SYMBIOSIS (2006) 41, 71–79 ISSN 0334-5114

Travelling through time and space on wings of beetles: A tripartite insect-fungi-nematode association

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(Received February 16, 2006; Accepted March 30, 2006)

Abstract

Here we report a previously undescribed symbiotic association involving spruce beetles, *Dendroctonus rufipennis* Kirby, several species of fungi, and a nematode, *Ektaphelenchus obtusus* Massey. The nematodes and fungi occur within special pocket-like structures, hereafter termed "nematangia", on the insects' hind wings. The abundance of nematangia varied seasonally, ranging from 60% incidence in beetles overwintering within trees to 17% in beetles flying in late spring. Nematangia incidence was similar between beetle genders. The nematangia were orange-brown with a melanized appearance and leathery consistency. Ultrastructural analysis suggested that the walls of the nematangium are composed of a granular matrix that is infiltrated by microbial spores and secreted from the insects' wing veins. The presence of this granular substance and spores within the hollow wing veins, the surface of which is riddled with minute pores, is a novel observation. We identified four predominant microbial species: two *Ophiostoma* spp., most closely matching *Ophiostoma abiocarpum* (82% incidence) and *Ophiostoma penicilliatum* (24% incidence), and two yeasts in the genera *Candida* (88% incidence) and *Pichia* (82% incidence) based on ITS sequencing. One or both *Ophiostoma* spp. were present in all nematangia sampled. All spruce beetle wings observed were covered with mucilaginous secretions and fungal spores and/or mycelia. However, nematangia were only found when nematodes were present. This suggests a crucial role of the nematodes in their formation. Nematangia were not found associated with *Dendroctonus ponderosae* Hopkins or *Ips pini* (Say).

Keywords: Bark beetle, *Dendroctonus*, *Ips*, fungus, *Ophiostoma*, nematode, *Ektaphelenchus*

1. Introduction

Bark beetles (Coleoptera: Curculionidae) exert significant economic impacts on forest products, and pose important challenges to the sustainable management of natural resources. These insects develop entirely in the phloem tissue of woody plants (Wood, 1982) and can cause severe economic and environmental losses in managed and natural forest systems. Various species differ in their preferred host species range, generation times, overwintering strategies, and symbionts.

Bark beetles have a wide variety of symbiotic interactions with numerous microorganisms across different kingdoms (Paine et al., 1997; Six and Paine, 1998; 1999a, b; Moser et al., 2005). These associates include a diverse assemblage of fungi (Paine et al., 1997; Klepzig and Six, 2004), bacteria (Bridges et al., 1984; Delalibera et al., 2005), mites (Moser et al., 2005; Hofstetter et al., 2006) and nematodes (Massey et al., 1956; Moser et al., 2005).

In some cases the roles of these microorganisms are relatively well defined (Klepzig et al., 2004), but more often their roles are either matters of ongoing debate (Krokene and Solheim, 1998; Franceschi et al., 2005; Harrington, 2005) or are viewed as context-dependent (Eckhardt et al., 2004; Klepzig and Six, 2004; Kopper et al., 2004). Bark beetle-fungal associations are often maintained through elaborate transport systems, such as beetle mycangia and sporothecae of associated mites (Klepzig et al., 2001; Klepzig and Six, 2004; Hofstetter et al., 2006).

Nematodes are common associates of bark beetles, with each bark beetle species appearing to have its unique assemblage. These associations can be phoretic, commensal or parasitic (Rühm, 1956; Massey, 1956; Tomalak et al., 1984; Lieutier and Vallet, 1982). In many cases these nematodes have evolved special adaptations that allow them to overcome starvation or desiccation while associated with their hosts. The role of most of these nematodes in the beetles' life cycle and fitness, if any, is uncertain.

While examining the symbiotic community associated with bark beetles, we discovered the occurrence of novel

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pocket-like structures housing nematodes on the beetles' hind wings. Herein we describe research undertaken to 1) determine the incidence of structures containing nematodes on 3 bark beetle species, *Dendroctonus rufipennis* Kirby, *Dendroctonus ponderosae* Hopkins and *Ips pini* Say; 2) characterize the morphology and ultrastructure of nematode harboring structures; and 3) isolate and identify the nematodes and other microorganisms associated with these structures.

