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Early detection of the oak wilt fungus (*Bretziella fagacearum*) using trapped nitidulid beetle vectors

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Abstract

As the range of the oak wilt fungal pathogen expands, it will become increasingly important for land managers to have early detection tools to find the disease before it becomes locally established and causes environmental and economic losses. New York State has been at the leading edge of the disease's range since 2008 and intensively surveys for and manages the disease to protect the state's oak resource. The oak wilt fungus was successfully detected using double-nested PCR on nitidulids wet-collected in multi-funnel traps, suggesting we can find it on the landscape before symptomatic trees are detected. From 2019 to 2021, 10–13 percent of beetle samples were PCR positive for the oak wilt fungus, including sites in New York where oak wilt had been treated and 'early detection' sites where infected trees had never been found. Our method is also useful for identifying local associates, such as *Carpophilus lugubris* in New York. While some obstacles exist, such as the efficiency of sampling and the ability to trace positive nitidulid samples back to infected trees, with more development this tool will be another useful option for land managers in detecting and managing oak wilt.

KEYWORDS

Bretziella fagacearum, *Carpophilus sayi*, *Colopterus truncatus*, double-nested PCR, invasive forest diseases, oak disease

1 | INTRODUCTION

Oak wilt (*Bretziella fagacearum* [Bretz]), a fungal disease lethal to oak trees, has killed tens of thousands of trees in the Midwest and millions in Texas since its discovery (Juzwik et al., 2011). Removal costs from oak wilt-infected trees were predicted to be $18–60 million over 10 years in one county in Minnesota, where the disease is widespread (Haight et al., 2011). Control is also highly expensive where the disease is established, with Texas spending nearly $10 million in 12 years (1988–2000) and still only controlling a fraction of infections (Wilson, 2001). Although oak wilt occurs more sporadically and acts differently in the Appalachian region and northeast (Juzwik et al., 2011), land managers continue to be concerned about the disease as its range expands northwards. In areas where the disease has not been found, such as Canada, oak wilt is a pathogen of regulatory concern, and modelled estimates of removal and replacement costs were hundreds of millions of Canadian dollars (Pedlar et al., 2020). Early detection is crucial to management success, as finding and removing infected trees before the fungus can reproduce allows managers to stop local spread (Juzwik et al., 2011).

Our native oak trees contribute numerous timber, wildlife and aesthetic benefits, as well as improving the quality of our environment.

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In New York, oak is associated with $55 million of revenue for private landowners annually (NYS DEC Division of Lands and Forests, 2016) and is also an important export to Canada, with over 85,000 MBF being sent in 2018 alone (NYS DEC Division of Lands and Forests, 2020). Oak is the fourth most abundant tree species in New York timberland by sawtimber volume and has increased by over 20% since 2007 (Widmann et al., 2015). Oak wilt was first found in the state in 2008 in the town of Glenville, over 250 miles from the closest known infection. Since then, New York has remained the northeastern extent of the disease's known range, and the disease is limited to small infection sites in just five counties statewide (Figure 1). Approximately 75% of New York's infection centres consist of one or two infected trees when they are discovered, and an average of one infection centre is found per year.

Due to the limited geographic extent of oak wilt in New York, the NYS Department of Environmental Conservation (DEC) has sought to detect and remove all infected trees across the state to reduce disease spread. Trees are detected via aerial, ground and roadside surveys and through public reports of diseased oaks informed by outreach webinars, pamphlets, mailings and online resources. Finding these small, satellite infections over such a large area presents funding and logistical challenges. In addition to time and labour-intensive surveys and responding to public reports, DEC sought a more passive approach to detecting oak wilt through trapping nitidulid beetles (Coleoptera: Nitidulidae).

Nitidulid beetles are vectors in the 'aboveground spread' of oak wilt when they carry the disease spores from infected to healthy trees. Nitidulids are attracted to sweet-smelling things such as the oak wilt fungus' reproductive spore mat, where larva and adults of different species have been documented feeding (Curl, 1955, Kyhl, 2006 in Jagemann et al., 2018). The beetles are also attracted to sap from tree wounds, and as they feed in the wounds, the spores can be deposited and cause new infections. Fungal spore mats have been found most abundantly in spring and fall in other parts of the disease range (Juzwik et al., 2011), and high populations of the vectors are present in April–July based on concurrent work by the authors (NYS DEC trapping data, unpublished). We believe this aboveground spread is the main method of infection in New York, as our infection centres are small and scattered in limited areas of the state.

