Characterization of the Entomopathogenic Fungal Species Conoideocrella luteorostrata on the Scale Insect Pest Fiorinia externa Infesting the Christmas Tree Abies fraseri in the USA

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Characterization of the entomopathogenic fungal species *Conoideocrella luteorostrata* on the scale insect pest *Fiorinia externa* infesting the Christmas tree *Abies fraseri* in the USA

Hector Urbina[1], and Muhammad Z. Ahmed[2,]*

Abstract

We characterized the entomopathogenic fungal species, *Conoideocrella luteorostrata* (Zimm.) D. Johnson, G.H. Sung, Hywel-Jones & Spatafora (Hypocreales: Clavicipitaceae), on the elongate hemlock scale, *Fiorinia externa* Ferris (Hemiptera: Diaspididae), infesting Fraser fir Christmas tree, *Abies fraseri* (Pursh) Poir (Pinaceae). Fraser fir Christmas trees that were cultivated in Michigan, North Carolina, and Virginia were intercepted in Florida during plant inspection. This study is based on the isolation in pure culture, and morphological and molecular characterization using a 4-locus (ITS, LSU, SSU, *tef1*) and represents the first record of *C. luteorostrata* on *F. externa*. In addition, we reviewed all previously reported natural enemies of *F. externa* in the USA, discussed their potential as biological control agents, and concluded the need to explore a new natural enemy of *F. externa*. We recommend using *C. luteorostrata* as a biocontrol agent for *F. externa*. We also suggest that our isolate could be a source of new uncharacterized active compounds and could be used in the biological control of whiteflies and scale insects, as demonstrated in other *C. luteorostrata* strains. We also discussed the importance of investigating biological control agents in pest and pathogen interception samples.

Key Words: biological control agents; elongate hemlock scale; interception; plant inspection

Resumen

En esta investigación nosotros presentamos la caracterización del hongo entomopatógeno *Conoideocrella luteorostrata* (Zimm.) D. Johnson, G.H. Sung, Hywel-Jones & Spatafora (Hypocreales: Clavicipitaceae) encontrado sobre el insecto escama *Fiorinia externa* Ferris (Hemiptera: Diaspididae) que infestaba árboles de navidad de la especie *Abies fraseri* (Pursh) Poir (Pinaceae) cultivados en Michigan, North Carolina y Virginia, y que fueron interceptados en Florida. Este estudio está basado en la obtención en cultivo puro y el estudio morfológico y molecular usando cuatro genes (ITS, LSU, SSU, *tef1*) y además representa el primer registro de *C. luteorostrata* en *F. externa*. En este artículo se recomienda el uso de *C. luteorostrata* como agente biocontrol de mosca blancas e insectos escamas, que son considerados las pestes de plantas más importantes. Este aislado caracterizado por nosotros representa una nueva fuente de metabolitos secundarios aun por caracterizar, como se ha demostrado en otras cepas de *C. luteorostrata*. También se discute la importancia de la exploración de agentes de control biológico presentes en cargamentos interceptados de plantas.

Palabras Claves: agentes de control biológico; insectos escamas; intercepción; inspección de plantas

The genus *Fiorinia* (Hemiptera: Diaspididae) is comprised of 70 species of armored scale insects, including several exotic invaders of the USA: *Fiorinia externa* Ferris, *Fiorinia phantasma* Cockerell & Robinson, *Fiorinia proboscidaria* Green, and *Fiorinia theae* Green (Miller & Davidson 2005; Ahmed 2018). The elongate hemlock scale, *F. externa*, is native to Asia and feeds on coniferous trees (Ferris 1942). It was found first in the USA in 1908 in the state of New York, and has since dispersed throughout the eastern states (García et al. 2016), where infestations were associated with tree mortality of eastern hemlock, *Tsuga canadensis* (L.) Carrière (Pinaceae).

In addition to its primary host, eastern hemlock, *F. externa* is also a pest of Fraser fir, *Abies fraseri* (Pursh) Poir. (Pinaceae) (Dale et al. 2020). Fraser fir, one of the most common Christmas tree species available in the USA, is grown principally in and distributed from North Carolina (NASS 2017). Trees are grown outdoors for 6 to 10 yr before harvesting (McKinley & Hazel 2019) and are shipped along with many inhabitant organisms, including *F. externa*. Since *F. externa* is not established in Florida, regulatory efforts have been implemented to prevent its introduction from imported cut Fraser fir Christmas trees (Stocks 2016).

There have been reports of several natural enemies, including predators, parasitoids, and entomopathogenic fungi, feeding on or attacking *F. externa* in the last 65 yr (Davidson & McComb 1958; McClure 1977a, b, c, 1978, 1979; Lambdin et al. 2005; Lynch et al. 2006; Mayer et al. 2008; Marcelino et al. 2009a, b; Abell & Driesche 2012). However, only a few have shown potential to be used as biological control agents (Table 1).

For several yr, many Fraser fir Christmas tree shipments were rejected by the Florida Department of Agriculture and Consumer Ser-

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Table 1. Literature review of incidences and potential of natural enemies of *F. externa* in the USA.

Natural enemy	Order: Family	Field report (FR) or tested (T)	Category for potential	Notes	Reference
Predators					
Atractotomus magnicornis (Fallén)	Hemiptera: Miridae	FR	4	It was indicated as nr. *buenoi* Knight in the literature	McClure (1977c, 1979)
Phytocoris sp.	Hemiptera: Miridae	FR	4	NA	McClure (1977c)
Coniopterix sp.	Neuroptera: Coniopterygidae	FR	4	NA	Lambdin et al. (2005), Lynch et al. (2006)
Conwentzia pineticola Enderlein	Neuroptera: Coniopterygidae	FR	4	NA	McClure (1977c), Lynch et al. (2006)
Hemerobius stigma Stephens	Neuroptera: Hemerobiidae	FR	4	NA	Lambdin et al. (2005)
Chilocorus stigma (Say)	Coleoptera: Coccinellidae	FR	4	NA	McClure (1977c, 1979), Lambdin et al. (2005), Lynch et al. (2006)
Chilocorus kuwanae Silvestri	Coleoptera: Coccinellidae	FR	3	Introduced from Asia, successfully established in several parts of the USA. It was cross referenced in Lambdin et al. 2005, Lynch et al. 2006, and Mayer et al. 2008, but no information about its report from the USA in the cited references. Lambdin et al. 2005 found it feeding on scale in the USA	Lambdin et al. (2005)
Cybocephalus nipponicus Endrödy-Younga	Coleoptera: Coccinellidae	FR	3	Imported from China and Korea, reared and mass released in New Jersey, USA	Mayer et al. (2008)
Harmonia axyridis Pallas	Coleoptera: Coccinellidae	FR	4	Appeared as a generalist predator	Lambdin et al. (2005), Lynch et al. (2006)
Rhyzobius lophanthae (Blaisdell)	Coleoptera: Coccinellidae	FR	3	NA	Lambdin et al. (2005), Lynch et al. (2006)
Scymnillus horni (Gordon)	Coleoptera: Coccinellidae	FR	3	NA	Lambdin et al. (2005), Lynch et al. (2006)
Scymnus loweii Mulsant	Coleoptera: Coccinellidae	FR	4	Appeared as a generalist predator	Lynch et al. (2006)
Unidentified lady beetle species	Coleoptera: Coccinellidae	FR	4	NA	Lambdin et al. (2005)
Spiders	Araneida	FR	4	NA	McClure (1978)
Parasitoids					
Aphytis aonidiae (Mercet)	Hymenoptera: Aphelinidae	FR	2	*Density-dependent parasitism up to 30% (only on males), it was indicated as nr. *aonidiae* in the literature	McClure (1978, 1979)
Encarsia citrina (Craw)	Hymenoptera: Aphelinidae	FR, T	1	*Density-dependent parasitism up to 72% (only on females), its population is asynchronous with that of *F. externa*, its synonym, *Aspidiotiphagus citrinus* (Craw), was used in the literature	McClure (1977b, 1978), Lambdin et al. (2005), Abell & Driesche (2012)
Prospaltella sp.	Hymenoptera: Aphelinidae	FR	2	Parasitism may be as high as 16.6%, it might be a misidentification of *E. citrina*	Davidson & McComb (1958)
Entomopathogenic fungus					
Beauveria bassiana (Bals.-Criv.) Vuill.	Hypocreales: Cordycipitaceae	FR	4	Reported entomopathogenicity to control *F. externa* in the field but was not tested	Marcelino et al. (2009a)
Botrytis sp.	Helotiales: Sclerotiniaceae	FR	4	Reported as a phytopathogen	Marcelino et al. (2009a)

