

**Rapid Molecular Detection and Population Genetics of *Pityophthorus juglandis*, a Vector of
Thousand Cankers Disease in *Juglans* spp.**

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ABSTRACT

Thousand Cankers Disease (TCD) is a disease complex involving the fungal pathogen *Geosmithia morbida*, an insect vector *Pityophthorus juglandis*, and the hosts, *Juglans* spp. and *Pterocarya* spp. Signs and symptoms of TCD include crown thinning due to branch dieback, yellowing and wilting of the leaves, appearance of epicormic shoots, numerous entrance/exit holes, gallery formation by *P. juglandis*, and the development of small, dark brown cankers underneath the bark. TCD originally described from western U.S., has now expanded to eastern U.S. and northwestern Italy. The disease complex is often difficult to diagnose due to the absence of symptoms or signs on the bark surface. Furthermore, disease symptoms can be confused with the impact of other abiotic or biotic agents. As a result, rapid molecular detection of TCD is necessary to improve our detection methods and prevent massive die-offs of these important trees. We also have limited knowledge regarding the genetic diversity of the TCD complex members. Therefore, understanding population dynamics, gene flow, and the spread of this disease is important in combating future outbreaks and potential large scale epidemics. In this study, we focused on two objectives: developing rapid molecular detection protocol for TCD, and evaluation of genetic diversity, spatial structure, and distribution of *P. juglandis* from subpopulations in the U.S. and Europe using microsatellite loci. Using previously developed species specific microsatellite loci for *G. morbida* and *P. juglandis*, our results provided a successful protocol with a high degree of sensitivity and outlined evidence that rapid molecular detection of TCD is feasible, effective, and time efficient. For population studies, *P. juglandis* specimens (n=839) from 40 subpopulations across northwestern, southwestern, and eastern U.S., and Italy were genotyped using twelve highly polymorphic *P. juglandis*

microsatellite loci. Our results indicated high genetic diversity, presence of population structure, and limited gene flow among these groups. Also, high levels of genetic diversity across all groups were explained by human mediated movement of infested plant material from multiple sources on multiple occasions. This supports an earlier hypothesis that the disease has been established in these areas for a longer period of time than previously expected.

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1 Introduction

Thousand Cankers Disease (TCD) of walnut is caused by interactions between the canker-producing fungal pathogen *Geosmithia morbida* M. Kolarik, E. Freeland, C. Utley, & N. Tisserat, an insect vector *Pityophthorus juglandis* Blackman, and the susceptible plant hosts, *Juglans* spp. L. and *Pterocarya* spp. Nutt. ex Moq (Tisserat et al. 2009; Kolarik et al. 2011; Utley et al. 2013; Hishinuma et al. 2016). Symptoms of TCD include crown thinning due to branch dieback, chlorosis and wilting of the leaves, the appearance of epicormic shoots, numerous entrance/exit holes, gallery formation by *P. juglandis*, and development of small, dark brown cankers underneath the bark (Kolarik et al. 2011; Tisserat et al. 2011). Due to the formation of a large number of coalescing cankers around the galleries and exit holes, the disease was eventually named Thousand Cankers Disease (Tisserat et al. 2009; Zerillo et al. 2014), which can result in rapid tree decline (Kolarik et al. 2011; Hadziabdic et al. 2014; Rugman-Jones et al. 2015; Daniels et al. 2016). Although TCD has caused significant mortality among native and non-native walnut populations, the disease origin, spread, and overall etiology of the pathogen is still largely unknown (USDA-FS-PPQ 2015).

Although the pathogen associated with TCD was identified in the late 2000s and documented in 2011 (Kolarik et al. 2011), the first disease occurrence, as associated with *P. juglandis* damage in *J. nigra* trees, was documented in Colorado in the early 2000s (Tisserat et al. 2009; Daniels et al. 2016). However, initial *J. nigra* mortality was reported in Utah in the mid-1990s, followed by severe declines in Oregon in 1997, and in New Mexico in 2001 (Tisserat et al. 2011; Daniels et al. 2016). Currently, TCD is found in most of western U.S. states, resulting in significant economical and environmental impact. The estimated value of standing *J. nigra* in

the U.S. is \$568 billion (USDA-APHIS 2009; USDA-FS-PPQ 2012). Wood of *J. nigra* is highly prized for woodworking, gunstocks, its ornamental and high-quality timber characteristics (Goodell 1984; Daniels et al. 2016), as well as a food source for animals (Vander Wall 2001).

In 2010, TCD was first discovered in eastern U.S. in Knoxville, Tennessee, representing the first report of this disease complex occurring within the native range of *J. nigra* (Grant et al. 2011), thus presenting a significant threat to the highly valuable native timber stands of eastern U.S. Since 2010, TCD has been spread or detected in seven additional eastern U.S. states (Grant et al. 2011; Fisher 2013; Hadziabdic et al. 2014; Daniels et al. 2016), and has been reported in southern Europe, which is the native range of *J. regia* L. (Montecchio and Faccoli 2014). The disease is often difficult to identify due to the rough bark surface that may not exhibit any TCD symptoms or can be confused with drought damage (Hadziabdic et al. 2014; USDA-FS-PPQ 2015). Recent research findings from the United States Forest Service Forest Inventory and Analysis inspections acknowledged that *J. nigra* resources in eastern U.S. were threatened by the TCD infestations across the native range (Randolph et al. 2013). However, initial tree starvation and canker development may appear long before crown die-back is apparent, making disease confirmation complicated (Randolph et al. 2013).

Morphological confirmation of TCD can be challenging, laborious, and often unreliable due to the competition with faster-growing fungi. Diversity of the myriad of fungal species that are associated with *Juglans* spp. may cause lesions similar to those of *G. morbida*, making traditional identification challenging and time-consuming (McDermott-Kubeczko 2016). Currently, disease management is unavailable, and no pesticides or control methods exist to

prevent the spread of TCD. Thus, the economic, social, and environmental consequences of further TCD epidemics could be devastating (Hadziabdic et al. 2014).

Progress has been made in understanding the biology and life cycle of *P. juglandis* (McDonald and Linde 2002; Stefansson et al. 2012; Nix 2013), yet details about key biological aspects, including the genetic diversity and spatial distribution presented by this species, remains limited (Rugman-Jones et al. 2015). Recently, Rugman-Jones et al. (2015) detected high levels of genetic diversity with evidence of two genetic lineages among *P. juglandis* in the U.S., suggesting that *P. juglandis* may be a species-group comprised of two morphologically indistinguishable species. They concluded that two major haplotypes of a single lineage are responsible for the spread of the *P. juglandis* range (Rugman-Jones et al. 2015).

In this study, our goals were to develop a protocol that will reduce disease confirmation time using molecular tools, as well as to elucidate levels of genetic diversity among *P. juglandis* subpopulations. To complete this, we proposed the following objectives: 1) to develop a rapid molecular detection protocol for TCD using species-specific microsatellite loci developed for *G. morbida* and *P. juglandis* and 2) to determine genetic structure and spatial distribution of *P. juglandis* populations across different geographical regions in the U.S. and Italy. A better understanding of genetic diversity and population structure is expected to provide insights into the species' evolutionary potential and may explain which populations could present the greatest risk and highest mortality rates to *Juglans* spp.

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2 Rapid Molecular Detection of Thousand Cankers Disease

2.1 Abstract

The Thousand Cankers Disease (TCD) complex involves host trees including *Juglans* spp. and *Pterocarya* spp., fungal pathogen, *Geosmithia morbida*, and vector, *Pityophthorus juglandis*. TCD, originally described from western United States (U.S.), has now expanded to eastern U.S. and northwestern Italy. The disease complex is often difficult to diagnose due to the absence of symptoms or signs on the bark surface. Furthermore, disease symptoms can be confused with the impact of other abiotic or biotic agents. Thus, there is a critical need for rapid TCD detection using molecular tools. Using previously developed species-specific microsatellite loci for *G. morbida* and *P. juglandis*, we developed a rapid molecular detection protocol for the TCD complex. A total of 120 *Juglans* bolts were collected from three geographical locations, either within (California, Tennessee) or outside (Missouri) the current distribution of TCD. Forty bolts were collected from each location. A total of 1600 drill cores were taken and shavings from these cores were used for DNA extraction. Following PCR amplification, DNA samples were analyzed with a QIAxcel capillary electrophoresis system. Data were analyzed using the program R, and the intraclass correlation coefficient was used for power analysis. Molecular detection outcomes among all sampled trees demonstrated that California samples had the highest rate of TCD complex member detection (85%, n=34) compared to trees from Tennessee (37.5%, n=15). Our results provided a successful protocol with high degree of sensitivity and outlined evidence that rapid molecular detection of TCD is feasible, effective, and time efficient.

2.2 Introduction

In the past two decades, widespread mortality of *Juglans nigra* L. in the United States was a result of the devastating disease identified as Thousand Cankers Disease (TCD) (Grant et

al. 2011; Kolarik et al. 2011). The disease complex involves most walnut species (*Juglans* spp.), and three species of wingnut (*Pterocarya* spp. Nutt. Ex Moq.) trees (Juglandaceae) as a host, the fungal pathogen *Geosmithia morbida* M. Kolarik, E. Freeland, C. Utley, & N. Tisserat (Ascomycota: Hypocreales: Bionectriaceae) and insect vector *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae), the walnut twig beetle (Kolarik et al. 2011; Tisserat et al. 2011; Freeland 2012; Seybold et al. 2012; Serdani et al. 2013; Utley et al. 2013; Hishinuma et al. 2016). Even though the severe decline of *Juglans* spp. was observed in the early 1990s in western U.S. (Tisserat et al. 2009; Zerillo et al. 2014), it was not until 2011 that the fungal symbiont, *G. morbida*, was identified and described (Kolarik et al. 2011; Daniels et al. 2016).

Symptoms of TCD include crown thinning due to branch dieback, yellowing and wilting of the leaves (flagging), appearance of epicormic shoots (Fig. 1), numerous entrance/exit holes and gallery formation by *P. juglandis* (Fig. 2), and development of small, dark brown cankers underneath the bark (Fig. 3) (Kolarik et al. 2011; Tisserat et al. 2011). The disease was eventually named Thousand Cankers Disease after due to the formation of a large number of coalescing cankers around galleries and exit holes that ultimately girdle the tree and prevent the flow of water and nutrients (Tisserat et al. 2009; Zerillo et al. 2014). *Geosmithia morbida* conidia surrounding bark beetle galleries can be dispersed by wind, water, and the insect vector which provides the most efficient movement of the disease agent (Kolarik et al. 2008). Walnut tree die-off is observed three to four years after the initial disease symptoms are detected (Kolarik et al. 2011; Hadziabdic et al. 2014). This is particularly troubling since the wood is highly prized for woodworking, gunstocks, ornamental and high-quality timber characteristics (Goodell 1984), as well as a food source for forest animals (Hadziabdic et al. 2014). The current

value of all standing *J. nigra* in the U.S. is estimated to be over \$568 billion (Hadziabdic et al. 2014; Daniels et al. 2016); in Tennessee alone, the value of black walnut is around \$1.37 billion in urban areas and \$1.47 billion in forest areas (Haun et al. 2010), making it an important species from both an environmental and economic perspective.

In 2010, TCD was discovered in Knoxville, Tennessee resulting in the first report of this disease complex in the native range of *J. nigra* (Fig. 4) (Grant et al. 2011), threatening the highly valuable native timber stands in eastern U.S. (Newton et al. 2009; Seybold et al. 2012). Though all *Juglans* spp. are susceptible to disease pressures, *G. morbida* fungal colonization was most severe in the native *J. nigra* trees (Utley et al. 2013). Currently, TCD has spread to seven eastern U.S. states – Indiana, Maryland, North Carolina, Ohio, Pennsylvania, Tennessee and Virginia (Grant et al. 2011; Fisher et al. 2013; Hadziabdic et al. 2014) – and to northwestern Italy, which is within the native range of *J. regia* L. (Montecchio and Faccoli 2014). It has been hypothesized that the movement of the pathogen and vector might also be human-assisted through the transfer of walnut bolts, logs, or commercial products, particularly for the recent Italian introductions (Haun et al. 2010; Hadziabdic et al. 2014; Zerillo et al. 2014; Rugman-Jones et al. 2015).

Geosmithia morbida is the first phytopathogenic species reported in this genus, although *Geosmithia* spp. have not previously been considered important plant pathogens (Kolarik et al. 2011). *Geosmithia morbida* differs from other *Geosmithia* spp. because of its inability to grow on Czapek-Dox Agar (CDA), its thermotolerance (Freeland 2012), and the monilioid or atypically branched base of the conidiophores (Kolarik et al. 2011). Fungal colonies on half strength potato dextrose agar (PDA) are cream colored to tan and tan to yellow-tan on

the reverse side of the petri dish with cylindrical to ellipsoid conidia that can form in chains (Fig. 5). Morphological confirmation of the species can be challenging since there is no specific medium for obtaining pure *G. morbida* cultures. Also, the pathogen is slow-growing in culture and often is outcompeted by other faster-growing fungi (Fig. 6). Even though *Penicillium* spp. and *Geosmithia* spp. conidia are not related, conidiophore morphology of *G. morbida* is similar in appearance to *Penicillium* spp. and can often be confused with the latter (USDA-FS-PPQ 2015) making diagnostic efforts more challenging. *Geosmithia morbida* is morphologically homogeneous but genetically variable (Kolarik et al. 2011) with high haploid genetic diversity (Hadziabdic et al. 2014; Zerillo et al. 2014).

The TCD vector, *P. juglandis*, is a small (ca. 2 mm long), yellowish to reddish brown, phloem-feeding insect (Kolarik et al. 2011) thought to be native to southwestern U.S. (Arizona, California, New Mexico) and northern Mexico (Chihuahua) (Fig. 7) (Blackman 1928; Bright 1981; Wood S.L. and D.E. 1992; LaBonte and Rabaglia 2012; Seybold et al. 2013). During the winter, *P. juglandis* can live as both larvae and adults within the galleries of *Juglans* spp. In eastern U.S., adults resume activity by late April and most fly to branches to mate and initiate new tunnels for egg galleries. Some may remain in the trunk and expand overwintering tunnels (Nix 2013). There are usually two overlapping generations per season in Colorado, and three generations can be observed in Tennessee (Klingeman, personal communication). Recent findings by Rugman-Jones et al. (2015) revealed high levels of genetic diversity and evidence of two genetic lineages among *P. juglandis* in the U.S., suggesting the presence of two morphologically indistinguishable species.

TCD galleries often contain diverse fungal communities. McDermott-Kubeczko (2016) indicated that fungal communities on *J. nigra* branches include *Aspergillus* and *Penicillium* species, which are plurivorous fungi. Also, *Fusarium solani* isolates that were recovered from TCD-infested branches can cause large necrotic areas on inoculated *J. nigra* (Tisserat et al. 2009; Montecchio et al. 2015) but the effect on the development of TCD is still ambiguous (Ploetz et al. 2013). Though we do not know how *F. solani* pathogenicity affects the development of TCD, it was found to commingle with *G. morbida*, suggesting that a two phytopathogen system may be aggressively colonizing dead or dying phloem in weakened trees (Ploetz et al. 2013; McDermott-Kubeczko 2016).

The disease is often difficult to identify because the bark surface may not exhibit any TCD symptoms, or symptoms can be confused with drought damage (Hadziabdic et al. 2014; USDA-FS-PPQ 2015). Recent research findings from the U.S. Forest Service Forest Inventory and Analysis inspections acknowledged that *J. nigra* resources in eastern U.S. were threatened by the TCD infestations across the native range (Randolph et al. 2013). They observed that less than 5% of evaluated trees were affected by TCD based on tree crown conditions (Randolph et al. 2013). However, initial tree starvation and canker development may appear long before crown die-back is apparent, making disease confirmation complicated. The authors concluded that lack of evidence of TCD confirmation might be superficial due to inability of the monitoring system to confirm disease presence based solely on current crown dieback (Randolph et al. 2013).

In past years we have observed a large number of new epicormic shoots formations on initially heavily infested TCD trees in eastern U.S. (Hadziabdic, personal communication).

Currently, we have limited knowledge of the ability of these “recovered” trees to survive another TCD attack or prevent further mortality caused by other opportunistic or aggressive diseases. Morphological confirmation of TCD can be challenging, time-consuming, and often unreliable due to competition with faster-growing fungi (Fig. 8). Plant disease diagnosis based on symptoms is rarely used as a single method of confirmation due to its limitations. Molecular techniques can provide valuable tools in this process and can be applied for simultaneous detection and identification of plant pathogens (Silva et al. 2016). Also, diversity of the myriad of fungal species that are associated with *Juglans* spp. may cause lesions similar to those of *G. morbida*, making traditional identification challenging and time consuming (McDermott-Kubeczko 2016). Currently, disease management is unavailable and no pesticide or control methods exist to prevent the spread of TCD. Thus, the economic, social, and environmental consequences of further TCD epidemics could be devastating (Hadziabdic et al. 2014). As a result, rapid molecular detection of TCD is necessary to prevent massive die-offs of this important tree.

Using molecular methods such as PCR has become the primary means for early detection in many fields such as agriculture and forestry, as well as human and animal health (Versalovic and Lupski 2002; Lamarche et al. 2015). Molecular methods are sensitive enough to detect minute amounts of DNA from the target organism even when the material is heterogeneous, and can lessen labor time needed to isolate and confirm pathogen of interest (Cawkwell et al. 1993; Hamelin et al. 2000; Lamarche et al. 2015; Silva et al. 2016). To reduce the time needed for disease complex member(s) confirmation using traditional culturing methods, this study utilized rapid molecular tools to identify TCD. Our study objective was to

develop a rapid molecular detection protocol for TCD using species-specific microsatellite loci developed for *G. morbida* and *P. juglandis*.

2.3 Materials and Methods

2.3.1 Collection of Walnut Branches

A total of 120 *Juglans* spp. bolts (~ 5-8 cm diameter and 35 – 45 cm long) were collected from three geographical locations, either within (California, Tennessee) or outside (Missouri) the current distribution of TCD (Fig. 9). Forty bolts were collected from each location and were kept inside a walking cooler (4°C) until processed. Two different types of drilling were used to obtain wood shavings from sampled branches: lesion-directed (n=1200) and feature-directed (n=400). A total of 1600 drill cores were taken (Fig. 10), and shavings from these cores were used for DNA extraction (Fig. 11).

2.3.2 Sampling Process

2.3.2.1 Lesion-directed Sampling

For lesion directed sampling, bark was removed from each bolt using a scalpel to expose cankers and phloem discoloration. Preliminary observations have revealed a broad diversity in discoloration of phloem ranging across tan and dark brown hues to nearly black tissues that can be associated with *G. morbida*-induced lesions. Ten cankers were identified, and drill shavings were collected into 1.5 ml safe-lock microcentrifuge tubes. Drill bits (7/64 inch- 0.28 cm diameter) were heat sterilized using a bead sterilizer (Steri 350, Fisher Scientific, Pittsburgh, PA) between each drilling point to prevent cross-contamination. A total of 10 lesion-directed samples were taken from each collected bolt (40 bolts/location, 10 drills/bolt at each location n=400, total n=1200). Using a sterile drill bit, a small end of a bottomless tube (microfunnel)

was placed against the exposed, marked cankers. The sterilized drill bit was placed through the microfunnel, and drilled through the wood to collect the shavings containing canker and/or beetle parts. Shavings collected in the microfunnel were transferred into a labeled 1.5 ml safe-lock microcentrifuge tube. The process was repeated until ~ 150 mg of drill shavings were collected. Samples were stored at -20°C until used for DNA extraction.