New York began trapping nitidulid beetles in 2017 as part of a multi-state effort to determine (1) which trap types collected the

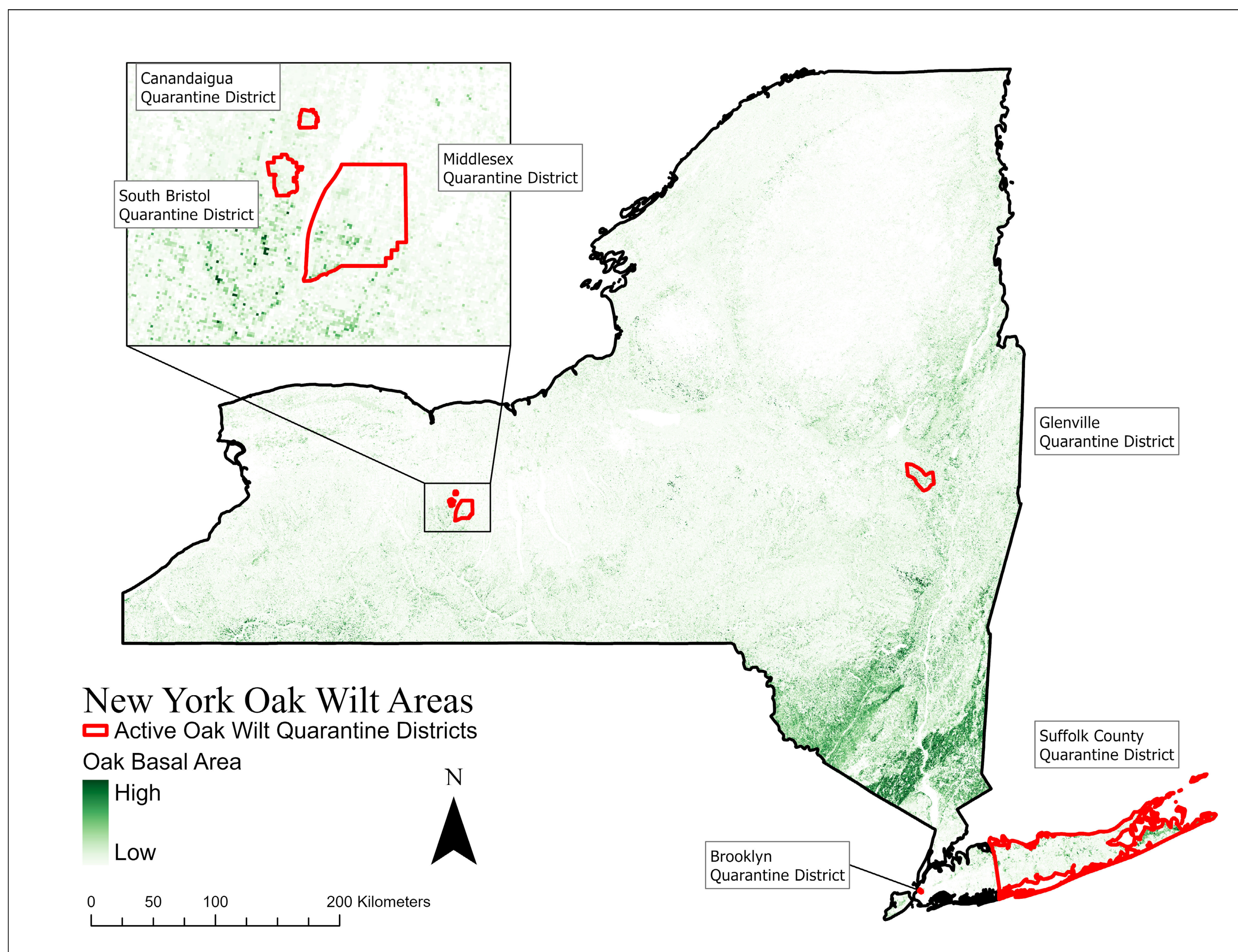

FIGURE 1 Oak wilt quarantine districts where at least one infected tree has been found with New York's oak basal area.

