Thousand Cankers Disease: Virulence of *Geosmithia morbida* isolates and potential alternative vectors of the fungus

A Thesis Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

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May 2018

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Dedication

To

My inspiration

The who inspired me to do my work, live peacefully and think emotionally for the goodness of everyone. I recall my Godman as food grower for the world and we need him three times a day – a Farmer! I am thankful especially to the ones who gave me values and sowed the seed of hard, honest and selfless work/service in me – my mom (Sardarni Sukhwinder Kaur Chahal) and dad (Sardar Jasdev Singh Chahal). I watched them closely and learned many lessons. The most important in my opinion- do your work with dedication without expecting a fruit of the outcome.

B.Sc. Agriculture (Hons.) 6 years program

That provided me the chance to explicit many knowledge and potential for joining one of the most prestigious Indian institute in agricultural sciences - Punjab Agricultural University (PAU), Ludhiana. I did get not only my bachelor's degree from here, but it also provided me the opportunities to become a handball athlete.

B.Sc. Agriculture (Hons.) 2009 batch (6 years)

Where, I met the most important people in my life. I will never forget the time and adorable memories I shared with them. Those 6 years were blessed to me; the most precious time in my life!

Acknowledgements

I would like to thank everyone who assisted me in this research project. I am grateful to my major adviser Dr. Mark T. Windham, for his mentorship and encouragement during the research project and academic courses. He has been an exceptional guide, editor and enthusiast for the research project. Also, I would like to thank to my committee members, Dr. William Klingeman, Dr. Paris Lambdin, Dr. Jerome Grant and Dr. Denita Hadziabdic for their assistance and worthy suggestions which helped me to complete the research project. It was my pleasure to work with all of you. A special thanks to Dr. Robert Trigiano for allowing me to use his lab equipment.

I am very grateful to Dr. Romina Gazis, post-doctoral fellow in Hadziabdic lab for her invaluable guidance during this research. I am thankful for her time and efforts to guiding me about new methodology and principles of novel methods and techniques. Special thanks to Windham lab managers, Lisa M. Vito, and Sara Collins for their help and training. I would like to thank Mr. Dave Paulsen for his assistance in field. I appreciate the help of Qunkang Cheng, Kristen Edds, Jordan Campbell, Patrick Daniel, Benjamin Meeks and Maxime Hanset. My heartiest thanks to Mr. Kevin Thompson (Director, Middle Tennessee Research & Education Center) and his friendly staff for their help in walnut wood collections. I appreciate the help of everyone from Entomology and Plant Pathology, UTK who shared their knowledge and ideas with me. I am thankful to my entire family and my all teachers for their guidance and support at every step of my life.

Abstract

The fungal pathogen, *Geosmithia morbida*, along with its vector, walnut twig beetle (WTB), *Pityophthorus juglandis* is the causal agent of the Thousand Cankers Disease complex (TCD) in *Juglans* spp. and *Pterocarya* spp. Disease outbreaks have been reported in the eastern and western United States (U.S.). Since TCD was detected in the western U.S. in early 2000s, the disease complex has spread to the eastern U.S. and Italy, Europe. In our preliminary data, *G. morbida* isolates from eastern and western states have been placed in five distinct genetic clusters, though geography was not correlated with origins of isolates of each cluster. In the eastern U.S., fungal pathogen had been detected on other insect species besides WTB. These insect species included two ambrosia beetles, *Xyleborinus saxesenii* and *Xylosandrus crassiusculus*, and a bark weevil, *Stenomimus pallidus*. Since TCD incidence and severity have been reported to be higher in the western U.S. states when compared to the eastern states, we hypothesized that differences in disease severity and incidence are due to variations in the fungal virulence at different geographical locations. To test our hypothesis, the proposed study had two specific objectives: 1) to determine if other wood boring beetle species could be potential vectors of *G. morbida* in Tennessee, and 2) to evaluate the level of virulence of five isolates from each of the five genetically distinct clusters of *G. morbida*. Our results indicated that eleven species of bark and ambrosia beetles were found to carry *G. morbida*. This finding raised concerns about the role these potential vector species play in the dissemination of the pathogen to healthy walnut trees. We also found that canker area was not correlated with geography or genetic clusters. The genetic Cluster 1 produced significantly larger cankers with the mean canker area 271 mm^2 (millimeter square) than isolates from the four other clusters. In addition, isolates from the same

genetic cluster exhibited significant variation in virulence. In conclusion, results from this study present potential implications to phytosanitary and quarantine efforts of TCD.

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1 Introduction

Thousand cankers disease (TCD) is a disease complex, resulting from the interaction of walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman, (Coleoptera: Curculionidae: Scolytinae) and canker causing fungal pathogen, *Geosmithia morbida* Kolarik, Freeland, Utley, and Tisserat, (Ascomycota: Hypocreales: Bionectriaceae) on susceptible hosts belongs to Juglandaceae, *Juglans* spp. L. and *Pterocarya* spp. Nutt. ex Moq (Hishinuma et al. 2016; Kolarik et al. 2011; Tisserat et al. 2009; Tisserat et al. 2011). The Juglandaceae constitute seven genera and 50 species distributed to the Americas, Asia and southeastern Europe (Manning 1978). Out of 21 *Juglans* spp. (Manning 1978), 16 *Juglans* spp. are native to the Americas (Manning 1978, Stone et al. 2009) and only six are endemic to the U.S. (Stone et al. 2009). These six native species are, *J. californica* S. Watson (Southern California black walnut), *J. cinerea* L. (butternut), *J. hindsii* Jeps. (Northern California black walnut), *J. major* Torr. (Arizona walnut), *J. microcarpa* Berland. (little walnut), and *J. nigra* L. (black walnut) (Stone et al. 2009). Many *Juglans* spp. including the six native species to U.S., showed various level of susceptibility to *G. morbida* inoculations (Utley et al. 2013). Black walnut, *J. nigra* was reported to be the most susceptible while fungal cankers produced on Arizona walnut, *J. major* were small and superficial (Utley et al. 2013). The authors also determined that three economically important, closely related hickory species, *Carya illinoinensis* (Wangenh) K. Koch, *C. aquatic* Nutt., and *C. ovata* (Mill) K. Koch, were immune to *G. morbida* inoculations (Utley et al. 2013). Although, many walnut species (*Juglans* spp., *Juglandaceae*) are primary host, three wingnut (*Pterocarya* spp., *Juglandaceae*) species, *Pterocarya fraxinifolia* (Poiret) Spach, *Pterocarya rhoifolia* Siebold and Zuccarini, and *Pterocarya stenoptera* C. de Candolle also serve as generic hosts for the pathogen and WTB (Hishinuma et al. 2016; Utley et al. 2013).

Black walnut mortality due to WTB feeding was reported in Utah in the mid-1990s (Tisserat et al. 201). These reports were followed by new discoveries in Oregon in 1997and New Mexico in 2001 (Tisserat et al. 2011; Daniels et al. 2016). An unknown fungal pathogen was isolated from WTB galleries located in phloem of dead black walnut trees in Colorado (Tisserat et al. 2009), and was subsequently identified as *G. morbida* (Kolarik et al. 2011). The report of TCD as insect-fungal complex, association of *G. morbida* with *P. juglandis*, was reported in Colorado in the early 2000s (Tisserat et al. 2009; Daniels et al. 2016). Since 2000s, TCD has been attributed to causing severe black walnut mortality in the western U.S. (Kolarik et al. 2011, Tisserat et al. 2009).

Geosmithia morbida is the first species in the genus reported to be plant pathogenic (Kolarik et al. 2011). Kolarik et al. (2011) specified three characters that distinguish it from other *Geosmithia* species: (1) thermotolerance of the pathogen (ability to grow at 37 ºC), (2) monilioid conidiophores with atypical branched base, and (3) lobed mycelial colonies resulting in white sporulation, and yellowish to tan colored appearance on the underside of the petri dish when grown on half strength PDA (hPDA) (Kolarik et al. 2011). Although thermotolerance is also found in two other *Geosmithia* species - *G. lavendula* and *Geosmithia* sp. 19 also, the cultures exhibit significant differences, including color and red spores on their cultured colonies (Kolarik et al. 2011). Optimal growth of *G. morbida* is at 31 ºC but reduced growth was also documented at 41 ºC. *Geosmithia morbida* is also able to grow on the high osmotic potential medium, Czapek-Dox Agar (CDA). *Geosmithia morbida* has also been reported to have a high level of genetic diversity and presence of different genetic clusters across wide geographical ranges (Hadziabdic et al. 2014; Zerillo et al. 2014). Sitz et al (2017) reported that virulence of *G. morbida* isolates were not determined by prior genetic groupings. They also reported that

Fusarium solani, a pathogen identified to co-infect TCD affected walnut trees (Tisserat et al. 2009), didn't yield synergistic affecting canker development when *G. morbida* was present (Sitz et al. 2017).

The WTB, is a small (~2 mm long) yellow to reddish brown, phloem-feeding bark beetle (Kolarik et al. 2011). These beetles are native to several western states including Arizona, California, New Mexico, as well as northern Mexico (LaBonte and Rabaglia 2012; Seybold et al. 2013). Hibernation of WTB can occur as larvae and adults within galleries found in host plants (Nix 2013). In Tennessee, the beetles emerge in late April and start tunneling new galleries for egg laying (Nix 2013). Rugman-Jones et al. (2015) reported two generic lineages of WTB with a high genetic diversity and proposed that there could be two morphological inseparable species.

Many symptoms of the TCD complex resemble symptoms associated with drought-stressed trees including wilting, yellowing (flagging) of upper canopy, followed by crown thinning and branch dieback, emergence of epicormic shoots from main stem, and WTB entrance/emergence holes in the outer bark (Tisserat et al. 2009; Tisserat et al. 2011). Internally, infections by *G. morbida* lead to numerous, small cankers that may coalesce. This proliferation of small cankers before the trees died led to the disease complex's name of Thousand Cankers (Tisserat et al. 2009). In the western U.S., TCD outbreaks caused death of many trees in 4-5 years after initial symptoms were observed (Tisserat et al. 2009). In the eastern U.S., these outbreaks have led to tree mortality but not at the rate or incidence as seen in the western U.S. (Klingeman, per. Comm.). However, in the eastern U.S., which is also native range of black walnut, TCD poses a serious threat since the value of timber is estimated at $568 billion (Newton et al. 2009). Although the disease complex threatens black walnut populations in the western and eastern regions of the U. S. (Grant et al. 2011; Kolarik et al. 2011; Tisserat et al. 2009), the

origin of TCD and much of the etiology and epidemiology of the pathogen remains ambiguous (USDA-FS-PPQ 2015).

In 2010, the first detection of TCD in the eastern U. S. (Knoxville, Tennessee) imposed a threat to urban and forest settings of black walnut within its native range (Grant et al. 2011). Since then, the TCD complex was reported from 6 other eastern states (IN, MD, NC, OH, PA, VA) (Daniels et al. 2016; Hadziabdic et al. 2014; Hansen et al. 2011; Seybold et al. 2013), as well as Italy, Europe (Montecchio and Faccoli 2014). The U.S. Forest Service Forest Inventory and Analysis (FIA) estimated that TCD was present in eastern U. S. for at least 10 years before the first report within the native range of black walnut (Randolph et al. 2013). However, the mortality of black walnut attributed to TCD has been very low in the eastern US as compared to mortality rates in western states (Newton et al. 2009; Randolph et al. 2013; Tisserat et al. 2011). Survey of black walnut canopies from the counties with known WTB infestations in the eastern U.S. revealed less than 5% mortality. In another study, black walnut trees that were reported to have TCD in Tennessee and Virginia had visual recovery from the disease (Griffin 2015). Griffin et al. (2015) observed individual symptomatic black walnut trees and reported static disease progression, however some of the trees recovered to normal growth over a time of three years. Differences in the disease incidence and severity within U. S. could be due to differences in pathogen virulence at different geographical locations. However, more studies are needed to make meaningful conclusions regarding potential recovery of TCD infested trees in the eastern U.S.

In 2014, the weevil species, *Stenomimus pallidus* Boheman was collected from a black walnut trees Indiana and *G. morbida* was detected on it (Juzwik et al. 2015). In 2015, two ambrosia beetles, *Xylosandrus crassiusculus* Motschulsky and *Xyleborinus saxesenii* Ratzeburg

and one weevil species, *S. pallidus* were reported to carry the fungus in Ohio (Juzwik et al. 2016). Other insect pests of black walnut could also be a concern to serve as potential alternative vectors of *G. morbida* (Dallara et al. 2012; Newton et al. 2009; Reed et al. 2015) although no other studies suggested likely candidates.

The objectives of this thesis were to determine if insect species other than WTB carry *G. morbida* on their bodies and to evaluate the virulence of isolates within previously identified *G. morbida* genetic clusters. To accomplish these objectives three hypotheses were tested: 1) insect species, other than WTB, that are associated with black walnut trees in Tennessee can carry propagules of *G. morbida* on their bodies, 2) isolates of *G. morbida* vary in virulence, and 3) geographic location and origin of *G. morbida* isolates play a role in in virulence.

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2 Association of *Geosmithia morbida* with Bark and Ambrosia Beetles Collected in the Canopies of Eastern Black Walnut*, J. nigra,* in East Tennessee

2.1 Abstract

The fungal pathogen, *Geosmithia morbida* Kolařík, Freeland, Utley, and Tisserat along with its vector, *Pityophthorus juglandis* (Blackman) are the causal agents of thousand cankers disease (TCD) in *Juglans* L. spp. and *Pterocarya* Nutt. ex Moq. spp. The disease has been reported from the eastern and western U.S. In the eastern U.S., *G. morbida* had been detected on other insect species, *Stenomimus pallidus* (Boheman), *Xyleborinus saxesenii* (Ratzeburg), and *Xylosandrus crassiusculus* (Motschulsky) in Indiana and Ohio. To better analyze the potential for other insect species to vector the fungus in east Tennessee, wood-boring beetles were collected using ethanol-lured traps. The traps were hung in the canopies of TCD-symptomatic trees. Two fungal detection methods were used, a culture based assay and a molecular based method. For the culture based assay, insects were washed in sterile water and the rinsate from individual insects was plated on half strength potato dextrose agar. Axenic *G. morbida* cultures were obtained for identification. For the molecular based method, *G. morbida* presence was validated by amplification of the previously developed species-specific microsatellite locus GS 004. Capillary electrophoresis was used to detect the amplified amplicons. 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), *X. saxesenii*, *X. crassiusculus* and *Xylosandrus germanus* (Blandford), *S. pallidus*, *Oxoplatypus quadridentatus* (Olivier), and *Xylobiops basilaris* (Say). These findings raise the awareness of alternative vectors that might contribute to the spread of *G. morbida* within the native range of eastern black walnut.

2.2 Introduction

Black walnut, *Juglans nigra* L., is an ecologically and economically important forest tree which is native to the eastern and midwestern U.S. (Grant el al. 2011; Manning 1978; Stone et al. 2009). The tree is highly prized for making veneer, furniture, cabinets and gun stocks (Goodell 1984). The nuts of black walnut are used for human consumption. The estimated value of standing black walnut in the U.S. is $568 billion (Newton et al. 2009). This important economic resource is under threat by an insect/fungal disease complex known as thousand cankers disease (TCD) (Kolarik et al. 2011; Tisserat et al. 2009, Grant et al. 2011). This insect/fungal disease complex can cause severe mortality of the black walnut, which is the most susceptible host (Kolarik et al. 2011; Tisserat et al. 2009; Utley et al. 2013).

In the last two decades, TCD has attributed to severe mortality and canopy die back of black walnut in the western U.S. (Kolarik et al. 2011; Utley et al. 2013). Thousand cankers disease complex involves a fungal pathogen, *Geosmithia morbida* Kolařík, Freeland, Utley, and Tisserat (Ascomycota: Hypocreales: Bionectriaceae), an insect vector, walnut twig beetle (WTB), *Pityophthorus juglandis* (Blackman) (Coleoptera: Curculionidae: Scolytinae), and the plant host, *Juglans* L. *spp*. and *Pterocarya* Nutt. ex Moq. spp. (Kolarik et al. 2011; Tisserat et al. 2009). TCD symptoms can be both external and internal. External symptoms include wilting, foliage chlorosis of the upper canopy (flagging), crown thinning and branch dieback, an emergence of epicormic shoots, and WTB entrance holes in the bark (Tisserat et al. 2009; Tisserat et al. 2011). Internal symptoms include numerous small cankers, and vertical and horizontal galleries produced by WTBs in the inner bark/ phloem (Kolarik et al. 2011; Tisserat et al. 2009; Tisserat et al. 2011). These numerous cankers can create large necrotic areas and girdle

the tree, which was the reason the complex was named Thousand Canker Disease (TCD) (Tisserat et al. 2009).