2. Materials and Methods

Presence and incidence of nematode harboring structures on different bark beetle species

Adult spruce beetles were collected from naturally infested trees on the Kenai Peninsula of Alaska, USA, and shipped to the Forest Entomology Laboratory at the University of Wisconsin, Madison, WI, USA. Adult mountain pine beetles, *D. ponderosae* were collected in baited funnel traps (Lindgren, 1983) set within lodgepole pine forests near Logan, UT. Pine engraver beetles, *I. pini*, were obtained from a laboratory culture that is derived from local populations and replenished with wild stock annually, and is maintained on red pine logs at the University of Wisconsin Department of Entomology. All insects were segregated by gender, and placed within 200 ml screw cap glass jars that contained crumpled Kimwipes® (Kimberly Clark, Roswell, GA) to absorb excess moisture. The insects were then stored at 4°C until needed for experiments.

To determine the incidence of nematodes and harboring structures, 40 insects (20 males + 20 females) of each species were killed by freezing and dissected. The body parts of each insect were carefully inspected under a dissecting stereoscope for the presence of nematodes and nematode-harboring structures. The number of such structures, their location, and beetle gender were recorded for each beetle species.

Based on results from the above study, we conducted more detailed studies on spruce beetles. Beetles were excavated from their overwintering sites within the bases of spruce trees in late October, 2004 (n=63), April (n=104) and May (n=47), 2005. Two additional samples were collected from baited funnel traps just south of Fairbanks, AK. These flight samples were collected in early May (n=64) and June (n=47), 2005. Spruce beetles were handled, freeze-killed, dissected and inspected individually as described previously. The incidence of nematangia, location on the insect body and beetle gender were recorded.

Characteristics of pockets harboring nematodes (=nematangia) on spruce beetles

The lengths and widths of 17 nematangia dissected from male (n=6) and female (n=8) spruce beetles were measured. The nematangia were then broken open, and the number and gender of the nematodes contained within each were recorded. Additional observations on the texture and color of the nematangia were also made.

For light microscopy, portions of the insects' wings containing nematangia were fixed in 4% paraformaldehyde in 0.1 M Na-phosphate buffer, pH 7.0 for 24 h and washed twice in 70% EtOH before being embedded in paraffin. Micro-sections (0.007 mm) of paraffin-embedded nematangia were made with an American Optic Spencer 820 microtome. Mounted sections were examined under a Leica DM LB2 phase contrast compound scope.

For electron microscopy, wings with pockets were fixed in 2% glutaraldehyde in 0.05 M Na-phosphate buffer, pH 7.0. Wings were washed in 0.05 M Na-phosphate buffer, postfixed in 2% OsO_4 for 1 h at room temperature, washed in phosphate buffer, and then dehydrated in a graded series of ethanol. Following dehydration, samples were transitioned in propylene oxide and infiltrated in a mixture of unaccelerated Poly/Bed812 resin (Polysciences Inc.) and propylene oxide. After resin infiltration, samples were polymerized for 48h at 70°C. Sections were produced using a Leica UC6 ultramicrotome, collected on carbon-coated Pioloform 2×1 mm slot grids, and then poststained with uranyl acetate and lead citrate. Sections were examined and documented with a Philips CM120 electron microscope. Images were collected with a SIS MegaView III digital camera (Soft Imaging Systems Corp.).

Isolation and identification of microorganisms within spruce beetle nematangia

The nematodes dissected from nematangia were collected, heat killed in a 60°C water bath, and preserved in 10% formalin 1% acetic acid solution mixed with equal parts of double-distilled H_2O. The preserved specimens were sent to the US Department of Agriculture's Nematology Laboratory (Beltsville, MD) for identification. The identification was confirmed based on morphological characters by Robin Giblin-Davis (University of Florida, Ft. Lauderdale, FL).

To characterize the microbial community within the nematangia, spruce beetles were dissected to collect individual nematangia under aseptic conditions. Nematangia were transferred to an autoclaved 1.5 ml micro-centrifuge tube containing 500 µl of sterilized double-distilled water. The microorganisms were extracted by crushing the nematangia with a sterile disposable pestle. Previous experiments indicated that the pressure exerted by pestle grinding was enough to rupture the nematangium, but not the nematodes. Serial dilutions of 10% and 1% were made from the original extract. Cultures of these dilutions and the original stock were made by transferring 100 µl aliquots onto 10% tryptic soy agar (TSA), made with 3 g tryptic soy broth (Difco™, Becton, Dickinson & Co., Sparks, MD) and 15 g agar per liter, poured onto disposable standard (10 × 1.5 cm) plates. The microbial growth on each culture

plate was evaluated after 5–7 days, and the microorganisms were classified based on macro-morphology (color, size, shape, texture). The presence of the different morphotypes on the different dilution plates was used to determine their percent occurrence.