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highest abundance and diversity of nitidulid beetles and (2) to determine if oak wilt could be cultured from beetles collected in wind-oriented dry collection traps (DiGirolomo et al., 2020). Oak wilt was not successfully cultured from dry-collected beetles during the study. The beetles collected in wet collection traps were not used for culturing due to the sterilizing ability of the propylene glycol commonly used in wet trapping systems. The results showed that multiple-funnel trap with wet collection cups caught higher abundances and more diverse groups of nitidulids in New York (DiGirolomo et al., 2020).

Wet collection multiple-funnel traps are more familiar to land managers as they are used for a variety of forest pests, so these traps would be ideal for an early detection system. The fact that these traps caught higher abundances and diversity of beetles also suggested that these traps would be the best way to collect nitidulids to screen for oak wilt. However, researchers had concerns that the propylene glycol used in wet collection traps would degrade DNA or wash the DNA off of the beetles. Knowing this, in 2019 DEC staff began discussing a new experiment with the oak wilt sample processing laboratory, the Plant Disease Diagnostic Clinic at Cornell University (CUPDDC). The goals of this study were to (1) determine whether double nested PCR testing of wet-collected beetles could successfully detect the oak wilt fungus and (2) determine whether these methods could be used as an early detection system, identifying oak wilt on beetles at sites where no symptomatic trees were detected but disease presence was likely.

2 | MATERIALS AND METHODS

Field Methods: In 2019, nitidulids were trapped near seven previously treated oak wilt infection centres (Table 1). These centres were sites of previous infected trees identified 2–3 years earlier that were treated via infected tree removal, stump grinding and herbicide treatment. Treated sites were monitored annually for symptoms and were managed between 2 months and 3 years previous to trapping. Ten multiple-funnel traps (Synergy Semiochemicals Corp.) were arranged in two transects of five traps each, with traps spaced 30 m apart. Traps were deployed from April to August and were baited with *Colopterus truncatus* (Randall) and *Carpophilus sayi* (Parsons) pheromone lures (Trécé Pherocon, Great Lakes IPM), changed monthly, and a fermenting malt extract (wort), changed biweekly. Traps were checked biweekly. In 2020, nitidulids were trapped at four previously treated oak wilt infection centres with the same trap setup. We also trapped at five sites labelled 'early detection sites' where oak wilt infected trees had never been found, but that were likely sites for new infections. Sites were chosen in a forested corridor between New York and Pennsylvania counties with known oak wilt infections and located on ridge tops where prevailing winds regularly cause damage to oaks that could attract nitidulids. Early detection sites had three traps each, positioned approximately 30 m apart. Traps were deployed from April to August in the northeastern New York and April through October in the rest of the state. Traps were checked and lures were changed monthly. In 2021, we trapped in eight early detection sites and one previously treated oak wilt infection centre. Early detection sites had three traps each, and the previous infection centre had five traps. Traps were deployed from May through October and checked biweekly.

Laboratory Methods: After collection, samples were frozen and sent to DEC's Forest Health Diagnostic Lab. Leaves, debris and other beetles were removed, and three groups of three nitidulid beetles were selected for testing from each collection. In 2021, three groups of five beetles were selected in hopes of having more opportunities to find the fungus. All other methods remained the same for the 3 years of testing. Samples were mailed to Cornell University Plant Disease Diagnostic Clinic (CUPDDC).

Methods at CUPDDC remained consistent for all 3 years of testing. When received at CUPDDC, the tubes were organized to note the field identification numbers, determine the number of beetles per tube and assigned a unique laboratory identification number. The beetles were stored in a −20°C freezer until sampling could be conducted.

The same procedure used at CUPDDC for plant tissue analysis was followed for the beetle samples. All suspect oak wilt plant tissue samples are processed two ways—symptomatic tissue is sub-sampled with half being plated on a nutritive agar, typically Acidified Potato Dextrose Agar (APDA), and the remaining half directly tested with the nested PCR procedure. If an oak wilt characteristic fungus grows on the agar plates, the isolate is also tested with the nested PCR. For the nitidulid testing, the laboratory staff followed the same DNA extraction kit and protocol, and the same nested PCR primers and procedures used for the plant tissue analysis.

