



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* Upadhyay & 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 *Knoxdaviesia* 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, Escudie & 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 responsible 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 unencapsulated mitochondrial 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* (Eichhoff), 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 arthropod-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 desiccation, (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.

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

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

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

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

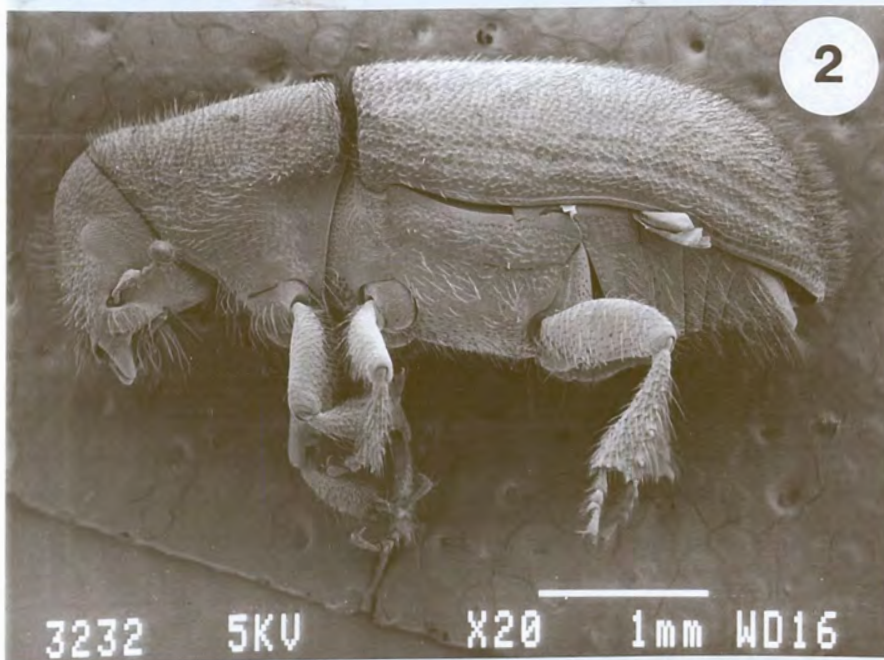
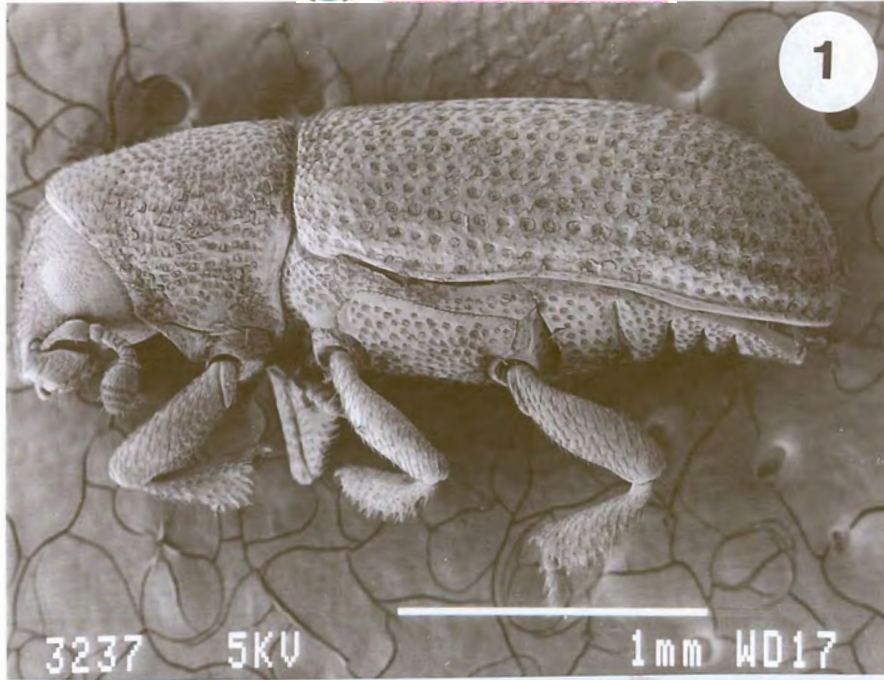
Table 3. Records of five pine bark beetle species in the Southern Hemisphere.

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

* This chapter was published as: 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.

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* Upadhyay & 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 McCartney 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, $F = (312 / 694) \times 100 \% = 45.0 \%$.

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 *Hyalorhinoclaadiella* 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 *Hyalorhinoclaadiella* 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 *Hyalorhinochlaediella* 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. elliotii*. 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 *Hyalorhinochlaeniella* 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.

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

	<i>Orthotomicus erosus</i>	<i>Hylastes angustatus</i>	<i>Hylurgus ligniperda</i>
<i>Ophiostoma ips</i>	399 (60.0 %)	12 (1.7 %)	25 (12.6 %)
<i>Leptographium lundbergii</i>	4 (0.6 %)	311 (44.8 %)	43 (21.6 %)
<i>L. serpens</i>	3 (0.5 %)	312 (45.0 %)	42 (21.1 %)
<i>O. galeiformis</i>	-	-	5 (2.5 %)
<i>O. pluriannulatum</i>	2 (0.3 %)	12 (1.7 %)	2 (1.0 %)
<i>O. stenoceras</i>	-	5 (0.7 %)	1 (0.5 %)
<i>O. piceae</i>	-	-	3 (1.5 %)
<i>Ceratocystiopsis minuta</i>	-	6 (0.9 %)	2 (1.0 %)
<i>Pesotum</i> spp.	11 (1.7 %)	27 (3.9 %)	12 (6.0 %)
<i>Sporothrix</i> sp.	2 (0.3 %)	6 (0.9 %)	3 (1.5 %)
<i>Hyalorhinocladiella</i> 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).

