Environmental Entomology, 48(4), 2019, 882–893
doi: 10.1093/ee/nvz064
Advance Access Publication Date: 30 May 2019
Research

Assessment of Alternative Candidate Subcortical Insect Vectors From Walnut Crowns in Habitats Quarantined for Thousand Cankers Disease

Karandeep Chahal,[1,2] Romina Gazis,[3] William Klingeman,[4,5] Denita Hadziabdic,[1] Paris Lambdin,[1] Jerome Grant,[1] and Mark Windham[1]

[1]Department of Entomology and Plant Pathology, University of Tennessee, 370 Plant Biotechnology Building, Knoxville, TN 37996, [2]Current address: Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, MI 48824, [3]Department of Plant Pathology, Tropical Research & Education Center, University of Florida, Homestead, FL 33031, [4]Department of Plant Sciences, University of Tennessee, 2431 Joe Johnson Dr., 252 Ellington Plant Sciences Building, Knoxville, TN 37996, and [5]Corresponding author, e-mail: wklingem@utk.edu

Subject Editor: David Onstad

Received 7 February 2019; Editorial decision 2 May 2019

Abstract

Thousand cankers disease (TCD) results from the combined activity of the fungal pathogen, *Geosmithia morbida* Kolařík, Freeland, Utley, and Tisserat and its principle vector, *Pityophthorus juglandis* (Blackman) (Coleoptera: Curculionidae: Scolytinae) in *Juglans* L. spp. and *Pterocarya* Kunth spp. host plants. TCD has been reported from the eastern and western United States. To evaluate potential for other beetle species to vector the fungus in east Tennessee, specimens were collected using ethanol-baited traps that were suspended beneath crowns of TCD-symptomatic trees. Associations of *G. morbida* with insect species collected in traps were assessed in an unsuccessful, preliminary culture-based fungal assay, and then with a molecular-based detection method. For culture-based assays, rinsate from washed, individual insects was plated on nutrient media and growing colonies were subcultured to obtain axenic *G. morbida* cultures for identification. For the molecular-based method, *G. morbida* presence was detected by amplifying the previously developed, species-specific microsatellite locus GS004. Capillary electrophoresis was used to detect the amplified amplicons and representative reactions were validated using Sanger sequencing. Eleven beetle species were found to carry *G. morbida*, including *Cnestus mutilatus* (Blandford), *Dryoxylon onoharaensum* (Murayama), *Hylocurus rudis* (LeConte), *Monarthrum fasciatum* (Say), *Monarthrum mali* (Fitch), *Xyleborinus saxesenii* (Ratzeburg), *Xylosandrus crassiusculus* (Motschulsky), *Xylosandrus germanus* (Blandford) (all Coleoptera: Curculionidae: Scolytinae), *Stenomimus pallidus* (Boheman) (Coleoptera: Curculionidae: Cossoninae), *Oxoplatypus quadridentatus* (Olivier) (Coleoptera: Curculionidae: Platypodinae), and *Xylops basilaris* (Say) (Coleoptera: Bostrichidae). These findings raise concerns that alternative subcortical insect species that already occur within quarantined habitats can sustain incidence of introduced *G. morbida* and contribute to spread within the native range of black walnut, *Juglans nigra* L., in the eastern United States.

Key words: subcortical insect, novel fungus-beetle association, *Geosmithia morbida*, pathogen vector, *Pityophthorus juglandis*

Thousand cankers disease (TCD) has been considered as an emerging threat to health of walnut (*Juglans* L.) and wingnut (*Pterocarya* Kunth) tree species (Utley et al. 2009, Hishinuma et al. 2016). TCD is a complex that involves a fungal pathogen, *Geosmithia morbida* Kolařík, Freeland, Utley, and Tisserat (Ascomycota: Hypocreales: Bionectriaceae), an insect vector, walnut twig beetle, *Pityophthorus juglandis* (Blackman) (Coleoptera: Curculionidae: Scolytinae), and *Juglans* spp. and *Pterocarya* spp. host plants. External symptoms of TCD include wilting, foliage chlorosis of the upper crown (flagging), crown thinning, and branch dieback followed by emergence of epicormic shoots (Tisserat et al. 2009). Internal symptoms include numerous small cankers, and vertical and horizontal galleries produced by walnut twig beetle in the inner bark/phloem (Kolařík et al. 2011, Tisserat et al. 2011). The many cankers can coalesce, creating large necrotic areas that girdle the tree (Tisserat et al. 2009). All examined *Juglans* spp. have demonstrated susceptibility to TCD; however, black walnut (*Juglans nigra* L.) is the most susceptible host to the pathogen (Utley et al. 2013).

In the last two decades, TCD has resulted in severe mortality and crown dieback of *Juglans* spp. in the western United States (Seybold

et al. 2016). *Juglans nigra* has an estimated value for standing timber in the United States that exceeds $568 billion (Newton and Fowler 2009). The tree is one of the most economically valuable North American hardwood species and has many uses including furniture, veneer, cabinets, interior architectural woodwork, flooring, and gunstocks (Manning 1978, Grant et al. 2011). In addition to the annual market consumption of walnut wood in the United States, in 2017, United States exported walnut lumber to more than 67 countries and walnut logs to more than 49 countries that were valued at $258 million, and $163 million, respectively (USDA-FAS 2018).

In Knoxville, Tennessee in 2010, TCD was detected within the native range of *J. nigra* (Daniels et al. 2016), and this discovery presents a serious threat to *J. nigra* within urban and forest settings across the distribution of susceptible *Juglans* spp. (Grant et al. 2011). Subsequent TCD detections in the eastern United States are now reported from Indiana, Maryland, North Carolina, Ohio, Pennsylvania, and Virginia (Hansen et al. 2011, Fisher et al. 2013, Seybold et al. 2013, Hadziabdic et al. 2014, Daniels et al. 2016), as well as Italy (Montecchio and Faccoli 2014, Moricca et al. 2019).

Fungi within the genus *Geosmithia* Pitt comprise genetically and ecologically diverse lineages with mainly asexual mode of reproduction. This genus has a global distribution, having been reported from North and South America, Europe, Asia, and Australia (Pitt 1979, Kolařík et al. 2008, Lin et al. 2016, Kolařík et al. 2017). *Geosmithia* spp. occupy diverse ecological niches with species that are thermophilic, mesophilic, or thermotolerant (Kolařík et al. 2004). Although most *Geosmithia* spp. are saprophytic, some species can act as weak pathogens (Schuelke et al. 2017). Some *Geosmithia* spp., including *G. morbida*, have been characterized as having strong associations with specific phloeophagous bark beetle species, or interactions that are limited within narrow taxonomic groups, that serve a critical function in vectoring these fungal species among healthy host trees (Kolařík et al. 2007, Huang et al. 2017). Other *Geosmithia* spp. show varying levels of specificity to their beetle vectors and tree hosts that range from generalists to single-species specialists (Kolařík et al. 2008, 2017). These symbiont-forced evolutionary associations between beetles and fungus are also believed to have resulted in morphological adaptations that enhance fungal dispersal and beetle feeding (Kolařík and Kirkendall 2010).