The DNA extraction step began with transferring each set of beetles to a 2.0 ml Matrix A Lysis tube, containing a ¼" ceramic bead and a sand-like garnet matrix recommended for grinding animal and plant tissues, nematodes, insects, fungi and bacteria. The beetles were ground using the FastPrep24 tissue homogenizer; a table-top, high-speed, matrix and bead-beating tissue and cell lysis instrument set at 6.0 m/s for 40 s. If the beetles did not appear completely ground, it was repeated for another 40-second run. The DNA extraction procedure used the Qiagen Fast DNA Stool Kit, recommended for bark and wood processing and followed the kit instructions as written.

The molecular analysis used a common diagnostic laboratory procedure, the oak wilt nested polymerase chain reaction (PCR) procedure by Yang et al. (2014). The nested procedure is defined as a conventional PCR with two consecutive rounds of amplification. The first round of PCR uses the commonly used general fungal primer set, ITS-1 and ITS-4. The second round used the oak wilt pathogen-specific primers, CF01 and CF02. (Table 2).

The DNA amplification program was identical for both Round 1 and Round 2 and was performed on a conventional thermocycler. The program was entered into the machine with the program names 'OW FRST' and 'OW SCND' and used the steps indicated in Table 3.

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TABLE 1 Traps deployed by site type and year

Year	Previously treated sites	Early detection sites	Total sites	Total traps
2019	7	0	7	70
2020	4	5	9	55
2021	1	8	9	29

TABLE 2 Primer names and designs used in oak wilt nested PCR procedure

Primer name:	Primer design:
ITS-1	5′–TCCGTAGGTGAACCTGCGG–3′
ITS-4	5′–TCCTCCGCTTATTGATATGC–3′
CF01	5′–GGCGACTTCTTTCTT–3′
CF02	5′–AAGGCTTGAGTGGTGAAA–3′

TABLE 3 DNA amplification program steps for oak wilt testing

Temp (°C)	Time	# Cycles	Description
95	3 min	1	Denaturation
95	30 s	35	Denaturation
60	30 s		Annealing
72	30 s		Elongation
72	5 min	1	Extension
4	Pause		Hold

A two percent agarose gel using GelRed dye was used to run out the PCR product from Round 2. The electrophoresis machine was set to 80 V for 60 min, then transferred to an imaging system to visualize the results. Samples with amplicons at the 280-base pair location were determined to be positive for the oak wilt fungus, *B. fagacearum*.

In the early sampling of suspect oak wilt plant tissue and beetles, PCR positives were confirmed with sequencing to verify the PCR results. The sequencing step was done on plant tissue samples from suspect trees in new locations in New York, typically at the county level. For the beetle project, the first few positive beetle samples from 2019 were confirmed with sequencing of the DNA and blasting the database for the closest matches. All sequences associated with this project, including suspect tree submissions, have matched the results of the nested PCR. In other words, all nested PCR samples that displayed a 280 base pair band were a sequence match to *B. fagacearum*.

3 | RESULTS

The oak wilt fungus (*B. fagacearum*) was successfully detected with nested PCR from nitidulids collected in propylene glycol, with ITS sequencing confirmations of the first few positive results. In 2019, 7 of 52 nitidulid samples were positive for the oak wilt fungus (11%). Five of the seven sites where beetles were trapped had positive samples. In 2020, 20 of 177 nitidulid samples were oak wilt positive (13%). Seven of the nine trapping sites had oak wilt positive samples, with two of the four previous oak wilt infection centres, and all five of the early detection sites having positive samples. In 2021, 17 of 162 nitidulid samples were positive for oak wilt (10%). Seven of the nine sites where beetles were trapped had positive samples, including one previous oak wilt infection centre site and six of the eight early detection sites. The repeated discovery of nitidulids that tested positive for the oak wilt fungus with PCR at sites across the state suggests that double-nested PCR testing of wet-collected nitidulids is a feasible method for detecting oak wilt (Figure 2).