Pure cultures representative of the different morphotypes were obtained from 3 different nematangia by transferring fungi to potato dextrose agar (PDA) (Difco™) and transferring bacteria to 10% TSA standard Petri plates. Ophiostomatoid fungi, as determined morphologically (Jacobs and Wingfield, 2001) were transferred to MEA amended with 200 mg/l of cyclohexamide (MP Biomedicals, LLC, Aurora, OH) and 100 mg of streptomycin sulfate (Sigma-Aldrich Co., St. Louis, MO) (Jacobs and Wingfield, 2001). All cultures were maintained in darkness at 25±2°C.

The identities of microorganisms that occurred in over 80% of the nematangia were determined by extracting DNA and sequencing of the 16S and internal transcribed spacer region (ITS) of the ribosomal DNA. Primers ITS1 (forward) and ITS4 (reverse) (White et al., 1990) were used to extend the desired region of the fungal rDNA. The sequences obtained with each primer were assembled and cleaned using DNASTAR Seqman (DNASTAR, Inc., Madison, WI) and then subjected to a Blast search in GenBank (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD) to identify the closest known sequence.

3. Results

Presence and incidence of nematode harboring structures on different bark beetle species

Nematodes were found to be associated with all three bark beetle species examined. In all three species, nematodes were found in anhydrous clusters under the elytra, commonly at the proximal ends, or on their abdominal tergites. On three of the *D. ponderosae* examined we found clusters of juvenile *Bursaphelenchus* sp. on their hind wings, but these were not contained within specialized structures. No wing clusters or nematode harboring structures were found on *I. pini*. In contrast, spruce beetles had pocket-like structures containing several female nematodes, to which we hereafter refer to as nematangia. These unusual structures always occurred within the jugal lobe area of membranous wings (hindwings). Nematangia, or other similar structures, were not found on any other part of the beetles' bodies, nor were they found on the other beetle species examined. In addition to containing nematodes, spruce beetle nematangia always harbored microbial spores.

Nematangia were found on 60% of the spruce beetles examined. These spruce beetles were collected from their host during the peak of their overwintering phase (February, 2004). Nematangia occurred mostly on one wing, but on rare occasions on both wings (5% of beetles having them). The incidence of nematangia did not vary significantly by gender: 48% of nematangia were on males and 52% were on females. There was no association with a particular wing: 49% of the nematangia were on the right wing and 51% were on the left wing.

The incidence of nematangia on spruce beetles varied seasonally. Nematangia were prevalent on insects collected from overwintering sites, which are in the bases of trees below the soil line, with the highest incidence being on insects collected in April 2004 (60%) and the lowest on overwintering insects collected in late May (27%) (Fig. 1). Interestingly, the incidence of nematangia on the beetles collected from flight traps was only 32% in early May and dropped to 17% by peak flight, in late May – early June.