The vector, WTB, is native to western U.S., including Arizona, California, New Mexico and northern Mexico (LaBonte and Rabaglia 2012; Seybold et al. 2013). The first report of black walnut mortality associated to WTB was recorded in Espanola valley of New Mexico (Tisserat et al. 2011). However, in early 1990s, high occurrence of mortality of black walnut in Utah and Oregon was attributed to WTB, but that work was never published or recorded (Tisserat et al. 2011). In early 2003, black walnut mortality in Colorado was observed and WTB were collected from dying trees (Tisserat et al. 2009). *Geosmithia morbida* was isolated from the area surrounding WTB galleries (Cranshaw and Tisserat 2008; Tisserat et al. 2009) and formally described by Kolarik et al. (2011). *Geosmithia morbida* is considered a weak pathogen as it requires a vector to penetrate in the black walnut (Ginzel and Juzwik 2014; Kolarik et al. 2011). Walnut twig beetles carry and deposit conidia in the phloem and subsequently infest the tree (Cranshaw and Tisserat 2008; Tisserat et al. 2009).

Although *G. morbida* is documented to be vectored only by with the WTB, scolytid beetles associated with black walnut trees have been suspected as vectors of the fungus (Newton et al. 2009). In 2014, *Stenomimus pallidus* (Boheman) weevils were collected from a black walnut in Indiana and *G. morbida* was detected using both morphological and molecular methods (Juzwik et al. 2015). Since this collection, no WTB have been collected in subsequent studies (June 14, 2017). Two ambrosia beetles (*Xylosandrus crassiusculus* Ratzeburg and *Xyleborinus saxesenii* Motschulsky) and one weevil species (*S. pallidus*) were reported to carry the fungus at a site in Ohio (Juzwik et al. 2016).

Black walnut wood, veneer, lumber and logs have been exported from the U.S. to more than 45 countries (Utley et al. 2013). There is potential that *G. morbida* can spread to other countries from the U.S. with the distribution of black walnut wood. Black walnut insect pests other than WTB can also be infested with the fungus and help in pathogen spread to other countries where the fungus is not reported yet. Therefore, it is critical to determine if other insect species, including those found in Indiana and Ohio, can carry propagules of *G. morbida* to other areas where TCD is not established. This study was conducted to better understand *G. morbida* dissemination by woodboring insect pests of black walnut in east Tennessee.

2.3 Materials and Methods

2.3.1 Collection of Coleopteran insects from TCD-symptomatic black walnut canopies

Modified soda bottle traps were used to collect Coleopteran woodborers from the canopies of black walnut trees displaying symptoms characteristic of TCD infection (Daniels et al. 2016). Two-liter plastic soda bottles were cut, 5 cm above the bottle's bottom, to make two rectangular 15 cm x 6 cm windows at opposite sides. Insect collection bottles, with the bottoms replaced by a screen (10 x 10 cm, 75-mesh size) to allow precipitation pass through but insects couldn't escape from traps, were attached to spout end of soda bottles. Black walnuts bolts, of ca. 15 cm in length and 3 to 5 cm in diameter, were used as lure chambers. Bolts were obtained from black walnut tree in Cumberland county TN, outside of TCD quarantined areas. Each bolt was drilled, using a 19.05 mm diameter drill bit, to a depth of ~10 cm. To attract wood-boring insects, 20 to 22 ml of 95% ethanol were added to the chamber of each bolt (Oliver and Mannion 2001) and later closed using rubber cork stoppers (size 2, top diameter 20 mm, bottom diameter 16 mm, length: 2.5 cm). (Fig. 1). Lure filled black walnut bolts were hanged inside soda bottle traps with a rope and the completed traps were hung within canopies of black walnut trees. Traps

were placed at three locations in east Tennessee: 1) Burkhart Road, Knoxville (at a private property in northeastern Knox county, Lat. 36.079501, Long. 83.858278), 2) Lakeshore Park, Knoxville (a public park in south of Knox county, Lat. 35.922767, Long. 83.991813) and 3) Maryville College, Maryville (forest land north of Blount county, Lat. 35.748671, Long. 83.961779). At each site, traps were placed on one tree, in a set in two levels (upper and lower canopy) and in four directions (east, west, north and south), for a total of eight traps. A total 24 traps were used for the entire study, which resulted in eight traps being used per each of the three sites. Traps remained in the field from 1 April to 18 November in 2016 and were inspected 1-3 times a week, according to insect abundance. Under field conditions, beetles were retrieved from the collecting bottles using forceps and placed in sterile vials, bolts were refilled with lure, and traps were wiped with 95% ethanol. Once in the laboratory, insects were placed individually in 0.6 mL microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA, U.S.) and kept at 4 C° until processed for *G. morbida* detection. Across these sites, the Lakeshore Park, Burkhart Road and Maryville College locations differed in tree density (any tree species) and number of black walnut trees present where traps were installed. The tree density at each site was determined with visual observation. At each site tree density was based on how other trees (any tree species) were apart and also proximity of black walnut from the tree which was used to install traps. At Maryville College, a forest site, tree density was very high, tree trunks from any other tree species (except black walnut) were 3-4 meters apart and canopies were unbroken; you couldn't see through the canopies. Proximity of the tree which was used to install traps to the nearest stand of black walnut trees were 5 – 10 meters. At Burkhard Road site, tree density was less than as observed at Maryville College and canopies were broken; you could see through it. Other tree species were 10 to 15 meters from the black walnut tree which was used to install traps. There

were not as many black walnut trees surrounding the traps's tree as comparative to Maryville College site. Proximity of other black walnut trees from trap's tree is estimated 20 – 25 meters. The third location was, Lakeshore Park, a public park were individual trees from any species were very scattered. There were no mixed canopies from tree settings, individual trees were estimated to be 25 apart among other. Nearest black walnut from the tree which was used to install traps was 35 – 40 meters apart.

2.3.2 Identification of insects

Collected insects were identified to species using morphological characters and taxonomic keys (Hulcr and Smith 2010; Wood 1982). For difficult to identify beetles, molecular identification was utilized (Hebert et al. 2003). To extract genomic DNA from the beetles, the Thermo Scientific GeneJet Genomic Purification Kit (Fisher Scientific) was used following the manufacture's protocol with modifications to improve DNA recovery (Gazis et al. *submitted*). Modifications included the addition of 2.3 mm diameter Zirconia/Silica beads (BioSpec Products, Bartlesville, OK, USA) to the 2 ml conical screw-cap microcentrifuge tubes containing 180 µl digestion solution and the use of a Bead Mill 24 homogenizer (Fisher Scientific). The Bead Mill 24 homogenizer operated for 2 cycles, each for 30 s and a 3 min stop time between the two cycles. Continued shaking rotations of the Bead Mill 24 homogenizer at high speed allowed Zirconia/Silica beads to crush the beetle specimens into very fine particles, liberating the soft tissue rich in genomic DNA. Further modifications included the use of 40 µl of proteinase K per sample and an overnight incubation at 56°C. Finally, the elution buffer (70°C) was heated before eluting the samples and applied twice in volumes of 45 µl per sample, with a 5 min incubation in between. Final DNA solution (90 µl/sample) was stored at -20°C until PCR amplification was performed. The cytochrome oxidase c subunit 1 gene region of the mitochondrial (CO1) was

amplified and sequenced using the primer pair LCO1490F and HCO2198R (Folmer 1984) for a fragment length of ca. 650 bp. Each PCR reaction-mix (final volume 25 µl) contained 12.5 µL GoTaq®G2 Hot Start Master Mix (Promega Corp., Madison, WI, USA), 1.25 µL 10 mM reverse primer, 1.25 µL 10 mM forward primer, 1 µL dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA), 1 µl of genomic DNA (~ 25 ng/µL) and 9 µL double-distilled water. The PCR thermal cycle started with initial denaturation step of 2 min at 94°C followed by 5 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). Amplifications of 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, USA) was used to assess the quality of the chromatograms and assemble the strands into contigs. COI 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).

2.3.3 Screening of insects for *Geosmithia morbida*

2.3.3.1 Culture-based detection method

Distilled sterile water (200 µl) was added to each of 0.6 mL microcentrifuge tube containing an individual insect specimen. The tube was then shaken thrice for 15-18 seconds using a Thermolyne Maxi Mix II, type 37600 mixer (Barnstead/Thermolyne, Dubuque, IA, USA) at 300 rpm. The rinsate was drawn off from the tubes using a sterile 200 µl pipette and poured dropwise onto 100mm x 15mm Petri dishes containing 20 ml of half strength Difco™ Potato Dextrose Agar (hPDA) (Difco Laboratories, Detroit, MI, USA) amended with antibiotics

[30 mg/L of chlortetracycline HCL (Sigma-Aldrich, St. Louis, MO, U.S.) and streptomycin sulfate (Fisher Bioreagents, Fair Lawn, New Jersey, U.S.)]. Rinsate drops were streaked on the media with an inoculation loop. Petri dishes were allowed to set for 1-2 hours to let the water absorb into the medium, before they were wrapped with Parafilm® and incubated under 16/8 hours of light/dark at room temperature (25ºC). Petri dishes were inspected for fungal growth every 24 hours and once hyphae or yeast colonies resembling *G. morbida* (Kolarik et al. 2011) were noticed, subcultures were made until axenic cultures were obtained (Fig. 2). Further subcultures were made on the same hPDA medium but without antibiotics. For DNA extraction, axenic cultures were grown in full strength Difco™ Potato Dextrose Broth at room temperature (25°C) for 3 weeks. Mycelium was harvested, and genomic DNA was extracted using aforementioned protocol. The genome region was amplified using the primers ITS1F (Gardes and Bruns 1993) and ITS4R (White et. al. 1990). PCR mix protocol were the same as described above for insect samples. PCR conditions started with an initial denaturation step of 2 min at 94°C followed by 15 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 65°C and primer extension for 1 min at 72°C; followed by further 30 cycles of 30 s at 94°C, 30 s at 48°C and 1 min at 72°C; and followed by a final elongation for 10 min at 72°C. 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.

2.3.3.2 Molecular-based detection method

DNA was isolated from insect species stored individually in 0.6 mL microcentrifuge tubes at 4ºC, these specimens were not assayed for culture-based detections. The DNA isolations

were performed as detailed in section 2.2. Microsatellite primers GS004, specifically designed for *G. morbida* and currently being used as a rapid molecular detection tool (Hadziabdic et al. 2012; Oren et. al 2017), were used to confirm the presence of the pathogen. PCR reactions were set as follows: 5 µl of sterile water, 4 µl GoTaq®G2 Hot Start Master Mix, 1 µl of 10 µM of each primer, 0.5 µL dimethyl sulfoxide (DMSO) and 1 µl of DNA (undiluted). To avoid false negatives, three concentrations of undiluted DNA were used (1 µl, 3 µl and 5 µl) in three separate reactions. 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 on the QIAxcel Capillary Electrophoresis System (Qiagen, Germantown, MD, U.S.) using an internal 25 base pair DNA size marker. QIAxcel ScreenGel software operated the QIAxcel Capillary Electrophoresis System. Beetles were considered positive for presence of *G. morbida* when a peak with a length between 201-234 bp. (Hadziabdic et al. 2012) was obtained (Fig. 4a) and negative when there was no amplification (Fig. 4b).

2.3.4 False positives: Cross-species amplification of the GS004 microsatellite locus

Oren et. al. (2017) confirmed the specificity of the GS004 locus against one hundred ascomycetous fungi isolated from *P. juglandis* galleries in different species of *Juglans* from Tennessee and California. In addition, we tested the marker against 57 *Geosmithia* Pitt isolates obtained as part of a project conducted by members of our working group (Gazis et al. unpublished/preliminary data). To harvest mycelium, *Geosmithia* isolates were grown in full strength Difco™ Potato Dextrose Broth at room temperature (25°C) for 2 weeks. DNA

extractions from the fungal isolates, PCR reactions and amplicon analyses were conducted using the above-mentioned protocols.

2.3.5 Screening of insect traps for *Geosmithia morbida*

To verify that field traps were not being contaminated with *G. morbida* from non-insect sources such as rain splash or air currents, one trap from each of the three sampling locations (Lakeshore Park, Maryville College and Burkhart Road) was randomly selected for *G. morbida* detection. Traps had been in the field from 1 April to 9 September in 2016. We tested two sections of the traps: 1) the spout end of the soda bottles (internal circumference near neck) where beetles hit before falling into the collection bottle, and 2) the mesh screen of the collection bottle where trapped insects lie and wind and precipitation pass through. The spout part of the cut soda bottles was rinse with sterile water and a sterilized cotton swab were used to rub the internal circumference until 25 ml of washout was collected in Falcon tube (Fisher Scientific). The bottom screen of the collection bottle was cut off from using sterile scissors and chopped into smaller pieces. The procedure was done under the hood and chopped pieces were placed inside sterile petri plates and then transferred to Falcon tubes and added 25 ml of sterile water (Fig. 5). A total of six samples were obtained, two samples from each trap; one sample from soda bottle spout end and another one from the collection bottle screen. Falcon tubes were centrifuged at 5000 rpm for 20 mins using a centrifuge (Sorvall RC-6 plus, Thermo Fisher Scientific Inc., Waltham, MA, U.S.) to settle down impurities/screen parts. After centrifugation two replicate samples (500 µl each) were taken separately from each of six 6 samples/Falcon tubes. DNA extraction protocol, PCR amplification and final detection was obtained following previously described protocols.

2.3.6 Establishment of positive and negative controls

Three separate experiments were performed to establish a positive control for our *G. morbida* detection test from traps. In other words, we wanted to be sure that if rain or water were carrying *G. morbida* propagules into the traps, our method was able to detect them. Experiments were performed separately and inside the laboratory (due to biological containment level 2 regulations on *G. morbida*) under a laminar flow hood. In the first experiment, spores and conidiophores of a sporulating *G. morbida* colony (8 wk old) were retrieved with a sterile cotton swab and rubbed on a 75-mesh size (0.18 x 0.25 mm) net screen (same mesh size screen used as collection bottle bottoms for field installed). In the second experiment, a 75-mesh size net screen (10 x10 cm) was placed on a sterile petri plate (10 cm dia., 1.5 cm depth) and a Petri dish containing a sporulating *G. morbida* colony (8 wk old), facing downwards, was tapped three times from a distance of 20 cm (Fig. 7) over the screen. The third experiment, was similar than the second one but the *G. morbida* cultures was tapped only once. Screens from the three experiments were processed for DNA extraction as indicated in the previous section.

Three modified traps were used as negative controls and installed on the trees (one trap per location) used to collect insects from 23 September to 21 October in 2016. Modifications included the addition of two net screens (0.18 x 0.25 mm) glued to either side of the trap's windows. This prevented the access of insects to the inside of the traps but allowed the entrance of air and rainwater. Therefore, allowing access to airborne and water-dispersed fungal propagules (Fig. 6). These traps were examined with the same protocol used in section 2.5, and were expected to be negative for the presence of *G. morbida*.

2.4 Statistical analysis

Incidence data was analyzed to measure the percentage of *G. morbida* carrying individuals from each insect species and compared among beetle species to elucidate if there is a species/s, from the local pool, that is more prone to carry *G. morbida*. Interaction between beetle species (*Cnestus mutilatus* Blandford, *X. saxesenii*, *X. crassiusculus*) and collection locations was also inspected to find potential relation between origin of the sample and *G. morbida* incidence. The logistic regression (binary) analyses were conducted for pairwise comparison of two variables (insect species and collection locations) using logistic procedure in SAS 9.4 TS1M3 (Windows 64x version, SAS Institute Inc., Cary, NC). Significant effects were identified at $P<0.05$. The *P* values are listed from Wald's chi square test, a pre-step for determining bivariate analysis with logistic regression. In our statistical analysis, we did not detect a significant relation from pairwise interaction of two variables using binary logistic regression. Hence, *P* values for non-significant differences were taken from Wald's chi square analysis.

2.5 Results

False positives: Cross-species amplification of the GS004 microsatellite locus

To determine if the GS004 microsatellite locus would test positive for other *Geosmithia* species, a collection of genetically diverse *Geosmithia* species (Table 4) as described in Gazis et al. (*in prep*) were screened with this satellite marker. None of the tested *Geosmithia* species demonstrated cross-transferability of 'GS 004' microsatellite locus. Therefore, we were able to use this marker to screen for *G. morbida* on insects collected in this study.

