Multipartite Symbioses Among Fungi, Mites, Nematodes, and the Spruce Beetle, *Dendroctonus rufipennis*

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ABSTRACT The spruce beetle, *Dendroctonus rufipennis*, is an eruptive forest pest of significant economic and ecological importance. *D. rufipennis* has symbiotic associations with a number of microorganisms, especially the ophiostomatoid fungus *Leptographium abietinum*. The nature of this interaction is only partially understood. Additionally, mite and nematode associates can mediate bark beetle–fungal interactions, but this has not yet been studied for spruce beetles. In this study, we found eight mite species associated with spruce beetles: *Tarsonemus ips*, *T. endophloeus*, *Histiogaster arborsignis*, *Dendrolaelaps quadrisetus*, *Proctolaelaps hytricoides*, *Trichouropoda alascae*, *T.* n. sp. nr *dalarenaensis*, and *Urobovella* n. sp 767. The most prevalent species was *H. arborsignis*. In addition, 75% of beetles examined carried nematodes, with six species represented. These included a new species of *Parasitorhabditis*, *Ektaphelenchus obtusus*, *Bursaphelenchus* n. sp. 727, *Aphelenchoides* n. sp., *Panagrolaimus* sp., and *Mykoletzkya ruminis*. *H. arborsignis* showed strong feeding and oviposition preferences for *L. abietinum* among four fungal species tested in laboratory assays. Information on our attempts to culture the various nematode species collected from *D. rufipennis* is also provided. *Bursaphelenchus* were cultured from *D. rufipennis* nematangia plated on agar containing *L. abietinum* but not sterile agar. Thus, *L. abietinum* plays an important role in these gallery communities, affecting the tree-killing bark beetle, its phoretic mites, and nematodes. These data add to our understanding of bark beetle–microorganism interactions.

KEY WORDS symbiosis, bark beetles, *Histiogaster*, Leptographium, Hyphomycetes

The spruce beetle, *Dendroctonus rufipennis* (Kirby), is a native species that occurs throughout North America wherever there is contiguous spruce (*Picea* spp.). Under most conditions, these beetles occur at low population levels and play a beneficial role by feeding on stressed or fallen trees. This provides food and habitat for wildlife and contributes to nutrient cycles necessary for a healthy forest. However, during eruptive conditions, they attack and kill healthy, vigorous trees and undergo vast region-wide outbreaks. For example, *D. rufipennis* killed ≈2.3 million hectares of spruce forests in Alaska and 3 million mature spruce in Utah in the 1990s alone (Dymerski et al. 2001). Females locate suitable hosts, bore through the bark and release aggregation pheromone that attract large numbers of conspecifics (Wood 1982). Mating occurs within the host and females oviposit in niches along the main parental gallery. These galleries become microhabitat for the myriad of microorganisms associated with these insects.

Bark beetles, including *D. rufipennis*, have intricate symbiotic associations with microorganisms. These associates include a diverse assemblage of fungi (Paine et al. 1997, Klepzig and Six 2004), bacteria (Bridges 1984, Delalibera et al. 2005, Cardoza et al. 2006a), mites (Moser et al. 2005, Hofstetter et al. 2006), and nematodes (Massey 1956, Moser et al. 2005, Cardoza et al. 2006b). The roles of some microorganisms, particularly certain fungi, are relatively well defined (Six 2003, Six and Bentz 2003, Klepzig and Six 2004, Cardoza et al. 2006a, Bentz and Six 2006). Some fungi and bacteria may facilitate digestion of host tissues, pheromone synthesis, or depletion of host defense (Brand et al. 1976, Ayres et al. 2000, DiGuistini et al. 2007). For phloeophagous species such as *D. rufipennis*, whose food is nutritionally poor, fungal symbionts may also provide an alternative source of nutrients (Six 2003, Harrington 2005, Bentz and Six 2006). Some bacterial symbionts may benefit the beetle hosts by modifying the microbial community, particularly by inhibiting antagonistic fungi (Cardoza et al. 2006a). Some associates likely exert both positive and negative effects (Eckhardt et al. 2004, Klepzig and Six 2004, Kopper et al. 2004) and hence may be context-dependent or conditional mutualists.