Geosmithia spp. have been isolated from a variety of beetle galleries in diverse host plants, including economically important coniferous trees; yet this fungal genus seems to be more commonly associated with hardwoods (Kolařík et al. 2017). Despite the ubiquity of *Geosmithia* spp. within bark beetle galleries, their ecological role, and the nature of the complex relationship between *Geosmithia* spp. and their beetle vectors, including the relationship between members of the TCD pathosystem, are not well understood (Huang et al. 2017). *Geosmithia morbida* is not reported to persist independently from its plant hosts (Tisserat et al. 2009) and this plant pathogenic species has close association with *P. juglandis* as a principal vector (Rugman-Jones et al. 2015, Seybold et al. 2016, Kolařík et. al. 2017). The fungal pathogen appears to rely primarily on *P. juglandis* for entry into host phloem, for spread within compromised host plant tissues, and for dissemination across larger distances (Tisserat et al. 2009, Kolařík et al. 2011). However, walnut twig beetle is considered a weak flier with an average flight distance of ~372 m (Kees et al. 2017). Therefore, the broad and recent range expansion of TCD has been attributed to human-related activities, including movement of firewood and transport of lumber and wood products trading across states (Newton and Fowler 2009, Audley et al. 2016). The human-mediated dispersal hypothesis has been supported by population genetic studies conducted on wide-scale sampling across the United States and Italian populations of walnut twig beetle (Hadziabdic et al. 2014, Zerillo et al. 2014, Rugman-Jones et al 2015, Montecchio et al. 2016).

Geosmithia morbida, and walnut twig beetle are native to Southwestern United States, where they are associated with *J. major* Torr.; a walnut species that has been shown to have a higher tolerance to the beetle and fungal damage (Utley et al. 2013). Subsequent range expansion and increase in disease severity can be explained by a switch of the pathogen to a naïve host and habitat (Tisserat et al. 2011). Although not yet demonstrated in TCD, successful vector switching in other important pathosystems in the United States have demonstrated negative consequences for native forested ecosystems (Wingfield et al. 2010, Saucedo-Carabez et al. 2018). Consequently, emergence of novel beetle associations occurring within a compromised habitat presents practical concerns about the role of other subcortical insect species that are associated with walnut in spreading and sustaining TCD within the native range of *J. nigra* (e.g., Juzwik et al. 2015, Klingeman et al. 2017).

Walnut twig beetle is considered the primary vector of *G. morbida* in the United States, yet other scolytid beetles associated with *J. nigra* trees in the western United States have been suspected as potential alternative vectors of the fungus (Newton and Fowler 2009). Using morphological and molecular methods, *G. morbida* was recovered from the weevil *Stenomimus pallidus* (Boheman) (Coleoptera: Curculionidae: Cossinae) collected in Brown County, Indiana (Juzwik et al. 2015), and *P. juglandis* was detected both in a trap and from logs at a sawmill in Franklin County, Indiana in 2014 (Indiana Department of Natural Resources 2015). Walnut twig beetle has not been recovered in Indiana since that time (Thousand Cankers Disease Research and Management Operational Meeting 2017). In Ohio, the fungus has been recovered from *S. pallidus* and two ambrosia beetle species, *Xylosandrus crassiusculus* Motschulsky and *Xyleborinus saxesenii* Ratzeburg (Coleoptera: Curculionidae: Scolytinae) (Juzwik et al. 2016). Association of *G. morbida* with other forest insect species has raised concerns of potential for more rapid fungal spread and pathogen persistence in the eastern United States (Juzwik et al. 2015, 2016). Domestic and international quarantine protocols have already been implemented to limit spread of the disease by regulating and pre-treating wood products that can contain the principle members of the TCD complex (Audley et al. 2016, Mayfield et al. 2018). Other insects associated with *J. nigra* may also play a role in disseminating *G. morbida* and sustaining active cankers in susceptible host plants. The extent to which other bark- and wood-associated insect species may associate with *G. morbida* has not been well examined within a quarantined area containing TCD-symptomatic trees. To address these knowledge gaps, the objectives of this study were 1) to assay beetle species that are active within the crowns of *J. nigra* trees where *G. morbida* has been identified to determine which species may be associating with *G. morbida*; and 2) to evaluate the frequency of these potential associations.

Materials and Methods

Trapping of Coleopteran Insects at Locations With TCD-Symptomatic *J. nigra* Trees

Subcortical insect species that were active in crown habitats of *J. nigra* were collected primarily from three locations in eastern Tennessee where trees displayed characteristic symptoms of TCD infection. Additional beetle species that were collected at three other locations (W. Klingeman, unpublished records) were also assayed for presence of *G. morbida*. For all insect collections, dry

cup traps were made for insect collection. Two-liter plastic soda bottles were modified into traps by cutting 5 cm above the bottom of the bottle to make two 15 × 6 cm openings on opposing sides of the bottle (Supp Fig. 1 [online only]). For venting, the bottoms of 250-ml Nalgene bottles (Mfr. No. 2103-0008, Thermo Fisher Scientific Inc., Rochester, NY) were cut and replaced with a 10 × 10 cm piece of 75-mesh size nylon screen. Modified Nalgene bottles were then attached to the spout end of the modified soda bottle trap. To create baits for insects that associate with subcortical tissues of walnut, healthy *J. nigra* limbs were obtained outside of the TCD quarantined area from a healthy *J. nigra* at The University of Tennessee Plateau Research and Education Center in Cumberland County, TN (Lat. 36°00′58″N, Long. 85°07′48″W). Limbs were confirmed to be negative for *G. morbida* infection, using lesion-directed sampling and the molecular protocol described in Oren et al. (2018). Limb sections were cut into 15 cm long (3–5 cm diameter) pieces that were cored about 10 cm deep using a 19 mm diameter drill bit. To attract other subcortical insect species active in walnut tree crowns and that respond to trees under stress, 20 ml of 95% ethanol were added to the chamber of each limb section and cores were stoppered with #2 rubber corks. Ethanol-filled *J. nigra* limb sections were suspended inside modified soda bottle traps with a nylon rope.

Three locations in eastern Tennessee were primary sampling sites (Table 1). At these locations, traps were suspended beneath the crown of one *J. nigra* at each of the three sites. Within each tree, eight modified soda bottle traps were suspended beneath *J. nigra* crowns and hung at two different heights (lower [3–8 m] and upper [10–15 m] crown) and four cardinal positions (east, west, north, and south). Traps were deployed at two heights because different species of bark and ambrosia beetles were active at different heights beneath the tree crown (Klingeman et al. 2017). A total of 24 traps were used in this study and traps were monitored from 1 April to 18 November during 2016. Trap contents were collected into sterile vials 1–3 times per wk, depending on weather conditions and previously observed insect activity. During specimen retrieval, ethanol chambers within branch sections were refilled and trap surfaces were wiped with 95% ethanol to avoid potential contamination of *G. morbida* propagules. In the laboratory, individual insects were transferred, using sterilized forceps, into 0.6-ml microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) and then held at 4°C until processing. For use in molecular-based detection of *G. morbida*, additional beetle species and samples were provided by WEK from collections made in different walnut trees at the same three locations as above, plus species collected at Choto Road, Hinton Drive, and Murphy Road sites (Table 1). Traps deployed by WEK did not include a walnut limb section, and instead included a lateral chamber, with insect excluding mesh, from which a 50 ml reservoir was suspended containing 95% ethanol.

Table 1. Locations from which beetles associated with *Juglans nigra* habitats were collected[a] for culture-based and *Geosmithia morbida* molecular detection assays during 2016

Tennessee county	Location	Site coordinates
Knox	Burkhart Rd.*	36°04′46″N, 83°51′29″W
Knox	Lakeshore Park*	35°55′22″N, 83°59′30″W
Blount	Maryville College*	35°44′55″N, 83°57′42″W
Knox	Choto Rd.	35°49′15″N, 84°09′12″W
Knox	Hinton Dr.	35°58′16″N, 83°59′33″W
Knox	Murphy Rd.	36°03′12″N, 83°52′33″W

[a]At primary sampling locations (noted with *), traps were placed on a single tree, set in two different levels (upper and lower crown) and in four cardinal directions (east, west, north, and south). Several other beetle species, which were also collected beneath walnut crowns at the other listed locations in 2016, were also screened for the presence of *G. morbida*.

