



Research

Development of a qPCR Assay for Species-Specific Detection of the Tar Spot Pathogen *Phyllachora maydis*

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Abstract

Phyllachora maydis is a fungal plant pathogen that causes tar spot of corn (*Zea mays*) in North and South America, causing devastating yield losses under favorable conditions. Although the causal agent is relatively easy to diagnose via macroscopic and microscopic observations, other diseases and conditions, such as insect frass, have been mistaken for tar spot of corn. Furthermore, conidia and ascospores in isolation can be difficult to visually distinguish from other fungi, and the development of signs and symptoms of the disease may not be observed until 12 to 20 days after infection. Therefore, we developed a TaqMan quantitative polymerase chain reaction (qPCR) assay for the detection and quantification of this pathogen to be used for diagnostics and airborne spore quantification. The assay was designed for the internal transcribed spacer region of *P. maydis*. The specificity of the assay was confirmed and tested against various nontarget *Phyllachora* species, corn pathogens, endophytes, and *P. maydis* samples from several states in the Midwest and from Mexico. The detection limit of this assay was determined to be 100 fg of genomic *P. maydis* DNA. To demonstrate the transferability of this technology, the assay was tested in different labs using various qPCR thermal cyclers. This assay can be used in downstream research involving latency period, disease prediction, and diagnostics.

Keywords: diagnostics, fungal pathogen, pathogen detection, *Phyllachora maydis*, qPCR assay, tar spot of corn

Phyllachora maydis is a fungal obligate parasite that causes tar spot of corn (*Zea mays*) and was first described in Mexico in the early 1900s (Maublanc 1904). Over the course of the 20th and 21st centuries, tar spot was found across the Americas and has caused significant crop production loss. Previous reports in Mexico noted that tar spot could cause up to 50% yield loss (Hock et al. 1989; Pereyda-Hernandez et al. 2009). Currently, tar spot of corn has been reported in 19 countries (da Silva et al. 2021;



Valle-Torres et al. 2020), with confirmation of tar spot in the continental United States in 2015. In only a few years, tar spot has spread across the United States and into Canada. Tar spot of corn is now found in 18 states in the United States, including all the top 10 corn-producing states (Collins et al. 2021; Dalla Lana et al. 2019; Malvick et al. 2020; McCoy et al. 2018; Moura et al. 2023; Pandey et al. 2022; Ruhl et al. 2016; Wise et al. 2023; <https://corn.ipmPIPE.org/tarspot/>). In 2021, yield losses in the United States due to tar spot were estimated at 5.97 million metric tons (235 million bushels), equivalent to 1.2 billion U.S. dollars in economic loss (Crop Protection Network Disease Loss Calculator; <https://loss.cropprotectionnetwork.org/>). Furthermore, data for corn hybrid performance trials performed in Illinois, Indiana, Michigan, and Wisconsin showed an estimated disease loss ranging from 1,130 to 2,605 kg/ha under high tar spot disease pressure (Telenko et al. 2019), with up to 6,725 kg/ha reported by farmers in severe situations (Chilvers, *personal observation*). Tar spot is now considered one of the top yield-reducing corn diseases in the United States.

Tar spot of corn is defined by the small, black stroma that form on the foliage of corn. These stroma typically range between 0.5 and 2.0 mm in size but can be up to 1.0 cm and are therefore readily identified by macroscopic observation (da Silva et al. 2021; Solórzano et al. 2023). Additionally, *P. maydis* stroma are embedded in the leaf, and stroma can often be seen on both the abaxial and adaxial sides of the leaf. The stroma are slightly raised above the plane of the corn leaf and cannot be brushed off the leaf surface. Although the signs of tar spot are distinct, several diseases, such as Physoderma brown spot (caused by *Physoderma maydis*) and common corn rust (caused by *Puccinia sorghi*) can be mistaken for tar spot (Solórzano et al. 2023). Furthermore, insect frass and pesticide damage have been mistaken for tar spot on both corn and sorghum (Solórzano et al. 2023; Chilvers, *personal observation*). To confirm that the observed signs are those of *P. maydis*, microscopic dissection should be undertaken to observe the presence of fruiting structures, perithecia that contain asci with uniseriate ascospores, and pycnidia that contain the asexual spores within the stroma. The ascospores of *P. maydis* are aseptate, hyaline, ellipsoid, and 9.5 to 14 by 5.5 to 8 μm (Maublanc 1904; McCoy et al. 2018), overlapping in characteristics of other pathogenic and cosmopolitan fungi. The asexual spores are slightly more distinct but can be difficult to observe, being hyaline and 0.2 by 10 to 15 μm (Maublanc 1904). Although diagnostic techniques for tar spot can be simple, these capabilities depend on having a high-quality sample, a compound microscope, and trained personnel. A molecular diagnostic assay may aid in the identification of difficult or ambiguous samples. No molecular diagnostic techniques existed prior to this study for *P. maydis*.

Many of the species in the order Phyllachorales, including *P. maydis*, cause tar spot on various host plants (Silva-Hanlin and Hanlin 1998). Most are considered obligate parasites, meaning they can only grow on their biological host and do not grow in axenic culture, and until recently, consistent greenhouse or growth chamber inoculation protocols were not available (Breunig et al. 2023). Due to the obligate parasitic nature of these fungi, classifications have relied heavily on microscopic observation and host plant identification. Further phylogenetic and classification work remains to confirm the species and host specificity within the order. Current phylogenetic studies show *Phyllachora graminis* to be one of the most closely related species to *P. maydis* (Broders et al. 2022; Mardones et al. 2017). Whereas *P. maydis* has high host specificity to corn, *P. graminis* is a species with a reported wide host range affecting members of the Poaceae family. According to the USDA fungus-host database, *P. graminis* has been identified on 76 genera, such as *Andropogon*, *Bromus*,

Calamagrostis, *Elymus*, *Muhlenbergia*, *Panicum*, *Paspalum*, and *Sporobolus* (Farr and Rossman 2023). Several of these hosts can also be infected by other *Phyllachora* species. For example, *Phyllachora vulgata* can infect similar hosts in the *Muhlenbergia* and *Sporobolus* genera (Farr and Rossman 2023). Among these possible discrepancies in classifications, there is a lack of DNA sequence data of *Phyllachora* species, due in part to the difficulty in working with these organisms and their previous insignificance to economically important agronomic crops. Genomes of *P. maydis* were recently published and are the first genomes to be sequenced within the order Phyllachorales (MacCready et al. 2023; Telenko et al. 2020). Many species within the order have only the internal transcribed spacer (ITS) sequence available, a common locus used in fungal identification and molecular detection assays (Crouch and Szabo 2011; Gardes and Bruns 1993), whereas many other species have no sequences available, making genetic work and molecular comparisons between species impossible.