Screening of insect traps, positive and negative controls for *Geosmithia morbida*

Positive and negative controls (as described in materials and methods) were used to determine if detection of *G. morbida* on collected beetles was due to contaminated traps. Using *G. morbida* species-specific microsatellite locus GS004, we confirmed the absence of *G. morbida* in the field installed traps. All representative samples, four from each trap (two from spout end and two of mesh screen), showed negative detections for the fungus. In the same manner, modified traps that were installed in the field to serve as negative control were also negative for fungal DNA. On surface-contaminated, positive control traps, fungal DNA was detected from all trap portions, so served as positive experimental control, confirming that *G. morbida* DNA could be detected from the soda bottle trap parts.

Culture-based detection method

A total of 1,596 wood-boring insects were screened to detect *G. morbida* with culture-based detection method. Out of 1,596 screened specimens, *G. morbida* was recovered from just one individual rinsate sample, and that specimen yielded two colonies of *G. morbida* on hPDA. The ITS sequences generated from isolates GM-TN-SP1 and GM-TN-SP2 were deposited in GenBank database, and given accession numbers MG008847 and MG008848, respectively.

Molecular-based detection method

Incidence data was analyzed to measure the percentage of individuals of 18 insect species collected, yielded positive detections for *G. morbida* DNA. Positive individuals out of total analyzed for each of 11 species were *C. mutilatus* (28/67), *Dryoxylon onoharaensum* Murayama (11/26), *Hylocurus rudis* LeConte (1/3), *Monarthrum fasciatum* Say (15/27), *Monarthrum mali* Fitch (5/15), *X. saxesenii* (45/90), *X. crassiusculus* (22/45), and *Xylosandrus germanus*

Blandford (6/8). Additionally, *G. morbida* DNA was recovered from *S. pallidus* (18/27) (Coleoptera: Curculionidae: Cossinae), *Oxoplatypus quadridentatus* Olivier (2/2) (Coleoptera: Curculionidae: Platypodinae), and *Xylobiops basilaris* Say (34/54) (Coleoptera: Bostrichidae). Specimens of *C. mutilatus, X. saxesenii,* and *X. crassiusculus* were collected proportional to the collections locations (Table 4). Interactions compared among beetle species to elucidate if there is a species/s, from the local pool, that is more prone to carry *G. morbida*. This pool of beetle species includes *C. mutilatus, X. saxesenii, X. crassiusculus* and collection locations was also inspected to find potential relation between origin of the sample and *G. morbida* incidence. The binary logistic regression analysis was conducted to determine differences in any two variables that were being compared. But none of the comparison was significant so values of Wald's Chi square were reported, which is pre-step to determine if LOGISTIC procedure results are significant or not before reporting a value of the interaction between to variables. Analyzed specimens of *C. mutilatus, X. saxesenii,* and *X. crassiusculus* indicated that these insect species shared a similar likelihood that either species would be carrying the fungal pathogen if trapped within a TCD-compromised walnut habitat (X^2 = 1.267, df = 2, P = 0.530). In fact, these three beetle species shared a similar likelihood that they could be carrying *G. morbida,* regardless of collection location. The three collection locations also responded in the same manner for the positive incidence of *G. morbida* on combined individuals of these tree species respective to the locations (X^2 = 0.3887, df = 2, P = 0.823). Though, the collection locations were different in tree density, number of black walnut trees surrounding the black walnut tree where traps were located, but incidence of the combined, fungus carrying individuals of *C. mutilatus, X. saxesenii, X. crassiusculus,* were same among three locations. The interactions within specimens of an

insect species for two locations and between insect species for a location yielded similar responses related to *G. morbida* incidence (X^2 = 5.335, df = 4, P = 0.254).

2.6 Discussion

False positives: Cross-species amplification of the GS004 microsatellite locus

Microsatellite primers GS004 (Hadziabdic et al. 2012) are specific to *G. morbida* and are currently being used in rapid molecular detection of the pathogen (Oren et. al 2017). In previous efforts, the species-specificity for the *G. morbida* locus GS004 was assessed as a step taken to minimize false positive samples. Potential for cross-amplification of the locus was tested against 57 *Geosmithia* isolates described in Gazis et al. (unpublished/preliminary data). The locus did not amplify with any of the *Geosmithia* species. Oren et al. (2017) also confirmed the specificity of the locus against one hundred taxonomically diverse Ascomycota lineages that were isolated from WTB galleries or lesions within black walnut phloem.

Screening of insect traps, positive and negative controls for *Geosmithia morbida*

Typically, *G. morbida* is vectored to a suitable host plant by WTB resulting in infection of host phloem and it is not reported to have a free-living stage outside of its hosts (Tisserat et al. 2009). Although, *G. morbida* is not expected to be distributed by wind, it is possible that rain-splash or bark decomposing from infected tree tissues could result in localized pathogen movement beyond beetle galleries. Consequently, spores from plant pathogens could be detected and quantified on surfaces where propagules collect (Aylor 1998). To test for this concern, and to limit the likelihood that beetles were cross-contaminated within the trap collection chamber, the inner surfaces of field installed traps and trap collection cups were analyzed for both screened and un-screened (exclusion) traps. All trap surfaces tested negative for detectable

presence of *G. morbida* DNA. *Geosmithia morbida* detections from positive control (insect collection cup screens, surface-contaminated) traps confirmed that the pathogen could be successfully recovered and amplified from contaminated trap surfaces.

Culture-based detection method

A total of 1,596 wood-boring insects were screened to detect *G. morbida* with culture-based detection method. *Geosmithia morbida* was recovered from only one specimen-*Stenomimus pallidus.* There could be many factors responsible for extremely low efficiency of this method comparative to molecular detection. One of the most likely explanations is that the pathogen is outcompeted by ubiquitous fungi associated with insect species (Fig. 8). Morphological identifications of some of the identified fungi on artificial medium showed presence of ophiostomatoid species and included the hyphomycetes *Aspergillus, Cladosporium*, *Fusarium, Penicillium,* and *Trichoderma* (personal observation). The presence of antagonistic fungi, *Trichoderma* spp., might have yielded detrimental effects on the growth of *G. morbida* (Gazis et al. in progress). *Geosmithia morbida* also grows slowly because of its pleomorphic characters of forming a yeast phase before growing into filamentous stage (Kolarik et al. 2011). *Geosmithia morbida* slower growth rate combined with the rapid growth rate of other fungi, such as ophiostomatoid and the hyphomycetes growing from beetle rinsates, could make it impossible to discern the presence of *G. morbida*. Moreover, the lack of a selective medium for detecting *G. morbida* made recovery of the fungus difficult from mixed mycelia of other fungi. Also, beetle rinsate could not have reached spores if they were transmitted endozoically (when spores are carried by insects inside their special body structures such as mycangium). *Geosmithia morbida* was not found on beetles in western U.S. using culture based-method (Kolarik et al. 2017), however, the above-mentioned factors such as being overgrown by other fungi, lack of a

selective medium, and inability of a culture based method to access *G. morbida* spores when transmitted endozoically by insects, may play a role for the lack of fungus presence in their study and very low detection incidence in this study. Because culture based detection method had a number of limitations, further diagnosis on insect species was completed with only the molecular-based method.

Detections of viable or non-viable *G. morbida* propagules

Using culture-based methods for detecting *G. morbida* carried by beetles, we were able to recover the alive pathogen from *Stenomimus pallidus*. Propagules carried in this manner may be abundant enough to induce a canker on a suitable host plant after visitation by the insect. Positive confirmations for *G. morbida* are also reported for ten different bark and ambrosia beetle species. Therefore, other insect species that were positive for *G. morbida*, might be carrying viable fungal propagules. However, positive molecular detection may not be sufficient evidence to support that *G. morbida* inoculum is present at a sufficient density to result in canker formation.

Molecular-based detection method

Geosmithia morbida was detected on 11 species of wood-boring beetles (Table 1). Many non-native, invasive species of bark and ambrosia beetles have been introduced into the continental U.S., and are a serious threat to native forest health (Aukema et al. 2010; Haack 2006). Alongside their fungal symbionts, previous studies also reported lateral transmission of tree pathogens by many of these bark and ambrosia beetles (Alamouti et al. 2009; Carrillo et al. 2014; Gibbs 1978). *Cnestus mutilatus*, *X. saxesenii* and *X. crassiusculus* are exotic species of ambrosia beetles and recognized as emerging pests of trees in forest settings, landscape, and in nurseries (Solomon 1995; Hudson and Mizell 1999; Oliver and Mannion 2001; Oliver et al.

2012; Weber and McPherson 1983). *Xylosandrus crassiusculus*, *C. mutilatus*, *X. saxesenii* were first time reported in South Carolina in 1974 (Anderson 1974), Mississippi in 1999 (Schiefer & Bright 2004), and in California in 1911 (Rabaglia et al. 2006; Haack and Rabaglia 2013), respectively. These non-native, polyphagous scolytine species have been spread in southeastern U.S. where they were reported to damage host tree species in forests and nurseries (Solomon 1995; Hudson and Mizell 1999; Oliver and Mannion 2001; Oliver et al. 2012; Weber and McPherson 1983). *Xyleborinus saxesenii*, which is one of the most destructive species in Xyleborini (Rabaglia et al. 2006), infests both healthy and stressed trees (Kovach and Gorsuch 1985; Oliver and Mannion 2001). *Xylosandrus crassiusculus* can infest more than 200 tree species including angiosperms and gymnosperms growing in nurseries and forests (Kovach and Gorsuch 1985; Oliver and Mannion 2001). Pest potential of *C. mutilatus* in the U.S. is not well understood (Schiefer & Bright 2004), but this species may become an important pest of tree settings in forest, landscape and nurseries (Schiefer and Bright 2004; Oliver et al. 2012; Leavengood 2013).

In this study, the incidence of *G. morbida* was similar for *C. mutilatus*, *X. saxesenii*, and *X. crassiusculus*. For any particular location, comparable response of these insect species for carrying the fungus were the same (see results), though all three locations were distinct in response to tree species density and number of black walnut trees (see materials and methods). For these three beetles, similarity results for *G. morbida* detection is probably due to their similarities for gallery formation, depth of constructed galleries, laying eggs and cultivation of ambrosia fungi. Individual beetles may become contaminated with *G. morbida* while boring through host phloem which had TCD cankers. The three collection locations yielded the same positive incidence of the fungus from the combined individuals of *C. mutilatus*, *X. saxesenii*, and

X. crassiusculus for perspective location (X^2 = 0.3887, df = 2, P = 0.823). These insect species may have same attractions for black walnut volatile, as black walnut reported to release ethanol from leaves (Kimmerer and Kozlowski 1982). Selection of host and invasion responses of *X. crassiusculus* led to a preference for ethanol releasing trees (Ranger et al. 2013).

Relatively high number of *X. saxesenii* and *X. crassisusculus* individuals were collected in Tennessee, and a gradual increase in abundance of *C. mutilatus* were reported from 2011 to 2013 (Klingeman et al. 2017). These three insect species have different preferences for branch diameter size, altitude of tree canopy for infestations on host species (Klingeman et al. 2017; Reding et al. 2010; Stone et al. 2007). *Cnestus mutilatus, X. saxesenii* and *X. crassiusculus* have different habitat preferences on the same host tree, which could help in reducing interspecific competition by partitioning the resources; and can benefits to build large populations in low competition for existence. High trap catches of these insect species from black walnut canopies in Tennessee indicate that *C. mutilatus, X. saxesenii* and *X. crassiusculus* could serve as competent vectors of *G. morbida* (Klingeman et al. 2017).

In the TCD complex, *G. morbida* is transmitted by the phloem-feeding WTB. This study reveals that *G. morbida* was carried by five species of exotic ambrosia beetles, three species of native ambrosia beetles, two species of native bark beetles, and one species of native bark weevil (Table 1). U.S. native *S. pallidus* weevils (66% or 18 out of 27) were found to carry *G. morbida*. *Stenomimus pallidus* live and reproduce beneath dead bark of *Carya, J. nigra* and *Quercus* species and in stressed black walnut trees (Ciegler and Wheeler 2010). Among the trap locations, most *S. pallidus* individuals (20 out of 27) were collected from the Maryville College site traps and *G. morbida* was confirmed from 12 individuals. This site was in the edge of a natural forest urban interface where many TCD symptomatic and dead black walnut trees were observed.

Stenomimus pallidus may prefer to inhabit stressed or died black walnut trees than healthy growing black walnut trees. At the second trap location, Lakeshore Park which is a managed public park with very low tree species density but has scattered *J. nigra* trees, no *S. pallidus* specimens were collected from this site. At the third location on Burkhart Road, had intermediate in tree density and tree species composition than the Maryville College and Lakeshore Park sites, five individuals of *S. pallidus* were collected. At Burkhart Road farm site, symptomatic trees were observed adjacent to the tree beneath which the live traps were suspended.

Thirty-four of 54 specimens of, *Xylobiops basilaris*, tested positive for presence of *G. morbida* DNA. This polyphagous bark beetle species is well distributed in the eastern U.S. (Fisher 1950; Solomon 1995). Juglandaceae (hickory, pecan) and Ebenaceae (persimmon) are preferred hosts (Baker 1972; Crandall and Baker 1950; Solomon 1995), but the beetle has not been documented on black walnut. This beetle is reported to inhabit dead trees but has been found attacking newly flooded *Quercus alba* L. trees (Ott 2007). Interactions between *X. basilaris* with black walnut are not understood and its threat as a vector may be low.

Geosmithia morbida was also reported from other 4 species of non-native ambrosia beetles. *Cnestus mutilatus*, *Dryoxylon onoharaensum*, *Xyleborinus saxesenii*, and *Xylosandrus crassiusculus* which have broad host range that includes black walnut (Reed et al. 2013; Solomon 1995) (Table 4). Colonization behavior of these polyphagous, exotic ambrosia beetles on stressed black walnut in IN, MO and OH suspected they might spread *G. morbida* propagules (Juzwik et al. 2016; Reed et al. 2015). *Cnestus mutilatus*, *D. onoharaensum*, *X. saxesenii*, and *X. crassiusculus* were also collected from black walnut canopies in east Tennessee (Klingeman et al. 2017). *Monarthrum fasciatum* was first reported from Indiana and Ohio in 2011 in black walnut. The same year, *M. mali* infestations on black walnut in Indiana were reported (Reed et

al. 2015). Both *Monarthrum* species were also found from girdled black walnut branches in east Tennessee (Klingeman et al. 2017). *Hylocurus rudis, O. quadridentatus* and *X. germanus* were other insect species that were collected in limited numbers but yielded positive *G. morbida* detection. *Hylocurus rudis* (Atkinson 2012; Reed et al. 2015) and *X. germanus* (Weber and McPherson 1984) have been previously reported to infest black walnut. However, *O. quadridentatus* has not been reported to infest black walnut (Atkinson 2012). Some insect species resulted in no detection for *G. morbida*, which could be due to small sample sizes of collected specimens (Table 2).

Trap collections across time from infested localities in east Tennessee suggest that populations of *P. juglandis* are in decline (Daniels et al. 2016). It is possible that other beetle species might serve as alternative vectors in the absence of WTB, or could perpetuate distribution of the pathogen to other host trees within an environment. This hypothesis is supported by observations in Brown County, Indiana where *G. morbida* was reported on *S. pallidus*, yet *P. juglandis* has not been detected (Ginzel and Juzwik 2014). WTB is only restricted to TCD susceptible hosts, *Juglans* spp. and *Pterocarya* spp. (Hishinuma et al. 2015; Utley et al. 2009). However, these bark and ambrosia beetles reported in this study to have *G. morbida* on them are polyphagous and can reproduce within broad ranges of hosts (Table 3). *Geosmithia morbida* contaminated insects could lead to the transfer of *G. morbida* to potentially new trees that could host the fungus. *Geosmithia morbida* could have a pathogenic or symbiotic relationship with any of the tree species on which the beetle species feed and reproduce. Even if *G. morbida* is isolated from the insect species (Table 1) where WTB is located, other insect species could be potential additional vectors which could enhance the spread of *G. morbida*. The bark and ambrosia beetles could bore through WTB galleries with *G. morbida* and become

infested. However, lateral transfer of *G. morbida* to other insect species is less likely than if the pathogen of TCD was a systemic and colonized the entire vascular system. However, due to the thousands of cankers associated with TCD in infected trees, the disease, though not systemic, may mimic the effects of a systemic infection.

