THESIS

INTRASPECIFIC VARIABILITY OF *GEOSMITHIA MORBIDA* THE CAUSAL AGENT OF THOUSAND CANKERS DISEASE, AND EFFECTS OF TEMPERATURE, ISOLATE AND HOST FAMILY (*JUGLANS NIGRA*) ON CANKER DEVELOPMENT

Submitted by

Emily Freeland

Department of Bioagricultural Sciences and Pest Management

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2012

Master's Committee:

Advisor: Ned Tisserat

Jan Leach
Whitney Cranshaw
James Klett

ABSTRACT

INTRASPECIFIC VARIABILITY OF *GEOSMITHIA MORBIDA*, THE CAUSAL AGENT OF THOUSAND CANKERS DISEASE, AND EFFECTS OF TEMPERATURE, ISOLATE AND HOST FAMILY (*JUGLANS NIGRA*) ON CANKER DEVELOPMENT

Since the mid-1990's widespread mortality of Black Walnut (*Juglans nigra*) in the western United States has been noted. This mortality is the result of aggressive feeding of the Walnut twig beetle (*Pityophthorus juglandis)* and subsequent canker development caused by the newly named fungus *Geosmithia morbida*. Thousand Canker Disease (TCD) has been confirmed in Oregon, Washington, Idaho, Utah, California, Colorado, New Mexico, Arizona and recently in the native range of *J. nigra*, in Tennessee, Pennsylvania, and Virginia. Intraspecific variability of isolates was determined using rDNA ITS partial sequences and partial beta tubulin sequences. Nested clade phylogeographic analysis was used to look for correlations between haplotype trees and geography of isolates collected in screenings for the disease. Patterns of restricted dispersal by distance were found for both markers and high variability was found in isolates from single locations. Indicating that the populations causing disease throughout the western United States are not the result of recent point introductions. *G. morbida* isolate and *J. nigra* family had inconsistent effects on canker development, while temperature had a consistent effect. At higher temperatures (32°C) canker development was reduced compared to 25°C. Several genetically different *G. morbida* isolates were compared and repeated differences in pathogenicity were produced.

TABLE OF CONTENTS

Chapter I. Recovery Plan For Thousand Cankers Disease of Black Walnut Caused by *Geosmithia morbida* And Vectored by the Walnut Twig Beetle (WTB) *Pityophthorus juglandis* 1

EXECUTIVE SUMMARY 2
INTRODUCTION 5
BIOLOGY AND SYMPTOMS 9
SPREAD 13
MONITORING AND DETECTION 15
RESPONSE 17
USDA PATHOGEN PERMITS 18
ECONOMIC IMPACT AND COMPENSATION 19
MITIGATION AND DISEASE MANAGEMENT 20
INFRASTRUCTURE AND EXPERTS 26
RESEARCH, EXTENTION AND EDUCATION PRIORITIES 28
TIMELINE FOR RECOVERY 30
REFERENCES 31

Chapter II. Genetic screening of *Geosmithia morbida* populations 34

INTRODUCTION 34
MATERIALS AND METHODS 37
2.1 Collection and Isolation of *Geosmithia morbida* isolates 37
2.2 DNA extraction, amplification and sequencing 37
2.3 Phylogeographic sequence analysis 38
RESULTS 39
2.4 rDNA ITS sequence analysis 39
2.5 Partial Beta tubulin sequence analysis 39
DISCUSSION 41
TABLES and FIGURES 44
REFERENCES 60

Chapter III. Effects of *Geosmithia morbida* isolate, *Juglans nigra* family and temperature on canker development 63

INTRODUCTION 63
MATERIALS AND METHODS 66
3.1 Effect of *Geosmithia morbida* haplotype on canker development in black walnut families 66
3.2 Effect of temperature on canker development 67
3.3 Effect of dormancy on canker development 68
RESULTS 69

3.4 Effect of *Geosmithia morbida* haplotype on canker development in black walnut families....69
3.5 Effect of temperature on canker development....69
3.6 Effect of dormancy on canker development....70
DISCUSSION....71
FIGURES....74
REFERENCES....80

APPENDIX I. NCPA INFERENCE KEY....82

CHAPTER I.

This recovery plan is one of several disease-specific documents produced as part of the National Plant Disease Recovery System (NPDRS) called for in Homeland Security Presidential Directive Number 9 (HSPD-9). The purpose of the NPDRS is to ensure that the tools, infrastructure, communication networks, and capacity required to minimize the impact of high consequence plant disease outbreaks are available so that a reasonable level of crop production is maintained.

Each disease-specific plan is intended to provide a brief primer on the disease, assess the status of critical recovery components, and identify disease management research, extension, and education needs. These documents are not intended to be stand-alone documents that address all of the many and varied aspects of plant disease outbreak and all of the decisions that must be made and actions taken to achieve effective response and recovery. They are, however, documents that will help the USDA to further guide efforts toward plant disease recovery.

EXECUTIVE SUMMARY

Widespread branch dieback and mortality of black walnut (*Juglans nigra*) has occurred in Colorado, Idaho, New Mexico Oregon, Utah, and Washington since the mid-1990s. Black walnut is not native to this region, but has been widely planted as an ornamental and nut tree species. Affected trees initially exhibit yellowing and wilting of the foliage followed by progressive branch dieback. Trees are killed within three to four years after initial symptoms develop. Tree mortality is the result of aggressive feeding by the walnut twig beetle, *Pityophthorus juglandis* (WTB) and subsequent canker development surrounding beetle galleries by the fungus *Geosmithia morbida*. The number of cankers formed on branches and the trunk is enormous, hence the name thousand cankers (TCD) to describe the disease. The WTB and *G. morbida* also have been identified in other walnut species including southern and northern California walnuts (*J. californica* and *J. hindsii* respectively) and Arizona walnut (*J. major*).

Walnuts in the United States have both a nut and timber economic value. Loss of the black walnut timber industry could have a large economic impact as well as significant ecological consequences. Black walnut in the eastern United States has an estimated value of over half a trillion dollars. Although Persian walnut (English walnut) appears not be as susceptible to the disease, TCD could pose a risk to some of the hybrid rootstocks. The Persian walnut crop in California was valued at over $1 billion in 2010.

In July 2010, TCD was identified in Tennessee and in the native range of black walnut. In 2011, TCD was discovered in Richmond Virginia and Bucks County, Pennsylvania. It is unlikely these outbreaks can be eradicated because there are no effective control methods developed. The economic and ecological impact of this disease could be staggering. It is

debatable whether there can be a successful ‘recovery’ plan for TCD. Our best hope is to prevent the rapid spread of the WTB by taking the following action:

- Delineate the current distribution of the WTB and *G. morbida* in North America and then prevent movement of infested logs and firewood into uninfested areas.
- Develop a national educational program on the threat of TCD
- Identify methods to sanitize infested logs so that they may be safely used for commercial purposes
- Identify potential prophylactic treatments (insecticides or fungicides) to preserve high value timber or landscape trees. Identify potential biological control agents.
- Identify potential sources of resistance in black walnut or related walnut species and consider developing a germplasm preservation program

A TCD technical working group, comprised of University research scientists, USDA Forest Service scientists and personnel, state and federal regulatory officials, and representatives of the Walnut Council and the forest lumber industry, convened in 2009 to discuss a national framework for dealing with this disease. The TCD working group is continuing to develop effective management strategies.

Contributors and Reviewers:

Recovery Plan For

Thousand Cankers of Black Walnut

Caused by

Geosmithia morbida

And Vectored by the

Walnut Twig Beetle (WTB) *Pityophthorus juglandis*

Contributors

- Colorado State University (Ned Tisserat, Emily Freeland and Whitney Cranshaw)

Reviewers:

Reviewed by the members of the Thousand Canker Disease Technical Working Group and the American Phytopathological Society.

INTRODUCTION

Black walnut (*Juglans nigra*) is one of the most highly valued timber species in North America (Harlow & Harrar, 1969). The wood is prized for use in cabinetry, gunstocks and other finished wood products. The nuts are also an important nutritional source for wildlife and as a food condiment. Black walnut is native to eastern North America, and is widely distributed on deep alluvial soils from New England and the Appalachian Mountains west to the Great Plains and from the Canadian border south into Texas and the Florida panhandle (Harlow & Harrar, 1969). It has been widely planted outside its native range in the western United States as an ornamental and timber tree, and for nut production.

Widespread morbidity and mortality of black walnut was first observed in the Wasatch Mountains of Utah and areas of the Columbia Gorge and Willamette Valley in Oregon as early as the 1990's. At that time mortality was not linked to a specific cause. The first published report of extensive dieback and death of black walnut was in New Mexico in 2001 (USDA Forest Service 2002). Mortality was reported to be associated with drought conditions and noted the presence of walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman, (Coleoptera Curculionidae, Scolytinae). By 2003 widespread black walnut mortality was observed in several Colorado municipalities and by 2008 the cause was determined to be the result of aggressive feeding by the WTB and subsequent canker development surrounding beetle galleries caused by a fungal symbiont of the beetle (Tisserat et al. 2009). The number of cankers formed on branches and the trunk is immense, hence the name thousand cankers (TCD) to describe the disease. The fungus was recently named *Geosmithia morbida* (Kolařík et al. 2011). Since the disease was first described, both the WTB and *G. morbida* have been collected from various *Juglans* species in counties in California, Oregon, Washington, Idaho, Utah, Arizona, New Mexico and Colorado

(Figure 1). Extensive mortality of black walnut has occurred wherever TCD has been found (Tisserat *et al.* 2011).

In July 2010, TCD was confirmed from dying and dead black walnuts in Knoxville, TN. The size of this infestation is currently being determined, but hundreds of walnuts with TCD have been identified in at least four TN counties. In 2011, TCD was discovered in Richmond, VA and Bucks County, PA. The extent of these outbreaks is still unknown. TCD now is apparently well-established in the native range of black walnut and poses a serious threat to this species.

The origin of TCD remains unclear. The WTB was first collected in 1896 in New Mexico (Blackman, 1928). In 1992 its range was reported as Arizona, New Mexico and Chihuahua, Mexico (Wood, 1992). This coincides with the northern native range of the Arizona walnut (*J. major*). In 2008 and 2009, *G. morbida* was consistently isolated from necrotic phloem surrounding WTB galleries in native stands of Arizona walnut in AZ and NM, but the fungus was not causing branch dieback or mortality in this species. This has led to speculation that WTB and its fungal symbiont *G. morbida* are native to the southwestern United States.

Other walnut species are also hosts of the WTB. The beetle was collected from southern California walnut (*J. californica*) at two cites in Los Angeles County in 1959 (Bright & Stark, 1973) but curiously was not found, or at least not reported on this species, until recently, from other sites in California. However, since 2008 the WTB has been widely associated with and collected from declining southern California walnuts in the foothills and mountains just north of the Los Angeles Basin (Seybold et al. 2010). Similarly, the WTB has recently been associated with widespread dieback of northern California walnut (*J. hindsii*) in the lower Sacramento Valley as well as in riparian zones along the tributaries to the Sacramento River. It has also been

found in northern California walnuts in isolated locations in central and northern California. *Geosmithia morbida* is consistently isolated from the small cankers surrounding galleries on trees at all of these locations. Because the first collection records in California pre-date those from Mexico and because of the native distributions of walnut in California, Seybold et al. (2010) have speculated that the WTB may be native to at least the southern California walnut (Seybold et al. 2010). However, the beetle has remained relatively inconspicuous in the collection record in California for approximately 50 years, and only recently has been associated with the dieback of California walnut trees.

California also has large commercial nut plantings of the non-native English walnut (*J. regia*). The WTB and *G. morbida* have been found on scattered trees in orchards, but they currently are not causing significant damage or mortality (approximately 30 cases documented so far). English walnut appears to be moderately too highly resistant to TCD, but most of the commercial trees are now grafted onto a walnut hybrid root stock called Paradox. Paradox is commonly defined as a hybrid between *J. hindsii* and *J. regia* but the black walnut component of the hybrid can include any black walnut (*J. nigra, J. californica* or *J. hindsii*) (Potter, Gao, Baggett, McKenna, & McGranahan, 2002). The genetic diversity in the rootstocks can therefore be very high and the resistance to TCD possibly variable. As a result, there have been observations of some Paradox rootstocks with symptoms of TCD.

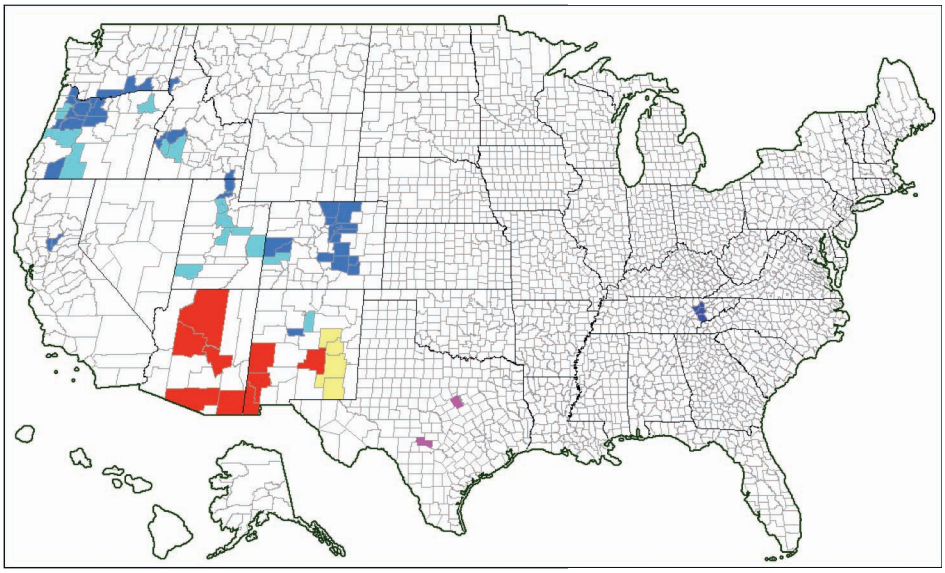

Fig 1. Distribution of the walnut twig beetle and *Geosmithia morbida* on *Juglans* species (excluding the native *J. hindsii* and *J. californica* in California) in the United States as of October, 2010. Counties highlighted in dark blue represent those in which *G. morbida* has been isolated from symptomatic trees. Counties highlighted in light blue represent those where symptoms of thousand cankers disease were observed or where the walnut twig beetle (WTB) was found but where isolation of *G. morbida* was not attempted because trees were removed before samples could be collected. Some of the trees sampled for confirmation of *G. morbida* in Oregon are hybrids of *J. nigra* with *Juglans hindsii*. Counties in red respresent those in which the WTB and fungus was recovered from *J. major*. The WTB was not observed in isolated pockets of *J. major* in the two Texas counties highlighted in pink or in *J. microcarpa* in New Mexico counties highlighted in yellow.

BIOLOGY AND SYMPTOMS

The WTB is a minute (1.5-2.2 mm) yellowish-brown bark beetle, about 3X as long as it is wide. It is the only *Pityophthorus* species associated with *Juglans* in the western U.S. but can be readily distinguished from other members of the genus by several physical features (Figures 2 and 3). Among these are 4 to 6 concentric rows of asperities on the prothorax, usually broken and overlapping at the median line. The declivity at the end of the wing covers is steep, very shallowly bisulcate, and generally flattened at the apex with small granules.

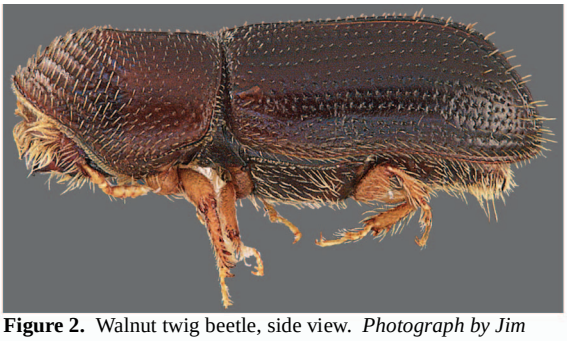

Figure 2. Walnut twig beetle, side view. *Photograph by Jim LaBonte, Oregon Department of Agriculture.*

Figure 3. Walnut twig beetle, top view. *Photograph by Jim LaBonte. Oregon Department of Agriculture.*

Despite its small size and common name, WTB rarely develops in twigs. Instead tunneling is almost always confined to branches greater than 2 cm diameter. Very large branches and even the trunk can be colonized during advanced stages of TCD.

The life history of the WTB is not completely understood. In areas with cold winters such as Colorado, overwintering is thought to be spent primarily in the adult stage, but some larval development also occurs. Overwintering may also occur in larval stages, particularly where winters are mild. Adults resume activity by late-April – beginning as early as February in California. Most fly to branches to mate within a chamber under the bark, and females initiate egg gallery tunnels (Figure 4). Larvae feed for 4-6 weeks under the bark in meandering tunnels

that run perpendicular to the egg gallery (Figure 5). Pupation occurs at the end of the tunnel and the adults emerge through minute, round exit holes.