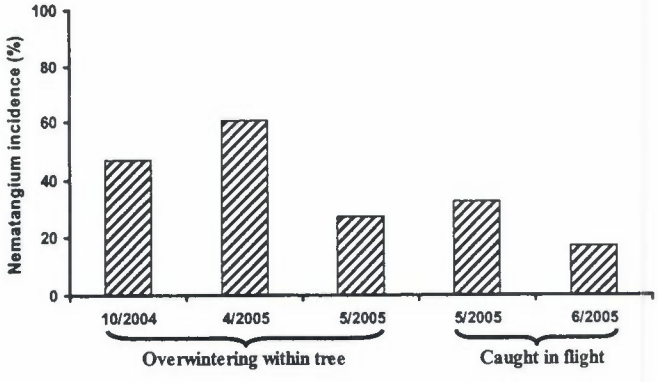
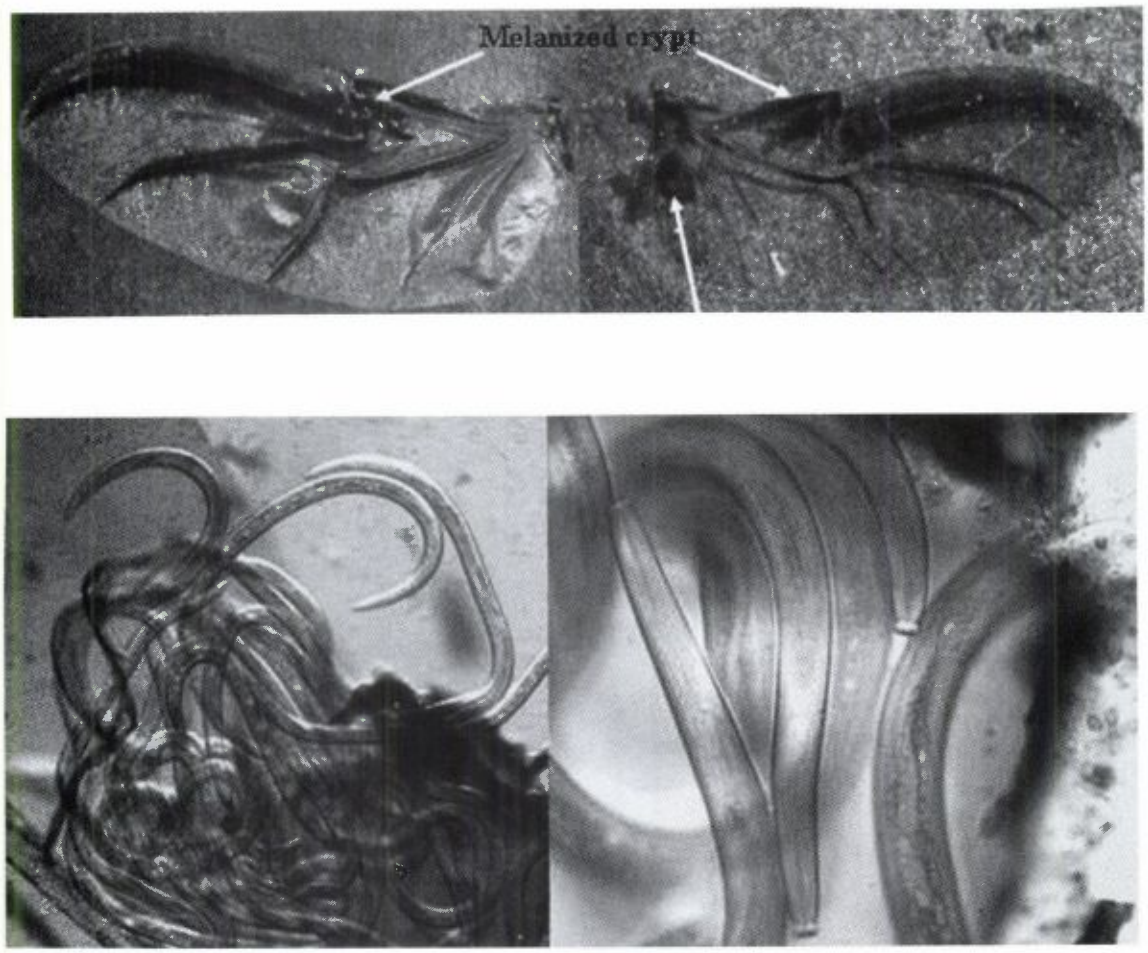


Figure 1. Incidence of nematode harboring structures, termed "nematangia", on overwintering and flying spruce beetles. The incidence was highest on overwintering beetles (October – May) and lowest during the peak of their flight period (June).

Characteristics of nematangia on spruce beetles

The nematangia were orange-brown, with a melanized appearance and apparent leathery consistency (Fig. 2A). Female nematodes were observed in all of these nematangia (Fig. 2B). The nematangia detached easily from the wings and thus, did not appear to be an integral part of the insects' wing tissue (Fig. 3A). Ultrastructural observations revealed that the nematangium appeared to be a composite of a granular matrix and fungal mycelia and spores (Fig. 3B). Individual nematangia were (Mean±SE) 1.0±0.202 mm long and 0.49±0.122 mm wide. On average each nematangium contained 27.7±6.24 female nematodes. No male nematodes were found within the nematangia. We only obtained 3 nematangia without nematodes from all the spruce beetles examined in this study. These nematangia were ruptured so conceivably, the nematodes had either exited or they had been eaten by a predator.



B)

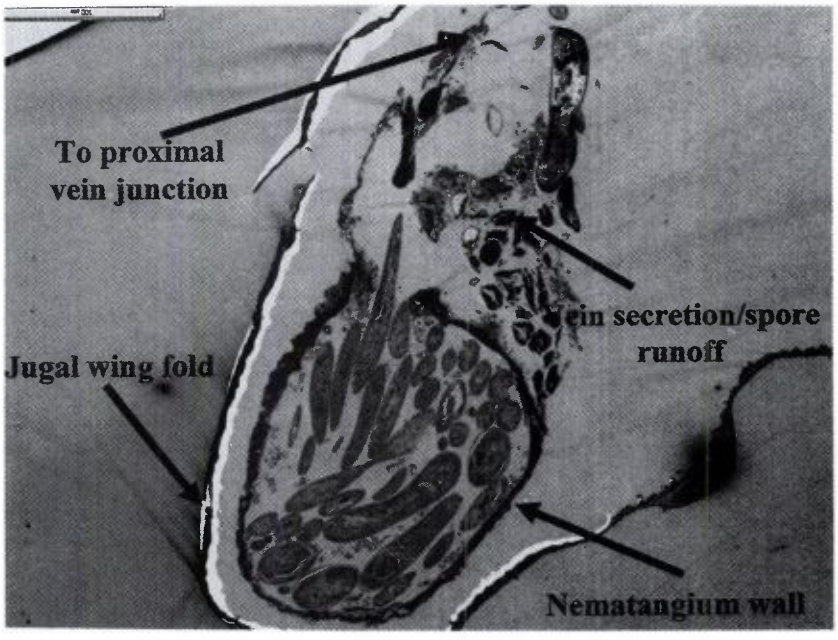
Figure 2. A) Spruce beetle wings without (left) and with (right) a nematangium (10×, bright field); B) female *Ektaphelechus obtusus* inside a disrupted nematangium (40×, bright field).

