Conoideocrella luteorostrata (Hypocreales: Claviciptaceae), a potential biocontrol fungus for elongate hemlock scale in United States Christmas tree production areas

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Abstract

The entomopathogenic fungus *Conoideocrella luteorostrata,* which is known to infect armored scale insects and whiteflies in Thailand and Japan, has been recently found causing natural epizootics in elongate hemlock scales (EHS) in Christmas tree farms in the eastern United States. The fungus was isolated from mycosed EHS, but not from the surrounding Fraser fir needles, which suggests that the life cycle is restricted to the insect host. This fungus is a promising candidate for biocontrol of EHS since it infects scales naturally and when applied under lab conditions, thus confirming pathogenicity of *Conoideocrella luteorostrata* against EHS, an essential first step in developing it as a biocontrol. Comparisons of multiple locus sequence data generated from isolates spanning multiple sites in North Carolina support a single clonal strain impacting United States EHS populations. Our results suggest that this biocontrol fungus merits further investigation and development as an agent targeting EHS in Christmas tree farms and surrounding hemlock forests.

1: Introduction

In the summer of 2020, the entomopathogenic fungus *Conoideocrella luteorostrata* was found causing a natural epizootic in elongate hemlock scales (EHS) in surveys of Christmas tree farms in Virginia and North Carolina. EHS (*Fiorinia externa)* were first documented in the

United States in 1908, in New York State (Sasscer, 1912), but they have spread throughout the northeastern states. Despite their name, their hosts include spruce, fir, and pine trees, in addition to eastern hemlock (*Tsuga canadensis*) (McClure & Fergione, 1977). The damage inflicted by these insect pests on tree farms and in wild stands is difficult to quantify, but the spread of this insect along with another non-native pest, hemlock woolly adelgid, has been associated with defoliation and decline of hemlocks in the United States (Royle & Lathrop, 2002). These host species are also significant to the Christmas tree production industry, where scale infection can decrease the ability of farmers to transport and sell their products across state lines (McClure & Fergione, 1977), since the transport of Christmas trees is a significant vector for this insect (Dale et al., 2020). The range of these insects was originally limited by their cold sensitivity, but adaptations that increased cold tolerance emerged in the 1970's which allowed for rapid northward expansion (Preisser et al., 2008)**.**

Chemical control efforts have limited efficacy in controlling this pest. Due to the high fertility of the insects and the vulnerability of their natural predators to chemical treatments, the population can rebound to even higher levels after treatment (McClure, 1977). Fungal biocontrol agents have several characteristics that could make them especially valuable for the management of these scales. Since they have natural redundancy in their infection process, scales are unlikely to evolve resistance to fungal pathogens (Khan et al., 2012). They are also often highly host-specific, so they may have fewer non-target effects than chemical pesticides (McClure, 1977). The fungus *Colletotrichum fioriniae* has been proposed as a biological control agent of EHS on eastern hemlock (Marcelino et al., 2009), but further research showed that it is primarily a leaf endophyte (Martin & Peter, 2021). It also has been reported as a post-harvest pathogen of apples (Chechi et al., 2019), other fruits (Ling et al., 2021; Ivic et al., 2013), and native plants (Kasson et al., 2014). Another putative fungal pathogen of elongate hemlock scale, *Metarhiziopsis microspora*, has also been described from the United States by Li and colleagues (2008), but its pathogenicity, ecology, and non-target effects have not been further

characterized. If a fungal biocontrol agent can be developed for elongate hemlock scale, it would be a valuable addition to an integrated pest management plan for Christmas tree growers.

Since 2019, the fungus *Conoideocrella luteorostrata,* has been independently confirmed from EHS infested Fraser fir (*Abies fraseri*) Christmas trees originating from Michigan, North Carolina, and Virginia, United States of America (Urbina & Ahmed, 2022; this study). The entomopathogenic fungus*,* previously known as *Torrubiella luteorostrata* and *Paecilomyces cinnamomeus*, has also been reported from Japan, Thailand, Indonesia, Cuba, Ghana and Russia (Gavrilov-Zimin & Borisov, 2020; Hywel-Jones, 1993; Luangsa-ard et al., 2008; Petch, 1923; Saito et al., 2012; Samson, 1974, p. 62) and is presumed native to Thailand based on the historic occurrence of *C. luteorostrata* and other *Conoideocrella* species (Luangsa-ard et al., 2008). The genus contains three formally described species: *Conoideocrella tenuis*, *Conoideocrella krungchingensis*, and *Conoideocrella luteorostrata* (Schoch et al., 2020)*. C. luteorostrata* is commonly found infecting multiple species in the hemipteran suborder Sternorrhyncha, including armored scale insects and whiteflies (Saito et al., 2012). When it infects, it forms characteristic orange-colored mats of mycelium on the surface of the infected insect and dense hyphal growth within the body.

The objective of this work is to morphologically and molecularly characterize the recently discovered United States population of *C. luteorostrata* and investigate its potential as a biocontrol agent of elongate hemlock scales. Though numerous surveys around the globe have confirmed the presence of *C. luteorostrata* on various hemipteran hosts, formal investigation of pathogenicity under lab conditions has not been pursued.

Methods

Surveying for fungal pathogens of elongate hemlock scale

Fungal surveys were conducted at three sites in Ashe County, North Carolina (Vannoy Farm, Upper Mountain, and Deal Family Farm) and two sites in Greyson County, Virginia (Mount Rogers Orchard, and Mount Rogers) on August 31 and September 1, 2020. A fungal epizootic was found in the crawler stage of elongate hemlock scale insects (EHS) on Fraser fir at all three North Carolina sites but not the Virginia sites, despite the presence of EHS in Mount Rogers Orchard. One infection was also observed in an adult female at Vannoy Farm, North Carolina. Affected insects were identifiable by the distinct orange mat of hyphae covering the cadavers, and were abundant, with multiple infected cadavers on each affected needle (Figure 1B: inset). Healthy branches were collected from all sites, branches with uninfected insects from the four EHS positive sites, and branches with mycosed insects from the three North Carolina sites. Insects found among branches were also preserved by site to allow for subsequent examination.

Collection and isolation of *Conoideocrella luteorostrata*

The fungus *Conoideocrella luteorostrata* was cultured from the insects by removing the infected nymph cadavers from the needle with a sterile point and placing them on a potato dextrose agar (PDA) plate supplemented with 0.1 g/L streptomycin and 0.01 g/L tetracycline (+ST) to prevent bacterial contaminants. Cultures were prepared from each sample that had infected scales.