2.3.2.2 Feature-directed Sampling

In addition to lesion-directed sampling, a subset of five samples was taken from each bolt as feature-directed cores. These samples were included to emulate field-collected samples in which a walnut branch section could be examined for the presence of the TCD complex members without having any evidence of pathogen or vector presence. Consequently, feature directed samples were taken adjacent to lenticels, secondary branch unions, bark furrows, and various other bark-wound sites without bark removal. Five different feature-directed drill shavings were collected into 1.5 ml safe-lock microcentrifuge tubes. Drill bits (7/64 inch- 0.28 cm diameter) were heat sterilized using a bead sterilizer (Steri 350, Fisher Scientific, Pittsburgh, PA) between each drilling point to prevent cross-contamination. A total of five feature-directed samples were taken from bolts collected in TCD confirmed locations – Tennessee and California (40 bolts/location, 5 drills/bolt at each locations n=200, total n=400). Missouri branches were used as a control and were drilled using feature-directed drilling only. Using a sterile drill bit, a small end of a bottomless tube (microfunnel) was placed against the exposed, marked cankers. The sterilized drill bit was placed through the microfunnel, and drilled through the wood to collect the shavings. Shavings collected in the microfunnel were transferred into a labeled 1.5

ml safe-lock microcentrifuge tubes. The process was repeated until ~ 150 mg of drill shavings were collected. Samples were stored at -20°C until used for DNA extraction.

2.3.3 DNA Extraction and PCR

DNA was extracted using either a QIAamp Fast DNA Stool Mini Kit or a DNA Stool Mini Kit (QIAGEN, Germantown, MD, US), according to the manufacturer protocol. PCR amplifications were performed using *P. juglandis*-specific microsatellite locus (WTB 192) (Hadziabdic et al. 2015) as well as using *G. morbida*-specific microsatellite locus (GS 004) (Hadziabdic et al. 2012). PCR amplification was performed in 11 µl reactions containing 1 µL DNA (undiluted), 4 µl Go-Taq G2 Hot Start Colorless Master Mix (Promega, Madison, WI, US), 1 µL of 10 µM of each primer, 0.5 µl DMSO and 3.5 µL of sterile water. Both positive and negative control samples were included for additional confirmation and accuracy of results (Fig. 12). PCR amplification was performed in a 96-well plate, using an Eppendorf Mastercycler pro Thermocycler (Eppendorf AG, Hamburg, Germany) with an initial denaturation step of 2 min at 95°C, followed by 30 cycles of 95°C for 30 s, 55°C for 45 s, 70°C for 1 min, and a final extension at 70°C for 1 min.

Following PCR amplification, DNA samples were analyzed via a QIAxcel Capillary Electrophoresis System (QIAGEN, Valencia, CA, US) using a 25 base pair DNA size marker. With the QIAxcel system, data are automatically generated and exported using QIAxcel ScreenGel software. The software facilitates rapid data interpretation and provides flexibility with data as results are displayed in both electropherogram and gel image view (Fig. 12).

2.3.4 Statistical Analysis

Presence/absence data were analyzed with R software (R-CoreTeam 2016). Intraclass correlation coefficient, as described by Zou (2012), was used for power analysis. Pearson's chi-square test ($\alpha = 0.05$) with Yates' continuity correction was performed to discern the predictive capability of the tested drilling protocols for the ability to detect the pathogen, *G. morbida* (*Gm*) and/or the beetle, *P. juglandis* (*Pj*). A logistic mixed model analysis also assessed the consistency of results across locations (CA, MO, TN) and between sample types (lesion-directed samples or feature-directed samples), as well as interaction between these factors using lme4 (Bates et al. 2015), lmerTest (Kuznetsova et al. 2016), lsmeans (Lenth 2016), and ggplot2 (Wickham 2016) packages.

2.4 Results

Molecular confirmation was based on positive results from DNA amplification of drilled bark samples and provided an efficient method of diagnosing *G. morbida* and *P. juglandis* using species-specific microsatellite loci. Pearson's Chi-square test with Yates' continuity correction was performed to infer efficiency of these markers in rapidly detecting TCD using both lesion- and feature-directed drilling methods. The relation between lesion- and feature-directed sampling was significant for all samples, [χ^2 (df=1, n=1600) = 14.11, $P < 0.001$] (Table 1). When the two drilling methods were analyzed separately, both lesion-directed [χ^2 (df=1, n=1200) = 14.24, $P < 0.001$] and feature-directed [χ^2 (df=1, n=400) = 11.16, $P < 0.01$] drilling procedures were competent methods/procedures to detect *Gm* and or *Pj* (Table 1). Effect of location was also assessed for both drilling methods (Table 1). Only California samples yielded an ability to detect either *Gm* or *Pj* using both lesion-directed and feature-directed drilling methods [χ^2

(df=1, n=400) = 6.68 $P < 0.001$, X^2 (df=1, n=200) = 5.99, $P < 0.01$, respectively] (Table 1). By contrast, neither drilling method was highly effective at detecting *Gm* or *Pj* for the samples taken in Tennessee (Table 1).

Another Pearson's Chi-square test with Yates' continuity correction was performed to determine the probability that any of the 10 lesion-directed or 5 feature-directed drill cores, within each of 40 trees per location, could confirm that a tree is positive for any of the TCD complex member (either *Gm* or *Pj* presence). This was examined under the following conditions: presence of *Gm* only, presence of *Pj* only, or joint confirmation of both *Gm* and *Pj* within a sample (Table 2). When drill sampling outcomes were pooled, both lesion-directed and feature-directed drilling methods were effective in detecting a TCD complex member in the California and Tennessee locations (X^2 [df=1, n=200] = 12.11, $P < 0.001$) (Table 2). When the two drilling methods were analyzed separately, both lesion-directed (X^2 [df=1, n=120] = 7.69, $P = 0.005$) and feature-directed (X^2 [df=1, n=80] = 6.79, $P = 0.009$) drilling methods were efficient molecular means of detecting TCD complex members (Table 2). However, when tested across localities and among trees, only California samples yielded similar capabilities for both lesion-directed and feature-directed drilling (X^2 [df=1, n=40] = 5.06, $P = 0.02$) to detect a TCD complex member (Table 2).

The logistic mixed model statistics revealed that lesion-directed drilling was almost 13 times more likely to yield a positive confirmation of *G. morbida* from the California-sourced *J. hindsii* trees than was observed among the Tennessee *J. nigra* samples (Table 3, Figs. 13 and 14). In addition, among California *J. hindsii* trees sampled the *Gm*-specific marker confirmed the presence of the pathogen by returning a "positive" detection outcome about seven times more

efficiently using lesion-directed sampling than was achieved by for feature-directed sampling (Table 3) (Fig. 13).

Molecular detection outcomes among all sampled trees (n=120) across the three different locations (n=40 trees/location), demonstrated that California samples had a higher rate of TCD complex member detection than trees from Tennessee (Fig. 14). The validity of the sampling techniques was supported, as the control location (Missouri) (Fig. 14) was negative for the presence of both the pathogen and beetle in all examined samples (Fig. 15). *Geosmithia morbida* was detected among 21 California trees, whereas in Tennessee, only 12 trees revealed presence of the pathogen (Fig. 14). *Pityophthorus juglandis* DNA was detected from just two California trees and one Tennessee tree (Fig. 14). However, 11 California trees and two Tennessee trees were confirmed to have both the pathogen and the beetle among drilled samples (Fig. 14). In summary, 37.5 percent (n=15) of sampled *J. nigra* trees in Tennessee and 85 percent (n=34) of sampled *J. hindsii* trees in California were confirmed to have either *Gm* or *Pj*, thus showing infestation by at least one of the TCD complex members (Fig. 15A). When data was partitioned based on the two drilling methods, 30 percent (n=12) of *J. nigra* trees and 80 percent (n=32) of *J. hindsii* trees in Tennessee and California respectively, were confirmed for presence of either *Gm* or *Pj* using the lesion-directed method (Fig. 15B); 15 percent (n=6) of *J. nigra* trees in Tennessee; and 37.5 percent (n=15) of *J. hindsii* trees in California were confirmed for the presence of either *Gm* or *Pj* using feature-directed method (Fig. 15C).

2.5 Discussion

The molecular based protocol used in this study provided an efficient, sensitive and cost-effective tool for rapid molecular detection of TCD complex members. Molecular detection

methods can enhance our ability to detect microbial pathogens rapidly and efficiently across a number of scientific fields including agriculture, forestry, and human and animal health (Versalovic and Lupski 2002; Lamarche et al. 2015). Here, we significantly reduced the confirmation time of disease complex member(s) from weeks to 4-6 hours using minute amounts of DNA from mixed sample that could include either plant host, pathogen(s) and/or vector(s). Rapid molecular detection of pathogens and the diseases they cause is widely used in different disciplines. For example, Audy et al. (1996) developed a quick PCR-based procedure for rapid detection of the casual agents of bean common blight *Xanthomonas campestris* pv. *phaseoli* and bean halo blight, *Pseudomonas syringae* pv. *phaseolicola*, in bean seed (Audy et al. 1996). Cawkwell et al. (1993) developed a rapid molecular technique for detecting allele loss in colorectal tumour samples using microsatellite loci (Cawkwell et al. 1993). Versalovic and Lupski (2002) used nucleic acid amplification techniques to detect *Chlamydia trachomatis*. Their methodology was further improved to detect other *Chlamydia* and *Mycoplasma* species (Versalovic and Lupski 2002).

Our ability to obtain pure *G. morbida* culture can take from a few days to several weeks, particularly from samples obtained in eastern U.S. region where *J. nigra* is native. This is due to a number of factors including lack of *G. morbida* specific medium, slow growing nature of the pathogen, and inability to outcompete other faster growing fungi such as *Fusarium* spp. or *Aspergillus* spp. (Kolarik et al. 2011; Hadziabdic et al. 2014). Differences between eastern and western U.S. isolates have been observed, in particular the amount of sub-culturing needed to obtain pure strains of *G. morbida* (Gazis, unpublished data). This process can be labor intensive, time consuming and expensive. As a result, rapid molecular detection is needed to establish

quarantine and buffer areas and mitigate further disease epidemics. Although instituting quarantines can be effective, it has not prevented TCD from spreading across the U.S. and Europe. To mitigate further spread of TCD into Canada, Canadian national plant protection organizations developed a real-time PCR based detection methods for 10 of the most unwanted alien forest pathogens in Canada, which includes *G. morbida* (Lamarche et al. 2015). Their methods were based on the Taq-Man technology using β -tubulin gene and represent a powerful tool to prevent introduction and establishment of potentially invasive species (Lamarche et al. 2015). Their research findings, however, did not focus on the insect vector or isolating the fungus from the infested tree branches, which makes detection more challenging and cumbersome. The same method was used to detect the dogwood anthracnose pathogen (*Discula destructiva*) (Miller et al. 2016). Even though *D. destructiva* is a slow growing fungus like *G. morbida*, the molecular detection method was successful in identifying the fungus from both dried herbarium and fresh *Cornus* spp. samples (Miller et al. 2016).

DNA extraction and PCR amplification for rapidly detecting the TCD pathogen or insect vector are reproducible, allowing for analysis of extremely heterogeneous environments such as infested branch samples (Vainio and Hantula 2000; Pennanen et al. 2001; Mumford et al. 2006). The PCR assay described here was specific to *G. morbida* and *P. juglandis*. We used two different approaches to ascertain our PCR protocol for rapidly detecting TCD complex member(s): (1) we tested the *G. morbida* specific primer (GS004) against several common contaminants of TCD galleries; and (2) we conducted simultaneous fungal isolations and PCR assays from two different Tennessee branches. These approaches confirmed that cross-transferability is not an issue for the *G. morbida* primer (GS004) used in this study, thus

eliminating the possibility of false positive in any given sample tested for the presence of the fungus. In addition, two branches were tested with both traditional and molecular methods, as described in this study. The outcome of this limited experiment was negative for the presence of pathogen using both methods, indicating that false negative was not an issue for this protocol.

In our preliminary studies, cross-transferability of *P. juglandis* microsatellites was observed; however, the study is still ongoing with several different *Pityophthorus* spp. across 18 different highly polymorphic *P. juglandis* species-specific microsatellite loci. According to Bright (1981), the genus *Pityophthorus* Eichhoff contains 220 species in North and Central America. To date, only three different *Pityophthorus* spp. (*P. lautus* Eichhoff, *P. pulicarius* Zimmermann, and *P. liquidambarus* Blackman) were tested across all *P. juglandis* specific microsatellites. Neither species had cross-amplified with the *P. juglandis* specific microsatellite locus, WTB192, used in this study. However, they cross-amplified across other loci [*P. lautus* (WTB 2, 13 and 19); *P. pulicarius* (WTB 1, 2, 12, 13, 14, 19, 128, and 130; and *P. liquidambarus* (WTB 1, 12, 13, 19, 128, and 130)].

We also tested three different DNA polymerase enzymes in this study: AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, U.S.), KOD Hot Start DNA polymerase (Millipore, Billerica, Massachusetts, U.S.), and Go-Taq G2 Hot Start Colorless Master Mix (Promega, Madison, WI, U.S.). All tested enzymes were successful in detection of the pathogen and/or vector from the heterogeneous samples (wood shavings) but GoTaq produced the most reliable products and was therefore used in further analyses. Lastly, estimated cost per sample,

which includes consumables only, is estimated at \$10.76, and the method required less than 4 hours from the drilling process to pathogen/vector confirmation.

Our results indicated that 34 out of 40 trees from California were positive for the presence of either *G. morbida* or *P. juglandis* using both lesion-directed and feature-directed samples, whereas only 15 out of 40 Tennessee trees were positive. Our results further revealed that lesion-directed drilling was almost 13 times more likely to yield a positive confirmation of the pathogen from the California-sourced *J. hindsii* trees when compared to *J. nigra* trees in Tennessee. Utley et al. (2013) tested the virulence of *G. morbida* across *J. ailantifolia*, *J. californica*, *J. cinerea*, *J. hindsii*, *J. major* (a native host of the *P. juglandis*), *J. mandshurica*, *J. microcarpa*, *J. nigra*, and *J. regia*. *Juglans* spp. response to *G. morbida* inoculations differed, indicating higher susceptibility of canker development in *J. nigra* when compared to *J. hindsii* and *J. major*, a native host of the beetle. The authors concluded that genetic variability in resistance to *G. morbida* was present among half-sibling families of *J. nigra* and *J. cinerea*. However, we need to take into account the ability of the beetle to attack different walnut species since it is a significant component in TCD resistance (Utley et al. 2013). Additionally, disease pressures and *P. juglandis* populations have been considerably higher in California than in Tennessee for the past few years (Gazis, unpublished data).

The lack of detection confirmation from Tennessee is not necessarily due to the inability of the technology to detect *Gm* and/or *Pj*. In this study, trees were collected from a region of Tennessee in which disease was present, but not every single tree was currently exhibiting signs and symptoms of TCD. However, California samples were taken from a single location in Yolo County, CA which has a high density of TCD complex members, whereas Tennessee samples

were taken from seven different TCD confirmed counties across a larger geographical area in East Tennessee. The other likely explanation for higher disease complex member(s) detection in western U.S. could be the contribution of recent drought conditions in California, which could have resulted in rapid tree mortality as a result of TCD (Utley et al. 2013). In contrast, according to Griffin (2015), initially infested trees in Tennessee produced new foliage and stem growth, which resulted in apparent “recovery” from TCD; this was related to high soil water potential in 2013 (Griffin 2015). In a recent study by McDermott-Kubeczko (2016) similar observations were noted, in which TCD confirmed trees had crowns that appeared healthy. Furthermore, our preliminary studies from eastern U.S. indicated that tree strata and location of the branch on a suspected tree may play a role in infection process and can result in the presence or absence of cankers.

One of the great advantages of using molecular techniques is detecting pathogens from asymptomatic plants (Llop et al. 2000). To simulate that, we examined feature-directed drilling methods that were taken without bark removal or identification of potential cankers. Our results using this method indicated that 15% of *J. nigra* trees in Tennessee and 37.5% of *J. hindsii* trees in California contained either *Gm* or *Pj*. The *Gm*-specific marker was about seven times more efficient in detecting lesion-directed samples in comparison to feature-directed samples across all locations. Although the success of feature-directed drilling method is not as robust as direct-drilling method, it can still be used to detect the presence of TCD complex members.

For both drilling methods, molecular confirmation of *Pj* was less successful when compared to *Gm*, which could be explained by the presence of other potential vectors involved

in dissemination of the *Gm*. To date, *G. morbida* has been associated only with *P. juglandis*, but the weevil *Stenomimus pallidus* Boheman and two ambrosia beetle species, *Xylosandrus crassiusculus* Motschulsky and *Xyleborinus saxeseni* Ratzeburg (Juzwik et al. 2015; Juzwik et al. 2016), have also been identified as TCD vectors. Our preliminary data indicated that other ambrosia beetles are associated with *G. morbida*, however, the work is currently ongoing to elucidate this finding (Chahal, unpublished data). Another likely explanation for the limited detection rate of *Pj* could be explained by the low quality of DNA in a drilled shaving sample (Silva et al. 2016), which could be below the detection limit of the *P. juglandis* specific primer.

In a conclusion, the PCR-based method used in this study provided an efficient, sensitive and cost-effective tool for rapid molecular detection of TCD complex members. Disease complex member(s) confirmation time can be reduced from weeks to hours using minute amounts of DNA from either pathogen and/or vector mixed with other environmental material including plant host and other potential vectors. Using species specific microsatellite loci, we were able to confirm the presence of the pathogen and insect vector alone or in combination with each other (due to some states' quarantine rules). This method can be replicated in other labs and can reinforce quarantine issues in cases when disease symptoms are not apparent. This methodology could be utilized for other similar invasive species and could prevent further spread of introduced pathogens which could result in significant environmental, social and economical damage.

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- 3 Evaluating genetic diversity, spatial structure and distribution of *Pityophthorus juglandis* from subpopulations in the United States and southern Europe using microsatellite loci

3.1 Abstract

Thousand Cankers Disease (TCD) of walnut is caused by the interactions between the canker-producing fungal pathogen *Geosmithia morbida*, an insect vector *Pityophthorus juglandis*, and the susceptible plant hosts, *Juglans* spp. L. and *Pterocarya* spp. Nutt. ex Moq. This disease is manifested by canopy thinning, which results from numerous cankers initiated during aggregated attacks on hosts and consequent excavation and tunneling by the bark beetle insect vector. When multiple cankers coalesce, subsequent branch die-back may lead to tree death. Although the disease has caused significant mortality among native and non-native walnut tree populations, we have limited knowledge regarding genetic diversity demonstrated within the TCD complex members. Broad-scale differences in genetic structure and regional diversity are indicative of the recent evolutionary history of a species. This study reports current estimates of the level of genetic diversity and structure demonstrated by *P. juglandis* subpopulations collected across a broadly representative geographical area where Thousand Cankers Disease occurs. *Pityophthorus juglandis* specimens from 40 subpopulations across northwestern, southwestern, and eastern US, and Italy were genotyped using 12 different, highly polymorphic, *P. juglandis*-specific microsatellite loci. Results demonstrate a high genetic diversity, presence of population structure, and limited gene flow that has occurred between these groups. High levels of genetic diversity present across all groups is likely to best be explained by human mediated movement of infested plant material from multiple source locations on multiple occasions. These findings further substantiate previous hypotheses that infer thousand cankers disease has been established in these different source locations for a longer period of time than was originally proposed.