Tree density (any tree species) and number of *J. nigra* trees differed across the Lakeshore Park, Burkhart Road, and Maryville College locations where traps were installed. At the Maryville College location, walnut trees were located within an urban forest setting with high density of diverse tree species. Visual observations were used to estimate that trunks of tree species (mixed stands consisting primarily of *Acer* L. spp., *Carya* Nutt. spp., *Quercus* L. spp., and *Ulmus* L. spp., plus *J. nigra*) were 3–4 m apart and canopies were contiguous. Within this mixed tree species environment, *J. nigra* selected for trapping were at least 5–10 m apart. Less tree density was present at the Burkhart Road site and canopies were intermittent. Walnut trees from which traps were suspended were 10–15 m away from trunks of other *J. nigra* and walnut crowns were independent of one another but may have been interspersed with limbs from another tree species. At the Lakeshore Park location, *J. nigra* used for trapping were 35–40 m apart and the nearest park trees had discrete (noncontiguous) crowns and were located at least 25 m away.

Sample Preparation and Insect Identification

Collected insect specimens were identified to species or the lowest taxonomic level using morphological characters and taxonomic keys (Wood 1982, Hulcr and Smith 2010). Insects with cryptic diagnostic characters were identified through molecular techniques, using the insect universal barcode *cytochrome c oxidase subunit 1* (CO1) and following protocols described in Rugman-Jones et al. (2012). Genomic DNA from beetles was extracted using the GeneJet Genomic Purification Kit (Fisher Scientific, Pittsburgh, PA) following protocol modifications by Oren et al. (2018). Briefly, individual beetles were placed into 2-ml conical screw-cap microcentrifuge tubes containing five to ten 2.3-mm diameter zirconia/silica beads (BioSpec Products, Bartlesville, OK) and 180 µl digestion solution. Samples were then homogenized using a Bead Mill 24 homogenizer (Fisher Scientific, Pittsburgh, PA), set for two 30-s-long cycles and with a 3-min rest period in between. Once homogenized, 40 µl of proteinase K (20 mg/ml) was added to the sample and incubated overnight at 56°C. The elution buffer was heated to 70°C before being added to the columns, with 45 µl buffer applied two times per sample, with a 5-min incubation period in between. Final DNA solution (90 µl/sample) was stored at –20°C until polymerase chain reaction (PCR) amplification was performed.

The CO1 regions were amplified and sequenced using the primer pair LCO1490F and HCO2198R (Folmer 1994), resulting in a fragment length of about 650 bp. Each PCR reaction contained 12.5 µl GoTaqG2 Hot Start Master Mix (Promega Corp., Madison, WI), 1.25 µl 10 mM reverse primer, 1.25 µl 10 mM forward primer, 1 µl dimethyl sulfoxide (DMSO, Sigma–Aldrich, St Louis, MO), 1 µl of template DNA (undiluted) and 10 µl double-distilled water yielding the 27 µl final reaction volume.

The PCR thermal cycle started with initial denaturation step of 2 min at 94°C followed by five cycles of denaturation for 30 s at 94°C, annealing for 1 min 30 s at 45°C and primer extension for 1 min at 72°C; followed by a further 35 cycles of 30 s at 94°C, 60 s at 51°C and 1 min at 72°C; and followed by a final elongation for 5 min at 72°C (Rugman-Jones et al. 2012). Amplified PCR products were confirmed with gel electrophoresis, and PCR products were sent to MCLAB laboratories (www.mclab.com) for cleaning and sequencing.

Sequencher TM 4.9 (Gene Codes Corp., Ann Arbor, MI) was used to assess the quality of the chromatograms and assemble the strands into contigs. CO1 sequences were assigned to taxa based on the results of the Basic Local Alignment Search Tool (BLAST), using the NCBI nucleotide database (www.ncbi.nlm.nih.gov/BLAST) and the International Barcode of Life project (iBOL, http://www.boldsystems.org). Sequences were confirmed to be at least 500 bp long and contained no missing or non-nucleotide characters. Generated sequences were submitted to GenBank with accession numbers MK224407–MK224420.

Geosmithia morbida Recovery and Detection from Coleopteran Insects Active in *J. nigra* Habitats

Culture-Based Screening for *G. morbida*

Initial fungal screening on culture medium was conducted with identified, individual insect specimens collected between 1 April and 21 July 2016. Distilled sterile water (200 µl) was added to each 0.6-ml microcentrifuge tube containing a single insect specimen. Tubes were vortexed three times, each for about 15 s, using a Thermolyne Maxi Mix II 37600 mixer (Barnstead/Thermolyne, Dubuque, IA) at 300 rpm. Under a laminar flow hood, the homogenized sample was pipetted and the entire 200 µl volume streaked using a sterile inoculation loop onto 100 × 15 mm Petri dishes containing half-strength Potato Dextrose Agar (PDA) (Difco Laboratories, Detroit, MI) amended with 30 mg/liter of chlortetracycline HCL (Sigma–Aldrich, St. Louis, MO) and 30 mg/liter streptomycin sulfate (Fisher Bioreagents, Fair Lawn, NJ) (1/2 PDA++). Petri dishes were left for 1–2 h to allow the inoculant solution to absorb into the medium (~200 µl), after which they were wrapped with Parafilm M (Bemis, Oshkosh, WI), and then incubated under 16:8 (L:D) h at room temperature (25°C). Petri dishes were inspected for fungal growth at 24-h intervals and once hyphae or yeast colonies resembling *G. morbida* (as described in Kolařík et al. 2011) were observed, subcultures were made until axenic isolates were obtained. Further subcultures were made on the same ½ PDA medium but without antibiotics. For DNA extraction, axenic cultures were grown in full strength Potato Dextrose Broth at room temperature (25°C) for 3 wk. Mycelium was harvested, and genomic DNA was extracted as described in Gazis et al. (2018). To confirm isolate identity, the Internal Transcribed Spacer (ITS) region of the ribosomal RNA operon was amplified, using the primers ITS1F (Gardes and Bruns 1993) and ITS4R (White et al. 1990). PCR protocol and thermocycler conditions were conducted as described in Gazis et al. (2018). Amplifications of PCR products were confirmed with gel electrophoresis and sent to MCLAB laboratories for cleaning and sequencing. Sequencher TM 4.9 was used to assess the quality of the chromatograms and assemble the strands into contigs. ITS sequences were compared to the ones deposited in NCBI nucleotide database through BLAST. Identified sequences were submitted to GenBank with accession numbers MG008847 and MG008848.

