Research

Detection of *Geosmithia morbida* on Numerous Insect Species in Four Eastern States

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Abstract

Thousand cankers disease is caused by the coalescence of numerous *Geosmithia morbida* cankers on branches and stems of *Juglans* species, leading to branch dieback and eventual tree death. The fungus sporulates in galleries of the walnut twig beetle (*Pityophthorus juglandis*), allowing for acquisition of pathogen propagules and its subsequent transmission to other branches or trees following adult emergence. Recently, *G. morbida* has been isolated from *Xylosandrus crassiusculus* and *Xyleborinus saxesenii* collected in Ohio and *Stenomimus pallidus* collected in Indiana. These beetles are known to colonize diseased *Juglans nigra* in these states. In this study, an operational trap survey for ambrosia beetles, bark beetles, and other weevils was conducted in four eastern states, and captured beetles were assayed to detect *G. morbida* using both culture and PCR-based methods. A new primer pair (GmF3/GmR13), based on the β-tubulin region, was designed for *G. morbida* DNA detection. The pathogen was detected on 18 insect species using molecular methods, and live cultures were isolated from two species. This is the first report of the pathogen in Illinois and Minnesota.

Keywords: diagnostics, trees, thousand cankers disease, *Juglans*, walnut, beetles, primer pair

Thousand cankers disease (TCD) of walnut (*Juglans*) species results from numerous cankers caused by *Geosmithia morbida* on branches and main stems of susceptible hosts (Tisserat et al. 2009). The fungus is introduced during attacks of *Juglans* spp. by its primary vector, the walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae). Branch dieback and crown death occur after extensive death of the phloem and cambium on branches and stems of affected trees, often taking several years to develop. The causal fungus of the disease was identified and the disease named in 2009 following investigation of widespread death of eastern black walnut in urban settings in Colorado in 2003 and subsequent years (Tisserat et al. 2009). The disease is now known to be present in nine western and five eastern (Maryland, North Carolina, Ohio, Pennsylvania, and Virginia) states. The WTB and the pathogen have been detected in separate locations in Indiana, but diseased trees have not been observed. TCD has been considered a serious threat to health of eastern black walnut and also an economic threat owing to walnut's high monetary value (Newton et al. 2009).

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Detection and monitoring surveys for TCD and/or the WTB have been conducted in multiple eastern states since 2010. These efforts are largely based on guidelines published by the USDA Forest Service and APHIS PPQ (USDA Forest Service and Plant Protection and Quarantine 2018). Insect funnel traps, girdled walnut trap trees, and log baits have been used for these surveys. Lindgren funnel traps baited with a commercially available WTB pheromone lure are most commonly used (Seybold et al. 2013). Collection cups associated with these traps may contain fluids ("wet cups") (e.g., propylene glycol or saline solution) or no fluid ("dry cups"). Stem sections from girdled trap trees or log sections used as bait traps are removed after specified exposure times, placed in emergence tubes or buckets, and any emerged insects regularly collected from dry cups associated with the emergence chamber. Insects obtained from funnel traps with dry cups or from emergence chambers can be used for detection of viable propagules of *G. morbida* or for DNA of the fungus, whereas those from wet cups may only be assayed for fungal DNA owing to the fluid killing the fungi. The pathogen has been isolated from and/or its DNA detected by molecular assay in macerated aqueous suspensions of bark weevils and ambrosia beetles emerged from girdled trap trees or from TCD-symptomatic trees in Indiana and Ohio, respectively (Juzwik et al. 2015, 2016). Thus, several eastern states have recently expanded their WTB monitoring programs to include selected weevil and ambrosia beetle species.

One pathogen detection study (Lamarche et al. 2015) reported on primers (GmF677/GmR878) based on the β-tubulin gene that were selective and sensitive for *G. morbida* detection in a real-time PCR protocol. More recently, microsatellite markers have been developed that are specific for the pathogen based on assays

conducted with wood shavings (Oren et al. 2018). Both these methods required specialized equipment; thus, we devised a PCR assay what was capable of being reproduced in a typical, basic diagnostic lab.

