0 (- 20.0) x 3.0 - 6.0 µm; rami tertiarii aseptati, 7.0 - 19.0 (- 24.0) x 2.0 - 5.0 µm; rami quartii (11.0 -) 14.0 - 17.0 (- 20.0) µm. Cellulae conidiogenae discretae, 2 vel 3 per ramum, cylindricae, apicem versus leviter attenuatae, (18.0 -) 23.0 - 26.0 (- 32.0) µm longae, 2.0 - 4.0 (- 6.0) µm latae. Conidia oblonga vel obovoidea, lasi truncata, ad apicem apparatus conidiogeni in massa guttulata mucilaginosa accumulata, (4.0 -) 7.0 - 8.0 (- 11.0) x 2.0 - 6.0 µm.

Colonies with optimal growth at 25 °C on 2 % MEA, reaching 13 mm in diameter in 7 days; no growth below 10 °C or above 30 °C; able to withstand high concentrations of cycloheximide, with no reduction in growth on 0.5 g/l cycloheximide after 7 days at 25 °C in the dark; on MEA dark olivaceous (19"f), with smooth margins; mycelia submerged or on top of agar with sparse aerial hyphae, dark olivaceous to hyaline, granular outer surface, not constricted at the septa, (2.0 -) 3.0 - 7.0(- 9.0) µm in diameter. Conidiophores occurring singly, arising directly from the mycelium, erect, macronematous, mononematous, 74.0 - 227.0 (- 233.0) µm in length; rhizoid-like structures absent; stipe light olivaceous, cylindrical, simple, with 0 - 4 septa, occasionally constricted, 11.0 - 66.0 (- 112.0) µm long (from first basal septum to below primary branches), 4.0 – 9.0 µm wide below primary branches; apical cells not swollen, (3.0 -)5.0 - 6.0(- 11.0) µm wide at base; basal cells not swollen (Figs. 1, 7a, b). Conidiogenous apparatus (40.0 -) 83.0 - 88.0 (- 127.0) long (excluding the conidial mass), with 2 to 4 series of cylindrical branches; primary branches light olivaceous to hyaline, smooth, cylindrical, with 0 - 1 septum, (9.0 -) 12.0 - 15.0 (- 20.0) µm long, 3.0 - 7.0 µm wide; secondary branches light olivaceous to hyaline, aseptate, (9.0 -) 13.0 - 15.0 (- 20.0) µm long, 3.0 - 6.0 µm wide; tertiary branches light olivaceous to hyaline, aseptate, 7.0 - 19.0 (- 24.0) µm long, 2.0 - 5.0 µm wide; quaternary branches (11.0 -) 14.0 - 17.0 (- 20.0) µm long, 2.0 - 5.0 µm wide (Figs. 2, 7b). Conidiogenous cells discrete, 2 - 3 per branch, cylindrical, tapering slightly toward the apex, (18.0 -) 23.0 - 26.0

(- 32.0) µm long, 2.0 - 4.0 (- 6.0) µm wide. Conidium development occurring through replacement wall building with holoblastic ontogeny, percurrent proliferation and delayed secession, giving the false impression of sympodial proliferation (Minter, Kirk &Sutton, 1982; Van Wyk, Wingfield & Marasas, 1988) (Fig. 4). Conidia accumulating in slimy droplets at the apex of conidiogenous apparatus, oblong to obovoid, with a truncate base, (4.0 -) 7.0 - 8.0 (- 11.0) x 2.0 – 6.0 µm (Figs. 5, 6, 7c).

Materials examined: Cultures on 2 % MEA, isolated from *T. piniperda* infesting *P. yunnanensis*, Yunnan, South-western China, collected by Xu Dong Zhou, Hui Ye and HuaSun Ding, March 1997, CMW 5304 (= PPRI 6907, living culture) (holotype: PREM 56579, dried culture); December 1996, CMW 5305 (= PPRI 6908, living culture) (paratype: PREM 56580, dried culture); March 1997, CMW 5153 (= PPRI 6923, living culture) (paratype: PREM 56578, dried culture); August, 1995, CMW5152 (=PPRI 6909, living culture), no PREM number for this isolate presently.

DISCUSSION

Leptographium yunnanense can easily be recognised by its small conidiophores, which are abundantly produced on the surface of MEA. As with other species in *Leptographium*, *L. yunnanense* can tolerate high concentrations of cycloheximide (Harrington, 1988). *Leptographium yunnanense* is typical of the other members of this genus: i.e., numerous conidia produced through annellidic conidium development and accumulated in slimy masses on the apices of the conidiophores. In older cultures, spore masses flow from the conidiophores,

become sticky and cover the entire structure. This makes the study of the conidiophore structure in older cultures extremely difficult.

Leptographium yunnanense is morphologically similar to the *Leptographium* anamorph of *O. crassivaginatum* (H. D. Griffin) T. C. Harrington and *L. pyrinum* R. W. Davidson. These species are all characterised by short robust conidiophores and hyphae that appear to have a granular surface. Furthermore, these species have all been isolated from conifers and are associated with insects (Griffin, 1968; Davidson, 1978).

Leptographium yunnanense, lacking the teleomorph, can be distinguished from the anamorph of *O. crassivaginatum* based on its slightly longer conidiophores (Table 1). *Leptographium yunnanense* and the anamorph of *O. crassivaginatum* have conidia of similar length, while those of *L. yunnanense* are almost twice as broad as those of *O. crassivaginatum* anamorph. This makes the conidia of *L. yunnanense* distinctly obovoid compared with the oblong conidia of *O. crassivaginatum* anamorph (Griffin, 1968).

Leptographium yunnanense can be distinguished from *L. pyrinum* based on the considerably longer conidiophores in the latter species (Table 1). *Leptographium yunnanense* and *L. pyrinum* have conidia of similar dimensions, but can be distinguished based on the pear-shaped conidia of *L. pyrinum*, compared with the obovoid conidia of *L. yunnanense*. *Leptographium pyrinum* is also characterised by conidiophores with rhizoids (Davidson, 1978), while these structures are absent in *L. yunnanense*. In addition, *L. yunnanense* is associated with *T. piniperda* in China, while *L. pyrinum* is associated with *Dendroctonus* species in the western USA (Davidson, 1978).

This is the first report on fungi associated with *T. piniperda* in China. The beetle has been considered to be a secondary pest, which usually colonizes weakened, stressed and recently killed trees (Långström & Hellqvist, 1993). However, in China, it can also attack healthy trees (Ye, 1991). *Leptographium yunnanense* occurs in China, not in Europe, where the beetles infest and

feed on shoots as well as colonise trunks of living trees. The insect, therefore, appears to be a much more serious pest in China than in Europe.

REFERENCES

Bakke, A. (1968) Ecological studies on bark beetle (Coleoptera: Scolytidae) associated with the Scots pine in Norway with particular reference to the influence of temperature. *Meddelelser fra Norsk Institutt for Skogforskning* **111:** 1-151.

Davidson, R. W. (1978) Staining fungi associated with *Dendroctonus adjunctus* in pines. *Mycologia* **70**: 35-40.

Furniss, M. M., Solheim, H. & Christiansen, E. (1990) Transmission of blue-stain fungi by *Ips typographus* (Coleoptera: Scolytidae) in Norway spruce. *Annals of the Entomological Society of America* **83**: 712-716.

Griffin, H. D. (1968) The genus *Ceratocystis* in Ontario. *Canadian Journal of Botany* **46**: 689-718.

Haack, B. & Kucera, D. (1993) New introduction-common pine shoot beetle, *Tomicus piniperda* L. *Pest Alert*, NA-TP-05-93. USDA, Forest Service, Northeastern Area: pp 2.

Haack, B. & Lawrence, R. K. (1997) *Tomicus piniperda* L. (Coleoptera: Scolytidae) reproduction and behaviour on Scotch pine Christmas trees taken indoors. *Great Lakes Entomologist* **30**: 19-31.

Harrington, T. C. (1988) *Leptographium* species, their distributions, hosts and insect vectors. In *Leptographium root diseases on conifers* (T. C. Harrington & Jr. F. W. Cobbs., eds): 1-39. American Phytopathological Society Press, St. Paul, Minnesota.

Harrington, T. C. (1993) Diseases of conifers caused by species of *Ophiostoma* and *Leptographium*. In *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 161-172. American Phytopathological Society Press, St. Paul, Minnesota.

Kaneko, S. & Harrington, T. C. (1990) *Leptographium truncatum* isolated from Japanese red and black pines. *Report of the Tottori Mycological Institute* **28**: 171-174.

Kendrick, W. B. (1962) The *Leptographum* complex. *Verticicladiella* S. Hughes. *Canadian Journal of Botany* **40**: 771-797.

Långström, B. & Hellqvist, C. (1990) Spatial distribution of crown damage and growth losses caused by recurrent attacks of pine shoot beetles in pine stands surrounding a pulp mill in southern Sweden. *Journal of Applied Entomology* **110**: 261-269.

Långström, B. & Hellqvist, C. (1993) Induced and spontaneous attacks by pine shoot beetles on young Scots pine trees: tree mortality and beetle. *Journal of Applied Entomology* **115**: 25-36.

Masuya, H., Kaneko, S., & Yamaoka, Y. (1998) Blue stain fungi associated with *Tomicus piniperda* (Coleoptera: Scolytidae) on Japanese red pine. *Journal of Forestry Research* **3**: 213-219.

Minter, D. W., Kirk, P. M. & Sutton, B. C. (1982) Holoblastic phialides. *Transactions of the British Mycological Society* **79**: 75-93.

Olchowecki, A. & Reid, J. 1974. Taxonomy of the genus *Ceratocystis* in Manitoba. *Canadian Journal of Botany* **52**: 1675-1711.

Paine, T. D., Raffa, K. F. & Harrington, T. C. (1997) Interactions among Scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* **42**: 179-206.

Perry, T. J. (1991) A synopsis of the taxonomic revisions in the genus *Ceratocystis* including review of blue-staining species associated with *Dendroctonus* bark beetles. *General Technical Report*, SO-86. New Orleans, LA: U. S. Department of Agriculture, Forest Service, Southern Forest Experiment Station, pp 16.

Rayner, R. W. (1970) A mycological colour chart. Commonwealth Mycological Institute and British Mycological Society, Kew.

Six, D. L. & Paine, T. D. (1996) *Leptographium pyrinum* is a mycangial fungus of *Dendroctonus adjunctus*. *Mycologia* **88**: 739-744.

Van Wyk, P. S., Wingfield, M. J. & Marasas, W. F. O. (1988) Differences in synchronisation of stages of conidial development in *Leptographium* species. *Transactions of the British Mycological Society* **90**: 451-456.

Wingfield, M. J. (1993) *Leptographium* species as anamorphs of *Ophiostoma*: progress in establishing acceptable generic and species concepts. In *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 43-51. American Phytopathological Society Press, St. Paul, Minnesota.

Wingfield, M. J. & Gibbs, J. N. (1991) *Leptographium* and *Graphium* species associated with pine-infesting bark beetles in England. *Mycological Research* **95**: 1257-1260.

Wingfield, M. J., Harrington, T. C. & Solheim, H. (1995). Do conifer bark beetles require fungi to kill trees? In *Proceedings from a symposium held at the Norwegian Forest Research Institute (NISK) in Ås, Norway, 31 July – 2 August, 1995* (E. Christiansen, ed). *Aktuelt fra Skogforsk* 6/95: 6.

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

Yamaoka, Y., Wingfield, M. J., Ohsawa, M. & Kuroda, Y. (1998). Ophiostomatoid fungi associated with *Ips cembrae* in Japan and pathogenicity to Japanese larch. *Mycoscience* **39**: 367-378.

Ye, H. (1991) On the bionomy of *Tomicus piniperda* L. (Coleoptera: Scolytidae) in the Kunming region of China. *Journal of Applied Entomology* **112**: 366-369.

Yin, H. F., Huang, F. SH. & Li, ZH. L. (1984) Economic insect fauna of China (Fascicle 29), (Coleoptera: Scolytidae). Science Press, Beijing.

Table 1. Comparison of morphological characteristics of *Leptographium yunnanense*, *Ophiostoma crassivaginatum* anamorph and *L. pyrinum*.

	L. yunnanense	*O. crassivaginatum anam.*	*L. pyrinum*
Substrate	*Pinus yunnanensis* *P. densata* *P. kesiya*	[a]*Picea mariana, P. glauca, Pinus resinosa, P. strobus, P. sylvestris, Populus grandidenta, P. tremuloides, Fraxinus nigra*	[c]*Pinus ponderosa*
Insect association	*Tomicus piniperda*	[b]*Trypodendron retusus*	[d]*Dendroctonus adjunctus*
Conidiophore length	74.0 - 227.0 (- 233.0) µm	25.0 - 106.0 (- 118.0) µm	(117.5 -) 215.0 - 236.5 (- 392.5)µm
Primary branches	2 - 3	2 – 3	2 - 4
Rhizoids	absent	absent	present
Conidium shape	obovoid	oblong to obovoid	oblong, almost pear-shaped
Conidium length	(4.0 -) 7.0 - 8.0 (- 11.0) µm	(4.0 -) 4.5 - 5.5 (- 10.0) µm	5.0 - 12.0 µm
Conidium width	2.0 - 6.0 µm	1.0 - 2.5 µm	4.0 - 6.0 µm
Granular hyphae	present	present	present
Teleomorph	absent	Present	absent

[a] Griffin (1968), Olchowecki and Reid (1974);
[b] Harrington (1988);
[c] Davidson (1978);
[d] Davidson (1978), Harrington (1988), Perry (1991), Six and Paine (1996).

Figs. 1 - 6. *Leptographium yunnanense* (CMW 5304) on MEA. Fig. 1. Conidiophores with dark olivaceous stipes and complex conidiogenous apparatuses (Bar = 10 µm). Fig. 2. Complex conidiogenous apparatus (Bar = 20 µm). Figs. 3, 4. Conidiogenous cells showing false sympodial, and annellidic conidiogenesis indicated by arrows (Bar = 10 µm in Fig. 3, 1 µm in Fig. 4). Figs. 5, 6. Conidia (Bar = 10 µm in Fig. 5, 1 µm in Fig. 6).

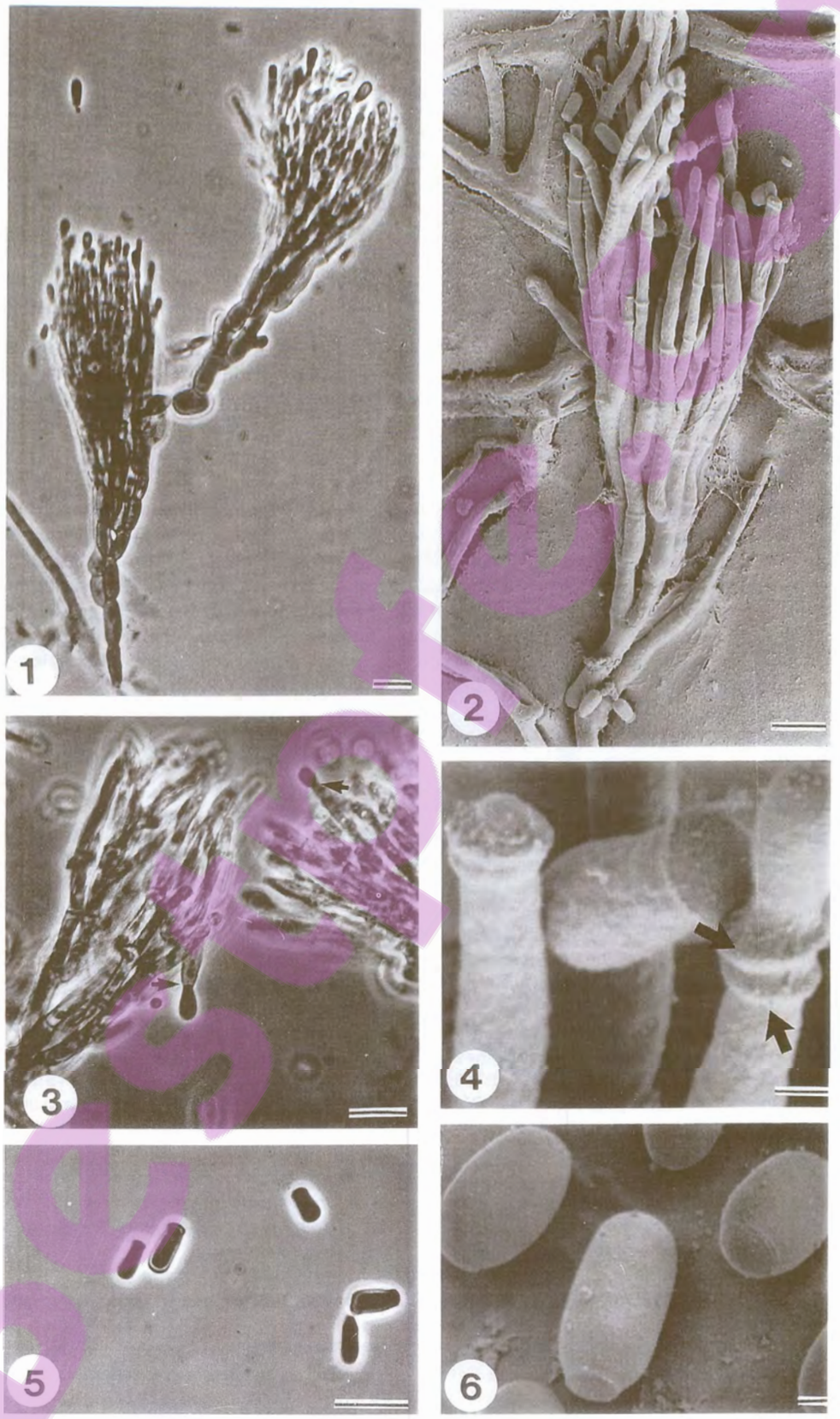
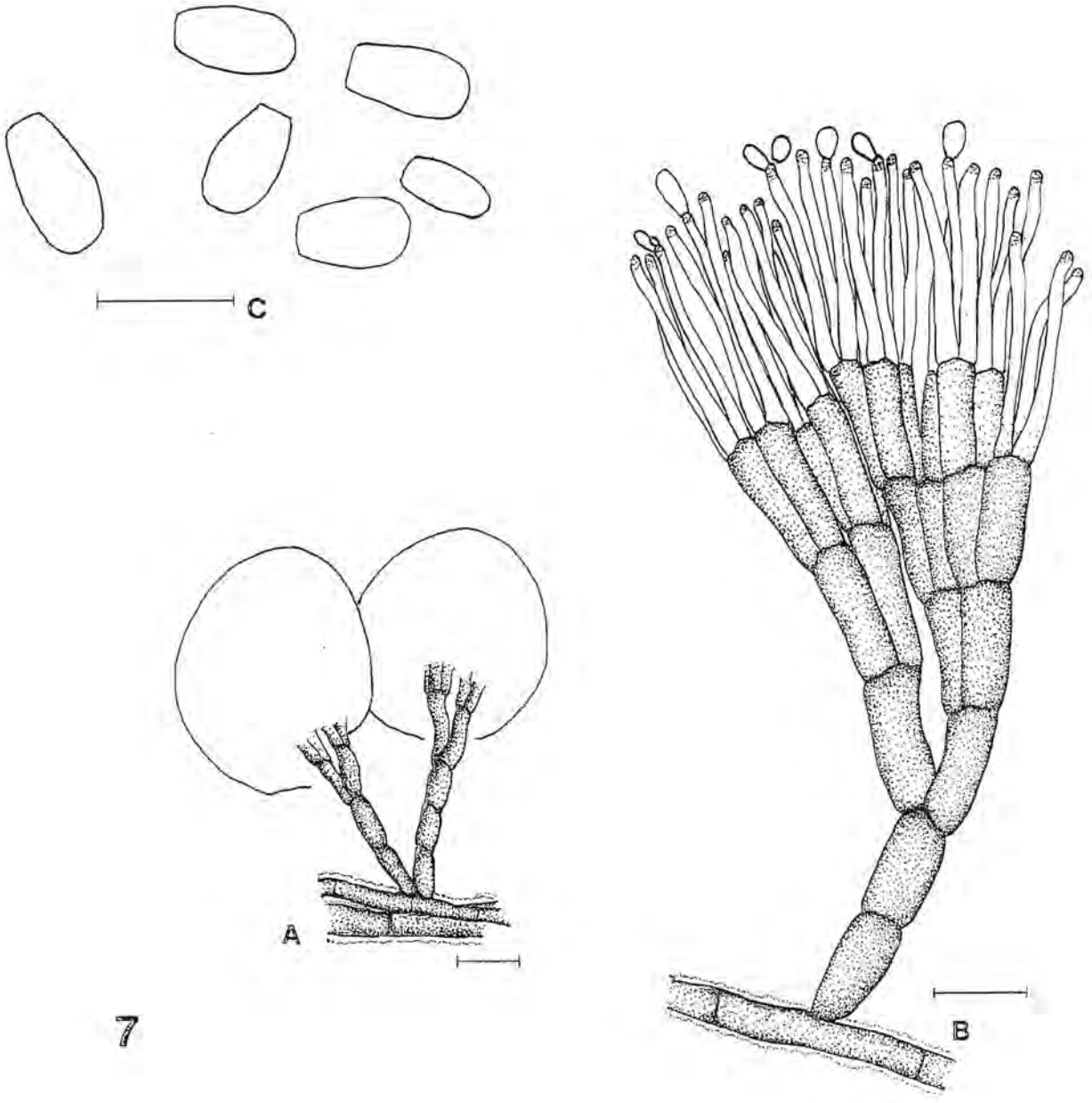