The timing of positive collections was not consistent from year to year. In 2019 and 2020, most of the nitidulids that tested positive for oak wilt were collected in the late summer and fall, starting in July in 2019 and August in 2020 (Figure 3). In contrast, most of the nitidulids that were positive for the oak wilt fungus via PCR testing in 2021 were collected in May and June. The earliest that oak wilt-positive beetles were collected was April 2020 and May 2021. The latest that oak wilt-positive beetles were collected was November in 2020 and October in 2021. Sites did not show a consistent pattern of when positive samples were collected. For example, Moss Hill State Forest, which was one of the early detection sites, had samples positive for the oak wilt fungus in April, August and November in 2020, and May and October in 2021.

Beginning in 2020, we recorded the nitidulid species that were sampled for the oak wilt fungus. In addition to *Colopterus truncatus* and *Carpophilus sayi*, our sampling showed that *Glischrochilus fasciatus* (Olivier) was also positive for the fungus via PCR, supporting previous research that this species is another vector of oak wilt (Juzwik & French, 1983). We also had one sample with only *Carpophilus lugubris* (Murray) that was PCR positive for the fungus. This species has not been previously documented as an oak wilt associate in the research we reviewed. Other species were in mixed samples containing multiple nitidulid species that were positive for oak wilt via PCR testing, but it is impossible to say which of the species in these samples were oak wilt associates.

From 2019 to 2021, we collected 44 different nitidulid species in New York State. It was not a goal of this project to determine associates of oak wilt but could be done using this method if samples were consistently separated by nitidulid species.

4 | DISCUSSION

Based on the success of our results, the New York State DEC has continued to use nitidulid trapping as an early detection method in 2022. The utility of nitidulid testing as an early detection tool for oak wilt depends on the specificity of the area of concern and the ability to extensively survey. From 2019 to 2021, trap data has not been successfully used to find a specific infected tree, but data have demonstrated that infected trees and positive beetles

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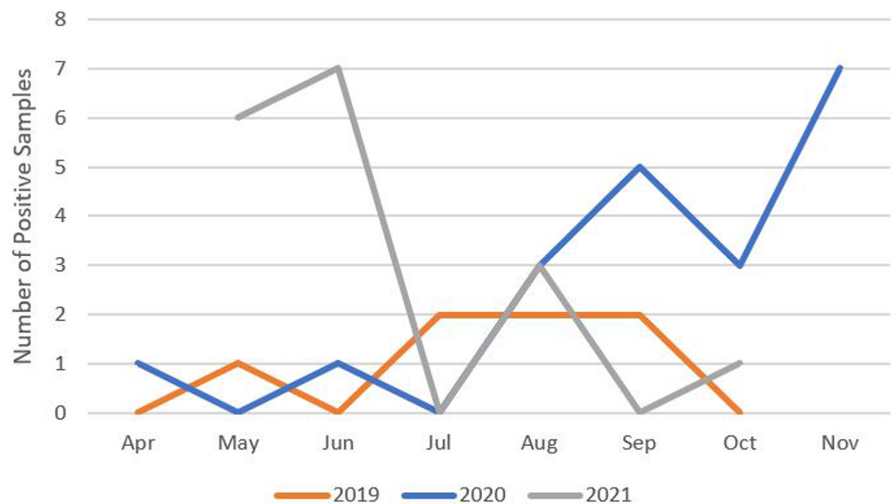
FIGURE 2 Location of oak wilt infected trees and PCR-positive nitidulid samples in New York

FIGURE 3 Collections of nitidulids that tested positive for the oak wilt fungus over time



can be found in the same area at the same time. In 2019, 10 oak wilt infected trees were found in Middlesex, NY, 6.4 km away from a trapping site with positive beetles in Bristol, NY (Figure 2). The infected trees were reported on July 30th, and oak wilt-positive beetles were collected on August 6th. An aerial survey of approximately 45,000 hectares by DEC identified another oak wilt infected tree 5.8 km from where oak wilt positive nitidulids were trapped. Infected trees were not found in other areas with oak wilt-positive beetles in any year, despite extensive surveys covering over 160,000 hectares annually.

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In addition to linking molecular detections of oak wilt from beetles back to infected trees, another area for improvement is sampling efficiency. The ability to process larger numbers of beetles with a more sensitive test would decrease the cost of analyses. Currently, we are only testing three beetles at a time from samples with hundreds of beetles, which provides incomplete information. We hope to increase the number of beetles that can be tested at once through artificial inoculation trials to better measure our test's sensitivity. Bilodeau et al. (2021)are also tackling this problem using a quantitative PCR process with new primers.