The objective of the study was to assay numerous scolytine and related insects trapped in operational WTB monitoring efforts for the presence of *G. morbida* (viable propagules and/or DNA). The detection of *G. morbida* on multiple ambrosia beetle, bark beetle, other weevils, and two related insect species in four eastern states and the techniques used in the detections are reported here.

Monitoring Sites and Trapping Protocol

Locations of concern for TCD were selected by cooperators in four eastern states and traps deployed (Fig. 1). In North Carolina, F1 traps were placed near eastern black walnut trees in campgrounds, parks, and wood products businesses in proximity to where TCD

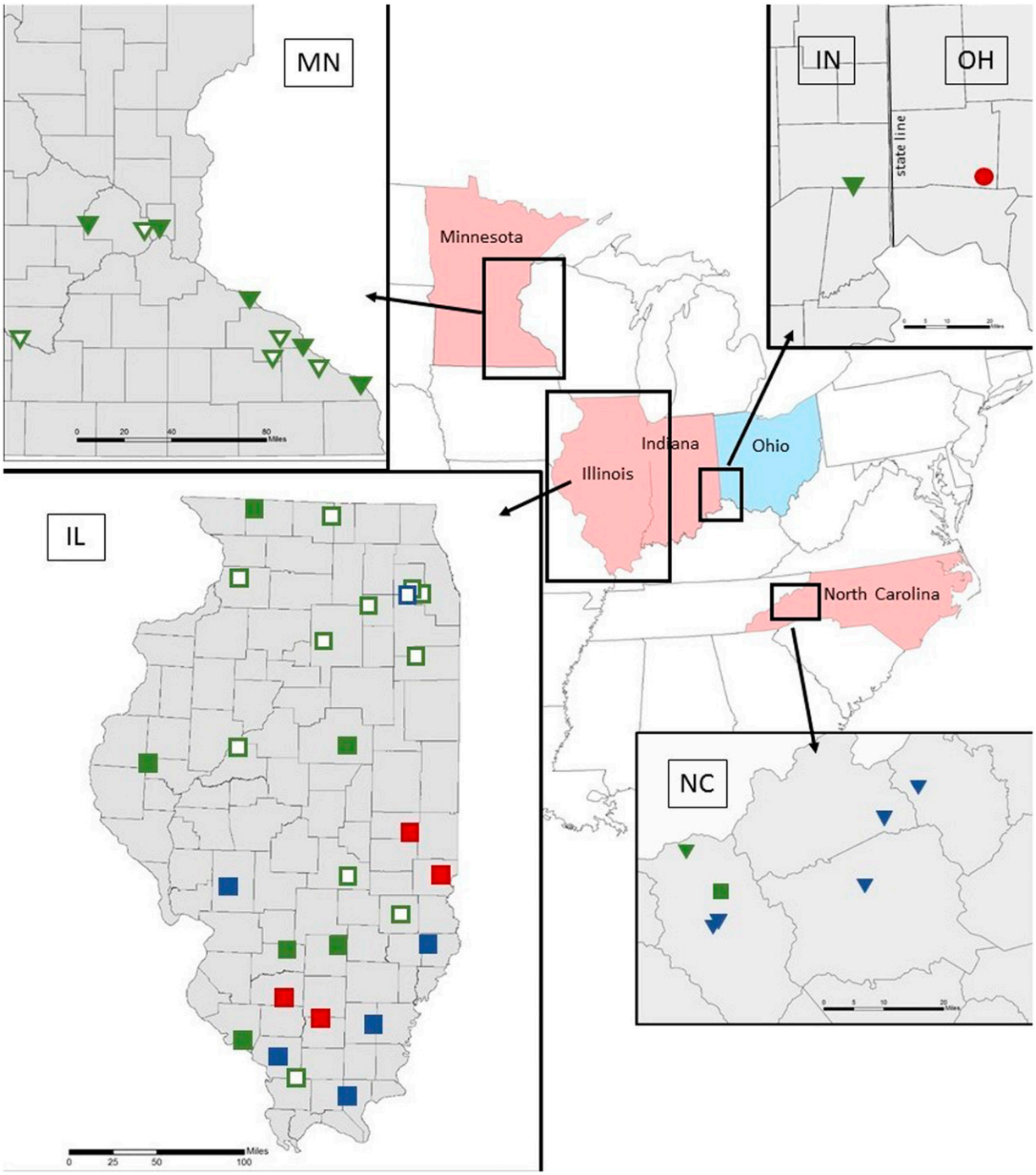

FIGURE 1

Map of survey areas and locations of traps for collecting insects, 2015 to 2017, for *Geosmithia morbida* detection assays. States with reported trap surveys are highlighted in red. State with thousand cankers disease (TCD) tree survey (Ohio) highlighted in blue. Symbol color: red = 2015; green = 2016; and blue = 2017. Symbol shape: ▼ = Lindgren funnel trap location; ■ = trap tree or trap log location; and ● = TCD tree location. Solid shape = positive detection for *G. morbida*; and outline only shape = no *G. morbida* detected.

was first detected in the state in 2013. Similarly, in Indiana traps were placed in and near a lumber mill located in Franklin County, which borders the county in Ohio where TCD is established. In Illinois, where TCD has not been detected, black walnut trap trees were chosen in representative parks throughout the state where black walnut is common, as part of TCD early detection surveys. Likewise, in Minnesota, traps were placed in areas where black walnut are common as part of an early detection program. At each location, the primary use of traps was to monitor for WTB, but other insects ("by-catch") such as ambrosia beetles were also captured in the process. Multiple insect species were captured from 2015 through 2017, with the most extensive trapping effort in 2016.