Category 1: Reported to be the most effective to control *F. externa*, but have hurdles to implement in the field; Category 2: Reported to be effective, but not enough; Category 3: Reported feeding on or causing mortality to *F. externa*, but their effectiveness is still not confirmed; Category 4: Reported in *F. externa* infested area, no noticeable reduction in *F. externa* population was linked with them. *Density-dependent parasitism can cause a decrease in parasitism rate and equilibrium in host density (Ives 1992).

Table 1. (Continued) Literature review of incidences and potential of natural enemies of *F. externa* in the USA.

Natural enemy	Order: Family	Field report (FR) or tested (T)	Category for potential	Notes	Reference
Predators					
Colletotrichum fioriniae (Marcelino & Gouli) Pennycook	Glomerales: Glomerellaceae	FR, T	1	Reported mortality rates of > 90 for crawlers and > 55% for settlers but was not tested against *F. externa* in the field. It might be because it shows plasticity in host choice from plants to insects, especially endophyticity against strawberries	Marcelino et al. (2009a, b), J. A. P. Marcelino (personal communication)
Cordyceps sp.	Hypocreales: Cordycipitaceae	FR	4	Reported entomopathogenicity to *F. externa* in the field but was not tested	Marcelino et al. (2009a)
Fusarium sp.	Hypocreales: Nectriaceae	FR	4	Reported as a phytopathogen	Marcelino et al. (2009a)
Lecanicillium lecanii Zare and Gams	Hypocreales: Cordycipitaceae	FR	4	Reported entomopathogenicity to *F. externa* in the field but was not tested	Marcelino et al. (2009a)
Mycosphaerella sp.	Capnodiales: Mycosphaerellaceae	FR	4	Reported as a phytopathogen	Marcelino et al. (2009a)
Myriangium sp.	Myriangiales: Myriangiaceae	FR	4	Reported entomopathogenicity to *F. externa* in the field but was not tested	Marcelino et al. (2009a)
Nectria sp.	Hypocreales: Nectriaceae	FR	4	Reported as a phytopathogen	Marcelino et al. (2009a)
Phialophora sp.	Chaetothyriales: Herpotrichiellaceae	FR	4	Reported as an endophyte	Marcelino et al. (2009a)

Category 1: Reported to be the most effective to control *F. externa*, but have hurdles to implement in the field; Category 2: Reported to be effective, but not enough; Category 3: Reported feeding on or causing mortality to *F. externa*, but their effectiveness is still not confirmed; Category 4: Reported in *F. externa* infested area, no noticeable reduction in *F. externa* population was linked with them. *Density-dependent parasitism can cause a decrease in parasitism rate and equilibrium in host density (Ives 1992).

vices for sale in Florida because of contamination with *F. externa*. Nine such shipments, originating from Michigan, North Carolina, and Virginia during the last 2 Christmas seasons (2019–2020), mostly revealed numerous dead individuals of *F. externa* covered with a dark orange fungal mass (Table 2). Those fungal masses were identified as *Conoideocrella luteorostrata* (Zimm.) D. Johnson, G.H. Sung, Hywel-Jones & Spatafora (Hypocreales: Clavicipitaceae) (Table 2; Fig. 1) (FDACS-DPI 2020).

Table 2. Detail of samples of *Fiorinia externa* intercepted between 2019 and 2020 with and without entomopathogenic fungus *Conoideocrella luteorostrata*.

Yr	Sample #	City	State	*Conoideocrella luteorostrata*
2019	6549	Jefferson	North Carolina	absent
2019	6550	Sparta	North Carolina	absent
2019	6449	Glade Creek	North Carolina	absent
2019	6450	Glade Creek	North Carolina	absent
2019	6474	Cullowhee	North Carolina	absent
2019	6547	Crumpler	North Carolina	present
2019	6581	Laurel Springs	North Carolina	absent
2019	6583	Laurel Springs	North Carolina	absent
2019	6582	Laurel Springs	North Carolina	absent
2019	6580	Laurel Springs	North Carolina	absent
2019	6593	Sparta	North Carolina	absent
2019	6594	Sparta	North Carolina	absent
2019	6632	Grassy Creek	North Carolina	absent
2019	6633	West Jefferson	North Carolina	absent
2019	6655	Cullowhee	North Carolina	absent
2019	6746	Ennice	North Carolina	absent
2019	6745	Ennice	North Carolina	absent
2019	6713	NA	North Carolina	absent
2019	6716	NA	North Carolina	absent
2019	6776	Kentwood	Michigan	present
2019	6772	NA	North Carolina	absent
2019	6773	Sparta	North Carolina	absent
2019	6771	Sparta	North Carolina	absent
2019	6806	Cullowhee	North Carolina	absent
2019	6805	Cullowhee	North Carolina	absent
2019	6808	Sparta	North Carolina	absent
2019	6807	Whitetop	Virginia	absent
2019	6829	Ennice	North Carolina	absent
2019	6827	Ennice	North Carolina	absent
2019	6828	Ennice	North Carolina	absent
2020	4603	Sparta	North Carolina	present
2020	4540	Jefferson	North Carolina	absent
2020	4653	Tuckasegee	North Carolina	absent
2020	4685	Tuckasegee	North Carolina	present
2020	4686	Tuckasegee	North Carolina	absent
2020	4705	Plumtree	North Carolina	absent
2020	4706	Plumtree	North Carolina	absent
2020	4707	Elk Creek	Virginia	present
2020	4731	Cullowhee	North Carolina	absent
2020	4730	Cullowhee	North Carolina	absent
2020	4726	NA	North Carolina	absent
2020	4750	NA	North Carolina	absent
2020	4749	NA	North Carolina	absent
2020	4766	NA	North Carolina	absent
2020	4765	Elk Creek	Virginia	present
2020	4777	Laurel Springs	North Carolina	present
2020	4778	Laurel Springs	North Carolina	present
2020	4825	NA	North Carolina	present