Frequent occurrence is not an only criterion to judge if an insect carrying a pathogen could vector it to susceptible host(s) (Table 1). A carrier organism should follow four principles originally described by Leach to determine its role as the vector (Leach 1940). Leach's first principle indicates that the insect species should have a close association with the diseased plants. Band ambrosia beetles are attracted to stressed trees which could be due to disease or abiotic factors or both disease and abiotic factors. Reed et al. (2015) investigated if diverse species of wood-borers could colonize a stressed black walnut trees simulating the conditions with TCD infected trees. Insect species reported here were also collected from stressed trees in Indiana and Missouri (Reed et al. 2015). Juzwik et al. (2016) also found equivalent results and collected eight species of bark and ambrosia beetles from symptomatic trees in Ohio and three of the insect species were carrying *G. morbida* (Juzwik et al. 2016). *Stenomimus* pallidus was previously reported to infest wounded (Juzwik et al. 2015) and stressed (Reed et al. 2015) black walnut trees. Other bark and ambrosia beetles are also attracted to stressed or TCD symptomatic black walnut trees (Reed et al. 2013; Reed et al. 2015). Juzwik et al. (2015) and Reed et al. (2015) speculated these beetles may contribute to the spread of TCD when these beetles are associated with *G. morbida* infected trees.

Leach's second principle demonstrates that a vector must visit/interact with the healthy plant species. Although most wood-boring beetles are thought to interact with stressed or dead trees with rare cases to infest living trees (Kuhnholz et al. 2001), exotic species are known to

attack healthy trees (Hulcr and Dunn 2011). Scolytid beetles were also reported to attack healthy chestnut in Tennessee (Oliver and Mannion 2001) and *C. mutilatus* is emerging as a nursery pest in the state (Oliver et al. 2012). *Cnestus mutilatus* has been reported to attack black walnut and also collected from the canopies of black walnut trees in Tennessee (Klingeman et al. 2017: Oliver et al. 2012). Three ambrosia beetles, *X. saxesenii*, *X. crassisuculus* and *X. germanus* are known to attack alive black walnut (Reed et al. 2013). *Xylosandrus germanus* is more associated with living black walnut trees comparative to *X. saxesenii* and *X. crassiusculus* (Reed et al. 2013; Weber and McPherson 1984). *Xylobiops basilaris* is previously known to attack dead trees, but have also been found attacking recently flooded oak, *Q. alba*, trees (Ott 2007). Ott (2007) suggested that *X. basilaris* has been acquiring a more aggressive habit to attack living, stressed trees. Results in this study also indicate that this species could be attacking living, stressed black walnut trees.

According to Leach's third rule, the disease-causing organism must be associated with the insect. In this study, among the reported species individuals from six species were carrying the fungus ≥ 50 % cases (Table 1). Most of the insect species (Table 1) follow Leach's first three rules in order to be potential alternative vectors for *G. morbida*. However, Leach's fourth rule for insect transmission of a plant pathogen needs to be demonstrated. According to fourth rule, the disease must be developed on plant host after visitation of insect species that are known for carrying the pathogen (Leach 1940). To address Leach's fourth rule, future studies will include exposing black walnut trees to potential alternative vectors in controlled environmental conditions with adequate checks. If symptoms of the pathogen appear following infestation of potential alternative vectors, the next step will be to isolate the pathogen from the host to validate Koch's postulates. Juzwik et al. (2015) speculated that these pathogen-carrying beetle species

may exacerbate the disease symptom progression in TCD infested areas. However, additional research is required to understand the role of these potential alternative vectors in TCD epidemiology.

Due to the relatively high number of collected individuals of *X. basilaris, S. pallidus, C. mutilatus, D. onoharaensum, M. fasciatum, X. saxesenii*, and *X. crassiusculus* that were positive for *G. morbida,* these species may play an important role in dissemination of the fungus from one location to another. This movement of the pathogen, associated with the TCD complex, via other potential insect vectors may make quarantines for just WTB is not insufficient to stop the movement of *G. morbida* in the native range of black walnut.

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3. Virulence of *Geosmithia morbida* isolates from the eastern and western United States

3.1 Abstract

The Thousand Cankers Disease (TCD) complex involves interactions between a fungal pathogen *Geosmithia morbida* Kolarik, Freeland, Utley, and Tisserat, the principal insect vector *Pityophthorus juglandis* (Blackman), and susceptible *Juglans* L. spp. and *Pterocarya* Nutt. ex Moq. spp. plant hosts. The disease was first documented from the western U.S. in 2007 and now has been spread to nine western and seven eastern states, as well as northern Italy. The disease has devastated landscape plantings of eastern black walnut in the urban areas of western states. In the eastern states, black walnut tree mortality has not been as severe as reported from the western states. Population genetics studies have revealed a high level of genetic diversity for *G. morbida*. To determine if differences in disease mortality could be due to the variations in pathogen virulence, five isolates from each of five genetic clusters (n=25 isolates) were tested for virulence. All isolates were inoculated on black walnut bolts, 2 - 3 cm diam. and 8 cm length. Each of *G. morbida* isolates and negative and positive controls were inoculated twice on opposite sides of black walnut bolts and replicated 6 times. The experiment was repeated once. Area of the individual cankers, developed by *G. morbida* isolates and negative control were measured at 7 days after inoculation. Significant differences in canker areas produced by fungus isolates from different genetic clusters were observed. The largest cankers were produced by fungal isolates of a genetic cluster designated as Cluster 1 with a mean canker area of 271 mm^2. Mean cankers areas produced by *G. morbida* isolates of Cluster 1 were significant larger than mean canker areas produced by the isolates of any other cluster. The mean canker areas produced by *G. morbida* isolates in Clusters 2, 3, 4, 5 were 185 mm^2, 165 mm^2, 160 mm^2 and 134 mm^2, respectively. The mean canker areas developed by *G. morbida* isolates from Clusters 3, 4, 5 were not different from each other. The mean canker area produced by *G. morbida* isolates of Cluster

2 were larger (t = -3.46; df = 35; *P* = 0.022) than mean canker area produced by fungal isolates of Cluster 5 but were not different from those produced by Clusters 3 and 4. (t = 1.31; df = 35; *P* = 0.844 and t = -1.51; df = 35; *P* = 0.734, respectively). For Clusters 2, 3, 4, and 5 significant differences in canker areas were observed for isolates within a cluster. However, no differences in canker areas developed by the isolates of Cluster 1 were observed. Canker areas from the isolates of the eastern and western U.S. were not different (t = 0.20; df = 10; *P* = 0.848) with the mean canker area 187 mm^2 and 182 mm^2, respectively. However, *G. morbida* isolates from the different U.S. states developed statistically different (F = 22.92; df = 9, 50; *P* < 0.001) canker areas. In conclusion, virulence of the *G. morbida* is not determined by the isolates from different genetic clusters of the fungus.

3.2 Introduction

Thousand cankers disease (TCD) is a complex, involves walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman, (Coleoptera: Curculionidae: Scolytinae) and a canker-causing fungal pathogen, *Geosmithia morbida* Kolarik, Freeland, Utley, and Tisserat, (Ascomycota: Hypocreales: Bionectriaceae) on the susceptible host plants, *Juglans* spp. L. and *Pterocarya* spp. Nutt. ex Moq (Hishinuma et al. 2016; Kolarik et al. 2011; Tisserat et al. 2009; Tisserat et al. 2011). Although, many walnut (*Juglans* spp., *Juglandaceae*) and wingnut (*Pterocarya* Nutt. ex Moq spp., *Juglandaceae*) species serve as generic hosts for the pathogen and WTB, black walnut, *J. nigra* L. is most susceptible to TCD (Hishinuma et al. 2016; Utley et al. 2013).

In 2010, the first detection of TCD in the eastern U.S. (Knoxville, Tennessee) indicated a potential threat to urban and forest settings of black walnut in its native range (Grant et al. 2011). Subsequently, the TCD complex reported from 7 other eastern states (PN, VA, NC, OH, IN and MD) (Daniels et al. 2016; Hadziabdic et al. 2014; Hansen et al. 2011; Seybold et al. 2013). The U.S. Forest Service Forest Inventory and Analysis (FIA) estimated that TCD was present in eastern US for at least 10 years before the first report within the native range of black walnut (Randolph et al. 2013). However, the mortality of black walnut attributed to TCD has been very low in the eastern U.S. (5%) when compared to mortality rates in western states (Newton et al. 2009; Randolph et al. 2013; Tisserat et al. 2011). In another study, black walnut trees symptomatic of TCD in Tennessee and Virginia had visual recovery from the disease and the recovery symptom pattern has not been outlined from the western U.S. (Griffin 2015). In the eastern U.S., Griffin (2015) also observed a dormant/quiescent TCD development condition after

the initial TCD detection from individual black walnut trees while such cases were not reported after the development of initial TCD symptoms to the death of observed trees in western U.S.

Differences in the disease expression, severity and incidence between the eastern and western US (Randolph et al. 2013) could be due to differences in host distribution, heterogeneity of black walnut habitats in the native range (Williams 1990). In the native range of black walnut, non-host vegetation between black walnut patches in forest act as buffer areas for the pathogen and WTB invasion and reduce disease progression (Wiggins et al. 2014). Wiggins et al. also proposed low WTB colonization pressure in the eastern states limited disease incidence. Abiotic factors such as drought, precipitation, available soil water, were reported to influence the trees to produce TCD associated symptoms among different geographical locations in the eastern and western US; Tisserat et al. 2009). Low precipitation, 60-64 cm in 2011 and 2012 believed to cause TCD progression in Richmond, VA while no new TCD symptoms were observed from the area in 2013 when higher precipitation (79-130 cm) was occurred (Griffin 2015). Griffin 2015 also reported increased foliage growth and recovery of black walnut trees from TCD in Knoxville, TN from 2011 to 2013 associated with high annual precipitation over the period of three years. Gazes et al. (2017) proposed different fungal communities co-existing with WTB galleries in the eastern and western US could impact the progression of TCD.

The objective of this study was to evaluate whether differences observed in TCD incidence and severity within the U.S. can be at least partially explained by differences in five distinct genetic profiles presented by fungal isolates taken from different geographical locations where TCD occurs (Hadziabdic et al. not published/preliminary data). Therefore, experiments were undertaken to; a) measure canker growth resulting from inoculation with isolates of *G.*

morbida vary and b) to compare these lesions on the basis of genetic clustering evident from their DNA.

3.3 Materials and Methods

3.3.1 Analysis for the presence of *G. morbida* on black walnut branches

Two black walnut trees from Middle Tennessee Research & Education Center, Spring Hill, Tennessee were selected to obtain branches for the experiment. Neither organism of TCD complex have been reported from the area. To confirm that collected branches were not already infected with *G. morbida*, samples from the trees were verified to be free of *G. morbida* using a rapid molecular detection procedure (Oren et al. 2017). Branches from the both trees were cut and brought in lab in separate containers. Ten branches (~1 m length) were randomly selected from each of the tree. Outer bark layer of branches was peeled to find potential cankers, phloem discoloration and necrotic areas that represent symptoms (Fig. 8) associated with infection by *G. morbida*. Drill bits (7/64 inch or 0.28 cm diam.) were sterilized using a bead sterilizer and then passed though, bottomless 1.5 ml centrifuge tubes. And used to collect small pieces of wood from potential infected tissues. The drill bit shavings were transferred to sterile 1.5 ml microcentrifuge tube until approximately 150 mg of drill shavings were collected. Three separate wood samples were taken from the selected 10 branches of each tree. Collected samples were stored at -20ºC until DNA extractions were done. Extractions of DNA, amplifications of the samples and amplicons were detected using above-mentioned method as describe in chapter 2.

3.3.2 Selection of *Geosmithia morbida* isolates

To determine if differences in the disease severity and incidence were due to differences in the virulence within the pathogen population, isolates from different regions of the country

that fall into different genetic clusters should be evaluated for virulence. In preliminary analyses, Hadziabdic et al. (unpublished) grouped *G. morbida* population across different geographical locations into five different genetic clusters using 15 polymorphic microsatellite loci (unpublished data). From these five differently clustering genetic pools, five separate isolates were chosen to maximize genetic diversity available across the available distribution of *G. morbida* (Table 6). The five genetic clusters of the fungus (Hadziabdic et al. unpublished) were color-coded, wherein Cluster 1, Cluster 2, Cluster 3, Cluster 4 and Cluster 5, are graphically depicted as Yellow, Pink, Red, Green and Blue genetic clusters, respectively (Fig. 10).

3.3.3 Molecular confirmation of selected axenic isolates

After examining morphological characters, all selected isolates were confirmed as *G. morbida* using molecular techniques. Mycelial plugs from each isolate were grown in Difco™ Potato Dextrose Broth at room temperature (25°C) for 3 weeks. After harvesting of mycelium, DNA was extracted using protocols described above (2.3.1). The ITS region was amplified using the primers ITS1F (Gardes and Bruns 1993) and ITS4R (White et al. 1990) to confirm the identity of *G. morbida*. Each PCR reaction-mix (final volume 25 µl) contained 12.5 µL GoTaq®G2 Hot Start Master Mix (Promega Corp., Madison, WI, USA), 1.25 µL 10 mM reverse primer, 1.25 µL 10 mM forward primer, 1 µL dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA), 1 µl of genomic DNA (~ 25 ng/µL) and 10 µL double-distilled water. The PCR thermal cycle started with initial denaturation step of 2 min at 94 °C followed by 15 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 65 °C and primer extension for 1 min at 72 °C; followed by 30 cycles of 30 s at 94 °C, 30 s at 48 °C and 1 min at 72 °C; and followed by a final elongation for 10 min at 72 °C. Amplifications of PCR products were confirmed with gel electrophoresis. PCR products were sent to MCLAB laboratories (www.mclab.com) for cleaning

and then sequencing. Sequencher TM 4.9 (Gene Codes Corp., Ann Arbor, MI, USA) was used to assess the quality of the chromatograms and assemble the strands into contigs. Basic Local Alignment Search Tool (BLAST), using the NCBI nucleotide database (www.ncbi.nlm.nih.gov/BLAST) searches from ITS sequences, results are provided in Table 7.

3.3.4 Preparation of black walnut bolts

Virulence experiments were performed on *J. nigra* bolts, length 8 cm, dia. 2-5 cm, in the laboratory. Black walnut branches, cut on May 25, 2017, were stored at 5ºC once they were returned to the lab until used. One day before inoculation, black walnut branches (~1 m length) were cut into standardized pieces in laboratory using a hand saw. Branch external surfaces were disinfested using ethanol (70%) sprays (Fig. 9). Once bolts dried, two wounds (~2 mm deep) were created on opposite sides of each branch piece using an electric drill (Lithium 18V, Ryobi tools (USA) and bit (3.175 mm)

3.3.5 Inoculations

Single spore cultures were obtained from all 25 *G. morbida* isolates. The fungal isolates were grown at 25ºC for 4 weeks in petri dishes (10 cm dia., 1.5 cm depth) containing 20 ml of hPDA. Petri dish containing only hPDA without fungus was used to make plugs for negative control. One isolate of *G. morbida* (GM 17) was used a positive control. This isolate had been reported to cause cankers in a previous study (Gazis et al. 2017). Prior inoculations, bolts with measured length of 8 cm and diameter 2-3 cm, were selected randomly. To inoculate wounded bolts, 4 mm diameter fungal plugs were obtained using sterile Humboldt brass cork borers (Fisher Scientific, 4 mm outer dia.) Fungal plugs were then inserted upside-down in each wound using a sterile scalpel. After inoculation, each wound was wrapped with Parafilm (M Laboratory

Film, Bemis, Neenah, WI) and diameter of each inoculated bolt was measured with a digital caliper. Each bolt was inoculated twice, on the opposite sides. Each of the 25 isolates and both controls were replicated 6 times. Cut ends of inoculated bolts were dipped in paraffin wax and then wrapped with aluminum foil. Inoculated bolts were placed in a tray and incubated at 25ºC for 7 days. At 7 DAI, a sterile scalpel was used to peel bark from the wounds exposing cankers. Digital images of exposed cankers along with a reference scale were taken immediately. The entire experiment was repeated once.

3.3.6 Canker measurements

Image processing and analysis software, ImageJ (https://imagej.nih.gov/ij/) (Schneider et al. 2012) was used to measure canker areas from digital photographs. The software was calibrated to standardize each photograph from the reference scale included in each photograph.

3.3. 7 Confirmation of the pathogen from cankers

To confirm re-isolation of *G. morbida* from cankers, rapid molecular detection method was used. Out of the six replicated bolts, three were randomly selected to obtain samples. From the selected three bolts, one random canker was drilled out of two inoculations from each bolt. Wood shavings were collected from a canker area excluding the wound, where fungus was inoculated. Protocol to take sample, DNA extractions, PCR amplifications and detections of amplicons were same as described in (3.3.1).

