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Article

Next-Generation Sequencing to Investigate Existing and New Insect Associations with Phytopathogenic Fungal Propagules

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Abstract: Understanding ecological interactions is a key in managing phytopathology. Although entomologists rely mostly on both traditional molecular methods and morphological characteristics to identify pests, next-generation sequencing is becoming the go-to avenue for scientists studying fungal and oomycete phytopathogens. These organisms sometimes infect plants together with insects. There are many relationships yet to be discovered and much to learn about how these organisms interact with one another. Considering the growing number of exotic insect introductions in Canada, a high-throughput strategy for screening those insects is already implemented by the Canadian Food Inspection Agency (CFIA). However, no plan is deployed to investigate the phytopathogenic fungal and oomycete species interacting with insects. Metagenomics analysis was performed on the preservation fluids from CFIA's insect traps across Canada. Using the Ion Torrent PGM technology and fusion primers for multiplexing and indexing, community profiling was conducted on the different semiochemicals used in the insect traps and the various areas where these traps were placed. Internal transcribed spacer 1 (fungi and oomycetes) and adenosine triphosphate synthase subunit 9-nicotinamide adenine dinucleotide dehydrogenase subunit 9 spacer amplicons were generated. Although direct links between organisms could not be established, moderately phytopathogenic fungi (e.g., *Leptographium* spp. and *Meria laricis*) and oomycetes (mainly *Peronospora* spp. and *Pythium* spp.) unique to every type of semiochemical were discovered. The entomopathogenic yeast *Candida michaelii* was also detected. This project demonstrated our ability to screen for unwanted species faster and at a higher scale and throughput than traditional pathogen diagnostic techniques. Additionally, minimal modifications to this approach would allow it to be used in other phytopathology fields.

Keywords: Insects, Forest, Fungi, ITS1, metagenomics, NGS, Oomycete, Phytopathogens, *Phytophthora*, vectors

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1. Introduction

The Era of Globalization has dramatically and consistently increased international cargo shipments since 1970 [1]. Solid wood packaging material (SWPM) such as pallets, crates, and boxes are used to transport products all over the world. Bark and wood-boring insects, such as bark beetles, longhorned beetles, woodwasps, jewel beetles, weevils, and ambrosia beetles are often intercepted in SWPM [2-5]. Even with the implementation of International Standards for Phytosanitary Measures (e.g., ISPM No. 15), which states the need to treat wood products shipped abroad in order to prevent the spread of insects and diseases, live wood-boring insects are still intercepted in SWPM at Canadian and American borders [6,7]. Emerald ash borer (*Agrilus planipennis*), brown spruce longhorned beetle (*Tetropium fuscum*), sirex woodwasp (*Sirex noctilio*), and pine shoot beetle (*Tomicus piniperda*) are just a few examples of species recently introduced and established in Canadian forests [8-12].

The transmission of exotic phytopathogenic propagules, an important threat to forest health, figures among the many issues associated with the introduction of exotic insects in Canada, especially because some of these wood-boring insects proliferate within common North American tree species such as pine and spruce [13-16]. Aside from killing or damaging trees, insects can also transmit different phytopathogenic species (e.g., fungal spores) to their respective plant host. A noteworthy example is the fungus *Ophiostoma ulmi*, one of the causative agents of Dutch elm disease, transported to elm trees by bark beetles [15]. This fungus has devastated North American forests and also occurs in Asia and Europe [16,17]. Researchers have also previously reported potential links between insect and arthropod excreta and *Phaeoacremonium* spp. in grapevine infection cycle [18-20]. There are also numerous yeast associated with insects. More specifically, *Candida* spp. and *Cladosporium* spp. have been linked with bark and rove beetles [21-25]. Oomycetes contain numerous plant pathogens responsible for considerable damage to the environment as well [16,26-29]. Their propagative structures can remain viable for further plant infection even after ingestion and defecation by invertebrates [30]. For instance, chlamydospores of *Phytophthora ramorum* can still infect leaves after passing through the digestive tract of snails [30]. Oomycetes have also been associated with indirect interactions (positive or negative) with insects. For instance, while ants can transport *P. palmivora* and *P. megakarya* to cocoa trees, which can subsequently become infected [31], oviposition of the moth *Spodoptera littoralis* is enhanced after *P. infestans* modifies the volatile compounds emitted by the host plant [32]. In contrast, the reproductive output by aphids is inversely proportional to the level of *Phytophthora* infection [33-35].

Insects can also benefit from mutualistic relationships with plant pathogens that overwhelm the plant's defenses (e.g., thousand cankers disease), or induce a plant's cell suicide response [14,36,37]. Contrarily, the damage caused by insects can indirectly predispose plants to microbial attacks. For example, in addition to the maize crops losses caused by the African pink stem borer (*Sesamia calamistis*) and the false codling moth (*Thaumatotibia leucotreta*), it was observed that aflatoxin (produced by *Aspergillus* spp.) concentrations were proportional to the number of insects that came in contact with these crops after storage [38-40]. Many bark and ambrosia beetles even rely on a fungal symbiosis to fulfill their nutrition needs [41].

While there are numerous studies of insects transmitting plant viruses [42-44] and bacteria [45,46], there is a need for additional research on associations between forest insects and microorganisms. Although there has been research on pinewood nematode [37,47], ophiostomatoid fungi [14,15,31,48], and their insect vectors, there are likely more associations to be discovered, including transmission by vectors. For instance, additional fungal species never previously found to be associated with insect species may also be unexpectedly transmitted in this way.

The Canadian Food Inspection Agency (CFIA) conducts annual surveys using traps baited with semiochemicals to detect nonnative wood-boring insects in high-risk areas such as industrial and commercial zones [49-52]. Semiochemicals are communication chemicals to induce inter- or intraspecific interactions between organisms (e.g., decaying trees produce a kairomone that attracts bark beetles) [53]. They have been extensively studied for their ability to attract specific insects [47,54-60], especially for monitoring particular groups of beetles and the microorganisms associated

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with them. With the advances associated with next-generation sequencing (NGS), scientists have used the power of metagenomics for the diagnosis of phytoviruses [42], fungi, and oomycetes [61], and the detection of exotic fungi on asymptomatic live plant material imported into Canada [62]. In addition to its high-throughput sequencing capacity and high sensitivity, NGS also allows for the analysis of hundreds of environmental samples in a fraction of the time compared with traditional methods [63-65].

Taking advantage of a well-established, nationwide entomological survey that uses preservation fluids within insect traps, this project aimed to use a metagenomics approach to screen for the presence of potentially phytopathogenic oomycetes and fungi in order to fill in a gap in plant pathogens detection. The approach could potentially help forest pathology stakeholders to orientate surveys for disease monitoring and management at a large scale. In attempting to partially decipher complex tree infection processes (e.g., insect-fungi) by extracting additional and valuable information from the insect trap samples, the project also addressed the potential for wood-boring insects to actively, or incidentally carry phytopathogenic propagules into baited insect traps. Evaluation of commonalities between the areas of collection or the specific semiochemicals used and the respective fungal or oomycete diversity was also conducted.

2. Materials and Methods

Insect traps: During the summers of 2013 to 2015, CFIA inspectors installed traps at 41 sites in industrial and commercial zones, landfills, and SWPM disposal facilities (Supplementary Figure S1). These areas are end points for international SWPM and dunnage, and are considered high-risk areas for the introduction of nonnative pests. At each site, 12-unit funnel traps with wet collection cups (Synergy Semiochemicals Corporation, Burnaby, BC, Canada) were suspended between trees. Each trap's collection cup was positioned at approximately 30 to 200 cm above ground, according to the height of the understory vegetation. Depending on local temperatures, baited traps were placed in forested areas between March and April, and taken down at the end of September. To assess the fungal communities (background noise) in the sampled areas, non-baited air samples were collected using Johnson and Barnes (JB) rainfall collectors (J. L. Johnson, *personal communication*) as previously done by Barnes et al., Szabo et al. and Hambleton et al. [66-69]. Because un-baited 12-unit trap could not be used within an operational program, these JB air samplers were employed as negative controls.

Semiochemicals: In 2013 and 2014, CFIA inspectors placed six traps per site. Half the traps were baited with one combination of lures, while the other half was baited with a different set. Inspectors attached one lure type to each trap with each lure dispensed from individual release devices. Additional details pertaining to the semiochemicals (chemical composition, purity, packaging, and release rate) used in this project are provided in Supplementary Material S1.

The first semiochemical combination (C_6C_8) contained ethanol, as well as aggregation pheromones of some longhorned beetles in the Cerambycinae subfamily [54]. The second semiochemical combination (UHR_E_AP) contained ethanol and alpha-pinene, which are attractive to a wide range of bark and wood-boring insects [49,55,56]. Traps baited with C_6C_8 were suspended between coniferous or broadleaf trees, whereas traps containing UHR_E_AP were primarily placed between coniferous trees.

In 2015, CFIA inspectors implemented two new semiochemical sets in order to target different insect taxa. The first semiochemical (i.e., General Longhorn) was attractive to longhorned beetles in the Spondylidinae [57] and Lamiinae subfamilies [70] but, could also capture various bark and ambrosia beetles, due to the addition of ethanol [58]. The second semiochemical set (Pine Sawyer) was attractive to *Monochamus* (longhorned beetles) species from North America, Europe, and Asia [47,59,60] but the inclusion of ethanol and alpha-pinene also makes it attractive to bark and ambrosia beetles.

The semiochemical sets were deployed in different areas depending on the forest type. In British Columbia, each site had four traps baited with the Pine Sawyer lures, and two traps baited with the General Longhorn. In Ontario and Quebec, 75% of the sites were in broadleaf forests or

mixed forests, and all six traps were baited with General Longhorn lure. The remaining sites, composed primarily of coniferous trees, were baited with the Pine Sawyer lure. In the Atlantic provinces (i.e., New Brunswick, Newfoundland and Labrador, Nova Scotia, and Prince Edward Island), each site had three traps with Pine Sawyer lure and three traps with General Longhorn lure. Traps baited with the Pine Sawyer lure were suspended between two coniferous trees, whereas traps baited with the General Longhorn lure were placed between coniferous or broadleaf trees. Collected fluids from each trap (i.e., insect traps and air samplers) were kept and analyzed separately. Lures were replaced approximately every 90 days.

