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Differential Virulence Among *Geosmithia morbida* Isolates Collected Across the United States Occurrence Range of Thousand Cankers Disease

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Thousand cankers disease (TCD), first documented in the western United States in the early 2000s, has spread into nine western and seven eastern states in the United States and northern Italy. TCD incidence and severity differ between eastern and western United States outbreak localities. Black walnut (*Juglans nigra*) trees, introduced into both urban and plantation settings in the western United States, have been severely impacted as evident by the documented high disease incidence and mortality. However, in eastern United States localities, where *J. nigra* is native, host-pathogen-vector interactions resulted in two different outcomes: trees either die or partly recover followed by infection. Recent genetic studies on the TCD causal agent, *Geosmithia morbida*, indicate the spatial genetic structure and high levels of genetic diversity among United States populations. Using detached branch inoculation assays, we reported differential virulence among 25 *G. morbida* isolates collected across the current distribution range of the disease. As a proxy for virulence, the canker area was measured to 7 days after inoculation. Varying degrees of virulence were observed among tested *G. morbida* isolates, which was partly explained by their genetic provenance (genetic clusters). Isolates that grouped within genetic cluster 2 ($n = 7$ from the eastern United States and $n = 6$ from the western United States; mean = 210.34 mm^2) induced significantly larger cankers than isolates that grouped within genetic cluster 1 ($n = 12$; all western United States isolates; mean = 153.76 mm^2). Canker sizes varied among isolates within each genetic cluster and were not correlated with a geographic region (eastern vs. western United States) but rather to the isolated state of origin. Mean canker size also differed in response to isolates that originated from different tree host species. *G. morbida* isolates collected from *Juglans major* induced statistically smaller cankers when compared to isolates recovered from undetermined *Juglans* species but not from *J. nigra*. In sum, the increased mortality reported for western United States walnut tree populations cannot be explained by a higher virulence of local *G. morbida*. Plausible explanations for the observed disparity include environmental conditions, such

as prolonged drought, greater population densities of walnut twig beetle causing a higher number of inoculation events to individual trees, and multiple introductions of *G. morbida* originating from multiple locations. Future experimental evaluation should be undertaken to quantify the influence of these factors on the local epidemics.

Keywords: cankers, *Geosmithia morbida*, pathogenicity, thousand cankers disease, virulence, *Pityophthorus juglandis*, walnut twig beetle, *Juglans nigra*

INTRODUCTION

Thousand cankers disease (TCD) is a disease complex that involves interactions between walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae), a canker-causing fungal pathogen, *Geosmithia morbida* Kolarik, Freeland, Utley, and Tisserat (Ascomycota: Hypocreales: Bionectriaceae), and susceptible host plants, walnut (*Juglans* spp. L.) and wing nut (*Pterocarya* spp.) Kunth (Tisserat et al., 2009, 2011; Kolarik et al., 2011; Hishinuma et al., 2016). Although most *Juglans* and *Pterocarya* species are susceptible, symptoms are more severe in *Juglans nigra* L., the eastern United States native black walnut (Utley et al., 2013; Rugman-Jones et al., 2015; Hishinuma et al., 2016; Hefty et al., 2018). External symptoms of TCD include wilting, flagging, crown thinning, and branch dieback followed by the emergence of epicormic shoots (Tisserat et al., 2009). Internal symptoms include numerous localized small cankers within the phloem that can coalesce and girdle the tree (Kolarik et al., 2011; Tisserat et al., 2011). Vertical and horizontal phloem-restricted galleries beneath the bark provide evidence of beetle feeding and reproduction (Tisserat et al., 2011; Seybold et al., 2019).

Fungal members of the genus *Geosmithia* Pitt are ubiquitous and have been reported from North and South America, Europe, Asia, and Australia (Kolarík et al., 2008, 2017; Chahal et al., 2017). The majority of *Geosmithia* spp. are associated with bark and ambrosia beetles infesting diverse host plants, including coniferous and hardwoods (Chahal et al., 2017; Kolarík et al., 2017; Schuelke et al., 2017; Huang et al., 2019). Although most *Geosmithia* spp. are saprophytes, a few species can act as weak pathogens (Schuelke et al., 2017), including *G. morbida* (Kolarik et al., 2011; Kolarík et al., 2017), which was the first pathogenic species described in this genus. The second one was *Geosmithia* sp. 41 (initially identified as *Geosmithia pallida* by Lynch et al., 2014), the causal agent of foamy bark disease on *Quercus agrifolia* Nee. (California live oak) and other oak species (Huang et al., 2019). Although the associations of *Geosmithia* spp. with bark and ambrosia beetles are documented (Huang et al., 2017; Jankowiak and Bilański, 2018), the details of the ecological interactions among *Geosmithia* spp., its beetle vector(s), and host plant(s) are not fully understood (Huang et al., 2019). This is true for the TCD pathosystem, in which the nutritional role of *G. morbida* is still largely unknown. *G. morbida* has only been reported to colonize hosts in the *Juglandaceae* family (Tisserat et al., 2009). However, the fungus has been recently recovered from other subcortically active beetles besides WTB (Chahal et al., 2019; Moore et al., 2019), suggesting that the fidelity of *G. morbida* to WTB as the most functional beetle associate may have been overestimated. Additional work is needed to elucidate the potential role of other insect species in the efficient transmission of the pathogen and on the development of disease on susceptible hosts (Chahal et al., 2019).