Molecular-Based Detection of *G. morbida* DNA

The screening method used to identify *G. morbida* on insect specimens was shifted to molecular-based detection following limited success observed via culture-based screening. Insect specimens that were collected from 22 July to 18 November 2016 were analyzed for *G. morbida* DNA presence using a molecular-based detection method as described in Oren et al. (2018). Primers GS004F and GS004R, designed to amplify the *G. morbida* specific-microsatellite locus GS004 (Hadziabdic et al. 2012), were used to detect the presence of *G. morbida* in the samples. The GS004 locus has been screened against 100 taxonomically diverse Ascomycota lineages that were isolated from walnut twig beetle galleries (Oren et al. 2018). Cross-amplification of these fungal species DNA with the GS004 marker was not detected and this diagnostic probe has been developed into a rapid molecular toolkit for detecting *G. morbida* directly from TCD-infected walnut branches (Oren et al. 2018). Recently discovered, genetically diverse *Geosmithia* species have associations with different beetle and host plant species in the southeastern United States (Chahal et al. 2017, Huang et al. 2019). If other *Geosmithia* spp. DNA can be recovered with the GS004 marker, it is possible that cross-amplification could result in false-positive *G. morbida* detections. To address this concern, GS004 primers were tested against 57 additional *Geosmithia* isolates representing at least seven clades within the genus. Of the 57 *Geosmithia* isolates examined, 45 had been obtained from subcortical insect species that were also found to carry *G. morbida* in this study (*data not shown*). These 45 *Geosmithia* isolates were obtained as part of an independent project conducted by members of our working group (Chahal et al. 2017, Gazis et al. unpublished records). DNA extractions, PCR reactions, and analysis of the ITS region of the *Geosmithia* isolates were conducted as described in Gazis et al. (2018). No cross-amplifications occurred, thus, the likelihood of false-positive *G. morbida* detections using this approach is minimal.

Insect specimens that were stored individually in 0.6-ml microcentrifuge tubes at 4°C were homogenized, as described above, to yield extraction of total genomic DNA. Each PCR reaction contained 5 µl of sterile water, 4 µl GoTaqG2 Hot Start Master Mix, 1 µl of 10 µM of each primer, 0.5 µl dimethyl sulfoxide (DMSO) and 3 µl of template DNA (undiluted) to yield the 14.5 µl final reaction volume. To guard against false negatives when only small amounts of *G. morbida* DNA are present, three different volumes of undiluted template DNA were screened (1, 3, and 5 µl) in separate reactions using GS004 primers. In cases where *G. morbida* DNA could not be detected at any of the three volumes tested, the reaction was considered a negative detection and reported as such, DNA was not quantified following extractions using this protocol because quantification values would reflect combined sample DNA (e.g., from beetles and many other associated organisms, including endosymbiotic fungi and bacteria), rather than the concentration of *G. morbida* DNA. Thermocycler conditions started with initial denaturation at 94°C for 3 min, followed by 35 cycles each of, denaturation at 94°C for 40 s, annealing at 55°C for 40 s, elongation at 72°C for 30 s, and final extension at 72°C for 4 min (Hadziabdic et al. 2012). After DNA amplification, PCR products were analyzed with QIAxcel Capillary Electrophoresis System (Qiagen, Valencia, CA) using an internal 25 bp DNA size and a 15–600 bp external alignment markers. QIAxcel Capillary Electrophoresis System utilizes QIAxcel ScreenGel software, which generates both gel view image and chromatograms for data visualization. Simply, amplicons could be visualized as bands or as 'peaks'. Beetles were considered positive for the presence of *G. morbida* when a peak (clearly distinguishable from background noise) with a base pair length between 221 and 254 bp (Hadziabdic et al. 2012) was observed (Supp Fig. 4A [online only]) and negative when there was no evidence of amplification in any of the three PCR reactions (1, 3, and 5 µl) (Supp Fig. 4B [online only]). As a validation step, nine representative positive reactions corresponding to unique associations of beetle with *G. morbida* were sequenced and compared to the GenBank GS004 microsatellite sequence (accession JN580435, Hadziabdic et al. 2012).

Screening Insect Traps for *G. morbida* DNA—Positive and Negative Controls

To control for the possibility that traps deployed in the field had become contaminated with environmental *G. morbida* hyphae or

conidia, thus providing a source of DNA that could be transferred to trapped insects, one trap from each of the three primary sampling locations was randomly screened for presence of *G. morbida* DNA (negative control) using *G. morbida* specific GS004 primers (Hadziabdic et al. 2012, Oren et al. 2018). Two trap portions were assayed: 1) the spout end of the soda bottle that directed trapping beetles into the collection container, and 2) the mesh screen from the base of the collection bottle (Supp Fig. 2 [online only]). Sterile distilled water was used to collect rinsate from the spout, and a sterilized cotton swab was used to dislodge residues from the internal trap surfaces until 25 ml of washout and swab residues were collected into a 50-ml Falcon tube (Fisher Scientific, Pittsburgh, PA). Using sterilized scissors in a laminar flow hood, the bottom screen from collection bottles was removed and screen pieces were transferred to Falcon tubes containing 25 ml of sterile distilled water. A total of six samples were obtained: two samples from each trap with one trap examined for each treatment. Falcon tubes containing the screen pieces and the cotton swabs were vortexed for 2 min to dislodge and rupture cells from any potential conidia or hyphae. Falcon tubes were then centrifuged at 5,000 rpm for 20 min (Sorvall RC-6 plus, Thermo Fisher Scientific Inc., Waltham, MA) to precipitate impurities/screen parts. After centrifugation, 1 ml of the supernatant was extracted per sample and used for pathogen detection with GS004 primers. The DNA extraction protocol, PCR amplification, and final GS004 detection steps were described as above, with screening performed using 1, 3, and 5 µl of the DNA suspension.

To further test if rain or wind could carry *G. morbida* propagules into traps, the same molecular detection methods described above were used to screen for *G. morbida* DNA in three different assays designed as positive control tests. The first experiment used a sterile cotton swab under a laminar flow hood to brush the surface of an 8-wk old, sporulating *G. morbida* colony. The inoculated cotton swab was then rubbed across a 0.18 mm × 0.25 mm piece of nylon hardware cloth screen. In a second experiment, a 10 × 10 cm nylon screen was placed on a sterile, empty 10-cm-diameter Petri dish. A PDA amended Petri dish containing an 8 wk old sporulating *G. morbida* colony was inverted 20 cm above the sterile, empty Petri dish containing 10 × 10 cm nylon screen and then tapped three times to dislodge conidia and hyphae onto the screen (Supp Fig. 3 [online only]). In a third experiment, the same procedure was followed as described in the second experiment with the exception of tapping once onto sporulating *G. morbida* plate instead of three times. Screens from these three experiments were processed for DNA extraction and GS004 amplification as previously described. Finally, insect exclusion (negative control) traps were modified by gluing two 0.18 × 0.25 mm, 75-mesh size screens to either side of trap openings so that insects were prevented access to the inside of traps, but wind and rainwater that could carry fungal propagules were able to enter. These traps were deployed from 23 September to 21 October 2016, beneath three different trees where insects had also been trapped. After 28-d deployment, traps were assayed as described above.

Statistical Analysis

Incidence data were analyzed to measure the percentage of individuals from each insect species from which *G. morbida* was recovered. For several scolytine beetle species, including *Cnestus mutilatus*, *X. saxesenii*, and *X. crassiusculus,* comparisons were made among beetle species to determine whether any of these species had an association with *G. morbida* that was greater than would be expected by chance. These same beetle species were also screened from the Burkhart Rd., Lakeshore Park, and Maryville College collection locations that were TCD-compromised habitats, to assess whether locations yielded different numbers of *G. morbida* positive specimens within insect species. Interaction effects between *C. mutilatus*, *X. saxesenii*, and *X. crassiusculus* and the three collection locations were also examined to determine whether the effects of location and insect species on *G. morbida* incidence were interdependent. Data were analyzed with beetle association status with *G. morbida*, either as positive or negative) for individuals of each insect species as the response variable, while collection location and insect species were independent variables using binary logistic regression. Analyses were conducted using PROC LOGISTIC in SAS9.4 (SAS Institute 2017). No significant interactions were observed among the independent variables (range: $X^2 = 1.10$ to 0.34, df = 2, $P = 0.57$ to 0.84); thus, P values were taken from Wald's χ^2 test, a pre-step for binary logistic regression analysis. Observed differences were reported as significant for tested variables when $P < 0.05$.