The disease cycle of tar spot also has uncertainties. Specifically, *P. maydis* overwinters on corn residue left in the field (Groves et al. 2020; Kleczewski et al. 2019). Under high humidity (greater than 75%) and moderate temperatures (17 to 22°C), *P. maydis* spores are exuded and then dispersed via wind and rain splash. Ascospores land onto the host plant tissue and germinate (Hock et al. 1995). Using an appressorium infection structure, the fungus penetrates the leaf surface and begins colonizing the plant (Dittrich et al. 1991). Tar spot stroma then develop approximately 12 to 20 days after the initial infection occurs (da Silva et al. 2021). Development of disease over the course of infection, specifically at early stages, has not been extensively observed with microscopy, and the detection of the disease depends on macroscopic observation of tar spot stroma. Therefore, predictions of disease development can be misconstrued, as the infection period cannot be tracked before symptoms develop. Research is being conducted to determine the spore load observed over the season using spore trapping methods. Using microscopy or a molecular detection assay, one can determine the timing of spore release and describe relationships between weather events and spore release (Falacy et al. 2007; Prados-Ligero et al. 2003). Efforts are being made to develop a predictive disease risk model for tar spot of corn. Using multiple-parameter weather data and corn growth stage, one can assess the risk of *P. maydis* infection and tar spot development in a field and determine if management actions need to be made to prevent disease and protect yield (Webster et al. 2023). Disease risk models have also correlated weather data with real-time detection of spores in other pathosystems to improve prediction model accuracy (Newlands 2018; Wen et al. 2017). However, microscopic identification of *P. maydis* spores in isolation would be difficult to distinguish in environmental samples. Therefore, a molecular assay is needed for earlier detection of this pathogen to help in tar spot predictions.

In this study, a species-specific DNA detection assay was designed based on the ITS region for *P. maydis* using a TaqMan probe-based qPCR assay. The specificity of the assay to *P. maydis* was validated against *Phyllachora* species and other common foliar corn pathogens, sensitivity was tested against known amounts of DNA and spores, and transferability of the assay was assessed across PCR platforms and laboratories.

Materials and Methods

Sample collection

Phyllachora maydis-infected leaf tissue was collected from corn fields in 2021 in Michigan, Indiana, Wisconsin, and Minnesota in the United States and Puebla and Oaxaca in Mexico. *Phyllachora graminis*-infected leaf tissue was collected from

various grass species in Michigan and Kansas in 2020. In addition, historical *Phyllachora*-infected plant specimens were received from the USDA National Fungus Collection (BPI), consisting of herbarium specimens collected from around the world from 1893 to 2004 (Farr and Rossman 2023). The *Phyllachora* herbarium species included the following: *P. maydis*, *P. graminis*, *P. vulgata*, *P. sorghi*, *P. paspalicola*, *P. acaciae*, *P. ambrosiae*, *P. americana*, *P. andropogonis*, *P. chardonii*, *P. cornispora*, *P. luteomaculata*, *P. miscanthi*, and *P. wilsonii*. In addition, various fungal organisms were isolated from infected corn leaves in Michigan during the 2018, 2019, and 2020 field seasons. The isolates or samples used in this study can be found in Supplementary Table S1.

DNA extractions

For herbarium specimens, 5 to 10 mg of dried leaf tissue with visible tar spot stroma were sampled and homogenized using lysing Matrix A in a FastPrep homogenizer (MP Biomedicals, Irvine, CA, U.S.A.). DNA was extracted using 2% cetyltrimethylammonium bromide (CTAB) buffer and a modified phenol/chloroform protocol (Hallen-Adams et al. 2011). Similar DNA extraction methods were performed on the contemporary samples of *P. maydis* and *P. graminis* collected from Michigan, Kansas, Indiana, Wisconsin, and Mexico. The *P. maydis* sample from Minnesota was extracted using the DNeasy Plant Mini Kit (Qiagen, Venlo, the Netherlands). Pure fungal isolates of other common foliar corn pathogens were grown on potato dextrose agar (PDA) and transferred to potato dextrose broth. Mycelia were vacuum filtered and subsequently homogenized as described above. After homogenization, DNA was extracted using either the CTAB method described above (Hallen-Adams et al. 2011) or the FastDNA Kit (MP Biomedicals). The DNA extraction method used for each isolate or specimen is listed in Supplementary Table S1.

ITS amplification and sequencing

Polymerase chain reactions (PCRs) were performed on all *Phyllachora* DNA samples. The ITS locus was sequenced using the ITS primers 1F and 4A (Gardes and Bruns 1993; Larena et al. 1999) or using a novel set of ITS primers (*Phyllachora* ITS F1 or F2 and *Phyllachora* ITS R) designed based on *Phyllachora* alignments (Table 1). Samples that produced a single PCR amplification product were cleaned using exonuclease I and shrimp alkaline phosphatase. Amplicons were submitted for Sanger sequencing at Michigan State University's Genomics Core. High-quality sequences were aligned using Geneious Prime v.2021.2.2 (Dotmatics, Boston, MA, U.S.A.), and primer and probe align-

ments were produced using the ggmsa R package (Zhou and Yu 2022). A single gene tree was made with MEGA11 using a maximum likelihood kimura-2 parameter plus gamma distribution model with 1,000 bootstrap replicates (Kimura 1980; Tamura et al. 2021).

qPCR primer and probe design

The ITS locus of the ribosomal DNA (rDNA) was chosen for the design of the real-time qPCR assay, as it is a multicopy target locus and there is availability of more sequence data for this locus than other loci. Sequences of the ITS region from *Phyllachora* species were downloaded from the National Center for Biotechnology Information (NCBI) GenBank nucleic acid sequence database (Sayers et al. 2022) and aligned using Geneious Prime (Dotmatics) and MEGA11 (Tamura et al. 2021). Primers were designed for the ITS2 region to amplify a 199 base pair (bp) region, and a probe was designed in the middle of the amplicon. Primers were designed with a few single-nucleotide polymorphisms (SNPs), and the probe was designed to contain several SNPs not found in nontarget *Phyllachora* spp. to increase specificity to *P. maydis*. The primers (*Phyllachora maydis* qPCR F and *Phyllachora maydis* qPCR R) have similar melting temperatures (T_m) of 58.8 and 58.6°C, respectively, and the probe (*Phyllachora maydis* qPCR probe) has approximately a 5°C higher T_m at 65.9°C. The probe was designed to have a 5' 6-carboxyfluorescein (6-FAM) and 3' black hole quencher (BHQ1). The primer and probe sequences used in this study are noted in Table 1.