Leptographium abietinum is the ophiostomatoid fungus most frequently associated with spruce beetles, being found on 80–100% of adults (Six and Bentz 2003,

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Aukema et al. 2006). Despite this high incidence, the cost/benefit to the beetle remains to be determined. Astigmatid mites (Arachnida: Acaridae) and various species of nematodes are also predominant associates of *D. rufipennis.* They have been observed in significant numbers along the galleries of the beetles and seem to thrive on galleries pervaded by blue-stain fungi (Massey 1956, Rühm 1956, Cardoza et al. 2006a).

This study furthers our exploration of bark beetle–microorganism interactions by evaluating the effects of *L. abietinum* on the survival and performance of *D. rufipennis* under laboratory conditions. The feeding preference of the most prevalent astigmatid mite associated with *D. rufipennis* was evaluated. Finally, we collected, identified, and attempted to establish cultures of the various nematode species associated with *D. rufipennis.* The latter was done with the main purpose of facilitating future studies on the interactions between these nematode species and their beetle hosts.

Materials and Methods

Insects, Mites, and Fungi. Adult spruce beetles were collected from naturally infested trees on the Kenai Peninsula of Alaska, and shipped to the Forest Entomology Laboratory at the University of Wisconsin, Madison, WI. The collection area is described by Holsten et al. (1999, 2003). On arrival, the insects were segregated by sex, placed in 200-ml screw cap glass jars, and provided with pieces of excised spruce phloem for food and crumpled Kimwipes (Kimberly Clark, Roswell, GA) to absorb excess moisture and provide insulation and a surface for walking. The insects were stored at 4°C until needed for experiments.

Astigmatid mites were collected from field-caught *D. rufipennis* and were maintained on *L. abietinum* malt extract agar (MEA; Difco, Detroit, MI) culture plates in the laboratory. All mites used for these experiments were obtained from laboratory colonies and were newly emerged adults. Other mite associates were collected for identification by placing 20 beetles (10 males + 10 females) in 70% EtOH and vortexing for 3 min to dislodge them from their host beetle. Additional mites were collected from galleries of the beetles made on phloem sandwiches, as described below. Mites collected in this manner were transferred to a watch glass, sorted by morphology, stored in 70% EtOH, and identified.

The fungal species used were *L. abietinum, Aspergillus nomius, A. fumigatus,* and *Trichoderma harzianum/atroviridae,* isolated and identified by Cardoza et al. (2006a). Fungal cultures were started by placing a 1-cm-diameter mycelial plug in the center of MEA plates. The fungi were allowed to grow for 5–7 d in complete darkness.

Effects of *L. abietinum* on Feeding and Performance of *D. rufipennis*. Spruce beetles feeding on *L. abietinum* were tested in no-choice feeding experiments. Male or female beetles were individually weighed and allowed access to a 1-cm MEA plug with or without *L. abietinum* for 48 h. At the end of this period, the survival and weight of the beetles were determined. Feeding by a total of 24 beetles of each sex was evaluated on either sterile MEA (control) or MEA colonized by *L. abietinum* mycelium. Beetle feeding on fungal plugs was determined based on plug size reduction, presence of chewing marks, and production of fecal pellets containing plug material.