Fig. 7. *Leptographium yunnanense* (CMW 5304) on MEA. A. Habit sketch of the conidiophores (Bar = 50 µm). B. Conidiogenous apparatus (Bar = 10 µm). C. Conidia (Bar = 10 µm).



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Chapter 6

Epitypification of *Ophiostoma galeiformis* and phylogeny of species in the *O. galeiformis* complex

Ophiostoma galeiformis was first described from *Larix kaempferi* in Scotland in 1951, where it was associated with *Hylurgops palliatus*, *Dryocoetes autographus*, and *Trypodendron lineatum*. The taxonomy of this fungus has, however, been uncertain due to a lack of sexual structures on the type specimen and contamination of a preserved ex-type culture. The aim of this study was to designate an epitype for *O. galeiformis*, and to consider phylogenetic relationships of the species. Eighteen *O. galeiformis*-like isolates from different parts of the world were included in the study, including collections from *Pinus sylvestris* infested with *Tomicus piniperda* in Scotland. Both light microscopic study and ITS rDNA sequencing were used to study these fungi. Morphological characteristics of isolates from Scotland, Sweden, South Africa and Chile corresponded well with those described for *O. galeiformis* and an isolate from Scotland was designated as an epitype. An extended description is provided for this species, which should facilitate identification in the future. ITS rDNA sequence data showed that the isolates representing *O. galeiformis*, were phylogenetically separated from three isolates from the USA and Mexico that had been identified as this species. The latter fungi probably represent an undescribed taxon.

Keywords: bark beetle, sapstain, ITS, *Tomicus piniperda*.

INTRODUCTION

Bark beetles (Coleoptera: Scolytidae) commonly occur in most forest ecosystems and several species are regarded as important forest pests (Wood & Bright, 1992). Most bark beetles species act as vectors of fungi, especially ophiostomatoid fungi (Whitney, 1982; Beaver, 1989; Wingfield, Seifert & Webber, 1993; Paine, Raffa & Harrington, 1997). The genus *Ophiostoma* includes some primary tree pathogens as well as sapstain agents (Lagerberg, Lundberg & Melin, 1927; Brasier, 1979, 1991; Harrington, 1993; Seifert, 1993; Brasier & Mehrotra, 1995).

Ophiostoma galeiformis (Bakshi) Mathiesen-Käärik, originally described as *Ceratocystis galeiformis* Bakshi, is a sapstain fungus that was first described from Scotland (Bakshi, 1951; Mathiesen-Käärik, 1953). This fungus was isolated from the bark of *Larix kaempferi* infested with *Hylurgops palliates* (Gyll.), *Dryocoetes autographus* (Ratzeburg), and *Trypodendron lineatum* (Olivier) (Bakshi, 1951). Later, the fungus was found on *Picea* infested with *Hylastes cunicularius* (Errichson) in Sweden (Mathiesen-Käärik, 1953, 1960), and from unknown pine-infesting bark beetles (Hunt, 1956). *Ophiostoma galeiformis* is also associated with pine-infesting bark beetles occurring in Chile and South Africa (Chapter 2, 3). A single, *O. galeiformis*-like isolate has also recently been isolated from *Dendroctonus mexicanus* (Hopkins) infesting *Pinus pseudostrobus* in Mexico (Chapter 4).

The taxonomy of *O. galeiformis* has been confused for many years. This is largely due to the fact that the type specimen lacks sexual structures (Hunt, 1956; Upadhyay, 1981). Although Hunt (1956) included the species in his study of the genus *Ceratocystis*, Upadhyay (1981) and Seifert, Wingfield & Kendrick (1993) considered it a species of uncertain status. The International Code of Botanical Nomenclature (Art. 9.7) (Greuter *et al.*, 2000), allows the designation of a specimen and/or a culture as an epitype where the holotype does not show the necessary distinguishing characters. The epitype would then serve as the holotype in determining characteristics that

cannot be obtained from the holotype. The aim of this study was to reconsider the taxonomic status of *O. galeiformis* and designate an epitype for the species. Light microscopy was employed, and the ITS (internal transcribed spacer) region of the ribosomal RNA operon was sequenced to confirm its phylogenetic relationships.

MATERIALS AND METHODS

Fungal isolates and morphological investigation

The holotype of *O. galeiformis* (IMI20168) was studied based on available morphological structures. We intended to include the ex-type culture (CBS137.51) in this study, but it was found to be contaminated and repeated attempts to purify it failed. Seventeen other isolates, identified as *O. galeiformis* based on descriptions of the fungus (Bakshi, 1951; Hunt, 1956) and ecology, were included in the study (Table 1). Single conidial cultures were prepared for all these isolates and were grown on 2 % MEA (20 g Biolab malt extract, 20 g Biolab agar and 1000 ml distilled water), and examined. For the isolate producing perithecia (CMW5290), 25 sexual and asexual structures were examined and measured using a light microscope, and the ranges and averages were computed. All cultures are maintained in the culture collection (CMW) of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Mating experiments

Five isolates (CMW241 and CMW567 from South Africa, CMW4442 and CMW4444 from Scotland, and CMW9988 from Sweden) (Table 1) resembling *O. galeiformis* were chosen and crossed in every possible combination. All crossed cultures, including control crosses of isolates against themselves, were incubated on MEA with pine twigs as described by Harrington *et al.* (2001).

DNA sequencing and phylogenetic analysis

DNA extraction, PCR amplification, sequencing reactions, and phylogenetic analysis were conducted in a similar way as those described in Chapter 3. Eighteen single hyphal tip cultures of selected isolates were prepared for sequencing (Table 1). DNA was extracted using a modified version of the extraction method developed by Raeder and Broda (1985). The ITS1 and ITS2 (internal transcribed spacer) regions, including the 5.8S gene of the ribosomal RNA operon, were amplified, using primers ITS1-F (Gardes & Bruns, 1993) and ITS4 (White *et al.*, 1990). PCR products were sequenced with the same primers used for PCR, as well as two additional internal primers, CS2 (Wingfield *et al.*, 1996), and ITS3 (White *et al*, 1990). The obtained sequences were aligned using Sequence Navigator version 1.01 (ABI PRISM, PerkinElmer). Aligned data were analysed using PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1998). Bootstrap analysis (1000 replicates) was run to determine confidence intervals of the branching points.

RESULTS

Anamorph morphology

Morphological comparisons showed no differences between the anamorph structures of the isolates from Scotland, Sweden, Chile and South Africa and those present on the holotype (IMI20168). *Pesotum* was dominant, and measurements of fruiting structures corresponded well with those described previously (Table 2). The three *O. galeiformis*-like isolates from Mexico (CMW9490), Georgia, USA (C527), and California, USA (C1293), were similar but differed slightly from isolates of *O. galeiformis* from Scotland, Sweden, Chile, and South Africa. Colony color of the isolates from Mexico and the USA was lighter than that of other isolates.

Mating experiments

Perithecia were formed on crosses using the five isolates from South Africa, Scotland and Sweden. The crosses of two South African isolates (CMW241 and CMW567), and two isolates from South Africa and Scotland (CMW567 and CMW4442), produced the most perithecia.

DNA Sequencing analysis

DNA fragments approximately 510 bp in size were amplified for all the isolates (Table 1), except the ex-type culture (CBS137.51), which had a fragment length of 463 bp. This sequence was subjected to a BLAST search, which revealed that it was a species of *Phialophora* and thus a contaminant. Manual alignment of the remaining sequences resulted in a total of 546 characters. Of these, 20 were parsimony-informative, 92 parsimony-uninformative, and 434 constant. Heuristic searches using *O. cucullatum* as the outgroup taxon, resulted in three most parsimonious trees (CI = 0.967, RI = 0.913, HI = 0.033) of which one (Fig. 1) was chosen for presentation here. Two main clades (Fig. 1) were evident in the three phylogenetic trees. The first clade, including two sub-clades, represented the *O. galeiformis* group. Isolates from Chile, South Africa and Sweden formed one subclade with a bootstrap support of 67 %, while the five isolates from Scotland formed the other subclade with a bootstrap support of 64 %. The second clade, with a bootstrap support of 100 %, included the three isolates from Mexico and the USA.

TAXONOMY

The five *O. galeiformis* isolates from Scotland are morphologically almost identical. The anamorph characteristics are also indistinguishable from those on the holotype specimen. These isolates share the same ITS sequences, and differed only by two base pairs from the isolates from

Chile, South Africa and Sweden. All these isolates are, therefore, considered to represent a single species whose morphological characteristics agree well with the original description of *O. galeiformis* (Bakshi, 1951; Mathiesen-Käärik, 1953). One of the Scottish isolates (CMW5290), is designated as the epitype strain of *O. galeiformis* since it originated from the same geographical region as the holotype specimen, and has been deposited in the culture collection (CMW5290) of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and in the Centraalbureau voor Schimmelcultures (CBS***), Utrecht, Holland. The epitype specimen, a dried culture grown on 1.5 % MEA with pine twigs, and bearing both perithecia and the asexual *Pesotum* state has been deposited in the National Collection of Fungal Specimens of South Africa (PREM***). The species description provided below is based on the epitype specimen (PREM***) and culture of *O. galeiformis* (CMW5290).

Ophiostoma galeiformis (Bakshi) Mathiesen-Käärik, Meddn. St. Skogsfor. Sk. Inst. 43: 47. 1953. = *Ceratocystis galeiformis* Bakshi, Mycol. Pap. 35: 13. 1951.

Colonies reaching 30 mm in diameter in 10 days on 2 % MEA at 25 °C. Colonies light grey (19''d) to dark brown (13'''''k) (Rayner, 1970) with age, appressed with yeasty appearance. *Perithecia* rarely produced in culture. *Perithecial bases* globose, dark brown to black, (105-) 340 (-545) µm in diameter (Fig. 2A), with few ornamental hyphae. *Perithecial necks* dark brown to black, (260-) 560 (-840) µm long, (20-) 50 (-93) µm wide at base, (8-) 27 (-54) µm wide at the apex. *Ostiolar hyphae* absent (Fig. 2B). *Asci* not observed. *Ascospore masses* in tendrils at the apex of perithecial necks (Fig. 2C). *Ascospores* hyaline, aseptate, with brim, bean shaped in side and face view, (2-) 3.5 (-6) x (1-) 1.7 (-2.0) µm (Fig. 2D).

Leptographium anamorph: rarely produced in culture. *Conidiophores* up to seven septate, 60 – 92 (- 130) µm long (Fig. 2E). *Conidia* hyaline, cylindrical to ellipsoid, with a truncate base, (1.9-) 3.6 (-4.7) x (1.0-) 1.4 (-2.2) µm (Fig. 2F).

Pesotum anamorph: predominant in culture. *Conidiophores* with apex hyaline to light grey, stalk brown, 50 – 140 (- 300) µm long, (7-) 19 (-29) µm wide at base, (10-) 47 (-190) µm wide at head (Fig. 2G). *Conidia* hyaline, cylindrical, (2.6-) 4.2 (-6.4) x (1.1-) 2.0 (-3.0) µm (Fig. 2H).

Additional strains examined: CMW4426, Scotland, Elgin, isolated from *P. sylvestris* infested with *T. piniperda*, 29 August 1997, M. J. Wingfield, CBS*** = PREM***. CMW4447, Scotland, Elgin, isolated from *P. sylvestris* infested with *T. piniperda*, 29 August 1997, M. J. Wingfield, CBS*** = PREM***.

Holotype: IMI20168, isolated from bark of *Larix kaempferi* (Japanese larch), associated with bark beetles *Hylurgops palliatus* and *Dryocoetes autographus*, Blaire Atholl, Perthshire, Scotland, 1951, B.K. Bakshi.

Epitype: PREM*** [CBS***: CMW5290; epitype designated herewith], Scotland, Elgin, isolated from *P. sylvestris* infested with *T. piniperda*, 29 August 1997, T. Kirisits.

DISCUSSION

In this study, we have confirmed previous reports that the holotype of *O. galeiformis* only contains the anamorph state of the fungus. We have, furthermore, shown that the ex-type culture deposited in the CBS is contaminated and cannot be used in taxonomic studies. An epitype based on a

collection from the same geographical area where *O. galeiformis* was first collected has thus been designated. This should ensure that future studies on the fungus are based on material known to represent the species. Results of this study have also confirmed that *O. galeiformis* occurs in Chile, South Africa, and Sweden. It, furthermore, represents the first report of the association between this fungus and *Tomicus piniperda*. Three isolates from the USA and Mexico thought to represent this species are different and probably represent an undescribed taxon.

In the descriptions of *O. galeiformis* by Bakshi (1951) and Hunt (1956), the conidial states were assigned to three genera: *Graphium, Leptographium*, and *Cephalosporium*. Both Mathiesen-Käärik (1953) and Hunt (1956) mentioned that the fungus formed a continuum of conidiophore structures varying from single, simple conidiophores to true synnemata, typical of the genus *Graphium*. Wingfield (1993) stated that it was difficult to assign a generic name to the anamorph of *O. galeiformis* since the species has both synnematous and mononematous states. Scanning electron microscopy studies further showed that there is a continuum in patterns of conidium development including those typical of *Sporothrix*, *Hyalorhinocladiella*, and *Graphium* (now *Pesotum*) (Benade, Wingfield & Van Wyk, 1997). In this study, both the *Pesotum* and *Leptographium* forms of the anamorph of *O. galeiformis* were observed, but the *Pesotum* form was predominant. We do not believe that it is necessary to provide a formal name for the anamorph but if these were selected, we would preferentially refer to it as *Pesotum*, because this state is dominant in cultures.

Analysis of sequence data of *O. galeiformis* isolates from Chile and South Africa presented in this study has shown that these isolates are closely related to those from Scotland and Sweden. Occurrence of mating between the isolates from South Africa, Scotland, and Sweden indicated that these isolates could represent a single species. Ophiostomatoid fungi in countries such as South Africa and Chile are carried by bark beetles, which were accidentally introduced into these countries from Europe (Ciesla, 1988; Tribe, 1992). *Ophiostoma galeiformis* is apparently

common in Europe associated with a wide range of bark beetles and it would have been introduced into South Africa and Chile with one or more of these insect species. In South Africa, *O. galeiformis* is associated with *Hylurgus ligniperda* (Fabricius) (Zhou *et al.*, 2001) and in Chile we have commonly isolated it from *Hylastes ater* (Chapter 3). None of these insects have been connected with *O. galeiformis* in their natural European habitat but this is probably due only to the lack of studies of fungi associated with these insects in Europe.

REFERENCES

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

Beaver, R. A. (1989) Insect-fungus relationships in the bark and ambrosia beetles. In *Insect-Fungus Interactions* (N. Wilding, N. M. Collins, P. M. Hammond & J. F. Webber, eds): 121-143. Academic Press, London.

Benade, E., Wingfield, M. J. & Van Wyk, P. S. (1997). Conidium development in *Sporothrix* anamorphs of *Ophiostoma*. *Mycological Research* **101**: 1108-1112.

Brasier, C. M. (1979) Dual origin of recent Dutch elm disease outbreaks in Europe. *Nature* **281**: 78-79.

Brasier, C. M. (1991) *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. *Mycopathologia* **115**: 151-161.

Brasier, C. M. & Mehrotra, M. D. (1995) *Ophiostoma himal-ulmi* sp. nov., a new species of Dutch elm disease fungus endemic to the Himalayas. *Mycological Research* **99**: 205-215.

Ciesla, W. M. (1988) Pine bark beetles: a new pest management challenge for Chilean foresters. *Journal of Forestry* **86**: 27-31.

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

Greuter W., McNeill, J., Barrie, F. R., Burdet, H. M., Demoulin, V., Filgueiras, T. S., Nicolson, D. H., Silva, P. C., Skog, J. E., Trehane, P., Turland, N. J. & Hawksworth, D. L. (2000) *International Code of Botanical Nomenclature (Saint Louis Code)*. Adopted by the Sixteenth

International Botanical Congress St. Louis, Missouri, July-August 1999. *Regnum Veg.* **138**: 1-474.

Harrington, T. C. (1993) Diseases of conifers caused by species of *Ophiostoma* and *Leptographium*. In *Ceratocystis and Ophiostoma:. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 161-172. American Phytopathological Society Press, St. Paul, Minnesota.

Harrington, T. C., McNew, D., Steimel, J., Hofstra, D. & Farrell, R. (2001) Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia* **93**: 111-136.

Hunt, J. (1956) Taxonomy of the genus *Ceratocystis*. *Lloydia* **19**: 1-59.