qPCR conditions

Real-time qPCR assays were performed on the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Reactions were performed in a total volume of 20 µl. The reagents for each reaction were as follows: 8 µl of 2.5× PerfeCTa multiplex qPCR ToughMix (Quantabio, Beverly, MA, U.S.A.), 2 µl of 25 mM magnesium acetate, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 1 µl of 10 µM probe, 1 µl of DNA, and 6 µl of water (Supplementary Table S2). Thermal cycling conditions were 95°C for 3 min and 40 cycles at 95°C for 10 s and 60°C for 30 s. Fluorescent imaging was monitored after every cycle. Cycle threshold values were determined using the auto-threshold for the instrument. The assay was also tested for use with an inexpensive nonmultiplex mix (PerfeCTa qPCR ToughMix) using the same cycling conditions. For this reaction mix, the reagents for each reaction were as follows: 10 µl of PerfeCTa qPCR Toughmix (Quantabio), 2 µl of 25 mM magnesium acetate, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 1 µl of 10 µM probe, 1 µl of DNA, and

TABLE 1

Primers and probes used in this study

Primer/probe	Sequence (5' to 3')	Length (bp)	T _m (°C)
Sequencing primers			
ITS1F ^a	CTTGGTCATTAGAGGAAGTAA	22	49.2
ITS4A ^b	CGCCGTTACTGGGGCAATCCCTG	23	62.4
<i>Phyllachora</i> ITS F1	GGAAGTAAAGTCGTAACAAGGTCT	25	54.4
<i>Phyllachora</i> ITS F2	AACTTCAACAACGGATCTCTTG	24	54
<i>Phyllachora</i> ITS R	CCGCTTATTGATATGCTTAAGTTCAG	26	54.8
qPCR assay primers			
<i>Phyllachora maydis</i> qPCR F	GCATGCCTGTTCGAGCGTCATCT	23	58.8
<i>Phyllachora maydis</i> qPCR R	TGCCTGATCCGAGGTCAACGTG	22	58.6
qPCR assay probe			
<i>Phyllachora maydis</i> qPCR probe	[FAM]AGCGYAGTAGCACACACCYCGCTCGC[BHQ1]	26	64.3 to 67.4

^a Primer from Gardes and Bruns (1993).

^b Primer from Larena et al. (1999).

4 µl of water. A brief and easy-to-use protocol for both master mixes is supplied in Supplementary Document S1.

qPCR specificity testing

To evaluate specificity, the *P. maydis* assay was tested against a panel of 83 *Phyllachora* spp. and multiple other fungal isolates (Table 2). The panel included 13 different *Phyllachora* spp., several fungi causing common diseases of corn, endophytic species, and *P. maydis* contemporary and historical samples from various geographical locations. The DNA concentrations for each sample were standardized to be tested at 0.5 ng for leaf tissues and 0.1 ng for pure culture isolates. Samples were run with three technical replicates using the PerfeCTa multiplex qPCR ToughMix (Quantabio), and cycle thresholds were determined based on the RFU auto-threshold set by the CFX96 Touch Real-Time PCR Detection System software (Bio-Rad Laboratories).

qPCR sensitivity testing

For qPCR assay sensitivity testing based on DNA concentration, *P. maydis* conidia were collected directly from cirrhi on the leaf surface to reduce contaminants (MacCready et al. 2023). DNA was extracted using manual homogenization with liquid nitrogen and mortar and pestle, and a modified phenol/chloroform extraction as described above. DNA concentration was measured using a Quant-iT dsDNA broad range assay kit (Invitrogen, Waltham, MA, U.S.A.), and a DNA standard of 10 ng/µl was amended with 1 ng/µl of salmon sperm DNA to prevent DNA degradation and sticking of DNA to plastic at low concentrations. A 10-fold serial dilution ranging from 10 ng to 1 fg was tested to determine the sensitivity of the qPCR assay. Each dilution was tested in three technical replicates using both the multiplex and nonmultiplex PerfeCTa qPCR ToughMix (Quantabio) master mixes.

For qPCR assay sensitivity testing against spores, a mixed spore suspension containing ascospores and conidia were produced by rehydrating approximately 500 mg of dried corn leaf tissue infected with *P. maydis* in 200 ml of sterile water with agitation on a shaking table at approximately 225 rpm at 22 to 24°C for 4 h (Groves et al. 2020). Ascospores and conidia were counted separately twice using a hemocytometer to obtain an accurate estimation of total spore concentration of the suspension. A spore suspension containing 1.52×10^9 total spores/ml (1×10^6 ascospores/ml and 1.52×10^9 conidia/ml) was used to make 10-fold serial dilutions. Aliquots of 100 µl were spiked onto 9-mm corn leaf disks for each dilution, resulting in concentrations of 1.52×10^8 to 152 spores. The leaf disks were sampled from nondiseased greenhouse corn plants. DNA was extracted from three replicates of each spore concentration by homogenization with lysing Matrix A in a FastPrep homogenizer (MP Biomedicals), followed by the CTAB and phenol/chloroform extraction described above. Each extraction was tested in two technical replicates with the qPCR assay using the multiplex PerfeCTa qPCR ToughMix (Quantabio) master mix.

Linear regressions were made based on $\log_{10}$ concentration of DNA or number of spores and cycle thresholds (Ct). PCR efficiency was calculated as $10^{\frac{-1}{\text{slope}} - 1}$. The limit of detection (LOD) was determined as the lowest DNA concentration or number of spores that could be reliably detected in 95% of replicates. The average Ct at the LOD was set as the threshold for positive amplification in tested samples.

qPCR transferability testing

To test the transferability of the assay, a subset of DNA samples was tested in laboratories at the following institutions: Michi-

gan State University (MSU), Michigan State University Plant and Pest Diagnostics Lab (MSU Diagnostics), Purdue University (Purdue), University of Minnesota (UMN), University of Wisconsin-Madison (UW-Madison), and the International Maize and Wheat Improvement Center (CIMMYT). Each lab performed the qPCR assay on the same subset of DNA samples as well as a standard DNA 10-fold dilution series ranging from 10 ng to 10 fg with three technical replicates for each sample and repeated in two separate experiments ($n = 6$). Serial dilutions were prepared from a 10 ng/µl genomic DNA stock at each institution separately. Assays were performed using the PerfeCTa multiplex qPCR ToughMix (Quantabio) under the conditions defined above. The thermal cycler platforms used at each institution are listed in Table 3. Cycle threshold values were determined using the auto-threshold for each platform. LOD was determined as described above across both experiments (Table 3). PCR efficiency and R^2 were calculated separately for each experiment (Supplementary Table S2).