On a separate experiment, the performance of spruce beetles on phloem sandwiches inoculated with either sterile MEA or *L. abietinum* was also studied. The experimental arena consisted of an 8 by 8-cm piece of spruce phloem with bark sandwiched between two 10 by 10-cm Plexiglas plates. The edges around the Plexiglas plates were sealed using 1.0 by 0.25 by 200-cm vinyl foam weatherseal strips (Thermwell Products Co., Mahwah, NJ) cut to fit each side of the arena. The arenas were bound together with two 2.54-cm binder clips on each side. These arenas were adapted from "phloem sandwich" designs described by Taylor et al. (1992). The phloem pieces were obtained from white spruce logs ≈30 cm diameter cut 1–3 wk in advance of set up. Logs were covered with molten paraffin wax at both ends immediately after tree felling to prevent desiccation and stored at 4°C until needed.

Phloem was surface sterilized by dipping into a Bleach:EtOH:H_2O (1:5:94) solution for 1 min and then air dried within a laminar flow hood for ≈15–20 min, after which no bleach or EtOH could be detected by smell. The sterile phloem pieces were then inoculated with five 1 cm diameter plugs of each fungus by placing a plug at each of the corners and one at the center of the phloem square so that the fungus side would be in direct contact with the phloem surface. Arenas mock-inoculated with sterile MEA plugs served as controls. The inoculated pieces of phloem were then assembled into the arenas as described previously. Two male-female pairs of spruce beetles were introduced per arena immediately after fungal inoculation. The beetles were allowed to feed and oviposit for 4 wk. Beetle performance was evaluated based on number of galleries, total gallery length (cm), number of eggs laid and percent adult mortality. This experiment was set up in duplicate and repeated using different groups of insects and phloem sources to obtain a total of 14 replicates.

Mite Identification and Effects of Fungi on Feeding Preference of Astigmatid Mites. Mite feeding preferences were tested in dual choice assays in which 10 newly emerged adult mites (5 females + 5 males) were placed within an MEA media plate containing 1-cm plugs from sterile MEA control or from cultures of the four fungal species mentioned above. Pair combinations were designed so all the fungi were tested against each other, as well as against a sterile MEA control. The two plugs on each plate were placed opposite each other against the plate walls (for maximum spatial separation). Mites were released in the center of each dish, and the number of mites and eggs at either of the plugs were counted and recorded at 4, 24, and 48 h.

Location, Identification, and Culturing of Nematode Associates. Nematodes were obtained by dissecting 328 live beetles (142 males + 183 females). Nematode load for each insect was estimated as none (no nematodes observed on any body part), low (1–50), moderate (50–100), and high (>100). Nematodes were tabulated based on the body part on which they were found and prepared for bioassays.

Before culturing, nematodes were rinsed twice in watch glasses containing autoclaved ddH_2O, and transferred onto MEA plates with or without *L. abietinum.* Five nematodes of similar morphology were placed in the center of each plate (10 no fungus controls and 10 *L. abietinum*). Plates were individually sealed with two layers of Parafilm tape and kept under complete darkness for 30 d. After this time, plates were inspected for nematode activity.

Samples of nematodes collected directly from beetles body parts and from the above cultures were placed in autoclaved H_2O, immobilized in a 60°C water bath for 3–5 min, and fixed in FA 4:1 solution (10 ml of 40% formaldehyde, 1 ml glacial acetic acid, 89 ml autoclaved H_2O). These samples were sent to Drs. Lynn Carta and Zafar Handoo (USDA–ARS, Nematology Laboratory, Beltsville, MD) and Dr. Robin Giblin-Davis (FLREC, University of Florida, Fort Lauderdale, FL) for morphological identification.

Statistical Analyses. Data were found to conform to the assumptions of normality using a Box-Cox power of transformation test. The effect of beetle sex, plug treatment, and sex × plug treatment on weight gain and survival of spruce beetles was tested using analyses of variance (ANOVAs; PROC GLM; SAS Institute 1996). The effects of fungus inoculation on the number of spruce beetle galleries, total gallery length, number of eggs laid, and adult percent survival were also evaluated using ANOVAs (PROC GLM; SAS Institute 1996). ANOVAs with significant P values ($P \leq 0.05$) for treatment effects were followed by Tukey's mean separation tests. The feeding and oviposition preferences of the mites between fungal pairs were tested using paired t-tests (PROC MEANS; SAS Institute 1996) if mite and egg count values for two compared treatments were higher than zero.