Lagerberg, T., Lundberg, G. & Melin, E. (1927) Biological and practical researches into blueing in pine and spruce. *Svenska Skogsvardsföreningens Tidskrift* **25**: 145-272.

Mathiesen-Käärik, A. (1953) Eine Übersicht über die gewöhnlichsten mit Borkenkärfern assoziierten Bläuepilze in Schweden und einige für Schweden neue Bläuepilze. *Meddelanden Från Statens Skogforskningsintitut* **43**: 3-74.

Mathiesen-Käärik, A. (1960) Studies on the ecology, taxonomy and physiology of Swedish insect-associated blue-stain fungi, especially the genus *Ceratocystis*. *Oikos* **11**:1-25.

Paine, T. D., Raffa, K. F. & Harrington, T. C. (1997) Interactions among Scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* **42**: 179-206.

Raeder, U. & Broda, P. (1985) Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**: 17-20.

Rayner, R. W. (1970) A mycological colour chart. Commonwealth Mycological Institute and British Mycological Society, Kew.

Seifert, K. A. (1993) Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*. (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 141-151. American Phytopathological Press, St. Paul, Minnesota.

Seifert, K. A., Wingfield, M. J. & Kendrick, W. B. (1993) A nomenclator for described species of *Ceratocystis*, *Ophiostoma*, *Ceratocystiopsis*, *Ceratostomella* and *Sphaeronaemella*. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*. (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 269-287. American Phytopathological Press, St. Paul, Minnesota.

Swofford, D. L. (1998) PAUP*: Phylogenetic Analysis Using Parsimony (* and other methods). Version 4.0. Sinaur Associates: Sunderland, Massachusetts.

Tribe, G. D. (1992) Colonization sites on *Pinus radiata* logs of the bark beetles, *Orthotomicus erosus*, *Hylastes angustatus* and *Hylurgus ligniperda* (Coleoptera: Scolytidae). *Journal of the Entomological Society of Southern Africa* **1**: 77-84.

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

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR protocols: genes for phylogenetics. (M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 315-322. Academic Press, San Diego, California.

Whitney, H. S. (1982) Relationships between bark beetles and symbiotic organisms. In *Bark beetles in North American conifers*. (J. B., Mitton & K. B., Sturgeon, eds): 183-211. Austin: University of Texas.

Wingfield, M. J. (1993) *Leptographium* species as anamorphs of *Ophiostoma*: progress in establishing acceptable generic and species concepts. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*. (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 43-51. American Phytopathological Press, St. Paul, Minnesota.

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

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

Wood, S. L., & Bright, D. E. (1992) A catalog of Scolytidae and Plapodidae (Coleoptera), Part 2: Taxonomic index. *Great Basin Naturalist Memoirs* **A (13)**: 1-6.

Zhou X. D., De Beer Z. W., Wingfield B. D. & Wingfield M. J. (2001) Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. *Sydowia* **53**: 290-300.

Table 1. Fungal isolates included in this study.

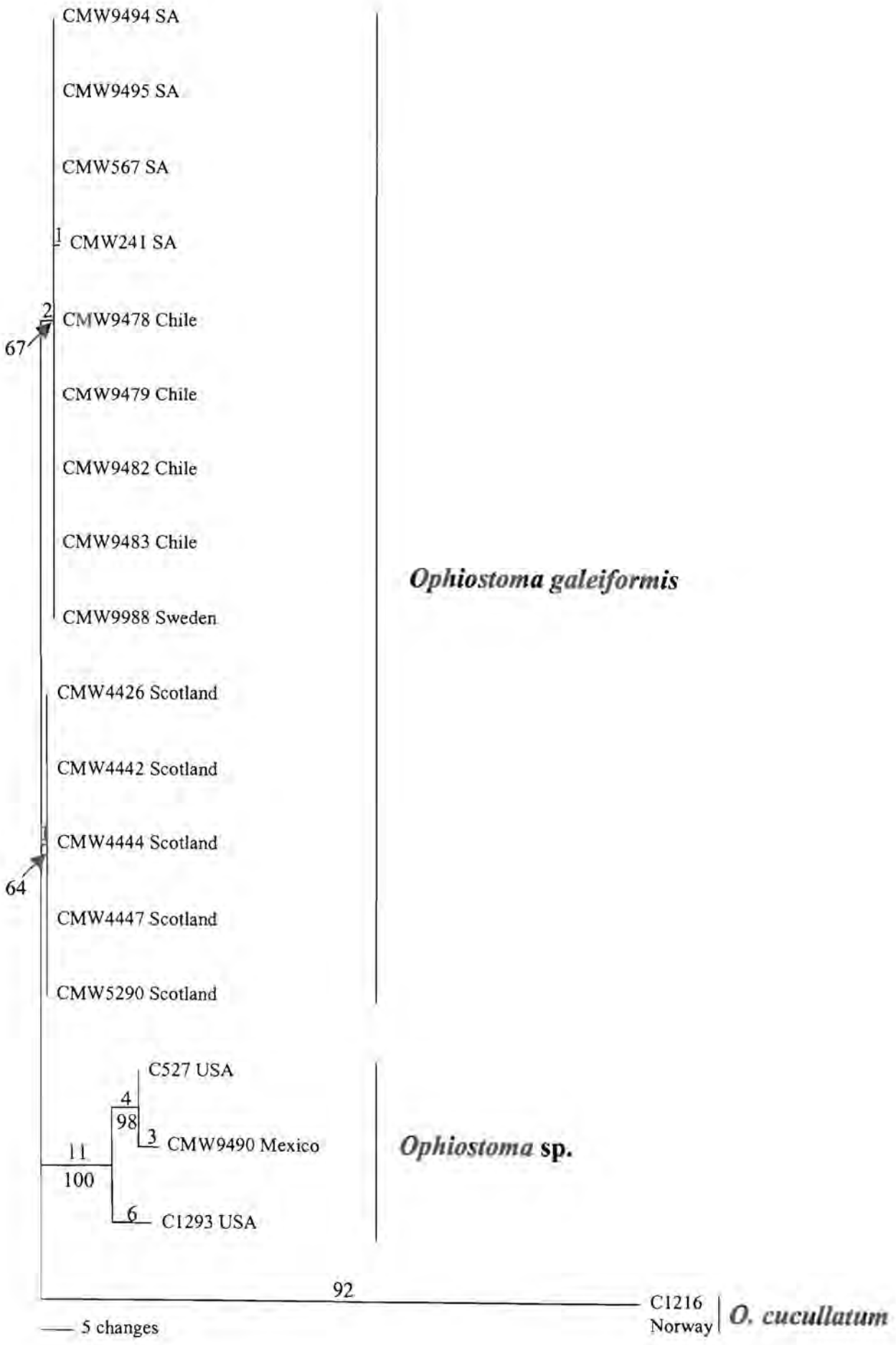
Species	Isolate No.	Other No.	Herbarium	Collector or supplier	Host	Insect	Origin	Sexual state	rDNA sequence
Ophiostoma	[1]CMW4426			MJ Wingfield & T Kirisits	*Pinus sylvestris*	*Tomicus piniperda* (Linnaeus)	Elgin, Scotland		
galeiformis	CMW4442			MJ Wingfield & T Kirisits	*P. sylvestris*	*T. piniperda*	Elgin, Scotland		
	CMW4444			MJ Wingfield & T Kirisits	*P. sylvestris*	*T. piniperda*	Elgin, Scotland		
	CMW4447			MJ Wingfield & T Kirisits	*P. sylvestris*	*T. piniperda*	Elgin, Scotland		
	CMW5290		[2]PREM???	MJ Wingfield & T Kirisits	*P. sylvestris*	*T. piniperda*	Elgin, Scotland	present	
	CMW9478			MJ Wingfield & XD Zhou	*P. radiata*	*Hylastes ater* (Paykull)	Valdivia, Chile		
	CMW9479			MJ Wingfield & XD Zhou	*P. radiata*	*H. ater*	Valdivia, Chile		
	CMW9482			MJ Wingfield & XD Zhou	*P. radiata*	*Hylurgus ligniperda* (Fabricius	Valdivia, Chile		
	CMW9483			MJ Wingfield & XD Zhou	*P. radiata*	*H. ligniperda*	Valdivia, Chile		
	CMW9494			XD Zhou	*P. elliiottii*	*H. ligniperda*	KwaZulu-Natal, South Africa		
	CMW9495			XD Zhou	*P. elliiottii*	*H. ligniperda*	KwaZulu-Natal, South Africa		
	CMW241			MJ Wingfield	*P. pinaster*		Grabouw, South Africa		
	CMW567			MJ Wingfield	*P. pinaster*		Grabouw, South Africa		
	CMW9988	[3]CBS150.54		A Mathiesen-Käärik	*Picea abies*	*Hylastes cunicularius*	Västerbotten, Sweden		
Ophiostoma		[4]C527		M Baldwin	*Pinus taeda*		Georgia, USA		
galeiformis-like		C1293		D Hofstra	*P. radiata*		California, USA		
	CMW9490			MJ Wingfield & XD Zhou	*P. pseudostrobus*	*Dendroctonus mexicanus*	Chiapas, Mexico		
O. cucullatum		C1216		H Solheim	*Picea abies*	*Ips typographus*	Norway		

Notes: [1]CMW is the Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. [2]PREM – The National Collection of Fungi, South Africa. [3]CBS – Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; [4]C – Culture Collection of T.C Harrington, Department of Plant Pathology, Iowa State University, Iowa, USA.

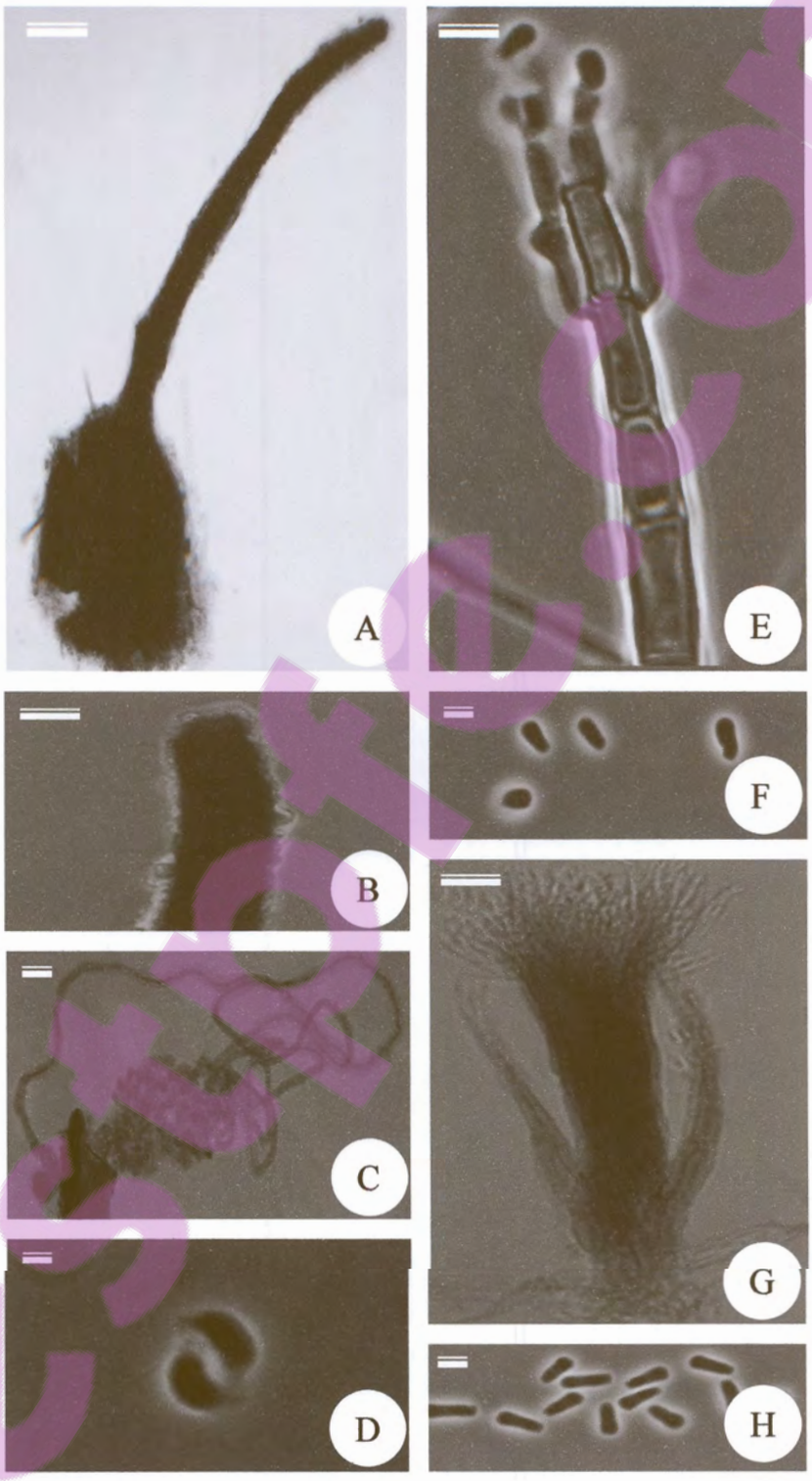
Table 2. Comparison of the epitype specimen and previous descriptions of *O. galeiformis* (all measurements in µm).

			Epitype	Bakshi, 1951	Mathiesen-Käärik, 1953	Hunt, 1956
Teleomorph	Perithecial base	Colour	Dark brown to black	Brown to black	Black	Same as those of Bakshi (1951)
		Diameter	(105-) 340 (-545)	(182-) 218 (- 273)	(184-) 221 (-255)	
		Ornamentation	Very few hairs	Present	Hairless or with few single hairs	
	Perithecial neck	Length	(260–) 560 (–840)	(539-) 640 (-700)	(620-) 760 (-930)	
		Base width	(20–) 50 (-93)	(39-) 49 (–60)	(45–) 51 (–65)	
		Apex width	(8-) 27 (-54)	(15–) 25 (-28)	(22–) 25 (-28)	
	Ostiolar hyphae		Absent	Absent	Absent	
	Ascospores	Colour	Hyaline	Hyaline	Hyaline	
		Septation	Absent	Absent	Absent	
		Shape	Bean shaped, with brim	Bean shaped, with brim	Bean shaped, with appendages	
		Size	(2.0-) 3.5 (– 6.0) x (1.0-) 1.7 (– 3.0)	(4.0–) 4.6 (-5.3) x (2.1–) 2.5 (-3)	3.8 – 2.0 um, without sheath	
Anamorph	*Pesotum* anamorph		Conidia (2.0–) 4.0 (- 6.0) x (1.0–) 2.0 (-3.0)	Conidia one-celled, elongate, (4.0-) 4.6 (-5.2) x (1.9-) 2.0 (-2.2)	Real synnemata: stipe 200 – 400 long, head up to 100 - 500, hyaline or light greenish.	Stalks brown to black at base, hyaline in the upper part, up to 300 x 60, conidia same as those of *Leptographium*.
	Leptographium anamorph		Conidia cylindrical to ellipsoid (2.0–) 3.6 (-4.7) x (1.0-) 1.5 (–2.0)	Conidia one-celled, elongate, (4.0-) 4.6 (-5.2) x (1.9-) 2.0 (-2.2)	Simple conidiosphore: stipe 150 – 250 long, 4 - 8 septate, head up to 50 - 60, conidia egg-shaped, (4.0-) 4.8 (-5.5) x (2.0-) 2.2 (-2.4)	Stalks brown, thick-walled, up to 8 septate, up to 300 x 3-5, head up to 50, conidia hyaline, cylindrical to ellipsoid, 5 – 6 x 2.5 - 3
	'Cephalosporium' anamorph			Conidia one-celled, oval, hyaline, (2.2-) 2.7 (-3.1) x (1.5-) 1.7 (-2.0)	*Cephalosporium*-like head: one celled, hyaline, ova, (2.2-) 2.8 (-3.6) x (1.2-) 1.9 (- 2.3)	Hyaline (conidiphores and conidia), spores ellipsoid to oval, 3 - 3.5 x 2 - 2.5

Fig. 1. Phylogram of the *Ophiostoma galeiformis* complex based on analyses of ITS sequences (ITS1 and ITS2 regions, as well as 5.8S rRNA gene). *Ophiostoma cucullatum* was used as outgroup. Base substitution numbers are indicated above the branches and the bootstrap values (1000 bootstrap repeats) below the branches.



Figs. 2A – 2H. *Ophiostoma galeiformis* (CMW 5290) on 1.5 % MEA with pine twigs. A. Dark perithecia with long neck (Bar = 85 µm). B. Apex of the neck without ostiolar hyphae (Bar = 15 µm). C. Tendril of ascospore masses (Bar = 50 µm). D. Bean shaped ascospores (Bar = 2.5 µm). E. *Leptographium* anamorph (Bar = 7 µm). E. Conidia of *Leptographium* anamorph (Bar = 5 µm). F. *Pesotum* anamorph (Bar = 23 µm). G. Conidia of *Pesotum* anamorph (Bar = 4 µm).



Chapter 7

Pathogenicity of *Ophiostoma ips*, *Leptographium serpens* and *L. lundbergii* to pines in South Africa*

Three exotic bark beetles (Coleoptera: Scolytidae), *Hylastes angustatus*, *Hylurgus ligniperda*, and *Orthotomicus erosus*, infest *Pinus* spp. in South Africa. These beetles are generally considered as secondary pests, but can also act as vectors of ophiostomatoid fungi. In South Africa, at least 12 ophiostomatoid fungi are associated with the three beetle species, of which *Ophiostoma ips*, *Leptographium serpens*, and *L. lundbergii*, occur most frequently. The aim of this study was to test the pathogenicity of the three fungi to pines in South Africa. Two isolates of each fungus were inoculated on various species of pines in different areas of South Africa. The inoculated fungi caused resin exudation and sapwood discoloration around inoculation points. There were significant differences in lesion length between species inoculated, times of inoculation and plantation areas. Although *O. ips* gave rise to longer lesions than *L. serpens* and *L. lundbergii*, our results suggest that none of these species should be considered as serious pathogens.

Keywords: *Pinus*, bark beetles, *Hylastes angustatus*, *Hylurgus ligniperda*, *Orthotomicus erosus*.

* Zhou, X. D., De Beer, Z. W., Wingfield, B. D & Wingfield, M. J. (2002) Infection sequence and pathogenicitiy of *Ophiostoma ips*, *Leptographium serpens* and *L. lundbergii* to pines in South Africa. In: Fungal Succession (eds. K. D. Hyde and E. B. G. Jones). *Fungal Diversity* 10: 229-240.

INTRODUCTION

Three exotic bark beetle species, *Hylastes angustatus* (Herbst), *Hylurgus ligniperda* (Fabricius), and *Orthotomicus erosus* (Wollaston), native to Europe and the Mediterranean Basin, infest *Pinus* spp. in South Africa (Tribe, 1992). *Hylurgus ligniperda* and *O. erosus* are generally considered as secondary pests. *Hylastes angustatus*, however, is more aggressive than the other two bark beetle species, and is considered as a primary pest. This insect damages pine seedlings during maturation feeding and thus, causes significant losses in newly established pine plantations (Anonymous, 1946; Tribe, 1992).

