Conventional Gel Electrophoresis and TaqMan Probes Enable Rapid Confirmation of Thousand Cankers Disease from Diagnostic Samples

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Abstract

Thousand cankers disease (TCD) is caused by the fungal pathogen *Geosmithia morbida* and vectored by the walnut twig beetle *Pityophthorus juglandis*. In infected walnut and butternut (*Juglans* spp.) hosts and wingnut species (*Pterocarya* spp.) hosts, tree decline and death results in ecological disruption and economic losses. A rapid molecular detection protocol for TCD using microsatellite markers can confirm the presence of insect vector or fungal pathogen DNA, but it requires specialized expensive equipment and technical expertise. Using four different experimental approaches, capillary and conventional gel electrophoresis, and traditional polymerase chain reaction (PCR) and quantitative PCR (qPCR), we describe simplified and inexpensive processes for diagnostic confirmation of TCD. The improved and rapid detection protocols reported in this study reduce time and equipment

costs associated with detection of molecular pest and pathogen DNA by (1) using conventional gel electrophoresis or TaqMan molecular probes to elucidate the detection limits for *G. morbida* and *P. juglandis* DNA and (2) identifying resources that allow visualization of positive test results for infected host plant tissue samples. Conventional gel electrophoresis and TaqMan molecular probe protocols detected presence of DNA from TCD-associated fungal and insect samples. These procedural improvements can be readily adopted by diagnostic end-users and adapted for use with other complex disease systems to enable rapid pest and pathogen detection.

Keywords: *Geosmithia morbida*, *Juglans nigra*, microsatellites, *Pityophthorus juglandis*, TaqMan, thousand cankers disease, visualization

Thousand cankers disease (TCD) is an emerging plant disease complex that results from interactions between the host, walnut and butternut (*Juglans* spp.) and wingnut species (*Pterocarya* spp.), the principal bark beetle vector, *Pityophthorus juglandis* Blackman, and the fungal pathogen *Geosmithia morbida* Kolařík, Freeland, Utley, and Tisserat (Hishinuma et al. 2016; Kolařík et al. 2011; Seybold et al. 2013; Tisserat et al. 2009). Although most *Juglans* spp. can be affected by TCD, *Juglans nigra*, black walnut, is the most susceptible host across the current range of pathogen distribution in the United States. The disease was introduced into Europe and in Italy, where it infests native English walnut, *J. regia*, as well as *J. nigra*, which is an introduced tree species (Montecchio et al. 2014, 2016; Moricca et al. 2018; Seybold et al. 2019; Tisserat et al. 2009). The disease is spread by adult *P. juglandis* beetle attacks and larval gallery excavation within the phloem and vascular cambium of the tree. Although *P. juglandis* is the primary vector of TCD, Chahal et al. (2019), Moore et al. (2019), and Juzwik et al. (2016) have reported detection of *G. morbida* in six different eastern U.S. states in association with 20 species of bark- or wood-inhabiting beetles in seven different insect subfamilies. Vectors distribute *G. morbida* conidia and hyphae, which contribute to fungal expansion and dark, necrotic cankers beneath the

bark. As the multiple cankers expand, they coalesce and girdle the tree, causing wilting, yellowing leaves, and dieback that progresses across 3 to 4 years and can eventually result in tree mortality. Excellent discussion of TCD disease etiology and symptoms is available (Daniels et al. 2016; Griffin 2015; Kolařík et al. 2011; Newton and Fowler 2009; Seybold et al. 2013, 2019; Tisserat et al. 2009).

Early detection of TCD is difficult; initial symptoms are often mistaken for drought damage because nearly all visible signs of the pathogen are hidden beneath the bark (Daniels et al. 2016). In addition, efforts at culture-based detection of *G. morbida*, which were made by screening 1,596 subcortical insects that had been collected beneath walnut tree crowns in TCD-impacted areas, yielded recovery of only two *G. morbida* isolates. When paired with molecular detections achieved using additional specimens sampled from the same locations (Chahal et al. 2019), this limited culture-based recovery outcome provides additional evidence that a better molecular detection protocol is needed to confirm pathogen presence. Traditional and molecular methods for detecting *G. morbida* (Gazis et al. 2018; Oren et al. 2018; Tisserat et al. 2009) have been extremely useful in facilitating state or federal regulatory activity.

To isolate and confirm the pathogen using traditional tools, a small branch section from a potentially infested tree is collected. After carefully removing the bark, including part of the phloem, necrotic tissue from suspected cankers is excised and incubated on an agar medium (Gazis et al. 2018; Oren et al. 2018). Fungi are then subcultured until axenic cultures are obtained. Confirmation of *G. morbida* identity is completed using morphological and molecular approaches (Hadziabdic et al. 2014). This process can be extremely laborious and time consuming, taking up to 5 weeks for eastern U.S. isolates that may be more difficult to isolate because of fewer instances of fungal recovery from infected plant tissues (Oren et al. 2018). The principal vector *P. juglandis* is identified using a morphological key (Bright 1981), aided by a descriptive guide (Seybold et al. 2013), and can then be confirmed using *P. juglandis*-specific microsatellite loci markers (Hadziabdic et al. 2015).

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An alternative molecular approach has been developed to identify the presence of TCD complex members (*G. morbida* and *P. juglandis*) in infected host plant tissues (Oren et al. 2018) using species-specific microsatellite loci (Hadziabdic et al. 2012, 2015; for details about DNA detection enhancement, see Oren et al. 2018). This process eliminates the need to isolate the pathogen, which can be laborious because of lack of selective media (Oren et al. 2018).

In addition to detection of species-specific microsatellite loci, two other effective and sensitive molecular detection methods are available that both use quantitative polymerase chain reaction (qPCR) assays (Lamarche et al. 2015; Rizzo et al. 2020). Similar to the approach by Oren et al. (2018), these authors used different environmental samples, including symptomatic and asymptomatic wood, insect frass, entire adult individual beetles, and fungal mycelia that were obtained from pure culture. Both studies used β -tubulin genes for identification of the pathogen (Lamarche et al. 2015; Rizzo et al. 2020), and Rizzo et al. (2020) used mitochondrial cytochrome oxidase subunit 1 (COI) genes to detect the insect vector. A third approach has been developed by Moore et al. (2019) that uses conventional PCR and gel visualization for detecting *G. morbida*; however, the study noted some limitations. The authors were able to detect β -tubulin regions of fungal DNA using pathogen-specific primers GmF2 and GmR13; however, detection capability was reduced for low concentrations of *G. morbida* DNA, and test accuracy was 86%, which could lead to false-positive results (Moore et al. 2019).

Using the published protocol (Oren et al. 2018), molecular detection can be accomplished within a day, and like the other studies, completing the procedures requires expensive equipment, specialized technical training, and sampling expertise. Consequently, a critical need remains to modify our existing methodological practices by creating a user-friendly and cost-effective procedure that would enhance adoption by diagnostic labs for the microsatellite loci approach to detecting target species. In addition, modifications to the current protocol will reduce the need for costly and specialized equipment and allow diagnostic success from personnel who may have less technical training. In this study, we compared previously used automated capillary electrophoresis to a conventional gel system and considered the use of TaqMan probes to simplify the process and reduce confirmation time.