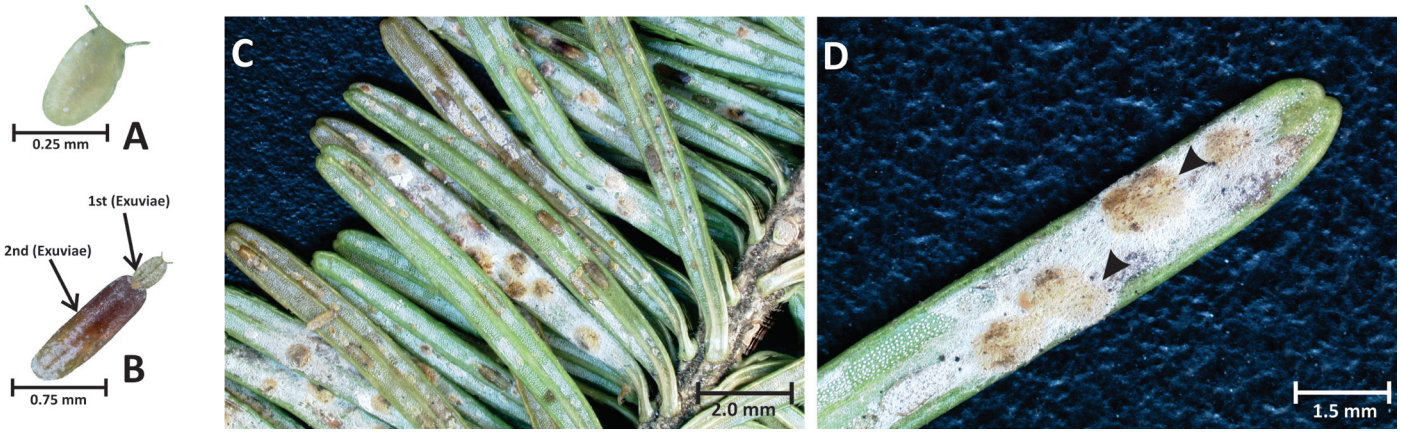

Fig. 1. Features of *Fiorinia externa*: (A) 30× view of alive first instar (crawler); (B) 30× view of adult female body (inside cover) with exuviae of first and second instar; (C) naked eye view of entomopathogenic fungus *Conoideocrella luteorostrata* on different stages of *F. externa* (black arrow heads); (D) close-up of *C. luteorostrata* covering *F. externa* (black arrow heads).

There have been 5 entomopathogenic fungal species reported from *F. externa* in the USA (Table 1). So far, only 1, *Colletotrichum fioriniae* (Marcelino & Gouli) Pennycook (Phyllachorales: Phyllachoraceae) was found to be effective. However, *C. fioriniae* was reported to cause endophyticity towards plants (Marcelino et al. 2009 a, b; Table 1; JAP Marcelino, personal communication). The objectives of this study are: (1) morphological and molecular characterization of entomopathogenic fungal species, *C. luteorostrata* from scale insect species, *F. externa*; (2) morphological diagnostics of *F. externa* and its comparison with 2 closely related species, *F. fioriniae* and *F. phantasma*; and (3) a comprehensive review of potential natural enemies of *F. externa*. This study provides useful information regarding the potential of *C. luteorostrata* as a biological control agent for *F. externa* and discusses the importance of *C. luteorostrata* as a new potential biological control agent for other scale insects and whiteflies in the USA.

Materials and Methods

In Dec 2019 and 2020, inspectors at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry intercepted shipments of Fraser fir Christmas trees originating from Michigan, North Carolina, and Virginia destined for sale in Florida due to the presence of a scale insect pest (Table 2). The samples were sent to Florida Department of Agriculture and Consumer Services, Division of Plant Industry (DPI) headquarters, Section of Entomology in Gainesville, Florida, USA, for scale insect identification.

Adult female specimens were prepared and slide-mounted following the method in Ahmed et al. (2021a) and Ahmed (2018). The scale insects were identified as *F. externa* using the taxonomic key from Ahmed et al. (2021b), as well as a comparison of morphological characteristics with the original description and illustration from Ferris (1942). Numerical values for taxonomic characters were taken from a minimum of 5 specimens of *F. externa* from as many localities as possible, and were compared with descriptions of the closely related species *F. fioriniae* and *F. phantasma* to observe intra- and interspecific variations. The specimens were deposited in the Florida State Collection of Arthropods (Table 2). During microscopic examination, we noticed that many of the dead individuals were covered with dark-orange fungal masses; consequently, a subsample of infected scales was submitted to the Section of Plant Pathology for identification (Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Plant Pathology sample numbers 2019–102372; 2020–105882, 105940, 105941).

The isolation of entomopathogenic fungus was carried out by culturing small fragments extracted from the interior of stromatic tissue covering dead scale insects into sterile potato dextrose broth (DB Difco™, Franklin Lakes, New Jersey, USA) amended with antibiotics; after several d of incubation at room temperature, mycelium was transferred onto plates containing sterile potato dextrose agar (DB Difco™, Franklin Lakes, New Jersey, USA) and oatmeal agar made from scratch using a recipe: oatmeal flakes, 30 g; agar-agar, 15 g; distilled water, 1 L; and incubated at room temperature. Dehydrated axenic culture together with infected scale insects of the first sample received (2019–102372) were deposited in the Division of Plant Industry Herbarium (specimen number 14812). DNA extractions were carried out individually from 2 wk old colonies on agar plates and the stroma on scale insect by using DNeasy® Plant Mini Qiagen kit following manufacturer protocol (Germantown, Maryland, USA). Molecular identification was done by PCR amplification using the following markers (primers forward/reverse, product size): small subunit (SSU, NS1/NS4, 985 bp, White et al. 1990); the internal transcribed spacer (ITS, ITS1F/ITS4, 568 bp, White et al. 1990; Gardes & Bruns 1993); and large subunit (LSU, LR0R/LR3, 467 bp, Hopple & Vilgalys 1999) of the ribosomal RNA genes; as well as the protein coding gene, transcription elongation factor 1 [*tef1,* (elf728F/ef1-986R, 321 bp), (ef1a-983F/elf1a-1567R, 273 bp) (Rehner 2001)] with the recommended protocols. All the PCR reactions were carried out in 25 µL of final volume containing 1X GoTaq® Master mix (Promega, Madison, Wisconsin, USA), 2.5 pmol of each primer and 3 µL of total DNA and carried out in Applied Biosystems GeneAmp PCR System 9700 thermocycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Purified PCR products were sequenced bidirectionally in-house using an Applied Biosystems SeqStudio platform (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with BigDye Terminator v. 3.1 cycle sequencing chemistry (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Contig sequences were generated in Geneious v.11.0.4+11 (Kearse et al. 2012), and compared to GenBank by using MegaBlast (Chen et al. 2015) and deposited in the GenBank (MT796333–MT796336). Phylogenetic placement of the entomopathogenic fungus isolated here was assessed by using a 4-locus-concatenated dataset (3,353 bp) aligned with available conspecific and congener sequences downloaded from GenBank (accession numbers shown in phylogenetic tree). Alignments were generated for each locus in Geneious v.11.0.4+11 using the MAFFT (Katoh et al. 2002) algorithm; phylogeny was estimated in a Maximum Likelihood (ML) framework in RAxMLv8.0.0 (Stamatakis 2014), under a per-locus General Time Reversible model of

