THESIS

THE BIOLOGY OF *GEOSMITHIA MORBIDA* AND SUSCEPTIBILITY OF WALNUT AND HICKORY SPECIES TO THOUSAND CANKERS DISEASE

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ABSTRACT

THE BIOLOGY OF *GEOSMITHIA MORBIDA* AND SUSCEPTIBILITY OF WALNUT AND HICKORY SPECIES TO THOUSAND CANKERS DISEASE

Since 2001 widespread mortality of black walnut (*Juglans nigra*) has been reported in Colorado, USA. Affected trees initially show a yellowing and thinning of leaves in the upper crown, followed by twig and branch dieback and ultimately tree death. We report that this mortality is the result of a combination of an expanded geographic range of the walnut twig beetle (*Pityophthorus juglandis*), its aggressive feeding behavior on black walnut, and extensive cankering caused by a filamentous ascomycete in the genus *Geosmithia* (Ascomycota: Hypocreales). Thirty seven *Geosmithia* strains collected from *J. californica*, *J. hindsii*, *J. major*, and *J. nigra* in eight USA states (AZ, CA, CO, ID, OR, UT, WA) were compared using morphological and molecular methods (ITS rDNA sequences). Strains had common characteristics including a yellowish color of conidia *en masse*, growth at 37°C, and absence of growth on Czapek-Dox agar and belonged to a single species described here as *G. morbida*. *G. morbida* is the first *Geosmithia* species documented as a plant pathogen. We also tested the susceptibility of hickory and walnut species to *G. morbida* in greenhouse and field studies. *Carya illinoinensis*, *C. aquatica*, and *C. ovata* were immune. All walnut species tested, including *J. ailantifolia*, *J. californica*, *J. cinerea*, *J. hindsii*, *J. major*, *J. mandshurica*, *J. microcarpa*, *J. nigra* and *J. regia* developed cankers following inoculation with *G. morbida*. *J. nigra* had the largest cankers, whereas *J. major*, a native host of the WTB and presumably *G. morbida*, had smaller and more superficial cankers. Canker size differed among maternal half-sibling families of *J. nigra* and *J. cinerea*, indicating genetic variability in resistance to *G. morbida*. Our

inoculation studies with *G. morbida* have corroborated many of the field observations on susceptibility of hickory and walnut species to TCD, although the ability of the WTB to successfully attack and breed in walnuts is also an important component in TCD resistance.

ACKNOWLEDGEMENTS

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CHAPTER 1. Black Walnut Mortality in Colorado Caused by the Walnut Twig Beetle and Thousand Cankers Disease.

Preface

In this first chapter where we describe Thousand Cankers Disease was published as: Black walnut mortality in Colorado caused by the walnut twig beetle and thousand cankers disease. Online in Plant Health Progress doi:10.1094/PHP-2009-0811-01-RS, 2009, by [1]Tisserat, N., Cranshaw, W., Leatherman, D., Utley, C., and Alexander, K. 2009. My contributions included many observations regarding the lifecycle, attack habits, and branch selection of *P. juglandis* in *J. nigra.* I peeled many branches to expose the cankers to determine canker growth progression through the phloem of black walnuts showing different degrees of canopy symptoms. I also contributed to the collection of photographic evidence. I collected numerous *Geosmithia* isolates from sampled diseased trees, and conducted the fungal vector test experiment with live walnut twig beetles.

Introduction

Black walnut (*Juglans nigra*) is one of the most highly valued timber species in North America (8). The wood is prized for use in cabinetry, gunstocks and other finished wood products. The nuts are also an important nutritional source for wildlife. Black walnut is native to the eastern North America, and is widely distributed on deep alluvial soils from New England

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and the Appalachian Mountains east to the Great Plains and from the Canadian border south into Texas and into the Florida panhandle (8). It has been widely planted outside its native range in the western United States as an ornamental and timber tree, and for nut production.

As early as 2001 arborists and foresters in Boulder and Colorado Springs, Colorado noted decline and mortality of black walnut. Affected trees initially showed a yellowing and thinning of leaves in the upper crown, followed by twig and branch dieback (Fig. 1.1). Over a period of several years, progressively larger branches were killed and affected trees eventually died. Trees typically were killed within two years after initial symptoms developed although smaller trees (<10-cm diameter at breast height), or those growing on sites prone to drought stress, declined more rapidly. Basal sprouts often developed on trees in advanced stages of decline, or from stumps of removed trees, but these sprouts also wilted and died within one or two years after emergence.

By fall 2008 over 700 trees were killed and removed in Boulder alone, representing the majority of black walnut in that municipality. Similar mortality rates occurred in Colorado Springs, which now has few surviving black walnuts. Tree decline is now occurring in several communities in the Metro Denver area. The disease was also observed on black walnut in the city of Delta approximately 400 km west of Denver.

Black walnut mortality was initially attributed to a drought that persisted from 2000-2003. However, tree deaths commonly occurred at sites receiving supplemental irrigation, and mortality rates accelerated after annual precipitation returned to normal in 2004. This suggested an alternate cause for the unusual tree mortality. We report here on the association of the walnut twig beetle and the canker fungi *Geosmithia* and *Fusarium solani* with mortality of black walnut

in Colorado, and the potential threat this disease complex could have on black walnut if it were introduced into the native range of this tree species.

Walnut Twig Beetle

Pityophthorus juglandis is a minute (1.5-1.9 mm) yellowish-brown bark beetle. It is one of only a few species in the genus *Pityophthorus* that is associated with hardwoods and the only one associated with Juglans (7). It can be readily distinguished from other members of the genus by several physical features (Fig. 1.2). 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 at the apex it is generally flattened with small granules.

The walnut twig beetle is apparently native to North America and was originally described in 1928 from a collection in the area of "Lone Mountain", New Mexico, presumably in Lincoln County (1). Wood and Bright (19) reported the insect from *Juglans* in New Mexico, Arizona, and Chihuahua, Mexico, a distribution that largely coincides with Arizona walnut (*J. major*). The walnut twig beetle was also recovered from both black walnut and southern California walnut (*J. californica*) in Los Angeles County in 1959 (2).

The walnut twig beetle was first associated with black walnut mortality in the Espanola Valley of New Mexico in 2001 (15). The beetle was subsequently collected in Boulder and the adjacent community of Westminster Colorado in 2004 and in Colorado Springs in 2005 on declining black walnut (16). Prior to these reports, the walnut twig beetle had never been associated with any significant *Juglans* dieback or mortality. Drought was originally suspected as the cause of the decline and death of trees, with the beetle as a secondary pest.

The life cycle of this insect has not been fully determined. Despite its small size, attacks by adult walnut twig beetles are not confined to small diameter twigs. In fact, the minimum size of infested limbs was approximately 2 cm diameter and tunneling regularly was found in branches of 10 cm diameter or larger and even in trunks of large trees, a behavior considered unusual for this genus. Results of sticky panel trapping in Boulder during 2006 indicated a flight of adult beetles from late April through October (data not shown). Initiation of adult tunneling was observed in the field during early May and a single generation was completed within 7 weeks in infested logs held at room temperature. This suggests that two or more generations are completed annually in Colorado and that they overlap.

Fungal Cankers

Two different cankers were observed on declining walnut trees. In the early stages of tree decline, small, roughly circular to oblong cankers developed around *P. juglandis* galleries in twigs, branches and even the trunk (Fig. 1.3). Cankers were usually not visible until a thin layer of outer bark was removed. In some cases, a dark amber stain formed around the beetle entrance hole on the bark surface. Bark cracks sometimes formed near galleries on small diameter branches giving them a rough and somewhat bumpy appearance. Nevertheless, branches with numerous beetle galleries and cankers often showed no outward appearance of bark damage, except for the small beetle entrance holes, even when leaf wilting was present.

Cankers surrounding the beetle galleries in thicker-barked branches or the trunk often were initially restricted to the phloem and outer bark and did not extend into the cambium. With time, cankers expanded in the phloem and outer bark, became more diffuse, and caused a dark brown to black maceration of the tissue. Cambial discoloration was observed only after

extensive bark colonization by the fungus had occurred. Beetle galleries and associated cankers often were scattered every 2- to 5-cm in bark during advanced stages of decline. Thus, the total number of cankers on each declining tree was enormous. Eventually multiple cankers coalesced and girdled twigs and branches, resulting in dieback. A dusty, white to tan fungus was often found in the insect galleries and on adult beetles that had died in galleries (Fig. 1.4).

A second canker type was observed on the trunks of five dissected black walnut trees in advanced stages of decline. These diffuse cankers were much larger and more continuous than those observed on branches during the early stages of the disease. Trunk cankers often exceeded two meters in length, extended from the ground into the scaffold branches, and sometimes encompassed more than half the circumference of the trunk (Fig. 1.5). The bark remained firmly attached to the trunk such that cankers were not visible without first removing the outer bark. On some trees a dark brown to black stain on the bark surface or in bark cracks indicated the presence of a trunk canker. The inner bark and cambium were water-soaked and stained dark brown to black. The wood adjacent to the cambium was also stained. Walnut twig beetle entrance holes were present in bark crevices, and tunneling was observed beneath the bark surface in the canker face. In addition, small round entrance holes (2-3 mm) of the exotic ambrosia beetle *Xyleborinus saxeseni* Ratzeburg were sometimes observed in sapwood beneath the trunk cankers.

Branch and trunk samples with cankers were collected and the outer bark was peeled back with a sterile scalpel to expose diseased tissue. 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 agar amended with 100 mg/L streptomycin sulfate and 100 mg/L chloramphenicol (¼ PDA++). Emerging fungal colonies were transferred to half strength PDA.

Twelve adult walnut twig beetles were excised from their galleries in branches on three trees and placed directly on ¼ PDA++ without surface disinfestation.

A fungus was consistently isolated from canker margins, from discolored phloem lining beetle galleries and from beetles removed from galleries on branches and the trunk. Fungal colonies on half strength PDA were cream-colored to tan, and tan to yellow-tan on the reverse side of the plate (Fig 1.4). The fungus initially grew very rapidly out of the wood chips and colonies commonly exceeded 20-40 mm in diameter after 3-5 days at 25 °C. However, colonies generally were attenuated (<20-30 mm diameter after several weeks) with appressed margins following successive transfers on ½ strength PDA. The fungus sporulated profusely in culture producing dry conidia on multi-branched, verticillate, verrucose conidiophores. Conidia were tan *en masse*, cylindrical to ellipsoid (2)2.7(6) x (6) 6.5(14) µm and formed in chains. Morphological characteristics of the conidiophores and conidia were consistent with those described for the genus *Geosmithia* (11, 12, 13).

In addition to *Geosmithia*, *Fusarium solani* (Mart.) Sacc. was consistently isolated from cambial tissue collected from the margins of large trunk cankers during the final stages of the disease but not from cankered bark tissue surrounding walnut twig beetle galleries on branches. DNA was extracted from two putative single spore isolates of *Geosmithia* (1217 and 1218) grown on modified yeast extract broth for 10 days. DNA amplification of the ITS1, 5.8s ,and ITS2 regions of the rDNA were performed using the ITS 1 and ITS 4 universal primers (18). Sequences of the two isolates were identical to one another except at one base pair and similar to (97% sequence similarity) but not identical with *G. flava* (AM181457) and *G. obscura* (AM181460) (BLASTN, NCBI 2.2.1.5). Thus, the *Geosmithia* isolated from black walnut in Colorado may be an unnamed species. Its origin has not yet been determined. Similarly, the

identities of two isolates (917 and 1179) of *F. solani* based on morphological characteristics were supported by ITS sequencing results. Isolates 1217 (CBS 124663) and 1218 (CBS 124664) of *Geosmithia* and isolates 917 (CBS 124665) and 1179 (CBS 124666) of *F. solani* have been deposited in the Centraalbureau voor Schimmelcultures.

Pathogenicity Tests

One–year-old bare root black walnut trees approximately 100 cm in height and 7-12 mm diameter at ground level were used for pathogenicity tests. Dormant trees were planted in 3.8 liter pots in February 2008 in a commercial nursery mix and placed in a greenhouse. Inoculations were made in March after the trees had resumed growth and leaves had fully emerged. Two isolates each of *Geosmithia* (1217 and 1218) and *F. solani* (917 and 1179) were grown for 3 weeks on ½ strength PDA.

Inoculations were made by slicing down through the bark with a sterile scalpel at three sites on each stem. Resulting wounds were approximately 0.5-1.0 cm wide and 1 cm long with the flap of bark still attached to the stem at the base of the wound. Wounds were made at approximately 15-cm intervals along the stem starting 15 cm above the soil line. A plug of sterile ½ strength PDA approximately 0.5 cm^2 was inserted under the bark flap and against the wood on the middle wound on each tree. An agar plug of similar size but colonized by one of the fungal isolates was then inserted under the bark flap on the top and bottom wound. Four trees were inoculated with each isolate. All wounds were sealed with Parafilm® (American National Can, Menasha, WI) and the trees were randomly placed on a greenhouse bench. The Parafilm® was removed after 3 wk.

After 8 wk, all trees were harvested and the outer bark was shaved from the wounds with a sterile scalpel to expose the extent of bark discoloration. Wounds in which sterile agar was inserted were sealed by callus and there was no evidence of bark discoloration beyond the original wound. Cankers developed at all wounds inoculated with *Geosmithia* and at 38% of the wounds inoculated with *F. solani* (Table1.1). Cankers caused by *Geosmithia* were both longer than those caused by *F. solani* and had more diffuse margins (Fig. 1.6). Both fungi were consistently isolated from the canker margins.

Vector Test

An experiment was conducted to demonstrate that *P. juglandis* is a vector of *Geosmithia*. Ten adult *P. juglandis* beetles were collected as they emerged from a cankered black walnut log that had been placed in the laboratory. The beetles were transferred to a sealed plastic box containing a recently cut, 9.5-cm-long and 2.5-cm-diameter black walnut stem placed on a moistened paper towel. The cut ends of the stem were sealed with Parafilm® to prevent moisture loss. The stem was collected from a healthy black walnut in a region of the state where thousand cankers had not yet become established. No cankers or beetle galleries were visible prior to beetle exposure. Within 24 hours beetles had begun to burrow into the stem. *Geosmithia* was subsequently isolated from frass pushed out of the tunnels. After 4 wk the outer stem bark was carefully removed around the beetle galleries as previously described. Cankers similar to those observed on naturally infected trees had developed around the beetle galleries and *Geosmithia* was isolated from canker margins (Figure 1.7).

Summary

We report for the first time that an unnamed *Geosmithia* associated with the walnut twig beetle is a cause of a lethal canker disease of black walnut. Many *Geosmithia* species have been reported to be associated with bark beetles of hardwood and conifer trees (10,11,12,13). For example *G. flava* and *G. obscura* have been isolated from scolytid beetle galleries in several tree species in Europe (11,13). *Geosmithia* species have not previously been reported to cause cankers on trees although Čížková et al. (4) found that *G. langdonii* and *G. pallida* reduced stem growth of germinating seedlings of garden cress (*Lepidium sativum* L. var. *capitatum*).