Trapping fluid (200 to 300 mL of USP/FCC grade 1,2-propanediol (propylene glycol) (Fisher Scientific, Hampton, NH, USA), (Denatonium benzoate or Bitrex® = [Benzyl-diethyl (2,6-xylylcarbamoyl methyl) ammonium benzoate]) (Sigma-Aldrich, Saint-Louis, Mo., USA), and PhotoFlo 200 (surfactant) (Fisher Scientific) were poured into the collection cups of each trap, and traps were spaced at least 25 m from each other [71,72]. All of the solution from each trap was collected every two to three weeks. The contents were poured onto fine-gauged sieves and insects were removed because only the collection fluid was analysed in this study. The liquids were then filtered on 0.45-µm cellulose paper filters and stored at 4°C until processed. The filter papers were cut in half to preserve a section as a back-up, while the other half was put in Tris buffer, heated at 65°C and sonicated (40 kHz). The solution containing the DNA was then centrifuged (10,000 rpm, 2 min) and extracted with the FastDNA kit for soil (MP Biomedicals, Santa Ana, CA, USA). To remove PCR inhibitors, purification of the extracted DNA was done using magnetic particles (Bio-Nobile, Österнäsvägen, Finland).

Then, PCR was performed bidirectionally to amplify DNA and add unique identifier (barcodes) to each sample using Ion Torrent PGM fusion primers. Detailed sequences for fungi and oomycete fusion primers, as well as PCR cycling and parameters can be found in Tremblay et al. [61]. Fusion primers allowed for multiplexing environmental samples and generating internal transcribed spacer 1 (ITS1) fungi and oomycete amplicons. In addition, when an oomycete band was visualized by electrophoresis, another PCR targeting the adenosine triphosphate synthase subunit 9-nicotinamide adenine dinucleotide dehydrogenase subunit 9 (ATP9-NAD9) spacer was performed to allow for the proper downstream resolution of *Phytophthora* species, considering that it is better-suited than the ITS to resolve species within this genus [29,73-75]. Products were visualized on a 1.5% agarose gel with a Gel Doc XR+ Gel Documentation System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primer-dimers and other smaller sized fragments (<100bp) were removed with Agencourt AMPure XP magnetic beads at a 0.7:1 beads:DNA ratio (Agencourt Bioscience, Beverly, MA, USA) [76]. Sequencing libraries were quantified with the Ion Universal Library Quantitation qPCR Kit, (Life Technologies, Carlsbad, CA, USA), and then pooled at the equimolar concentration of 16 pM. The Ion Personal Genome Machine (PGM) Template OT2 Kit 400 bp (Life Technologies) and the Ion PGM sequencer (Life Technologies) were used to perform NGS [77].

Bioinformatics: The raw data output from the sequencer was analysed with the pipeline previously described by Tremblay et al. [61]. FASTQ files were converted into sequence (FASTA) and quality score (QUAL) files using fastqutils [78]. Quality trimming based on sequence quality and length was done with Mothur (version 1.37.2) [79] using the trim.seqs function parameters minlength = 120, maxambig = 0, and maxhomop = 8. ITS extraction was done with ITSx (version 1.0.11) [80]. Operational taxonomic unit (OTU) tables were generated with QIIME (version 1.7.0) [81]. Taxonomic assignment was done using the UNITE database (version 31.01.2016) [82] for fungi and the National Center for Biotechnology Information (NCBI) nucleotide database for oomycetes. The resolution power of the ATP9-NAD9 region for *Phytophthora* species relies on a custom-built database including the majority of all currently described *Phytophthora* species (NCBI accessions numbers JF771616.1 to JF772053.1 and JQ439009.1 to JQ439486.1) [29,83]. To evaluate species alpha diversity, evenness, and the proportion of different organisms within the sample types, statistical analyses were done with R (version 3.1.3) [84] using the RAM package (version 1.2.1.3) [85]. This package was also used to evaluate common elements between dataset (Venn diagrams), to assess similarities through Principal Coordinates Analysis (PCoA) plots, and to generate sampling maps.

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To visualize the distribution of the type of trees that were used to hang traps at the collection sites, a data aggregation plot was built using UpsetR version 1.3.3 [86] in R.

Species subtraction. Species subtraction hereafter refers to the dataset excluding species that were commonly detected in both the control and insect traps. Insect trapping procedures were already established by the CFIA biologists and sites were selected by CFIA inspectors prior to this project [87]. Although CFIA entomologists screened and identified all wood-boring insects, due to time constraints, they only reported the non-indigenous insects captured during the survey. Considering the physical design of the insect traps, the passive collection of fungal spores suspended in air was inevitable. In an attempt to extract unique features associated with insects attracted to the different types of semiochemicals, NGS data from JB collector air samples, collected in similar context as insect traps (i.e., same areas, sampling time, sites, year, season, and so on), was used to identify which fungal OTU were solely found in insect traps. Although the JB collectors were passive spore samplers, through species subtraction it was possible to determine which fungi or oomycetes species were unique to the insect traps and may be associated with an insect vector.

Following BLAST alignments of the OTU with the respective reference databases, the remaining species were screened against a text-formatted database to determine their fungal biological functions [88], and the resulting file was parsed to identify fungi of interest, mostly those known to be plant pathogens and rot fungi. Ectomycorrhizal fungi, saprotrophs, mycoparasites, lichenized fungi, and most yeast were discarded.

To assess the depth of sequencing and diversity of the subtracted and negative control data (i.e., spore trap), and to compare them with the original insect trap data, rarefaction curves were generated using the functions "diversity" and "rarefy" from the R package Vegan (version 2.5-2) [89]. This step was performed only on fungal data as the oomycete dataset was too small to obtain relevant rarefaction curves.

3. Results

Samples. A total of 108 samples originating from British Columbia, New Brunswick, Newfoundland and Labrador, Nova Scotia, Ontario, Prince Edward Island and Quebec were collected over three years (2013 to 2015). From those samples, 39 were baited with UHR_E_AP, 36 with C_6C_8, 17 with General Longhorn, and 16 with Pine Sawyer (Supplementary Table S1). As well, Figure 1 presents the type of trees to which sample traps were hung for sampling. However, the data does not fully represent the type of forest surrounding sampling sites because traps were only suspended from two trees.

PCRs. Fungal amplicons (ITS1) generated a visible band via gel electrophoresis in ≥83% of samples, whereas oomycete amplicons (ITS1) were observed in only 11% of samples (Table 1). Forty percent of oomycete amplicons generated bands for *Phytophthora* spp. following the PCR amplification of the ATP9-NAD9 spacer (Table 1).

Fungal OTU, prior to species subtraction. At the phylum level, the most abundant fungi for all semiochemical treatments were members of the Ascomycota division, followed by Basidiomycota, unidentified OTU, OTU "unclassified below kingdom", Chytridiomycota, Zygomycota, and then Glomeromycota (Table 2). A few Rozellomycota members were associated with all lures except for General Longhorn (Table 2). Ascomycetes and basidiomycetes were evenly distributed between the samples baited with UHR_E_AP and C_6C_8 semiochemicals but, there were larger proportions of ascomycetes compared with the proportion of basidiomycetes found in the General Longhorn and Pine Sawyer-baited samples (Table 2). At the genus level, the most abundant fungal OTU remained unidentified regardless of the semiochemical analysed but, *Phoma* sp., *Leptographium* sp., and *Mycosphaerella* sp., while below 5% in proportion, are genera that include many forest pathogens (Table 3). The UHR_E_AP and C_6C_8 semiochemicals contained higher percentages of *Rhodotorula* sp. and *Cystobasidium* sp. compared with the General Longhorn and Pine Sawyer semiochemicals, which only contained traces of these genera. Instead, the two latter semiochemicals had more *Cryptococcus* sp. and *Leptographium* sp. All four semiochemicals had low amounts of *Epicoccum* sp.

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Analysis of the top ten species in relation to lure type revealed a high number of "unidentified fungal OTU" and "OTU unclassified below genus" in all four attractants (Table 4). Other species, including *Rhodotorula mucilaginosa*, *Cystobasidium slooffiae*, *Leptographium piriforme*, *Cladosporium exasperatum*, and *Aureobasidium pullulans* were frequent across the different semiochemical treatments (Table 4).

From the 2439 different species OTU detected prior to the species subtraction, 1057 (43%) were common to all semiochemical types, 228 species (9%) were unique to the UHR_E_AP semiochemical, 112 (4.6%) were unique to General Longhorn, 105 (4.3%) were unique to Pine Sawyer, and 118 (4.8%) were unique to the C_6C_8 semiochemical (Figure 2a).

To visualize sampling depth, examples of the rarefaction curves obtained for the spore traps (negative control) and their respective original insect dataset, and subtracted insect dataset are shown in Supplementary Figure S2. The spore trap data demonstrated the highest sequencing depth as saturation was obtained for all of the samples tested with a sequence number per sample ranging between approximately 5,000 and 25,000. The lowest species number obtained by all samples tested was just below 150. In contrast, only part of the samples from both the original insect and the subtracted data reached saturation in their respective rarefaction curves.

Fungal OTU, after species subtraction. A total of 1,527 species remained once the species detected in the negative control were discarded, of which 368 species (approximately 25%) were common to all semiochemical types, 220 species (approximately 14%) were unique to UHR_E_AP, 109 (7.1%) were unique to General Longhorn, 99 (6.5%) were unique to Pine Sawyer, and 116 (7.6%) were unique to the C_6C_8 lure (Figure 2b). Following the approach of Tedersoo et al. [88] to investigate fungal species functions, the data revealed the occurrence of some species of moderate concern in each of the semiochemical treatments (Supplementary Table S2). In Pine Sawyer-baited samples, three rot fungi and six phytopathogenic fungi (including *Ambrosiella ferruginea*, *Leptographium* sp., and *Phaeoacremonium inflatipes*) were detected. From the General Longhorn samples, five rot fungi and eight phytopathogenic causal agents, including *Echinodontium tinctorium*, *Siroccocus conigenus*, and *Pucciniastrum circaeae* were detected. The C_6C_8 semiochemical treatment featured unique fungi of interest as well: a single white rot, the yeast *Candida michaelii*, which is associated with the gut flora of handsome fungus beetles (Endomychidae), and eight phytopathogens including *Phyllosticta minima*, *Podosphaera clandestina*, and *Ciborinia Whetzelii*. Finally, the UHR_E_AP semiochemical had six rots along with thirteen pathogenic fungi including *Colletotrichum fructi*, *Podosphaera leucotricha*, and *Strelitziana mali*.