nucleotide evolution and CAT approximation of rate heterogeneity (GTRCAT). Nodal support was assessed with 1,000 bootstrap replicates. Tree and alignment were deposited in TreeBase project (S26962).

Results

There were 48 samples of *F. externa* intercepted in the last 2 Christmas seasons. Nine were found covered with fungi from Michigan, North Carolina, and Virginia. There were 30 in yr 2019 (2 with fungi, 1 from Michigan, and 1 from North Carolina) and 18 in 2020 (7 with fungi, 5 from North Carolina, and 2 from Virginia) (Table 2; Fig. 2).

Field characters used in tentative identification of *F. externa* during interceptions were pupillarial adult female completely enclosed in second instar exuviae; that is elongate reddish brown anteriorly and light brown to yellow posteriorly; the first instar exuviae barely touches second instar exuviae and form a distinct indentation between attachment of first and second instar exuviae (Fig. 1). Slide-mounted specimens were with 4 to 6 macroducts on each side of body and the number of macroducts unequal between both sides of body. There are 5 to 6 macroducts in *F. externa* reported in Miller and Davidson (2005) and Ahmed et al. (2021b). We found 3 out of 5 specimens with 4 macroducts on 1 side of the body. However, there were either 5 or 6 on opposite sides of the body in these specimens. The presence of 4 macroducts on 1 side overlaps with that of its closely related species *F. fiorinae* in which 3 to 4 on each side of body commonly are found (Fig. 3A, D). The width of macroduct was 7.5 to 10 µm contrary to 2 to 3 µm in *F. phantasma* (Fig 3E, I). The number of perivulvar pores were ranged between 39 and 47 (with average of 42.6), and their numbers were unequal between sides of body (Fig. 3 A, D) as compared of that in *F. fiorinae* in which they range between 21 and 36 (26) according to Miller and Davidson (2005). The antennae located on submargin with a short spur of the length of 8 to 9 µm making them more or less as long as wide (Fig. 3B). This is contrary to antennae on margin with a lot longer spur of the length of 25 to 27.5 µm in *F. fioriniae* (Fig. 3F). There is no processing between antennae (contrary to crown-shaped processing between antennae in *F. phantasma*; Fig. 3G).

Microscopic characteristics of the stromatic tissue of the strain of *C. luteorostrata* studied here include a compact mycelium with twisted dark-orange hyphae, smooth to finely roughed wall up to 1 µm in thickness, 3 to 4 µm in diam (Fig. 4A, B); in 10% KOH, hyphae turn dark blue in masses. In pure culture, colonies were felt-like, with hyaline to pale-yellow in the border and dark-orange to cinnamon in the center, with thin-wall hyphae when young, to thick-wall when old (up to 1 µm in thickness), with slow growth, 1 cm diam per mo in PDA (Fig. 4C). The *Paecilomyces*-asexual state described for *C. luteorostrata* was formed after at least 2 mo of incubation only on oatmeal agar; hyaline, unbranched-conidiophores longer than 200 µm in length, smooth walls, septate with hyaline flask-shape phialides with verticillate, 9 to 15 × 2 to 3 µm, producing hyaline to yellowish fusiform-conidia in chain, lemon shape 6 to 7 × 2 µm (Fig. 4D, E).

The ITS sequences obtained from DNA extracted from both the stromatic tissue and a purified culture were identical to each other with the exception of a single intra-genomic heterogeneity at the nucleotide 151 (C/T) in the ITS1 sequences obtained from the culture and the stroma. The genomic peculiarity of heterogeneity in the ITS1 sequences obtained from the culture and the stroma also was observed in the ITS1 and ITS2 in other ascomycete- and basidiomycete-fungi (Wipf et al. 1996; Zhao et al. 2015; He et al. 2017).

Molecular identification based on ITS showed 99.03% similarity to a well-characterized strain NHJ 12516 (JN049860) of *C. luteorostrata*. Also the molecular markers SSU and LSU of the rRNA genes exhibit 100% sequence identity (EF468994 and EF468849) and the *tef1* gene 99% similarity (EF468800) to the same isolate NHJ 12516 (Johnson et al. 2009). In concordance with the morphological characterization and MegaBlast results, the multilocus phylogenic analysis shows that the strain isolated here is circumscribed within *C. luteorostrata* (Fig. 5).