Bark beetles are well-known vectors of fungi, and particularly *Ophiostoma* and *Ceratocystis* spp. (Münch, 1907; Whitney, 1982; Harrington, 1988; Beaver, 1989; Wingfield *et al.*, 1993; Paine *et al.*, 1997; Jacobs and Wingfield, 2001). These fungi generally sporulate in the galleries of their bark beetle vectors and are either carried in mycangia, on the exoskeletons, or in the guts of the beetles (Beaver, 1989; Paine *et al.*, 1997). The relationship between ophiostomatoid fungi and their bark beetle vectors, however, varies among different hosts, fungal species and their insect vectors (Harrington, 1993a; Wingfield *et al.*, 1995; Paine *et al.*, 1997).

Many ophiostomatoid species cause sapstain of freshly cut wood (Münch, 1907; Lagerberg *et al.*, 1927; Seifert, 1993). Several species are also pathogenic to plants. *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, which cause Dutch elm disease, have killed millions of elm trees in the Northern Hemisphere during the past century (Brasier, 1990; Brasier & Mehrotra, 1995). Three host-specific varieties of *Leptographium wageneri* (Kendrick) M. J. Wingfield, which cause black stain root disease of conifers, have led to severe losses to forestry in United States and Canada (Harrington and Cobb, 1988). Less pathogenic species such as *O. minus* (Hedgcock) H. & P. Sydow, *L. wingfieldii* M. Morelet and *L. terebrantis* Barras & Perry,

can cause significant lesions, or even kill the trees when mass inoculated (Wingfield, 1986; Harrington, 1993b; Solheim *et al.*, 1993).

In South Africa, at least 12 ophiostomatoid species are associated with the three pine-infesting bark beetles (Zhou *et al.*, 2001). Of the 12 species, *Ophiostoma ips* (Rumb.) Nannf., *L. lundbergii* Lagerb. & Melin and *L. serpens* (Goid.) M. J. Wingfield, are most frequently encountered (Zhou *et al.*, 2001). These three species have been reported to be pathogenic to conifers in many parts of the world (Mathre, 1964; Lorenzini and Gambogi, 1976; Lieutier *et al.*, 1989; Kaneko and Harrington, 1990; Otrosina *et al.*, 1997).

A number of preliminary pathogenicity trials have been conducted with these species on pines in South Africa (Wingfield and Knox-Davies, 1980; Wingfield and Marasas, 1980, 1983; Wingfield and Swart, 1989; Dunn *et al.*, 2002). Little is, however, known regarding their relative importance or pathogenicity to pines in the area. The aim of this study was, therefore, to test and compare the pathogenicity of the three most frequently encountered fungal associates of pine-infesting bark beetles in South Africa. These tests were conducted on two-year-old pines representing a number of key species and in two different geographic areas.

MATERIALS AND METHODS

Screening of fungal isolates

All isolates used in this study were obtained during a survey of ophiostomatoid fungi associated with the three pine infesting bark beetle species in South Africa (Zhou *et al.*, 2001). Fungal isolates were selected based on their relative growth rate in culture, because this was shown in preliminary trials (Wingfield, unpublished) to correlate strongly with pathogenicity. Initially, 139 isolates of *O. ips*, 116 of *L. serpens*, and 138 of *L. lundbergii* were screened on 2 % MEA (Malt Extract Agar: 20 g Biolab malt extract, 20 g Biolab agar and 1000 mL distilled water) at

25 C in the dark for two weeks. The two fastest-growing isolates of each species were chosen for the pathogenicity trials. All isolates used in this study are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, Republic of South Africa.

Inoculation experiments

Pinus spp., which are known hosts of the bark beetles and the three fungal species, were chosen for the pathogenicity trials. The availability of trees, as well as locations where the bark beetles occur, were also considered. Field inoculations were conducted in two-year-old plantations in the Western Cape province (Knysna, 23° 04' 00" E, 33° 56' 00" S) and Mpumalanga province (Sabie, 30° 39' 00" E, 25° 08' 00" S), South Africa. In Knysna, two pine species, *P. radiata* and *P. elliottii*, were selected for inoculations. In Sabie, *P. elliottii*, and a hybrid of *P. elliottii* and *P. caribaea*, were used.

Inoculum was prepared by growing fungal isolates on 2 % MEA at 25 C in the dark for two weeks. After this period, cultures had commenced sporulation and the agar surface was covered with dark mycelium.

Twenty trees of each pine species were inoculated with each of the six isolates. An equal number of trees of each species served as controls. One branch per tree, with an average of 20 mm in diameter, was inoculated. A plug of bark was removed, using a sterile 10 mm-diameter cork borer, to expose the cambium. An agar plug of equal size, bearing the test fungus, was placed mycelium side down, in each wound. Sterile agar plugs were used as controls. All inoculation points were sealed with masking tape to reduce desiccation. Six weeks after first inoculation, all trials were repeated by inoculating a second branch of the same trees inoculated during the first trial.

Branches were examined six weeks after inoculation by removing the bark and exposing the cambium. Lesion lengths and branch diameters were measured. Reisolations were done by transferring pieces of freshly cut, discoloured cambium to 2 % MEA. Cultures were incubated at 25 C for two weeks, after which they were microscopically examined to confirm that the lesions had been caused by the inoculated fungi.

Data analysis

All data sets were analysed separately. Isolates belonged strictly to a specific fungal species and measurements were done on two different inoculated branches of the same tree. Therefore, a hierarchical ANOVA was employed for the analysis. The treatment variances differed somewhat, and to improve the accuracy of the ANOVA by eliminating the effect of branch diameter, branch diameter was used as a covariate in an ANCOVA. However, branch diameter was non-significant, and had no influence over lesion measurements. Differences between times of trials, species and isolates were evaluated by using a multiple comparison method adjusted to maintain the accuracy of the comparisons (Tukey – Kramer) (Anonymous, 1989).

RESULTS

Screening of fungal isolates

The fastest growing isolates of *L. lundbergii* (CMW6185 and CMW6186) and *L. serpens* (CMW6187 and CMW6188), originated from *H. angustatus* infesting *P. patula* in Mpumalanga province. Isolates of *O. ips* selected, however, came from *O. erosus* infesting *P. patula* in Mpumalanga province (CMW6189), and *P. elliottii* in Kwazulu-Natal (CMW6190), respectively.

Inoculation experiments

Six weeks after inoculation, resin exudation was visible, and the inoculated fungi caused discoloration of sapwood on inoculated branches. However, no signs of dieback were seen. The branches inoculated with test fungi had more resin around inoculation points than controls. Reisolations from inoculated branches consistently yielded the inoculated fungi.

Ophiostoma ips was more pathogenic than *L. serpens* and *L. lundbergii*, and generally gave rise to longer lesions. The lesion length average of *O. ips* from the four sites was 33.3 mm, varying between 28.7 mm and 48.5 mm. For *L. serpens*, lesion length average was 27.8 mm (between 15.2 mm and 44.9 mm), and for *L. lundbergii*, it was 29.3 mm (between 15.4 mm and 37.2 mm) (Tables 1, 2). In the Sabie area, however, *O. ips* (with lesion length average 31.1 mm) caused slightly shorter lesions than *L. serpens* (32.7 mm) and *L. lundbergii* (31.4 mm) (Table 2).

The hybrid of *P. elliottii* and *P. caribaea* was generally more susceptible to the test fungi than *P. elliottii* and *P. radiata*. This hybrid had a lesion length average of 32.6 mm, while the average lesion lengths for *P. radiata*, and *P. elliottii* in Sabie, and *P. elliottii* in Knysna, were 28.9 mm, 30.1 mm, 28.9 mm, respectively. Interestingly, *P. elliottii* in Sabie (with lesion length average 29.6 mm) was more resistant to *O. ips* than it was in Knysna (38.9 mm). However, *P. elliottii* was more susceptible to *L. serpens* in Sabie (31.9 mm), than it was in Knysna (21.4 mm) (Table 2).

In general, longer lesion lengths were recorded in the Sabie area than in the Knysna area. In Sabie, the lesion length average was 31.4 mm, while it was 28.9 mm in the Knysna area. *Ophiostoma ips*, however, gave rise to a different trend. In the Knysna area, the fungus had a lesion length average of 36.5 mm, while it was 31.1 mm in the Sabie area (Table 2).

Multiple comparisons of lesion length showed that there were no significant differences between the lesion lengths for the two trials with *L. lundbergii* and *L. serpens*, or for *O. ips* in the

Sabie area (Table 3). However, for *O. ips*, there were significant differences on the two pine species in the Knysna area (P = 0.0012, P = 0.0001) (Table 3).

Combined analysis of variance (Table 4) for lesion length of the two trials at each of the four sites, showed that there are significant differences between experiment site (p = 0.0003), species (p = 0.0001), site x species (p = 0.0001), times of trials (p = 0.0001), species x times of trials (p = 0.0009), site x times of trials (p = 0.0002), and site x species x times of trials (p = 0.0364). No significant differences were found between trees at each site (Table 4).

DISCUSSION

Results of this study showed that *O. ips*, *L. serpens* and *L. lundbergii* can cause lesions in the cambium of *Pinus* spp. in South Africa. However, none of the three species inoculated caused outward symptoms such as die-back on trees. This suggests that they are weak pathogens and confirms the results of previous studies where these fungi have been tested separately on a limited number of tree species (Wingfield and Knox-Davies, 1980; Wingfield and Marasas, 1980, 1983; Wingfield and Swart, 1989). Of the three species tested, *O. ips* caused the longest lesions. *Leptographium serpens* and *L. lundbergii* gave rise to similar lesion lengths, which were generally shorter than those associated with *O. ips*.

Our results have shown that *O. ips* can cause lesions, but is not particularly pathogenic to pines in South Africa. This is in agreement with the studies of Wingfield and Marasas (1980), Rane and Tattar (1987), Parmeter *et al.* (1989), and Dunn *et al.* (2002). There are other studies, however, showing that the fungus was pathogenic to pines. In western Japan, *O. ips*, the associate of an *Ips* sp. infesting *P. densiflora* and *P. thunbergii*, infests the roots and has been reported to cause death of living pine trees in forests (Nisikado and Yamauti, 1933). The fungus

has also been shown to significantly inhibit sapflow of infected *Pinus ponderosa* (Mathre, 1964). In France, it is pathogenic to Scots pines and possibly plays a role in the establishment of *Ips sexdentatus* (Boerner) on trees (Lieutier *et al.*, 1989). In the United States, *O. ips*, together with *L. terebrantis* and *L. procerum* (Kendrick) M. J. Wingfield, is important in the dynamics of susceptibility of southern pines to the attack by the southern pine beetle, *Dendroctonus frontalis* (Zimmermann) (Otrosina *et al.*, 1997).

Neither *L. lundbergii* nor *L. serpens* was pathogenic to living healthy pines in South Africa. This is interesting, since *L. serpens* has been recorded to be associated with a root disease of *P. pinea* in Italy (Lorenzini and Gambogi, 1976), and *P. pinaster* and *P. radiata* in South Africa (Wingfield and Knox-Davies, 1980; Wingfield *et al.*, 1988). *Leptographium lundbergii* has been found to be weakly pathogenic to severely stressed red and black pines in Japan (Kaneko and Harrington, 1990).

Ophiostoma ips, which was more pathogenic than *L. serpens* and *L. lundbergii*, is primarily vectored by the non-aggressive *O. erosus* (Zhou *et al.*, 2001). The two *Leptographium* spp. are mainly isolated from *H. angustatus*, which is considerably more aggressive than *O. erosus* (Zhou *et al.*, 2001). This situation, where the less aggressive bark beetle carries the more virulent fungus, has also been observed in other studies (Owen, 1987; Harrington, 1993a, b). Owen (1987) found that the more virulent fungus, *L. terebrantis*, was vectored by a less aggressive bark beetle, *Dentroctonus valens* (LeConte). There are, however, also studies indicating that more aggressive conifer–infesting bark beetle species vector more virulent fungi (Krokene and Solheim, 1998; Solheim *et al.*, 2001). For example, *Ophiostoma canum* (Münch) H. & P Sydow, the major associate of *Tomicus minor* (Hartig), was found to be less virulent than *L. wingfieldii* and *O. minus*, the main associates of *T. piniperda* (Linnaeus) (Solheim *et al.*, 2001). Långström and Hellqvist (1993) showed that *T. minor* is less aggressive than *T. piniperda*.

In our study, the hybrid of *P. elliottii* and *P. caribaea* was more susceptible to the test fungi than *P. elliottii* and *P. radiata*. *Pinus elliottii* was more resistant to *O. ips*, while more susceptible to *L. serpens* in the Sabie area than in the Knysna area. These results suggest that different hosts differ in their response to fungal penetration. This is in agreement with the study of Raffa and Smalley (1995), where *P. resinosa* and *P. banksiana* showed different response patterns to *O. ips* and *O. nigrocarpum* (R. W. Davidson) De Hoog.

In the Sabie area, the tested fungi caused longer lesions than in the Knysna area, with the exception of *O. ips*. This might be explained by interactions between hosts, fungal species, climatic and other conditions in the two areas. This would be consistent with the fact that forest stand density has an influence on the infection by blue-stain fungi (Christiansen, 1985), that high water tables can increase the rate of black-stain root disease (Kulhavy *et al.*, 1978), and that stand conditions affect the expression of host resistance (Peter and Lorio, 1993).

Our results have shown that between the first and second trials, there were no significant differences in lesion length for *L. serpens* and *L. lundbergii* in the two areas, and of *O. ips* in the Sabie area. However, lesion lengths for the two trials using *O. ips* differed significantly in the Knysna area. The differences could be due to the interactions of hosts, fungi, and stand conditions, rather than seasonal difference. This is in agreement with the study of Parmeter *et al.* (1989), though other reports suggest seasonal difference affects the host response (Paine, 1984; Lorio, 1986).

Analysis of the combined ANOVA confirmed that interactions were significant, not only between sites, times of trials, fungal species inoculated, but also between site x times of trials, site x species, species x times of trials, and site x times of trials x species. Similar results have been found by Dunn *et al* (2002). They showed that pathogenicity of *O. piliferum* (Fr.) H. & P. Sydow, *O. ips*, and *Sphaeropsis sapinea* (Fr.: Fr.) Dyko & Sutton interacted strongly with host species, location, and season.

Overall, our results have confirmed that *O. ips*, *L. serpens* and *L. lundbergii* should not be considered as serious pathogens of above ground parts of *P. elliottii*, *P. radiata*, or the *P. elliottii* / *P. caribaea* hybrid in South Africa. But both *O. ips* and *L. lundbergii* are well-known sapstain agents on pines (Lagerberg *et al.*, 1927; Davidson, 1935; Gibbs, 1993; Seifert, 1993; Farrell *et al.*, 1997). Therefore, the ophiostomatoid fungi, together with their bark beetle vectors, should be taken into account when disease resistant clones, or control strategies against sapstain, are developed.

REFERENCES

Anonymous. (1946) The black pine beetle (*Hylastes ater*) and other closely allied beetles. *Forestry Commission Leaflet No. 4*. His Majesty's Stationery Office, London: 1-10.

Anonymous. (1989) SAA/STAT Users Guide, SAS INSTITUTE INC., Version 6.12, 4th Edition, Volume 1 and 2, Cary, NC, USA. ISBN 1-55544-376-1.

Beaver, R. A. (1989) Insect-fungus relationships in the bark and ambrosia beetles. In *Insect-Fungus Interactions* (N. Wilding, N. M. Collins, P. M. Hammond & J. F. Webber, eds): 121-143. Academic Press, London.

Brasier, C. M. (1990) China and the origins of Dutch elm disease: an appraisal. *Plant Pathology* **39**: 5-16.

Brasier, C. M. & Mehrotra, M. D. (1995) *Ophiostoma himal-ulmi* sp. nov., a new species of Dutch elm disease fungus endemic to the Himalayas. *Mycological Research* **99**: 205-215.

Christiansen, E. (1985) *Ceratocystis polonica* inoculated in Norway spruce: Blue-staining in relation to inoculum density, resinosis and tree growth. *European Journal of Forest Pathology* **15**: 160-167.

Davidson, R. W. (1935) Fungi causing stain in logs and lumber in the Southern States, including five new species. *Journal of Agricultural Research* **50**: 789-807.

Dunn, C., Wolfaardt, F. & Wingfield, M. J. (2002) Pathogenicity of *Ophiostoma piliferum* compared with that of other South African sap-staining fungi. *South African Journal of Science* **98**: 401-403.

Farrell, R. L., Hadar, E., Kay, S. J., Blanchette, R. A. & Harrington, T. C. (1997) Survey of sapstain organisms in New Zealand and albino anti-sapstain fungi. Biology and prevention of sapstain. A conference sponsored by the Department of Forest Products, Oregon State University, held May 25, 1997, Delta Whistler Resort, Whistler, British Columbia, Canada: 57-62.

Gibbs, J. N. (1993) The biology of Ophiostomatoid fungi causing sapstain in trees and freshly cut logs. In *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 153-160. American Phytopathological Society Press, St. Paul, Minnesota.

Harrington, T. C. (1988) *Leptographium* species, their distributions, hosts and insect vectors. In *Leptographium root diseases on conifers* (T. C. Harrington & Jr. F. W. Cobbs., eds): 1-39. American Phytopathological Society Press, St. Paul, Minnesota.

Harrington, T. C. (1993a) Biology and taxonomy of fungi associated with bark beetles. In *Beetle-Pathogen Interactions in Conifers Forests* (T. D. Schowalter & G. M. Filip, eds): 37-58. Academic Press, New York.

Harrington, T. C. (1993b) Diseases of conifers caused by species of *Ophiostoma* and *Leptographium*. In *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 161-172. American Phytopathological Society Press, St. Paul, Minnesota.

Harrington, T. C. & Cobbs Jr., F. W. (1988) *Leptographium* root diseases on conifers American Phytopathological Society Press, St. Paul, Minnesota.

Jacobs, K. & Wingfield, M. J. (2001) *Leptographium* species - tree pathogens, insect associates and agents of blue-stain. American Phytopathological Society Press, St. Paul, Minnesota.

Kaneko, S. & Harrington, T. C. (1990) *Leptographium truncatum* isolated from Japanese red and black pines. *Report of the Tottori Mycological Institute* **28**: 171-174.

Krokene, P. & Solheim, H. (1998) Pathogenicity of four blue-stain fungi associated with aggressive and non-aggressive bark beetles. *Phytopathology* **88**: 39-44.

Kulhavy, D. L., Partridge, A. D. & Stark, R. W. (1978) Mountain pine beetle and disease management in lodgepole pine stands: inseparable. In *Theory and practice of mountain pine beetle management in lodgepole pine forests*. Symposium Proceedings. Forest, Wildlife, and Range Experiment Station, University of Idaho, Moscow: 177-181.