3.4 Statistical Analysis

Mixed model analysis was conducted using PROC MIXED in SAS9.4 TS1M3 with models fit using PROC RANK transformation (SAS Institute Inc., Cary, NC). Lesion area data were ranktransformed before the analysis, to account for non-normality and unequal variances

observed. Actual canker area values are reported. Canker area was a response variable and genetic cluster, isolates were treated as independent variable and diameter of the bolts as the covariates. The effect of other factors such state of isolate origin, geography of isolate (eastern and western), host from the isolate was obtained and time-in culture were analysed on the canker area. Repetition, and replications within the experiment were included as the random effects to account for blocking. Multiple comparisons for genetic cluster and isolates were conducted and corrected with Tukey's HSD. Significant effects for canker area from the variables were identified at $P<0.05$.

3.5 Results

Analysis of the tree branches for *G. morbida*

Molecular detection of *G. morbida* from representative branch samples of both trees, were used to obtain bolts, resulted in absence of the pathogen DNA indicating that prior to this experiment, selected bolts were free from the fungus.

Confirmation of Koch's postulates

To confirm Koch's postulates, cankers that were developed by the isolates of *G. morbida* were diagnosed with molecular detection method. Most of the analyzed samples gave positive detections of the pathogen from cankers, 94% (147/156). Wood samples obtained from the bolts that were treated as negative control for the experiment confirmed absence of *G. morbida*.

Effect of the variation in bolt diameter on canker area

Though, bolts were standardized from 2 - 3 cm in diameter, the effect of small variations that were measured maximum for 10 mm among used bolts, on canker area were analyzed.

Variations in the diameter of standardized inoculated bolts did not affect on canker area (F = 2.32; df = 1, 280; *P* = 0.128) when data were analyzed to observe differences in canker areas from the isolates nested with different genetic clusters of the fungus. The effect of bolt diam. on canker area was analyzed with while determining the other fixed variables in this study. There was no significant effect of bolt diam. on canker area when analyzed for individual isolates (F = 2.03; df = 1, 160; *P* = 0.156), origin of isolates at state level (F = 1.36; df = 1, 238, *P* = 0.245), host from an isolate was obtained (F = 0.58; df = 1, 274; *P* = 0.448), geographical origin of isolate (F = 0.28; df = 1, 286; *P* = 0.596) and time-in-culture for isolates (F = 0.130; df = 1, 291; *P* = 0.624).

Effect of the pathogen genetic groupings on canker area

Significant differences were observed in canker area produced by the isolates from different genetic clusters of *G. morbida* (F = 24.96; df = 6, 35; *P*= <0.0001) (Table 8, Fig. 10). *Geosmithia morbida* isolates from Cluster 1 produced significant larger cankers than isolates from other clusters and the mean canker area was 271 mm^2. Mean canker area produced by isolates from Clusters 2, 3, 4, 5 were 185 mm^2, 165 mm^2, 160 mm^2 and 134 mm^2, respectively. The average canker areas by isolates from Clusters 3, 4, 5 were not significantly different from each other. However, canker area was significantly larger for isolates of Cluster 2 than the mean canker area produced by isolates in Cluster 5 (t = -3.46; df = 35; *P*=0.022), but were not different for canker area for cankers produced by isolates of Clusters 3 and 4 (t = 1.31; df = 35; *P* = 0.844 and (t = -1.51; df = 35; *P* = 0.734, respectively).

Significant variations in canker area also developed among isolates within genetic clusters except in Cluster 1. For example, mean canker area for two isolates, GM186 and GM140 of Cluster 5 were 177 and 74 mm^2, respectively, and these were significantly different from each

other (t = -4.60; df = 135; $P = 0.003$). Whereas canker areas among isolates in Cluster 1 were not significantly different among each other. The mean canker areas from the genetic clusters of *G. morbida* were large than the negative control. But cankers developed by some isolates individually were not significant larger than negative control. Cluster 5 had two isolates that did not produce cankers different in area from the negative control and Clusters 2 and 3 had one isolate each that did not produced cankers larger than those produced by the negative control. All isolates from Cluster 1 and 4 produced cankers larger than those in the negative control.

3.6 Discussion

Canker area produced by *G. morbida* isolates from different genetic clusters of the fungus were variable. Isolates of Cluster 1 produced the largest cankers than any other cluster group and all isolates of Cluster 1 produced cankers of similar area. However, for the other four genetic clusters, significant differences in canker area by the specific isolates were observed within each cluster. *Geosmithia morbida* isolates used in this study were originally isolated between 2009 and 2014 (Table 4). We suspect that some isolates, especially those that produced no cankers larger than were observed for the negative control (agar-only) inoculations, might have lost virulence following repeated sub-culturing on synthetic media. And that reduction of virulence might also be responsible for significant variations in canker area from isolates within a genetic cluster. For example, isolate GM11 from Cluster 2 had been sub-cultured since 2010 and may have lost virulence. Other plant pathogenic fungi have been reported to lose capability to infect host plants, or have had reduced virulence and aggressiveness due to continuous sub-culturing and maintenance in culture (Awuah and Lorbeer 1988; Gramss 1991; Krokene and Solheim 2001; Newcombeet et al. 1990). Still, isolate GM301 from Cluster 1 has been maintained on synthetic media since 2009, yet produced the 5th largest mean canker area among all isolates,

irrespective of genetic cluster. Data from canker areas of *G. morbida* isolates when analyzed by time-in-culture of isolates, were not significant for canker area ($F = 0.09$; df = 1, 291; $P = 0.769$). Hence, year of isolate collection or duration of being in culture did not yield effect on canker area by the isolates. It seems unlikely that some isolates lost virulence due to continuous sub-culturing. Isolates that produced small cankers or cankers no larger than observed in negative control may have always been less virulent than other isolates. In a previous study (Sitz et al. 2017), specific isolates within a genetic cluster differed significantly for the canker aresa (Sitz et al. 2017). However, Sitz et al. 2017 did not observe significant differences in canker area for different genetic clusters. The authors used two isolates from each of three genetic clusters of *G. morbida* as illustrated in Zerillo et al. (2014). Zerillo et al. (2014) described four genetic clusters of the fungus corresponding to three geographical regions using single nucleotide polymorphisms (SNPs) and microsatellite loci. The difference in results between their study and this one could be due to the relative small sample area of isolates in Sitz et al. (2017).

In this study, the data were analyzed for geography (eastern or western) of the isolates that did not show statistical significance (Fig. 13). Cankers produced by eastern isolates had a mean canker area 187 mm^2 and that mean canker area was not statistically ($t = 0.20$; df = 10, $P = 0.848$) different from the isolates collected in western U.S., which had a mean canker area of 182 mm^2. This lack of significance could be due to unbalanced sample proportions for geographical locations because isolates were only prioritized according to genetic groupings of the fungus (Hadziabdic et al. unpublished/preliminary data). Results indicates that geographical origin of isolates, the eastern and western U.S., perhaps not playing a role in virulence within pathogen populations. Moreover, from one state in U.S., isolates from different genetic clusters can be found (Table 6). However, it is difficult to draw conclusions from this research because

sample size from eastern and western isolates were not proportional - six eastern isolates were selected due to sampling constraints, compared to selection of 19 western isolates. Significant differences ($F = 22.92$; $df = 9, 50$; $P<0.0001$) in canker production according to the collection location/state of isolate were observed (Table 9, Fig. 12). But, isolates from a state also produced statistically different cankers (Fig. 11). For example, isolate GM101 and GM140 that were collected in Arizona and grouped with Cluster 5 produced statistically distinct ($t = 5.93$; $df = 135$; $P<.0001$) cankers. Therefore, virulence of *G. morbida* isolates didn't correspond to the state of isolate collection. Regardless of genetic clusters, isolates from each state produced distinct cankers except isolates from Oregon and Utah. The mean canker area of Pennsylvania, Ohio, Utah, Indiana, Oregon and Utah isolates were not statistically distinct regardless of genetic cluster. Mean canker area developed by isolates from Colorado, New Mexico, Arizona, Tennessee, and North Carolina were not statistically distinct regardless of genetic cluster. Isolates from Pennsylvania, Ohio, Indiana, Oregon and Utah produced significant larger than cankers produced by isolates from Colorado, New Mexico, Arizona, Tennessee, and North Carolina. Sample size from eastern and western states was not equal within each genetic cluster. Likewise, isolates did not represent all states which contributed isolates to each genetic cluster. Largest canker area was produced by isolates of Cluster 1 however isolates from this cluster represented three states OR and UT (two isolates each) and Ohio (one isolate).

Data were analyzed to determine if hosts from which the isolates were originally obtained could have an effect on canker area. *Geosmithia morbida* isolates used in this study were isolated from *Juglans major*, *J. microcarpa*, and *J. nigra*. Information about the host, from which some isolates were obtained is missing, they were designated to being isolated from *Juglans* spp. as listed in Table 6. Canker size was significantly different for isolates grouped by the host they

were obtained from ($F = 16.50$; df = 3, 20; $P<0.0001$). But, the differences may have been due to unequal number of isolates from each host species (Table 6). Mean canker area of isolates collected from *Juglans major* produced statistically smaller canker than all the host species, whereas isolates from unknown *Juglans* species, designated in the collection record only as *Juglans* spp. produced mean highest canker area. Isolates derived from either *J. microcarpa* and *J. nigra* produced similarly sized (area) cankers. However, canker size was significantly variable (some smaller and some larger) for isolates obtained from each host. For example, isolates GM158 and GM186 were obtained from *J. nigra* a and were from the same state, genetic cluster, and isolated in the same year but produced statistically distinct cankers with mean canker area 78 mm^2 and 177 mm^2, respectively ($t = -4.67$; df = 135; $P = 0.002$).

From this research, we have concluded that the fungus virulence is not determined by the genetic cluster of *G. morbida*. The gene(s) responsible for virulence and avriulence are not linked with microsatellite markers used in population grouping in Hadziabdic et al. (unpublished/preliminary data) research. It might be possible that the markers are not evenly distributed or not able to capture the whole genetic heterogeneity in the pathogen. Significant variations in virulence among isolates from same host, state and geographical regions indicate that virulence in the fungal species is also not explained by these variables. The variations in disease severity with geographical regions of U.S. might be a result of differences in tree health and biotic and abiotic factors. Further research is required to analyze the relation of the pathogen virulence with the aggressiveness phenotypically and genetically how they could be related to host decline.

3.7 Literature cited

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4 Conclusion

From the potential vectors screening study, eleven species of bark and ambrosia beetles were detected carrying *G. morbida* out of total eighteen screened species. Eight insect species are reported for the first time to carry *G. morbida*. If *Geosmithia morbida* is associated with other beetle species, these insects may disseminate *G. morbida* within black walnut populations is the eastern U.S. From these findings, I accept the hypothesis (Chapter 2) that insect species, other than walnut twig beetles, that associated with black walnut trees carry propagules of *G. morbida* in Tennessee. These findings also raise the awareness that the presence of *G. morbida* propagules on insect species other than walnut twig beetles could have future implications on quarantine and or phytosanitary regulations.

From the virulence experiment data, significant differences were observed in canker area produced by different genetic clusters of *G. morbida*. Isolates from Cluster 1 produced significant large cankers (mean canker area 271.49 mm^2) than rest of the four clusters. Isolates from Clusters 2, 3, 4 and 5 produced mean canker areas of 184.38 mm^2, 165.32 mm^2, 160.38 mm^2 and 134.31 mm^2, respectively. However, mean canker area was not different for all genetic clusters and thus all genetic clusters could not be separated from each other based on canker area. Differences in the canker development from different genetic clusters of *G. morbida* were also noted in some genetic clusters and not others. which could have effect on the varying TCD incidence within U.S. Based on these results the hypotheses (Chapter 3) that isolates of *G. morbida* vary in virulence is accepted. However, the hypothesis that genetic clusters of isolates differ in virulence is rejected. This rejection is based on that significantly different cankers were observed within genetic clusters of the fungus except Cluster 1.

5 Directions for future research

Most of the insect species follow Leach's first three rules in order to be potential alternative vectors for *Geosmithia morbida* (see Discussion). However, Leach's fourth rule for insect transmission of a plant pathogen still needs to be demonstrated. According to the fourth rule, the disease must be developed on plant host following visitation of insect species that are known for carrying the pathogen (Leach 1940). To address this question, future studies should include an experiment in which black walnut trees will be exposed to potential alternative vectors in controlled environmental conditions with adequate checks. If symptoms of the pathogen appear following infestation of potential alternative vectors, the next step will be to isolate the pathogen from the host to validate Koch's postulates.

It is important to determine the factors involve in successful fungal transmission by these insect species. Successful *G. morbida* transmission to *Juglans* spp. via these insect species may depends upon combination of many factors such as proportion of individuals contaminated with *G. morbida*, quantity of spores per individual, mechanism of *G. morbida* transmission, biology and feeding behavior of different insect species. In this study DNA of the pathogen was detected on individual specimens of different insect species to determine sample proportions carrying the fungus. Proportions of *G. morbida* contaminated individuals were varying among tested insect species, indicating the capabilities of the insect species could differ in fungal transmission. Different insect species might be carrying different *G. morbida* spore loads and their phoresy rates could have relationship to their body size or feeding, breeding behavior. For example, the relationship between fungal spore load and size of beetles and breeding behavior have been observed in vectors of Dutch elm disease (Webber et al. 1987; Webber 1990, 2000). The pathogen spore load per beetle could be quantified using qualitative PCR (qPCR) from

representative insect species using species specific primers (Fang and Ramaraja 2015). Suspensions of varying concentrations of *G. morbida* spores can be used to extract DNA which could serve as reference standards for DNA based quantification of spore loads. Schweigkofler et al. (2005) detected and quantified black-stain root disease pathogen, *Leptograpghim wageneri* using qPCR with DNA from bark beetle species. The estimation of spore loads on the potential alternative vectors could also be compared with the quantified spore loads on WTB specimens. In such case, *G. morbida* spore loads on WTB also need to be determined. Quantification of the pathogen will be used to determine, how these tested insect species varies in *G. morbida* carrying capability among each other, and comparative to WTB. It would be informative to study at what spore/propagule load other potential vectors can successfully transmit *G. morbida*. For instance, in the case of Dutch elm disease caused by *Ophiostoma novo-ulmi*, a minimum of 1×10^3 conidia required to be carried by beetle vectors for infection on moderately susceptible elm species (Webber 1990; Webber and Gibbs 1989). Estimations of fungal spore counts needed to induce infection may reveal that some insect species are more important potential vector than others thus play varying roles in TCD epidemiology.

This study did not evaluate how potential alternative vectors carry *G. morbida*, whether it is phoretic/epizoic transmission or endozoic transmission were occurred (Batra 1963; Beaver et al. 1989). Of the three common fungal inoculum; spores, sclerotia and vegetative mycelium, spores are the most common fungal inoculum and very well adapted to insect transmission (Leach 1940, Kluth et al. 2002). Fungal spores are generally resistant to environment, adhere readily to the external body surface of an insect and might also brushed off easily (Leach 1940). An example of phoretic transmission is *Geosmithia morbida* vectored by walnut twig beetle externally, but possible endozoic transmission may also occur (Daniels et al. 2016; Cranshaw and Tisserat

2009). Endozoic transmission occurs when spores of fungi carried inside special body cavities such as mycangia and/or pass through the insect's intestinal track uninjured. The latter transmission is usually a criterion for primary ambrosial fungi associated with bark and ambrosia beetles. Bark and ambrosia beetles are associated with their major species of ambrosial fungi and inoculum of these fungal species maintain, grow inside insect's body (Beaver et al. 1989). But, unwanted or "weed" fungal species which are accidentally ingested inside mycangia are eliminated with secreted chemicals that inhibit the growth, and maintain the populations/growth of symbiotic fungi (Happ et al., 1971). Also, toxins produced by primary fungi could be responsible to eliminate the contamination by other microorganisms (Nakashima et al., 1982). If *Gesomithia morbida* has been spread endozoically by the potential alternative vectors, the fungus could be eliminated at some extent from mycangia and other body cavities of bark and ambrosia beetles. But, fungal propagules usually pass uninjured through the digestive system of phytophagous insects (Leach 1940; Taubenhaus and Christensen 1936).

The following objectives might be addressed in the future studies focused on alternative vectors of *Geosmithia morbida*-

1. To determine if potential alternative vectors initiate *G. morbida* infection following their visitations to the host species?
2. To determine spore load on different tested insect species and its relation to their size and feeding, breeding behavior and minimum spore load required to initiate the infection?
3. Mode of fungal transmission by potential alternative vectors?