All wing veins had pores along their entire length, appeared hollow, and were filled with spores, hyphal masses, and an unstructured granular material (Fig. 4A,B). The pores were particularly numerous at the proximal and basal (just above the line of main fold) ends of the sub-costal vein (Fig. 4C,D). The sub-costal vein appeared to have an additional structure, a melanized crypt-like enlargement just above the line of the wings' main fold, on the dorsal wing margin (Fig. 4C,D). This structure contained spores, hyphal masses and the granular material but no nematodes. Crypts were present on both wings all *D. rufipennis* examined. Whether or not nematangia were present, all spruce beetle wings observed were covered with mucilaginous secretions and fungal spores and/or mycelia, as visualized by bromophenol blue staining (Fig. 5A,B).

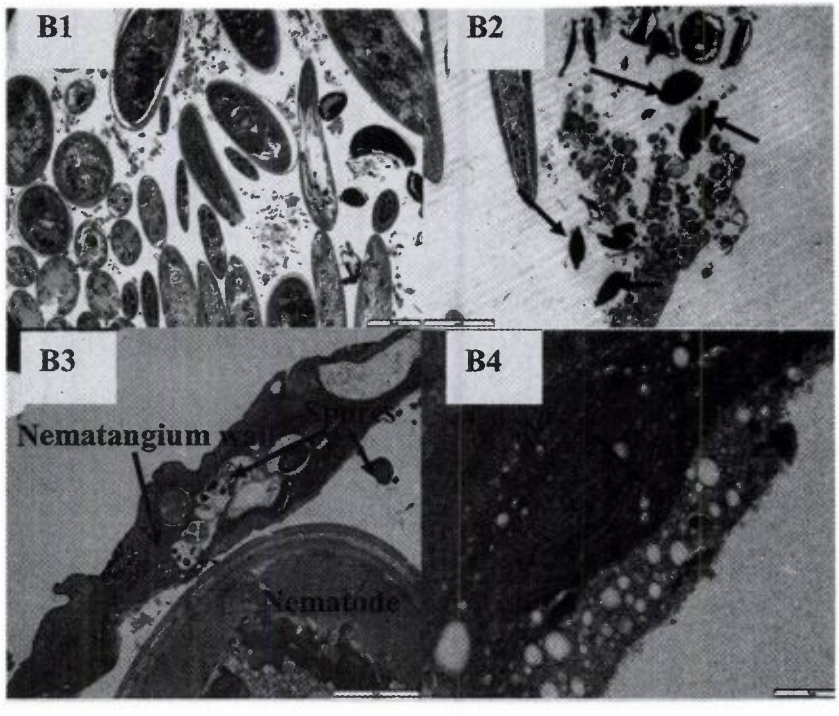
Isolation and identification of microorganisms within spruce beetle nematangia

The nematode species consistently associated with nematangia were morphologically determined to be females of *Ektaphelenchus obtusus* Massey. The nematodes within these structures were lethargic but responsive to disturbance, and appeared well hydrated. They were crowded and coiled-up within the nematangia and they were never observed penetrating the nematangium wall or the insects' bodies with their stylets.

In addition to the nematodes, 15 different microbial morphotypes were recovered from cultures made from nematangium extracts. Of these, 4 morphotypes were present in more than 80% of the nematangia examined. The macro-morphological descriptions of these were: 1) Colonies dark brown, reverse dark brown, with hazel spreading edges. Mycelium mostly submerged and white aerial mycelium, when present. No conidiophores or perithecia produced on CSMA at 25°C after 3 months; 2) Colonies hyaline, reverse hyaline, mycelium submerged, no aerial mycelium observed. No conidiophores or perithecia produced on CSMA at 25°C after 3 months; 3) Colonies mucoid, white, opaque, round, spreading, and slightly convex; 4) Colonies same as 3, but pale yellow. All other morphotypes had an incidence of 30% or lower.



