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Short Communication

Walnut Witches'-Broom Disease Threatens Butternut Restoration Efforts in Indiana

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Abstract

Due to the devastating effects of butternut canker disease, efforts to protect the endangered butternut (*Juglans cinerea*) tree through resistance breeding have been a primary focus of forest restoration efforts. Walnut witches'-broom (WWB) disease poses a serious threat to these restoration efforts. This study sought to confirm the presence of the WWB disease phytoplasma, '*Candidatus* Phytoplasma pruni', in butternut research plantings in Indiana using molecular methods and Sanger sequencing and to identify butternut families affected by the disease. We also sought to better understand the incidence of the WWB phytoplasma in asymptomatic trees and asymptomatic branches of symptomatic trees to better direct management decisions. Sanger sequencing confirmed the presence of the WWB phytoplasma in the butternut restoration plantings, the first confirmation in Indiana based on sequencing to our knowledge, in both symptomatic and some asymptomatic trees. In addition, the WWB phytoplasma was detected in asymptomatic branches of symptomatic trees, indicating that phytoplasma infection is not necessarily localized to symptomatic tissues in a tree. Trees with positive molecular confirmation of the WWB phytoplasma consisted of 23 different butternut families and one family of Japanese walnut (*J. ailantifolia*), which is considered to be one of the most susceptible species to WWB disease. Based on these findings, future studies should prioritize identifying the hybridity and pedigrees of families and their susceptibility to WWB disease to aid in butternut restoration efforts.

Keywords: etiology, *Juglans cinerea*, pathogen detection, phytoplasma

Walnut bunch disease, also known as walnut witches'-broom (WWB), is a surreptitious disease affecting many species within the *Juglans* genus in the eastern and central United States. Symptoms of the disease were first reported in the early 1930s, but it is likely that it was present in forests prior to this time and was not reported (McKay and Harley 1951; Waite 1933). The disease is caused by '*Candidatus* Phytoplasma pruni' in the subgroup 16SrIII-G (Marcone 2015), which causes symptoms characteristic of other phytoplasma diseases, including foliar chlorosis, stunted leaves, deformed stems resembling brooms, bunched foliage, and dieback (Seliskar 1976; Fig. 1). While the phytoplasma can attack many *Juglans* species, the most susceptible tree species reported are Japanese (*J. ailantifolia* Carr.) and Manchurian (*J. mandshurica* Les. Hoz.) walnuts, while butternut (*J. cinerea* L.), Persian walnut (*J. regia* L.), and black walnut (*J. nigra* L.) are generally considered less susceptible (Seliskar 1976).

Although butternut is considered less susceptible to WWB disease than other *Juglans* species, it readily hybridizes with Japanese walnut. Butternut × Japanese walnut hybrids (hereafter referred to as hybrids) can grow intermixed with native butternut trees and in some stands only hybrids remain (Boraks and Broders 2014). The abundance of hybrids is due to their apparent resistance to butternut canker disease (BCD), a lethal disease that is estimated to have a mortality rate of 90% and is found throughout butternut's natural range (LaBonte et al. 2015; Schultz 2003). A study by Boraks and Broders (2014) found that hybrids had an average of 2.5 cankers per tree while pure butternuts had an average of 4.5. Therefore, efforts to restore butternut, an IUCN endangered species (Stritch and Barstow 2019), may rely at least in part on hybrid trees (McKenna et al. 2013; Michler et al. 2006; Pike et al. 2021). However, since the susceptibility of these hybrids to WWB disease has not been documented, WWB has the potential to threaten ongoing restoration efforts in managed butternut plantings.