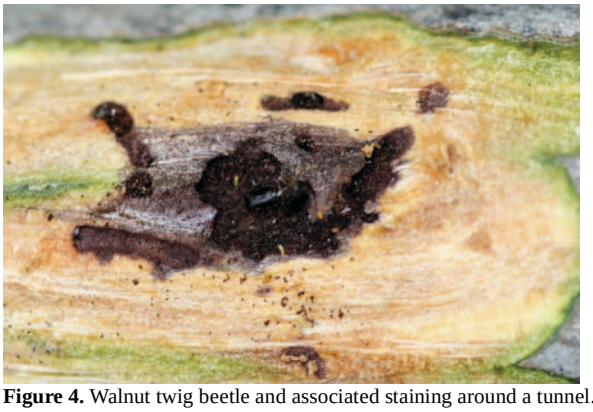

Figure 4. Walnut twig beetle and associated staining around a tunnel.

Figure 5. Walnut twig beetle tunneling under the bark of a large branch.

Adults emerge to produce a second generation in early summer. Peak flight activity of adults occurs from mid-July through late August and declines by early fall as the beetles enter hibernation sites. A small number of beetles produced from eggs laid late in the season may not complete development until November and some larval development may continue throughout winter, particularly in warmer areas. In warmer climates a third generation may be possible.

Geosmithia spp. (Ascomycota, Hypocreales) are dry-spored fungi that are almost exclusively found in association with bark beetles and subcortical insects (Kolařík et al., 2004). Although these fungi are generally found sporulating within beetle galleries on both hardwood and coniferous species, they produce hydrophobic conidia common to air borne fungi (Kolařík et al, 2007). The genus contains seven previously described species as well as at least nine operational taxonomic units (OTUs) that are newly described species (Kolařík et al., 2005, Kolařík et al, 2007, 2009). Based on a comparison of the ITS region, as well as morphological observations, *G. morbida* from *Juglans* species do not fit any previously described species. Morphological identification and ITS region (ITS1-5.8S-ITS2) rDNA sequences have confirmed

that this is a new species, subsequently named *G. morbida* (Kolařík et al., 2011).

The new species of *Geosmithia* causing cankers on black walnuts is the first in this genus to be pathogenic (Čížková, et al., 2005). Although *G. morbida* is the only known *Geosmithia* species to be directly pathogenic, an unknown *Geosmithia* sp. was recovered from an elm tree that was infected with dutch elm disease (DED). The isolate produced cerato-ulmin (CU). CU is a protein produced by *Ophiostoma* fungi that cause DED and is thought to be a pathogenicity factor and key for isolate fitness (Scala et al., 2007). Neither the CU protein or the gene has been isolated from any other *Geosmithia* spp. isolates leading to the hypothesis that horizontal gene transfer is responsible for the CU production. Commonly, *Geosmithia* species sporulate on the surface of bark where both wind and water could be effective means of transmission of the fungi. However, these *Geosmithia* spp. are found in complex communities that could not be maintained by simple random dispersal by environmental factors. This suggests an insect vector is necessary for effective transmission (Kolařík et al., 2007).

Initially infected walnut trees show symptoms in the upper crown with a yellowing and thinning of leaves leading to twig and branch die-back. Progressively larger branches die and eventually the tree is killed. The total length of time for TCD to kill a mature walnut tree (i.e. from initial beetle infestation to tree death) is not known, but trees generally die within four years after symptoms develop.

Galleries of *P. juglandis* are only visible after the outer bark is removed. They are surrounded by diffuse brown to black cankers that extend beyond beetle galleries in the phloem and only in late stages into the cambium. Infection with *G. morbida* is not systemic, as cankers remain localized to the areas within approximately 4 cm beyond beetle galleries. Although symptoms initially present themselves in small diameter branches of the upper crown, removal of

the outer bark reveals galleries and cankers in twigs, branches and the trunk and are not confined to small diameter branches or weakened branches typical of twig beetle attack (Figs 6-9). After beetle colonization, numerous cankers occur every 2 to 5 cm at each gallery or entrance hole. The cankers eventually coalesce, leading to girdling of limbs (Tisserat et al., 2009).

Fig 6. Large, oval shaped cankers in bark. Walnut twig beetle tunnels are present in the center of each canker.

Fig 7. Shallow tunnels produced by the walnut twig beetle can usually be seen in the center of cankers.

Fig 8. Outer bark removed to expose coalescing cankers in phloem that eventually girdle branches.

Fig. 9. Bark completely removed to show discoloration of sapwood during advanced stages of the disease. Note the white, dusty appearance of *Geosmithia morbida* at the canker margin

SPREAD

The greatest threat of movement of the WTB into the native range of black walnut is from the sales and movement of raw logs that harbor infective beetles. Black walnut wood is highly prized for both lumber and veneer logs. This wood is often sold on small scale by woodworkers for musical instruments, gun stocks, woodturners and furniture. Black walnut standing wood prices range from $200 to 4,000 per thousand board feet and $500 to 6,000 per thousand board feet depending on quality and suitability for veneer wood to mills in Illinois (ILDNR, 2008). These values are representative of prices throughout the United States (Hoover, 1995). As well as intact logs, burls also have a high value for veneer wood and are shipped to east coast mills from the west with intact bark on a regular basis (Newton et al., 2009). These long distance human mediated events could potentially introduce the TCD complex into the native range of black walnut from which it could spread by natural dispersal. The movement of trees from the western to eastern United States by individuals is not well documented but internet searches show many instances of walnut logs for sale to individuals (Newton et al., 2009).

Although black walnut wood is a high value timber, many logs are also used for firewood when they are not suitable for woodworking or mill wood. The risk of movement of WTB infested firewood long distances from the western United States to the eastern range of black walnut is probably low because firewood is generally not shipped and sold over great distances (Newton et al., 2009). However, campers are known to move firewood over several states which could lead to introduction of the WTB into campgrounds containing walnut trees. A small survey of campgrounds by the Kansas Department of Agriculture found that campers had brought firewood in from states as far as California, indicating that introduction of WTB by firewood is possible (Kansas Department Forestry, 2007). The risk of moving the WTB in

infested wood from areas where TCD is established is very high. For example, the WTB has been found in black walnut firewood sold along the front range of Colorado (W. Jacobi, personal communication). Firewood movement also poses a significant threat in areas of Tennessee.

The risk of introduction of the WTB or *G. morbida* into new areas by movement of nuts is low. *G. morbida* is not systemic, it is not a seed borne pathogen and the WTB doesn't feed on the nuts. The possibility of moving the WTB in pallets is low because walnut wood is valuable and not likely used in the manufacturing of pallets. Another possible pathway for interstate movement is movement of infected nursery stock. More research on the suitability of young trees as hosts for the WTB is needed.

The estimated longest flight distance of the WTB is 2 miles, limiting the distance of natural dispersal of the TCD complex (Newton et al., 2009). Although the Great Plains could provide a natural barrier between areas of native black walnut forests and areas of confirmed TCD, several bridges may be present. The WTB is small and may be carried longer distances by weather events and possibly to susceptible hosts. Long distance travel between wide spread hosts is not likely based solely on insect dispersal. However, spread along riparian areas such as the South Platte and Arkansas rivers is a potential concern and these areas should be monitored. As well as spread on black walnuts, the range of little walnut (*J. microcarpa*) overlaps with the native range of black walnut and may provide a natural bridge for infected WTB. However, all of these concerns may be moot now that the WTB has become established in Tennessee.

MONITORING AND DETECTION

Survey and monitoring of TCD has primarily been coordinated at the state level with the assistance, in some cases, of federal agencies. Surveys were conducted in late summer and fall of 2010 in Tennessee and were primarily targeted in Knox and adjacent counties looking for evidence of branch dieback or mortality associated with TCD. Further surveys are critical to ascertain the distribution of the disease in Tennessee for management and regulatory decisions. This is necessary to limit the spread through movement of diseased wood as well as natural pathways.

A detailed survey of black walnut trees in eastern Colorado (W. Cranshaw, unpublished) and western Kansas (Kansas Department of Agriculture) was conducted in 2009. A survey of the distribution of the WTB on several walnut species in California has been conducted. However, there is no current coordinated survey plan to identify the distribution of TCD on black walnut outside the native range of this species

The WTB and *G. morbida* were widely collected on Arizona walnut in Arizona and New Mexico during surveys in 2009 and 2010 (Cranshaw and Leatherman, personal communication). No decline or mortality was observed on infested trees. The farthest east that the beetle and fungus were found on this species was in Lincoln County, NM. TCD was not observed in little walnut (*J. microcarpa*) in a survey of Eastern New Mexico in 2011 (Leatherman, personal communication) and in eastern New Mexico and Texas in 2011 (A.D. Graves, personal communication). Additional surveys of Arizona and little walnut in their extreme eastern ranges (Texas).

The WTB is not attracted to ethanol-baited, or colored sticky traps. Therefore efforts to monitor insect movement have been unsuccessful. Continued research is needed to better

develop trapping methods to effectively monitor the WTB's presence. Development of pheromone traps would allow monitoring in locations along the western front of the native range of black walnuts and in Tennessee.

A Standard Operating Procedure (SOP) for NPDN diagnostic labs is under development (Snover-Clift, 2010). The SOP contains information on isolation techniques for *G. morbida* and characteristics for morphological identification. Molecular confirmation of *G. morbida* is performed by sequencing the rDNA ITS region by PCR.

RESPONSE

A TCD technical working group comprised of University research scientists, USDA Forest Service Personnel, state and federal regulatory officials, and representatives of the Walnut Council and the forest lumber industry convened in 2009 to discuss a national framework for dealing with this disease. The TCD working group is continuing to develop management strategies.

In July 2010, prior to the discovery of the WTB in Tennessee, USDA APHIS PPQ decided not to impose a federal quarantine on TCD. Therefore, individual states are responsible for establishing their own quarantines. Many states including Kansas, Nebraska, Iowa, Minnesota, and Missouri have imposed external quarantines to prevent movement of black walnut wood from states known to have TCD from entering their state. This would include Colorado and all states to the west of Colorado, and Tennessee. Tennessee has also imposed an internal quarantine designed to slow the spread of WTB between counties and maintain marketability of Tennessee walnut products. Of great concern is the impact of quarantines, internal and external, on the marketability and commercial value of black walnut in Tennessee.

USDA PATHOGEN PERMITS

Plant Protection Act of 2000 (codified at 7 CFR Part 330) regulates registration and permit requirements for UDSA, APHIS and PPQ permits. A PPQ 526 Permit is required for laboratories receiving suspect infected plant materials and or plant pests from out-of-state regardless of the quarantine status of the suspect organism. A PPQ 526 permit is required for interstate movement and importation of infected plant material, vectors, pure cultures and diagnostic samples. Permit information can be found at the PPQ permit website (http://www.aphis.usda.gov/ppq/permits/, the PPQ permit services (301-734-0841 or 866-524-5421) or by email (Pest.Permits@aphis.usda.gov).

ECONOMIC IMPACT AND COMPENSATION

Loss of the black walnut timber industry could have a large economic impact as well as significant ecological consequences. Black walnut in the eastern United States has an estimated value of over half a trillion dollars (Newton et al., 2009). The impact of TCD on the walnut timber industry in Kansas alone showed losses of over $9.5 million annually to landowners, loggers and mills, as well as indirect losses to the economy and 46 jobs. The nut industry in Kansas is worth approximately $600 thousand a year. A third loss to the state is the cost to remove infected urban and park trees and their replacement which is valued at over $65 million for the state (Treiman et al., 2010). Other states in the native range of black walnut may experience similar economic losses if TCD becomes established in these areas.

Walnuts in the United States have both a nut and timber economic value. California walnuts were valued at over $527 million in 2008 (USDA-NASS, 2009). English walnut and the Paradox root stocks most commercially grown walnuts are grown on may or may not be susceptible. If TCD infects these walnut species, the results could be devastating and potentially eliminate the industry in California. The value of black walnut wood is estimated at over $41 million and is exported to over 67 countries world wide.

MITIGATION AND DISEASE MANAGEMENT

Exclusion

The response and mitigation of TCD is dependent on the walnut species affected. On black walnut, there are no known effective control measures once TCD becomes established. Thus, exclusion should be considered the only means to prevent introduction of the WTB and subsequent development of TCD. Surveys to delineate the current distribution of TCD as well as regulations to control the movement of WTB-infested walnut logs to non-infested areas are the highest priority. Mitigation efforts may slow, but probably not stop, TCD progression in black walnut, but may have more positive effects in other walnut species.

Surveys

Since exclusion of TCD from a community or forest is the only means to effectively manage this disease, it is critical to determine the current distribution of TCD. Exclusion is no longer an option where TCD is already present. Nevertheless, if the distribution of the disease can be determined, and then contained, then the ultimate course of TCD may be limited to the destruction of walnut trees within communities where the disease has already become established.

An intensive visual survey should continue in Tennessee to determine the current extent of the outbreak in that state. Trees suspected of TCD should be confirmed by identifying the WTB and associated cankers caused by *G. morbida*. Verification of TCD by culturing is not necessary if the WTB is present except in the case of new county or state records since it can be assumed that all WTB are infested with *G. morbida*.

Surveys should be conducted throughout the native range of black walnut. The most likely source of introduction of the WTB is on logs and firewood. This is likely how the beetle was introduced into the Knoxville, Tennessee area. Therefore, surveys should initially be focused in urban areas and near sawmills where satellite infections are most likely to occur. These are locations where early detection might be easier.

Surveys of nearby forests adjacent to TCD outbreaks should be conducted annually to delimit infestation. Examinations may begin with landowners or other non-professionals and escalate to site visits by foresters or others trained to take tree samples if the situation dictates. When infested trees are removed, periodic monitoring of nearby forests is warranted.

Surveys should also intensify in the Western United States. The WTB continues to spread in Colorado, Idaho, Oregon, California and Washington, yet there are no formal surveys being conducted in many of these states.

Education

An intensive education program is needed to inform arborists, foresters, and tree owners on the identification of TCD symptoms. It is also critical that education materials be developed to stress the importance of proper handling of infested wood to prevent spread to new locations. If the disease is widespread and *Juglans* plantings occur throughout the municipality, there is little chance to stop the course of the disease by tree removals. Education and proper handling of TCD-infective wood should be the primary emphasis.

Sanitation and Eradication

Freshly cut wood is highly attractive to the WTB and can support its development.

Successful larval development will require wood of sufficient moisture, and drying ultimately will make wood an unsuitable habitat. Because of the small size of the beetles, development may continue in small pockets within logs. Where drying is slow, logs may remain suitable for breeding for 2 or 3 years after felling.

Preemptive harvesting of black walnut in the forest or plantations is not recommended except to contain a known infestation. Removal of infested and potentially infested black walnut within the stand or on the same property is advised. Sanitation harvests, partial harvests or clear-cuts are appropriate methods of treatment. Walnut tree tops should be chipped and left in the woods or burned. Walnut logs should be separated from other logs when loaded for hauling. Walnut logs or products with bark intact in a quarantined county or buffer county should not be taken outside the quarantine area.

In an urban situation, aggressive sanitation may have a role in management of TCD. This is largely due to two factors: 1) the long lag time between tree infestation and TCD symptom expression that allow for undetected local spread; and 2) the consistent association of the pathogen with essentially all bark beetles. Because of this, once TCD has become established in a city, eradication is unlikely without clear cut although some slowing of spread may be realized. The removal and destruction of WTB-infested wood can potentially reduce the accidental spread to other cities.

Chipping diseased trees will hasten bark drying and beetle destruction. However not all WTB are killed by normal chipping therefore chipped infested wood should also be handled with care. During warm periods, active beetles potentially may be dispersed from cut wood as it is moved from the site. Therefore, care should be given in routing trucks hauling TCD-infected wood to avoid areas of healthy, uninfected walnut.

Because of the very high value of black walnut logs, salvage often will be attempted. If logs cut from TCD-infected trees are recovered, they should be handled to prevent beetle dispersal until the wood no longer supports walnut twig beetle development. Until sufficiently dried, logs should be isolated. Isolation can be achieved by stockpiling wood in a site that is distant from healthy walnuts, particularly walnuts located downwind. Storage of logs in buildings can achieve beetle containment. Tarping logs with clear plastic also may contain beetles within logs. Tarping to achieve solarization may be a means to kill developing beetles.

Protection of trees with insecticides

Trunk/branch insecticide sprays (e.g., permethrin, bifenthrin) typically used for bark beetle control do not appear to be effective in preventing TCD, although detailed experiments have not been performed. The large number of WTB present over an extended period (May-September) and the large areas of the tree that may be attacked are all significant impediments to effective coverage. It is possible that late summer trunk sprays directed at beetles seeking overwintering shelter in the trunk may be useful in reducing populations. This may have some value in slowing TCD development and spread; however, this has not been demonstrated.

The value of soil applied systemic insecticides in TCD management has not been formally evaluated. Limited observations indicate that imidacloprid (i.e., Merit, Marathon, Touchstone, etc.) is ineffective after symptoms have developed. Anecdotal accounts suggest that disease progress may be slowed by imidacloprid if applications are made before extensive cankers have been formed; however, it is unlikely that currently available systemic insecticides can prevent TCD. Successful inoculations of *G. morbida* likely can occur even if the walnut twig beetle is subsequently killed. Cankers resulting from infection will produce pockets within the

tree where future movement of systemic insecticides will be limited and allow successful development of twig beetles at these sites. Areas under the bark where beetles will be protected from systemic insecticides will increase with time as cankers expand and new cankers are initiated. The more water soluble insecticide dinotefuran (Safari) may provide improved coverage; however, it has not been evaluated.