Cultures with morphological characteristics consistent with *C. luteorostrata,* including a striking red metabolite secreted by the fungus and hyaline, smooth-walled, aseptate conidia with acute ends (Johnson et al. 2009), were retained for further morphological and molecular investigation (Figure 2).

Morphological studies of *C. luteorostrata*

A total of 13 strains of *Conoideocrella luteorostrata* recovered from mycosed EHS spanning three locations in North Carolina, United States of America were assessed. Studies included conidial measurements, colony growth rates, appressoria production, and blastospore production.

Slides for measuring spores were prepared from two-week-old cultures. Slides were prepared with lactophenol cotton blue, hematoxylin, or lactic acid to assess the impact of different dyes on spore measurements. Slides were sealed with clear nail polish and stored for several weeks before measurements were conducted. Additionally, slides from several different strains were also prepared with lactic acid to assess any differences among strains.

The growth rate was measured by using a sterile 1.78 cm diameter cork-borer to cut evenly sized colonized agar plugs from a two-week-old fungal culture. These cores were plated mycelium side down on PDA. The plates were wrapped in foil so they would be consistently dark. Ten plates each were placed in 10˚C, 22˚C, or 30˚C environments. The perimeter of the colony was marked at four equidistant points at 2 pm every two days. After two weeks, the distance between each point and the perimeter of the original plug was measured and recorded. Average daily growth rate was calculated as half of two-daily growth observations during linear growth (i.e., during days 4-14). Plates with satellite colonies or abnormal colony growth (e.g., outside of a circular perimeter) were excluded from the study.

Slides for observing appressoria and conidium development were prepared by mixing conidia into solution with cooling PDA, then placing a small volume onto a sterile slide (~5 microliters). To allow observation of appressoria upon the glass surface, a sterile glass coverslip was placed above before the PDA had completely cooled. This slide was incubated in a sterile petri dish for three days: until fungus was observed growing out onto the coverslip. The fungus was grown in liquid culture shaken at 150 rpm for seven days in potato dextrose broth in an attempt to trigger blastospore production.

DNA extraction, PCR, sequencing, and phylogenetic studies of of *Conoideocrella* spp.

DNA was extracted using the protocol previously described by Macias et al. (2020). Fungal tissue (mycelium and conidia) were aseptically removed from 1-2 week old active cultures, added to 1.5 ml microcentrifuge tube with 600 µL of Nuclei Lysis Solution (Promega, Madison, Wisconsin) and macerated with sterile micropestiles. These samples were incubated in a 65˚C water bath for 30 min, vortexing for 5 min at the 15- and 30-minute timepoints. 200 µL of Protein Precipitation Solution (Promega) was added and mixed in by vortexing for 10 sec once sampled were at room temperature. The solution was centrifuged for 3 min at 14,000 rpm, and the supernatant was collected and added to new microcentrifuge tubes containing 600 µL of 99.9% isopropanol, while the protein pellet was discarded. Samples with isopropanol were gently mixed by pippetting and centrifuged again for 1 min. Supernatant was discarded. 600 µL of 70% ethanol were added to the DNA pellet remaining in each tube and mixed by centrifuging for 1 min. Finally, this supernatant was discarded and the tube with the DNA pellet was inverted for 30 minutes to air dry. DNA was resuspended in 100 µL of the Elution Buffer (Alfa Aesar, Ward Hill, Massachusetts) which had been warmed to 65˚C and the extracted DNA was stored at −20˚C.

We targeted sequencing of the universal fungal barcoding gene, the ribosomal internal transcribed spacer region (ITS), which includes ITS1, 5.8S, and ITS2, D1–D2 domains of nuclear 28S rRNA gene (28S), and elongation factor 1-alpha (EF1-α), for each sample listed in Table 1. We also used existing data from NCBI GenBank for 13 reference strains. Primer names, sequences, and annealing temperatures are listed in Supplemental Table 1. PCR components and post-PCR clean-up are as described in Macias et al. 2020. The amplified purified products were sequenced using Sanger sequencing by Eurofins Genomics (Huntsville, AL, United States of America) using the same primer pairs.

The chromatograms were manually inspected, clipped and corrected. For the phylogenetic analysis, sequences were compared across three species that comprise the

genus: *Conoideocrella tenuis*, *Conoideocrella krungchingensis*, and *Conoideocrella luteorostrata*. We included sequences of multiple *C. luteorostrata* isolates from Japan and Thailand in an effort to determine the geographic origin of our samples (Table 1). Quality-checked novel sequences and available comparative sequences (downloaded from GenBank) were aligned in MEGA11 using the default alignment options for non-protein-coding sequences (Tamura et al., 2021) to check their compatibility, trim the ends, and look for unspecified loci. Next, sequences were unaligned and uploaded to the Guidence2 server, masking residues below a 0.5 confidence level (Sela et al., 2015). Residues below a 0.5 confidence were masked. Indels on the ends of this alignment were masked in MEGA11, and the best models were selected by the Akaike information criterion corrected (Alcc) score. The determined optimal models were HKY for ITS and GTR for all other trees. Single-gene alignments were created for ITS, LSU, and EF-1 alpha, and then concatenated into a combined alignment for tree building. Maximum Likelihood (ML) trees were created using RAxML version 8 (Stamatakis, 2014) with 1000 bootstraps. Bayesian Inference (BI) trees were created in Mr. Bayes 3.2 (Ronquist et al., 2012), using 1 million generations. Finally, the ML tree was annotated with BI support values where topology was in agreement (Figure 3).

Culturable mycobiome of fir needles

The culturable fungal mycobiomes of the fir needles were studied from needles from each sample, including needles from all three groups (i.e., no EHS, EHS only, and EHS infected with fungus). These fungi were isolated following surface sterilization. Needles were sterilized in 10% sodium hypochlorite for 20 seconds, placed on paper towels to dry for one minute, and then plated onto PDA+ST. Cultures were isolated according to their morphology and inhibitory interactions until all unique culturable fungi were isolated. These fungi were sorted into groups by morphology and selected representative strains were identified by genetic sequencing of the internal transcribed spacer (ITS) region, as seen in Supplemental Table 2.