Trap type and timing varied by the needs and interests of the individual state cooperators. Trap trees were small-diameter walnut trees that were girdled early in the growing season (May or June) by two encircling cuts (15 cm apart) made into the outer xylem. These trees are attractive to bark-inhabiting beetles that colonize declining trees. In mid-August the trees were felled, and stem sections (approximately 30 cm long) were placed in emergence buckets. Collection cups were inspected twice per week, and all emerged insects were collected as they appeared. Trap logs were individual branches or small stem sections hung in or near living walnut trees from mid-May to mid-June (about 4 weeks) and then collected and placed in emergence buckets.

Lindgren funnel traps were baited with a commercial WTB pheromone lure and equipped with either wet (antifreeze containing propylene glycol and ethanol) or dry collection cups with kill strips inserted. Most Lindgren traps were deployed in the early summer and the beetles collected every 1 to 2 weeks through mid-July. A 2-week period of collection was also done in early fall in Minnesota and North Carolina in 2016.

Insect Identification and Assay for Pathogen

Insect handling. Following collection from traps or emergence buckets, insects were initially frozen, identified to species, and placed singly in 1.5-ml microcentrifuge tubes before being stored at –20°C until further processing. To avoid contamination across insects in an individual collection cup, kill strips were placed in dry collection cups, forceps were sterilized, and new filter paper was used between insects when performing species identification. Generally, insects were identified to species at labs associated with the surveying agency and then frozen and sent to the St. Paul lab location for the fungal assays. The samples were processed within 6 to 12 months of their collection date.

