

## Survey of Ophiostomataceae associated with *Hylurgus ligniperda* (Curculionidae: Scolytinae) in New Zealand

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### Abstract

A survey of Ophiostomataceae isolated and identified from *Hylurgus ligniperda* adults, pupae and larvae was undertaken in ten sites in *Pinus radiata* cutover forest in the central North Island. Members of this family are associated with sapstain and other plant diseases. This survey found that these fungi were common on both the surface of the beetles, as well as internally. In total, eight species of fungi belonging to this family were isolated from the beetles sampled. Identification was undertaken using morphological and physiological characteristics. Two *Ophiostoma* species, *O. galeiforme* and *O. huntii* were isolated most often, and were found in 50-97% of samples (groups of 10 adults, pupae or larvae from each stump). The remaining species, *O. floccosum*, *O. setosum*, *O. ips*, *O. quercus*, *Leptographium procerum*, and *L. truncatum* were isolated less frequently. The most dominant of these species, *O. floccosum* was isolated from 14% of surface sterilised *H. ligniperda* larval samples. These results implicate *H. ligniperda* in the movement of staining and other fungi throughout exotic forests in New Zealand.

**Keywords:** bark beetles, *Ophiostoma*, *Leptographium*, *Pinus radiata*, sapstain

### Introduction

Sapstain is a significant economic concern, especially to the *Pinus radiata* forest industry in New Zealand (Butcher 1968, Farrell *et al.* 1998). Sapstain is the discolouration of sapwood caused by several taxonomic groups of fungi including members of the Ophiostomataceae family and *Sphaeropsis sapinea*. The discoloration may be grey, black or brown reflecting the pigment of the fungal hyphae (Seifert, 1993). Staining is caused by melanin granules on the outer surface of the

fungus wall and in the medium surrounding the cells (Wheeler 1983, Zink & Fengel 1988, Brisson *et al.* 1996, Eagen *et al.* 1997). Saprophytic sapstain fungi are of great economic significance to the forest industry, as this group invades wood after the tree has been harvested (Seifert 1993), but only stain the timber when conditions are favourable for fungal growth, such as in wood with high sapwood content in a warm humid climate (Seifert 1993, Farrell *et al.* 1998). In contrast, staining due to pathogenic and endophytic fungi is apparent when the tree is harvested and wood may be discarded prior to processing (Seifert 1993).

The Ophiostomataceae family are ascomycetes, and a large number are insect associated species that colonise sapwood. *Leptographium*, *Pesotum*, *Sporothrix* and other anamorphic genera are known to have *Ophiostoma* teleomorphs or are thought to be closely related to *Ophiostoma* species based on DNA sequence analysis (Hausner *et al.* 2000, Jacobs *et al.* 2001).

A few species of bark beetles are notoriously capable of massive forest destruction. Adult beetles usually bore through the bark to the cambium of suitable hosts. Females lay eggs in galleries they excavate between the bark and the wood. Some species may bore further into the wood of hosts. Bark beetles are well known vectors of fungi and the interactions between aggressive bark beetles and fungi are well documented. While most bark beetles attack weak or dying trees, aggressive species are able to overwhelm and kill healthy mature trees, and may destroy large areas of forest during outbreak conditions (e.g., members of the genus *Dendroctonus*) (Klepzig *et al.* 1991, Raffa 1991, Paine *et al.* 1997). Many bark beetles carry fungal spores in specialised structures called mycangia that are typically simple pits found on the head, pronotum or elytral areas, or may lie internally within the digestive tracts (Harrington 1993, Paine *et al.* 1993, Solheim 1993, Klepzig

1998). Once introduced into trees these fungi may colonise the sapwood and disrupt the flow of water to the crown, killing the tree.