3.2 Introduction

Thousand Cankers Disease (TCD) of walnut is caused by the interactions between the canker-producing fungal pathogen *Geosmithia morbida*, an insect vector *Pityophthorus juglandis* (walnut twig beetle), and the susceptible plant hosts, *Juglans* spp. L. and *Pterocarya* spp. Nutt. ex Moq. (Tisserat et al. 2009; Kolarik et al. 2011; Utley et al. 2013; Hishinuma et al. 2016). Disease signs and symptoms include canopy thinning due to branch die-back, discoloration of the leaves, upon close inspection presence of numerous entrance and exit holes caused by *P. juglandis* on the bark, and the formation and persistence of small, dark lesions within and beneath the bark (Kolarik et al. 2011; Tisserat et al. 2011) (Fig. 16). After the initial disease symptoms are detected, tree die-off may be observed within three to four years (Kolarik et al. 2011; Hadziabdic et al. 2014; Rugman-Jones et al. 2015; Daniels et al. 2016). This rapid rate of tree mortality, particularly within western U.S., results from the movement, feeding, and tunnel formation produced by the bark-beetle vector, *P. juglandis*, which leads to *G. morbida* canker development (Tisserat et al. 2009; Kolarik et al. 2011; Utley et al. 2013; Hadziabdic et al. 2014). Even though TCD has caused significant mortality among native and non-native walnut populations, the disease origin, spread, and overall etiology of the pathogen is still largely unknown (USDA-FS-PPQ 2015). Thousand Cankers Disease may be categorized as a "black swan" event, in which a rare plant disease, that is unpredictable in its epidemiology, can have extremely negative consequences, including significant economical and environmental impacts to native species and the conservation of biodiversity (Taleb 2010; Ploetz et al. 2013).

Although the fungal symbiont associated with TCD, *G. morbida*, was identified in the late 2000s and documented in 2011 (Kolarik et al. 2011), the first consequences of the disease, as

associated with *P. juglandis* damage in eastern black walnut, *J. nigra* L. trees, was documented in Colorado in the early 2000s (Tisserat et al. 2009; Daniels et al. 2016). The first record of *J. nigra* mortality, however, occurred in Utah in the mid-1990s, followed by tree mortality reports in Oregon in 1997, and in New Mexico in 2001 (Tisserat et al. 2011; Daniels et al. 2016). TCD now occurs in most of western U.S. states, with detection progression reported from Idaho (2003), Washington (2008), California (2008), to Nevada (2011) (USDA-FS 2002; Cranshaw 2011; Daniels et al. 2016) (Fig. 17). In 2010, TCD was first discovered in the eastern U.S. in Knoxville, Tennessee, representing the first report of this disease complex occurring within the native range of *J. nigra* (Grant et al. 2011), thus presenting a significant threat to the highly valuable native timber stands of eastern U.S. Since 2010, TCD has been spread or detected in seven additional eastern U.S. states (Grant et al. 2011; Fisher 2013; Hadziabdic et al. 2014; Daniels et al. 2016), and has been reported in southern Europe, which is the native range of *J. regia* L. (Montecchio and Faccoli 2014).

Early estimates from the U.S. Forest Service Forest Inventory and Analysis (FIA) indicated that TCD could have been present in the native range of *J. nigra* for at least ten years prior to the first disease detection within eastern U.S. (Randolph et al. 2013). FIA data were used to conclude that 306 million live black walnut trees were present in eastern U.S. forests. However, no evidence was noted of tree decline consistent with TCD symptomology, on the basis of changing crown condition and tree mortality rates (Randolph et al. 2013). The authors have acknowledged, however, that the apparent lack of TCD-consistent decline could also reflect an inability within the inventory and monitoring system methodologies to adequately discern TCD presence in eastern U.S. (Randolph et al. 2013). However, a recent study indicated

that the fungus could be isolated from asymptomatic branches in eastern U.S. (McDermott-Kubeczko 2016). Although *P. juglandis* was collected in 2004 and 2005 from dying *J. nigra* trees in western U.S., the species was recorded as a secondary pest because it had never previously been described as an important factor in pathogen spread or in causing tree mortality (Tisserat et al. 2009). Rapid declines and mortality in *J. nigra* trees were eventually confirmed as resulting from the tunneling and feeding by *P. juglandis* within the galleries in the phloem, followed by pathogen canker development near those galleries (Tisserat et al. 2009; Kolarik et al. 2011). It is highly probable that relatively high populations of *P. juglandis* are required before TCD can induce severe tree decline or mortality (Seybold et al. 2013).

Pityophthorus juglandis is a small (~ 2 mm long), phloem-feeding insect (Seybold et al. 2016) that is yellowish to reddish brown in color, and native to southwestern U.S. (Arizona, California, New Mexico) and northern Mexico (Chihuahua) (Blackman 1928; LaBonte and Rabaglia 2012). Male *P. juglandis* are similar in appearance to females, with a diagnostic exception of the presence of a tuft of fine short blonde setae on the front of the head in adult females. Elytral punctures occur in rows (Blackman 1928) (Fig. 18), with punctures on female beetles that may appear more coarse (Nix 2013). Male beetles may have distinct but small granules that occur on both the elytral suture and on lateral convexities of the elytra (Blackman 1928). *Pityophthorus juglandis* has a steep declivity at the posterior of the elytra, as well as 4 to 6 concentric rows of pronotal asperities that are usually broken and that may overlap at the median line (Blackman 1928; Bright 1981; Tisserat et al. 2009).

Seybold et al. (2012) discovered that *P. juglandis* flight activity peaks at temperatures between 23°C - 24°C and beetles cease flying when temperatures drop below 17°C - 18°C.

Larvae feed for 1-1.5 months under the bark, before they pupate at the ends of gallery tunnels (Seybold et al. 2012b). There are usually two overlapping generations per season in Colorado, and three generations can be observed in Tennessee (Klingeman, personal communication). Nix's (2013) investigation of the life history of *P. juglandis* revealed that egg and immature stage (larval and pupal) development occurs within the brood chambers initiated by the female beetles that excavate egg niches laterally to the entrance tunnel galleries and at the interface of bark and cambial tissues. Larval tunnels extend perpendicularly to the main gallery made by adult female beetles. The 3rd-instar is the last larval stadium, and this instar expands the end of the gallery prior to pupation. During the winter, *P. juglandis* may be found as larvae and adults within the galleries of *Juglans* spp. Adults resume activity by late April where most emerge and then fly to branches to mate, after which the females initiate new entry tunnels and egg galleries. Some adult beetles may remain in the trunk and continue to expand overwintering tunnels (Nix 2013).

The fungal agent of TCD is considered to be the first phytopathogenic fungus within the *Geosmithia* genus (Tisserat et al. 2009). *Geosmithia morbida* M. Kolařík, E. Freeland, C. Utley, & N. Tisserat (Ascomycota: Hypocreales: Bionectriaceae) causes a multitude of cankers that have been known to coalesce and girdle in branches or twigs (Leslie et al. 2009), thus the name of thousand cankers disease (Tisserat et al. 2009; Zerillo et al. 2014). *Geosmithia morbida* can be found in various habitats associated with bark and ambrosia beetles (Kolarik et al. 2007; Kolarik et al. 2008; Kolarik and Kirkendall 2010) (Fig. 19). When the pathogenic fungus is associated with the insect vector, *P. juglandis*, the result can be devastating to *Juglans* sp. (Ploetz et al. 2013). In the past few years, progress has been made to investigate the spatial structure and

genetic diversity of *G. morbida* using molecular tools. Kolařík et al. (2011) demonstrated that *G. morbida* is genetically variable but morphologically and ecologically homogenous (Kolarik et al. 2011). Using both single nucleotide polymorphisms (SNPs) from three genomic regions and microsatellite loci, Hadziabdic et al. (2014) and Zerillo et al. (2014) have provided evidence of high haploid genetic diversity and significant differentiation among genetic clusters that correspond to different geographical regions. There has been no evidence of sexual reproduction or genetic recombination in any population. Both Zerillo et al. (2014) and Hadziabdic et al. (2014) indicated that *G. morbida* was most likely to have been introduced to different geographic regions in several different instances and from several different sources. From the large number of haplotypes and the genetic complexity presented by *G. morbida*, a plausible hypothesis has been inferred that the pathogen has evolved in association with at least one *Juglans* spp. and its insect vector, *P. juglandis*, and that this occurred long before the first reports of the disease were made (Hadziabdic et al. 2014; Zerillo et al. 2014).

Although *Juglans* spp. are the primary TCD host, a recent study by Hishinuma et al. (2016) provided evidence that *Pterocarya* spp. Nutt. ex Moq., *Pterocarya fraxinifolia* (Poiret) Spach, *Pterocarya rhoifolia* Siebold and Zuccarini, and *Pterocarya stenoptera* C. de Candolle (Juglandaceae) might also function as competent TCD hosts. Out of 16 *Juglans* spp. that are native to the Americas (Stone et al. 2009; Rugman-Jones et al. 2015), only six species (*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.) are found in the U.S. Utley et al. (2013) demonstrated that susceptibility to *G. morbida* inoculations differed among *Juglans* spp. *Juglans nigra* was determined to be the

most susceptible walnut species to *G. morbida*, while inoculations of *J. major*, southwestern U.S. native host, yielded smaller and more superficial cankers (Utley et al. 2013). Utley and colleagues also confirmed that three closely related species (*Carya illinoensis* (Wangenh) K. Koch, *C. aquatic* Nutt., and *C. ovata* (Mill) K.Koch), which are important both economically and ecologically, were unaffected by *G. morbida* inoculations (Utley et al. 2013).

Wood of *J. nigra* is highly prized for woodworking and gunstocks, as well as for its ornamental and high-quality timber characteristics (Goodell 1984; Daniels et al. 2016). *Juglans* spp. nuts are a crucial food source for a variety of animals, especially squirrels (Vander Wall 2001). Trees are also important for the shade, aesthetics, and wildlife forage they provide in urban areas (Randolph et al. 2013). Currently, the estimated value of standing *J. nigra* in the US is \$568 billion (USDA-APHIS 2009; USDA-FS-PPQ 2012) and wood, veneer, lumber and logs are exported to over 45 countries (Utley et al. 2013), making it an important species from both an environmental and economic perspective (Daniels et al. 2016). Although limited research is available regarding *Pterocarya* spp. Nutt. ex Moq. across the broad geographical distribution TCD, the potential for further spread of *P. juglandis* beyond Europe and into Asia could be devastating for the conservation of native *Juglans* and *Pterocarya* species in those regions.

Progress has been made in understanding the biology and life cycle of *P. juglandis* (McDonald and Linde 2002; Stefansson et al. 2012; Nix 2013), yet details about key biological aspects, including the genetic diversity and spatial distribution presented by this species, remains limited (Rugman-Jones et al. 2015). Recently, Rugman-Jones et al. (2015) detected high levels of genetic diversity with evidence of two genetic lineages among *P. juglandis* in the US, suggesting that *P. juglandis* may be a species-group comprised of two morphologically

indistinguishable species. They concluded that two major haplotypes of a single lineage are responsible for the spread of the *P. juglandis* range (Rugman-Jones et al. 2015). A better understanding about genetic structure and spatial distribution of *P. juglandis* populations is expected to provide insights into the species' evolutionary potential and may explain which populations could present the greatest risk and highest mortality rates to *Juglans* spp.

Insecticide and fungicide control options to manage the pathogen and its vector are currently limited or unavailable, and methods to prevent the spread of TCD rely on regulatory containment and enforcement of quarantines (Daniels et al. 2016).

To test the hypothesis that the recent geographic range expansion of *P. juglandis* can be explained by human mediated movement associated with multiple introductions, this study will be using previously developed *Pityophthorus*-specific microsatellite loci (Hadziabdic et al. 2015) to assess the genetic diversity, differentiation, and spatial structure of *P. juglandis* subpopulations across the U.S. and Italy. This objective will be accomplished by addressing the following questions: (1) How much genetic diversity exists in these geographically fragmented subpopulations? (2) How is the genetic diversity spatially structured across the entire species distribution range? and, (3) Is there any evidence of gene flow among these subpopulations? Having some understanding regarding the interactions between the host, pathogen, and vector, as well as their origin and distribution, can provide an insight into the best management practices and potentially prevent further global spread of TCD.

3.3 Materials and Methods

3.3.1 Specimen Collection

Pityophthorus juglandis individuals were trapped or collected from individual *Juglans* spp. across wide geographical area in the U.S. and Italy during 2014, 2015 and 2016, where TCD has been reported (Table 4). The trapping methodology described by Seybold et al. (2013) was employed for specimen collection, and *P. juglandis* samples were stored in 70% ethanol until used for the population study (Fig. 20). Briefly, multiple funnel traps were baited with lures containing synthetic aggregation pheromone, which are produced by male *P. juglandis*. Captured insects were collected into a trap cup, containing ~250 ml of alcohol free, low toxicity antifreeze (RV & Marine Antifreeze, SPLASH, Saint Paul, MN, USA) to preserve the insects. Traps were checked every 14 days in TN or until enough specimens were collected from the collection sites and pheromone lures were replaced every two months (USDA-FS-PPQ 2015).

Pityophthorus juglandis adults were transferred from the collection cup into a leak proof vial containing 70% non-denatured ethanol. City, county, state, coordinates, collector, and tree host were recorded. Collected samples were identified morphologically using a *P. juglandis* identification guide (Seybold et al. 2013) and confirmed by Dr. William Klingeman. Across Tennessee field sites in 2015, *P. juglandis* trapping began at the beginning of May and ended in the middle of October. For 2016, trapping started in late May and ended at the beginning of August, due to low *P. juglandis* flight activity. Shipment of infested material and/or *P. juglandis* was conducted in accordance with USDA-issued permits (P526P-14-04158, P526P-15-03500 and P526P-14-04158).

3.3.2 DNA Isolation

Prior to DNA isolation, *P. juglandis* specimens were placed in a Petri dish containing distilled water for 15 min and then placed onto filter paper to allow for the evaporation of residual ethanol. Samples were then placed in 2 ml conical screw-cap microcentrifuge tubes (Fisherbrand, Pittsburgh, PA, USA) containing 2.3 mm diameter Zirconia/Silica beads (BioSpec Products, Bartlesville, OK, USA). Samples were placed in a Bead Mill 24 homogenizer (Fisher Scientific, Pittsburgh, PA, USA) operating at 5 m/s for 30 seconds with 2 cycles, each with a 3 min stop time between cycles. DNA was extracted using Thermo Scientific MagJET Genomic DNA Kit (Fisher Scientific, Pittsburgh, PA, USA), according to manufacturer protocol with slight modifications. Modifications that yielded optimal DNA recovery included: an additional 20 µl of proteinase K/sample prior to overnight incubation at 56°C in a digital dry bath/heating block (Fisher Scientific, Pittsburgh, PA, USA) the elution buffer, which was heated to 70°C, was added twice (45 µl/each elution) with 5 min incubation at room temperature between elution; finally, the eluted sample was centrifuged for 1 min at 8000 rpm each time, resulting in final DNA volume of 90 µl/each sample.

PCR amplifications were performed using 12 *P. juglandis*-specific microsatellite loci (Hadziabdic et al. 2015) (Table 5). PCR amplification was performed in 11 µl reaction solutions containing 1 µL of DNA (undiluted), 4 µl of Go-Taq G2 Hot Start Colorless Master Mix (Promega, Madison, WI, USA), 1 µL of each 10 µM primer, 0.5 µl of DMSO and 3.5 µL of sterile water. PCR amplification was performed 96-well plates, using an Eppendorf Mastercycler Pro Thermocycler (Eppendorf AG, Hamburg, Germany) with an initial denaturation step of 2 min at

95°C, followed by 30 cycles of 95°C for 30 secs, 55°C for 45 secs, 70°C for 60 secs, and a final extension at 70°C for 1 min.

Following amplification, DNA samples were analyzed via a QIAxcel Advanced Capillary Electrophoresis System (QIAgen, Valencia, CA, USA) using an internal 25 base pair DNA size marker. With the QIAxcel Advanced system, data are automatically generated and exported using QIAxcel Advanced ScreenGel software. This software facilitates rapid data interpretation, and provides flexibility with data in which the results are displayed in both an electropherogram and a gel image view. Both positive and negative control samples were included for additional confirmation and to validate accuracy of results. After analyzing all individuals using the 12 polymorphic *P. juglandis* specific microsatellite loci, individuals that failed to amplify across at least 6 microsatellite loci were excluded from further analyses.

3.3.3 Data Analyses

The program FLEXIBIN (Amos et al. 2007) was used for automated binning of the allelic data by converting raw allele lengths into allele classes. The programs GenAEx 6.5 (Peakall and Smouse 2012) and R package gstudio (Dyer 2016; R-CoreTeam 2016) were used for calculating the standard measures of genetic diversity among *P. juglandis* subpopulations.

Population structure of *P. juglandis* subpopulations was investigated using two different Bayesian-based clustering methods: STRUCTURE and TESS (Pritchard et al. 2000; Caye et al. 2016). To infer the number of *P. juglandis* subpopulations occurring across a wide geographical area, the program STRUCTURE version 2.3.4 (Pritchard and Donnelly 2001; Hubisz et al. 2009) was utilized using twenty independent runs for each *K* value between *K* = 1-10 and an initial burn-in of 200,000 iterations, followed by 600,000 Markov chain Monte Carlo (MCMC) steps. In

addition, an admixture model with correlated allele frequencies was selected, which assumes no prior information of origin of these populations. The appropriate number of clusters was evaluated using Evanno's method (Evanno et al. 2005) and visualized in STRUCTURE HARVESTER (Earl and Vonholdt 2012) and POPHELPER (Francis 2016). The second Bayesian-based approach utilized TESS using a R platform (Caye et al. 2016) incorporating 10,000 iterations with 20 replicates for each K value between $K = 1-10$. TESS can estimate spatial population structure and evaluate ancestry coefficients using algorithms that are based on least-squares optimization, as well as geographically-constrained, non-negative matrix factorization (Caye et al. 2016). The optimal number of ancestral populations was determined using the deviance information criterion (Caye et al. 2016) and data was plotted and visualized in R.

Genetic differentiation was calculated using the analysis of molecular variance (AMOVA) in ARLEQUIN v. 3.5.2.2 (Excoffier and Lischer 2010) and three different analyses were performed. The first analysis grouped all samples into one hierarchical group. Subsequent analyses grouped individuals based on their STRUCTURE and geographical assignments. The significances of variance components for each hierarchical comparison were assessed using a 10,000 permutations and the 95% confidence interval.

The program Bottleneck 1.2 (Piry et al. 1999) was used to test if *P. juglandis* subpopulations have undergone a recent bottleneck (excess in gene diversity) or population expansion (deficit in gene diversity). We grouped *P. juglandis* subpopulations into four geographical regions (northwestern U.S., southwestern U.S., eastern U.S. and Italy). Both the Sign and Wilcoxon tests were used to detect any significant excess or deficit in gene diversity (Cornuet and Luikart 1996) under the infinite allele model (IAM), stepwise mutation model

(SMM), and two-phase mutation models (TPM), using default settings. Lastly, correlation between genetic and geographic distance was evaluated using the adegenet package (Jombart 2008) in the R program, and statistical significance was evaluated using a Mantel test with 1,000 permutations.

3.4 Results

Our results revealed the presence of population structure, high genetic diversity and differentiation among *P. juglandis* individuals from the subpopulations taken from eastern and western U.S., and Italy. For this study, a total of 1007 *P. juglandis* individuals, across 40 subpopulations from different geographical regions, were analyzed using twelve microsatellite loci (Hadziabdic et al. 2015). After removing samples that failed or did not amplify across a number of loci, a total of 839 *P. juglandis* individuals were used in subsequent data analyses. Gene diversity (H_e) across all *P. juglandis* individuals and each population was high (0.60), with values ranging from 0.49 in the Italian subpopulation IT3 (Sandrigo, Vicenza, IT) to 0.67 in the Oregon subpopulations OR8 and OR9 (Mossier and The Dalles, Wasco Co., OR) (Table 6). The mean number of alleles (N_a) across all loci and subpopulations was 4.85 (Table 6). In general, subpopulations from northwestern U.S. had greater gene diversity than those in southwestern and eastern U.S. and Italy. Allelic richness across 40 subpopulations was 2.44, ranging from 2.17 in the Indiana subpopulation to 4.59 in Sandrigo, Vicenza, Italy (IT3) subpopulation (Table 6). The total number of alleles across all loci was 164, with an average of 14 alleles per locus (alleles ranged from 9-20) (Table 7). Shannon's diversity index across 40 subpopulations was 1.15 and ranged from 0.87 (IT3) to 1.42 (OR8) (Table 6). The presence of private alleles, defined as an allele found in only one subpopulation, was observed across all loci, and across 13 out of

the 40 subpopulations (Table 6). The *P. juglandis* subpopulation from Walnut Creek Center, Yavapai County, Arizona (AZ) had the highest number of private alleles when compared to other subpopulations (Table 6). Subpopulations from western U.S. had a higher occurrence and number of private alleles when compared to subpopulations from eastern U.S. (Table 6).