Conventional gel systems require minimal training and expertise and are readily available to many diagnostic laboratories across the United States. They also have the advantage of being a less expensive alternative to the automated capillary electrophoresis system that was used in an earlier detection protocol (Oren et al. 2018). Moore et al. (2019) used a conventional gel approach for *G. morbida* detection and reported detection confidence with 86% certainty when using primer pairs developed for the pathogen's β -tubulin region. Although their approach simplified preliminary detection, the process benefits from an additional sequencing step to achieve a higher degree of confirmation accuracy. We examined a conventional gel platform to determine if this system could be optimized to detect *G. morbida* and *P. juglandis* DNA in a suspect TCD tissue sample rather than relying on an automated capillary gel system. The novel use of this approach stems from visualization of the presence of a minute amount of target plant pathogen or vector DNA within the "dirty" complex of DNA from the host plant sample, which contains mostly host plant DNA. Fluorescing molecular probes are designed to elicit a specific light spectrum when illuminated with an excitation wavelength that allows PCR results to be visualized, thus eliminating the need for further steps or sequencing of PCR product.

TaqMan probes incorporate modified, short oligonucleotide sequences that are adapted to identify the presence of small amounts of a specific target DNA sequence (Tyagi and Kramer 1996; Lamarche et al. 2015). More importantly, they can be designed to test multiple reactions (multiplexing) of different samples in a single tube or array, thus reducing time, labor, and costs associated with molecular detection (Lamarche et al. 2015; Rizzo et al. 2020). In conventional usage, TaqMan probes have been designed to work in conjunction with a qPCR machine that is calibrated to digitally measure the level of fluorescence emitted by molecular probes or dyes that become bound to target DNA sequences during amplification. Using a molecular approach with TaqMan probes, Lamarche et al. (2015) successfully detected DNA of

pathogens that currently are not found in Canada, creating a tool that can be used for rapid confirmation of submitted suspect samples, for example, to assist with regulatory monitoring or quarantine decisions.

A few studies have noted that the fluorescence of molecular beacons sometimes can be viewed without using a qPCR thermocycler (Kostrikis et al. 1998; Qian et al. 2018). The study reported here builds upon those observations and tests the extent to which positive test results using fluorescent probes can be visualized independently by traditional PCR methods. To our knowledge, an end-user's ability to directly visualize a TaqMan probe test outcome outside of a qPCR thermocycler, which documents this visualization action internally, has not been reported.

This study detailed methods by which molecular diagnosis of TCD can be improved beyond currently available protocols and assessed the corresponding equipment costs that are available for use with conventional electrophoresis and probe visualization. The following objectives for the experiments reported here were to (1) optimize the existing TCD PCR diagnostic protocol for use with conventional electrophoresis and (2) document sensitivity of test detection capability and resources needed to achieve a positive test result using TaqMan probes (i.e., visualization of target species' DNA from within a complex host plant tissue sample).

Materials and Methods

Diagnostic approaches. For this study, four separate experiments investigated the lowest limits of DNA source material collected from TCD complex members consisting of the fungal pathogen *G. morbida* and the primary insect vector *P. juglandis*. The pathogen and insect vector can be detected with a previously published protocol that uses species-specific microsatellite markers (Oren et al. 2018). In this study, stepwise dilution series of subject DNA were analyzed in a QIAxcel Advanced System (Qiagen, Germantown, MD), which provides fully automated, sensitive, high-resolution capillary electrophoresis with a ScreenGel software-derived electropherogram on 96 samples per run. These results were compared with those achieved using a conventional gel electrophoresis system.

A parallel approach validated use of the same species-specific microsatellite markers that were modified to include TaqMan probes to enable a visible fluorescent signal at a specific wavelength, revealing a positive test result. Visualization of fluorescence was recorded within a QuantStudio 6 Flex Real-Time PCR System that was used for qPCR (hereafter referred to as qPCR) (Applied Biosystems, Foster City, CA). These results were compared with fluorescence reporting that was achieved using traditional PCR using a SimpliAmp Thermal Cycler (Applied Biosystems) followed by direct visualization using a NightSea Dual Fluorescent Protein Flashlight (NightSea, Lexington, MA) light source (details discussed later). Success with this latter visualization step would demonstrate that positive test results could be visualized independently without the qPCR system, thus eliminating the need for specialized, expensive equipment.

DNA sources, extraction, dilution series, and visualization in four different experiments. *Experiment 1.* Source DNA for the first experiment was extracted directly from either *G. morbida* mycelia grown in axenic cultures (four isolates: GM27, GM33, GM35, and GM47) (Hadziabdic et al. 2014) or from an individual *P. juglandis* beetle (four samples using an entire adult individual beetle), which had been identified based on insect morphology. The goal of this experiment was to test the lowest amount of detectable DNA using each of the four approaches described earlier. DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen), per the manufacturer's protocol. For the first two diagnostic approaches (QIAxcel Advanced system and conventional gel tests), DNA from the four *G. morbida* isolates was serially diluted in sterile water into 12 concentrations ranging from 0.75 to 0.00125 ng/ μ l (Table 1). The four *P. juglandis* DNA samples were tested separately, and each sample was diluted into 11 concentrations ranging from 0.75 to 0.0006 ng/ μ l (Table 1). To test our third and fourth diagnostic approaches (qPCR and traditional PCR), dilutions were made using one-to-one serial dilutions in sterile water from known starting concentrations of DNA, and then samples were assayed using blue light visualization tests (see later discussion). Two technical replications for each of *G. morbida* and *P. juglandis* concentration series

were performed. For *G. morbida*, 14 different DNA concentrations ranging from 14.0 to 0.0008 ng/μl were used to test feasibility of this approach (Table 1). All samples were tested until DNA was no longer detectable (a negative response) or recorded amplifications were not detected after the 38-cycle set threshold in qPCR.

To visualize TaqMan probe fluorescence in blue light visualization tests, the samples were illuminated with a 440- to 460-nm excitation wavelength generated externally by a handheld NightSea Dual Fluorescent Protein Flashlight (NightSea). In response to the excitation wavelength, the fluorescent molecule, or fluorophore, emitted a 495- to 525-nm spectral wavelength that could be viewed by eye when the excitation spectrum was blocked with light-filtering glasses (NightSea) (Fig. 1). The glasses also can be placed in front of a camera lens to facilitate image capture. For this project, we used an FAM fluorophore that has an excitation of 495 nm and an emission of 520 nm, and ROX (a fluorescing reference dye) that has a 575-nm excitation wavelength and a 602-nm emission wavelength. FAM was used to assay a sample for presence of a specific section of target DNA, whereas ROX was used as a fluorescing, passive reference dye. In the blue light visualization tests, a negative response was recorded when the samples appeared red when illuminated with the excitation wavelength.