Superficial survey for fungal infections in co-occurring arthropods

Non-sessile arthropods (i.e., insects, arachnids, and collembolans) found in EHS infested and EHS free Fraser fir branch materials were collected, examined for conspicuous fungal infections, and stored in ethanol for subsequent morphological identification. A higher level (i.e., class, subclass, or order) and, where possible, a lower level (down to species) identification was provided for arthropods, as seen in Supplemental Table 3.

Elongate hemlock scale pathogenicity assays

Branches with elongate hemlock scale crawlers were collected from hemlock trees in Monongahela County, West Virginia. Infested branchlets were mixed to randomize and distributed evenly into three replicates of two treatments: dipped into 10^7 *C. luteorostrata* conidia in water or dipped into water with no spores. Treatments were done for 10 seconds, ensuring all parts of the branch were submerged in inoculum. Branchlets were held at 31˚C in square petri dishes in separate plastic containers. Damp paper towels were provided in containers to prevent needles from drying out. These treatments were monitored daily for 13 days, except on day 9, to assess emergence and mortality. The results of this bioassay were subjected to Kaplan-Meier log-rank survival analysis to test for significant differences.

To fulfill Koch's postulates, a refined infection protocol was used. Here, the branches were stored at 31˚C in a sealed container with a damp paper towel. Once sufficient crawlers had emerged after 2-4 days, infection assays were set. Fresh branches without EHS were prepared by dipping in a solution of 10^7 *C. luteorostrata* spores suspended in water. Then 15 emerged crawlers for each of the four petri plates of branches were gently moved to these branches using a small, sterile paintbrush. Cadavers from the bioassay were removed 14 days later, surface sterilized with 10% sodium hypochlorite for 10 seconds, and plated on PDA. The resulting isolates were identified through spore and colony morphology and sequencing of the

ITS region. All data and code for analyses included in this manuscript are available on GitHub: https://github.com/HanaBarrett/EHS-CL-Analysis.

Results

C. luteorostrata isolation and molecular identification

Elongate hemlock scale (EHS) was present in all sites, except Mount Rogers. A fungal epizootic was found in the crawler stage of EHS on Fraser fir in Ashe County, North Carolina at all sites. No mycosed EHS were present in Mount Rogers Orchard despite recovering EHS from this location. At the Vannoy Farm site in North Carolina, *C. luteorostrata* was observed and isolated from one adult female. Infected insects were identifiable by the distinct orange mat of hyphae covering the cadavers, and were abundant, with multiple infected cadavers on each affected needle (Figure 1B: inset).

NCBI GenBank pairwise blastn results of LSU sequences between *C. luteorostrata* strains from Urbina & Ahmed (2022) including FDACS-DPI 2019–102372 from North Carolina, U.S.A. (MT796334) and FDACS-DPI 2020-105941 from Virginia, U.S.A. (MW419877) and 7 North Carolina, U.S isolates from this study were 100% identical. Pairwise blastn results of ITS sequences between *C. luteorostrata* strains FDACS-DPI 2019–102372 from NC (MT796335), FDACS-DPI 2020-105882 (MW419875), and FDACS-DPI 2020-105940 from NC (MW419876) and 7 NC isolates from this study were 99.62 – 99.81% identical. Urbina & Ahmed (2022) deposited a partial EF1-α sequence (MT796336) for strain FDACS-DPI 2019–102372 from North Carolina, U.S.A., of which 100/695 bp were represented by Ns (N= any of A,C,G,T). However, a 220 bp partial sequence was 99.09 – 100% identical to 7 North Carolina, U.S.A. isolates from this study. However due to the reduced length, sequences deposited by Urbina & Ahmed (2022) were excluded from phylogenetic analysis.

Phylogenetic analysis of *Conoideocrella* spp.

To examine phylogenetic relationships among *Conoideocrella* spp., phylogenetic analyses were conducted for single-gene and combined datasets using two methods of phylogenetic inference, maximum likelihood (ML) analysis and Bayesian inference (BI) analysis, for a total of eight analyses. The three individual gene trees (EF1-α, 28S, and ITS), as well as the concatenated 3-gene tree (EF1-α + 28S + ITS), resolved *C. luteorostrata* as a well-supported (>90% bootstrap support) monophyletic lineage encompassing all our United States isolates (FIG. 2). The EF1-α and concatenated 3-gene tree both showed additional resolution with two well-supported (>70% bootstrap support) clades within *C. luteorostrata*: one containing all United States and Japanese strains from armored scales and whiteflies and a second containing two strains from unknown hosts in Thailand. Sequence data for all United States *C. luteorostrata* strains were identical except for strain VF11, which contained a single SNP in the EF1-α region. The lack of genetic variation between multiple isolates combined with the complete absence of perithecia recovered from mycosed EHS cadavers suggests that sexual reproduction is not present and the population is likely clonal. Since we sampled several sites in several counties, and *C. luteorostrata* has not been previously reported in the United States, this likely represents an introduced species. Our concatenated 3-gene tree reflects expected species relationships (Johnson et al., 2009), with the four included *C. luteorostrata* isolates from Thailand at the base of the clade for this species.

Conoideocrella krungchingensis and *C. tenuis* were also shown to be strongly supported (>90% bootstrap support except for combined tree support for *C. tenuis* = 69%*)* genealogically exclusive lineages in all four analyses with one exception (FIG. 2). In the 28S *tree, C. tenuis* formed a single clade with one outgroup taxon, *T. petchii* NHJ 6240, but the remainder of *Conoideocrella* was left monophyletic.

Fungal morphology

Young cultures of *Conoideocrella luteorostrata* have a distinct yellow color. The fungus grows optimally around 22°C, with an average radius increase of 0.95 mm/day on PDA media, but does not actively grow on artificial media at 10°C or 30°C, though the fungus can resume normal growth if returned to 22°C after 7 days exposure to both of these temperatures. After about a week of growth the fungus begins to secrete a red metabolite from the older growth (Figure 2B) and gradually develops a red surface. After several weeks the surface is covered with green conidia (Figure 2C). Ovular appressorial growth was observed along the underside of a glass coverslip after 10 days. Blastospores were not observed in liquid culture over time.