Results

Effects of *L. abietinum* on Feeding and Performance of *D. rufipennis*. Beetle feeding on plugs was determined based reduction of plug size, chewing marks, and production of fecal pellets containing plug material. All male and female spruce beetles that had been provided inoculated plugs were observed feeding on *L. abietinum.* There was no significant difference in weight gain or survivorship between beetles feeding on control MEA or *L. abietinum.* Initial weights averaged 15.7 ± 0.36 (SE) mg for females and 16.8 ± 0.30 mg for males. Female beetles gained ≈1.2 mg more than their male counterparts, 4.0 ± 0.29 and 3.9 ± 0.28, respectively. Percent survival was 72 ± 6.48 for females and 50 ± 7.29 for male beetles. Although differences were not significant, it is worth noting that there was a tendency for female beetles to gain more weight when feeding on *L. abietinum* plugs and survival was 17 and 13% higher for *L. abietinum* fed males and females, respectively, compared with the respective clean MEA controls.

Table 1. Performance of *D. rufipennis* in experimental phloem arenas inoculated with *L. abietinum*

Parameter	Treatment (mean ± SE)	
	Control	*L. abietinum*
Number of galleries	2.7 ± 0.30[a]	1.9 ± 0.28
Gallery length	15.7 ± 2.85[a]	9.1 ± 1.27
Number of eggs	28.1 ± 7.07[a]	18.2 ± 7.16
Percent mortality	42.5 ± 5.33	36.4 ± 9.74

[a] Significantly different between treatments, Tukey's mean separation test ($P \leq 0.05$).

Inoculation of phloem arenas with *L. abietinum* significantly affected the number of galleries ($F = 16.64$; df = 1,26; $P = 0.004$), total gallery length ($F = 12.66$; df = 1,26; $P = 0.0015$), and number of eggs constructed and laid ($F = 8.78$; df = 1,26; $P = 0.0064$) by *D. rufipennis* (Table 1). Beetles constructed fewer and shorter galleries and deposited fewer eggs on *L. abietinum* than on MEA mock-inoculated phloem (Table 1). However, adult survival did not differ significantly between the treatments (Table 1).

Mite Identification and Effects of Fungi on Feeding by Astigmatid Mites. Mites collected from *D. rufipennis* were identified as: *Tarsonemus ips* and *T. endophloeus* (Acari: Tarsonemidae), *Histiogaster arborsignis* (Acari:Acaridae), *Dendrolaelaps quadrisetus* (Acari: Digamasellidae), *Proctolaelaps hystricoides* (Acari:Ascidae), two species of *Trichouropoda* (Acari:Trematuridae), *T. alascae* and *T.* n. sp. nr *dalarenaensis*, and *Urobovella* n. sp. 767 (Acari: Uropodidae). Pictures of live and mounted specimens of these species are provided in Fig. 1.

Histiogaster arborsignis was the astigmatid mite most commonly associated with *D. rufipennis* galleries. In our experiments, these mites settled on the provided fungal plugs within 4 h, and their preference for a given treatment remained constant throughout the experiment. For this reason, only data for the 48-h count are presented (Fig. 2). In our two-choice assays, this mite species showed clear feeding and oviposition preference for *L. abietinum* (Fig. 2). These mites also showed a strong feeding preference for *L. abietinum* compared with all other fungi, followed by *T. harzianum/atroviridae* compared with either of the *Aspergillus* species (Fig. 2). Mites showed no preference between either of the two *Aspergillus* species (Fig. 2). Mite oviposition was not observed on any of the substrates at 4 h. By 24 h, however, eggs were seen on both *L. abietinum* and *T. harzianum* (Table 2). Oviposition preference followed the same pattern as feeding preference. Mites laid significantly higher numbers of eggs on *L. abietinum*, regardless of the other fungal choice. The second most preferred fungus was *T. harzianum* (Table 2). Eggs were not deposited on sterile MEA or on either of the *Aspergillus* species. The highest mean