Experiment 2. Simulated bark samples were created using solutions of DNA extracted from drilled bark shaving samples taken from disease-free trees. This experiment, which tested the recovery capability for known amounts of target species DNA from a sample containing host plant bark tissue, used frozen drill shaving samples from Missouri-sourced *J. nigra* host plant branches that tested negative for presence of either *G. morbida* or *P. juglandis* DNA. Host plant DNA from bark shaving samples stored in 2-ml Eppendorf tubes was extracted using a QIAamp Fast DNA Stool Mini Kit and then quantified using a NanoDrop 1000 Spectrophotometer (ThermoFisher, Waltham, MA). Host plant DNA samples were standardized to a final *J. nigra* DNA concentration of 7 ng/μl per sample. To complete the simulated bark samples, serial dilutions containing a known (quantified) amount of fungal or vector DNA was added to the host plant DNA stock solution. These simulated bark samples are descriptively referred to as “Type 1

simulated bark samples.” For the first two diagnostic approaches (QIAxcel Advanced system and conventional gel tests), each Type 1 simulated bark sample received DNA from one of four *G. morbida* isolates using eight concentrations of DNA that ranged from 0.15 to 0.001 ng/μl (Table 2). For Type 1 simulated bark samples receiving *P. juglandis* DNA, the nine tested concentrations ranged from 0.15 to 0.0005 ng/μl (Table 2). Four sets of simulated bark samples containing *G. morbida* and four sets of simulated bark samples containing *P. juglandis* were created and tested. To test our third and fourth diagnostic approaches (qPCR and traditional PCR detection), followed by blue light visualization tests for Type 1 simulated bark samples, DNA from three *G. morbida* isolates were diluted into six different concentrations that ranged from 0.6 to 0.008 ng/μl. Type 1 simulated bark samples that contained *P. juglandis* DNA were tested across nine concentrations ranging from 0.15 to 0.0005 ng/μl (Table 2). For this particular test, individual DNA samples from only three adult *P. juglandis* beetles were used.

Experiment 3. A third experiment assayed drilled bark shaving DNA samples that had been extracted from trees in California and Tennessee that were previously confirmed to be positive for the presence of *G. morbida* or *P. juglandis* DNA (samples from Oren et al. 2018). The goal of this experiment was to reassess field-collected samples from which *G. morbida* or *P. juglandis* DNA had been detected but from which it was not possible to quantify the amount of DNA in the target species. These samples most closely reflected the type of resource that would be submitted to a diagnostic laboratory. Samples were drilled around the *G. morbida* canker areas and beetle galleries. Collected drill shavings were used for DNA isolation. Unlike the Type 1 bark sample construct, this DNA source contained an unknown (unquantifiable) amount of mixed fungal, vector, or host plant DNA. Hereafter, we refer to this DNA source simply as confirmed drilled bark shaving samples. Serial dilutions from drilled bark shaving samples were performed in one-to-one, stepwise dilutions from the original recommended 1-μl volume (=100%) without known concentrations (Table 3), resulting in ranges from 100 to 0.4%. For this test, we only used the QIAxcel Advanced system and conventional gel diagnostic approaches.

Table 1. Experiment 1 test results from dilution assays with DNA extracted from four different axenic *Geosmithia morbida* cultures (GM27, GM33, GM35, GM47) and four different samples of individual adult *Pityophthorus juglandis* beetles^a

	QIAxcel Advanced Conventional gel system test results (no. positive samples per test type)	Tested concentrations (ng/μl)	qPCR Traditional PCR blue light visualization test results (no. positive samples per test type)
Amplification of <i>G. morbida</i> DNA			
0.75	4 4 ^b	14.0	4 4
0.56	4 4	3.5	4 4
0.28	4 4	1.8	4 4
0.11	4 4	0.9	4 4
0.08	4 4	0.4	4 4
0.06	3 4	0.2	4 4
0.04	3 4	0.1	4 4
0.02	3 3	0.05	4 4
0.01	3 2	0.025	4 0
0.005	2 2	0.013	4 0
0.0025	1 1	0.006	4 0
0.00125	1 0	0.003	0 0
		0.0016	0 0
		0.0008	0 0
Amplification of <i>P. juglandis</i> DNA			
0.75	4 4	0.75	4 4
0.37	4 4	0.37	4 4
0.18	4 4	0.18	4 4
0.09	4 4	0.09	4 4
0.04	4 4	0.04	4 0
0.02	4 4	0.02	4 0
0.01	4 4	0.01	4 0
0.005	3 4	0.005	4 0
0.0025	3 1	0.00125	0 0
0.00125	0 1	0.0006	0 0
0.0006	0 0		

^a Efficiencies of four methods are presented for parallel diagnostic approaches used detect target DNA.

^b Counts of positive test results reflect confirmation at the last concentration before the QIAxcel Relative Fluorescence Units fell below 0.05 ng/μl (after which test results were considered negative). A negative conventional gel result was conferred when there was no visible band on the gel.

G. morbida-positive drilled bark shaving samples were used from four *J. hindsii* trees in California and four *J. nigra* trees in Tennessee; three *P. juglandis*-positive samples from California also were used (Table 3).

Experiment 4. It is possible that host plant bark chemistry could inhibit re-extraction of *G. morbida* DNA when a solution containing extracted DNA is added to drilled bark shavings in the simulated bark samples. To test for this effect, a fourth experiment examined the ability to detect serially diluted concentrations of extracted *G. morbida* or *P. juglandis* DNA that was added to drilled bark shavings taken from disease-free trees and then re-extracted. These samples are referred to as “Type 2 simulated bark samples.” Drilled

bark shavings (80 to 100 mg) that were obtained from TCD-free *J. nigra* branches cut from trees in Davidson County, Tennessee (near Nashville), were mixed with 400 µl of nanopure water in each 2-ml Eppendorf tube. DNA extracted from *G. morbida* or *P. juglandis* was serially diluted to yield four concentrations of target species DNA that ranged from 30 to 0.03 ng/µl, which were added to sample tubes. These concentration rates were assayed for QIAxcel Advanced system and conventional gel tests as well as for qPCR and traditional PCR, followed by blue light visualization tests (Table 4). After *G. morbida* or *P. juglandis* DNA volumes were added to each tube, the QIAamp Fast DNA Stool Mini Kit was

Fig. 1. Details in steps and times needed to achieve *Geosmithia morbida* detection using a standard methodology, a preliminary molecular detection approach (Oren et al. 2018), and molecular protocol enhancements describing conventional gel, blue light TaqMan probe, and qPCR TaqMan probe detection methods.

used to (re-)extract the combined DNA. Five replications were used for each tested concentration (Table 4). Two different negative controls were included in PCR after extractions were performed: nanopure water and a sample of DNA from nontarget *Helianthus verticillatus* (whorled sunflower). These two different negative controls were used to ensure that viable target DNA was amplified and could be retrieved with this approach and that no cross-amplification would occur (data not shown).