For *C. luteorostrata,* we observed whorled conidiophores (Figure 2D) with hyaline, smooth-walled, aseptate conidia with acute ends (Figure 2E). No significant differences were observed in spore width on lactophenol cotton blue, hematoxylin, or lactic acid stained slides, or between spores widths from cultures isolated from different sites (Figure 4). Spore length revealed minimal differences between stains, with spores from strain VF11 stained with lactophenol cotton blue being slightly smaller than other stains: 4.87 micrometers versus 5.57 in lactic acid and 5.81 in hematoxylin (Figure 4). Though not significantly different, the width of this strain in lactophenol cotton blue was measured to be the smallest at 1.46 micrometers. Among the four strains we measured in lactic acid, two lengths were significantly different (5.97 micrometers for BAF11 and 5.57 micrometers for VF11), but the measured length of the two other strains were indistinguishable from other strains. Overall, our results suggest that there is measurable variation in conidial length on artificial medium among our isolates, but these are practically indistinguishable (i.e., two strains spanned significant differences between two other strains). In lactic acid, we estimate a mean conidial length of 5.75 micrometers and a mean conidial width of 1.60 micrometers.

Surveying for *C. luteorostrata* within Fraser fir needles

Other EHS entomopathogens are known to also be leaf endophytes, so we sought to determine if *C. luteorostrata* could be re-isolated from needles as well. We isolated the culturable mycobiome of the needles from samples of Fraser fir from each site and sample type (i.e., EHS present/absent and *C. luteorostrata* present/absent). These isolates included many commonly observed environmental and needle-dwelling fungi, with isolates from the genera *Pestalotiopsis*, *Trichoderma*, and *Nigrospora* (Baird et al., 2009). *C. luteorostrata* has a distinct colony morphology in culture, which was not observed, even though it is readily culturable on this isolation medium (PDA+ST) (Supplemental Table 2).

Laboratory pathogenicity bioassays

In our first bioassay, we treated infested needles, allowing crawlers to emerge over the course of our bioassay. Elongate hemlock scales are known to have continuous emergence across the season, and this was observed in our laboratory bioassays. Across our entire two-week bioassay, we found crawlers continuously emerged. This presented challenges for counting moving crawlers as numbers rose above ~20 individuals, and it also resulted in continuous exposure to *C. luteorostrata*: some crawlers were initially infected on day 0, while others were infected on day 13 (Figure 5A). This continuous emergence resulted in percent survival increasing on some days (Figure 5B). We recorded crawler vitality daily for both treatments and formatted this data for Kaplan-Meier survival analysis. We found a significant difference ($p=0.03$) between *C. luteorostrata* treated EHS and controls using a log-rank test. Our survival curve (Figure 5B) documenting daily percent survival in this bioassay revealed lower survival in *C. luteorostrata* treatment on days 10-13, but survival in the treatment never fell below 50% in these bioassays.

In our second bioassay, crawlers were introduced onto treated branchlets and cadavers were collected two weeks after exposure. Figure 6 shows an asymptomatic crawler (Figure 6A) a treated crawler exhibiting melanization (Figure 6B), which is expected during infection and a

cadaver with visible fungal outgrowth (Figure 6C) *C. luteorostrata* was isolated from three of five surface-sterilized crawler cadavers and identified through colony morphology, spore morphology, and genetic sequencing of the ITS region. *C. luteorostrata* was not isolated from any of the three surface-sterilized crawlers in the control group.

Superficial arthropod survey

In this survey, we collected all non-sessile arthropods that we encountered among our samples. No fungal infections were observed in these arthropods. We documented 70 arthropods living among these needles (Supplemental Table 3). The most common arthropod encountered was the beetle *Cybocephalus nipponicus*, a known predator of EHS which was collected from Vannoy Farm and Deal Family Farm: both of these sites were not being managed for production during collection and as such both exhibited higher EHS populations. Two parasitoid wasps were also collected, one of the two was observed ovipositing into an EHS adult female prior to collection. We identified these parasitoid wasps as *Encarsia citrina*, a known parasitoid of EHS. EHS-parasitoid wasps were also observed developing within the tests of collected female elongate hemlock scales under microscopic examination. None of these natural enemies of EHS or other arthropods exhibited *C. luteorostrata* mycosis.

Discussion

Our discovery of the naturally occurring epizootic of *Conoideocrella luteorostrata* on elongate hemlock scales is promising since these insects are an economically significant pest on Christmas tree farms and do not currently have a commercially available fungal biocontrol option. A recently published independent survey by Urbina & Ahmed (2022) supports the relatively recent expansion of this fungus across EHS populations impacting Christmas tree production in VA, NC, and MI. We expanded on the current understanding of the U.S. population of *C. luteorostrata* with a mycological investigation that included bioassays using

elongate hemlock scales and a rigorous phylogenetic analysis of multiple strains from across the survey area. We additionally examined the culturable mycobiome of fir needles to confirm whether *C. luteorostrata* is also a needle endophyte, since a previously proposed biocontrol agent for these insects (Marcelino et al., 2009), *Colletotrichum fioriniae*, is now known to be primarily a leaf endophyte (Martin & Peter, 2021). *C. luteorostrata* was not isolated from Fraser fir needles, even though it is readily culturable.

When we commenced our study, *C. luteorostrata* had not been reported in the United States. Concurrently, this pathogen was identified during plant pest screening as causing epizootics in elongate hemlock scale across multiple states: Michigan (Urbina & Ahmed, 2022), Virginia and North Carolina. The identification of this pathogen in this entomological survey and our own suggests that targeted sampling of elongate hemlock scale may expand the range of this pathogen in the United States. The morphology we observed was consistent with the literature on this species. Our spore measurements are within the range found in Hywel-Jones' 1993 description of *C. luteorostrata*, which was 5-7.5 micrometers in length and 1.5-2.5 in width. The presence of appressorium, which are swollen hyphal tips or differentiated cells used by fungi to breach the plant or insect cuticle (Talbot, 2004), could allow the fungus to infect by penetrating the exoskeleton or outer covering (test) of the insect. The similarity of the sequences of *C. luteorostrata* samples from three different sites suggests that a single clonal strain of *C. luteorostrata* was introduced to the United States. The United States introduction may originate from the natural range of the fungus in Japan since our isolates were genetically more similar to strains from Japan than strains from Thailand.