To better assess the threat of WWB disease to butternut restoration efforts, the main objective of this study was to confirm whether or not the WWB phytoplasma is present in existing butternut research plantings and identify the butternut families affected by the disease. While WWB is widely known to affect butternut plantings across Indiana, to our knowledge no one has confirmed the presence of the WWB phytoplasma based on sequence data. Given that not much is known about the lifestyle of the WWB phytoplasma on butternut trees, we also wanted to examine phytoplasma incidence in asymptomatic trees and asymptomatic branches of symptomatic trees to guide managers in making more informed decisions. Current management for WWB relies on the observation of WWB symptoms followed by removal of symptomatic branches or entire trees.

To answer these questions related to WWB in butternut plantings, we sampled trees from two butternut research plantings, referred to as

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plot 51 and plot 53, established by the Hardwood Tree Improvement and Regeneration Center (HTIRC) at Martell Forest, Purdue University, West Lafayette, IN (40.424484, −87.041656). The plantings were established in 2010 (plot 51) and between 2011 and 2013 (plot 53) with 1-year-old seedlings from seed collected across the range of butternut and from trees in the HTIRC breeding program. Each planting is comprised primarily of open-pollinated butternut and hybrid butternut families. Black walnut is interspersed throughout plot 51 and plot 53, and Japanese walnut families were also planted within plot 53. Incidence of WWB disease has been reported in each plot with 0.2% of trees showing symptoms in plot 51 and 5% in plot 53 in 2022. Symptoms are actively managed by removing symptomatic branches and entire trees (in severe cases) at both plantings.

In order to identify the WWB phytoplasma and better understand its lifestyle, we established three leaf symptom types to compare: (i) leaves with WWB symptoms sampled from symptomatic trees, (ii) leaves without WWB symptoms sampled from symptomatic trees (i.e., phenotypically healthy leaves collected from branches without brooming symptoms), and (iii) leaves without WWB symptoms sampled from asymptomatic trees. We collected three compound leaves from trees of each of the three symptom types at both plot 51 and plot 53, and the leaves were bulked as one sample per symptom type for a total of 53 samples (Table 1). Among the symptomatic and asymptomatic trees were three samples from grafted trees (grafted in 2022) with WWB symptoms and three samples from grafted trees without symptoms. The scions for grafted trees came from butternut ortets in plot 51 and all rootstocks were black walnut. Grafted trees were tested for WWB phytoplasma to confirm pathogen presence in symptomatic material and to determine whether the WWB phytoplasma is cryptically present in asymptomatic grafts, which is important for butternut restoration efforts. We also collected samples from the black walnut tree (cv. Thomas) that served as a source of seed for rootstock to determine if it was a source of the WWB phytoplasma. The tree was asymptomatic and located at Purdue University's Lugar Farm, also in West Lafayette, IN. All leaf samples were placed on dry ice immediately after collection and stored at −80°C.

Table 1. Number of bulked samples per symptom type submitted for the metabarcoding analysis

Leaf symptom type	Tree symptom type	Number of bulked samples
Symptomatic	Symptomatic	31
Asymptomatic	Symptomatic	13
Asymptomatic	Asymptomatic	19
Total		53

The bulked leaf samples were ground in liquid nitrogen, and DNA was extracted using the Qiagen DNeasy Plant Mini kit (QIAGEN, Germantown, MD) following the manufacturer's protocol with the addition of 8 µl of beta-mercaptoethanol to the AP1 starting buffer. The DNA, along with a negative control and a positive control containing DNA from the elm yellows phytoplasma collected by personnel at Pennsylvania State University, was amplified using a nested PCR with universal phytoplasma primers P1 and P7 (Smart et al. 1996) followed by R16F2n and R16R2 (Gundersen and Lee 1996). The PCR product was purified using QIAquick PCR purification kit (QIAGEN), and the purified product was sequenced separately using the forward P1 primer, the forward R16F2n primer, and the reverse R16R2 primer through the Sanger sequencing platform. The sequences were assembled into one contiguous sequence using the CAP3 assembly program (Huang and Madan 1999) to examine the target 1.2-kbp fragment of the 16S rRNA region. Phytoplasma reads were identified and classified using two bioinformatic platforms: *i*PhyClassifier (Zhao et al. 2009) and the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990).

