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

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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: Bark beetles, South Africa, sapstain, *Ophiostoma*, *Leptographium*.

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 & al., 1993; Marais & al., 1998; Viljoen & al., 2000). Although morphologically similar, these genera are phylogenetically distantly related (Spatafora & Blackwell, 1994; Viljoen & al., 1999). Anamorph genera associated with these teleomorph genera are: *Pesotum* Crane & Schoknecht *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 & al., 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 & al., 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 & al., 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 carry out 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 sterilized tweezers 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 dissecting 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 characterized using light microscopy.

Frequency of occurrence

From each sample, only one isolate per fungal species was recorded and subsequently used for frequency calculations. 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

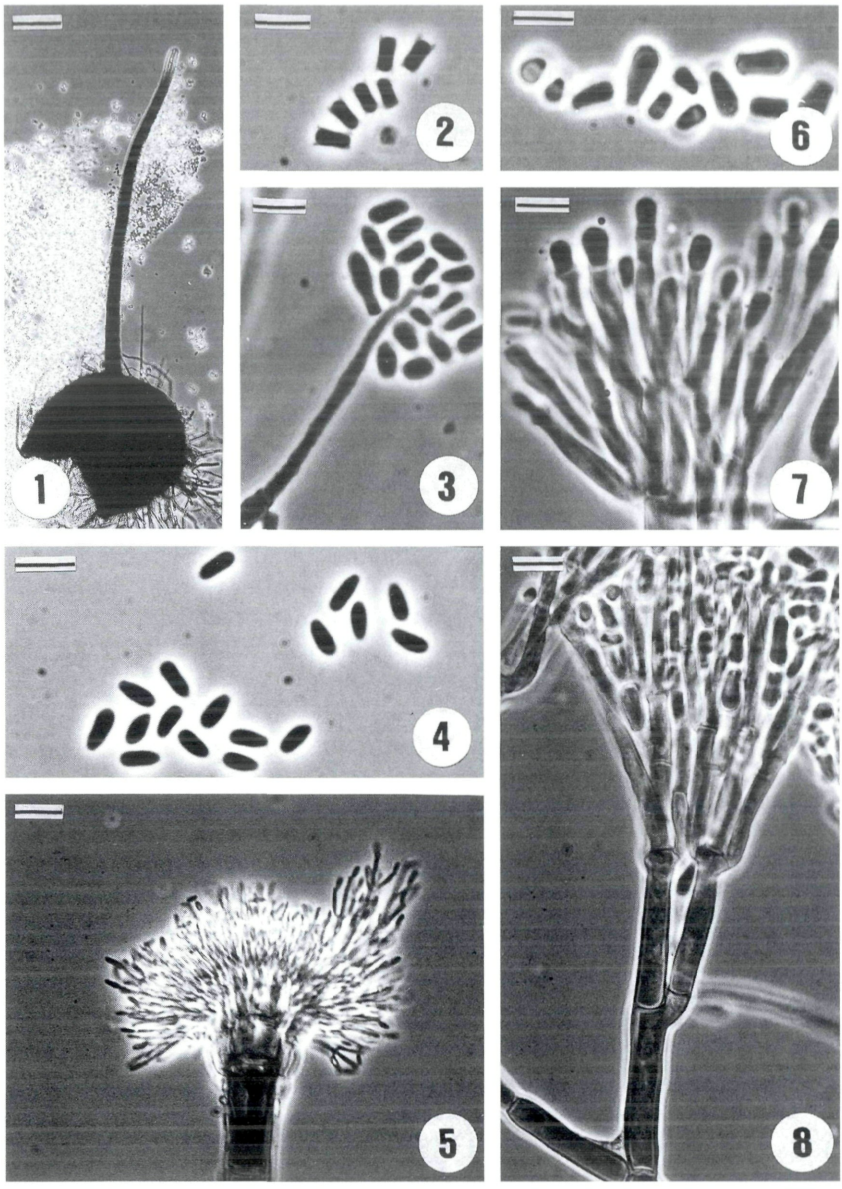
Collection of bark beetles and galleries

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. & Kendrick, 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



Figs. 1–8 – *Ophiostoma ips*. – 1. Ascocarp (Bar = 105 µm). – 2. Ascospores (Bar = 10 µm). – 3. *Hyalorhinocladiella* anamorph (Bar = 10 µm). – 4–5. *Leptographium serpens*. – 4. Conidia (Bar = 10 µm). – 5. Conidiogenous apparatus (Bar = 15 µm). – 6–8. *Leptographium lundbergii*. – 6. Conidia (Bar = 10 µm). – 7. Conidiogenous apparatus (Bar = 10 µm). – 8. Conidiophore (Bar = 10 µm).

Tab. 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

[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 calculation.
– Not present.

found on both *H. angustatus* and *H. ligniperda*. Frequency of occurrence of *O. ips* from *O. erosus* was 60%. For *L. lundbergii* and *L. serpens*, it was both approximately 45% for each species respectively from *H. angustatus*, and 22% and 21% 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 among the three bark beetle species could be linked, to some extent, 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* and *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, pers. comm.), 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, pers. comm.) 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, as 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 & al., 1995). *L. theobromae* was, however previously identified as the main cause of sapstain on pine logs exported from South Africa (De Beer & al., 2001). Apart from these two species, species such as *L. lundbergii*, *O. ips*, *O. pluriannulatum*, *O. piceae* and *Cop. minuta*, could also be considered as potentially serious sapstain agents. Therefore, together with the associated bark beetles, these species should be taken into consideration when control measures for sapstain are developed for the South African forestry industry.

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