Two introduced bark beetles both of European origin, are commonly found in New Zealand pine forests where they are found colonising *P. radiata* stumps after harvest. *Hylastes ater* (Paykull) (Curculionidae: Scolytinae) and *Hylurgus ligniperda* L. (Curculionidae: Scolytinae) are secondary bark beetles that typically colonise dead or dying hosts and both species may be found together in the same host. *Hylastes ater* is of more importance as a pest, as offspring emerge from stumps and feed on pine seedlings that may be planted nearby (Reay *et al.* 2005). This maturation feeding can result in large areas of seedling mortality in new plantings. *Hylurgus ligniperda* does not feed on seedlings, but may vector sapstain and other fungi.

In a recent survey of Ophiostomataceae at ten sites in second rotation *P. radiata* forests in the central North Island, Reay *et al.* (2005) demonstrated that *H. ater* vectored sapstain fungi to *P. radiata* seedlings during sub-lethal feeding damage. No research to date has directly looked at the potential vectoring of sapstain fungi by *H. ligniperda* in New Zealand. The present study investigated whether the many *H. ligniperda* individuals found during the Reay *et al.* (2005) study carried Ophiostomatid fungi, and determined whether these fungi were similar to those vectored by *H. ater* individuals collected from the same stumps.

## Materials and Methods

Ten sites were selected within a second-rotation *P. radiata* forest in the central North Island, New Zealand in 2001. The approximate distances between the sites ranged from 0.5 km to 20 km. Trees at six sites were harvested between February and April, and at four sites between June and September. Approximately six to nine months after harvest, 10-20 *P. radiata* stumps were randomly selected in each site. *Hylurgus ligniperda* individuals of any life stage, found when the bark of stumps was removed, were collected into sterile containers (one container for each stump) and placed into cool storage until they were taken back to the laboratory. The same stumps that were sampled during the Reay *et al.* (2005) survey of

sapstain fungi associated with *H. ater* were used in this survey. Where adults and pupae of both species were collected from the same stumps they were stored in separate containers. Larvae from each stump were collected into the same container and were separated into species in the laboratory with the aid of a microscope. Collection and isolation of fungi on *H. ligniperda* was carried out using the same methodology as described by Reay *et al.* (2005) and was undertaken at the same time as this study so that comparisons of the fungi isolated from the two species could be made.

Two media were used to isolate fungi. The first was a general isolation medium, malt extract agar (2.0% malt extract and 2.0% agar), and the second, malt extract agar (0.2% yeast extract, 2.0% malt extract and 2.0% agar) supplemented with 200 µg/ml chloramphenicol, 100 µg/ml streptomycin sulphate and 400 µg/ml cycloheximide, was selective for the Ophiostomataceae.

Fungi were isolated from the different life stages of *H. ligniperda* as follows: Five individual adult beetles collected from each infested stump were placed on one plate of each of the two media and allowed to move freely for 10 minutes before being removed (unsterilised adults). A further ten adults per stump were surface sterilised by soaking in 5% hypochlorite solution for two minutes, and then rinsed twice using distilled water. Each adult was crushed using sterile tweezers. Five of the crushed adults were placed on a plate of the selective medium and five were placed on a plate of the non-selective medium. Ten pupae/larvae from each stump were surface sterilised, as described above, crushed using sterile tweezers and five individuals were placed on a plate of each of the two media as described above.

The plates were incubated at 25°C for up to 30 days and checked daily for fungal growth. As fungi developed, cultures were transferred aseptically onto fresh plates of the same medium. Members of the Ophiostomataceae family were identified on the basis of morphological and physiological characteristics into putative species using classical microbial techniques. Morphological and physiological features of the Ophiostomataceae included: *Pesotum*, *Leptographium*, *Sporothrix* (*Ophiostoma* anamorphs) structures or perithecia (*Ophiostoma* teleomorphs) structures. Sexual compatibility testing (Thwaites *et al.* 2005)

with known representatives from the Northern Hemisphere or New Zealand, was used to confirm unknown species. Members of this family were recorded as being isolated from a sample if identified from either of the two media plates containing the beetle/pupae/larvae sample from a particular stump. The data were analysed as fungi being present or absent on a sample of beetles, pupae or larvae.