We conducted a comprehensive review of natural enemies of *F. externa* in this study and elaborated on the need for a new natural enemy of *F. externa* in the USA (Table 1). There are 12 identified and 1 unidentified predator species reported from 3 families (Coccinellidae, Miridae, and Neuroptera) in *F. externa* infested areas in the USA (Table 1). This includes 8 ladybird beetles (Table 1). Only 4 were found feeding on *F. externa*, including *Chilocorus kuwanae* Silvestri (Coleoptera: Coccinellidae), *Cybocephalus nipponicus* Endrödy-Younga (Coleoptera: Cybocephalidae), *Rhyzobius lophanthae* (Blaisdell) (Coleoptera: Coccinellidae), and *Scymnillus horni* (Gordon) (Coleoptera: Coccinellidae). *Chilocorus kuwanae* is an introduced species and was found established in the USA (Table 1). *Cybocephalus nipponicus* was imported, mass-reared, and released to control *F. externa* in the USA (Table 1). There was no noticeable reduction in *F. externa* population in the presence of these predators (see the column of category for potential in Table 1). Three parasitoid species have shown parasitism for *F. externa* (Table 1). *Prospaltella* sp. was reported by one of the earliest studies and might be a misidentification of *Encarsia citrina* (Craw) (Table 1). *Aphytis aonidiae* (Mercet) and *E. citrina* were reported multiple times in the literature and have shown potential to control *F. externa* (Table 1). Ten fungi have been recovered from *F. externa* including 4 entomopathogenic fungi (*Beauveria bassiana* (Bals.-Criv.) Vuill. [Cordycipitaceae]; *Cordyceps* sp. (L.) Fr. [Cordycipitaceae]; *Lecanicillium lecanii* Zare and Gams [Cordycipitaceae]; *Myriangium* sp. Mont. & Berk. [Myriangiaceae]), 1 endophyte, and 5 phytopathogens (Table 1), with 1 isolate of phytopathogen, *Colletotrichum fioriniae* (Marcelino & Gouli) Pennycook (Glommerellaceae) showing higher entomopathogenic potential for *F. externa* (Table 1). We categorized the natural enemies based on their control potential and concluded that 2 parasitoids, *A. aonidiae* and *E. citrina*, and 1 entomopathogenic fungus, *C. fioriniae* were the most effective in reducing the population of *F. externa* (Table 1). However, the effectiveness of *A. aonidiae* alone is not enough and *E. citrina* population is asynchronous with that of *F. externa* in the USA (Table 1). In addition, both parasitoid species showed density-dependent parasitism causing a decrease in parasitism rate and an equilibrium in host density (Table 1). On the other hand, the entomopathogenic fungus, *C. fioriniae* shows plasticity in host choice from plants to insects (Table 1).

Discussion

Following isolations, morphological and molecular characterization of the entomopathogenic fungal species, we present the first record of *C. luteorostrata* infecting the scale insect *F. externa* in the USA. We did not detect any sexual fungal structures on dead scale insects on the underside of Fraser fir leaves, consistent with a previous report of *C. luteorostrata* (Hywel-Jones 1993) that pointed out that the production of perithecia and ascospores were detected only on samples collected during the wet season, and that the sexual state generally occurs on the underside of leaves of diverse dicotyledonous plants, and not while infecting its insect host (Mongkolsamrit et al. 2016). *Conoideocrella luteorostrata* was described first under another genus as *Torrubiella luteorostrata* Zimm. in 1901 from Java on an unidentified scale insect (Coccomorpha) (Hywel-Jones 1993). Only 2 more species

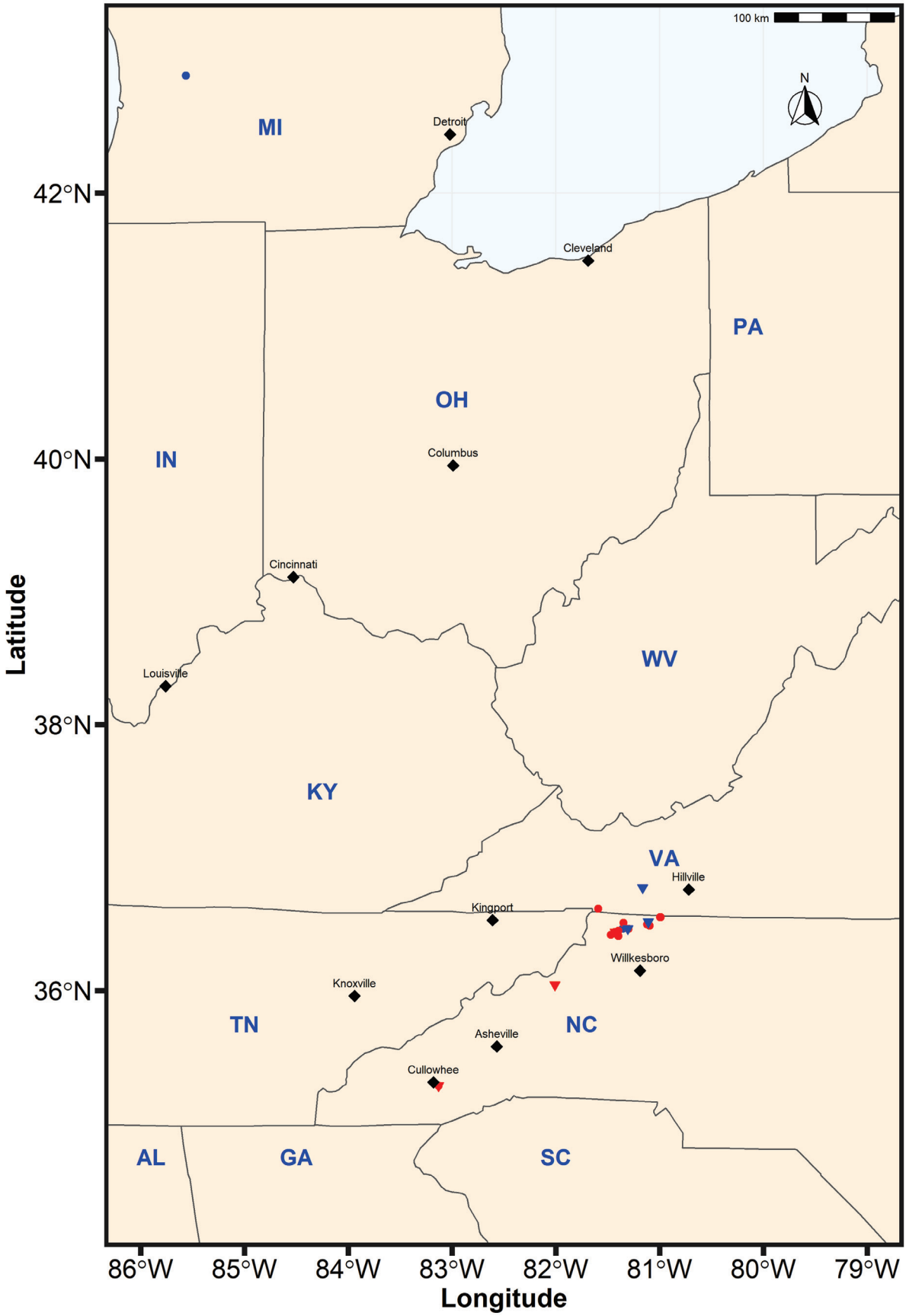

Fig. 2. Original localities of intercepted shipments of Christmas trees in 2019 (shown as circle) and 2020 (triangle). Samples with entomopathogenic fungus *Conoideocrella luteorostrata* are colored in blue and without fungus in red. Major cities are shown as black diamonds.