Results

Sequencing of the ITS region and qPCR assay design

Primers and a probe specific to *P. maydis* were designed for areas of polymorphism within the ITS region observed in the alignment of publicly available ITS sequences from members of the genus *Phyllachora*. Based on the heterogeneity within the *P. maydis* ITS sequence, the probe was designed with two degenerate pyrimidine bases (cytosine or thymine). Due to the lack of sequence data for the genus *Phyllachora*, sequences of the ITS region were generated from various *Phyllachora* species to confirm the design of the assay (Supplementary Table S1; Fig. 1). Alignments of the primer and probe regions for the qPCR assay confirmed the SNPs in the 199-bp amplicon from the ITS2 region among *Phyllachora* species (Fig. 1; Supplementary Fig. S1). *Phyllachora* species exhibit a high similarity to the designed forward primer and moderate similarity to the reverse primer. However, there are several SNPs within the probe sequence making the assay specific to *P. maydis* based on the available sequence data. Furthermore, the *P. maydis* sequences show complete sequence similarity to the designed assay, and the pyrimidine degenerate bases in the probe allow for detection of the differing *P. maydis* ITS sequences. The alignments also confirmed that the assay would detect historical and contemporary isolates from various geographies. A single-gene tree was constructed with these sequenced *Phyllachora* spp. ITS regions (Supplementary Fig. S2), which demonstrated grouping by species. Interestingly, grouping by location was observed in the *P. maydis* samples regardless of sample age. Specifically, the South and Central American *P. maydis* samples grouped separately from the North American samples.

Sensitivity and specificity

To test the sensitivity of the assay, serial dilutions of *P. maydis* DNA were tested, and linear regressions were made (Fig. 2A). The 10 ng concentration amplified at an average Ct of 18.5. The LOD was determined as 100 fg, as it was the lowest concentration that was reproducibly amplified in greater than 95% of samples. Amplification was observed at 10 fg but only detected 75% of the time, whereas no amplification was observed at 1 fg. The cycle cutoff was set at 35.2 using the 100 fg LOD average Ct. The R^2 of the regression was 0.9965, the slope was -3.32 , and the PCR efficiency was 100.08% (Fig. 2A). To determine the limit of spore detection, a 10-fold serial dilution of a *P. maydis* spore suspension ranging from 1.52×10^8 to 152 spores was tested with the qPCR assay. The spore LOD was determined to be 152

spores amplifying at an average 35.2 Ct. The spore regression line had an R^2 value of 0.9955, slope of -3.66 , and PCR efficiency of 87.6% (Fig. 2B). Sensitivity testing was repeated using a nonmultiplex qPCR mix (PerfeCTa qPCR ToughMix) to

show the flexibility of the assay and for user preference. The genomic DNA linear regression using the nonmultiplex qPCR mix showed similar efficiencies to the above results. The LOD when using the nonmultiplex qPCR mix was 100 fg (R^2 of 0.9975, and

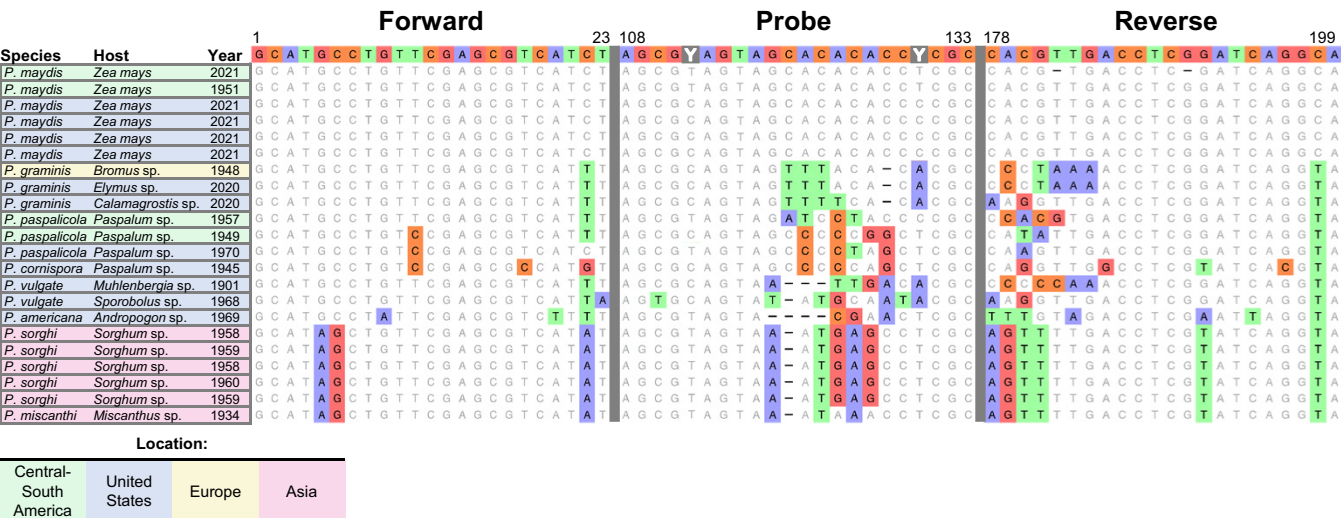


FIGURE 1 Multi-sequence alignment of the qPCR primers and probe regions for *Phyllachora* spp. from this study. Primers and probe sequences from the 199-bp amplicon are at the top of the alignment. The forward primer is 1 to 23 bp, the probe is 108 to 133 bp, and the reverse primer is 178 to 199 bp from the qPCR amplicon in the ITS region. *Phyllachora* spp. sequences are listed below and show single-nucleotide polymorphisms (SNPs) in color and sequence similarity in gray. For each specimen, the host species and year collected are included. The location where the specimen was collected is colored in the table: Central and South America (green), United States (blue), Europe (yellow), Asia (red). Y = pyrimidine base C or T.