The arthropods collected included *Encarsia citrina*, the parasitic wasp, and the beetle predator of elongate hemlock scale *Cybocephalus nipponicus*. The beetle has been introduced in various regions of the United States as a biocontrol agent for elongate hemlock scales and other scale insects (Blumenthal et al., 2005). The beetles were particularly abundant on the Deal Family Farm and Vannoy Farm sites, which has not been heavily managed. This may be

due to the pesticide sensitivity of these insects (Smith & Cave, 2006). The arthropods collected in our survey, particularly the natural predators, would be good candidates for a non-target bioassay with *C. luteorostrata*. Further study is needed on whether the fungus could impact these species and whether they may be a vector for fungal disease. This fungus is reported to have natural specificity to armored scale insects (family Diaspididae) and whiteflies (family Aleyrodidae) (Saito et al., 2012), which was supported by the fact that we did not find any evidence of infection in other collected arthropods.

Our bioassays were designed to mimic a spray treatment by Christmas tree growers, but the biology of the scale prevented us from describing the full biocontrol potential of our isolate. In particular, the continuous emergence of individuals clearly diluted the observed scale mortality and presented issues for accurately counting live/dead individuals. Despite significant challenges, our bioassays revealed that surface treatment of needles leads to increased mortality of elongate hemlock scale. We refined our approach in our second bioassay where we gently introduced crawlers into fungus-treated, healthy hemlock needles. Koch's postulates were confirmed with this approach since, following conidial treatment, we were able to re-isolate this pathogen from the interior (i.e., following surface sterilization) of infected crawlers. This result, coupled with the fact that it was found to cause a natural epizootic, shows that *C. luteorostrata* can effectively infect elongate hemlock scales in the field and under lab conditions. Our laboratory bioassays and field observations suggest that *C. luteorostrata* is a promising biocontrol agent against the mobile crawler stage. Due to the specialized lifecycle of this insect, it may be necessary to perform future bioassays directly on living branches instead of in a laboratory setup requiring multiple considerations and caveats.

Since *C. luteorostrata* can maintain itself in the environment over long periods of time, both on a farm and in nearby wild trees, it can provide a source of passive control. In this way, a fungal biocontrol agent causing natural epizootics can provide long-term control. The fungus and insect predators can work in concert to keep elongate hemlock scale populations at a stable

low level and suppress outbreaks. Since the crawler stage is most commonly infected, this fungus has the potential to significantly reduce the spread of these insects between trees. Our study and a recent report of this fungus in multiple states (Urbina & Ahmed, 2022) suggest that *C. luteorostrata* is already naturally controlling elongate hemlock scale populations in Christmas tree operations in the United States: intentional application of this fungus should broaden its efficacy.

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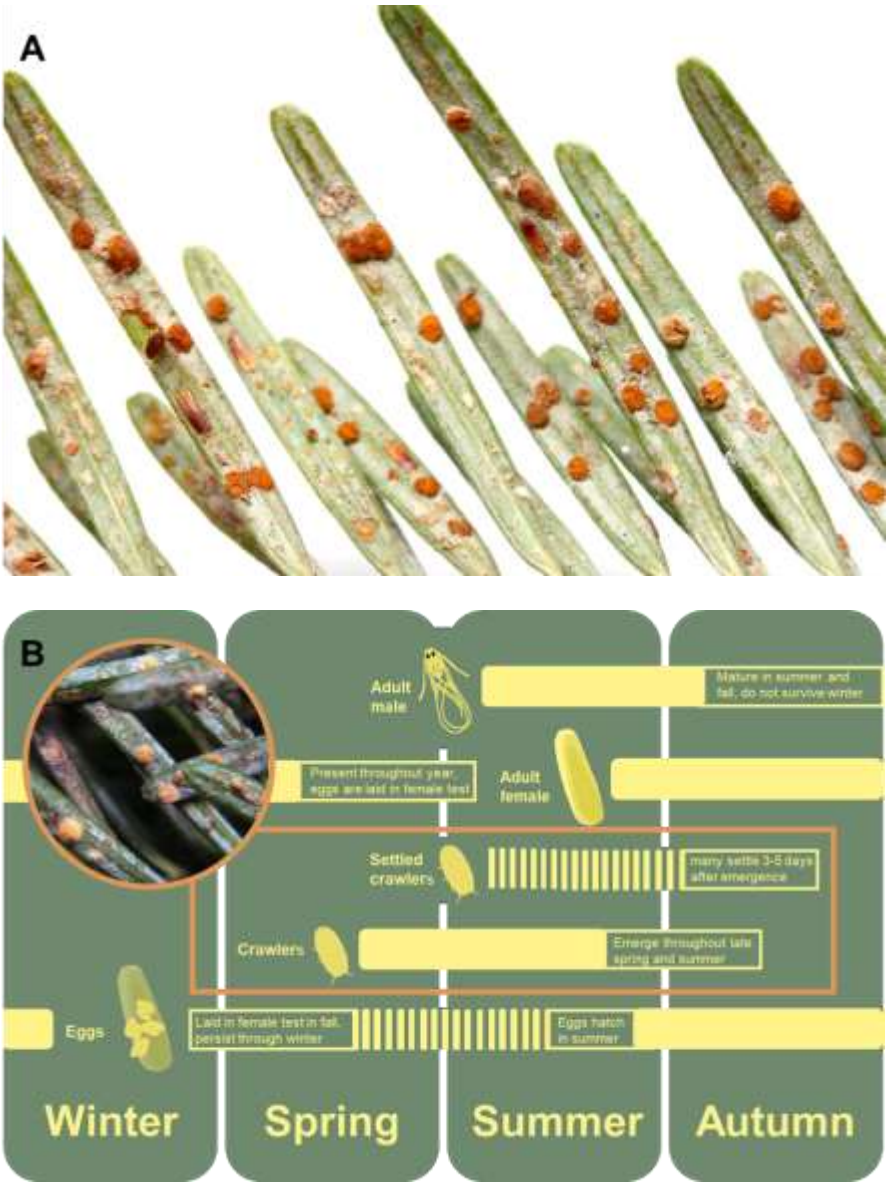

Figure 1. Natural infections of *C. luteorostrata with characteristic* orange stroma produced over elongate hemlock scale crawler cadavers on the underside of Fraser fir needles in North Carolina (A). Photo credit to Dr. Matt Bertone. Diagram representing the lifecycle of elongate hemlock scale on Fraser fir (B) with inset showing life stages most impacted by *C. luteorostrata* infection. Crawlers hatch throughout the spring and summer. Most settle 3-4 days after emergence. Once settled, they secrete a waxy coating that protects them as they mature. Males emerge from their coating throughout the summer and fall, while females remain protected by their waxy covering, called a test. Eggs are laid under the female test, where they remain

through the winter. Life cycle layout based on hemlock wooly adelgid life cycle from Limbu et al. 2018.