Several *Fusarium* species including *F. solani* have been associated with elongate, annual cankers of black walnut in North America (5, 6, 14, 17) and English walnut in South Africa (3) although the epidemiology of these cankers is poorly understood. Trees are thought to be predisposed to canker formation by stress factors including suboptimal site conditions, improper pruning and adverse weather (5,6,14). Kessler (9) and Weber (17) hypothesized that an apparent symbiosis between ambrosia beetles and *Fusarium* fungi resulted in canker formation on black walnut. Although we have observed ambrosia beetles in diseased black walnut wood in Colorado, we have not determined whether they are serving as vectors for *F. solani*.

Carlson et al. (5) questioned whether *F. solani* was the primary cause of cankers on mature black walnuts and suggested it may be present only as a saprophyte or as a facultative parasite. We noted that trees exhibiting elongate trunk cankers already had extensive tunneling in the trunk bark caused by *P. juglandis* and *Geosmithia* was isolated from discolored tissue surrounding these galleries. Thus we believe *Geosmithia* had already extensively colonized the bark and was the primary pathogen associated with walnut mortality.

Nevertheless, *F. solani* was consistently isolated from the cambium and outer sapwood at the margins of elongate trunk cankers. This observation as well as our pathogenicity studies suggests a role, even if minor, for *F. solani* in tree mortality.

We propose the name thousand cankers disease to describe this lethal disease of black walnut because trees are killed by the cumulative effects of numerous, coalescing twig, branch and trunk cankers that are associated with walnut twig beetle galleries. The speed at which this disease is eliminating black walnut along the Front Range of Colorado is alarming. We believe thousand cankers disease poses a grave risk to black walnut throughout its native range in eastern North America should the walnut twig beetle/*Geosmithia* complex be introduced. The distribution of thousand cankers disease and relative susceptibility of other native and exotic *Juglans* species to *Geosmithia* needs to be determined.

Tables and Figures

Table 1.1 Canker formation in black walnut 8 weeks after inoculation with isolates of *Fusarium solani* and *Geosmithia*.

Isolate	Number of inoculation sites developing cankers [x]	Average and range ( ) of canker lengths (mm)
Fusarium solani 917	2/8	19.0 (16-22)
Fusarium solani 1179	6/8	21.0 (10-40)
Geosmithia 1217	8/8	57.0 (29-130)
Geosmithia 1218	8/8	46.5 (31-60)

[x] Two inoculation sites on each of 4 trees.

Figure 1.1 Symptoms of thousand cankers disease in *Juglans nigra*. **A,** Yellowing of leaves and branch dieback during early stages of disease. **B,** extensive branch dieback and **C,** wilting and collapse of entire crown prior to death. Note that the bark on branches and trunk remains firmly attached even in final stages of disease and there is no external evidence of cankers.

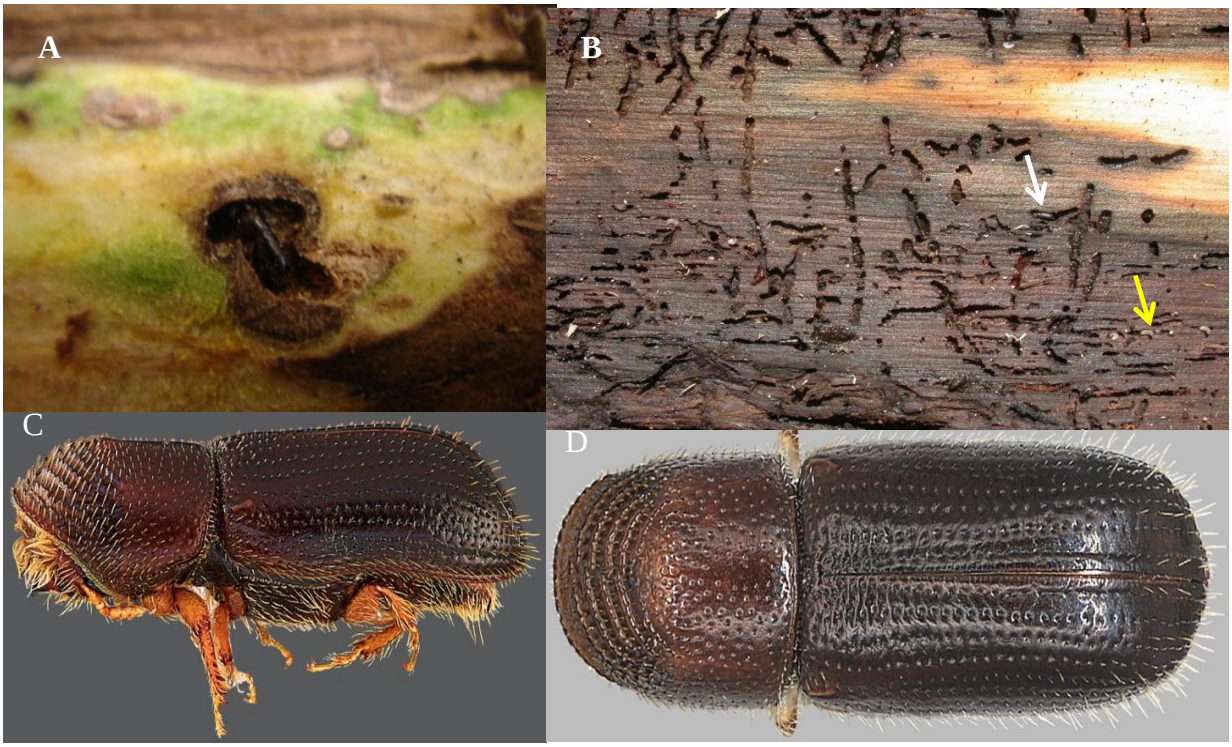

Figure 1.2 A, Gallery formation caused by walnut twig beetle adult in *Julgans nigra* branch. **B,** Extensive tunneling in inner trunk bark caused by walnut twig beetle adults and larvae. Note the extensive discoloration surrounding galleries caused by *Geosmithia*. **C,** Walnut twig beetle, side view (*Photograph by Jim LaBonte, Oregon Department of Agriculture*). **D,** Walnut twig beetle, top view (*Photograph by Jim LaBonte, Oregon Department of Agriculture*).

Figure 1.3 Cankers caused by *Geosmithia* are visible only after the outer bark is removed. **A,** Oblong to elliptical cankers surround galleries of the walnut twig beetle. **B,** Coalescing cankers eventually girdle the branch resulting in dieback. **C,** Multiple cankers caused by *Geosmithia* developing on the trunk approximately 1 m from the ground.

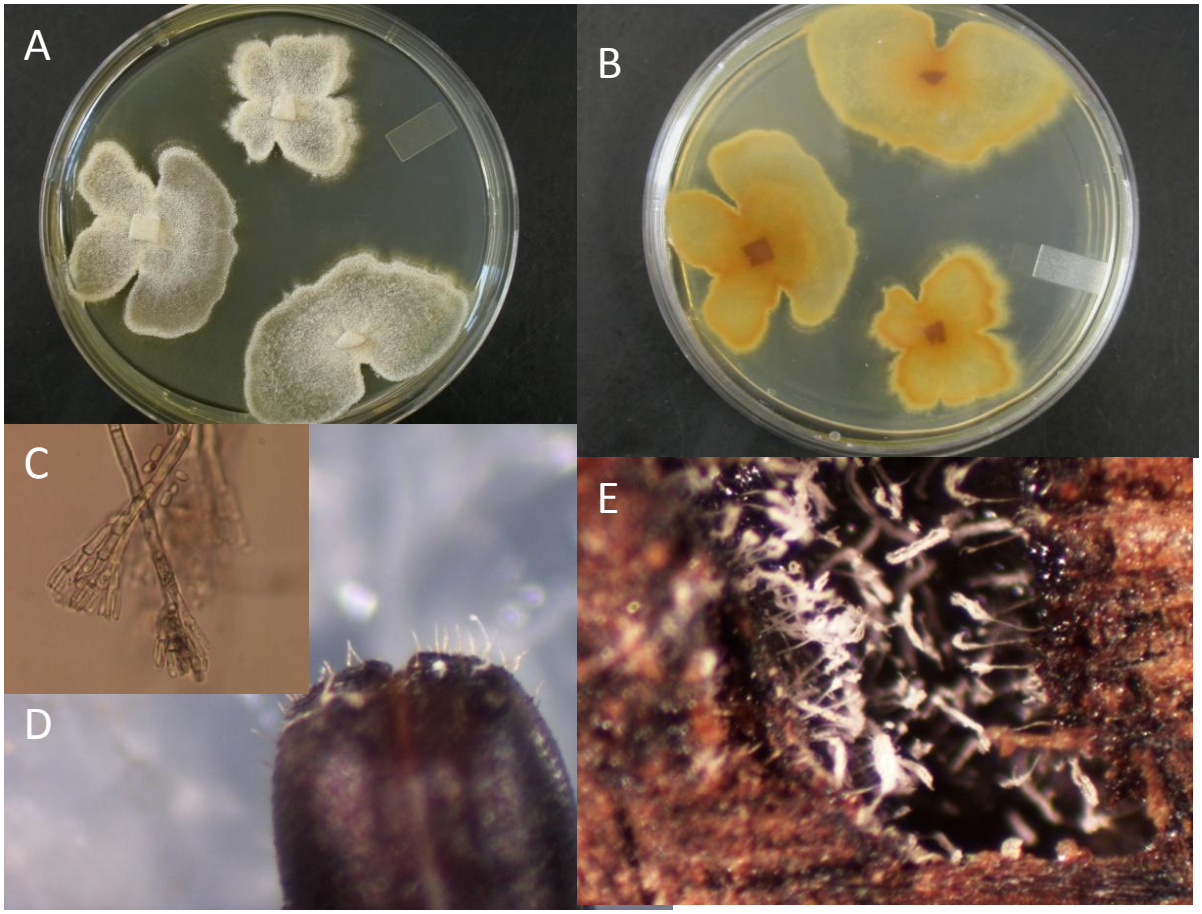

Figure 1.4 A, Colony morphology of *Geosmithia* on ½ strength potato dextrose agar **B,** Colony morphology on reverse side of plate **C,** verticillate conidiophores and conidia **D,** conidiophores on elytra of dead walnut twig beetle excised from gallery and **E,** *Geosmithia* sporulating in walnut twig beetle gallery.

Figure 1.5 A, and **B**, Bark has been removed to reveal the elongate, diffuse trunk cankers that develop during the final stages of the disease. *Geosmithia* was consistently isolated from the discolored bark whereas both *Geosmithia* and *Fusarium solani* were isolated from the cambium at the canker margins. **C**, Extensive bark and cambial necrosis on trunk at a height of 1m. Approximately ¾ of the trunk circumference (clockwise from top to bottom arrow) has been killed.

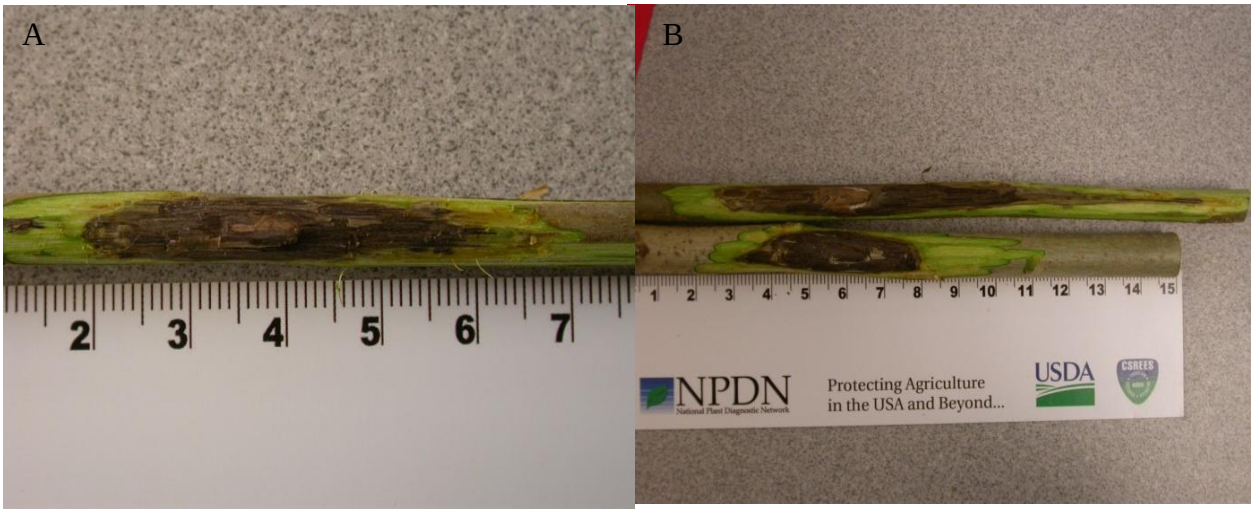

Figure 1.6 Canker development in *Juglans nigra* 8 weeks after inoculation with **A,** *Fusarium solani* and **B,** *Geosmithia*.

Figure 1.7 Gallery and canker development 4 weeks after placement of adult walnut twig beetles emerged from *Juglans nigra* with thousand cankers disease in a cage containing a branch segment from a healthy black walnut.

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CHAPTER II. *Geosmithia morbida* sp. nov. A New Phytopathogenic Species Living in Symbiosis with the Walnut Twig Beetle (*Pityophthorus juglandis*) on *Juglans* in the USA

Preface

In this paper where we name and describe the new fungal pathogen *Geosmithia morbida*, was published as: *Geosmithia morbida* sp. nov. a new phytopathogenic species living in symbiosis with the walnut twig beetle (*Pityophthorus juglandis*) on *Juglans* in the USA. In Mycologia 103:325-332, 2011 by, [2]Kolařík, M., Freeland, E., Utley, C., and Tisserat, N. My contributions included the collection and cataloguing of two of the tested *G. morbida* haplotypes, observations and description of the yeast or budding phase of *G. morbida* and the temperature experiments which are included in the differential characters.

Introduction

Widespread branch dieback and mortality of *Juglans nigra* (black walnut) has occurred in several western states including Colorado (CO), Idaho (ID), New Mexico (NM), Oregon (OR), Utah (UT), and Washington (WA) of the USA since the mid-1990s. *Juglans nigra* 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

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result of aggressive feeding by the walnut twig beetle, *Pityophthorus juglandis* Blackman, (Col. Curculionidae, Scolytinae) and subsequent canker development surrounding beetle galleries in the phloem. The number of cankers formed on branches and the trunk is enormous, hence the name thousand cankers to describe the disease. A previously undescribed species of *Geosmithia* (Ascomycota: Hypocreales) was consistently isolated from the canker margins, beetle galleries and adult beetles on *Juglans nigra*. Artificial inoculations of *Juglans nigra* demonstrated this fungus was responsible for canker development (21). *P. juglandis* and *G. morbida* have not been reported in the native range of *J. nigra* in the eastern United States.

