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Chemodiversity and biodiversity of fungi associated with the pine weevil *Hylobius abietis*

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ABSTRACT

The pine weevil *Hylobius abietis* is a severe pest of conifer seedlings in reforestation areas. Weevils lay eggs in the root bark or in the soil near roots of recently dead trees and cover the eggs with frass (feces combined with chewed bark), possibly to avoid conspecific egg predation. The aim of the present investigation focused on isolation, identification, and volatile production of fungi from pine-weevil feces and frass. Fungi were isolated from weevil frass and feces separately, followed by identification based on ITS sequencing. Fifty-nine isolates belonging to the genera *Penicillium*, *Ophiostoma*, *Mucor*, *Leptographium*, *Eucasphaeria*, *Rhizosphaera*, *Debaryomyces*, and *Candida* were identified. Volatile organic compounds (VOCs) produced by the fungal community and fungal isolates cultured on weevil-frass broth were identified by SPME-GCMS. Major VOCs emitted from the fungal community and pure isolates were species- and strain specific and included isopentylalcohol, styrene, 3-octanone, 6-protoilludene, methyl salicylate, 3-methylanisole, 2-methoxyphenol, and phenol. Some of these are known to influence the orientation of pine weevils when tested among highly attractive newly planted conifer seedlings.

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Abbreviations; GC–MS, gas chromatography–mass spectrometry; HS, head space; ITS, internal transcribed spacer; PDA, potato-dextrose agar; SPME, solid-phase micro extraction; WFA, weevil-frass agar; WFB, weevil-frass broth

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Introduction

The pine weevil, *Hylobius abietis* (L.) (Coleoptera: Curculionidae), is an important pest of conifer reforestation (Leather *et al.* 1999; Day *et al.* 2004). The weevil preferably feeds on tender bark of actively growing parts of conifers (Örlander *et al.* 2000); however, stem and root bark of recently dead trees are also utilized (Wallertz *et al.* 2006). The pine weevils breed in the stump roots of recently dead trees and their numbers remain at a relatively low level in self-regenerated forests (Leather *et al.* 1999). However, since the mid of last century the weevil numbers have increased considerably due to the use of silvicultural practices of clear cutting and reforestation on vast areas. These practices provide the weevils a constantly high supply of suitable breeding substrate (Örlander *et al.* 1997; Nordlander *et al.* 2011).

In the managed reforestation areas, adult weevils feed on the bark of planted conifer seedlings resulting in large feeding scars that commonly girdle and kill the seedling. If no proper preventive measures are employed, the mortality of small plants due to pine weevil feeding may reach over 80 % every year, leading to considerable economic losses (Von Sydow 1997; Petersson & Örlander 2003). The risk of damage to newly grown seedlings is highest during the first three years after clear-cutting, subsequently declining during the fourth and fifth years (Örlander *et al.* 1997).

During spring, pine weevils migrate in search of new areas where they can find fresh stumps for breeding (Örlander *et al.* 2000). They are attracted by the wood-degradation product ethanol together with α-pinene and other host monoterpenes (Nordlander 1991). A female weevil lays about 70–80 eggs in one season (Bylund *et al.* 2004), which she lays in moist soil near the roots of fresh stumps or in the bark of the roots (Nordlander *et al.* 1997). She constructs a hole in the root bark with her snout, places an egg and feces in it and adds chewed bark (Fuchs 1912; Borg-Karlson *et al.* 2006). Similar egg-laying behavior has been reported for other *Hylobius* species (Wilson & Millers 1983; Wen *et al.* 2004) and also for other bark- and wood-feeding weevils (Trudel *et al.* 2001; Zhang *et al.* 2004; Tanahashi *et al.* 2009).

It is not fully understood why the pine-weevil females cover the eggs with frass (mixture of feces and chewed bark); it is possible that there are feeding deterrents in the feces/frass that keep feeding conspecifics away from egg-laying sites (Bylund *et al.* 2004). Short term anti-feedant activity towards weevils has been demonstrated for methanol extracts of female weevil-feces (Borg-Karlson *et al.* 2006) indicating the presence of active compounds in the feces. After chromatography and anti-feedant tests, the most active fraction contained several oxygenated aromatic compounds, presumably originating from lignin degradation (Borg-Karlson *et al.* 2006). Under natural conditions, an egg takes one to four weeks to hatch (Eidmann 1974), thus the oviposition site needs continuous protection for a long period. We hypothesize that the pine-weevil frass contains microorganisms that grow in the moist environment at the oviposition site and continuously produce compounds responsible for the deterrence of conspecifics from the egg-laying site (Azeem *et al.* 2013, 2015). Covering eggs with feces as a protection against conspecific competitors or against various predators has been described in many insects (Blum & Hilker 2008).

In this paper, we present the profile of volatile organic compounds (VOCs) emanating from a fungal community and pure cultures associated with pine-weevil frass/feces. VOC analysis of the fungal community and isolated cultures was carried out using solid-phase micro-extraction and GC–MS after cultivating on weevil-frass broth. Fungal cultures isolated from different samples of frass/feces were grouped based on their VOC profile and finally identified by molecular techniques. The effect of fungal VOCs on pine-weevil behavior is generally discussed in terms of deterrence.

Materials and methods

Collection of pine-weevil frass and feces

Pine weevils (*Hylobius abietis*) of both sexes were field-collected in their natural environment near Uppsala, Sweden and brought to the laboratory in clean boxes to avoid contamination. The weevils were maintained on freshly cut *Pinus sylvestris* twigs till used for frass/feces collection. The collection of weevil frass and feces was performed as described in Azeem *et al.* (2013). Briefly, the apparatus and weevil food were autoclaved or surface sterilized with 70 % ethanol before using them for frass/feces collection. Two-hundred weevils of mixed sexes were starved for 24–48 h; however, water was provided on a wet filter paper. The starved weevils were placed in a container with a metallic sieve at the bottom and fitted into another container. Freshly cut *P. sylvestris* twigs (15–30 mm diameter and 100 mm length) were provided as food. The pine-weevil frass (mixture of weevil feces and pine-bark particles chewed by weevils) fell through the sieve into the underlying container. The frass was collected aseptically in a glass vial from the collecting chamber.

After frass collection, fully fed weevils were placed in a similar container used for frass collection with only wet filter paper for another 24 h. The feces were collected from the container below and aseptically transferred to a glass vial. The collected frass and feces were stored at 5 °C until used for fungal isolation. Frass and feces collection was repeated at least five times using new weevils each time and fresh twigs of *P. sylvestris* picked from different self-regenerated, healthy trees from the Lunsen forest located south of Uppsala, Sweden (59°46′N, 17°40′E) and in a forest located in Stockholm, Sweden (59°19′N, 18°3′E) on different occasions during 2 y.

Volatiles from the fungal community associated with weevil frass

Aseptically collected weevil frass (WF) particles (5 g) were wetted with 25 ml water in a beaker and incubated at room temperature (22 ± 2 °C) after closing with aluminum foil. In order to evaluate the possibility of contamination from air, a beaker containing potato dextrose agar (PDA) was also prepared and incubated at similar conditions. After 10 d of incubation, a fungal community had grown on the frass surface; however, no microbe appeared in the PDA beaker. A representative sample