The results of the Sanger sequencing indicated that 20 of the 31 symptomatic samples successfully produced a PCR product with a DNA sequence that matched '*Candidatus* Phytoplasma pruni' *rrnA* operon in the subgroup 16SrIII-G (GenBank accession no. JQ44395) with similarities between 99.6 and 99.8% according to the *i*PhyClassifier, and 99.7 to 100% similarity according to NCBI's BLAST (Table 2). Five of the sample's sequences did not meet the 1.2 kbp length requirement for *i*PhyClassifier and were only confirmed using NCBI BLAST. From the 13 asymptomatic samples collected from symptomatic trees, 4 samples produced a PCR product associated with phytoplasmas. Those sequences matched with similarities between 99.4 and 99.7% to the '*Candidatus* Phytoplasma pruni' *rrnA* operon in the subgroup 16SrIII-G (GenBank accession no. JQ44395) according to *i*PhyClassifier, and 99.7 to 100% similarity according to NCBI BLAST. Two of the samples' sequences did not meet the length requirement for *i*PhyClassifier and were confirmed using NCBI BLAST only. Seven of the 19 asymptomatic samples from asymptomatic trees amplified phytoplasma DNA, which were identified with 99.7% similarity to the '*Candidatus* Phytoplasma

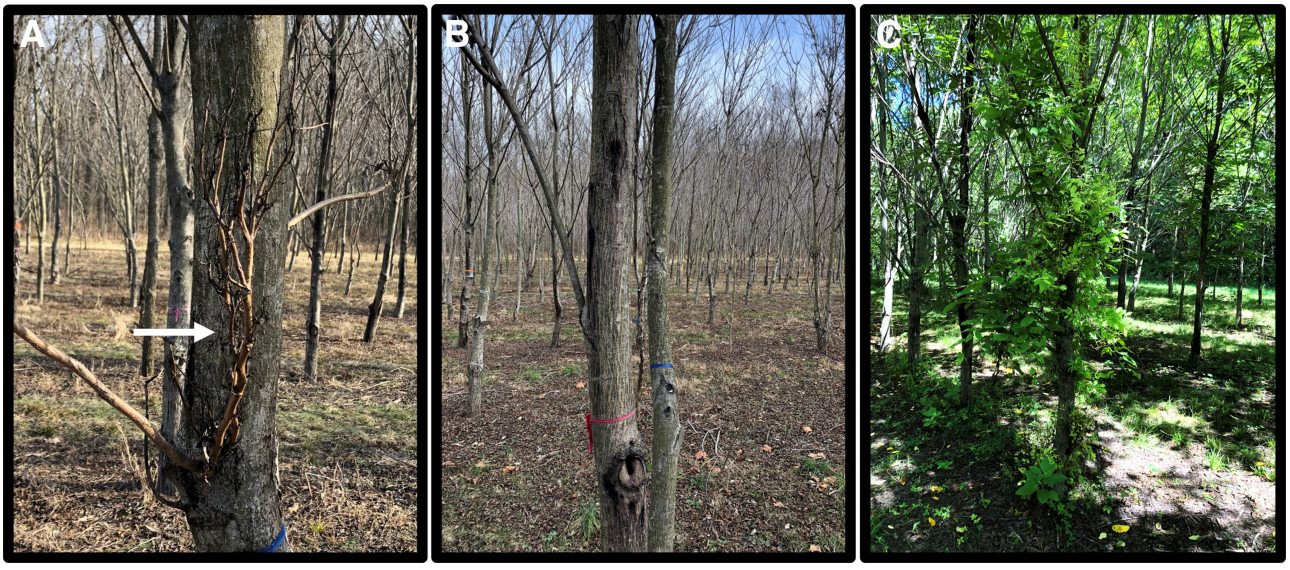

Fig. 1. Characteristic symptoms of walnut witches'-broom (WWB; **A**; white arrow) compared to WWB-free trees (**B**) during leaf off and during the growing season (**C**; symptomatic tree in foreground).