Individual insects were macerated in a microtube in 30 µl of sterile molecular-grade water using a sterile micropestle. Following maceration, three sterile 2-mm glass beads were added to each tube, and the tubes were vortexed for 30 s. For the molecular assay, 110 µl of cetyltrimethylammonium bromide (CTAB) lysis buffer was added to each tube (Lindner and Banik 2009), and the tube was frozen at –20°C. For beetles used for the dual (both live dilution plating and molecular) assay, 60 µl of sterile water was used for the maceration and vortexing. Following maceration, 30 µl of the macerate was placed in 110 µl of CTAB, and 30 µl was used for serial dilution plating (SDP) (described below). Insects from five of the nine collections assayed were obtained from dry cups or emerged from trap trees or logs and used in the dual assay (Table 1). T1

Detection of *G. morbida*. Half of the macerated insect suspension was used to create a dilution series (30 µl of suspension in 300 µl of sterile water; 30 µl of the first dilution in 300 µl of sterile water). Three 100-µl aliquots of each dilution were dispensed and spread evenly on each of three plates containing quarter-strength potato dextrose agar (PDA) amended with streptomycin and chloramphenicol. Suspect colonies of *G. morbida* growing on the incubated plates (25°C; ambient fluorescent lighting) were transferred to half-strength PDA and subcultured as necessary to obtain pure isolates. Small amounts of mycelium were placed in 110 µl of CTAB in 0.6-µl tubes for DNA extraction and standard PCR (GeneClean with modifications by Lindner and Banik [2009]) using the following primer pair: GmF3, CAGGCGAGGAGAAACGAGAA; and GmR13, GAGTCAGTGTTCTGACCGCA (hereafter referred to as "GmP" primers). This primer pair was developed specifically for *G. morbida* detection using the β-tubulin region. PCR products were visualized on 1.5% agarose gel with ethidium bromide and viewed under UV light. Standard Sanger sequencing and a BLASTn search of the NCBI GenBank database were performed to confirm *G. morbida* identity using a 97% cutoff.

Total genomic DNA was extracted from CTAB portions of the above-described macerates using the Lindner and Banik (2009) method. Amplification of extracted DNA was based on the protocol

TABLE 1
Sources of insects, years, and trap types used for detection of *Geosmithia morbida* and comparison of molecular results with culturing (serial dilution plating [SDP]) method when performed Q:5

State	Year	Trap type	Number of insects assayed	Number of insects positive for *G. morbida* based on	
				PCR plus DNA sequencing[a]	SDP
Illinois	2015	Trap trees	35	11	NA[b]
Illinois	2016	Trap trees	349	88	1
Indiana	2016	Lindgren funnel, dry cup	17	6	0
Minnesota	2016	Lindgren funnel, wet cup	58	24	NA[c]
North Carolina	2016	Trap logs	55	42	NA[d]
North Carolina	2016	Lindgren funnel, dry cup	246	75	NA[d]
North Carolina	2016	Lindgren funnel, dry cup	109	41	0
Illinois	2017	Trap trees	148	69	1
North Carolina	2017	Lindgren funnel, dry cup	149	53	0

[a] GmP primer assay using primer pair GmF3/GmR13 and confirmed by Sanger sequencing.
[b] Not applicable: insects were stored in 70% ethanol after collection; thus, it was not possible to culture fungi.
[c] Not applicable: insects were trapped in propylene glycol and unable to culture fungi.
[d] SDP not performed.

of Juzwik et al. (2015) with the following modifications. In the PCR reaction mixture, each primer (the above-mentioned GmP primer pair) had a final concentration of 0.4 μM, and supplemental 25 mM magnesium solution was added to create a final concentration of 2.5 mM. No extra water was added to the PCR mix; therefore, all of the water (9.8 μl of the 15 μl reaction mixture) was from the original DNA extract, in order to maximize the chance of detecting minute quantities of target DNA. The thermocycler conditions were as follows: initial denaturing at 94°C for 10 min; 40 cycles of denaturing at 94°C for 40 s, annealing at 53°C for 40 s; extension at 72°C for 1.5 min; and a final extension step of 72°C for 10 min. Visualization of the PCR product was done on a 1.5% agarose gel with ethidium bromide incorporated under UV light.