PCR procedures, conditions, and visualization using conventional gel electrophoresis and the QIAxcel Advanced system. To evaluate the current protocol, the DNA sources used in the four experiments described earlier were tested via the following PCR protocol: each reaction contained 2 µl of DNA with various concentrations (sample dependent), 8 µl of Go-Taq G2 Hot Start Colorless Master Mix (Promega, Madison, WI), 2 µl of each species-specific microsatellite primers, 1 µl of dimethyl sulfoxide (ThermoFisher), and 5 µl of sterile water to obtain final volume of 20 µl. Each PCR was run using the previously published settings in Oren et al. (2018) and included a positive DNA control, which consisted of DNA extracted from an axenic *G. morbida* culture or *P. juglandis* beetle tissues, and a sterile water negative control. PCR amplifications were temperature cycled using a SimpliAmp Thermal Cycler (ThermoFisher) in 96-well plates using the settings described in Oren et al. (2018).

To visualize our samples using conventional gel system, 2% agarose gels were stained with SYBR Safe DNA Gel Stain (ThermoFisher) and loaded with 10 µl of PCR product and 1 µl of 0.1× loading dye, for a total of 11 µl per sample. Samples, which included 1× TAE buffer, were run on a Bio-Rad Power Pack 300 (Bio-Rad, Hercules, CA) at 100 volts for 45 min. Results were visualized on a Typhoon Trio (GE Healthcare Bio-Sciences, Pittsburgh, PA) variable-mode imager that produces a digital image of a fluorescing sample. For conventional gel electrophoresis visualization, the lowest visible dilution was considered the sample that was detectable under the Typhoon Trio fluorescence, whereas an electropherogram was viewed in QIAxcel Advanced System (Qiagen, Valencia, CA) outputs with a relative fluorescent unit (RFU) of 0.05 established as the lowest detection limit for any given sample (lowest detection threshold). The QIAxcel Advanced system was used to visualize samples

following the protocol detailed in Oren et al. 2018 using an internal 25-bp size marker for each 96-well plate.

TaqMan probe design. TaqMan probes were designed to target microsatellite loci within the *G. morbida* and *P. juglandis* genomes previously reported to work in detection (Oren et al. 2018): GS004 (*G. morbida*-specific primers) and WTB09 (*P. juglandis*-specific primers) (Hadziabdic et al. 2012, 2015) (Table 5). TaqMan probes aligning with these microsatellite markers were designed using the PrimerQuest design program (Integrated DNA Technologies, IDT, Coralville, IA) and ordered from IDT (Table 5). TaqMan probes were designed with the 5′ fluorophore FAM attached as the reporter and 3′ Iowa Black fluorescence quencher with ZEN as a dual quencher placed within the center of the sequence.

Cross-transferability studies for TaqMan probes. To ensure that the new *G. morbida* and *P. juglandis* TaqMan probes amplified only DNA from the target species, the probes were tested against other fungal and beetle species. For *G. morbida*, this test included samples of 23 different fungal species as well as five *Geosmithia* species, including *G. morbida*, tested against the GS004 primers and probe using qPCR (Table 6; adapted from Oren et al. 2018). Next, DNA extracted from 20 different beetle species was tested against the WTB09 primers and probe using qPCR (Table 7; adapted from Oren et al. 2018).

TaqMan reactions and amplification using qPCR and traditional PCR, followed by visualization using a blue light spectrum. TaqMan qPCR reactions contained 10 µl of TaqMan Fast Advanced Master Mix (ThermoFisher), 2 µl of each primer (10 µM), 2 µl of TaqMan probe (10 µM), 2 µl of DNA, and 2 µl of sterile nanopure water, for a total reaction volume of 20 µl. Each run included a positive control of beetle-derived or fungal-derived DNA and a negative control of master mix and sterile water. A QuantStudio 6 Flex Real-Time PCR System was used for qPCR with the following program: 50°C for 2 min, 95°C for 20 s, and then 20 cycles of denaturation at 95°C for 3 s and annealing/extension at 60°C for 30 s, followed by 20 cycles of denaturation at 95°C for 3 s and annealing/extension at 55°C for 30 s. Fluorescence was measured digitally, internally during the 60/55°C annealing/extension steps. These different temperatures, which reflect optimization for microsatellite loci that were developed for the pathogen and the

Table 2. Experiment 2 test results with “Type 1 simulated bark samples” using DNA extracted from host tissue (disease-free drilled bark shavings) to which different concentrations of fungal (*Geosmithia morbida*) or insect vector (*Pityophthorus juglandis*) DNA was added (spiked material)^a

QIAxcel Advanced Conventional gel system test results (no. positive samples per test type)		qPCR Traditional PCR blue light visualization test results (no. positive samples per test type)	
Tested concentrations (ng/µl)		Tested concentrations (ng/µl)	
Bark samples plus <i>G. morbida</i> DNA ^{b,c}			
0.15	4 4	0.6	3 3
0.075	4 4	0.15	3 2
0.037	4 4	0.08	3 2
0.018	4 4	0.04	3 1
0.009	4 4	0.02	3 1
0.005	3 4	0.008	0 0
0.002	3 3		
0.001	3 3		
Bark samples plus <i>P. juglandis</i> DNA ^{c,d}			
0.15	4 4	0.15	3 3
0.064	4 4	0.08	3 0
0.032	4 4	0.04	2 0
0.016	4 4	0.02	2 0
0.008	4 4	0.008	0 0
0.004	4 4	0.004	0 0
0.002	4 3	0.002	0 0
0.001	4 2	0.001	0 0
0.0005	2 0	0.0005	0 0

^a Efficiencies of four methods are presented for parallel diagnostic approaches used to detect target DNA.

^b For the qPCR and traditional PCR blue light visualization assays with *G. morbida* DNA, GM47 DNA was not available; therefore, only DNA from *G. morbida* isolates GM27, GM33, and GM35 was used.

^c Counts of positive test results reflect confirmation at the last concentration before the QIAxcel Relative Fluorescence Units fell below 0.05 ng/µl (after which test results were considered negative). A negative conventional gel result was conferred when there was no visible band on the gel.

^d For the qPCR and traditional PCR blue light visualization assays with *P. juglandis* DNA, DNA samples from only three individual adult *P. juglandis* beetles were used.

vector, respectively (Hadziabdic et al. 2012, 2015), resulted in independent reaction runs for detection of each target species. Samples were considered positive if the amplification exceeded the background noise threshold between cycles 26 and 38 in duplicate samples. TaqMan probe fluorescence was also quantified using traditional PCR, following the traditional protocol described earlier. For blue light visualization tests, results were visualized using the Night-Sea Dual Fluorescent Protein Flashlight with filtering glasses.