Data was further partitioned into four geographical regions (northwestern, southwestern, and eastern U.S., and Italy) and two groups based on clusters identified by STRUCTURE. The results indicated high gene diversity across four regions ($H_e=0.69$) and two genetic clusters ($H_e=0.70$) (Tables 8 and 10). The northwestern U.S. partition yielded the highest gene diversity, yet the highest allelic richness was observed in southwestern U.S. subpopulation (Table 8). Similar to the previous findings, southwestern and northwestern U.S. subpopulations had the highest number of private alleles (42% and 39% respectively) when compared to eastern U.S. and Italy (Table 8). For all data sets, a significant amount of inbreeding was observed across all subpopulations ($F_{IS} = 0.65, 0.68$ and 0.69 for 40 subpopulations, four geographical regions and two genetic clusters, respectively) (Tables 7, 9 and 11).

The proportion of genetic diversity attributed to population differentiation across 40 subpopulations was high across all loci ($F_{ST}=0.20$), ranging from 0.10 (WTB19) to 0.33 (WTB 130) (Table 7) with limited gene flow ($N_m=1.18$), ranging from 0.52 (WTB130) to 1.44 (WTB16) (Table 7). When data was partitioned into four geographical locations and two genetic clusters, it was observed to have a slightly different pattern of lower population differentiation and increased gene flow ($F_{ST}=0.07$, $N_m=4.36$ and $F_{ST}=0.06$, $N_m=11.42$, respectively) (Tables 9 and 11).

Pairwise population differentiation (F_{ST}) across subpopulations ranged from 0.013 to 0.245, indicating a moderate to high genetic differentiation between the 40 subpopulations of *P. juglandis*. The lowest genetic differentiation was found between two Tennessee subpopulations (TN5 and TN6) ($F_{ST}=0.01$). The largest differentiation was observed between Colorado (CO2) and Italian (IT3) subpopulations ($F_{ST}=0.25$), and this comparison represents the most geographically distant sampling of subpopulation pairs (data not presented). When data was partitioned across four geographical locations, limited genetic differentiation was evident among the groups, ranging from 0.01 between northwestern and eastern U.S. subpopulations to 0.07 between Italy and southwestern U.S. (Table 12). In addition, gene flow was present among all four geographic regions, yet the highest values were observed among northwestern and eastern U.S. subpopulations ($N_m=20.29$) (Table 13). A positive correlation was also indicated between geographic and genetic distances ($P=0.002$, Fig. 21). Although geographic distance between these subpopulations is the likely contributor to genetic differentiation among them, other factors such as genetic drift and natural selection can be of interest as well.

Two genetic clusters ($\Delta K = 2$) were detected using program STRUCTURE with Evanno's method (Evanno et al. 2005) (Fig. 22). Twenty runs of the same K, used for the STRUCTURE analysis, produced highly consistent individual assignment probabilities. The two clusters included southwestern U.S. subpopulations (AZ, NM and CO) as one group and northwestern U.S., eastern U.S. and Italy subpopulations as the second group (Figs. 23 and 24A). The model-based clustering results produced by TESS indicated the presence of three genetic clusters based on the lowest DIC value that corresponds well to geographical regions (Fig. 24B). Although TESS indicated presence of 3 possible clusters, biological data were better explained

by the presence of two clusters, and therefore the two clusters model was used in further analyses.

The analysis of molecular variance (AMOVA) was performed using three different analyses, in which subpopulations were: i) combined in one hierarchical group across all subpopulations (839 individuals across 40 subpopulations), ii) grouped based on the two clusters identified by STRUCTURE, and iii) combined into four geographical groups: northwestern, southwestern, and eastern U.S., and Italy. Using AMOVA, most of the genetic variation between the 40 subpopulations was explained by individual variation rather than divergence across 40 subpopulations, 84.08 % and 15.92 % ($F_{ST} = 0.16$, $P < 0.01$), respectively (Table 14). Similarly, data were analyzed using two inferred genetic clusters, most of the variation was individually based (87.36%); only 12.64% of the variation was attributed to differentiation among two genetic clusters ($F_{ST} = 0.13$, $P < 0.01$) (Table 14). When *P. juglandis* individuals were grouped based on four geographical regions, only 6.71% of the variation was among the groups, 10.95% among subpopulations within the groups and 82.35% within the subpopulations ($F_{CT} = 0.07$, $F_{SC} = 0.12$, $F_{ST} = 0.18$, $P < 0.01$) (Table 14).

Since TCD has been newly distributed into eastern U.S., population bottleneck or expansion was tested using four geographical regions across all *P. juglandis* individuals. Two different tests, Sign and Wilcoxon were utilized to detect the presence of a significant deficit or excess in gene diversity. In northwestern and eastern U.S. groups, our results indicated a significant excess in gene diversity under the IAM model, but also a significant deficit under the SMM model indicating both population bottleneck and expansion (Table 15). However, the

mode-shift indicator followed a normal L-shaped distribution, which is expected under mutation-drift equilibrium.

3.5 Discussion

We used highly polymorphic species-specific microsatellite loci to evaluate population structure of *P. juglandis* based upon the analyses of 839 *P. juglandis* specimens representing 40 subpopulations across 10 U.S. states and Italy. This approach has provided greater understanding of the genetic composition and demography of *P. juglandis* and provides valuable insight into the spatial structure and distribution of *P. juglandis*, both in the U.S. and Italy, spanning the current distribution of TCD in Europe. Rugman-Jones et al. (2015) analyzed genetic diversity and spatial distribution of *P. juglandis* using mitochondrial cytochrome oxidase c subunit 1 gene (COI). Their results revealed the existence of two morphologically undistinguishable divergent lineages with high levels of polymorphism. By their interpretation, one of these lineages is likely to be responsible for spreading TCD around the U.S. (Rugman-Jones et al. 2015). Our data, which indicates high levels of genetic diversity and population differentiation among the 40 subpopulations of *P. juglandis* across different geographic regions, supports their finding of a high genetic diversity observed among *P. juglandis* specimens around the U.S. We also found a high inbreeding coefficient among the 40 subpopulations with limited gene flow and an admixture between 40 subpopulations.

High levels of genetic diversity are usually typical of an organism's native area (Rugman-Jones et al. 2015). Previous research suggests that the species origin of *P. juglandis* may be Arizona, California and New Mexico (Blackman 1928; Rugman-Jones et al. 2015). Rugman-Jones et al. (2015) hypothesized that the *P. juglandis* L1 lineage could have spread into the native

range of *J. major*, extending further south into Mexico region. Our data shows high genetic diversity among all subpopulations, indicating that the vector may have existed in those areas longer than expected. Additionally, due to the inconsistent distribution of *P. juglandis* and the limited flight capacity of adult *P. juglandis*, our data indicate spread of *P. juglandis* from multiple sources on multiple occasions and supports the working hypothesis that the spread of both TCD and *P. juglandis* are most likely human-mediated (Seybold et al. 2012a; Utley et al. 2013; Rugman-Jones et al. 2015; Daniels et al. 2016), as is observed for *G. morbida* (Hadziabdic et al. 2014; Zerillo et al. 2014).

It would be expected that newly-established TCD areas, such as both eastern U.S. and Italian *P. juglandis* subpopulations should have lower genetic diversity and reduced allelic richness when compared to *P. juglandis* subpopulations where TCD has become well established (Grant et al. 2011; Montecchio and Faccoli 2014). However, our data detected high allelic richness with a high gene diversity compared to northwestern and southwestern U.S. Consequently, it is possible that both *P. juglandis* and potentially *G. morbida* existed in these regions undetected for longer than has been believed. We also found the highest gene flow between northwestern and eastern U.S., which supports the previous findings that grouped northwestern and eastern *G. morbida* isolates together as one cluster (Hadziabdic et al. 2014).

Private alleles are unique to the population in which they are detected and can be a useful tool in conservation genetics applications (Kalinowski 2004). In a larger, genetically diverse populations, the probability of a higher number of private alleles is expected (Kalinowski 2004). This principle helps explain why western subpopulations of our data, which are represented by a larger sample size, also tended to have more private alleles per locus than

those populations in eastern U.S. and Italy. Bayesian clustering analyses among the 40 subpopulations of *P. juglandis* reflect the history of the recent spread of the vector and also supports our hypothesis of multiple introductions throughout our geographical sampling area, similar to outcomes from the *G. morbida* studies (Hadziabdic et al. 2014; Zerillo et al. 2014).

Although the TCD outbreak in Italy was recently discovered (Montecchio and Faccoli 2014), our analyses suggest the presence of two clusters within the Italian subpopulations, with relatively equal representation of both identified clusters. The northwestern and eastern U.S. and Italy subpopulations grouped as one cluster and southwestern U.S. grouped as the second cluster. These findings support our hypothesis that the movement of TCD may have taken place from western U.S. to Italy and eastern U.S., but we cannot ascertain the source of the spread from our results. In this study, we found no evidence of origin for the recent expansion, however, the level of connectivity among eastern and western U.S. subpopulations, which includes the Italian subpopulation, suggests multiple introductions from multiple sources. Although the model-based clustering results provided by TESS indicated the presence of three clusters, the model did not fit the data.

The analysis of molecular variance (AMOVA) showed similar patterns of population structure among *P. juglandis* subpopulations. A majority of genetic variation occurs among individuals rather than among subpopulations, similar to the genetic variation that was observed in *G. morbida* subpopulations (Hadziabdic et al. 2014). Regardless of hierarchical grouping, AMOVA analysis identified moderate to high genetic differentiation among *P. juglandis* subpopulations. When all subpopulations were analyzed as one hierarchical group, high genetic differentiation and limited gene flow were apparent among *P. juglandis*

subpopulations. This observation suggests that the natural barriers presented by the study area, for example the Rocky Mountains in western U.S. and the Appalachian Mountains in eastern U.S. may be functioning to deter or limit gene flow (Allendorf and Luikart 2007; Tang et al. 2016).

Our results are similar to those obtained from studies of other insect pests. For example, Alvarez et al. (2005)'s study concluded that the spread of *Acanthoscelides obtectus* Say was due to human-mediated movement (Alvarez et al. 2005) and Kebe et al. (2016) found that the beetle *Callosobruchus maculatus* has limited gene flow. The major factor limiting the gene flow between subpopulations may be due to geographic isolation (Kebe et al. 2016), which was present in our study. However, when *P. juglandis* were grouped based on geographical origin or STRUCTURE analysis, results showed low genetic differentiation and high levels of gene flow. Generally speaking, differentiation of plant-insect herbivore species pairs should be largely driven by factors affecting the host distribution, given that an asymmetry of interdependence exists (for example, that the plant can survive without its insect herbivore, but not vice versa) (Nason et al. 2002).

Currently, there are two different hypotheses regarding TCD disease complex origin: (1) that the principal vector insect and plant pathogenic fungus co-evolved with *J. major* and the disease then spread to northwestern and eastern U.S. or, (2) that the vector insect and fungus co-evolved with *J. californica* and then moved to northwestern and eastern U.S. (Zerillo et al. 2014). *Juglans nigra* is native to neither Colorado nor Oregon, but has been imported from its native range in eastern U.S. and are utilized as street trees that are adapted to urban landscape habitats across southwestern U.S. Consequently, the host-pathogen interaction might be

responding differently than expected to disease pressure (Hadziabdic et al. 2014). According to Utley et al. (2013), the host among the *Juglans* spp. most susceptible to *G. morbida* is *J. nigra*. Another likely explanation for the movement of TCD from western to eastern U.S. might be due to the natural spread of *Juglans* spp. (Zerillo et al. 2014). Lastly, walnut trade, and seed marketing and exchange can provide an additional explanation for rapid movement of both the vector and the pathogen across wide geographical areas. This is evidenced by the relative closeness of the TCD infested area in northern Italy to a sawmill importing walnut logs from North America (Montecchio and Faccoli 2014; Rugman-Jones et al. 2015).

Our data shows a high level of inbreeding among the 40 subpopulations of *P. juglandis*. Keller et al. (2011) also found high levels of inbreeding among their genetic species. Previous research related to *P. juglandis* suggested that hybridization in evolutionary terms was a recent event, confirming two morphologically indistinguishable lineages (Rugman-Jones et al. 2015). The authors also suggested that in sympatric areas of two existing lineages, evidence of interbreeding should be expected, yet this condition was not found in their study (Rugman-Jones et al. 2015).

Future research should focus on understanding the biodiversity of both the fungus and the walnut twig beetle populations in infected black walnut communities within the current disease distribution. Understanding the genetic structure and spatial distribution of *P. juglandis* will improve our knowledge regarding the disease epidemiology and eliminate further outbreak of the disease.

3.6 References

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4 Conclusion

Using previously developed species-specific microsatellite loci for *G. morbida* and *P. juglandis*, we developed a rapid molecular detection protocol for the TCD complex. Molecular detection outcomes among all sampled trees demonstrated that California samples had the highest rate of TCD complex member detection compared to trees from Tennessee. The results provided a successful protocol with a high degree of sensitivity and outlined evidence that rapid molecular detection of TCD is feasible, effective, and time efficient. Results further indicated high genetic diversity, presence of population structure, and limited gene flow has occurred between 40 subpopulations of *P. juglandis* from U.S. and Italy. High levels of genetic diversity across four geographical regions are best explained by a human mediated movement of infested plant material that has been introduced from multiple source locations on multiple occasions. These findings further substantiate previous hypotheses that infer thousand cankers disease has been established in these different source locations for a longer period of time than was originally proposed.

Appendix

Table 1. Pearson's Chi-square test with Yate's continuity correction comparisons of molecular detection among drill cores taken at sites where *Geosmithia morbida* (*Gm*) and *Pityophthorus juglandis* (*Pj*) are known to occur. Presented data was quantified across collection locations and sample type whether lesion-directed or feature-directed on walnut branch sections.

Location	Sample Type	N ^A	<i>Pj</i> ⁽⁻⁾ / <i>Gm</i> ⁽⁻⁾	<i>Pj</i> ⁽⁺⁾ / <i>Gm</i> ⁽⁻⁾	<i>Pj</i> ⁽⁻⁾ / <i>Gm</i> ⁽⁺⁾	<i>Pj</i> ⁽⁺⁾ / <i>Gm</i> ⁽⁺⁾	χ^2 (df=1) ^B	P-value
CA, TN	LD&FD ^C	1600	1420	28	141	11	14.11	< 0.001
CA, TN	LD	1200	1065	4	126	5	14.24	< 0.001
CA, TN	FD	400	355	24	15	6	11.16	< 0.001
CA	LD&FD	600	449	23	117	11	1.96	NS ^D
TN	LD&FD	600	571	5	24	0	6.50	NS
CA	LD	400	287	1	107	5	6.68	< 0.001
CA	FD	200	162	22	10	6	5.99	< 0.01
TN	LD	400	378	3	19	0	<0.0001	NS
TN	FD	200	193	2	5	0	<0.0001	NS

^ANumber of individuals.

^BPearson's chi-square test ($\alpha= 0.05$) with Yates' continuity correction.

^CLD = lesion-directed drill sampling; FD = feature-directed drill sampling.

^DNS = not statistically significant.

Table 2. Pearson's chi-square test with Yates' continuity correction comparison of molecular detection in any of the drilled samples (per tree) could yield a confirmation that a sample (tree) is positive for a TCD complex member [e.g., either pathogen, *Geosmithia morbida* (*Gm*), or beetle *Pityophthorus juglandis* (*Pj*) presence]. Data were quantified across collection locations and sample type, whether from lesion-directed or feature-directed sampling of walnut branches.

Location	Sample Type	N ^A	<i>Pj</i> ⁽⁻⁾ / <i>Gm</i> ⁽⁻⁾	<i>Pj</i> ⁽⁺⁾ / <i>Gm</i> ⁽⁻⁾	<i>Pj</i> ⁽⁻⁾ / <i>Gm</i> ⁽⁺⁾	<i>Pj</i> ⁽⁺⁾ / <i>Gm</i> ⁽⁺⁾	χ^2 (df=1) ^B	P-value
Combined thousand cankers disease-infested localities ^C								
CA, TN	LD & FD ^D	200	135	7	45	13	12.11	< 0.001
CA, TN	LD	120	76	1	36	7	7.68	0.005
CA, TN	FD	80	59	6	9	6	6.79	0.009
Among trees ^E								
CA	LD or FD	40	25	4	5	6	5.05	0.02
TN	LD or FD	40	34	2	4	0	< 0.0001	NS ^F

^ANumber of individuals.

^BPearson's chi-square test ($\alpha=0.05$) with Yates' continuity correction.

^CTallied observations across 10 lesion-directed or 5 feature-directed drill cores per tree.

^DLD = lesion-directed drill sampling; FD = feature-directed drill sampling

^ETallied observations, irrespective of lesion-directed or feature-directed drilling effort, noted within 40 branch samples (each serving as a single-tree representative).

^FNS = not statistically significant.

Table 3. Logistic mixed model outcomes observed by region and among drill core samples taken from California and Tennessee (Thousand Cankers Disease infested sites), and MO (un-infested, control) locations.

Location	Sample Type	N ^A	LS Mean Estimate ^B	Lower 95% CI	Upper 95% CI	Contrast Estimate	P-value
Analysis of All (Combined) Sampling Methods							
CA	LD & FD ^C	600	-2.28	-2.82	-1.76		
TN	LD & FD	600	-4.23	-5.00	-3.45		
MO	FD	400	NA	NA	NA		
CA vs TN						1.94 ^D	< 0.001
LD vs FD						-1.92	< 0.001
Analysis of Each Sampling Method							
CA	LD	400	-1.33	-1.88	-0.77		
TN	LD	400	-3.89	-4.71	-3.06		
MO	FD ^E	400	-20.86	-61.96	20.25		
CA	FD	200	-3.25	-4.10	-2.40		
TN	FD	200	-4.57	-5.74	-3.40		
MO	LD	NA	NA	NA	NA		
CA, LD vs TN, LD						2.55	< 0.001
CA, LD vs CA, FD						1.92	< 0.001
CA, LD vs TN, FD						3.24	< 0.001
CA, FD vs TN, FD						0.64	NS (0.85) ^F
CA, FD vs TN, LD						1.32	NS (0.37)
TN, LD vs TN, FD						0.68	NS (0.90)

^ANumber of individuals.

^BValues are given in logit (not the response) scale.

^CLD = lesion-directed drill sampling; FD = feature-directed drill sampling.

^DDetection recovery probabilities are calculated from contrast estimates of comparisons reported. Thus, compared with samples taken in Tennessee, the California drill core samples were 6.96 times more likely to yield a positive detection of *Geosmithia morbida* or *Pityophthorus juglandis* (e.g., where exponential log [1.94] = 6.96).

^EBecause no disease symptoms were present among Missouri samples, drilling was directed toward most-likely features observed on the bark surface. No other drilling was performed for these samples.

^FNS = not statistically significant.

Table 4. *Pityophthorus juglandis* subpopulations used to evaluate genetic diversity, spatial structure and distribution in the U.S. and Italy.