Future studies on determination of *Geosmithia morbida* virulence

The study on virulence of *G. morbida* isolates revealed that the virulence is not represented by the genetic clusters. Virulence was also not explained by host, and geographical regions of the isolates. Further research is required to characterize gene(s) responsible for virulence and avirulence in *G. morbida*. Most virulent and avirulent isolates from this research could be used for genome sequencing to determine the potential differences in genetic composition. The potential differences in genetic composition among isolates may reveal differences in GC (Guanine-Cytosine) content, genome length and number of genes (Chiapello et al. 2015; Jelen et al. 2016; Xue et al. 2012). Hence, future research could characterize the gene(s) responsible for virulence and avirulence the isolates. It would be also informative to conduct histology of canker development from virulent and avirulent isolates on different susceptible *Juglans* species. This could reveal the potential differences in colonization, depth and necrosis of cankers etc., by different isolates on susceptible walnut hosts. It could help to understand the mechanism(s) in different host species to fungus and their significance in disease severity and incidence (Milholland 1970; Rayachhetry et al. 1996). Analysis of the aggressiveness of pathogen isolates is also crucial to study for determination of its relation to virulence (Sakr 2011). The virulent isolate in this study might be aggressive in nature, on different host species regardless of genetic cluster or might not have a relationship to aggressiveness. It might contribute to address the question of different disease severity and incidence within the U.S.

The following objectives might be addressed in the future studies to understand variations in the virulence of *Geosmithia morbida* isolates-

1. Do virulent and avirulent isolates differ in genetic composition

2. If genetic variation among isolates is due to differences in gene(s) then which gene(s) inherit virulence and avirulence?
3. Relationship between virulence and aggressiveness of most virulent and avirulent isolates and histology on different susceptible hosts.

5.1 Literature cited

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Appendix

Table 1. Detection results from the insect species that were reported to be associated with *Geosmithia morbida*

Insect species[a]	**Taxonomic classification**[b]	**Feeding guild**[c]	**Origin**[d]	**Analyzed specimens**[e]	**Positive incidence**[f]	**Percentage of positive incidence**[g]	**Detection report**[h]
Xylobiops basilaris	Coleoptera: Bostrichidae: Bostrichinae	BB	Native	54	34	62	First report
Stenomimus pallidus	Coleoptera: Curculionidae: Cossoninae	BW	Native	27	18	66	Previously reported (Juzwik et al. 2015)
Oxoplatypus quadridentatus	Coleoptera: Curculionidae: Platypodinae	AB	Native	2	2	100	First report
Cnestus mutilatus	Coleoptera: Curculionidae: Scolytinae	AB	Exotic	67	28	41	First report
Dryoxylon onoharaensum	Coleoptera: Curculionidae: Scolytinae	AB	Exotic	26	11	42	First report
Hylocurus rudis	Coleoptera: Curculionidae: Scolytinae	BB	Native	3	1	33	First report
Monarthrum fasciatum	Coleoptera: Curculionidae: Scolytinae	AB	Native	27	15	55	First report
Monarthrum mali	Coleoptera: Curculionidae: Scolytinae	AB	Native	15	5	33	First report
Xyleborinus saxesenii	Coleoptera: Curculionidae: Scolytinae	AB	Exotic	90	45	50	Previously reported (Juzwik et al. 2016)
Xylosandrus crassiusculus	Coleoptera: Curculionidae: Scolytinae	AB	Exotic	45	22	48	Previously reported (Juzwik et al. 2016)
Xylosandrus germanus	Coleoptera: Curculionidae: Scolytinae	AB	Exotic	8	6	75	First report

[a] Insect species that were reported to carry *G. morbida* DNA in this study

[b] Insect species are presented according to taxonomical arrangements (order: family: subfamily)

[c] Feeding guild of the insect species, AB: ambrosia beetle, BB: bark beetle, BW: bark weevil

[d] Insect species native or exotic to United States

[e] Total number of tested specimens for the presence of *G. morbida* from each insect species

[f] Number of specimens confirmed the presence of *G. morbida* out of the total tested individuals for an insect species

[g] Percentage of specimens that confirmed the presence of *G. morbida* out of the total tested individuals for an insect species

[h] Insect specie's association with *G. morbida* from previous research

Table 2. List of the insect species that were not reported to associate with *Geosmithia morbida*

Insect Species[a]	**Taxonomic classification**[b]	**Analyzed specimens**[c]	**Positive incidence**[d]	**Percentage of positive incidence**[e]
Neoclytus acuminatus	*Coleoptera: Cerambycidae: Cerambycinae*	1	0	0
Lepturges confluens	*Coleoptera: Cerambycidae: Lamiinae*	1	0	0
Madoniella dislocatus	*Coleoptera: Cleridae: Epiphloeinae*	2	0	0
Ambrosiophilus atratus	*Coleoptera: Curculionidae: Scolytinae*	2	0	0
Xyleborus ferrugineus	*Coleoptera: Curculionidae:Scolytinae*	1	0	0
Euwallacea validus	*Coleoptera: Curculionidae:Scolytinae*	8	0	0
Microsicus parvulus	*Coleoptera: Zopheridae: Colydiinae*	7	0	0

[a] Insect species that were reported not to carry *G. morbida* DNA in this study

[b] Insect species are presented according to taxonomical arrangements (order: family: subfamily)

[c] Total number of tested specimens for the presence of *G. morbida* from each insect species

[d] Number of specimens confirmed the presence of *G. morbida* out of the total tested individuals for an insect species

[e] Percentage of specimens that confirmed the presence of *G. morbida* out of the total tested individuals for an insect species

Table 3. Status, distribution, feeding guild and host range of insect species that were analyzed positive for *Geosmithia morbida*

Insect species[a]	**Taxonomic classification**[b]	**Native Status in U.S.**[c]	**Reported distribution among U.S. states**[d]	**Feeding guild**[e]	**Host Plant Famil(ies)**[f]
Xylobiops basilaris[h]	Coleoptera: Bostrichidae: Bostrichinae	Native	[h] AR, DE, DC, FL, GR, IL, IN, IO, KS, KY, LS, MD, MA, MS, MO, NJ, NY, NC, OH, PA, SC, TN, TX, VA, WV, WI	BB	Anacardiaceae, Ebenaceae, Fabaceae, Fagaceae, Juglandaceae, Moraceae, Myrtaceae, Oleaceae, Pinaceae, Poaceae, Rosaceae, Rutaceae, Ulmaceae, Vitaceae,
Stenomimus pallidus[g]	Coleoptera: Curculionidae: Cossoninae	Native	DC, IL, IN, MD, OH, PA, VA, MO	BW	Fagaceae, **Juglandaceae**
Oxoplatypus quadridentatus	Coleoptera: Curculionidae: Platypodinae	Native	AL, AZ, AR, FL, GR, IN, LS, MD, MS, NC, OK, SC, TN, TX, VA, WA	AB	Fagaceae, Hamamelidaceae, Hippocastanaceae, Pinaceae
Cnestus mutilatus	Coleoptera: Curculionidae: Scolytinae	Exotic	AL, AR, FL, GA, IL, IN, KY, LS, MD, MS, NC, PA, SC, TN, TX, VA	AB	Aceraceae, Anacardiaceae, Betulaceae, Cornaceae, Cupressaceae, Fagaceae, **Juglandaceae**, Lauraceae, Melastomataceae, Oleaceae, Papilionaceae, Styracaceae, Theaceae
Dryoxylon onoharaensum	Coleoptera: Curculionidae: Scolytinae	Exotic	AL, AR, CT, DE, FL, GA, IN, KY, LS, MD, MA, MS, NJ, NY, NC, OH, PA, SC, TN, TX, VA	AB	Aceraceae, Fagaceae, Magnoliaceae, Salicaceae
Hylocurus rudis	Coleoptera: Curculionidae: Scolytinae	Native	AL, AR, DE, DC, FL, GR, 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	Coleoptera: Curculionidae: Scolytinae	Native	AL, AR, DE, DC, FL, GR, IL, IN, IA, KS, KY, LS, ME, MA, MI, MS, MO, NE, NJ, NY, NC, OH, OK, OR, PN, RI, SC, TN, TX, VT, VA, WV, WI	AB	Aceraceae, Fagaceae, **Juglandaceae**, Mimosaceae, Nyssaceae, Pinaceae, Rosaceae
Monarthrum mali	Coleoptera: Curculionidae: Scolytinae	Native	AL, AR, CA, CT, DE, DC, FL, GR, IL, IN, IA, KS, KY, LS, ME, MA, MI, MN, MS, MO, NE, NH, NJ, NY, NC, OH, OK, PN, RI, SC, TN, TX, VT, VA, WV, WI	AB	Aceraceae, Betulaceae, Burseraceae, Cornaceae, Fagaceae, Hamamelidaceae, **Juglandaceae**, Mimosaceae, Nyssaceae, Oleaceae, Sapotaceae, Tiliaceae
Xyleborinus saxesenii	Coleoptera: Curculionidae: Scolytinae	Exotic	AL, AZ, AR, CA, CO, CT, DE, DC, FL, GR, ID, IL, IN, IO, KS, KY, LS, MN, MD, MA, MI, MS, MO, NE, NV, NH, NJ, NM, NY, NC, OH, OK, OR, PA, RI, SC, SD, TN, TX, UT, VT, VA, WA, WV, WI,	AB	Aceraceae, Actinidiaceae, Anacardiaceae, Annonaceae, Apocynaceae, Betulaceae, Casuarinaceae, Cornaceae, Cupressaceae, Ebenaceae, Ericaceae, Fagaceae, **Juglandaceae**, Lauraceae, Magnoliaceae, Meliaceae, Moiraceae, Myrtaceae, Pinaceae, Rosaceae, Salicaceae, Taxodiaceae, Tiliaceae

Table 3 Continued.

Insect species[a]	Taxonomic classification[b]	Native Status in U.S.[c]	Reported distribution among U.S. states[d]	Feeding guild[e]	Host Plant Famil(ies)[f]
Xylosandrus crassiusculus	Coleoptera: Curculionidae: Scolytinae	Exotic	AL, AR, CT, DE, FL, GR, IL, IN, KS, KY, LS, MD, MA, MI, MS, MO, NE, NY, NC, 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
Xylosandrus germanus	Coleoptera: Curculionidae: Scolytinae	Exotic	AL, AR, CT, DE, FL, GR, IL, IN, KS, KY, LS, ME, MD, MA, MI, MS, MO, NH, NJ, NY, NC, OH, OR, PA, RI, SC, TN, TX, VT, VA, 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] Insect species that were carrying *G. morbida* DNA in this study

[b] Insect species are presented according to taxonomical arrangements (order: family: subfamily)

[c] Status is from Wood (1982), Wood and Bright (1992), and Rabaglia et al. (2006)

[d] First record and [e]Feeding guild (AB: Ambrosia beetle, BB: Bark beetle) are from Haack and Rabaglia 2013

[e] Distribution in U.S. states according to Atkinson (2012)

[f] Host family(ies) information is taken from Atkinson (2012) except [g] is from O' Brien et al. (1982) and [h] from Fisher (1950)

Juglandaceae, specifically stated to include *Juglans nigra*

Table 4. Abundance of *Cnestus mutilatus*, *Xyleborinus saxesenii*, and *Xylosandrus crassiusculus* specimens correspond to collection locations

Insect species[a]	Location[b]	Analyzed specimens[c]	Positive incidence[d]	Percentage of positive incidence[e]
Cnestus mutilatus	Burkhart	21	8	38
Cnestus mutilatus	Lakeshore	19	7	36
Cnestus mutilatus	Maryville	25	12	48
Xyleborinus saxesenii	Burkhart	30	19	63
Xyleborinus saxesenii	Lakeshore	30	13	43
Xyleborinus saxesenii	Maryville	30	13	43
Xylosandrus crassiusculus	Burkhart	15	5	33
Xylosandrus crassiusculus	Lakeshore	15	8	53
Xylosandrus crassiusculus	Maryville	14	8	57

[a] Insect species that were collected in balanced proportions from each collection location

[b] Three collection locations where insect traps were installed

[c] Total number of tested specimens for the presence of *G. morbida* from each insect species

[d] Number of specimens confirmed the presence of *G. morbida* out of the total tested individuals for an insect species

[e] Percentage of specimens that confirmed the presence of *G. morbida* out of the total tested individuals for an insect species

Table 5. Information about genetic cluster, state of collection, mean canker area, host, geography and isolation year of *Geosmithia morbida* isolates used in the experiment.

Isolate[a]	Genetic cluster[b]	State of collection[c]	Mean canker area[d]	Host[e]	Geography[f]	Year[g]
GM101	Cluster 5	AZ	208.41	*J. major*	Western	2013
GM140	Cluster 5	AZ	73.101	*J. major*	Western	2013
GM216	Cluster 5	NM	134.94	*J. major*	Western	2013
GM158	Cluster 5	CO	78.5342	*J. nigra*	Western	2013
GM186	Cluster 5	CO	177.58	*J. nigra*	Western	2013
GM106	Cluster 4	AZ	131.5	*J. major*	Western	2013
GM133	Cluster 4	AZ	113.84	*J. major*	Western	2013
GM236	Cluster 4	NM	163.39	*J. major*	Western	2013
GM156	Cluster 4	CO	228.73	*J. microcarpa*	Western	2013
GM170	Cluster 4	CO	165.51	*J. nigra*	Western	2013
PDA	Negative Control	*n.a.*	11.6516			
GM66	Cluster 2	IN	236.36	*J. nigra*	Eastern	2014
GM67	Cluster 2	IN	193.66	*J. nigra*	Eastern	2014
GM50	Cluster 2	PA	288.92	*J. nigra*	Eastern	2011
GM11	Cluster 2	TN	37.9639	*J. nigra*	Eastern	2010
GM25	Cluster 2	TN	169.84	*J. nigra*	Eastern	2011
GM17	Positive Control	TN	152.18	*J. nigra*	Eastern	2011
GM104	Cluster 3	AZ	153.04	*J. major*	Western	2013
GM188	Cluster 3	CO	119.73	*J. nigra*	Western	2013
GM59	Cluster 3	NC	98.5078	*J. nigra*	Eastern	2012
GM269	Cluster 3	UT	210.65	*J. nigra*	Western	2013
GM250	Cluster 3	OR	245.59	*Juglans* sp.	Western	2013
GM259	Cluster 1	UT	283.75	*J. nigra*	Western	2013
GM252	Cluster 1	OR	231.66	*Juglans* sp.	Western	2013
GM293	Cluster 1	OH	280.85	*J. nigra*	Western	2013
GM277	Cluster 1	UT	280.23	*J. nigra*	Western	2013

Table 5 Continued

Isolate[a]	Genetic cluster[b]	State of collection[c]	Mean canker area[d]	Host[e]	Geography[f]	Year[g]
GM301	Cluster 1	OR	277.95	*Juglans* sp.	Western	2009

[a] *Geosmithia morbida* isolates presented with lab codes, used to determine the virulence of fungi

[b] Representation of genetic cluster to each isolate as per Hadziabdic et al. (unpublished/preliminary data)

[C] State from where *G. morbida* isolate was obtained originally

[d] Mean canker size of the isolate calculated from 24 observations

[e] Host tree species from which the isolate was obtained originally

[f] Representation of isolate from eastern or western United States

[g] Year in which the isolate was obtained and being cultured on synthetic medium

Table 6. Molecular confirmation of *Geosmithia morbida* isolates used in determining the fungus virulence. GenBank accession numbers are presented along with the fungus isolate codes in the table. Table presented the identity and query cover of the isolates matched to previously submitted *G. morbida* sequences on BLAST.

Isolate ID[a]	**GenBank accession number**[b]
GM101	MG008832
GM104	MG008838
GM106	MG008840
GM11	MG008846
GM133	MG008842
GM140	MG008845
GM156	MG008830
GM158	MG008844
GM170	MG008836
GM186	MG008834
GM188	MG008841
GM216	MG008839
GM236	MG008837
GM250	MG008827
GM252	MG008829
GM259	MG008823
GM25	MG008835
GM269	MG008831
GM277	MG008825
GM293	MG008824
GM301	MG008826
GM50	MG008822
GM59	MG008843
GM66	MG008828

Table 6 Continued.