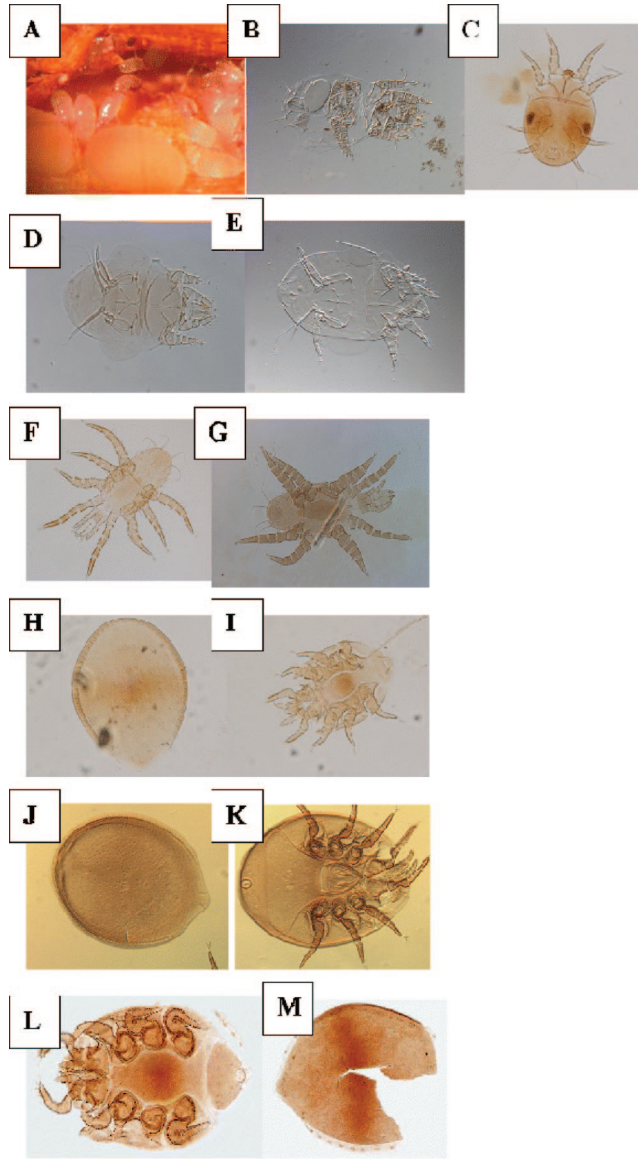

Fig. 1. Mites associated with *D. rufipennis*. *H. arborsignis*: active feeding stage and beetle eggs within galleries (A), female with egg (B), deutonymph (C); *T. endophloeus* (D); *T. ips* (E); *D. quadrisetus* deutonymph (F); *Proctolaelaps* sp. male (G); *Trichoroupoda* n. sp. nr *dalarenaensis*, deutonymph dorsal (H) and ventral (I) views; *T. alascae*, deutonymph dorsal (J) and ventral (K) views; *Urobovella* n. sp. 767 deuteronymph ventral (L) and dorsal (M) views. All images taken with a compound scope, ×100–200 in light phase.

number of eggs was observed on *L. abietinum* when the mites were choosing between it and *A. nomius*. The lowest oviposition rate on *L. abietinum* occurred when it was paired with *A. fumigatus* (Table 2). In contrast, the highest mean egg deposition on *T. harzianum* was when this fungus was paired with *A. fumigatus* (Table 2).