Subpopulation Code	Sample Site*	Host	Latitude	Longitude	Collection Date	Collector**
AZ	Walnut Creek Center, Yavapai Co., AZ	<i>J. major</i>	34.923553	-112.843744	2016	RC
CO1	Fort Collins, Larimer Co., CO	<i>J. nigra</i>	40.57775	-105.08141	2013	DH
CO2	Laporte, Larimer Co., CO	<i>J. nigra</i>	40.630604	-105.14609	2013	DH
IN	Brookville, Franklin Co., IN	<i>J. nigra</i>	39.386535	-85.05253	2015	TS
IT1	Sandrigio, Vicenza, IT	<i>J. regia</i>	45.636754	11.591358	2014	MF
IT2	Thiene, Vicenza, IT	<i>J. regia</i>	45.667146	11.50568	2014	MF
IT3	Sandrigio, Vicenza, IT	<i>J. regia</i>	45.650623	11.599902	2014	MF
IT4	Bressanvido, Vicenza, IT	<i>J. regia</i>	45.659338	11.650444	2014	MF
IT5	Dueville, Vicenza, IT	<i>J. regia</i>	45.61096	11.50117	2014	MF
IT6	Castelfranco Veneto, Treviso, IT	<i>J. regia</i>	45.66061	11.89805	2014	MF
NM1	Reserve, Catron Co., NM	<i>J. major</i>	33.704656	-108.75905	2016	TD
NM2	Reserve, Catron co., NM	<i>J. major</i>	33.713348	-108.75808	2016	TD
NV1	Reno, Washoe Co., NV	<i>J. nigra</i>	39.523995	-119.79693	2015	SJ
NV2	Sparks, Washoe Co., NV	<i>J. nigra</i>	39.529725	-119.77642	2015	SJ
NV3	Sparks, Washoe Co., NV	<i>J. nigra</i>	39.527867	-119.77747	2015	SJ
OH	Hamilton, Butler Co., OH	<i>J. nigra</i>	39.413994	-84.608295	2013	WK
OR1	Barlow, Clackamas Co., OR	<i>J. nigra</i>	45.250833	-122.72361	2013	JP
OR2	Corvallis, Benton Co., OR	<i>J. nigra</i>	44.591389	-123.21833	2013	JP
OR3	Corvallis, Benton Co., OR	<i>J. nigra</i>	44.591389	-123.21833	2013	JP
OR4	Sauvie Island, Multnomah Co, OR	<i>J. nigra</i>	45.703611	-122.79111	2013	JP
OR5	Medford, Jackson Co., OR	<i>J. nigra</i>	42.406389	-122.94	2013	JP
OR6	Salem, Marion Co. OR	<i>J. nigra</i>	44.943611	-123.00861	2013	JP
OR7	Eugene, Lane Co. OR	<i>J. nigra</i>	44.072222	-123.12111	2013	JP
OR8	Mosier, Wasco Co., OR	<i>J. nigra</i>	45.676667	-121.40333	2013	JP
OR9	The Dalles, Wasco Co., OR	<i>J. nigra</i>	45.654722	-121.21611	2013	JP

Table 4. Continued.

Subpopulation Code	Sample Site*	Host	Latitude	Longitude	Collection Date	Collector**
PA1	Doylestown, Bucks Co., PA	<i>J. nigra</i>	40.35011325	-75.127792	2014	PS
PA2	Doylestown, Bucks Co., PA	<i>J. nigra</i>	40.351433	-75.128374	2014	PS
TN1	Spring City, Rhea Co., TN	<i>J. nigra</i>	35.6956	-84.85745	2015	EÖ
TN2	Hinton, Knox Co., TN	<i>J. nigra</i>	35.971833	-83.992056	2014	WK
TN3	Warrior Trail, Knox Co., TN	<i>J. nigra</i>	35.82225	-84.146638	2015	WK
TN4	Church, Knox Co., TN	<i>J. nigra</i>	35.9765	-84.001944	2015	WK
TN5	Murphy Rd., Knox Co., TN	<i>J. nigra</i>	36.0539167	-83.876194	2015	WK
TN6	Burkhart Rd., Knox Co., TN	<i>J. nigra</i>	36.0742222	-83.856472	2015	WK
TN7	Lakeshore Park, Knox Co., TN	<i>J. nigra</i>	35.9253611	-83.991778	2012	WK
TN8_9	Pearson Springs Park, Blount Co., TN	<i>J. nigra</i>	35.73753	-83.97828	2012	WK
TN10	Sandy Springs Park, Blount Co., TN	<i>J. nigra</i>	35.74028	-83.97561	2012	WK
TN11	UT Farm Rd., Blount Co., TN	<i>J. nigra</i>	35.84025	-83.94997	2014	WK
TN12	Marion Co., TN	<i>J. nigra</i>	35.25626	-85.44903	2015	EÖ
TN13	Dayton, Rhea Co., TN	<i>J. nigra</i>	35.4866	-85.02054	2015	EÖ
WA	Walla Walla, Walla Walla Co., WA	<i>J. nigra</i>	46.01722	-118.2955	2016	MG

*Sample site includes location, county and state information.

**Collectors: Royce Carlson (RC), Denita Hadziabdic (DH), Tyler Stewart (TS), Massimo Faccoli (MF), Tracy Drummond (TD), Jeff Knight (JK), William Klingeman (WK), Jay Pscheidt (JP), Paul Smith (PS), Emel Ören (EÖ), Matthew Ginzel (MG).

Table 5. Genbank accession numbers, primer sequences, number of alleles, and allelic ranges (bp) of twelve microsatellite loci used to assess genetic diversity of *Pityophthorus juglandis* individuals.

Locus	Repeat Motif	Primer sequences (5' - 3')	Allele size range (bp)	Alleles	Genebank accession number
WTB01	(TG) ₈	F: CTAAGGCGTTCTAGGTGCTGAT R: AGACCGAAGTAGCCAGACAAAG	402 – 415	10	KP091439
WTB9	(AT) ₈	F: CATTTTCGGTTTCGCCTCAAGTT R: TCCCAAATCCGGACTTTAAGGG	372 - 379	9	KP091442
WTB13	(AT) ₁₀	F: GCACGCACATTCACACGTAATA R: AGAACATTTTCGTGCATCTTGCA	353 - 362	9	KP091445
WTB14	(AC) ₁₀	F: GCCAAAAGTTTAAAGTGCTCGCT R: TACACACGCACACATTCACAAC	356 - 366	10	KP091446
WTB15	(AC) ₈	F: GGCTTTCACCTTTCTGCCAAAT R: TTCACATGGCTTCAGACCACTT	350 - 378	8	KP091447
WTB16	(AG) ₈	F: AGTGCACTTTGGCTTGTTTTCA R: CCCAGTTCACCCTTCAAGAAGA	352 - 365	10	KP091448
WTB19	(AG) ₈	F: CCCCAGGAACCTTGATCAAGAA R: CCTGAACGTGTTTCAATTGTCA	334 - 371	14	KP091449
WTB128	(CAG) ₇	F: TAAAGGGGCGCAGAATAGTGC R: AAGAAAGAGGAGCCATGACAGG	380 - 389	6	KP091450
WTB130	(TAA) ₈	F: GCGACTAAATTGAATAAATCCGACC R: CTCCATATGCGGACTACACGAA	282 - 292	6	KP091451
WTB189	(AACA) ₆	F: AAAGGGAGACGACGTTTCGATAG R: GTTAAAAACGATGCCGCTTTCA	134 - 142	7	KP091453
WTB191	(ACCA) ₇	F: ACGAGAAGCCCATGAACTGG R: TTTTGTCCATCAAATGTCCGGC	223 - 240	10	KP091455
WTB192	(AAAT) ₆	F: ATTTTAGCAGCAACATCGAGGC R: TATGAAGGTCGGTTCATCACA	268 - 277	7	KP091456

All loci Ta (°C) is 55.

Table from Hadziabdic et al. (2015)

Table 6. Diversity measures across 839 *Pityophthorus juglandis* individuals from 40 different subpopulations using twelve microsatellite loci.

Subpopulation Code	N ^a	Na ^b	Ar ^c	I ^d	H _o ^e	H _e ^f	Pa ^g
Northwestern U.S.							
NV1	15	4.00	2.28	1.04	0.18	0.56	0
NV2	5	3.00	2.33	0.91	0.21	0.53	0
NV3	19	4.75	2.54	1.26	0.15	0.66	0
OR1	8	3.25	2.31	1.00	0.14	0.57	0
OR2	26	5.58	2.53	1.31	0.19	0.66	1
OR3	24	5.17	2.38	1.18	0.15	0.61	2
OR4	7	3.83	2.46	1.10	0.21	0.58	0
OR5	26	5.25	2.51	1.28	0.23	0.66	0
OR6	26	5.08	2.28	1.11	0.20	0.57	2
OR7	14	3.83	2.29	1.02	0.14	0.56	0
OR8	30	6.83	2.59	1.42	0.30	0.67	4
OR9	6	4.50	2.75	1.30	0.33	0.67	0
WA	37	6.08	2.50	1.30	0.25	0.65	3
Southwestern U.S.							
AZ	48	5.92	2.31	1.17	0.16	0.57	5
CO1	29	5.50	2.42	1.23	0.27	0.60	3
CO2	7	3.42	2.27	0.97	0.25	0.53	2
NM1	30	6.00	2.51	1.31	0.29	0.62	4
NM2	30	6.42	2.45	1.30	0.24	0.61	0
Eastern U.S.							
IN	24	4.42	2.17	1.00	0.24	0.53	0
OH	29	5.00	2.33	1.15	0.13	0.58	0
PA1	26	4.75	2.26	1.07	0.20	0.55	0
PA2	26	5.00	2.26	1.10	0.21	0.56	0
TN1	16	4.17	2.27	1.03	0.19	0.56	0
TN2	29	5.92	2.42	1.25	0.15	0.62	0
TN3	30	5.33	2.37	1.18	0.20	0.61	1
TN4	15	4.33	2.43	1.16	0.11	0.62	0
TN5	22	5.00	2.27	1.10	0.17	0.55	1
TN6	29	5.00	2.23	1.08	0.18	0.54	0
TN7	29	5.83	2.38	1.21	0.22	0.60	0
TN8_9*	31	6.00	2.52	1.33	0.18	0.65	1
TN10	29	6.17	2.56	1.36	0.22	0.66	0
TN11	27	5.67	2.50	1.31	0.17	0.64	0

Table 6. Continued.

Subpopulation Code	N ^a	Na ^b	Ar ^c	I ^d	H _o ^e	H _e ^f	Pa ^g
TN12	14	4.83	2.28	1.08	0.27	0.55	0
TN13	8	4.08	2.51	1.12	0.17	0.60	0
Italy							
IT1	10	3.42	2.22	0.96	0.20	0.54	0
IT2	11	4.50	2.42	1.15	0.27	0.59	0
IT3	7	2.92	4.59	0.87	0.12	0.49	0
IT4	10	4.00	2.33	1.10	0.28	0.57	0
IT5	13	4.67	2.40	1.16	0.32	0.58	0
IT6	17	4.75	2.33	1.12	0.26	0.57	1
Overall Mean	21	4.85	2.44	1.15	0.21	0.6	0.75

N^a – number of *P. juglandis* individuals

Na^b – mean number of different alleles

Ar^c – allelic richness

I^d – Shannon's information index

H_o^e – observed heterozygosity

H_e^f – expected heterozygosity

Pa^g – number of private alleles in each population

*TN8_9 samples were pulled together due to small number of individuals in TN 8 subpopulation (n=3).

Table 7. Diversity measures, fixation indices and gene flow calculations across 839 *Pityophthorus juglandis* from 40 different subpopulations using twelve microsatellite loci.

Locus	N ^a	H _o ^b	H _e ^c	F _{IS} ^d	F _{ST} ^e	Nm ^f
WTB1	13	0.23	0.61	0.62	0.15	1.40
WTB9	10	0.10	0.53	0.81	0.16	1.37
WTB13	15	0.25	0.58	0.58	0.22	0.89
WTB14	11	0.18	0.58	0.68	0.14	1.50
WTB15	20	0.23	0.53	0.56	0.28	0.63
WTB16	16	0.12	0.62	0.81	0.15	1.44
WTB19	18	0.36	0.71	0.50	0.10	2.30
WTB128	13	0.09	0.54	0.84	0.31	0.56
WTB130	9	0.09	0.49	0.82	0.33	0.52
WTB189	11	0.24	0.60	0.60	0.21	0.96
WTB191	15	0.25	0.72	0.65	0.16	1.36
WTB192	13	0.37	0.60	0.38	0.17	1.19
Overall Mean	14	0.21	0.60	0.65	0.20	1.18

N^a – number of alleles observed at each locus

H_o^b – observed heterozygosity

H_e^c – expected heterozygosity

F_{IS}^d - inbreeding coefficient

F_{ST}^e - the variance among subpopulations relative to the total variance

Nm^f – the number of migrant individuals entering a subpopulation each generation

Table 8. Diversity measures across 839 individuals from four geographical regions using twelve microsatellite loci.

Subpopulation Code	N ^a	Na ^b	Ar ^c	I ^d	H _o ^e	H _e ^f	Pa ^g
Northwestern U.S.	243	10.33	8.17	1.57	0.22	0.73	16
Southwestern U.S.	144	10.67	8.21	1.62	0.22	0.71	17
Eastern U.S.	384	10.17	7.68	1.47	0.19	0.68	7
Italy	68	6.92	6.76	1.30	0.26	0.63	1
Overall Mean	210	9.52	7.90	1.49	0.22	0.69	10.25

N^a – number of *P. juglandis* individuals

Na^b – mean number of different alleles

Ar^c – allelic richness

I^d – Shannon's information index

H_o^e – observed heterozygosity

H_e^f – expected heterozygosity

Pa^g – number of private alleles in each subpopulation

Table 9. Diversity measures, fixation indices and gene flow calculations across 839 *Pityophthorus juglandis* from four geographical regions using twelve microsatellite loci.

Locus	N ^a	H _o ^b	H _e ^c	F _{IS} ^d	F _{ST} ^e	Nm ^f
WTB1	13	0.24	0.69	0.65	0.05	4.40
WTB9	10	0.10	0.53	0.82	0.05	4.83
WTB13	15	0.24	0.64	0.62	0.13	1.70
WTB14	11	0.19	0.67	0.72	0.05	4.77
WTB15	20	0.21	0.66	0.68	0.16	1.32
WTB16	16	0.14	0.70	0.80	0.04	6.07
WTB19	18	0.42	0.78	0.46	0.03	8.45
WTB128	13	0.11	0.74	0.86	0.09	2.68
WTB130	9	0.08	0.63	0.87	0.13	1.66
WTB189	11	0.29	0.73	0.61	0.06	4.13
WTB191	15	0.26	0.79	0.67	0.04	6.39
WTB192	13	0.39	0.70	0.45	0.04	5.95
Overall Mean	14	0.22	0.69	0.68	0.07	4.36

N^a – number of alleles observed at each locus

H_o^b – observed heterozygosity

H_e^c – expected heterozygosity

F_{IS}^d - inbreeding coefficient

F_{ST}^e - the variance among subpopulations relative to the total variance

Nm^f – the number of migrant individuals entering a subpopulation each generation

Table 10. Diversity measures across 839 *Pityophthorus juglandis* individuals from two genetic clusters using twelve microsatellite loci.

Cluster	N ^a	Na ^b	Ar ^c	I ^d	H _o ^e	H _e ^f	Pa ^g
Cluster1	612	11.83	10.16	1.51	0.20	0.70	30
Cluster2	227	11.16	11.06	1.58	0.25	0.71	22
Overall Mean	420	11.50	10.61	1.55	0.22	0.70	26

N^a – number of *P. juglandis* individuals

Na^b – mean number of different alleles

Ar^c – allelic richness

I^d – Shannon's information index

H_o^e – observed heterozygosity

H_e^f – expected heterozygosity

Pa^g – number of private alleles in each subpopulation

Table 11. Diversity measures, fixation indices and gene flow calculations across 839 *Pityophthorus juglandis* from two genetic clusters using twelve microsatellite loci.

Locus	N ^a	H _o ^b	H _e ^c	F _{IS} ^d	F _{ST} ^e	Nm ^f
WTB1	13	0.26	0.75	0.65	0.01	37.26
WTB9	10	0.09	0.59	0.84	0.01	31.82
WTB13	15	0.22	0.63	0.66	0.12	1.87
WTB14	11	0.18	0.67	0.74	0.04	5.77
WTB15	20	0.23	0.69	0.67	0.15	1.36
WTB16	16	0.15	0.69	0.79	0.03	8.36
WTB19	18	0.43	0.78	0.44	0.04	5.40
WTB128	13	0.10	0.67	0.85	0.17	1.18
WTB130	9	0.09	0.74	0.88	0.01	20.44
WTB189	11	0.30	0.75	0.60	0.05	5.17
WTB191	15	0.27	0.81	0.67	0.02	12.29
WTB192	13	0.34	0.68	0.51	0.04	6.10
Overall Mean	14	0.22	0.70	0.69	0.06	11.42

N^a – number of alleles observed at each locus

H_o^b – observed heterozygosity

H_e^c – expected heterozygosity

F_{IS}^d - inbreeding coefficient

F_{ST}^e - the variance among subpopulations relative to the total variance

Nm^f – the number of migrant individuals entering a subpopulation each generation

Table 12. Pairwise population differentiation (F_{ST}) of *Pityophthorus juglandis* from four geographic regions using twelve microsatellite loci.

	Northwestern U.S.	Southwestern U.S.	Eastern U.S.	Italy
Northwestern U.S.	0			
Southwestern U.S.	0.05	0		
Eastern U.S.	0.01	0.06	0	
Italy	0.05	0.07	0.06	0

Table 13. Pairwise gene flow (Nm) values for 839 *Pityophthorus juglandis* individuals from four geographic regions using twelve microsatellite loci.

	Northwestern U.S.	Southwestern U.S.	Eastern U.S.	Italy
Northwestern U.S.	0			
Southwestern U.S.	4.68	0		
Eastern U.S.	20.29	3.86	0	
Italy	4.83	3.37	4.26	0

Table 14. Analysis of molecular variance (AMOVA) for 839 *Pityophthorus juglandis* individuals across twelve microsatellite loci.

Variance partition	Degrees of freedom	Sum of squares	Variance component	% Variation	P value	F-statistic
(i)						
Among subpopulations	39	519.23	0.28 Va	15.92	$P < 0.01$	$F_{ST} = 0.16$
Within subpopulations	1638	2451.76	1.50 Vb	84.08	$P < 0.01$	
Total	1677	2970.99	1.78			
(ii)						
Among subpopulations	1	162.33	0.24 Va	12.64	$P < 0.01$	$F_{ST} = 0.13$
Within subpopulations	1676	2808.66	1.68 Vb	87.36	$P < 0.01$	
Total	1677	2970.99	1.92			
(iii)						
Among groups	3	169.96	0.12 Va	6.71	$P < 0.01$	$F_{CT} = 0.07$
Among subpopulations within groups	36	349.28	0.20 Vb	10.95	$P < 0.01$	$F_{SC} = 0.12$
Within subpopulations	1638	2451.76	1.50 Vc	82.35	$P < 0.01$	$F_{ST} = 0.18$
Total	1677	2970.99	1.81			

The analysis included all individuals across 40 subpopulations as one hierarchical group and partitioning subpopulations into two groups identified by the program STRUCTURE and regions.

F_{ST} – the variance among subpopulations relative to the total variance

F_{SC} – the variance among subpopulations within groups

F_{CT} – the variance among groups relative to the total variance

Table 15. Bottleneck determination by Sign and Wilcoxon tests for 839 *Pityophthorus juglandis* individuals grouped by the four geographic regions using twelve microsatellite loci.

Region	Mutation models ^a		
	I.A.M. (<i>P</i> value)	T.P.M. (<i>P</i> value)	S.M.M. (<i>P</i> value)
Northwestern U.S.	1/11 ^b (0.02)	7/5 (NS)	12/0 (< 0.01)
Southwestern U.S.	3/9 (NS)	6/6 (NS)	12/0 (< 0.01)
Eastern U.S.	1/11 (0.02)	6/6 (NS)	12/0 (< 0.01)
Italy	3/9 (NS)	6/6 (NS)	10/2 (< 0.01)

^a I.A.M. = infinite allele model; T.P.M. = two-phase mutation models; S.M.M. = stepwise mutation model

^b Number of loci in deficit (population expansion)/excess (population bottleneck)



Figure 1. Thousand Cankers Disease symptoms on *Juglans nigra* trees. Symptoms include yellowing of the leaves, crown and branch dieback and formation of epicormic shoots. Image provided by Dr. Denita Hadziabdic.