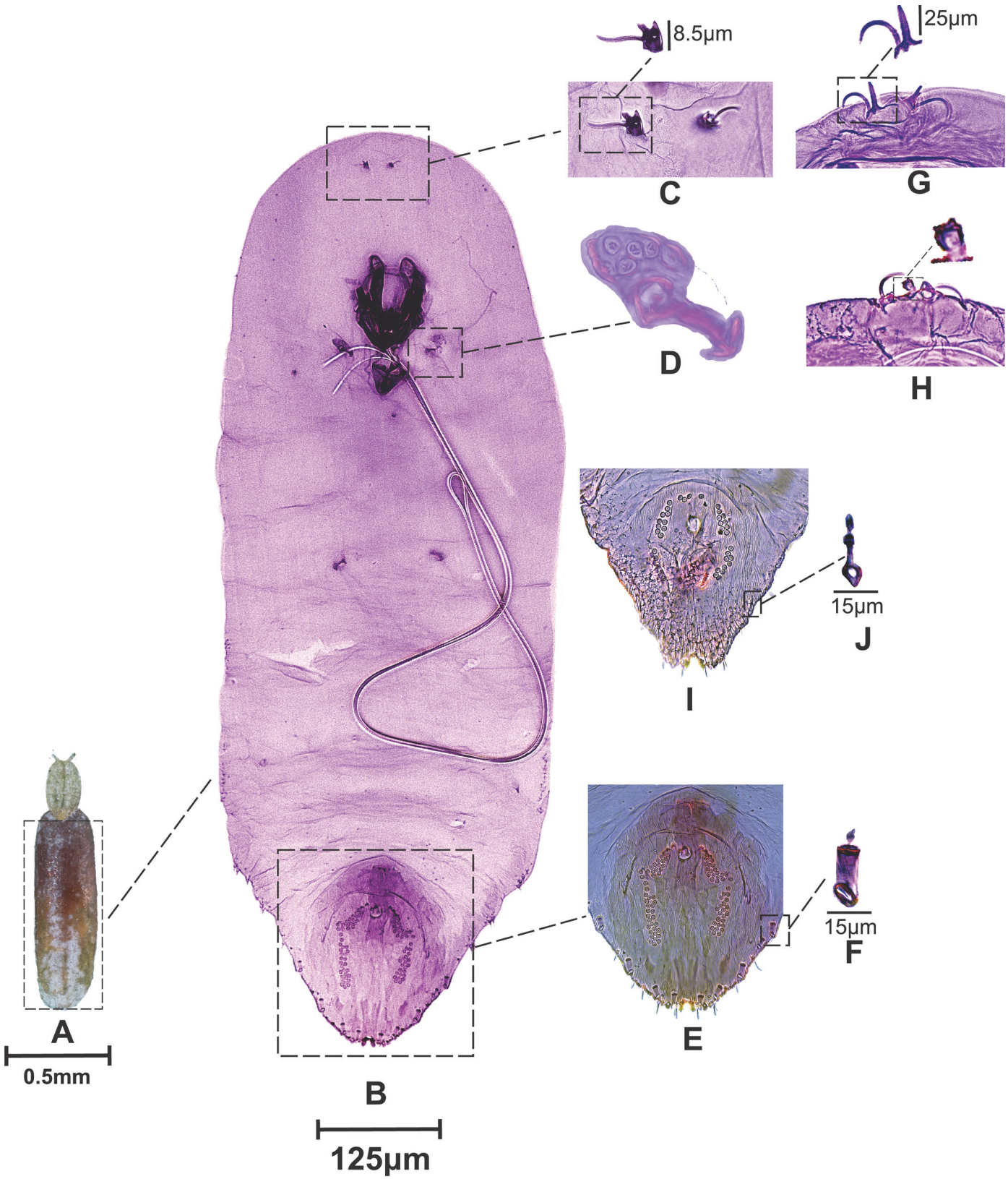

Fig. 3. Field view of *Fiorinia externa* collected on *Abies fraseri* from Glade Creek, North Carolina, USA (FDACS-DPI, sample #2019-6449) (A); its slide-mounted view (B); antennae on submargin of the head with short spur (C); anterior spiracle with pores (D); pygidium with five marginal macroducts (E); close-up of wide macroduct (F); antennae on the margin of head, with a long spur, of *F. fioriniae* collected on *Chamaerops humilis* from Ocala, Florida, USA (2019-4546) (G); antennae on the margin of head, with a short spur and processing between antennae, of *F. phantasma* collected on *Ligustrum japonicum* from Boynton Beach, Florida, USA (2020-1365) (H); pygidium with 4 marginal macroducts (I); close-up of narrow macroduct (J).