Species ^{a,b}	Host species	Location	Year collected ^c	Average Ct ^d
<i>Phyllachora</i> spp.				
<i>Phyllachora</i> sp. BPI 636012	<i>Andropogon</i> sp.	U.S.A. - Florida	1974	ND
<i>P. acacia</i> BPI 636157	<i>Acacia farnesiana</i>	Cuba - Vento	—	ND
<i>P. acacia</i> BPI 636166	<i>Vachellia farnesiana</i>	Trinidad and Tobago - Hastings	1946	ND
<i>P. ambrosiae</i> BPI 636220	<i>Ambrosia artemisifolia</i>	U.S.A. - Alabama	1935	ND
<i>P. ambrosiae</i> BPI 636224	<i>Ambrosia artemisifolia</i>	U.S.A. - Wisconsin	1962	ND
<i>P. ambrosiae</i> BPI 636242	<i>Iva imbricata</i>	U.S.A. - Florida	1969	ND
<i>P. americana</i> BPI 636252	<i>Andropogon</i> sp.	U.S.A. - Florida	1969	ND
<i>P. andropogonis</i> BPI 636286	<i>Andropogon sorghum</i>	Indonesia - Bogur	1955	ND
<i>P. chardonii</i> BPI 636727	<i>Paspalum</i> sp.	Liberia - Linkor	1951	ND
<i>P. cornispora</i> BPI 636831	<i>Paspalum langii</i>	U.S.A. - Texas	1945	ND
<i>P. cornispora</i> BPI 640270	<i>Paspalum langii</i>	U.S.A. - Texas	1945	ND
<i>P. ischaemi</i> BPI 638240	<i>Miscanthus sinensis</i>	Japan	1971	ND
<i>P. luteomaculata</i> BPI 638411	<i>Andropogon furcatus</i>	U.S.A. - Maryland	1895	ND
<i>P. miscanthi</i> BPI 638659	<i>Miscanthus</i> sp.	China - Yen-Hsien	1934	ND
<i>P. miscanthi</i> BPI 638661	<i>Miscanthus</i> sp.	Korea	1960	ND
<i>P. paspalicola</i> BPI 638876	<i>Paspalum</i> sp.	U.S.A. - Florida	1970	ND
<i>P. paspalicola</i> BPI 638877	<i>Paspalum</i> sp.	Bolivia - Temporal Cochabamba	1957	ND
<i>P. paspalicola</i> BPI 638907	<i>Paspalum conjugatum</i>	Costa Rica - Turrialba	1949	36.55*
<i>P. sorghi</i> BPI 639668	<i>Sorghum</i> sp.	Cambodia - Prek Leap	1960	38.09*
<i>P. sorghi</i> BPI 639672	<i>Sorghum</i> sp.	Philippines - Irosin	1916	ND
<i>P. sorghi</i> BPI 639673	<i>Sorghum halepense</i>	Madagascar - Java	1958	ND
<i>P. sorghi</i> BPI 639677	<i>Sorghum halepense</i>	Philippines - Mt. Maquiling	1913	ND
<i>P. sorghi</i> BPI 639678	<i>Sorghum sudanense</i>	Cambodia	1958	ND
<i>P. sorghi</i> BPI 639679	<i>Sorghum vulgare</i>	Philippines - Laguna	1966	ND
<i>P. sorghi</i> BPI 639680	<i>Sorghum vulgare</i>	Cambodia - Prek Leap	1959	ND
<i>P. sorghi</i> BPI 639681	<i>Sorghum vulgare</i>	Cambodia - Prek Leap	1959	ND
<i>P. sorghi</i> BPI 639683	<i>Sorghum vulgare</i>	India - Coimbatore	1953	ND

(Continued on next page)

^a *Phyllachora* spp. DNA was extracted from leaf samples and qPCR tested at 0.5 ng genomic DNA concentration. Other nontarget commonly encountered fungi DNA was extracted from pure mycelial cultures and qPCR tested at 0.1 ng genomic DNA concentration. FIESC = *Fusarium incarnatum-equiseti* species complex. FTSC = *Fusarium tricinctum* species complex.

^b USDA National Fungal Collection herbarium specimens are indicated with their respective BPI identifier.

^c Year collected information not available indicated by ‘—’.

^d Average cycle threshold (Ct) values across three technical replicates. ND = Not Detected; * = 1/3 replicates amplified; ** = 2/3 replicates amplified.

PCR efficiency of 97.43%) of pure DNA (Supplementary Fig. S3). To test the specificity, the assay was performed on a panel of 83 DNA samples, including 47 nontarget *Phyllachora* spp. from 13 different species, 16 target *P. maydis* samples, and 18 fungal isolates associated with corn leaves (Table 2; Supplementary Table S1). All six contemporary *P. maydis* samples were reliably amplified. The *P. maydis* historical herbarium samples were inconsistently detected, with four of the ten samples being not detected (ND) or past the cycle cutoff (Ct > 35.2). All nontarget samples were negative on the assay, with either no amplification or past the cycle cutoff.

Cross-platform and institution validation

A subset of *Phyllachora* spp. and other fungal DNA samples was used to test the transferability of the assay. False positive and

false negative amplifications were counted from each institutional lab (Table 3; Supplementary Table S2). No false negatives were recorded at four of the participating institutions, including MSU, indicating that all *P. maydis* samples were detected on the qPCR assay. At lab number five, the *P. maydis* sample from Michigan was not detected before the Ct threshold in one replicate. At lab number four, one of the *P. maydis* samples was never detected, whereas all other *P. maydis* samples were not detected in 1 or 3 replicates ($n = 6$). Further inspection of the data from lab number four showed that a single run had later amplification in the DNA standards and target *P. maydis* samples. No false positives were detected at three of the institutions, including MSU, indicating no amplification of nontarget samples. In the three remaining labs, false positives were detected in the negative control. In lab numbers one and three, there were false positives of nontarget

TABLE 2 (Continued from previous page)