P. juglandis was first described from *J. major*, the apparent native host of the beetle, in the south-western United States (1, 23). In 2008 and 2009 *G. morbida* was isolated from necrotic phloem surrounding *P. juglandis* galleries in *J. major* in native stands in AZ and NM, but the fungus was not causing branch dieback or mortality in this species. Both *P. juglandis* and *G. morbida* also have recently (2008) been associated with dieback of *J. hindsii* and *J. californica* in their native range in CA. However, pathogenicity of *G. morbida* to these species has not yet been documented. *Geosmithia* is a genus of mitosporic filamentous fungi with a world-wide distribution containing 22 published species (3, 7, 8, 9, 11, 12), and at least 20 more unpublished species. Certain *Geosmithia* species sporadically occur on broad range of substrates, including plant debris, cereals and in soil (12, 16), however, most are exclusive associates of subcortical insects including scolytids (Coleoptera, Curculionidae, Scolytinae) and bostrichids (Col., Bostrichidae). *Geosmithia* spp. are typically found in association with phloeophagous bark beetles (7, 10, 11), but also with wood-boring ambrosia beetles, where they can act as primary or auxiliary ambrosia fungi (8). While ambrosial *Geosmthia* spp. provide the main nutritional source for their vectors and represent an extreme example of nutritional mutualism, little is

known about interactions of other symbiotic *Geosmithia* species, or their vectors and host plants. Several attempts to evaluate phytopathogenicity of *Geosmithia* have been conducted. A *Geosmithia* sp. associated with *Cryphalus piceae* (Col., Curculionidae, Scolytinae) was non-pathogenic to *Abies alba* seedlings (6). Čížková (2005) reported that the *Quercus* inhabiting *G. langdonii* and *G. pallida* inhibited the growth of *Lepidium sativum*, but were non-pathogenic to *Quercus* seedlings. Scala et al. (2007) found that the strain of *Geosmithia pallida* obtained from wilting *Ulmus* in Italy possessed a cerato-ulmin toxin, the protein involved in Dutch elm disease (DED). This strain was unable to cause DED symptoms on inoculated *U. glabra* trees. Nevertheless *Geosmithia* spp. co-occur regularly with phytopathogenic *Ophiostoma* species on elms and their contribution to DED complex is little explored and deserves further study.

We identified a set of morphological and molecular genetic characteristics of *Geosmithia* isolates collected from *J. nigra, J. major, J. californica* and *J. hindsii* throughout the western USA that were unique from published and unnamed *Geosmithia* species in the collection of the first author. The species from *Juglans* is described here as *Geosmithia morbida* sp. nov.

Materials and Methods

Fungal Cultures and Fungal Morphology

Thirty seven fungal isolates from *Juglans* spp. throughout the western United States (AZ, CA, CO, NM, ID, OR UT, WA) were isolated on ½ strength PDA (½ PDA, Difco Corp., Sparks, MD) containing 100mg/L chloramphenicol and streptomycin sulfate from *Juglans* spp. exhibiting disease symptoms (21). These single spore strains were characterized using ITS rDNA sequences (Fig. 2.1) and a subset of 12 strains representing main ITS rDNA haplotypes and places of origin were selected for the morphological characterisation (Table 2.1). The ex-type

and other representatives of *Geosmithia* from walnut were deposited in the Culture Collection of Fungi (CCF), Prague, Czech Republic and Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. A dried herbarium specimen of the holotype was deposited in the herbarium of the Mycological Department, National Museum in Prague (PRM). All measurements and observations were done on fungal structures grown 7-14 d on malt extract agar (MEA, malt extract Oxoid 20 g L-1, glucose 20 g L-1, peptone 1 g L-1, agar 20 g L-1) and incubated in the dark or in incidental light at 25°C. Other media used for colony descriptions were Czapek yeast agar, (CYA) Czapek-Dox agar (CDA) (12) or ½ PDA. Fungal structures were mounted both in lactophenol with cotton blue and in water for better observation of the cell wall surface. Structures were examined with phase and differential interference contrast (Olympus BX-51 with digital camera) and measured using Quick Photo® software. Measurements are reported as the maximum, average and minimum values of 50 observations.

Temperature Studies

Isolates CBS124663 and CBS124664 were grown on sterile wheat seeds for 10 days at 21 ^{o} C in a manner previously described (22). A 3-mm-diameter sterile cork borer was used to remove an agar plug from the center of an 80-mm-diameter Petri plates containing ½ PDA and a colonized wheat berry of one of the *Geosmithia* isolates was inserted. Three plates of each isolate were then incubated in the dark at 13, 21, 25, 30 or 41 ^{o}C and the maximum diameter of fungal growth was recorded daily. The experiment was repeated with similar results. The growth of *Geosmithia* isolates on MEA and CYA at selected temperatures were determined by measuring diameter growth at 7 and 14 d after placement of agar containing mycelium in the center of the plate.

DNA Analysis

Single spore isolates were grown in yeast extract broth for 5-7 days on a rotary shaker and the mycelium collected. Genomic DNA was isolated from the mycelium using Easy DNA Kit (Invitrogen Carlsbad, CA) according to the manufacturer's instructions. A 560 bp nuclear rDNA fragment containing the internal transcribed spacers (ITS1 and ITS2) and 5.8S subunit was amplified with the universal primers ITS1 and ITS4 according to White et al. (1990). PCR products were purified using Pure Link PCR Purification kit (Invitrogen Carlsbad, CA) and sequenced directly using ABI 3130xL Genetic Analyzer to process sequencing samples prepared with ABI's BigDye® Terminator v3.1 sequencing chemistry. A genotype network of the strains from *Juglans* was constructed by using statistical parsimony in TCS 1.21 (2). Sequences were aligned to representatives of published *Geosmithia* spp. in MUSCLE (4). Maximum likelihood (ML) analyses were performed in PHYML (4) using default settings and Bayesian analyses were performed with MrBayes v3.1 (17). The latter was based on the substitution models determined using MrModeltest 2.2 (14), metropolis-coupled Markov chain Monte Carlo search algorithm with 1,000,000 generations, and calculation of Bayesian posterior probabilities after discarding a burn in of 500 generations. Analyses using Minimum evolution (ME) method and LogDet distance algorithm were performed in MEGA 4 (13). All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). Support for branches in ML and NJ trees were estimated using 1,000 bootstrap replicates. *Acremonium alternatum* Link, ancestrally related to *Geosmithia* (18), was chosen as the outgroup. Sequences were submitted to GenBank (see Fig. 2.1 and Table 2.1 for Accession numbers) and sequence alignment was deposited in the Treebase (www.treebase.org/) database under the submission code SN4939.

Results

We detected eight rDNA ITS haplotypes from 37 *Geosmithia* isolates collected throughout the western USA (Fig. 2.1). The aligned ITS rDNA haplotypes of the *Juglans G. morbida* isolates showed six variable positions (five of them in the poly-C region) in the ITS1 region. Distribution of haplotypes was not correlated with the geographic site or the *Juglans* species from which isolates were collected. Multiple haplotypes were present in each state and tree species, whereas identical haplotypes often were found in different states and species. A subset of isolates exhibiting the various ITS rDNA haplotype sequences were then compared to sequences of species and unnamed isolates of *Geosmithia* from other hosts (Fig. 2.2). This manually adjusted ITS alignment contained 47 *Geosmithia* sequences each containing 582 nucleotid bases (including alignment gaps). Of these, 430 were conserved, 143 were variable and 85 were parsimony-informative. The ME, ML and MP analyses showed agreement in topology of highly supported nodes only (≥60, 0.7). Sequences of *Geosmithia* from *Juglans* formed a well-supported clade that was distinct from all other *Geosmithia* spp. Strains from *Juglans* together with *G. fassatiae* and *G. flava* formed a well supported phylogenetic group characterized by white to yellow colonies and association with bark beetles living on hardwoods. Strains from *Juglans* represent an easily morphologically recognisable and homogenous group, distinct from other species. Variability in growth rate, and conidial and phialide sizes were noted but these differences were not correlated with specific haplotypes identified by ITS sequences (Table 2.1).

Growth on ½ PDA was faster than on MEA and was optimal at 31°C with limited growth at 41°C (Fig. 2.3). In a subsequent study we did not detect any growth of isolates CBS124663 and CBS124664 on ½ PDA at 48 °C (data not shown). However, when the colonized wheat seeds exposed to 48 °C were transferred to fresh ½ PDA and returned to 31°C mycelial growth resumed normally.

Taxonomy

Morphological characteristics and DNA sequence analyses of the *Juglans* isolates indicated they represent a single and undescribed species which is described here.

Geosmithia morbida M. Kolařík, E. Freeland, C. Utley, & N. Tisserat sp. nov. MycoBank Will be added Fig. 2.4

Coloniae in agaro matli (MEA) post septem diebus in 25°C diam 18–40 mm attingentes; post septem diebus in 37°C diam 15–25 cm cresecentes; in agaro Czapekii (CDA) conidia non germinantia; pars aversa coloniae culturarum MEA velutina; conidiogenesis flava. Conidiophora penicillata, verruculosa, biverticillata usque pentaverticillata; stipes 20–250 µm longi; penicilli 30–60 µm longi; conidia cylindrica, (4.0–)4.5–6(–8) × (1.5–) 2(–2.5) µm; portata in columnis usque 200 mm longis.

Etym.: refers to its pathogenicity to *Juglans nigra*

Specimens Examined:

USA. Colorado: Boulder, 40°00´41.45´´N, 105°16´07.57´´W, elev. 1620 m. Isolate from an adult of *Pityophthorus juglandis*, VII-2008, N. Tisserat, (HOLOTYPE PRM 915940, ISOTYPES PRM 915941-2, culture ex-type CCF3879, CBS124664). For additional material examined see Table 2.1.

Habitat: galleries of *Pityophthorus juglandis* and adjacent phloem

Distribution: Western USA from California to Colorado

Teleomorph: Unknown

Colonies on MEA attaining diam of 18–40 mm in 7d, 30–55 in 14d at 25°C; 15-25 in 7d and 30-40 in 14d at 37°C. On CYA at 25°C attaining diam 30–45 in 7d, 55–65 in 14d. No growth observed on CDA. Colony on MEA highly lobate, low and plane; mycelium hyaline; substrate mycelium dense, monilioid, often with numerous yeast-like and inflated globose cells (5–10 µm, originating from conidia) and conidia produced in the medium (substrate conidia) forming together a tough mass resulting in a slimy appearance of young colonies; yeast colonies also may originate from conidial suspensions placed on MEA; conidiogenesis moderate, ochre yellow; exudate absent; soluble pigment yellowish to ochre; reverse yellowish to ochre. Colony at 37°C differing by a regular margin, presence of sparse aerial mycelium and cream sporulation. Colony on CYA with regular or slightly lobate margin, plane and low, surface consisting of sterile substrate mycelium or forming floccose areas with abundant sporulation in the central area and highly floccose and less sporulating marginal area; conidiogenesis low to moderate, cream; exudate absent; soluble pigment ochre to light brown; reverse light brown to dark brown. Conidiophores roughened to distinctly verrucose, penicillate; stipe 20–200 × 2.5–3 µm, base (peg foot) consisting of curved and atypically branched cells (Figs. 2.4q, s, v) or of single or

several inflated globose cells (Fig. 2.4m); penicillus 30–60 µm, terverticillate or quaterverticillate, rarely more branched, symmetric or asymmetric, rami (1. branch) 15–35 × 2–3 µm, metulae (last branch) 9–11 × 2–2.5 µm, phialides 8–15 × 2–2.5 µm, 3–6 per cluster; conidia narrowly cylindrical to ellipsoid (4.0–)4.5–6(–8) × (1.5–)2(–2.5) µm, in persistent chains up to 200 µm long, conidial chains tangled, not parallel and forming a compact crust; synanamorph with conidial heads on acremonium-like conidiophores present (substrate conidia according to Kolařík et al. 2004[12]), cylindrical to ellipsoidal with truncate base, 8–15 × 2–4 µm.

Intraspecific Variability.

The above description is a consensus of all strains. Intraspecific variability included differences in conidium and phialide size and shape and colony growth rate (Fig 2.2, Table 2.1). Conidial sizes (arithmetic mean) varied from 5 × 2 µm (with phialides 8–10 × 2 µm) in CBS124664 to 5.6 × 2.35 µm (with phialides 9.5–15 × 2.5 µm) in 1276 (Fig. 2.4i). Size of conidia also varied within a single strain; e.g. in strain 1260 chains of conidia with diameters of 5.0 µm or 6.5 µm were observed

Differential Characters

The species exhibits three remarkable differential characters. It is unable to grow on CDA that is otherwise characteristic for *Geosmithia* associated with bark beetles infesting trees from the family Pinaceae (unpublished), but is unique among *Geosmithia* from hardwoods. The presence of growth at 37°C and thermotolerance is an unusual character typically found only in red-spored *G. lavendula* and its relative *Geosmithia* sp. 19 (7). The base of the condiophores often are atypically branched or monilioid.

G. morbida is similar in colony color and micromorphology to *G. flava* and the unnamed *Geosmithia* sp. 13 (11) but is easily distinguished from these species based on ITS rDNA data. These species occur in the same geographic region as *G. morbida* but have different hosts. *Geosmithia* sp. 13 has similar yellowish colonies on MEA with a lobate margin and identical arrangement of conidial chains but differs by its slower growth (MEA, 20–25 mm, 14 d at 25°C). Colonies of *G. flava* tend to have regular margins and abundant sporulation with parallel conidial chains forming a deep and compact crust.

Discussion

G. morbida is presented here as a genetically variable but morphologically and ecologically homogenous species clearly separated from other species. This is the first report of a phytopathogenic species in this genus and in the Bionectriaceae. Members of this family typically are fungicolous, myxomyceticolous, coprophilous or saprotrophic on plant material (19).

G. morbida has only been isolated from *P. juglandis* or from necrotic phloem associated with *P. juglandis* feeding on *Juglans* species in the western United States. *G. morbida* was not isolated from 33 species of subcortical insects associated with 40 plant species representing all main tree hosts and *Geosmithia* insect vectors in the same area (CA, CO) during a survey in 2009 (unpublished data). The host range of *G. morbida* is thus limited to *P. juglandis* (or to other non-studied bark and wood boring insects associated with *Juglans*).