A)



B)

Figure 3. Electron microscopic images of A) jugal wing fold and nematangium tissue slice, bar on upper left corner denotes 100 μm; and B) Pocket sections showing nematodes and microbial spores (B1, bar=20 μm), microbial spores and spore-containing capsules (B2, bar=0 μm), wall section made of granular matrix and embedded microbial spores (B3, bar=5 μm), and wall filamentous infolding (B4, bar=1 μm).

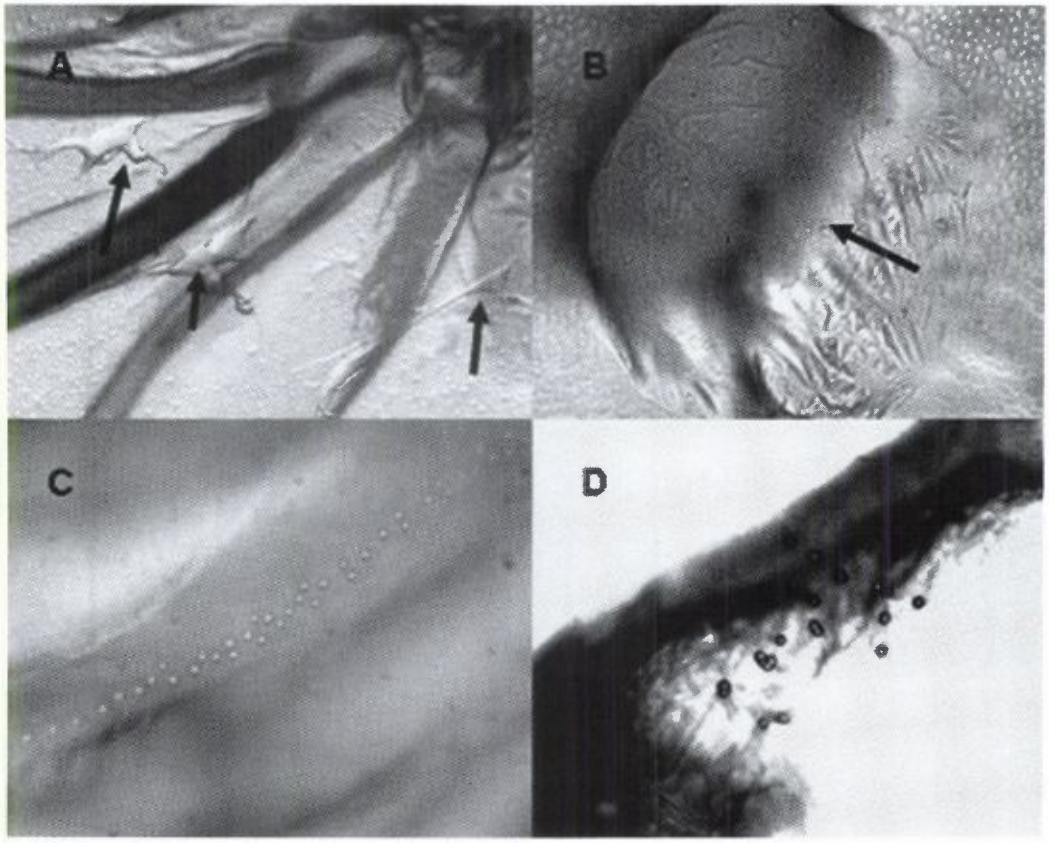


Figure 4. Light microscopic images of pores on proximal end of a wing vein A); basal end of the sub-costal melanized crypt (B) (40×, bright field); wing vein junction with secretions (C) (10×, bright field) and; secretion droplet with spores (D) (100×, bright field).

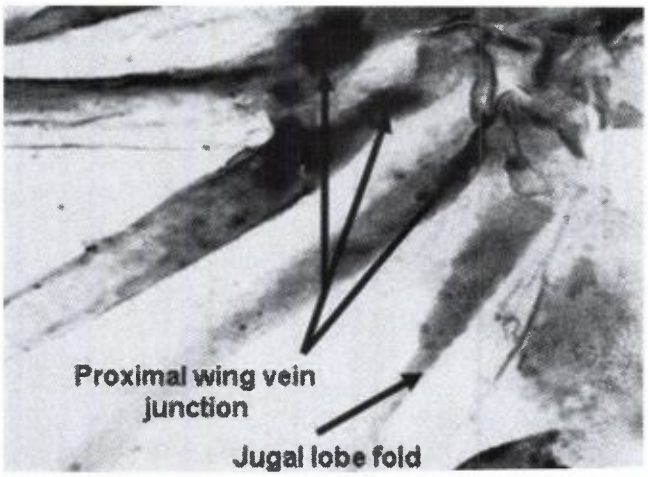
Sequencing of the ITS rDNA region yielded close matches to two *Ophiostoma* spp.: *Ophiostoma abiocarpum* (98% match for 573 base pairs) and *Ophiostoma penicilliatum* (97% match for 535 base pairs). Sequencing of isolates for the other two yielded close matches for two yeast species in the genera *Candida* (95% match for 155 base pairs) and *Pichia* (95% match for 218 base pairs). One or both *Ophiostoma* spp. were present in all of the nematangia sampled. The morphotype closely matching *O. abiocarpum* was present in 82% and the morphotype closely matching *O. penicilliatum* was present in 24% of the pockets examined. The *Candida* and *Pichia* yeast morphotypes were found in 88 and 82% of the nematangia, respectively.