Preliminary tests of this method revealed that at very low concentrations the *G. morbida* DNA was not always detected, and there were occasional false negatives. Therefore, each DNA sample was subjected to PCR with the GmP primers for two equal but independent runs, and a specimen was deemed tentatively positive if at least one run had a distinct band on the agarose gel at approximately 250 bp (Fig. 2). All PCR products were sequenced as described above to confirm *G. morbida* identity. The most commonly matched (>99%) GenBank accession number was KJ148224.1. Identification was possible with approximately 86% certainty for agarose gel bands that were initially visualized as positive, using a 97% cutoff for sequence match. Approximately 4.4% of sequences matched *G. morbida* in the GenBank database, but the match was below the cutoff. Occasionally a tentative positive would not be confirmed owing to unreadable or incomplete sequencing results (5.4%). The detection rate increased to 95% when unreadable sequences were removed and *G. morbida*

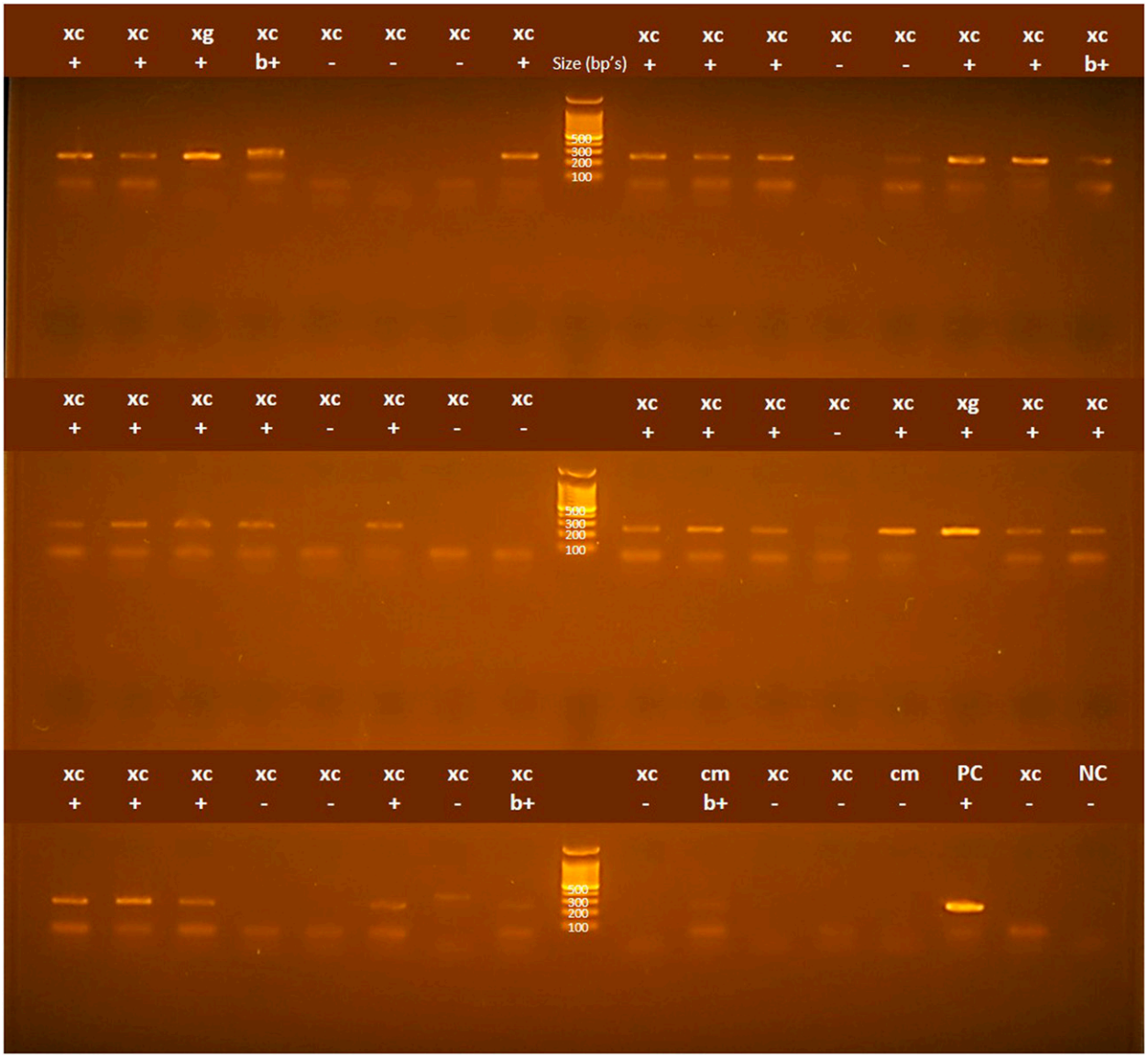

FIGURE 2

Image of PCR products in a 1.5% agarose gel following electrophoresis typical of band patterns observed in this study. Products derived from fungal DNA extracted from variously trapped insects. Positive band is approximately 250 bp. xc = *Xylosandrus crassiusculus*; xg = *X. germanus*; cn = *Cnestus mutilatus*; PC = positive control (*Geosmithia morbida* DNA); NC = negative control water blank; + = positive band; – = negative band (or too faint); and b+ = borderline size or brightness. All positive bands confirmed as *G. morbida* by sequencing using a 97% cutoff.

matching sequences under the 97% cutoff were added. Some (2.3%) of the readable sequences did not match any known organism in the database (85% cutoff). Only 1.8% matched another fungal or bacterial species.

Pathogen Detection on Assayed Insects