Results

Adapting the existing QIAxcel Advanced protocol for use with conventional gel electrophoresis and assessing DNA detection capabilities. DNA samples in the first experiment consisted of DNA that was extracted directly from either *G. morbida* cultures or *P. juglandis* specimens. With the QIAxcel Advanced System, the lowest detectable sample concentration of *G. morbida* DNA that achieved the 0.05 RFU threshold contained 0.00125 ng/μl of sample DNA (Table 1). The conventional gel system (Fig. 1) yielded a discrete band and detection of 0.00125 ng/μl of *G. morbida* DNA, which

Table 3. Experiment 3 test results for serial dilutions of DNA collected from previously tested samples^a that were positive for *Geosmithia morbida* or *Pityophthorus juglandis*^b

Tested proportion of a 1-μl mixed-DNA starting sample concentration ^c (%)	QIAxcel Advanced Conventional gel system test results (no. positive samples per test type)	
	CA	TN
Dilutions of DNA extracted from bark samples that tested positive for <i>G. morbida</i> DNA ^d		
100	4 4 ^e	4 4
50	4 4	4 4
25	4 4	4 4
12.5	4 4	4 4
6.25	4 4	4 4
3	0 3	N/A N/A ^f
1.6	0 1	N/A N/A
0.8	0 0	N/A N/A
0.4	0 0	N/A N/A
Dilutions of DNA extracted from bark samples that tested positive for <i>P. juglandis</i> DNA ^g		
100	3 3	— ^h
50	3 3	—
25	3 3	—
12.5	3 3	—
6.25	3 3	—
3	0 2	—
1.6	0 1	—
0.8	0 0	—

^a Data from Oren et al. (2018).

^b Efficiencies are presented for QIAxcel Advanced and conventional gel approaches that were conducted in parallel.

^c Concentrations of each target species DNA cannot be determined from a mixed-species DNA extraction sample that also contained *Juglans nigra* DNA. Therefore, concentration values presented reflect the proportion of diluted sample from a 1-μl starting mixed-DNA extraction sample volume (=100%).

^d DNA extracted from bark samples taken from TCD-infested trees in California ($n = 4$) and Tennessee ($n = 4$). All tested DNA extractions were from samples that tested positive in Oren et al. (2018).

^e Counts of positive test results reflect confirmation at the last concentration before the QIAxcel Relative Fluorescence Units fell below 0.05 ng/μl (after which test results were considered negative). A negative conventional gel result was conferred when there was no visible band on the gel.

^f N/A, samples were not tested at this dilution rate.

^g DNA extracted from three drill shaving samples from TCD-infested trees in California were tested (samples from Oren et al. 2018).

^h —, no bark samples were available for testing from Tennessee that contained *P. juglandis* DNA.

was almost two times more sensitive than the capillary system that detected 0.0025 ng/μl of *G. morbida* DNA. Both systems were able to detect *P. juglandis* DNA samples with dilution concentrations to as low as 0.0025 ng/μl, although the conventional gel system was capable of detecting a slightly lower quantity at 0.00125 ng/μl (Table 1).

In the second experiment, Type 1 simulated bark samples that contained known amounts of *G. morbida* plus *J. nigra* DNA were detected for concentrations of fungal DNA down to 0.001 ng/μl for both the QIAxcel Advanced System (Fig. 1) and conventional gels (Table 2). The detection limit for samples containing *P. juglandis* plus *J. nigra* DNA was 0.0005 ng/μl using the QIAxcel Advanced System and 0.001 ng/μl using the conventional gel (Table 2).

DNA sources in the third experiment retested extracted DNA in dilution series from stored drilled bark shaving samples that had previously tested positive for the target species of interest. Results of these tests yielded differences in detection limits from the two different sources. In bark shaving samples from California, *G. morbida* DNA was detected by the QIAxcel Advanced System down to a 6% dilution from the original 1 μl of mixed DNA concentration and down to a 1.6% dilution of the mixed DNA concentration in the conventional gel. Samples from Tennessee were detected in dilutions down to 6% with both the QIAxcel Advanced and conventional gel systems (Table 3). The QIAxcel Advanced System and conventional gels were able to detect *P. juglandis* DNA concentrations that diluted to 6 and 1.6%, respectively, from the original 1 μl of DNA volume (Table 3).

In the fourth experiment, using sample dilutions of Type 2 simulated bark samples that contained DNA from either *G. morbida* or *P. juglandis*, all *J. nigra* host plant and target species DNA was re-extracted from the sample. From the re-extracted DNA, *G. morbida* DNA was detected down to 3 ng/μl with both the QIAxcel Advanced and conventional gel systems; *P. juglandis* DNA in this experiment could not be detected across methods for any supplemented concentration (Table 4).

Validating PCR protocol outcomes using a qPCR platform and enhancing sample DNA detection with traditional PCR, followed by visualization using a blue light spectrum. In experiment 1, DNA extracted from *G. morbida* yielded a qPCR threshold of 0.006 ng/μl, whereas visualization using traditional PCR with a blue light-enabled detection (Figs. 1 and 2) to the lowest threshold of 0.05 ng/μl (Table 1). For *P. juglandis* DNA, the qPCR threshold was 0.005 ng/μl, and the blue light threshold was visualized at 0.09 ng/μl (Table 1).

For the second experiment, the lowest qPCR and the traditional PCR with blue light visualization thresholds for *G. morbida* in Type 1 simulated bark samples were 0.02 ng/μl. The lowest qPCR detection threshold for *P. juglandis* DNA was 0.02 ng/μl, whereas threshold DNA detection level in the traditional PCR visualized using the blue light spectrum was 0.15 ng/μl (Fig. 2; Table 2).

For the third experiment, which assayed *G. morbida* DNA-confirmed drilled bark shaving samples using *J. nigra* samples from Tennessee and *G. morbida* and *P. juglandis* DNA-confirmed *J. hindsii* samples from California (see results in Oren et al. 2018), all branch samples tested positive for presence of *G. morbida* DNA with the optimized capillary electrophoresis and conventional gel protocols (Fig. 1; Table 3).

For the fourth experiment, DNA from Type 2 simulated bark samples (DNA of *G. morbida* or *P. juglandis* added to bark shavings and then re-extracted) yielded a lower qPCR threshold level for *G. morbida* at 3 ng/μl, whereas traditional PCR visualized with the blue light spectrum indicated positive detection at 30 ng/μl only (Table 4). The qPCR detection and blue light visualization for *P. juglandis* DNA were negative, with no concentrations yielding positive results (Table 4).

To provide current costs of these approaches (2020 catalog pricing in U.S. dollars), an estimate was made of the unnegotiated costs of materials and supplies associated with qPCR, conventional gel, and blue light visualization procedures. These values and totals are provided in Supplementary Tables S1 to S3.