Results

Culture-Based Detection of *G. morbida*

A total of 1,596 subcortical insects were screened for the presence of *G. morbida* through a culture-based detection approach. Culturing of rinsate from specimens yielded high numbers of yeast-like species and fast-growing fungi. Because *G. morbida* grows slow in artificial media (Oren et al. 2018), no efforts were made to further characterize these other fungal and yeast isolates. Only two *G. morbida* isolates were recovered from the rinsate of a single *S. pallidus* specimen collected in Maryville, TN.

Molecular-Based Detection of *G. morbida* DNA

Poor recovery from agar-based culture media prompted extended seasonal trapping to provide specimens used to assay for presence of *G. morbida* DNA using a molecular detection protocol. Between 22 July and 18 November 2016, 389 specimens representing 18 different beetle species were collected and assayed for presence of *G. morbida* DNA. Of these, 187 individuals (47%) tested positive for the presence of *G. morbida* using GS004 primers. From 18 beetle species collected in traps, 11 yielded positive *G. morbida* detection outcomes. The number and proportion of positive individuals examined across these 11 species were: *C. mutilatus* (28 [42%]), *Dryoxylon onoharaensum* Murayama (11 [42%]), *Hylocurus rudis* LeConte (1 [33%]), *Monarthrum fasciatum* Say (15 [56%]), *Monarthrum mali* Fitch (5 [33%]), *X. saxesenii* (45 [50%]), *X. crassiusculus* (22 [49%]), and *Xylosandrus germanus* Blandford (6 [75%]) (All Coleoptera: Curculionidae: Scolytinae). Additionally, *G. morbida* DNA was recovered from *S. pallidus* (18 [67%]) (Coleoptera: Curculionidae: Cossinae), *Oxoplatypus quadridentatus* Olivier (2 [100%]) (Coleoptera: Curculionidae: Platypodinae), and *Xylobiops basilaris* Say (34 [63%]) (Coleoptera: Bostrichidae) (Table 2).

Specimens of *C. mutilatus*, *X. saxesenii*, and *X. crassiusculus* were collected in sufficient numbers across all primary locations (Table 2) to enable comparisons of their relative likelihood that each may be carrying *G. morbida* within the sampled TCD-compromised habitats. When interactions were assessed between these three beetle species across trap locations, the predicted incidence of *G. morbida* detection was consistent; at each location, a given beetle species had a similar predicted probability of being associated with fungal DNA compared with either of the other species ($X^2 = 5.34$, df = 4, $P = 0.25$). When pooled across locations, each of those scolytine beetle species had a consistent probability that an individual would be carrying *G. morbida* fungal inoculum

Table 2. *Geosmithia morbida* detection incidence from 387 individuals representing 18 beetle species that were trapped in modified soda bottle traps deployed beneath *Juglans nigra* crowns in eastern Tennessee between 22 July and 18 November 2016

Coleopteran species[a]	Trap locations[b]						
	Burkhart Rd.	Lakeshore Park	Maryville College	Choto Rd.	Hinton Dr.	Murphy Rd.	*Geosmithia morbida* incidence[c] (%)
Bostrichidae: Bostrichinae							
Xylobiops basilaris	21 (14)[d]	21 (9)	9 (8)	3 (3)	—	—	63
Curculionidae: Cossoninae							
Stenomimus pallidus	5 (4)	—	20 (12)	2 (2)	—	—	67
Curculionidae: Platypodinae							
Oxoplatypus quadridentatus	—	—	—	—	—	2 (2)	100
Curculionidae: Scolytinae							
Ambrosiophilus atratus	2 (0)	—	—	—	—	—	0
Cnestus mutilatus	21 (8)	19 (7)	25 (12)	2 (1)	—	—	42
Dryoxylon onoharaensum	4 (2)	13 (7)	8 (2)	—	1 (0)	—	42
Euwallacea validus	6 (0)	—	—	—	2 (0)	—	0
Hylocurus rudis	—	2 (0)	1 (1)	—	—	—	33
Monarthrum fasciatum	13 (6)	12 (9)	2 (0)	—	—	—	56
M. mali	6 (1)	—	9 (4)	—	—	—	33
Xyleborinus saxesenii	30 (19)	30 (13)	30 (13)	—	—	—	50
Xyleborus ferrugineus	1 (0)	—	—	—	—	—	0
Xylosandrus crassiusculus	15 (5)	15 (8)	14 (8)	1 (1)	—	—	49
X. germanus	8 (6)	—	—	—	—	—	75
Zopheridae							
Microsicus parvulus	2 (0)	3(0)	2(0)	—	—	—	0
Cerambycidae							
Lepturges confluens	—	—	—	—	—	1 (0)	0
Neoclytus acuminatus	—	—	—	—	1 (0)	—	0
Cleridae							
Madoniella dislocatus	—	—	2 (0)	—	—	—	0

[a]Insect species that were screened for *Geosmithia morbida*.
[b]Locations from which insects were collected.
[c]Incidence values reported indicate the proportion of the species, pooled among locations, that yielded positive *G. morbida* detection.
[d]For species in each location, positive *G. morbida* detection counts are given in parentheses.

(X^2 = 1.27, df = 2, P = 0.53). For the beetle species examined, all locations presented a similar probability that the beetles encountered would be carrying *G. morbida* (X^2 = 0.39, df = 2, P = 0.82). In other words, despite differences in the numbers of walnut trees and variety of other tree species present across locations, similar numbers of each of these scolytine beetle species yielded detectable *G. morbida* DNA (Table 2).

Screening Insect Traps for *G. morbida* DNA

Amplification efforts with GS004 failed to detect presence of *G. morbida* on all sampled portions of field-deployed traps with emphasis on the spout end of the soda bottle and mesh screens (as negative controls). Similarly, modified insect exclusion traps that were installed in the field were tested and found to be negative for detectable presence of *G. morbida* DNA. *Geosmithia morbida* DNA was detected from all trap portions of intentionally surface-contaminated traps that were established as positive experimental controls and confirmed that the diagnostic procedure was capable of detecting *G. morbida* DNA.

Discussion

Increased globalization of commercial trade has accelerated the movement and expanded the geographic distribution of forest insect pests and their associated plant pathogens (Aukema et al. 2010, Santini et al. 2013). Once established in new localities, fungal associates, including symbiotic organisms, may be transferred laterally to other arthropods (native, naturalized, or exotic) enhancing their survival and increasing their spread potential (Wingfield et al. 2010, 2016). Lateral transmission by novel vector associations can result in greater threats to susceptible host trees than would have been expected from a single insect vector. These novel interactions may also provide the pathogen with access to more susceptible host plant species than were available in its native range, promoting the encounter with naïve hosts (Wingfield et al. 2016). Therefore, these novel associations can play a critical role in the severity, maintenance, and expansion of emerging infectious diseases in forest tree species, yet mechanisms by which these new relationships form remain poorly understood.

Where consequences of expanded range distribution of an emerging plant pathogenic threat intersect with greater susceptibility among new host plant species, a critical need exists to quickly understand the patterns and processes underlying disease development and severity. This need is particularly crucial when susceptible host plants include economically and ecologically important species and crops (Wingfield et al. 2010, Ploetz et al. 2013). When conditions like these arise, it also becomes critical to quickly assess the severity of the emerging threat and to employ, or develop, reliable and highly specific techniques that will detect and diagnose incipient infectious stages. To mitigate the consequences of new plant pathogen introductions, all of these steps must be accomplished before the new associations between pest and plant pathogens become established within urban landscape and forest habitats and threaten naïve plant species (Wingfield et al. 2010, 2016).