Oomycetes OTU, prior to species subtraction. At the phylum level, BLAST alignments of the OTU sequences with the ones in the NCBI (nucleotides) database showed that OTU from ITS1 amplicons using oomycete primers predominantly aligned with Oomycota sequences. However, the UHR_E_AP (26.1%) and C_6C_8 (26.6%) semiochemicals had moderate percentages of OTU which could not be identified below the kingdom taxonomic rank (Table 5). On the other hand, the majority of sample sequences from traps baited with General Longhorn (99.9%) and Pine Sawyer (96.9%) corresponded to Oomycota members (Table 5). Among all genera detected that could be identified, *Peronospora* spp. was the most abundant in all semiochemical treatments, except for C_6C_8, where *Phytophthora* spp. was the most abundant identified genus (Table 5). Prior to species subtraction, of the total 54 different OTU, there were 21 species detected in all semiochemical types but, few were unique to each treatment (Figure 2c). *Pythium monospermum* was unique to traps baited with the UHR_E_AP semiochemical. *Pythium oligandrum* was unique to traps baited with the Pine Sawyer semiochemical. Five species were unique to traps baited with the General Longhorn semiochemical (*Peronospora* sp. UPS F-119986, *P. flava*, *P. sparsa*, *Pythium carolinianum*, and *Phytophthora* spp.). No unique species were recovered from traps baited with the C_6C_8 semiochemical (Figure 2c). Within the top ten most abundant species identified, all semiochemicals except C_6C_8 (0.67%) had a high percentage of *Peronospora manshurica* (Table 6). The UHR_E_AP (26.1%) and C_6C_8 (26.6%) semiochemicals had a considerably higher number of OTU unclassified below genus compared with General Longhorn (traces) and Pine Sawyer (3.02%).

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Oomycetes OTU, after species subtraction. Following species subtraction, fifteen species remained, from which no unique oomycete species were detected in either the Pine Sawyer or UHR_E_AP baited traps (Figure 2d). Eleven species were unique to General Longhorn including *Peronospora* sp., one was unique to C_6C_8 (*Pythium sp.* CAL-2011e), and three species were shared with the two latter semiochemicals (Supplementary Table S3).

***Phytophthora* spp. OTU, prior to species subtraction:** No *Phytophthora* sp. were unique to the insect traps, or any semiochemical treatment. Additionally, ATP9-NAD9 OTU from the original dataset generated prior to the species subtraction could only recover *Phytophthora* spp. from traps baited with the C_6C_8 and UHR_E_AP semiochemicals. *Phytophthora cryptogea* could only be detected from traps baited with the UHR_E_AP semiochemical, whereas *P. foliorum*, *Phytophthora* sp. "*kelmania*" [83] and *P. syringae* were associated with both C_6C_8 and UHR_E_AP lures.

Diversity: fungi. The fungal species evenness (Shannon index [90]) was very similar between all four semiochemicals, with median relative values ranging between 0.5 and 0.75 (Supplementary Figure S3a). The true diversity median values for fungi (Shannon index, per unit of number of species [90,91]) were also evenly distributed among all semiochemical types, ranging between approximately 20 and 40 units of number of species (Supplementary Figure S3b).

Diversity: oomycetes. Oomycete species evenness (Shannon index) revealed a notable variation between the different semiochemicals (Supplementary Figure S3c). While species from the C_6C_8 (median relative value of approximately 0.45) and the UHR_E_AP (median relative value of approximately 0.49) lures were evenly distributed, species from Pine Sawyer (median relative value of approximately 0.15) and the General Longhorn-baited traps (median relative value of approximately 0.2) were much less evenly distributed. These differences help explain the true diversity (Shannon) variation also observed in Supplementary Figure S3d.

Areas of collection. After discarding fungal and oomycete species that were most likely passively, or incidentally captured in the insect traps, we observed certain community aggregations associated with geographic regions. The sampling sites were split in three areas of Canada: West Coast (i.e., British Columbia), Eastern Canada (i.e., Quebec and Ontario) and the Atlantic Region (i.e., New Brunswick, Newfoundland and Labrador, Nova Scotia, and Prince Edward Island). The PCoA plot generated at the order level for fungi (Supplementary Figure S4a) demonstrated a clear similarity (i.e., clustering) between OTU data from the West Coast, a clustering trend for Eastern Canada data, and a lack of similarity within the Atlantic Region. At the class level, however, the OTU clustered more clearly based on the geographic region (Supplementary Figure S4b). Such observation was not possible for oomycetes because not enough species remained following the species subtraction (data not shown). Although, there were oomycete species unique to the Eastern zone, and aside from *Saprolegnia* sp. SAP1 and *Hyaloperonospora cochleariae*, all others were either *Peronospora* spp. (*P. farinosa*, *P. sparsa*, *Peronospora* sp. UPS F-119986, *P. viciae*, and *Peronospora* sp. isolate 079405,59), or *Pythium* species (*Pythium* aff. *hypogynum*, *Pythium* sp. CAL-2011f, *Pythium* sp. AvdB-2012, *Pythium* sp. P19300/1/3, and *Pythium* sp. BP2013k). One species—*Pythium* sp. BG02—was unique to the West Coast data but, there were no species uniquely associated with the Atlantic Region.

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3.1. Figures, Tables and Schemes

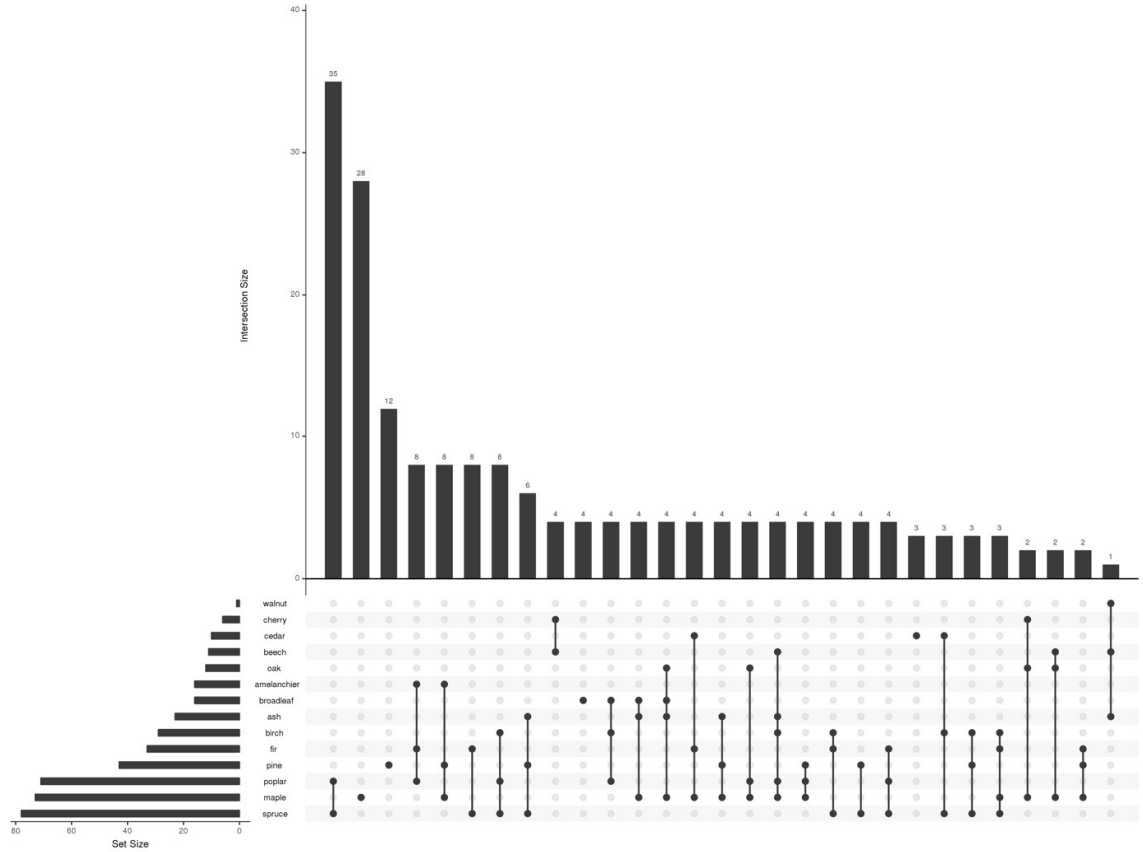

Figure 1. Upset plot to visualize the type of trees from which traps were suspended. The intersection size number represents the number of times a specific tree combination was found (similar to a Venn diagram), and the set size number corresponds to the number of samples surrounded by a specific type of tree. Most samples were collected from traps placed in forested areas comprising more than one tree species.

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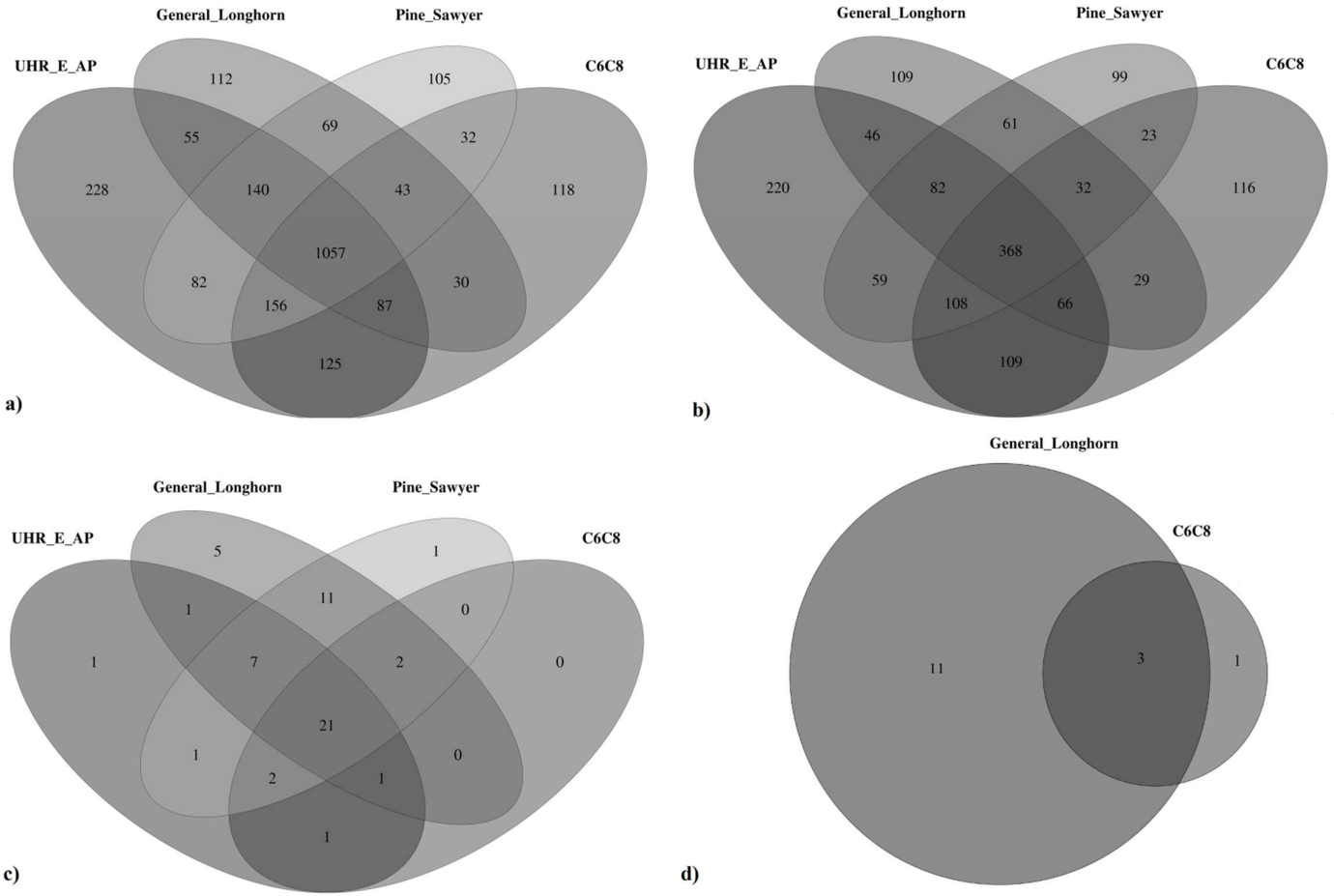