Pesticide label use restrictions will be an important limitation to the use of insecticides and fungicides in TCD managment. Any pesticide (insecticide, fungicide) considered in TCD management must comply with use restrictions for walnuts grown for nut-crops. At present, there are food tolerances for imidacloprid in walnut meat since this insecticide (Provado formulation) is used in commercial nut production. Dinotefuran currently has no established tolerance in walnut meat and no formulations are labeled for this crop.

Resistance

There currently are no known sources of resistance to TCD in black walnut. Although surviving black walnut trees in TCD-affected regions in Colorado have been identified, they have not been evaluated for resistance to *G. morbida* or the WTB. There is also no coordinated effort to identify and preserve these putatively resistant trees. An efficient screening method for resistance needs to be developed. Some other walnut species appear to have moderate to high levels of resistance to *G. morbida* and may be useful in future breeding programs.

Germplasm preservation

There are several plantings for walnut germplasm preservation and development. These include facilities at the University of Missouri (primarily for nut germplasm), the USDA - Forest

Service, HTIRC facility at Purdue University (primarily for black walnut and butternut timber germplasm), the National Center for Germplasm Resources at UC Davis (NCGR facility primarily for *Juglans* species other than black walnut), and the Improving Perennial Plants for Food and Bioenergy (IPPFB) in Richmond, Utah (primarily black walnut collections from the western United States). TCD has been found in the NCGR and IPPFB plantings and will be difficult, if not impossible to eradicate at these locations. The best course of action may be to let the disease run its course and identify resistance in surviving trees. TCD has not been observed in trees at the University of Missouri or HTIRC in Indiana. These plantings contain improved timber and nut genotypes. Every effort should be made to protect and preserve the germplasm in these plantings. Unfortunately, these resources are located within the native range of black walnut, and could potentially be impacted by TCD in the future. It may be feasible to graft scion material from these important germplasm resources and relocate them to a region outside the native ranges of all North American species of walnuts and isolated from any infestation of the WTB. This would require committed long-term funding and a coordinated effort among various organizations throughout the nation.

There are current efforts to store walnut germplasm by cryopreservation of scion material (Mark Coggeshall, Univ. Missouri, personal communication). This research is in its infancy and will be expensive and labor intensive on a large scale.

Nut collection and storage may provide some short-term germplasm preservation. Nuts can be stored for at least four years without significant loss in germination (Williams, 1971). This may not be a viable long-term strategy.

INFRASTRUCTURE AND EXPERTS

Vector

- Whitney Cranshaw, Colorado State University (Whitney.Cranshaw@colostate.edu)
- Steven Seybold, USDA Forest Service, PSW Stateion Davis,CA (sjseybold@gmail.com)
- Andrew Graves, USDA Forest Service, Albuquerque, NM (adgraves@fs.fed.us)
- Dave Leatherman, Colorado State Forest Service (retired) (daleatherman@msn.com)
- Sky Stevens, Colorado State Forest Service (Sky.Stevens@colostate.edu)

Pathogen

- Ned Tisserat, Colorado State University (Ned.Tisserat@colostate.edu)
- William Jacobi, Colorado State University (William.Jacobi@colostate.edu)
- Miroslav Kolarik, Institute of Microbiology CAS, Czech Republic <miroslavkolarik@seznam.cz>

Other

- Keith Woeste – USDA Forest Service , Purdue (woeste@purdue.edu)
- Mark Coggeshall, University of Missouri (CoggeshallM@missouri.edu)
- Scott Schlarbaum, University of Tennessee (tenntip@utk.edu)
- Jim McKenna, USDA Forest Service, Purdue (jrmckenn@purdue.edu)
- Tim Ford, Improving Perennial Plants for Food and Bioenergy (TCFORD3@aol.com)
- Bruce Moltzan, USDA Forest Service, (bruce.moltzan@gmail.com)

Thousand Canker working team

- **Michael Brown,** APHIS-PPQ, Missouri State Plant Health Director
- **Phil Cannon,** FHP Regional Forest Pathologist, Pacific Southwest Region R5
- **Mark Coggeshall,** Research Analyst/Tree Improvement Specialist, University of Missouri
- **Whitney Cranshaw,** Professor & Ext. Specialist, Colorado State University
- **Dennis Haugen,** FHP FS NA Entomologist
- **Robert Lawrence,** Forest Entomologist**,** Missouri Department of Conservation
- **Phil Marshall,** Indiana DNR
- **Jim McKenna** USDA Forest Service Purdue
- **Jay Pscheidt,** Professor, Oregon State University
- **Steven Seybold,** Research Entomologist**,** USDA FS, Pacific Southwest Research Station
- **Eric Smith,** FHTET
- **Carla Thomas,** NPDN
- **Sharon Dobish,** GPDN, 4606 Throckmorton, Manhattan, KS 66506-5502
- **Ned Tisserat,** Professor & Ext. Specialist, Colorado State University
- **Jerry VanSambeek,** USDA Forest Service
- **Colin Wamsley,** Missouri Dept. of Agriculture, P. O. Box 630, Jefferson City, MO 65102-0630
- **Yun W,** FHTET

RESEARCH, EXTENSION AND EDUCATION PRIORITIES

Research Priorities

Research is needed in many areas of this new disease including aspects of the WTB, *G. morbida* and the *Juglans* host and interactions between all three. The following key priorities have been identified as research goals.

- Investigate the life history and physiology of the WTB: number of generations per year, flight times, host range, tolerance to temperature extremes and temperature requirements for development.
- Isolate and identify WTB aggregation pheromones for monitoring behavior and trapping WTB. This includes identifying behaviorally active compounds, synthesizing these compounds and testing them in field and laboratory trials.
- Develop genetic markers for screening *G. morbida* populations throughout the western United States and Mexico to determine the origin and population structure of the pathogen and assist in identifying strains differing in pathogenicity or other features key to development of the disease.
- Develop genetic markers to determine the population structure and origin of WTB, especially in non-native plantings of *Juglans* species.
- Identify long-term methods for germplasm preservation. This may include establishment of new germplasm orchards outside TCD-affected areas, cryopreservation techniques, or long term storage of nuts
- Determine the host range for the WTB. Observe WTB colonization of various hosts in areas where many species are planted such as the National Clonal Germplasm Repository in Winters CA as well as field observations where multiple hosts occur.

- Determine means of disinfestation of wood products to permit salvaging diseased wood for safe transport throughout the United States.
- Investigate control measures such as chemical controls (fungicides and insecticides), biological controls, and measures to eradicate the complex from newly infected areas.
- Determine the host range of *G. morbida* and screen susceptible hosts for possible individual resistance for breeding purposes. Initial screening can be done in the greenhouse but this work should be followed by field testing.
- Determine the epidemiology of the disease including a timeline of disease progression and factors such as effects of stress level on disease transmission

Education and Extension Priorities

- Expansion of training for early detectors of TCD to include: master gardeners, foresters, extension specialists, walnut producers and arborists.
- Preparation of training materials for identification and diagnostic procedures to include: WTB identification, *G. morbida* culture procedures and identification criteria.
- Preparation and distribution of educational materials for woodworkers and members of the timber industry.
- Develop Best Management Practices (BMP) for both urban and forested landscapes.

TIMELINE FOR RECOVERY

Recovery is unlikely to be accomplished in one year.

Disease resiliency factors: pathogen and vector are present on much of the range of the host, management of vector and pathogen are presently poorly understood, and we do not even know the present range of the vector or pathogen.

The current distribution of the WTB and *G. morbida* in North America needs to be delineated and then movement of infested logs and firewood needs to be prevented from infesting non infested areas. A national educational program on the threat of TCD needs to be developed and implemented. Methods need to be identified to sanitize infested logs so that they may be safely used for commercial purposes and prophylactic treatments (insecticides or fungicides) to preserve high value timber or landscape trees needs development. Management methods need to be developed for the biological control of WTB and resistance in black walnut or related walnut species to *G. morbida*. These projected approaches will take several years to implement in order to achieve a reasonable level of our former production of black walnut.

REFERENCES

Blackman, M. (1928). The genus *Pityophthorus* Eichh. in North America: A revisional study of the Pityophthori, with descriptions of two new genera and seventy-one new species. (Technical, pp. 3-6). Syracuse, NY. New York State College of Forestry, Syracuse Univ.

Bright, D., & Stark, R. (1973). The Bark and Ambrosia Beetles of California (Scolytidae and Platypodidae). (Volume 16.). University of California, Berkley, Bulletin of the California Insect Survey.

Čížková, D., Šrůtka, P., Kolařík, M., Kubátová, A., & Pažoutová, S. (2005). Assessing the pathogenic effect of *Fusarium, Geosmithia* and *Ophiostroma* fungi from broad-leaved trees. Biological Control 50(1): 59-62.

Cranshaw, W. (2011). Recently recognized range extensions of the walnut twig beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae), in the Western United States. The Coleopterist's Bulletin 65: 48-49.

Harlow, W. and Harrar, E. (1969) Textbook of Dendrology. 5th ed. McGraw-Hill. 511 pp.

Hoover, W. L. (1995). Predicting Black Walnut Log Prices. West Lafayette, IN.

ILDNR. (2008). Illinois timber prices. Illinois Department of Natural Resources, Urbana.

KDF. (2007). Firewood survey methods and results. Kansas Department of Forestry, Manhattan.

Kolařík, M., Freeland, E., Utley, C. and Tisserat, N. (2011). *Geosmithia morbida* sp. nov., a new phytopathogenic species living in symbiosis with the walnut twig beetle (*Pityophthorus juglandis*) on *Juglans* in the USA. Mycologia 103: 325–332.

Kolařík, M., Kostovčík, M., & Pažoutová, S. (2007). Host range and diversity of the genus *Geosmithia* (Ascomycota: Hypocreales) living in association with bark beetles in the Mediterranean area. Mycological Research 111(11): 1298-1310. doi: 10.1016/j.mycres.2007.06.010.

Kolařík, M., Kubátová, A., Cepicka, I., Pazoutova, S., & Srutka, P. (2005). A complex of three new white-spored, sympatric, and host range limited *Geosmithia* species. Mycological Research 109(12): 1323-1336. doi: 10.1017/S0953756205003965.

Kolařík, M., Kubátová, A., Hulcr, J., & Pažoutová, S. (2007). *Geosmithia* fungi are highly diverse and consistent bark beetle associates: Evidence from their community structure in temperate Europe. Microbial Ecology 55(1):65-80. doi: 10.1007/s00248-007-9251-0.

Kolařík, M., Kubátová, A., Pažoutová, S., & Srutka, P. (2004). Morphological and molecular characterisation of *Geosmithia putterillii*, *G. pallida* comb. nov. and *G. flava* sp. nov., associated with subcorticolous insects. Mycological Research 108(9): 1053-1069. doi: 10.1017/S0953756204000796.

Kolařík, M., Stodulková, E., Kvresinová, Z., Kuzma, M., Sulc, M., Man, P., et al. (2009). Hydroxylated anthraquinones produced by *Geosmithia* species. Folia Microbiologica 54(3): 179–187.

Newton, L. P., Fowler, G., Neeley, A. D., Schall, R. A., & Takeuchi, Y. (2009). Pathway Assessment: *Geosmithia* sp. and *Pityopthorus juglandis* Blackman movement from the western into the eastern United States. Distribution. http://tn.gov/agriculture/publications/regulatory/tc_pathwayanalysis.pdf

Potter, D., Gao, F., Baggett, S., McKenna, J. R., & McGranahan, G. H. (2002). Defining the sources of Paradox: DNA sequence markers for the North American walnut (*Juglans* L.) species and hybrids. Scientia Horticulturae 94:157-170.

Scala, A., Comparini, C., Tegli, S., & Scala, F. (2007). A non-ophiostoma fungus expresses the gene encoding the hydrophin cerato-ulmin. Journal of Plant Pathology 89(582):233-240.

Seybold, S. J., D. Haugen, J. O'Brien, and A. Graves. (2010). Pest Alert: Thousand Cankers Disease. USDA Forest Service, Northeastern Area, State and Private Forestry. Rev. August 2010.

Tisserat, N., Cranshaw, W., Leatherman, D., Utley, C., & Alexander, K. (2009). Black Walnut mortality in Colorado caused by the Walnut Twig Beetle and Thousand Cankers Disease. Plant Health Progress, (August). doi: 10.1094/PHP-2009-0811-01-RS.

Tisserat N., Cranshaw W., Putnam, M., Pscheidt, J. Leslie, C.A., Murray, M., Hoffman, J., Barkely, Y., Alexander, K., and Seybold, S.J. (2011). Thousand cankers disease is widespread on black walnut, *Juglans nigra*, in the western United States. Online. *Plant Health Progr*. (June 30, 2011) doi:10.1094/PHP-2009-0811-01-RS.

Treiman, T., Atchison, B., McDonnell, T., Barden, C., & Moser, W. K. (2010). Economic Loss Associated with the Introduction of Thousand Cankers Disease of Black Walnut to Kansas. Kansas.

USDA-NASS. (2009). 2009 California Walnut Objective Measurement Report. Office. California.

Wood, S. A. (1992). A Catalog of Scolytidae and Platypodidae (Coleoptera), Part 2.: Taxonomic Index Volume B. (No.6., p. 1553). Great Basin Naturalist Memoirs.

USDA Forest Service 2002. Forest Insect and Disease Conditions of the Southwestern Region, (2001). USDA-FS Southwestern Region, Forestry and Forest Health Publication R3-02-01.

Williams, R. D. (1971). Stratified walnut seed still viable after four years in storage. Tree Plant. Notes 22: 1-2.

Wood S. L., and Bright D. E. 1992. A catalog of Scolytidae and Platypodidae (Coleoptera) 2. Taxonomic index. Great Basin Nat Mem 13:1–1533.

CHAPTER II. Genetic screening of *Geosmithia morbida* populations

INTRODUCTION

Over the past decade, a new fungal/ insect complex called thousand cankers disease (TCD) has threatened *Juglans spp.* throughout the western United States. Walnut trees in the West have been affected by colonization by the walnut twig beetle (WTB) (*Pityophthorus juglandis*) and the vectored fungus *Geosmithia morbida* (Kolařík et al., 2011). *G. morbida* causes cankers to form in the phloem around beetle galleries; these cankers eventually coalesce leading to tree mortality. The disease was first observed in Colorado in 2003 and has since been found to be widely distributed throughout the western United States on several *Juglans* species (Tisserat et al 2011). More recently TCD has been confirmed on black walnut (*Juglans nigra*) in areas of Virginia (VDACS 2011), Pennsylvania (Pennsylvania Dept. of Agriculture 2011) and Tennessee (Treiman et al., 2010). Black walnut is the most susceptible *Juglans* species to the disease (Utley et al. 2009) and this has led to major concern about the future survival of black walnut in its native range in the eastern United States. Black walnut is a major component of the eastern forests.

Although the fungus and the beetle appear to be endemic to the United States a conclusive link has not been established for a native range of the fungus and the cause of the apparent sudden shift to new *Juglans* hosts and localities is not clear. There are several ways the fungus could have been spread to new localities and hosts. These include; 1) a sudden large distance dispersal of the walnut twig beetle carrying the fungus during a large scale weather event; or 2) in movement of WTB infected wood long distances by humans (Rachowicz et al. 2005). Another hypothesis is that both the fungus and WTB are endemic to native walnut

species (Arizona and northern and southern California walnuts) in the Western United States and that the current epidemic may be attributed to environmental changes (e.g. heat or drought). In addition a change in the aggressiveness of the WTB or pathogenicity of *G. morbida* to *Juglans* spp. mpay have developed.

Determination of the genetic structure of novel as well as established pathogens allows for the implementation of more effective control strategies (McDonald 1997). Fungal pathogens, especially those without visible fruiting structures or other means of assessing spread of individuals can be difficult to determine population boundaries. Kolarik et al. (2011) suggested that the population structure of *G. morbida* was complex based on analysis of the rDNA ITS sequences. However their work was limited in the number of isolates and the geographic range from which they were collected.

The known range of TCD is continually changing as new areas of disease are discovered. Depending on location the disease seems to have progressed on different time scales and the time between initial introduction of the disease and symptoms of decline is unknown. Thus determination of the age of populations can be difficult to define. For example, the recently discovered populations in Tennessee are estimated to have been present between 10 and 20 years (Haun et al. 2010). Historical patterns underlying the current spread of populations of *G. morbida* will be critical to understanding the spread of TCD.

Linkage between microevolutionary theory and phyloenetics led to the coinage of the term intraspecific phylogeography (Avise et al. 1987). Since the introduction of the term in 1987, statistical methods for testing hypotheses about phylogeography have emerged. One such method, Nested Clade phylogenetic analysis (NCPA), correlates haplotype trees with geography and time without using a molecular clock (Templeton et al. 1995). A haplotype map is created

using the statistical parsimony method (Templeton et al. 1992) and analyzed based on the spatial distribution of the genetic variation (Templeton 1998). Since the original publication of NCPA, concerns have arisen about validation of the method, especially the apparent abundance of false positives (Beaumont and Panchal 2008; Petit 2008). However, the method continues to be modified and is extensively used to identify phylogeographic patterns(Templeton 2009, 2002, 2010).

The purpose of the current study is to determine the amount of intraspecific variability in *G. morbida* of using rDNA ITS and BT sequences. The intraspecific haplotype data can be used to look for patterns with geography using Nested Clade Phylogeographic Analysis (NCPA).