Collection, Identification, and Culturing of Nematode Associates. Seventy-five percent of the beetles examined ($n = 328$) were found to carry nematodes. Overall nematode loads were similar between male and female beetles (Fig. 3). Nematodes associated with *D. rufipennis* could be found for the most part in anhydrous clusters under the elytra, mainly attached to the proximal end, or within nematangia associated with their membranous wings (Cardoza et al. 2006b). Collections from the elytra clusters were found to be immature aphelenchoid-like, and andult *Mikoletzkya* and *Panagrolaimus* sp. cultures of nematodes could be obtained on both sterile MEA and *L. abietinum* plates, depending on the species. Nematodes cultured from beetle elytra were identified as a new species of *Parasitorhabditis* (Fig. 4A), a species of *Aphelenchoides* (Fig. 4B), and *Mikoletskya ruminis* (Fig. 4C). The

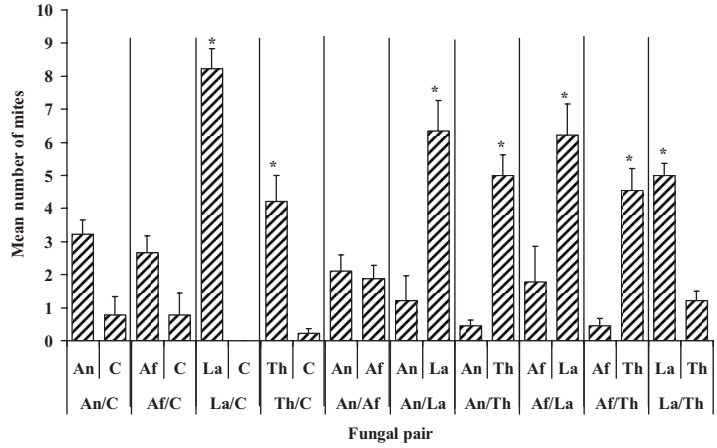

Fig. 2. Dual choice *H. arborsignis* feeding assay. Mean number of mites located on plugs of either of the paired fungi. *Significant difference within fungal pairs (paired *t*-test, $P \leq 0.05$). Error bars denote SE.

Parasitorhabditis and *Aphelenchoides* species were recovered from *L. abietinum* cultures, whereas *M. ruminis and Panagrolaimus* sp. were collected from sterile MEA plates. These plates, it should be noted, showed evidence of bacterial and yeast-like growth, most likely originating from spores transferred with or on the nematodes. Four of 10 plates onto which five nematodes from beetle nematangia were placed on *L. abietinum* yielded nematode cultures. None of the 10 sterile MEA plates onto which this type of nematode was placed yielded cultures. The nematodes collected from the latter cultures were identified as a *Bursaphelenchus* spp. (Fig. 4D) and not *Ektaphelenchus obtusus* (observed previously, Cardoza et al. 2006b) (Fig. 4E).

Table 2. Oviposition preference by *H. arborsignis* in laboratory dual-choice fungal assays

Fungal pair	Treatment[a]	No. eggs (mean ± SE)	
		24 h	48 h
AfC	Af	0.0 ± 0.00	0.0 ± 0.00
	C	0.0 ± 0.00	0.0 ± 0.00
Af/La	Af	0.0 ± 0.00	0.0 ± 0.00
	La	0.4 ± 0.24	1.8 ± 0.62*
Af/Th	Af	0.0 ± 0.00	0.0 ± 0.00
	Th	0.6 ± 0.56	1.0 ± 1.00
An/C	An	0.0 ± 0.00	0.0 ± 0.00
	C	0.0 ± 0.00	0.0 ± 0.00
An/La	An	0.0 ± 0.00	0.0 ± 0.00
	La	2.9 ± 1.45	5.0 ± 2.13[a]
An/Th	An	0.0 ± 0.00	0.0 ± 0.00
	Th	0.2 ± 0.22	0.4 ± 0.34
La/C	La	1.3 ± 0.58	3.3 ± 1.09[a]
	C	0.0 ± 0.00	0.0 ± 0.00
La/Th	La	0.7 ± 0.44	2.0 ± 1.05[a]
	Th	0.1 ± 0.11	0.2 ± 0.15
Th/C	Th	0.0 ± 0.00	0.4 ± 0.44
	C	0.0 ± 0.00	0.0 ± 0.00
AfAn	Af	0.0 ± 0.00	0.0 ± 0.00
	An	0.0 ± 0.00	0.0 ± 0.00

[a] Significant differences based on paired-*t*-test comparisons ($P \leq 0.05$, Proc Means; SAS Institute 1996).