Figure 2. *Pityophthorus juglandis* galleries in the phloem (upper image) and exit holes (lower image in *Juglans regia* branches (arrows). Adult *P. juglandis* create entrance and emergence holes the size of a pinhead (0.64-0.75 mm) with galleries approximately 2.5 to 5 cm long. Images provided by Dr. Denita Hadziabdic.



Figure 3. *Geosmithia morbida* induced elliptical cankers underneath *Juglans nigra* branches. Individual cankers (upper image) can coalesce and form large necrotic lesions underneath the bark (lower image) thus girdling the branch and resulting in rapid tree mortality. The cankers are visible only after the outer bark is removed. Images provided by Dr. Denita Hadziabdic.

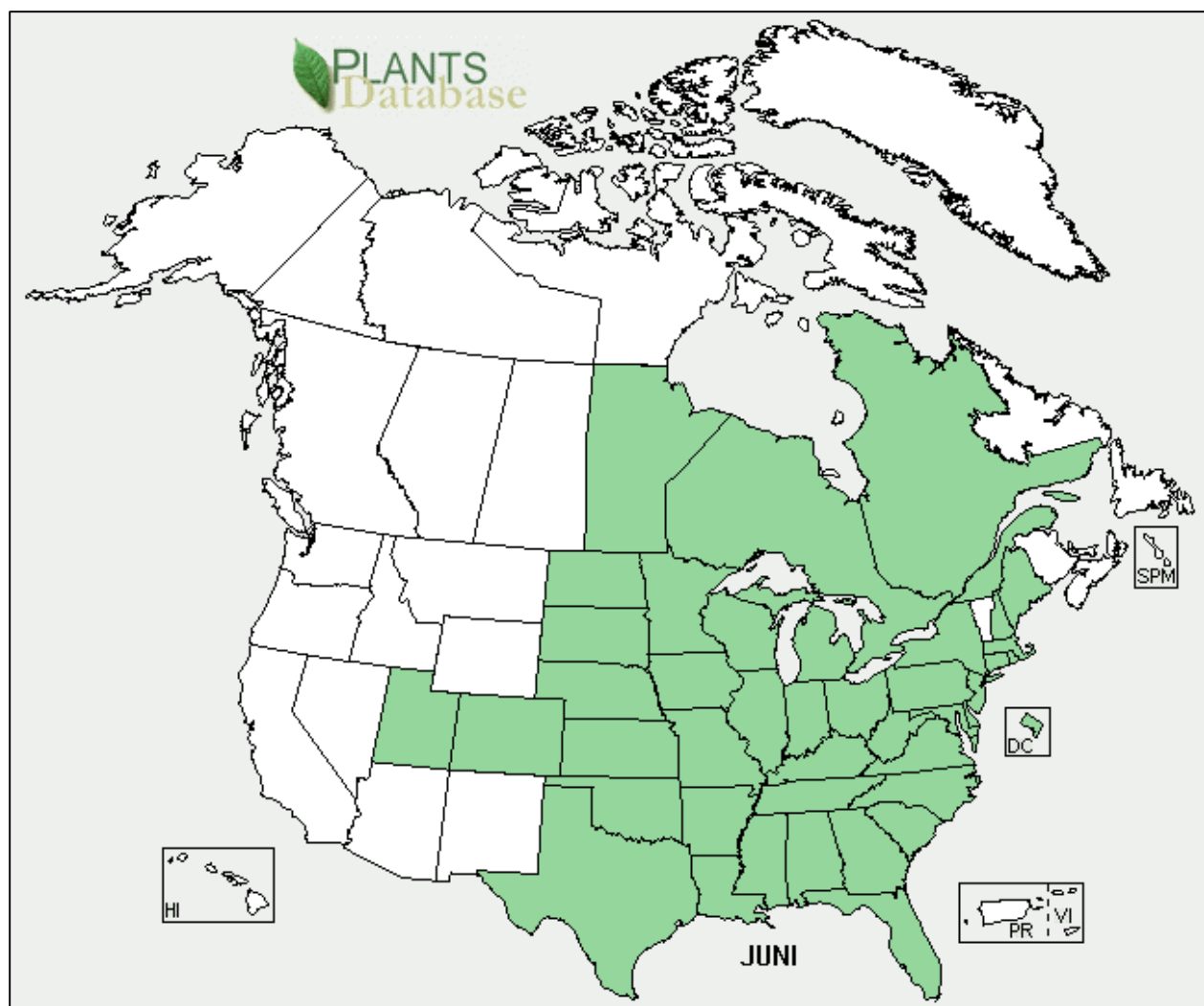


Figure 4. The native range of *Juglans nigra* (green shading). The map courtesy of USDA-NRCS Plant Database.

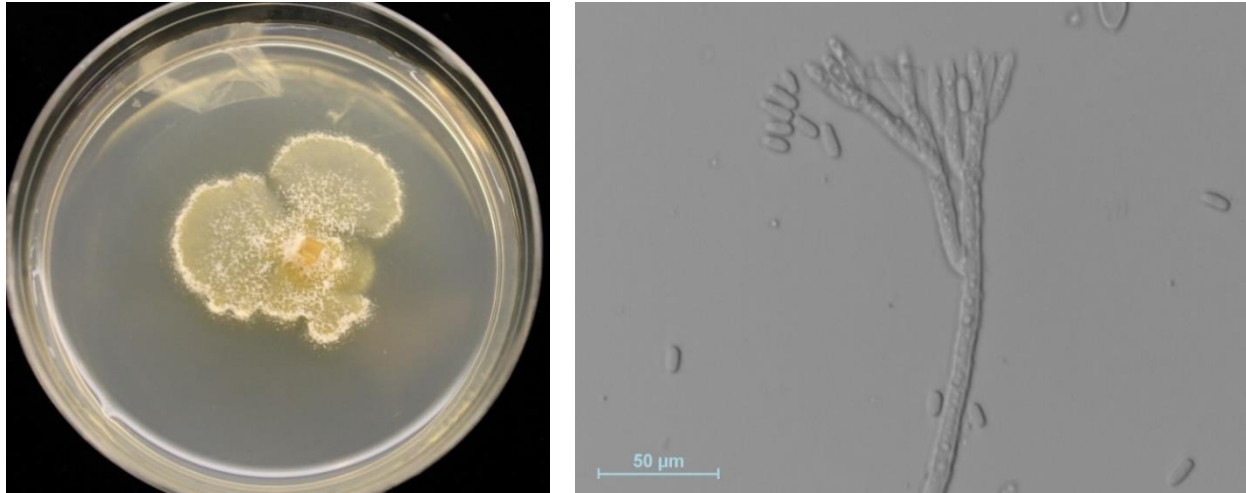


Figure 5. *Geosmithia morbida*. Cream colored to tan colonies of *Geosmithia morbida* grown on half strength potato dextrose agar (PDA) (left image). Conidia and conidiophore of *G. morbida* (right image). Images provided by Dr. Denita Hadziabdic and Tyler Edwards.



Figure 6. Mixed cultures collected from infested *Juglans* spp. galleries. *Geosmithia morbida* is slow growing pathogen that is often outcompeted by other faster growing fungi. Images provided by Dr. Romina Gazis.



Figure 7. *Pityophthorus juglandis*. The body length is less than two mm (A); the pronotal asperities from the middle to the anterior margin form two or more well-defined concentric rows; anterior margin of pronotum with more than twelve asperities (B); apex of elytra evenly rounded (C). Images courtesy of Steven Valley.

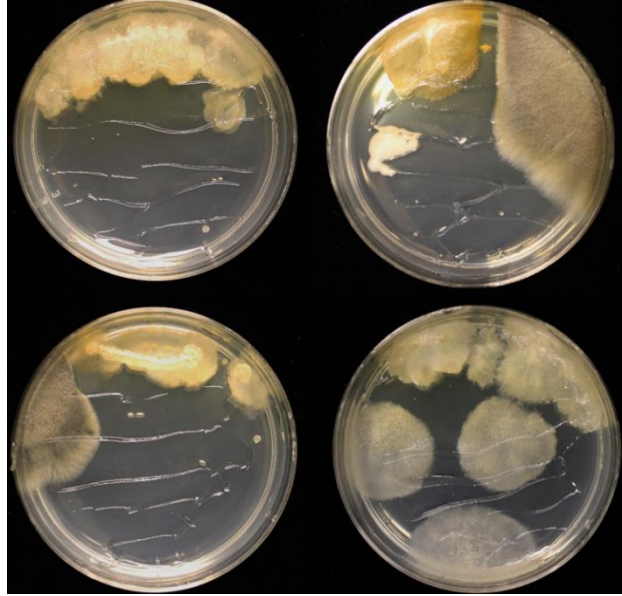


Figure 8. Mixed cultures from *Pityophthorus juglandis* colonized galleries. Detection methods of *Geosmithia morbida* is difficult due to other fungi that can grow faster and outcompete slow growing *G. morbida*. Image provided by Dr. Romina Gazis.

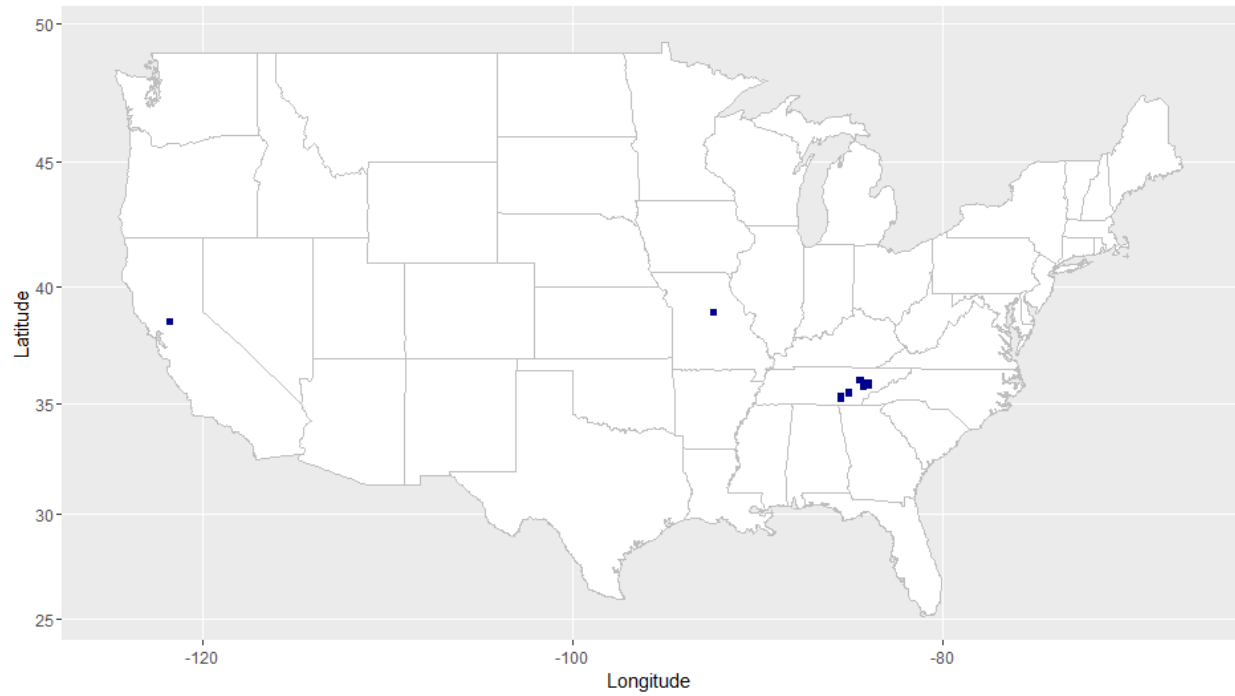


Figure 9. Three geographical locations used in rapid molecular detection study. The samples were collected from either within (California, Tennessee) or outside (Missouri) the current distribution of Thousand Cankers Disease.



Figure 10. Drilling of *Juglans* spp. branches to obtain wood shavings used for DNA isolation. Drilling potential cankers for DNA isolation and molecular confirmation of presence/absence of Thousand Cankers Disease.



Figure 11. Batches of walnut drill shavings were collected into 1.5 ml safe-lock microcentrifuge tubes (left); solutions of isolated DNA were used for PCR amplification (right).

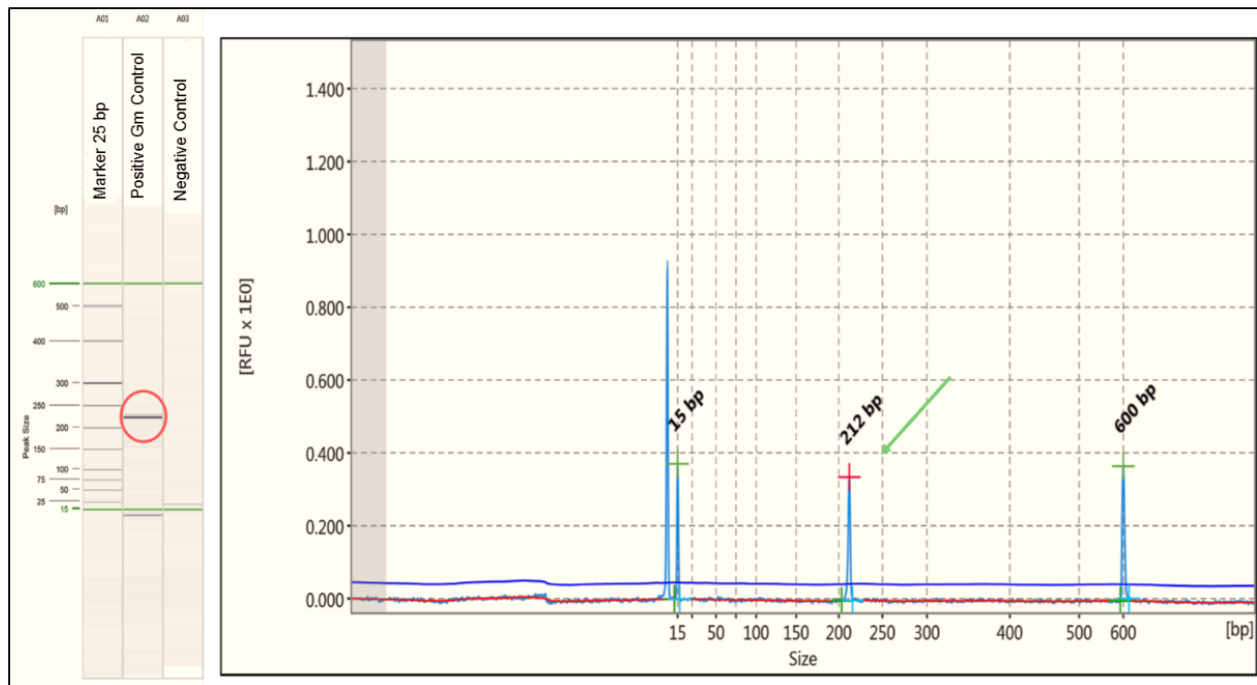


Figure 12. Thousand Cankers Disease molecular confirmation. Gel image of positive control (*Geosmithia morbida*) (circle) and negative control (water) based on 25 base pairs marker; electropherogram from Tennessee walnut shavings amplified with *Geosmithia* specific microsatellite locus, GS004.

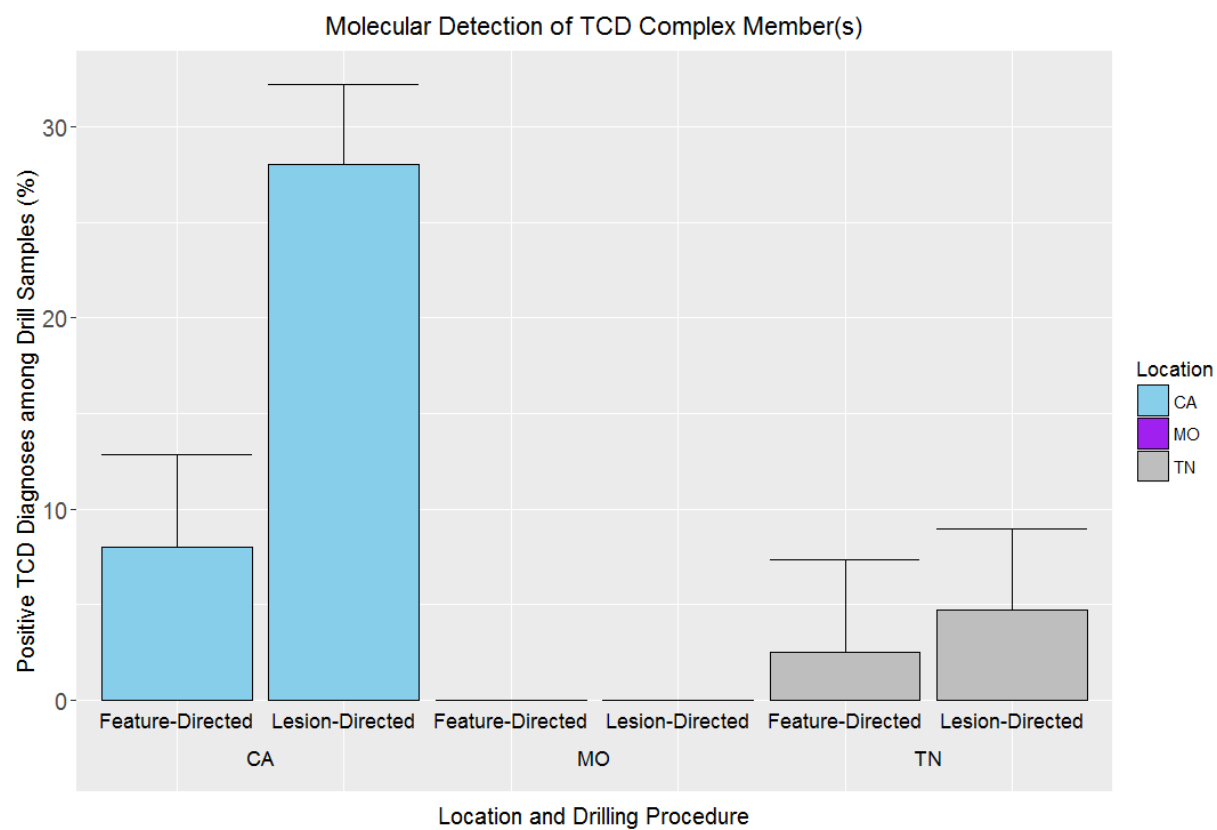


Figure 13. Molecular detection of Thousand Cankers Disease complex member(s) percentage of positive drill samples from both feature-directed (n=400) and lesion-directed (n=800) samples from California, Missouri, and Tennessee.

