Clade-Specific Biosurveillance of *Pseudoperonospora cubensis* Using Spore Traps for Precision Disease Management of Cucurbit Downy Mildew

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ABSTRACT

Pseudoperonospora cubensis is an obligate oomycete and cause of cucurbit downy mildew (CDM), the most destructive foliar disease affecting cucurbit hosts. Annual epidemics develop throughout the United States as windborne sporangia travel great distances and survive prolonged exposure to solar radiation. Recent genomic evidence suggests that *P. cubensis* isolates display host adaptation based on their respective clade. Early detection is key for fungicide application timing, and identification of the host-adapted clade provides information on the risk of infection for specific cucurbit crops. In this study, a multiplex quantitative PCR assay was developed based on species- and clade-specific nuclear genomic markers. The assay detected as few as 10 sporangia or DNA at 100 fg/ml for both clades and was validated in the

field by deploying rotorod spore samplers in cucurbit sentinel plots located at two research stations in North Carolina. Using this assay, sporangia DNA was detected in spore trap sampling rods before signs of *P. cubensis* or CDM symptoms were observed in the sentinel plots. Both clade 1 and clade 2 DNA were detected in late-season cucumber and watermelon plots but only clade 2 DNA was detected in the early-season cucumber plots. These results will significantly improve disease management of CDM by monitoring inoculum levels to determine the cucurbit crops at risk of infection throughout each growing season.

Keywords: disease control and pest management, epidemiology, oomycetes, pathogen detection

Pseudoperonospora cubensis is an obligate oomycete and the causal agent of cucurbit downy mildew (CDM), a devastating disease of cucurbits worldwide (Salcedo et al. 2020). In 2004, a population shift in *P. cubensis* resulted in an epidemic that devastated cucurbit crops along the Atlantic coast of the United States (Holmes et al. 2015). Previously resistant cucumber varieties and fungicides that had been effective for CDM management were no longer effective after 2004 (Savory et al. 2011). Due to the impact of the CDM epidemic on U.S. agriculture (Beans 2018), several studies were completed to characterize pathogen populations (Naegel et al. 2016; Quesada-Ocampo et al. 2012; Summers et al. 2015b; Wallace and Quesada-Ocampo 2017; Wallace et al. 2020) and generate new management strategies (Call et al. 2012; Goldenhar and Hausbeck 2019; Summers et al. 2015a; Withers et al. 2016).

Recently, two host-specialized clades of *P. cubensis* were described: clade 1 and clade 2. Clade 1 isolates preferentially infect acorn squash (*Cucurbita pepo* L.), pumpkin (*C. maxima* Duchesne.), butternut squash (*C. moschata* Duchesne ex Poir.), watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai), and the wild hosts bitter melon (*Momordica charantia* L.) and balsam apple (*M. balsamina* L.). Clade 2 isolates preferentially infect cucumber (*Cucumis sativus* L.), cantaloupe (*C. melo* L.), and the wild host bottle gourd (*Lagenaria siceraria* (Molina) Standl.) (Wallace et al.

2020). In addition to host preference, *P. cubensis* clades showed some fluctuation by season and location within North Carolina (Wallace et al. 2020), highlighting the importance of local cucurbit acreage in influencing inoculum composition at a given time or location (Ojiambo et al. 2015). Currently, management of CDM relies heavily on frequent fungicide applications with effective products (Goldenhar and Hausbeck 2019). To reduce fungicide applications, producers are recommended to begin applying fungicides preventatively only once the disease has been reported in a neighboring state through the CDM ipmPIPE (Ojiambo et al. 2011). However, previous studies have shown that 5% of reports do not correspond with the expected movement of the disease from southeastern to northeastern U.S. states along the Atlantic coast (Ojiambo and Holmes 2011). This may be due, in part, to year-round greenhouse production acting as a source of inoculum in some regions (Naegel et al. 2016), or to wild cucurbits serving as reservoirs of *P. cubensis* (Runge and Thines 2009; Wallace and Quesada-Ocampo 2015; Wallace et al. 2014). The CDM ipmPIPE relies on reported outbreaks of commercial cucurbits but does not account for other sources of inoculum; as a result, researchers have developed complementary alert systems for detecting *P. cubensis* airborne inoculum (Granke and Hausbeck 2011; Granke et al. 2014; Rahman et al. 2017; Summers et al. 2015a). Spore traps have been used successfully to detect *P. cubensis* sporangia in air currents; however, increasing acreage of hop in the eastern United States has become a confounding factor to the detection of *P. cubensis* (Crandall et al. 2018; Rahman et al. 2017). The hop downy mildew pathogen, *P. humuli*, is a sister species to *P. cubensis* that cannot be distinguished morphologically and has impeded positive identification in areas where both cucurbit and hop are produced (Purayannur et al. 2020). This hindrance has prompted efforts to generate species-specific molecular assays for both pathogens (Rahman et al. 2019; Summers et al. 2015a; Withers et al. 2016). While describing species-specific markers for *P. cubensis*, Withers et al. (2016) identified marker c2555.3e7 that displayed variation depending on the host of origin for individual *P. cubensis* isolates.

In this study, we confirm that the observed polymorphism in marker c2555.3e7 corresponds to the host-adapted *P. cubensis*

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clades described by (Wallace et al. 2020). We then build on this finding in order to (i) develop a *P. cubensis* clade-specific assay to estimate the risk of specific cucurbit crops becoming infected by *P. cubensis*, (ii) develop *P. cubensis* clade-specific multiplex quantitative PCR (qPCR) assays for detecting and quantifying *P. cubensis* airborne sporangia, and (iii) validate the assays by sampling airborne sporangia in cucurbit fields using rotorod spore traps. This work is the first step toward developing a comprehensive biosurveillance system for *P. cubensis* that combines information on inoculum level with risk of infection for specific cucurbit crops and will be useful to inform crop-specific fungicide application timing based on the host-adapted clade. Such a strategy will allow for fungicides to be applied only to at-risk crops, which is likely to reduce economic losses and preserve fungicide efficacy by reducing unnecessary selection for fungicide resistance.