Cross-transferability studies for TaqMan probes. The micro-satellite primers that were designed to detect *G. morbida* and *P. juglandis* DNA maintained their species specificity. None of the 23 samples of DNA from different fungal species amplified using the *G. morbida* TaqMan probe (Table 6). Cross-amplification also did not occur when other *Geosmithia* spp. DNA samples were amplified, and only the *G.*

morbida-positive control DNA was recovered using the probe. Only *P. juglandis* beetle DNA amplified using the TaqMan probe designed for that species. None of the other beetle species that were screened resulted in any DNA amplification from the probe (potential false-negative results) (Table 7). Finally, none of these samples returned a positive test result when other beetle species DNA was assayed using qPCR or screened via the blue light visualization test (data not shown).

Discussion

This study described a simplified molecular detection technique that can be used to detect TCD-positive samples. Results from our study affirmed that all four diagnostic methods tested (QIAxcel Advanced, conventional gel, qPCR systems, and the blue light visualization test following traditional PCR amplification) can provide reliable diagnostic detection of TCD complex members. The QIAxcel Advanced and conventional gel systems successfully confirmed the presence of the pathogen/vector with all sample types. When comparing the two systems, the conventional gel approach was easier to use, required less specialized equipment, and was generally already available for rapid molecular detection among state diagnostician and other regulatory agency laboratories. TaqMan probes and blue light visualization also yielded successful detection of *G. morbida* from all four DNA sources tested, and *P. juglandis* DNA was detected from three of the four DNA sources tested. *P. juglandis* DNA was not amplified after re-extraction of host plant and vector DNA from Type 2 bark sample constructs. We acknowledge that the primers used for *P. juglandis* detection may require additional optimization to enable consistent detection of low amounts of *P. juglandis* DNA.

Our approaches for molecular detection using microsatellite markers within conventional gel and blue light visualization are capable of distinguishing small amounts of pathogen- or vector-specific DNA contained within a heterogeneous extracted DNA sample. In this model system, we replicate the type of sample obtained when DNA is extracted from host plant drill shavings received as a diagnostic submission (e.g., from susceptible plant parts containing suspicious lesions). As demonstrated in this study, optimizing the detection protocols by using conventional gel and blue light visualization procedures makes these approaches applicable as diagnostic tools for screening of target species DNA in suspect samples. Although diluting was performed in these experiments to ascertain lower detection thresholds, diluting samples should not be necessary for diagnostic purposes. It is likely, however, that the proportional amount of DNA from target species within samples would be functionally diluted if sample submissions were to include multiple drill shaving collections that were combined to obtain a complex sample (e.g., provide a mixed sample intended for detection purposes that assays multiple points along an entire branch or an entire log from a TCD-suspect tree). Dilution assays

demonstrated that only a limited amount of *G. morbida* or *P. juglandis* DNA is necessary within drill shavings to yield positive results and confirm pathogen/vector presence.

Importantly, with the second DNA source, Type 1 simulated bark samples, the protocol was able to detect *G. morbida* or *P. juglandis* DNA even in the presence of large quantities of nontarget *J. nigra* host plant DNA. Similar findings of successful identification of mixed environmental samples were reported by Lamarche et al. (2015), including no false-positive results. In our study, sample detection from the simulated bark samples was comparable to the fungal culture-derived and vector-derived DNA samples, in some cases yielding lower detection capability than was observed for *G. morbida* or *P. juglandis* DNA alone. We caution that accepting only the lowest limit thresholds from different sample types presents challenges in interpretation of results and could result in false-positive or -negative test outcomes because of presence of other inhibitors extracted from environmental samples (Lamarche et al. 2015).

Because the initial extracted host plant samples in our study contained mixed species DNA, including potentially minute amounts of fungal species target DNA that could be quantified within the original

Table 5. Microsatellite marker-based primers and probe sequences designed for detection of *Geosmithia morbida* and *Pityophthorus juglandis* DNA

Name	Specificity	Amplicon	Sequence 5'–3'
JN580435.1		211 bp	
GS004-F	<i>G. morbida</i>	– ^a	TAG ACG AAT AAC AGC GCA AGA A
GS004-R	<i>G. morbida</i>	–	CCC GCT ACC GTA TGA AGG TAT T
GS004-Probe	<i>G. morbida</i>	–	/56-FAM/CG TTT GTC G/ZEN/G TTC TTG CGT CC/3IABkFQ ^b
KP091442.1		379 bp	
WTB09-F	<i>P. juglandis</i>	–	CAT TTC GGT TTC GCC TCA AGT T
WTB09-R	<i>P. juglandis</i>	–	TCC CAA ATC CGG ACT TTA AGG G
WTB09-Probe	<i>P. juglandis</i>	–	/56-FAM/AG GTC AAG T/ZEN/C GTT GGT GGC TTC TC/ 3IABkFQ ^b

^a Amplicon length values apply only to the whole microsatellite locus for the entire primer that was used to amplify the probes for *G. morbida* using GS004 (JN580435.1) or *P. juglandis* using WTB09 (KP091442.1).

^b The /56-FAM/ code within the probe sequence refers to the attachment position for the FAM fluorophore; /ZEN/ is the position at which the dual quencher was attached, whereas the /3IABkFQ/ code is associated with Iowa Black fluorescence quencher.

Table 4. Experiment 4 test results of “Type 2 simulated bark samples” comprised of drilled, disease-free *Juglans nigra* bark shavings and nanopure water, into which different concentrations of fungal (*Geosmithia morbida*) or insect vector (*Pityophthorus juglandis*) DNA were added^a

QIAxcel Advanced Conventional gel system test results (no. positive samples per test type)		qPCR Traditional PCR blue light visualization test results (no. positive samples per test type)	
Tested concentrations (ng/μl)		Tested concentrations (ng/μl)	
TCD-free <i>J. nigra</i> bark plus <i>G. morbida</i> DNA was (re-)extracted			
30	5 5 ^b	30	5 5
3	3 3	3	5 0
0.3	1 0	0.3	0 0
0.03	0 0	0.03	0 0
TCD-free <i>J. nigra</i> bark plus <i>P. juglandis</i> DNA was (re-)extracted			
30	0 0 ^b	30	0 0
3	0 0	3	0 0
0.3	0 0	0.3	0 0
0.03	0 0	0.03	0 0

^a The mixed-sample DNA was re-extracted using established protocols, and efficiencies are presented for four methods run in parallel diagnostic approaches to detect target DNA.

^b Counts of positive test results reflect confirmation at the last concentration before the QIAxcel Relative Fluorescence Units fell below 0.05 ng/μl (after which test results were considered negative). A negative conventional gel result was conferred when there was no visible band on the gel.

sample, the stepwise dilutions (e.g., for re-extraction of DNA from Type 2 simulated bark samples in Experiment 4) were likely to result in extremely small amounts of *G. morbida* or *P. juglandis* material. Accuracy for detection of target DNA in such samples also could be compounded when technical or mechanical error is introduced (e.g., when working with samples containing low starting concentrations of fungal or beetle DNA). Additional testing would help further quantify the detection constraints imposed by functional dilution of target species DNA. These constraints may occur when users wish to pool multiple drill shaving samples per branch or tree and perform a

whole-branch or whole-tree assessment of TCD infection status. Similarly, a bulk sample collected from multiple host logs (e.g., received from multiple suppliers or locations or logs intended for export) could be screened in a batch. If a positive test result is found, subsequent testing could yield trace-back detection to the infected location or supplier. Although additional testing to address this potential use was outside the scope of this article, we do not expect this application to be a practical limitation for the protocol.