Although, by 2009, TCD was documented in most of the states of western United States (Tisserat et al., 2009, 2011), the first detection in the eastern United States (Knoxville, Tennessee) was recorded in 2010, representing an imminent threat to *J. nigra* in urban and forest settings (Grant et al., 2011). The initial occurrence of TCD in the western United States is spatially pertinent since it overlaps with the native range of the TCD vector, WTB (Kolarik et al., 2011; Sitz et al., 2021). Following the initial discovery of TCD in Tennessee, members of this disease complex were subsequently reported in Indiana, Maryland, North Carolina, Ohio, Pennsylvania, and Virginia (Hansen et al., 2011; Hadziabdic et al., 2014; Daniels et al., 2016), as well as in Italy (Montecchio and Faccoli, 2014; Moricca et al., 2019). Forest Inventory and Analysis (FIA) projections estimated that TCD could have been present in the eastern United States at least 10 years before the first report was made (Randolph et al., 2013). In the eastern United States, *J. nigra* mortality attributed to TCD has been very low (<5%) in comparison with mortality rates in western states where ∼60% of *J. nigra* populations have been removed (Newton et al., 2009; Tisserat et al., 2011; Randolph et al., 2013; Seybold et al., 2019). Furthermore, recovery of some *J. nigra* trees following TCD infection has been documented in the eastern United States (Griffin, 2015; Seybold et al., 2019). Slow TCD progression and partial recovery have also been observed in affected populations distributed in the western United States (Seybold et al., 2019) but at lower rates compared to the eastern United States. In the western United States, the high tree mortality of *Juglans* spp. has been observed in diverse habitats, including agricultural plantations, urban forests, and wildland settings (Seybold et al., 2019). These patterns were not observed in the eastern United States, where the majority of affected trees are distributed in the urban landscape, along roads, and in recreational parks, suggesting that resistance to *G. morbida* may be naturally present in native, *J. nigra* trees (Griffin, 2015; Seybold et al., 2019; Sitz et al., 2021). We hypothesized that differences in TCD outcome observed between eastern and western United States are due to differential virulence present in *G. morbida* populations.

Population studies have revealed high genetic diversity and spatial structure among *G. morbida* populations distributed across the United States (Hadziabdic et al., 2014; Zerillo et al., 2014). *G. morbida* populations collected across the entire

geographic distribution of TCD can be grouped into two distinct genetic clusters (Hadziabdic et al., 2014; Hadziabdic et al., unpublished). This grouping not only indicates the complexity of the pathogen inoculum but also supports earlier suggestions that *G. morbida*, such as WTB, is likely native to North America (Sitz et al., 2021). The current working hypothesis states that *G. morbida* evolved in close association with at least one native *Juglans* species within the western United States range of WTB and that *G. morbida* was present, but undetected, in the eastern United States for a long time before it was first documented (Hadziabdic et al., 2014; Zerillo et al., 2014; Sitz et al., 2021). The study by Sitz et al. (2021) supported this hypothesis by reporting regional resistance and variation in host responses to *G. morbida* infestations in *J. nigra* trees. Their results indicated higher resistance in *J. nigra* populations in the western and central ranges of the native distribution, where TCD has not been documented (Sitz et al., 2021).

The objective of this study was to determine whether isolates collected from different localities have different virulence profiles. We hypothesized that differences in *G. morbida* virulence are correlated to their genetic cluster assignment and that their genetic grouping can explain the difference in TCD incidence and severity observed between eastern and western states. Using canker size as a proxy for virulence, our specific objectives were a) to measure canker size resulting from *G. morbida* inoculation into *J. nigra* host branches and b) to compare canker size variation as a function of genetic clustering and geographic and host origin.

MATERIALS AND METHODS

Selection of *G. morbida* Isolates

Preliminary genetic profiling from early examinations of this data set led to the selection of the 25 *G. morbida* isolates that were used in this study. Isolates were chosen based on their genetic diversity and assignment probability to belong to one of the assigned genetic clusters (Hadziabdic et al., unpublished data; **Supplementary Figure S1**). More specifically, these isolates were chosen as representatives of five putative genetic clusters and were used to initiate the experiments. Subsequent addition of *G. morbida* isolates from additional states led to a reanalysis of the data and reorganization of genetic variability across the United States into two genetic clusters (Hadziabdic et al., unpublished data; **Supplementary Figure S1**) that are consistent with the previous study (Zerillo et al., 2014). Thus, experiments reported here were conducted with 12 isolates from genetic cluster 1 (all western United States isolates) and 13 isolates representing genetic cluster 2 ($n = 7$ from the eastern United States and $n = 6$ from the western United States) (**Table 1**).

TABLE 1 | *Geosmithia morbida* isolates selected for inoculation experiment per genetic groupings of Hadziabdic et al. unpublished.

Genetic cluster[a]	Isolate number[b]	Geographic origin[c]	Collection location[d]	Host[e]	Genbank accession[f]
Cluster 1	GM101	Western	AZ	*J. major*	MG008832
Cl. 1	GM104	Western	AZ	*J. major*	MG008838
Cl. 1	GM106	Western	AZ	*J. major*	MG008840
Cl. 1	GM133	Western	AZ	*J. major*	MG008842
Cl. 1	GM140	Western	AZ	*J. major*	MG008845
Cl. 1	GM156	Western	CO	*J. microcarpa*	MG008830
Cl. 1	GM158	Western	CO	*J. nigra*	MG008844
Cl. 1	GM170	Western	CO	*J. nigra*	MG008836
Cl. 1	GM186	Western	CO	*J. nigra*	MG008834
Cl. 1	GM216	Western	NM	*J. major*	MG008839
Cl. 1	GM236	Western	NM	*J. major*	MG008837
Cl. 1	GM269	Western	UT	*J. nigra*	MG008831
Cluster 2	GM188	Western	CO	*J. nigra*	MG008841
Cl. 2	GM301	Western	OR	*Juglans* sp.	MG008826
Cl. 2	GM250	Western	OR	*Juglans* sp.	MG008827
Cl. 2	GM252	Western	OR	*Juglans* sp.	MG008829
Cl. 2	GM259	Western	UT	*J. nigra*	MG008823
Cl. 2	GM277	Western	UT	*J. nigra*	MG008825
Cl. 2	GM66	Eastern	IN	*J. nigra*	MG008828
Cl. 2	GM67	Eastern	IN	*J. nigra*	MG008833
Cl. 2	GM59	Eastern	NC	*J. nigra*	MG008843
Cl. 2	GM293	Eastern	OH	*J. nigra*	MG008824
Cl. 2	GM50	Eastern	PA	*J. nigra*	MG008822
Cl. 2	GM11	Eastern	TN	*J. nigra*	MG008846
Cl. 2	GM25	Eastern	TN	*J. nigra*	MG008835
Negative_ Control	Neg_Cntrl	-	-	-	
Positive_ Control	GM17	Eastern	TN	*J. nigra*	MG008849