Species ^{a,b}	Host species	Location	Year collected ^c	Average Ct ^d
<i>P. vulgata</i> BPI 640219	<i>Muhlenbergia cuspidata</i>	U.S.A. - Nebraska	1940	ND
<i>P. vulgata</i> BPI 640224	<i>Muhlenbergia cuspidata</i>	U.S.A. - Nebraska	1940	ND
<i>P. vulgata</i> BPI 640251	<i>Muhlenbergia glomerata</i>	U.S.A. - Kansas	1893	ND
<i>P. vulgata</i> BPI 640260	<i>Muhlenbergia mexicana</i>	U.S.A. - Iowa	1901	ND
<i>P. vulgata</i> BPI 640283	<i>Muhlenbergia racemosa</i>	U.S.A. - New Mexico	1936	ND
<i>P. vulgata</i> BPI 640324	<i>Sporobolus contractus</i>	U.S.A. - Arizona	1968	ND
<i>P. vulgata</i> BPI 640327	<i>Sporobolus cryptandrus</i>	Canada - Ontario	1953	ND
<i>P. vulgata</i> BPI 880278A	<i>Muhlenbergia schreberi</i>	U.S.A. - Virginia	2004	ND
<i>P. wilsonii</i> BPI 640366	<i>Paspalum stramineum</i>	U.S.A. - Kansas	1957	ND
<i>P. graminis</i> BPI 637651	<i>Panicum virgatum</i>	U.S.A. - New York	1917	ND
<i>P. graminis</i> BPI 637652	<i>Bromus ramosus</i>	Germany - Oberbayern	1949	ND
<i>P. graminis</i> BPI 637833	<i>Bromus ramosus</i>	Germany - Mittelfranken	1948	ND
<i>P. graminis</i> BPI 637979	<i>Eragrostis</i> sp.	U.S.A. - New Mexico	1939	ND
<i>P. graminis</i> BPI 637984	<i>Paspalum</i> sp.	U.S.A. - Texas	1967	ND
<i>P. graminis</i> BPI 638044	<i>Triticum repens</i>	Czechoslovakia - Hranice	1936	ND
<i>P. graminis</i> BPI 638045	<i>Triticum repens</i>	Czechoslovakia - Hranice	1911	ND
<i>P. graminis</i>	<i>Poa</i> sp.	U.S.A. - Kansas	2020	ND
<i>P. graminis</i>	<i>Elymus repens</i>	U.S.A. - Michigan	2020	ND
<i>P. graminis</i>	<i>Calamagrostis arundinaceae</i>	U.S.A. - Michigan	2020	ND
<i>P. graminis</i>	<i>Elymus repens</i>	U.S.A. - Michigan	2020	ND
<i>P. maydis</i>	<i>Zea mays</i>	Mexico - Puebla	2021	27.48
<i>P. maydis</i>	<i>Zea mays</i>	Mexico - Oaxaca	2021	26.76
<i>P. maydis</i>	<i>Zea mays</i>	U.S.A. - Michigan	2021	28.48
<i>P. maydis</i>	<i>Zea mays</i>	U.S.A. - Indiana	2021	23.45
<i>P. maydis</i>	<i>Zea mays</i>	U.S.A. - Minnesota	2021	24.88
<i>P. maydis</i>	<i>Zea mays</i>	U.S.A. - Wisconsin	2021	24.97
<i>P. maydis</i> BPI 638549	<i>Zea mays</i>	Dominican Republic - Pueblo Viejo	1918	ND
<i>P. maydis</i> BPI 638560	<i>Zea mays</i>	Mexico - Rincon	1905	37.65**
<i>P. maydis</i> BPI 638562	<i>Zea mays</i>	Costa Rica	1953	32.57
<i>P. maydis</i> BPI 638564	<i>Zea mays</i>	Mexico	1977	29.11
<i>P. maydis</i> BPI 638568	<i>Zea mays</i>	Costa Rica - Isla Bonito	1947	ND
<i>P. maydis</i> BPI 638572	<i>Zea mays</i>	Guatemala	1940	31.79
<i>P. maydis</i> BPI 638573	<i>Zea mays</i>	Colombia - Rio Negro	1951	30.31
<i>P. maydis</i> BPI 638577	<i>Zea mays</i>	Bolivia - Coroico	1943	32.51
<i>P. maydis</i> BPI 638584	<i>Zea mays</i>	Guatemala - Antigua	1905	ND
<i>P. maydis</i> BPI 638586	<i>Zea mays</i>	Peru - Lima	1929	34.74
Nontarget fungi				
<i>Alternaria alternata</i>	<i>Zea mays</i>	U.S.A. - Michigan	2020	ND
<i>Bipolaris/Curvularia</i>	<i>Zea mays</i>	U.S.A. - Michigan	2020	ND
<i>Epicoccum nigrum</i>	<i>Zea mays</i>	U.S.A. - Michigan	2020	ND
<i>Fusarium</i> FIESC	<i>Zea mays</i>	U.S.A. - Michigan	2020	ND
<i>Fusarium</i> FTSC	<i>Zea mays</i>	U.S.A. - Michigan	2018	ND
<i>Fusarium</i> FTSC	<i>Zea mays</i>	U.S.A. - Michigan	2018	36.70*
<i>Fusarium</i> FTSC	<i>Zea mays</i>	U.S.A. - Michigan	2018	ND
<i>Fusarium</i> FTSC	<i>Zea mays</i>	U.S.A. - Michigan	2018	ND
<i>Fusarium graminearum</i>	<i>Zea mays</i>	U.S.A. - Michigan	2020	ND
<i>Fusarium graminearum</i>	<i>Zea mays</i>	U.S.A. - Michigan	2020	ND
<i>Fusarium sporotrichioides</i>	<i>Zea mays</i>	U.S.A. - Michigan	2020	ND
<i>Fusarium verticillioides</i>	<i>Zea mays</i>	U.S.A. - Michigan	2019	ND
<i>Microdochium bolleyi</i>	<i>Zea mays</i>	U.S.A. - Michigan	2020	ND
<i>Paraphaeosphaeria neglecta</i>	<i>Zea mays</i>	U.S.A. - Michigan	2019	ND
<i>Paraphaeosphaeria neglecta</i>	<i>Zea mays</i>	U.S.A. - Michigan	2019	ND
<i>Cercospora zea-maydis</i>	<i>Zea mays</i>	U.S.A. - Michigan	2020	ND
<i>Colletotrichum graminicola</i>	<i>Zea mays</i>	U.S.A. - Michigan	2020	ND
<i>Exserohilum turcicum</i>	<i>Zea mays</i>	U.S.A. - Michigan	2020	ND

samples. Although false positives were detected in three of the participating labs, no pattern between DNA sample and location was observed. These institutions also included a standard serial dilution of *P. maydis* DNA ranging from 10 ng to 10 fg to determine the sensitivity and efficiency (Table 3; Fig. 3). Two institutions had an LOD of 100 fg, and three institutions had an LOD of 1,000 fg, with inconsistent amplification across experiments observed at 100 fg (Supplementary Table S2). The fifth lab had an LOD of 1 pg (10,000 fg). All labs had an R^2 of the linear regression above 0.88, with four labs above 0.98 (Fig. 3; Table 3). PCR efficiencies ranged from 62.39 to 104.76%, with five labs above 85% and two labs above 99%.

Discussion

In this study, we developed the first species-specific qPCR assay for *P. maydis* and demonstrated the validation of its use. The assay displayed specificity to *P. maydis*, having been tested against closely related species and commonly encountered corn pathogens. Furthermore, *P. maydis* was reliably detected in contemporary and historical tar spot samples, as well as in samples collected from various geographical locations. The assay is sensitive, having a demonstrated limit of detection of 100 fg of *P. maydis* genomic DNA and 152 spores. Additionally, the assay is transferable across platforms in other labs, having been cross validated at six separate institutions, including several land-grant universities, a National Plant Diagnostics Network lab, and an international lab. The assay was also demonstrated to work across qPCR thermal cyclers and master mixes.

Choosing a multicopy locus as a molecular target can increase the specificity and sensitivity of a DNA detection assay (Groth-

Helms et al. 2023). Common multicopy loci used for development of qPCR assays for phytopathogen detection include the ribosomal DNA tandem repeats, specifically the ITS region and intergenic spacer (IGS) region, as well as mitochondrial DNA (Bilodeau et al. 2012, 2014; Chilvers et al. 2007; Crouch and Szabo 2011; Duong et al. 2022; Higgins et al. 2022; Roth et al. 2020). For *P. maydis*, the ITS region was chosen, as it had sufficient polymorphism to design a species-specific assay, and ITS was the only locus sequenced for a high number of closely related *Phyllachora* species. Heterogeneity has been detected in the ITS region of *P. maydis*, with three ITS variants identified (Broders et al. 2022). Additionally, in an amplicon sequencing experiment, multiple operational taxonomic units (greater than 97% sequence similarity) for *P. maydis* were identified based on data from the ITS locus (McCoy et al. 2019). This variation in the ITS region has also been seen in other fungal species (Rush et al. 2019; Simon and Weiß 2008; Smith et al. 2007). For example, the obligate-parasite *Phakopsora pachyrhizi*, causal agent of soybean rust, has been found to have intragenomic variation of the ITS region within a single sorus, which can have ramifications in designing species-specific diagnostic assays, with increases in false positive and false negative amplification (Rush et al. 2019). To account for the known ITS haplotypes in *P. maydis*, the qPCR probe was designed with degenerate bases that could encode for both pyrimidine bases, cytosine and thymine. With the use of these two degenerate bases, the qPCR assay was able to detect the ITS haplotypes, as verified by ITS sequencing.