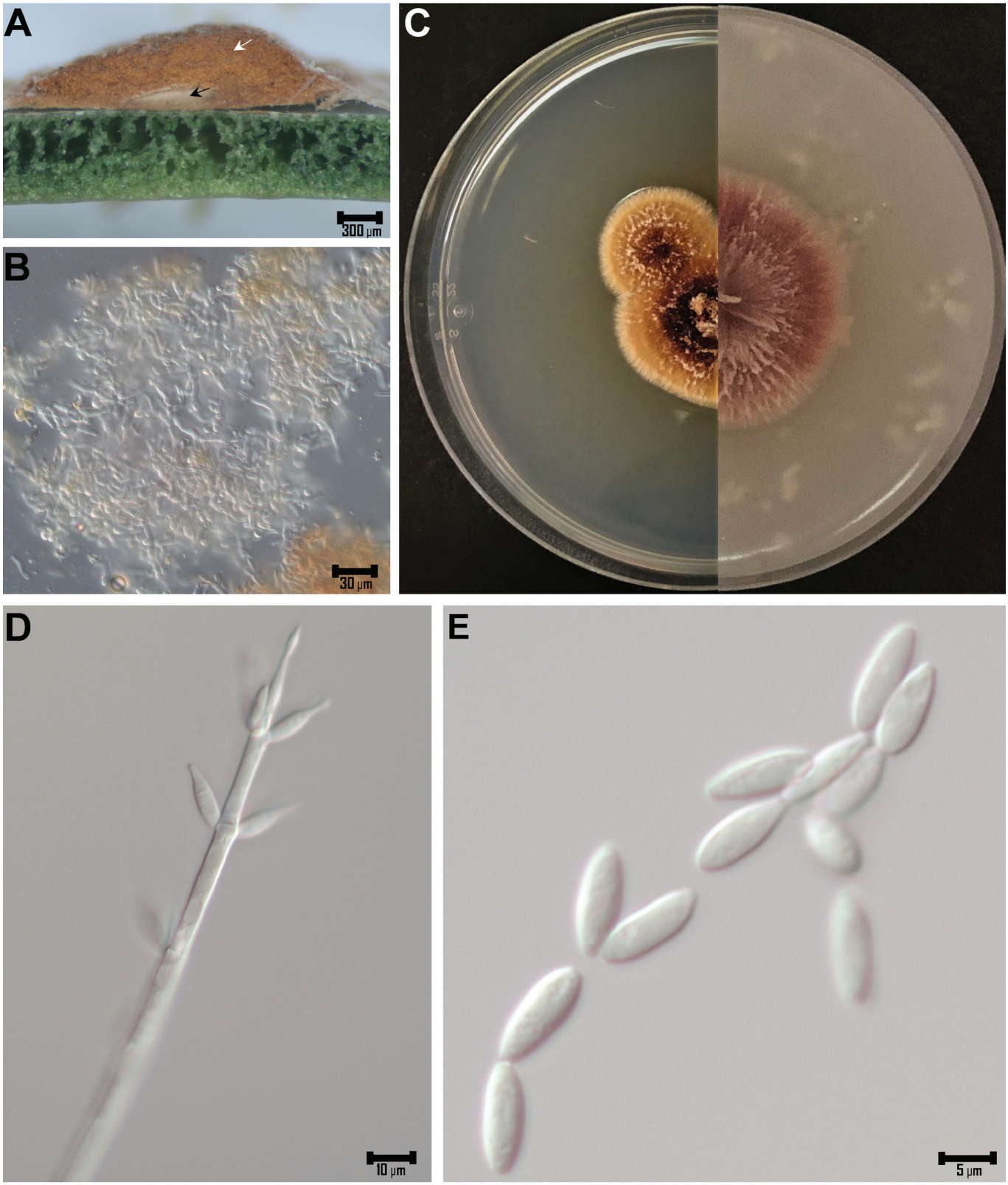

Fig. 4. Features of *Conoideocrella luteorostrata*: (A) stromatic tissue (white arrow) on *Fiorinia externa* (black arrow); (B) details of stromatic hyphae on 10% KOH, 40×; (C) 1 mo old culture on PDA (left) and oatmeal agar (right); (D) conidiophore; and (E) spores, 100×.

have been circumscribed within the genus *Conoideocrella*: *C. krungchingensis* Mongkols., Thanakitp. & Luangsa-Ard, and *C. tenuis* (Petch) D. Johnson, G.H. Sung, Hywel-Jones & Spatafora (known sister species of *C. luteorostrata* [Mongkolsamrit et al. 2016]) (both Hypocreales: Clavicipitaceae). Both species were described from unidentified scale insects in Thailand.

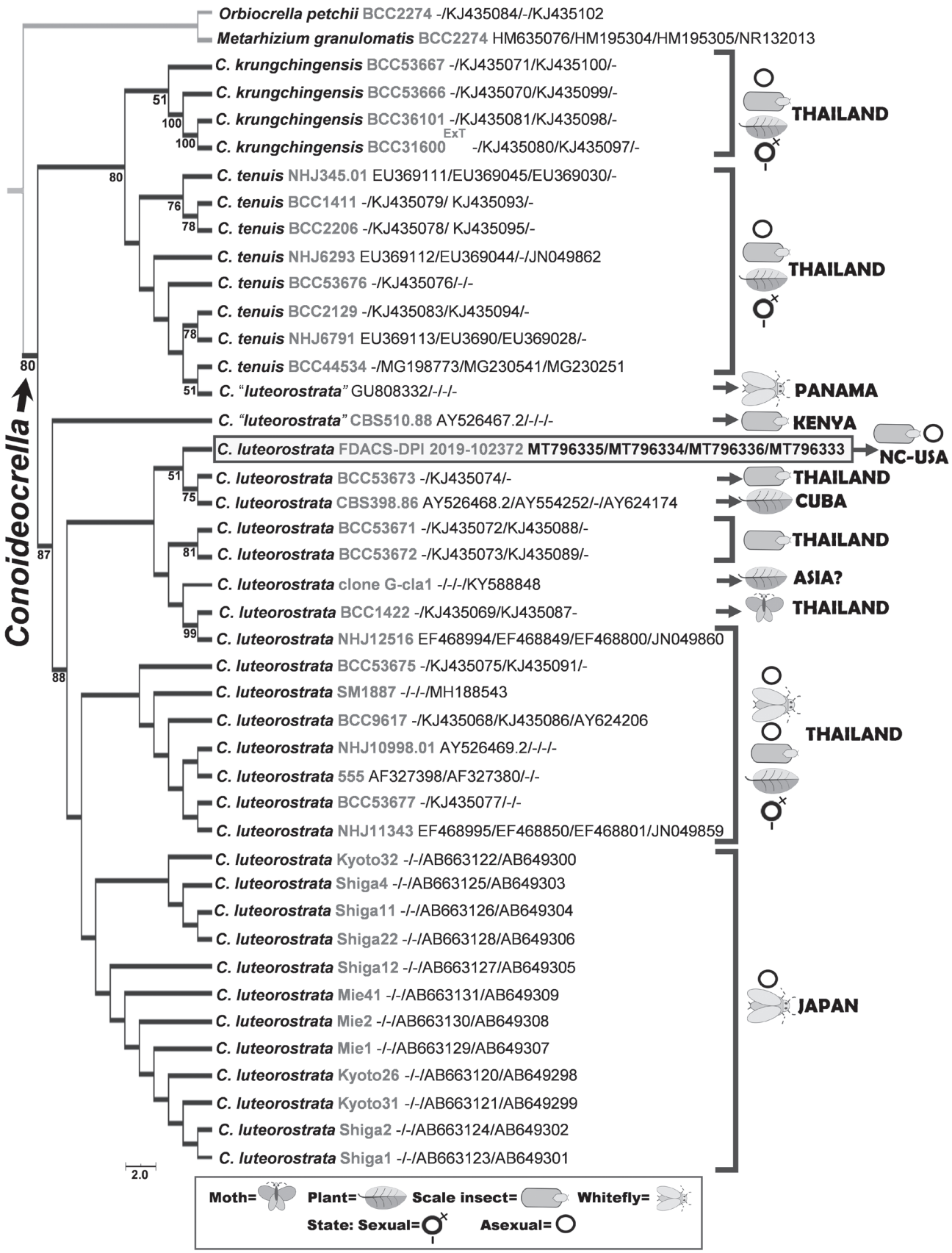

Fig. 5. Maximum Likelihood phylogenetic reconstruction of *Conoideocrella* species, using an SSU-LSU-tef1-ITS concatenated dataset with *Metarhizium granulomatis* (Sigler) Kepler, S.A. Rehner & Humber (Clavicipitaceae) as designated outgroup taxon, and showing host, sexual state and county of isolation. Ex-type species denoted as ExT.

The asexual state of *C. luteorostrata* (described as *Paecilomyces cinnamomeus*) has been reported principally from Asia (Thailand and Japan) and from North America (Cuba) on various scale insect species (Hywel-Jones 1993), whiteflies (Samson 1974; Isaka et al. 2019) and moths (strain BCC 14222, unpublished) (Fig. 5). Particularly, in the USA, Samson (1974) detected the asexual state of this entomopathogenic fungus on whitefly species infesting *Citrus* × *aurantiaum* L. (Rutaceae) in Louisiana, with morphological and culturable characteristics similar to the strain isolated in this study.