Collectively, molecular detection approaches for TCD (Lamarche et al. 2015; Moore et al. 2019; Rizzo et al. 2020) and those presented

Table 6. DNA of fungal species that were assayed for cross-amplification with *Geosmithia morbida* primers^a

Strain code	Amplification with GS004	BLAST ID accession	GenBank
NGM35-21	No	<i>Alternaria alternata</i> strain ATCC 6663	KU729030
NGM36-12	No	<i>Alternaria arborescens</i> strain MUCL 44260	DQ242505
NGM33-16	No	<i>Alternaria destruens</i> ATCC 204363	NR_137143
NGM33-9	No	<i>Aureobasidium pullulans</i> strain CBS 584.75	KT693733
CheatPM	No	<i>Blumeria graminis</i>	MF508973
NGM34-3	No	<i>Ceratocystiopsis collifera</i> (<i>Ophiostoma colliferum</i>) CBS 126.89	NR_103597
NGM33-2	No	<i>Chaetothyrium agathis</i> MFLUCC 12-0113	NR_132914
NGM36-17	No	<i>Cladosporium cladosporioides</i> strain ATCC 6721	KP780438
NGM34-18	No	<i>Cladosporium pini-ponderosae</i> strain CBS 124456	NR_119730
NGM35-10	No	<i>Diatrypella atlantica</i> voucher HUEFS 194228	KM396615
NGM36-20	No	<i>Epicoccum nigrum</i> strain UAMH 3247	AY625064
VP609B	No	<i>Erysiphe pulchra</i>	N/A ^b
Greengold PM2	No	<i>Golovinomyces spadiceus</i>	MF460422
DH1	No	<i>Insolibasidium deformans</i>	MH790966
Henbit PM2	No	<i>Neoerysiphe galeopsidis</i>	N/A
PT14	No	<i>Peronospora tabacina</i>	N/A
NGM35-12	No	<i>Pezicula sporulosa</i> strain CBS 225.96	KR859262
PC	No	<i>Phytophthora capsici</i>	N/A
NGM35-22	No	<i>Phaeoacremonium viticola</i> strain CBS 101738	KF764574
NGM36-19	No	<i>Pichia hampshirensis</i> strain ATCC 62896	FJ381694
NGM33-13	No	<i>Tremella aurantia</i> strain CBS 6965	AF444315
NGM34-32	No	<i>Yamadazyma mexicana</i> CBS 7066	EF568069
Uncoded ^c	No	<i>Rasamsonia</i> (syn. <i>Geosmithia</i>) <i>argillacea</i> CBS102.69	MH859267
Uncoded	No	<i>Geosmithia lavendula</i>	MG733658
6LN5	No	<i>Geosmithia pallida</i>	HF546259
NRRL 2024	No	<i>Geosmithia putterillii</i>	AF033384
GM17 (positive control)	Yes	<i>Geosmithia morbida</i>	MG008849

^a Adapted from Oren et al. (2018).

^b N/A, fungal sample originated from laboratory cultures identified by DH and RNT and maintained in association with ongoing (unpublished) work. A GenBank accession number is not assigned.

^c Uncoded, no strain code was assigned to the fungal sample.

Table 7. Non-*Pityophthorus juglandis* bark beetle-derived DNA samples taken from *Juglans nigra* wood samples that were assayed for cross-amplification^a

Beetle (order: family: subfamily and genus species)	Collection location (county, state)	Amplification of WTB09
Coleoptera: Curculionidae: Cossinae		
<i>Stenomimus pallidus</i>	Knox & Blount, TN	No
<i>Stenoscelis brevis</i>	Anderson, TN	No
Coleoptera: Curculionidae: Scolytinae		
<i>Cnestus mutilatus</i>	Anderson & Knox, TN	No
<i>Euwallacea validus</i>	Anderson, TN	No
<i>Hypothenemus crudiae</i>	Knox, TN	No
<i>Monarthrum mali</i>	Knox, TN	No
<i>Monarthrum fasciatum</i>	Knox, TN	No
<i>Phloeotribus liminaris</i>	Marshall, IN	No
<i>Pityophthorus lautus</i>	Knox, TN	No
<i>Xyleborinus saxesenii</i>	Knox, TN	No
<i>Xyleborus affinis</i>	Anderson, TN	No
<i>Xyleborus ferrugineus</i>	Anderson, TN	No
<i>Xylosandrus crassiusculus</i>	Anderson, TN	No
<i>Xylosandrus germanus</i>	Knox, TN	No
Coleoptera: Bostrichidae		
<i>Xylobiops basilaris</i>	Anderson, TN	No
Coleoptera: Platypodidae		
<i>Euplatypus compositus</i>	Anderson, TN	No
<i>Oxoplatypus quadridendatus</i>	Anderson, TN	No
Coleoptera: Buprestidae		
<i>Chrysobothris sexsignata</i>	Knox, TN	No
Coleoptera: Cerambycidae		
<i>Lepturges confluent</i>	Knox, TN	No
<i>Neoclytus acuminatus</i>	Knox, TN	No

^a Adapted from Oren et al. (2018).

here provide powerful and directly applicable tools for detecting *G. morbida* and *P. juglandis* DNA. Rapid and accurate molecular confirmation is paramount to preventing further spread and making informed quarantine and regulatory decisions in both North America and Europe, where TCD is currently found (Gazis et al. 2018; Montecchio et al. 2014, 2016; Moricca et al. 2018; Seybold et al. 2019). Native *J. nigra* stands in the eastern United States and *J. nigra* and *J. regia* plantations in the nonnative, western United States and European and Mediterranean Plant Protection Organization region are of particular concern because of their environmental and economic importance (Commission Implementing Regulation [EU] 2019; Rizzo et al. 2020).

The blue light visualization protocol provides a readily adoptable method that diagnostic users can use to quickly differentiate between test results that are positive, or visibly green, and negative, or visibly red, for the species-specific DNA of interest. The visualization protocol allows for immediate result identification without the need for a conventional gel, access to a QIAxcel Advanced System, or additional sequencing (Moore et al. 2019). Adopting this protocol makes these diagnostic tests easier to perform and provides results that are more easily interpreted by end-users. Although a conventional gel does reduce the cost of equipment by thousands of (U.S.) dollars, it is not as cost-effective as the blue light visualization test. Conventional gel procedures are widely practiced in diagnostic labs, and blue light visualization requires less specialized training and is a user-friendly method.