Figure 2: Venn diagram of a) fungal species shared or unique to the semiochemical type employed in insect traps, b) fungal species shared or unique to the semiochemical type employed in insect traps after species subtraction, c) oomycete species shared or unique to the semiochemical type employed in insect traps, d) oomycete species shared or unique to the semiochemical type employed in insect traps after species subtraction. All were obtained by amplifying the ITS1 genic region.

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Table 1: Presence or absence of amplification as detected by gel electrophoreses using barcoded PCR products from 108 environmental insect samples by targeted organisms, and the percentages of positive reactions obtained.

Primer used to append barcodes[a]	Organism	Target region[b]	Positive PCR	Total PCR	Positive (%)[c]
ITS1-Forward	Fungi	ITS1	90	108	84
ITS1-Reverse	Fungi		98	108	91
ITS1-Forward	Oomycete		12	108	11
ITS1-Reverse	Oomycete		12	108	11
ATP9-NAD9- forward	*Phytophthora* sp.	ATP9-NAD9	8	20	40
Total	…	…	220	452	Average: 47

[a]Each PCR included a set of primers but, as presented by Tremblay et al. [61], bidirectional sequencing required one primer per direction to append the sample and organism index (i.e., barcode).
[b]ITS1 = internal transcribed spacer 1 and ATP9-NAD9 = adenosine triphosphate synthase subunit 9-nicotinamide adenine dinucleotide dehydrogenase subunit 9 spacer.
[c]Percentage of the number of positive PCR over the total number of reactions done.

Table 2: Operational Taxonomic Units: fungal identification proportion (%) by semiochemical type at the Phylum taxonomic level using the ITS1 genic region.

Semiochemical[a]	**UHR_E_AP**	**C_6C_8**	**General Longhorn**	**Pine Sawyer**
Phylum				
Ascomycota	39.6	41.6	63.9	68.5
Basidiomycota	39.5	40.9	24.2	23.0
Unidentified OTU	17.5	14.2	9.3	5.6
OTU unclassified below kingdom	3.1	3.1	2.4	2.4
Chytridiomycota	0.3	0.1	0.1	0.2
Zygomycota	0.1	0.1	0.1	0.2
Glomeromycota	traces[b]	1.0	traces	traces
Rozellomycota	traces	0.5	absent	traces

[a]**UHR_E_AP** = Ultra-high release ethanol and ultra-high release alpha-pinene.
C_6C_8 = Racemic 3-hydroxyhexan-2-one (K6), racemic 3-hydroxyoctan-2-one (K8), and ultra-high release ethanol.
General Longhorn = Ultra-high release ethanol, (*E*)-6,10-dimethyl-5,9-undecadien-2-ol (*E*-fuscumol), and (*E*)-6,10-dimethyl-5,9-undecadien-2-yl (*E*-fuscumol acetate).
Pine Sawyer = 2-undecyloxy-1-ethanol (monochamol), ultra-high release ethanol, ultra-high release alpha-pinene, and racemic 2-methyl-6-methylene-7-octen-4-ol (ipsenol).
[b]Below 0.01% or not in the top 10 for this semiochemical.

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Table 3: Operational Taxonomic Units: fungal identification proportion (%) by semiochemical type at the genus taxonomic level using the ITS1 genic region.

Semiochemical[a]	UHR_E_AP	C_6C_8	General Longhorn	Pine Sawyer
Genus				
Unidentified OTU	40.5	34.2	29.5	30.0
Rhodotorula	10.0	11.0	traces[b]	3.2
Cystobasidium	7.5	5.5	traces	traces
Cryptococcus	4.3	4.1	5.5	6.1
OTU unclassified below family	3.1	3.1	2.4	2.4
Alternaria	2.9	traces	traces	traces
Epicoccum	2.5	5.2	3.3	2.2
Phoma	2.2	3.2	traces	traces
Scopuloides	1.8	traces	traces	traces
Verticillium	1.8	2.2	traces	traces
Hannaella	traces	2.3	traces	traces
Wickerhamomyces	traces	1.8	traces	traces
Aureobasidium	traces	traces	5.3	traces
Leptographium	traces	traces	5.2	4.4
Cladosporium	traces	traces	3.9	7.3
Neurospora	traces	traces	3.2	traces
Kluyveromyces	absent	traces	3.0	traces
Torulaspora	traces	traces	2.3	traces
Candida	traces	traces	traces	8.0
Mycosphaerella	traces	traces	traces	3.1
Geopyxis	traces	traces	traces	2.0

[a]**UHR_E_AP** = Ultra-high release ethanol and ultra-high release alpha-pinene.
C_6C_8 = Racemic 3-hydroxyhexan-2-one (K6), racemic 3-hydroxyoctan-2-one (K8), and ultra-high release ethanol.
General Longhorn = Ultra-high release ethanol, (*E*)-6,10-dimethyl-5,9-undecadien-2-ol (*E*-fuscumol), and (*E*)-6,10-dimethyl-5,9-undecadien-2-yl (*E*-fuscumol acetate).
Pine Sawyer = 2-undecyloxy-1-ethanol (monochamol), ultra-high release ethanol, ultra-high release alpha-pinene, and racemic 2-methyl-6-methylene-7-octen-4-ol (ipsenol).
[b]Below 0.01% or not in the top 10 for this semiochemical.

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Table 4: Operational Taxonomic Units: fungal identification proportion (%) by semiochemical type at the species taxonomic level (top 10 species) using the ITS1 genic region.

Semiochemical[a]	UHR_E_AP	C_6C_8	General Longhorn	Pine Sawyer
Species				
fungi sp.	17.5	14.2	9.3	5.6
Rhodotorula mucilaginosa	8.2	8.5	traces[b]	2.7
Cystobasidium slooffiae	6.4	3.3	traces	traces
Ascomycota sp.	5.3	4.5	2.8	5.9
OTU unclassified below genus	9.6	3.1	2.4	2.4
Epicoccum nigrum	2.5	5.2	3.3	2.2
Alternaria alternata	2.5	traces	traces	traces
Scopuloides hydnoides	1.8	traces	traces	traces
Verticillium dahliae	1.8	2.2	traces	traces
Cystobasidium pinicola	traces	2.2	traces	traces
Hannaella luteola	traces	1.9	traces	traces
Wickerhamomyces anomalus	traces	1.8	traces	traces
Leptographium piriforme	traces	traces	5.2	4.4
Aureobasidium pullulans	traces	traces	5.1	traces
Cladosporium exasperatum	traces	traces	3.9	6.9
Neurospora terricola	traces	traces	3.2	traces
Kluyveromyces wickerhamii	absent	absent	2.9	traces
Torulaspora delbrueckii	absent	traces	2.3	traces
Candida sp.	traces	traces	traces	7.9
Mycosphaerellaceae sp.	traces	traces	traces	3.0

[a]**UHR_E_AP** = Ultra-high release ethanol and ultra-high release alpha-pinene.
C_6C_8 = Racemic 3-hydroxyhexan-2-one (K6), racemic 3-hydroxyoctan-2-one (K8), and ultra-high release ethanol.
General Longhorn = Ultra-high release ethanol, (*E*)-6,10-dimethyl-5,9-undecadien-2-ol (*E*-fuscumol), and (*E*)-6,10-dimethyl-5,9-undecadien-2-yl (*E*-fuscumol acetate).
Pine Sawyer = 2-undecyloxy-1-ethanol (monochamol), ultra-high release ethanol, ultra-high release alpha-pinene, and racemic 2-methyl-6-methylene-7-octen-4-ol (ipsenol).
[b]Below 0.01% or not in the top 10 for this semiochemical.

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Table 5: Operational taxonomic units: oomycete identification proportion (%) by semiochemical type at the Phylum and Genus taxonomic levels using the ITS1 genic region.

Semiochemical[a]	UHR_E_AP	C_6C_8	General Longhorn	Pine Sawyer
Phylum				
Oomycota	73.9	73.4	99.9	96.9
OTU unclassified below kingdom	26.1	26.6	0.01	3.02
Genus				
Peronospora	38.7	8.55	64.1	9.39
OTU unclassified below family	26.1	26.6	0.01	0.30
Phytophthora	18.4	46.5	16.3	absent
Pythium	13.1	14.4	17.5	0.14
Hyaloperonospora	1.90	2.68	0.87	0.15
Plasmopara	1.72	absent	0.13	absent
Basidiophora	0.13	1.34	1.07	0.01
Saprolegnia	absent	absent	traces[b]	traces

[a]**UHR_E_AP** = Ultra-high release ethanol and ultra-high release alpha-pinene.
C_6C_8 = Racemic 3-hydroxyhexan-2-one (K6), racemic 3-hydroxyoctan-2-one (K8), and ultra-high release ethanol.
General Longhorn = Ultra-high release ethanol, (*E*)-6,10-dimethyl-5,9-undecadien-2-ol (*E*-fuscumol), and (*E*)-6,10-dimethyl-5,9-undecadien-2-yl (*E*-fuscumol acetate).
Pine Sawyer = 2-undecyloxy-1-ethanol (monochamol), ultra-high release ethanol, ultra-high release alpha-pinene, and racemic 2-methyl-6-methylene-7-octen-4-ol (ipsenol).
[b]Below 0.01% or not in the top 10 for this semiochemical.