METHODS AND MATERIALS

2.1 Collection and isolation of *G.morbida* isolates

Branches or trunk sections with WTB galleries and associated cankers were collected from *Juglans* species in one of 35 locations in nine states (Tables 2.1 and Figure 2.1). In some locations more than one isolate was collected, and in one case (Wheat Ridge, CO) multiple isolates (from different cankers) were collected from one tree.

Small bark chips approximately 5-10 mm long and 3-5 mm wide were removed from canker margins and placed directly on quarter strength potato dextrose amended with 100mg/L streptomycin sulfate and 100 mg/l chloramphenicol (1/4 PDA++). Single spore isolates were transferred to half strength PDA and placed at 4°C for short term storage or were immersed in both 10% and 15% glycerol at -80 °C for long term storage.

2.2 DNA extraction, amplification and sequencing

Isolates were grown for 7-10 days in yeast extract broth and the mycelium was harvested as described by Tisserat et al. 2009. DNA was extracted with the Easy DNA kit (Invirtogen, Carlsbad CA) according to the manufactures instructions. Universal primers ITS1, ITS1F, ITS4 and ITS5 were used to amplify internal transcribed spacers 1 and 2 and the 5.8s subunit of rDNA (White et al., 1990). Beta tubulin gene sequences were amplified using primers T1 (5'-AACAT-GCGTGAGATTGTAAGT-3'), T2 (5'-TAGTGACCCTTGGCCCAGTTG-3') and T22 (5'-TCTGGATGT- TGTTGGGAATCC-3')(O'Donnell and Cigelnik 1997). Pure Link PCR purification kit (Invitrogen Carlsbad, California) was used to purify PCR products. After purification all products were prepared with ABI BigDye® Terminator 3.1 sequencing chemistry

and sequenced with a ABI 3130xL genetic analyzer. All sequence chromas files were checked for quality in 4peaks (A. Griekspoor and Tom Groothuis, mekentosj.com) and then aligned and trimmed with ClustalW (Larkin et al. 2007). Sequences were then converted to Nexus sequential format using ALTER (Glez-Peña et al. 2010).

2.3 Phylogeographic sequence analysis

Nexus sequential formatted multiple sequence alignments were used to complete haplotype maps and phylogeographic analyses using AneCA (Clement, Posada, and Crandall 2000; Panchal 2007). The haplotype map was then analyzed using nested clade phylogeographic analysis (NCPA) and loops were resolved by hand. Geodis v 2.6 was used for geographic analysis (Panchal 2007). Significant clade distance (D_C) and nested clade distance (D_N) derived using the Dunn-Sidak correction for multiple comparisons were used to determine inferences. The inference key for NCPA created January 6th 2011 was used (Appendix 1).

RESULTS

2.4 rDNA ITS analysis

The rDNA ITS sequences of 141 *G. morbida* isolates were compared. Twelve haplotypes with nine variable positions in the 504 bp sequence including gaps were identified (Table 2.2 and 2.3). Fifty-four percent of all isolated belonged to haplotype 1 (Table2.3). This haplotype was found in all states except in AZ and NM. Two haplotypes (7 and 10) were found only on Arizona Walnut in AZ whereas haplotype 8 represented by just one isolate, was identified in CO on black walnut. The greatest diversity of haplotypes was observed in CO and the lowest diversity was found in AZ and NM on Arizona walnut. Four haplotypes were represented from the eight isolates collected from black walnut in Tennessee.

The relationship among the rDNA ITS haplotypes is shown in Figure 2.2. All haplotypes were linked to one another based on a succession of one bp mutational changes with the exception of one missing intermediate haplotype connecting haplotype 4 to haplotype 1. Haplotype 9, Clade 1-1 and 1-4 clade distances were significantly small and clade 1-4 nested clade distance was significantly small (Table 2.4). Thus the null hypothesis of no geographical association of haplotypes within the specified clades was rejected. Inferences from these significant values led to conclusions of restricted dispersal by distance for clades with significant values (Table 2.5).

2.5 Partial beta tubulin sequence analysis

Partial beta tubulin sequences of fifty-six *G. morbida* isolates collected from 26 locations in nine states were analyzed. Ten haplotypes were found with 11 variable positions in 495 bp

(Table2.7) The haplotypes were connected to one another by single mutational changes with the exception of Haplotype 9 which was connected to the other haplotypes through 5 inferred intermediates not present in this sample. Haplotypes were grouped into four one-step clades and three two-step clades (Figure 2.4). Clade distance and nested clade distances were significantly small for clade 1-2. Based on the coalescent interior clades are expected to be older than tip clades and can be compared. Interior tip comparison for clade 2-2 D_D and D_N were significantly large (Table 2.8). Clade 2-2 showed patterns of significance consistent with restricted dispersal by distance (Table 2.9).

DISCUSSION

There was substantial variation in the rDNA ITS and beta tubulin sequences among *G. morbida* isolates. Intraspecific variation showed that *G. morbida* was not the result of a genetic bottleneck or limited variation in geographically isolated populations. Average ITS rDNA variability was slightly over 1%. Of the known members in the genus *Geosmithia*, the levels of intraspecific variation range from identical ITS rDNA sequences in two species (*G. microcorthyli* and *Geosmithia* sp. 8) to up to 4.3% variation in *G. lavendula* (Kolařík et al. 2011; Kolařík and Kirkendall 2010).

Isolates of *G. morbida* collected from a single tree in Wheatridge, CO belonged to 3 ITS rDNA haplotypes and 3 BT haplotypes. Of four isolates with both loci sequenced all had different di-locus genotypes. This indicates a high level of variation, which was not expected at the outset, especially with no known *Geosmithia* teleomorph or previous evidence for recombination in this species. However, many clonal organisms can have limited recombination events that can drastically change population genetic structure (Halkett et al. 2005). For example, evidence for extensive recombination was found in *Botrytis cinerea* isolates collected from grape, even though no sexual stage has been observed (Giraud and E. Fournier 2008).

Separate analyses of ITS rDNA data and BT data revealed patterns of restricted dispersal throughout the known range of TCD. Populations from Arizona and New Mexico were visibly different than those from other states (Figure 1.3). Most noticeably, several haplotypes were found only in AZ and the most common ITS haplotype (Haplotype 1) was not found in either AZ or NM. This was confirmed with NCPA showing restricted dispersal by distance. Validation

through more markers as well as in depth sampling of individuals will be necessary to determine the patterns of dispersal and origin of *G. morbida*.

These results are based on small sample sizes collected from screenings for the presence of TCD. As more data is collected about dispersal and density of disease, better estimations of populations can be determined. Attempts to classify population boundaries, sexual structure, and amount genetic variation both within and among populations can be difficult for fungi (Giraud et al. 2008). Multilocus sequence typing (MLST) including multilocus microsatellite typing (MLMT) can be highly useful in discriminating patterns of emerging fungal diseases (Taylor 2003). This includes ancestral populations, emergence of a virulent genotype, host or pathogen population shift, as well as others. Although MLMT can be highly useful because of their power to define evolutionary relationships, they can be difficult to isolate in fungi. Unlike other methods of mulitlocus typing, MLMT loci need to be isolated for each new species. Compared to other groups, fungal microsatellites are more difficult to isolate and are less polymorphic (Dutech et al. 2007)

Dispersal patterns will also affect population structure. In the case of TCD, two methods of natural dispersal of the fungus are possible; movement by airborne spores or by the WTB. Although *Geosmithia* species commonly sporulate on the surface of bark where they could be dispersed by wind and water, they often are found in complex communities that could not be maintained by simple random dispersal by environmental factors and are insect-fungal symbioses are stable and necessary for maintaining these commuities (Kolařík et al. 2007). Furthermore, in the case of *G. morbida,* the fungus sporulates in beetle galleries that remain covered with outer bark. Therefore, the spores are less likely to be dispersed by wind or water.

This suggests an insect vector is necessary for effective transmission. Estimated flight distance of the WTB is between one and two (Newton et al. 2009) and natural spread of TCD by WTB flight may affect population structure. Long distance dispersal by movement of infected wood by humans could have more dramatic consequences to population structure and dispersal to new areas. At this time it is unclear the amount of movement caused by long distance human-mediated dispersal. However, patterns of current known dispersal of TCD occur along major transport routes and suggests that TCD movement is aided by humans (Newton et al. 2009).

TABLES AND FIGURES

Table 2.1 Host and number of *G. morbida* rDNA ITS and beta tubulin sequences determined from isolates collected from locations in nine states.

location ID	State	Host Species	ITS Sequences	BT Sequences	Latitude, Longitude	Sample area radius (km)
1	CA	*J. californica*	8	5	34.36, -119.15	16
2	CA	*J. californica, J hindsii*	10	5	38.55, -121.73	32
3	CA	*J. hindsii, Paradox*	3	0	36.07, -119.81	34
4	CA	*J. regia*	4	1	36.23, -118.8	45
5	CA	*J. californica, J. hindsii*	3	1	34.05, -118.25	35
6	CA	*J. hindsii*	2	1	38.27, -121.94	33
7	NM	*J. major*	1	1	33.55, -105.70	10
8	CA	*J. hindsii X J. nigra*	2	0	39.04, -121.69	22
9	CA	*J. hindsii*	11	1	39.09, -122.76	30
10	CA	*J. californica*	4	2	34.54, -120.03	32
11	CA	*J. hindsii*	1	0	37.93, -121.95	30
12	CA	*J. californica, J. regia*	7	3	36.75, -119.65	20
13	CA	*J. californica*	2	1	37.65, -121.91	25
14	AZ	*J. major*	4	2	32.91, -107.87	35
15	AZ	*J. major*	1	0	34.74, -112.12	10
16	NM	*J. major*	1	1	33.71, -108.75	15
17	AZ	*J. major*	6	1	34.92, -112.84	22
18	AZ	*J. major*	5	1	34.53, -112.47	22
19	OR	*J. hindsii*	1	0	45.06, -121.23	10
20	AZ	*J.major*	1	0	34.99, -111.73	10
21	AZ	*J.major*	2	1	32.07, -111.48	15
22	UT	*J. nigra*	3	3	41.92, -111.81	25
23	ID	*J. nigra*	1	1	43.61, -116.21	10
24	CO	*J. nigra*	2	1	38.92, -108.29	35
25	CO	*J. nigra*	26	15	39.87, -105.01	35
26	CO	*J. nigra*	2	0	38.83, -104.82	10
27	CO	*J. nigra*	2	1	38.05, -103.72	15
28	CO	*J. nigra*	2	0	38.43, -105.22	10
29	OR	*J. hindsii*	2	1	42.33, -122.76	20
30	OR	*Juglans hybrid*	6	4	45.66, -121.30	17

31	OR	*Juglans hybrid*	4	1	45.20, -122.77	25
32	WA	*J. nigra*	1	1	46.21, -119.76	15
33	WA	*J. hindsii*	1	0	45.88, -120.97	10
34	WA	*J. nigra*	2	0	46.06, -118.34	15
35	TN	*J. nigra*	8	1	35.96, -83.92	20

Table 2.2 The locations (state) haplotype grouping of 141 *Geosmithia morbida* isolates based on 504 bp rDNA ITS sequence. The four digit isolate numbers are followed after the decimal by their locations listed in Table 1.1 and Figure 1.1.

Hap	Northern CA	Southern CA	CO	WA/OR	AZ	NM	TN	ID/UT
1	1227.2 1249.2 1355.2 1380.2 1365.2 1424.2 1426.2 1427.8 1385.8 1484.9 1485.9 1489.9 1490.9 1491.9 1492.9 1459.11 1428.13	1263.1 1262.1 1268.1 1266.1 1264.1 1275.1 1403.3 1405.3 1410.4 1414.4 1352.5 1493.5 1348.5 1383.5 1387.6 1473.10 1481.10 1482.10 1520.12 1267.12 1270.12 1261.12 1521.12	1368.24 1359.24 1535.27 1496.28 1217.25 1224.25 1250.25 1274.25 1279.25 1298.25 1306.25 1315.25 1391.25 1417.25 1418.25 1308.25 1225.25	1534.29 1432.30 1345.30 1349.30 1384.30 1441.31 1440.31 1439.31 1236.32 1530.33 1528.34 1529.34			1525.35 1523.35 1507.35 1524.35	1223.22 1245.23
2	1228.2 1233.2 1487.9	1272.1 1370.6 1415.3	1393.25	1346.30 1533.29	1395.2		1508.35	
3			1239.25 1271.25					
4			1305.25 1311.25 1246.26 1273.25 1285.24 1316.25			1540.7 1362.14 1558.14		1309.22 1222.22
5	1229.2 1486.9	1409.4 1407.4 1476.10 1518.12 1494.5	1218.25 1318.27	1532.19	1461.21 1462.21		1509.35 1506.35	
6	1483.9	1269.1 1519.12	1350.25	1430.31			1522.35	
7					1550.17 1307.18 1412.17 1299.18 1310.18 1303.18 1234.17			
8			1247.26					

9	1321.25 1301.25 1248.25	1358.17 1388.17 1554.15 1302.18	1373.14 1557.14 1401.16
10		1411.17	
11	1402.3		
12	1497.28		

Table 2.3 rDNA ITS polymorphisms of 12 haplotypes identified from 141 *Geosmithia morbida* isolates.

Haplotype	Frequency (%)	Position of polymorphism								
		22	24	25	31	32	40	90	408	466
1	54	T	C	C	-	-	-	-	A	T
2	8	T	T	C	-	-	-	-	A	T
3	1	T	-	-	-	-	-	T	A	T
4	8	T	-	-	-	-	-	-	A	T
5	10	T	-	C	-	-	G	-	A	T
6	4	T	C	C	-	C	-	-	A	T
7	5	T	C	C	-	T	-	-	A	T
8	0.5	T	T	C	-	-	G	-	A	T
9	7	C	C	C	-	-	-	-	A	T
10	0.5	T	C	C	C	T	-	-	A	T
11	0.5	T	C	C	-	-	-	-	G	T
12	0.5	T	-	-	-	-	-	-	A	C

Table 2.4 Clade (D_c) and nested clade (D_n) distances in kilometers for rDNA ITS haplotypes and clades for 141 isolates with loops resolved in haplotype tree. Significant D_c or D_n are indicated by super scripted S (significantly small value) and L (a significantly large value). Significance is based on the Dunn-Sidak correction which accounts for multiple comparisons within the tree. Significant values indicate a geographical association.

Within Total			**Within one Step Clades**		
Clade	D_C	D_N	Haplotype	D_C	D_N
1-1	397^S	784	4	425	424
			3	0	553
			12	0	220
			I-T	425	115
1-2	721	757	1	669	699
			11	0	35
			6	1079	1020
			9	214^S	770
			I-T	162	-163
1-3	811	813	5	895	890
			2	651	696
			8	0	852
			I-T	-298	-181
1-4	27^S	457^S	7	25	26
			10	0	29
			I-T	25	-3
I-T	160	9			

Table 2.5 Inferences and conclusions based on D_c or D_n values derived from rDNA haplotype data and collection locations of 141 isolates of *Geosmithia morbida*. The inference key (Appendix 1) was used to determine conclusions of inferences of geographical patterns.

Clade	Chain of Inference	Conclusion
1-1	No significant D_C or D_N values within clade	
1-2	1-2-3-4	Restricted Dispersal by Distance in Non-sexual species
1-3	No significant D_C or D_N values within clade	
1-4	No significant D_C or D_N values within clade	
Total Cladogram	1-2-3-4	Restricted Dispersal by Distance in Non-sexual species

Table 2.6 Locations (state) and beta tubulin haplotype of 56 *Geosmithia morbida* isolates. The four-digit isolate numbers are followed after the decimal with their locations listed in Table 1.1 and Figure1.1.

Hap	Northern CA	Southern CA	CO	WA/OR	AZ	NM	TN	ID/UT
0			1248.25 1274.25					
1			1217.25					
2	1233.2	1272.1	1301.25 1239.25 1273.25 1305.25	1384.30	1302.18	1437.16		1309.22
3	1249.2	1262.1 1378.5 1476.10 1261.12			1462.21			
4						1373.14 1374.14		
5				1440.31 1236.32				
6	1227.2 1370.6 1494.9 1428.13 1228.2	1269.1 1264.1 1268.1 1267.12 1270.12 1478.10	1364.25 1285.24 1224.25 1225.25 1315.25 1298.25 1318.27 1279.25	1369.30 1346.30		1252.7	1522.35	1245.23 1222.22
7	1229.2	1407.4	1306.25	1432.30 1533.29				1223.22
8			1393.25					
9					1234.17			

Table 2.7 rDNA ITS polymorphisms of 10 haplotypes identified from beta tubulin sequences of *Geosmithia morbida* isolates.

Hap	Frequency (%)	Position of polymorphism										
		35	66	67	68	124	139	145	208	237	347	428
0	4	C	G	G	A	A	G	T	C	G	C	G
1	2	C	G	-	A	A	G	T	T	G	C	A
2	18	C	-	-	A	A	G	T	T	G	C	G
3	10	C	G	-	A	A	G	T	T	G	C	G
4	4	C	-	-	A	A	G	T	T	G	T	G
5	4	C	G	-	A	A	G	T	T	G	T	G
6	45	C	G	G	A	A	G	T	T	G	T	G
7	10	T	G	G	A	A	G	T	T	G	T	G
8	2	T	G	G	A	A	G	T	T	G	C	G
9	2	C	G	G	-	T	T	C	T	A	C	G

Table 2.8 -Clade (D_c) and nested clade (D_n)distances for beta tubulin haplotypes and clades with loops resolved in the haplotype tree. Significant D_c or D_n are indicated by super scripted S (significantly small value)and L (a significantly large value). Significance is based on the Dunn-Sidak correction which accounts for multiple comparisons within the tree. Significant values indicate a geographical association. I-T represents an interior-tip comparison.