Molecular techniques have been used to detect novel associations between plant pathogens and insects (Roets et al. 2006, Persson et al. 2009, Lamarche et al. 2015). DNA-based detection can provide major advantages for rapid, sensitive, and specific diagnosis of plant pathogens on insects yet may not answer questions about the viability or pathogenic competence of the organism detected (Schweigkofler et al. 2005). Where the latter objective is the specific goal, detection of plant pathogenic fungi on bodies of forest insects is more frequently achieved by isolating fungi using traditional culture-based methods (Krokene and Solheim 1996, Six and Bentz 2003). Reliance on culture-based methods, however, can present challenges in identification of potential insect vectors of some fungal pathogens. For instance, the target fungal species may not be culturable (obligate symbiont) or, like in the case of *G. morbida*, be overgrown or out-competed by other beetle-associated fungi that are transported on the beetles' exterior or as endosymbionts. Our culture-based study revealed a high incidence of these rapid growing fungi. Based on colony morphology, we were able to distinguish *Aspergillus, Cladosporium, Fusarium, Penicillium, Trichoderma* among other generalist ascomycetes (*data not shown*). The latter was not surprising as a high diversity of fungi has been detected in TCD-compromised trees from both native and introduced regions (Gazis et al. 2018). The presence of potentially antagonistic isolates of *Trichoderma* spp. may also have restricted the in vitro growth of *G. morbida* (for discussion, see Gazis et al. 2018). Regardless, culture-based recovery efforts for the slow-growing *G. morbida* pathogen yielded few detections and it is considered inefficient, given the time, labor, and material resources invested. However, culture-based methods are the only ones that can confirm the viability of the inoculum.

In this study, molecular assays revealed the presence of *G. morbida* DNA on 11 insect species that may have subcortical associations with *Juglans* spp. hosts and that were active in the crowns of walnut trees in habitats containing TCD infested host plants (Table 2). Among these beetles, *G. morbida* was recovered from five exotic ambrosia beetle species and three ambrosia beetle species native to the eastern United States, as well as a species of native bark beetle, a native woodborer species, and one species of native bark dwelling weevil (Table 2). The fact that *G. morbida* was not detected on some insect species known to be associated with *J. nigra* (Table 3) may be explained, in part, to small sample sizes collected for those species (Table 2). *Geosmithia morbida* was not found on beetles collected in western U.S. forests following attempts to recover *Geosmithia* spp. on agar-based culture medium (Kolařík et al. 2017). The low detection power of culture-based methods is confirmed by comparing our culture-based methods versus DNA detection results. This difference in detection suggests that *G. morbida* might have been present on the beetle species assayed in Kolařík et al. (2017) study but was not detected due to the technique used to screen the beetles. The study reported here examined beetle species that were collected only from quarantined habitats where TCD has been documented. Huang et al. (2019) surveyed bark beetle species to document associations with other *Geosmithia* spp. in locations across the southeastern United States. Several species of *Geosmithia* were recovered, and these did not include *G. morbida* from the locations where bark beetles were trapped. It would be informative as a follow on to this study to expand trapping and *G. morbida* detection protocols into counties in Tennessee and adjacent states that containing walnut trees, but where TCD has not been documented. It is possible that *G. morbida* might be recovered more quickly by surveying for other insect species using the molecular approach employed in this report.

We caution that positive detection of *G. morbida* DNA does not provide sufficient evidence to support that the *G. morbida* propagule detected is viable or would be capable of initiating a canker if conveyed to tissues of a susceptible host plant. In other words, the insects we report to be carrying *G. morbida* DNA, that were recovered adjacent to *J. nigra* in TCD-compromised landscape and urban forest settings in eastern Tennessee, may not be competent or reliable vectors of *G. morbida* to *J. nigra*. However, *J. nigra* is reported as a reproductive host for eight of the 11 insect species that tested positive for *G. morbida* DNA (Table 3, after Atkinson 2018). Although most subcortical beetles interact preferentially with stressed or dead trees, many species can occasionally infest live, apparently healthy trees (e.g., Kühnholz et al. 2001), including several exotic bark beetle species that can attack healthy trees (Hulcr and Dunn 2011). Two related studies evaluated reproductive host plant capabilities for insects associated with *J. nigra* in Indiana and Missouri. Opportunistic beetle species that attacked and emerged from walnut trees were documented, including trees that had been stressed by girdling to simulate TCD symptoms (Reed et al. 2013, 2015). From these studies, *D. onoharaensum, X. saxesenii*, *X. crassiusculus*, and *X. germanus* have a broad host range that includes *J. nigra* (Reed et al. 2013) (Tables 2 and 3). *Xylosandrus germanus* seems to be more frequently associated with living *J. nigra* than *X. saxesenii* and *X. crassiusculus*, which preferred stressed trees (Weber and McPherson 1984, Reed et al. 2013). *Hylocurus rudis, M. fasciatum, M. mali, X. saxesenii*, and *X. germanus* have also been detected in high abundance in actively growing black walnut orchards in North Carolina and Illinois (Weber and McPherson 1991) and co-occur with walnut trees in eastern Tennessee (Klingeman et al. 2017).

Cnestus mutilatus, X. saxesenii, *X. crassiusculus*, and *X. germanus* are nonnative, polyphagous scolytine ambrosia beetle species that are recognized as emerging pests of trees, including *J. nigra*, in orchards, forests, landscapes, and nurseries (Weber and McPherson 1984, Kovach and Gorsuch 1985, Oliver and Mannion 2001, Oliver et al. 2012, Leavengood 2013, Klingeman et al. 2017). *Xyleborinus saxesenii* is one of the most destructive species in *Xyleborini* (Rabaglia et al. 2006) and infests both healthy and stressed trees (Kovach and Gorsuch 1985, Oliver and Mannion 2001). In Tennessee, a gradual increase in *C. mutilatus* abundance was observed between 2011 and 2013 (Klingeman et al. 2017). Females of these ambrosia beetles bore horizontally into sapwood to construct galleries where eggs are laid, hatched larvae continue boring and feed on ambrosia fungi growing on the walls of galleries (Weber and McPherson 1984, Kovach and Gorsuch 1985, Oliver and Mannion 2001, Oliver et al. 2012, Leavengood 2013). Newly emerged adults, mostly female overwinter in parent galleries and emerge from the galleries in favorable weather to start new infestations (Weber and McPherson 1984, Kovach and Gorsuch 1985, Oliver and Mannion 2001, Rabaglia et al. 2006, Oliver et al. 2012, Leavengood 2013).