Isolate ID	GenBank accession number
GM67	MG008833
GM17 (positive control)	MG008849

[a] *Geosmithia morbida* isolates presented with lab codes, used to determine the virulence of fungi

[b] GenBank accession number(s) of the nucleotide sequence(s) generated from each isolate

Table 7 Mean canker areas of representative isolates within different genetic clusters of *G. morbida* for canker area on *Juglans nigra* branches

Genetic Cluster[a]	Mean Canker area[b]	Letter Group[c]
Negative Control	11.3999	A
Cluster 5	134.31	B
Positive Control	153.59	BC
Cluster 4	160.38	BC
Cluster 3	165.32	BC
Cluster 2	184.38	C
Cluster 1	271.49	D

[a] Five distinct genetic clusters of *Geosmithia morbida*, negative and positive control

[b] Mean canker area produced by representative isolates within each cluster from 120 observations

[c] Genetic clusters sharing the same assigned letter are not statistically producing distinct cankers, PROC MIXED model analyses was conducted using Tukey's HSD and significant differences were identified at $P < 0.05$

Table 8. Mean canker area developed by the isolates from representative states of U.S. according to isolates from different collection locations

Collection location[a]	Number of Isolates[b]	Mean Canker area[c]	Letter Group[d]
PA	1	288.99	A
OH	1	280.91	A
UT	3	258.56	A
OR	3	251.23	A
IN	2	217.69	A
CO	5	154.19	B
NM	2	149.18	B
AZ	5	136.18	B
TN	2	102.29	B
NC	1	99.0255	B

[a] Isolates obtained from ten states

[b] Number of isolates used in this study from the representative state

[c] Mean canker area produced by representative isolates from each state

[d] Collection locations sharing the same assigned letter are not statistically producing distinct cankers, PROC MIXED model analyses was conducted using Tukey's HSD and significant differences were identified at $P<0.05$



Figure 1. Preparation of modified soda bottle traps. A. Drilling of black walnut bolts to make lure chambers. B. Cut ends of drilled bolts dipped in paraffin wax. C. Rectangular windows (15 cm tall x 6 cm wide) were cut on soda bottles using a razor blade. D. Preparation of insect collection bottles (Bottoms were replaced with screen). E. A modified soda bottle trap. Photo Credits. Qunkang Cheng and Karandeep Chahal



Figure 2. Axenic culture of *Geosmithia morbida* isolated from *Stenomimus pallidus* following culture-based detection method. Photo Credits: Karandeep Chahal

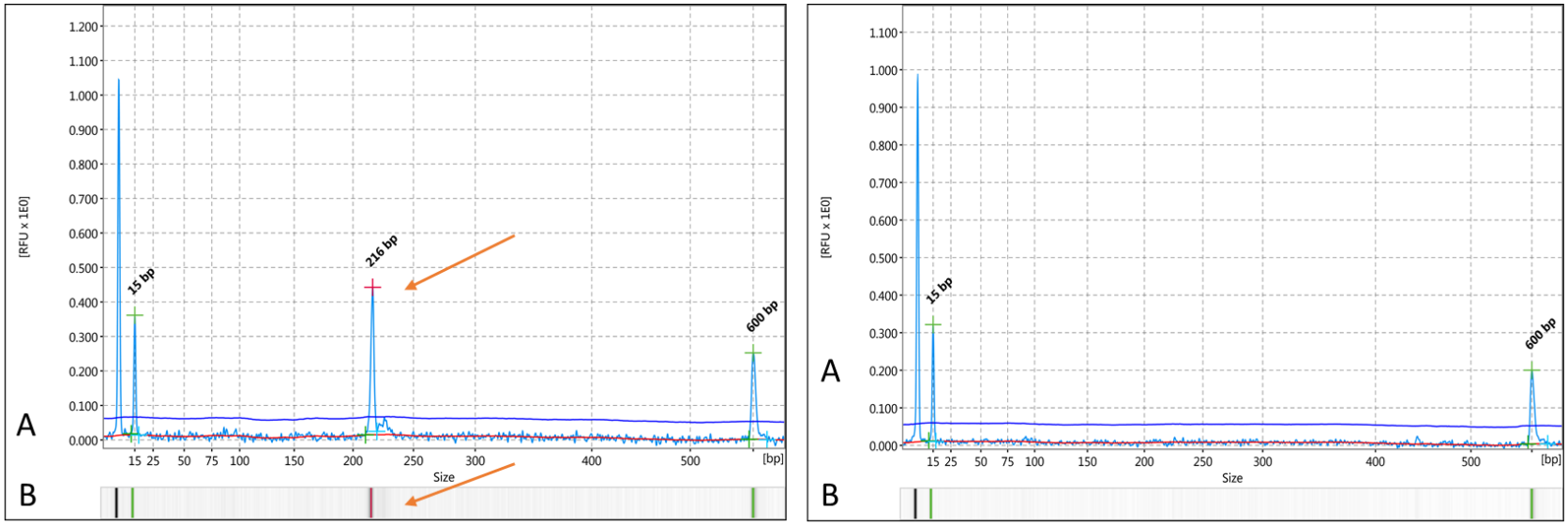


Figure 3. *Geosmithia morbida* molecular detection. Left figure showing results for a positive sample. A. Electropherogram showing peak (indicated by arrow) of amplified DNA (216 bp), B. Gel image of amplified DNA (band indicated by arrow). Right figure showing results for negative sample, absence of peak and band on gel image.

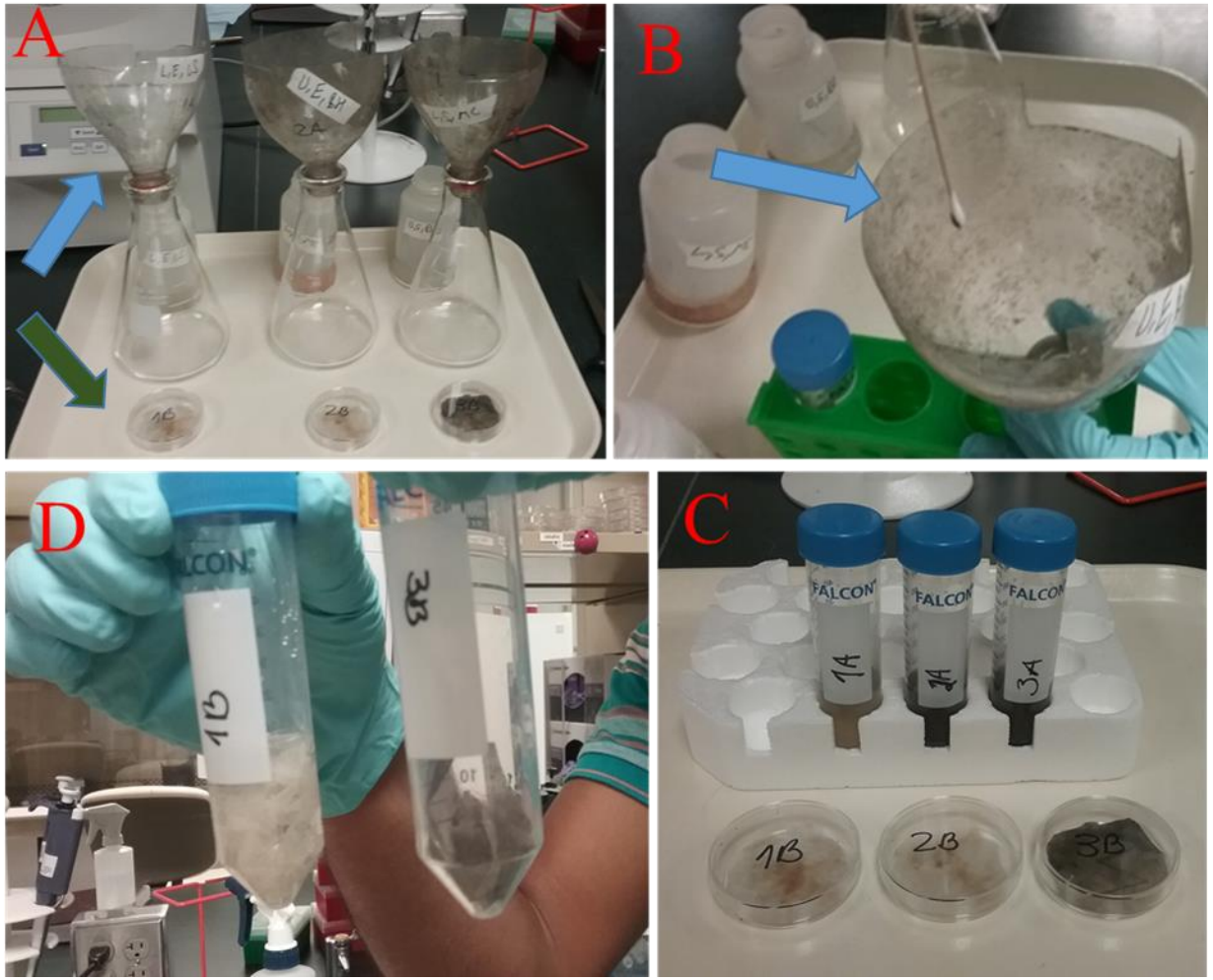


Figure 4. Detection of *Geosmithia morbida* from field installed traps. A. Cuttings of spout end (blue arrow) of trap bottles and collection-bottle bottom screens (green arrow). B. Internal circumference of soda bottle spout ends wiped and rinsed. C. Rinsed washout from soda bottle casings/spout ends collected in falcon tubes. D. Chopped pieces of collection bottle bottom screens in falcon tubes. Photo Credits: Dr. Romina Gazis and Karandeep Chahal

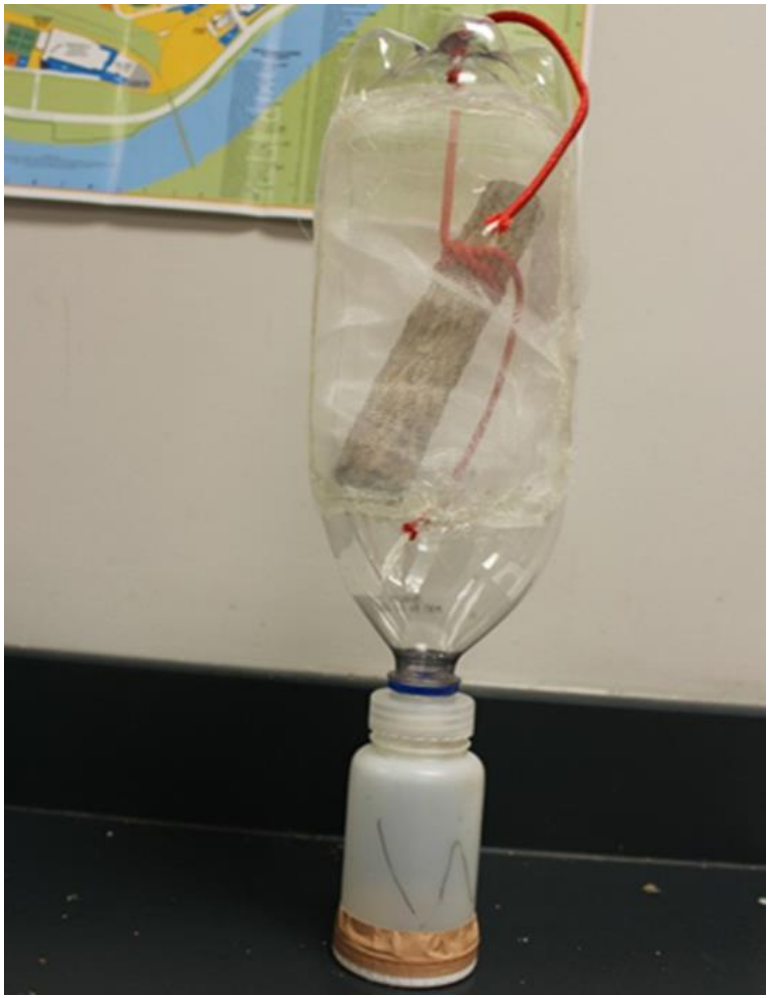


Figure 5. A model of traps which were used to serve as negative controls in the field. Photo Credit. Karandeep Chahal

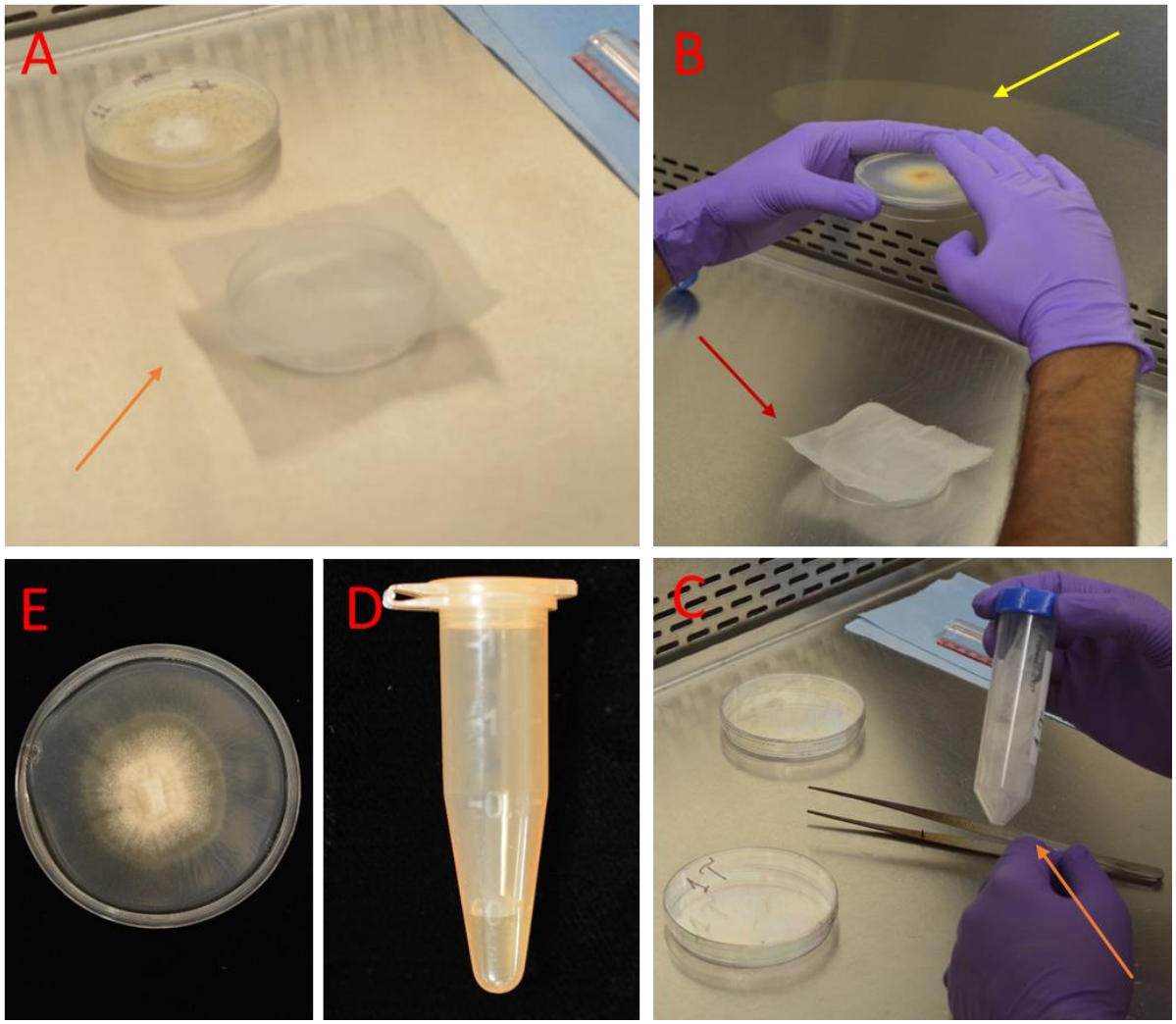


Figure 6. *Geosmithia morbida* detection of traps, positive control experiment. A. Screen (10 X 10 cm, 75 mesh size) placed on sterile petri plate B. Petri dish with sporulated *G. morbida* colony (yellow arrow) tapped on the screen (red arrow). C. Chopped screen in sterile falcon tube. D. Extracted DNA from the screen sample. E. The fungal colony used for this experiment. Photo Credits: Karandeep Chahal