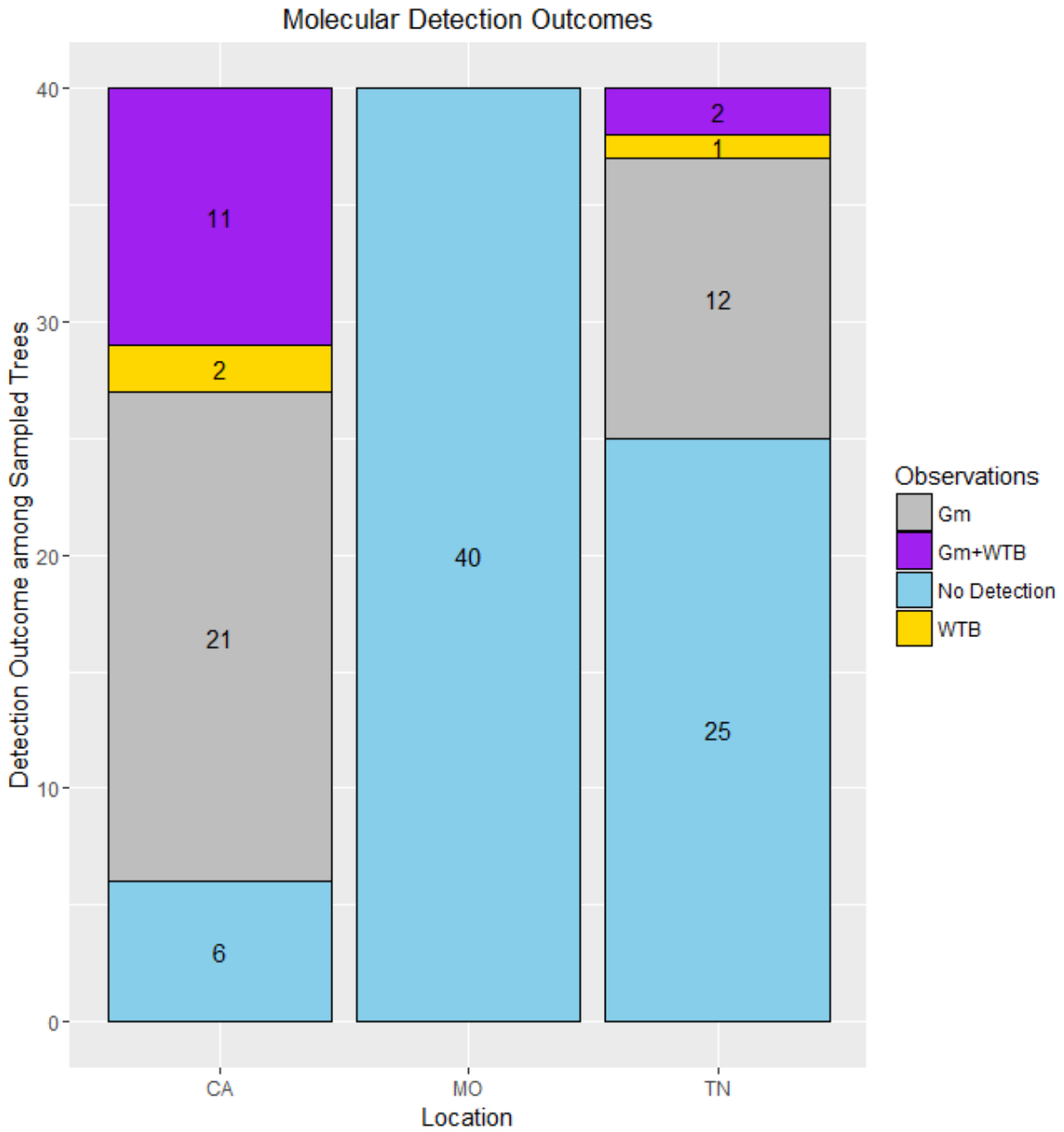


Figure 14. Molecular detection outcomes among 40 *Juglans nigra* and *J. hindsii* samples from California, Missouri (control) and Tennessee.

Figure 15. Molecular detection of Thousand Cankers Disease complex member(s) among positive trees for all samples and both drilling methods (A), among positive trees for all samples using lesion-directed drillings (B), and among positive trees for all samples using feature-directed drillings (C).

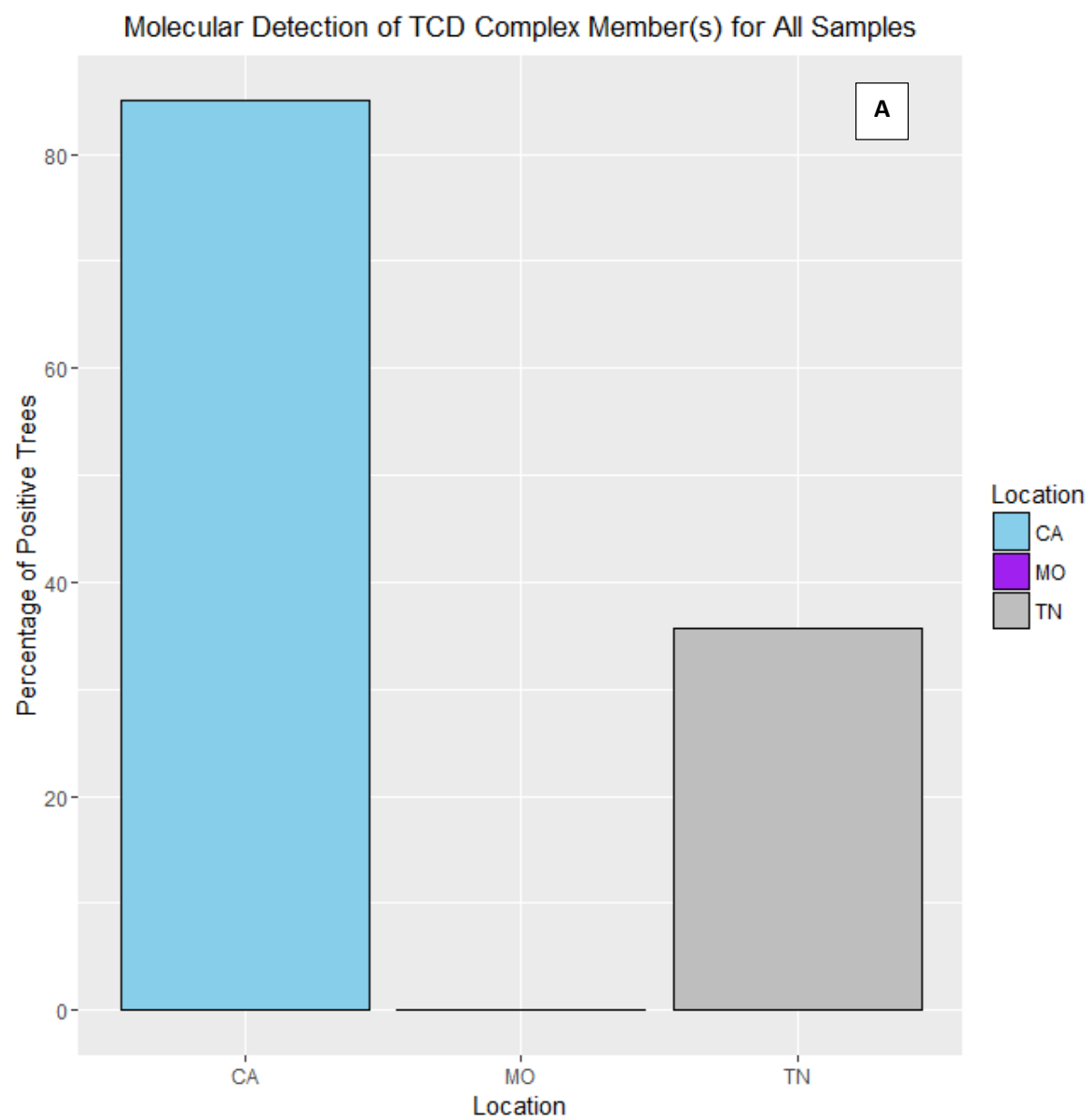


Figure 15. Continued.

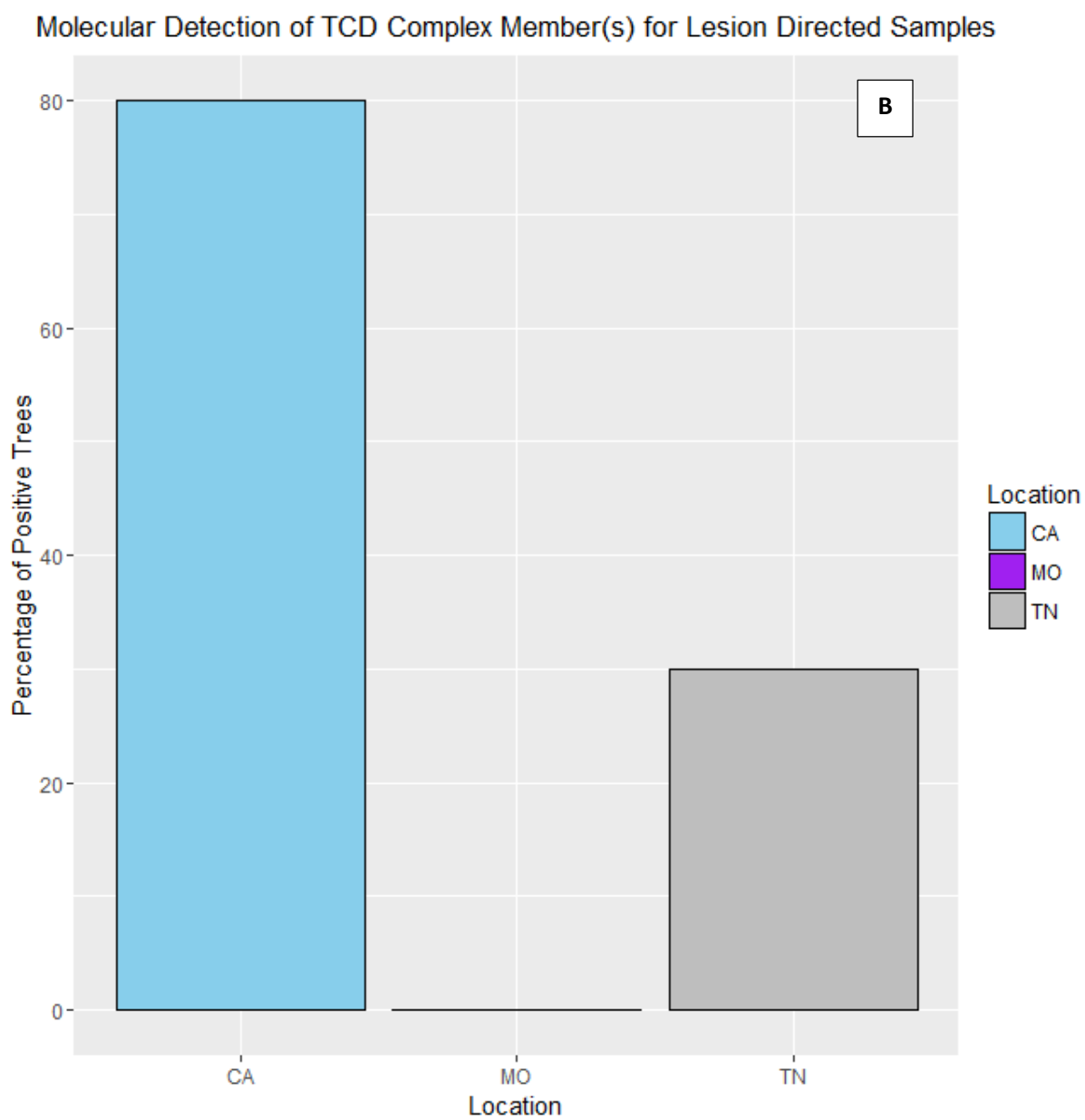


Figure 15. Continued.

Molecular Detection of TCD Complex Member(s) for Feature Directed Samples

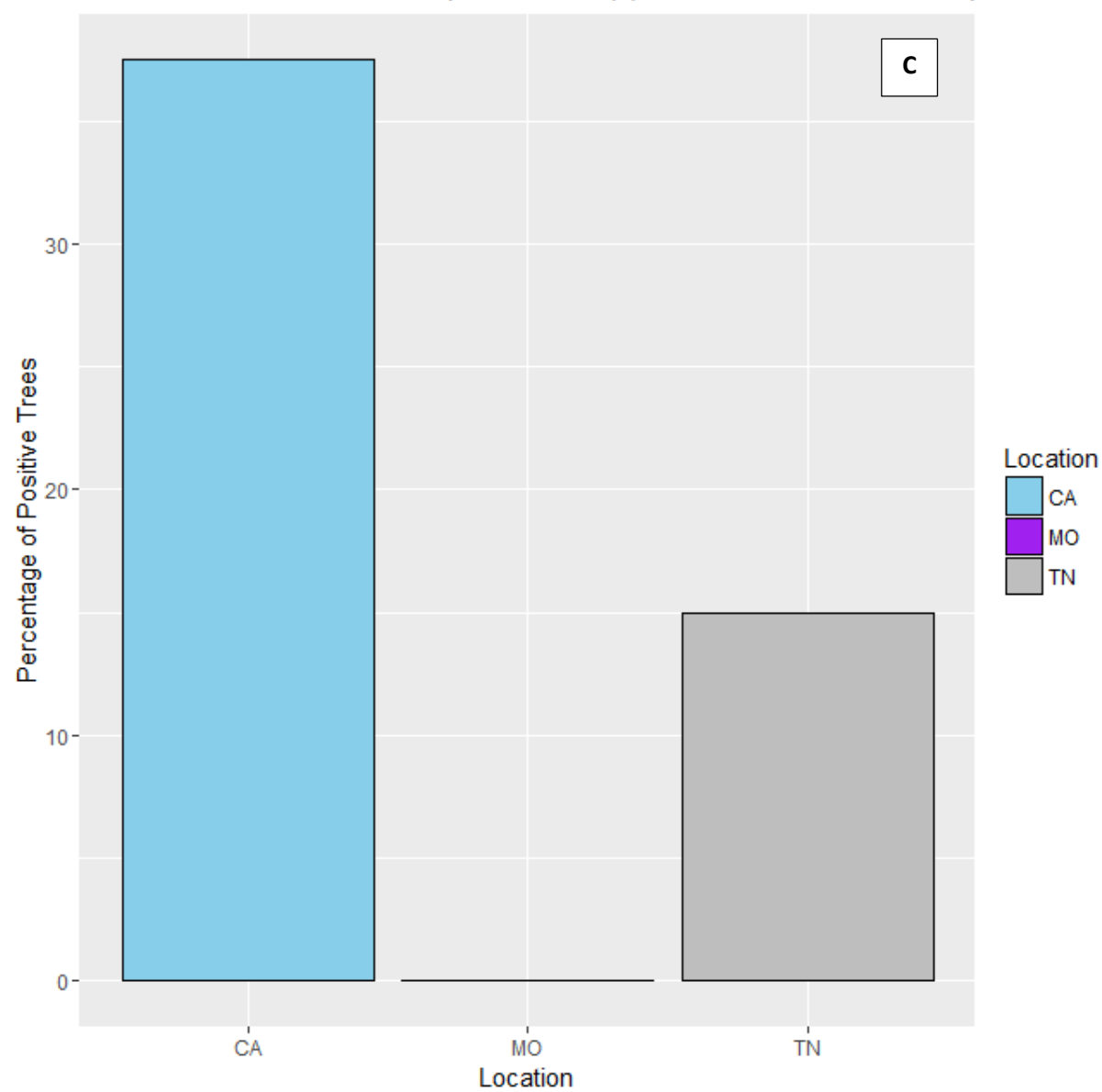


Figure 15. Continued.



Figure 16. *Geosmithia morbida* induced canker areas under *Juglans* spp. bark. Elliptical cankers in the phloem caused by *G. morbida* are visible only after the outer bark is removed. Image provided by Dr. William Klingeman.

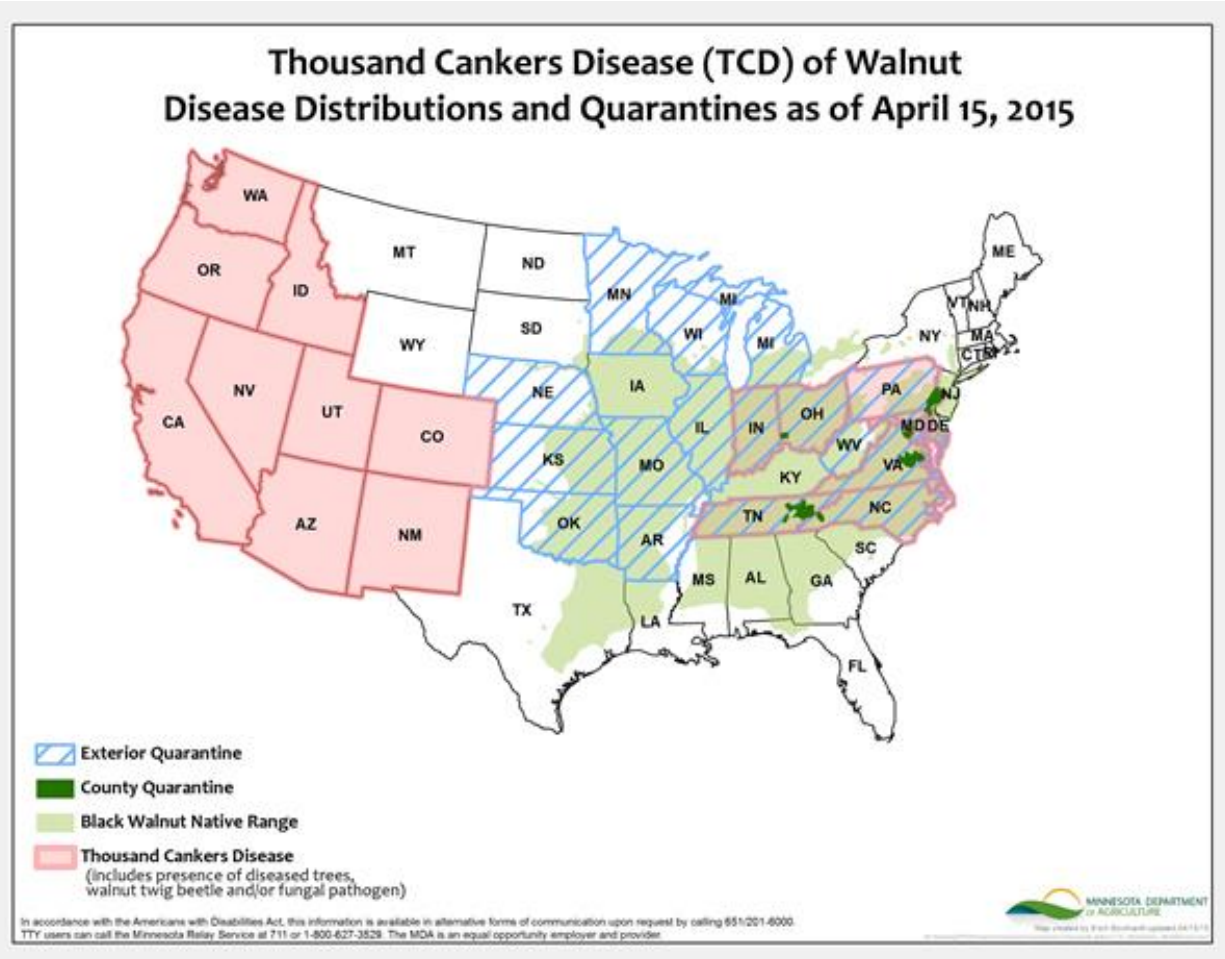


Figure 17. Thousand Cankers Disease distributions and quarantines areas as of April 15, 2015. Map courtesy Minnesota Department of Agriculture.