MATERIALS AND METHODS

Analyzing polymorphic diagnostic marker c2555.3e7 for assay development. The previously identified candidate genomic marker c2555.3e7 of *P. cubensis* exhibited polymorphism by host of origin (Withers et al. 2016) and was used for the development of a diagnostic multiplex qPCR assay. Two host-adapted clades were described for *P. cubensis* by Wallace et al. (2020), and 10 isolates representing each clade were identified for assay design and validation in our study (Table 1). To confirm clade correspondence for these 20 isolates, marker c2555.3e7 was amplified using primers sw552 and sw553 in conjunction with genomic DNA that had previously been extracted and genotyped (Wallace et al. 2020; Withers et al. 2016). Once the polymorphism of marker c2555.3e7 was confirmed (Fig. 1), DNA of *P. cubensis* isolates in our long-term collection were used to amplify c2555.3e7 and identify a representative isolate for each clade that could be used to propagate sporangia for subsequent experiments. Single-sporangium isolates on infected leaves and DNA are stored at -80°C in our long-term collection. This initial experiment resulted in *P. cubensis* isolates SC1982 and MSU-1, being selected as representatives of clade 1 and clade 2, respectively, for assay development (Fig. 1).

Products obtained after amplification of c2555.3e7 in clade 1 SC1982 and clade 2 MSU-1 were sequenced using Sanger technology on a 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, U.S.A.) by the North Carolina State Genomic Sciences Laboratory (Raleigh, NC, U.S.A.). The resulting sequences were aligned to the MSU-1 genome sequence (Savory et al. 2012a,b) and the universal forward primer ar1517 was designed to be used with the sw553 reverse primer for amplifying the polymorphic region of c2555.3e7 in an additional 97 *P. cubensis* isolates. These isolates were collected from multiple host plants grown in different regions of the United States (Supplementary Table S1; Fig. 2). After confirming clade correspondence, the polymorphic region of c2555.3e7 was used to develop a multiplex qPCR assay to detect and quantify the specific clades of *P. cubensis* as described below.

Propagating, counting, and extracting genomic DNA from *P. cubensis* sporangia. Isolates of *P. cubensis* were propagated for sporangia development following the same method as described previously (Withers et al. 2016). For each isolate, a sporangial suspension of 1 to 5×10^4 sporangia/ml was spray inoculated on the abaxial surface of detached cucumber leaves placed upside down on moist sterile paper towels in plastic boxes. Inoculated leaves were incubated at 21 and 18°C with a cycle of 12 h of light and 12 h of darkness, respectively, in a precision plant growth chamber (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Using a Preval sprayer (Preval, Bridgeview, IL, U.S.A.), sterile water was used to dislodge sporangia from leaves; this leaf wash was passed through sterile $40\text{-}\mu\text{m}$ cell strainers (Genesee Scientific, San Diego, CA, U.S.A.) and collected into a sterile 50-ml tube. The spores from the leaf wash were then centrifuged at $10,000 \times g$ for

5 min at 4°C , the supernatant was decanted, and the spore pellet transferred to a 1.5-ml microcentrifuge tube. All spore pellet tubes were stored at -20°C until needed for further experiments.

Eight distinct *P. cubensis* sporangia counts (1 , 10 , 20 , 50 , 100 , 200 , 500 , and $1,000$ sporangia) were generated on rotorod spore sampling rods coated in vacuum grease (Dow Corning, Sigma-Aldrich, Inc., St. Louis, MO, U.S.A.) from both SC1982 and MSU-1. To obtain counts of 1 , 10 , 20 , and 50 fresh *P. cubensis* sporangia, a handmade sterile glass needle method (Goh 1999) was used to collect individual sporangium from sparsely sporulating cucumber leaves. Under a dissecting microscope (Leica M205C stereomicroscope; Leica Microsystems Inc., Chicago, IL, U.S.A.), a single sporangium on the ultimate branchlets of sporangiophores was picked up using the tip of a glass needle and dragged on a grease-coated sampler rod. Dark melanized *P. cubensis* sporangia added to colorless silicone grease-coated white sampler rods were counted under the dissecting scope. Sporangiophores were very rarely encountered in this process and, whenever present, or if an undesired number of sporangia were accidentally added to the rod, the rod was discarded. To obtain counts of 100 , 200 , 500 , and $1,000$ sporangia, a suspension of *P. cubensis* sporangia was quantified using a hemocytometer and an appropriate aliquot was dispensed on grease-coated sampler rods. For each count of *P. cubensis* sporangia, 10 sampler rods were prepared. Each prepared sampler rod was placed in a sterile 2-ml screw-cap microcentrifuge tube and stored at -20°C until use. Genomic DNA extracted from silicone grease-coated sampler rods without the presence of any sporangia was used as a negative control.