Within total Clade			**Within Two Step Clades**			**Within One Step Clades**		
Clade	D_c	D_n	Clade	D_C	D_N	Haplotypes	D_C	D_N
2-1	0	363						
2-2	710	725	1-2	516^S	582^S	1	0	969
						2	520	562
						3	348	460
						I-T	-124	-138
			1-5	816	988	4	0	1232
						5	129	608
						I-T	129	-623
			I-T	300^L	405^L			
2-3	840	840	1-3	894	880	0	0	676
						6	896	897
						I-T	896	220
			1-4	485	680	7	463	468
						8	0	1201
						I-T	463	-733
			I-T	409	199			
I-T	162	130						

Table 2.9 – Inferences and conclusions based on D_c or D_n values derived from beta tubulin haplotype data and collection locations of isolates *of Geosmithia morbida*. The NCPA inference key (appendix 1) was used to determine conclusions.

Clade	Chain of Inference	Conclusion
1-1	No significant D_C or D_N values within clade	
1-2	No significant D_C or D_N values within clade	
1-3	No significant D_C or D_N values within clade	
1-4	No significant D_C or D_N values within clade	
1-5	No significant D_C or D_N values within clade	
1-6	No significant D_C or D_N values within clade	
2-1	No significant D_C or D_N values within clade	
2-2	1-2-3-4	Restricted Dispersal by Distance in Non-sexual species
2-3	No significant D_C or D_N values within clade	

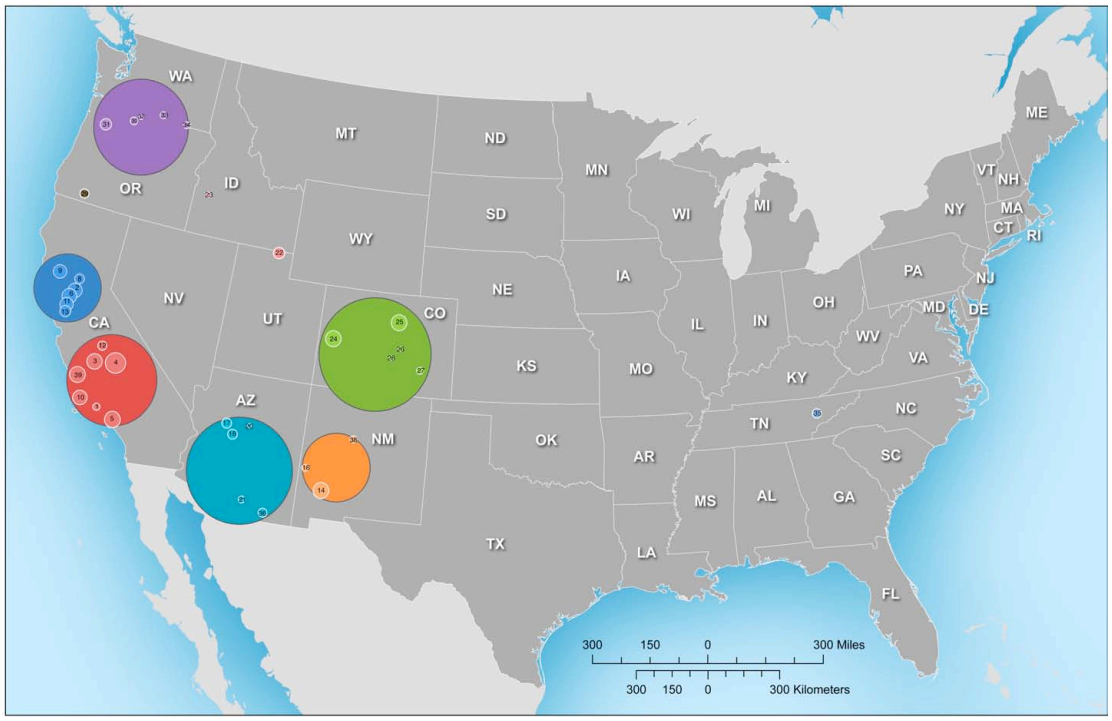

Figure 2.1 Sampling sites for *G. morbida.* Small circles represent sampling locations and were used in Nested Clade Phylogeographic Analysis and large circles represent groupings of sampling locations used for haplotype maps.

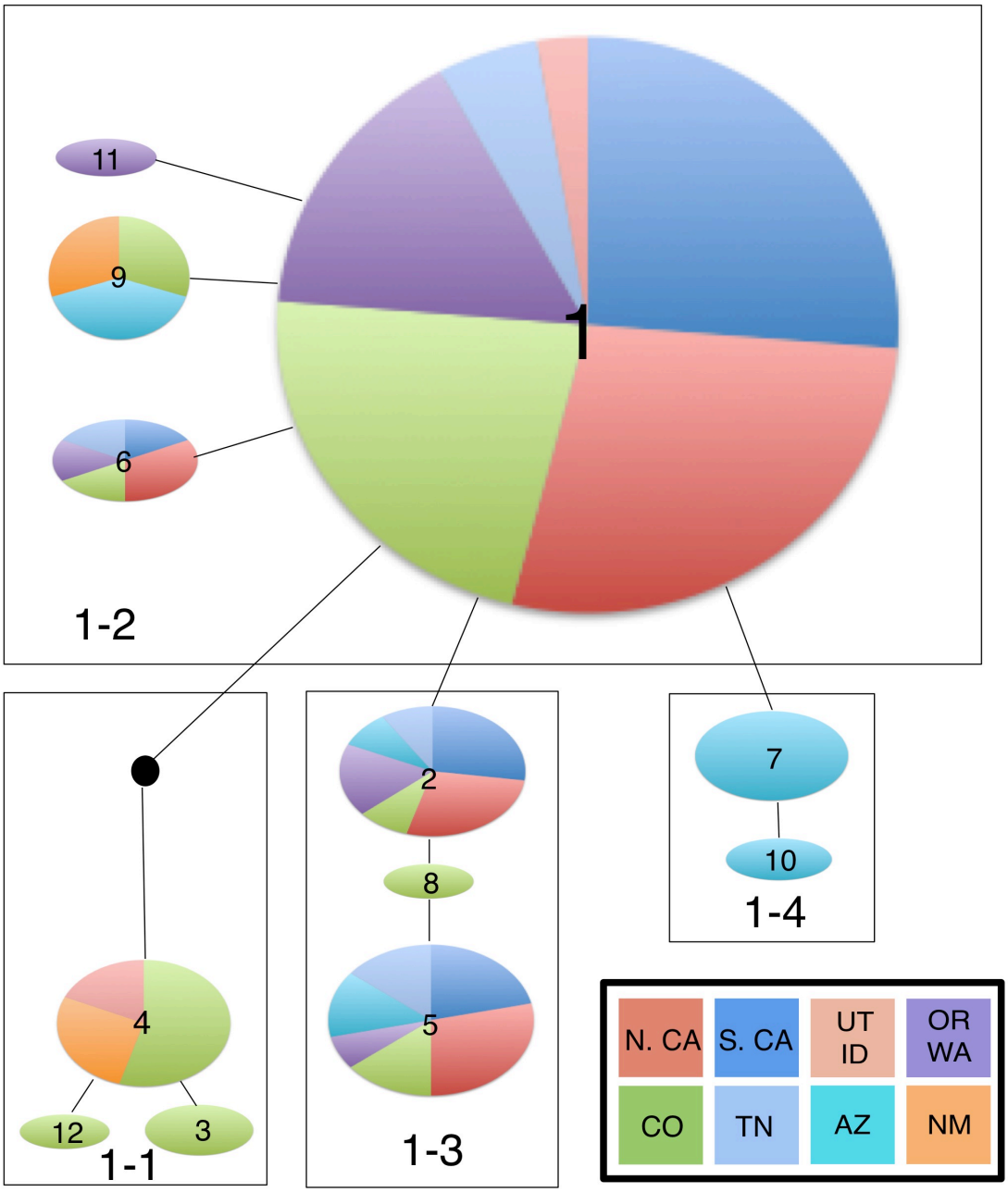

Figure 2.2 The relationships among rDNA ITS haplotypes of *Geosmithia morbida*. Each circle represents a unique ITS haplotype. The size of the circle is proportional to the number of isolates of that haplotype and the colors indicate the states from which the isolates were collected. Each line represents a one bp change in the sequence. Using Nested clade phylogeographic analysis, the haplotypes were grouped into four clades. The black circle in clade 1-1 represents an as yet unrecovered intermediate haplotype linking haplotype 4 to haplotype 1.

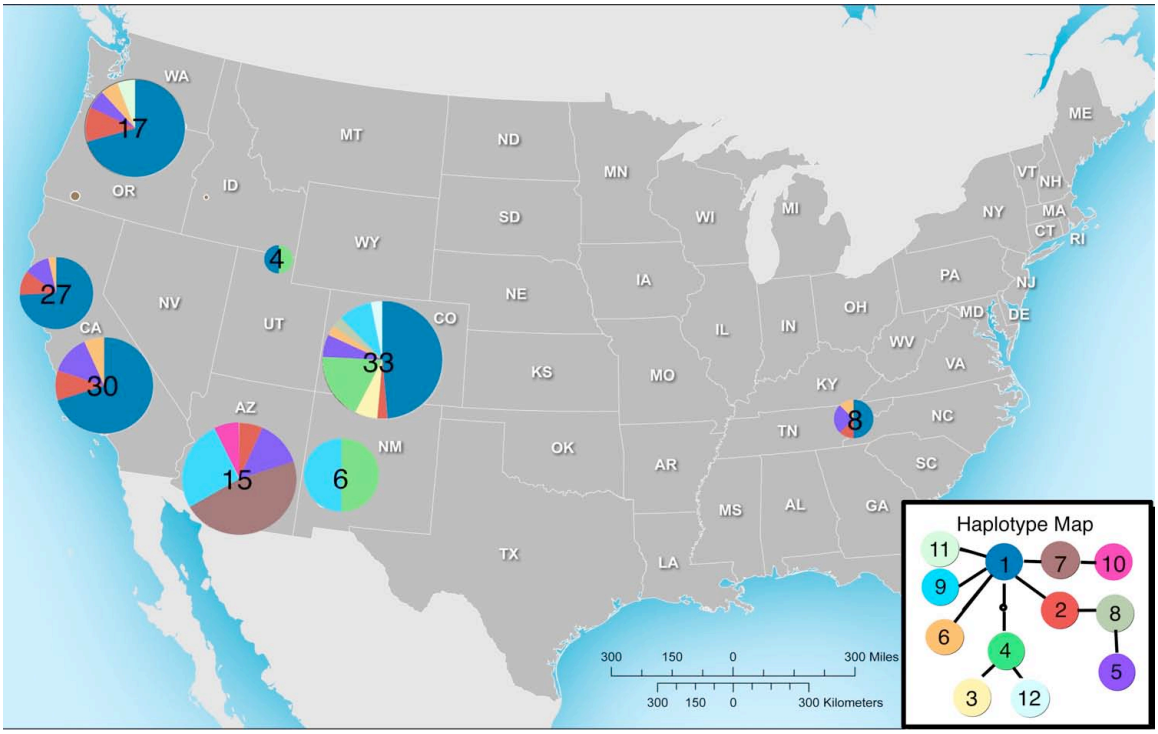

Figure 2.3 Distribution and frequency of the 12 haplotypes (colors from haplotype key) of *Geosmithia morbida* based on ITS rDNA sequences. Numbers within the circles represent the number of isolates analyzed from each location.

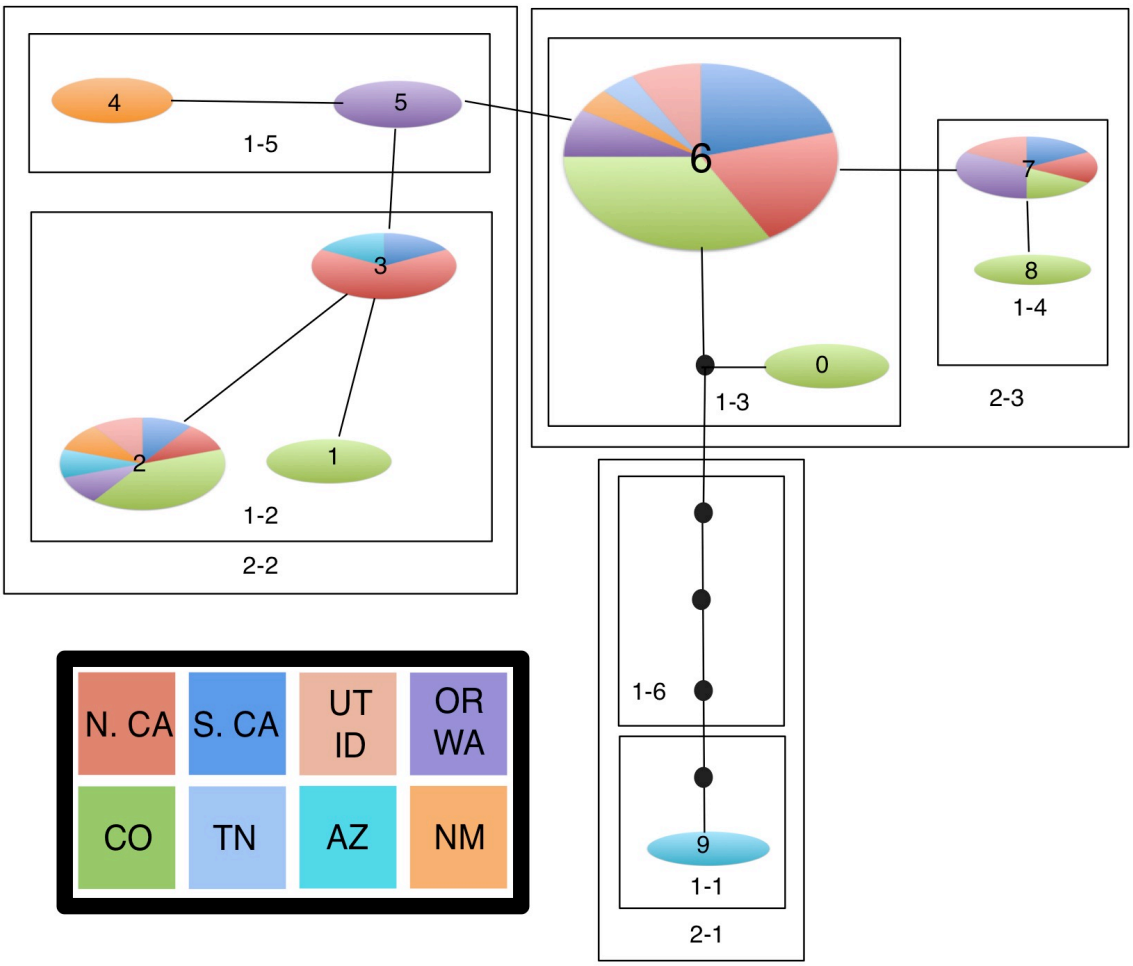

Figure 2.4 The relationships among beta tubulin haplotypes of *Geosmithia morbida*. Each circle represents a unique Beta tubulin haplotype. The size of the circle is proportional to the number of isolates of that haplotype and the colors indicate the states from which the isolates were collected. Each line represents a one bp change in the sequence. Using nested clade phylogeographic analysis, the haplotypes were grouped into six clades. Black circles represent an as yet unrecovered intermediate haplotype linking haplotypes.

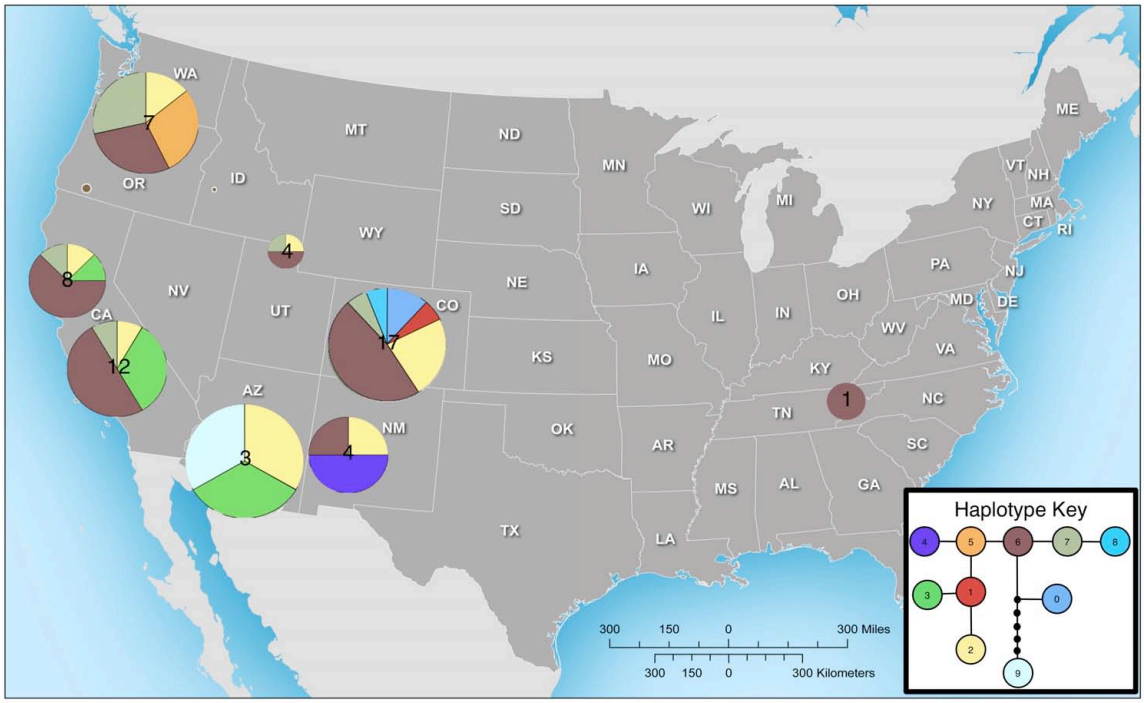

Figure 2.5 Distribution and frequency of the 10 haplotypes (colors from Haplotype key) *of Geosmithia morbida* based on partial beta tubulin sequences. Numbers within the circles represent the number of isolates analyzed from each location.