Lagerberg, T., Lundberg, G. & Melin, E. (1927) Biological and practical researches into blueing in pine and spruce. *Svenska Skogsvårdsföreningens Tidskrift* 25: 145-272.

Långström, B. & Hellqvist, C. (1993) Induced and spontanous attack by pine shoot beetles on young Scots pine trees: tree mortality and beetle performance. *Journal of Applied Entomology* **115**: 25-36.

Lieutier, F., Yart, A., Garcia, J., Ham, M. C., Morelet, M. & Levieux, J. (1989) Champignons phytopathogènes associés à deux coléoptères Scolytidae du Pin sylvestre (*Pinus sylvestris* L.) et étude préliminaire de leur agressivité envers l'hôte. *Annales des Sciences Forestieres* **46**: 201-216.

Lorenzini, G. & Gambogi, P. (1976) Decline of *Pinus pinea* associated with the presence of *Verticicladiella* sp. (preliminary note) (Italian). *Informatore Fitopatologico* **5**: 5-8.

Lorio, P. L., Jr. (1986) Growth-differentiation balance: A basis for understanding southern pine beetle-tree interactions. *Forest Ecology and Management* **14**: 259-273.

Mathre, D. E. (1964) Pathogenicity of *Ceratocystis ips* and *Ceratocystis minor* to *Pinus ponderosa*. *Contributions Boyce Thompson Institute* **22**: 363-388.

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

Nisikado, Y. & Yamauti, K. (1933) Contributions to the knowledge of the sap stains of wood in Japan. I. Studies on *Ceratostomella ips* Rumbold, the cause of a blue stain of pine trees in western Japan. *Berichte des Ohara Instituts für Landwirtschaftliche Forschungen* **5:** 501-538.

Otrosina, W. J., Hess, N. J., Zarnoch, S. J., Perry, T. J. & Jones, J. P. (1997) Blue-stain fungi associated with roots of southern pine trees attacked by the southern pine beetle, *Dendroctonus frontalis*. *Plant Disease* **81**: 942-945.

Owen, D. R., Lindahl, K. Q., Wood, D. L. & Parmeter, J. R. Jr. (1987) Pathogenicity of fungi isolated from *Dentroctonus valens*, *D. brevicomis* and *D. ponderosae* to ponderosa pine seedlings. *Phytopathology* **77**: 631-636.

Paine, T. D. (1984) Seasonal response of ponderosa pine to inoculation of the mycangial fungi from the western pine beetle. *Canadian Journal of Botany* **62**: 551-555.

Paine, T. D., Raffa, K. F. & Harrington, T. C. (1997) Interactions among Scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* **42**: 179-206.

Parmeter, J. R., Slaughter, G. W., Chen, M.-M., Wood, D. L. & Stubbs, H. A. (1989) Single and mixed inoculations of ponderosa pine with fungal associates of *Dendroctonus* spp. *Phytopathology* **79**: 768-772.

Peter, L. & Lorio, J. R. (1993) Environmental stress and whole-tree physiology. In *Beetle-pathogen interactions in conifer forests* (T. D. Schowalter & G. M. Filip, eds): 81-101. Academic Press, New York.

Raffa, K. F. & Smalley, E. B. (1995) Interaction of pre-attack and induced monoterpene concentrations in host conifer defense against bark beetle-fungal complexes. *Oecologia* **102**: 285-295.

Rane, K. K. & Tattar, T. A. (1987) Pathogenicity of blue stain fungi associated with *Dendroctonus terebrans*. *Plant Disease* **71**: 879-883.

Seifert, K. A. (1993). Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 141-151. American Phytopathological Press, St. Paul, Minnesota.

Solheim, H., Krokene, P. & Långström, B. (2001) Effect of growth and virulence of associated blue-stain fungi on host colonization behaviour of the pine shoot beetles *Tomicus minor* and *T. piniperda*. *Plant Pathology* **50**: 111-116.

Solheim, H., Långström, B. & Hellqvist, C. (1993) Pathogenicity of the blue-stain fungi *Leptographium wingfieldii* and *Ophiostoma minus* to Scots pine: effect of tree pruning and inoculum density. *Canadian Journal of Forest Research* **23**: 1438-1443.

Tribe, G. D. (1992) Colonization sites on *Pinus radiata* logs of the bark beetles, *Orthotomicus erosus*, *Hylastes angustatus* and *Hylurgus ligniperda* (Coleoptera: Scolytidae). *Journal of the Entomological Society of Southern Africa* **1**: 77-84.

Whitney, H. S. (1982) Relationships between bark beetles and symbiotic organisms. In *Bark beetles in North American conifers*. (J. B. Mitton & K. B. Sturgeon, eds): 183-211. University of Texas Press, Austin, Texas.

Wingfield, M. J. (1986) Pathogenicity of *Leptographium procerum* and *L. terebrantis* on *Pinus strobus* seedlings and established trees. *European Journal of Forest Pathology* **16**: 299-308.

Wingfield, M. J., Capretti, P. & MacKenzie, M. (1988) *Leptographium* spp. as root pathogens on conifers. In *Leptographium root diseases on conifers* (T. C. Harrington & F. W. Cobb, eds): 113-128. American Phytopathological Society Press, St. Paul, Minnesota.

Wingfield, M. J., Harrington, T. C. & Solheim, H. (1995) Do conifer bark beetles require fungi to kill trees? In *Proceedings from a symposium held at the Norwegian Forest Research Institute (NISK) in Ås, Norway, 31 July – 2 August, 1995* (E. Christiansen, ed). *Aktuelt fra Skogforsk* 6/95: 6.

Wingfield, M. J. & Knox-Davies, P. S. (1980) Root-disease, associated with *Verticicladiella alacris*, of pines in South Africa. *Plant Disease* **64**: 569-571.

Wingfield, M. J. &. Marasas, W. F. O. (1980) *Ceratocystis ips* associated with *Orthotomicus erosus* (Coleoptera: Scolytidae) on *Pinus* spp. in the Cape Province of South Africa. *Phytophylatica* **12**: 65-69.

Wingfield, M. J. & Marasas, W. F. O. (1983) Some *Verticicladiella* species, including *V. truncata* sp. nov., associated with root diseases of pine in New Zealand and South Africa. *Transactions of the British Mycological Society* **80**: 231-236.

Wingfield, M. J., Seifert, K. A. and Webber, J. F. (1993) *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology and Pathogenicity. American Phytopathological Society Press, St. Paul, Minnesota.

Wingfield, M. J. & Swart, W. J. (1989) Relative pathogenicity of fungi associated with pine root-infesting insects in South Africa. In *Proceedings of the Seventh International Conference Root and Butt Rots* (D. J. Morrison, ed): 381-391. British Columbia, Canada.

Zhou, X. D., De Beer, Z. W., Wingfield, B. D. & Wingfield, M. J. (2001). Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. *Sydowia* **53**: 290-300.

Table 1. Lesion length means (mm) for different isolates of *Ophiostoma ips*, *Leptographium lundbergii*, and *L. serpens* at the various inoculation sites.

Fungal Species	Isolate No.	Trial [a] No.	No. of trees	Lesion Length Mean			
				Sabie (*P. elliottii* x *P. caribaea*)	Sabie (*P. elliottii*)	Knysna (*P. radiata*)	Knysna (*P. elliottii*)
L.Lundbergii	CMW6185	1	20	28.4	24.6	22.3	15.4
L. lundbergii	CMW6186	1	20	36.6	37.2	34.0	33.8
L. lundbergii	CMW6185	2	20	36.2	26.2	28.6	27.8
L. lundbergii	CMW6186	2	20	34.4	27.9	26.9	28.5
L. serpens	CMW6187	1	20	31.0	28.1	21.3	15.2
L. serpens	CMW6188	1	20	27.8	33.7	21.4	20.2
L. serpens	CMW6187	2	20	30.1	30.0	25.6	25.2
L. serpens	CMW6188	2	20	44.9	35.8	29.9	25.1
O. ips	CMW6189	1	20	31.6	29.7	28.8	32.4
O. ips	CMW6190	1	20	29.7	29.2	28.8	33.6
O. ips	CMW6189	2	20	31.8	30.6	44.5	48.5
O. ips	CMW6190	2	20	29.1	28.7	34.3	41.2

[a] The first set of trials at each site is referred to as 1, and the repetition of the complete trial on the second branch of each tree, is referred to as 2.

Table 2. Lesion lengths (mm) associated with inoculation of *Ophiostoma ips*, *Leptographium lundbergii*, and *L. serpens* onto various pine species in two geographic areas of South Africa.

	Area			Area			
Species	Sabie (*P. elliottii* x *P. caribaea*)	Sabie (*P. elliottii*)	Mean (Sabie)	Knysna (*P. radiata*)	Knysna (*P. elliottii*)	Mean (Knysna)	Mean
L.Lundbergii	33.9	29.0	31.4	28.0	26.4	27.2	29.3
L. serpens	33.5	31.9	32.7	24.6	21.4	23.0	27.8
O. ips	30.6	29.6	31.1	34.1	38.9	36.5	33.3
Mean	32.6	30.1	31.4	28.9	28.9	28.9	

Table 3. Comparison of the differences between lesion lengths (mm) after inoculations with *Ophiostoma ips*, *Leptographium lundbergii*, and *L. serpens* at two different times.

Species	Index	Sabie (*P. elliottii* x *P. caribaea*)	Sabie (*P. elliottii*)	Knysna (*P. radiata*)	Knysna (*P. elliottii*)
L. lundbergii	[a] D	-2.7 ([c] 32.5, [d] 35.2)	3.9 (30.9, 27.0)	0.2 (28.1, 27.9)	-3.1 (25.0, 28.1)
	[b] P	1.0000	0.9931	1.0000	0.9997
L. serpens	D	-7.9 (29.3, 37.2)	-2.0 (30.9, 32.9)	-6.4 (21.3, 27.7)	-7.4 (17.7, 25.1)
	P	0.1123	1.0000	0.4476	0.1650
O. ips	D	0.2 (30.6, 30.4)	-0.3 (29.4, 29.7)	-10.6 (28.8, 39.4)	-11.8 (33.0, 44.8)
	P	1.0000	1.0000	0.0012	0.0001

[a] D - Difference of the lesion length means between the first and second trials.
[b] P - Probability value.
[c] Lesion length mean of the first trial.
[d] Lesion length mean of the second trial.

Table 4. Combined ANOVA for lesion length measurements of the two trials at each of the four sites.

	[b] DF	[c] SS	[d] MS	[e] F	[f] P
Site	3	2001.37	667.12	6.33	0.0003
Trees at each site	76	8597.14	113.12	1.07	0.3193
Species	2	5085.55	2542.77	24.13	0.0001
Site x Species	6	12382.22	2063.70	19.58	0.0001
[a] Times	1	3771.78	3771.78	35.79	0.0001
Species x Times	2	1487.75	743.88	7.06	0.0009
Site x Times	3	2115.42	705.14	6.69	0.0002
Site x Species x Times	6	1425.77	237.63	2.26	0.0364

[a] Times - Initial and repeated inoculations
[b] DF - Degree of Freedom
[c] SS - Sum of Squares
[d] MS - Mean Square
[e] F - F value
[f] P - Probability Value

Chapter 8

Development of polymorphic microsatellite markers for the tree pathogen and sapstain agent, *Ophiostoma ips*[*]

Twelve pairs of simple sequence repeat markers (SSR) were developed using a single ascospore isolate of *Opiostoma ips*, isolated from the bark beetle, *Orthotomicus erosus*, infesting *Pinus elliottii* in South Africa. All markers were found to be polymorphic when tested on 7 isolates of *O. ips* collected from Austria, Chile, Israel, Mexico, South Africa, Sweden, and USA.

Keywords: SSR, *Orthotomicus*, *Pinus*, polymorphic.

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Opiostoma ips, is a fungus commonly associated with pine-infesting bark beetles in the Northern Hemisphere. These insects are important forest pests and their associated fungi degrade wood through sapstain (Seifert, 1992) and might also contribute to tree death (Zhou *et al.*, 2001). *Ophiostoma ips* has been introduced into Southern Hemisphere pine-growing countries such as South Africa, together with bark beetles (Wingfield & Marasas, 1980; Tribe, 1990). The close association between the beetles and the fungus, provides a unique system to examine the frequency and number of introductions into a new area. In order to understand patterns of introduction and spread, the diversity of the fungal population must be studied. The most effective means to achieve this goal is to develop co-dominant molecular markers. Thus, the aim of this study was to develop polymorphic microsatellite markers for *O. ips*.

DNA from the single ascospore isolate (CMW6418) was randomly amplified using ISSR primers 5' DHB$(CGA)_5$, 5' DDB$(CCA)_5$, 5' DBD$(CAC)_5$, 5' NDB$(CA)_7$C, 5' NDV$(CT)_8$, 5' HBDB$(GACA)_4$, and M13 (Meyer & Mitchell, 1995; Buscot *et al.*, 1996; Hantula, Dusabenyagasani & Hamelin, 1996). PCR volume of 50 uL consisted of 5 ng DNA, 0.2 mM of each dNTP, 0.6 uM primer, 3.5 U Expand High Fidelity PCR System enzyme mix, and 5 uL of Expand HF buffer, 10 X conc., with 15 mM $MgCl_2$ (Roche Molecular Biochemicals, Alameda, CA). PCR reactions were performed on an Eppendorf Mastercycler® Personal (Perkin-Elmer, Germany), and conditions were as follows: 95°C for 2 min followed by 40 cycles of 30 s at 95°C, 45 s at 48°C, and 2 min at 95°C, and a final step at 72°C for 10 min. PCR products were visualised under UV illumination on 1 % agrose gel (Promega, Madison, Wisconsin), purified using High Pure PCR Product Purification Kit (Boehringer, Mannheim, Germany), and different sizes of products were cloned using the pGEM®-T Easy Vector System (Promega Corporation, Madison, Wisconsin, USA).

Bacterial colonies containing recombinant plasmids were selected using the technique described by Burgess *et al.* (2001), and plasmid DNA was recovered using alkaline lysis

(Sambrook & Russell, 2001). Plasmid DNA was then digested with *Eco* RI (Roche Molecular Biochemicals, Alameda, CA) to release the inserts. Different sized inserts were sequenced using an ABI PRISM™ 377 Autosequencer (Applied Biosystems, Inc., Forster City, Calif.) with the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) using the T7 and Sp6 primers.

Sequence electropherograms were analysed using Sequence Navigator version 1. 0. 1 (Applied Biosystems) and screened for microsatellite regions. SSR primer pairs were then designed to flank the microsatellite regions. For some sequences, the microsatellite region of interest was at the beginning or end of the insert. In these cases, genome walking was used to obtain the full repeat sequence (Burgess *et al.*, 2001). In total, twelve SSR primer pairs were designed, based on these sequences, to amplify a DNA fragment in the range of between 180-450 bp (Tab. 1). The primer pairs were designed to amplify a variety of tandem repeats including GA, CT, GT and GTT (Tab. 1).

SSR-PCR was conducted with DNA from 7 isolates of *O. ips*, 5 isolates believed to be native in Austria, Israel, Mexico, Sweden, and USA, and 2 isolates from introduced populations in Chile and South Africa. PCR volume of 25 uL consisted of 2 ng DNA, 0.1 mM of each dNTP, 0.3 uM primer, 0.7 U Expand High Fidelity PCR System enzyme mix, and 2.5 uL of Expand HF buffer, 10 X conc., with 15 mM $MgCl_2$ (Roche Molecular Biochemicals, Alameda, CA). PCR conditions were the same as those described by Barnes *et al.* (2001), except that an annealing temperature of 60°C was used for all primers. Polymorphisms of primer pairs were identified on PAGE gel (6 % acrylamide in 50 mM Tris-borate-EDTA buffer, 7 h at 140 v) followed by silver staining (Blum, Beier & Gross, 1987). All twelve primer pairs proved to be polymorphic (Tab 1) and one of each primer pair was labelled with a phosphoramidite fluorescent dye, HEX or FAM (MWG, Ebersberg, Germany).

Fluorescent-labelled PCR products of those 7 isolates with 12 primer pairs were separated on an ABI PRISM™ 377 sequencer as described by Burgess *et al.* (2001). Sizes of alleles were determined by using a combination of GeneScan® 2.1 analysis software (Perkin Elmer Corp.) and Genotyper® 3.0 (Perkin Elmer Corp.), comparing to the TAMRA internal size standard.
At each of the 12 loci amplified by the markers, 2-4 alleles were amplified to give a total of 35 alleles across all loci (Tab. 2). For each isolate, a data matrix of characters was compiled by scoring the presence or absence of each allele at each locus. Parsimony analysis was performed on the data set using PAUP* (Swofford, 1998). The most parsimonious trees were obtained using heuristic searches with random addition in 1000 replicates, with the tree bisection-reconnection branch-swapping option "on" and the steepest-descent option "off". Bootstrap consensus trees were constructed using the same conditions.

The data matrix comprised of 35 characters, each character representing an individual allele at one of the 12 polymorphic SSR loci. Of the 35 characters, 13 were parsimony-informative. Heuristic searches using parsimony resulted in 9 trees of 39 steps (Fig 1). Bootstrap analysis supported strong branches separating the American from the European isolates. Isolates from introduced populations in South Africa and Chile clustered with the European isolates, in particular the isolate from Austria.

The primary aim of this study was to produce microsatellite markers for future *O. ips* population analyses. However, patters emerging from this preliminary study suggest that isolates from different geographic regions have different profiles. If proves to be the case, it should be possible to determine the origin of introduced populations in the Southern Hemisphere. The primers developed can now be used in population studies of *O. ips* from many parts of the world.

REFERENCES

Barnes, I., Gaur, A., Burgess, T., Roux, J., Wingfield, B. D. & Wingfield, M. J. (2001) Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt pathogen, *Ceratocystis fimbriata*. *Molecular Plant Pathology* **2**: 319-325.

Blum, H., Beier, H. & Gross, H. J. (1987) Improved silver staining of plant proteins, RNA and DNA in poluacrylamide gels. *Electrophoresis* **8**:93-99.

Burgess, T., Wingfield, M. J. & Wingfield, B. D. (2001) Simple Sequence Repeat Markers Distinguish among Morphotypes of *Sphaeropsis sapinea*. *Applied and Environmental Microbiology* **67**: 354-362

Buscot, F., Wipf, D., Battista, C. D., Munch, J. C., Botton, B. & Martin, F. (1996) DNA polymorphism in morels: PCR/RFLP analysis of the ribosomal DNA spacers and microsatellite-primed PCR. *Mycological Research* **100**: 63-71.

Hantula, J., Dusabenyagasani, M. & Hamelin, R. C. (1996) Random amplified microsatellites (RAMS) – a nove method for characterizing genetic variation within fungi. *European Journal For Forestry Pathology* **26**: 159-166.

Meyer, W. & Mitchell, T. G. (1995) Polymerase chain reaction fingerprinting in fungi using single primers specific to minisatellites and simple repetitive DNA sequences: Strain variation in *Crytococcus neoformans*. *Electrophoresis* **16**: 1648-1656.