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Table 6: Operational Taxonomic Units: oomycete identification proportion (%) by semiochemical type at the species taxonomic level (top 10 species) using the ITS1 genic region.

Semiochemical[a]	UHR_E_AP	C_6C_8	General Longhorn	Pine Sawyer
Species				
Peronospora manshurica	26.7	0.67	61.3	84.9
OTU unclassified below genus	26.1	26.6	traces[b]	3.02
Phytophthora sp.	18.04	46.2	16.3	traces
Peronospora aestivalis	8.20	2.82	1.36	1.47
Pythium sp. CAL-2011e	4.61	11.4	NA[c]	NA
Pythium hypogynum	4.14	1.63	traces	traces
Peronospora alta	2.11	4.59	0.002	3.16
Pythium sp. BG01	1.80	NA	17.1	NA
Plasmopara viticola	1.72	NA	traces	traces
Hyaloperonospora brassicae	1.42	traces	0.75	0.39
Hyaloperonospora parasitica	traces	2.43	0.001	1.10
Basidiophora entospora	traces	1.34	1.07	traces
Pythium catenulatum	NA	0.32	0.23	traces
Peronospora polygoni	0.01	traces	0.49	2.95
Peronospora variabilis	traces	traces	0.40	0.83
Peronospora sepium	traces	traces	0.19	traces
Pythium sp. 3862	traces	traces	traces	0.96
Peronospora arthurii	traces	NA	traces	0.29

[a]**UHR_E_AP** = Ultra-high release ethanol and ultra-high release alpha-pinene.
C_6C_8 = Racemic 3-hydroxyhexan-2-one (K6), racemic 3-hydroxyoctan-2-one (K8), and ultra-high release ethanol.
General Longhorn = Ultra-high release ethanol, (*E*)-6,10-dimethyl-5,9-undecadien-2-ol (*E*-fuscumol), and (*E*)-6,10-dimethyl-5,9-undecadien-2-yl (*E*-fuscumol acetate).
Pine Sawyer = 2-undecyloxy-1-ethanol (monochamol), ultra-high release ethanol, ultra-high release alpha-pinene, and racemic 2-methyl-6-methylene-7-octen-4-ol (ipsenol).
[b]Below 0.01% or not in the top 10 for this semiochemical.
[c]Not applicable or not in the top 10 for this semiochemical.

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4. Discussion

This study demonstrated that the described metagenomics approach to investigate insect trap fluids makes it possible to detect airborne, or insect-vectored fungal species even at very low abundances (i.e., ≤10 OTU). A unique aspect of this study is that despite the fact that the CFIA has used insect traps in its national survey for many years, this is the first time that the formerly discarded trap preservation fluids have been analyzed, and the information extracted was highly valuable.

The project aimed to expand knowledge on phytopathogenic fungi and oomycetes by studying insect trap fluid samples, and this was assessed using the subtracted species datasets. More specifically, the project searched for interactions between phytopathogenic fungi and insects, and results, though ambiguous in certain cases, surely showed that there may be some novel insect-fungal relationships and effect of lure type that deserve further inquiry. Traditional methods such as cloning and culturing make it difficult for scientists to screen species at a larger scale but, our metagenomics approach now provides the opportunity to do so. Indeed, this project can provide a good overall assessment of the communities without having to isolate all organisms for identification purposes. This is a tool that regulatory agencies and other stakeholders could use for primary screening and disease monitoring. This approach is essentially a general detection survey for non-indigenous pathogens that partially fills a large gap that is a key step in the battle against invasive pathogens. By taking advantage of an established survey conducted by CFIA inspectors, other than shipment of fluid samples to the lab, no additional resources were required, thereby making this a cost-effective surveillance method. Just as air trap samplers are used to capture various pathogens in a given environment, our results suggested that insect traps can actively and passively gather worthy information from the environment.

Despite the fact that using ITS1 for fungal communities profiling does not always allow for resolution below the genus level for certain organisms [92], like it was presented in Supplementary Table S2, species subtraction filtered the data from background noise and highlighted numerous species with the potential to damage trees or other plants. Species detected in control traps were least likely to be vectored by insect andwere discarded, revealing several potentially phytopathogenic entities remaining that were more likely to be insect-transmitted. For instance, species unique to the various semiochemical types were from genera composed of important plant pathogens. Among others, *Phoma glomerata*, the causal agent of blight, leaf spots, and fruit rot of many plants, was uniquely detected in C_6C_8 samples, whereas *Mycosphaerella areola* (mildew)—despite the fact that it typically infects wheat leaves [93,94], field peas [95], and cruciferous vegetables [96]—was only detected in General Longhorn-baited trap samples. This semiochemical is an aggregation pheromone for longhorned beetles in the Spondylidinae [57] and Lamiinae subfamilies [70]. Similarly, the genera *Mortierella* and *Phyllosticta* (counting, *P. minima*: the causal agent of leaf spot in maple), which also contain numerous phytopathogenic species [97], were recovered from C_6C_8 semiochemical, which is an aggregation pheromone for longhorned beetles in the Cerambycinae subfamily.

Data collected from Pine Sawyer samples revealed fungi typically associated with insects such as Ceratocystidaceae, *Ambrosiella* spp., and *Leptographium* spp. [98-100]. Detection of *Leptographium*, a genus that includes the causal agent of blue stain in conifers, was not unexpected given its known association with bark beetles [99,101]. For example, *L. piriforme* is vectored by *Tomicus piniperda* (exotic), as well as other native bark beetles species [102]. Interestingly, the proportion of *Leptographium* species detected in the General Longhorn and Pine Sawyer semiochemical traps prior to species subtraction were greater than for C_6C_8 and UHR_E_AP. This could suggest an association with particular insect groups or demes. Because the phylogenetic analysis of *Leptographium* for species inference is usually done using a combination of at least three genic regions [103], more sequencing data, or alternate standardized assay (e.g., qPCR) would be required in order to validate down to the species level in this case (e.g., *L. piriforme*).

Also, two considerable plant pathogen genera were observed solely within the Pine Sawyer lure subtracted data: *Taphrina* (specifically *T. padii*, the causal agent of cherry fruit deformation) and *Phellinus* (*P. ferrugineovelutinus* is the causal agent of wood rot in alder and maple).

Given that some of the aforementioned fungal groups detected were less likely to actually encounter wood-boring insects, the presence of genera such as *Phoma* spp., *Taphrina* spp., and *Mycosphaerella* spp. could also be explained by the occurrence of other insects caught into the insect traps. In fact, flies, wasps, bees, dragonflies, moths, and other insects, which are regularly found by the CFIA inspectors in the traps (Troy Kimoto, *personal communication*), could have incidentally come in contact with infected plants prior to be captured, and, therefore, transport fungal propagules. For instance, honey bees are known vectors of bacteria, viruses, and fungus during foraging activities [104-108].

Likewise, some insects attracted to UHR_E_AP may be involved in the transmission of powdery mildews (*Erysiphe* spp.) and anthracnoses (*Colletotrichum* spp.) considering that these known pathogenic fungi were not detected in any other semiochemical treatment. However, because the addition of ethanol makes this lure attractive to a wide range of insects, there could be another reason for the detection of such pathogens in those samples, possibly being (i) the forest type or province of origin or weather conditions given that mildews are wind and water dispersed [109], or (ii) the capture of other insects including pollinators.

Similarly, the General Longhorn semiochemical (mainly attracts longhorned beetles and bark beetles) was associated with important phytopathogenic genera, including *Pucciniastrum* (among others, the rust *P. circaeae*), *Sirococcus* (specifically *S. piceicola*, and *S. conigenus*, two shoot blight causal agents), and *Trametes* (reduces wood value as a decaying agent) [97]. Additionally, while some *Verticillium* species are pathogenic to insects [110], other phytopathogenic *Verticillium* species are transported by jewel beetles (e.g. *Agrilus* spp.) and bark beetles [111]. For example, in Europe, *V. dahliae* is transported to *Quercus* spp. by the bark beetle *Scolytus intricatus* (does not occur in North America) and the ambrosia beetle *Anisandrus dispar* (F.) [111]. Therefore, the presence of *V. isaacii* (wilt in multiple plants) in samples baited with C_6C_8 may be due, in part, to ethanol contained in the lure because it is highly attractive to ambrosia beetles. However, not all *Verticillium* species can be resolved using the ITS1 region [112,113] so, the detection of *V. isaacii* using ITS1 was not conclusive at this point. Still, alignments of ITS1 sequences were done for the potential *V. isaacii* OTU with reference sequences (data not shown) and revealed a 100% match with *V. isaacii*, *V. tricorpus*, and *V. klebahnii*, which was expected. For the abovementioned reasons, complementary tests shall be performed to validate sensitive data.

There were similarities in communities between traps baited with UHR_E_AP and C_6C_8 (Tables 3, 4 and 6), and between the communities detected in Pine Sawyer and General Longhorn-baited samples. In contrast, differences occurred between the communities detected by the two former lure types versus the two latter. Such variation could be due to the fact that there were much fewer UHR_E_AP and C_6C_8 samples compared with the number of Pine Sawyer and General Longhorn samples, rendering comparison between the datasets unbalanced. The main reason for this difference is because the two former lures were used only during one season (2013), whereas the latter two were used for two collecting seasons (2014 and 2015). The forest type, the chemical composition, and the seasonal weather (temperature and rain) may also have influenced the communities retrieved.

Compared with fungi, the fewer number of identifiable oomycetes OTU in this study may be explained by the fact that the number of taxonomically described oomycetes is much lower [114-116]. One outstanding aspect of the oomycete analysis is that, following the species subtraction, most remaining species were unique to the General Longhorn samples (Supplementary Table S3) and none were recovered from the Pine Sawyer semiochemical. There were no *Phytophthora* species remaining after species subtraction but, *Pythium* (broad host range, mainly affecting roots or leaves) and *Peronospora* species (broad host range, mainly causing mildews) were dominant. Once again, this could contribute in demonstrating novel observations between plants, insects, and oomycetes.

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The ITS1 *Phytophthora* OTU (i.e., non-subtracted data) revealed *Phytophthora foliorum* (*Rhododendron* spp. and Azalea leaf blight [117,118]), *Phytophthora* sp. *"kelmania"* (affects gerbera [119] and Christmas trees [120]), and *P. syringae* (has wide host range causing numerous diseases [121]) but, because this intergenic spacer does not contain sufficient variation for species resolution, unlike ATP9-NAD9, those identifications were not conclusive. In contrast, the ATP9-NAD9 region allowed identification of *P. cryptogea* (numerous hosts causing different diseases [121]), *P. foliorum*, *Phytophthora* sp. *"kelmania"*, and *P. syringae* but, these were discarded following species subtraction. This might indicate that *Phytophthora*-insects associations are not as frequent compared with other oomycetes such as *Pythium* spp.