Xylobiops basilaris, which was frequently collected in traps during 2016, is a polyphagous woodboring beetle species that is distributed across the eastern and southern United States (Fisher 1950). Adults bore into sapwood and make tunnels across the grain which result girdling of limbs and trunks of small diameter (Baker 1972). Larvae mostly feed on sapwood, bore along the wood grain, and overwinter in tunnels that are filled with fine, powder-like wood dust (Baker 1972). Although Juglandaceae (including hickory and pecan) and Ebenaceae (e.g., persimmon) are preferred hosts for *X. basilaris* (Baker 1972), this beetle has not yet been associated specifically with *J. nigra*. *Xylobiops basilaris* generally attacks damaged trees, but Ott (2007) found these beetles attacking recently flooded oak trees,

Table 3. Coleopteran species that yielded positive *Geosmithia morbida* diagnostic detections at locations within thousand cankers disease quarantine areas in Tennessee during 2016–2017, their native status within the United States with reported state and district distributions, feeding guilds, and family-level host plant range

Coleopteran species[a]	Native status in the United States[b]	Reported U.S. distribution[c]	Feeding guild[d]	Host plant families[e]
Bostrichidae: Bostrichinae				
Xylobiops basilaris[f]	Native	AR, DE, DC, FL, GA, IA, IL, IN, KS, KY, LA, MA, MD, MO, MS, NC, NY, OH, PA, SC, TN, TX, VA, WI, WV	BB	Anacardiaceae, Ebenaceae, Fabaceae, Fagaceae, **Juglandaceae**, Moraceae, Myrtaceae, Oleaceae, Pinaceae, Poaceae, Rosaceae, Rutaceae, Ulmaceae, Vitaceae
Curculionidae: Cossoninae				
Stenomimus pallidus[g]	Native	DC, IL, IN, MD, MO, OH, PA, VA	BW	Fagaceae, **Juglandaceae**[b]
Curculionidae: Platypodinae				
Oxoplatypus quadridentatus	Native	AL, AZ, AR, FL, GA, IN, LS, MD, MS, NC, OK, SC, TN, TX, VA, WA	AB	Fagaceae, Hamamelidaceae, Hippocastanaceae, Pinaceae
Curculionidae: Scolytinae				
Cnestus mutilatus	Exotic	AL, AR, FL, GA, IL, IN, KY, LA, MD, MS, NC, PA, SC, TN, TX, VA	AB	Aceraceae, Anacardiaceae, Betulaceae, Cornaceae, Cupressaceae, Fagaceae, **Juglandaceae**, Lauraceae, Melastomataceae, Oleaceae, Papilionaceae, Styracaceae, Theaceae
Dryoxylon onoharaensum	Exotic	AL, AR, CT, DE, FL, GA, IN, KY, LA, MD, MA, MS, NC, NJ, NY, OH, PA, SC, TN, TX, VA	AB	Aceraceae, Fagaceae, Magnoliaceae, Salicaceae
Hylocurus rudis	Native	AL, AR, DC, DE, FL, GA, IL, IN, IA, KS, KY, LS, MD, MI, MS, MO, NE, NJ, NY, NC, OH, OK, PA, SC, TN, TX, VA, WV	BB	Aceraceae, Betulaceae, Fagaceae, **Juglandaceae**, Magnoliaceae, Papilionaceae
Monarthrum fasciatum	Native	AL, AR, DE, DC, FL, GA, IA, IL, IN, KS, KY, LA, MA, ME, MI, MO, MS, NC, NE, NJ, NY, OH, OK, OR, PA, RI, SC, TN, TX, VA, VT, WI, WV	AB	Aceraceae, Fagaceae, **Juglandaceae**, Mimosaceae, Nyssaceae, Pinaceae, Rosaceae
Monarthrum mali	Native	AL, AR, CA, CT, DC, DE, FL, GA, IL, IN, IA, KS, KY, LA, MA, ME, MI, MN, MO, MS, NC, NE, NH, NJ, NY, OH, OK, PA, RI, SC, TN, TX, VA, VT, WI, WV	AB	Aceraceae, Betulaceae, Burseraceae, Cornaceae, Fagaceae, Hamamelidaceae, **Juglandaceae**, Mimosaceae, Nyssaceae, Oleaceae, Sapotaceae, Tiliaceae
Xyleborinus saxesenii	Exotic	AL, AR, AZ, CA, CO, CT, DC, DE, FL, GA, ID, IL, IN, IO, KS, KY, LS, MA, MD, MI, MO, MN, MS, NC, NE, NH, NJ, NM, NY, NV, OH, OK, OR, PA, RI, SC, SD, TN, TX, UT, VT, VA, WA, WI, WV	AB	Aceraceae, Actinidiaceae, Anacardiaceae, Annonaceae, Apocynaceae, Betulaceae, Casuarinaceae, Cornaceae, Cupressaceae, Ebenaceae, Ericaceae, Fagaceae, **Juglandaceae**, Lauraceae, Magnoliaceae, Meliaceae, Moiraceae, Myrtaceae, Pinaceae, Rosaceae, Salicaceae, Taxodiaceae, Tiliaceae
Xylosandrus crassiusculus	Exotic	AL, AR, CT, DE, FL, GA, IL, IN, KS, KY, LA, MA, MD, MI, MO, MS, NC, NE, NY, OH, OK, OR, PA, RI, SC, TN, TX, VA, WA	AB	Agavaceae, Anacardiaceae, Annonaceae, Apocynaceae, Arecaceae, Burseraceae, Caesalpinaceae, Cannabaceae, Capparaceae, Caprifoliaceae, Clusiaceae, Combretaceae, Convolvulaceae, Cornaceae, Cucurbitaceae, Dilleniaceae, Dipterocarpaceae, Ebenaceae, Eleaocarpaceae, Euphorbiaceae, Fagaceae, Hamamelidaceae, **Juglandaceae**, Lauraceae, Leguminosae, Lythraceae, Magnoliaceae, Melastomataceae, Meliaceae, Mimosaceae, Moraceae, Myristicaceae, Myrtaceae, Nolinoideae, Olacaceae, Papilionaceae, Phyllanthaceae, Pinaceae, Poaceae, Proteaceae, Rosaceae, Rutaceae, Salicaceae, Sapindaceae, Sapotaceae, Sterculiaceae, Styracaceae

Table 3. *Continued*

Coleopteran species[a]	Native status in the United States[b]	Reported U.S. distribution[c]	Feeding guild[d]	Host plant families[e]
Xylosandrus germanus	Exotic	AL, AR, CT, DE, FL, GA, IL, IN, KS, KY, LA, MA, ME, MD, MI, MO, MS, NC, NH, NJ, NY, OH, OR, PA, RI, SC, TN, TX, VA, VT, WA, WV, WI	AB	Aceraceae, Anacardiaceae, Betulaceae, Caesalpinaceae, Caprifoliaceae, Cornaceae, Cupressaceae, Ebenaceae, Ericaceae, Fagaceae, Hippocastanaceae, **Juglandaceae**, Lauraceae, Magnoliaceae, Moraceae, Myricaceae, Nyssaceae, Oleaceae, Pinaceae, Platanaceae, Rhamnaceae, Rosaceae, Salicaceae, Styracaceae, Taxodiaceae, Theaceae, Tiliaceae

[a]Coleopteran species that were carrying *G. morbida* in this study.

[b]Native status is from Wood (1982), and Rabaglia et al. (2006) except [f], which is from Fisher (1950) and [g], which is from Ciegler and Wheeler (2010).

[c]Distribution in U.S. states according to Atkinson (2018) except [f], which is from Fisher (1950) and [g], which is from Ciegler and Wheeler (2010).

[d]Feeding guilds (AB: Ambrosia beetle, BB: Bark beetle) are from Haack and Rabaglia (2013) except [f], which is from Fisher (1950) and [g], which is from Ciegler and Wheeler (2010).

[e]Host plant Families information is taken from Atkinson (2018) except [f], which is from Fisher (1950) and [g], which is from Ciegler and Wheeler (2010).

[h]**Juglandaceae** (in bold) is the plant Family containing *Juglans* spp. and other closely related plant genera. Where **Juglandaceae** is followed by [h], the reproductive host plant was specifically stated to include *J. nigra*.

Quercus alba L., and suggested that *X. basilaris* has been acquiring a more aggressive habit. Healthy growing hardwoods are also occasionally attacked by *X. basilaris* when in close proximity of heavily infested trees (Baker 1972). Results reported here from walnut habitats in Tennessee suggests that this species is interacting with living *J. nigra* under stress from TCD.