In Illinois, where TCD has not been observed, the rates of *G. morbida* detection on insects collected from trap trees and assayed using the GmP primer assay were 31, 25, and 47% for collections made in 2015, 2016, and 2017, respectively (Table 1). In Minnesota, another state with no known TCD, the detection rate was 41% (Lindgren funnel traps, wet cup). In North Carolina, where TCD has been documented, *G. morbida* DNA was detected using the GmP assay in 2016 on 75% of assayed beetles from trap logs compared with 35% of beetles from Lindgren funnel traps with dry cups. Similarly, in 2017, 36% of the insects from the North Carolina dry cups of funnel traps were positive for *G. morbida* DNA.

When the SDP procedure was used on a subset of five insect collections (Table 1), *G. morbida* was detected only twice (0.26%) compared with much higher rates found for the same insects using the GmP assay (35%). The SDP method detects live propagules, whereas the GmP assay detects DNA of live or dead *G. morbida*. The detected DNA could be coming from nonviable, dead, or even fragmentary sources, but it confirms the presence of *G. morbida* in the environment.

Assay of Insects from TCD Trees

Rates of *G. morbida* detection based on SDP were compared with those from the GmP assay method used for WTB and weevil species collected from emergence buckets containing branch and main stem sections (1.0 m long) of four TCD-symptomatic *Juglans nigra* (25 to 35 cm diameter at breast height) in Butler Co., Ohio, in 2015. The previously described dual assay system was used to process 41 weevils (representing four species) and two *P. juglandis* (Table 2). *G. morbida* was detected more frequently (χ^2 $P = 0.0283$) using GmP (37.2%) than SDP (16.3%). GmP results are based on DNA sequencing. Positive detection by both methods (11.6%) was obtained for five specimens represented by three species (*P. juglandis, Stenomimus pallidus,* and *Himatium errans*).

TABLE 2
Comparison of *Geosmithia morbida* detection on adults of four weevil and one bark beetle (*Pityophthorus juglandis*) species by two assay methods[a]

Insect species	Number of insects assayed	Number of insects with detectable *G. morbida*[b]		
		SDP	GmP	Both methods
Himatium errans	25	5	8	3
Stenomimus pallidus	9	1	5	1
Conotrachelus retentus	3	0	2	0
Pityophthorus juglandis	2	1	1	1
Acamptus rigidus	4	0	0	0

[a] Insects were emerged from branch and main stems sections taken from thousand canker disease-symptomatic *Juglans nigra* in Ohio, September 2015.