Following species subtraction, there was a number of fungal species found that can degrade timber, some of which were associated with specific lures (Supplementary Table S2). Despite the fact that these fungi are not highly virulent, they can still damage or stain wood, thereby reducing timber marketability. The methodology used here appears to have the capacity to detect more harmful organisms if they had been present, because genera containing virulent pathogens were detected. The collection areas also seemed to have a role when profiling the fungal communities as OTU aggregated to geographic region (Supplementary Figure S4).

Considering that the spore trap samples reached the most sequences per sample compared with both the insect traps and the subtracted insect trap datasets, it refutes the possibility of having mistakenly discarded OTU due to spore trap undersampling. Because the control samples were highly diverse, it is more likely that the species remaining after subtraction are actually unique to the insect traps. There are logically fewer species in the subtracted data versus the original one, which is likely why those remaining had a rarefaction curve approaching saturation. Interestingly, the fact that only a part of the original insect data was sequenced deeply enough (i.e., rarefaction curve saturation) suggests that, for future NGS runs, sequencing fewer multiplexed fusion primer samples at a time would probably yield a more representative diversity analysis. In contrast, it appears that it was beyond sufficient for the spore traps, meaning more samples could be tested at once.

Given that numerous yeast are commonly associated with insects [122], the presence of *Aureobasidium* sp., *Candida* sp., *Cladosporium* sp., *Cystobasidium* sp., *Cryptococcus* sp., *Hannaella* sp., *Kluyveromyces* sp., *Rhodotorula* sp., *Torulaspora* sp., and *Wickerhamomyces* sp. in the unsubtracted dataset was expected (Table 4). *Candida* spp. are natural biocontrols agents of fruit and vegetable pests [122-124], and are also associated with bark beetles [21,23-25]. Following species subtraction, our results showed the presence of *Candida michaelii* only in the samples baited with the C_6C_8 semiochemical, which primarily attracts longhorned beetles, but the addition of ethanol makes it attractive to bark beetles as well.

Furthermore, the fact that the entomopathogenic species *Colletotrichum nymphaeae* was retrieved from the samples is promising for entomologists, because they could consider using our method to screen for either insect pathogens, or new biological pest controls. As a matter of fact, beneficial fungal endophytes have been studied for their ability to help plant's defenses [125]. For example, *Beauveria bassiana* infection has reduced populations of emerald ash borer (*Agrilus planipennis*) [126,127]. The gut-associated species detected (*Candida michaelii* [handsome fungus beetle]) demonstrated the robustness of our metagenomics approach in studying fungus-insect relationships regardless of the niche they occupy. Plus, this high-throughput method is more efficient compared with traditional assays because many sites could be sampled simultaneously due to the high volume of samples processed at a time, thus increasing the likelihood of detecting new nonnative fungal species.

Although some of the fungal genera or species that were detected in this project are already reported to be affiliated with insects, the results suggest that there may be other fungal and oomycetes species transported to potential host trees by insects. Based on the unique fungal and oomycete species detected within a given lure, our data suggests that there may be previously unrecorded associations between insects, fungi, and oomycetes. Each of the different semiochemicals employed in this study was attractive to a certain range of insect group or deme but, inevitably, passive or incidental collection of other insects

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(e.g., pollinators) contaminated by phytopathogens which they came across, though they would otherwise never transmit it to the plant host, occurred. The ethanol added also increased the chances of such event to happen.

All considered, making a direct link between the fungal species detected based on the semiochemical, the collection area, and the insects caught is very complex when solely using the presented approach. Nevertheless, if an organism of potential interest was detected, this method would provide stakeholders with location data that would narrow the target area for follow-up surveys. These targeted surveys would involve searching for symptomatic hosts, collecting samples, and performing validated low throughput assays. It is important to note that because the material studied consisted of airborne material rather than symptomatic trees, the detection of a given species does not automatically translate in the occurrence of diseased trees.

Despite the fact that this method may lack in providing forest stakeholders with a definitive answer, the observations shed light on new potential insect-pathogen associations. Yet, the approach to quickly screen species at a large geographic scale would be highly useful to (i) efficiently sample the environment for the presence of any new fungi or (ii) to obtain primary data in order to provide guidance to those who monitor and manage phytopathogens over large jurisdictions. Finally, future research could examine the fungal communities associated with specific wood-boring insects to determine if there are undiscovered relationships with these organisms and their host trees.

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Supplementary Material S1: Semiochemicals' composition and additional details.

Each lure set (i.e., C_6C_8, UHR_E_AP, General Longhorn, and Pine Sawyer) was placed on separate traps.

First semiochemical set: C_6C_8.

The first combination (C_6C_8) consisted of racemic 3-hydroxyhexan-2-one (C_6) (Bedoukian Research Inc., Danbury, CT, USA), racemic 3-hydroxyoctan-2-one (C_8) (Bedoukian Research Inc.), and ultra-high release ethanol (UHR EtoH), where each chemical was placed within individual release devices. C_6 and C_8 were verified 99% pure by gas chromatography–mass spectrometry (GC-MS) by the Canadian Forest Service, and loaded into polyethylene pouches by Contech Inc (Delta, BC, Canada). Each pouch contained 1.4 g of either C_6 or C_8. The release rates (at 20˚C) were 20 mg/d for C_6, and 25 mg/d for C_8. Two C_6 and two C_8 pouches (semiochemicals) were both placed on a trap to obtain cumulative release rates of 40 to 50 mg/d.

Second semiochemical set: UHR_E_AP.

The second semiochemical set (UHR_E_AP) consisted of UHR EtoH and UHR alpha-pinene. The two chemicals were loaded into separate release devices and placed on an insect trap. The UHR ethanol (95% purity, 121.5 g loaded/pouch) and UHR alpha-pinene [95% (-) enantiomer, 172 g/pouch] lures (Contech Inc., Delta, BC, Canada) had release rates (at 20˚C) of 275 mg/d and 2 g/d, respectively. The UHR ethanol and UHR alpha-pinene chemicals were exactly the same throughout this project.

Third semiochemical set: General Longhorn.

The lure set consisted of UHR EtoH, (*E*)-6,10-dimethyl-5,9-undecadien-2-ol (*E*-fuscumol) and (*E*)-6,10-dimethyl-5,9-undecadien-2-yl (*E*-fuscumol acetate). Both *E*-fuscumol and *E*-fuscumol acetate were synthesized by Bedoukian Research Inc. and placed into polyethylene bubble caps by Contech Inc.; 130 mg/bubble cap of *E*-fuscumol (release rate = 1 mg/d), and 200 mg/bubble cap of *E*-fuscumol acetate (release rate = 2 mg/d).

Fourth semiochemical set: Pine Sawyer.

The Pine Sawyer lure set included four separate components consisting of 2-undecyloxy-1-ethanol (monochamol), UHR EtoH, UHR alpha-pinene and racemic 2-methyl-6-methylene-7-octen-4-ol (ipsenol). Monochamol (99.3% purity, 0.025 g/bubble cap) and racemic ipsenol (>99% purity, 0.04 g/bubble cap) were also purchased from Contech Inc. and had release rates (20˚C) of 0.2 mg/d and 0.4 mg/d, respectively.

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Supplementary Table S1: Summary of the samples collected from 2013 to 2015.

Semiochemical[a]	Number of samples	Canadian provinces
UHR_E_AP	39	British Columbia, New Brunswick, Newfoundland and Labrador, Nova Scotia, Ontario, Prince Edward Island, and Quebec
C_6C_8	36	
General Longhorn	17	
Pine Sawyer	16	
Total:	108	7 Canadian provinces

[a]**UHR_E_AP** = Combination of two semiochemicals (ultra-high release (UHR) ethanol and UHR alpha-pinene) that attract a wide range of bark and wood-boring insects [49,55,56].

C_6C_8 = Combination of three semiochemicals (racemic 3-hydroxyhexan-2-one, racemic 3-hydroxyoctan-2-one, and UHR ethanol). The first two chemicals are aggregation pheromones of some longhorned beetles in the Cerambycinae subfamily [54] but, the addition of UHR ethanol increases attraction to other wood-boring insects.

General Longhorn = Combination of three semiochemicals (UHR ethanol, (E)-6,10-dimethyl-5,9-undecadien-2-ol (E-fuscumol), and (E)-6,10-dimethyl-5,9-undecadien-2-yl (E-fuscumol acetate)) used to attract Spondylidinae, Lamiinae, and Scolytinae beetles [57,58,70].

Pine Sawyer = Combination of four semiochemicals (2-undecyloxy-1-ethanol (monochamol), UHR ethanol, UHR alpha-pinene, and racemic 2-methyl-6-methylene-7-octen-4-ol (ipsenol)) used to attract longhorned beetles (*Monochamus*) and bark and ambrosia beetles due to the added ethanol and alpha-pinene [47,59,60].

Supplementary Table S2: Exotic and native fungal species of interest that are unique to a semiochemical (i.e., post species subtraction), and grouped by the potential damage (= trophic status of concern) associated with those fungi. Also included is a risk level scale in terms of virulence. Identifications based on the ITS1 sequences obtained.