[a] *Genetic cluster association of Geosmithia morbida isolates were characterized using our unpublished data and combining eastern and western isolates (294 samples); Hadziabdic et al., unpublished.*
[b] *Alphanumeric laboratory identification code for Geosmithia morbida isolates.*
[c] *Source of isolate origin from either eastern or western United States.*
[d] *State in which the G. morbida isolate was collected.*
[e] *Juglans host species (where known) from which the G. morbida isolate was collected.*
[f] *GenBank accession number.*

Molecular Confirmation of Selected Axenic Isolates

Axenic single-spore cultures were obtained from all the 25 *G. morbida* isolates following established protocols (Hadziabdic et al., 2014). All isolates were confirmed as *G. morbida* through morphological (prior to this study) and molecular identification. In brief, mycelial plugs from each isolate were grown in Difco™ Potato Dextrose Broth at room temperature (25°C) for 3 weeks following established protocols (Gazis et al., 2018). DNA was extracted from harvested mycelium as described previously (Gazis et al., 2018; Chahal et al., 2019). The Internal Transcribed Spacer (ITS) region was amplified using the primers ITS1F (Gardes and Bruns, 1993) and ITS4R (White et al., 1990). 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 double-distilled water to complete the total volume of 25 μl. The PCR thermal cycle 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 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. The amplifications of PCR products were confirmed with gel electrophoresis. 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. Basic Local Alignment Search Tool (BLAST) was used to compare the ITS sequences deposited in the NCBI nucleotide database. Generated ITS sequences were deposited to GenBank (**Table 1**).

Preparation of *J. nigra* Branch Sections

Detached branch assays were used to limit the potential confounding effects inherent in using field trees, such as differences in host genotype and microclimates. Two mature (>35-year-old) *J. nigra* trees located at Middle Tennessee Research & Education Center, Spring Hill, Tennessee, USA (latitude/longitude 35°43'01.3" N, 86°57'19.3" W; 35°43'03.0" N, 86°57'18.7" W) were selected as a source of branch sections for each of the two inoculation experiments that were repeated. For each inoculation treatment, each of two replicates was completed using branch sections taken from a single tree. None of the TCD complex members have been reported in Spring Hill, or from Maury and Williamson counties in Tennessee where Spring Hill is located. Symptoms of TCD were not observed on trees at the Spring Hill site. Once branches were detached, 10 cut branch sections (∼1 m length) from each tree were visually inspected for the presence of TCD signs and symptoms. During the examination, a portion of the outer bark layer from branch sections was peeled to verify that cankers, phloem discoloration, lesions, or necrotic areas nor galleries of other wood-boring beetles were present. Collected tree branches were also verified to be free of *G. morbida* using the molecular protocol described by Oren et al. (2018). In brief, three individual samples each containing approximately 150 mg of drilled inner wood shavings were collected from portions of 10 different branches that were harvested from each tree (**Supplementary Figure S2**). Samples were stored at −20°C until processed. DNA extractions, PCR amplifications, and amplicons were conducted as described by Oren et al. (2018). Molecular detection of *G. morbida* from source branches was negative for pathogen DNA, indicating that prior to this experiment, selected branch sections were free from *G. morbida* infection. The cut branches were stored at 4°C (∼5 days) prior to inoculation. Branches were cut into branch sections (length = 8 cm, diameter = 2–3 cm) using a handsaw and surface-sterilized with 70% ethanol (**Supplementary Figure S3**). Cut ends of inoculated branch sections were then dipped into paraffin wax and wrapped with aluminum foil to prevent evaporation and preserve moisture.

Inoculations of *J. nigra* Branch Sections With *G. morbida* Isolates

Fungal isolates were grown in Petri dishes (10 cm diameter, 1.5 cm depth) containing 20 ml of half-strength PDA at 25°C for 4 weeks. *G. morbida* isolates GM17, which is a well-characterized strain used in multiple previous studies (e.g., host-plant inoculations, development of molecular detection protocols, and comparative genetics and genomics experiments) was used as a positive control. Agar-only plugs were inserted into wounds as a negative control. Once branch pieces had dried following surface sterilization, sections were chosen randomly for *G. morbida* inoculation experiments (**Supplementary Figure S3**). Using a cordless drill with a 3.175-mm diameter bit, two ∼2-mm-deep wounds were offset on opposing sides of six separate branch sections per fungal isolate. Branch sections were inoculated by using a sterile, 4-mm diameter (o.d.) Humboldt Brass Cork Borer (Fisher Scientific, Pittsburgh, PA, USA) to remove a piece of fungal agar plug, which was then picked using a sterile scalpel, inverted, and inserted completely into each of the two opposing wounds per branch section. Colonized agar plugs were obtained from the edge of the growing colonies, and all plugs were completely covered by the fungus. Wounded branch sections were wrapped with Parafilm (M Laboratory Film, Bemis, Neenah, WI, USA), and the diameter of each inoculated branch section was recorded using a digital caliper (Lyman Products Corp., Middletown, CT, USA). Inoculated branch sections were incubated at 25°C in darkness for 7 days.