DNA extraction from *P. cubensis* sporangia and infected or uninfected leaf tissue was performed using a phenol-chloroform DNA extraction method as previously described (Withers et al. 2016), with minor modifications. A $50\text{-}\mu\text{l}$ sporangial suspension, a 2- to 3-mm infected leaf segment, or other oomycete mycelial tissue was collected in a 1.5-ml microcentrifuge tube with approximately $200\text{ }\mu\text{l}$ of 425- to 600-mm acid-washed glass beads (Sigma-Aldrich, Inc.) and $500\text{ }\mu\text{l}$ of extraction buffer (200 mM Tris-HCl [$\text{pH } 8$], 250 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate, and 0.5% Triton X-100). Additionally, three 2.3-mm Zircon beads (Biospec, Bartlesville, OK, U.S.A.) were added to infected leaf lesions tubes. Sporangia tubes were vortexed horizontally for 5 min whereas leaf lesion tissues were lysed using an OMNI International BeadRuptor (OMNI International, Tulsa, OK, U.S.A.). Following tissue lysis, RNase (30 mg/ml) and proteinase K (50 mg/ml) (G-Biosciences, St. Louis, MO, U.S.A.) were added and incubated at 65°C for 30 min in two subsequent steps. Subsequently, β -mercaptoethanol ($20\text{ }\mu\text{l/ml}$) was added to all samples and centrifuged at $21,000 \times g$ for 15 min; the resulting aqueous phase was then subjected to an equal volume of saturated phenol ($\text{pH } 8$) and to chloroform-isoamyl alcohol ($24:1$) extraction. DNA was then precipitated with 0.8 volume of 100% ice-cold isopropanol (30 to 60 min at -20°C), washed with $500\text{ }\mu\text{l}$ of 70% ethanol, dried, and resuspended in $100\text{ }\mu\text{l}$ of 10 mM Tris-HCl. Extracted DNA was quantified using the Qubit dsDNA HS assay (Life Technologies, Carlsbad, CA, U.S.A.). DNA extractions from spore trap sampler rods were performed using the NucleoSpin Plant II kit according to manufacturer's instructions, with minor modifications (Klosterman et al. 2014). In brief, pairs of sampler rods collected in 2-ml screw-cap tubes were first centrifuged at $21,000 \times g$ for 20 min; then, the sampler rods were removed and acid-washed glass beads were added along with $300\text{ }\mu\text{l}$ of buffer PL1, vortexed horizontally for 5 min, and incubated at 65°C for 10 min. RNase A was added to the samples (according to the manufacturer's instructions), which were then heat treated and vortexed before further processing, as described by Klosterman et al. (2014).

Conventional end-point PCR and cleanup for sequencing. Primers for PCR and sequencing (Table 2) were ordered through IDT (Integrated DNA Technologies, Coralville, IA, U.S.A.) or Eton Bioscience (Research Triangle, NC, U.S.A.). The PCR assays were

completed as previously described (Withers et al. 2016), except the annealing temperatures (T_a) were set according to Table 2. Amplicon clean-up from PCR assays and agarose gel extraction were completed using ExoSAP-IT (Thermo Fisher Scientific) and the Promega Wizard SV Gel and PCR Cleanup System (Fisher Scientific, Hampton, NH, U.S.A.), respectively, according to manufacturer's instructions.

Design of primers, probes, and the qPCR assay. Clade-specific primers and locked nucleic acid (LNA) probes were designed using Geneious 10.2.3 (Biomatters, Auckland, New Zealand) to target the polymorphic region of c2555.3e7 (Table 2; Fig. 2) following the previously described guidelines (Bustin et al. 2009; Rodriguez et al. 2015) and analyzed for homo- and heterodimer binding strength using the OligoAnalyzer Tool (Integrated DNA Technologies, Inc.). The LNA probe specific for clade 1 isolates was HEX dye labeled (ar1497) whereas, for clade 2, it was FAM dye labeled (ar1529), and both had Iowa BlackO as a quencher. The internal control (IC) probe was labeled with Cy5, with Iowa BlackO Dark as a quencher (Table 2). A 20- μ l multiplexed qPCR mix contained 1 $\times$ PerfeCta Multiplex qPCR ToughMix (QuantaBio, VWR, Beverly, MA, U.S.A.), 2 mM $MgCl_2$, 750 nM universal forward primer, 500 nM each reverse primer, 100 nM each LNA probe, and 2 μ l of template DNA (1 μ l from each clade for mixed DNA or from spore trap samples) or 1 μ l of DNA when only one clade was used, unless otherwise stated. For a singleplex qPCR, a similar 20- μ l reaction volume, 500 nM primers, and 100 nM LNA probe (either clade 1 or 2) were used. The CFX 96 Touch qPCR system (Bio-Rad, Hercules, CA, U.S.A.) and Bio-Rad CFX Manager software (Bio-Rad) were used to run all qPCR assays in hard-shell 96-well plates (Bio-Rad recommended), with a reaction profile of 5 min at 95°C for initial denaturation followed by 38 cycles of 95°C for 15 s and 65°C for 1 min.

Specificity and detection limits of the qPCR assay. DNA from different sporangial counts and 10-fold serial dilutions of total genomic DNA ranging from 1 ng/ μ l to 1 fg/ μ l from both SC1982 (clade 1) and MSU-1 (clade 2) were used to determine the specificity and detection limits of the multiplex qPCR assay. All

other infected plant tissue samples tested were adjusted to a DNA concentration of 1 ng/ μ l before testing. All total genomic DNA concentrations for each clade isolate were tested individually and in all possible concentration combinations with the opposing clade DNA. Additionally, SC1982 and MSU-1 DNA from the different sporangial counts were used in singleplex and multiplex qPCR assays to confirm the specificity and determine the detection sensitivity of clade-specific sporangial DNA.

Spore trap setup, sampler rod collection, and CDM disease monitoring. Battery-powered impaction-type rotorod spore samplers (Model 82; Sampler Technologies, Inc.) were placed in cucurbit sentinel plots for trapping *P. cubensis* aerial sporangia on two sampler rods (2 by 2 by 32 mm) (Fig. 3) using a procedure described previously (Neufeld et al. 2013). The sentinel plots were planted twice per year in 2016 and 2017 at the Central Crops Research Station (CCRS; located in Clayton, NC) and the Cunningham Research Station (CURS; located in Kinston, NC), in late April to early May and again in early August of both years. One spore trap was placed adjacent to the cucumber plot and another one adjacent to the butternut squash plot. The traps were operated from May to September each year, and sampler rods from each spore trap at the CCRS were collected 4 days per week (Tuesday to Friday) and 2 days per week at the CURS (Tuesday and Friday). The collected sampler rods were stored at -20°C until DNA extraction, which was then analyzed using the clade-specific multiplex qPCR assay to detect the presence of airborne sporangia. As mentioned above, two samplers were placed at each plot and the sampler rods with the lower cycle threshold (C_q) value for each clade-specific probe on each collection date were selected to run the qPCR assays in triplicate. The first detection of *P. cubensis* sporangia for each clade was determined based on C_q values and disease development was monitored in sentinel plots based on visual inspection every other day until symptoms and signs were observed. After the initial detection of CDM, disease progress in each plot was monitored twice per week for 1 to 3 weeks and disease severity was recorded. Disease outbreaks in the surrounding counties and states monitored by the CDM ipmPIPE system