One key validation step in a detection assay is to test the sensitivity (Cardwell et al. 2018; Groth-Helms et al. 2023). The LOD is defined as the lowest amount amplified in greater than or equal to 95% of replicates (Bustin et al. 2009; Groth-Helms et al. 2023).

TABLE 3

Cross-institution and platform validation of *Phyllachora maydis* species-specific qPCR assay

Sample	Testing location ^a					
	MSU	1	2	3	4	5
False negatives ^{b,c}						
<i>Phyllachora maydis</i> - IN	0	0	0	0	3	0
<i>Phyllachora maydis</i> - MI	0	0	0	0	6	1
<i>Phyllachora maydis</i> - MN	0	0	0	0	1	0
<i>Phyllachora maydis</i> - WI	0	0	0	0	3	0
<i>Phyllachora maydis</i> - Mexico	0	0	0	0	3	0
False positives ^{c,d}						
<i>Phyllachora graminis</i> - MI 2020	0	0	0	1	0	0
<i>Phyllachora graminis</i> BPI 637979	0	0	0	0	0	0
<i>Phyllachora sorghi</i> BPI 639680	0	0	0	1	0	0
<i>Phyllachora vulgata</i> BPI 640219	0	0	0	1	0	0
<i>Exserohilum turcicum</i> (NLB)	0	0	0	0	0	0
<i>Cercospora zea-maydis</i> (GLS)	0	0	0	0	0	0
<i>Fusarium</i> sp. FTSC (Fisheye)	0	1	0	0	0	0
<i>Paraphaeosphaeria neglecta</i> (Fisheye)	0	0	0	0	0	0
<i>Zea mays</i> only	0	0	0	0	0	0
NTC	0	1	1	2	0	0
Statistics ^e						
PCR efficiency	100.08%	104.76%	85.09%	87.31%	89.02%	62.39%
R^2	0.9965	0.9817	0.9927	0.9181	0.9968	0.8856
Limit of detection (LOD)	100 fg	1,000 fg	100 fg	1,000 fg	1,000 fg	10,000 fg
LOD cutoff Ct	35.2	35.5	37.1	35.8	32.9	37.7
qPCR machine	Bio-Rad CFX96	Bio-Rad CFX96	Bio-Rad CFX96	Bio-Rad CFX96	Applied Biosystems	Bio-Rad CFX96

^a Testing locations: MSU = Michigan State University, Dr. Martin Chilvers Lab. Five other institutions performed the assay: University of Wisconsin – Madison, Dr. Damon Smith Lab; Michigan State University Plant and Pest Diagnostic Lab, Laura Miles; Purdue University, Dr. Darcy Telenko Lab; International Maize and Wheat Improvement Center, Drs. Amos Alakonya and Akshaya Biswal Labs; University of Minnesota, Dr. Dean Malvick Lab, where assay efficiency and LOD improved significantly with the ordering of a new probe stock.

^b False negative counts for target *P. maydis* samples when the sample did not amplify before the cycle threshold cutoff.

^c False amplification counts based on six replicates ($n = 6$).

^d False positive counts for nontarget samples when samples did amplify before the cycle threshold cutoff.

^e Parameters based on linear regressions of standard *P. maydis* genomic DNA and qPCR thermal cycler platform.

In this study, the LOD was determined as 100 fg of genomic *P. maydis* DNA, as it had consistent amplification in 100% of replicates. Although the assay detected at lower concentrations (10 fg), the amplification was only detected in 75% of replicates and therefore did not meet the LOD definition. The PCR efficiency of an assay is also an important characteristic to benchmark the performance of a qPCR assay (Bustin et al. 2009). Using genomic DNA, the PCR efficiency of this assay was found to be 100.08%. Overall, this indicates a highly sensitive and efficient assay similar to many published assays on other pathosystems (Bilodeau et al. 2014; Rojas et al. 2017; Roth et al. 2020; Wang et al. 2015). Determining the LOD on a known number of spores is standard in phytopathogen quantification assays for monitoring of epidemics (Bello et al. 2022; Kunjeti et al. 2016), and further testing was performed to demonstrate the assay's use in detection of airborne

spores for future epidemiology studies. Difficulties in extracting DNA from *P. maydis* spores occurred, likely due to poor lysing of cells. Therefore, the spore sensitivity was tested by spiking known amounts of spores onto nondiseased corn leaf disks. The corn leaf disks served as carrier DNA and helped improve the DNA extraction efficiency from low numbers of spores (data not shown). The spore sensitivity was determined as 152 spores with an 87.6% PCR efficiency. DNA extraction efficiency can affect the PCR efficiency and is the plausible cause of the reduction from the spore linear regression (Groth-Helms et al. 2023). Improvements to the spore DNA extraction may be necessary in future studies.

Development of a qPCR detection assay relies on validation of specificity by testing against nontarget samples and confirming amplification of target samples (Groth-Helms et al. 2023). Although many *Phyllachora* species exist around the world, these