Canker Measurements

To visualize cankers in the phloem of cut branches before extensive tissue oxidation occurred, lesions were assessed to 7 days after inoculation (7 DAI). A sterile scalpel was used to shave the outer bark from around the wounding site exposing the cankers (**Supplementary Figure S3**), and the digital images of exposed cankers beside a reference scale were taken immediately. Image processing and analysis software, ImageJ (https://imagej.nih.gov/ij/) (Schneider et al., 2012), was used to measure canker area from digital photographs. The software was calibrated to standardize each photograph to the reference scale included in each photograph.

Confirmation of Pathogenicity *via* Modified Koch's Postulates

To confirm *G. morbida* as the causal agent of the observed cankers, a modified version of Koch's postulates was conducted using the rapid molecular detection method described by Oren et al. (2018). Three of six replicated branch sections used in each isolate inoculation were randomly selected, and one of the inoculation sites was selected for molecular screening. Under sterile conditions, drill shavings of phloem and wood tissues were collected only from the margins of the cankers, excluding the inoculation wound site. Sample collection, DNA extraction, and PCR amplification for pathogen detection were performed as described by Oren et al. (2018).

Statistical Analysis

The effects of the genetic cluster, isolates, *Juglans* host, location (state), and region (eastern vs. western United States) (**Table 1**) on canker size were each analyzed using the mixed model analysis for split-plot design with the individual *J. nigra* trees as the experiment unit for the whole plot effects. Diagnostic

analysis was conducted to assess model assumptions. The rank transformation was applied if diagnostic analysis exhibited violation of normality and equal variance assumptions. *Post-hoc* multiple comparisons were performed with Tukey's adjustment. Statistical significance was identified at $P < 0.05$. Analyses were conducted in SAS 9.4 TS1M7 for Windows 64x (SAS Institute Inc., Cary, NC, USA).

RESULTS

Confirmation of Koch's Postulates

From 150 samples tested (25 *G. morbida* isolates across six replicates), 147 were positive for the presence of *G. morbida* DNA in tissues collected from the margins of cankers (98% *G. morbida* detection rate). Phloem margins from darkened damage-response areas of six agar-only control samples were also tested, and *G. morbida* DNA was not detected from any of these samples.

Effect of the Variation in Branch Section Diameter on Canker Area

Branch sections of 2–3 cm diameter (average of 2.5 cm) were used for inoculations. The potential effect of variation in branch section diameter on the canker area was analyzed. Canker area did not differ in response to branch diameter ($P = 0.569$); therefore, branch diameters were pooled for further analyses.

Effect of Genetic Cluster, State, and Host Origin of Fungal Isolate on Canker Size

Overall, *G. morbida* isolates from genetic cluster 2 induced significantly larger cankers than the ones from cluster 1, yielding a mean canker area of 210.34 mm^2 and 153.76 mm^2, respectively