		Semiochemical[a]							
	Known damage	Pine sawyer	General Longhorn	C_6C_8	UHR_E_AP	Presence status[b]	Risk level[c]	Known host(s)	References
plant pathogen									
Ambrosiella ferruginea	galleries and wounds caused by insect vector (mycangia)	x				N, C	2	conifers and deciduous trees	[128]
Ciborinia whetzelii	anthracnose and ink spot disease			x		N, C	2	aspen and cottonwood	[129-131]
Colletotrichum fructi	anthracnose				x	N	2	over 30 plant genera	[132,133]
Colletotrichum nymphaeae	anthracnose, leaf spot, and bitter rot				x	N	2	grapevine, pepper, black locust, strawberry, water lily, apple, crab apple and protea	[134-137]
	insect pathogen							citrus orthezia	[138]
Devriesia americana	unknown			x		N	2[d]	unknown	[139,140]

 doi:10.20944/preprints201810.0333.v1

Devriesia strelitziicola	death of leaves				x	E	2	*Strelitzia* spp.	[141-144]
Erysiphe adunca		x				N, C	2	*Populus* spp. and willow	[88,145]
Erysiphe convolvuli					x	N, C	2	*Calystegia* spp. and *Convolvulus* spp.	[121]
Erysiphe cruciferarum				x		N, C	2	mustard, cabbage, bok choy, and turnip	[146]
Erysiphe diffusa					x	N	2	soybean and legumes	[147,148]
Erysiphe elevata	powdery mildew		x			N	2	flowering trees	[149,150]
Golovinomyces depressus			x			E	2	numerous plants in the Asteraceae family	[88,121,145]
Neoerysiphe galeopsidis					x	N, C	2	wild basil, nettle, white turtlehead, and mint	[129,146,151,152]
Podosphaera clandestina				x		N, C	2	serviceberry, hawthorn, purple loosestrife, crab	[97,129,151]

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								apple, apricot, cherry, plum, peach and spirea	
Podosphaera leucotricha					x	N, C	2	apple, and crab apple	[97,129,146]
Podosphaera lini					x	E	2	flax	[121,145,153]
Podosphaera negeri				x		E	2	flowering shrubs	[145,154]
Echinodontium tinctorium	heart rot and brown stringy rot		x			N, C	3	hemlock, fir, and cedar	[97,129,155,156]
Leptographium sp.	blue stain and sapstain	x				N, C	1?	conifer and hardwood	[99-103,157]
Lirula macrospora	needle cast		x			N, C	3	spruce	[129,158]
Meria laricis		x				N, C	3	larch	[97,159]
Mollisia dextrinospora	eyespot of cereal				x	E	2	cereals	[160-162]
Mycosphaerella areola	areolate mildew		x			N	3	cotton	[121,163]
Phaeo-acremonium inflatipes	wilt and decline	x				N	1?	*Quercus* spp., *Nectandra* spp., whitebeam, vine, and	[18,121,164-166]

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								quince	
Phoma glomerata	blight, leaf spots, and fruit rot			x		N, C	2	over 80 different plants	[121,129,167-169]
Pucciniastrum circaeae	rust		x			E	2	shrubs, fir and *Circaea* spp.	[121,170]
Septoria gladioli	leaf spot and hard rot				x	N, C	2	flowers and corn	[97,129,171]
Sirococcus conigenus	shoot blight		x			N, C	2	pine, spruce, fir, and hemlock	[97,172,173]
Sirococcus piceicola			x			N, C	2	spruce	[173,174]
Stagonospora pseudopaludosa					x	E	3	grass	[175]
Teratosphaeria xenocryptica	leaf spot				x	E	2	eucalytpus	[176,177]
Phyllosticta minima				x		N, C	3	maple	[88,97,129,178,179]
Strelitziana mali	sooty blotch				x	E	3	apple and vine	[180-182]
Taphrina padi	fruit deformation	x				E	2	cherry	[183-185]
Verticillium isaacii	vascular wilt			x		N	2	artichoke, tomato, spinach,	[112,186]

 doi:10.20944/preprints201810.0333.v1

								lettuce, cauliflower, eggplant, pepper, and strawberry	
insect gut associated									
Candida michaelii	N/A[e]			x		N	N/A	handsome fungus beetle	[187]
loss of wood value									
Donkioporia albidofusca		x				U	5	decaying wood	[88,188]
Melastiza chateri			x			U	5	decaying wood	[88]
Perenniporia luteola					x	U	5	decaying wood	[189]
Phlebiopsis sp.		x				N, C	5	decaying wood	[190,191]
Pleurotus ostreatus	white rot				x	N, C	5	decaying wood	[97,129,131, 151,192]
Pluteus eludens				x		U	5	decaying wood	[88]
Pluteus phlebophorus					x	U	5	decaying wood	[88]
Ramaria pinicola			x			U	5	decaying wood	[88]
Steccherinum oreophilum					x	N, C	5	decaying wood	[129,193]
Trametes cubensis			x			N	5	decaying wood	[88,194-196]

Antrodia albobrunnea	brown rot		x		N, C	5	decaying wood	[197,198]
Sidera lunata				x	E	5	decaying wood	[88,199]
Diatrype disciformis	beech barkspot			x	N, C	3	decaying hardwood trees	[129,151,200]
Hyphodontia microspora	other wood rots		x		E	5	decaying wood	[201,202]
Phellinus ferrugineovelutinus		x			N, C	3	maple and alder	[97,203,204]

[a] **UHR_E_AP** = Ultra-high release ethanol and ultra-high release alpha-pinene.

C_6C_8 = Racemic 3-hydroxyhexan-2-one (K6), racemic 3-hydroxyoctan-2-one (K8), and ultra-high release ethanol.

General Longhorn = Ultra-high release ethanol, (*E*)-6,10-dimethyl-5,9-undecadien-2-ol (*E*-fuscumol), and (*E*)-6,10-dimethyl-5,9-undecadien-2-yl (*E*-fuscumol acetate).

Pine Sawyer = 2-undecyloxy-1-ethanol (monochamol), ultra-high release ethanol, ultra-high release alpha-pinene, and racemic 2-methyl-6-methylene-7-octen-4-ol (ipsenol).

[b] N = the organism is native or reported to be present in North America, C = the organism is native or reported to be present in Canada, E = the organism is not reported or present in North America (exotic), and U = unknown status because information is lacking for Canada and North America.

[c] Risk associated with the organism on a 1 to 5 scale. 5 = a riskless saprophyte fungus, 4 = a saprophyte fungus capable of causing damages to plants 3 = a weakly-virulent pathogenic fungus, 2 = a moderate virulent pathogenic fungus but common in Canada, and 1 = a highly-virulent pathogenic fungus.

[d] Assumption based on the impact of species within the same genus.

[e] Not applicable.

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Supplementary Table S3: Unique oomycete species detected in the different semiochemicals after proceeding with species subtraction and using the ITS1 genic region.

	Semiochemical[a]	
General Longhorn	**C_6C_8**	**C_6C_8 and General Longhorn**
Species		
Peronospora farinosa	*Pythium* sp. CAL-2011e	*Pythium* sp. BG01
Peronospora sp. isolate 079405,59		*Pythium* sp. P3862
Peronospora sp. UPS F-119986		No blast hit
Peronospora sparsa		
Peronospora viciae		
Pythium aff. hypogynum		
Pythium sp. AvdB-2012		
Pythium sp. BP2013k		
Pythium sp. CAL-2011f		
Pythium sp. P19300/1/3		
Saprolegnia sp. SAP1		
Total: 11	1	3

[a] **General Longhorn** = Ultra-high release ethanol, (*E*)-6,10-dimethyl-5,9-undecadien-2-ol (*E*-fuscumol), and (*E*)-6,10-dimethyl-5,9-undecadien-2-yl (*E*-fuscumol acetate).
C_6C_8 = Racemic 3-hydroxyhexan-2-one (K6), racemic 3-hydroxyoctan-2-one (K8), and ultra-high release ethanol.

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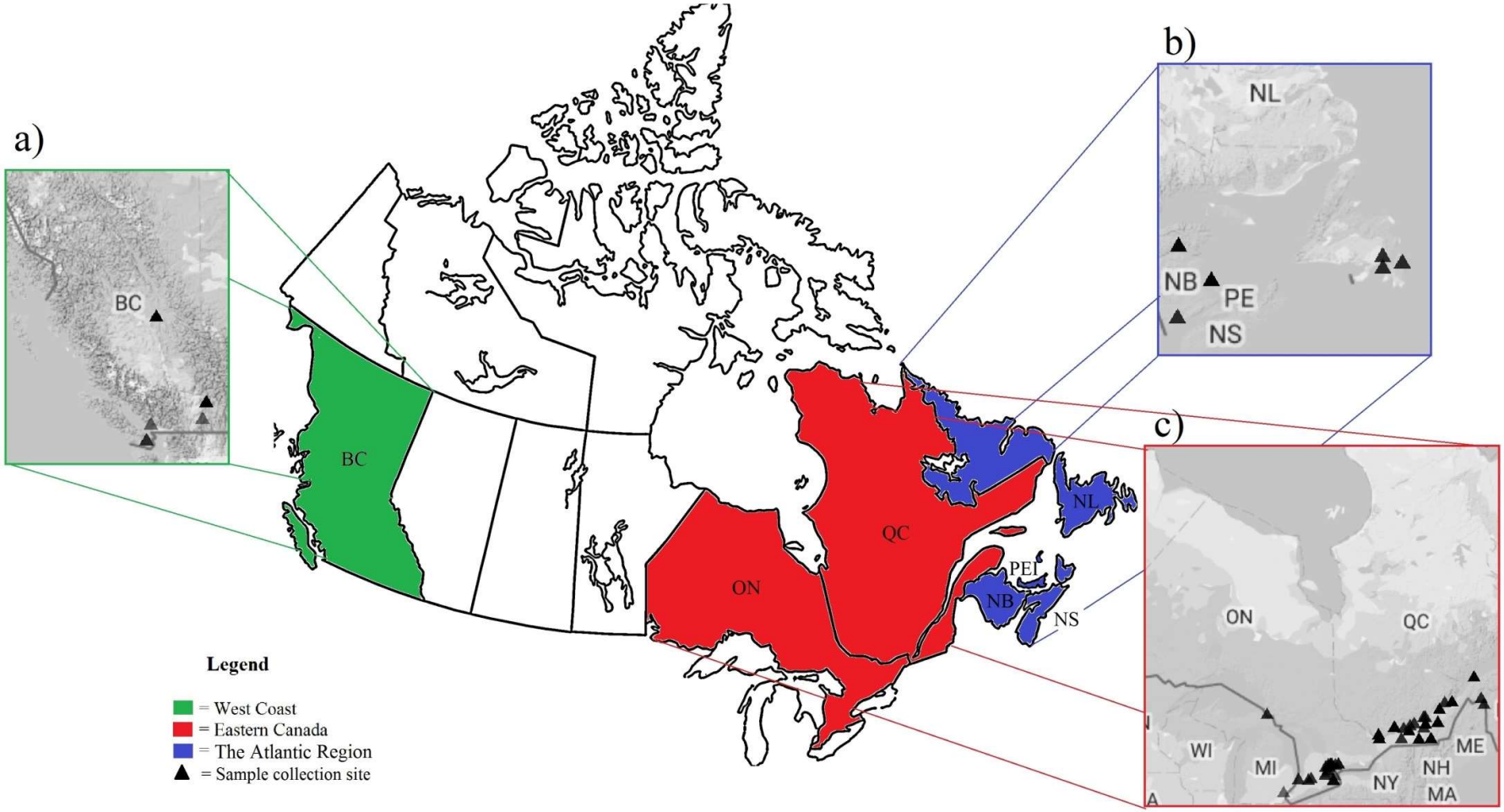

Supplementary Figure S1**: Sampling sites in Canada; a) West Coast, b) the Atlantic Region, and c) Eastern Canada. Adapted from Google Earth.**