Sambrook, J. & Russell, D. W. (eds). (2001) Molecular Cloning-A laboratory manual. Third Edition. Cold Spring Harbour Laboratory Press: New York, **1**: 1.32-1.34.

Seifert, K. A. (1993) Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In *Ceratocystis and Ophiostoma. Taxonomy, Ecology and Pathogenicity* (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 141-151. American Phytopathological Society Press, St. Paul, Minnesota.

Swofford, D. L. (1998) PAUP*: Phylogenetic Analysis Using Parsimony (* and other methods). Vers: 4. Sinaur Associates: Sunderland, Massachusetts.

Tribe, G. D. (1990) Phenology of *Pinus radiata* log colonization and reproduction by the European bark beetle *Orthotomicus erosus* (Wollaston) (Coleoptera: Scolytidae) in the south-western Cape Province. *Journal of the Entomolgical Society of Southern Africa* **2**: 117-126.

Wingfield, M. J. &. Marasas, W. F. O. (1980) *Ceratocystis ips* associated with *Orthotomicus erosus* (Coleoptera: Scolytidae) on *Pinus* spp. in the Cape Province of South Africa. *Phytophylatica* **12**: 65-69.

Zhou, X. D., De Beer, Z. W., Wingfield, B. D. & Wingfield, M. J. (2001) Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. *Sydowia* **53**: 290-300.

Table 1. Characteristics of polymorphic microsatellite markers designed for plant pathogen and sapstain agent, *Ophiostoma ips*.

SSR Primer Pair	PO	Sequence	Flourescent Label	Expected Size(bp)	Calculated T_m (°C)	Annealing Temp (°C)	Core Sequence	Band Pattern
OI-1	F	5' CAA GGT GAA GTG GTG GGG AC	FAM	340	64	60	(GGAAGGAGGA)$_2$(AG)$_2$*(AG)$_3$	Single; Polymorphic
OI-2	R	5' CGC CCC TGA TTT CCC GAT TC			64	60	(GAGGA)$_2$*(GA)$_4$, and rich in G, A	Single; Polymorphic
OI-3	F	5' CAC CTT GCG CAG CCA GTT AC	FAM	210	64	60	GA$_3$GA$_8$G$_3$A$_9$GA$_5$GAG$_4$A	Single; Polymorphic
OI-4	R	5' CGT AGC GGT GGA GTC AAG CG			66	60		Single; Polymorphic
OI-5	F	5' CCA CTC ACC TCT CTT TAC GAC	FAM	441	64	60	CT$_4$CT$_7$CT$_5$CT$_6$*(CTT)$_3$*(TC)$_3$*	Single; Polymorphic
OI-6	R	5' CTC CTC TGC AAA CTC GTC CC			64	60	(TTTG)$_3$*(CT)$_3$, and rich in T	Single; Polymorphic
OI-7	F	5' GCT GTG GCG AGA CGA TGT CG	HEX	318	66	60	(GA)$_3$(GT)$_3$*(GGA)$_6$*(AGG)$_3$*	Single; Polymorphic
OI-8	R	5' CAT GCC AGC CGT TTC ATG TGC			66	60	(AGC)$_3$*(AGG)$_4$	Single; Polymorphic
OI-9	F	5' GAT GTC GCG GAG AAT GAC GG	HEX	221	64	60	(GTT)$_2$*T$_3$G$_2$T$_3$GTGT$_2$G$_2$T$_6$G$_2$T$_2$*	Single; Polymorphic
OI-10	R	5' GAT ATT AAA TCG CCC CCT CCC			62	60	(GT)$_3$*(TAGG)$_2$	Single; Polymorphic
OI-13	F	5' GCC TGG ACC GCT TCA TTG TCG	FAM	346	68	60	(CG)$_4$(CCG)$_2$*(TGC)$_6$*	Single; Polymorphic
OI-14	R	5' GAC GGT TTC GCC AGC GAG TAG			68	60		Single; Polymorphic
OI-17	F	5' CAT CCT GGC CAA CCG ACT GG	FAM	253	66	60	(GGC)$_2$*(TTC)$_2$*AGA$_9$GAT$_2$A$_9$*	Single; Polymorphic
OI-18	R	5' CTC CGA ATC TGG AGA GCC AG			64	60	(AC)$_3$, and rich in A	Single; Polymorphic
OI-19	F	5' GAG GAG AGA GAT GCG CCA GC	HEX	235	66	60	T$_8$CAT$_5$CAT$_7$AT$_4$*(CACTTTTT)$_2$*	Single; Polymorphic
OI-20	R	5' GTC TGC GTC GAA ATT GCC CC			64	60	(CTT)$_3$*(TTA)$_2$ *,and rich in T	Single; Polymorphic
OI-23	F	5' CAC GCG CAA GTT TGC CGA GG	FAM	184	66	60	(GGC)$_6$*(CGG)$_2$ *, and rich in G	Single; Polymorphic
OI-24	R	5' GCA CGT TGT TGT AGT ACC GCG			66	60		Single; Polymorphic
OI-25	F	5' GCT CCA TCC ACC ACT TAC AAC	HEX	365	64	60	(CCACCACAT)$_3$*(ACTTCCACC)$_2$*	Single; Polymorphic
OI-26	R	5' GCC GGT CAA GGA GAC AGT AAG			66	60	(CCACCACAT)$_2$(CCA)$_2$	Single; Polymorphic
OI-27	F	5' GGG CAT CGC CAT TGC CCT G	FAM	242	64	60	(GTT)$_7$*(TGG)$_5$*(GC)$_3$*	Single; Polymorphic
OI-28	R	5' GAG GTA CTC GAC CTG GAA CG			64	60		Single; Polymorphic
OI-31	F	5' CAG GTA CAG CGA GGG CGT G	HEX	320	64	60	(GT)$_3$*(GTT)$_3$*(GGT)$_6$	Single; Polymorphic
OI-32	R	5' GAC ACC TCC CCT AGC TCT AG			64	60		Single; Polymorphic

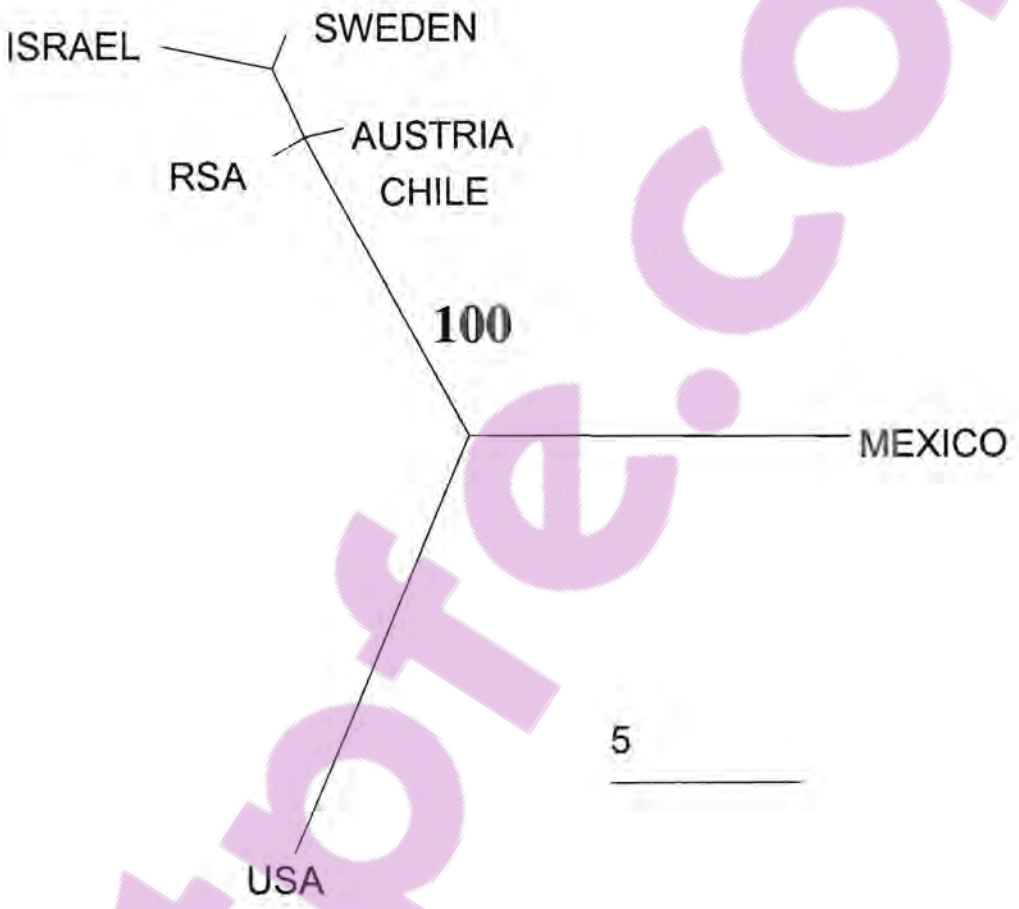
Notes: PO, primer orientation; F, forward; R, reverse primer.

Table 2. Alletic properties of designed polymorphic primers on 7 different isolates of *Ophiostoma ips*.

Isolate No.	Other No.	Origin	OI-1/2	OI-3/4	OI-5/6	OI-7/8	OI-9/10	OI-13/14	OI-17/18	OI-19/20	OI-23/24	OI-25/26	OI-27/28	OI-31/32
CMW7076	CBS 151.54	Sweden	329	209	447	317	223	346	253	235	185	363	239	318
CMW1173	SFP 215	Israel	329	209	455	317	223	346	253	235	185	354	239	318
CMW7079	CBS 438.94	Austria	337	209	447	317	223	346	253	235	185	354	239	318
CMW6416		Chile	337	209	447	317	223	346	253	235	185	354	239	318
CMW6418		South Africa	337	209	447	317	223	346	253	235	185	363	239	318
CMW7075	CBS 137.36	USA	337	210	391	318	214	318	259	249	191	332	213	295
CMW9020		Mexico	340	203	438	314	223	327	259	235	189	346	233	319

Notes: CMW – the Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, Republic of South Africa.

Figure 1. Unrooted phylogram of one of the 9 most parsimonous trees generated from SSR polymorphic data showing strong bootstrap support separating the European from American isolates.



Chapter 9

Microsatellite (SSR) markers reveal genetic diversity among isolates of *Ophiostoma ips* from South Africa, Chile, Europe, and the USA

In the Northern Hemisphere, many conifer-infesting bark beetles (Coleoptera: Scolytidae) are vectors of the tree pathogen and sapstain agent, *Ophiostoma ips* (Rumb.) Nannf. In the Southern Hemisphere, *O. ips* has been introduced into exotic pine-growing countries together with bark beetles. Very little is known regarding the population biology of *O. ips*. The aim of this study was to evaluate the genetic diversity of populations of the fungus using SSR markers in order to gain an understanding of its introduction patterns. Twelve pairs of polymorphic markers were used to examine the population structure for five populations of *O. ips*, one each from Chile, Europe, and the USA, and two from South Africa. The SSR markers produced a total of 74 alleles across the 12 loci examined. The highest gene diversity was found in the USA population, while the lowest was observed for the European population, together with the introduced Mpumalanga population. Forty-four genotypes were found across all populations. The highest genotypic diversity was found in the USA population while the lowest was observed for the European population. A high gene flow was observed between Mpumalanga and KwaZulu-Natal populations, while moderate gene flow was present between the other populations. A neighbour-joining dendrogram showed that the three introduced populations from Chile and South Africa clustered more closely with the European population than with the USA population. The USA population had the highest genetic diversity, and is most likely to be the centre of origin of *O. ips*.

Keywords: Polymorphic marker, population genetics.

INTRODUCTION

Ophiostoma spp. represent an important fungal group that includes many primary tree pathogens (Harrington & Cobb, 1988; Brasier, 1990). *Ophiostoma ips* (Rumbold) Nannfeldt has been reported as pathogenic to conifers (Lieutier *et al.*, 1989; Otrosina *et al.*, 1997), killing living pine trees in extreme cases (Nisikado & Yamauti, 1933). Other studies, however, showed that *O. ips* is not severely pathogenic, causing lesions, but not killing trees, when artificially inoculated (Parmeter *et al.*, 1989; Zhou *et al.*, 2002a). Like many other *Ophiostoma* spp., *O. ips* causes sapstain on logs and freshly cut wood that leads to significant losses to the forestry industry globally (Rumbold, 1931; Hutchison & Reid, 1988; Stone & Simpson, 1990; Seifert, 1993; Marmolejo & García-Ocañas, 1993; Farrell *et al.*, 1997).

Ophiostoma ips has been found casually associated with different conifer-infesting bark beetles such as *Ips* spp. (Rumbold, 1931; Upadhyay, 1981; Lieutier *et al.*, 1991), *Dendroctonus* spp. (Rumbold, 1931; Hunt, 1956; Upadhyay, 1981; Perry, 1991), *Orthotomicus* spp. (Mathiesen-Käärik, 1960; Wingfield & Marasas, 1980), and *Tomicus piniperda* (Linnaeus) (Mathiesen, 1950; Mathiesen-Käärik, 1953; Masuya *et al.*, 1999). The fungus has typically been reported from Northern Hemisphere countries such as the USA, France, Sweden, and Japan (Mathiesen-Käärik, 1960; Lieutier *et al.*, 1991; Perry, 1991; Masuya *et al.*, 1999). It was also introduced into exotic pine-growing countries in the Southern Hemisphere such as Chile and South Africa (Chapter2, 3), together with bark beetles of European origin.

The bark beetle vectors of *O. ips* in Chile and South Africa include *Hylurgus ligniperda* (Fabricius), *Hylastes ater* (Paykull), *H. angustatus* (Herbst), and *Orthotomicus erosus* (Wollaston) (Chapter 1). Although some research has been conducted on the taxonomy and biology of the fungi associated with these beetles (Chapters 1, 2, 3), nothing is known regarding the population structure of *O. ips* or indeed any of these fungi.

Population structure generally refers to the amount and distribution of genetic variation within and between populations. Fungal populations with higher levels of genetic variation are likely to adapt more rapidly to fungicides or resistant hosts (McDonald & McDermott, 1993; Milgroom, 1996; McDonald, 1997). Understanding population structure of pathogens and pests is, therefore, an important component of effective disease and pest management. One of the most effective tools for studying population structure is using co-dominant markers, such as simple short repeats (SSR) markers (Burgess, Wingfield & Wingfield, 2001). In a previous study, twelve pairs of polymorphic SSR markers were developed for *O. ips* (Zhou *et al.*, 2002b). The objective of the present study was to use these markers to compare genetic diversity in different populations of *O. ips*. The genetic variation, genetic distance, and mode of reproduction within and between the different populations were considered.

MATERIALS AND METHODS

Fungal Isolates

Five populations of *O. ips* were investigated in this study (Table 1). The two South African populations (30 isolates each) were isolated from three exotic pine-infesting bark beetle species, *H. ligniperda*, *Hylastes angustatus* and *O. erosus*, during a two-year survey of *Ophiostoma* spp. associated with these beetles in two geographic regions (Chapter 2). In Mpumalanga, the beetles infested *P. patula*, while they were found on *P. elliottii* in KwaZulu-Natal. The Chilean population consisted of 21 isolates from *H. ligniperda* infesting *Pinus radiata* in the Valdivia area. The European population was comprised of 15 isolates from *Ips acuminatus* (Gyllenhal) infesting *P. sylvestris* in Sweden, and 15 from *I. sexdentatus* (Boerner) also infesting *P. sylvestris* in France. Two additional isolates (CBS151.54 and CBS 438.94) from Sweden and Austria respectively, and one (CMW1173) from Israel, were also included in the European

population. A further six isolates originated from the USA, isolated from *P. resinosa* and *P. ponderosa*, and the bark beetle *I. integer* (Eichhogg), were also included. All cultures used in this study are maintained in the culture collection (CMW) of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

DNA Extractions

A single hyphal tip culture of each isolate was grown in 2 % ME (20 g Biolab malt extract, and 1000 ml distilled water). DNA was extracted using a modified version of the extraction method developed by Raeder and Broda (1985) as described in detail in Chapter 3.

SSR-PCR

SSR-PCR was conducted with all isolates using twelve pairs of polymorphic fluorescent-labelled microsatellite primers designed for *O. ips* (Zhou *et al.*, 2002b). PCR reaction mixtures and conditions were the same as those described previously (Zhou *et al.*, 2002b). PCR products were visualized, but not purified.

Genescan analysis

Fluorescent-labelled PCR products were arranged according to the expected size of the PCR product and fluorescent label type (HEX or FARM) attached to the primers. SSR PCR products were then separated and analysed as described previously (Zhou *et al.*, 2002b).

Gene and Genotypic Diversity

For each isolate, a data matrix of multistate characters was compiled by assigning each allele at each of the 12 loci a different letter (e.g. AABDCGDAFBGB). Two functions of gene and genotypic diversities were used to evaluate the genetic variation within the populations studied.

Gene diversity was determined by allele frequencies at each locus, while genotypic diversity was determined by the number and frequency of combinations of alleles at multiple loci. Gene diversity of each population was calculated according to the formula, $H = 1 - \Sigma_k x_k^2$, where x_k is the frequency of the k^{th} genotype (Nei, 1973), using POPGENE version 1.31 (http://www.ualberta.ca/~fyeh/fyeh). Genotypic diversity of the populations was calculated using the formula, $G = 1 / \Sigma [f_x (x/n)^2]$, where n is the sample size, and f_x is the number of genotypes occurring x times in the sample (Stoddart & Taylor, 1988). To compare the genotypic diversities between populations, the maximum percentage of genotypic diversity was obtained using the formula $\hat{G} = G / N*100$, where N is the population size (McDonald *et al.*, 1994).

Genetic distance

$\delta\mu^2$ and D_{AD} were used to calculate the genetic distance between populations based on microsatellite data. $\delta\mu^2$ is the square of the mean distance between two populations, and D_{AD} is based on absolute distance, which is not squared (Goldstein *et al.*, 1995). Genetic distances between populations were calculated using the programme MICROSAT (http://human.stanford.edu/microsat), and neighbour-joining trees were constructed in MEGA version 2.1 (http://www.megasoftware.net).

Population differentiation and gene flow

θ and G_{ST} were calculated to evaluate the level of population differentiation. θ was calculated in Multilocus version 1.2 (http://www.bio.ic.ac.uk/evolve/software/multilocus), and an estimate of F_{ST}, using the equation $\theta = Q - q / 1 - q$, where Q is the probability that two alleles from the same population are the same, and q is the probability that two alleles from different populations are the same. A value of θ equal to 0 indicates no population differentiation, while a value of θ equal to 1 indicates totally different alleles between two populations. The significance of θ was

determined by comparing the observed value to that of 1000 randomized datasets in which individuals were randomized across populations. The null hypothesis of no population differentiation can be rejected where the P value is significant.

Another measurement, G_{ST}, was calculated in POPGENE version 1.31 (http://www.ualberta.ca/~fyeh/fyeh), using the equation $G_{ST} = (H_T - H_S) / H_T$, where H_T is the mean total gene diversity and H_S is the mean within population diversity. A value of G_{ST} closer to zero reflects less genetic variation between populations, while values of 0 to 0.05 reflect little, and values above 0.25 large genetic differentiation.