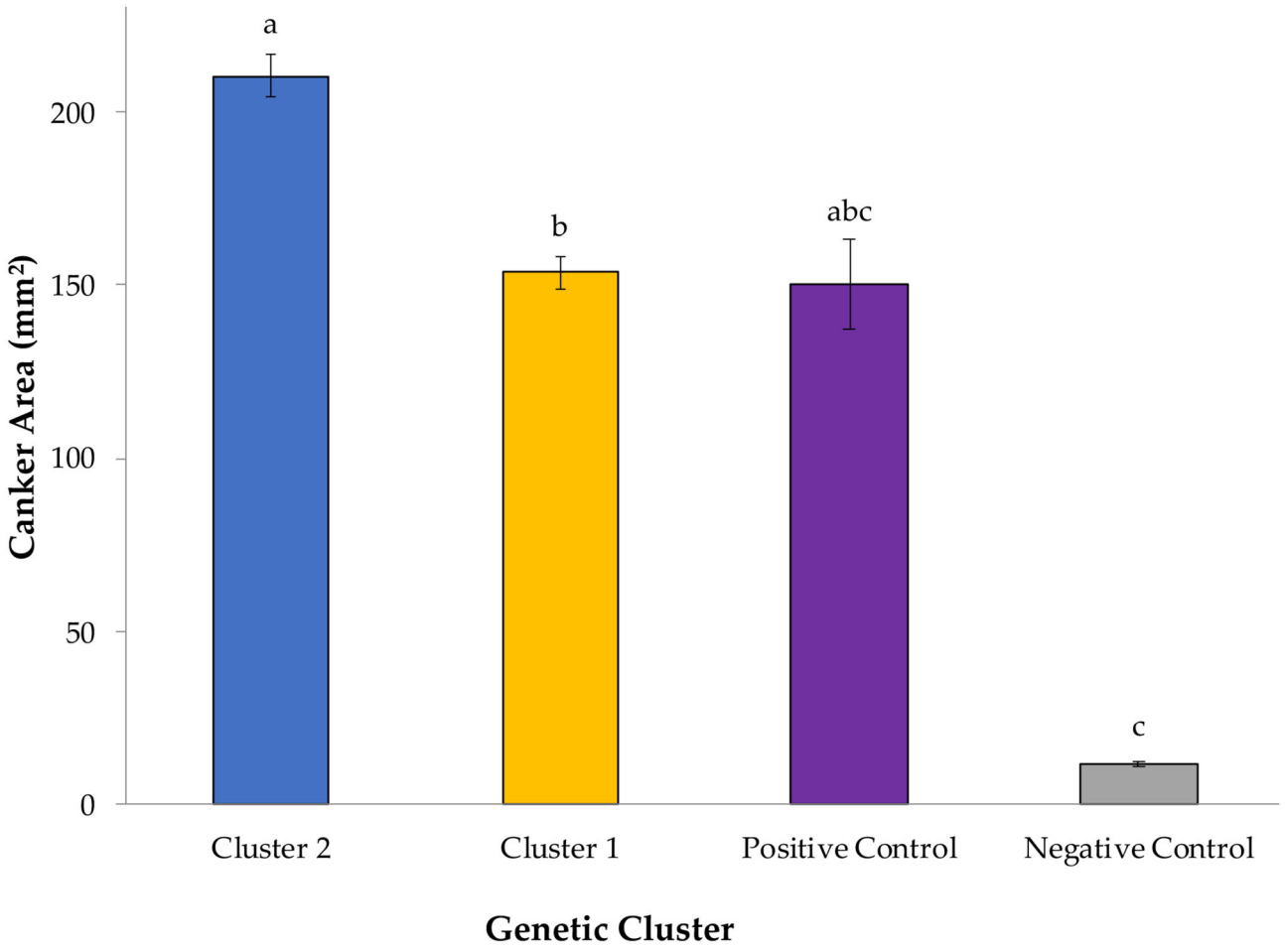

FIGURE 1 | Mean canker sizes were observed at 7 days after inoculation (DAI) with *Geosmithia morbida* isolates representing 2 genetic clusters, as were characterized in the studies by Hadziabdic et al. (2014) and Hadziabdic et al. (unpublished data, **Supplementary Figure S1**). In this study, 12 *G. morbida* isolates represented genetic cluster 1 and 13 *G. morbida* isolates represented genetic cluster 2. Within cluster or control group, treatment mean separations were made using least square mean estimates calculated from rank-transformed canker areas and differ significantly ($P < 0.05$; Tukey's honestly significant difference) when indicated with different letters. Mean canker areas and standard errors of genetic clusters and controls depicted here reflect non-transformed data measured across assays performed on both assayed trees.

($P = 0.034$; **Figure 1**). Canker sizes did not differ significantly among isolates within cluster 1 ($P = 0.080$), but canker sizes differed among isolates within cluster 2 ($P = 0.0003$). All *G. morbida* isolates resulted in the formation of cankers with areas that were significantly larger than responses observed in the negative control (effect of wound in isolation), except for isolates GM140 ($P = 0.184$) and GM158 ($P = 0.196$) (affiliated with cluster 1) and GM11 ($P = 0.623$) and GM59 ($P = 0.063$) (affiliated with cluster 2) (**Figure 2**). These isolates induced cankers; however, their smaller lesion areas were not statistically different from lesions produced by the wounding alone (**Figure 2**). Irrespective of genetic cluster association, all *G. morbida* isolates were variable in the mean canker sizes observed 7 DAI ($P < 0.001$). Despite variability observed across mean canker sizes from all isolates (Figure 2), differences were observed in mean canker sizes when analyzed according to the states in which the *G. morbida* isolates were collected ($P < 0.001$). At 7 DAI, the largest mean canker areas were induced by isolates GM 293 (OH), GM 277 (UT), GM 50 (PA), GM 259 (UT), and GM 301 (OR) (**Figure 2**). The largest canker sizes were observed across a mixture of western (OR and UT) with eastern (IN, OH, and PA) states. Similar variability was also evident in the state origins of isolates that induced smaller canker areas. However, we caution that for several states (OH, PA, and NC), only a single isolate could be obtained for assays, which restricts the validity of predictions that may be inferred regarding isolates from these states. When the results from these three state isolates were removed from data analysis, the effect of canker size on collection location (AZ, CO, IN, NM, OR, TN, and UT) was significant ($P = 0.0019$). To address this limitation to the experimental design, a comparison of canker areas was analyzed according to the eastern vs. western geographic region from which isolates originated and canker sizes among *G. morbida* isolates did not statistically differ ($P = 0.927$). Thus, our results indicate that the geographic origin of isolates is not a good predictor of expected virulence. Mean canker areas observed between geographic groups were 188 ± 8.6 mm^2 for eastern and 181 ± 4.4 mm^2 for western isolates (data not shown).

Finally, the observed mean canker areas differed when isolates were grouped by origin from different *Juglans* sp. hosts ($P = 0.048$). Mean canker area of isolates collected from *Juglans major* induced statistically smaller cankers when compared to isolates recovered from undetermined *Juglans* spp. ($P = 0.045$) but not from *J. nigra* ($P = 0.134$) (**Figure 3**). Cankers induced by the isolate recovered from *Juglans microcarpa*, which were excluded