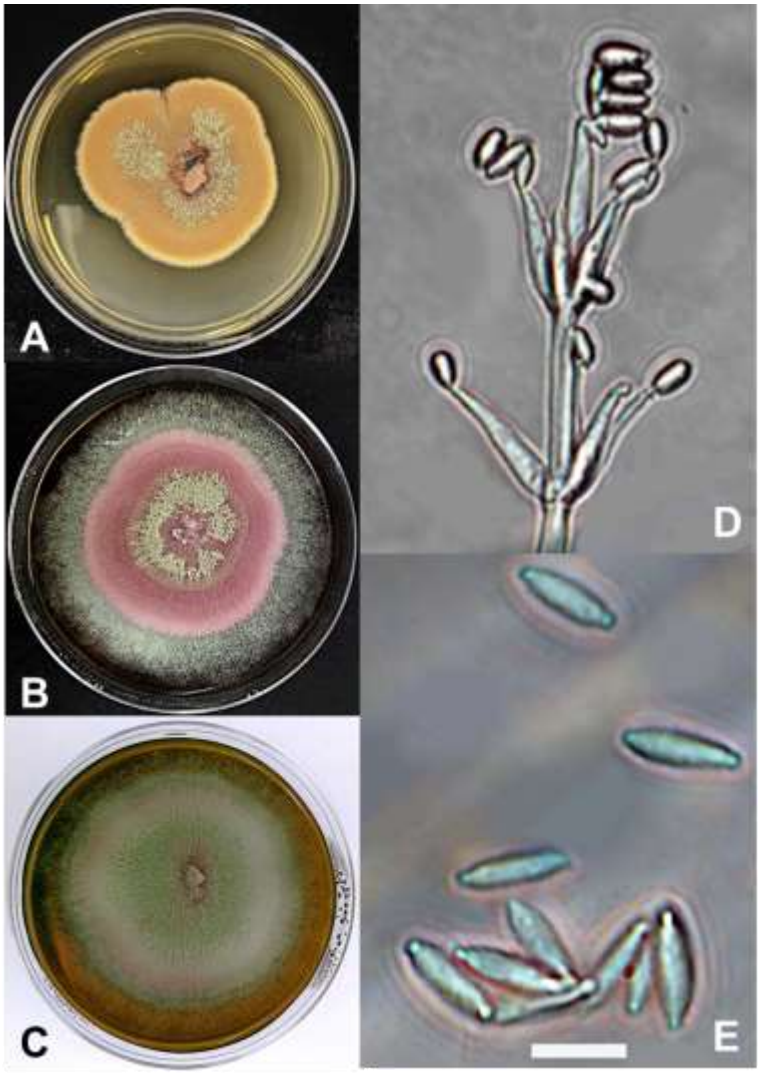

Figure 2. Representative cultures of *C. luteorostrata* on PDA+ST at 1-2 weeks (A), 3-4 weeks (B), and 5+ weeks of age (C). *C. luteorostrata* conidiophore with phialides (D) and representative conidia (D-E; 1000x magnification). Scale bars = 5 µm for panel E.