TABLE 1. Isolates of *Pseudoperonospora cubensis* collected from North Carolina used to validate clade-specificity of molecular markers and assays

Isolate ^b	Year	Host ^c	K value ^a		Clade	
			K1	K2	PCR	qPCR ^d
KIN-1-7	2013	<i>Cucumis sativus</i>	0.010	0.990	2	2
KIN-1-10	2013	<i>C. sativus</i>	0.009	0.991	2	2
CLE-1-2	2013	<i>C. sativus</i>	0.009	0.991	2	2
14CLE2-1-2B	2014	<i>C. sativus</i>	0.008	0.992	2	2
14CLE2-1-11A	2014	<i>C. sativus</i>	0.008	0.992	2	2
14KIN-3-5D	2014	<i>C. melo</i>	0.009	0.991	2	2
14KIN-3-1C	2014	<i>C. melo</i>	0.007	0.993	2	2
14KIN2-3-2A	2014	<i>C. melo</i>	0.008	0.992	2	2
14CLE-3-8	2014	<i>C. melo</i>	0.006	0.994	2	2
14WAY2-3-1B	2014	<i>C. melo</i>	0.010	0.990	2	2
KIN2-6-4	2013	<i>Cucurbita moschata</i>	0.990	0.010	1	1
14KIN2-6-6A	2014	<i>C. moschata</i>	0.989	0.011	1	1
14WAY2-6-2A	2014	<i>C. moschata</i>	0.985	0.015	1	1
WAY2-4-15	2013	<i>C. pepo</i>	0.983	0.017	1	1
WAY2-4-14	2013	<i>C. pepo</i>	0.992	0.008	1	1
14KIN2-4-3B	2014	<i>C. pepo</i>	0.972	0.028	1	1
WAY2-5-14	2013	<i>C. maxima</i>	0.990	0.010	1	1
14CLE2-5-4C	2014	<i>C. maxima</i>	0.990	0.010	1	1
CLE-7-11	2013	<i>Citrullus lanatus</i>	0.992	0.008	1	1
CLE-7-7	2013	<i>C. lanatus</i>	0.991	0.009	1	1

^a K groupings determined through Bayesian clustering and allele frequency correlations as described by Wallace et al. (2020).

^b Isolates collected as part of a study described by Wallace et al. (2020).

^c *Cucumis sativus* = cucumber, *C. melo* = cantaloupe, *Cucurbita moschata* = butternut squash, *C. pepo* = acorn squash, *C. maxima* = pumpkin, and *Citrullus lanatus* = watermelon.

^d Quantitative PCR.

Fig. 1. Confirmation of c2555.3e7 described by Withers et al. (2016) as a marker of *Pseudoperonospora cubensis* clades described by Wallace et al. (2020). Gel with a 100-bp ladder (M) showing the c2555.3e7 PCR amplicons of clade 2 (C2) isolate MSU1 and clade 1 (C1) isolate SC1982 using primers sw552 and sw553 designed by Withers et al. (2016).

(Ojiambo et al. 2015) were also compared with the timing of *P. cubensis* sporangia detection and with visual disease diagnosis in the sentinel plots.

Analysis of qPCR inhibition and IC. Silicone grease and other particles were routinely captured by the sampling rods. Thus, the efficiency of the multiplex qPCR assay was determined by amplifying genomic DNA of SC1982 and MSU-1 sporangia in the presence of 2 ml of eluate from CDM disease-free field sampler rods and analyzing as previously described (Kunjeti et al. 2016). Additionally, an IC, as described previously (Haudenshield and Hartman 2011; Kunjeti et al. 2016), was designed to identify any alterations in amplification efficiency in the presence of field spore trap sample eluate. Primers and probes for the amplification and

quantification of the IC were designed with a similar Ta as that of clade 1 and clade 2. A standard curve of IC was prepared using 10-fold serial dilutions ranging from 100 fM to 1 aM using IC-specific primers and probes in a singleplex qPCR (Table 2; Supplementary Fig. S2). Afterward, 10 aM of IC oligonucleotide was used as a target in singleplex qPCR assays with each of the 2-ml spore trap samples to identify any significant shift in IC quantification cycle (Cq) (Supplementary Fig. S3).

Fig. 2. Gel images of c2555.3e7 PCR amplicons of clade 1 (829 bp, top) and clade 2 (128 bp, bottom) *Pseudoperonospora cubensis* sporangia DNA using primers ar1517 and sw553. Clade 1 and clade 2 isolates used in the gel are (from left to right) as follows. Clade 1: KIN2-6-4, 14KIN2-6-6A, 14WAY2-6-2A, WAY2-4-15, WAY2-4-14, 14KIN2-4-3B, WAY2-5-14, 14CLE2-5-4C, CLE-7-11, and CLE-7-7. Clade 2: KIN-1-7, KIN-1-10, CLE-1-2, 14CLE2-1-2B, 14CLE2-1-11A, 14KIN-3-5D, 14KIN-3-1C, 14KIN2-3-2A, 14CLE-3-8, 14WAY2-3-1B.

Fig. 3. A, *Pseudoperonospora cubensis* sporangia and sporangiophores on cucumber leaf surface; B, cucumber leaf infected with cucurbit downy mildew and showing characteristic chlorotic, angular lesions delimited by leaf veins; and C, a rotorod spore trap setup in a cucumber sentinel plot at the Central Crops Research Station in Clayton, NC (early to mid-May 2016).