Variability in the ITS rDNA sequence in *Geosmithia* is species dependent and cannot be used as the sole criterion for species identification. An extreme example of minute variability in rDNA between species is *G. microcorthyli* and *Geosmithia* sp. 8. These species can be clearly

distinguished based on morphology, host range, distribution, β-tubulin or TEF-1α sequences, but they have identical ITS rDNA sequences (8). In contrast, a comprehensive analysis of *G. lavendula* populations from Euroasia and Africa revealed seven haplotypes and 4.3% variation in ITS rDNA sequence (unpublished). Similarly, *G. morbida* has 1% variability in rDNA and at least eight haplotypes. These haplotypes are not correlated with phenotypic characters, geographic origin of the isolates or their plant hosts. The apparent complex genetic structure of *Geosmithia* suggests that its presence in the western United States was unlikely the result of recent introduction events and that fungus and beetle may have been established outside the range reported by Wood and Bright(1992) for some time. More detailed population genetics study supported by multilocus data and detailed sampling should elucidate the evolutionary history of this fungus.

Tables and Figures

Table 2.1. Characterisation of the strains used in the description of *Geosmithia morbida.* Only characters showing intraspecies variability are presented.

Source	Origin, host,collection´s date, collector	Growth rate [mm] on MEA, 25°C, 14d	Conidium size [µm]	GenBank Accession Number
1268	CA, *J. californica*, 2008 ,Seybold	30	(4.5–)5.3(–6) × 2	FN434076
1260	OR, *Juglans* sp., 2008 Pscheidt		(4.5–)5.3(–8) × 2	FN434075
1271	CO, *J. nigra*, 2008, Utley	38	(4.3–)5.0(–5.5) × 2	FN434077
1223	UT, *J. nigra*, 2008, Funk,	50	(4.3–) 5.0(–5.5) × 2	FN434080
1276	CO, *J. nigra*, 2008, Utley	30	(4.6–)5.6(–7) × 2.3	FN436020
CCF3880 (1234)	AZ, *J. major*, 2008,Cranshaw,	50	(4–)4.7(–5.2) × 2.2	FN434072
CBS124663 (CCF3881, 1217)	CO, J. nigra, 2007, Tisserat	30-45	(4–)4.7(–6)×2.2	FN434082

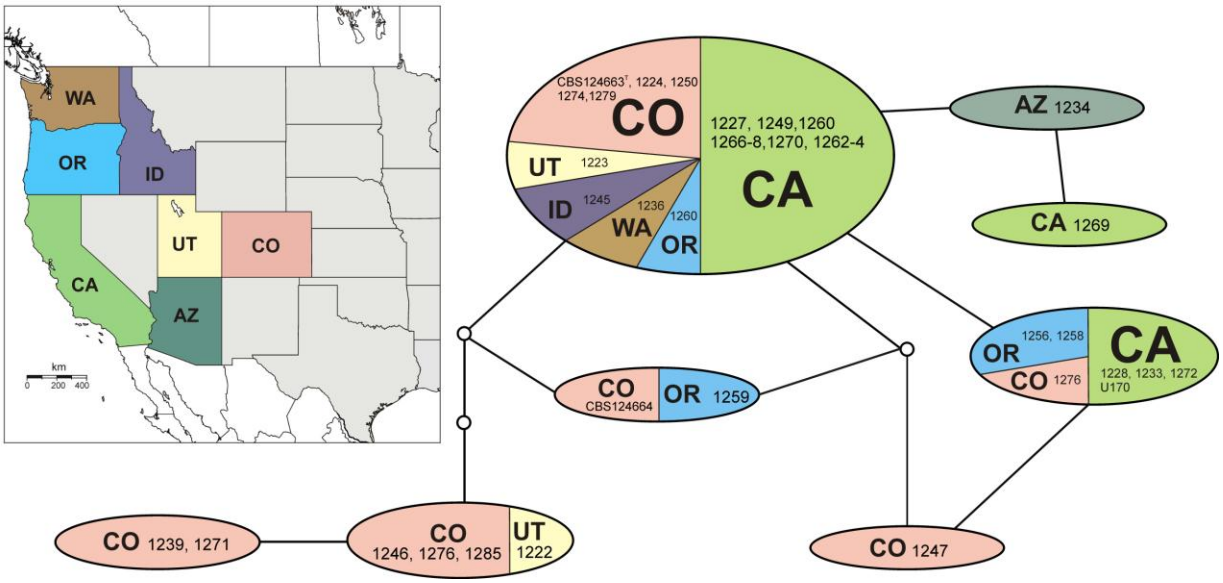

Fig. 2.1. Haplotype network of *G. morbida* ITS rDNA from 37 strains. The colored circles refer to haplotypes detected in the samples and their size is proportional to the sampled haplotype frequency. The shadings indicate the geographical locations as specified in the map. Isolates were collected in the states of of Arizona (AZ), California (CA), Colorado (CO), Idaho (ID), Oregon (OR), Utah (UT) and Washington (WA). Hosts of the isolates were as follows: 1227, 1228, 1261- 1264, 1266-1270 and 1272 from *J. californica*; 1233 from *J. hindsii*, 1234 from *J. major*, and 1258-1260 from undetermined *Juglans* species or hybrids; all other isolates were recovered from *J. nigra*. The line between the haplotypes represents one base change and the very small circles represent haplotypes not present in the sample. GenBank accession number representing each haplotype are given below the circles.

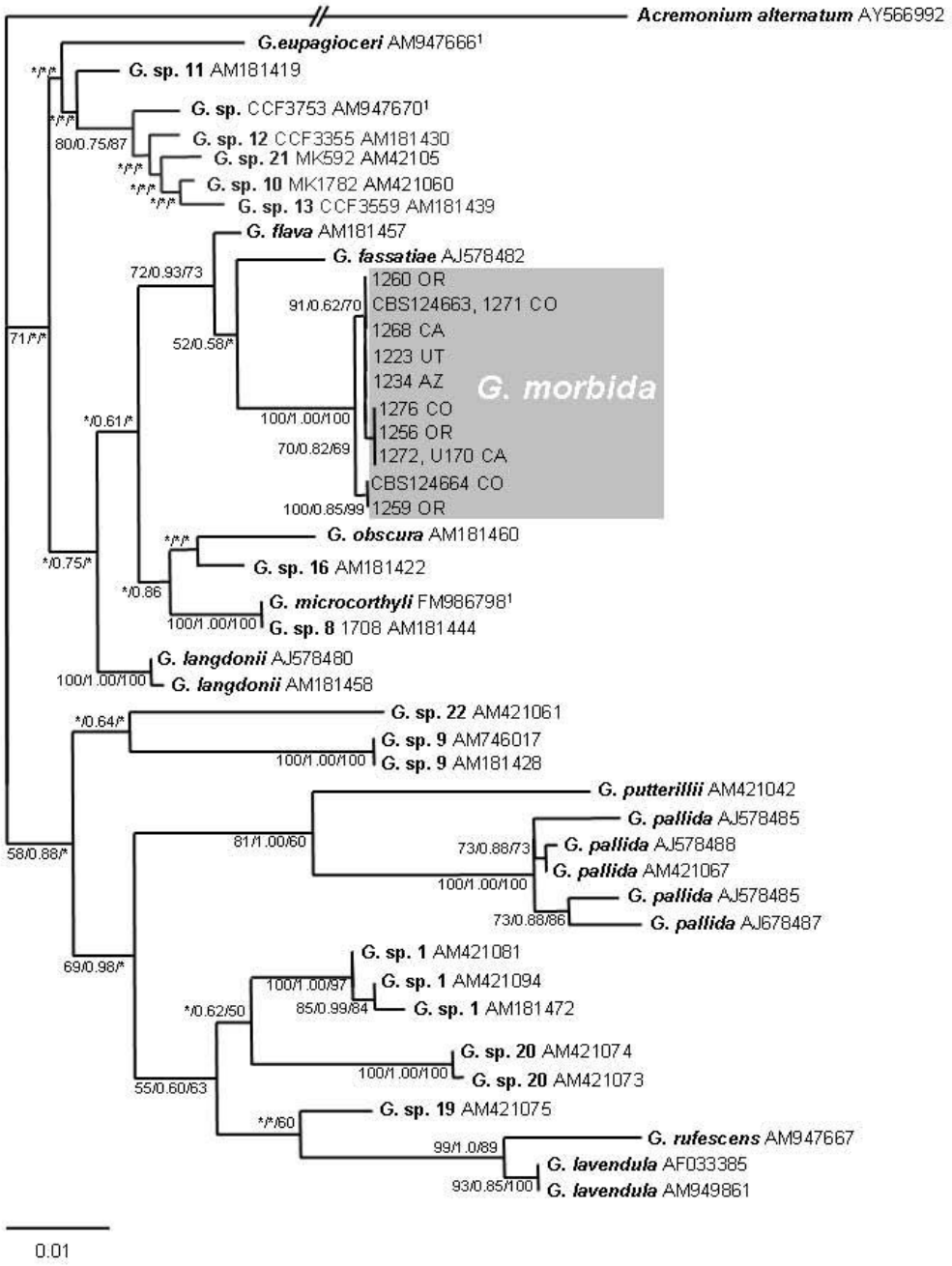

Fig. 2.2. A phylogeny of *Geosmithia* based on ITS rDNA sequence data representing all published species and *G. morbida*. The best tree resulting from heuristic maximum-likelihood analysis in PHYML is presented. Numbers beside the internal nodes are maximum likelihood bootstrap, Bayesian MCMC posterior probabilities and Minimum evolution bootstrap. Branch leading to the outgroup sequences is one-fourth actual length. The species numbering is from Kolařík et al. (2007; 2008).

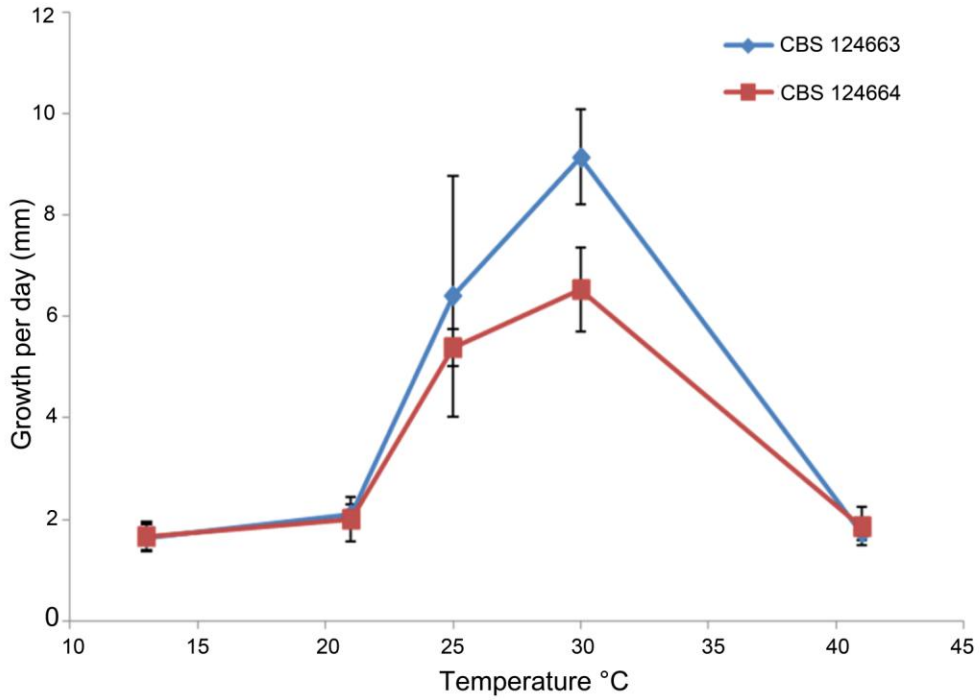

Fig. 2.3. Average daily diameter growth of *G. morbida* isolates CBS124663 and CBS124664 on ½ PDA at various temperatures. Bars represent the standard errors.

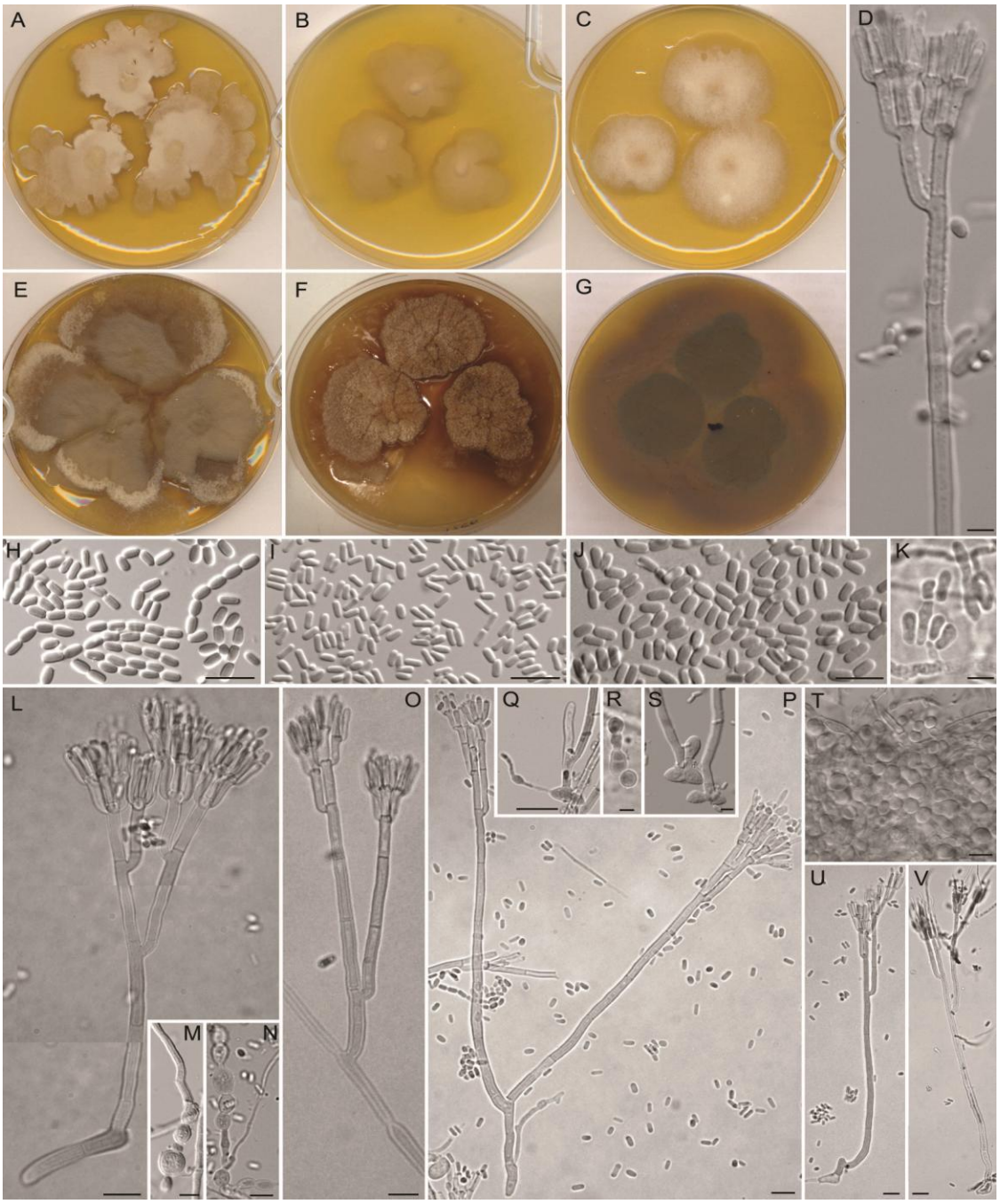

Fig. 2.4. *Geosmithia morbida*. Two-week old colonies grown on MEA (A–C) and CYA (E, G) (at 25°C unless otherwise noted): A. CBS124664. B. CBS124663. C. CBS124664 (37°C). E. CBS124664. F. 1256. G. 1234. Conidiophores: D. 1221. L. 1260. O. 1268. P. 1271. U. 1271. V. 1268, phialides are bearing hyphae instead of conidia. Conidia: H. CBS124663. I. 1276. J. CBS124664. Substrate conidia: K. 1260. Conidophores´ bases: M, Q. 1217. S. 1276. Monillioid mycelium and budding and inflated cells forming the basis of the colony: N. 1221. r. CBS124664. Yeast stage: T. CBS124663. Bars: D, K, R, S = 5 µm; H–J, L–Q, T–V = 10 µm.