This method can provide localized insights on disease movement, biology and nitidulid associates, leading to more efficient surveys and better management decisions. In 2019 and 2020, most oak wilt-positive beetles were trapped later in the season, but in 2021, more positive beetles were trapped in the early season, suggesting mat formation may peak at different times in different years (Figure 3). One sample in 2020 with only *Carpophilus lugubris* was positive for the oak wilt fungus with PCR. This species has not yet been formally documented as a vector or phoretic carrier of *B. fagacearum* and highlights the ability of this test to identify local nitidulid species associated with oak wilt. Testing nitidulids for the presence of the oak wilt fungus with PCR can also help determine when beetles are carrying oak wilt, which can inform local pruning guidelines. While significant obstacles exist, the technology shows promise as a new tool for detecting an otherwise difficult to find disease.

The method tested in the current study also has advantages over traditional culturing methods, as it can be used even if the fungus is killed during collection and can give more rapid results than culture growth. Beginning in the 1980s, nitidulids could be tested for oak wilt spores via culturing the fungus or visually checking nitidulids for spores using microscopy methods (Juzwik & French, 1983). Ultimately, these methods are time-consuming, technical and often require careful trapping and lab techniques to maintain the fungus' viability and prevent contamination. In contrast, our test is a relatively quick and sensitive procedure, that works even after the fungus may be killed during the collection process. This makes it a better fit for early detection surveys, where the rapidity of results leaves more time to deploy more intensive survey efforts, rather than waiting four to six weeks for culture results.

A successful oak wilt infection requires a nitidulid carrying viable spores, a fresh wound made at the appropriate depth, and a susceptible host. Ultimately, we can only capture part of these requirements, and trapping beetles with the fungus does not always mean disease is imminent. However, we believe that this method still has value at the leading edge of the disease's range because it demonstrates that infection could be possible in areas that are otherwise unaware that the oak wilt fungus is present at all. For much of the northeastern United States, where oak wilt infections are not widespread, this would be valuable information that could help argue for funding, prioritize survey areas and establish management frameworks proactively to be better prepared if infections are found. In New York State, where oak wilt has been intensively managed in the few locations it is found, we continue to use PCR testing of trapped nitidulids to prioritize areas for survey and identify areas at risk. Given the high intensity and cost of surveying to detect single infected trees, the additional information provided by this oak wilt early detection method has led to a better allotment of resources.

Oak wilt has not been widely studied in the northeast since it was researched in Pennsylvania in the 1960–80s (examples Merrill, 1967, Jones, 1971, Bowen & Merrill, 1982). Through our surveys and management, we have learned that the fungus' behaviour appears different than in the Midwest, where it is more heavily studied. In the Midwest, root graft spread is the main method of infections due to the oak-heavy nature of their forests and deep, sandy soils that promote root grafting (Juzwik et al., 2011). In the northeast, aboveground spread seems to be much more significant, and many New York infections occurring on steep slopes with rocky soils see less root graft spread. We have less information on spore mat formation since infected trees are destroyed as they are found to prevent spread. This method gives land managers in the northeast a relatively passive method that can inform them of whether the oak wilt fungus is in their area, when beetles with it are most abundant, and even what species are most frequently associated. This information will provide a better understanding of the fungus as it moves into new areas on the landscape.

We successfully detected the oak wilt fungus from wet-collected nitidulid samples with nested PCR, demonstrating that this is a plausible early detection method. The discovery of positive beetles around a new infection in 2019 suggest that this testing can accurately reflect when oak wilt is present. Oak wilt positive samples at 11 of our 13 early detection sites in 2020 and 2021 demonstrate that this early detection method can be used over large areas to passively detect oak wilt. It is more difficult to use this method to find specific infected trees for management, as positive beetles do not necessarily mean infected trees are in the immediate vicinity. Over time, this technology should continue to develop until it is operational and ready for wider use. We hope this will help land managers better understand when oak wilt is entering their area, its local biology and associated nitidulids, and when the highest risk of spread occurs. Knowing this information will help managers take better preventative and therapeutic actions to slow the spread of this devastating disease.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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