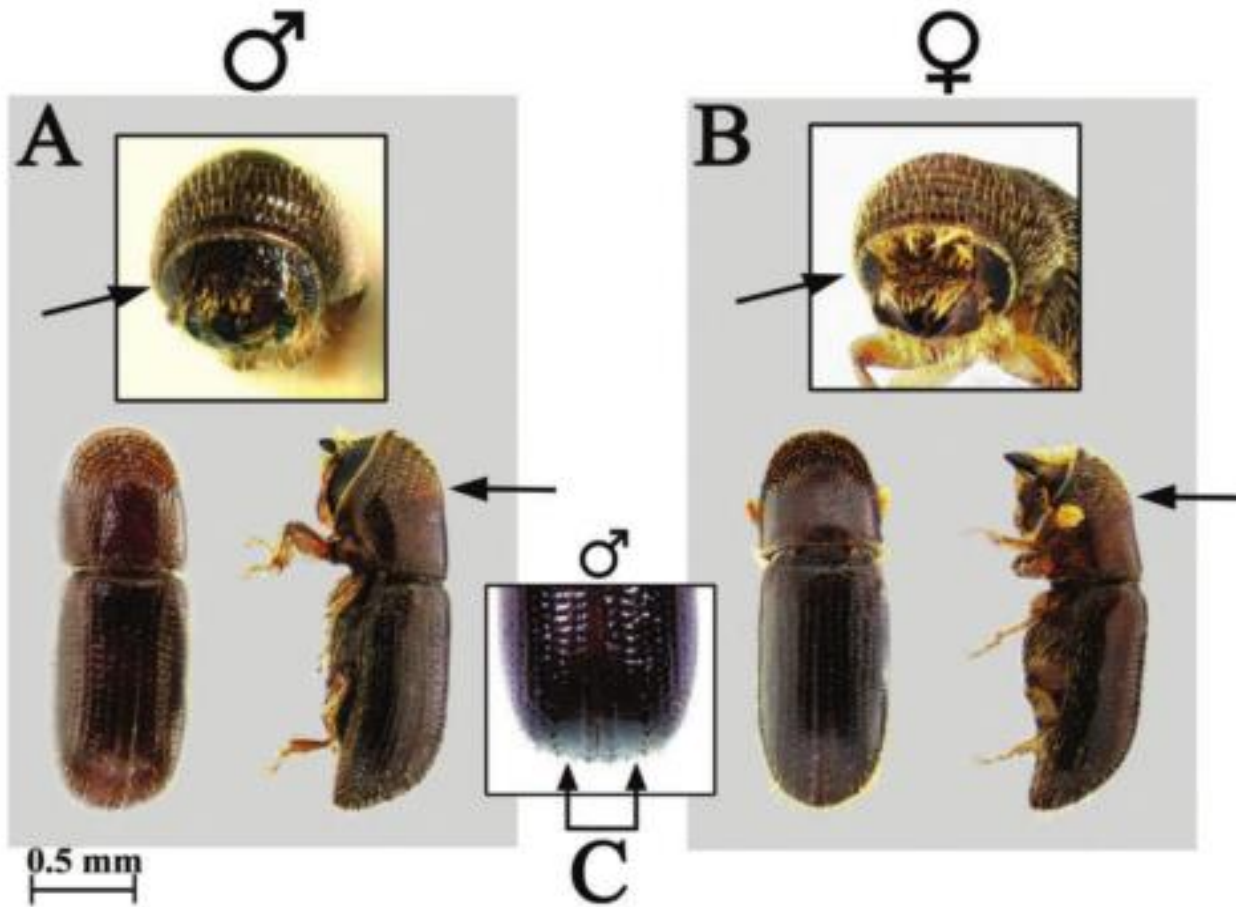


Figure 18. *Pityophthorus juglandis*. Comparison of morphological characters of male (A) and female (B) WTB. Arrows indicate the degree of pubescence on the male and female frons; the apex, which occurs before the midpoint on the anterior half of the pronotum of males and females; and granules on the male elytral declivity (C). Image provided by S. M Hishinuma and A. D. Graves. Images from Seybold et al. 2013.

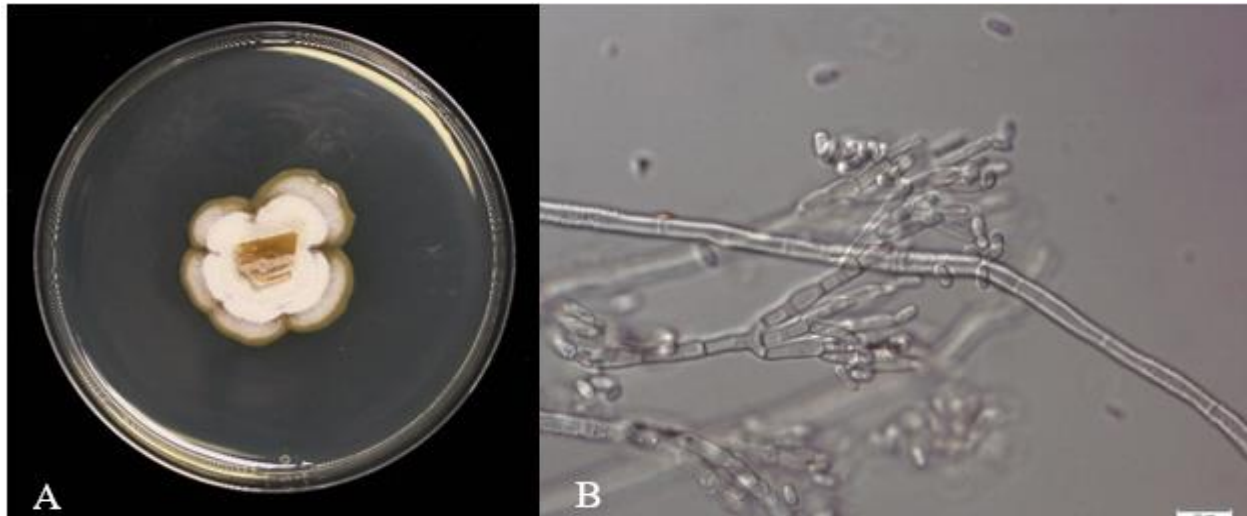


Figure 19. *Geosmithia morbida*. Four weeks old colonies of *G. morbida* grown on half strength potato dextrose agar (PDA) (A); conidia and conidiophore (B). Images provided by Dr. Romina Gazis (A), Dr. Denita Hadziabdic (B).



Figure 20. Placing the trap about 2.5 to 4.5 m from the main stem of suspect walnut tree, 1.5 to 3 m from the live branches of that tree's crown and hang the trap on a 3 m pole. Image provided by Dr. Denita Hadziabdic.

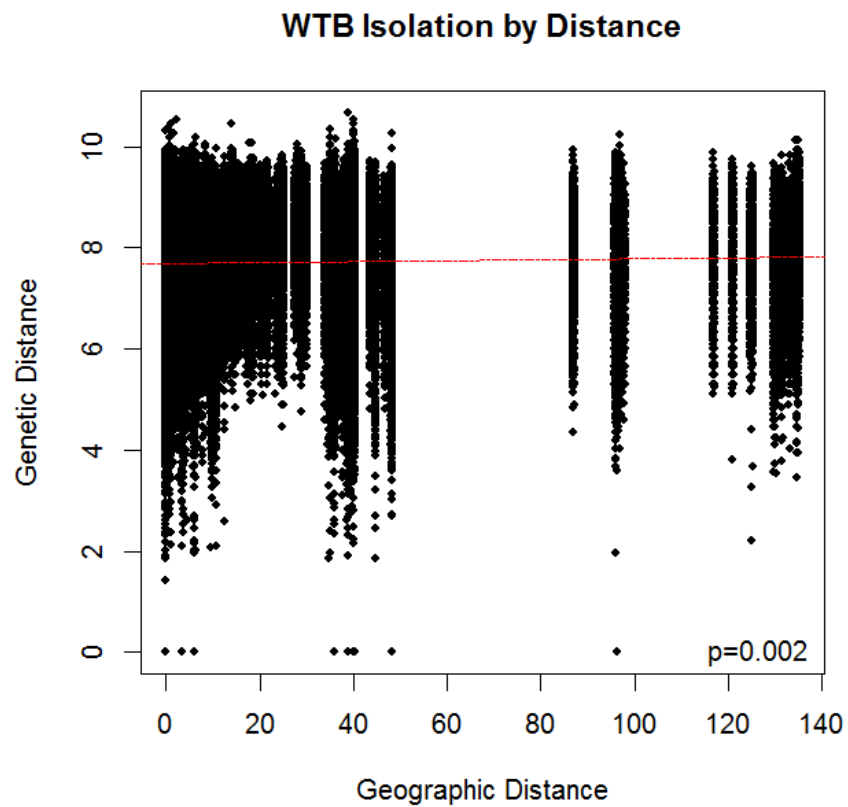


Figure 21. Isolation by distance plot across 40 subpopulations of *Pityophthorus juglandis*. Correlation between genetic and geographic distance was evaluated using a Mantel test with 1,000 permutations.

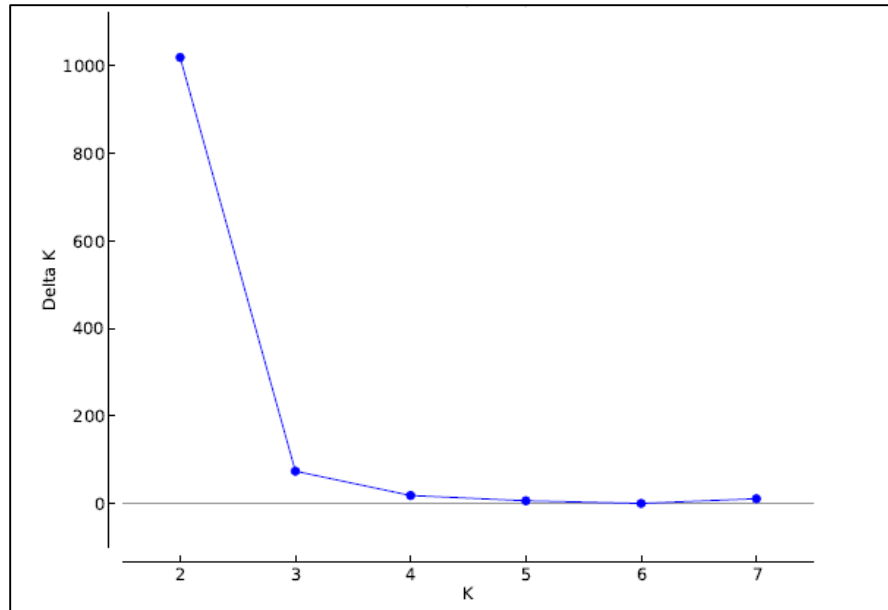


Figure 22. Plot of Delta K, generated by program STURCURE. Maximum ΔK at $K = 2$ estimated using Evanno's method for 839 *Pityophthorus juglandis* individuals across twelve microsatellite loci.

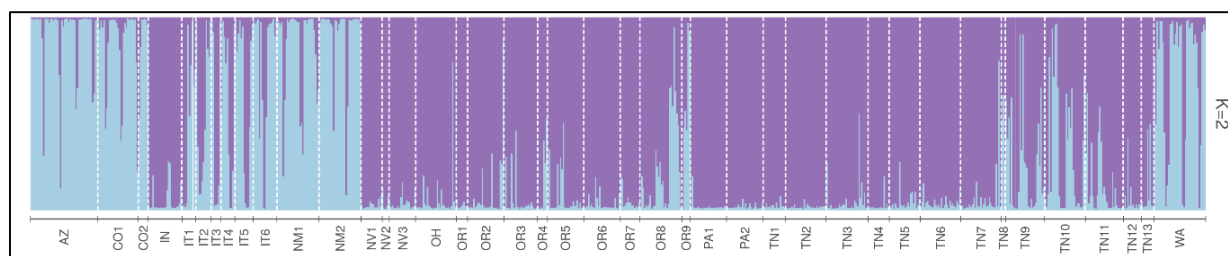


Figure 23. Structure bar graph representing two genetic clusters of 839 *Pityophthorus juglandis* individuals from four geographic regions (northwestern, southwestern, and eastern U.S., and Italy) using twelve microsatellite loci.

Figure 24. The STRUCTURE results revealed two distinct clusters among *Pityophthorus juglandis* populations representing 40 subpopulations, southwestern U.S. as one cluster and northwestern, eastern U.S. with Italy subpopulations as the second cluster (A). The model-based clustering results produced by TESS3 indicated the presence of three genetic clusters based on the lowest DIC value (B). Above map shows U.S. subpopulations, below map shows Italy subpopulations.

A

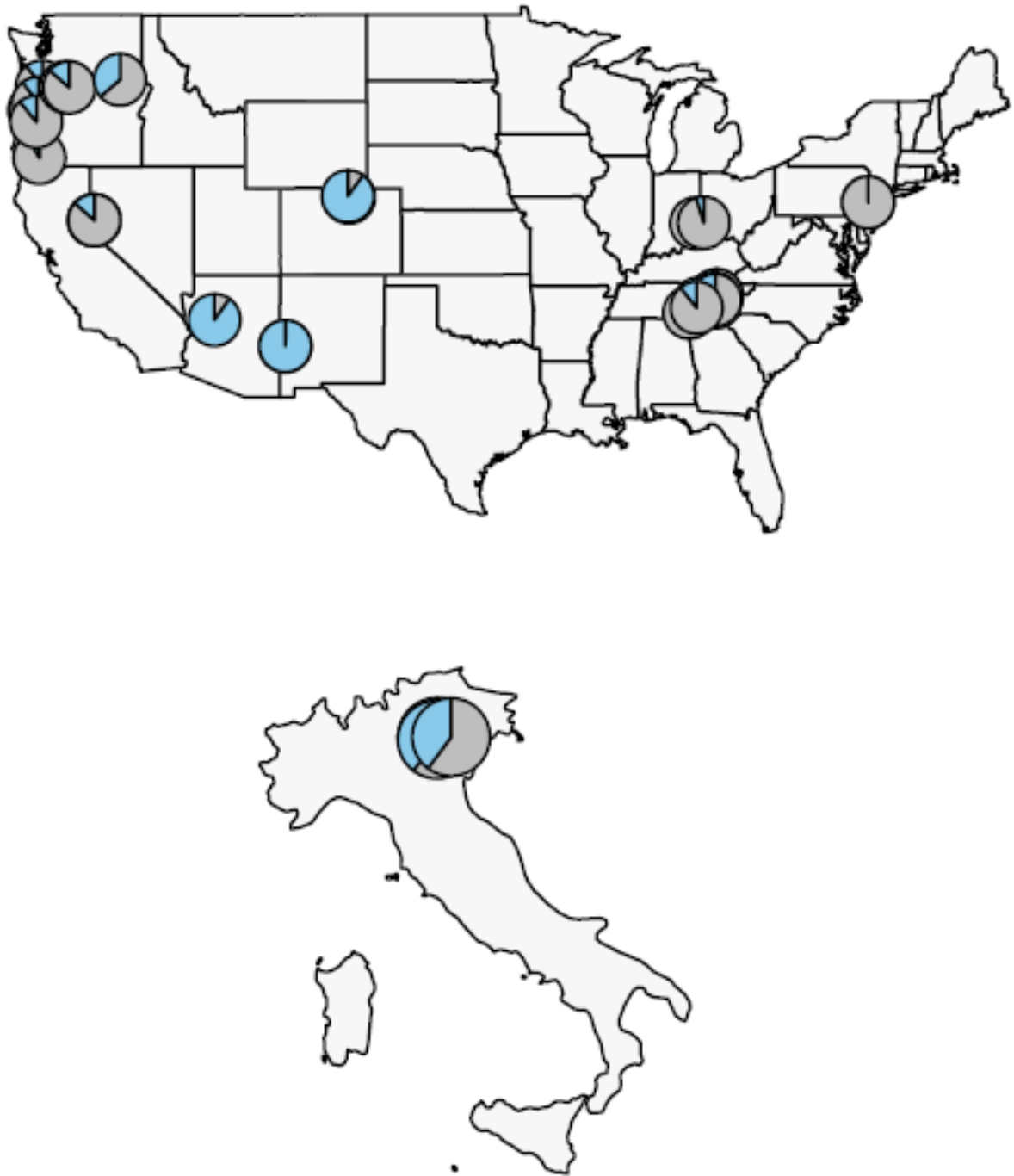


Figure 24. Continued.

B

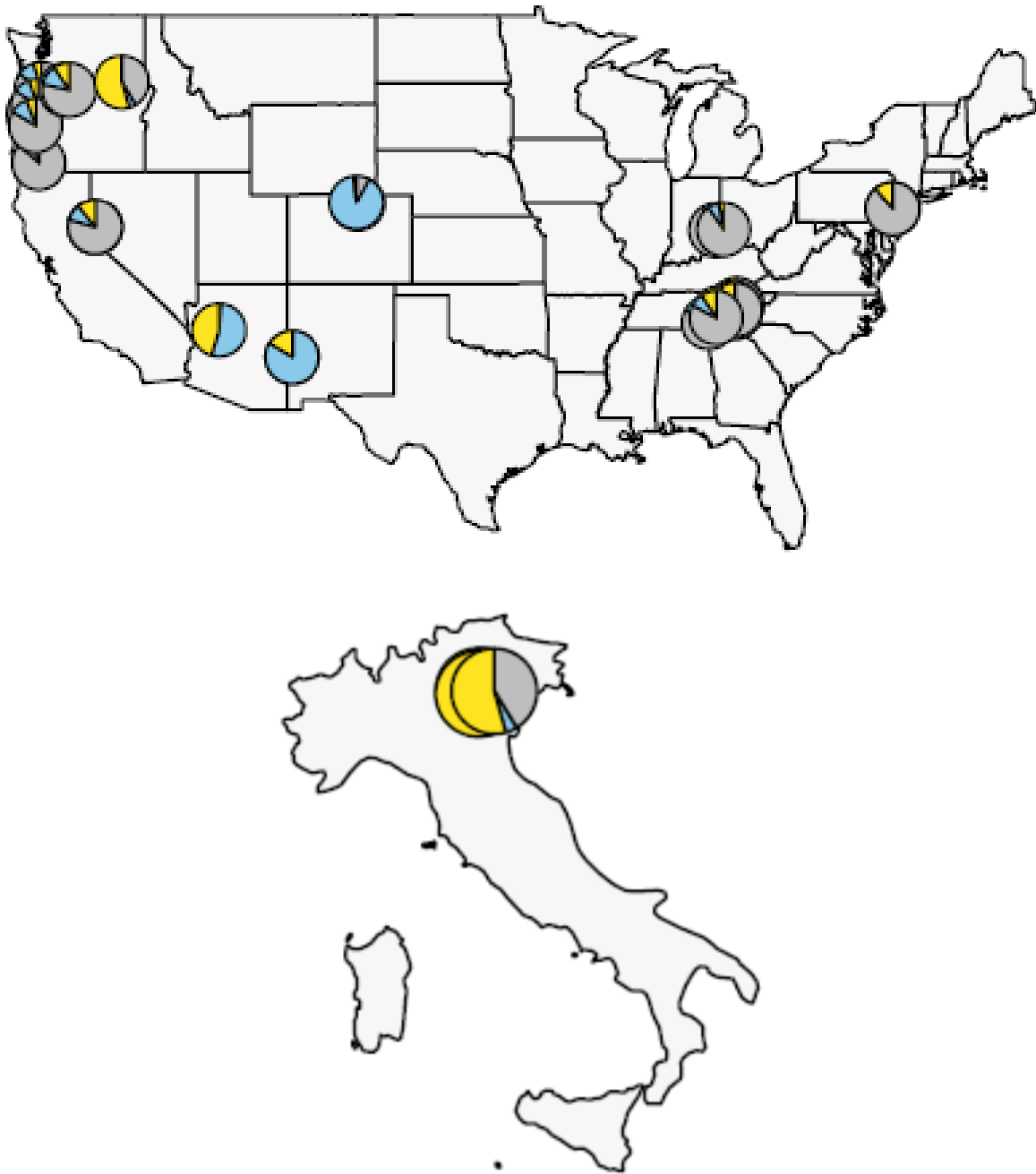


Figure 24. Continued.

Vita

Emel Oren was born in Mugla, Turkey, to the parents of Selma and Feyzullah. She attended Emirbeyazit Primary School and continued to Mugla Anatolian High School in Mugla, Turkey. After graduation, she headed to Aydin, Adnan Menderes University where she was introduced to Agricultural Engineering program, with a major in plant protection. After completing her undergraduate degree, she received a fellowship for a Master of Science in the United States. She studied at the English Language Institute in University of Delaware, Newark, Delaware. Afterwards, she started her Master's with Dr. Denita Hadziabdic at the University of Tennessee, Knoxville. During her Master's education, she placed 2nd in student paper competition and student poster competition for the Tennessee Entomological Society and Southern Chapter of International Society of Arboriculture, respectively. Emel graduated with a Master of Science degree in Entomology and Plant Pathology in December, 2016.