La, *L. abietinum;* Af, *A. fumigatus;* An, *A. nomius;* Th, *T. harzianum;* C, control.

Discussion

These results support the view that *L. abietinum* may have both positive and negative effects on *D. rufipennis* and that this association may be a context-dependent mutualism. Bentz and Six (2006) have recently found that phloem tissue infected with blue-stain fungi, including *L. abietinum*, have higher levels of sterols than noninfected tissue. Insects need exogenous sources of sterols to support normal physiological functions such as molting and reproduction (Hobson 1935). In addition, related fungi seem capable of metabolizing plant allelochemicals, which could benefit beetles by circumventing host tree defenses (Paine et al. 1997). The lower mortality observed in females feeding on *L. abietinum* may likewise reflect a benefit. That is, *L. abietinum* mycelia and, perhaps fruiting bodies, may provide sustenance, albeit limited, to female beetles when confronted with unfavorable host conditions. However, our findings on the negative impact of *L. abietinum* on spruce beetle gallery construction and oviposition indicate that this relationship also has its antagonistic aspects.

Mites associated with bark beetles have been observed transporting, disseminating, and feeding on blue-stain fungal species (Moser et al. 2005). Hofstetter et al. (2006) also found that *Tarsonemus* spp. car-

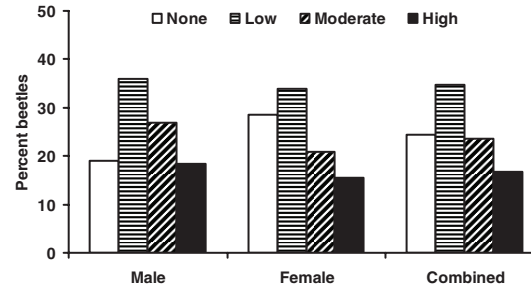

Fig. 3. Mean percent nematode incidence on *D. rufipennis* and observed abundance in elytra anhydrous clusters.

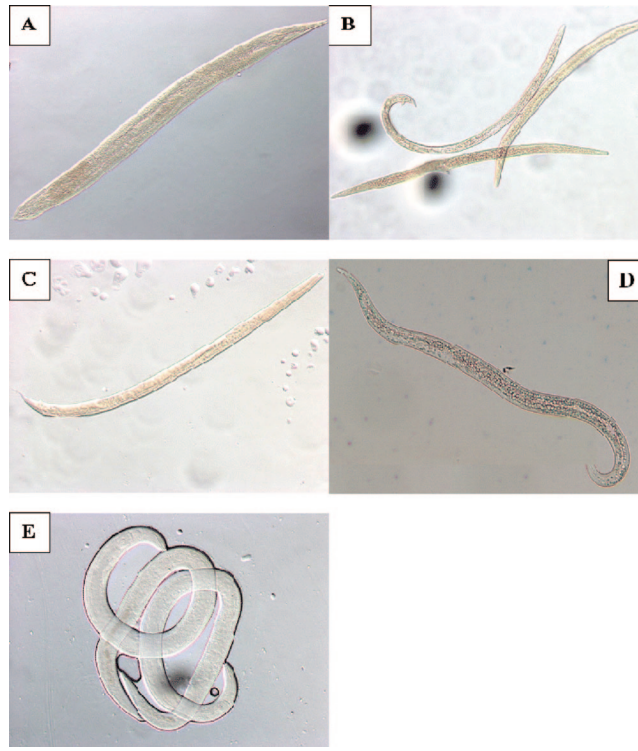

Fig. 4. Full body image of nematodes associated with *D. rufipennis*: *Parasitorhabditis* sp. (A, ×100), *Aphelenchoides* sp. (B, ×200), *M. ruminis* (C, ×200), *E. obtusus* (D, ×400), and *Bursaphelenchus* n. sp. 727 (E, ×200). Images taken with a compound scope in light phase.