[b] Dual assay system used for macerated insects suspended in molecular-grade water. SDP = serial dilution plating on agar medium; and GmP = extracted fungal DNA amplified with GmF3/GmR13 primers in PCR reaction, results confirmed by Sanger sequencing.

When comparing results of the SDP method only, the probability of detecting the fungus was greatest for insects obtained in Ohio, where TCD-symptomatic trees occur (16.3%), compared with Illinois (0.26%), where TCD trees have not been found (χ^2 $P < 0.0001$).

Summary of Insect Species on Which *G. morbida* Was Detected

G. morbida (either live propagules via SDP assay or fungus DNA via GmP assay and sequencing) was detected on 18 species of ambrosia beetles, bark beetles, other weevils, and two other taxa through our surveys (Table 3). The most frequently trapped and assayed insect species was *Xylosandrus crassiusculus*. One of the live *G. morbida* isolates obtained from the Illinois survey collection came from an *X. crassiusculus* specimen, whereas the other was obtained from *Pseudopityophthorus minutissimus*. Other frequently trapped insects testing positive for *G. morbida*, from multiple states, include *Xyleborinus saxesenii, Xylosandrus germanus,* and *Monarthrum mali.* For insect taxa represented by eight or more individuals, rates of *G. morbida* detection ranged from 15 to 60%, and frequencies of detection were different among these taxa (χ^2 $P = 0.0048$). The highest detection rates were for two species not previously known to colonize walnut and were found in trap trees in Illinois in 2017: the oak bark beetle, *P. minutissimus* (60%), and the red-shouldered bostrichid, *Xylobiops basilaris* (56%). Another species found occasionally to carry *G. morbida* in Illinois in 2017 was *Neoclytus acuminatus*, the red-headed ash borer, a cerambycid that has been known to colonize walnut (Solomon 1995).

Implications and Conclusions

The disease known as TCD has been viewed as a disease complex involving the WTB and its symbiotic fungus, *G. morbida*. First reports of TCD in a state are based on the (i) detection of WTB, (ii) isolation of *G. morbida*, and (iii) presence of TCD-symptomatic trees (USDA Forest Service and Plant Protection and Quarantine 2018). Previously, *G. morbida* had been considered a unique associate of the WTB, which is native to the southwestern United States and invasive to newly detected areas. However, the discovery of *G. morbida* on the insect *S. pallidus* in Indiana in the absence of symptomatic walnut trees (Juzwik et al. 2015) led to the hypothesis that the *G. morbida*/WTB association was not unique and G. *morbida* may be more widespread in the United States than previously considered. Our current findings provide further evidence for "de-coupling" *G. morbida* from WTB.

New evidence has been reported that *G. morbida* DNA has also been found on ambrosia beetles captured by Lindgren funnel traps in Missouri (Doerhoff and Diesel 2018). Detections were based on results of assays using the GmP primers and were confirmed by sequencing (Josephine Mgbechi-Eseri, *personal communication*). To date, *G. morbida* DNA has been detected on non-WTB insects in five states, and live propagules have been found in two. The fact that *G. morbida* DNA has now been found in so many locations supports the hypothesis that the fungus may be widespread throughout the eastern United States and occurs in the absence of TCD-symptomatic walnut or WTB.

Live, culturable *G. morbida* has now been detected on three insect species in non-TCD areas: *S. pallidus* (Juzwik et al. 2015) and now *X. crassiusculus* and *P. minutissimus*. However, the frequency of the pathogen isolation has been rare from dispersing insects captured as "by-catch" in WTB pheromone-baited traps or emerged from trap trees or logs. In contrast, *G. morbida* has been

TABLE 3
Insects for which *Geosmithia morbida* DNA was detected using the GmP primer assay[a]