4. Discussion

We identified a unique structure on spruce beetle wings that harbors nematodes which we have termed "nematangium". Nematangia have not been described from any other insect-nematode association. The only prior description of a nematode-harboring structure is that of "leathery cocoons" containing female *Ektaphelenchus* spp. found under the elytra or on the abdominal tergites of several bark beetle species (Rühm, 1956). Interestingly, one of the beetles listed as having such structures was *Dendroctonus engelmanni* (=*D. rufipennis*, Wood, 1982). In contrast, the structures we observed were found exclusively within the jugal wing folds. Also in contrast to the leathery cocoons described by Rühm (1956), these pockets were only observed on *D. rufipennis*.

Associations of *Ektaphelenchus* species with bark beetles have been described previously (Massey, 1956). However, their roles in the insects' life histories or gallery communities have not been studied. Rühm (1956) suggested that *Ektaphelenchus* were parasitic on the insects and that the "leathery cocoons" in which they occur might be either produced by the insect in response to the nematode feeding or secreted vaginally by the female nematodes.

The species of fungi recovered from these nematangia were unexpected, because they have not been reported as *D. rufipennis* associates to date. Because the sequence matches for the ITS region were below 99%, we cannot conclusively ascertain their identities to the species level and further morphological and molecular analyses are needed.



A)

B)

Figure 5. A) Spruce beetle wing stained to show presence of fungal spores and mycelium, particularly abundant inside wing veins (10×, bright field). B) Stained fungal spores could be easily visualized on wing vein surfaces (40×, bright field).

Although the morphotype closely matching *Ophiostoma abiocarpum* was recovered from 82% of the nematangia, this species has not been recovered from *D. rufipennis* previously, despite extensive study of this system (e.g., Solheim, 1995; Wingfield et al., 1997; Six and Bentz, 2003), including a recent survey of 1000 beetles from the same region by ourselves (Aukema et al., 2005). Similarly, the morphotype closely matching *O. penicilliatum* has only been reported once from *D. rufipennis*, in Colorado (Davidson, 1955). Interestingly, *Leptographium abietinum* (Peck) Wingfield, which is typically recovered from 80–90% of *D. rufipennis* in large-scale studies (Six and Bentz, 2003; Aukema et al., 2005; Cardoza et al., submitted), was not recovered from the nematangia. Because sequences for the ITS regions of ophiostomatoid morphotypes obtained from *D. rufipennis* in a previous study yielded close sequence match (98%) to *L. abietinum* (Cardoza et al., submitted), we are confident that the species recovered from the nematangia are different from this prevalent associate. Aukema et al. (2005) reported 3 species of *Ophiostoma*, denoted A, D and E, which were not resolved to the species level. Direct morphological and molecular comparisons between these species and those recovered from the nematangia will be needed to determine their similarities.

The nematangia appear to be the product of interactions between substances secreted through pores on the insects' wing veins, fungi, and nematodes. The jugal fold provides a propitious area for vein secretions to collect, particularly when the insect is at rest, such as during their overwintering phase. Fungal mycelia and/or spores covered the wing veins and wing surfaces of all insects examined during this study, and likewise occurred around and inside the nematangia. The structure of the nematangium wall is reminiscent of yeast sporogeneous organs (Robinow and Johnson, 1991). Thus, it is possible that the *Pichia* and *Candida* species isolated from the nematangia may partake in the wall formation process. Since all insects examined contained secretions and fungal spores, but nematangia occurred only when nematodes were present, the nematodes likely play an important role in nematangium formation. The pool of secretions combined with accumulated mycelia and spores could conceivably serve as attractants for the nematodes. Hardening (sclerotization) of the outer surface may occur by oxidation or desiccation, giving rise to the nematangium.