REFERENCES

Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., Reeb, C. A, and Saunders, N. C. (1987). Intraspecific Phylogeography: The Mitochondrial DNA Bridge Between Population Genetics and Systematics. Annual Review of Ecology and Systematics *18*, 489–522.

Beaumont, M. A., and Panchal, M. (2008). On the validity of nested clade phylogeographical analysis. Molecular ecology *17*, 2563–2565.

Clement, M., Posada, D., and Crandall, K. A. (2000). TCS: a computer program to estimate gene genealogies. Molecular ecology *9*, 1657–1659.

Dutech, C., Enjalbert, J., Fournier, E., Delmotte, F., Barrès, B., Carlier, J., Tharreau, D., and Giraud, T. (2007). Challenges of microsatellite isolation in fungi. Fungal genetics and biology : FG & B *44*, 933–949.

Giraud, T., Enjalbert, J., Fournier, E., Delmotte, F., and Dutech, C. (2008). Population Genetics of Fungal Diseases of Plants. Parasite *15*, 449–454.

Giraud, T., and Fournier, E. (2008). Sympatric genetic differentiation of a generalist pathogenic fungus, Botrytis cinerea, on two different host plants, grapevine and bramble. Journal of Evolutionary Biology *21*, 122–132.

Glez-Peña, D., Gómez-Blanco, D., Reboiro-Jato, M., Fdez-Riverola, F., and Posada, D. (2010). ALTER: program-oriented format conversion of DNA and protein alignments. Nucleic Acids Research.

Halkett, F., Simon, J.-C., and Balloux, F. (2005). Tackling the population genetics of clonal and partially clonal organisms. Trends in ecology & evolution *20*, 194–201.

Haun, G., Powell, S., Strohmeier, C., and Kirksey, J. (2010). State of Tennessee Thousand Cankers Disease Action Plan.

Kolařík, M., Freeland, E., Utley, C., and Tisserat, N. (2011). Geosmithia morbida sp. nov. a new phytopathogenic species living in symbiosis with the walnut twig beetle (Pityophthorus juglandis) on Juglans in the USA. Mycologia *103*, 325–332.

Kolařík, M., Kubátová, A., Hulcr, J., and Pažoutová, S. (2007). Geosmithia Fungi are Highly Diverse and Consistent Bark Beetle Associates: Evidence from their Community Structure in Temperate Europe. Microbial Ecology *55*, 65–80.

Kolařík, M., and Kirkendall, L. R. (2010). Evidence for a new lineage of primary ambrosia fungi in Geosmithia Pitt (Ascomycota: Hypocreales). Fungal biology *114*, 676–689.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. a, McWilliam, H., Valentin, F., Wallace, I. M., Wilm, a, Lopez, R., et al. (2007). Clustal W and Clustal X version 2.0. Bioinformatics (Oxford, England) *23*, 2947–2948.

McDonald, B. A. (1997). The Population Genetics of Fungi: Tools and Techniques. Phytopathology *87*, 448–453.

Newton, L. P., Fowler, G., Neeley, A. D., Schall, R. A., and Takeuchi, Y. (2009). Pathway Assessment: Geosmithia sp. and Pityopthorus juglandis Blackman movement from the western into the eastern United States.

O'Donnell, K., and Cigelnik, E. (1997). Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus Fusarium are nonorthologous. Molecular phylogenetics and evolution *7*, 103–116.

Panchal, M. (2007). The automation of Nested Clade Phylogeographic Analysis. Bioinformatics (Oxford, England) *23*, 509–510.

Pennsylvania Depeartment of Agriculture (2011). Agriculture Department Announces Detection of Thousand Cankers Disease in Pennsylvania Trees, Enacts Quarantine to Prevent Spread.

Petit, R. J. (2008). The coup de grâce for the nested clade phylogeographic analysis? Molecular ecology *17*, 516–518.

Rachowicz, L. J., Hero, J.-M., Alford, R. a., Taylor, J. W., Morgan, J. T., Vredenburg, V. T., Collins, J. P., and Briggs, C. J. (2005). The Novel and Endemic Pathogen Hypotheses: Competing Explanations for the Origin of Emerging Infectious Diseases of Wildlife. Conservation Biology *19*, 1441–1448.

Taylor, J. (2003). Fungal multilocus sequence typing — it's not just for bacteria. Current Opinion in Microbiology *6*, 351–356.

Templeton, A. R. (2010). Coalescent-Based, Maximum Likelihood Inference in Phylogeography. Molecular ecology *19*, 431–435.

Templeton, A. R. (2002). Out of Africa again and again. Nature *416*, 45–51.

Templeton, A. R. (2009). Why does a method that fails continue to be used? The answer. Evolution; international journal of organic evolution *63*, 807–812.

Templeton, A. R., Crandall, K. A., and Sing, F. (1992). Cladistic Analysis of Phenotypic Associations With Haplotypes Inferred From Restriction Endonuclease Mapping and DNA Sequence Data. 111. Cladogram Estimation. Genetics *132*, 619–633.

Templeton, A. R., Routman, E., and Phillips, C. a (1995). Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, Ambystoma tigrinum. Genetics *140*, 767–782.

Templeton, A. R. (1998). "Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history." *Molecular ecology* 7(4):381-97.

Tisserat N., Cranshaw W., Putnam, M., Pscheidt, J. Leslie, C.A., Murray, M., Hoffman, J., Barkely, Y., Alexander, K., and Seybold, S.J. 2011. Thousand cankers disease is widespread on black walnut, *Juglans nigra*, in the western United States. Online. *Plant Health Progr*. (June 30, 2011) doi:10.1094/PHP-2009-0811-01-RS.

Treiman, T., Atchison, B., McDonnell, T., Barden, C., and Moser, W. K. (2010). Economic Loss Associated with the Introduction of Thousand Cankers Disease of Black Walnut to Kansas (Kansas).

Utley, C., Cranshaw, W., Seybold, S., Graves, A., Leslie, C., Jacobi, W., and Tisserat, N. (2009). Susceptibility of Juglans and Carya species to Geosmithia; a cause of thousand cankers disease. Phytopathology *99*.

VDACS. 2011. VDACS - Plant and Pest Services - Thousand Cankers Disease. Retrieved November 17, 2011, from http://www.vdacs.virginia.gov/plant&pest/disease-tcd.shtml

White, T. J., Bruns, T., Lee, S., and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols A guide to Methods and Applications, M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. (Academic Press), pp. 315–322.

CHAPTER III. Effects of *Geosmithia morbida* isolate, *Juglans nigra* family and temperature on canker development

INTRODUCTION

Thousand cankers disease (TCD) of black walnut (*Juglans nigra*) is the result of aggressive feeding by the walnut twig beetle (WTB) (*Pityophthorus juglandis*) and extensive cankering caused by the insect symbiont *Geosmithia morbida* (Kolařík et al. 2011; Tisserat et al. 2009). The disease is widespread in black walnut in the western United States (Tisserat et al. 2011) and more recently has been confirmed in Tennessee (Grant et al. 2011; G. Haun et al. 2010), Virginia (VDAC 2011) and Pennsylvania (Pennsylvanya Department of Agriculture 2011). TCD now is well-established in the native range of black walnut and poses a serious threat to this species.

TCD has been found in a number of *Juglans species*. *Geosmithia morbida* has been isolated from WTB galleries in *J. major* throughout its northern range in Arizona and New Mexico. In this species the fungus causes very small cankers in the phloem and is not associated with major dieback or mortality. The WTB was first collected in 1896 in New Mexico(Blackman 1928) in *J. major* and this has led to speculation that this species may be the native host for the beetle and fungus. TCD has also been reported in *J. californica* and *J. hindisii* in their native ranges in California (Seybold et al. 2010), and the introduced species *J. nigra* and *J. regia* throughout the West (Tisserat et al. 2011). The susceptibility of the these walnut species to TCD is variable based on field observations and greenhouse inoculation studies, although none are as susceptible as *J. nigra* (Utley et al. 2009).

Surviving black walnuts have been found in cities (e.g. Boulder CO) severely impacted by TCD (Tisserat et al. 2011). It is not known whether these surviving trees are escapes, or are resistant to *G. morbida*, the WTB or both. Field inoculations of selected families of *J. nigra* with *G. morbida* resulted in high variability in canker development within individual trees in a family and no discernible resistance, however the scope of this study was limited (Utley et al. 2009). Further testing of these surviving trees, as well as walnuts selected for improved nut or timber qualities is needed.

G. morbida is genetically complex, with multiple haplotypes occurring on the same tree (Kolarik et al., 2011). Furthermore, there is apparently no correlation between haplotypes and the host or geographic region from which the fungus was isolated. It is not known if there is a link between diversity within *G. morbida* and its virulence in different genetic backgrounds of *J. nigra*. Haplotype and genetic diversity of neutral markers have been shown to correlate to virulence differences in different fungi (Mahuku and Riascos 2004; Pascual et al. 2000; Sugimoto 2003). These factors could influence the current spread of the disease, disease movement and disease management strategies.

TCD was first observed in Colorado following severe heat and drought conditions in the early 2000s (Tisserat et al. 2009). This led to speculation that high temperatures may have played a role in the epidemic. *G. morbida* is thermotolerant (optimal growth at 31 °C, limited growth at 41 °C) (Kolařík et al. 2011), but the interaction between canker development and temperature is unknown.

Several studies were undertaken to look at interactions between temperature, genetic background of *J .nigra* trees and genetic background, host origin and origin location of *G. morbida*.

MATERIALS AND METHODS

3.1 Effect of *Geosmithia morbida* haplotype on canker development in black walnut families

One-year-old black walnut trees representing three families (119, 135 and 288) were obtained from Purdue University (Jim McKenna, USDA Forest Service, Purdue University West Lafayette IN, 47907) in May 2009. Trees (half-siblings) within a family were grown from nuts collected from the same mother tree. Trees were placed in pots containing a soilless mix and maintained in the greenhouse at temperatures between 20°C and 26°C with 70% relative humidity. In October 2009 six, 3 mm-diameter holes were punched into the bark to the xylem in six half-siblings from each walnut family. The bark plug was removed and replaced with a plug of sterile half-strength potato dextrose agar (PDA) of a similar size, or plugs colonized by *Aspergillus niger* or *G. morbida* isolates 1217 (representing rDNA ITS haplotype 1), 1218 (representing haplotype 5), and 1274 (representing haplotype 1) and 1271 (representing haplotype 3). All isolates were originally collected from *J. nigra* in Colorado. The six inoculation sites on each tree were spaced approximately 10 cm apart along the stem with isolates arranged in a Latin square design to test the effect of stem position on canker development. Inoculation sites were sealed with Parafilm®. The Parafilm® was removed from wounds after three weeks and after six weeks the stems were harvested and the outer bark was removed to expose phloem discoloration associated with the cankers (Figure 3.1). Trees were photographed and canker areas were determined using ImageJ software (Abramoff, Magelhaes, and Ram 2004). Data analysis of canker area was performed using SAS software (SAS Institute Inc., Cary, NC, USA). Inoculations were repeated in May 2010 on half siblings from the same walnut families, and using the same experimental design with the exception that isolate 1234

(representing haplotype 7, and collected from *J. major* in Arizona,) was substituted for isolate 1274.

3.2 Effect of temperature on canker development

One-year-old, bare-rooted, half-siblings representing three black walnut families were obtained from Purdue University. Trees were placed in pots and maintained in the greenhouse as previously described. In June 2010, five trees in family OSU1 were placed in one of two growth chambers. Both chambers were set a day/night cycle of 16/8 hours respectively with a night temperature of 20°C. The daytime temperature of the chambers were maintained at 25 °C and 32 °C, respectively .Trees were acclimated to growth chambers over a 10 day period and then inoculated with isolates 1217, 1218,1234 and 1222 (representing haplotype 4, collected from *J. regia* in Utah) in a Latin square design (isolate position on stem) as previously described. The experiment was repeated in August 2010 with half-siblings representing family OSU295 and in October with trees from family Green 280.

In another series of temperature experiments, one- year-old, open- pollinated black walnut trees were purchased from Kansas State Forest Service (Manhattan KS, 66508) and potted as previously described. Trees were placed in growth chambers at either a constant 20° C or at 25°C day/22.5° C night (12 hour day/night cycle) for 10 days prior to inoculation. Trees were inoculated with *G. morbida* isolates 1217 and 1218 as previously described. Since previous experiments indicated that position of inoculation on the stem had no effect on canker development, isolate 1217 was always placed in the bottom and 1218 in the top position of stem wounds. Stems were harvested and canker areas determined after six weeks. The experiment

was repeated, except that temperatures in the two growth chambers remained a constant 20° C and 25 °C (12 hour day/night cycle) for the duration of the experiment.

3.3 Effect of dormancy on canker development

Open-pollinated, one- year-old, bare-rooted trees obtained from KSU Forestry (Manhattan KS, 66508) were potted and placed in the greenhouse as previously described. After three days, six, dormant trees were placed in a growth chamber at 25 °C light and 20 °C dark with a 12 hour photoperiod. PDA or PDA colonized with *G. morbida* isolates 1217 and 1218 were placed in wounds as previously described. Trees broke dormancy three weeks into the experiment and were fully leafed by experiment completion.

RESULTS

3.4 Effect of *Geosmithia morbida* haplotype on canker development in black walnut families

Canker size was not affected ($P>0.10$) by stem position in the two experiments. In both experiments sterile PDA plugs and *A. niger* produced significantly smaller areas of phloem discoloration than all *G. morbida* isolates ($P<0.05$). There were no differences in canker areas among *G. morbida* isolates tested in October 2009. However, in May 2010 isolate 1234 produced smaller ($P<0.05$) cankers than isolate 1218 (Fig 3.2). Half-siblings in family 135 had smaller ($P<0.05$) cankers than walnuts belonging to the other two families in October, but differences among families weren't observed when the experiment was repeated (Figure 3.3)

3.5 Effect of temperature on canker development

There were no differences ($p>0.05$) in canker area based on stem position in any of the experiments. In each experiment, canker areas were larger ($P<0.05$) when trees were incubated at 25°C compared to 32°C. However, canker size varied among the three walnut families ($P<0.001$) and the experiments were therefore analyzed separately. In family OSU 1, there were no differences in canker size among *G. morbida* isolates but in OSU 295, cankers produced by isolate 1234 were smaller ($P < 0.05$) at both temperatures. In family 280, there was an interaction ($P=0.017$) between temperature and isolate. At 25°C isolate 1218 caused larger cankers than other isolates whereas at 32°C cankers caused by 1218 were larger than those of isolates 1222 and 1217, but not 1234 (Figure 3.4). In a second set of experiments, canker area was larger at a day/night temperature of 25/22.5 °C compared to a constant 20° C (Figure 3.5). No differences were found between isolate 1217 and 1218. In the second experiment comparing

canker size at constant temperatures of 20°C and 25°C no differences in canker area were found based on temperature or isolate.

3.6 Effect of dormancy on canker development

Cankers were caused by *G. morbida* in black walnut trees that were dormant at the time of inoculation, but broke dormancy after 3 weeks and fully leafed out after 6 weeks. Canker sizes caused by *G. morbida* isolates 1217 and 1218 were not different ($P>0.05$). Overall, canker sizes in this experiment were similar to those in an experiment conducted the previous fall (October) but approximately 25% of the size of those in the June and August 2010 experiments (Figure 3.6).