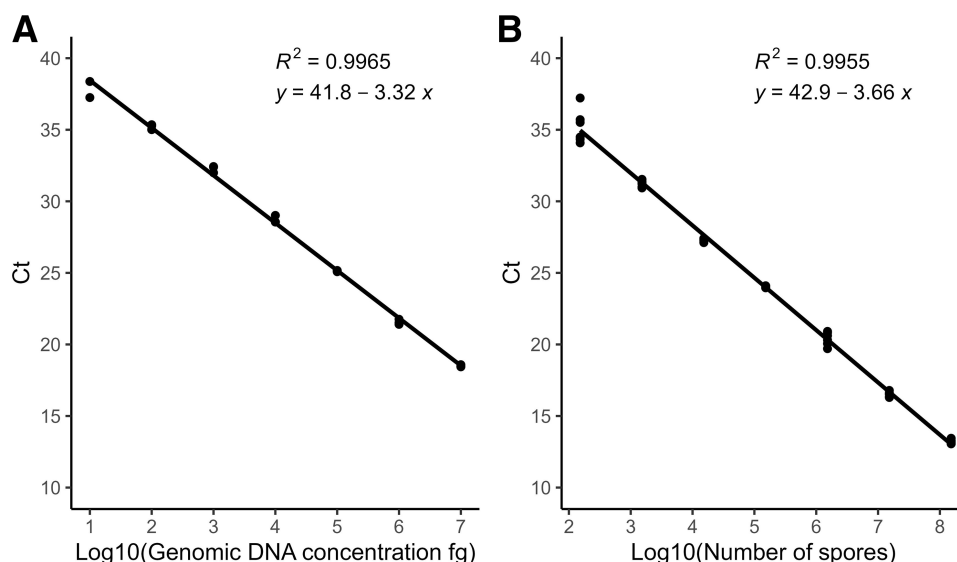
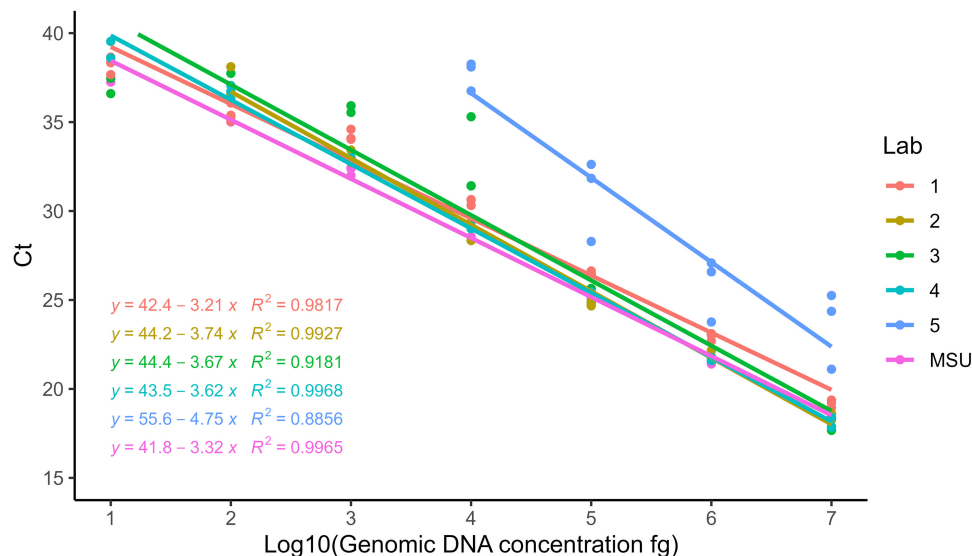


FIGURE 2 Standard curves for *Phyllachora maydis* qPCR. The formula and R^2 values are shown for each linear regression of the log-transformed concentration (fg or spores) plotted against the cycle threshold (Ct) values. **A**, Linear regression of serially diluted genomic DNA concentration from 10 ng to 10 fg. Three replicates are plotted for each concentration. **B**, Linear regression of serially diluted spore concentration from 1.52×10^8 to 152 spores. DNA was extracted from three replicates per spore concentration, and qPCR was replicated twice for each concentration. These six replicates are plotted for each concentration.

FIGURE 3 Cross-institution validation of the *Phyllachora maydis* qPCR. Using DNA concentrations serially diluted from 10 ng to 10 fg, log-transformed DNA concentration (fg) is plotted against the cycle threshold (Ct) values. Three replicates are plotted for each concentration. The formula and R^2 values are shown for the linear regressions for each institution, respectively.



fungi are largely understudied, and the geographic distribution and host range of these fungi remain largely unknown (Silva-Hanlin and Hanlin 1998). *P. maydis* and *P. graminis* samples were found and collected; however, more *Phyllachora* species were needed for robust specificity testing. Due to the constraints in finding *Phyllachora* samples, herbarium specimens from the USDA National Fungus Collection were acquired, which allowed for testing the assay against 53 samples representing 14 *Phyllachora* spp., including *P. maydis*, from around the world, collected between 1893 and 2004 (Farr and Rossman 2023). Herbarium specimens have allowed for sequencing of several obligate plant pathogens for phylogenetic and evolutionary studies (Bradshaw and Tobin 2020; Bradshaw et al. 2023; Yoshida et al. 2014). Through the use of these herbarium samples, we verified sequence specificity and validated the assay. When tested against the assay, the nontarget herbarium samples and fungal isolates from corn had no reproducible amplification. All contemporary *P. maydis* samples amplified and produced a strong signal, whereas the *P. maydis* herbarium specimens amplified with varying results. Compared with the contemporary *P. maydis* samples, *P. maydis* herbarium specimens had later amplification, with a few having no detection. To further validate the specificity and expand the sequence data availability, the ITS region was sequenced. All specimens produced amplicons; however, only 15 out of 53 herbarium samples produced good-quality ITS sequences, with most samples having either poor ITS amplification, bad-quality sequences, or non-*Phyllachora* sequences. These herbarium specimens could have low quality due to their age, resulting in the poor ITS amplification from highly fragmented DNA (Weiß et al. 2016). Additionally, low quantity DNA from herbarium and leaf tissue samples contain plant or other nontarget DNA, which further reduces the target *Phyllachora* species DNA for amplification. Improvements in sequencing amplicons from herbarium samples could be done using a nested PCR approach, and future phylogenetic analyses may instead rely on whole-genome sequencing for more reliable comparative data (Bradshaw and Tobin 2020; Lang et al. 2019). Although there were difficulties working with these samples, the herbarium specimens allowed for the validation of assay specificity across more *Phyllachora* species and significantly improved the scope of this project.

Testing the robustness of a qPCR assay is important to determine that conditions can be reproduced using other platforms and hands (Cardwell et al. 2018; Groth-Helms et al. 2023). To test the transferability of the assay, a subset of DNA samples including a standard and blind test panel was sent to various institutions. Overall, the assay was shown to perform well at the different institutions, including the use of both Bio-Rad and Applied Biosystems qPCR instrument platforms. In a few labs, false positive amplification of nontarget samples was detected in single replicates, but no pattern was observed, indicating random amplification that was not reproducible. One *P. maydis* sample had false negative amplification at two of the institutions. This sample also had later Ct values compared with the other *P. maydis* samples in all labs. Although this could be a reduction in the assay efficacy, the specific DNA aliquot could be of lower quality or quantity, causing later amplification and false negative results. Linear regressions of DNA standards generally demonstrated acceptable results, with PCR efficiencies above 85.09% and an LOD of 100 or 1,000 fg, except for one lab that had reduced sensitivity of 10,000 fg (10 pg) and efficiency at 62.39%. Troubleshooting of the assay in this lab was further conducted, and assay performance for sensitivity and efficiency was improved following the reordering and use of the probe reagent from another vendor (data not shown). This suggests that additional troubleshooting

of reagents, the thermal cycler, or lab equipment will likely allow for the assay to perform up to required specifications across diverse labs (Groth-Helms et al. 2023). The acceptable efficiency and sensitivity in most labs and platforms demonstrate the transferability of the assay.

Overall, this study validated a species-specific qPCR assay for quantifying *P. maydis*. This assay can be used to detect the pathogen for diagnostic purposes and at early stages of disease development, as well as beneficial in epidemiology studies to assess the corn-tar spot pathosystem. Currently, this assay is being used at various institutions for detection of spores or plant infection to improve understanding of the biology and epidemiology of this pathogen, including spore release dynamics, to improve disease risk prediction models.

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