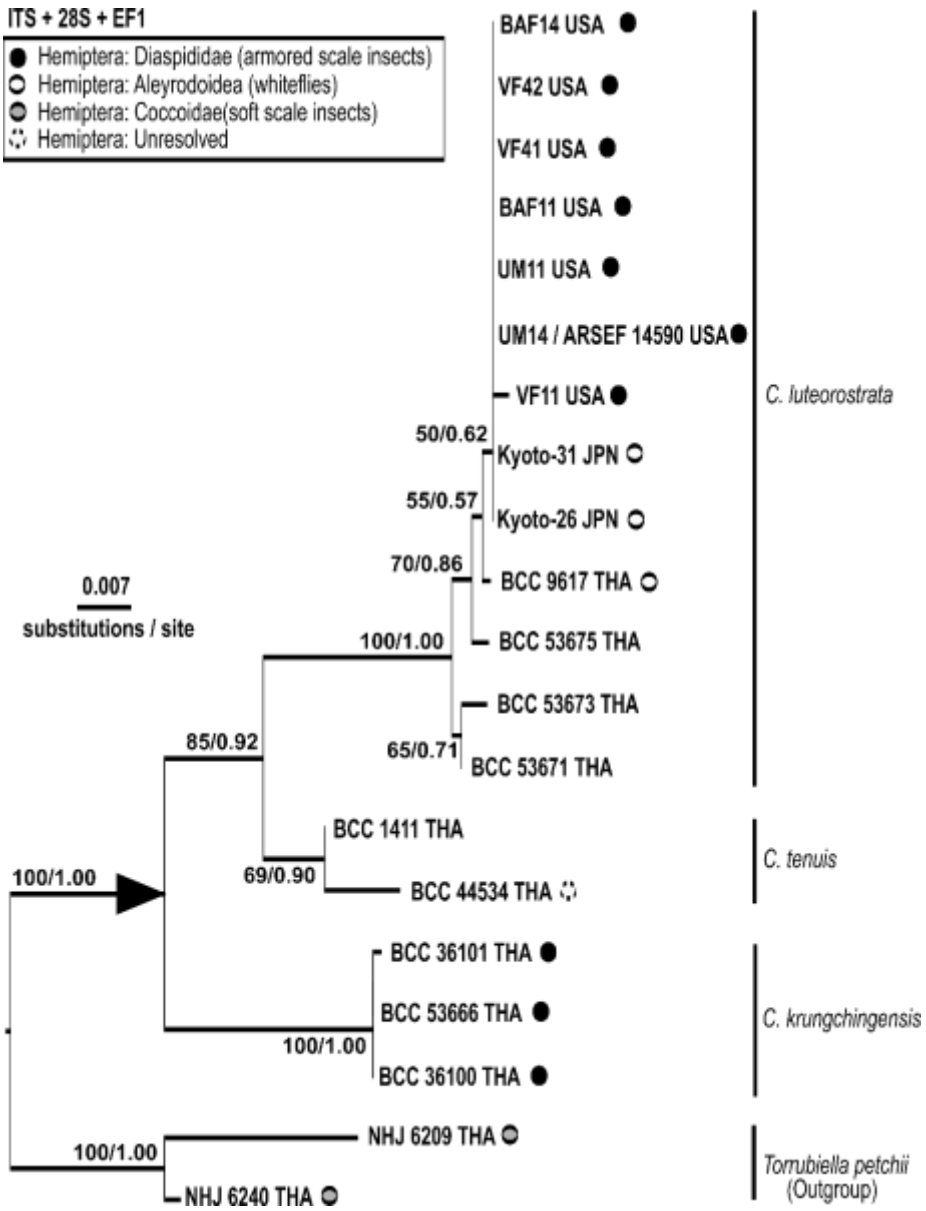

Figure 3. Phylogenetic tree of *Conoideocrella* spp. based on a concatenated (EF1-α + 28S + ITS) dataset. Tree topology and branch lengths shown are from the ML analysis. Bootstrap support and posterior probabilities are indicated for each node supported in the ML analysis (ML/BI). Arrow indicates the most recent common ancestor of the genus *Conoideocrella*. Country of origin and insect host associations are denoted for all taxa where data are available. Strain metadata including GenBank accession numbers are listed in Table 1.

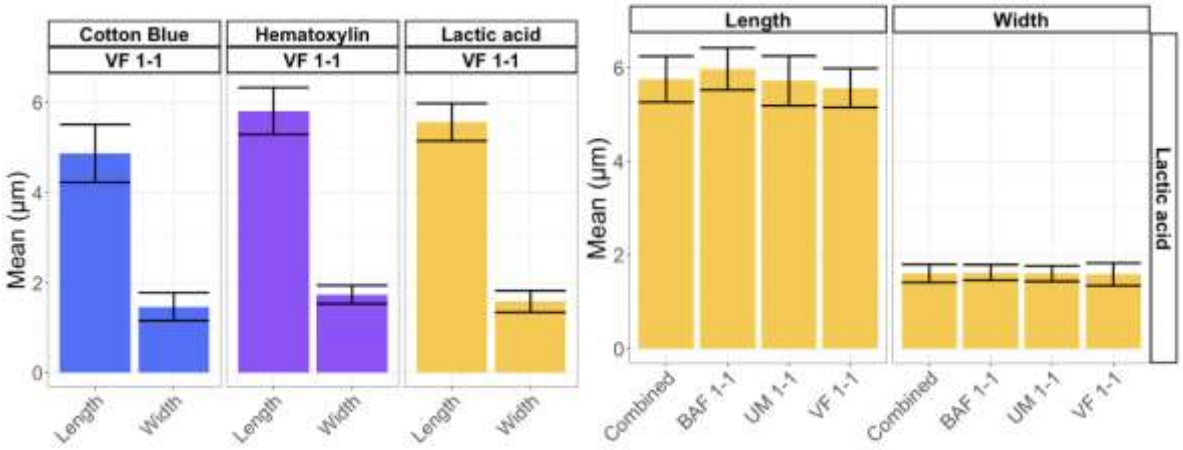

Figure 4. Mean spore measurements compared across multiple stains / mountants (lactophenol cotton blue, hematoxylin, and lactic acid) and representative *C. luteorostrata* strains (BAF11, UM11, and VF11) in lactic acid.

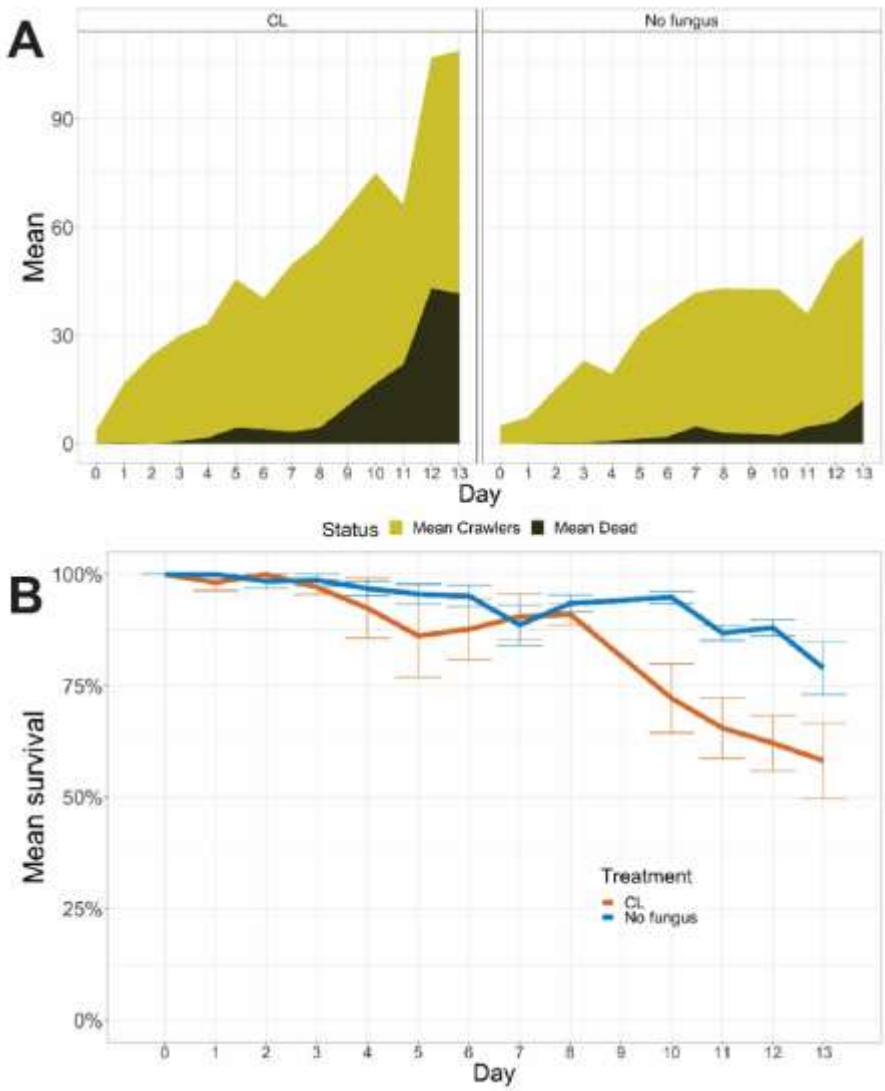

Figure 5. Crawler emergence by treatment over the course of our bioassay (A). Mean total crawlers (across 3 replicates) are represented by the yellow area whereas mean dead crawlers are represented in black. B) Calculated percent survival for each treatment (B). CL denotes EHS were exposed to *C. luteorostrata.*