TABLE 2. Primers and probes used in this study^a

Assay, ID	Description	Sequence (5' to 3')	Ta (°C)	Product size (bp)
Multiplex ar1517	Multiplex forward primer	AACGGTGATCCATGCAGCTTTA	60.6	N/A
Clade 1 (SC1982)				
ar1488	Clade 1 uniplex forward primer	GGAGACTTTGTGCTCTGGTCGTT	61.2	68
ar1495	Universal clade 1 reverse primer	CGCAGTGGTTGGGTGTGT	60.5	111
ar1497	Clade 1 universal probe	HEX-CAG+TAGCA+TAACCCAAG+ACTTCGT-IABkFQ	69.4	N/A
Clade 2 (MSU-1)				
ar1493	Clade 2 uniplex forward primer	TCGAACGGTGATCCATGCAG	60.5	72
ar1494	Clade 2 uniplex reverse primer	GGATAGGCTCTTGTCATGCTC	58.9	72
ar1498	Clade 2 uniplex probe	FAM- TG+CTGGAGA+CT+TTGTGCTCT-IABkFQ	59.2	N/A
ar1529	Clade 2 multiplex probe	FAM-TGGTCGAGC+ATTGACAAGAGCCTATCC-IABkFQ	69.9	N/A
ar1531	Clade 2 multiplex reverse primer	CCATCAAGCCAGCAACTTGTT	59.7	93
ar357	Sequencing primer of L- and S-SC1982	CGTTGTCCAGGCAATATAAAT	...	N/A
ar538	Sequencing primer of L- and S-SC1982	GTATCTTCTCGACCATCTCTTC	...	N/A
ar907	Sequencing primer of L-SC1982	AGTGGACGTACGAATTCATA	...	N/A
ar957	IC forward primer	ATCCGCTTCCTGTACGAGTCAA	60.6	121
ar958	IC reverse primer	GCCAATTCAAGGTTGGAGCCA	60.5	...
ar956	IC probe	Cy5-GGTTTCAGCTCAGGAGGCACATGCT-TAO/3' Iowa BlackÖ Dark	66.4	N/A
sw552	c2555.3e7 forward primer	GACGCAACCCAGCATAACAAG	60.1	787
sw553	c2555.3e7 reverse primer	TGCGTCGAGTATCTTCTACC	59.4	...
ar1492	Forward primer psc-contig 1588	CCAGACCATGCATTAGCGGAT	60.5	899
ar1479	Reverse primer psc-contig 1588	CCAGTCGAGAACAAGTTTGATACGA	61.1	...

^a Product sizes and annealing temperatures (Ta) are indicated for primers used for amplification. All primers and probes were designed herein, with the exception that primers sw552 and sw553 were designed by Withers et al. (2016). For probes, +A or +T indicate locked nucleotides in the probes. IC = internal control, IABkFQ = Iowa BlackÖ, and N/A = not available.

Data analyses. Samples were run in triplicate with at least three biological replicates whenever possible. Data analysis was performed as described previously (Klosterman et al. 2014; Kunjeti et al. 2016). The relationship between Cq values and number of sporangia or genomic DNA was determined using linear regression. Numbers of sporangia per sample were estimated based on standard curves derived from the regression analysis. Efficiency of the qPCR was analyzed using the formula $[-1 + 10^{(-1/\text{slope})}] \times 100$.

RESULTS

Sequence analyses of the marker gene c2555.3e7 polymorphic DNA region. The primers sw552 and sw553 amplified single PCR fragments for isolates collected from cucumber, cantaloupe, and buffalo gourd, whereas isolates from watermelon, pumpkin, and butternut squash generated two amplicons; one of a similar size and an additional, larger amplicon. Similar results were observed when these primers were used to amplify genomic DNA of isolates SC1982 (clade 1) and MSU-1 (clade 2). Upon sequence analysis, MSU-1 produced a single fragment of 787 bp in length and SC1982 produced two fragments, one of 785 bp (called small or S-SC1982) and a second of 1,488 bp (called large or L-SC1982) (Fig. 1). The polymorphic nucleotide region in c2555.3e7 among these three sequenced fragments was identified and aligned to the MSU-1 genome (Supplementary Fig. S1). The conserved nature of the first 710 bp of c2555.3e7 between L-SC1982 and MSU-1 allowed for universal forward primer ar1517 to be designed for further validation of the polymorphism identified (Table 2). However, the c2555.3e7 nucleotide sequence from 711 to 790 bp of MSU-1 and SC1982 exhibited considerable polymorphism, with a total of 92.3% sequence identity. Substantially lower sequence identity was observed between L-SC1982 and S-SC1982 (77%) and S-SC1982 and MSU-1 (80.5%). Additionally, the nucleotide sequences between 790 and 1,440 bp of L-SC1982 were almost identical with part of psc-contig-1588 of MSU-1.

Clade-specific assay specificity. Sequence variability between SC192 and MSU-1, near the 3' end of c2555.3e7, allowed for primers ar1517 and sw553 to amplify the marker and determine the host-related clade of the additional 97 *P. cubensis* isolates. When DNA from isolates collected from watermelon, pumpkin, butternut squash, and acorn squash were PCR amplified, an 829-bp fragment was observed, whereas a fragment of 128 bp was noted in all isolates from cucumber and cantaloupe (Fig. 2; Table 1; Supplementary Table S1). None of the other oomycete or host plant sample PCR

amplifications generated any matching PCR products. These 97 *P. cubensis* isolates were also tested for clade identity using the multiplex qPCR assay. The two tests were in agreement for all isolates collected from cucumber and watermelon; however, 18 isolates collected from pumpkin, butternut squash, cantaloupe, and acorn squash exhibited Cq values for primers specific to both clades.

Specificity, sensitivity, and efficiency of the qPCR assay. The amplification of the multiplex qPCR assays for sporangial DNA of isolates SC1982 and MSU-1 exhibited a significant linear response, with an efficiency of 105.04% ($R^2 = 0.996$) and 100.5% ($R^2 = 0.998$), respectively (Fig. 4). Linearity and efficiency of the singleplex qPCR for both isolates were also similar (data not shown). Simultaneous detection using equal and unequal concentrations of DNA from both clades consistently demonstrated unhindered linearity at all tested DNA concentrations. No significant differences in Cq values were observed for SC1982 and MSU-1 when DNA was combined compared with that of single-clade DNA in a multiplex reaction (data not shown). However, detection of genomic DNA concentrations <100 fg was unreliable; therefore, the lower limit of detection of all qPCR assays was considered to be 100 fg of *P. cubensis* genomic DNA regardless of clade. Although the HEX-labeled LNA probe for clade 1 on rare occasions exhibited nonspecific Cq values for clade 2 total genomic DNA at a concentration ≥ 1 ng (Cq > 45), reducing the cycle number to 38 for the multiplex qPCR eliminated any nonspecific detection without losing sensitivity.