## Results

In total, 163 samples of *H. ligniperda* individuals (mostly adults) were sampled for the presence of Ophiostomataceae (Table 1). Between 75-98% of all *H. ligniperda* samples (groups of 10 adults, pupae or larvae) contained Ophiostomataceae. On average, 1.3-2.1 species were isolated from each sample. *Ophiostoma galeiforme* and *O. huntii* were isolated most frequently, and were found in 50-97% of all samples. In contrast, *O. floccosum* (0-7%), *O. setosum* (0-14%), *O. ips* (0-5%), *O. quercus* (0-6%), *L. procerum* (0-9%) and *L. truncatum* (0-2%), were isolated less frequently. All eight species were isolated from unsterilised adult beetles, and all species except for *L. truncatum* were isolated from sterilised adult and larval samples. Only two species, *O. galeiforme* and *O. huntii*, were isolated from the eight pupal samples.

*Ophiostoma* and *Leptographium* species developed on both selective and non-selective media. Other fungal species developed on the

non-selective medium only and these included *Penicillium* species, *Cladosporium* species, *Trichoderma* species and *Mucor* species. Non-selective medium was used to isolate other sapstain fungi including *Sphaeropsis sapinea* and members of the Ophiostomataceae family (*Ceratocystis* species) that do not grow in the presence of cycloheximide (Harrington 1981). No *Sphaeropsis sapinea* or *Ceratocystis* species were isolated on the non-selective media.

## Discussion

The results of this study showed that species of *Ophiostoma* and its anamorph form *Leptographium* were carried either internally or on the surface of *H. ligniperda*. A similar situation was described by Reay *et al.* (2005) for members of the Ophiostomataceae family associated with *H. ater*, and Zhou *et al.* (2004) for *H. ligniperda* and *H. ater* from Chile. The *H. ligniperda* individuals used in this study were collected from the same stumps that were sampled during the Reay *et al.* (2005) survey. Collection and isolation of the fungi on *H. ligniperda* was carried out using the same methodology as described by Reay *et al.* (2005). Therefore, it was possible to directly compare the species of fungi associated with the two beetle species.

This survey of beetles and larvae showed that eight sapstain fungi were associated with *H. ligniperda*. In contrast, Reay *et al.* (2005) isolated

**Table 1. The occurrence of members of the Ophiostomataceae family in samples of *H. ligniperda*.**

	Number of samples*	Occurrence of samples with any Ophiostomataceae species (%)*	Mean number of Ophiostomataceae species per sample*	The occurrence of individual Ophiostomataceae species (%)							
				<i>O. galeiforme</i>	<i>O. huntii</i>	<i>O. floccosum</i>	<i>O. setosum</i>	<i>O. ips</i>	<i>O. quercus</i>	<i>L. procerum</i>	<i>L. truncatum</i>
<i>H. ligniperda</i> adult unsterilised	60	97	2.1	96.7	86.7	5.0	6.7	5.0	1.7	5.0	1.7
<i>H. ligniperda</i> adult sterilised	60	98	2.0	95.0	80.0	5.0	5.0	5.0	1.7	8.3	0.0
<i>H. ligniperda</i> pupae sterilised	8	75	1.3	50.0	75.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>H. ligniperda</i> larvae sterilised	35	89	1.9	74.3	77.1	14.3	5.7	2.9	5.7	8.6	0.0