Insect species	Insect classification		Number of insects	
	Common name category	Subfamily	Total assayed[b]	*G. morbida*-positive
Xylosandrus crassiusculus	Ambrosia beetle	Scolytinae	735	250
Xyleborinus saxesenii	Ambrosia beetle	Scolytinae	198	77
Xylosandrus germanus	Ambrosia beetle	Scolytinae	34	12
Monarthrum mali	Ambrosia beetle	Scolytinae	33	9
Pseudopityophthorus minutissimus	Bark beetle	Scolytinae	30	18
Cnestus mutilatus	Ambrosia beetle	Scolytinae	23	11
Ambrosiophilus atratus	Ambrosia beetle	Scolytinae	17	4
Monarthrum fasciatum	Ambrosia beetle	Scolytinae	7	3
Ambrosiodmus obliquus	Ambrosia beetle	Scolytinae	5	1
Xyleborus californicus	Ambrosia beetle	Scolytinae	5	1
Pityophthorus juglandis	Bark beetle	Scolytinae	3	1
Himatium errans	Other weevils	Cossoninae	27	4
Stenomimus pallidus	Other weevils	Cossoninae	20	8
Stenoscelis brevis	Other weevils	Cossoninae	1	1
Conotrachelus retentus	Other weevils	Molytinae	5	3
Dryophthorus americanus	Other weevils	Dryophthorinae	1	1
Xylobiops basilaris	Powder-post beetle	Bostrichinae	32	18
Neoclytus acuminatus	Long horned beetle	Cerambycinae	20	4

[a] Results are combined from trap catches in four states over three years and assay results confirmed by DNA sequencing.
[b] Extracted fungal DNA amplified with β-tubulin primers in GmP (GmF3/GmR13) PCR reaction and results confirmed by Sanger sequencing.

commonly isolated from WTBs and several ambrosia beetle and weevil species emerged from TCD-affected walnut trees (Juzwik et al. 2016; this study; Moore and Juzwik, unpublished data), suggesting that the fungus is cryptic, doing little or no damage unless introduced into the numerous wounds created by the WTB.

In this study, *G. morbida* DNA was found on 18 species of insects, indicating the fungus has at least casual contact on a broad range of species. The granulate ambrosia beetle, *X. crassiusculus*, was by far the most common insect intercepted on or near eastern black walnut in our studies. This species is a widespread invasive pest that has been present in the eastern United States since at least 1974, and it has been implicated as a destructive pest of fruit trees and ornamentals, capable of mass attacking small trees (Ranger et al. 2016). *X. crassiusculus* has recently been reported on stressed *J. nigra*, as have *X. saxesenii* and *X. germanus* (Reed et al. 2015), two of the more frequently captured species in our study. *X. germanus* is also a widespread pest that has been in the United States since at least 1932 and is known to damage black walnut in nursery settings (Ranger et al. 2010; Weber and McPherson 1983). For any of these insects to be a major contributor to the TCD problem, however, they would have to consistently carry live *G. morbida* propagules as well as being capable of introducing enough inoculum through mass attack to affect the health of mature black walnut trees. At the present time, WTB alone is known to transmit the canker fungus to apparently healthy eastern black walnut trees. The relative importance of these other species in transmission of the pathogen and subsequent TCD development is not known and warrants further investigation.

Lastly, we developed a primer pair based on the β-tubulin gene for use within a traditional PCR protocol to detect DNA of *G. morbida.* This primer pair is a significant improvement over previously published tools that utilize the internal transcribed spacer (ITS) region. We were unable to devise a primer pair using the ITS region that was specific enough to detect *G. morbida* alone (unpublished results). This is consistent with other researchers' observations that the ITS region is not sufficiently informative to distinguish certain *Geosmithia* species (Kolařík et al. 2017). An 86% accuracy level for detection of *G. morbida* was determined for this protocol based on DNA sequencing results. Our protocol is suitable for use by plant disease diagnostic laboratories with basic molecular biology equipment; however, it requires DNA sequencing of the PCR product for confirming *G. morbida*. The sequencing confirmation is particularly important for regulatory situations and first detections in a state.

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AUTHOR QUERIES

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