Significant linear relationships were observed between sporangial count DNA and Cq values for both clades of *P. cubensis* in the multiplex qPCR assay. The amplification of the multiplex qPCR assays for clade 1 isolate SC1982 and clade 2 isolate MSU-1 sporangial counts exhibited a linear response with an efficiency of 109.81% ($R^2 = 0.911$) and 95.5% ($R^2 = 0.933$), respectively (Fig. 5). However, none of the qPCR assays were able to detect <10 sporangia and, therefore, the detection threshold was considered to be the DNA concentration associated with ≥ 10 sporangia. Similar linear relationships were also observed in the singleplex qPCR assays (data not shown). Simultaneous detection of equal and unequal sporangia counts for DNA from both clades did not show any effect on the Cq values for each clade (data not shown).

Detection and quantification of *P. cubensis* sporangia from spore traps. In total, 84 and 79 total spore samples were collected in 2016 and 2017, respectively, from the sentinel plots located at the CCRS and CURS. The clade-specific multiplex qPCR

Fig. 4. Standard curves of the multiplex quantitative PCR based on $\log_{10}$ -transformed DNA concentrations for **A**, SC1982 (clade 1) and **B**, MSU-1 (clade 2). A linear trend is observed in both cases, the shaded areas above the line represent 95% confidence intervals, Cq = cycle threshold, and the error bars represent the standard deviations (SD).

Fig. 5. Standard curves of the multiplex quantitative PCR based on $\log_{10}$ -transformed sporangia counts for **A**, SC1982 (clade 1) and **B**, MSU-1 (clade 2). A linear trend is observed in both cases, the shaded areas above the line represent 95% confidence intervals, Cq = cycle threshold, and the error bars represent the standard deviations (SD).

assay detected the presence of airborne *P. cubensis* sporangia from these samples (Fig. 6; Supplementary File S1) and the resulting Cq values were then compared with the previously described standard curves to estimate an approximate sporangia count (Fig. 5). In 2016, clade 2 sporangia were first observed on 7 and 5 July in spore samples collected from the early-season sentinel plots located at the

CCRS and CURS, respectively, and the resulting mean Cq values were equal to approximately 67 and 50 sporangia/sample at CCRS and CURS, respectively. Likewise, in the late-season sentinel plots, clade 2 sporangia were first observed in spore samples collected on 7 September at the CCRS and on 23 August at the CURS, with mean Cq values equivalent to approximately 43 and 50 sporangia/sample,

Fig. 6. *Pseudoperonospora cubensis* clade 1 and clade 2 sporangia detected with quantitative PCR in spore trap samples collected at the Central Crops Research Station (CCRS) and the Cunningham Research Station (CURS) in 2016 and 2017. Cq = cycle threshold. The gap between plots represents two plantings of the sentinel plots. Solid vertical lines indicate the first report to the cucurbit downy mildew ipmPIPE of a host susceptible to a clade in North Carolina. Dotted vertical lines indicate visual confirmation (<5% disease) of infection of a host susceptible to a clade in the sentinel plots.

respectively. Clade 1 sporangia were not observed in the early-season sentinel plots but were detected in the late-season planting on 9 September at the CCRS and on 26 August at the CURS, with mean Cq values equivalent to approximately 51 and 54 sporangia/sample, respectively. In 2017, the early-season sentinel plots saw clade 2 sporangia arrive at the CCRS on 20 June and at the CURS on 6 June, with mean Cq values equivalent to approximately 31 and 23 sporangia/sample, respectively. Clade 2 sporangia were first detected in the late-season sentinel plots on 1 September at the CCRS and on 5 September at the CURS, with mean Cq values equivalent to 30 and 17 sporangia/sample, respectively. Similar to 2016, clade 1 sporangia were not detected in the early-season sentinel plots at either location but were observed in the late-season sentinel plots at the CCRS on 1 September and at the CURS on 5 September, with mean Cq values equivalent to 23 and 26 sporangia/sample, respectively (Fig. 6).

When comparing detection of clade 1 or clade 2 sporangia using the spore samplers and the clade-specific assay with visual confirmation of downy mildew in the sentinel plots and outbreak reports made to the CDM ipmPIPE, we found that disease or sporangia were not detected in the sentinel plots in spite of CDM being reported in the state (Fig. 5). Based on the public records of the CDM ipmPIPE, the first detection of disease in North Carolina was on 1 June 2016 and 5 June 2017 in cucumber in Duplin County, with cucumber being a clade 2 host. The first detection of CDM on a clade 1 host (watermelon for both reports) was on 17 June 2016 in Nash County and 27 June 2017 in Carteret County. In contrast, disease was first confirmed in the cucumber (clade 2) sentinel plot on 13 July and 13 September 2016 for the first planting and 23 June and 6 September 2017 for the second planting at the CCRS, and on 12 July and 30 August 2016 for the first planting and 13 June and 8 September 2017 for the second planting at the CURS (Fig. 5). CDM was confirmed in the squash (clade 1) sentinel plot on 14 September 2016 and 6 September 2017 for the second planting at the CCRS, and on 2 September 2016 and 8 September 2017 for the second planting at the CURS (Fig. 5).

Symptom development (disease severity < 5%) in the sentinel plots was observed between 2 and 4 days after sporangia for the respective clades were detected using the spore samplers and multiplex qPCR (Fig. 6). Based on the spore sampler detection trends, CDM symptoms were typically observed within 1 to 2 days after Cq values became lower than approximately 36.7 for both clades and locations, which corresponds to approximately 10 to 20 sporangia (Fig. 5). For both clades, the number of detected sporangia increased as CDM progressed throughout the sentinel plots, as indicated by the gradual decrease of Cq values in the assay.