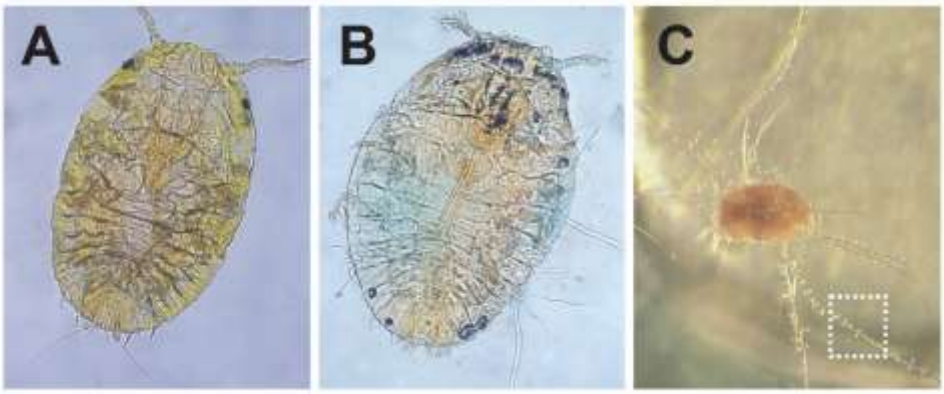

Figure 6. Asymptomatic EHS crawler from control treatment at 40x magnification (A) compared to crawler from *C. luteorostrata* treatment (40x) exhibiting melanization, which was associated with infection (B). Dead mycosed crawler with *C. luteorostrata* outgrowth under dissecting microscope (C). Box in C highlights whirled conidiophore characteristic of *C. luteorostrata*.

T**able 1:** Isolates used in phylogenetic and morphological studies and their associated metadata. Additional sequence data for included strains as well as other strains not used in this study are available in Supplemental Table 4. Collection abbreviations and countries of origin are as follows: [1] United States Department of Agriculture, ARS Emerging Pests and Pathogens Research Unit [2]Forestry and Forest Products Research Institute, Tsukuba, Ibaraki, Japan. [3]United States of America. [4]Japan. [5]Thailand.

Genus	Species	Strain	Collection	Location	Host	Source	ITS	28s rRNA	EF1-alpha
Conoideocrella	*luteorostrata*	BAF14	-	USA[3]	*Fiorinia externa*	This study	ON081996	ON082758	ON249021
Conoideocrella	*luteorostrata*	VF42	-	USA	*Fiorinia externa*	This study	ON081999	ON082759	ON249026
Conoideocrella	*luteorostrata*	VF41	-	USA	*Fiorinia externa*	This study	ON081998	ON082757	ON249025
Conoideocrella	*luteorostrata*	BAF11	-	USA	*Fiorinia externa*	This study	ON081995	ON082753	ON249020
Conoideocrella	*luteorostrata*	UM11	-	USA	*Fiorinia externa*	This study	ON081993	ON082754	ON249022
Conoideocrella	*luteorostrata*	UM14	ARSEF[1] 14590	USA	*Fiorinia externa*	This study	ON081994	ON082755	ON249023
Conoideocrella	*luteorostrata*	VF11	-	USA	*Fiorinia externa*	This study	ON081997	ON082756	ON249024
Conoideocrella	*luteorostrata*	Kyoto-31	FFPR[2]	JPN[4]	Hemiptera: Aleyrodidae	Saito et al. 2012	AB649299	-	AB663121
Conoideocrella	*luteorostrata*	Kyoto-26	FFPR	JPN	Hemiptera: Aleyrodidae	Saito et al. 2012	AB649298	-	AB663120
Conoideocrella	*luteorostrata*	BCC 9617	NHJ11343.01	THA[5]	Hemiptera: Aleyrodidae	Isaka et al. 2007	-	KJ435068	KJ435086
Conoideocrella	*luteorostrata*	BCC 53675	BBH 33685	THA	Unknown	Tasanathai et al. unpublished	-	KJ435075	KJ435091
Conoideocrella	*luteorostrata*	BCC 53673	BBH 32355	THA	Unknown	Tasanathai et al. unpublished	-	KJ435074	KJ435090
Conoideocrella	*luteorostrata*	BCC 53671	BBH 32353	THA	Unknown	Tasanathai et al. unpublished	-	KJ435072	KJ435088
Conoideocrella	*tenuis*	BCC 1411	NHJ00345.01	THA	Unknown	Tasanathai et al. unpublished	-	KJ435079	KJ435093
Conoideocrella	*tenuis*	BCC 44534	BCC 44534	THA	Hemiptera: unresolved	Saepua et al. 2015	MG230251	MG198773	MG230541
Conoideocrella	*krungchingensis*	BCC 36101	BBH27004	THA	Hemiptera: Diaspididae	Mongkolsamrit et al. 2016	-	KJ435081	KJ435098
Conoideocrella	*krungchingensis*	BCC 53666	BBH32349	THA	Hemiptera: Diaspididae	Mongkolsamrit et. al. 2016	-	KJ435070	KJ435099
Conoideocrella	*krungchingensis*	BCC 36100	BBH27004	THA	Hemiptera: Diaspididae	Mongkolsamrit et. al. 2016	-	KJ435080	KJ435097
Torrubiella	*petchii*	NHJ 6209	-	THA	Hemiptera: Coccoidea	Johnson et al. 2009	JN049861	EU369039	EU369023
Torrubiella	*petchii*	NHJ 6240	-	THA	Hemiptera: Coccoidea	Johnson et. al. 2009	-	EU369038	EU369022

Supplemental information:

Supplemental tables and code are available at https://github.com/HanaBarrett/EHS-CL-Analysis.

Supplemental Table 1: Primer sequences, PCR parameters, and sources for each primer pair used to generate sequence data

Supplemental Table 2: Raw data for mycobiome morphogroups from Fraser fir needle sterilization experiment

Supplemental Table 3: Counts and identifications for arthropods from Fraser fir needle surveys

Supplemental Table 4: Expanded strain table and associated metadata