\*Each sample consisted of 10 individuals collected from one stump

six species from *H. ater*. The two species that were found on *H. ligniperda*, but not *H. ater*, were *O. ips* and *L. truncatum*. *Ophiostoma ips* was isolated in this study from both sterilised and unsterilised adult beetles and sterilised larvae. *Leptographium truncatum* was only isolated from unsterilised adult beetles. However, more *H. ligniperda* samples (60 samples of sterilised & unsterilised adult beetles) than *H. ater* samples (40 & 38 samples of unsterilised and sterilised adult beetles) were collected in this survey (Reay *et al.* 2005). This was because both species were not always present in every stump sampled. It is possible that the absence of *O. ips* and *L. truncatum* from *H. ater* samples was a result of sampling variation, rather than these species not being associated with *H. ater*. The Reay *et al.* (2005) survey found that the eight species isolated in this study were also found in the wood of the stumps from which these beetles collected, and that the stump fungi composition was similar in the ten sites. *Ophiostoma ips* was also isolated from *H. ater* adults by Reay *et al.* (2002) and has been reported as a pathogen of *Pinus* species (Thwaites *et al.* 2005). The similarity between *Ophiostoma* species and *Leptographium* species isolated from the two beetle species and the stumps from which they were collected indicated that these beetles may opportunistically carry a range of Ophiostomataceae. Furthermore, the presence or absence of a fungus on a beetle may depend more on a beetle coming into contact with the fungus while it is sporulating, rather than a beetle being only able to carry a particular fungus. It is likely that additional members of the Ophiostomataceae family (Thwaites *et al.* 2005) present in New Zealand would be isolated from these beetles, if a wider survey was to be undertaken.

By contrast, Zhou *et al.* (2004), found only three Ophiostomataceae members (i.e., *Ceratocystiopsis minuta*, *O. galeiforme* and *O. ips*) from 80 unsterilised *H. ligniperda* individuals collected in Chile. They also isolated three species (i.e., *O. galeiforme*, *O. huntii* and *O. quercus*) from 34 unsterilised *H. ater* specimens. With the exception of *C. minuta* (which has not been isolated from New Zealand (Thwaites *et al.* 2005), these species have now been isolated from both *H. ligniperda* and *H. ater* in New Zealand (Reay *et al.* 2002, Reay *et al.* 2005).

The fungal species isolated in this survey were

similar, whether from the surface of beetles or from surface sterilised and crushed beetles, pupae and larvae. *O. galeiforme* and *O. huntii* were most commonly isolated from all of the beetle life stages sampled. They were also isolated most frequently from 67-100% of *H. ater* samples by Reay *et al.* (2005). The remaining *Ophiostoma* or *Leptographium* species found from both beetle species were isolated less frequently, in both the Reay *et al.* (2005) and the present study; and in both studies only *O. galeiforme* and *O. huntii* were isolated from pupae of both species. It is uncertain whether this was a reflection of the low number of pupal samples or that these are the only two species associated with the pupal stage.

Reay *et al.* (2005) demonstrated that *H. ater* vectored *O. galeiforme*, *O. huntii*, *O. floccosum*, *O. setosum*, *O. quercus*, *O. pluriannulatum*, *L. procerum* and *L. truncatum* to *P. radiata* seedlings during sub-lethal feeding activity. *Hylurgus ligniperda* has not been observed to feed on pine seedlings in New Zealand (Bain, 1977). However, it frequently colonises logs soon after harvesting when it may vector sapstain fungi. Bain (1977) reported that *H. ligniperda* was associated with minor sapstain in the outer sapwood of logs. Of the eight sapstain species isolated from *H. ligniperda* in this survey, *O. galeiforme* produces a greyish blue discolouration which was found to be severe on Scots pine, mild on spruce and light or no stain on larch (Bakshi, 1951). *O. huntii* and *L. truncatum* are both dark stainers on *P. radiata* (Thwaites, 2003). *Ophiostoma ips* was found to produce a severe and extremely blue-black stain in conifer sapwood (Griffen, 1968). Thwaites (2003) failed to induce *O. setosum*, *O. quercus* and *O. floccosum* to stain *P. radiata*, possibly because of unsuitable temperature conditions.

The results of this study supports the notion that introduced bark beetles vector sapstain fungi in New Zealand, and that these beetles may play an important role in the movement of these fungi around New Zealand exotic forests, as well as other areas where logs may be stored Reay *et al.* (2002, 2005).

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