Monarthrum fasciatum, *M. mali* are native ambrosia beetles and host generalists among many hardwood species (Wood 1982) (Table 3). *Monarthrum fasciatum* was first reported from Indiana and Ohio in 2011 colonizing *J. nigra*. The same year, *M. mali* was reported infesting *J. nigra* in Indiana (Reed et al. 2015). Both *Monarthrum* spp. were also found from girdled *J. nigra* branches in East Tennessee (Klingeman et al. 2017). More than half of *M. fasciatum* specimens (15 of 27 individuals screened) tested positive for *G. morbida* DNA (Table 2). Finally, *Hylocurus rudis, O. quadridentatus* and *X. germanus* were other subcortically active insect species that were collected in small numbers during 2016 yet tested positive for presence of *G. morbida* DNA. *Hylocurus rudis* (Reed et al. 2015, Atkinson 2018) and *X. germanus* (Weber and McPherson 1984) have been previously reported to develop within *J. nigra*. *Hylocurus rudis* is native bark beetle that breeds in hardwood trees (Wood 1982) and hosts include *J. nigra* (Table 3). *Oxoplatypus quadridentatus* has not yet been reported to reproduce within *J. nigra* (Atkinson 2018). Biology, ecology of *O. quadridentatus* is poorly understood, with beetles often associated with *Quercus* spp. (Atkinson 2018). *Dryoxylon onoharaensum* is an exotic ambrosia beetle that has become well established in the southeastern United States (Atkinson 2018). The biological and ecological associations that are occurring within southeastern U.S. forest and landscape systems are not well documented. *Stenomimus pallidus* is a bark dwelling weevil native to the United States that lives and reproduces beneath dead bark of *Carya* and *Quercus* species, as well as wounded or stressed *J. nigra* trees (Ciegler and Wheeler 2010, Juzwik et al. 2015, Reed et al. 2015). *Geosmithia morbida* has also been isolated from individuals of *S. pallidus* that emerged from TCD symptomless *J. nigra* trees in Brown County, Indiana (Juzwik et al. 2015).

Most of these insect species would be attracted to volatile ethanol, including when used in traps to monitor for scolytine and other beetle presence (e.g., Klingeman et al. 2017). *Juglans nigra* is also capable of releasing ethanol from its leaves (Kimmerer and Kozlowski 1982), however, the conditions required to initiate ethanol release from walnut trees are not well articulated. Regardless, many of the infrequently collected scolytine beetle species are unlikely to be significant contributors to *G. morbida* spread due to the scarcity at which they are collected in traps (e.g., Klingeman et al. 2017). Still, we hypothesize that these scolytine, woodborer, and bark dwelling weevil species could have acquired *G. morbida* DNA if individual beetles interacted with tunnels or galleries excavated by walnut twig beetle, e.g., by boring through or transiting these spaces while creating or exiting their own reproductive galleries within infected walnut host plant tissues. Progenies of these subcortical insect species that emerge from parental galleries and developmental chambers could transmit fungal propagules to susceptible host species and sustain low levels of *G. morbida* infection within a compromised habitat.

In support of this hypothesis, for seven species caught in numbers that exceeded 25 individuals, *G. morbida* DNA was detected more than 40% of the time. Included among these were exotic *C. mutilatus*, *X. crassiusculus*, and *X. saxesenii* ambrosia beetle species. More than 60% of *X. basilaris* and *S. pallidus* individuals native to the eastern United States tested positive (Table 2). These findings are relevant in the eastern United States because previous studies from infested localities in east Tennessee indicate that walnut twig beetle populations are declining (Daniels et al. 2016). That the relatively low numbers of individuals examined means that the proportional incidence of *G. morbida* detected may be higher than would be encountered from many habitats. The sampling deployed also does not allow observation of seasonality regarding probability of species associations with *G. morbida*.

Colder winter temperatures in the eastern United States could also help limit geographical distribution and persistence of introduced walnut twig beetle populations in regions of native distribution of black walnut. Lower lethal temperatures of adult walnut twig beetle exposed to cold ranged from –14 to –23°C (Hefty et al. 2017). Based on 30-yr-mean annual extreme low temperatures recorded from the counties of eastern United States where walnut twig beetle has been detected (e.g., US-NWS 2018), Hefty et al. (2017) forecasted a range in annual overwintering mortality of between <50%, to up to 75% of walnut twig beetle populations in locations where lower lethal cold temperatures are reached. During winters from 2010/2011 (December 2010 to February 2011) to 2017/2018

(December 2017 to February 2018), lower lethal temperatures in this range were met in Knoxville, TN for brief periods during the winters of 2013/2014 and 2014/2015 (US-NWS 2018). Compared with trap captures in previous years, fewer walnut twig beetle were collected in traps deployed in eastern Tennessee during the 2014 and 2015 growing seasons (P. Lambdin, unpublished data). Similarly, following a colder than usual winter in Ohio (Marinaro et al. 2015), no individual walnut twig beetle were recovered in pheromone-baited traps during 2014 trapping in that state (Hefty et al. 2017). It is possible that these colder than usual winters have played a partial role in observed decline of walnut twig beetle populations in eastern United States. Other factors, including interaction of walnut twig beetle with natural enemies that have been observed in eastern Tennessee (Lambdin et al. 2015, W. Klingeman, unpublished data), would also effect *P. juglandis* populations in infested locations. Although cold tolerance is less understood for most of the other potential beetle vector species reported here (Table 3), *X. saxesenii* is more tolerant of cold than walnut twig beetle (Luna et al. 2013) and has continued to be collected in abundance in eastern Tennessee (Klingeman et al. 2017, W. Klingeman, unpublished data).

If subcortical insect species that are active in walnut habitats in the eastern United States carry *G. morbida* propagules within and between susceptible host plants, and can sustain TCD presence in infested habitats, then these species may be of regulatory interest as a potential threat to black walnut in its native range. A maintenance hypothesis for *G. morbida* is supported by observations in Brown County, Indiana, where the pathogen was recovered from an individual *S. pallidus*, yet efforts to capture *P. juglandis* at that location have continued to be unsuccessful (Ginzel and Juzwik 2014, Ginzel, personal communication).

Future research efforts by our team will be necessary to assess whether *G. morbida* cankers develop on *J. nigra* following visitation by *G. morbida*-contaminated individuals of these TCD vector-candidate species. If direct disease transmission by some of these species can be demonstrated, then the role that these potential alternative vectors may play in TCD epidemiology will need to be examined, particularly as they might influence future regulatory or quarantine restrictions to interstate and international movement of plants and wood products. Additionally, it would be important to quantify the relative conidia load of *G. morbida* on alternative candidate insect vectors, and to relate those findings to respective beetle size, as well as feeding, reproductive, and gallery formation behaviors within *J. nigra*.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

Acknowledgments

We thank Grace Pietsch (The University of Tennessee-Knoxville [UTK]) for technical assistance and help with the bioassays conducted at UTK. We are thankful to David Paulsen (UTK) for his assistance in the field, and to Sun Xiaocun (UTK) for statistical consulting and analyses. This research was conducted in partial fulfillment of a MS degree for K.C. from The University of Tennessee and was funded in part by the Woodtiger Foundation as part of the *Evaluating Impact of Thousand Cankers Disease on Black Walnut: Risk Assessment, Biological Control and Development of Novel Assessment Tools* project. Additional funding support was provided by USDA National Institute of Food and Agriculture Hatch project #1009630; TEN00495 and Cooperative Agreement 15-CA-11272139-050 between the USDA FS Pacific Southwest Research Station and The University of Tennessee.

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