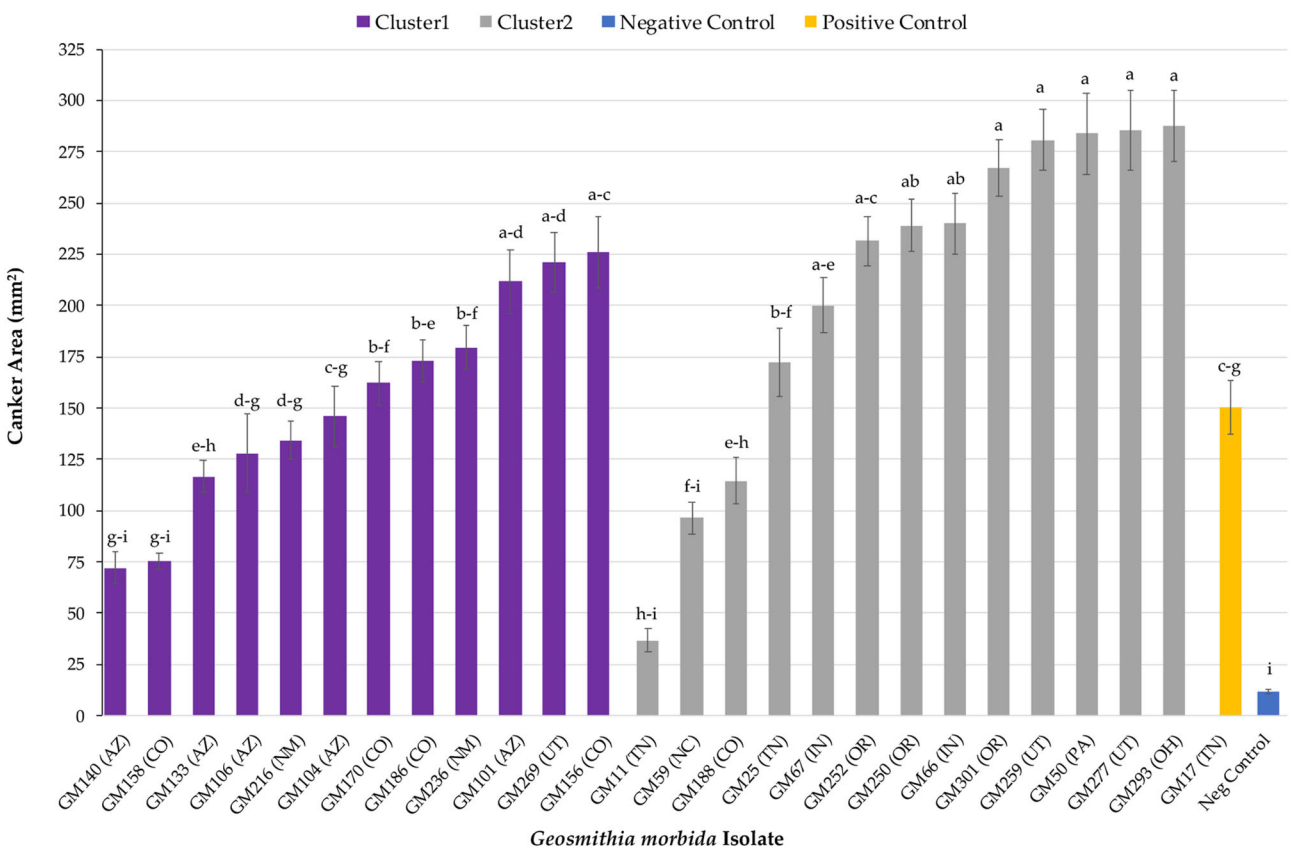

FIGURE 2 | Mean canker area observed across all *G. morbida* isolates. For each isolate, treatment mean separations were made using least square mean estimates calculated from rank-transformed canker areas and differ significantly ($P < 0.05$; Tukey's honestly significant difference) when indicated with different letters. Mean canker areas and standard errors depicted here reflect non-transformed data measured across both assayed trees.

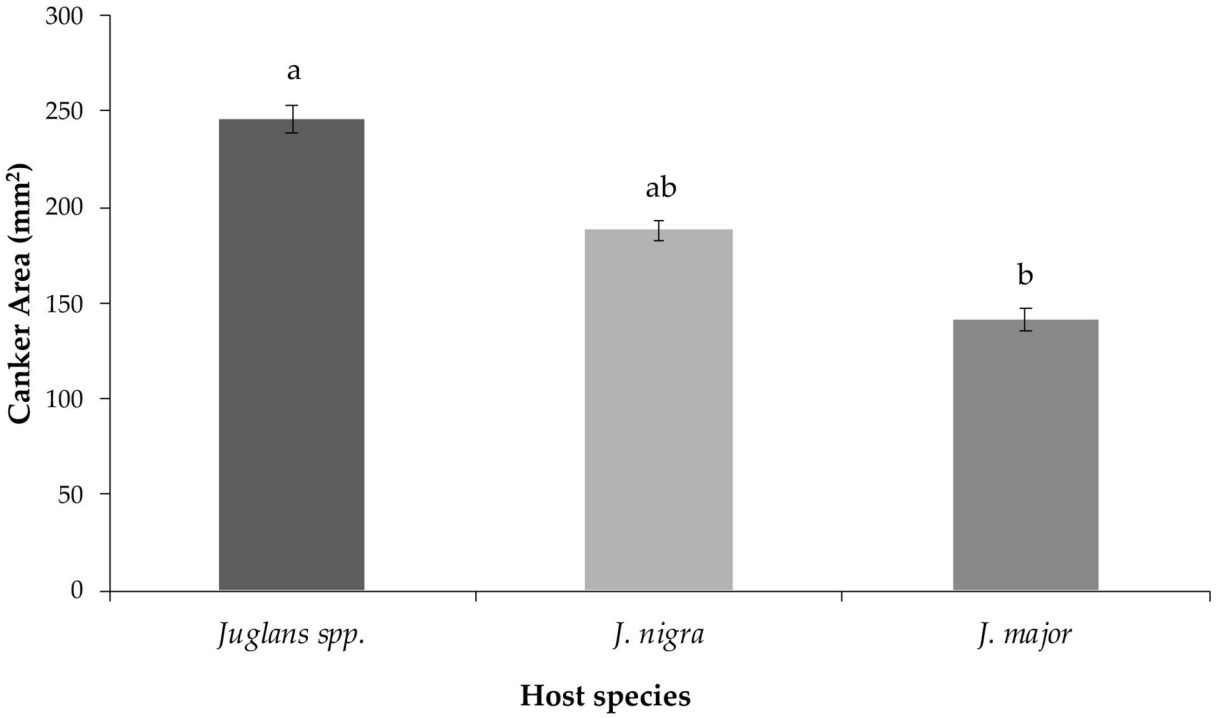

FIGURE 3 | Mean canker area measured for lesions induced by *G. morbida* isolates that were grouped based on *Juglans* species host source as follows: undetermined *Juglans* spp., *Juglans major*, and *Juglans nigra*. Within species, treatment mean separations were made using least square mean estimates calculated from rank-transformed canker areas and differ significantly ($P < 0.05$; Tukey's honestly significant difference) when indicated with different letters. Non-transformed data and standard errors are presented.

from statistical comparisons due to limited sample size, yielded intermediate-sized cankers averaging 225.81 ± 17.28 mm^2.