pruni' *rrnA* operon in the subgroup 16SrIII-G (GenBank accession no. JQ44395) according to *i*PhyClassifier and with similarities between 99.6 and 100% according to NCBI BLAST. Five of the samples' sequences did not meet the length requirement for *i*PhyClassifier and were confirmed using only NCBI BLAST. Among the seven samples positive for the WWB phytoplasma were two samples from asymptomatic grafts. The sample from black walnut Thomas, which was the source of seed for rootstocks, did not produce a PCR product.

These sequencing results confirmed the presence of WWB disease in butternut restoration plantings in Indiana. Furthermore, the 31 trees that were confirmed to have the WWB phytoplasma represented 23 different families (Table 2). Also among these trees was one Japanese walnut located within plot 53. Family 781 had the greatest incidence of positive molecular confirmations for the WWB phytoplasma. The seeds for Family 781 originated from a planting within Martell Forest and seedlings of Family 781 were planted in plot 53 in 2012 and 2013. Based on morphology, 781 is likely a hybrid, but future research should focus on using genotyping to confirm the proportion of butternut genome in hybrid trees within these families to clarify the relationship between butternut hybridization and WWB susceptibility.

Based on these results, we confirmed that the WWB phytoplasma is present in Indiana, but symptoms may not always manifest since the phytoplasma was also detected in some asymptomatic samples. This confirms the findings by Yun and Harrington (2011), who determined that the phytoplasma can be detected in asymptomatic trees, and indicates that the phytoplasma has cryptic behavior, a common trait of phytoplasmas that makes them difficult to manage (Olivier et al. 2009). We also confirmed that the WWB phytoplasma can be detected in not only symptomatic leaves but also asymptomatic leaves from the same tree, the first report of this in butternut. This indicates that the phytoplasma infection is not necessarily localized, and WWB management techniques that include pruning only infected branches may not be sufficient to remove potentially infectious tissues.

Moreover, additional research on the disease etiology and symptomology of WWB disease is needed in order to better direct management decisions, as current management only occurs after symptoms are present. This information is particularly important given that the WWB phytoplasma was positively identified in two of the asymptomatic grafted trees. Preventive screening for the WWB phytoplasma via molecular methods of asymptomatic scions for grafting from areas where WWB symptoms have been observed prior to out-planting may be needed to prevent the spread of WWB.

In conclusion, to our knowledge this is the first confirmed report of the WWB phytoplasma in Indiana based on sequencing. This study also confirmed that the phytoplasma is not localized to only branches bearing symptomatic leaves and can exist cryptically, so the efficacy of symptomatic branch versus whole-tree removal for disease management should be evaluated. This study indicated that specific butternut families may have higher incidences of the WWB phytoplasma than others. Therefore,

Table 2. Information on the individual trees with positive phytoplasma amplification and the percent similarity to '*Candidatus* Phytoplasma pruni' *rrnA* operon in the subgroup 16SrIII-G (GenBank accession no. JQ44395) over a 1.2 kbp and 680 bp minimum for *i*PhyClassifier and BLAST, respectively[a]