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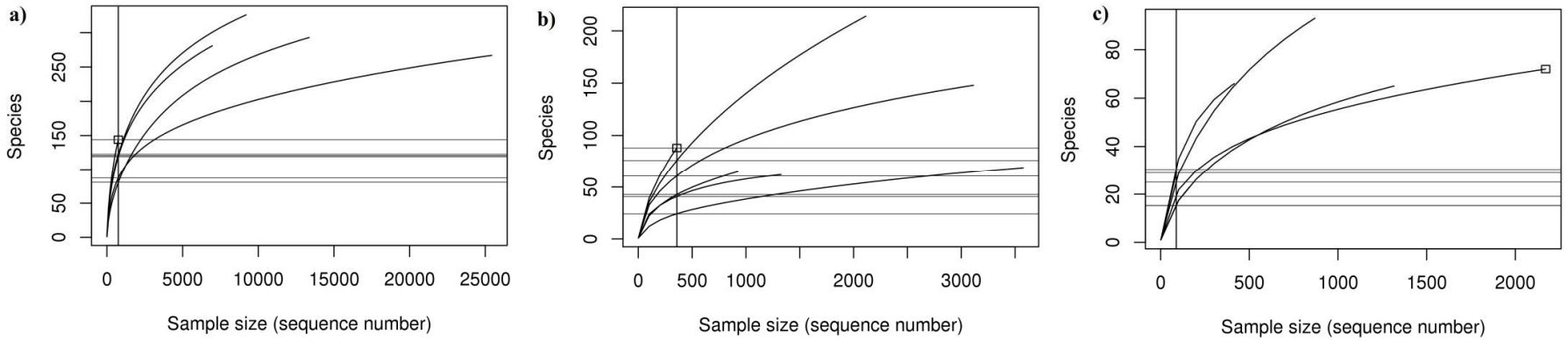

Supplementary Figure S2: Rarefaction curves (number of sequences obtained for each species) for a) spore trap samples and their respective b) insect trap samples (original data), and c) insect trap samples (subtracted data) to visualize sequencing depth.

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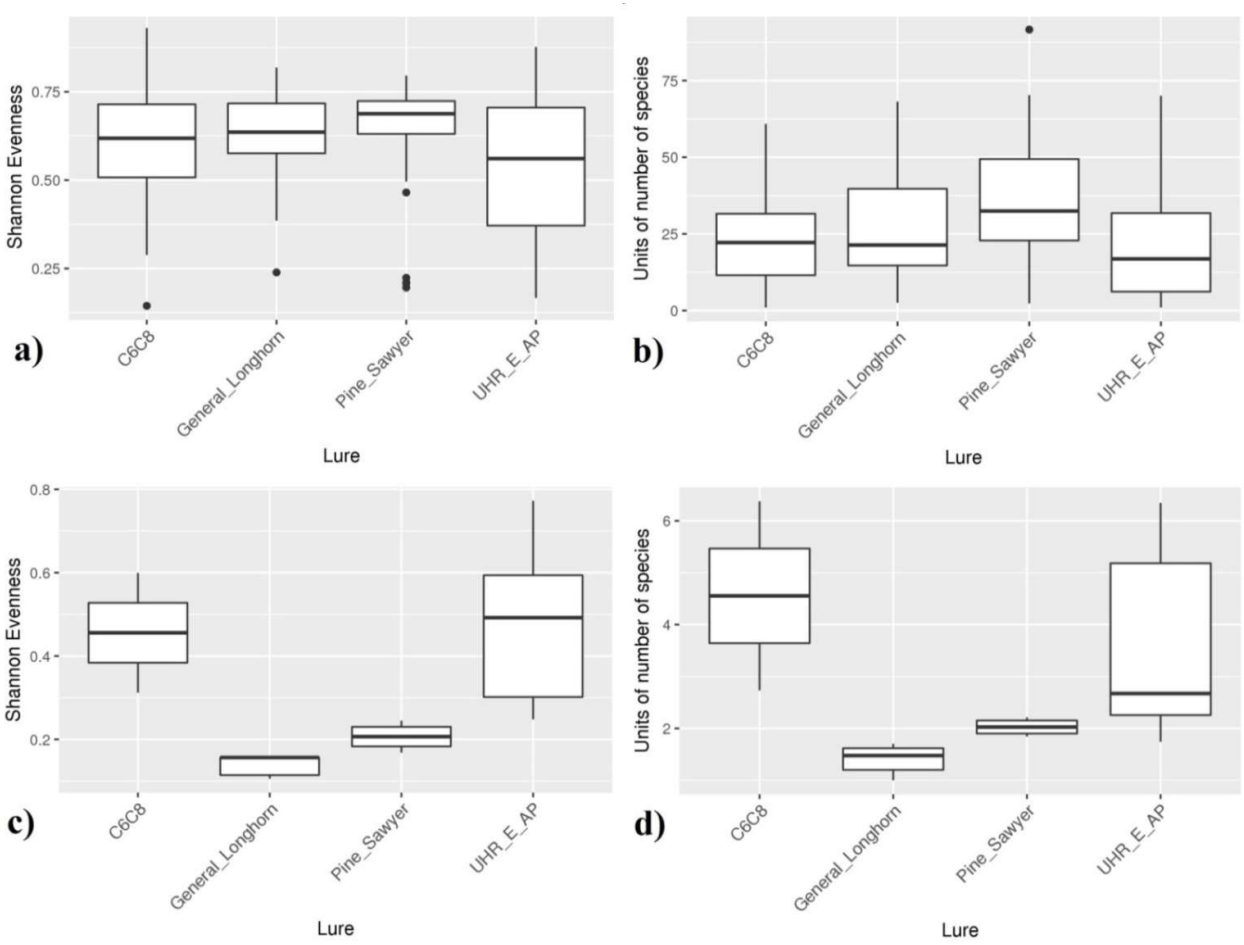

Supplementary Figure S3: fungal species a) evenness (Shannon) and b) true diversity (Shannon) by semiochemical type, and oomycete species c) evenness (Shannon) and d) true diversity (Shannon) by semiochemical type. The ITS1 sequences were used.

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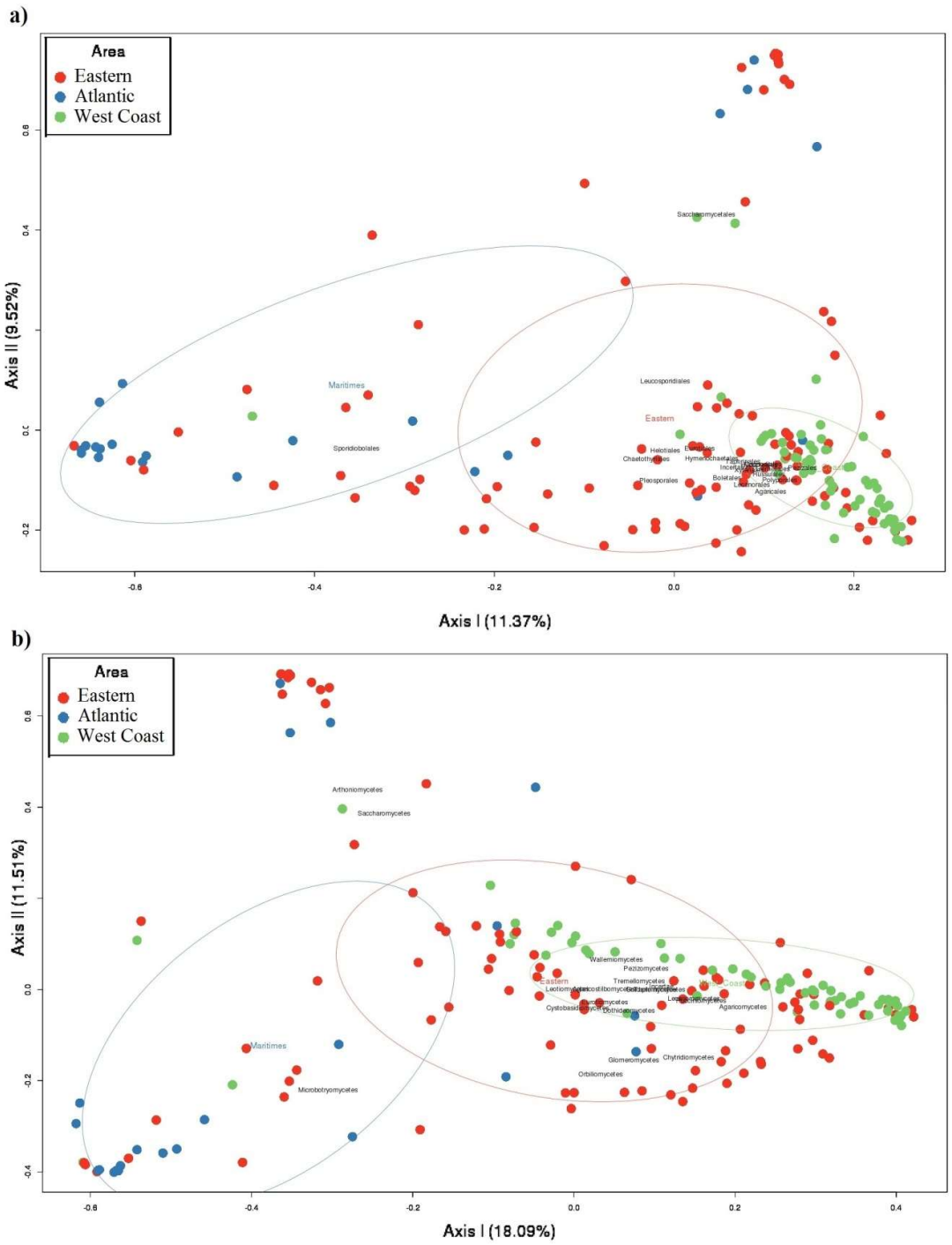

Supplementary Figure S4: Principal Coordinate Analysis of the fungal Operational Taxonomic Unit (ITS1) found in three Canadian geographic regions; the West Coast (BC), the Atlantic Region (PEI, NL, NS, and NB), and Eastern (QC and ON) at the a) order and b) class taxonomic ranks.

Author Contributions: Émilie D. Tremblay performed formal analysis, methodology development, visualization, programming, and data curation. Troy Kimoto drafted operational protocols for placement of insect traps in the field by inspection staff. Both É. D. Tremblay and T. Kimoto conducted investigation and wrote the original draft of this article. Dr. Guillaume Bilodeau conceptualized and supervised this study. Drs. Jean Bérubé and G. Bilodeau provided resources, and reviewed and edited the article.

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