DISCUSSION

All tested *G. morbida* isolates induced canker formation. However, the geographic origin of the isolates (eastern vs. western origin) was not correlated to their virulence and could not be used to provide meaningful insights into the different disease outcomes observed between the two regions. While our data indicate that *G. morbida* isolates assigned to genetic cluster 2 (mixture of eastern and western isolates) yielded cankers with a larger mean size than isolates in cluster 1 (western isolates only), the virulence of *G. morbida* may only be partially explained by their genetic grouping into distinct clusters (clustering herein was based on *G. morbida* microsatellites, Hadziabdic et al., 2014, unpublished data). Isolates belonging to the same cluster induced a wide range of canker sizes. After 7 days, canker areas induced by *G. morbida* isolates in cluster 2 ranged in size from 36 to 287 mm^2 (from isolates GM11 and GM293, respectively) compared with canker areas from 72 to 225 mm^2 (isolates GM140 and GM156, respectively) induced by *G. morbida* isolates from cluster 1. Overall, *G. morbida* isolates from genetic cluster 2 yielded cankers that were more variable in size than isolates from genetic cluster 1. Significant differences were observed among mean canker sizes according to the states from which the *G. morbida* isolates were collected. Given the variability observed in canker sizes at 7 DAT, however, state provenance of the isolates was not a reliable predictor of potential virulence. This observation is further evidence in support of the hypothesis that multiple introductions of *G. morbida* have occurred across time and which have originated from multiple locations (Hadziabdic et al., 2014; Zerillo et al., 2014). Given the lack of statistical significance observed when comparing canker sizes induced by western vs. eastern isolates, our hypothesis for explaining the greater TCD severity seen in the western localities was not supported by these laboratory assays. Similarly, results do not support anecdotal observations that *G. morbida* has been less virulent in the eastern United States. Factors, including environmental conditions, host-plant genetics, and interactions with other fungi and microorganisms, are likely to contribute to virulence across the distributed range of *G. morbida*. We also acknowledged that the lack of resolution could be partly due to the low number of isolates from eastern ($n = 7$) in comparison with western ($n = 18$) states.

Detached branch and stem assays are often used in pathogenicity assays for fungi affecting shrubs (Wagner et al., 2006), woody ornamentals (Guo et al., 2015), and forest trees (Amponsah et al., 2011; Sessa et al., 2017) as rapid alternatives to *in planta* inoculations. Detached branch assays can be as reliable as *in vivo* assays for pathogenicity, virulence, and germplasm screening experiments (Smith, 1996; Kohpina et al.,

2000; Dodd et al., 2005; Stewart et al., 2005; Baskarathevan et al., 2012). These assays are also particularly appropriate when regulated or quarantined organisms, such as *G. morbida*, are being studied. In addition, detached tissue assays are easier to handle and offer greater reproducibility by enabling the use of branch sections with uniform diameters allowing appropriate replication, consistent inoculum delivery, and greater accuracy in the quantification of disease symptoms (Kohpina et al., 2000; Hüberli et al., 2002). However, this system is not without its flaws or biological shortcomings. Disadvantages of detached tissue assays are that host-defense mechanisms may be compromised and detached branches may not adequately reflect the whole plant response (Sessa et al., 2017). For example, larger cankers were observed on detached branches of necrotic grapevine tissue compared to results from *in vivo* branch inoculations, which shows the importance of host-plant defense response in mitigating disease severity (Amponsah et al., 2011).

Virulence can play an important role in pathogen emergence and reemergence, potential host switches, and range expansion, thus making it very challenging to control and contain further spread (Sacristán and García-Arenal, 2008). Our study demonstrated variable virulence outcomes for isolates from both of our genetic clusters. This mixed result is consistent with the variable virulence that was observed by Tran et al. (2020), in which the virulence of the fungal pathogens *Monilinia fructicola* and *Monilinia laxa* on Japanese plum, *Prunus salicina* cv. "Fortune," did not correlate to pathogen genotypes. Similar results have been reported in other studies using *in planta* and detached branch assays, wherein differences in virulence were not correlated with genetic diversity of canker-causing fungal pathogens (Baskarathevan et al., 2012; Billones-Baaijens et al., 2013; Elena et al., 2015). The geographic origin of fungal isolates also was not associated with the virulence of canker-forming *Neonectria ditissima* fungus in apple trees (Campos et al., 2017) nor for various canker-forming Botryosphaeriaceae fungal pathogens that infect multiple host plants, including softwood and hardwood tree species (Mohali et al., 2007; Piškur et al., 2011; Félix et al., 2017).

Zerillo et al. (2014) used both single nucleotide polymorphisms and microsatellite markers to genotype *G. morbida* isolates across 17 geographic regions distributed across eastern and western United States with results that revealed the presence of four genetically distinct groups that were clustered into three different geographic regions. Based on this information, Sitz et al. (2017) conducted an *in planta* pathogen virulence field study using isolates from three genetic clusters. In their study, the virulence of *G. morbida* (using canker size as proxy) isolates was evaluated when inoculated into branches of standing trees. The authors also assessed canker formation when branches were co-inoculated with *Fusarium solani* species complex (FSSC). This latter step was undertaken because FSSC members have been recovered with *G. morbida* and might be associated with disease severity in the late stages of the disease (Tisserat et al., 2009; Montecchio et al., 2015).

Similar to our study, canker sizes induced by *G. morbida* (only) isolates from Colorado were not significantly correlated within the genetic cluster of the isolate (Sitz et al., 2017). Unlike our study, however, no significant difference in canker size was observed between genetic clusters (Sitz et al., 2017). The 183 mm^2 canker area that was averaged among all *G. morbida* isolates used in our study was much larger than the 87 mm^2 mean canker area reported by Sitz et al. (2017). From the co-inoculation efforts of Sitz et al. (2017) with *G. morbida* and FSSC members, the authors found that these pathogens do not yield a synergistic response. In our own preliminary studies on co-inoculation of juvenile *J. nigra* trees with *F. solani* and with eastern United States (TN), the isolates of *G. morbida* yielded contradictory results (data not shown). We caution that the specific role of FSSC members in tree mortality remains under-examined and should be a focus of future pathogenicity efforts. In sum, other factors including differences in environmental conditions, inoculation method, incubation period, host-plant age and condition, and *in planta* vs. detached branch assays all are likely to have contributed to different results found between Sitz et al.'s (2017) study and our study. Ideally, our ability to resolve these questions would require in-field, *in planta* assays using isolates from across the distributed range of *G. morbida* and inoculated into clonal *Juglans* germplasm.