DISCUSSION

Neutral markers, such as ITS rDNA haplotypes, correlate to virulence differences in plant pathogens (Pascual et al. 2000). In this study, all *G. morbida* haplotypes tested were pathogenic to *J. nigra*, and in some cases there were small, but significant differences in aggressiveness among haplotypes. However, these differences were not always consistent among experiments. Haplotype 1, represented by isolate 1217, is the most frequent haplotype recovered from *Juglans nigra* and its hybrids throughout the western United States (N. Tisserat, personal communication). Yet this isolate was no more aggressive than other haplotypes tested. Thus the frequency of this haplotype in the population is likely not associated with virulence factors. Similarly, haplotype 7, represented by isolate 1234, has only been collected from *J. major* in Arizona. Isolate 1234 was pathogenic in all studies but was slightly less aggressive than certain haplotypes collected from *J. nigra* in Colorado in some experiments. Thus, aggressiveness of *G. morbida* is not highly correlated with its haplotype. However, this indicates a possible range in pathogenicity and may have an effect on the rate of disease development. Furthermore, it is unlikely that a single, highly aggressive haplotype is responsible for the current TCD epidemic. Not all haplotypes were tested nor was variability in aggressiveness among isolates assessed within the same haplotype. However, these results suggest that selection of a specific isolate of *G. morbida* for epidemiological or genetic studies is not critical. Canker formation is consistent and repeatable on individual trees, and canker formation is not influenced by its position (height) on the stem. Therefore, multiple inoculations on a small, single-stemmed tree can be performed without affecting canker development.

Black walnut is an open pollinated (wind dispersed pollen) species and seedlings grown in forest nurseries are collected from a number of different trees. Genetic diversity is very high

with very low genetic differentiation in populations. This suggests extensive pollen flow over broad geographic areas (Victory et al. 2006). To reduce variability in canker formation resulting from walnut genetic diversity in *G. morbida* haplotype and temperature studies, I used half-siblings grown from nuts collected from the same tree. As a result, canker formation could be compared among different black walnut families. Canker formation occurred on all trees and in all families. While there were differences in average canker sizes among families in some experiments, the results were inconsistent. This inconsistency may have been caused by the screening procedure used. Because the sampling was destructive (i.e. the stems are destroyed in measuring cankers), each experiment contained only trees from one family and different families were used in subsequent experiments. Difference in genetic makeup between families of trees may have influenced canker development. Alternatively, inoculations done at different times of the year and likely at different physiological conditions of the tree may have affected canker development. This hypothesis is supported by the fact that canker sizes varied between experiments conducted at different times of the year (Figure 3.6). It is possible that trees going into or coming out of dormancy are less prone to canker development, although other explanations, such as variability in host susceptibility between experiment or the attenuation in aggressiveness of the fungal isolates over time, are plausible. Even so, differences in canker sizes among families were relatively small. It isn't clear whether a slight decline in canker size would substantially alter the development of TCD in a natural infection process.

Canker formation in black walnut occurred at all temperatures tested, but they were consistently smaller at 32°C. This is surprising considering *G. morbida* is thermotolerant, has optimal growth *in vitro* near 30°C, and is apparently native to the southwestern United States where summer temperatures can be extreme. Furthermore, higher temperatures would likely

stress to black walnut trees that are native to more northern regions of the United States, and may therefore be adapted to cooler daytime temperatures. High temperatures have been shown to predispose *Populus* to *Cytospora* canker (Worrall, Adams, and Tharp 2010). My results suggest high summer temperatures are not necessarily needed for bark colonization by *G. morbida*, and that this is unlikely to be a limiting factor in TCD development.

FIGURES

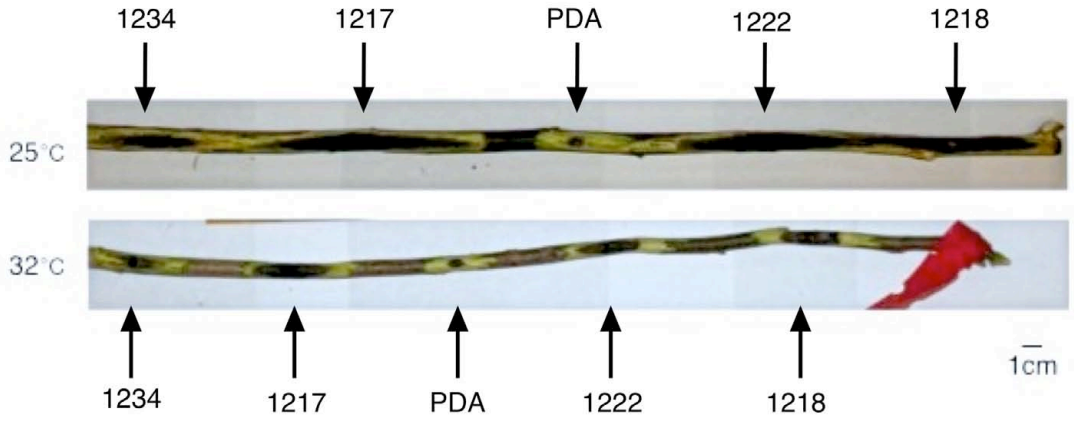

Figure 3.1Canker formation in *Juglans nigra* (family OSU295) six weeks after inoculation with four isolates of *Geosmithia morbida*, representing four rDNA ITS haplotypes at 25° and 32° C, in August 2010.

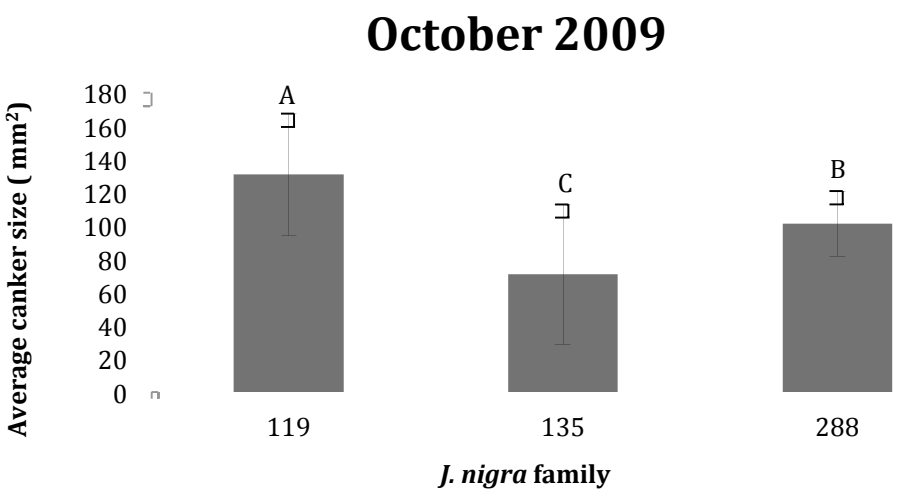

Figure 3.2. Average canker area in black walnut families following inoculation with four isolates of *Geosmithia morbida*, representing 3 rDNA ITS haplotypes, under greenhouse conditions in October 2009. Values were averaged over four *G. morbida* isolates. Bars represent standard deviations.

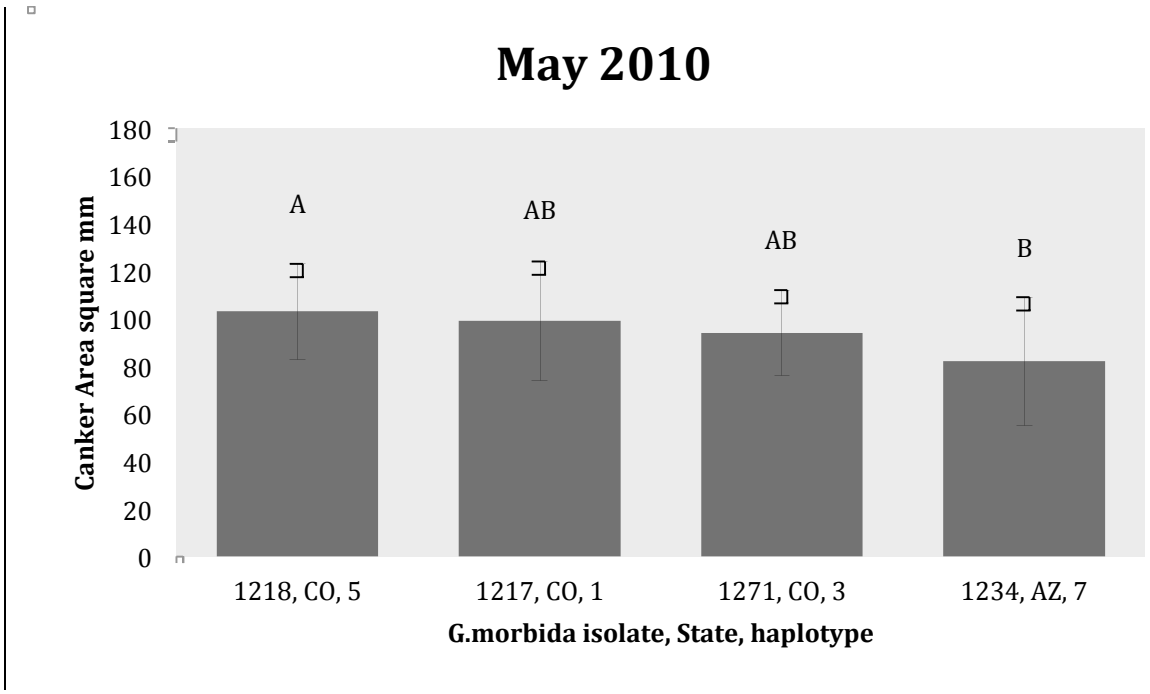

Figure3.3. Average canker area in black walnut trees following inoculation with four *Geosmithia morbida* isolates representing different rDNA ITS haplotypes, under greenhouse conditions in May 2010. Means not followed by the same letter are different (P=0.05) using Tukey HSD adjustment for multiple comparisons. Values were averaged over three black walnut families of *J. nigra*. Bars represent standard deviations.

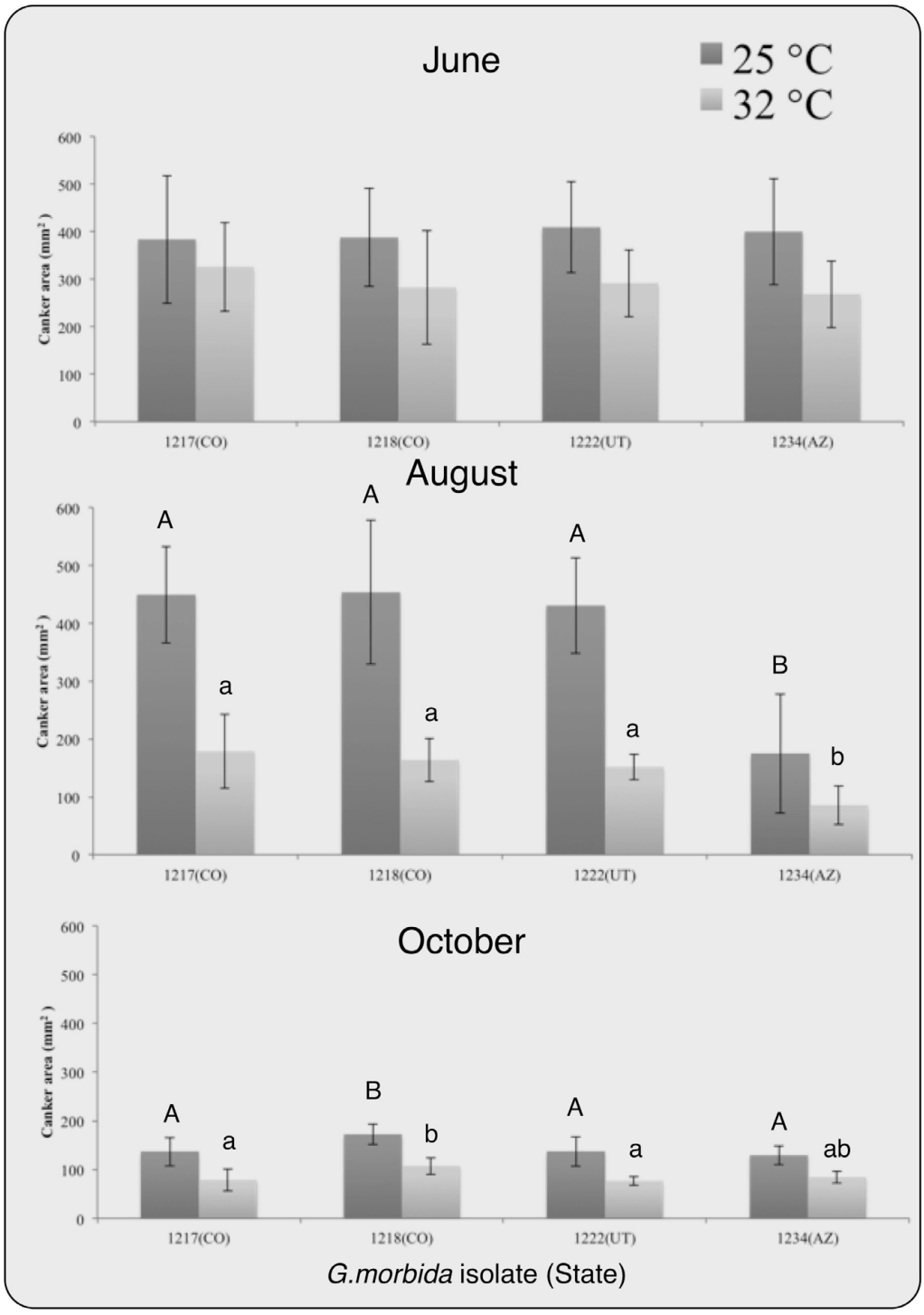

Figure 3.4 Average canker area in black walnut trees following inoculation with four *Geosmithia morbida* isolates representing different rDNA ITS haplotypes and at two incubation temperatures in May 2010(Family OSU1). The experiment was repeated in August (FamilyOSU295) and October 2010 (Family Green 280). Temperature differences were significant ($P<0.05$) in each month. Within each temperature, isolate means not followed by the same letter are different (P=0.05) by Tukey's HSD test. Bars represent standard deviations.

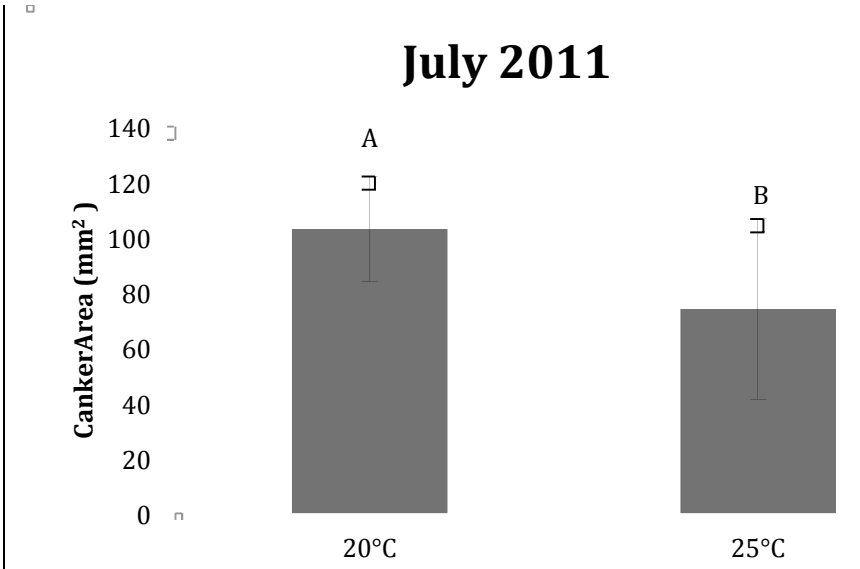

Figure 3.5 Average canker area in black walnut trees following inoculation with two *Geosmithia morbida* isolates (1217 and 1218). Means not followed by the same letter are different (P=0.05). Bars represent standard deviations.

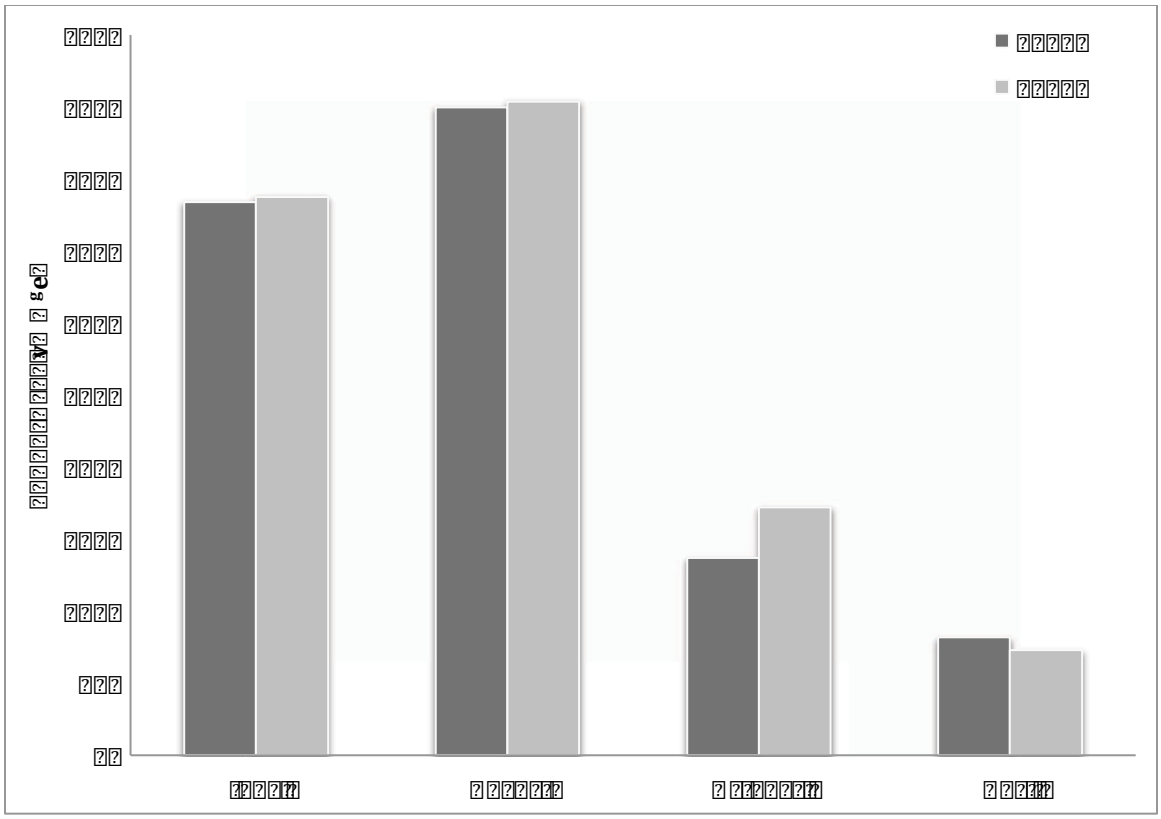

Figure 3.6. Differences in canker areas caused by two isolates of *Geosmithia morbida* and incubated at daytime temperatures of 25°C.