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CHAPTER III. Susceptibility of Walnut and Hickory Species to *Geosmithia morbida*.

Preface

In this Chapter we evaluated many other Juglandaceae family trees to determine if members of *Carya* and *Juglans* species were susceptible to thousand cankers disease as compared to a control wound and *J. nigra*. We felt it was important to determine what tree species may serve as potential alternative hosts of *G. morbida.* I designed and tested the inoculation technique using a core tool to remove a precise circle of bark that was used throughout this study. I conducted all of the greenhouse experiments as well as the Colorado and Utah/Idaho field experiments between February 2008 and April 2010 and wrote up my initial findings as part of a larger manuscript written collaboratively. This manuscript has now been accepted for publication Titled: Susceptibility of walnut and hickory species to *Geosmithia morbida*. Plant Disease: in Press. By, [3]Utley, C., Nguyen, T., Roubtsova, T.V., Coggeshall, M. Ford, T. C., Grauke, L.J. Graves, A.D., Leslie, C.A, McKenna, J., Woeste, K., Yaghmour, M.A., Cranshaw, W., Seybold, S.J., Bostock, R.M., and Tisserat, N.

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Introduction

Juglans nigra, commonly called black walnut or eastern black walnut, is one of the most highly valued timber species in North America. The wood is prized for use in cabinetry, gunstocks and other finished wood products, the nuts are an important nutritional source for wildlife, and the nut shells are used for a variety of industrial applications (17, 26). The greatest volume of black walnut growing stock on timberland, is found in Missouri, Ohio, Iowa, Indiana, Illinois, Tennessee, West Virginia, Kansas, Pennsylvania, Virginia, and Michigan (18, 26). In addition to the multi-million dollar US market for walnut wood, walnut lumber and logs are exported internationally to over 45 countries (33).

Juglans nigra

Juglans nigra is not native to the western U.S., but it has been widely planted there as an ornamental and nut-bearing tree, and to a limited extent for wood products. In the early 1990's *J. nigra* mortality was observed in Oregon and Utah but the cause was not determined (22, 30). Subsequent death of *J. nigra* in the late 1990's in Utah (22) and in 2001 in New Mexico was attributed to drought and attack by the walnut twig beetle (WTB), *Pityophthorus juglandis* (32). In 2008 *J. nigra* death in Colorado was determined to be the result of aggressive feeding by the WTB on large branches and even the trunk, and subsequent canker development around beetle galleries caused by a newly described fungal symbiont of the beetle, *Geosmithia morbida* (15, 29). *Fusarium solani* may also contribute to tree mortality during the latter stages of TCD by forming cankers on the main trunk (29). The disease was given the common name of thousand cankers disease (TCD) because of the enormous number of coalescing cankers that are formed in the bark of severely affected trees (29). The WTB and TCD is now widespread in *J. nigra* in the western United States and has caused extensive mortality (5,30).

The disease recently has become established in the native range of this species in Tennessee, Virginia and Pennsylvania (9,13,20,36) and poses a potential threat to J. nigra in those states.

Other Juglans Species

Sixteen walnut species are native to the Americas and six of these, including *J. californica* (southern California black walnut), *J. cinerea* (butternut), *J. hindsii* (northern California black walnut), *J. major* (Arizona walnut), *J. microcarpa* (little walnut) and *J. nigra* have ranges in the U.S. (16, 28) and are potentially at risk from TCD. The WTB is native to *J. major* in AZ and NM (2), and perhaps to *J. californica* in CA (3), but has expanded its range to include 90 U.S. counties (71 in the West and 19 in the East) in 12 states (24). *G. morbida* has been consistently isolated from small, superficial cankers surrounding the beetle galleries in *J. major* throughout its range in AZ and NM (Tisserat and Bostock, unpublished data) but the fungus has not caused widespread dieback or mortality in this species (5,11) as it has with *J. nigra*. Similarly, *G. morbida* has been isolated from WTB galleries in declining *J. californica* and *J. hindsii* in California (6, 24), but the relative susceptibility of these species to the fungus from controlled experiments has not been reported previously. TCD has recently been reported in a single *J. cinerea* tree in Oregon (23). The *J. cinerea* population throughout the eastern USA and Canada has been decimated by butternut canker caused by *Ophiognomonia clavigignenti-juglandacearum* (1, 4, 19) and further damage or mortality caused by TCD could be serious. Other walnut species exotic to the U.S. also may be impacted by TCD. *J. regia* (Persian or English walnut), native to Eurasia, is widely planted in North America and is a commercially important orchard crop in California. *G. morbida* has been isolated from individual *J. regia* trees exhibiting cankers in California orchards, but the potential impact of TCD on this crop is still unknown (6,12).

J. ailantifolia (Japanese walnut), introduced into North America from Asia, has become naturalized in areas of the eastern USA and in some locations has hybridized with *J. cinerea* (14).

Carya Species

Numerous hickories (*Carya* spp.), also members of the Juglandaceae, are native to North America and are important ecologically and commercially. For example, *Carya illinoinensis* (pecan) is native to the southern USA but is widely planted for nuts outside its natural range. In 2011, over 251 million pounds of pecans (in shell) were harvested (30). TCD has not been reported in any hickory species, although the ability of WTB and *G. morbida* to colonize hickories without causing significant damage could have important epidemiological consequences (e.g., serving as an inoculum reservoir) since many of their ranges overlap with those of *J. nigra* and *J. cinerea*. The objective of this study was to determine the relative susceptibility of selected walnut and hickory species to *G. morbida*. A preliminary assessment of some species was published (34).

Materials and Methods

Greenhouse Inoculations

For all greenhouse experiments, one- to two-year-old dormant trees were planted between February and April in each year in 3.8 or 7.6 L plastic pots containing a soilless mix (P4, Conrad Fafard, Inc., Agawam, MA). Trees were placed on a greenhouse bench at 20-25 °C and allowed to resume growth for at least one month prior to inoculation.

G. morbida isolates 1217(CBS 124663) and 1218 (CBS 124664), originally isolated from *J. nigra* in Colorado, were used as inocula in all experiments in Colorado. Isolates were grown

on half-strength PDA (½ PDA) for approximately 7 days prior to use. For inoculations in all greenhouse experiments, wounds were made in the bark as deep as the xylem in the main stem of each tree by using a 3-mm-diameter metal punch. Wounds were spaced approximately 10-15 cm apart, with the lowest wound on the stem approximately 15 cm from the tree base. The number of wounds varied with the experiment. Following wounding, bark inside the wound was removed and replaced with ½ PDA or ½ PDA colonized by one of the *G. morbida* isolates. Wounds were sealed with Parafilm® and trees were placed on the greenhouse bench in a randomized complete block design. Parafilm® was removed 3 wk after inoculation. After 6 wk stems were harvested and the outer bark was removed to expose phloem necrosis associated with wounding and/or canker development. Discolored areas were determined from digital images by using the Java software ImageJ 1.42; (http://rsb.info.nih.gov/ij/index.html). Canker areas that developed following inoculations with *G. morbida* were compared by paired *t*-tests to areas of discoloration associated with wounds in which ½ PDA was inserted. Canker area differences among tree species resulting from *G. morbida* inoculations were compared by analysis of variance (MINITAB 14, http://www.minitab.com/en-US/).

In May 2009, walnut and hickory species were obtained from various sources (Table 3.1). In some cases, the set of trees representing certain species (e.g. *C. illinoinensis* and *J. nigra*) were grown from open-pollinated nuts and therefore were not necessarily related to one another. In other species (e.g. *J. californica*) replicate trees were grown from nuts collected from a single parent tree. The set of maternal-half sibling trees representing these species were designated as families. Four wounds were made in the stems of five *C. ovata* (shagbark hickory) trees and six trees each of the other species. Stem diameters ranged from 4-11 mm. Bark plugs in the second and third from the bottom wounds were removed and replaced with plugs of ½ PDA or ½ PDA

colonized by *Aspergillus niger*, respectively. This fungus, commonly found in WTB galleries, was included as a control to compare wound discoloration associated with a non-pathogenic fungus to that of *G. morbida*. Holes at the other two wound sites were filled with ½ PDA colonized by one of the two *G. morbida* isolates such that each isolate was placed in the top hole on three replicate trees and the bottom hole on three others. In September 2009 a second set of open-pollinated or maternal half-sibling trees representing the same species, and collected from the same sources, were inoculated and incubated in the same manner.

In May 2010, stems from six arbitrarily-selected trees representing six maternal, half-sibling *J. nigra* families supplied by the Hardwood Tree Improvement and Regeneration Center (HTIRC, USDA Forest Service, Purdue University West Lafayette IN, 47907), were wounded in three places. A ½ PDA plug was inserted in the bottom wound of each tree and ½ PDA plugs colonized by the two *G. morbida* isolates, were randomly placed in the other two wounds. The experiment was repeated in September 2010 by inoculating six additional trees from the same families. In a separate September experiment, but using the same methods, three trees each of *C. illinoinensis* 'Peruque' and 'Riverside' and 6 trees of *C. aquatica* (water hickory), obtained from the USDA ARS Pecan Breeding & Genetics collection (Sommerville, TX, 77879), and six trees in each of nine maternal half-sibling *J. nigra* families, obtained from the University of Missouri (Columbia, 65205) were inoculated *with G. morbida*. The experiment was not repeated.

Six trees each in nine *J. cinerea* and three hybrid (*J. cinerea* x *ailantifolia)* families obtained from HTIRC were inoculated in May 2011 and evaluated as previously described except that only one *G. morbida* isolate (1217) was used. The experiment was not repeated.

Field Inoculations

Colorado

In June 2008, two 3-mm-diameter wounds, spaced approximately 15 cm apart, were made with a metal punch on each of four to six branches of a single *J. cinerea, J. mandshurica* (Manchurian walnut) and *J. microcarpa* tree located in the Colorado State University arboretum Fort Collins, 80523). Trees were approximately 20 yr old and the diameters of inoculated branches ranged from 7-15 mm. Half-strength PDA or ½ PDA colonized by *G. morbida* isolate 1217 was randomly inserted into a wound on each branch; wounds were wrapped with Parafilm® and the film was removed after 3 wk. Inoculated branches were cut from the trees after 8 wk and canker areas were determined as previously described.

Utah and Idaho

Field inoculations of walnut and hickory species were conducted in June 2009 at two plantings near Richmond, UT and one planting near Dayton, ID maintained by the Center for Improving Perennial Plants for Food and Bioenergy (IPPFB, Richmond UT, 84333). Trees were 1 to 6 yr old (field age) and had been planted in blocks (half-sibling families) at one location or were arbitrarily scattered (individual species) at the three locations. The number of trees representing each species or half-sibling family within a species that were inoculated varied from 1-5. Three, 3-mm-diameter wounds spaced 10 – 15 cm apart were made on three branches on each tree using a metal punch. Tree branches were approximately the same size (10-20 mm in diameter) and at the same tree height (1-2 m). Half–strength PDA was inserted into the center wound and ½ PDA colonized with *G. morbida* isolates 1222 and 1223, originally isolated from *J. regia* and *J. nigra* respectively at the IPPFB, were randomly placed in either the top or bottom wound. Following inoculation, wounds were wrapped with Parafilm®; the film was

removed after 3 wk. After 5 wk, all inoculated branches were removed from the trees and canker areas were determined as previously described. No differences ($P > 0.10$) in canker size between the two isolates on each tree species or family were detected. Therefore, canker areas formed in response to the two isolates were first averaged for each branch, then for all branches on the same tree. Mean canker area for each tree were then used to determine mean canker sizes for each tree species and family except where only one tree of a species was inoculated. In those cases, mean canker size was determined by averaging canker areas from the three inoculated branches. Inoculations were repeated in June 2011 on some, but not all of the same trees. The number of trees representing each species or half-sibling family within a species that were inoculated varied from 1-6. The experiment was modified from 2009 such that one branch on each tree (two branches for an individual tree representing a species) was inoculated at three locations with *G. morbida* isolate 1222 as described. Half-strength PDA was placed in a fourth wound located at the base of each branch. Branches were harvested in November and canker areas for each species were analyzed as previously described. Cankered areas that formed in response to *G. morbida* on branches within a species or half-sibling family in both years were compared in paired t-tests to areas of discoloration in response to wounds treated with ½ PDA. Canker sizes among species were not directly compared by analysis of variance because the number of tree replicates for each species and family was variable and trees were scattered across three locations.

California

In 2010 five walnut species (*J. ailantifolia*, *J. californica, J. major, J. microcarpa* and *J. regia*) from the *Juglans* Collection of the USDA-ARS National Clonal Germplasm Repository (NCGR), established in 1984 at Winters, CA, were evaluated for susceptibility to *G. morbida*.

On 8 Jul three 5-mm-diameter wounds spaced 10-15 cm apart were made with a cork borer into the bark to the xylem on two branches in each of 5 trees of each walnut species. Branches ranged from 2-4 cm diameter and were 2-4 m above the ground. Half–strength PDA was inserted into the center wound on each branch and ½ PDA colonized with *G. morbida* isolates GM-1 and GM-3, originally isolated from *J. hindsii* and *J. regia* in California respectively, were randomly placed in one of the two outer wounds. Wounds were covered with Parafilm® for the duration of the experiment. On 19 Aug inoculated branches were excised and canker lengths were determined. A second trial on a set of five different trees representing each species was conducted from 21 Jul – 1 Sep 2010. Two additional trials were conducted from 8 Jul to 19 Aug and 26 Jul to 8 Sep in 2011 in a similar manner except that five trees representing *J. hindsii* and *J. mandshurica* were also included at each date. To the extent possible, the same groups of trees representing each species were used in both years. In all trials, the length of discoloration associated with wounds filled with ½ PDA was subtracted from bark necrosis associated with *G. morbida* inoculations. Lesion length data were $\log_{10}$-transformed to establish a normal distribution and then analyzed as a mixed model with PROC MIXED (SAS ver. 9.1; SAS Institute, Cary, NC). Walnut species was treated as a fixed effect, while year, trial, isolate and replicate tree were treated as random effects.