Forest pathosystems are complex, especially when an insect vector is involved or more than one pathogen species contribute to the disease, as occurs in Ceratocystis wilt of ʻohiʻa (Hughes et al., 2020), beech leaf disease (Burke et al., 2020; Ewing et al., 2021), black pod of cocoa (Guest, 2007), and TCD on walnuts and wing nuts (Tisserat et al., 2009; Montecchio et al., 2015). The potential for multiple pathogen interactions makes it difficult to identify and document the role of each organism in tree decline. Interestingly, the *F. solani* isolate that was originally recovered from Colorado (Tisserat et al., 2009) did not yield a synergistic response in terms of canker size when co-inoculated with *G. morbida* on *J. nigra* branches (Sitz et al., 2017). Sitz et al. (2017) found that cankers induced by *Fusarium* alone were not different from the cankers induced by negative control inoculations. Members of FSSC have also been found in diseased *J. nigra* and *Juglans regia* trees in Italy where TCD has been recently introduced (Montecchio et al., 2015). Montecchio et al. (2015) observed that cankers induced by a *Fusarium* isolate from FSSC-25 alone were similar to cankers induced by *Fusarium* and *G. morbida* co-inoculations and were significantly larger than negative control inoculations. Hence, their results suggest *Fusarium* as a contributing pathogen to early stages of TCD in Italy (Montecchio et al., 2015). Differences in virulence results of *Fusarium* isolates in both studies (Montecchio et al., 2015; Sitz et al., 2017) are similar to our observed differences in *G. morbida* inoculation trails. These inconsistencies may be explained by the differences in *F. solani*/*G. morbida* strains used in testing trials, genotype, and age of host, environmental conditions, type of propagules used as inoculum, and incubation period. In future, a large-scale study that involves the inoculations of multiple strains of *Fusarium* from different TCD-associated FSSC groups on diverse *J. nigra* genotypes could help articulate the functional role of *Fusarium* in TCD etiology and epidemiology.

We also examined if virulence was correlated to the tree host species. If TCD members (host, pathogen, and vector) exist in isolation, pathogen populations could have adapted to particular hosts and, therefore, respond differently when inoculated into *J. nigra*. The isolates used in this study were recovered from cankers

on *J. major*, *J. microcarpa*, *J. nigra*, and unidentified *Juglans* spp. Canker sizes were significantly different when isolates were analyzed by host. Isolates collected from *J. major* induced significantly smaller cankers compared to isolates collected from other *Juglans* spp. but not from *J. nigra*. This finding is not surprising since the TCD vector, i.e., WTB, is native to the southwestern United States, and it has been hypothesized that both *G. morbida* and WTB have coevolved in a native host, Arizona walnut (*J. major*) (Zerillo et al., 2014; Rugman-Jones et al., 2015; Seybold et al., 2019). One plausible explanation is that *G. morbida* isolates from *J. major* might have reduced virulence on that particular host due to a coevolutionary relationship, and the concept of host-jumping into other *Juglans* spp. could provide further clues into the current virulence and migration patterns of *G. morbida*. This idea was further elaborated in a recent study by Sitz et al. (2021) that included 640 trees from wild and selected *J. nigra* families and found that improved seedlings (improved selections for enhanced growth and timber quality) exhibited larger canker sizes when compared to wild trees from the same provenance. Unfortunately, breeding efforts (Beineke, 1989; McKenna and Coggeshall, 2018; Sitz et al., 2021) may have resulted in nontargeted selection for reduced defense response against *G. morbida* infection. The authors further proposed that differences in TCD dynamics between *J. nigra* tree populations distributed in the western range of the species natural distribution vs. populations distributed in the central region (wild trees in the regions without TCD) reflect enhanced genetic resistance as a result of coevolution with *G. morbida* (Sitz et al., 2021).

CONCLUSION

Our results indicated varying degrees of virulence among tested *G. morbida* isolates, which was partly explained by their genetic provenance (state-of-origin). From the variables we tested here, genetic clustering (*G. morbida* isolates from genetic cluster 2 induced significantly larger cankers), state of origin, and *Juglans* host species could provide some explanation regarding the differences observed in *G. morbida* canker sizes. Although significant differences in canker size were noted when results from isolates were pooled within genetic cluster, host, geographic, and state-of-origin factors, a high level of variability was observed across most of these tested variables. Differential TCD severity and incidence between the western and eastern regions of the United States might instead result from other biotic (Gazis et al., 2018; Chahal et al., 2019) and abiotic factors (Griffin, 2015), or a combination of both (Seybold et al., 2019). The infestation level of WTB is likely a critical factor in determining tree mortality, as tree decline and death occur due to cambium-girdling caused by the coalescence of *G. morbida* cankers formed as a consequence of numerous beetle inoculations and WTB gallery formations (Tisserat et al., 2011). Hence, the lower levels of WTB infestation will result in less *G. morbida* inoculations and less likelihood of lesion coalescence to occur. In the eastern United States, WTB populations have been declining (Chahal et al., 2019), which in turn may be limiting the impact of TCD where *J. nigra* is native (Seybold et al., 2019).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon request, without undue reservation.

AUTHOR CONTRIBUTIONS

DH, RG, WK, and MW conceived and designed the experiments including the major conceptual ideas and proof outline. KC and MW collected samples. KC carried out the experiments. RG contributed to the processes. All authors contributed to the interpretation of the results, manuscript writing, editing, and provided critical feedback to shape the experiments, analyses, and finally produce the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2022.726388/full#supplementary-material

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