REFERENCES

Abramoff, M. D., Magelhaes, P. J., & Ram, S. . (2004). Image Processing with ImageJ. *Biophotonics International*, *11*(7), 36-42.

Blackman, M. W. (1928). *The genus Pityophthorus Eichh. in North America: A revisional study of the Pityphthori, with descriptions of two new genera and seventy-one new species.* (Technical ., pp. 3-6). Syracuse, NY: . New York State Coll. of Forestry, Syracuse Univ.

Grant, J. F., Windham, M. T., Haun, W. G., Wiggins, G. J., & Lambdin, P. L. (2011). Initial Assessment of Thousand Cankers Disease on Black Walnut, Juglans nigra, in Eastern Tennessee. *Forests*, *2*(3), 741-748. doi:10.3390/f2030741

Haun, G., Powell, S., Strohmeier, C., & Kirksey, J. (2010). *State of Tennessee Thousand Cankers Disease Action Plan*. *October*. Retrieved from *www.tn.gov/agriculture/publications/.../TN_TCD_ActionPlan.pdf*

Kolařík, M., Freeland, E., Utley, C., & Tisserat, N. (2011). Geosmithia morbida sp. nov. a new phytopathogenic species living in symbiosis with the walnut twig beetle (Pityophthorus juglandis) on Juglans in the USA. *Mycologia*, *103*(2), 325-332. doi:10.3852/10-124

Mahuku, G. S., & Riascos, J. J. (2004). Virulence and Molecular Diversity within Colletotrichum lindemuthianum Isolates from Andean and Mesoamerican bean Varieties and Regions. *European Journal of Plant Pathology*, *110*(3), 253-263. doi:10.1023/B:EJPP.0000019795.18984.74

Pascual, C. B., Toda, T., Raymondo, A. D., and Hyakumachi, M. 2000. Characterization by conventional techniques and PCR of *Rhizoctonia solani* isolates causing banded leaf sheath blight in maize. Plant Pathology 49: 108-118.

Pennsylvania Department of Agriculture (2011). Agriculture Department Announces Detection of Thousand Cankers Disease in Pennsylvania Trees, Enacts Quarantine to Prevent Spread Retrieved from http://www.agriculture.state.pa.us

Seybold, Steven, Haugen, D., O'Brien, J., & Graves, A. (2010). Pest Alert: Thousand Cankers Disease. *USDA Forest Service, Northeastern Area, State and Private Forestry*.

Sugimoto, M. (2003). Genetic, morphological, and virulence characterization of the entomopathogenic fungus Verticillium lecanii. *Journal of Invertebrate Pathology*, *82*(3), 176-187. doi:10.1016/S0022-2011(03)00014-4

Tisserat, N., Cranshaw, W., Leatherman, D., Utley, C., & Alexander, K. (2009). Black Walnut Mortality in Colorado Caused by the Walnut Twig Beetle and Thousand Cankers Disease. *Plant Health Progress*, (August). doi:10.1094/PHP-2009-0811-01-RS.Abstract

Tisserat, N., Cranshaw, W., Putnam, M. L., Pscheidt, J., Leslie, C. A., Murray, M., Hoffman, J., et al. (2011). Thousand Cankers Disease is Widespread in Black Walnut in the Western United States Plant Health Progress Plant Health Progress. *Plant Health Progress*, (June). doi:10.1094/PHP-2011-0630-01-BR.Black

Utley, C., Cranshaw, W., Seybold, S., Graves, A., Leslie, C., Jacobi, W., & Tisserat, N. (2009). Susceptibility of Juglans and Carya species to Geosmithia; a cause of thousand cankers disease. *Phytopathology*, *99*(S133).

VDACS. (2011). VDACS - Plant and Pest Services - Thousand Cankers Disease. Retrieved November 17, 2011, from http://www.vdacs.virginia.gov/plant&pest/disease-tcd.shtml

Victory, E. R., Glaubitz, J. C., Rhodes Jr, O. E., & Woeste, K. E. (2006). Genetic homogeneity in Juglans nigra (Juglandaceae) at nuclear microsatellites. *American Journal of Botany*, *93*(1), 118-126.

Worrall, J. J., Adams,G., and Tharp, S.C.. 2010. “Summer heat and an epidemic of cytospora canker of Alnus.” *Canadian Journal of Plant Pathology* 32(3):376-386.

Appendix 1

Inference Key for the Nested Haplotype Tree Analysis of Geographical Distances

Start with haplotypes nested within a 1-step clade and work up to clades nested within the total tree. If the tree is not rooted through an outgroup or if none of the clades nested at the total tree level have the sum of the outgroup probabilities of their haplotypes greater than or equal to 0.95, regard all clades nested at the total tree level as tips. When rooting is deemed reliable, interiors should also refer to the older clades in a nesting category, and tips to their evolutionary descendants.

This key is applied only if there are some significant values for D_c, D_n, or I-T within the nesting clade. If there are no statistically significant distances within the clade, the null hypothesis of no geographical association of haplotypes cannot be rejected (either panmixia in sexual populations, extensive dispersal in non-sexual populations, small sample size, or inadequate geographical sampling). In that case, move on to another clade at the same or higher level.

When performing a single-locus nested clade analysis, the significance of the test results should be corrected for multiple testing with the Dunn-Sidak correction, which is now incorporated into the program. When performing multi-locus nested clade analysis, the Dunn-Sidak correction is not needed, and the original probability values should be used to determine significance. In the case of multi-locus nested clade analysis, the false positive rate is corrected by cross-validation across loci.

1. Are all clades within the nesting clade found in separate areas with no overlap?
- NO – Go to step 2.
- YES - Go to step 19.

2. Is at least one of the following conditions satisfied?
   a. The D_c's for one or more tips are significantly small and the D_c's for one or more of the interiors are significantly large or non-significant.
   b. The D_c's for one or more tips are significantly small or non-significant and the D_c's for some but *not* all of the interiors are significantly small.
   c. The D_c's for one or more interiors are significantly large and the D_c's for the tips are either significantly small or non-significant
   d. The I-T D_c is significantly large.
- NO - Go to step 11.
- YES - Go to step 3.
- Tip/Interior Status Cannot be Determined - **Inconclusive Outcome.**

3. Is at least one of the following conditions satisfied?
   a. Some D_n and/or I-T D_n values are significantly reversed from the D_c values.
   b. One or more tip clades show significantly large D_n's.
   c. One or more interior clades show significantly small D_n's.
   d. I-T has a significantly small D_n with the corresponding D_c value non-significant.
- NO - Go to step 4.
- YES - Go to step 5.

4. Are both of the following conditions satisfied?
   a. The clades (or 2 or more subsets of them) with significantly small D_c or D_n values have ranges that are completely or mostly non-overlapping with the other clades in the nested group (particularly interiors).
   b. The pattern of completely or mostly non-overlapping ranges in the above condition represents a break or reversal from lower level trends within the nested clade series (applicable to higher-level clades only).

- NO - **Restricted Gene Flow with Isolation by Distance (Restricted Dispersal by Distance in Non-sexual species).** This inference is strengthened if the clades with restricted distributions are found in diverse locations, if the union of their ranges roughly corresponds to the range of one or more clades (usually interiors) within the same nested group (applicable only to nesting clades with many clade members or to the highest level clades regardless of number), and if the D_c values increase and become more geographically widespread with increasing clade level within a nested series (applicable to lower level clades only).
- YES - Go to step 9.

5. Are both of the following conditions satisfied?
   a. The clades (or 2 or more subsets of them) with significantly small D_c values have ranges that are completely or mostly non-overlapping with the other clades in the nested group (particularly interiors).
   b. The pattern of completely or mostly non-overlapping ranges in the above condition represents a break or reversal from lower level trends within the nested clade series (applicable to higher-level clades only).

- NO - Go to step 6.
- YES - Go to step 15.

6. Do clades (or haplotypes within them) with significant reversals or significant D_n values without significant D_c values define two or more geographically concordant subsets.

- No - Go to step 7.
- YES - Go to step 13.
- TOO FEW CLADES (≤ 2) TO DETERMINE CONCORDANCE - **Insufficient Genetic Resolution to Discriminate between Range Expansion/Colonization and Restricted Dispersal/Gene Flow** - Proceed to step 7 to determine if the geographical sampling is sufficient to discriminate between short versus long distance movement.

7. Are the clades with significantly large D_n's (or tip clades in general when D_n for I-T is significantly small) separated from the other clades by intermediate geographical areas that were sampled?

- NO - Go to step 8.
- YES - **Restricted Gene Flow/Dispersal but with some Long Distance Dispersal.**

4. Are both of the following conditions satisfied?
   a. The clades (or 2 or more subsets of them) with significantly small D_c or D_n values have ranges that are completely or mostly non-overlapping with the other clades in the nested group (particularly interiors).
   b. The pattern of completely or mostly non-overlapping ranges in the above condition represents a break or reversal from lower level trends within the nested clade series (applicable to higher-level clades only).

- NO - **Restricted Gene Flow with Isolation by Distance (Restricted Dispersal by Distance in Non-sexual species).** This inference is strengthened if the clades with restricted distributions are found in diverse locations, if the union of their ranges roughly corresponds to the range of one or more clades (usually interiors) within the same nested group (applicable only to nesting clades with many clade members or to the highest level clades regardless of number), and if the D_c values increase and become more geographically widespread with increasing clade level within a nested series (applicable to lower level clades only).
- YES - Go to step 9.

5. Are both of the following conditions satisfied?
   a. The clades (or 2 or more subsets of them) with significantly small D_c values have ranges that are completely or mostly non-overlapping with the other clades in the nested group (particularly interiors).
   b. The pattern of completely or mostly non-overlapping ranges in the above condition represents a break or reversal from lower level trends within the nested clade series (applicable to higher-level clades only).

- NO - Go to step 6.
- YES - Go to step 15.

6. Do clades (or haplotypes within them) with significant reversals or significant D_n values without significant D_c values define two or more geographically concordant subsets.

- No - Go to step 7.
- YES - Go to step 13.
- TOO FEW CLADES (≤ 2) TO DETERMINE CONCORDANCE - **Insufficient Genetic Resolution to Discriminate between Range Expansion/Colonization and Restricted Dispersal/Gene Flow** - Proceed to step 7 to determine if the geographical sampling is sufficient to discriminate between short versus long distance movement.

7. Are the clades with significantly large D_n's (or tip clades in general when D_n for I-T is significantly small) separated from the other clades by intermediate geographical areas that were sampled?

- NO - Go to step 8.
- YES - **Restricted Gene Flow/Dispersal but with some Long Distance Dispersal.**

8. Is the species absent in the non-sampled areas?

- NO - **Sampling Design Inadequate to Discriminate between Isolation by Distance (Short Distance Movements) versus Long Distance Dispersal**
- YES - **Restricted Gene Flow/Dispersal but with some Long Distance Dispersal over Intermediate Areas not Occupied by the Species; or Past Gene Flow Followed by Extinction of Intermediate Populations.**

9. Are the different geographical clade ranges identified in step 4 separated by areas that have not been sampled?

- NO - **Allopatric Fragmentation.** (If inferred at a high clade level, additional confirmation occurs if the clades displaying restricted by at least partially non-overlapping distributions are mutationally connected to one another by a larger than average number of steps.)
- YES - Go to step 10.

10. Is the species absent in the non-sampled areas?

- NO - **Geographical Sampling Scheme Inadequate to Discriminate Between Fragmentation and Isolation By Distance.**
- YES - **Allopatric Fragmentation.** (If inferred at a high clade level, additional confirmation occurs if the clades displaying restricted by at least partially non-overlapping distributions are mutationally connected to one another by a larger than average number of steps.)

11. Is at least one of the following conditions satisfied?
    a. The D_c value(s) for some tip clade(s) is/are significantly large.
    b. The D_c value(s) for all interior(s) is/are significantly small.
    c. The I-T D_c is significantly small.

- NO - Go to step 17
- YES - **Range Expansion**, go to step 12.

12. Are the D_n and/or I-T D_n values significantly reversed from the D_c values?

- NO - **Contiguous Range Expansion.**
- YES - Go to step 13.

13. Are the clades with significantly large D_n's (or tip clades in general when D_n for I-T is significantly small) separated from the geographical center of the other clades by intermediate geographical areas that were sampled?

- NO - Go to step 14.
- YES – 1) **Long Distance Colonization, Past Larger Range Coupled with Subsequent Extinction in Some Intermediate Geographical Areas, or Past Range Expansion, All of Which Can Possibly Be Coupled with Subsequent Fragmentation** (subsequent fragmentation is indicated if the clades displaying restricted but at least partially non-overlapping distributions are mutationally

connected to one another by a larger than average number of steps) or 2) **Past Fragmentation Followed by Range Expansion**. To see if secondary contact is involved in scenario 2), perform the supplementary tests given in Templeton, Molecular Ecology **10**: 779-791, 2001. To discriminate the type of movement leading to this pattern in scenario 1), go to step 21.

14. Is the species present in the intermediate geographical areas that were not sampled?

- YES - **Sampling Design Inadequate to Discriminate between Contiguous Range Expansion, Long Distance Colonization, and Past Fragmentation.**
- NO - **Long Distance Colonization and/or Past Fragmentation** (not necessarily mutually exclusive). If inferred at a high clade level, fragmentation rather than colonization is inferred if the clades displaying restricted but at least partially non-overlapping distributions are mutationally connected to one another by a larger than average number of steps. If the branch lengths are short, a colonization event is inferred, perhaps associated with recent fragmentation. To discriminate the type of movement leading to this pattern, go to step 21.

15. Are the different geographical clade ranges identified in step 5 separated by areas that have not been sampled?

- NO - **Past Fragmentation and/or Long Distance Colonization** (not necessarily mutually exclusive). If inferred at a high clade level, fragmentation rather than colonization is inferred if the clades displaying restricted but at least partially non-overlapping distributions are mutationally connected to one another by a larger than average number of steps. If the branch lengths are short, a colonization event is inferred, perhaps associated with recent fragmentation. To discriminate the type of movement leading to this pattern, go to step 21.
- YES - Go to step 16.

16. Is the species present in the intermediate geographical areas that were not sampled?

- YES - Go to step 18.
- NO - **Allopatric Fragmentation.** If inferred at a high clade level, additional confirmation occurs if the clades displaying restricted by at least partially non-overlapping distributions are mutationally connected to one another by a larger than average number of steps.

17. Are either of the following conditions satisfied?

a. The D_c values for tip or some (but not all) interior clades are significantly small.
b. The D_c for one or more interior clades is/are significantly large.
c. The I-T D_c value is significantly large.

- NO - **Inconclusive Outcome.**
- YES - Go to step 4.

18. Are the clades found in the different geographical locations separated by a branch length with a larger than average number of mutational steps.

- NO - **Geographical Sampling Scheme Inadequate to Discriminate Between Fragmentation, Range Expansion, and Isolation By Distance.**
- YES - **Geographical Sampling Scheme Inadequate to Discriminate Between Fragmentation and Isolation By Distance.**

19. Is the species present in the areas between the separated clades?
- NO – **Allopatric Fragmentation.** If inferred at a high clade level, additional confirmation occurs if the clades displaying restricted by at least partially non-overlapping distributions are mutationally connected to one another by a larger than average number of steps.
- YES - Go to step 20.

20. Was the species sampled in the areas between the separated clades?
- NO – **Inadequate Geographical Sampling.**
- YES – Go to step 2.

21. Are all of the following true?
    a. Is it biologically realistic that the organism could have undergone long-distance movement?
    b. Are the nested haplotypes that mark a potential long-distance colonization event within a clade that shows evidence of population growth by other methods (such as mismatch distributions)?
    c. At the level of the entire cladogram, does the clade *not* inferred to have produced long-distance colonization *not* show evidence of past population growth with other methods?
- YES – **Long-distance movement.**
- NO – Insufficient evidence to discriminate between long-distance movements of the organism and the combined effects of gradual movement during a past range expansion and fragmentation. If the case against long-distance movement is compelling, then the inference is **past gradual range expansion followed by fragmentation or a past larger range followed by extinction in intermediate areas.**