Tree ID	Research planting ID	Family	Leaf symptom type	Tree symptom type	*i*PhyClassifier percent similarity[b]	BLAST percent similarity	GenBank accession number
53-2011-1-52	Plot 53	36	Symptomatic	Symptomatic	N/A	99.8	OQ275122
53-2011-1-58	Plot 53	OS-184	Symptomatic	Symptomatic	99.6	99.8	OQ275121
53-2011-2-43	Plot 53	781	Symptomatic	Symptomatic	99.7	99.7	OQ275105
53-2011-4-56	Plot 53	Japanese walnut	Symptomatic	Symptomatic	99.7	99.8	OQ275106
53-2011-5-49	Plot 53	780	Symptomatic	Symptomatic	N/A	99.8	OQ275123
53-2011-9-8	Plot 53	781	Symptomatic	Symptomatic	99.8	99.9	OQ275107
53-2011-9-20	Plot 53	OS-165	Symptomatic	Symptomatic	N/A	99.8	OQ275124
53-2011-9-23	Plot 53	781	Symptomatic	Symptomatic	99.7	99.8	OQ275108
53-2011-9-23	Plot 53	781	Asymptomatic	Symptomatic	N/A	99.9	OQ275133
53-2011-9-32	Plot 53	781	Symptomatic	Symptomatic	99.7	99.6	OQ275109
53-2011-10-59	Plot 53	11062	Symptomatic	Symptomatic	N/A	99.8	OQ275125
53-2011-14-17	Plot 53	OS-52	Symptomatic	Symptomatic	99.7	99.8	OQ275110
53-2011-14-63	Plot 53	Rose Rd	Symptomatic	Symptomatic	99.7	99.7	OQ275111
53-2011-15-36	Plot 53	Zoller 2	Symptomatic	Symptomatic	99.7	99.7	OQ275112
53-2011-17-34	Plot 53	Brim N	Symptomatic	Symptomatic	N/A	99.8	OQ275126
22-124 (graft)	Plot 51[c]	11273	Symptomatic	Symptomatic	99.7	99.8	OQ275113
22-106 (graft)	Plot 51[c]	11495	Symptomatic	Symptomatic	99.8	99.7	OQ275103
22-106 (graft)	Plot 51[c]	11496	Asymptomatic	Asymptomatic	N/A	99.7	OQ275128
22-122 (graft)	Plot 51[c]	11316	Symptomatic	Symptomatic	99.7	99.7	OQ275114
22-110 (graft)	Plot 51[c]	14RD	Symptomatic	Symptomatic	99.7	99.7	OQ275104
22-101 (graft)	Plot 51[c]	11273	Symptomatic	Symptomatic	99.7	99.7	OQ275115
22-115 (graft)	Plot 51[c]	11262	Symptomatic	Symptomatic	99.7	99.7	OQ275116
53-2011-12-21	Plot 53	999	Asymptomatic	Symptomatic	99.7	99.8	OQ275119
53-2011-9-52	Plot 53	11062	Asymptomatic	Symptomatic	99.4	99.7	OQ275118
53-2011-2-44	Plot 53	11062	Asymptomatic	Symptomatic	99.7	99.6	OQ275117
51-18-18	Plot 51	11423	Asymptomatic	Asymptomatic	N/A	99.6	OQ275129
51-12-70	Plot 51	11409	Asymptomatic	Asymptomatic	99.7	99.8	OQ275120
53-2011-14-20	Plot 53	OS-35	Asymptomatic	Asymptomatic	N/A	99.9	OQ275130
53-2011-13-15	Plot 53	Zoller 1	Asymptomatic	Asymptomatic	N/A	99.9	OQ275131
22-121 (graft)	Plot 51[c]	11488	Asymptomatic	Asymptomatic	99.7	99.8	OQ275127
51-9-31	Plot 51	11329	Asymptomatic	Asymptomatic	N/A	98.9	OQ275132

[a] The DNA from 11 symptomatic leaves from symptomatic trees, 12 asymptomatic leaves from asymptomatic trees, and 9 asymptomatic leaves from symptomatic trees did not produce a positive phytoplasma amplification.
[b] N/A indicates the sequences were not of sufficient length to meet *i*PhyClassifier's standards of 1.2 kbp.
[c] Indicates from which research planting the scion was collected. (Note: Scion for graft 22-110 was collected from outside the research plantings.)

future efforts should be made to better understand the susceptibility of specific butternut families to WWB disease and identify the families more resistant to both WWB and BCD in order to assist in butternut restoration.

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