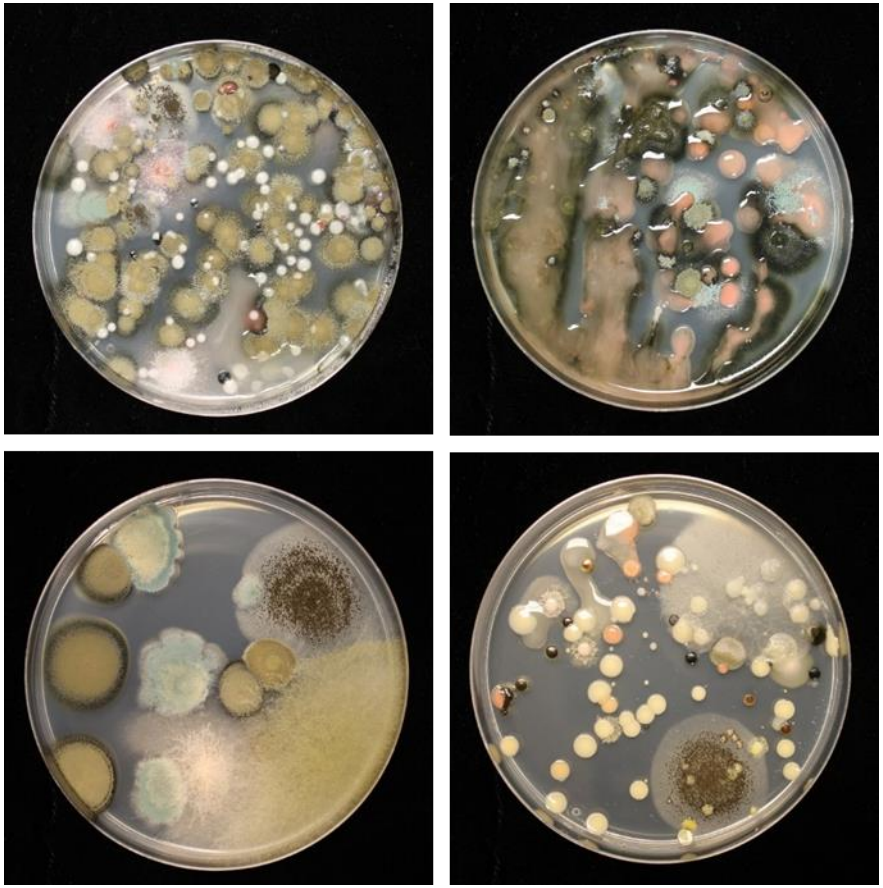


Figure 7. Ubiquitous fungi growing from ‘rinste’ on hPDA

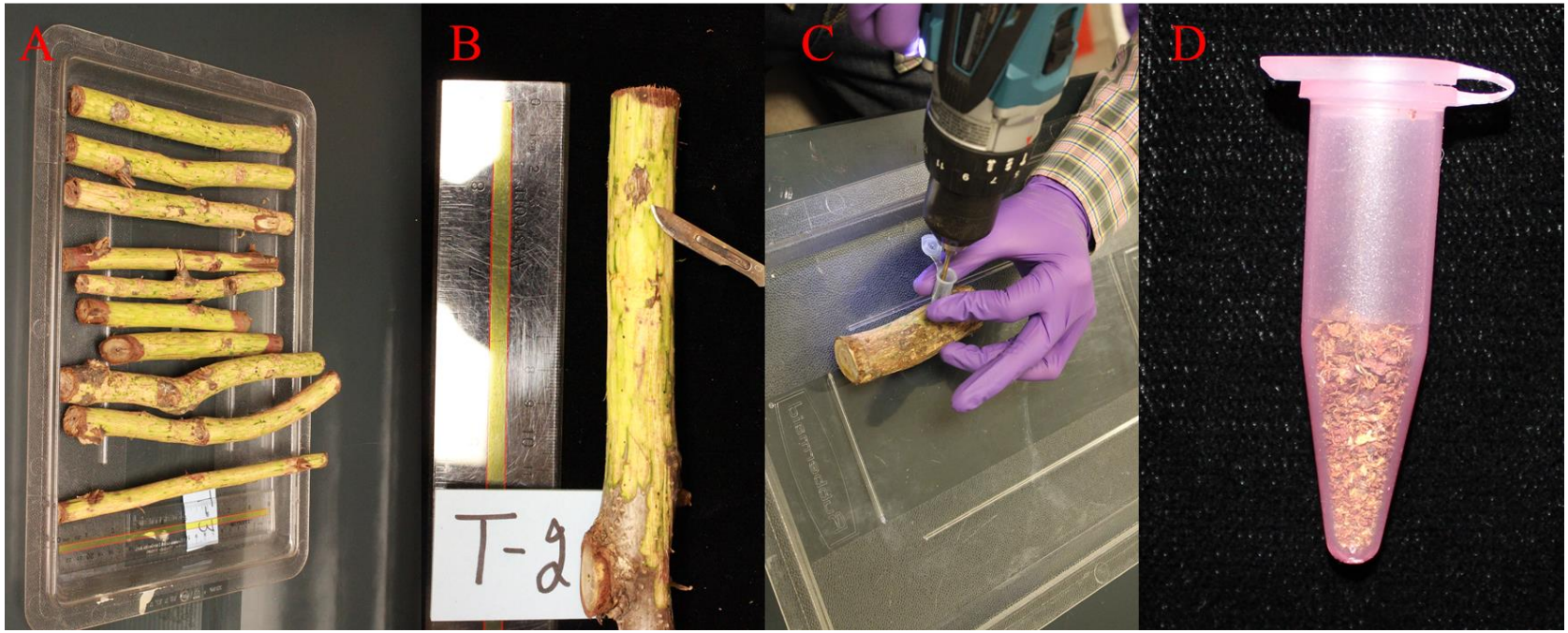


Figure 8. Procedure to take samples for *Geosmithia morbida* confirmation from collected wood branches. A. Bark was peeled to expose potential phloem discoloration. B. Potential symptoms (indicated by scalpel blade tip) resemble phloem necrosis. C. Drilling to collect wood shavings D. A collected sample Photo Credits: Karandeep Chahal and Sara Collins



Figure 9. Inoculation of *Geosmithia morbida* A. Collection of black walnut limbs from TCD free trees B. Preparation of bolts C. Surface disinfestation of bolts with ethanol D. Preparation of fungal plugs E. Inoculation of fungal plugs into wounds on bolts E. Excision of cankers. Photo Credits: Karandeep Chahal and Patrick Daniel

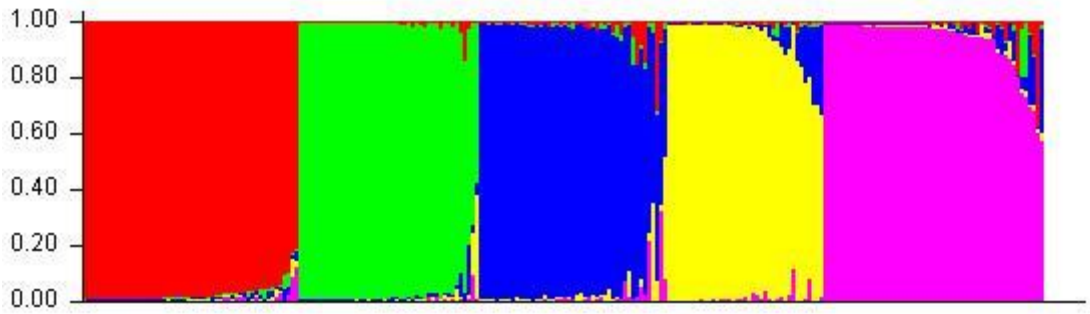


Figure 10. Distinct five genetic clusters of *G. morbida* as per Hadziabdic et al. (unpublished/preliminary data) study. Genetic clusters are determined by the STRUCTURE analysis. For Dk= 5, the different colors represent red, green, blue, yellow and pink distinct clusters.

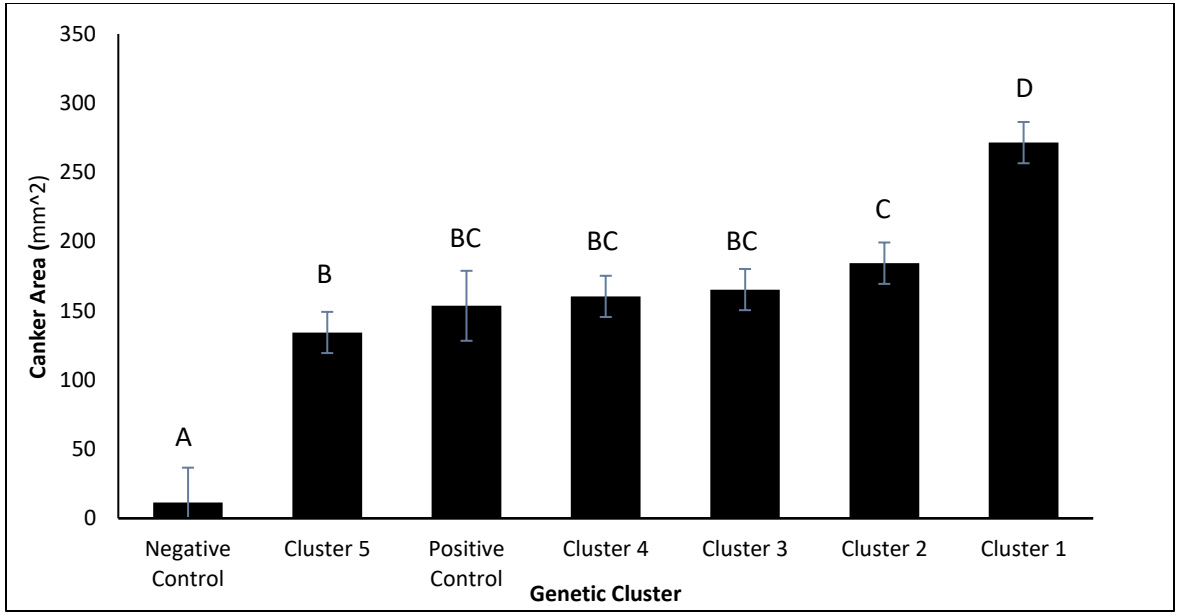


Figure 11. Mean canker areas observed for pooled representative isolates within *Geosmithia morbida* genetic clusters (Hadziabdic et al. unpublished/preliminary data). Differences in the letter groupings indicate statistical differences among canker area means at $p<0.05$ (Tukey's honest significant difference)

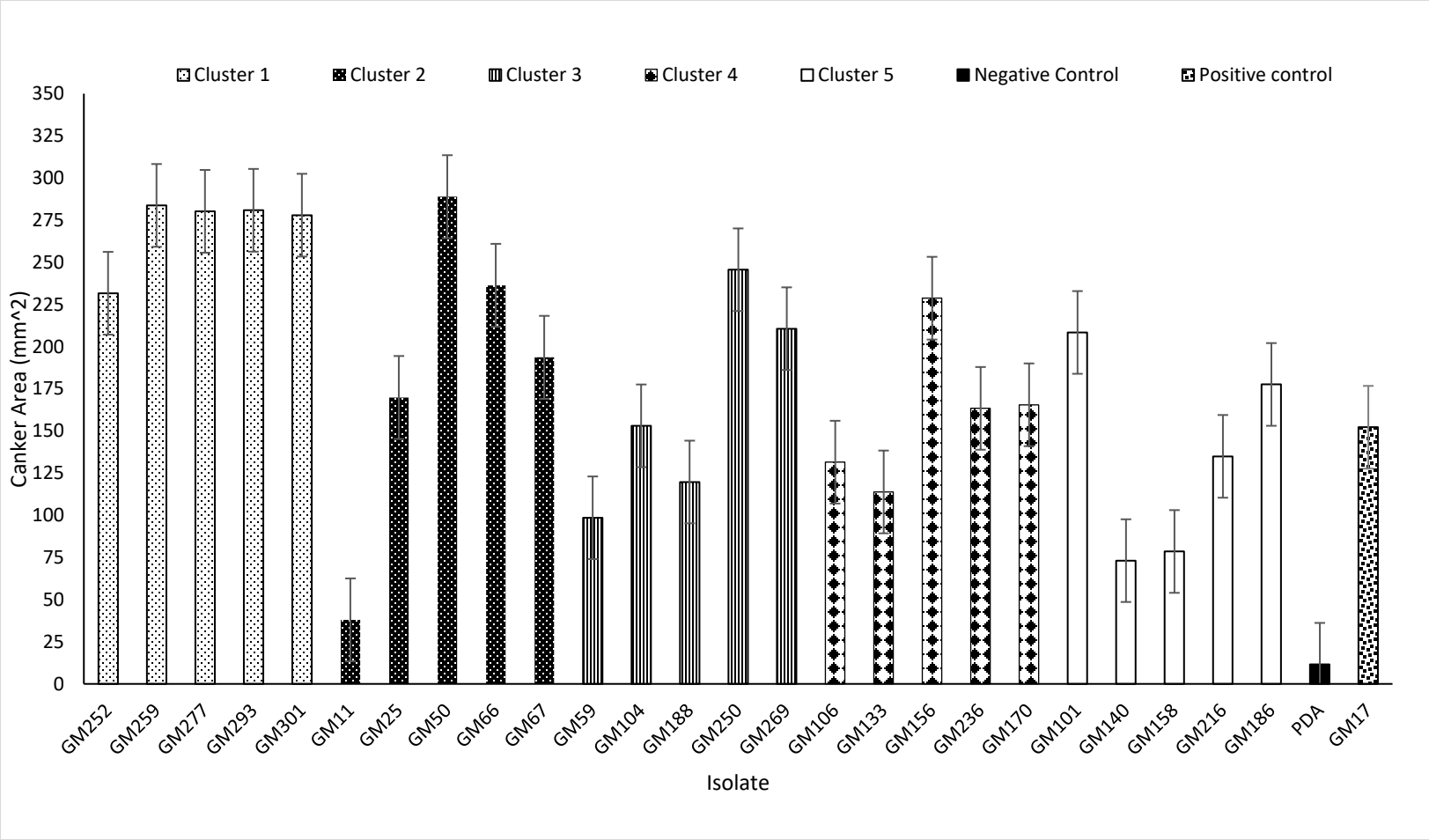


Figure 12. Mean canker areas observed across all *Geosmithia morbida* isolates.

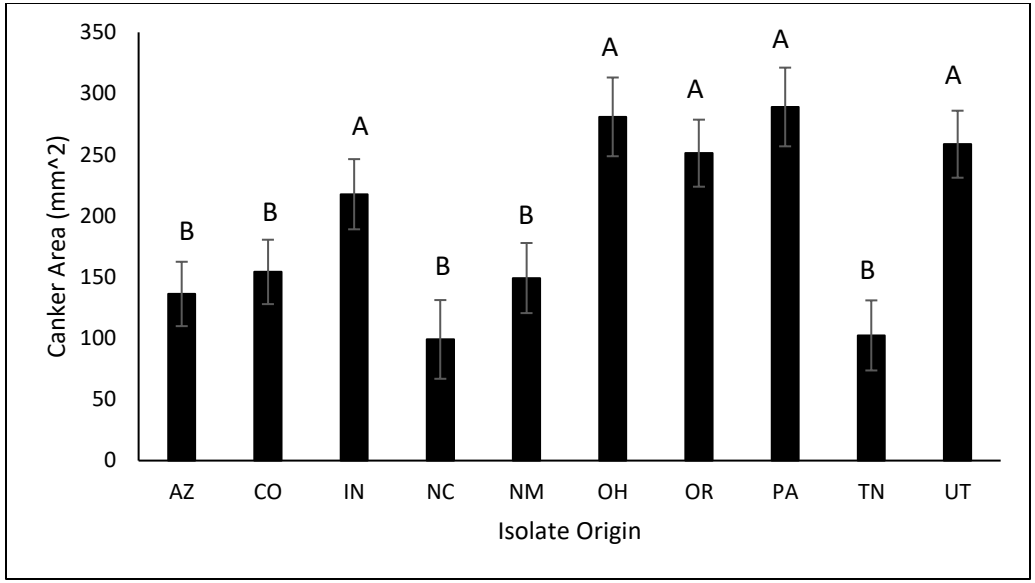


Figure 13. Mean canker areas measured following *Juglans nigra* inoculations using the *Geosmithia morbida* isolate(s) from representative collection states. Differences in the letter groupings indicate statistical differences among canker area means at $p<0.05$ (Tukey's honest significant difference)

Vita

Karandeep Singh Chahal was born in Punjab, India, to the parents of Sr. Jasdev Singh and Sukhwinder Kaur. After his 10^{th} grade he skipped high school and joined Punjab Agricultural University (PAU), Ludhiana, India for his BS in Agriculture (Hons.) and graduated in June 2015. He was awarded with Gold medal for his outstanding performance in academia as crop protection major. As a professional handball player for PAU, he received College and University merit certificates. For his MS in plant pathology, Karandeep joined the 'Woody Ornamental Disease Lab' at University of Tennessee, Knoxville in Fall 2015 under the guidance of Dr. Mark Windham. Karandeep is going to graduate with a MS degree in Plant Pathology in August 2017. For his Ph.D. in Plant Pathology, He will be joining Michigan State University, East Lansing.