Conoideocrellais luteorostrata is confirmed entomopathogenic fungi for 2 families of scale insects, the soft scales (Coccidae) and the armored scales (Diaspididae) (Evans & Prior 1990; Evans & Hywel-Jones 1997). Most of the studies referred to the hosts of *C. luteorostrata* as scale insects because the identification of the host in *Conoideocrella* infections has been troublesome due to the high degree of destruction of the host body. In our study, the connection was established because the plant host was infected with a single species of scale insect, and not all the life stages or individuals of *F. externa* were infected with *C. luteorostrata*. In addition, we also successfully retained the body of the scale insect species under fungal masses and mounted using the protocol from Ahmed et al. (2021a, b). Therefore, for the first time, we successfully identified *C. luteorostrata* and its host species simultaneously.

In the USA, extensive work has been conducted to isolate entomopathogenic fungi to be used as biocontrol agents against *F. externa* and from which fungal strains in 10 genera (*Beauveria*, *Botrytis*, *Colletotrichum*, *Cordyceps*, *Fusarium*, *Phialophora*, *Lecanicillium*, *Mycosphaerella*, *Myriangium*, and *Nectria*) have been recovered (Marcelino 2007; Marcelino et al. 2009a, b; Table 1). Among them, only 1 species of *Colletotrichum*, *C. fioriniae*, showed high pathogenicity against *F. externa* with mortality rates of 55% or higher (Table 1). However, *C. fioriniae* has been identified as a causal agent of disease in economically important crops, including blueberries, eggplants, hazelnuts, hemp, and Satsuma mandarin among others (Pszczółkowska et al. 2016; Sezer et al. 2017; Xu et al. 2018; Szarka et al. 2020; Table 1) restricting its use as a biocontrol agent.

Conoideocrella luteorostrata has been considered an unsuitable species for biocontrol due to its slow growth in vitro, production of sexual and asexual propagules influenced by weather conditions, and high susceptibility to common antagonistic compounds used in agriculture (e.g., fungicides and insecticides) (Hywel-Jones 1993; Saito et al. 2012). However, *C. luteorostrata* species has not been found in association with any plant disease, and it shows high pathogenic specificity against 2 important plant pest groups, whiteflies and scale insects. The majority of *F. externa* individuals found in these interceptions were colonized by *C. luteorostrata*, suggesting its high pathogenicity rate to control *F. externa*. More studies should be conducted to reevaluate the efficacy of *C. luteorostrata* and its active compounds in the biological control of whiteflies and scale insects.

So far, all attempts to control *F. externa* have been in vain due to scale cover that protects it against insecticides, natural enemies, and adverse climatic conditions (Marcelino et al. 2009a; Table 1). The use of insecticides has been associated with increased scale insect populations and outbreaks (Luck & Dahlsten 1975; Frank 2012). Chemical control affects predation and parasitism of scale insects (Luck & Dahlsten 1975; McClure 1977a, b, c; Frank 2012). Broad-spectrum insecticides (e.g., pyrethroids) commonly used for pre- or post-harvest Fraser fir pest control do not effectively control armored scales, but instead reduce natural enemy populations (Luck & Dahlsten 1975; McClure 1977a, b, c; Raupp et al. 2001; Frank 2012). Therefore, insecticide applications to Fraser fir that disregard the conservation of natural enemies may lead to their successful off-site dispersal. Predators are generalist and feed indiscriminately on different pest species, thereby reducing their effectiveness (Table 1). However, parasitoids are usually species-specific (Ahmed et al. 2017). In general, parasitoids along with other natural enemies have been shown to be ineffective in controlling *F. externa* (Abell & Van Driesche 2012; Table 1), a phenomenon attributed to asynchrony between armored scales and their parasitoids, triggered by overlapping *F. externa* generations (Table 1). Current management practices and future research should incorporate the use of entomopathogenic fungi or extracts of secondary metabolites with insecticidal properties that are compatible with parasitoids to maximize natural Fraser fir pest control during harvest and shipment. The combined use of parasitoids and entomopathogenic fungi has shown higher efficacy in whitefly control (Ou et al. 2019).

The aesthetic value of Christmas trees might be compromised by using entomopathogenic fungus. Nevertheless, the covers of *Fiorinia* species remain on the leaves long after the scales themselves have died naturally (Ahmed & Stocks 2020). Infestation of *F. externa* not only destroys the aesthetic value but also results in rejection of exported cut Christmas trees. A minimal reduction in aesthetic value is a tradeoff to the use of entomopathogenic fungus to control *F. externa*, which may minimize economic loss due to rejections of cut Christmas tree shipments in Florida because interception does not apply if scale insects are dead. Further, application of extracted mycotoxins produced by *C. luteorostrata* could eliminate the reduction of aesthetic value associated with fungal growth. Several studies that have characterized bioactive compounds produced by *C. luteorostrata* (e.g., antimalarial, antibacterial, antitumor cyclohexadepsipeptide) show significant differences in the production of these compounds among strains of the same species (Isaka et al. 2005, 2007a, b, 2019). Therefore, the strain of *C. luteorostrata* isolated here constitutes a new source of study of active compounds.

Non-native, invasive species pose major global threats to natural and anthropogenic ecosystems as well as economic interests and in fact could eliminate some agricultural industries altogether (Crooks 2002; Pimentel et al. 2005). Regardless of improved screening and sanitation practices (Meyerson & Reaser 2002; Mehta et al. 2007; Sanchirico et al. 2009), the international movement of humans and plant material is predicted to be doubled by 2035 (IATA 2017), which likely will result in higher incidence of exotic invasions. In the USA, there are an estimated 50,000 exotic invasive species (Pimentel et al. 2005), and in the state of Florida alone over 24 new species are being recognized as potentially established yearly (FDACS-DPI 2020). Data suggest that Florida receives and harbors more exotic species than any other state in the USA, largely due to the state's tourism industry, trade, and climate (Paini et al. 2010). Classical biological control involves the introduction of co-evolved natural enemies to control invasive pests (DeBach & Schlinger 1964) and is the best alternative approach to using pesticides. There are many documented cases where natural enemies entered along with invasive pest species (Ahmed et al. 2015, 2017). For example, Ahmed et al. (2017) found a new parasitoids species, *Baeoentedon balios* Wang, Huang & Polaszek (Hymenoptera: Eulophidae), in the New World in 2014 attacking the fig whitefly, *Singhiella simplex* (Singh) (Hemiptera: Aleyrodidae). It appears that both parasitoids and fig whitefly share the same origin.

A process to explore biological control agents during import inspection is needed urgently to expedite the most time-consuming steps in establishing biological control of exotic invasive species. It could be rewarding tremendously in the case of the intercepted samples of the pests of regulatory concern. The presence of parasitoids, predators, and pathogens associated with the mortality of the pests should be examined regularly in such samples. If found, biological control agents should be sent to respective experts for species-level identification. Af-

terward, the original location of those biological control agents should be traced, and researchers interested in further evaluating their biological control potential should be informed. In addition, studying already established entomopathogenic fungi in the impacted areas where invasive species already are established or being established could further strengthen the biological control of non-native, invasive pests.

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