Results

Greenhouse Inoculations

Canker formation in walnut and hickory species in the 2009 experiment was not influenced (P>0.10) by *G. morbida* isolate or by the position of inoculation on the stem; therefore data were pooled for analysis in both experiments (Table 3.1). However, differences in canker development in some species were observed between the two experiments and those

results are presented separately. No cankers developed in *C. illinoinensis* and *C. ovata* following inoculation with *G. morbida*. After 6 weeks, wounds had sealed and the fungus could not be isolated from the callus. In contrast, cankers developed in all inoculated *J. nigra* trees. Cankers, sometimes reaching 12 cm in length, often were irregular in shape with small, finger-like streaks of necrotic phloem radiating from their margins. These cankers were similar to those observed in naturally infected branches. *Geosmithia morbida* was consistently isolated from canker margins in these trials. Reactions of other walnut species to inoculations with *G. morbida* were variable. *Juglans californica*, *J. hindsii*, *J. major*, *J. microcarpa*, and *J. regia* developed small cankers in both experiments and they were larger ($P<0.05$) than the area of discoloration associated with wounds in which ½ PDA had been inserted. *Geosmithia morbida* was consistently isolated from discolored tissue in these species. In general, *J. cinerea* (except in the second experiment) and *J. ailantifolia* developed larger cankers than the other walnut species except *J. nigra*. Inoculation of trees with *A. niger*, a common non-pathogenic inhabitant of necrotic *J. nigra* bark, did not result in greater phloem discoloration ($P>0.10$) than wounds in which ½ PDA had been inserted.

There were no isolate or experiment interaction effects ($P>0.10$) on canker development following inoculation of *J. nigra* trees representing maternal half-sibling families from the HTIRC in 2010, so data from these experiments were combined for analysis. Cankers formed in all trees and differences ($P<0.05$) among maternal half-sibling families were observed. Canker areas in the most susceptible family were approximately 50% larger on average than the least susceptible family (Fig. 3.1). In a similar experiment in the same year, *J. nigra* families from the University of Missouri exhibited variation in mean canker size (Fig. 3.2). No cankers developed in C. *aquatica* or two varieties of *C. illinoinensis* in this experiment. In the experiment conducted in 2011, *J. cinerea* and its hybrids with *J. ailantifolia* also exhibited variation in

canker development (Fig. 3.3). Cankers in OS 60R were only slightly larger (P<0.05) than wound discoloration alone. Trees in other maternal half-sibling families had larger cankers, especially those representing *J. cinerea* hybrids.

Field Inoculations

Colorado

Cankers formed on branches of *J. microcarpa* (169 mm^2), *J. cinerea* (206 mm^2) and *J. mandshurica* (564 mm^2) 8 wk after inoculation with *G. morbida*. Bark necrosis surrounding branch wounds inoculated with *G. morbida* in each tree was greater (P <0.05) than wounds in which ½ PDA had been inserted. *Geosmithia morbida* was consistently isolated from canker margins on each tree in this trial.

Utah and Idaho

Juglans and *Carya* species planted at the IPPFB sites exhibited variable susceptibility to *G. morbida* in 2009 (Fig. 3.4*).* *Carya illinoinensis, C. ovata* and *J. ailantifolia* did not develop cankers; wounds had sealed with callus after 5 wk and *G. morbida* could not be isolated. *Juglans major* and *J. nigra* X *regia* had small necrotic lesions surrounding inoculation sites, but these were not larger (P>0.10) than discoloration associated with the wounding process. Nevertheless, *G. morbida* was isolated from canker margins in each species. Similarly *J. hindsii* and *J. regia* developed small cankers from which *G. morbida* could be isolated. Successively larger cankers were found in *J. microcarpa* and in maternal half-sibling families of *J. nigra*.

Results from the 2011 Utah inoculations were similar to 2009 (Fig. 3.5), except that canker areas tended to be larger in most species as a result of the longer period between inoculation and canker assessment (June-November). No or very little canker formation was

observed in *C. illinoinensis*, *C. ovata* or *J. major*. Small, but significant (P<0.05) areas of necrotic phloem developed at inoculation sites in *J. regia*, *J. hindsii* and *J. nigra* X *regia* and *G. morbida* was isolated from canker margins in this trial. Cankers developed in all *J. microcarpa* and *J. nigra* trees and variation in canker area was observed among the *J. nigra* families representing the different nut cultivars.

California

All walnut species tested at the NCGR developed cankers and *G. morbida* was consistently isolated from canker margins in these trials. There was no effect (P>0.10) of experimental trial, year, or their interactions with canker formation. *Juglans californica* and *J. microcarpa* had the longest cankers, with *J. hindsii*, *J. major*, and *J. regia* intermediate, and *J. ailantifolia* and *J. mandshurica* with the shortest cankers ($P \leq 0.05$; Fig. 3.6). There was a small difference ($P = 0.005$) in canker lengths between the two *G. morbida* isolates, with GM-3 (27.6 ± 1.1 mm) more virulent than GM-1(23.2 ± 1.1 mm).

Discussion

Thousand cankers disease is an emerging tree disease in North America that is capable of damaging multiple walnut species. In this study, we provide an initial comparison of a number of native and introduced walnut species as well as several hickory species for the reaction of their phloem to infection by *G. morbida*. A number of these species and their hybrids are important for rootstock development in commercial walnut production. Since effective disease management will most likely include deployment of resistant host material, identification of the potential variation in this regard among species and breeding lines is important.

Susceptibility to *G. morbida* among species of *Juglans* and *Carya* was assessed by lesion length (CA) or lesion area (CO and UT) following controlled inoculation of defined isolates of *G. morbida*. All *J. nigra* tested developed cankers following inoculation with *G. morbida* and it was the most susceptible of the walnut species. However differences in canker sizes among maternal half-sibling families in both greenhouse and field trials were identified. In most families, cankers extended from the phloem to the cambium and exhibited irregular margins with no indication of a host defense response (e.g., callus formation). Cankers in a few families (e.g., 280) were small and superficial with uniform margins that were bordered by suberized tissue, indicating an induced host defense response. These results support previously observed differences in canker formation among *J. nigra* families following inoculation with *G. morbida* (7, 8). *Juglans nigra* families included in our studies represented maternal half-siblings from trees with superior nut, growth or tree form characteristics; they were not selected based on empirical knowledge of their reaction to *G. morbida*. Thus, variation in canker development among these families was unexpected. Healthy *J. nigra* free of WTB galleries or cankers have been found in CO and UT in areas severely impacted by TCD (30). While these surviving trees have not yet been tested for resistance, their presence, along with results of our inoculation trials, suggest that there may be even greater genetic differences in *J. nigra* to canker formation caused by *G. morbida*. *Juglans nigra* is an open pollinated species with high genetic diversity (35). Its range overlaps or is at least contiguous with that of *J. major* and *J. microcarpa* in the southwestern United States (10). *Juglans major* is a native host of the WTB (2) and is less susceptible to *G. morbida* based on our inoculations and field observations. Introgression of resistance genes from *J. major* to *J. nigra* or from *J. major* to *J. microcarpa* and then to *J. nigra* via hybridization in this region is plausible. For example, natural hybrids between *J. major* and

J. nigra have been observed in TX (10). Further testing of these hybrids as well as other *J. nigra* accessions from TX and throughout eastern North America, will provide insights as to possible sources of resistance to *G. morbida*.

The WTB and *G. morbida* have not been detected in the native range of *J. microcarpa* in eastern NM (11) even though the beetle and fungus are widely distributed on *J. major* in the western part of that state. Exploratory surveys in TX have also yielded no evidence of either organism on *J. microcarpa*. This was surprising because *J. microcarpa* is susceptible to *G. morbida.* Furthermore, WTB galleries and natural cankers caused by *G. morbida* have been observed in *J. microcarpa* at the NCGR planting at Winters, CA, and this species proved to be among the most susceptible of the seven species tested there (Fig. 3.6). It is possible that *J. microcarpa* is isolated geographically from the native range of WTB, and hence *G. morbida*, in NM, although both the beetle and *G. morbida* were collected from a *J. major* tree located in Lincoln County, NM and within the native range of *J. microcarpa*. Alternatively, in native habitats, the WTB may have difficulty locating and colonizing *J. microcarpa* or the beetle may be unable to complete its life cycle on this host. In any case, further surveys for the presence of WTB and *G. morbida* throughout the native range of *J. microcarpa* including TX, OK, and KS are needed.

TCD was recently confirmed in a naturally infected *J. cinerea* in OR (23) and therefore poses a potential threat to this species in its native range. Our results support the general susceptibility of this species to *G. morbida*, although some half-sibling families were resistant. There was a trend for half-siblings of *J. cinerea* hybridized with *J. ailantifolia* to be more susceptible to *G. morbida* and further testing is needed to verify this observation.

If true, this could be important because *J. ailantifolia* is being used in butternut breeding programs as a potential source of resistance to the butternut canker pathogen (37).

In greenhouse trials, trees of both *J. californica* and *J. hindsii* developed cankers following inoculation by *G. morbida* but not to the same extent as *J. nigra*. The WTB was first collected in California from *J. californica* in Los Angeles County in 1959 (3) and since 2008 the insect and *G. morbida* have been widely collected on this species in southern CA (6, 24, 25). Thus, *G. morbida* may have always been associated with *J. californica*, causing cankers and contributing to branch dieback of stressed trees attacked by the WTB much in the same manner it does on *J. major*. The WTB and *G. morbida* are also widespread on declining *J. hindsii* in the lower Sacramento Valley (6, 24, 25) although other factors including mistletoe and drought may be contributing to the general dieback in this region. Rapid mortality in *J. hindsii*, similar to what is occurring in *J. nigra*, has not been observed, perhaps due in part to the lower aggressiveness of *G. morbida* on this host. Nonetheless, among the species tested, both *J. californica* and *J. hindsii* were relatively susceptible to *G. morbida* in branch inoculation trials at the NCGR (Fig. 3.6).

Juglans regia developed cankers following inoculation with *G. morbida*, but its susceptibility varied depending on experiment. These results are consistent with field observations. For example, two *J. regia* trees naturally infected with TCD were killed in CO in 2010, but healthy *J. regia* were found adjacent to *J. nigra* killed by TCD in Richmond UT in the same year. The WTB and *G. morbida* also have been found in *J. regia* trees in orchards throughout the major production areas of California's Sacramento and San Joaquin valleys, and the numbers of damaged trees that have been identified are increasing. Nevertheless, widespread mortality of *J. regia* in CA has not yet occurred and the potential threat of TCD in commercial orchards is still being assessed. *J. regia* trees often are grafted onto a walnut hybrid root stock

called Paradox. Paradox is commonly defined as a hybrid between *J. hindsii* and *J. regia* but may also include hybrids between *J. nigra* or *J. californica* and *J. regia* (21). We tested only a few *J. nigra* x *regia* hybrids in UT and their reaction varied from not forming cankers in one year to formation of small cankers in the next. It is likely that Paradox rootstocks will show substantial variation in susceptibility to *G. morbida* depending on parentage especially if *J. nigra* is used as one of the parents. Further screening of commercial hybrid rootstocks is warranted.

We did not exhaustively test hickory species for susceptibility to *G. morbida*, but *C. illinoinensis*, *C. aquatica* and *C. ovata* did not develop cankers following inoculation nor were we able to isolate *G. morbida* from the callus surrounding wounds. These results are consistent with field observations in CO and CA where mature *C. ovata* and *C. illinoinensis* appeared healthy, with no evidence of WTB galleries or cankers even though they were growing next to *J. nigra* (CO) and *J. hindsii* (CA) respectively, with advanced symptoms of TCD. This indicates these species, and perhaps all hickories, will not be susceptible to *G. morbida* or are they likely to serve as an inoculum reservoir as certain oaks do for the chestnut blight fungus (27).

Our inoculation studies with *G. morbida* have corroborated many of the field observations on susceptibility of hickory and walnut species to TCD. One caveat is that *G. morbida* is incapable of killing walnut species, even the highly susceptible *J. nigra*, on its own. In nature, individual cankers caused by *G. morbida* rarely exceed 20 cm in length and even though the fungus produces enormous numbers of dry conidia that could be airborne, there is no direct evidence that infection occurs anywhere on trees except at WTB feeding sites or in and around galleries. Thousands of coalescing cankers resulting from inoculations by the WTB are necessary to kill a mature tree.

Therefore the ability of the WTB to successfully find, colonize and breed in walnuts is also an important component in TCD resistance in *Juglans* species or genotypes. Ongoing research at the NCGR is documenting the potential for differential host selection behavior by the WTB on many walnut species.

Tables and Figures

Table 3.1. Canker development in hickory and walnut species six weeks after inoculation with *Geosmithia morbida* in the greenhouse in June and September 2009.

Species	Genotype[x]	Source	Canker area (mm^2)[y]	
			June	September
Carya ovata	Open pollinated	Cold Stream Farm, MI	4.3 a	--
Carya illinoinensis	Open pollinated	Kansas State Forest Nursery	12.8 a	8.6 a
Juglans major	Open pollinated	Walnut Creek, AZ	10.9 a	28.3 a
Juglans hindsii	Multiple families	UC Davis, CA	18.9 a	40.4 ab
Juglans californica	Family B-2-23	UC Davis, CA	19.2 a	111.0 c
	Family A-3-24		37.2 ab	----
	Family A-3-40		---	72.8 bc
Juglans microcarpa	Open pollinated	Concord, NE	30.0 a	19.9 a
Juglans regia	Open pollinated and multiple families	Cold Stream Nursery, TN, UC Davis CA	36.0 a	26.0 a
Juglans ailantifolia	Multiple families	Univ. TN	66.0 b	79.7 c
Juglans cinerea	Multiple families	Univ. TN	82.2 bc	30.4 a
Juglans nigra	Open pollinated	Kansas State Forest Service, KS	121.3 c	191.2 c

[x]Maternal half-sibling trees within a family were grown from nuts collected from a single tree. In the case where only two or three individuals of a family were obtained, trees from multiple families were arbitrarily selected for each experiment. Trees were 1-2 yr old (4-11 mm stem dia.) at the time of inoculation.

[y]Means in same columns not followed by the same letter are different ($P = 0.05$) by Fisher's LSD test. The area of phloem discoloration caused by *G. morbida* was greater ($P \leq 0.05$) in all species except *C. ovata* in June and *C. illinoinensis* in June and September than discoloration surrounding wounds in which half-strength potato dextrose agar was inserted.