ried spores of *Ophiostoma minus* on which they fed. Therefore, our findings that *H. arborsignis* preferentially fed and oviposited on *L. abietinum* suggest that similar relationships may exist in this system. During its phoretic association with *D. rufipennis*, *H. arborsignis* can be found all over the insects' body, so it is possible for spores to be transferred to the beetle through direct contact. Although *H. arborsignis* are primarily fungal feeders, they have been observed eating *D. rufipennis* eggs in our laboratory arenas, particularly under conditions unfavorable for beetle development (Y.J.C., unpublished data). It is unknown if they punctured the chorion of live eggs or fed on eggs that had previously been degraded. *Tarsonemus* mites are known fungal feeders (Hofstetter et al. 2006). The specific feeding habits of many bark beetle associates, other than *H. arborsignis*, have not been studied. However, we have observed fungal spores on the bodies and in the guts of the *Tarsonemus*, *Trichouropoda*, and *Histiogaster* species. *Proctolaelaps* and *Dendrolaelaps* mites have been found in association with beetles, including bark beetles, and they have been reported to be nematophagous in nature (Moser 1975, Kinn 1984, Krantz and Poinar 2004).

Results from our culturing assays suggest that some of the nematodes associated with *D. rufipennis* are microbial feeders, at least for part of their life cycle. For example, the *Aphelenchoides* species were successfully maintained on *L. abietinum.* The *Mikoletskya* and *Parasitorhabditis* species seem to thrive on microbial growth that had a bacterial/yeasty appearance. In controlled bioassays, the species of *Bursaphelenchus* sp. from nematangia could not be cultured on sterile MEA but was successfully cultured on *L. abietinum.*

Interestingly, we did not successfully culture the *Ektaphelenchus obtusus* morphs even though they were the main inhabitants of the nematangia (Cardoza et al. 2006b). Instead, the cultures obtained from nematangia nematodes were consistently determined to be a previously unidentified species of *Bursaphelenchus* n. sp. 727. This raises the possibility of either a second cryptic species of nematodes living in the nematangia or of *E. obtusus* being a phoretic or dormant morphology of the *Bursaphelenchus* sp. Other authors have reported very similar morphological and ecological characteristics between *Ektaphelechus* and *Bursaphelenchus* species, including a new species of *Bursaphelenchus* showing *Ektaphelenchus*-like morphological characteristics, which is also housed in a nematangium-like structure in a bark beetle (Braasch 2004, Penas et al. 2006). Resolving this issue is particularly important because *Bursaphelenchus* is the genus containing the pine wood nematode, *B. xylophilus*, an internationally important forest pest (Mota et al. 2006). We are currently exploring this taxonomic issue in a collaborative effort with Dr. Giblin-Davis (University of Florida, Ft. Lauderdale, FL).

To our knowledge, this is one of only a few descriptions of the mites and nematodes associated with a single bark beetle species (Balazy et al. 1987, Moser et al. 2005, Furniss and Kegley 2006). The data presented here provide an important complement to our previous publications documenting the fungal, bacterial, and other nematode species associated with *D. rufipennis* (Cardoza et al. 2006a, b). This information will contribute to a better understanding of the microecological interactions that underlie the eruptive population dynamics of important forest pests (Hofstetter et al. 2006). Further studies are needed to elucidate the complexity of spruce beetle interactions with its many microorganismal associates.

Acknowledgments

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