The level of gene flow (N_m) was calculated from the estimate of G_{ST}, where $N_m = 0.5 (1 - G_{ST}) / G_{ST}$. A N_m value of zero indicates that populations are completely isolated, and share no alleles.

Mode of reproduction

The Index of Association (I_A) (Maynard *et al.*, 1993) was used to determine the mode of reproduction for each population. I_A was calculated for populations including all isolates, while the clone corrected population contains only one representative of each genotype. The tests were performed on a data matrix of multistate characters for each allele at each locus, and calculated using Multilocus version 1.2. The observed data were compared with the expected data for a 1000 randomly recombining data sets calculated. Recombination within the population was then determined, comparing the observed data to the distribution range of the recombined data (Taylor, Jacobson & Fisher, 1999). If the observed data fall within the distribution range, the population is likely to be undergoing recombination. The population is, however, not undergoing recombination where the observed data fall outside the distribution range.

RESULTS

Allelic diversity

One hundred and twenty isolates of *O. ips* were amplified with 12 pairs of SSR markers, and the markers produced a total of 74 alleles across the 12 loci examined (Table 2). Individually, there were 22 alleles in the Chile population, 18 in Europe, 20 in Mpumalanga, 28 in KwaZulu-Natal, and 46 in the USA population.

Monomorphic alleles were present at locus OI1 (Mpumalanga and USA), OI3 (Europe and Mpumalanga), OI7 (Chile, Europe, and Mpumalanga), OI9 (Chile and Europe), OI13 (Chile, Europe, KwaZulu-Natal, and Mpumalanga), OI17 (Europe and Mpumalanga), OI19 (Europe), OI23 (Chile, KwaZulu-Natal, and Mpumalanga), OI27 (Chile), and OI31 (Mpumalanga) (Table 2).

Unique alleles were observed in the different populations. In total, the Chilean and Mpumalanga populations each had two unique alleles over two loci. The European population had three unique alleles over three loci, the KwaZulu-Natal population had four over four loci, and the USA population had 26 over 11 loci (Table 2).

The gene diversity estimated from the SSR profile of isolates varied greatly between populations (Table 2). The highest gene diversity (h = 0.63) was found in the USA population, while the lowest (h = 0.10) was observed for the Mpumalanga and European populations. Moderately high diversities were found in the populations from Chile (h = 0.16) and KwaZulu-Natal (h = 0.20).

Genotypic diversity

Forty-four genotypes, representing isolates of *O. ips* studied, were obtained: eight in the Chile population, six in Europe, 15 in KwaZulu-Natal, nine in Mpumalanga, and six in the USA population (Table 3).

A number of genotypes were shared across the populations (Table 3). For instance, genotype OI24 was shared by six isolates from Chile, 17 isolates from Europe, four isolates from KwaZulu-Natal, and seven isolates from Mpumalanga. None of genotypes obtained from the USA isolates was shared with other isolates (Table 3).

The genotypic diversity estimated from the SSR profile of isolates varied greatly among populations (Table 3). The highest maximum genotypic diversity ($\hat{G}$ = 100 %) was found in the USA population while the lowest ($\hat{G}$ = 8.5 %) was observed for the European population. Moderately high diversities were found in the three introduced populations from Chile ($\hat{G}$ = 21.2%), KwaZulu-Natal ($\hat{G}$ = 21.5 %), and Mpumalanga ($\hat{G}$ = 15.2 %).

A neighbour-joining dendrogram based on the genotypes of each population, showed that there were no specific groups based on hosts, insect vectors and geographic areas in the populations (Figure 1). Most genotypes presented clustered closely, except the three isolates from USA, which were very far from the rest (Figure 1).

Genetic distance

The neighbour-joining dendrogram based on the genetic distance between the populations showed that there were two main clades in the populations studied (Figure 2). In the first clade, the two introduced South African populations (Mpumalanga and KwaZulu-Natal) clustered more closely with the introduced Chilean population than to the European population. The USA population, however, was distant to all the other populations.

Little genetic differentiation (G_{ST} = 0.02, θ = -0.03) was found between KwaZulu-Natal and Mpumalanga populations, and moderate genetic differentiation was observed between the other populations (Table 4).

The amount of gene flow varied between populations. High gene flow (N_m = 22.71) was observed between Mpumalanga and KwaZulu-Natal populations, while moderate gene flow was

found between the other populations (Table 4). The lowest gene flow existed between the USA and Europe, and the USA and Mpumalanga populations.

Mode of reproduction

The European population was found to be clonal, 30 isolates represented only 3 genotypes, while each of the other three isolates represented another genotype. The latter three isolates were from *Orthotomicus proximus* (Gyllenhal), *Ips sexdentatus* (Boerner), and *Crypturgus mediteranous*, respectively, and collected many years ago. The lack of diversity rendered these data unsuitable for analysis of IOA. For the USA population, linked alleles indicated clonal reproduction, or in case of fungi, homothallism. The introduced populations from Chile and KwaZulu-Natal appear clonal, while the Mpumalanga population appears to be undergoing some recombination (Figure 3).

DISCUSSION

To the best of our knowledge, this study is the first to consider the population structure of the tree pathogen and sapstain agent, *Ophiostoma ips*. SSR markers were used to evaluate the genetic diversity, genetic distance, and mode of reproduction of different populations from Chile, Europe, South Africa, and the USA. The USA population had the highest genetic diversity. The highest gene flow was observed between the two South African populations. The three introduced populations from Chile and South Africa clustered most closely with the European population, and were clearly separated from the USA population.

According to McDonald (1997), a population from the centre of origin of a species would be expected to have higher gene diversity than other populations (McDonald, 1997). In the case of

O. ips, the USA population had the highest genetic diversity, indicating that North America is most likely the centre of origin for this fungus, *O. ips*.

The relatively lower gene diversity (h = 0.10) observed in the native European population suggested that *O. ips* has developed clonal lineages. *Ophiostoma ips* is homothallic (Chapter 4), and one individual of a homothallic fungus is capable of producing sexual spores genetically identical to the parent strain (Taylor *et al.*, 1999), which supports the hypothesis of clonal lineages.

Neighbour-joining distance analysis showed that the three introduced populations from Chile and South Africa clustered more closely with the European population than with the USA population. The three introduced populations of *O. ips* were isolated from exotic bark beetles which are native to Europe (Swan, 1942; Neumann 1987). Our results support the view that the fungus was introduced into exotic pine-growing countries in the Southern Hemisphere by bark beetles originating from Europe. In addition, a much lower gene flow (N_m = 1.78) was found between the native European and USA populations.

The neighbour-joining dendrogram based on genotypes of each population showed that there were no specific groups within the populations. This indicates that there is, at present, no evidence of host or insect specialization influencing the evolution of *O. ips*. In this study, populations from South Africa were isolated from three bark beetle species infesting *P. patula* and *P. elliottii*, the Chilean population from *H. ligniperda* infesting *P. radiata*, the European population mainly from *I. acuminatus* and *I. sexdentatus* infesting *P. sylvestris*, and the USA population from *P. resinosa*, *P. ponderosa*, and *I. integer*. Other studies have, however, shown that host specialization can play an important role influencing the evolution of fungi such as *Magnaporthe grisea* (Hebert) Barr (Zeigler, 1998), and *Ceratocystis fimbriata* Ellis & Halst (Barnes *et al.*, 2001).

The high gene flow (N_m = 22.71) and little genetic differentiation (G_{ST} = 0.02) observed between the KwaZulu-Natal and Mpumalanga populations from South Africa probably resulted from the spread of bark beetles within the country. This is not surprising considering that there is no geographical barrier between the two populations. McDonald (1997) stated that absence of gene flow among populations could be used to define the geographic boundaries of populations. In our study, however, it appeared that there was not much genetic differentiation and gene flow (Table 4), between all other populations and the USA population. This is because G_{ST} is underestimated while N_m is overestimated for the USA population as a result of the small number of isolates in the population.

I_A tests for populations including all isolates, as well as for clone corrected populations, showed that most recombination occurred in the introduced Mpumalanga population. This population, however, had lower genetic diversity. Our hypothesis for this is that the population has been in this area for a longer period than in KwaZulu-Natal, and that a few specific genotypes have been selected during spread. Populations from Chile and KwaZulu-Natal, which had a higher genetic diversity and were not undergoing recombination, could have originated from multiple introductions of insect vectors. In the case of KwaZulu-Natal, the population was collected within a 30 km radius of the Richard Bay harbour, from where large volumes of pine logs from all over South Africa are exported annually. This would inevitably lead to a higher genetic diversity in that area.

The SSR markers used in this study represent powerful molecular tools, making it possible to understand the structure of fungal populations and introduction patterns. Our results support the view that *O. ips* was introduced into exotic pine-growing countries such as Chile and South Africa, together with the bark beetles native to Europe. The USA population, however, with the highest genetic diversity, is most likely to be the centre of origin for *O. ips*. Further investigation

with higher numbers of isolates from the USA, and populations from other parts of the world will be necessary to better understand the global patterns of spread of *O. ips*.

REFERENCES

Barnes, I., Gaur, A., Burgess, T., Roux, J., Wingfield, B. D. & Wingfield, M. J. (2001) Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt pathogen, *Ceratocystis fimbriata*. *Molecular Plant Pathology* **2**: 319-325.

Brasier, C. M. (1990) China and the origins of Dutch elm disease: an appraisal. *Plant Pathology* 39: 5-16.

Burgess, T., Wingfield, M. J. & Wingfield, B. D. (2001) Simple sequence repeat markers distinguish among morphotypes of *Sphaeropsis sapinea*. *Applied and Environmental Microbiology* **67**: 354-362.

Farrell, R. L., Hadar, E., Kay, S. J., Blanchette, R. A. & Harrington, T. C. (1997) Survey of sapstain organisms in New Zealand and albino anti-sapstain fungi. Biology and prevention of sapstain. A conference sponsored by the Department of Forest Products, Oregon State University, 25 May 1997, Delta Whistler Resort, Whistler, British Columbia, Canada: 57-62.

Goldstein, D. B., Ruiz-Linares, A., Cavalli-Sforza, L. L. & Feldman, M. W. (1995) An evaluation of genetic distances for use with microsatellite loci. *Proceedings of the National Academy of Science USA* **92**: 6723-6727.

Harrington, T. C. & Cobbs Jr., F. W. (1988) *Leptographium* root diseases on conifers. American Phytopathological Society, St. Paul, Minnesota.

Hunt, J. (1956) Taxonomy of the genus *Ceratocystis*. *Lloydia* **19**: 1-59.

Hutchison, L. J. & Reid, J. (1988) Taxonomy of some potential wood-staining fungi from New Zealand: 1. Ophiostomataceae. *New Zealand Journal of Botany* **26**: 63-81.

Lieutier, F., Garcia, J., Yart, A., Vouland, G., Pettinetti & Morelet, M. (1991) Ophiostomatales (Ascomycètes) associées à *Ips acuminatus* Gyll (Coleoptera: Scolytidae) sur le pin sylvestre (*Pinus sylvestris* L.) dans le sud-est de la France et comparaison avec *Ips sexdentatus*. *Agronomie* **11**: 807-817.

Lieutier, F., Yart, A., Garcia, J., Ham, M. C., Morelet, M. and Levieux, J. (1989) Champignons phytopathogènes associés à deux coléoptères Scolytidae du pin sylvestre (*Pinus sylvestris* L.) et étude préliminaire de leur agressivité envers l'hôte. *Annales des Sciences Forestieres* **46**: 201-216.

Masuya, H., Kaneko, S.,Yamaoka, Y. & Osawa, M. (1999) Comparisons of ophiostomatoid fungi associated with *Tomicus piniperda* and *T. minor* in Japanese red pine. *Journal of Forestry Research* **4**: 131-135.

Mathiesen, A. (1950) Über einige Borkenkäfern assoziierte Bläuepilze in Schweden. *Oikos* **2**: 275-308.

Mathiesen-Käärik, A. (1953) Eine Übersicht über die gewöhnlichsten mit Borkenkärfern assiziierten Bläuepilze in Schweden und einige für Schweden neue Bläuepilze. *Meddelanden Från Statens Skogforskningsintitut* **43**: 3-74.

Mathiesen-Käärik, A. (1960) Studies on the ecology, taxonomy and physiology of Swedish insect-associated blue-stain fungi, especially the genus *Ceratocystis*. *Oikos* **11**:1-25.

Marmolejo, J. G. & García-Ocañas, F. (1993) Contribuciones micologicas en homenaje al biologo Jose Castillo Tovar por su labor en pro de la micologia mexicana. *Reporte Cientifico, No. Especial* **13**: 155-170.

Maynard, S. J., Smith, N. H., O'Rourke, M. & Spratt, B. G. (1993) How clonal are bacteria? *Proceedings of the National Academy of Science USA* **90**: 4384-4388.

McDermott, J. M. & McDonald, B. A. (1993) Gene flow in plant pathosystems. *Annual Review of Phytopathology* **31**: 353-373.

McDonald, B. A. & McDermott, J. M. (1993) Population genetics of plant pathogenic fungi. *Bioscience* **43**: 311-319.

McDonald, B. A., Miles, J., Nelson, L. R. & Pettway, R. E. (1994) Genetic variability in nuclear DNA in field populations of *Stagonospora nodorum*. *Phytopathlogy* **84**: 250-255.

McDonald, B. A. (1997) The population genetics of fungi: tools and techniques. *Phytopathology* **87**: 448-453.

Milgroom, M. G. (1996) Recombination and the multilocus structure of fungal populations. *Annual Review of Phytopathology* **34**: 457-477.

Nei, M. (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences USA* **70**: 3321-3323.

Neumann, F. G. (1987) Introduced bark beetles on exotic trees in Australia with special reference to infestations of *Ips grandicollis* in pine plantations. *Australian Forestry* **50**:166-178.

Nisikado, Y. and Yamauti, K. (1933) Contributions to the knowledge of the sap stains of wood in Japan. I. Studies on *Ceratostomella ips* Rumbold, the cause of a blue stain of pine trees in western Japan. *Berichte des Ohara Instituts für Landwirtschaftliche Forschungen* **5**: 501-538.

Otrosina, W. J., Hess, N. J., Zarnoch, S. J., Perry, T. J. and Jones, J. P. (1997) Blue-stain fungi associated with roots of southern pine trees attacked by the southern pine beetle, *Dendroctonus frontalis*. *Plant Disease* **81**: 942-945.

Parmeter, J. R., Slaughter, G. W., Chen, M.-M., Wood, D. L. & Stubbs, H. A. (1989) Single and mixed inoculations of ponderosa pine with fungal associates of *Dendroctonus* spp. *Phytopathology* **79**: 768-772.

Perry, T. J. (1991) A synopsis of the taxonomic revisions in the genus *Ceratocystis* including a review of blue-staining species associated with *Dendroctonus* bark beetles. *General Technical Report*, SO-86. New Orleans, LA: U. S. Department of Agriculture, Forest Service, Southern Forest Experiment Station, 1-16.

Raeder, U. & Broda, P. (1985) Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**: 17-20.

Rumbold, C. T. (1931) Two blue-staining fungi associated with bark-beetle infestation of pines. *Journal of Agricultural Research* **43**: 847-873.

Seifert, K. A. (1993) Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In: *Ceratocystis* and *Ophiostoma* Taxonomy, Ecology and Pathogenicity. (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 141-151. American Phytopathological Press, St. Paul, Minnesota.

Stoddart, J. A. & Taylor, J. F. (1988) Genotypic diversity: estimation and prediction in samples. *Genetics* **118**: 705-711.

Stone, C. & Simpson, J. A. (1990) Species associations in *Ips grandicollis* galleries in *Pinus taeda*. *New Zealand Journal of Forestry Science* **20**: 75-96.

Swan, D. C. (1942) The bark beetle - *Hylastes ater* Payk. (Coleoptera: Scolytidae) attacking pines in South Australia. *Journal of Agriculture* **12**: 86-90.

Taylor, J. W., Geiser, D. M., Burt, A. & Koufopanou, V. (1999) The evolutionary biology and population genetics underlying fungal strain typing. *Clinical Microbiology Reviews* 126-146.

Taylor, J. W., Jacobson, D. J. & Fisher, M. C. (1999) The evolution of asexual fungi: reproduction, speciation and classification. *Annual Review of Phytopathology* **37**: 197-246.

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

Wingfield, M. J. &. Marasas, W. F. O. (1980) *Ceratocystis ips* associated with *Orthotomicus erosus* (Coleoptera: Scolytidae) on *Pinus* spp. in the Cape Province of South Africa. *Phytophylactica* **12**: 65-69.

Zeigler, R. C. (1998) Recombination in *Magnaporthe grisea*. *Annual Review of Phytopathology* **36**: 249-275.

Zhou, X. D., De Beer, Z. W., Wingfield, B. D & Wingfield, M. J. (2002a) Infection sequence and pathogenicitiy of *Ophiostoma ips*, *Leptographium serpens* and *L. lundbergii* to pines in South Africa. In *Fungal Succession* (K. D. Hyde & E. B. G. Jones, eds). *Fungal Diversity* **10**: 229-240.

Zhou, X. D., Burgess, T., De Beer, Z. W., Wingfield, B. D. & Wingfield, M. J. (2002b) Development of polymorphic microsatellite markers for the tree pathogen and sapstain agent, *Ophiostoma ips*. *Molecular Ecology Notes* **2**: 309-312.

Table 1: Isolates of *Ophiostoma ips* used in this study.

Isolate numbers (number of isolates)	Country of origin	Host	Insect vector
CMW6418 – 6423 (6), CMW6432 – 6434 (3), CMW6436 – 6440 (5)	KwaZulu-Natal, South Africa	*Pinus elliottii*	*Orthotomicus erosus* (Wollaston)
CMW6446 – 6454 (9), CMW6456, CMW6458 – 6460 (3), CMW6463, CMW6465		*P. elliottii*	*Hylurgus ligniperda* (Fabricius)
CMW7215		*P. elliottii*	*O. erosus*
CMW6442 – 6444 (3), CMW6470 – 6471 (2)	Mpumalanga, South Africa	*P. patula*	*Hylastes angustatus* (Herbst)
CMW6472 – 6474 (3), CMW6476 – 6478 (3), CMW6480 – 6488 (9), CMW6490 – 6495 (6), CMW7211 – 7214 (4)		*P. patula*	*O. erosus*
CMW6401 – 6417 (17), CMW5089, CMW5179, CMW7209 – 7210 (2)	Valdivia, Chile	*P. radiata*	*H. ligniperda*
CMW9005 – 9019 (15)	Uppsala, Sweden	*P. sylvestris*	*Ips acuminatus* (Gyllenhal)
CBS151.54	Dalarna, Sweden		*O. proximus* (Eichhoff)
CMW9310 – 9324 (15)	East France	*P. sylvestris*	*I. sexdentatus* (Boerner)
CBS438.94	Flatz, Austria		*I. sexdentatus*
CMW1173	Israel		*Crypturgus mediteranous*
CMW311 – 313 (3)	Minnesota, USA	*P. resinosa*	
CMW1760 – 1761 (2)	Idaho, USA	*P. ponderosa*	
CBS137.36	Oregon, USA		*I. integer* (Eichhoff)

Table 2: Allele size (bp) at 12 loci and allelic diversity for *Ophiostoma ips* collected from Chile, Europe, KwaZulu-Natal, Mpumalanga, and USA.