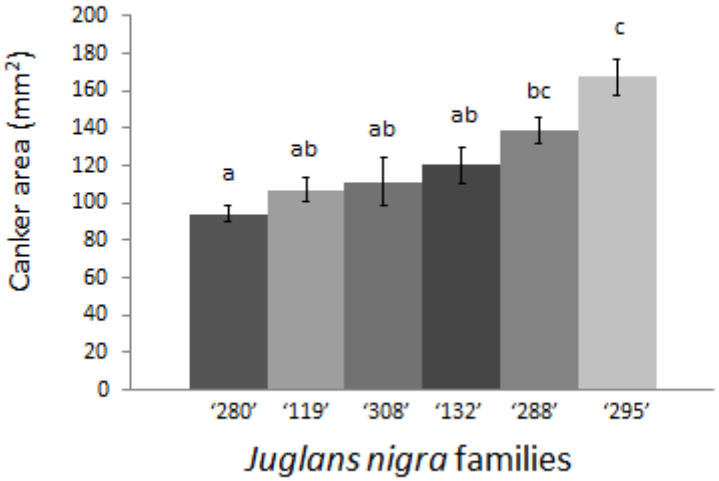

Figure 3.1. Canker development in *Juglans nigra* trees representing maternal half-sibling families 6 wk after inoculation with *Geosmithia morbida* in the greenhouse in 2010. Column means not followed by the same letter are different (P=0.05) by Fisher's LSD test and represent canker areas averaged across two *G. morbida* isolates and two experiments. Bars indicate standard errors.

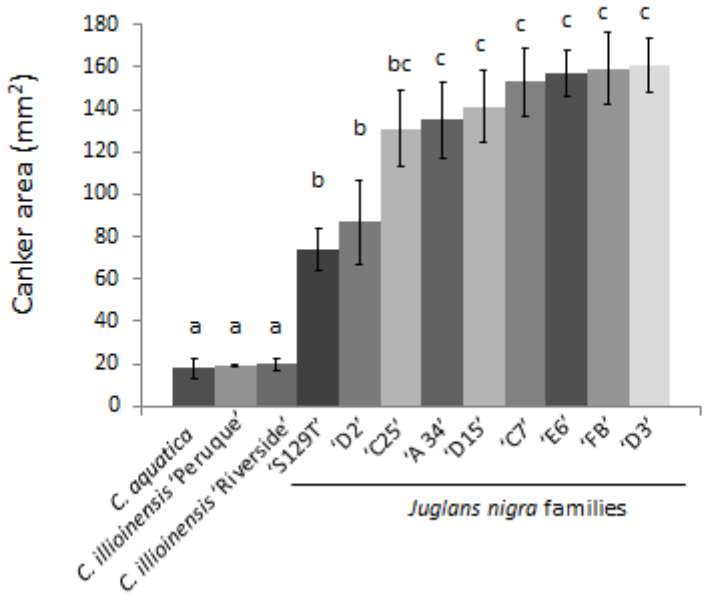

Figure 3.2. Canker development in *Carya aquatica*, *C. illinoinensis*, and *Juglans nigra* trees representing maternal half-sibling families 6 wk after inoculation with *Geosmithia morbida* in the greenhouse in 2010. Column means not followed by the same letter are different (P =0.05) by Fisher's LSD test and represent canker areas averaged for two *G. morbida isolates*. Bars indicate standard errors.

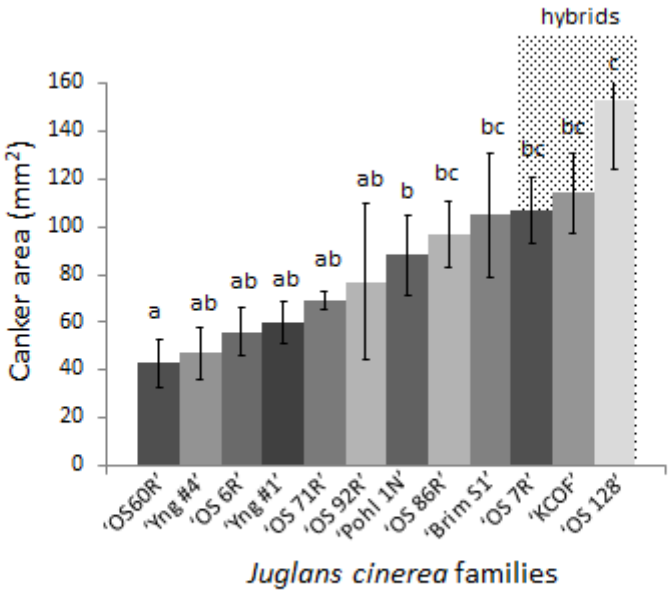

Figure 3.3. Canker development in *Juglans cinerea* and *J. cinerea* x *ailantifolia* hybrid (shaded columns) trees representing maternal half-sibling families 6 wk after inoculation with *Geosmithia morbida* in the greenhouse in 2011. Column means not followed by the same letter are different (P =0.05) by Fisher's LSD test and represent canker areas averaged for two fungal isolates. Bars indicate standard errors.

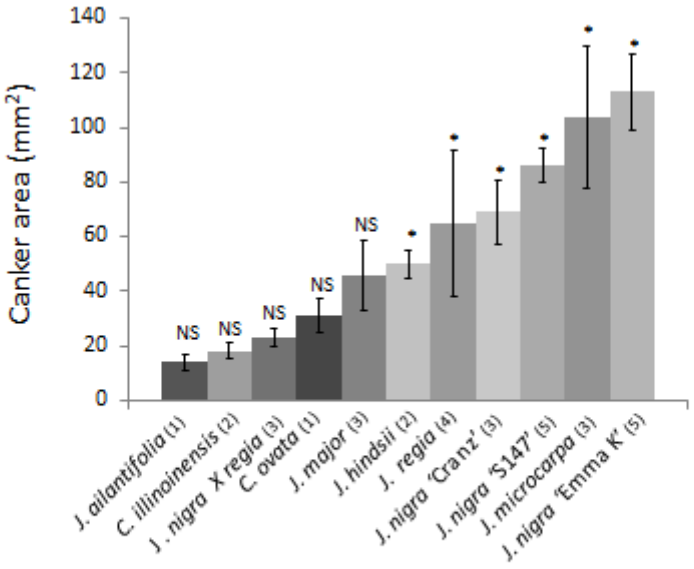

Figure 3.4. Canker development in branches of hickory and walnut species 5 wk after inoculation with *Geosmithia morbida* at the Center for Improving Perennial Plants for Food and Bioenergy field planting, Utah in May 2009. Bars represent standard errors and * ($P \leq 0.05$) = significant and NS ($P > 0.10$) = non-significant differences in bark necrosis around wounds inoculated with *G. morbida* compared to wounds amended with half-strength potato dextrose agar. Numbers in ( ) following species or family name represent the number of replicate trees inoculated.

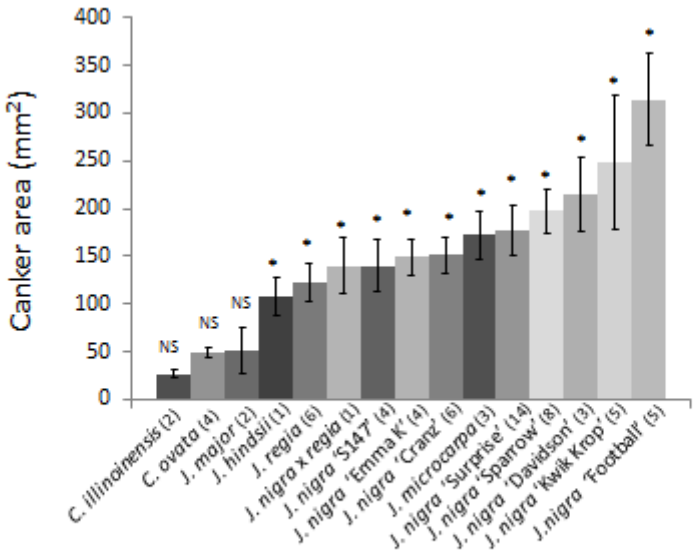

Figure 3.5. Canker development in branches of hickory and walnut species 5 mo after inoculation with *Geosmithia morbida* at the Center for Improving Perennial Plants for Food and Bioenergy field planting, Utah, June 2011. Bars represent standard errors and * ($P \leq 0.05$) = significant and NS ($P > 0.10$) = non-significant differences in bark necrosis around wounds inoculated with *G. morbida* compared to wounds amended with half-strength potato dextrose agar. Numbers in ( ) following species or family name represent the number of replicate trees inoculated.

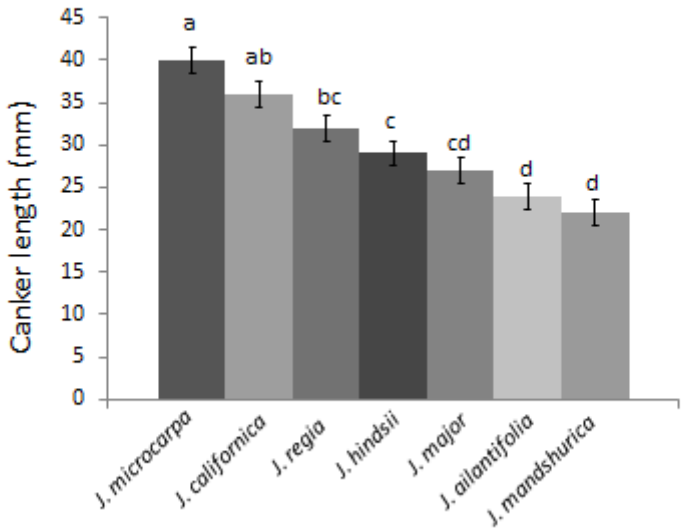

Figure 3.6. Canker development in branches of seven walnut species 6 wk after inoculation with *Geosmithia morbida* at the USDA-ARS National Clonal Germplasm Repository, California, in the summers of 2010 and 2011. Column means not followed by the same letter are different (*P* =0.05) by Fisher's LSD test and represent canker areas averaged for two fungal isolates. Bars indicate standard errors

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SUMMARY

This project began in 2004 as a series of field calls by two extension specialists at Colorado State University; Dr. Ned Tisserat and Dr. Whitney Cranshaw who visited Kathleen Alexander, Boulder city forester as she was removing a dying black walnut from city property. Now after 8 years we have done 3 great things to further scientific understanding. We described a new disease, thousand cankers disease; a symbiotic relationship between the fungus *Geosmithia morbida* and its vector; the tiny walnut twig beetle. This symbiosis (TCD) has had a detrimental effect on the health and well being of black walnuts and the industries created around *Juglans* throughout the Western United States and now also in the native range of black walnuts, a possibility we had hoped to avoid if at all possible. We have discovered and named a new pathogenic fungus, *Geosmithia morbida,* the causal agent of bark canker formation which was not previously known to science, from a genus of fungi not known to be pathogenic to its hosts. Because of *G. morbida's* pathogenicity we have also evaluated the potential host range of this new disease, while this work was not exhaustive, we did evaluate the most commonly encountered and economically important *Juglans* and *Carya* species found in the United States. Luckily we have determined that black walnut remains the most susceptible species to TCD. Due to the observed variability in resistance of certain black walnut breeding lines we are hopeful that superior trees exhibiting a high degree of genetic resistance against TCD will be found in the near future, sparing black walnut from facultative extinction from its center of diversity, a doomed fate experienced by the American chestnut.

APPENDIX 1. Ability of *Geosmithia morbida* to Survive in Bark After Exposure to -22° C

Introduction

The presence of thousand cankers disease in new locations must be confirmed by identifying the presence of the walnut twig beetle (WTB) and by culturing *Geosmithia morbida* from the phloem tissue. This is usually done by collecting bark samples and mailing them to plant disease diagnostic labs at land grant universities for inspection and culturing. To prevent the accidental escape of adult beetles during the mailing process, protocols were adopted in which the bark samples are frozen for at least 12 hours at -22° C. This temperature is lethal to the WTB. However, it was not known at the time (2009) whether the fungus would survive this temperature and thus be unable to be cultured from the bark. An experiment was conducted to determine whether *G. morbida* could survive the freezing process.

Materials and Methods

Wood was collected from a black walnut (*Juglans nigra*) trees affected by thousand cankers disease (TCD) in Denver on 23 Feb 2010. On 24 Feb 5-mm-long chips of necrotic phloem tissue were placed on ½ strength PDA using sterile technique. *Geosmithia morbida* was consistently observed growing from the chips by 1 Mar, confirming the presence of the fungus. Fifteen, 10-cm-long branch or trunk sections with bark attached and containing at least one *P. juglandis* hole were then cut with a band saw. Sections were prepared for freezing 3 different ways. Five arbitrarily selected sections were coated with 30 ml of 30% glycerol solution (glycerol), five were frozen in roughly 1 liter of deionized water (ice block) and remained untreated (dry). All wood sections were inserted in Ziploc® double zipper heavy duty freezer bags and placed in a standard -22 ^{o}C freezer in a randomized block design (shelves were blocks)

for 14 days. After 14 days samples were removed and allowed to thaw at room temperature overnight. Three bark chips from each section were removed and placed on ½ PDA. The presence of *G. morbida* on the bark chips was determined after 14 days by looking for the presence of the characteristic conidiophores and barrel-shaped conidia of the fungus.

Results

Geosmithia morbida was consistently isolated from bark chips in all treated samples except one of the dry and one of the glycerol samples. As sampling for *G. morbida* from each sample was not conducted before the samples were frozen these blocks may not have initially been colonized by the fungus.

This study demonstrates that *G. morbida* viability is unaffected by -22^{o} Celsius when stored either dry or wet. Freezing should serve as a means to store *J. nigra* wood samples waiting to be screened for the presence of thousand cankers disease.

Tables and Figures

Table A.1.1 Recovery of *Geosmithia morbida* from frozen bark.

Geosmithia morbida recovery[x]			
Wood section	Glycerol	Ice block	dry
1	3/3	3/3	2/3
2	2/3	3/3	0/3
3	3/3	2/3	3/3
4	2/3	3/3	3/3
5	0/3	3/3	3/3

[x]Proportion of bark chips removed from treated wood sections from which *Geosmithia morbida* was successfully isolated. Wood was coated with 30% glycerol, frozen in a block of ice or left untreated and then placed in a -22 ^{o}C freezer for 14 days.

Figure A1.1. Placement of branch sections treated with 30% glycerol, frozen in ice or left untreated in the freezer.

APPENDIX 2. Insecticide Bioassays with *Pityophthorus juglandis.*

Introduction

Thousand cankers disease (TCD) results from the combined activity of a canker producing fungus, *Geosmithia morbida*, and its insect vector, *Pityophthorus juglandis*. The disease progresses due to the cumulative effects of cankers that develop around beetle-produced wounds, which are produced in the course of feeding or during tunneling associated with breeding and egg production.