None of the DNA from other beetle species that were screened for potential cross-amplification with the *P. juglandis* probe resulted in amplification. This lack of amplification validates the species-specificity of the chosen probe and primer set as previously reported in Oren et al. (2018). Microsatellite markers are limited to yielding cross-amplification only in closely related species if cross-amplification occurs at all (Dutech et al. 2007; Hadziabdic et al. 2010, 2012, 2015; Oren et al. 2018). Our result is additionally relevant for the walnut system because many of the tested beetle species are active throughout the *J. nigra* crown, and a few of these species have been found to associate with *G. morbida* (Chahal et al. 2019; Hadziabdic et al. 2015; Klingeman et al. 2017).

Further testing of the *P. juglandis* probe using the protocol for samples of drilled bark shavings will assess the sensitivity of the TaqMan probe approach for detecting *P. juglandis* DNA taken from an infested, field-collected host plant. Our inability to reconfirm the *P. juglandis*-positive samples reported in Oren et al. (2018) is likely explained either by sample degradation during extended storage or by the low DNA volumes and low RFU (0.1 to 0.05; data not shown) that were observed when these samples were assayed with the QIAxcel Advanced System. We hypothesized that these samples contained *P. juglandis* DNA at levels too small for the qPCR to amplify. Another possibility is that microsatellite loci developed for *P. juglandis* are simply not as sensitive for detection methodologies.

Fig. 2. Visualization of negative (red) and positive (green) test results using *Geosmithia morbida* primers and TaqMan probe in a typical PCR setting. **A**, Negative water control in the left tube and a positive *G. morbida* sample in the right tube before PCR. **B**, The same two samples after PCR. The negative control remains red, attributed to presence of ROX reference dye in the solution, whereas a sample positive for detection of *G. morbida* DNA becomes green.

The conventional gel and blue light visualization methods each have advantages and disadvantages. Once DNA is amplified, visualizing test results via the conventional gel method takes 1 to 2 h to complete, whereas evaluating test results with blue light visualization can be concluded within a few minutes. Users also need to make or order gels before the assay can be performed. However, the conventional gel electrophoresis method is considerably more sensitive than the blue light visualization method. The conventional gel yields positive test results for bark sample construct Type 1 using both *G. morbida* and *P. juglandis* samples with target species DNA concentrations as low as 0.001 ng/μl. By contrast, the blue light visualization method detected positive results for the same bark sample constructs containing *G. morbida* and *P. juglandis* DNA at concentrations as low as 0.02 ng/μl and 0.15 ng/μl, respectively. Whereas blue light visualization is less sensitive than conventional gels, the method is faster, less expensive, and easier for end-users who may be challenged by equipment and technical expertise limitations. We also note that *P. juglandis* DNA was detected less consistently in supplemented host plant DNA samples assayed using the qPCR and light visualization procedures, which suggests need for additional optimization for this beetle species, particularly to meet future goals of multiplexing capability for using two probes in simultaneous screening of a single host plant tissue sample for presence of either *G. morbida* or *P. juglandis* DNA.

The costs of the starting equipment required for the QIAxcel Advanced System protocol (Oren et al. 2018) are considerably more than would be needed for use of the conventional gel and blue light visualization methods with modifications reported here. The QIAxcel Advanced System and supporting software together are currently priced between \$30,000 and \$45,000 (all prices are based on USD using fiscal year 2020 pricing). A conventional gel system, such as Invitrogen E-Gel Power Snap Electrophoresis System, costs approximately \$3,000. The qPCR system cost is similar to the cost of a QIAxcel Advanced System, so that method is not economically advantageous for simple detection assays. Alternatively, the blue light visualization protocol requires the purchase of a NightSea blue light and blue light-blocking glasses, together costing about \$815.

For the various detection methods detailed in this study, the cost of consumable supplies is similar (Supplementary Tables S1 to S3). However, it is important to understand that costs of materials may change across time and can differ depending on company and volume discounts. Materials costs associated with running a single sample using the QIAxcel Advanced System were estimated at \$10.60 per sample (Oren et al. 2018). In our study, reduced costs can be achieved after the initial expense of a conventional gel system. Using current pricing estimates, the materials cost for detection of the TCD pathogen/vector per sample using the QIAxcel system was estimated at \$11.98. For the conventional gel and blue light visualization protocols, per-sample supplies costs are similar, at \$11.33 and \$12.03, respectively.

Timesaving molecular techniques that can be used to quickly diagnose plant diseases are increasingly in demand, particularly for agricultural commodity crops. To assist cranberry (*Vaccinium macrocarpon* Alton) producers, traditional PCR techniques have been adapted into assays that enable detection of fungal DNA from up to 12 potential plant pathogen species that are implicated in cranberry fruit rot (Conti et al. 2019). The sensitivity of this multiplex approach yielded positive detection of target fungal DNA down to 0.00001 ng per sample (Conti et al. 2019). Sensitivity of their approach did exceed the 0.0025 ng per sample sensitivity of protocols used in this study for detecting pure *G. morbida* DNA. However, their approach used multicopy regions of internal transcribed spacers, large subunit regions, and translation elongation factor 1- α , which allowed for higher sensitivity, and required use of the QIAxcel system for confirmation.

Although using TaqMan probes for visual detection of pathogens is novel to this study, these probes have been used for disease detection in several studies using qPCR detection methods (Jeyaprakash et al. 2014; Lamarche et al. 2015). Laurel wilt is a complex disease resulting from interaction between the beetle *Xyleborus glabratus* Eichhoff, the fungal pathogen *Raffaelea lauricola* T.C. Harr. Fraedrich, and Aghayeva, and host species that include several important plants such as *Persea borbonia* (L.) Spreng (redbay), *P. americana* Mill (avocado), *Cinnamomum camphora* (L.) Sieb. (camphor), and *Sassafras albidum* (L.)

(sassafras) (Hughes et al. 2017). One current method to detect laurel wilt fungal DNA is accomplished using TaqMan probes to visualize amplification of the 28S multicopy gene sequence region from samples of finely powdered wood tissue (Jeyaprakash et al. 2014). Although this method is effective in rapidly diagnosing laurel wilt, the process still requires specialized equipment (qPCR) and trained personnel (Jeyaprakash et al. 2014). The laurel wilt fungus protocol is an example of a methodology that could be easily altered to fit the blue light visualization technique. Some diagnostic laboratories and technical evaluators may not have access to a qPCR system but may have access to the more common conventional PCR machine. These users could instead use this blue light visualization method rather than purchase a qPCR machine for tens of thousands of dollars. Ongoing technological developments and access to portable qPCR machines also can be adapted to enable in-field diagnostic determinations for presence of pathogen and other species' target DNA.

In conclusion, the novel methodologies described in this study are directly applicable and easily adaptable to diagnostic uses with other complex model systems. For example, this approach could be implemented where DNA from a target species, including bacterial, plant pathogen, and other co-occurring organisms or symbionts, may be present within a mixed sample of DNA from a potential host sample. We expect the diagnostic procedures outlined here to be readily adopted into use by municipal arborists, urban foresters, regulatory agents, and diagnosticians who can afford only limited laboratory time and relatively low laboratory equipment costs.

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