Locus	Allele	Chile	Europe	KwaZulu-Natal	Mpumalanga	USA
OI-1	329	-	2	-	-	-
	333	-	-	1	-	-
	337	20	30	26	30	6
	341	1	-	3	-	-
OI-3	179	-	-	-	-	1
	202	-	-	-	-	1
	205	-	-	1	-	-
	209	20	32	29	30	3
	210	-	-	-	-	1
	213	1	-	-	-	-
OI-5	391	-	-	-	-	1
	442	-	-	4	1	-
	444	-	-	1	-	-
	447	11	31	25	29	-
	448	-	-	-	-	2
	451	1	-	-	-	-
	455	9	1	-	-	1
OI-7	311	-	-	-	-	1
	317	21	32	28	30	4
	318	-	-	2	-	1
OI-9	210	-	-	-	-	1
	214	-	-	-	-	1
	217	-	-	-	-	2
	218	-	-	5	3	-
	223	21	32	25	27	2
OI-13	313	-	-	-	-	1
	318	-	-	-	-	1
	346	21	32	30	30	3
	349	-	-			1
OI-17	235	-	-	-	-	1
	247	-	-	-	-	1
	249	-	-	1	-	-
	253	20	32	27	30	-
	254	-	-	-	-	2
	257	1	-	2	-	-
	259	-	-	-	-	1
	263	-	-	-	-	1
OI-19	207	-	-	-	-	1
	226	-	-	-	-	1
	228	-	-	-	-	1
	230	-	-	1	-	-
	234	10	-	-	1	-
	235	10	32	28	29	2
	238	1	-	-	-	
	239	-	-	1	-	-
	249	-		-	-	1
OI-23	170	-	-	-	-	1
	179	-	-	-	-	3
	184	-	9	-	-	-
	185	21	23	30	30	1
	191	-			-	1
OI-25	332	-	-	-	-	1
	337	-	-	-	1	-
	350	-	-	-	-	1
	354	15	31	10	11	2
	355	-	-	-	-	1
	358	1	-	1	-	1
	359	-	-	2	-	-
	363	5	1	17	17	-
	367	-	-	-	1	-
OI-27	213	-	-	-	-	2
	223	-	-	-	-	1
	239	21	18	21	24	2
	240	-	14	-	-	-
	242	-	-	9	4	-
	243	-	-	-	2	-
	274	-	-	-		1
OI-31	278	-	-	-	-	1
	295	-	-	-	-	1
	296	-	-	-	-	1
	314	-	-	1	-	-
	318	20	31	29	30	3
	322	1	-	-	-	-
	327	-	1	-	-	-
Population size		21	32	30	30	6
No. of alleles		22	18	28	20	46
No. of unique alleles		2	3	4	2	26
No. of polymorphic loci		7	6	10	5	11
Gene diversity		0.16	0.10	0.20	0.10	0.63

Table 3: Multilocus genotypes and genotypic diversity obtained for *Ophiostoma ips* isolates from Chile, Europe, KwaZulu-Natal, Mpumalanga, and USA.

	Chile	Europe	KwaZulu-Natal	Mpumalanga	USA
OI1	-	-	-	1	-
OI2	3	-	10	11	-
OI3	-	1	-	-	-
OI4	1	-	-	-	-
OI5	-	-	1	-	-
OI6	-	-	1	-	-
OI7	-	-	3	4	-
OI8	-	-	1	-	-
OI9	-	-	1	-	-
OI10	1	-	-	-	-
OI11	1	-	-	-	-
OI12	-	1	-	-	-
OI13	7	-	-	-	-
OI14	1	-	-	-	-
OI15	-	-	1	-	-
OI16	-	-	1	-	-
OI17	1	-	-	1	-
OI18	-	1	-	-	-
OI19	-	-	-	2	-
OI20	-	-	2	-	-
OI21	-	9	-	-	-
OI22	-	4	-	-	-
OI23	-	-	-	1	-
OI24	6	17	4	7	-
OI25	-	-	1	2	-
OI26	-	-	1	-	-
OI27	-	-	1	-	-
OI28	-	-	1	-	-
OI29	-	-	1	1	-
OI30	-	-	-	-	1
OI31	-	-	-	-	1
OI32	-	-	-	-	1
OI33	-	-	-	-	1
OI34	-	-	-	-	1
OI35	-	-	-	-	1
	-	-	-	-	-
Number of isolates	21	33	30	30	6
Number of genotypes	8	6	15	9	6
Genotypic diversity (G)	4.46	2.80	6.44	4.56	6
Max. genotypic diversity ($\hat{G}$)	21.2%	8.5%	21.5%	15.2%	100%

Table 4: Amount of gene flow between populations.

	KwaZulu-Natal	Chile	Europe	USA
Mpumalanga	[a] $\theta = -0.03$	$\theta = 0.092$	$\theta = 0.055$	$\theta = 0.258$
	$(P = 0.423)$	$(P = 0.038)$	$(P = 0.096)$	$(P < 0.001)$
	[b] $G_{st} = 0.02$	$G_{st} = 0.16$	$G_{st} = 0.18$	$G_{st} = 0.23$
	[c] $N_m = 22.71$	$N_m = 2.65$	$N_m = 2.21$	$N_m = 1.70$
KwaZulu-Natal		$\theta = 0.097$	$\theta = 0.069$	$\theta = 0.213$
		$(P = 0.017)$	$(P = 0.075)$	$(P < 0.001)$
		$G_{st} = 0.13$	$G_{st} = 0.15$	$G_{st} = 0.19$
		$N_m = 3.35$	$N_m = 2.77$	$N_m = 2.14$
Chile			$\theta = 0.118$	$\theta = 0.208$
			$(P = 0.02)$	$(P = 0.002)$
			$G_{st} = 0.19$	$G_{st} = 0.20$
			$N_m = 2.07$	$N_m = 2.02$
Europe				$\theta = 0.204$
				$(P = 0.006)$
				$G_{st} = 0.22$
				$N_m = 1.78$

[a] θ = Population differentiation (Agapow & Burt, 2000);
[b] G_{st} = Genetic differentiation coefficient (Nei, 1973);
[c] N_m = Estimate of gene flow from G_{st} (McDermott & McDonald, 1993).

Figure 1: Neighbor-joining dendrogram of *Ophiostoma ips* isolates from Chile, Europe, KwaZulu-Natal, Mpumalanga, and the USA based on genotypes (Duplicate genotypes within a population were removed in the analysis).

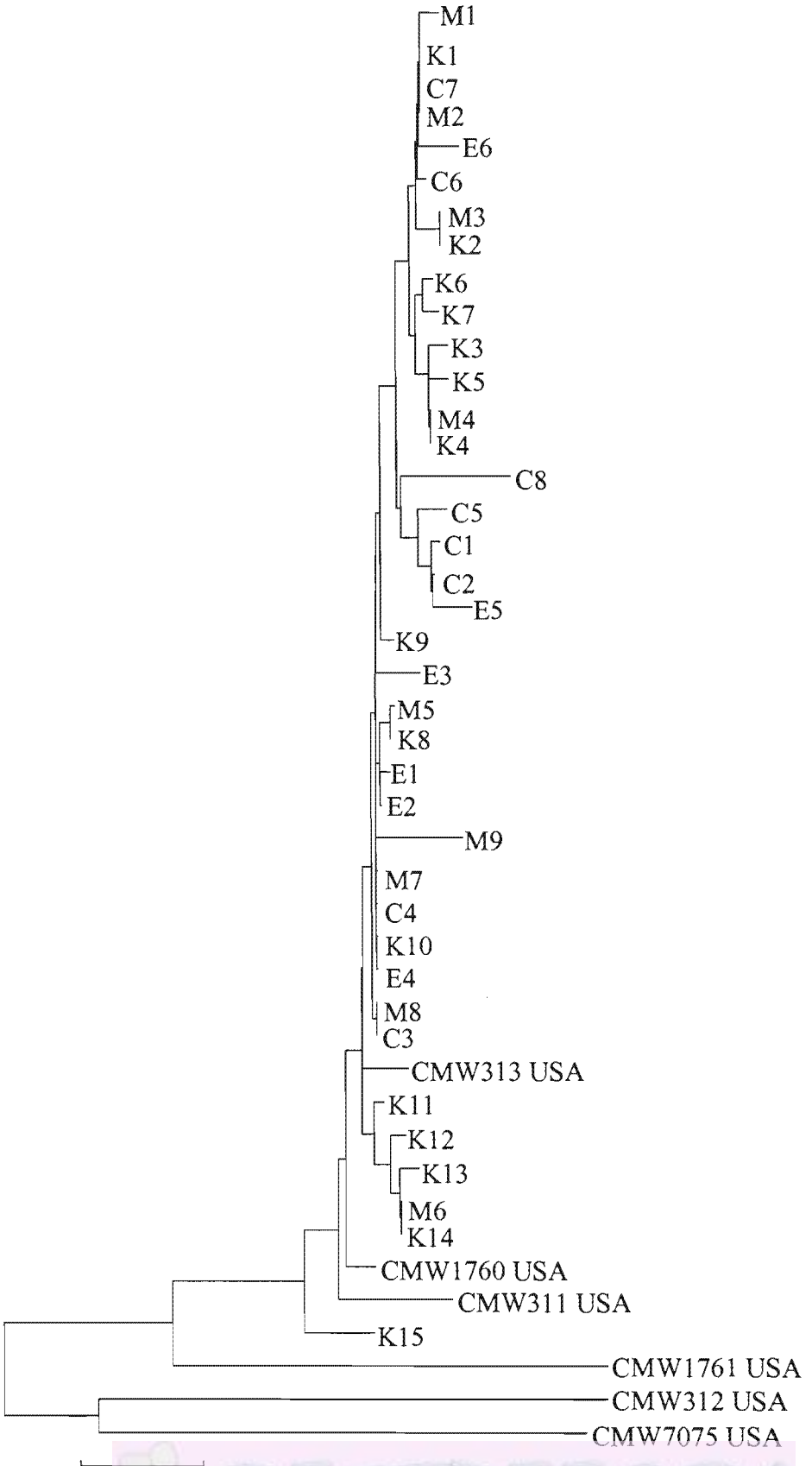


Figure 2: Neighbor-joining dendrogram of *Ophiostoma ips* populations from Chile, Europe, KwaZulu-Natal, Mpumalanga, and the USA.

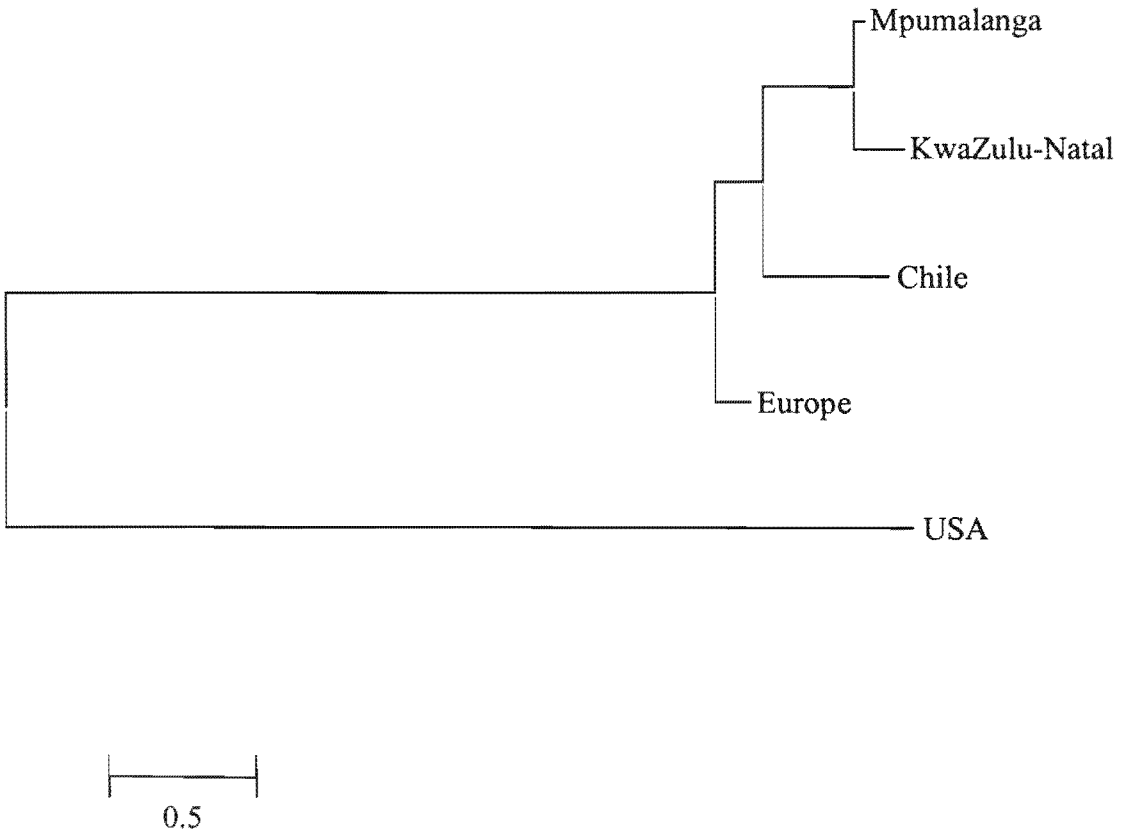
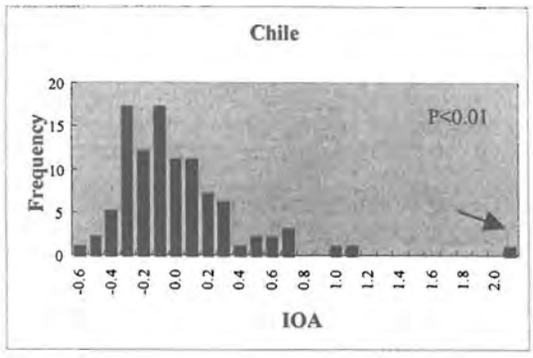
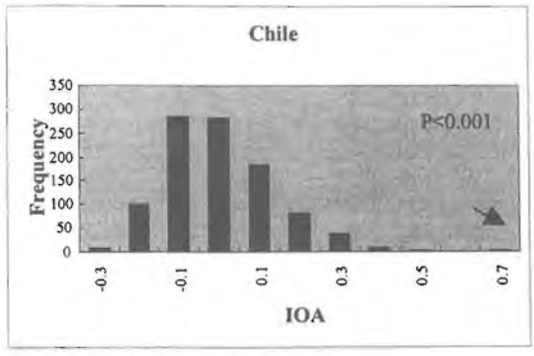
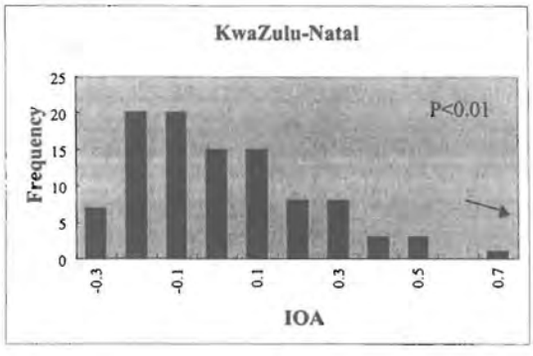
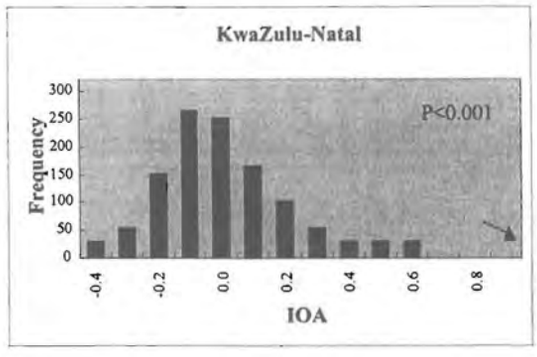


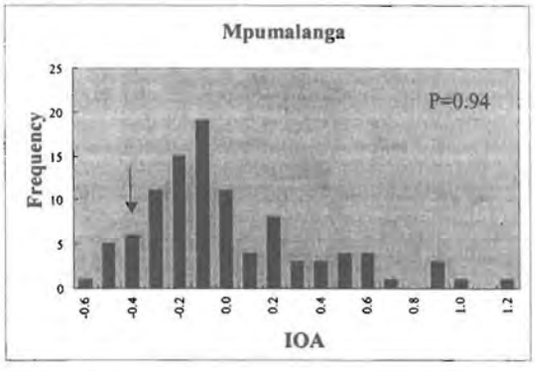
Figure 3: Histograms representing the distribution range of randomly recombining populations using the index of association (I_A).

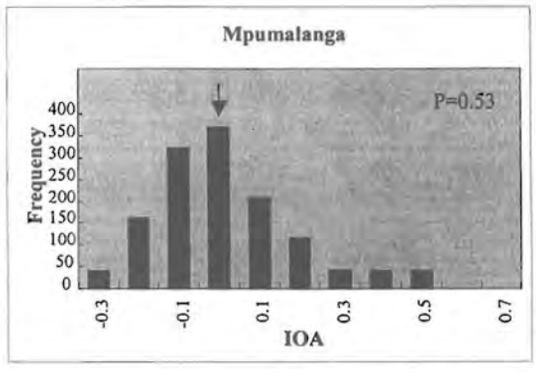


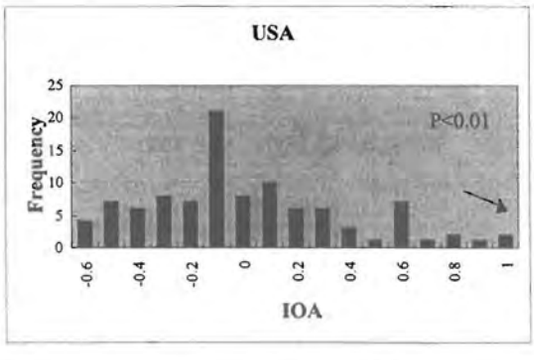


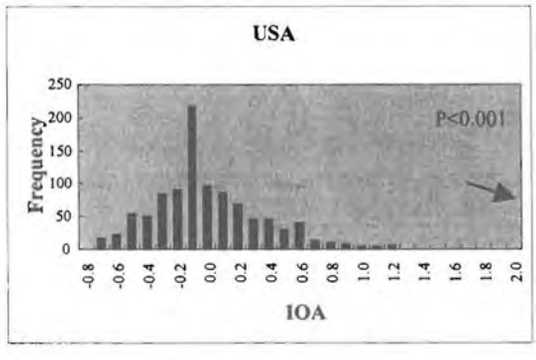












Clone corrected populations

Population including all isolates