Therefore, a reduction in the production of new beetle produced wounds should be able to alter the course of disease development, slowing its progress. Several insecticides are used prophylactically to control bark beetles, applied as surface applications to trunks and branches to prevent entry of adult beetles as they attempt to construct egg galleries under the bark. A preliminary bioassay was conducted to assess the insecticidal activity of these products against *P. juglandis*. In addition, evaluations were also conducted with imidacloprid, a systemic insecticide widely used as a soil application on shade trees.

Materials and Methods

Four different insecticides including bifenthrin, carbaryl, permethrin and imidacloprid were compared to a distilled water control in this experiment. Insecticides were tested at 3 different concentrations, 1X, 0.2X and 0.04X. The highest concentration (1X) corresponded to the highest concentration consistent with label use instructions for the formulation used.

For carbaryl, permethrin, and bifenthrin this involved relatively high rates used for control of bark beetles; imidacloprid was used at the highest labeled rate for use as a foliar spray on woody plants. Specific formulations and use rates are summarized in Table A.A1.

Bioassays involved the use of insecticide treated Whatman filter paper disks (90mm Grade #1) placed in the lid of a (100 x 15mm) plastic petri dish (2). Each paper received 1ml of test dilution, which was sufficient to completely wet the paper. Four replications were prepared for each treatment. The paper was allowed to air dry for 1 hour, then was covered with the plastic lid and placed in a -20^{o} C freezer until ready for use.

Beetles used in the study had recently emerged from felled log bolts that were originally collected in January 2009 from Prospect Park in Wheat Ridge, Colorado. The logs were stored in plastic tubs and the trial was initiated on September 3, 2009 when a sufficient number (520) of newly emerged beetles became available. Ten live beetles were transferred into each dish and the dishes were maintained at room temperature (21^{o}C) during the course of subsequent observation. Evaluations of mortality were made at 3, 6, 24, 48, 72, 96, and 120 hours from the time the beetles were exposed to the treatment arenas. Beetles were examined microscopically to determine health status. At each time interval, plates were read in the same order as initial beetle-transfer to experimental arenas, to better maintain consistency in the intervals between examinations. Beetles were only considered to have been killed when there was no observable movement of legs, antennae and mandibles. Beetles were considered incapacitated at 3 and 6 hours post exposure if observed on their backs and unable to right themselves (Fig. A2.3).

Mortality data was analyzed in SAS Version 9.2 (SAS Corp., Cary, NC.) using a generalized linear model procedure and defining the data's distribution as binomial with repeated measurements. Analysis included insecticide rate effects, treatment comparisons and treatment

by insecticide rate interactions. True differences were evaluated by Chi-squared values of the least-squared means. Secondary statistical analysis was done; comparing treatment effects at specific time points using a mixed model with an arcsin transformation of the proportions dead.

Results and Discussion

All of the insecticides (bifenthrin, carbaryl, permethrin) that are used for control of other bark beetles produced complete mortality of walnut twig beetles (Table A2.1). At the highest concentration (1X) 100% mortality was noted at within 48 hours for permethrin, bifenthrin and carbaryl, respectively. All tested lower concentrations (0.2X, 0.04X) also produced 100% mortality in time. These results suggest that these insecticides could be used to kill adult beetles in field applications.

Most of the insecticide exposures showed more immediate behavioral effects on walnut twig beetles. Greater than 90% knockdown was achieved in the first 6 hours of beetle exposure to permethrin and carbaryl at all rates, and bifenthrin at the highest rate, rendering the beetles moribund (Fig. A2.3).

Insecticide Rate Differences

A P value of 0.05 was used to determine differences between insecticidal rates. There was no statistical difference between the 0.04X rate and 0.2X rate of bifenthrin on beetle mortality; there was a statistical difference between the 0.2X and the 1X rate . All bifenthrin rates were statistically more effective at killing beetles than the water control.

There was a statistical difference between the 0.04X and 0.2X rates of carbaryl; however, no statistical difference was detected between the 0.2X and 1X rate. All rates of carbaryl killed all test subjects in 48 hours.

Bifenthrin and permethrin treatments at the 0.2X rate provided the same level of control. Permethrin was significantly more effective than carbaryl at the 0.2X and 1X rates. There was a statistical difference between all rates of permethrin. Permethrin was statistically superior to all other insecticides at the full label rate. Imidacloprid was not statistically different than the water control (Fig. A2.2).

Imidacloprid failed to produce significant mortality. Imidacloprid is typically used on shade trees as a soil applied systemic insecticide and it has been found to be effective at controlling buprestid beetle larvae developing under the bark (1). However, the lack of activity by imidacloprid in this study indicates that it is unlikely to be useful in the management of thousand cankers disease.

Although several insecticides are clearly able to kill *P. juglandis* under the tested bioassay conditions, their effectiveness under field conditions can differ. There are several difficulties in achieving control of *P. juglandis* with bark applications, notably the extended period during which the adults are active and difficulties in providing coverage of large trees throughout the flight season. These difficulties are anecdotally supported by reports from regional tree care companies of failure to arrest TCD progress with applications of bark beetle bark sprays of permethrin or bifenthrin (Whitney Cranshaw, personal communication, 2012).

Tables and Figures

Table A2.1. Active ingredient, formulation and use rates of insecticides used in bioassays of walnut twig beetle.

Active Ingredient	Formulation Trade Name	Formulation % a.i.	Labeled Rate (1X)	% a.i. in 1X Rate
bifenthrin	Onyx	23.4	2 pts/100 gal	0.0585
carbaryl	Garden Tech Sevin Concentrate	22.5	1 gal/100 gal	0.225
imidacloprid	Merit 2F	21.4	1.5 fl oz/100 gal	0.00252
permethrin	Astro	36.8	2 qts/100 gal	0.184

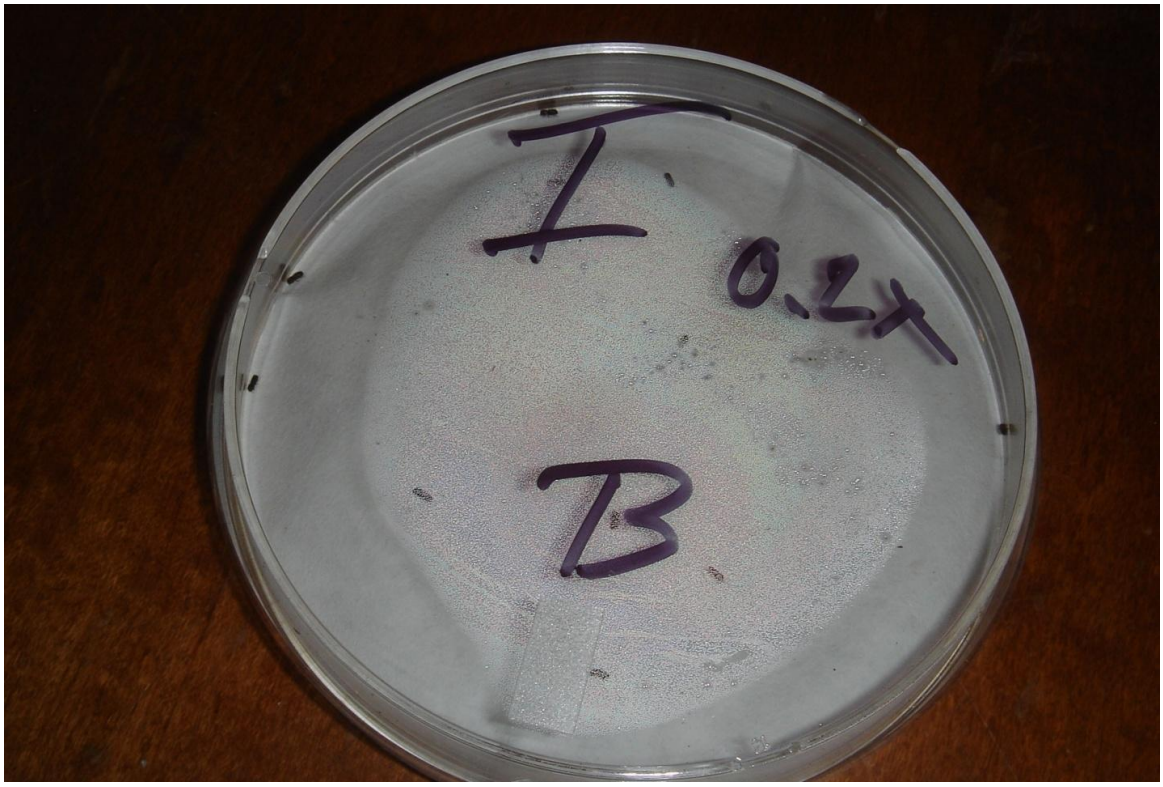

Figure A2.1. This is an example of a test arena; in this case 1ml of imidacloprid was applied to the filter paper floor at 0.2 times the label recommended rate of dilution. This plate was replication "B".

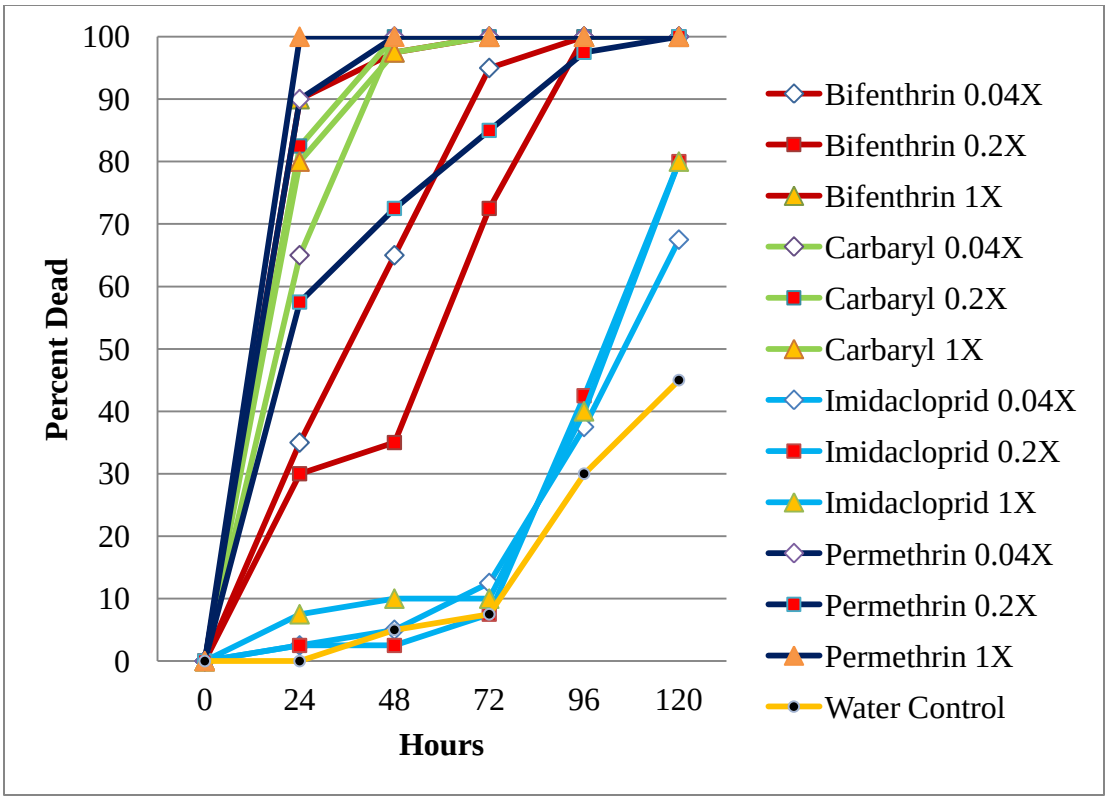

Figure A2.2. Mortality of *Pityophthorus juglandis* to four different insecticides (bifenthrin, carbaryl, permethrin, imidacloprid) applied at 3 different rates and evaluated over time. Each insecticide compound shares a common line color. Each insecticide rate shares a common line symbol and the 1X rate corresponds to the highest concentration allowed consistent with label instructions. A water check served as the control.

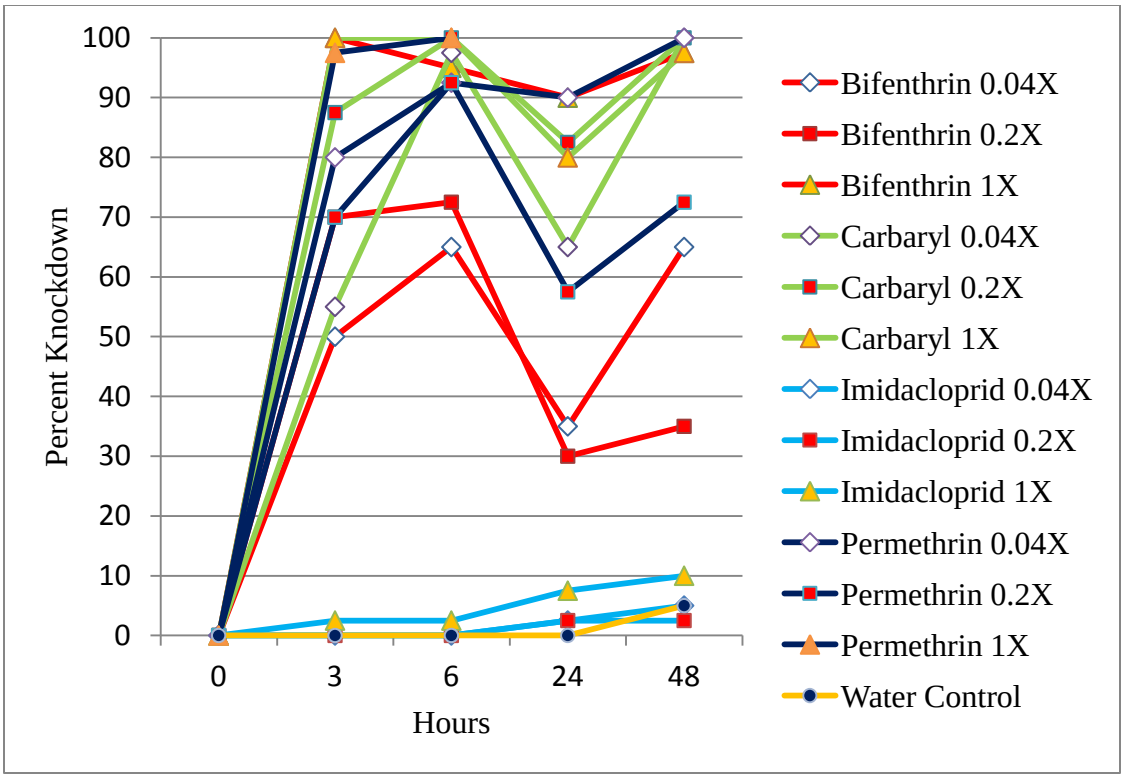

Figure A2.3 Effects on *Pityophthorus juglandis* adults during the first 48 hours of exposure to insecticides in a petri dish bioassay. There was quick knockdown effects observed with permethrin, carbaryl, and, to a lesser extent, bifenthrin.

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