The high incidence of nematangia on overwintering beetles and the low incidence on pre-flight and flying beetles suggest these structures may serve as an overwintering domatium for the nematodes. This hypothesis is further supported by the abundance of microbial spores (particularly yeasts and fungi) within the nematangia, which we theorize serve as food inoculum for the nematodes upon arrival to new host trees. While still in the nematangia, the nematodes are in a resting/dormant stage, tightly coiled and sluggish, and do not appear to feed. The role of these structures in nematode overwintering is further supported by differences between spruce beetle and mountain pine beetle and pine engraver, in which they do not occur. Specifically, of these three species, only spruce beetles spend lengthy periods within their host plant as adults, often over a year (Werner and Holsten, 1985; Hansen and Bentz, 2003). In contrast, mountain pine beetles overwinter as larvae and pine engravers are multivoltine throughout the flight season and overwinter as adults in the soil (Bentz et al., 1991, 2001; Logan and Bentz, 1999; Ayres et al., 2001; Aukema et al., 2005).

Ophiostomatoid-bark beetle associations are well documented and intensely studied, but there is disagreement over their role, with reports ranging from mutualistic to antagonistic (Paine et al., 1997; Six, 2002; Franceschi et al., 2005; Harrington, 2005; Hofstetter et al., 2006). Most

likely these relationships vary among different systems, and in some cases are context-dependent. That is, some fungi may assist beetles in overcoming tree defenses during the colonization phase but compete with brood during the development phase (Klepzig and Six, 2004). Similarly, nematode species have variable associations with bark beetles, including parasitic, commensal and phoretic (Massey, 1956). Ascomycetous yeasts, with hat-shaped ascospores, such as *Pichia* and *Candida*, have reported associations with wood feeding insects, including Scolytinae beetles, and are thought to benefit their hosts by enhancing the nutritional composition of their food and by mediating intra-specific communication (Leufven et al., 1984; Leufven and Nehls, 1986; Kurtzman, 2000; Lim et al., 2005; Suh et al., 2004a, b; Suh and Blackwell, 2005).

The observation that all spruce beetles had fungal masses and spores on the wing surfaces and/or within the veins suggests these veins may substitute for, or even be analogous to, a mycangium, which is considered absent in *D. rufipennis*. If these fungi benefit the beetles, the nematodes may antagonize their hosts by feeding on the fungi. Alternatively, the nematode could be mutualistic to the insects and fungi, by providing spore transportation to new host trees via these pockets, or improving the efficiency of fungal inoculation. If these fungi are antagonistic to the beetles, as has been shown with some ophiostomatoid species (Barras, 1970; Bridges et al., 1985; Six, 2003), fungal feeding by the nematodes would benefit the insects by functioning as biological control agents (Klepzig et al., 2001). The latter possibility is consistent with a context-dependency hypothesis, because the nematodes would control fungi growing along the brood galleries during larval development. Further work on the interactions among the beetles, fungi, and nematodes is needed to test these hypotheses, and to fully characterize this tripartite association.

Further experiments are needed to shed light on the chemical nature of the nematangium wall to confirm its true origin. Similarly, the sequence of events leading to the formation of nematangia within the spruce beetle hind wings is a subject that merits further exploration. The role and importance of the microorganisms on the nematode and beetle life cycles also needs to be investigated.

Acknowledgements

This work was funded by grants from the USDA NRI (2003-3502-13528), National Science Foundation (DEB0314215), USDA Forest Service, Southern Research Station, and the University of Wisconsin-HHMI Symbiosis Summer Program. We are grateful for insightful discussions with Archana Vasanthakumar (Entomology Department, UW-Madison) Ann MacGuidwin (Department of Plant Pathology, UW-Madison), Thomas Harrington (Department of Plant Pathology, Iowa State University) and Michael Wingfield (FABI, Pretoria, South Africa). We thank Lynn Carta and Zafar Handoo (USDA, Nematology Laboratory, Beltsville, MD) and Robin Giblin-Davis (University of Florida, Ft. Lauderdale, FL) for identification of the nematodes. We benefited from invaluable technical support by Evan H. Whalen and Jason Jones (Department of Entomology, UW-Madison, Madison, WI). The assistance of Ed Holsten and Ken Zogas (USDA Forest Service, Anchorage, AK); James Kruse and Angie Ambourn (USDA Forest Service, Fairbanks, AK); Jose Negron (USDA Forest Service, Rocky Mountain Research Station, Fort Collins, CO), Barbara Bentz and Matthew Hansen (USDA Forest Service, Logan, UT) in collecting bark beetles is greatly appreciated. We thank Randall Massey of the University of Wisconsin Electron Microscopy Facility for assistance with electron microscopy. The critical reviews of two anonymous reviewers are greatly appreciated.

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