Analysis of multiplex qPCR inhibition. Genomic DNA extracted from spore samples collected in early May from the CCRS and CURS was used as *P. cubensis* sporangia DNA-free eluate to evaluate any inhibition of the multiplex qPCR assay. During that period of time, sentinel plots had just been planted and no CDM disease was recorded in the state (Fig. 6). When the eluates were added to the sporangial DNA of either SC1982 or MSU-1, the 10-fold serial dilution series showed no significant change in Cq values (data not shown). To test whether the *P. cubensis* sporangia DNA-positive spore samples had any qPCR inhibitors present, an IC control singleplex qPCR method was employed. The standard curve of IC that was generated using 10-fold serial dilutions ranging from 100 fM to 1 aM exhibited complete linearity, with a slope of -3.26 (Supplementary Fig. S2). When 10 aM of IC was used as the template DNA in the IC qPCR assays with each of the spore-trap-positive samples, no significant change in Cq values was observed (Supplementary Fig. S3).

DISCUSSION

In this study, a polymorphic diagnostic marker, c2555.3e7 (Withers et al. 2016), was used to develop an end-point PCR and probe-based multiplex qPCR assay to detect and quantify sporangia

of *P. cubensis* belonging to host-specialized clades (Wallace et al. 2020). Our assay had a detection limit of 100 fg greater than described previously for *P. cubensis* (Summers et al. 2015a); however, it exhibited enhanced specificity and sensitivity when using different sporangial count DNA. The Summers et al. (2015a) multiplex qPCR assay based on a single nucleotide polymorphism (SNP) in the mitochondrial *cox2* region allowed for the differentiation of *P. cubensis* and *P. humuli* isolates (Summers et al. 2015a). The Summers et al. (2015a) assay was also used to detect airborne sporangia captured in rotorod spore trap samples with a detection limit as low as 1 fg of *P. cubensis* DNA and 10 sporangia, similar to our assay. However, the Summers et al. (2015a) assay did not differentiate between the *P. cubensis* clades in airborne sporangia because they were only recently reported (Wallace et al. 2020). The assays developed here are a crucial first step toward a biosurveillance system that will help to better inform fungicide application timing on specific cucurbit crops based on the host-adapted clade of *P. cubensis*.

Real-time or qPCR assays have been developed to detect airborne downy mildew propagules and are typically based on SNPs in a particular nuclear or mitochondrial gene. The c2555.3e7 marker used here is a species-specific, single-copy nuclear gene identified through comparative genomics, validated against multiple distant and closely related oomycete species, and with no cross-detection to closely related species (Withers et al. 2016). In contrast, a real-time PCR assay utilizing an SNP in the 18S ribosomal DNA to detect propagules of the spinach downy mildew pathogen *Peronospora effusa* showed cross detection to the sugar beet downy mildew pathogen *P. schachtii* at a limit of 100 fg in air currents using spore samplers (Klosterman et al. 2014). Single-copy markers based on nuclear DNA, like c2555.3e7, or low-copy number markers based on nuclear ribosomal DNA (nrDNA) such as the internal transcribed spacer, may also offer more accuracy for sporangia quantification compared with high-copy target markers based on mitochondrial DNA (mtDNA). Recently, an nrDNA-based marker provided more accurate quantification of multiple arbuscular mycorrhizal fungi than an mtDNA marker due to the correlation between nrDNA copy number and fungal biomass (Voříšková et al. 2017). The number of mitochondria in an individual oomycete sporangium is not well documented but may vary from 50 to 200 in individual yeast cells (Freel et al. 2015). Although this variability may allow for mtDNA markers to detect sporangia at lower DNA concentrations, the accuracy in detecting specific amounts of sporangia may be greatly reduced. Thus, using single-copy nuclear DNA markers in qPCR assays to quantify airborne sporangia may provide greater quantification capabilities than mtDNA markers, which is critical when developing a biosurveillance system that depends on accurate estimation of clade-specific *Pseudoperonospora cubensis* inoculum to determine the likelihood of CDM on specific cucurbit crops.

Genomic DNA extracted from *P. cubensis* sporangia and from CDM-symptomatic leaf lesions were analyzed using the end-point PCR and multiplex qPCR assays. When leaf lesions from cucumber and watermelon were evaluated, only DNA of clade 1 was observed in watermelon leaf lesions, and only DNA of clade 2 was observed in cucumber leaf lesions. These results support previous findings that isolates of *P. cubensis* display host adaptation (Quesada-Ocampo et al. 2012; Summers et al. 2015b; Thomas et al. 2017) and provides further evidence that host preference in isolates of *P. cubensis* is linked with the two clades described by Wallace et al. (2020). Previous research revealed that clade 2 isolates of *P. cubensis* preferentially infect cucumber and cantaloupe, with clade 1 isolates occurring at lower levels (<2% in cucumber and <20% in cantaloupe) in these hosts (Wallace et al. 2020). In this study, when analyzing 58 DNA samples collected from pumpkin, butternut squash, acorn squash, and cantaloupe leaf lesions, 18 were found to be positive for both clade 1 and clade 2 corresponding to 56% of pumpkin, 26% of acorn squash, and 28% of cantaloupe samples. As reported by Wallace et al. (2020), our findings show

that *P. cubensis* clades display host adaptation but not complete host specificity. At the present time, it is unclear whether pathogen, host, or environmental factors allow for a specific cucurbit host to harbor both clades. Throughout the course of this study, detached cucumber leaves were infected with *P. cubensis* isolates to maintain a constant source of sporangia. The older cucumber leaves seemed to support greater infection and sporulation of clade 1 isolates than did the younger leaves; clade 2 isolates were able to infect and sporulate on detached cucumber leaves regardless of their age and size (data not shown). Plant host resistance can vary with plant age or tissue maturity but evidence of a shift from resistance to susceptibility seems to be lacking (Develey-Rivière and Galiana 2007), and whether similar circumstances will lead to both clades of *P. cubensis* infecting pumpkin and squash should be explored further.

Spore trapping has been utilized to detect *P. cubensis* sporangia previously and provides an early warning system for fungicide application timing (Neufeld et al. 2013). However, combining spore traps with real-time qPCR has provided greater precision when identifying sporangia in air currents for other downy mildew pathogens (Klosterman et al. 2014; Kunjeti et al. 2016). Thus, in this study, sentinel plots were planted to cucumber and watermelon for two cropping periods within each growing season (spring to summer and summer to fall) and the clade-specific multiplex qPCR assay was validated by capturing airborne sporangia using rotorod impaction spore samplers. Interestingly, the results generated through assay validation provide further evidence of host preference as well as seasonal differences between the clades at both research stations.

Managing CDM requires the frequent application of effective fungicides but efficacy tests are often performed solely on cucumber, with the results applied to other cucurbit crops (Goldenhhar and Hausbeck 2019; Holmes et al. 2015; Keinath et al. 2019). Improving our knowledge of fungicide efficacy on other cucurbit hosts such as those preferred by clade 1 isolates (e.g., watermelon) could lead to fungicide recommendations for the specific cucurbit hosts based on clade. At present, cucurbit growers are encouraged to initiate preventive fungicide applications for downy mildew when an outbreak in a neighboring state has been reported to the CDM ipmPIPE, regardless of specific cucurbit host plant (Ojiambo et al. 2011). The host preferences described here and previously (Wallace et al. 2020) indicate that CDM should be managed based on the specific host affected. Managing CDM in a host-specific manner could reduce unnecessary fungicide applications by recommending that applications be initiated only after a report occurs on a specific crop in an adjacent state, which could also slow the development of fungicide resistance by reducing selective pressure.

Seasonal variation, where clade 2 sporangia and CDM on a clade 2 host was observed earlier in the growing season compared with clade 1 sporangia or CDM on a clade 1 host, occurred in both years of this study. Indeed, clade 2 sporangia were observed prior to CDM symptoms in the early-season cucumber plots at both field sites in both years. Likewise, clade 2 sporangia were observed prior to CDM symptoms in the late-season cucumber plots at both field sites in both years. Symptoms were not observed in early-season watermelon plots at either field site over the 2 years but CDM did occur in late-season watermelon shortly after clade 1 sporangia were identified in the spore traps. These results corroborate those of a previous study, where clade 1 sporangia were more prevalent in the fall and clade 2 sporangia were more prevalent in the summer (Wallace et al. 2020). However, in that study, symptomatic leaf tissue was collected at only two time points (summer and fall) per year for 2 years. Thus, using the spore samplers to collect sporangia every 2 to 4 days from May to September provided greater precision and confirmed the previously observed seasonality of the two clades (Wallace et al. 2020). As mentioned previously, CDM management is heavily reliant on chemical control but fungicide efficacy appears to vary by state and year (Keinath et al. 2019). The geographic variation in efficacy could be related to clade, though that has not been formally investigated. Additionally, these fungicide

applications could be preferentially controlling clade 1 isolates early in the growing season, resulting in epidemics caused by clade 2 isolates. However, whether that seasonal variation is the result of isolates of the respective clades differing in their response to fungicides is unknown and warrants further investigation. The seasonal differences observed here could also be a side effect of the total area planted to hosts susceptible to these two clades in North Carolina over the 2 years. Cucurbit producers in North Carolina typically plant greater hectareage to cucumber compared with watermelon and, in 2016, approximately 4,452 ha were planted to cucumber compared with only 2,671 ha of watermelon (NASS 2018). Similarly, in 2017, approximately 4,249 ha were planted to cucumber compared with 2,711 ha planted to watermelon (NASS 2018). That nearly twice as many hectares were planted to cucumber may have allowed for clade 2 sporangia to proliferate and spread across the state much faster than clade 1 sporangia. Neither clade 1 sporangia nor CDM symptoms on watermelon were observed in the sentinel plots before August of either year, despite being observed in North Carolina by late June of both years. Whether this phenomenon occurs in other states where cucurbit production occurs remains to be seen but future nationwide monitoring using the clade-specific marker described in this study would be useful to fully understand seasonal dynamics in the respective *P. cubensis* clades.

Utilizing the observed polymorphism in marker c2555.3e7 described by Withers et al. (2016), an end-point PCR and probe-based multiplex qPCR assay were developed to detect and quantify *P. cubensis* sporangia belonging to the host-adapted clades (Wallace et al. 2020). The multiplex assay was used in conjunction with rotorod impaction spore samplers and detected sporangia of both clade 1 and clade 2 between 2 and 4 days prior to disease onset on watermelon and cucumber sentinel plots, respectively. Current CDM management recommendations call for protective fungicide applications to protect all cucurbits, regardless of host, once a report has been submitted to the CDM ipmPIPE from a neighboring state. In 2016, CDM was first reported in North Carolina on 1 June and was observed on cucumber test plots at the CCRS and CURS a little over a month later but wasn't observed on watermelon plots at either station until 26 August. Similarly, CDM was reported in North Carolina on 5 June 2017 and was observed the next day at the CURS and within 2 weeks at the CCRS; however, once again, CDM was not observed on watermelon until 1 September, likely due to seasonal variation of the respective clades. With the exception of the cucumber plots at the CURS in 2017, all observations of CDM in cucumber or watermelon sentinel plots occurred beyond the recommended interval between fungicide applications, which would have been unnecessary due to the lack of inoculum in the local air currents. Thus, deploying spore sampling systems, either stationary or attached to unmanned aerial vehicles, and analyzing the samples with the multiplex qPCR assay developed here will act as a complement to the CDM ipmPIPE and allow for significant improvements in the detection of *P. cubensis* sporangia and prediction of CDM epidemics. Such improvements will enable CDM disease management practices such as fungicide applications to be employed only on crops at risk of infection at any given time throughout the growing season, based on detection of the specific *P. cubensis* clade.

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